UNIVERSITY OF CALIFORNIA SAN DIEGO

# Design and Optimization of a Probe-Free PCR Assay for Human Herpes Viruses

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by

Anshu Gupta

Committee in charge:

Professor Stephanie I. Fraley, Chair
Professor Shelley M. Lawrence
Professor Nicole F. Steinmetz
Professor Kun Zhang

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The thesis of Anshu Gupta is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| Congenital cytomegalovirus infection…….. | cCMV |
| Cycle threshold…….. | Ct |
| Deoxyribonucleotide triphosphate……. | dNTP |
| Digital polymerase chain reaction……. | dPCR |
| High resolution melt…….. | HRM |
| Human herpes simplex virus 1…….. | HSV1 |
| Human herpes simplex virus 2……... | HSV2 |
| Human cytomegalovirus…… | HCMV |
| Limit of detection…….. | LOD |
| Limit of quantification……... | LOQ |
| Neonatal herpes simplex virus infection…… | nHSV |
| No template control……... | NTC |
| Polymerase chain reaction……. | PCR |
| Quantitative polymerase chain reaction……. | qPCR |

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ABSTRACT OF THE THESIS

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by

Anshu Gupta

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Professor Stephanie I. Fraley, Chair

Human cytomegalovirus and Herpes simplex viruses 1 and 2 are double stranded DNA viruses that establish lifetime latency in a host upon initial infection[1–3]. Primary infection or reactivation of the latent virus in a pregnant woman can transmit the virus *in utero* or during birth to the fetus causing high neonatal morbidity[4,5]. Majority of these infections are asymptomatic at birth, but may present later with potential lethal infection and disseminated disease (HSV) or long term neurodevelopmental sequelae including sensorineural hearing loss (HCMV)[1,4,5]. The infection can, however, be symptomatic where it may present as non-specific neurologic conditions for HCMV or sepsis-like condition for HSV or severe HCMV cases[4,6], which may not result in the physician ordering immediate testing for these viruses[4].

An early detection of HCMV/HSV is thus necessary to decrease morbidity for asymptomatic infections. Their specific detection against other disease-causing agents is also required to inform correct patient treatment for non-specific symptoms or polymicrobial infections. An ideal test should be rapid to aid in clinical decision-making and broad based to include these viruses with other common neonatal infectious agents with similar symptoms and for which tests are commonly ordered. This work discusses the development of a rapid quantitative PCR based viral assay for HSV and HCMV that can be multiplexed with sepsis or other infectious disease panels with its probe-free chemistry, in order to allow early and specific detection of these viruses in neonates.

# Design and Optimization of a Probe-Free PCR Assay for Human Herpes Viruses

## 1. Introduction

### 1.1.Human cytomegalovirus

Human cytomegalovirus is the most common cause of congenital viral infection in humans and the leading non-genetic cause of neurodevelopmental sequelae in children including intellectual disability, cerebral palsy, seizures, visual defects and sensorineural hearing loss[4,7–13]. HCMV seroprevalence among women of reproductive age is high, ranging from 45-100% worldwide[2]. In the United States, an estimated 58.3% of pregnant women harbor the virus[14], with an additional 27,000 new CMV infections reported in pregnant women each year[15]. The infection is usually asymptomatic but active viral replication in pregnant women, resulting from new (primary) infections or reinfection/ reactivation of latent virus (non-primary infection) can lead to congenital CMV infection or cCMV in the fetus. While the risk for cCMV from non-primary infections is <3%, it can range from 30-70% for primary infection[2]. This leads to one in every 200 babies born in the US to have cCMV, with around 30,000 annual cases[16].

Only 10-20% of infants who acquire cCMV from primary maternal infection will be symptomatic[1], more than half of which will develop long term neurodevelopmental challenges[16]. These symptoms are not specific to HCMV and a diagnostic test for CMV may not be ordered upon their presentation[4], which could either lead to false diagnosis and an ineffective treatment or a high healthcare cost to conduct multiple tests for identifying the actual cause. More than 90% of infants with cCMV, however, are asymptomatic, 10%

of which still develop late onset health conditions, including sensorineural hearing loss (SNHL)[1]. More than half of these cases that lead to SNHL are progressive in nature and are missed by hearing screening at the time of birth[13]. cCMV infections also need to be diagnosed within the first three weeks life, after which, congenital infections cannot be distinguished from postnatally acquired infections[17].

Rapid, inexpensive, specific and early detection of cCMV is thus required to manage disease progression and inform early intervention. First, earlier CMV diagnosis can help initiate timely therapy and prevent/decrease the development of sequelae, particularly in case of asymptomatic and late onset conditions. Second, cCMV screening would help to monitor future hearing loss and provide timely intervention, which has been known to improve receptive and expressive language skills[4]. Third, early diagnosis would incur significant cost savings by avoiding extraneous diagnostic tests or therapies in the quest to find the actual source of CMV caused neurodevelopmental impairments[4]. Fourth, a precise and rapid CMV diagnostic test would also aid in antiviral clinical trials by identifying the neonates that may benefit from this therapy.

### 1.2.Herpes simplex virus

There are two types of herpes simplex viruses. While HSV1 causes oral, labial and facial lesions, HSV2 has been the traditionally known cause of genital herpes. Recent studies, however, have shown that HSV1 also causes genital herpes in 20-50% cases[5,18]. HSV, like HCMV, establishes latency in the host with a predilection for neurons of dorsal root ganglia and autonomic nervous system[3].

Seroprevalence of HSV infections in women of ages 15-49 in the US is 50.9% for HSV1 and 15.9% for HSV2[19]. Two in three women are either asymptomatic or show ambiguous symptoms which makes it difficult to diagnose and treat the infection[20,21]. HSV can be transmitted from a pregnant woman to the neonate trans-placentally *in utero* in 5% cases, through contact with infected genital secretions peripartum in 85% cases or postnatally in 10% infections[22]. Like CMV, transmission risk is higher for primary infections at 57% as compared to 25% for non-primary infections and 2% for recurring infections[21]. Unlike HCMV, however, HSV transmission does not occur via ingestion of breast milk[23].

Neonatal HSV infection (nHSV) is less common than cCMV, with occurrence in every 1 in 3200 births annually in the US, or around 1500 nHSV cases every year[21,23]. nHSV may present as skin-eye-mouth disease (SEM; 45%) such as keratitis and conjunctivitis, disseminated disease (25%) or neurologic disease (30%), including meningitis and encephalitis[23,24]. Without therapy, the mortality rate associated with disseminated disease is 85% and 50% for central nervous system (CNS) disease which reduces to 31% and 6% respectively with antiviral therapy[25]. This underscores the need for early diagnosis and timely therapy for nHSV. HSV is also the major cause of viral sepsis and has many common non-specific symptoms with other sepsis causing pathogens. It may, therefore, be difficult to differentiate HSV from more common pathogens that cause neonatal sepsis, particularly in the absence of skin lesions[26]. The gold standard in sepsis detection is blood culture[27], which cannot detect HSV and other viral agents. A viral detection technology, multiplexed with bacterial and fungal pathogens that cause sepsis, is thus required for correct detection and treatment.

An early and rapid test for HSV is therefore necessary for the following reasons. First, it will allow early diagnosis and intervention of otherwise asymptomatic infections in neonates. Second, it can help specific detection and correct treatment for neonatal sepsis in case of non-specific symptoms, which may otherwise be misdiagnosed or left undiagnosed. Third, it can be used to rapidly screen laboring women for active HSV infection that will inform the safest mode of delivery. In case of infection, Caesarean delivery could be ordered to reduce peripartum transmission risk through the birth canal.

### 1.3.Innovation

The aim of this work was to develop a probe-free universal real time PCR based assay to detect HCMV and HSV1 and 2 through high resolution melt (HRM) technology. This assay would also have the ability to be multiplexed with other neonatal infectious disease assays, which allows specific detection in a broad-based format, particularly in clinically ambiguous symptomatic cases.

The current gold standard for HCMV and HSV1 and 2 diagnosis is viral culture[28], which does not satisfy the requirements for a rapid test for early neonatal detection as discussed above. These tests have slower turn-around times that makes them unsuitable as a screening tool, have decreased sensitivity[29], higher costs than molecular tests and require larger sample volumes. Multiplexed molecular tests are also used in some laboratories. They usually utilize universal primers or multiple pathogenic primers added together in the same reaction, along with nested PCR (same external but different species-specific internal primers) or sequence specific fluorescent probes to differentiate each species

(specificity)[30]. This limits the number of pathogens that can be detected in a single reaction chemistry due to a cap on the maximum number of different oligonucleotides (primers or probes) that can be used effectively in the same reaction. Some assays split multiple primers/probes across different reactions, leading to a larger sample volume requirement to maintain assay sensitivity.

This work hypothesizes the use of probe-free assays with universal primer chemistry and specificity based on high-resolution melt technology to alleviate the above limitations on broad based pathogenic detection. Instead of sequence specific probes, species level identification is accomplished by high resolution melt signature that uses non-specific fluorescent intercalating dyes. The use of probe free assays would also allow extension of the assay to include more pathogens without redesigning new probes for each new species. Multiplexing universal primers for different pathogen groups, for instance, bacterial, fungal and viral, could further maximize the number of pathogens that could be detected through a single assay by attaining probe free specificity through melt signatures. This would result in a single multiplexed diagnostic panel for different pathogens that cause similar non-specific symptoms.

In this work, a universal probe free quantitative PCR (qPCR) assay was designed to detect HCMV, HSV1 and HSV2 utilizing high-resolution melt technology. The work involves primer design from viral sequences, qPCR assay optimization for the primers and its analytical characterization. The assay helps in improving neonatal disease management though early detection and timely intervention, specifically in cases of asymptomatic infections including sensorineural hearing loss. The inherent rapidity of the qPCR test as compared to viral culture and its higher sensitivity and cost effectiveness also allow it to

be used as a screening tool for maternal viral infections during pregnancy or for universal newborn screening for these viruses.

Proof of concept multiplexing experiments were also performed on the viral assay with bacterial and fungal sepsis assays developed in our lab. Multiplexing the probe free assay with sepsis panel or with other neonatal infectious agents facilitates simultaneous detection of various pathogens. It tackles the current clinical practice of primarily focusing on bacterial etiologies of infection which causes herpesviruses to go undetected or misdiagnosed. This would also reduce the burden on the rapidly evolving antibiotic resistance crises of broad-spectrum antibiotics and would result in significant cost savings by providing a single test for correct pathogen detection in place of separate panels for each infectious etiology.

A future aim for the viral or multiplexed assay would be its translation to our lab's digital molecular technology (digital PCR or dPCR) that would increase its sensitivity to single genome level along with broad based detection, employing the lab's machine learning algorithm to "learn" unique melt signatures of a multitude of amplicons. This would also allow the usage of a smaller sample volume than traditional PCR.

## 2. Materials and Methods

### 2.1.Design of primers

To find universal primers for the three human herpesviruses, 6 gene families whose products are evolutionarily conserved in these viruses were considered (Table 1)[31]. Each gene from the six selected gene families was manually aligned for the three viruses (HCMV, HSV1 and HSV2) using online Benchling alignment tool (http://www.benchling.com) to find all possible primers conserved in the three species. The primers were analyzed for homo-dimerization, hetero-dimerization and hairpin formation *in silico* using online tools: IDT oligo analyzer, Sigma Aldrich OligoEvaluator and PREMIER Biosoft NetPrimer. They were also assessed for specificity against human DNA through Primer BLAST tool to obtain 51 primer pairs, of which one is already found in literature[32] (Table 2). Barring one primer pair from the Glycoprotein B gene, all the primers are from the highly conserved DNA Polymerase gene of the viruses[30]. The primers range in length from 18-30 bases.

**Table 1:** List of Herpesvirus gene families

| |
|---|
| Capsid |
| Tegument and cytoplasmic egress |
| Envelope |
| Regulation |
| DNA Replication, recombination and metabolism |
| Capsid assembly, DNA encapsidation and nuclear egress |

**Table 2:** List of custom designed primers

| No. | Name | Forward Primer | Reverse Primer |
|---|---|---|---|
| 1 | p2 | GACTTTGCCAGCCTGTACC | GTCCGTGTCCCCGTAGATG |
| 2 | p4 | GTGTTCGACTTTGCCAGCC | GCACCAGATCCACGCCCTT |
| 3 | p4p2 | GTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATG |
| 4 | p5 | GAGGGCATCGTGGTGGTCT | TCGTTGTCCTCCCCCAGYTG |

**Table 2 (continued):** List of custom designed primers

| No. | Name | Forward Primer | Reverse Primer |
|---|---|---|---|
| 5 | p6p2 | GACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATG |
| 6 | p6p4 | GACTTTGCCAGCCTGTACCC | GCACCAGATCCACGCCCTT |
| 7 | p4p7 | GTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGA |
| 8 | p6p7 | GACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATGA |
| 9 | p8p14 | GTGTTCGACTTTGCCAGCCTCTACCC | GTCCGTGTCCCCGTAGATGACCCGC |
| 10 | p9p14 | CATCATCCTGGCCCACAACCTSTGC | GTCCGTGTCCCCGTAGATGACCCGC |
| 11 | p10p14 | CGAATACGAGATGCTGCTGGCCTTYATG | GTCCGTGTCCCCGTAGATGACCCGC |
| 12 | p11p14 | CATCTATGACGGACAGCAGATCCG | GTCCGTGTCCCCGTAGATGACCCGC |
| 13 | p12p14 | ACCCCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGACCCGC |
| 14 | p13p14 | CCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGACCCGC |
| 15 | p15p14 | GTGTTCGACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATGACCCGC |
| 16 | p8p16 | GTGTTCGACTTTGCCAGCCTCTACCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 17 | p9p16 | CATCATCCTGGCCCACAACCTSTGC | GTCCGTGTCCCCGTAGATGATCCGC |
| 18 | p10p16 | CGAATACGAGATGCTGCTGGCCTTYATG | GTCCGTGTCCCCGTAGATGATCCGC |
| 19 | p11p16 | CATCTATGACGGACAGCAGATCCG | GTCCGTGTCCCCGTAGATGATCCGC |
| 20 | p12p16 | ACCCCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 21 | p13p16 | CCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 22 | p15p16 | GTGTTCGACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 23 | p2p16 | GACTTTGCCAGCCTGTACC | GTCCGTGTCCCCGTAGATGATCCGC |
| 24 | p4p16 | GTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 25 | p6p16 | GACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATGATCCGC |
| 26 | p8p2 | GTGTTCGACTTTGCCAGCCTCTACCC | GTCCGTGTCCCCGTAGATG |
| 27 | p8p4 | GTGTTCGACTTTGCCAGCCTCTACCC | GCACCAGATCCACGCCCTT |
| 28 | p8p7 | GTGTTCGACTTTGCCAGCCTCTACCC | GTCCGTGTCCCCGTAGATGA |
| 29 | p9p2 | CATCATCCTGGCCCACAACCTSTGC | GTCCGTGTCCCCGTAGATG |
| 30 | p9p4 | CATCATCCTGGCCCACAACCTSTGC | GCACCAGATCCACGCCCTT |
| 31 | p9p7 | CATCATCCTGGCCCACAACCTSTGC | GTCCGTGTCCCCGTAGATGA |
| 32 | p10p2 | CGAATACGAGATGCTGCTGGCCTTYATG | GTCCGTGTCCCCGTAGATG |
| 33 | p10p4 | CGAATACGAGATGCTGCTGGCCTTYATG | GCACCAGATCCACGCCCTT |
| 34 | p10p7 | CGAATACGAGATGCTGCTGGCCTTYATG | GTCCGTGTCCCCGTAGATGA |
| 35 | p11p2 | CATCTATGACGGACAGCAGATCCG | GTCCGTGTCCCCGTAGATG |
| 36 | p11p4 | CATCTATGACGGACAGCAGATCCG | GCACCAGATCCACGCCCTT |
| 37 | p11p7 | CATCTATGACGGACAGCAGATCCG | GTCCGTGTCCCCGTAGATGA |
| 38 | p12p2 | ACCCCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATG |
| 39 | p12p4 | ACCCCGTGGCGGTGTTCGACTTTGCCAGCC | GCACCAGATCCACGCCCTT |
| 40 | p12p7 | ACCCCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGA |
| 41 | p13p2 | CCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATG |
| 42 | p13p4 | CCGTGGCGGTGTTCGACTTTGCCAGCC | GCACCAGATCCACGCCCTT |
| 43 | p13p7 | CCGTGGCGGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGA |
| 44 | p15p2 | GTGTTCGACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATG |
| 45 | p15p4 | GTGTTCGACTTTGCCAGCCTGTACCC | GCACCAGATCCACGCCCTT |

**Table 2 (continued):** List of custom designed primers

| No. | Name | Forward Primer | Reverse Primer |
|---|---|---|---|
| 46 | p15p7 | GTGTTCGACTTTGCCAGCCTGTACCC | GTCCGTGTCCCCGTAGATGA |
| 47 | p19p2 | CCGTGGTCGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATG |
| 48 | p19p4 | CCGTGGTCGTGTTCGACTTTGCCAGCC | GCACCAGATCCACGCCCTT |
| 49 | p19p7 | CCGTGGTCGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGA |
| 50 | p19p14 | CCGTGGTCGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGACCCGC |
| 51 | p19p16 | CCGTGGTCGTGTTCGACTTTGCCAGCC | GTCCGTGTCCCCGTAGATGATCCGC |

## 2.2.Human and viral DNA

Human genomic DNA for specificity experiments was extracted from frozen human cord blood samples obtained from Rady Children's Hospital. The frozen blood was thawed and DNA extracted in multiple aliquots using Promega Wizard Genomic DNA Purification kit using the vendor recommended protocol for whole blood to obtain an average of 160µg/mL DNA in PCR purified water. Extracted viral DNA was obtained from ATCC with the following product details: HCMV strain AD-169- ATCC VR-538D, HSV1 strain KOS- ATCC VR-1493D and HSV2 strain G- ATCC VR-734.

## 2.3.Testing of primer specificity against human DNA

All 51 primer pairs were tested against human DNA amplification. Each primer set was tested in four reactions, two with no template (no template controls or NTC) and two with extracted human genomic DNA as the template. All reaction volumes were 15µL with 1x NEB Q5 buffer, 2.5x EvaGreen, 0.2mM dNTPs, 0.02U/µL of NEB Q5 Polymerase and 0.5µM each of forward and reverse primers. Human DNA was used at a final reaction concentration of 3.2ng/µL. Thermocycling was performed in Thermo Fisher QuantStudio Real-Time PCR System at the following conditions: 1.5 minutes of initial denaturation at $98^0$C, 55x cycles of 10 seconds denaturation at $98^0$C, 30 seconds annealing at varying

temperatures and 30 seconds elongation at $72^0$C, followed by a 5-minute final extension at $72^0$C. Post the amplification cycles, the reactions were heated to $95^0$C followed by a melting curve temperature gradient from $60^0$C to $99.9^0$C. The annealing temperatures were estimated from NEB Tm calculator (tmcalculator.neb.com) to be $69^0$C for primer sets 1-8, $72^0$C for primer sets 9-22 and 47-51 and $70^0$C for primer sets 23-46.

Amplification and melt curves for each primer pair were analyzed and gel electrophoreses performed to find primers that do not amplify human DNA. The selected primer pairs were then re-subjected to PCR in 12 reactions- 2 as NTCs and 10 with human DNA as the template. Primer sets that did not amplify human DNA were selected for further testing.

## 2.4.Real time PCR assay development for herpes viruses

Selected primer sets that did not amplify human DNA were tested for HCMV and HSV1 amplification in duplicates with 2 more reactions as NTC. The thermocycling conditions for amplification and melting are the same as followed previously. Analysis was performed on their melt and amplification curves and gel electrophoresis. The primer set showing specific amplification of the two viruses were chosen and retested in qPCR in replicates of 6 for each virus. HSV2 testing was conducted after the designed and selected primer assay was optimized, since HSV2 genome is similar to HSV1.

## 2.5.Optimization of assay with the selected primer pair

The selected primer assay was optimized for various parameters, including the DNA Polymerase, primer annealing temperature, primer concentration, amplification

thermocycling temperature ramp rates and extension times. All the optimization reactions were conducted with HCMV and HSV1 DNA.

For comparing the DNA Polymerase enzymes, 10X serial dilutions of HCMV and HSV1 ranging from 10^5 to 10^2 genome copies/reaction were amplified in triplicates each with NEB Phusion Hot Start DNA Polymerase and NEB Q5 High Fidelity DNA Polymerase. The reaction conditions were as previously stated with an annealing temperature of 70$^{o}$C for both Polymerases.

For annealing temperature optimization, 10X serial dilutions of HCMV and HSV1 ranging from 10^4 to 10^2 genome copies/reaction each were amplified in duplicates using the Polymerase selected from the previous step. The experiment was carried out in Bio-Rad CFX96 qPCR instrument for 6 different temperatures ranging from 71.7$^{o}$C to 67.4$^{o}$C as follows: 71.7$^{o}$C, 71.2$^{o}$C, 70.2$^{o}$C, 69.0$^{o}$C, 68.0$^{o}$C and 67.4$^{o}$C.

Primer concentration experiments were performed with HSV1 by designing a matrix of different primer concentrations ranging from 0.05 to 0.5µM/reaction for forward and reverse primers. Both asymmetric as well as symmetric primer concentrations were tested. Ramp rates and extension time were also optimized for maximum sensitivity.

## 2.6. Analytical characterization of the assay to assess the limit of quantification, dynamic range and reproducibility

Using the selected primer pair, standard curves were created for eight 10X serial dilutions in triplicates for HCMV and HSV1: 10^7 to 10^0 genome copies/reaction and seven 10x serial dilutions for HSV2: from 10^6 to 10^0 genome copies/reaction. The qPCR standard

curves were used to find the linear dynamic range, limit of quantification (LOQ), Ct corresponding to LOQ and the reaction efficiency. The reproducibility of the reaction was found by inter and intra reaction variability.

## 2.7. Multiplexing viral assay with bacterial and fungal assays

Initial experiments were performed as three singleplex reactions: bacterial primers with *E. coli*, fungal primers with a species of *Candida* and an old viral primer (p5) with HCMV. The bacterial and fungal primers and DNA were obtained from our lab. The three primer sets for bacteria, fungus and virus were then multiplexed together to perform 4 reactions: first as NTC and then with bacterial, fungal and viral DNA as templates in each subsequent reaction. These reactions were performed with Phusion DNA Polymerase at an annealing temperature of 64$^{o}$C.

# 3. Results

## 3.1.Design of universal primers

Of the 51 primer pairs designed, only eight were found to be experimentally specific against human genomic DNA and did not amplify it in initial as well as repeat experiments. However, four of these (p12p2, p12p7, p13p2 and p13p14) formed multiple dimers and/or extraneous PCR products, as observed from the multiple melt curve peaks and multiple bands in gel electrophoresis (Figure 1).

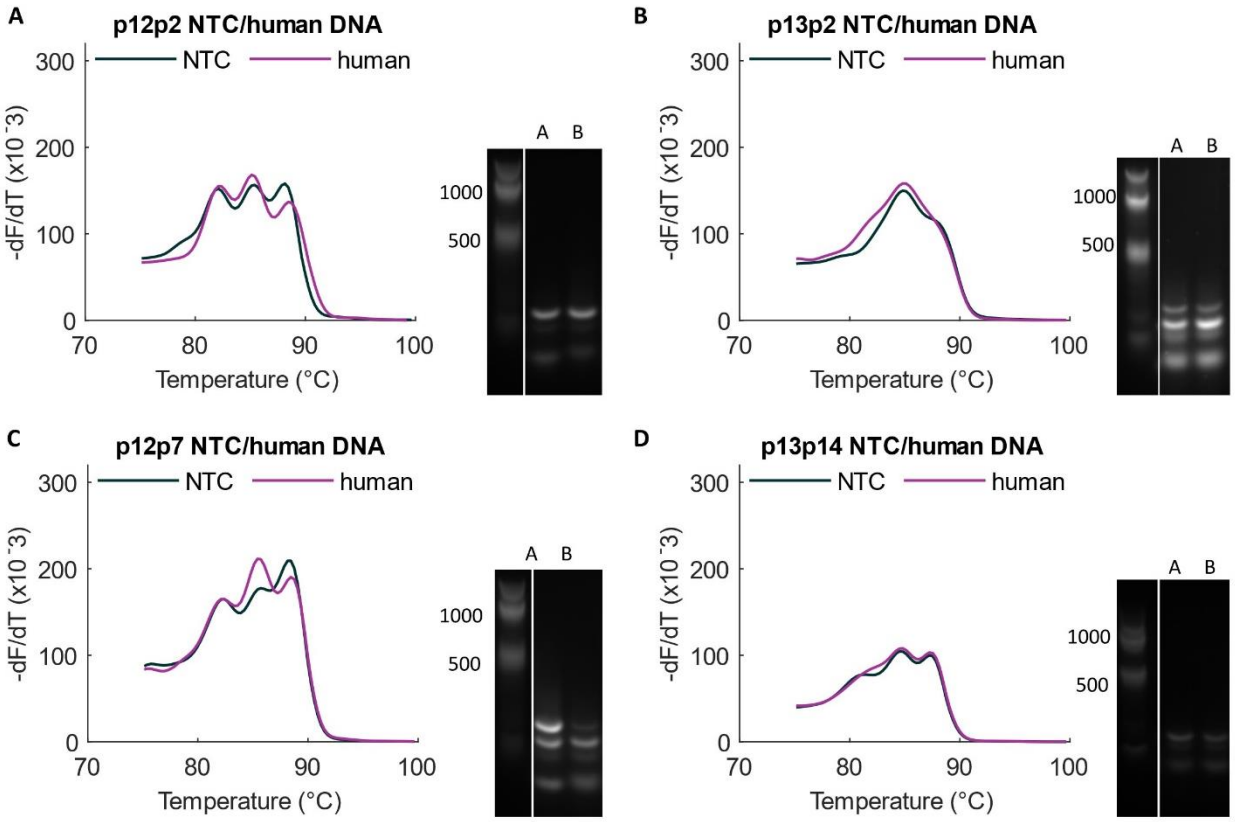


**Figure 1:** Primer dimer formation by custom primers. (A to D) Melt curves and gels showing formation of multiple primer dimers by p12p2, p13p2, p12p7 and p13p14, respectively. Columns A and B in the gels represent NTC and Human DNA as template, respectively.

The remaining four of the eight selected primer pairs were tested with HCMV and HSV1. With three of these (p11p2, p11p7 and p11p16), the forward primer, p11, bound to HSV1 at more than one position giving multiple products, as observed by multiple product peaks in the melt curves and bands on gel electrophoresis.

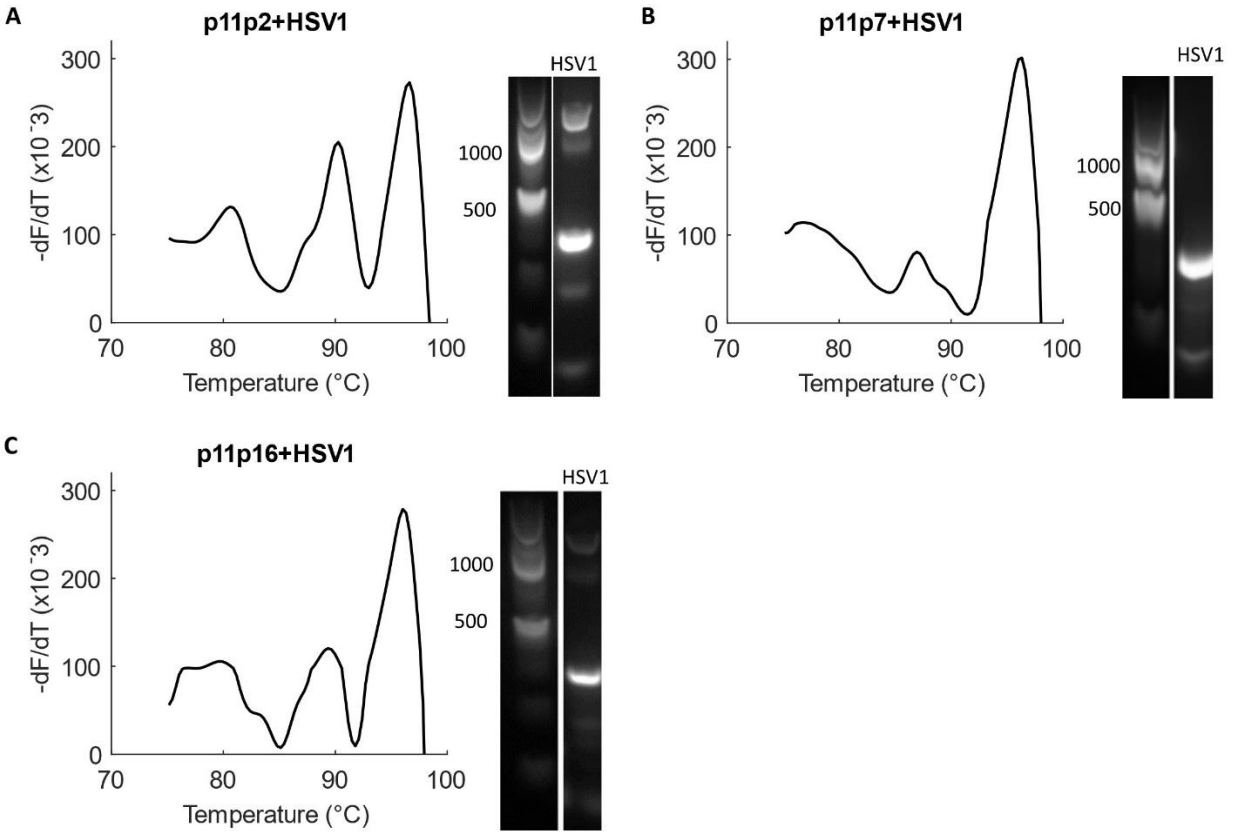


**Figure 2:** Primer p11 forms multiple products with HSV1. (A to C) p11p2, p11p7, p11p16 form multiple products with HSV1 as can be seen from their multiple melt peaks as well as bands in gel electrophoresis. Part of the forward primer, p11, was found to bind at multiple positions to HSV1 giving more than one product. The actual product in all cases was 829 bp long whereas both longer and shorter products are also observed.

The remaining eighth primer set, labelled p19p4, showed specific amplification of all three viruses without amplification of human DNA (Figure 3). The probe-free specificity can be observed as distinct melting signatures for the three viral amplicons with

different average melting temperatures as follows: 94.33±0.32ºC for HCMV, 95.70±0.28ºC for HSV1 and 96.00±0.14ºC for HSV2.

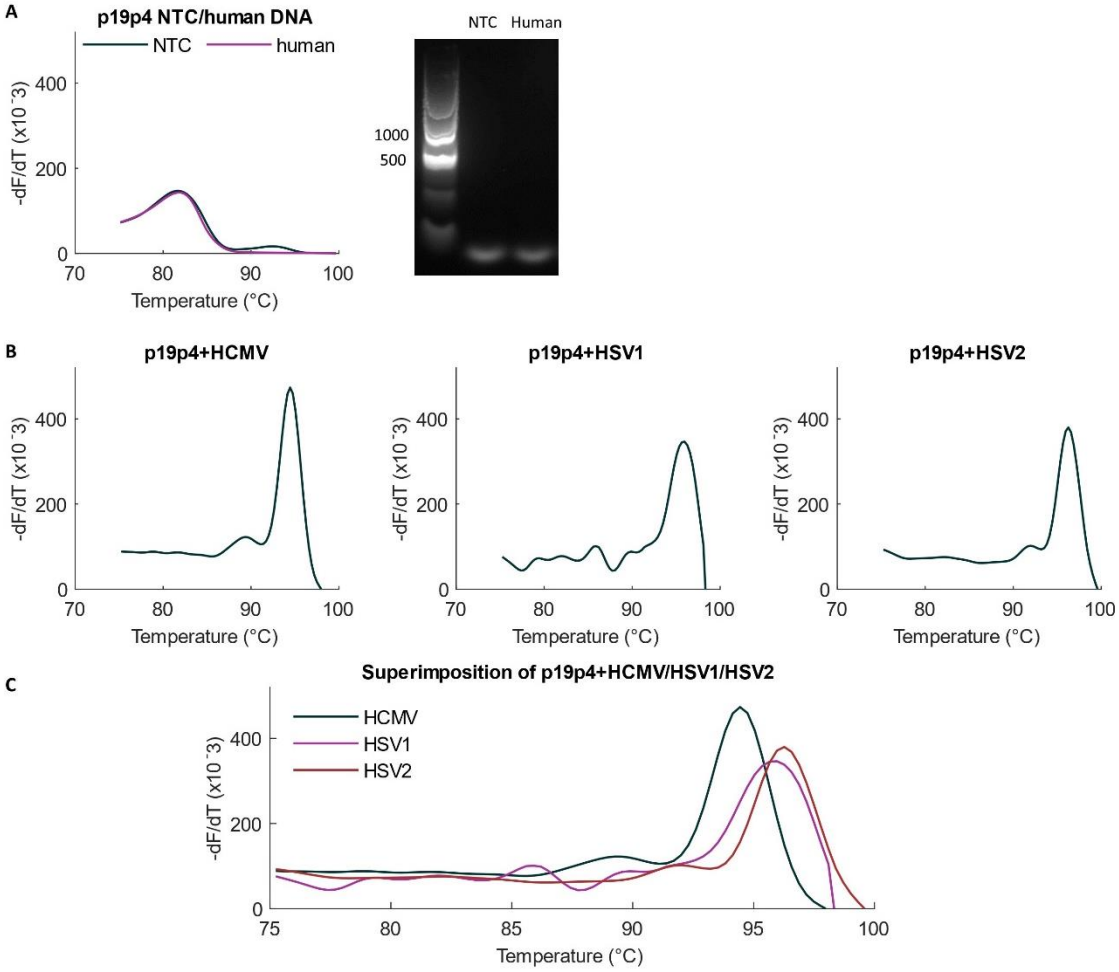


**Figure 3**: Amplification with primer pair p19p4. (A) The primer pair named p19p4 is specific against human DNA amplification as shown by the alignment for melt curves in NTC and human DNA as template. There was no product melt peak observed with human DNA although the primer forms a primer dimer. (B) Melt curves for each virus with the primer pair p19p4. (C) The melt curves for the three viruses are superimposed together to show their distinct signatures.

## 3.2.Optimization of probe free assay chemistry for p19p4

### 3.2.1.Optimizing DNA Polymerase chemistry

Our lab's molecular detection technology chemistry uses NEB Phusion Hot Start DNA Polymerase in the individual bacterial and fungal sepsis assays since they are to be multiplexed together. However, initial HCMV amplification with published primers was not transferable to Phusion chemistry and the results were inconsistent. Other DNA Polymerases, including NEB Luna Universal qPCR Master Mix (with Hot Start Taq DNA Polymerase) and NEB Q5 High Fidelity DNA Polymerase, were therefore tested. Q5 was chosen for further testing due to consistency in results, shorter reactions times, presence of 3'-5' exonuclease activity and 280x fidelity as compared to Luna (www.neb.com).

The initial working assay with custom primer pair p19p4 was then retested for optimization with Q5 and Phusion. The objective for retesting the viral assay with Phusion was to enable future multiplexing with the lab's bacterial and fungal assays, both of which use Phusion chemistry. With Phusion, the lowest concentration at which product melt peaks were observed, or the limit of detection (LOD), was 10^4 viral genome copies/reaction for both HCMV and HSV1. LOD for Q5 was an order lower (better) at 10^3 viral genome copies/reaction for both viruses (HCMV in Figure 4 and HSV1 in Figure A1).

Q5 Polymerase showed better experimental sensitivity for the viral assay and has a higher theoretical fidelity (neb.com) than Phusion. Shifting from Phusion to Q5 chemistry for the bacterial and fungal assays as well could therefore enable multiplexing of the three assays with a better overall sensitivity.

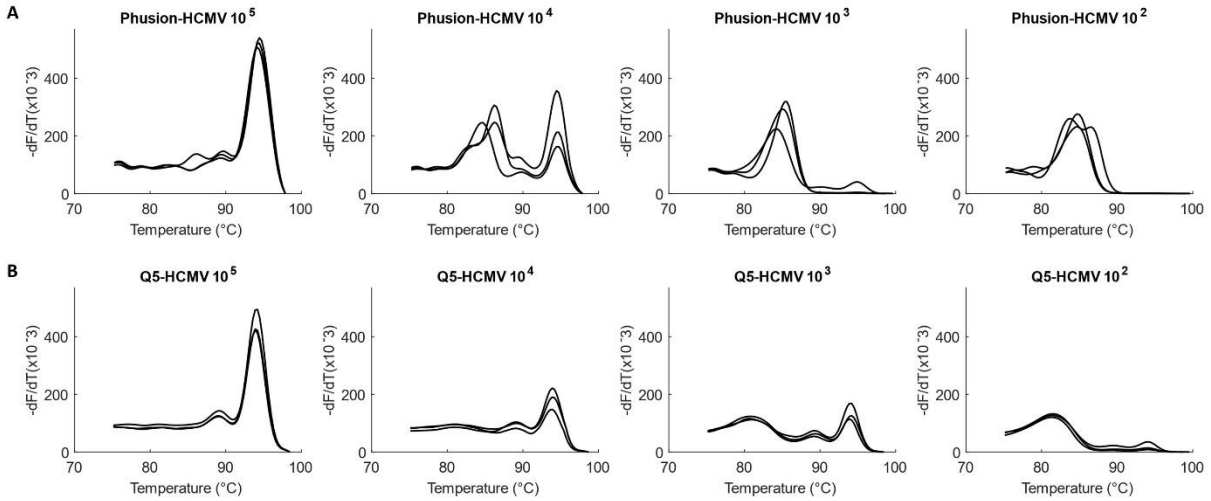


**Figure 4:** Comparison of Phusion vs Q5 DNA Polymerases for amplification of HCMV. Amplification of serially diluted (10^5 to 10^2 copies/reaction) HCMV in triplicates with (A) Phusion- LOD 10^4 copies and with (B) Q5- LOD 10^3 copies.

### 3.2.2.Annealing temperature optimization

A temperature gradient PCR was performed with HCMV and HSV1 to find the optimum annealing temperature. At the highest annealing temperature of 71.7$^{o}$C, amplification was observed at all concentrations of the viruses, including at 10^2 copies/reaction. As the annealing temperature decreased to 67.4$^{o}$C in a gradient, amplification at 10^2 copies/reaction decreased until the melt peak was not observed at all. Simultaneously, at lower temperatures, the occurrence and concentration of primer dimers increased. Thus, 71.7$^{o}$C, approximated to 72$^{o}$C, was taken as the optimized annealing temperature for the assay. Figure 5 shows melt curves for HCMV at the gradient temperatures. Similar results were observed for HSV1, except that the LOD was 10^3 even at the highest annealing temperature (Figure A2).

Another observation was the improvement of HCMV LOD at 70$^{o}$C from 10^3 copies/reaction in section 3.2.1 (Figure 4) to 10^2 copies/reaction in the present

section. The current experiment was performed in Bio-Rad CFX96 qPCR instrument that was found to have a higher temperature ramp rate during thermocycling. The experiment in section 3.2.1, on the other hand, was performed on ThermoFisher Scientific QuantStudio where a lower ramp rate had been utilized. Therefore, in future experiments, a higher ramp rate was employed in the QuantStudio instrument, which improved the HCMV LOD to 10^2 copies.

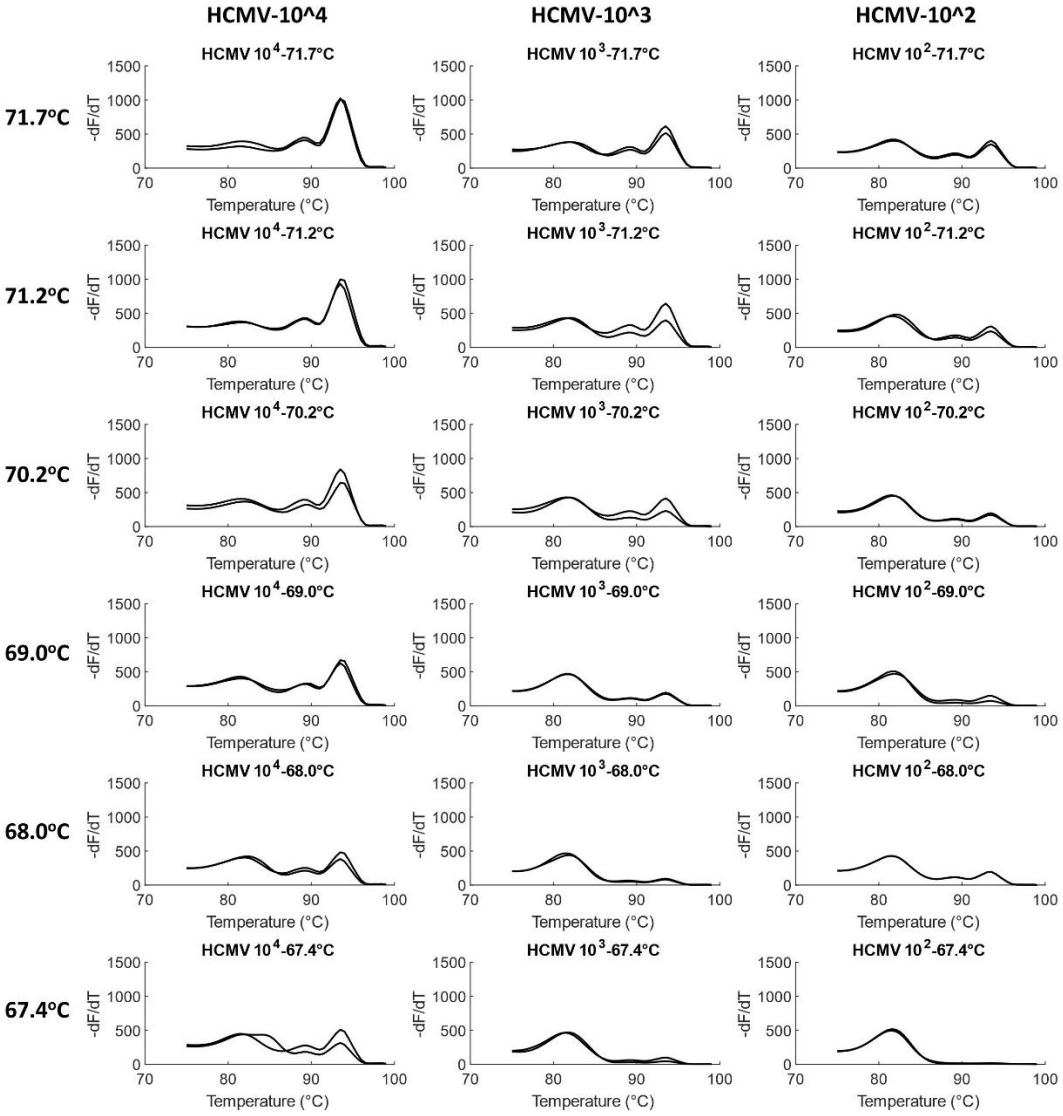


**Figure 5:** Optimization of annealing temperature. Temperature gradient experiments were performed for HCMV in duplicates from 72ºC to 67ºC (rounded off). Product concentration decreases with decrease in temperature, including for the lowest concentration of 10^2 copies/reaction. ~72ºC was thus chosen as the optimum annealing temperature.

### 3.2.3.Primer concentration optimization

Initial standard curve experiments for both HCMV and HSV1 showed the limit of quantification an order higher (poorer) than the limit of detection observed in the previous experiments- for HCMV, LOQ was 10^3 copies/reaction in standard curve while LOD from optimization experiments was 10^2 and for HSV1, LOQ was 10^4 and LOD, 10^3. At concentrations lower than the LOQ, the standard curve showed a long horizontal plateau phase, but the fluorescence did not decrease to zero, as it is expected to in ideal chemistries. This occurs since the primer, p19p4, forms primer dimers even in no template controls (Figure 3A). At template concentrations below LOQ, free and unutilized primers could be forming dimers, resulting in the fluorescence observed in standard curves below LOQ. Primer dimer bands in gel electrophoresis were also observed at a template concentration of 10^5 copies/reaction of virus for 0.5µM of each primer indicating that even at this high template concentration, either some amount of primers was left unutilized or primer dimer formation was completing with product amplification.

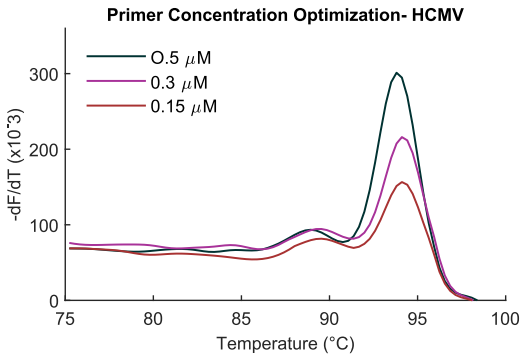


**Figure 6:** Optimizing primer concentration. Decreasing primer concentrations from 0.5 to 0.15µM decreased the amount of product formed.

Therefore, an experiment was designed to test if decreasing primer concentration resulted in decreased primer dimer formation such that the product concentration was unaffected. This would also improve the LOQ of the assay. It was observed that at certain lower concentrations of primers, even though primer dimer formation decreased, so did the amount of product formation (Figure 6). The standard curve LOQ worsened by 2 orders of magnitude at 0.15µM of each primer as compared to 0.5µM. Thus 0.5µM was considered the optimized concentration for both forward and reverse primers for the assay chemistry.

The optimized probe-free viral assay thus has 0.5µM/reaction of p19p4 primer and uses Q5 DNA Polymerase. The optimum thermocycling occurs for 30 seconds annealing at 72$^{o}$C and 30 seconds of extension. The ramp rates were 3$^{o}$C/s for initial denaturation and cycle denaturation stages, 2.5$^{o}$C/s annealing stage, 3$^{o}$C/s for extension stage and final extension after the cycling, and 0.2$^{o}$C/s for the final melting stage. The total run time of the assay is 1 hour 38 minutes, with an additional 10 minutes for initial ramping and final cooling.

A limitation of this optimized assay is the formation of primer dimers even in NTC (Figure 3A), which affects its analytical characteristics, as discussed in Section 3.4.

### 3.2.4. Testing the optimized assay with mock samples

The optimized assay was then validated with mock clinical samples to monitor the effect of common clinical inhibitors on its sensitivity. Although the viral DNA was obtained pre-purified from vendor, blood-extracted human DNA was

added to the reaction to simulate a mock clinical sample; an actual viral extraction from blood would result in a similar presence of human DNA and PCR inhibitors. 10X dilution series of HCMV DNA was created from 10^5 to 10^2 copies/reaction and 10^5 copies/reaction of extracted human DNA were added to each dilution. Each dilution was amplified in duplicates.

The addition of extracted human DNA to the assay did not inhibit the reaction chemistry. The melt curves and gel show similar sensitivity of the HCMV assay as was observed in previous sections (Figure 6). Specificity of the HCMV assay against human DNA is also underscored by this experiment.

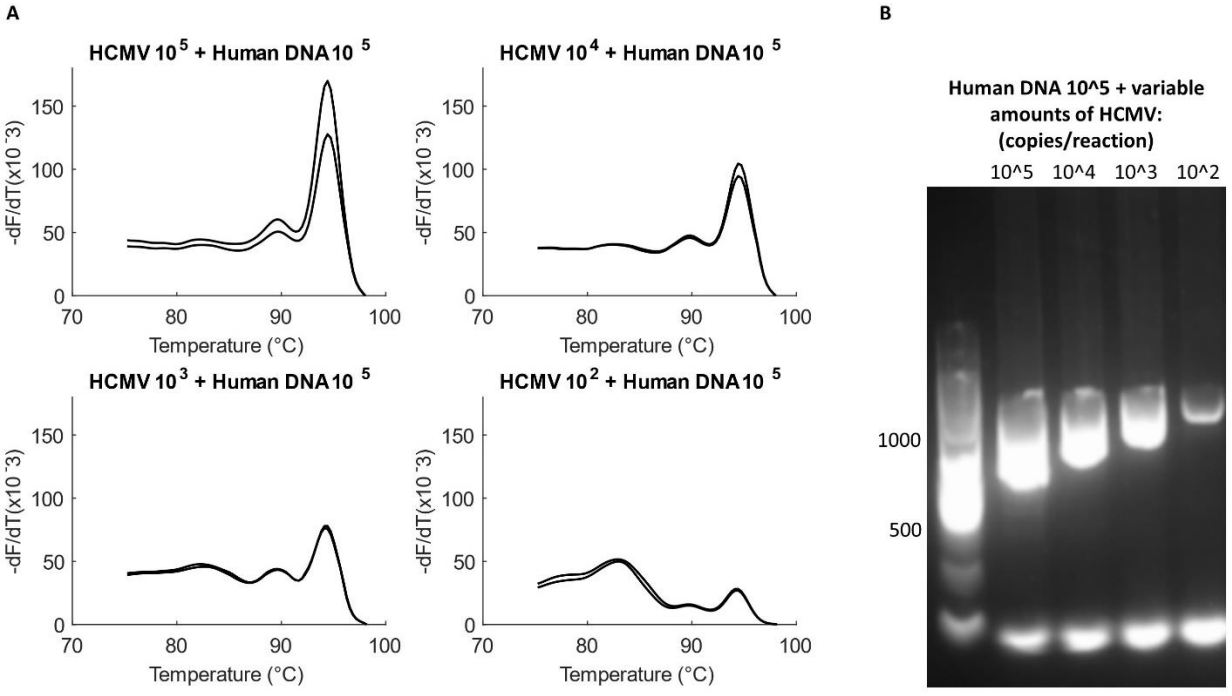


**Figure 7:** Testing the viral assay with mock samples. (A) Serial dilutions of HCMV added with the same amount of human DNA, tested in duplicates. HCMV product formation is uninhibited even at the lowest template concentration of 10^2 copies/reaction. (B) Gel does not show extra bands for amplification of human DNA. Products and primer dimers are both observed at all the tested concentrations of the virus.

### 3.3.Analytical characterization of the viral assay

Standard curves were created for the three viruses for the analytical characterization of the assay. The LOQ for HCMV is 10^3 genome copies/reaction while it is 10^4 copies each for HSV1 and HSV2. Figure 8 shows the standard curves for the three viruses, with the linear dynamic range marked with a linear fitted line and a plateau phase being observed at lower template concentrations. The linear dynamic range of HSV2 is poorer than the other two viruses since higher concentration of the virus showed inconsistent PCR generally attributed to the presence of inhibitors in the sample. The assay may need to be reoptimized to increase PCR performance for HSV2 or a new sample can be ordered to check the validity of the assay. Table 3 shows the characteristics of the assay, including $R^2$ values, efficiency and slope in addition to linear dynamic range and LOQ. The efficiency of the assay ranges from 85% for HSV1, 77% for HCMV to 58% for HSV2. The low efficiency could result due to utilization of longer amplicon chemistry or the formation of primer dimers.

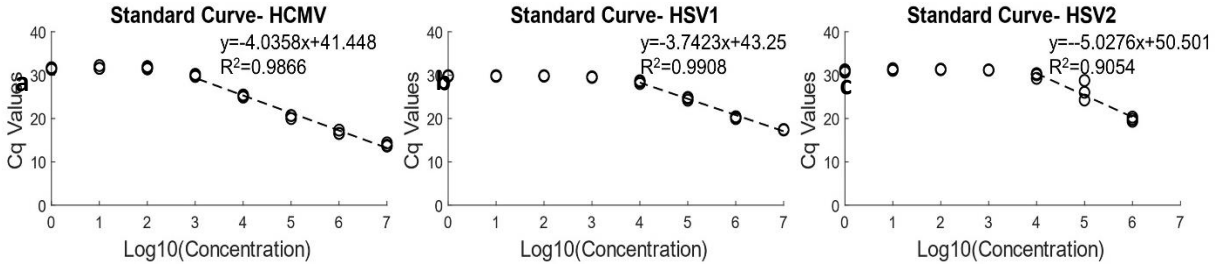


**Figure 8:** Standard curves for HCMV, HSV1 and HSV2. The quantification region (LOQ) is marked with dashed line while the plateau region at lower concentrations is due to primer dimers.

**Table 3:** Characteristics of the viral assay for HCMV, HSV1 and HSV2 amplification

| | Linear Dynamic Range | LOQ (copies/reaction) | $R^2$ | Efficiency (%) | Slope | Y intercept | Mean Cq at LOQ |
|---|---|---|---|---|---|---|---|
| HCMV | 10^7- 10^3 | 10^3 | 0.987 | 76.92 | -4.04 | 41.45 | 30 |
| HSV1 | 10^7- 10^4 | 10^4 | 0.991 | 85.022 | -3.74 | 43.25 | 28 |
| HSV2 | 10^6- 10^4 | 10^4 | 0.905 | 58.089 | -5.03 | 50.50 | 30 |

Intra and inter-assay variation was found with three repeat standard curve experiments each with triplicates. The inter- and intra-assay coefficient of variation for HCMV and HSV1 were low, ranging from 1.01 to 2.82% (intra assay) and from 3.01 to 5.98% (inter-assay). The HSV2 experiments had higher intra- and inter-assay variation (Table 4).

**Table 4:** Inter and intra-assay variation for the three viruses

| | **HCMV** | | **HSV1** | | **HSV2** | |
|---|---|---|---|---|---|---|
| | Mean Ct | %CV | Mean Ct | %CV | Mean Ct | %CV |
| Intra-assay | | | | | | |
| 1x10^6 | 13.95 | 2.63 | 17.41 | 1.17 | 19.84 | 2.46 |
| 1x10^5 | 20.45 | 1.96 | 24.59 | 1.37 | 26.35 | 8.61 |
| 1x10^4 | 25.20 | 1.01 | 28.43 | 1.18 | 29.90 | 1.92 |
| Inter-assay | | | | | | |
| 1x10^6 | 13.46 | 3.12 | 18.57 | 1.64 | 22.38 | 11.69 |
| 1x10^5 | 19.73 | 3.29 | 24.39 | 3.70 | 25.90 | 5.11 |
| 1x10^4 | 24.46 | 3.01 | 29.39 | 3.93 | 29.74 | 1.35 |

### 3.4.Multiplexing viral assay with bacterial and fungal assays

Proof of concept experiments to show multiplexing of the viral assay with our lab's bacterial and fungal sepsis assays were performed. Each singleplex assay has one primer pair with its corresponding DNA. The multiplexed assays have all three primer sets

(bacterial, fungal and viral) with one of the three DNA templates- *E. coli*, *Candida* or HCMV. The superimposition of singleplex and multiplex reactions for each pathogen group shows high degree of similarity of melt curves which concludes the specificity of primer sets for their respective templates, unaffected by multiplexing of the primers.

Melt curves for the three multiplex experiments are also superimposed together to show the distinct melt signature for each pathogen, enabling their identification. The three primers multiplexed together with no template (NTC) show the formation of a non-specific primer dimer, that may be used as an internal control upon further optimization.

The primer used for the viral assay here was p5 which has since been shown to cause human amplification. Multiplexing the custom primer pair p19p4 has not been tested which could solve the problem of human amplification and provide more specificity.

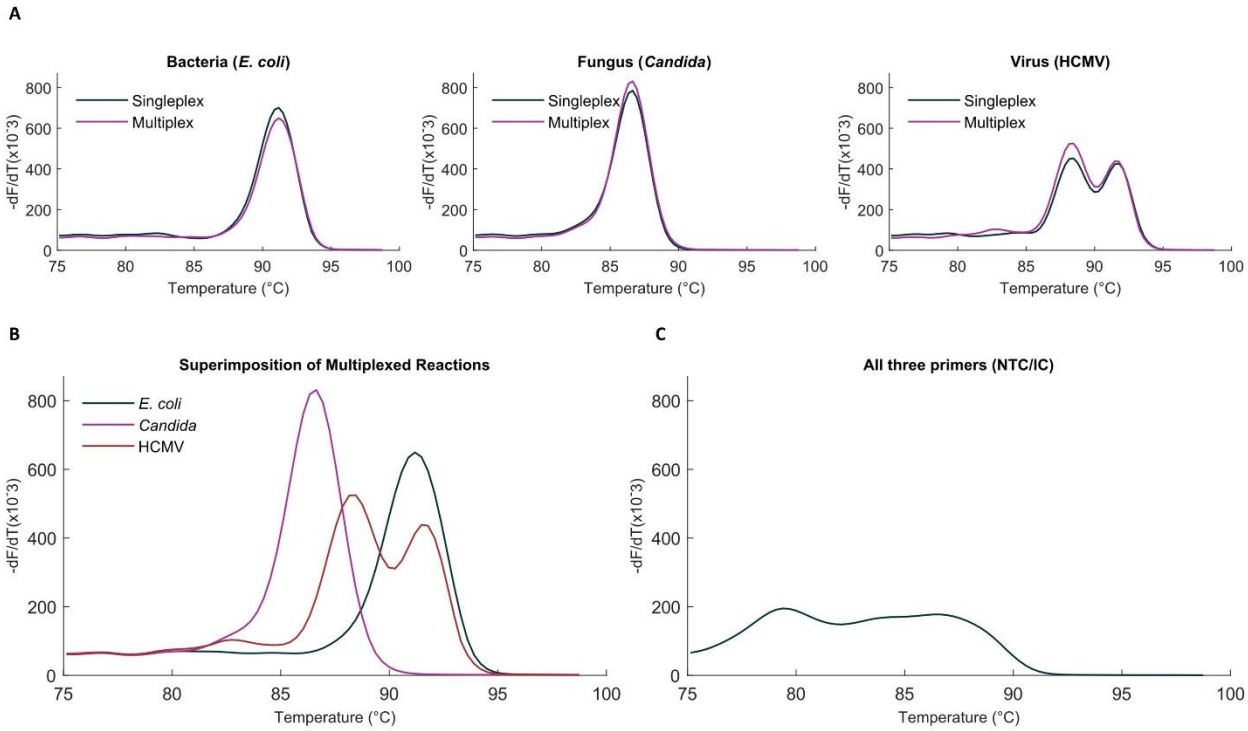


**Figure 9:** Multiplexing the viral assay with bacterial and fungal assays. (A) Superimposition of singleplex and multiplex assays showing that multiplexing does not affect specificity of the primers. (B) Superimposition of the three multiplex assays show distinct melt signatures for each pathogen. (C) Multiplexed primers as NTC show the formation of a non-specific primer dimer.

## 4. Discussion

HCMV, HSV1 and HSV2 are significant causes of congenital infections with potentially high morbidity[33,34]. These infections may present with non-specific symptoms or may be asymptomatic which may lead to either misdiagnosis or completely missing the infection, thus increasing the risk of late onset morbidity and/or mortality[1,4,6]. There is a need for a rapid, inexpensive, specific and multiplexed diagnostic technology for these viruses that can enable early screening and correct diagnosis for better patient management. The gold standard for herpesviral detection is viral culture[35,36] which has a higher turn-around time, higher costs and poorer sensitivity[36], that make it unsuitable as a tool for rapid and multiplexed screening. Probe-free PCR along with high resolution melt offers a rapid, inexpensive and specific detection technology amenable to multiplexing with other more common neonatal infectious panels that may alleviate some of the problems of current detection highlighted above.

The first part of this work's aim was to develop and optimize a probe-free viral assay for HCMV, HSV1 and HSV2. While a few universal primers for these viruses are available in literature[32,37], these are either non-specific against human DNA amplification or had high degeneracy/mismatches with the template that would affect the specificity and the stability of the reaction[38]. Custom primers were therefore designed for the assay and optimization performed. The optimized assay was found to portray specificity for each of the three viruses with distinct melt curve signatures for each (Figure 3C). Specificity against human DNA amplification was also observed. The optimized assay has a total turn-around time of <4 hours (2 hours for DNA extraction and sample preparation and <2 hours for thermocycling and melt imaging). The LOD of the assay is 10^2 copies/reaction for HCMV and 10^3 copies for HSV1

while the LOQ from standard curves was found to be 10^3 copies/reaction for HCMV and 10^4 for HSV1 (Figure 8).

For the second part of the aim, the viral assay was multiplexed with the Lab's bacterial and fungal assays. The multiplexed bacterial, fungal and viral primers show high specificity for their respective pathogens with distinct melt signatures for each (Figure 9).

Thus, a probe-free viral assay was developed that utilizes high resolution melt signatures for specific detection of each pathogenic species, removing the need for sequence-specific probes or nested PCR, and thus increasing the capacity for high throughput detection. The speed and affordability of the assay as compared to viral cultures would allow universal viral screening to be adopted, which is particularly significant for cCMV and maternal HSV infections[4,21]. Ability of the assay to be multiplexed with bacterial and fungal assays for sepsis make this technology a one-step diagnostic solution for neonatal sepsis, reducing the incidence of misdiagnosis and healthcare costs.

Notwithstanding the advantages of the assay in clinical neonatal diagnostics, more work needs to be done in addressing the limitations of the assay. First, the custom primer pair forms primer dimers in NTC (Figure 3A) that seems to be affecting the assay efficiency, LOQ and LOD and linear dynamic range. Second, the assay performs poorly for HSV2 as compared to the other two viruses, as observed by lower efficiency and poorer linear dynamic range for HSV2. Third, the viral primer used in multiplexing experiments, p5, was later to amplify human DNA and thus, provides reduced specificity. Fourth, the multiplexing reactions were performed with Phusion while the new optimized viral assay has a Q5 Polymerase chemistry.

Therefore, more work is required for optimizing the assay and enabling its multiplexing. First, the limitations of poor efficiency and sensitivity could be solved by translating the qPCR assay to a digital platform, which theorizes single genome sensitivity. The smaller reaction volumes may also bring the reactants closer together for better chance of collision and thus, amplification. Second, the assay requires more optimization particular to HSV2 which might improve its analytical characteristics. Third, the new viral assay utilizing p19p4 primer has been shown to not amplify human DNA. This primer could thus be used to test future multiplexing experiments. And fourth, the bacterial and fungal assays can be tested for sensitivity with Q5 chemistry like the viral assay, in order to enable their multiplexing.

The final goal is to translate the multiplexed qPCR assay to our lab's Digital Molecular Technology platform that combines the probe-free HRM principles described above with dPCR for broad-based detection, higher throughout and single genome sensitivity[27]. The machine learning algorithm used by the technology "learns" and detects species specific melt signatures for each pathogen and the probe-free technology enables extension of the platform to add new pathogens[27]. Digitizing the assay reduces sample volume requirement and improves current sensitivity. The processes can be automated for speed, ease of use and reduction in contamination.

A probe-free multiplexed viral, bacterial and fungal assay would thus provide a single clinical test for neonatal sepsis by concurrently detecting a multitude of sepsis-causing infectious agents, reducing the need for multiple panels. It would enable specific and correct diagnosis, faster clinical decision-making, reduce morbidity from late onset conditions by timely interventions and thus reduce overall healthcare costs.

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# 6. Appendix

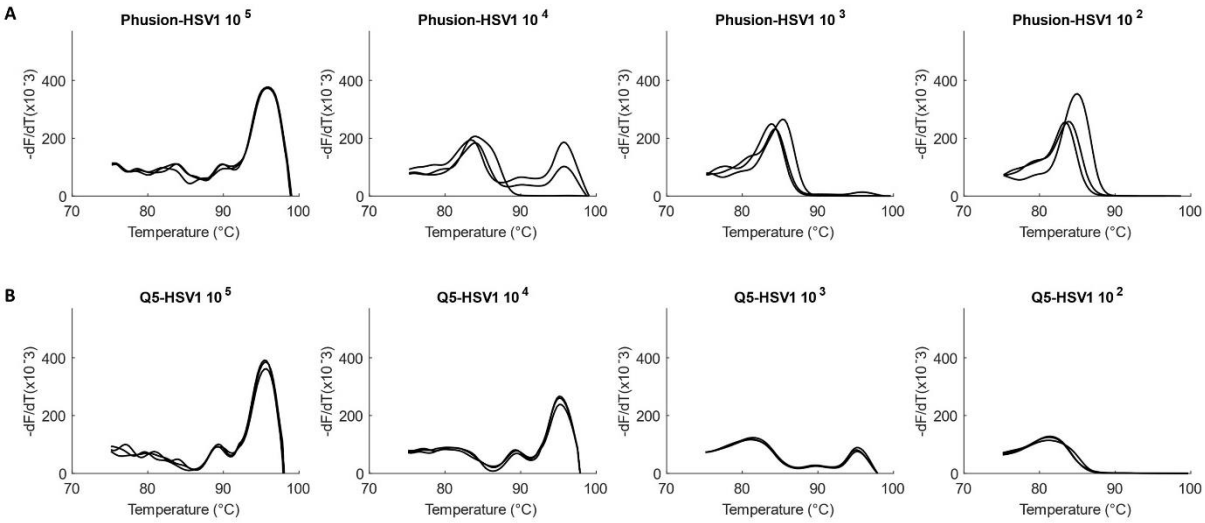


**Figure A1:** Comparison of Phusion vs Q5 DNA Polymerases for amplification of HSV1. Amplification of serially diluted (10^5 to 10^2 copies/reaction) HSV1 in triplicates with (A) Phusion- LOD 10^4 copies and with (B) Q5-LOD 10^3 copies.

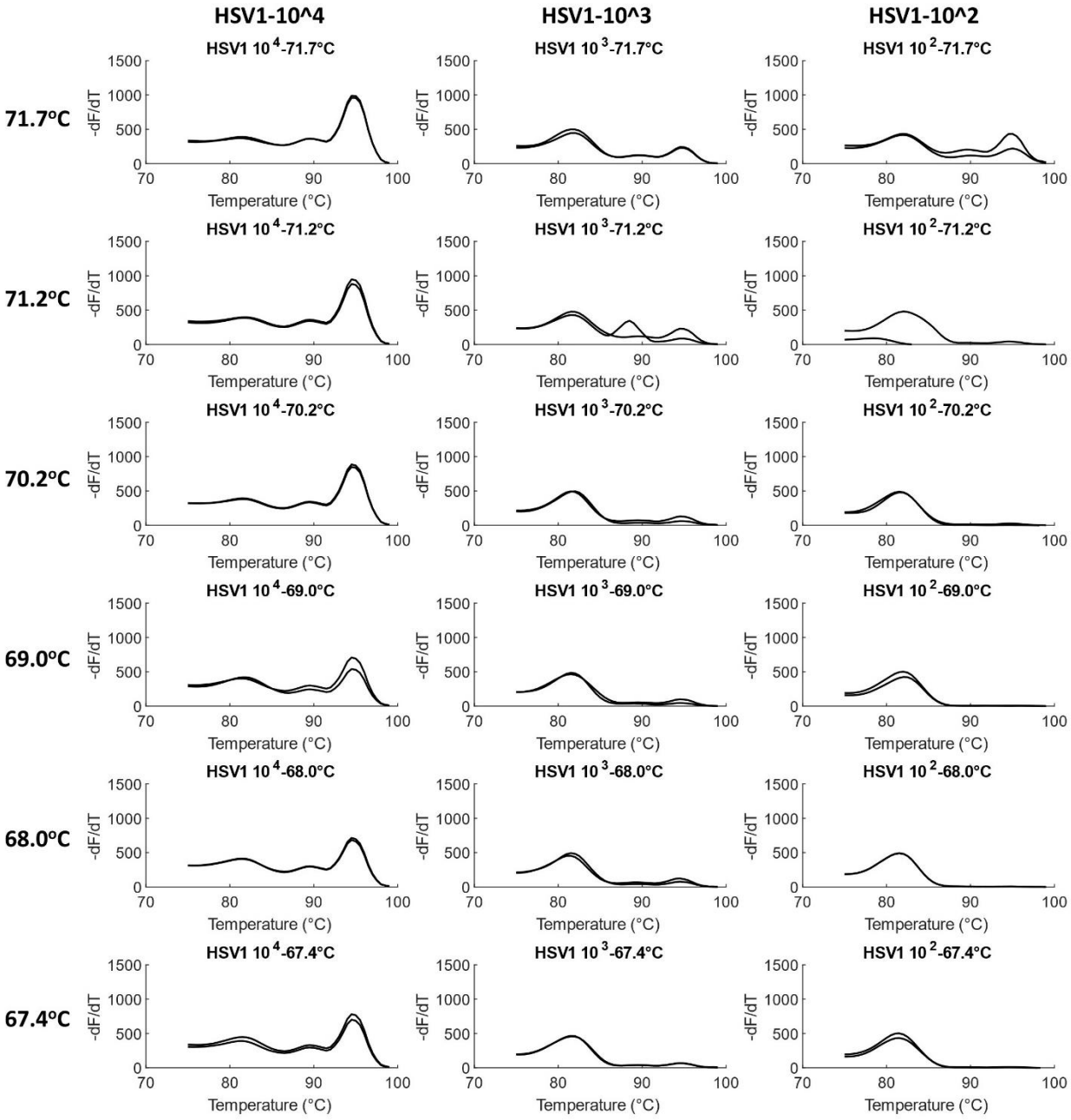


**Figure A2:** Optimization of annealing temperature. Temperature gradient experiments were performed for HSV1 in duplicates from 72ºC to 67ºC (rounded off). Product concentration decreases with decrease in temperature. For HSV1, the LOD is observed to be 10^3 copies/reaction, where concentration decreases with temperature decrease from $71.7^0$C. Thus, 72ºC is the ideal annealing temperature for HSV1.