stage of infection. In addition, real-time RT-PCR shortens the time for analysis and reduces the contamination risk. Using the MagNA Pure LC System for the preparation of nucleic acids, the resulting uninterrupted sample processing helps to accomodate larger sample numbers.

Due to the high specificity and sensitivity, the short analysis period, and high-throughput capacity of automated systems, real-time RT-PCR can be considered the screening method of choice, if clinical signs or the epidemiological situation require direct virus detection.

References

1. McGoldrick A et al. (1998) J Virol Meth 72: 125–135
2. Drew TW et al. (1999) Vet Microbiol 64: 145–154

Order INFO

Product	Pack Size	Cat. No.
LightCycler – RNA Master Hybridization Probes	1 kit (96 reactions)	3 018 954
High Pure Viral RNA Kit	1 kit (100 purifications)	1 858 882
High Pure Viral Nucleic Acid Kit	1 kit (100 purifications)	1 858 874
MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	3 038 505

Development of a Quantitative LightCycler Assay for the Detection of Epstein-Barr Virus DNA in Research Samples

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Introduction

The Epstein-Barr Virus (EBV), one of the eight human herpes viruses (HHV4), is a double-stranded DNA virus of ubiquitous spread. The virus is transmitted by salivary contact and most often individuals become infected during their childhood. In these cases, primary infections are mostly asymptomatic or reveal only mild nonspecific symptoms. When infection with EBV occurs during adolescence or young adulthood, it causes infectious mononucleosis in up to 50 % of all cases, an illness associated with fever and swollen lymph glands. In some cases, EBV is described as the causative agent of chronic fatigue syndrome.

Worldwide, EBV has a prevalence of about 90 %. Once acquired, the virus establishes a lifelong latent infection and remains in epithelial cells of the throat and in B-lymphocytes. In very rare events, EBV can cause severe complications, such as Burkitt's lymphoma or nasopharyngeal carcinoma mainly known to occur in Africans or Asians.

Normally, EBV is under the control of T-cells, which prevent the outgrowth in healthy individuals. Under immunosuppressive therapy, serious complications may occur after organ or bone marrow transplants. EBV can be reactivated leading to a severe post transplant lymphoproliferative disorder (PTLD), which can have a fatal progression. The involvement of EBV and its epidemiology in all of these diseases is the subject of many research projects.

The current practice of EBV testing includes several serological assays which detect antibodies to the viral capsid antigen (VCA), the early antigen (EA), and the EBV nuclear antigen (EBNA) based on IgG or IgM measurement. The serological assays mainly used for the detection of EBV have some significant disadvantages: they are cumbersome and slow, they usually do not correlate to the quantitative viral load, and the individual antibody status can sometimes result in misinterpretation.

In this article, we describe the development of a highly sensitive and quantitative polymerase chain reaction (PCR) assay for EBV DNA with the LightCycler Instrument for research purposes. This PCR test directly measures the viral DNA load in either plasma or whole blood

samples. This quantitative assay allows the user to combine extraction of EBV DNA with the MagNA Pure LC Instrument and the LightCycler Instrument's on-line PCR, resulting in a fully controlled, fast, reliable, and standardized system.

Material and Methods

Sample material

EBV-positive plasma and whole blood samples were generated by artificially spiking EBV seronegative specimens with quantified pure viral material (EBV B95-8; Advanced Biotechnologies Inc., Maryland, U.S.A.). Spiking of EBV-negative specimens ensures the performance of the assay can be evaluated with well-defined samples not influenced by other factors. This research material was used for the complete evaluation of our EBV quantitative assay.

Sample preparation

Nucleic acid preparation was performed using two different methods: first, manually by using the High Pure Viral Nucleic Acid Kit and, second, utilizing a fully automated sample preparation method applying the MagNA Pure LC Total Nucleic Acid Isolation Kit on the MagNA Pure LC Instrument. Sample input in both methods was 200 µl; nucleic acid was eluted in 100 µl.

LightCycler PCR

For amplification, we used a highly specific primer pair derived from a well-conserved part of the EBV genome. Five microliters of the purified material was used to amplify EBV DNA and internal control in the LightCycler Instrument, applying the "HybProbe technology": two pairs of specific hybridization probes – one for the internal control and one for the analyte – were present in the master mix. This provided a simultaneous and specific fluorescence detection of each amplicon during PCR (online detection). The EBV quantification assay was designed such that EBV was detectable in Channel 2 (640 nm) and the internal control in Channel 3 (705 nm), using the LightCycler Instrument.

In parallel to the samples, 5 different standards of known concentration were amplified in each LightCycler run. The crossing point (CP) values of these standards were used to generate an external standard curve, which provided accurate quantification of samples with unknown concentrations.

After amplification, the LightCycler Instrument was set up to perform a melting-curve analysis automatically. This additional analysis was used as a "quality control".

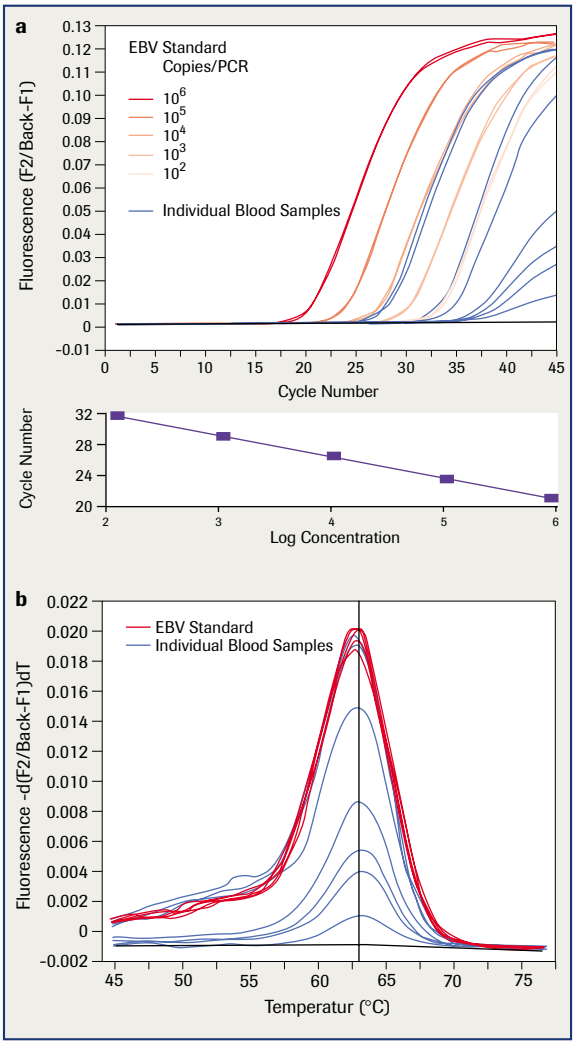

Figure 1:
a) LightCycler PCR amplification curves and linear regression line of the standard curve (10^6 – 10^2 copies/PCR). Individual whole blood samples were spiked with varying amounts of EBV.
b) Melting curve analysis of the EBV HybProbe pairs on its amplicon.

Results and Discussion

Internal control (IC)

Detection of viral nucleic acids requires a fully controlled PCR workflow from sample preparation to analyte detection. This has been set as a standard in virological labs for years. To support this with our EBV quantitative assay, we

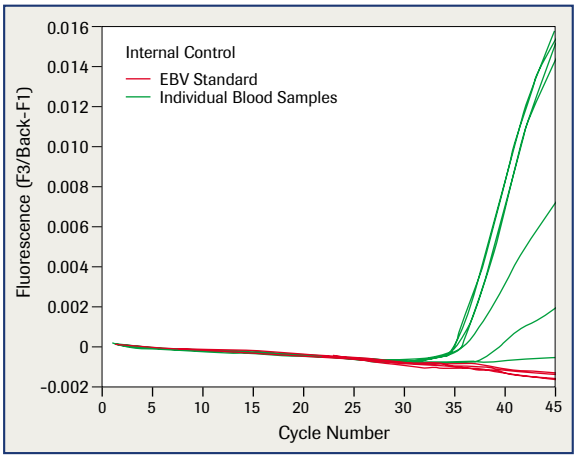

Figure 2:
LightCycler PCR amplification curves of the Internal Control (IC). IC is only present in the individual samples (green) not in the standards (red).

Common Profile Concept

The key to an efficient workflow in the laboratory is maximum uniformity and compatibility of different tests, the so-called "Common Profile". This allows the user to standardize procedures and thus increase the quality of the results. Furthermore, it allows combining of different analytes in one analytical procedure. We have maximized our efforts to ensure that all the LightCycler assays for the detection of herpes viruses (EBV, HSV 1, HSV 2, and future developments) can be combined in one LightCycler run. To guarantee this, we have standardized the PCR parameters, as well as the sample volumes and pipetting steps. This will result in high-throughput generation of reliable results in the PCR workflow system.

cloned an internal control (IC) precisely based on the amplicon sequence. Compared to the EBV PCR, the IC is amplified with the same primer pair; its amplicon is identical in length, has the same GC content, and therefore identical amplification efficiency. The IC can be differentiated from EBV by a unique binding region of two specific HybProbes, one of them labeled with the Red 705 fluorophore. This region has been introduced by site-directed mutagenesis and precisely replaces the EBV HybProbe binding region which is detected by Red 640.

The IC is introduced into the lysis buffer prior to sample preparation and therefore allows one to recognize putative failures during nucleic acid isolation, and monitor PCR inhibitors. IC should be positive in negative samples or low positive samples and can be out-competed in higher positive samples (Figure 2). This procedure ensures a fully controlled and standardized PCR workflow, and is totally in line with other (routine) PCR assays, such as hepatitis C virus (HCV), human immunodeficiency virus (HIV), or hepatitis B virus (HBV). A typical example of the LightCycler-EBV PCR and its melting curve performance is given in Figure 1.

Analytical sensitivity

In order to monitor EBV reactivation, a highly sensitive detection of the analyte in a sample is an important demand for an EBV PCR assay. We have determined the lower limit of detection (LOD) of our EBV quantitative assay by a statistical tool called Probit-analysis. Using artificially spiked reference material (plasma and whole blood) in either MagNA Pure or High Pure sample preparation, we were consistently able to determine the LOD below ten EBV genome equivalents per PCR (<10 geq/PCR) with a confidence interval of 95%.

Analytical specificity

The EBV genome seems to be less variable than the genome of other viruses, such as HIV or HCV. Nevertheless, primer and probes were selected from a highly conserved region within the EBV genome, compared to other known isolates, to ensure comparable performance on all isolates. In addition, primer and probes were designed not to cross-react with any other herpes virus or human DNA that would be part of the background in whole blood (data not shown).

In contrast to SYBR Green applications, the HybProbes detection format offers a higher degree of specificity, but we further pushed this specificity by analyzing the melting behavior of the EBV HybProbes. From the melting peak of about 63°C the user can see the uniformity of the amplicon. This is not a requirement as for SYBR Green applications, but is an added value to the user for "quality control" purposes (Figure 1b).

Linear dynamic range

A quantitative EBV assay needs a wide dynamic range since the viral load can vary to a large extent depending on the status of EBV infection, the EBV-related disorder, or its sample material. The external standard curve has been chosen between 10^2 to 10^6 geq/PCR, in which most of the samples are in-between and can therefore be accurately quantified. Nevertheless, the EBV quantification assay has a much broader linear dynamic behavior ranging from 10 geq/PCR to 10^{10} geq/ml (data not shown).

Conclusions

The LightCycler – EBV Quantification Kit is a fast and reliable tool for the detection and quantification of EBV genomes from biological research materials. The reliability of the complete nucleic acid detection system is granted by the co-amplification of an internal control to exclude false-negative results by inhibition. The assay is fully compatible with the PCR Workflow System (MagNA Pure LC and LightCycler Instruments), thus enabling complete automation of EBV quantification. The high sensitivity and wide dynamic range of the assay make it perfectly suited for a wide variety of research applications.

For background literature please contact author.

Order INFO

Product	Pack Size	Cat. No.
High Pure Viral Nucleic Acid Kit	1 kit (100 purifications)	1 858 874
MagNA Pure LC Total Nucleic Acid Isolation Kit	1 kit (192 isolations)	3 038 505
LightCycler EBV Quantification Kit	1 kit (48 samples)	3 330 028