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(54) Title: TITLE: INTEGRATION OF β-ACTIN GENE FOR SAMPLE QUALITY CHECK IN HSV-1 AND HSV-2 DIAGNOSTIC KIT

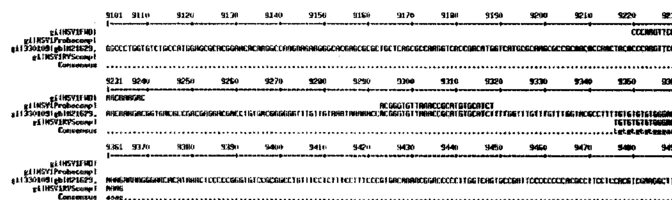


Figure : 1

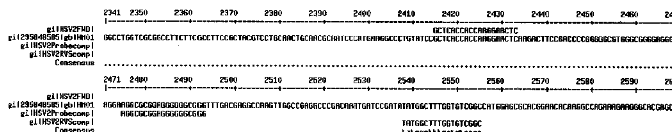
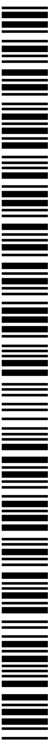


Figure : 2

(57) Abstract: Primers and probes for the in-vitro detection of Human HSV- 1 and HSV-2 virus in a sample comprising nucleotide sequences selected from the group comprising of or any combinations thereof: a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO: l and SEQ ID No. 4 or a part or a nucleotide having minimum 90% sequence identity with said SEQ ID NO: l, wherein the nucleotide sequence of SEQ ID NO: 1 or SEQ ID No. 4 represents forward primer to amplify human HSV- 1 virus; a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO: 2 and SEQ ID No. 5 or a part or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:2 and SEQ ID No. 5, wherein the nucleotide sequence of SEQ ID NO: l represents reverse primer to amplify human HSV- 1; and a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 and SEQ ID NO. 6 or a part or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:3 and SEQ ID No. 6, wherein the nucleotide sequence of SEQ ID NO:3 and SEQ ID No. 6 represents probes to detect human HSV- 1 and HSV-2. The nucleotide sequences enables high detection of all genotypes of Human HSV- 1 and HSV-2 wherein said nucleotide sequences enables detection of virus present in low copy number upto >102 IU/ml.



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TITLE: INTEGRATION OF β-ACTIN GENE FOR SAMPLE QUALITY CHECK IN HSV-1 AND HSV-2 DIAGNOSTIC KIT

FIELD OF THE INVENTION:

The present invention relates to in vitro for the detection of Herpes Simplex Virus in human samples. The present invention further provides a testing kit based on multiplex PCR, which enables simultaneous detection of herpes simplex virus-1 (HSV-1) and herpes simplex virus-2 (HSV-2), essentially comprising probes & primers for the detection of HSV1&2, with an internal control human gene for checking sample quality.

BACKGROUND AND THE PRIOR ART:

Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), also known as Human herpes virus 1 and 2 (HHV-1 and HHV-2), are DNA viruses of the family Herpesviridae. The viral genome is a linear, double stranded DNA molecule of ~152261 to ~154746 nucleotides that contains a single open reading frame which codes for 77 proteins. HSV infections in humans can cause lesions at a variety of sites, e.g., oral-facial, genital, visceral, ophthalmic, cutaneous and the central and peripheral nervous system. These lesions can be a result of the primary infection by the virus or they can result from a reactivation of the latent virus, causing recurrent episodes of the disease. HSV-1 and HSV-2 are genetically and antigenically distinct forms. HSV-2 is the most common cause of genital infections, due to venereal transmission; HSV-1 is commonly associated with other disease locations although both serotypes have been shown to cause disease in all locations of the body. Most HSV 1 infections are contracted during the childhood years, and many infections go completely unnoticed, as the symptoms can be minor or not apparent. HSV 1 is increasingly the cause of genital herpes infections as oral-genital contact becomes a more routine part of sexual expression. Studies have also shown an increasing prevalence of genital HSV infections with a concomitant increase of

the disease in neonates. The consequences of HSV infection can range from inconsequential (cold sores in otherwise healthy patients) to highly morbid and fatal (neonates).

The currently used methods for the diagnosis of HSV-1&2 is based on ELISA (Enzyme Linked immune sorbent assay) for detecting the serum markers such as, IgM and IgG specific to HSV1&2. Antibody takes a week or more to become detectable in body fluids. Old style blood tests (called crude antigen tests) could detect antibody to herpes simplex in general but were exceedingly poor at differentiating accurately between types 1 and 2. This inability to distinguish between the two viruses is called cross reactivity. Viral culture is considered to be gold standard but is expensive, time consuming, technically challenging and less sensitive. HSV PCR, with its consistently and substantially higher rate of HSV detection, has replaced other tests for diagnosis. PCR test takes a copy of viral genome and amplifies it many times in a visible product. Samples to be tested by PCR are less likely to be influenced by transport issues. Available HSV-1&2 PCR assays show cross reactivity among HSV-1 & HSV-2 due to genome similarity among HSV-1 & HSV-2. There are few assays for HSV-1 & HSV-2 detection in uniplex reaction and some have HSV-1, HSV-2 & VZV multiplex. These assays do not have internal quality control system. Moreover due to genotypic diversity many of the assays have limitations in picking up all the existing genotypes. These assays do not provide any provision which can provide assurance that the performed test has run successfully on a good quality sample. US20060141481 A1 (US 11/020,676, Brian Mariani), WO 2011133811 A2 (PCT/US2011/033488, Getman et al) discloses a detection method and kit for detecting HSV-1 and HSV-2 viruses in a test sample. However, the test does not have an internal control which provides validity to the reaction. These tests also have limitation of detecting low copy numbers.
Since ELISA based methods, culture and available PCR tests have their inherent limitations there is a need for

i) a sensitive, reliable and cost effective diagnosis in the first few weeks of infection,

ii) a test kit which helps in detecting the samples infected with low copy number of viruses,

iii) a test kit which comprises an internal control which enables the checking of the validity of the reaction so as to be ensured that the test has run successfully on a good quality sample,

iv) a reaction mixture for the multiplex PCR which enables the equal intensity of detection of HSV-1 and HSV-2 viruses separately i.e. the higher presence of one type of herpes virus does not affect the detection of the other herpes virus in the sample.

OBJECTS OF THE INVENTION:

It is therefore, an object of the present invention is to provide a set of probes and primers for the specific detection of human herpes simplex virus 1 and herpes simplex virus 2 simultaneously.

Another object of the present invention is to provide a highly specific detection of human herpes simplex virus 1 and herpes simplex virus 2 with no cross reactivity

Another object of the present invention is to provide a testing kit using the aforesaid probes and primers with high sensitivity, specificity and cost effectiveness enabling the simultaneous detection of HSV-1 and HSV-2 virus.

Another object of the present invention is to provide a test kit which can help in detecting almost all strains of HSV-1 and HSV-2.

Another object of the present invention is to provide a test kit which helps in detecting the sample with low copy number viruses.

Another object of the present invention is to provide a test kit which comprises an internal control which enables the checking of the validity of the reaction so as to be ensured that the test has run successfully and the sample quality is good.

Yet another object of the present invention is to provide a reaction mixture for the multiplex PCR which enables the equal intensity of detection of HSV-1 and HSV-2 viruses separately i.e. the higher presence of one type of herpes virus does not affect the detection of the present other herpes virus in the sample.

SUMMARY OF THE INVENTION:

According to this invention, there is provided Primers and probes for the in-vitro detection of Human HSV-1 virus in a sample comprising:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:1 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:1, wherein the nucleotide sequence of SEQ ID NO:1 represents forward primer to amplify human HSV-1 virus;
b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:2 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:2, wherein the nucleotide sequence of SEQ ID NO:1 represents reverse primer to amplify human HSV-1; and

c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:3, wherein the nucleotide sequence of SEQ ID NO:3 represents probes to detect human HSV-1;

wherein the said nucleotide sequences enables high detection of all genotypes of Human HSV-1. Wherein said nucleotide sequences enables detection of virus present in low copy number upto $\geq 10^2$ IU/ml.

A reaction mixture for multiplex real time PCR for the simultaneous detection of human herpes virus 1 and 2 comprising:

nuclease free water present in an amount of 4.2 μl;
buffer present in an amount ranging from 12.5μl;
HSV-1 Primer Forward (10pm) present in an amount ranging from 0.35μl;
HSV-1 Primer Reverse (10pm) present in an amount ranging from 0.35 μl;
HSV-2 Primer Forward (10pm) present in an amount ranging from 0.35 μl;
HSV-2 Primer Reverse (10pm) present in an amount ranging from 0.35 μl;
β-actin Primer Forward (10pm) present in an amount ranging from 0.20 μl;
β-actin Primer Reverse (10pm) present in an amount ranging from 0.20 μl;
HSV-1 Probe (10pm) present in an amount ranging from 0.175 μl;
HSV-2 Probe (10pm) present in an amount ranging from 0.175 μl;
β-actin Probe (10pm) present in an amount ranging from 0.15 μl;
enzyme present in an amount ranging from 1 μl; and

template present in an amount ranging from 5 μl;

wherein the reaction mixture enables equal intensity detection of all genotypes of human herpes virus-1&2.

BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS:

Fig 1 shows the alignment of HSV-1 primers and probe with UL36.

FIG 2 shows alignment of HSV-2 primers and probe with UL27 (gβ gene).

FIG 3 shows alignment of internal control primers and probe with Human β-actin gene.

FIG 4 shows a Real time plot of HSV-1 positive samples using SEQ ID No. 1, 2 &3 A showing HSV-1 amplification and B line showing No Template Control.

FIG 5 shows a Real time plot of HSV-2 positive samples using SEQ ID No. 4, 5 &6 A showing HSV-2 amplification and B line showing NTC.

FIG 6 shows a Real time plot of β-actin positive samples using SEQ ID No. 7, 8&9 A showing Human β-actin amplification and B line showing NTC.

FIG 7 shows a Real time plot of HSV-1 positive and HSV-2 negative sample in a multiplex reaction using SEQ ID No. 1to9 (a) HSV-1 positive with NTC (filter 465-510), (b) HSV-2 negative with NTC (filter 618-660) and (c) Human β-actin with NTC (filter 533-580)

FIG 8 shows a Real time plot of HSV-1 negative and HSV-2 positive sample in a multiplex reaction using SEQ ID No. 1to9 (a) HSV-1 negative with NTC (filter 465-510), (b) HSV-2 positive with NTC (filter 618-660) and (c) Human β-actin with NTC (filter 533-580).

FIG 9 shows a Real time plot of HSV-1 negative and HSV-2 negative sample in a multiplex reaction using SEQ ID No. 1to9 (a) HSV-1 negative with NTC (filter 465-510), (b) HSV-2 negative with NTC (filter 618-660) and (c) Human β-actin with NTC (filter 533-580).

FIG 10 shows a Real time plot of HSV-1 and HSV-2 mixed sample in a multiplex reaction using SEQ ID No. 1to9 (a) HSV-1 positive with NTC (filter 465-510), (b) HSV-2 positive with NTC (filter 618-660) and (c) Human β-actin with NTC (filter 533-580).

DETAILED DESCRIPTION OF THE INVENTION:

According to this invention there is provided an in-vitro detection of Herpes simplex virus in human samples. The present invention provides a multiplex real-time polymerase chain reaction for simultaneous detection of Human Herpes simplex virus 1 and 2 with Human β-actin gene as internal control.

The invention provides a test kit based on multiplex real time PCR for the simultaneous detection of Human Herpes virus 1 & 2. The kit advantageously helps in detecting all the genotypes of HSV1&2, shows no cross reactivity among HSV1 & HSV2 and also provides probes and primers that are efficient enough to detect sample with low copy number of viruses. Further, the invention extends to provide a reaction mixture for the multiplex PCR which enables the equal intensity detection of HSV1&2 viruses individually i.e. the higher presence of one type of herpes virus does not affect the detection of the

other herpes virus in the sample. The kit comprises nucleotide sequences selected from the group comprising of or any combinations thereof:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:1 (Table1) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:1;
b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:2 (Table1) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:2;
c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 (Table1) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:3;
d) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:4 (Table2) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:4;
e) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:5 (Table2) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:5; (Please check if 90% sequence identity is appropriate)
f) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:6 (Table2) or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:6;
g) an internal control gene;

wherein said nucleotide sequences enable high detection of all strains of HSV-1 and HSV-2 virus.

The nucleotide sequences enables detection of HSV-1 and HSV-2 virus present in low copy number upto $\geq 10^2$ IU/ml and $\geq 10^2$ IU/ml respectively.

The invention also provides primers and probes for the detection of Human HSV-1 virus in a sample comprising:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:1 or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:1, wherein said nucleotide sequence of SEQ ID NO:1 represents forward primer to amplify herpes simplex virus 1;
b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:2 or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:2, wherein said nucleotide sequence of SEQ ID NO:1 represents reverse primer to amplify herpes simplex virus 1;
c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:3, wherein said nucleotide sequence of SEQ ID NO:3 represents probes to detect herpes simplex virus 1;
and wherein said nucleotide sequences enables the high detection of all strains of HSV-1.

The nucleotide sequences enable detection of Herpes simplex virus-1 present in low copy number upto $\geq 10^2$ IU/ml and $\geq 10^2$ IU/ml respectively.

The invention also provides primers and probes for the detection of Human herpes simplex virus 2 in a sample comprising:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:4 or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:4, wherein said nucleotide sequence of SEQ ID NO:4 represents forward primer to amplify Human herpes simplex virus 2;
b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:5 or a part of it or a nucleotide having at least 90% sequence

identity with said SEQ ID NO:5, wherein said nucleotide sequence of SEQ ID NO:5 represents reverse primer to amplify Human herpes simplex virus 2;

c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:6 or a part of it or a nucleotide having at least 90% sequence identity with said SEQ ID NO:6, wherein said nucleotide sequence of SEQ ID NO:6 represents probes for the detection of Human herpes simplex virus 2;

wherein said nucleotide sequences enables high detection of all genotypes of Human herpes simplex virus 2 and wherein said nucleotide sequences enables detection of virus present in low copy number upto $\geq 10^2$ IU/ml.

The invention further provides a reaction mixture for multiplex real time PCR for the simultaneous detection of Human Herpes virus 1&2 comprising:

a) Nuclease free water present in an amount of 4.2 µl
b) Buffer present in an amount of 12.5µl
c) HSV-1 Primer Forward (10pm) present in an amount of 0.35µl
d) HSV-1 Primer Reverse (10pm) present in an amount of 0.35 µl
e) HSV-2 Primer Forward (10pm) present in an amount of 0.35 µl
f) HSV-2 Primer Reverse (10pm) present in an amount of 0.35 µl
g) β -actin Primer Forward (10pm) present in an amount of 0.20 µl
h) β-actin Primer Reverse (10pm) present in an amount of 0.20 µl
i) HSV-2 Probe (10pm) present in an amount of 0.175 µl
j) HSV-2 Probe (10pm) present in an amount of 0.175 µl
k) β -actin Probe (10pm) present in an amount of 0.15 µl
l) Enzyme present in an amount of 1 µl
m) Template present in an amount of 5 µl.

The reaction mixture advantageously enables equal intensity detection of all genotypes of Human Herpes virus 1&2.

DESIGNING THE PRIMERS & PROBES

HSV-1

Sequences representing different genotypes of HSV-1 were downloaded from the GenBank nucleotide database and aligned using the program multalin. A highly conserved region of HSV-1 (UL36) is selected for the design of real-time PCR primers and probe. Different genotypes were aligned and also checked for cross reactivity with human HSV-1 virus. No degeneracies are done as the primers and probes were binding completely to amplify all genotypes of HSV-1. The cross reactivity with the HSV-2 genome was tested. The probe is tagged with FAM as a reporter and IABkFQ as a quencher at 3' end. The primers and probes sequences for HSV-1 are mentioned in table 1:

Table 1:

Sequence ID	**Polarity**	**Oligonucleotide sequence 5'-3'**	**Length**	**Product size**
1. HSV-1 Fwd	Forward	CCCAAGTTCCCAACAAAGAC	20	143-151bp
2. HSV-1 Rvs	Reverse	CTTTCTTCCCACACACACA	19	
3. HSV-1 Probe (-)		/5FAM/AGATGCACATGCGGTTTAACA CCCGT/3 IABkFQ /	26	

HSV-2

Nucleotide sequence of the glycoprotein B gene (UL27) region of the human HSV-2 genome from different genotypes was analyzed from large number of isolates obtained throughout the world. The sequences of all the genotypes were aligned using multalin[13] to find conserved sequence of HSV-2 and primers& probe was designed. An alignment of primers & probe with nucleotide sequence of the glycoprotein B gene (UL27) from different HSV-2 genotypes which we studied is presented in Fig.2. No degeneracies are done yet the primers and probe are suitable for all the genotypes. The probe is tagged with Cy5 as a reporter and IAbRQSp as a quencher at 3' end. The primers and probe sequences for HSV-2 are mentioned in table 2.

Table 2:

Sequence ID	**Polarity**	**Oligonucleotide sequence 5'-3'**	**Length**	**Product size**
4. HSV-2 Fwd	Forward	GCTCACCACCAAGGAACTC	19	141bp
5. HSV-2 Rvs	Reverse	GCCGACACCAAAGCCATA	18	
6. HSV-2 Probe		5'-Cy5/CCCGCCCCCTCCGCGCCT/3IAbRQSp /-3'	19	

Human β-actin

Housekeeping gene is typically a constitutive gene that is required for the maintenance of basic cellular functions and expressed in all cells of an organism. β-actin (gene name ACTB) is one of six different actin proteins. Actins are highly conserved proteins that are involved in cell motility, structure, and integrity. Actin is a major constituent of the contractile apparatus and one of the two non-muscle cytoskeletal actins. Human β-actin gene is expressed at relatively constant levels. An alignment of primers & probe with nucleotide sequence of β-actin, using multalin is shown in Fig. 3. The probe designed is tagged with HEX as reporter and double quenched with ZEN and IABkFQ. The primers and probe sequences for Human β-actin are mentioned in table 3.

Table 3:

Table 3:				
Sequence ID	**Polarity**	**Oligonucleotide sequence 5'-3'**	**Length**	**Product size**
7. β-actin Fwd	Forward	ACCGAGCGCGGCTACAG	17	60bp
8. β-actin Rvs	Reverse	CTTAATGTCACGCACGATTTCC	22	
9. β-actin Probe		5'-/5HEX/TTCACCACC/ZEN/ACGGCCGAGC/3IABkFQ/-3'	19	

TESTING THE SENSITIVITY & SPECIFICITY OF PROBES AND PRIMERS

Culture fluid for HSV1 and HSV2 was tested with the respective primers and probes and desired results were obtained. Further the amplified product was cloned in a cloning vector, quantified and detection limit was tested by serial dilution. Primers and probes for HSV-1&2 of the invention exhibit an absence of hybridization to sequences contained in human RNA and DNA. This is being confirmed theoretically by BLAST analysis (NCBI), and empirically by testing selected primer sets against human total nucleic acid under both reverse transcription (RT) and/or PCR conditions. Additionally, the claims probes and primers lack cross reactivity against other non-HSV genomes that could be present in clinical samples. This may also be confirmed theoretically in a BLAST search, and empirically using for example, CMV, EBV, HHV6, HIV, HCV, HBV, enteroviral or parvoviral genomic material.

A known Human Herpes virus 1&2 controls were amplified with the aforesaid primers and cloned in pTZ57R/T cloning vector using commercial kit then the cloned plasmid was amplified using M13/pUC sequencing primers (Fwd & Rvs), then the amplified product was sequenced using capillary sequencer to get exact cloned sequence. Further the sequenced segment was checked for the sequence similarity using NCBI Blast program and it was confirmed that the cloned sequence belonged to Human Herpes virus 1&2.

A batch of 48 samples was prepared by mixing different viruses in different combinations including Human Herpes virus 1&2 as well as HBV, HCV, Human Parvovirus B19, Human Parvovirus 4, Adenovirus, Dengue, Japanese encephalitis, Influenza virus, Enteroviruses, Varicella zoster virus and was tested for Human Herpes virus by currently designed primer and probes. Results showed that there is no cross reactivity of the designed primers and

probes with the above mentioned viruses.

The sensitivity of this Real Time based assay was estimated based on a cloned and standardized HSV1&2 DNA with known copy number. Detection of positive samples containing viral load from 10^7 to 10^2copies/ml, of the HSV1&2 standard in the assay was 100% (10 of 10 tests) respectively, while samples with ≤10 copies/ml were tested positive 9 out of 10 times for HSV-1 and 7 out of 10 times for HSV-2. These results shows that the assay has a very high sensitivity and specificity required for detecting Human Herpes virus 1&2.

TESTING THE INFLUENCE OF HIGH VIRAL LOAD OF ONE VIRUS OVER THE AMPLIFICATION OF LOW VIRAL COPY NUMBER OF OTHER VIRUS:

Another set of experiment was done to ensure that there is no effect of high viral load of one virus over the amplification of low copy number of the other virus in multiplex reaction. Sample with high viral load of HSV-1 was mixed with 10 fold dilution of HSV-2 sample starting with 10^7 viral load and it was seen that there was no such effect on HSV-2 detection and reaction was able to pick upto $\geq 10^2$ IU/ml copy numbers of HSV-2. The same experiment was also done for HSV-1 and the results were similar i.e. up to $\geq 10^2$ IU/ml copy numbers were picked up. It was also assured that the detection of both the viruses is not affected by simultaneous detection of Human β-actin gene.

COMPARISON OF UNIPLEX & MULTIPLEX PCR IN CLINICAL SAMPLES

A batch of 165 samples was tested for HSV-1 and HSV-2 by currently designed primer and probes both in uniplex and multiplex. Results are shown in table no. 4. Real time plot of HSV-1, HSV-2 and Human β-actin positive samples using SEQ ID No. 1,2 & 3, 4,5&6 and 7,8&9 respectively in uniplex reaction are shown in figure 4, 5 & 6, red line showing amplification in sample and

green line showing no template control.

Same lot of 165 (serum/plasma/CSF/scraping) samples were tested in multiplex reactions using the same primer and probe (SEQ ID No. 1 to 9) as shown in figure 7,8,9&10. Red line in figure is showing amplification & green line is no template control. Results are shown in table 4. Figure 7 shows test plot of HSV-1 positive and HSV-2 negative sample. Figure 8 shows test plot of HSV-1 negative and HSV-2 positive sample. Figure 9 shows plot of HSV-1 negative and HSV-2 negative samples, while figure 10 is showing plot of sample positive for both HSV-1 and HSV-2.

Total 2 samples tested positive for HSV-1 while none tested positive for HSV-2. Human β-actin gene was amplified in 162 samples in both uniplex and multiplex reaction. Human β-actin gene had mean Cp of 27.57. All 165 samples were tested in a singleplex & multiplex reaction using real time PCR. Results were 100% concordant (table 4).

Table 4: Comparative results of claimed primers and probes in uniplex and multiplex reaction (n=165)

	Uniplex with our newly designed Probes and Primers	Multiplex with our newly designed Probes and Primers
HSV-1 (positives/ number tested)	2/165	2/165
HSV-2 (positives/ number tested)	0/165	0/165
Human β-actin (positives/ number tested)	162/165	162/165

Although the invention herein are described with various specific embodiments, it will be obvious for a person skilled in the art to practice the embodiments herein with modifications. However, all such modifications are deemed to be within the scope of the claims.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the embodiments described herein and all the statements of the scope of the embodiments which as a matter of language might be said to fall there between.

WE CLAIM:

1. Primers and probes for the in-vitro detection of Human HSV-1 virus in a sample comprising nucleotide sequences selected from the group comprising of or any combinations thereof:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:1 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:1, wherein the nucleotide sequence of SEQ ID NO:1 represents forward primer to amplify human HSV-1 virus;

b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:2 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:2, wherein the nucleotide sequence of SEQ ID NO:1 represents reverse primer to amplify human HSV-1; and

c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:3, wherein the nucleotide sequence of SEQ ID NO:3 represents probes to detect human HSV-1;

wherein the nucleotide sequences enables high detection of all genotypes of Human HSV-1. Wherein said nucleotide sequences enables detection of virus present in low copy number upto $\geq 10^2$ IU/ml.

2. The primers and probes as claimed in claim 1, wherein the nucleotide sequences enables detection of virus present in low copy number upto $\geq 10^2$ IU/ml.

3. The primers and probes as claimed in claim 1, wherein the probes and primers are sensitive to detect the sample with the viral load of $\geq 10^2$ IU/ml.

4. The primers and probes for the in-vitro detection of Human herpes virus-2 in a sample comprising:

    a. a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:4 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:4, wherein the nucleotide sequence of SEQ ID NO:4 represents forward primer to amplify human HSV-2 virus;
    b. a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:5 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:5, wherein the nucleotide sequence of SEQ ID NO:5 represents reverse primer to amplify human HSV-2 virus; and
    c. a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:6 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:6, wherein the nucleotide sequence of SEQ ID NO:6 represents probes for the detection of human HSV-2 virus;

    wherein the said nucleotide sequences enables high detection of different genotypes of human HSV-2 virus.

5. The primers and probes as claimed in claim 4, wherein the nucleotide sequences enables detection of virus present in low copy number upto $\geq 10^2$ IU/ml.

6. The primers and probes as claimed in claim 4, wherein the probes and primers are sensitive to detect the sample with the viral load of $\geq 10^2$ IU/ml.

7. A reaction mixture for multiplex real time PCR for the simultaneous detection of human herpes virus 1 and 2 comprising:

   nuclease free water present in an amount of 4.2 μl;

   buffer present in an amount ranging from 12.5μl;

   HSV-1 Primer Forward (10pm) present in an amount ranging from 0.35μl;

   HSV-1 Primer Reverse (10pm) present in an amount ranging from 0.35 μl;

   HSV-2 Primer Forward (10pm) present in an amount ranging from 0.35 μl;

   HSV-2 Primer Reverse (10pm) present in an amount ranging from 0.35 μl;

   β-actin Primer Forward (10pm) present in an amount ranging from 0.20 μl;

   β-actin Primer Reverse (10pm) present in an amount ranging from 0.20 μl;

   HSV-1 Probe (10pm) present in an amount ranging from 0.175 μl;

   HSV-2 Probe (10pm) present in an amount ranging from 0.175 μl;

   β-actin Probe (10pm) present in an amount ranging from 0.15 μl;

   enzyme present in an amount ranging from 1 μl; and

   template present in an amount ranging from 5 μl;

wherein the reaction mixture enables equal intensity detection of all genotypes of human herpes virus-1&2.

8. The reaction mixture as claimed in claim 6, wherein HSV-1 Probe comprises of FAM as a reporter and IABkFQ as a quencher at 3'end.

9. The reaction mixture as claimed in claim 6, wherein HSV-2 Probe comprises Cy5 as a reporter and IAbRQSp as a quencher at 3'end.

10. The reaction mixture as claimed in claims 6-9, wherein β-actin Probe comprises HEX as a reporter and ZEN and IABkFQ as quencher at 3'end.

11. The primers and probes as claimed in claim 1, wherein the probes and primers are designed from highly conserved region of the HSV-1 genome.

12. The primers and probes as claimed in claim 4, wherein the probes and the primers are designed from the glycoprotein β gene region of the HSV-2 genome.

13. A test kit based on multiplex real time PCR for the simultaneous detection and of Human herpes virus- 1&2, the kit comprises nucleotide sequences selected from the group comprising of or any combinations thereof:

a) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:1 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:1;
b) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:2 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:2;
c) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:3 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:3;
d) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:4 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:4;

e) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:5 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:5;

f) a nucleic acid molecule that encodes a nucleotide sequence of SEQ ID NO:6 or a part of it or a nucleotide having minimum 90% sequence identity with said SEQ ID NO:6;

g) an internal control gene;

wherein said nucleotide sequences enable high detection of all genotypes of Human herpes virus-1&2.

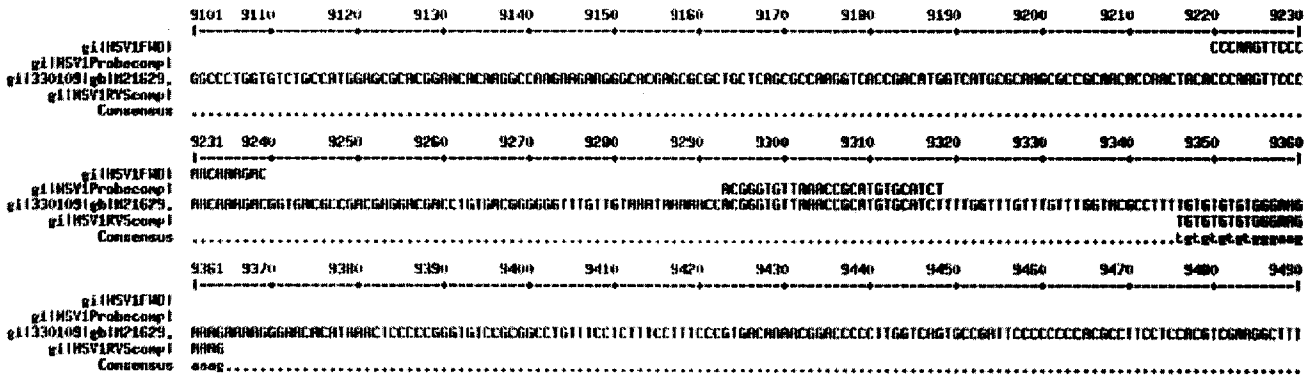

Figure : 1

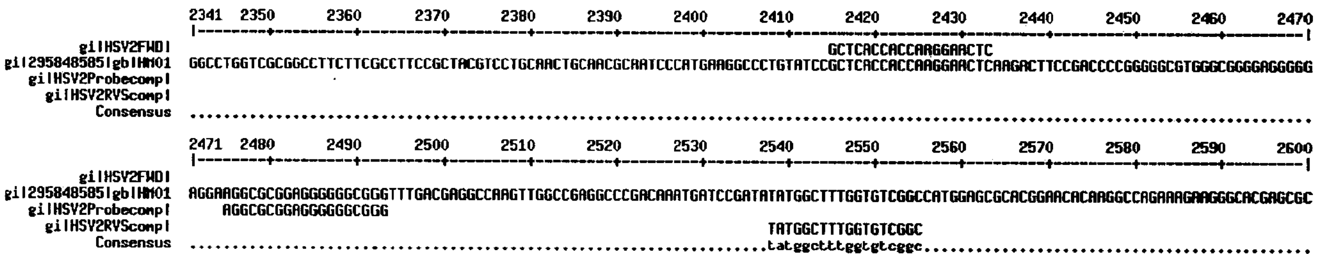

Figure : 2

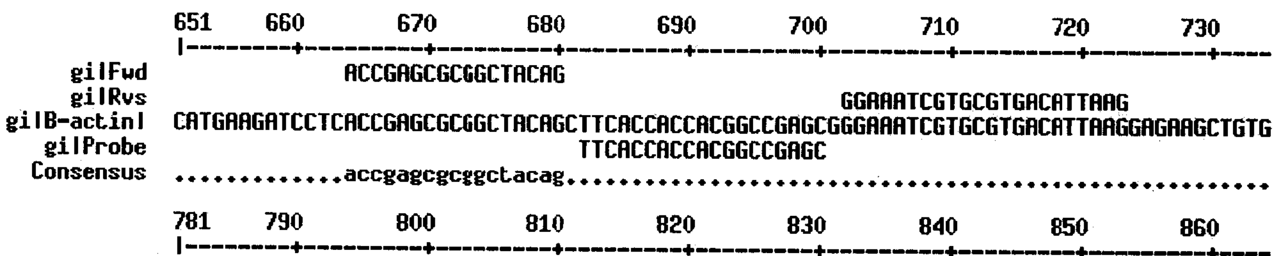

Figure : 3

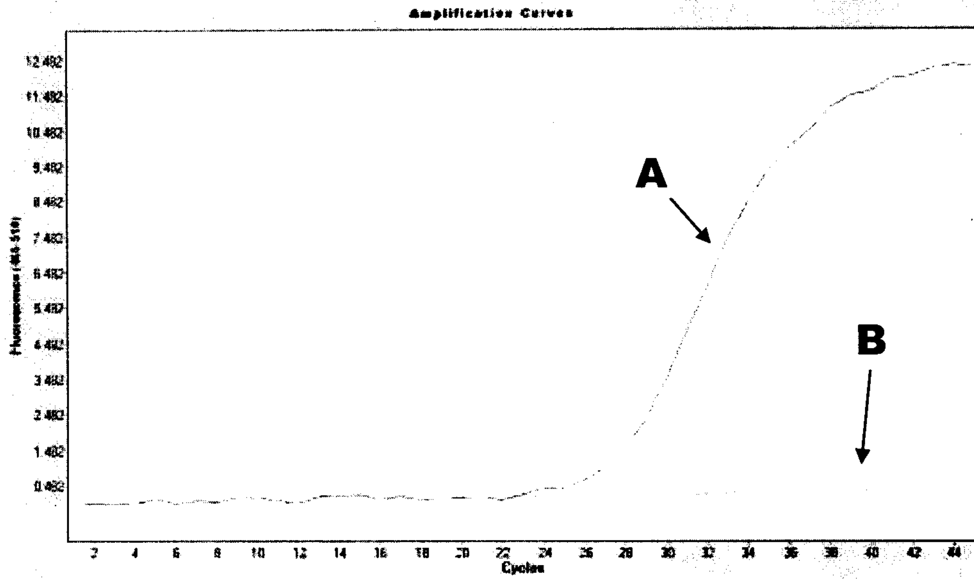

Figure : 4

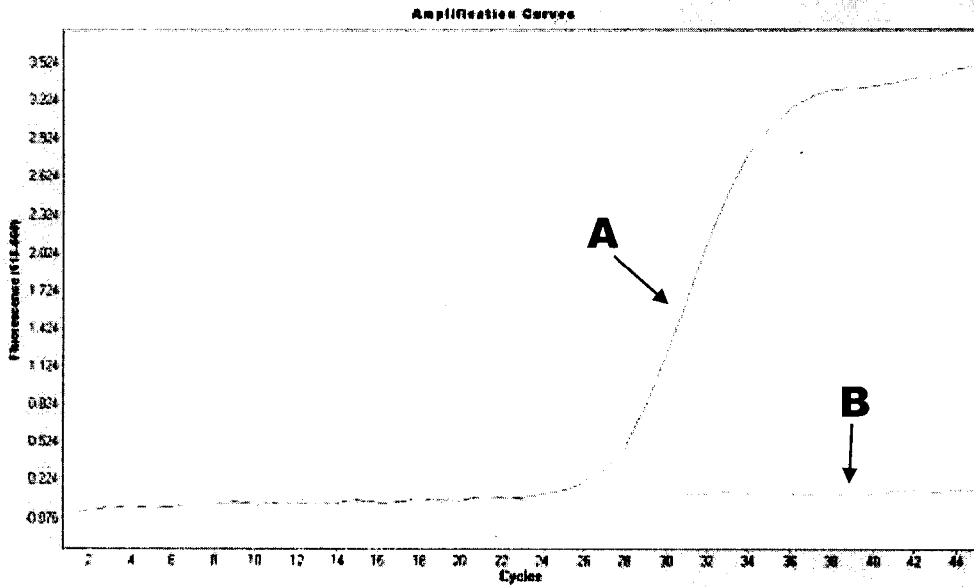

Figure : 5

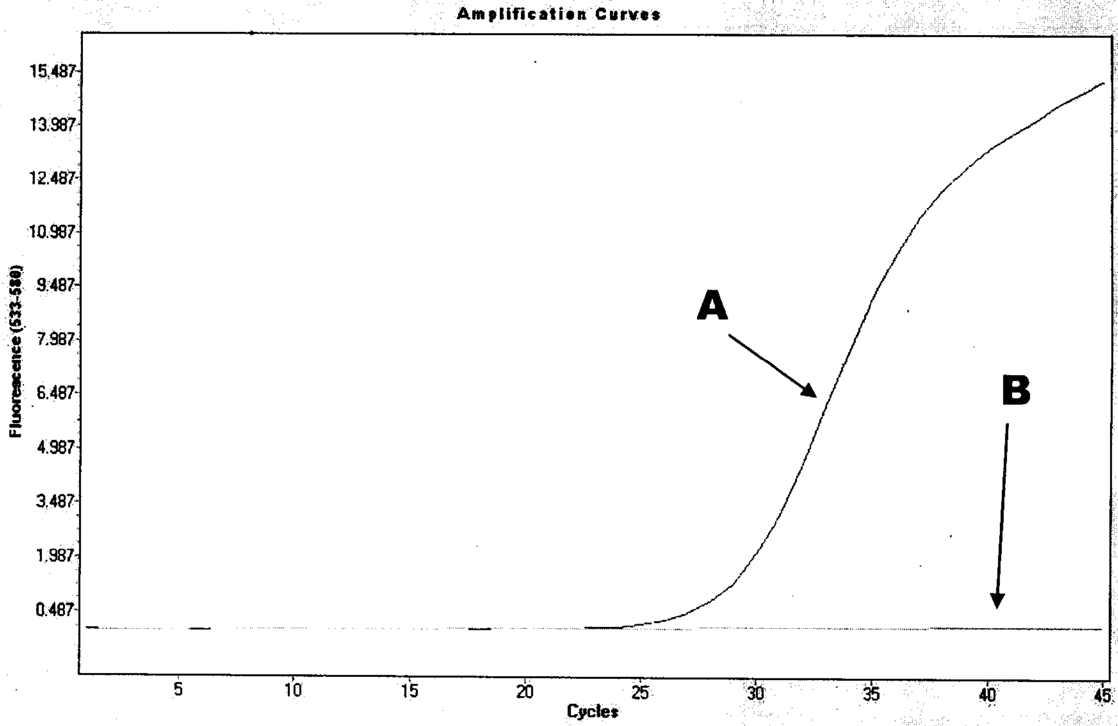

Figure : 6

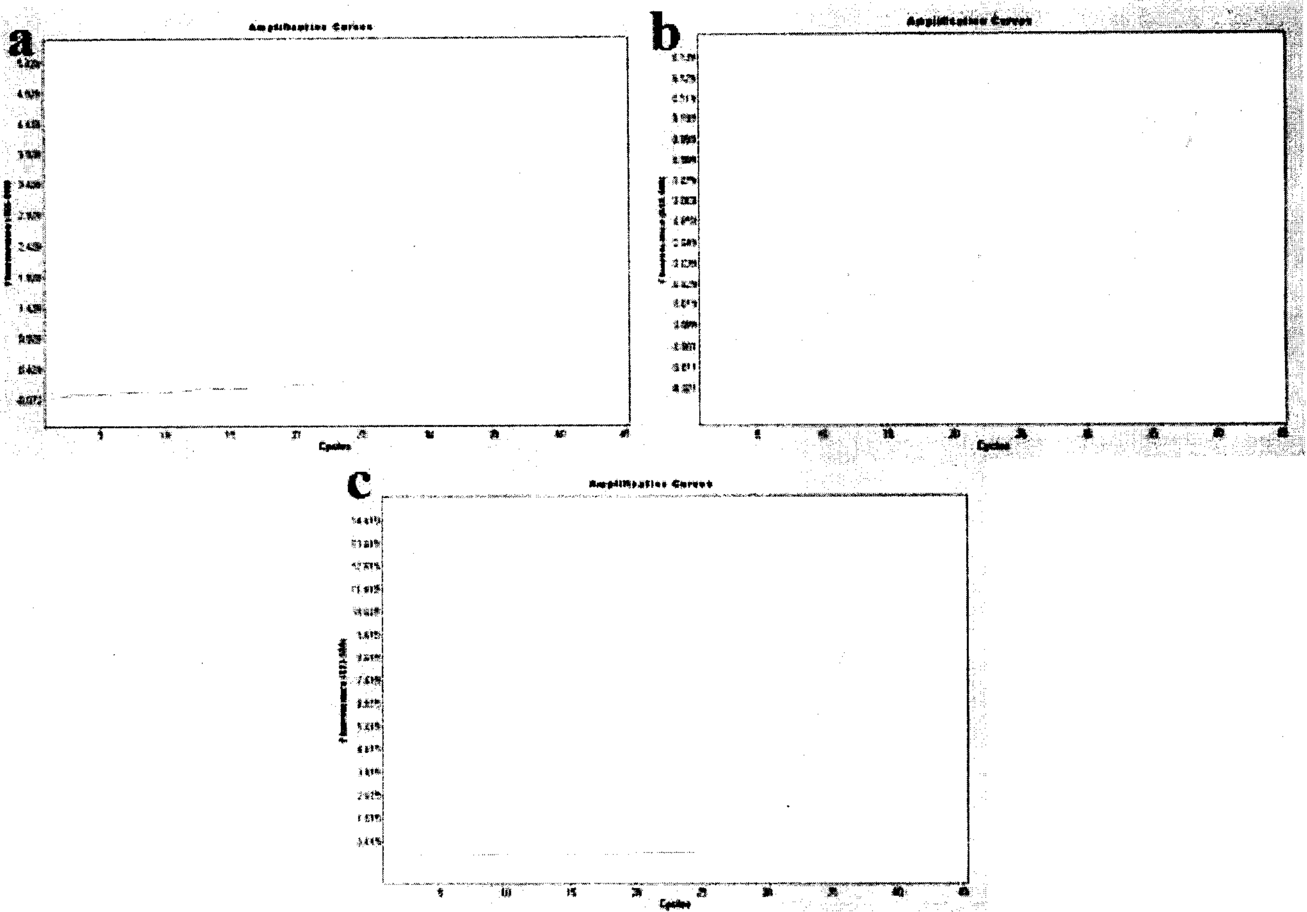

Figure : 7

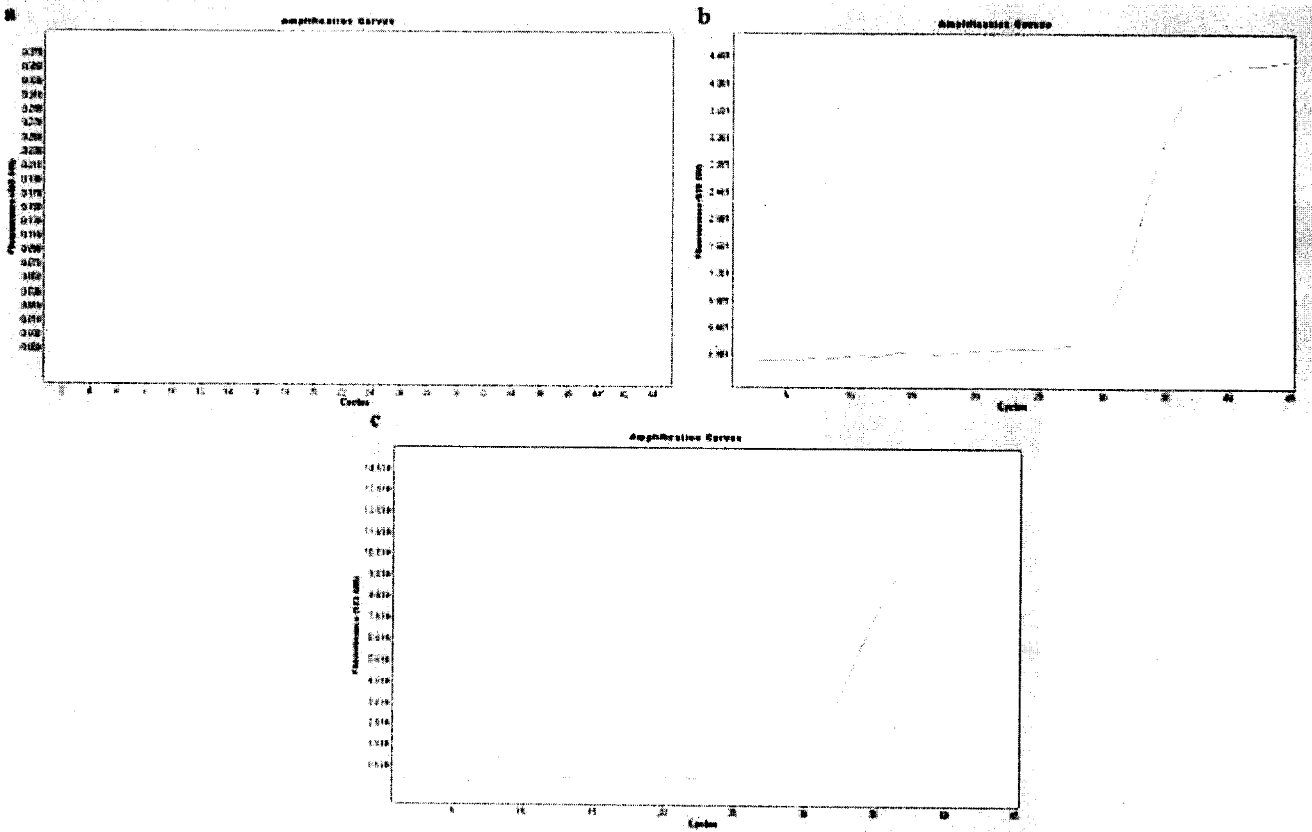

Figure : 8

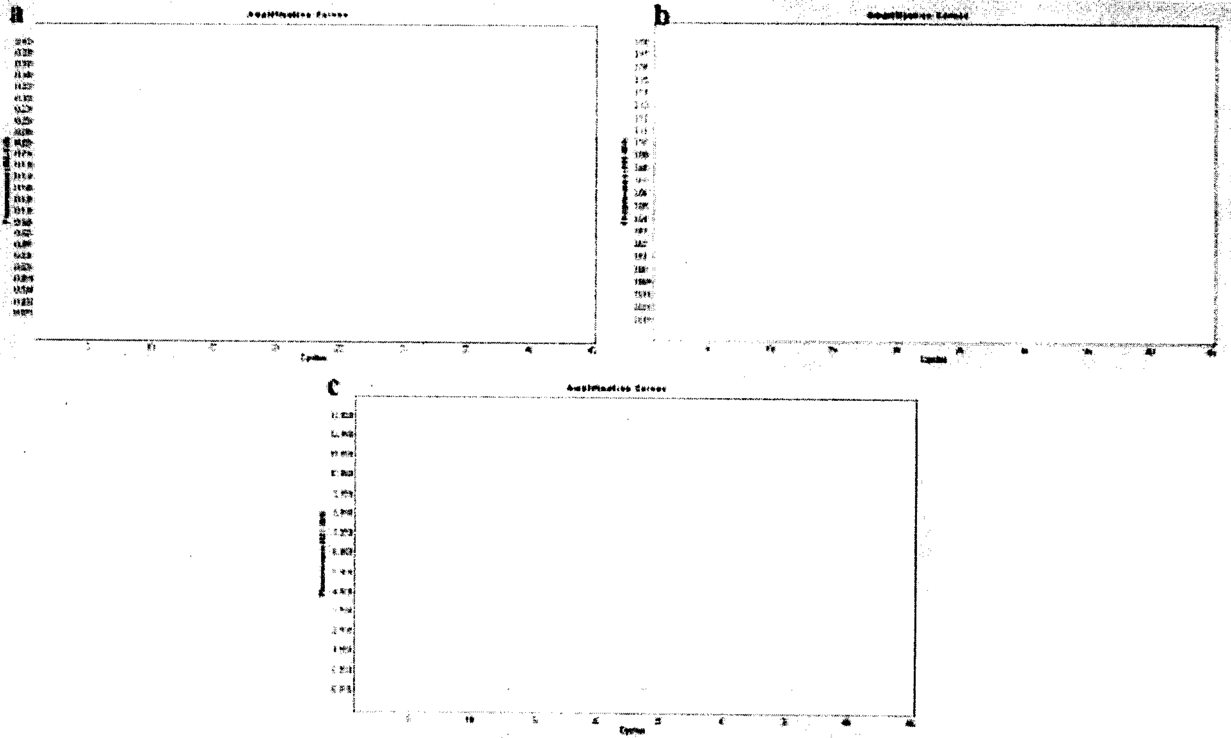

Figure : 9

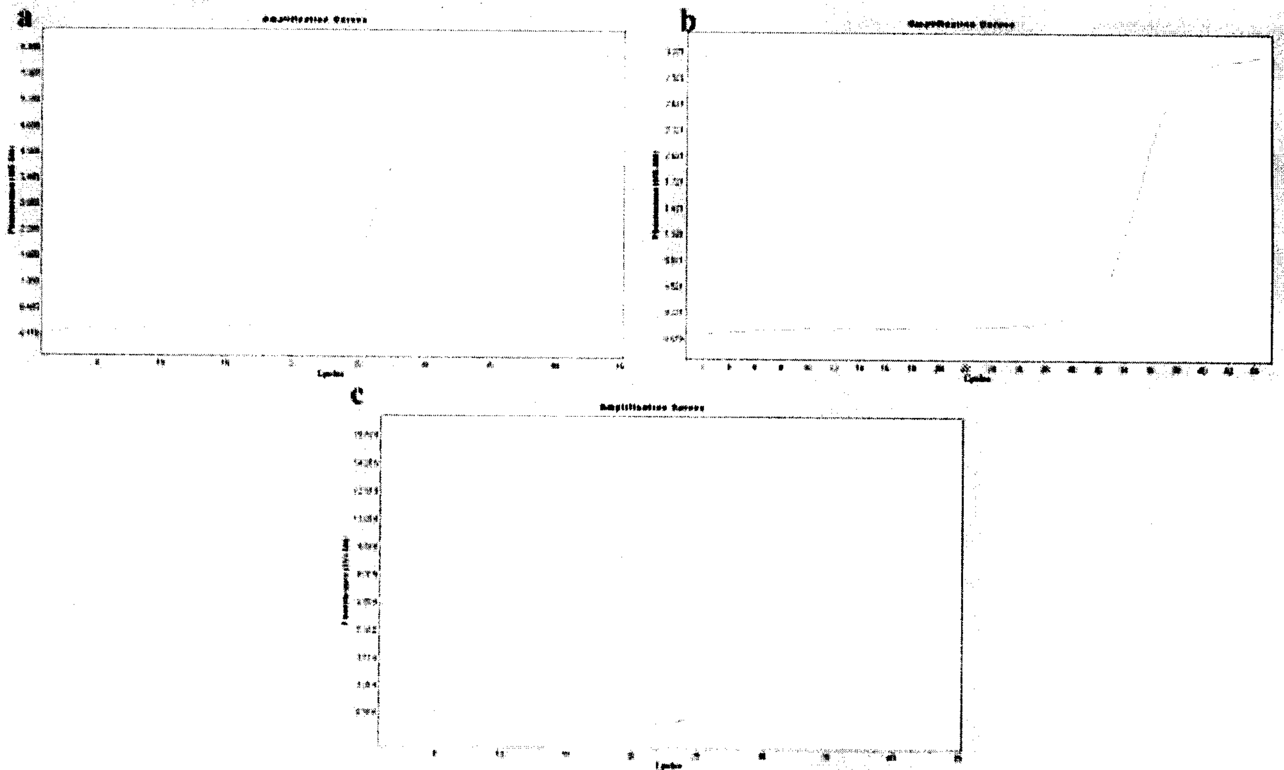

Figure : 10