A multi-template multiplex PCR assay for hepatitis B virus and human p-globin

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Abstract

The Hepatitis B surface antigen (HBsAg) is the hallmark of HBV infection. Detection of antibodies to HBs and the core (i.e. HBsAg and HBcAb) are primary serological algorithms in the laboratory diagnosis of HBV. Detection of HBsAg DNA is an important supplement to serological diagnosis especially in clinical cases. Simultaneous amplification of internal cellular controls is a good indicator of sample quality. Human P-globin is a well characterised housekeeping gene (HKG) that is often applied as internal controls (IC) in molecular diagnosis. In this study, individual plasmid clones of the human P-globin and HBs genes were constructed. These plasmid constructs have been applied to characterise a multiplex PCR assays for HBs and P-globin genes. The findings suggest detection limits of less than 10 genome copies of either template In vitro using conventional and multiplex PCR conditions. Under the multiplex conditions, co-amplification of P-globin and HBsAg DNA had a resultant effect on assay sensitivity. This study further highlights the importance of molecular diagnosis in HBV infectious individuals. If fully optimised, this assay could provide a possible diagnostic complement to serological detection in developing countries.

Keywords: Hepatitis B, HBs gene, Multiplex Polymerase Chain Reaction, Housekeeping Genes, Molecular diagnosis, Sensitivity

Introduction

Hepatitis B Virus (HBV) infection is a common cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma which may lead to death [1, 2]. Annually, approximately 4.5 million new cases occur worldwide, 25% of which progress to chronic liver disease. Approximately 5% of the world's populations are chronic carriers with an estimated 620,000 annual deaths as a result of acute and chronic HBV. Although two main vaccines against HBV have been in use for over three decades, HBV infections still remain highly endemic in sub-Saharan Africa and East Asia with dire public health concerns [3, 4].

HBV is an enveloped DNA virus of the family Hepadnaviridae. It has a relaxed-circular 3.2 kb genome, which is partially double-stranded (rcDNA). The HBV genome is replicated by reverse transcription of an RNA intermediate. It encodes four overlapping open reading frames that are translated into the viral core protein (C-gene), a highly conserved surface protein (S-gene), the viral polymerase (P-gene), and the X protein (X-gene) [5]. In part, due to the poor fidelity of the viral reverse transcriptase there is broad sequence-variability of the HBV genome with eight main genotypes (A-H) distributed worldwide [5-7]. In addition to other possible human genotypes [8, 9], HBV has also been reported in non-human primates including chimpanzees, woolly monkeys, orangutans and gorillas [10-12], while non-human HBV-like viruses have been reported in rodents and birds [13-15].

Serological and genetic markers are employed as primary algorithms for the screening and diagnosis of various stages and genotypes of HBV infections. The choice diagnostic specimen for HBV infection is the blood and the most significant targets are the viral envelope and capsid [16, 17]. Repeated isolation of the S-antigen (HBsAg) from the blood of infected persons for over 6 months is a diagnostic marker for chronicity, while ongoing infections are diagnosed by the presence of the e-antigen (HBeAg) in the blood [17]. HBV genome quantification in serum or plasma samples is an effective means of measuring viral

load over time and remains most informative in diagnosing a carrier state [18]. This has been demonstrated by PCR, which is able to detect less than 10 HBV genome copies/mL of serum or plasma in chronic cases [17]. Despite the high sensitivity of PCR, sensitivity or specificity could be adversely affected by a variety of factors such as poor sample quality or genomic extraction procedures; therefore the application of HKGs as ICs in molecular biology is vital. HKGs are indigenous genes expressed in each cell of an organism under any condition and typically play important roles in many basic cellular functions. There are numerous human HKGs, many of which have been characterised and are often applied as ICs in experimental studies as a result of their consistent levels of expression and high conservation despite infection or other factors [19, 20]. In laboratory and clinical diagnosis, the diagnostic ability of a test is expressed as percentages of real cases that are accurately diagnosed as positive and percentages of non-existent cases that are accurately diagnosed as negative i.e. sensitivity and specificity of the test. The occurrence of false negative (Type I error) and/ or false positive (Type II errors) test results largely determine how effectively an assay could diagnose a condition [21, 22].

In many developing economies, HBV diagnosis is predominantly serological [23, 24] despite alarming levels of prevalence reported even among blood donors [25-28], which suggests possibilities of misdiagnosed cases. The ideal molecular diagnostic procedure should be highly sensitive and reliable as well as relatively cheap and cost-effective. PCR was first reported by Karry Mulis in 1985 for the amplification of individual target DNA segments in a genome [29, 30]. In 1988, a multiplex PCR that combined several primer pairs in the same reaction to amplify different specific amplicons was reported [31]. The use of multiplex PCR is applicable to the detection of multiple amplicons, thus saving resources and substantially reducing turnaround time [32]. In this study a multiplex PCR assay for the detection of HBs gene and human P-globin is characterised as a molecular diagnostic model with ICs. Findings

are discussed.

Materials and Methods

Cell culture

The human lung epithelial cell line A549 and hepatoma cell line Huh7 were a generous gift from Professor Mark Harris (University of Leeds, UK). Human embryonic kidney epithelial HEK293T and cervical epithelial HeLa cell lines were a generous gift from Professor Nicola Stonehouse (University of Leeds, UK). Cells were maintained as monolayers in DMEM supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin and 100 U/ml streptomycin. All cells were incubated at 37°C with 5% CO2 and passaged upon confluence according to standard methods [33].

DNA extraction

Cells were harvested at 80% confluency by trypsinisation with Trypsin EDTA. Total genomic DNA was extracted using TRIZOL (phenol-chloroform method) following standard protocols [34].

Plasmid clones

The HBsAg open reading frame flanked with NotI restriction sites and 6 base pairs (bp) linkers was synthesised commercially (Invitrogen, UK). The synthesised HBsAg gene was ligated into a ^twI-linearized pCR-Blunt vector plasmid. The ligated plasmid was transformed into chemically competent E.coli DH5a cells and colonies screened by digestion with NotI. Nucleotide BLAST of all four cell line extracts matched NCBI reference human P-globin (NC_018922.2) [35].

The human P-globin gene from A549 cells was amplified by PCR, purified by gel extraction and ligated into linearized pGEM T-easy plasmid vector (Life Technologies), following manufacturer's instructions. Plasmid vectors were then transformed into chemically competent E.coli DH5a cells and colonies screened by digestion with EcoRI.

For quantitative analyses, plasmid copy number (PCN) was determined by standard methods [36].

PCR reagents, primers and thermocycling conditions

All PCR reactions were carried out using QIAGEN fast cycling PCR kit, following manufacturer's protocol. HBs gene was amplified using forward primer HBsAgF (5' GTAGTAGCCGGCCGCATGGAAGGCAT) with reverse primer HBsAgR (5' TACATTTAGCGGCCGCGTGTA). Human P-globin was amplified using modified GH20 upstream (5' GTAGTAGCGGCCGCGAAGAGCCAAGGACAGGTAC) and PC04 downstream (5' TACTACGCGGCCGCCCAACTTCATCCACGTTCACC) primers [37]. PCR reactions were cycled as follows; one-cycle initial denaturation at 95°C for 1 minute; 40 cycles of 5 second denaturation at 96°C, 5 seconds annealing at 55°C (for HBV template) or 68°C (for Human P-globin template), and extension at 68°C for a duration of 1 second per 10 base pairs. This was immediately followed by one cycle of final extension at 72°C for 1 minute.

Conventional PCR

Respective template PCN was determined as previously described. A set each of upstream and downstream HBs gene or P-globin primers was maintained at 0.5 pM, for respective templates, in a 20 pl reaction. Thermocycling conditions were maintained as previously described.

Mixed primer competition PCR

Template PCN was determined as previously described. Reactions were setup using increasing copies of P-globin or HBs gene templates. A set each of upstream and downstream HBs gene and P-globin primers was applied to final concentrations of 0.25 pM for each primer in a 20 pl reaction. Thermocycling conditions were maintained as previously described.

Multiplex PCR

Template PCN was determined as previously described. Multiplex PCR was set up with increasing copies of P-globin and HBs gene plasmid templates (Table 1). Reactions were setup using upstream and downstream HBs gene and P-globin primers as previously described at a final concentration of 0.25 pM for each primer in a 20 pl reaction. Thermocycling conditions included one-cycle initial denaturation at 95°C for 1 minute; 40 cycles of 5 second denaturation at 96°C, 5 seconds annealing at 55°C (i.e. to cover lower annealing temperature), and extension at 68°C for a duration of 75 minutes (i.e. to cover larger target size). This was immediately followed by one cycle of final extension at 72°C for 1 minute.

Results

Generating control materials for PCR.

The laboratory diagnose of clinical infections is more frequently employing molecular diagnostic techniques such as PCR. The use of IC in such techniques is essential for validating specimen quality and quantity, reducing the likelihood of false negative results. Targets typically used as ICs including HKG such as of P-globin, the abundance of which in mammalian cells is well characterised (10, 11).

In order to isolate the human P-globin gene to use as an IC in the molecular diagnosis of HBV in human samples, the total genomes were extracted from four human cell lines; Huh7, A549, HEK293T and HeLa. The human P-globin gene was amplified from each cell extract, using target-specific modified PCR primers GH20 and PC04. The PCR amplicon from each cell line was gel-purified and sequenced. The nucleotide BLAST against known sequences showed a match (100 %) of all cell lines with the human chromosome 11 (from region 5226978 to 5227216), alternate assembly CHM1_1.1 (NCBI sequence ID: re^NC_018922.2) [38]. The purified PCR amplicon from cells was subsequently introduced into the cloning vector pGEM T-easy (Figure 1A) to be employed in the downstream assays. The known sequence of the HBs gene was commercially synthesised as a blunt-ended DNA fragment and cloned into a ^twI-linearized pCR-Blunt vector plasmid (Figure 1B) for downstream assays. Relative sensitivity of p-globin and HBs gene PCR.

Stable plasmid clones containing the human P-globin or HBs gene allowed us to establish a controlled PCR for the detection of P-globin and HBs gene. In order to define the detection limits of these assays, respective PCR reactions were set up with a 10-fold serial dilution of HBs gene and human P-globin plasmid copies. PCR amplicons were separated through 2% agarose gel and product band intensities determined by densitometry (Figure 2).

For the human P-globin PCR, product was clearly detected with 10^2 to 10^{11} genomes copies per reaction, with a linear range of 10^2 to 10^6 genome copies per reaction. In comparison, the PCR for HBs gene demonstrated a reduced linear range of 10^1 to 10^5 genome copies per reaction. These results may suggest a 10-fold greater sensitivity for HBs gene amplification under these PCR conditions compared to human P-globin, albeit with a reduced overall linear range.

Mixed primers competition assays.

Multiplex PCR employs multiple primer sets for the simultaneous amplification of multiple amplicons. However, the use of multiple primer sets in a single reaction can result in reduced specificity and sensitivity in comparison to single target PCR, partly due to the reduced primer concentration as well as primer dimerization, which can significantly reduce template-binding [39]. In order to address this, assays were designed to establish whether multiplex PCR conditions reduced the relative sensitivity of P-globin and HBs gene amplification. Individual reactions were setup to amplify P-globin or HBs gene using multiplex PCR conditions and amplicons bands quantified by densitometry (Figure 3). For both the P-globin and HBs gene multiplex PCR, products were detected with greater than 2.8 x 10^2 genome copies per reaction. This result would suggest that the multiplex reaction conditions reduced the sensitivity of HBs gene PCR approximately 10-fold, even in the absence of any P-globin or other competitive templates.

Multiplex PCR.

In the application of HKG as ICs it is possible their consistent availability, which could be in greater amounts than target pathogens, can change the relative sensitivity of the PCR. In order to assess how an excess of host-specific P-globin [19, 20, 40, 41] could change the sensitivity of the multiplex assay, increasing copy numbers of human P-globin and HBs gene were co-amplified in multiplex PCR reactions. Both amplicons were co-analysed by gel

electrophoresis and intensities of the HBs gene (0.75 kb) and human P-globin (0.26 kb) DNA bands measured by densitometry (Figure 4).

A linear relationship between input multi-template genome copy number and amplified product within a multiplex PCR environment was observed. Like before, for the HBs gene under multiplex conditions in the absence of competing P-globin DNA (i.e. 0 P-globin gene copy), products were only detected with greater than 2.8×10^2 genome copies per reaction. Upon addition of an increasing amount of competing P-globin DNA, a stepwise increase in product was observed even with below-detectable levels of HBs gene targets. This is indicative of non-specific amplification under multiplex PCR conditions when both target templates are present. Despite such, non-specific amplification, a linear increase in product was observed with greater that 2.8×10^2 HBs genome copies up to 2.8×10^4 copies per reaction. Therefore, after accounting for the non-specific amplification, the multiplex PCR can still be specifically detected above 2.8×10^2 HBs genome copies per reaction.

Similar results were observed for the detection of P-globin target under multiplex conditions, with an addition of increasing amount of competing DNA resulting in a stepwise increase in product observed with below-detectable levels of HBs of P-globin target gene. Again, such observations would be suggestive of non-specific amplification. However, an increase in PCR product was observed with greater than 2.8×10^2 of P-globin gene copies per reaction. Thus, the multiplex PCR can detect greater than 2.8×10^2 copies of P-globin DNA even under greater quantity of competing DNA. Therefore the multiplex PCR conditions can specifically detect both HBs and P-globin at greater than 2.8×10^2 DNA copies per reaction.

Discussion

The data suggests that PCR-amplification of HBs gene may be a highly sensitive and reliable means of diagnosing hepatitis. Serological detection of HBV infection is often applied to screening, diagnosis, and immuno-surveillance [16] as an affordable option with a quick turnaround time, [42-44] however, it does not detect career status, levels of viremia or chronicity. The supplementary detection of the HBVs gene may be more robust for diagnosis; to determine viral load, HBV genotype; to screen for anti-viral resistance, drug/therapeutic efficacy [43], etc. Under conditions of conventional PCR, target genes are primed from single templates by a set of forward- and reverse-oriented gene-specific oligoes, and amplified by a DNA polymerase in the presence of dNTPs. With these assays, 10 genomic copies of HBs gene were detectable with a single set of primers from a single template in a conventional PCR. Thus, as would be anticipated, amplification of parts or all of the HBV genome by conventional PCR is very specific with low detection limits [43].

The large difference in size between HBs gene and P-globin amplicons allowed for easy differentiation of both PCR products with these assays at varied primer concentrations. The ability to co-amplify templates within a single PCR reaction could significantly reduce diagnostic turnaround time especially in clinical settings. HKGs are indigenous and host- specific [20]. They are ideal quality indicators in medical diagnostics. The multiplex PCR conditions reduced PCR sensitivity approximately 10-fold compared to conventional PCR, limiting the dynamic range from 2.8×10^2 up to 2.8×10^4 gene copies per reaction. This reduced dynamic range could suggest that a limited serial dilution of sample may be required for semi-quantitative diagnostic purposes. Under conditions where both PCR targets are present, the sensitivity of the multiplex PCR did not appear to change significantly. However, the specificity of the

12

multiplex PCR was reduced with some non-specific amplification observed in the

presence of high concentration of non-target DNA.

The multiplex assays suggest that, at optimal priming conditions, co-amplifying P-globin together with the gene of interest (e.g. HBs DNA) integrates a standard qualitative sample- based positive control, as an alternative to multiple PCR reactions, thereby avoiding risks of contamination. However these advantages come at the cost of a slightly reduced sensitivity, and importantly reduced specificity. This latter limitation could however be potentially accounted for by performing a small limiting dilution alongside standardised dilution series of P-globin and HBs target DNA clones. Furthermore, a dilution series of P-globin could be used to generate an ideal standard curve to quantitatively assess abundance of template in the sample.

In the laboratory diagnosis of HBV, PCR has been shown to have very high sensitivity and specificity [16]. In this study, the sensitivity [22], of conventional PCR was observed to be higher than the multiplex PCR assays and multi-primer competition PCR. This strengthens the submission that PCR efficiency is dependent on amounts of available primers and if they are in excess [45]. It further suggests that assay multiplex PCR assay could be ideal for multiple template amplification within a single reaction.

Conclusion

These findings suggest detection limits of less than 10 genome copies of either template in conventional and multiplex PCR conditions. With the use of multi-primer sets for the amplification of single and multi-template samples, these findings show that not only are ICs good indicators of quality assurance of sample processing, co-amplification of IC with gene of interest (HBs) may have a resultant effect on the viability of the chosen assay. If fully optimised, this assay would provide a diagnostic complement to serological diagnosis and/ or screening of HBV thus providing a semi-quantitative measurement of viral load. Such assays have been applied to diagnose multiple of organisms [46] or conditions from limited clinical samples within a single reaction.

Author's contributions

OOA conceived the study; OOA and MRH designed the study protocol; OOA carried out all experiments; OOA and MRH analysed and interpreted the data. All authors drafted the manuscript. All authors critically revised the manuscript for intellectual content. All authors read and approved the final manuscript.

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(A)

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                                                       40                                          80
A549          GAAGAGCCAA GGACAGGTAC GGCTGTCATC ACTTAGACCT CACCCTGTGG AGCCACACCC TAGGGTTGGC CAATCTACTC
HEK293T       GAAGAGCCAA GGACAGGTAC GGCTGTCATC ACTTAGACCT CACCCTGTGG AGCCACACCC TAGGGTTGGC CAATCTACTC
Huh7          GAAGAGCCAA GGACAGGTAC GGCTGTCATC ACTTAGACCT CACCCTGTGG AGCCACACCC TAGGGTTGGC CAATCTACTC
HeLa          GAAGAGCCAA GGACAGGTAC GGCTGTCATC ACTTAGACCT CACCCTGTGG AGCCACACCC TAGGGTTGGC CAATCTACTC
NC 018922.2   GAAGAGCCAA GGACAGGTAC GGCTGTCATC ACTTAGACCT CACCCTGTGG AGCCACACCC TAGGGTTGGC CAATCTACTC
              ********** ********** ********** ********** ********** ********** ********** **********
                                                      120                                         160
A549          CCAGGAGCAG GGAGGGCAGG AGCCAGGGCT GGGCATAAAA GTCAGGGCAG AGCCATCTAT TGCTTACATT TGCTTCTGAC
EK293T        CCAGGAGCAG GGAGGGCAGG AGCCAGGGCT GGGCATAAAA GTCAGGGCAG AGCCATCTAT TGCTTACATT TGCTTCTGAC
Huh7          CCAGGAGCAG GGAGGGCAGG AGCCAGGGCT GGGCATAAAA GTCAGGGCAG AGCCATCTAT TGCTTACATT TGCTTCTGAC
HeLa          CCAGGAGCAG GGAGGGCAGG AGCCAGGGCT GGGCATAAAA GTCAGGGCAG AGCCATCTAT TGCTTACATT TGCTTCTGAC
NC 018922.2   CCAGGAGCAG GGAGGGCAGG AGCCAGGGCT GGGCATAAAA GTCAGGGCAG AGCCATCTAT TGCTTACATT TGCTTCTGAC
              ********** ********** ********** ********** ********************* ********** **********
                                                      200                                         240
A549          ACAACTGTGT TCACTAGCAA CCTCAAACAG ACACCATGGT GCACCTGACT CCTGAGGAGA AGTCTGCCGT TACTGCCCTG
HEK293T       ACAACTGTGT TCACTAGCAA CCTCAAACAG ACACCATGGT GCACCTGACT CCTGAGGAGA AGTCTGCCGT TACTGCCCTG
Huh7          ACAACTGTGT TCACTAGCAA CCTCAAACAG ACACCATGGT GCACCTGACT CCTGAGGAGA AGTCTGCCGT TACTGCCCTG
HeLa          ACAACTGTGT TCACTAGCAA CCTCAAACAG ACACCATGGT GCACCTGACT CCTGAGGAGA AGTCTGCCGT TACTGCCCTG
NC 018922.2   ACAACTGTGT TCACTAGCAA CCTCAAACAG ACACCATGGT GCACCTGACT CCTGAGGAGA AGTCTGCCGT TACTGCCCTG
              ********** ********** ********** ********** ********************* ********** **********

A549          TGGGGCAAGG TGAACGTGGA TGAAGTTGG   269
HEK293T       TGGGGCAAGG TGAACGTGGA TGAAGTTGG   269
Huh7          TGGGGCAAGG TGAACGTGGA TGAAGTTGG   269
HeLa          TGGGGCAAGG TGAACGTGGA TGAAGTTGG   269
NC 018922.2   TGGGGCAAGG TGAACGTGGA TGAAGTTGG   269
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(B)
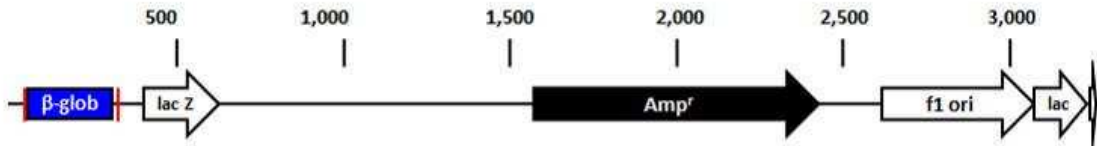
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GTAGTAGCGGCCGCATGGAAGGCATCACATCAGGATTCCTAGGACCCCTGCTCGTGTTACAGGCGGGGTTTTTCTTGTTGACAAAAATCCTCACAATACC
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CCTCTTGTCCTCCAATTTGTCCTGGCTATCGCTGGATGTGTCTGCGGCGTTTTATCATCTTCCTCTTCATCCTGCTGCTATGCCTCATCTTCTTGTTGGT
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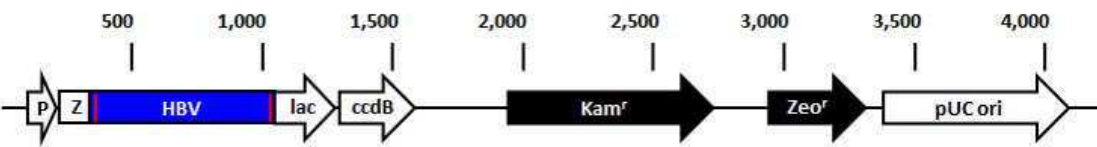
Figure 1. Plasmid constructs. (A) Total genomic DNA was extracted from A549, HEK293T, Huh7 and HeLa cell lines. The β-globin gene was amplified by PCR using a modification of upstream (PC04) and downstream (GH20) β-globin primers [37, 47, 48]. PCR amplicons were gel-purified and sequenced. Alignment of human β-globin gene extract against known human β-globin sequence are shown. The β-globin extract was ligated into an EcoRI-linearised pGEM T-easy vector plasmid. Plasmid map shows HBs gene (blue), restriction sites (red), and selection markers (black). Plasmid was transformed into chemically competent E. coli DH5α cells. Colonies were picked randomly, screened for inserts by digestion with EcoRI. (B) The HBV s-fragment gene was commercially synthesised with upstream and downstream NotI restriction sites (red letters) and ligated into a pCRBlunt vectors. Plasmid map shows HBs gene (blue), restriction sites (red), and selection markers (black). Plasmid was transformed into chemically competent (E. coli) DH5α cells. Twelve colonies were picked randomly, screened for inserts by restriction enzyme digest using NotI.
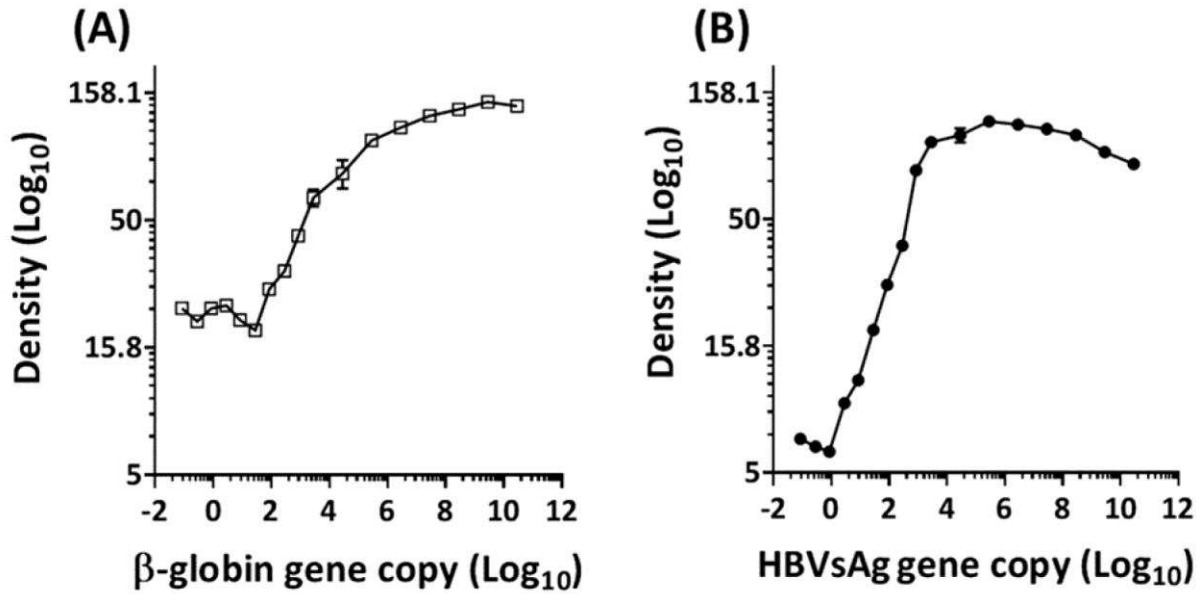
Figure 2. Input copy numbers assay showing densitometric plots of P-globin and HBs genes. In order to determine the linear ranges of conventional PCR for (A) P-globin and (B) HBs gene, the respective genes were amplified by PCR from a serial dilution of cloned targets with logarithmic increments in genome number. PCR amplicon was run through 2% agarose gel and DNA bands quantified by densitometry (n = 2 ± S.E.M).
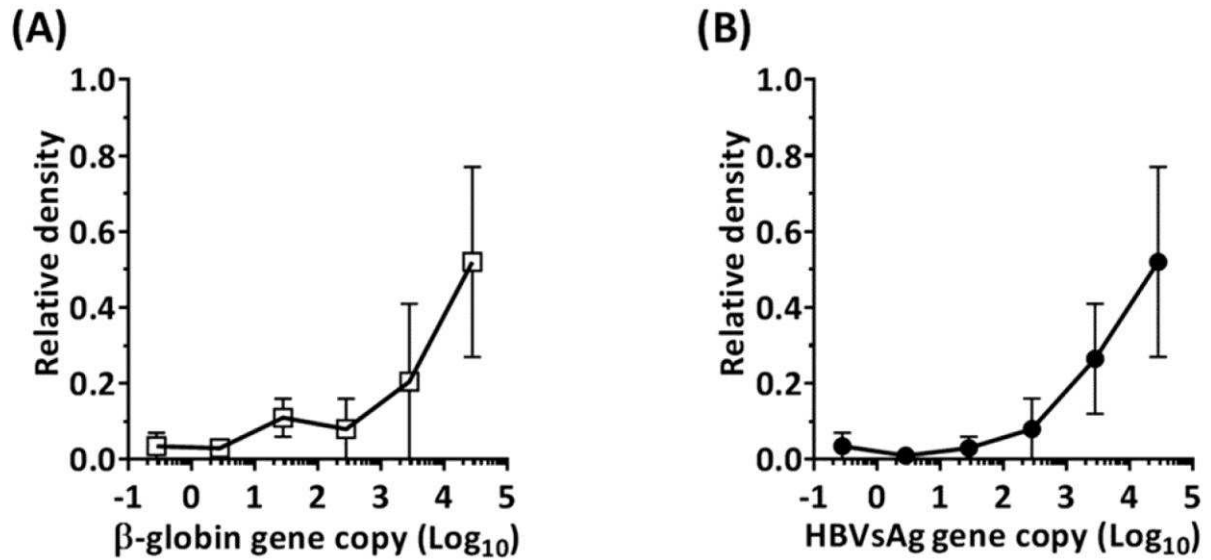
Figure 3. Mixed primers competition assays. To determine the effects of multiplex primers on PCR efficiency either (A) human P-globin or (B) HBsAg was amplified in a multiplex reaction containing equal concentrations of P-globin and HBsAg -specific primers. PCR products were analysed by agarose gel electrophoresis and DNA bands quantified by densitometry (n = 2 ± S.E.M).
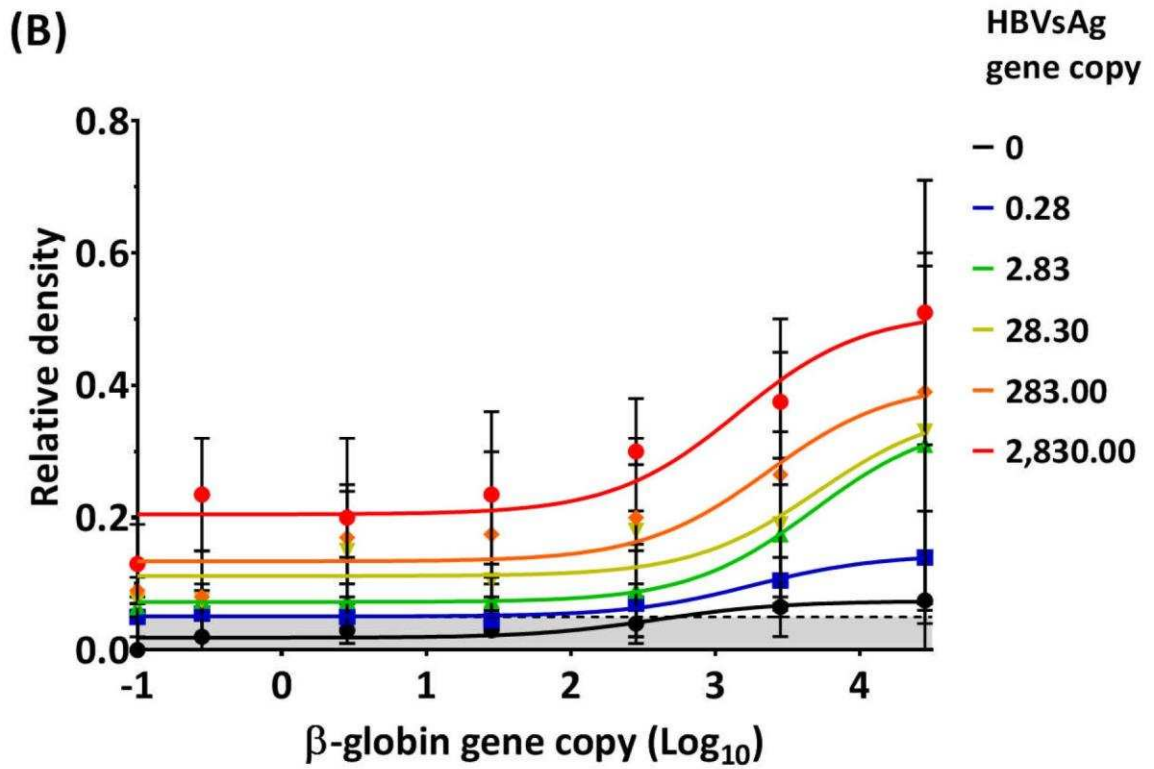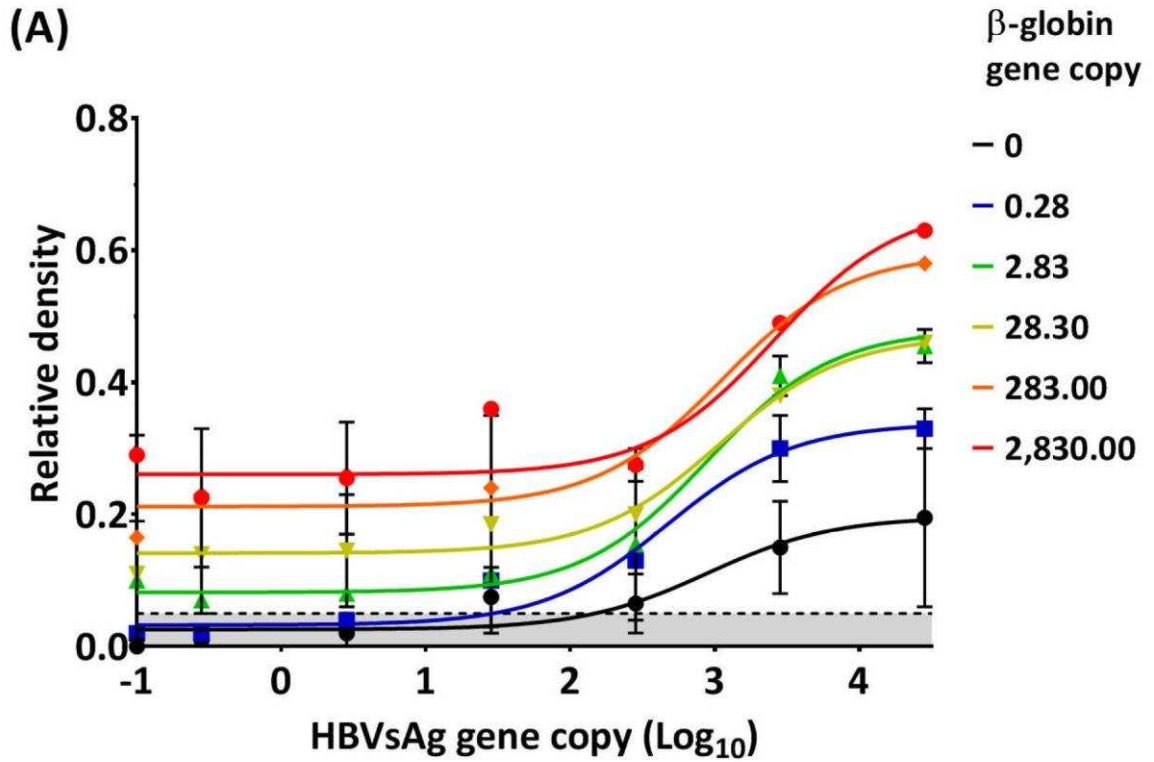
Figure 1. Multiplex PCR of P-globin and HBsAg with increasing genome copies. (A) Multiplex PCR of HBVsAg with increasing input copy number of P-globin. (B) Multiplex PCR of P-globin with increasing input copy number of HBVsAg. Both PCR amplicons were analysed by gel electrophoresis and DNA bands quantified by densitometry. A positive PCR was determined as having a relative density of above 0.05, based upon the data from the mixed primer competition assays. For clarity the area of the graph below 0.05 is shaded grey. (n = 2 ± S.E.M).

Table 1. Multiplex PCR template set up of HBsAg versus Human β-globin plasmid copies

Human β-globin plasmid copy (+)

0	+	++	+++	++++	+++++	++++++
*	* +	* ++	* +++	* ++++	* +++++	* ++++++
**	** +	** ++	** +++	** ++++	** +++++	** ++++++
***	*** +	*** ++	*** +++	*** ++++	*** +++++	*** ++++++
****	**** +	**** ++	**** +++	**** ++++	**** +++++	**** ++++++
*****	***** +	***** ++	***** +++	***** ++++	***** +++++	***** ++++++
******	****** +	****** ++	****** +++	****** ++++	****** +++++	****** ++++++

HBsAg plasmid copy (*)

Plasmid clones of HBsAg and human β-globin were added to multiplex PCR reactions at inversely proportional plasmid copies. Final primer concentrations of 0.25 μM was maintained in each reaction. Table shows a checked board setup of PCR reactions in a 96-well plate. HBsAg plasmid copies are represented as asterisks (*), while human β-globin plasmid copies are presented as pluses (+).