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Development of quantitative Q-LAMP technology generating a new model assay for detection and quantification of Epstein-Barr virus

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Il lavoro presentato in questa tesi è stato realizzato presso i laboratori di Diagnostica Molecolare DiaSorin, sotto la supervisione della Dr.ssa Giulia Minnucci e del Dott. Francesco Colotta.

Il materiale contenuto nella seguente tesi è strettamente confidenziale. E' stata inoltre presentata richiesta di embargo tesi per un periodo di 3 anni dalla data di conseguimento del titolo (vedi pag. 95).

INDEX

RIASSUNTO

Il virus di Epstein-Barr (EBV), detto anche human herpesvirus 4 (HHV-4) è un virus a DNA doppia elica in grado di infettare i linfociti B. EBV è classificato in 2 sottotipi: Tipo 1/A e 2/B a seconda della sequenza del gene EBNA-2.

Oltre il 90% della popolazione mondiale è stata esposta ad una infezione del virus EBV che è in grado di persistere in modo latente nell'ospite per tutta la vita, integrandosi nel suo genoma.

Nonostante la maggior parte delle infezioni primarie di EBV sono asintomatiche, il virus è l'agente eziologico della mononucleosi. EBV è associato a diverse malattie maligne, quali linfomi, disordini linfoproliferativi (post-trapianto PTLD), disordini legati all'immunodeficienza, carcinoma nasofaringeo (NPC) e carcinoma gastrico.

Diverse tipologie di test vengono utilizzate dai laboratori per diagnosticare un'infezione da EBV: test sierologici e metodi basati sulla PCR.

Nel corso degli ultimi decenni, sono state sviluppate varie tipologie di PCR, come multiplex PCR e RT-PCR, per poter rilevare la presenza del virus in modo rapido. Sempre di più vi è la necessità di quantificare la carica virale (VL), e non solo identificare la presenza del virus, poiché la quantificazione del DNA di EBV nel sangue, plasma, siero e liquido cerebrospinale (CSF) è essenziale per la diagnosi di infezione cronica di EBV, in particolare per la diagnosi di malattie linfoproliferative EBV-associate in soggetti immunocompromessi.

I test sierologici e i metodi basati su PCR hanno tempi di esecuzione elevati e comprendono numerosi passaggi che necessitano di personale specializzato e laboratori attrezzati.

Questa tesi descrive lo sviluppo di una tecnologia Loop Mediated Isothermal Amplification technology (Q-LAMP) quantitativa in grado di fornire un nuovo saggio modello per la rilevazione e la quantificazione dei due sottotipi clinicamente più rilevanti del virus EBV, in quattro matrici diverse (sangue intero, plasma, siero e CSF) e con un'alta sensibilità, specificità e precisione. Inizialmente la tecnologia Q-LAMP è stata sviluppata per fini qualitativi, grazie alle sue caratteristiche di essere semplice, rapida, precisa e sensibile, e pertanto facilmente applicabile nei laboratori clinici mediante l'utilizzo di semplici strumentazioni. Il saggio Q-LAMP EBV e stato disegnato sfruttando le proprietà della Q-LAMP qualitativa ed e stato migliorato per essere quantitativo, altamente sensibile, specifico e preciso su quattro diverse matrici.

Il gene EBNA-1 è stato identificato come potenziale regione target per il disegno del saggio, in quanto contiene regioni di elevata omologia tra i due sottotipi di EBV.

L'ottimizzazione è stata condotta sia su controlli plasmidici che su matrici negative estratte ed il saggio finale, in formato liofilo, è stato validato su campioni clinici. La tecnologia LAMP si è dimostrata molto specifica, sensibile e precisa. Inoltre, grazie al formato multiplex, è stato possibile introdurre in reazione un controllo che permette la valutazione dell'avvenuta estrazione del campione e la validazione del risultato dei

campioni negativi. L'amplificazione e la rilevazione del target e del controllo interno avvengono in real-time in un'unica provetta.

Per testarne il valore diagnostico e l'applicabilità nella pratica clinica il saggio è stato validato su campioni clinici positivi e negativi in comparazione con il metodo standard Real-Time PCR utilizzando come metodo di estrazione la piattaforma Liaison IXT (DiaSorin). La correlazione tra i due metodi si è dimostrata idonea alle richieste delle linee guida aziendali per i campioni di sangue intero e plasma.

Questi risultati hanno dimostrato come la tecnologia Q-LAMP sia quantitativa e il prototipo di saggio sviluppato sia adeguato ad una prossima industrializzazione per un futuro prodotto diagnostico.

ABSTRACT

Epstein-Barr virus (EBV) also called human herpesvirus 4 (HHV-4) is a double-stranded DNA virus that infects B cells. EBV is classified into two subtypes: Type 1 / A and 2 / B depending on the sequence of the EBNA-2 gene.

More than 90% of the world's population has been exposed to the EBV infection. Like all herpes viruses it is able to persist in the host for whole life with a silent infection by integrating in its genome. Although most primary EBV infections are asymptomatic, the virus is the causative agent of infectious mononucleosis. EBV is associated with a wide variety of malignant diseases such as lymphomas, post-transplant lymphoproliferative disorders (PTLD), immunodeficiency-related disorders, nasopharyngeal carcinoma (NPC) and gastric carcinoma.

Various laboratory tests are used to diagnose EBV infection: serological assay and PCR-based methods. During the past decades, various forms of PCR such as multiplex PCR and RT-PCR, have been developed to address the need for rapid identification of viruses. It has been suggested that quantitative Viral Load (VL) assessment is superior to qualitative detection, since the quantification of EBV DNA loads in blood, plasma, serum and cerebrospinal fluid (CSF) are essential for the diagnosis of chronic infection of EBV, especially for the diagnosis of EBV-associated lymphoproliferative disorders in immunocompromised subjects.

Serological assay and PCR-based methods are time consuming and need more steps for the set-up, therefore require specialized personnel and equipped laboratories.

This thesis describes the development of a quantitative Loop Mediated Isothermal Amplification technology (Q-LAMP) generating a new model assay for the detection and quantification of the two most clinically relevant subtypes of EBV virus, on four different matrices (whole blood, plasma, serum and CFS) and with high sensitivity, specificity and precision.

Initially the Q-LAMP technology has been developed only for qualitative purposes thanks to its features to be simple, rapid, specific and sensitive, therefore it is easily applicable in the clinical laboratories by using basic facilities. The Q-LAMP EBV assay has been evaluated to be also quantitative.

Exploiting the qualitative Q-LAMP properties and using EBV as a model assay, a Q-LAMP EBV assay has been developed that is quantitative with a highly sensitivity, specificity and precision on four different matrices.

EBNA-1 gene has been identified as a potential target region of Q-LAMP assay, since it contains a high level of homology region between the two EBV subtypes.

The optimization has been carried out both on plasmids and on negative extracted matrices and the final assay, in a freeze-dried format, has been validated on clinical samples. Thanks to the multiplex format it has been possible to introduce in the reaction a control that allows the assessment of the sample extraction and the validation of negative results. The amplification and the detection of the target and the internal control occur in real-time in a single tube.

To evaluate the diagnostic performance and the feasibility in clinical practice, the assay has been validated on positive and negative clinical samples compared with the standard method Real-Time PCR using as extraction method the Liaison IXT platform (DiaSorin). The correlation between the two methods on whole blood and plasma is acceptable and meets the requests of corporate guidelines, made in response to the market trend.

All these results have demonstrated that Q-LAMP technology can be used to be quantitative and Q-LAMP EBV assay is suitable for industrialization as a future diagnostic product.

INTRODUCTION

1. Epstein-Barr virus

Epstein-Barr virus (EBV) was discovered as a result of pioneering work in the 1950s, by Denis Burkitt. Who identified a previously unrecognized form of cancer which affected the jaws of young African children. Burkitt made the crucial insight that the distribution of this common tumor (now known as Burkitt's lymphoma) appeared to be influenced by climatic factors (in particular temperature and altitude). Burkitt theorized that the tumor might be due to a mosquito-born virus.

This discovery led Tony Epstein, Yvonne Barr and Burt Achong to examine freshly excised tumor biopsies for the presence of a virus. In 1964, using electron microscopy, they found herpesvirus-like particles in a small number of the biopsied cells, and they subsequently established that this was a new virus. Epstein-Barr virus was thus identified as the first candidate human tumor virus (Sugden, Sem. Virol. 5:197-205, 1994; Kieff, Chapter 74 of Fields' Virology, 3rd Edition Rickinson and Kieff, Chapter 75 of Fields' Virology, 3rd Edition).

EBV, also called human herpesvirus 4 (HHV-4), is a double-stranded DNA virus that infects B cells. The genome is about 172 kb in length and encodes more than 85 genes. EBV is classified into two subtypes: Type 1 / A and 2 / B based on the sequence in EBNA genes (Farrell et al., 2005; Rickinson and et al., 2007;Thompson et al., 2004). Initial classification was based on

differences in EBNA-2 identified in EBV isolates from lymphoblastoid cell lines. Additional differences between types 1 and 2 have been reported, including mismatched base pairs in genes encoding EBNA-3A, -3B and -3C (Sample et al., 1990). EBV Type 1 is more prevalent in Caucasian (74%) and in Asian (85%) populations. Type 2 is prevalent in Africa with 24% positive population. (Srivastava et al., 2000; Tiwawech et al., 2008; Zhou et al., 2001; Young et al., 1987; Chang et al.,2009).

1.1. EBV genes

EBV infects more than 90% of the world's population. Like all herpes viruses it is able to persist in the host for whole life as a silent infection (Crawford et al.,2001).

During this stage, EBV expresses a restricted set of genes, 2 EBV-encoded RNAs (EBER-1 and EBER-2), 6 EBV nuclear antigens (EBNA-1, EBNA-2,EBNA-3A, -3B and -3C, leader protein (EBNA-LP)), and 3 integral membrane proteins (LMP-1, LMP-2A and -2B) (Klein et al.,2010; Chang et al.,2009).

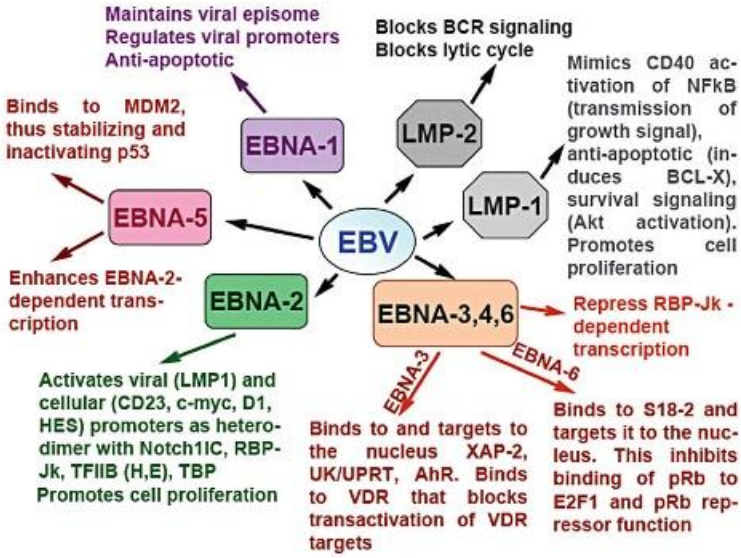


Figure 1: The main functions of the EBV-encoded proteins expressed in latent infection of B-lymphocytes (Klein et al., 2010)

During primary infection, latently infected B lymphoblasts circulate in blood and lodge in distant organs until they are controlled by the developing immunity. At this stage the natural, subclinical, childhood primary infection probably merges imperceptibly into lifelong persistence, but in older age the induction of the immune response often heralds immunopathological diseases (Crawford et al.,2001).

EBV genetic diversity has been identified in three gene regions: LMP-1, EBNA-1 and BZLF-1. They are the most frequently studied regions to date. In particular, LMP-1 has been studied extensively because it is thought to play a significant role in tumorigenesis. EBNA-1 because it is required for

maintenance of EBV in its latent form and is expressed in all EBV-associated tumors. EBNA-1 is unable to elicit an effective cytotoxic immune response, thus explaining partially why EBV is never eliminated from the body. BZLF-1 because it plays an important role in the switch from latent to lytic EBV infection (Crawford et al., 2001; Gulley et al., 2008).

1.2. EBV associated diseases

Although most primary EBV infections are asymptomatic, the virus is the causative agent of infectious mononucleosis, a mild and self-limited lymphoproliferative disease. EBV, like all herpes viruses, is able to persist in the host for whole life, but in the vast majority of healthy carriers the virus causes no disease. This is due to a delicate balance maintained between the host immune system, which limits production of virus particles, and the virus, which persists and is successfully transmitted in the face of host antiviral immunity. Disruption of this balance, resulting from primary or acquired immunodeficiency, may lead to the development of EBV-associated disease. EBV is associated with a wide variety of malignant diseases such as lymphomas, post-transplant lymphoproliferative disorders (PTLD), immunodeficiency-related disorders (Hodgkin's disease, and AIDS-related lymphoma), nasopharyngeal carcinoma (NPC) and gastric carcinoma (Williams et al.,2006; Gärtner et al., 2010; Gulley et al., 2008; Ruiz et al., 2005).

1.3. EBV diagnosis

Various laboratory tests have been used to diagnose EBV infection. In addition to tests for other diagnostically useful parameters (leukocytosis, lymphocytosis with atypical lymphocytes, abnormal liver function test, etc.), there are tests for detecting non-specific heterophilc antibodies and specific anti-EBV antibodies, as well as molecular biology methods used to detect EBV DNA.

The detection of antibodies is less useful in immunocompromised patients because of their immune system dysfunctions, and the fact that the type of antibody and its maintenance may vary over time depending on the dynamics of the disease, thus leading to atypical profiles. The variability observed in different patients (this may be complicated by the therapeutic use of immunoglobulins) indicated that a search for EBV DNA by molecular biology methods is useful for the diagnosis and follow-up of patients at risk of developing EBV-related lymphoproliferative disorders (De Paschale et al., 2012; Holmes et al., 2002). Serology is generally not decisive also in patients with EBV-associated tumors and a search for EBV DNA is essential (De Paschale et al., 2012).

1.3.1. Serological tests

Serological tests confirm primary infection and document remote infection. It is generally easy to distinguish acute and past infections in immuno-competent patients using only three parameters (Viral Capsid Antigen-IgG (VCA-IgG), VCA IgM and EBNA-1 IgG). The presence of VCA IgG and VCA IgM

in the absence of EBNA-1 IgG indicates acute infection, and the presence of VCA IgG and EBNA-1 IgG in the absence of VCA IgM is typical of past infection (De Paschale et al., 2012).

The most widely used serological assay, the heterophile antibody test, was first introduced in 1932, well before EBV was identified as the causative agent of infectious mononucleosis. Heterophile antibodies are antibodies that agglutinate cells of other animal species that are mainly associated with mononucleosis due to EBV but may be, albeit rarely, also detected in other diseases.

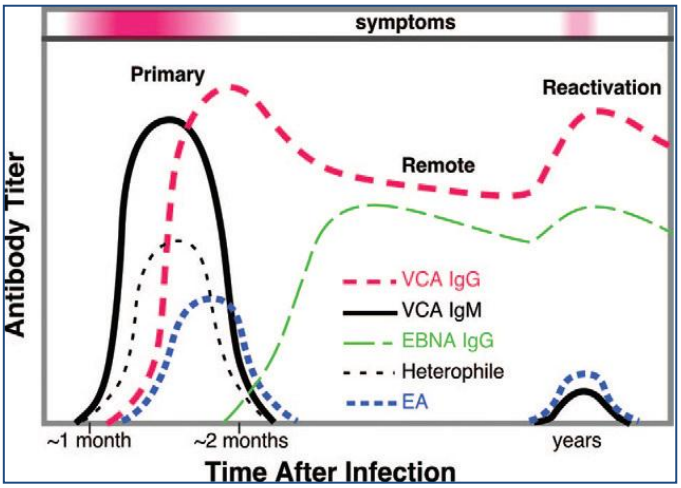


Figure 2: Serological titers distinguish primary infection from remote infection. IgG anti-VCA and IgM anti-VCA rise in concert with symptoms of primary infection and a positive heterophile test. After symptoms resolve, remote infection is characterized by EBNA and IgG anti-VCA without Early Antigen (EA), although EA and IgM may reappear with or without symptoms on viral reactivation or EBV-related neoplasia.

However, given the level of false negative results a search for specific antigen is useful. The specific tests for anti-EBV antibodies use different substrates or antigens and various technologies. Those commonly used for the routine screening of Early Antigen IgG (EA IgG), EBNA-1 IgG, VCA IgG and IgM are immunofluorescence assays (IFAs) or enzyme immunoassay (EIAs) with enzyme-linked immunosorbent assays (ELISA) and chemiluminescence immunoassay (CLIA) versions or newer multiplex flow immunoassay (MFI).

Serological assay remains the gold standard for diagnosis of primary EBV infection in immunocompetent patients. Nonetheless, despite the availability of several different serological markers, serology is not always able to accurately determine the stage of infection since false positive and false negative results are regularly observed (Gulley et al.,2008; Gartzonika et al., 2012; Matheson et al., 1990).

1.3.2. Molecular Biology tests

There is a continuous quest for novel diagnostic methods that can enhance accuracy, including molecular assays as EBV Polymerase Chain Reaction (PCR). During the past decades, various forms of PCR such as multiplex PCR and RT-PCR, have been developed to address the need for rapid identification of viruses. It has been suggested that quantitative Viral Load (VL) assessment is superior to qualitative detection. Quantitative EBV DNA measurement is essential for differentiating the low-level infection of healthy carriers from the high levels characteristic of EBV-related disease. Patients with active infection or EBV-related cancer tend to have high levels

of EBV DNA in the cell-free fraction of blood (plasma or serum), whereas in healthy carriers the virus is restricted to the intracellular compartment of the blood.

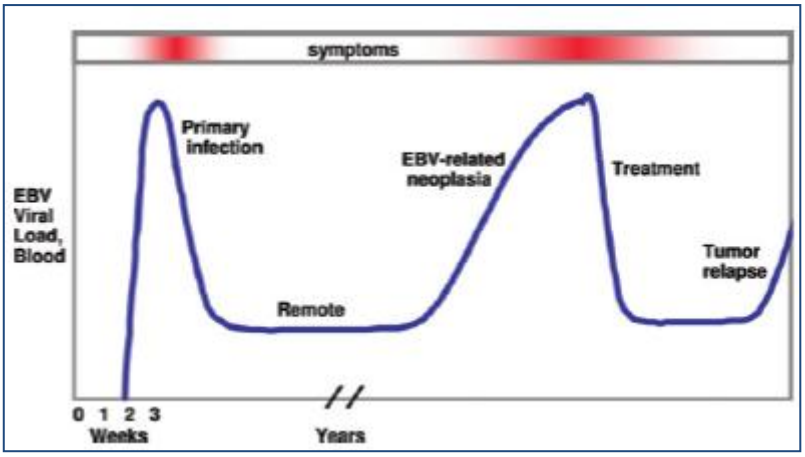


Figure 3: EBV viral load in whole blood reflects clinical status in patients with infectious mononucleosis, allogeneic transplant, and nasopharyngeal carcinoma. EBV DNA levels begin to rise within 2 weeks of primary infection and are already falling by the time the patient becomes symptomatic (due to interferon γ and other immune responses). Plasma or serum EBV DNA is undetectable in most remotely infected individuals; however, whole blood is low positive for the duration of life. If an EBV-related malignancy develops, levels may rise before clinical diagnosis, implying that high-risk patients benefit from routine monitoring. Successful therapy is marked by a decline to baseline, and rising levels may serve as a harbinger of relapse.

However, there is still no consensus concerning the best material to use, units of measurement, or the quantitative levels requiring intervention or

predicting prognosis (Gulley et al., 2008; Preiksaitis et al., 2009; Hayden et al., 2008). This means that particular care is necessary when comparing the data of different studies: for example, the units of measurement include copies per milliliter, copies per microgram of DNA, copies per 100,000 leukocytes, and copies per positive cell. The targets used may also vary from one method to another: LMP2, BKRF1 or EBNA-1, BNRF1 (membrane protein), BXLF1 (thymidine kinase), BZLF1 (ZEBRA), BALF5 (viral DNA polymerase) or BHRF-1 (transmembrane protein). In general, the best material used to search for EBV DNA depends on where it is, and varies during the course of the disease. The virions produced during primary infection spread in peripheral blood, and it is also possible to determine the EBV-free or fragmented DNA coming from apoptotic cells , and the B cells transformed during the latent phase also pass into the bloodstream. After the initiation of an immune response, viral load decreases slowly in PBMCs, but rapidly in plasma/ serum, and it becomes undetectable after 3-4 weeks, whereas memory cells with EBV may remain latent for a long time in blood.
The quantification of EBV DNA loads in blood, plasma, serum and CSF has a very important role, since it is primarily used for detecting EBV reactivation in immuno-compromised individuals, monitoring of response to therapy and development of antiviral resistance (Cesaro et al., 2005; Yang et al., 2000; Kanakry et al., 2016; Meerbach et al., 2008; Van Esser et al., 2001). Real-time PCR is a more rapid, sensitive, specific and reproducible method for detecting and monitoring the levels of EBV in comparison to conventional PCR (Niesters et al., 2000). Laboratory tests for EBV have improved and are increasingly used in diagnosis, prognosis, prediction, and

prevention of diseases ranging from infectious mononucleosis to selected subtypes of lymphoma, sarcoma, and carcinoma. Indeed, the presence of EBV is among the most effective tumor markers, supporting clinical management of cancer patients (De Paschale et al., 2012; Gulley et al., 2008).

The range of Nucleic Acid Amplification Technology (NAT) assays used in the diagnosis and management of EBV-associated diseases varies significantly. Laboratories use a range of DNA extraction and amplification methodologies including commercial kits, or analyte-specific reagents (ASR), and laboratory developed assays which differ in the reagents (including primers and probes) and instrumentation used. In addition, the quantification controls used to determine the concentration of viral DNA present vary. These may comprise either a plasmid clone of the PCR target, quantified viral DNA or virus particles, or cells containing specific copy numbers of the EBV genome, and may or may not be included in the extraction step. Consequently, results may be reported as EBV copies/mL, copies/µg, or copies/number of cells, depending on which control is used. Given the heterogeneity of these NAT-based assay systems, it is difficult to compare viral load measurements between different laboratories and to develop uniform therapeutic strategies. These highlighted the need for an internationally-accepted reference standard for EBV. In the absence of such a standard, individual centers apply their own management algorithms and these vary between centers. The WHO's Expert Committee on Biological Standardization (ECBS) establishes reference standards for biological substances used in the prevention, treatment or diagnosis of human

disease. WHO International Standards are recognized as the highest order of reference for biological substances, and are arbitrarily assigned a potency in International Units (IU). Their primary purpose is to calibrate secondary references used in routine laboratory assays, in terms of the IU, thereby providing a uniform result reporting system, and traceability of measurements, independent of the method used. The 1st WHO international standard for EBV was established in October 2011 from the National Institute for Biological Standards and Control (Hertfordshire, United Kingdom). The international standards is represented by EBV B95-8, with an assigned potency of 5×10^6 International Units (IU) (NIBSC code 09/260). It is intended to be used for the calibration of secondary reference materials, used in EBV NAT assays, in IU, thereby improving the comparability of patient viral load measurements (Fryer et al.,2011).

2. Loop-mediated Isothermal Amplification (LAMP)

The Loop-mediated isothermal AMPlification (LAMP) technology is an innovative non-PCR based nucleic acid amplification method performed using a strand-displacement polymerase under isothermal conditions. Both amplification and detection of nucleic acid sequences can be completed in a single step by incubating the mixture of sample, primers, DNA polymerase at a constant temperature, allowing the use of simple and cost effective reaction equipments (Notomi et al., 2000).

The amplification efficiency of LAMP is very high and the reaction proceeds rapidly as there is no need for initial heat denaturation of the template DNA or thermal cycling.

LAMP has been explored so far in hundreds of papers for the detection of DNA of many different organisms, such as bacteria (Nakao et al., 2010; Yang et al., 2009; Aryan et al., 2009), viruses (Kaneko et al., 2005; Yoda et al., 2009; Hong et al., 2004; Curtis et al., 2009), fungi (Sun et al., 2009; Uemura et al., 2008), parasites (Chen et al., 2009; Zhang et al., 2009; Nkouawa et al., 2009). LAMP is also suitable for the analysis of human DNA (Minnucci et al., 2012). Thanks to its peculiar characteristics, LAMP has shown to be an easy and rapid diagnostic tool in molecular medicine.

Moreover a Reverse Transcription (RT) LAMP has been developed for the detection and amplification of RNA by introducing a reverse transcriptase in the reaction mix, expanding the fields of applications for this technique (Parida et al., 2004; Ushio et al., 2005).

Thus, LAMP constitutes an attractive alternative to PCR for sequence detection, with a sensitivity and quantitative performance comparable to PCR, allowing different detection methods, simplex and multiplex reaction applications in a fast, easy and potentially cheap molecular diagnostics platform (Parida et al., 2008).

2.1. LAMP principle

LAMP is an isothermal DNA amplification method which relies on the use of a thermostable DNA polymerase with strand displacement activity (*Bst* polymerase from *Bacillus stearothermophilus*) and four primers specifically designed to recognize six distinct regions on the target gene, consisting in a pair of outer primers, F3 and B3, and a pair of inner primers, Forward Inner Primer (FIP) and Backward Inner Primer (BIP).

Both inner primers contain two distinct sequences (F2 + F1c for the FIP primer and B2 + B1c for the BIP primer), corresponding to the sense and antisense sequences of the target DNA, one for the initial priming and the other for the subsequent self-priming.

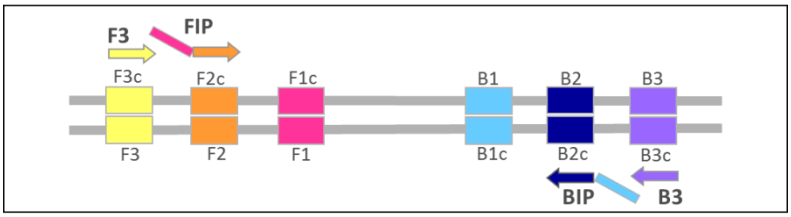


Figure 4: Schematic representation of the four primers, recognizing six distinct nucleotide sequences on the target gene, required for a basic LAMP reaction: two internal primers (FIP, BIP) and two external primers (F3, B3).

The mechanism of the LAMP amplification reaction includes three steps:

I. production of starting structure;
II. cycling amplification;
III. elongation and recycling.

All four primers are operative in the initial steps of the reaction, but in the later cycling steps only the inner primers are required for strand displacement DNA synthesis.

At a constant reaction temperature, inner primer FIB hybridizes to F2c in the target DNA and initiates complementary strand synthesis (structure 1). Outer primer F3 hybridizes to F3c in the target and initiates strand displacement of the new DNA chain (structure 2), releasing a FIP-linked complementary strand, which forms a looped-out structure at one end

(structure 4). This single stranded DNA serves as template for BIP-initiated DNA synthesis and subsequent B3-primed strand displacement DNA synthesis leading to the production of a dumbbell form DNA which is quickly converted to a stem-loop DNA (structure 6).

The dumbbell serves as the starting structure for LAMP exponential amplification, the second step of the LAMP reaction. The stem-loop DNA structure contains a double stem-loop which is the starting point for the whole LAMP amplification process as it promotes an amplification from its self-annealed 3' terminus and from a newly annealed internal primer (FIP or BIP) (structure 7). During amplification, FIP hybridizes to the loop in the stem-loop DNA and primes strand displacement DNA synthesis, generating as an intermediate one gapped stem loop DNA with an additional inverted copy of the target sequence in the stem, and a loop formed at the opposite end via the BIP sequence (structure 8). Subsequent self-primed strand displacement DNA synthesis yields one complementary structure of the original stem-loop DNA and one gap repaired stem-loop DNA (structure 10) with a stem elongated to twice as long and a loop at the opposite end (structure 9).

Both of these products then serve as templates for BIP-primed strand displacement in the subsequent cycles and in the elongation and recycling step. Thus, in LAMP the target sequence is amplified 3-fold every half cycle.

Amplification proceeds promoting itself, each strand being displaced by elongation of the new loops formed (Notomi et al., 2000).

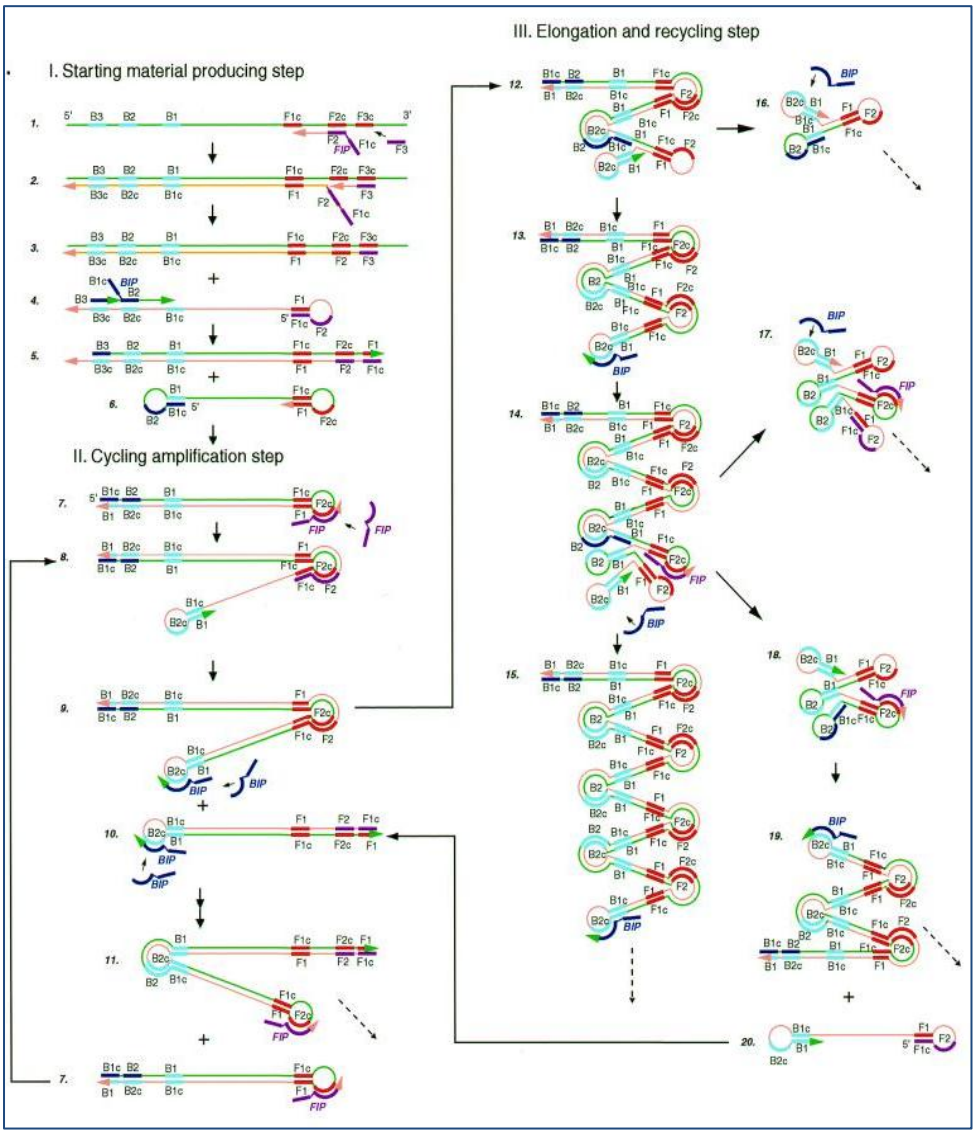


Figure 5: Schematic representation of the steps of a LAMP reaction. This figure shows the process that starts from primer FIP. DNA synthesis can also begin from primer BIP.

The final product is a mixture of stem-loop DNA with various stem length and cauliflower-like structures with multiple loops formed by annealing between alternately inverted repeats of the target sequence in the same strand.

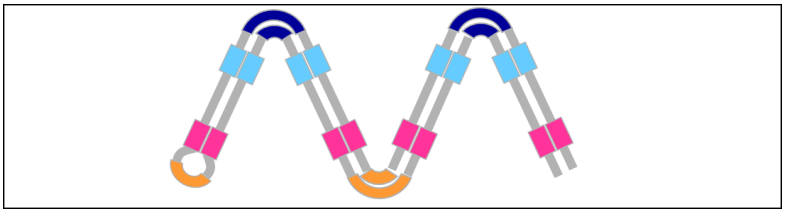


Figure 6: Schematic representation of inverted repeats concatamers that are the main amplification products of a LAMP reaction.

2.2. Loop primers

In order to accelerate the LAMP reaction, it is possible to introduce additional primers, called Loop Primers.

The Loop Primers LF (Loop Primer Forward) or LB (Loop Primer Backward) contain sequences complementary to the single stranded loop region (either between the F1 and F2 regions or between the B1 and B2 regions) on the 5' end of the dumbbell-like structure, providing increased number of starting points for DNA synthesis.

Loop primers hybridize to the stem-loops and prime strand displacement DNA synthesis.

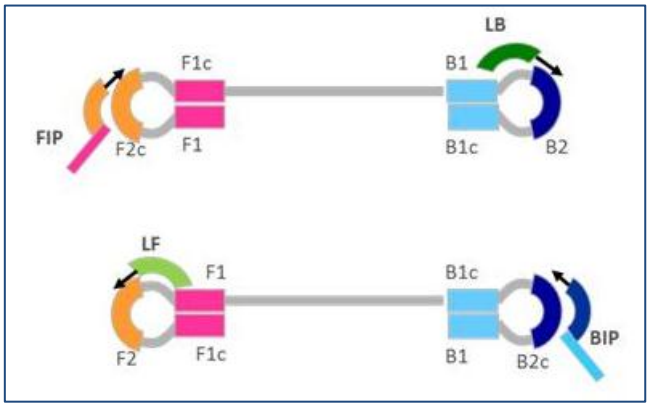


Figure 7: Schematic representation of the two stem loop structures (also called "dumbbell" structures) generated during a LAMP reaction: FIP and LB primers will hybridize on one dumbbell type, while BIP and LF primers will hybridize on the other dumbbell type.

The loop primers act as catalyzers of the amplification reaction, since their presence increases the rate of DNA production and therefore the sensitivity of the method, considerably decreasing the reaction time of the original LAMP method (Nagamine et al., 2002).

2.3. Detection of LAMP amplification products

Two principal methods have been widely used to detect LAMP amplification products and distinguish between positive and negative samples: turbidimetry or fluorescence.

Turbidimetry detection is made possible by the high amplification efficiency of LAMP reaction (Mori et al., 2004; Mori et al., 2001). This detection method consists in the measurement of turbidity of the reaction mix, due to the precipitation of magnesium pyrophosphate. This insoluble salt is formed by the interaction between Mg_{2+} present in solution and the inorganic pyrophosphate produced by the incorporation of dNTPs in the DNA growing strands. Thus, the amount of magnesium pyrophosphate is proportional to the amount of amplified DNA. The turbidity is visible by the naked eye and is measurable on a turbidimeter as a transmittance signal either at end-point for a qualitative analysis or in real-time, allowing quantitative applications.

Another approach for the detection of LAMP reaction products is represented by the use of **fluorescent intercalating dyes** that bind double-stranded DNA: they allow a real-time direct visualisation of the isothermal amplification by measuring the increase in fluorescence throughout the reaction correlated to the exponential synthesis of LAMP amplicons. The detection step has then to be followed by an annealing analysis to confirm the reaction product.

However, turbidimetry and fluorescent intercalating dyes are not very specific detection approaches, because they allow to detect double–stranded DNA without easy and rapid discrimination between specific and not specific products.

Other fluorescence approaches are being applied to LAMP technique, such as molecular beacons or quenching probes, relying on sequence-specific real-time detection of a desired LAMP product. The use of **fluorescent–**

labelled target-specific **probes** results in increased specificity and sensitivity. In addition, the use of different fluorophores specific for distinct targets allows to potentially amplify many sequences simultaneously.

2.4. Advantages of LAMP

The primary characteristic of LAMP is the ability to amplify nucleic acids at isothermal conditions, allowing the use of simple and cost-effective reaction equipments.

The use of a polymerase with strand-displacement activity increase exponentially the speed of reaction, unbinding the amplification to the extension step, as instead happens for PCR. This high efficiency of LAMP reaction also determines a deep sensitivity, comparable to that of nested PCR.

The multiplex format of Q-LAMP, makes possible the introduction of the internal control, and specific identification of different targets.

All these characteristics have made LAMP a very useful method for the detection of many different organisms, such as bacteria, viruses, fungi and parasites.

DiaSorin wanted to exploit all these advantages for develop a quantitative Q-LAMP using EBV as a model assay, able to detect and quantify the two most clinically relevant subtypes Type 1/A and 2/B.

MATERIALS AND METHODS

1. EBV sequence alignment

The NCBI database has been used to search for the reference strain sequences of EBV (*Human Herpesvirus 4)* Type 1 (NCBI accession number AJ507799) and Type 2 (NCBI accession number DQ279927). Different genes have been evaluated, performing a sequence alignment using the EMBL-EBI Clustal Omega tool, in order to assess the suitability for their use in the Q-LAMP assay. In particular 6 genes have been studied (BamHI W, EBNA-1, EBNA-2, LMP-1, LMP-2 and BZLF1) for both Type 1 and Type 2 EBV virus. The aim has been to find in both types a gene with an homologous region long enough to allow the Q-LAMP primer design. A high level of homology between the two strains on EBNA-1 gene has been identified as a potential target region for the Q-LAMP assay. To prevent the risk of not detecting both strains of EBV a nucleotide BLAST analysis in the NCBI website, using EBNA-1 gene sequence for EBV Type 1 and Type 2 has been performed, with the following parameters:

- Search set: nucleotide collection nr/nt
- Max target sequences: limited to 20000 results

From the results have been excluded: all the sequences that are not related to EBV and synthetic plasmid sequences.

2. cDNA plasmids

For the development of the EBV Q-LAMP assay has been necessary the use of plasmids containing the sequences of the EBNA-1 gene and of the negative control T4 phage. The size of the insertions for EBNA-1 and T4 phage were respectively 937 and 642 bp.

Plasmids have been produced by Life Technologies (GeneArt® Gene Synthesis). The DNA sequence was assembled from synthetic oligonucleotides, the fragment was cloned into pMK using PacI and AscI cloning sites. The plasmid DNA was purified from transformed bacteria and concentration determined by UV spectroscopy. The final construct was verified by sequencing. The sequence congruence within the used restriction sites was 100%.

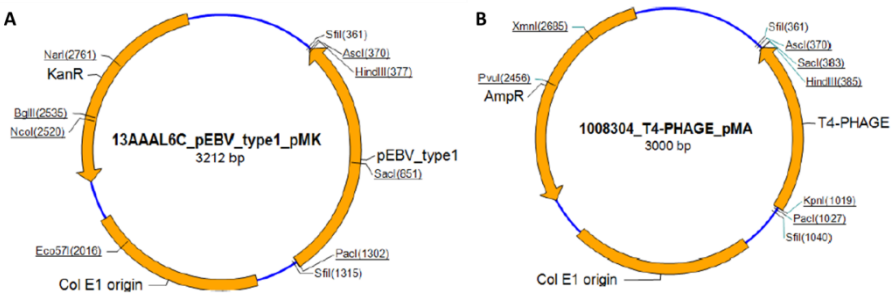


Figure 8: Plasmid's map provided by Life Technologies for EBNA-1 (A) and T4 bacteriophage (B).

The copy number per µL of each plasmid has been calculated based on the length and on the concentration. Plasmids have been diluted into a buffer containing Tris-HCl and Yeast RNA.

In order to assess the sensitivity, plasmids have been denatured by heating at 95°C for 10 minutes and immediately chilled on ice to prevent re-naturation.

In the final format of the assays the denatured plasmids have also been used as high and low calibrators and as negative control.

3. Clinical Samples

The final evaluation of relative sensitivity has been performed on 44 positive plasma and 45 positive whole blood clinical samples, previously tested with PCR. Relative specificity has been tested on 12 negative plasma and 27 negative whole blood clinical samples. These clinical samples were collected at Bologna Hospital from subjects who gave their informed consent.

4. NIBSC WHO International Standard (IS)

The 1st WHO International Standard for Epstein-Barr virus (EBV), NIBSC code 09/260, is intended to be used in the standardization of Nucleic acid Amplification Technique (NAT)-based assays for EBV. The reference comprises a whole virus preparation of the EBV B95-8 strain (type 1) (NCBI reference sequence: NC_007605.1), formulated in a universal buffer

comprising Tris-HCl, human serum albumin (HSA) and trehalose. The material has been lyophilized in 1 mL aliquots and stored at -20 °C. The material has been evaluated in a worldwide collaborative study involving 28 laboratories performing a range of NAT-based assays for EBV.

This material has been assigned a concentration of $5x10^6$ International Units (IU) when reconstituted in 1 mL of nuclease-free water, based on the results of a worldwide collaborative study. The assigned unitage could have a variability equal to +/- 23% due to the vial content and not to its calibration.

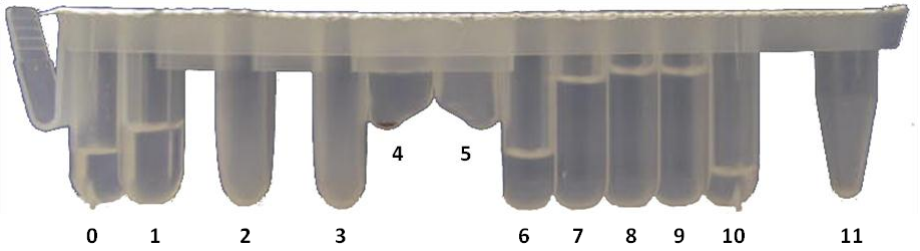
Each vial contains the lyophilized equivalent of 1 mL of EBV B95-8 strain (type 1) in 10 mM Tris buffer (pH7.4), 0.5% HSA, and 0.1% trehalose.

The IS should be used to calibrate secondary reference materials, for example, by determining the equivalent concentration of secondary reference reagent to be calibrated, against the International Standard, in parallel. The secondary reference reagent can then be assigned a concentration in terms of IU. Once reconstituted, the International Standard should be diluted in the matrix more appropriate for the material to be calibrated, and should be extracted prior to EBV DNA measurement.

For Q-LAMP EBV assay IS has been used to evaluate the LIAISON® Ixt extraction protocols, the assay sensitivity, determination of the Conversion Factor (CF) and to generate the EBV calibration curve.

5. LIAISON® Ixt platform

The extraction method is based on the LIAISON® Ixt platform (developed by DiaSorin). It is an automated, flexible and compact instrument for high quality DNA/RNA extraction and cell separation from a variety of specimen sample types. This extraction method is based on unique single use pump-tip device and cartridges containing magnetic beads.



Tube/Well	Content
Well 0:	Lysis/Binding buffer
Well 1:	Isopropanol
Well 2:	Working well
Well 3:	Empty
Well 4:	Beads
Well 5:	Empty
Well 6:	Lysis/Binding Buffer
Well 7:	70% EtOH
Well 8:	ddH2O
Well 9:	ddH2O
Well 10:	Elution buffer (10 mM Tris pH8)
Well 11:	Empty

Figure 9: LIAISON® Ixt DNA Cartridge filling

Extraction steps are described below:

1. Mount pumps on pipette tips and place in instrument.

2. Place the LIAISON® Ixt DNA cartridges in the tray, and then remove the seals.
3. Place sample tubes containing sample in the tray
4. Place empty tubes for eluate in the tray
5. Start protocol
6. Choose sample elution depending on the matrix used

Protocols run for 40-60 minutes depending on the protocol used.

5.1. DNA extraction protocols

Different protocols and chemistries have been evaluated for DNA extraction, depending on the matrix to be extracted: plasma, serum, Cerebrospinal Fluid (CSF) and whole blood. The protocols differ in: quantity and size of the magnetic beads, concentration of the buffers, time and temperature of incubation and power used by the piston on pumps.

For whole blood sample, a protocol with a concentration factor equal to 1x has been used. Sample volume: 200 µL + 50 µL Proteinase K (PK)+ T4 bacteriophage (Internal Control (IC)).

For plasma, serum and CSF samples, a protocol with a concentration factor equal to 4.8x has been used. Sample volume: 480 µL + 20 µL PK + IC.

Both protocols run for 50 minutes.

In order to assess the sensitivity, extracted DNA has been denatured by heating at 95°C for 10 minutes and immediately chilled on ice to prevent re-naturation.

6. Detection of EBV by PCR

The *artus* EBV RG PCR Kit (Qiagen) has been used as predicate device.

It is an in vitro nucleic acid amplification test for the quantification of Epstein-Barr virus (EBV) DNA in human plasma, serum, CSF, or blood cells. This diagnostic test kit utilizes the polymerase chain reaction (PCR) and is configured for use with Rotor-Gene Q Instruments. The EBV RG Master contains reagents and enzymes for the specific amplification of a 97 bp region of the EBV genome, and for the direct detection of the specific amplicon in fluorescence green channel.

The *artus* EBV RG PCR Kit has been performed in according to the protocol provided by the supplier.

7. Q-LAMP EBV assay

7.1. Primer design

A LAMP reaction employs at least 4 primers, which recognize 6 different regions on the target gene. F3 and B3 are the most external primers, which allow the strand-displacement activity of the enzyme at the beginning of the reaction. The inner primers are Forward Inner Primer (FIP) and Backward Inner Primer (BIP), made by 2 parts (F1 and F2; B1 and B2), respectively complementary to the sense and antisense sequence. This characteristic allows the self-annealing of the single strand product and the formation of the stem-loop structure, which represents the starting point for the LAMP reaction.

In addition, 2 more primers, Loop Forward (LF) and Loop Backward (LB), that anneal to the single strand sequence of the loops, can be employed in order to increase the speed of amplification.

In this work a fluorescent probe has been introduced, substituting one of the loop primers, which allows the real time detection of the amplification.

The primer sets for EBV and IC have been initially designed manually, analysing them with the software *Visual OMP* (DNASoftware, Ann Arbor, USA). The characteristic of this software is to provide also predictions of secondary structures, which can help in excluding primer sets that can give primer dimers amplification.

It performs the calculation of Tm by the "nearest neighbour" method, taking also in consideration the different factors influencing the reaction, such as temperature, primers, salts and glycerol concentration.

7.2. Primer sets selection

In silico selection has been based on the absence of primer dimers and non-specific binding to other portions of the target sequence.

Experimental selection has been performed testing each primer set at standard conditions on the specific plasmid (10,000, 1,000, 100 and 10 copies/reaction) to test the speed of amplification and on water samples to test the specificity. The primer sets that passed the selection had at least 98% of specificity and the amplification of the plasmids should be completed within 60 minutes.

7.3. Labelled probes

In the final set, one of the loop primers, has been substituted with a fluorescent probe that has the same sequence, but cannot be extended and is conjugated with a fluorophore. It allows the real time detection of the amplification and the discrimination between targets (EBV and IC).

For the assay, two different customized fluorophores have been used, specifically read by the channels of the Liaison IAM instrument. Customized fluorophores were produced by Cyanagen (Bologna, Italy), with different emission wavelength so that they could be read in two different channels of Liason IAM instrument without cross-talk effect.

For the duplex reaction EBV has been labelled with CHROMIS 500 and T4 with CHROMIS 530. CHROMIS 500 (C500) has an absorption wavelength of 496 nm and emits at 506 nm, CHROMIS 530 (C530) has an absorption wavelength of 529 nm and emits at 561 nm.

The primers and probes have been synthesized by SGS DNA.

7.4. Q-LAMP reaction

The Q-LAMP reactions with labelled probes have been performed on Liaison IAM instrument (DiaSorin) at 65°C for 60 minutes using standard reaction conditions.

Each reaction mixture contained the primer set specific for the target (F3/B3 0.2 µM, FIP/BIP 1.6 µM, LF or LB 0.8 µM) and the specific probes 0.8 µM, dNTPs 1.4 mM each, 4 mM $MgSO_4$, Tris-HCl 20 mM, KCl 10 mM, $(NH_4)_2SO_4$10 mM, tween 20 0.1%, enzyme 0.32 U/µL, 20 µL of plasmid and

distilled water (up to the final volume of 50 µL). Q-LAMP EBV assay with labelled probes were performed on Liason IAM instument (DiaSorin) at 64°C for 60 minutes using optimized LAMP reaction conditions. Each reaction mixture contained LAMP reagents, 20 µL of extracted sample and distilled water (up to the final volume of 50µL).

8. LIAISON Instrument

The Liaison IAM instrument has been specifically developed by DiaSorin for the execution of the Q-LAMP assays. It performs isothermal amplification and detection of nucleic acids, by real-time measuring of changes in fluorescence in up to three channels.

Liaison IAM detection system consists of three LED for excitation and three photodiodes for detection of three fluorescence channels. 8 optical fibres (one for each well) transmit the light for excitation of the fluorophores to the wells and consequently conduct the light emitted by the reaction for the elaboration.

The instrument is integrated with a *Liaison IAM Software* that recognises in which channel occurs the amplification and, according to the specific assay performed, is able to return a clear and objective result about the state of the patient (positive or negative) and, if positive, which specific transcript has been identified.

9. Freeze-drying

The freeze drying process has been performed on VirTis AdVantage Plus (SP Scientific) freeze-dryer.

The protocol consists of different phases that have been optimized during the development of the final products:

1. freezing, in which the reaction mix is taken at low temperatures (< -35°C) in order to immobilize the material and define the structure that will be dried;
2. primary drying through sublimation. In this phase vacuum is needed to reach the triple point of water, when the sublimation can occur;
3. secondary drying, in which the temperature increases to 20°C and a further drying of the product is obtained by desorption.

In order to create the solid organized structure that will remain at the end of the process Dextran70 is added to the reagent mix.

The Q-LAMP EBV duplex assay reaction mix has been lyophilized into one single cake, containing dextran70 plus dNTPs, enzyme, primers and probes.

Once freeze-dried, the cakes can be very easily reconstituted adding the remaining element of the liquid reaction mix: LAMP buffer.

RESULTS

1. EBV sequence alignment

A review of Nucleic Acid Test (NAT) assays for the detection of EBV illustrated the use of one of the following gene: BamHI W, EBNA-1, EBNA-2, LMP-1, LMP-2 and BZLF1. The NCBI database has been used to search for the reference strain sequences of EBV (*Human Herpesvirus 4)* Type 1 and Type 2. For each of the genes listed above a sequence alignment using the EMBL-EBI Clustal Omega tool of Type 1 and Type 2 has been performed to assess the suitability for their use in the Q-LAMP assay. In particular both types must have a homologous region long enough to allow the Q-LAMP primer design, 6 primers that are able to recognize 8 target regions. A high level of homology between the two strains on EBNA-1 gene has been identified as a potential target region for the Q-LAMP assay. To prevent the risk of not detecting both strains of EBV a nucleotide BLAST analysis using EBNA-1 gene sequence for EBV Type 1 and Type 2 has been performed. The results demonstrated synthetic plasmid sequences that have been excluded from the analysis. The results demonstrated also several sequences with only 31% coverage. These have been included in the alignment in order to increase the variability has to be consider for Q-LAMP primers design.

2. Primer set design and screening

Several EBV Q-LAMP primers have been manually designed (120 primers) on a conserved region of the multiple sequence alignments of EBNA-1 in order to amplify Type1 and Type2 with the same efficiency. These primers have been combined to obtain 20 sets. Each primer set is constituted by a number of 5 primers: 2 external primers (F3, B3), 2 internal primers (FIP, BIP), 1 loop primer (LB) and a specific probe labeled with a green fluorochrome, a novel detection strategy developed in DiaSorin, which allows the real time fluorescence detection.

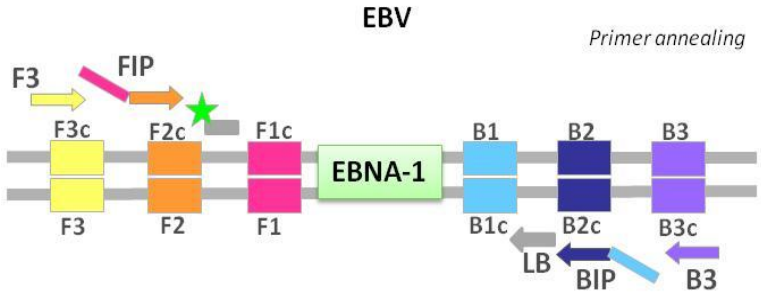


Figure 10: EBV primers design

2 external primers (F3, B3), 2 internal primers (FIP, BIP), 1 loop primer (LB) and a probe specific for the target (green labeled).

The use of the IC in Q-LAMP is very important to control the extraction process and to evaluate the presence of reaction inhibitors allowing to validate negative results.

The IC is made from T4 bacteriophage particles. T4 sequence has been taken as target for Q-LAMP primers manual design (90 primers). These primers have been combined to obtain 15 sets. As for the EBV target, each

primer set is constituted by a number of 6 primers: 2 external primers (F3, B3), 2 internal primers (FIP, BIP), 1 loop primer (LB) and a specific probe labeled with a yellow fluorochrome.

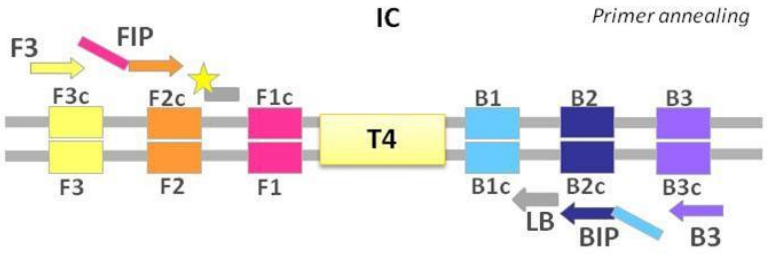


Figure 11: Internal control (T4) primers design

2 external primers (F3, B3), 2 internal primers (FIP, BIP), 1 loop primer (LB) and a probe specific for the IC (yellow labeled).

A next step in the primer selection has been the determination of melting temperatures of the primers and the study of interactions between primers using Visual-Omp software. The sets showing the presence of primer-primer interactions and potentially extending the 3' end, have been excluded.

After the *in-silico* screening, the remaining primer sets have been tested experimentally in the Q-LAMP reaction. The reaction conditions have been set according to Notomi et al. (Notomi et al.,2000). One plasmid containing the target region of EBV Type 1 and Type 2 and one plasmid containing the T4 target region have been generated to screen the primer set for EBV and IC.

Different EBV plasmid concentrations have been used to test the ability to detect low amount of plasmid (sensitivity) and the ability to quantify different amount of plasmid (precision).

The specificity has been evaluated on water samples for both primer sets and also on the T4 plasmid for EBV primer set, in order to mimic negative samples.

Only one primer set for EBV reached these characteristics and was therefore chosen as best candidate.

A summary showing the number of primer sets screened both *in-silico* and experimentally is shown in Figure 12.

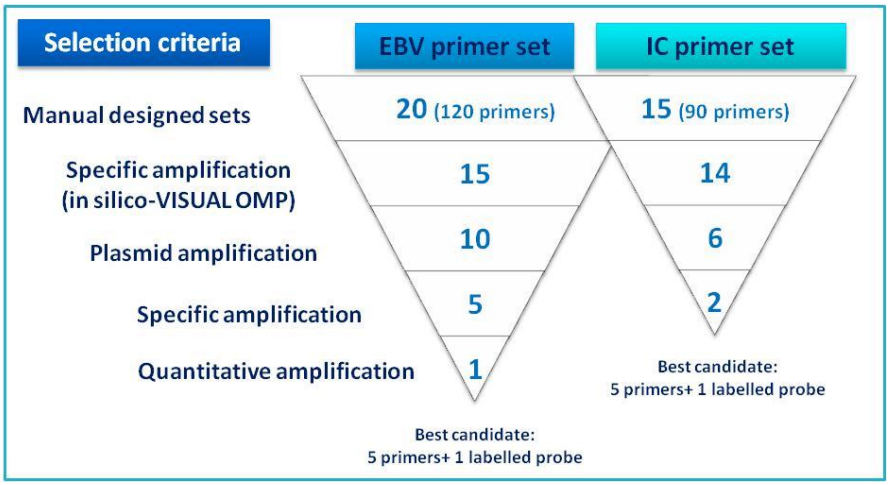


Figure 12: Primers selection

The pyramids show the number of primer sets successfully overcoming the different selection processes. The first step was a manual primer design: 120 primers for EBV has been designed and then combined together to obtain 20 primer set and 90 primers for T4 (IC) to obtain 15 primer sets. These primers have been analyzed *in-silico* with Visual OMP: 4 primer sets for EBV and 5 for IC have been excluded due to the presence of primer-primer interaction and potentially extending in the 3'end. Q-LAMP assay has been performed using standard conditions to evaluate the primer sets able to amplify plasmid and be specific and quantitative.

3. Cross- reactivity analysis

Sequence homology for each primer has been searched against all available human and viral sequences in the nucleotide BLAST database on the NCBI website. The cross reactivity analysis has been performed *in-silico* in order to assess the absence of primers annealing in not specific target region.

For all the 5 primers and 1 probe no cross reactivity has been found with Human DNA, Herpes viruses (HSV 1+2), Human herpesvirus 3, Human herpesvirus 5, Human herpesvirus 6, Human herpesvirus 7, Human herpesvirus 8, Human T cell leukemia virus Type 1 and Type 2 and Adenovius.

4. Q-LAMP EBV assay optimization

One primer set for the target and one for the IC have been selected to create a duplex assay (Q-LAMP EBV) able to amplify in the same reaction EBV and T4 respectively. The amplification of the IC has to occur only in negative samples in order to validate the results.

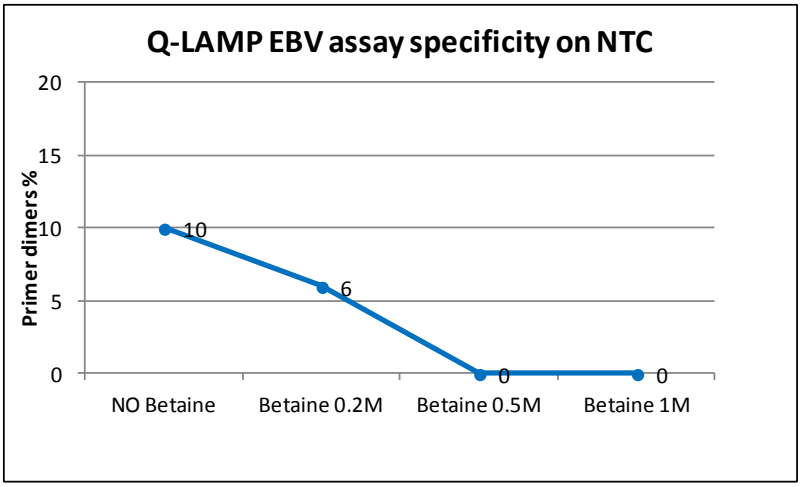
The optimization has been performed on different EBV plasmid dilutions, on water and negative matrices evaluating different parameters (i.e. primer concentration, temperature, etc.). The focus has been the ability to be specific, precise and, in terms of plasmid amounts, to be direct proportional to a Threshold Time (Tt).

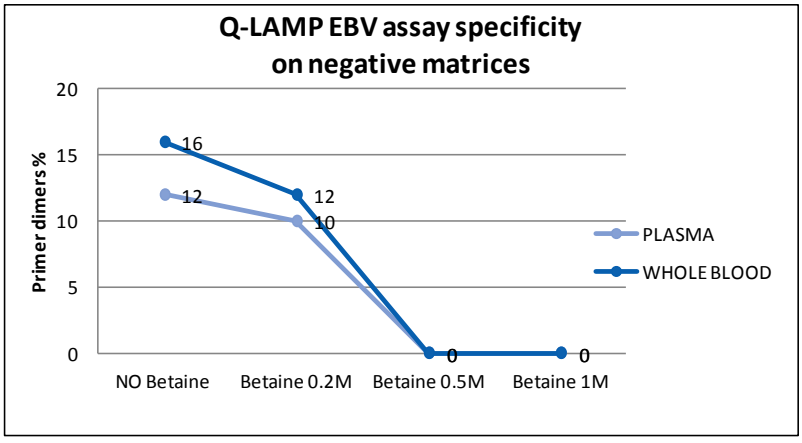
Despite the great number of reaction conditions tested, the optimization of the reaction by varying the standard parameters did not give good results in terms of precision and specificity.

Consequently has been explored the possibility to add enhancers in the reaction mix. A literature review has highlighted the use of betaine to have beneficial effects on some PCR amplifications, as for example increasing yield and specificity (S. Frackman Promega Notes Number 65, 1998, p.27).

To evaluate if these positive effects can be exploited also on a non-PCR method, Q-LAMP EBV assay has been tested with different concentrations of betaine (0M, 0.2M, 0.5M and 1M). The reaction has been performed at 64°C for 60 min on No Target Control (NTC, i.e. water samples) and negative matrices (plasma and whole blood) to evaluate specificity, and on different EBV plasmid dilutions (2e7cps/µL, 2e5cps/µL, 2e3cps/µL, 1e1cps/µL) to test precision and linearity. Without or with 0.2M of betaine the assay shows quite high level of primer dimers on both NTC and negative matrices. Also the linearity and precision are not optimal, with a R^2 between 0.81 and 0.95.

Using betaine at 0.5M has been observed an increased assay specificity, precision and linearity. Further increase of betaine concentration (1M) has shown the total absence of primer dimers on NTC and on negative plasma and whole blood. With betaine 1M, despite the good linearity , the assay shows to be less precise, with a higher standard deviation compared to betaine 0.5M.


Q-LAMP EBV assay specificity on NTC
Primer dimers %
20
15
10
5
0
10
6
0
0
NO Betaine
Betaine 0.2M
Betaine 0.5M
Betaine 1M


Q-LAMP EBV assay specificity on negative matrices
Primer dimers %
20
15
10
5
0
16
12
12
10
0
0
NO Betaine
Betaine 0.2M
Betaine 0.5M
Betaine 1M
PLASMA
WHOLE BLOOD

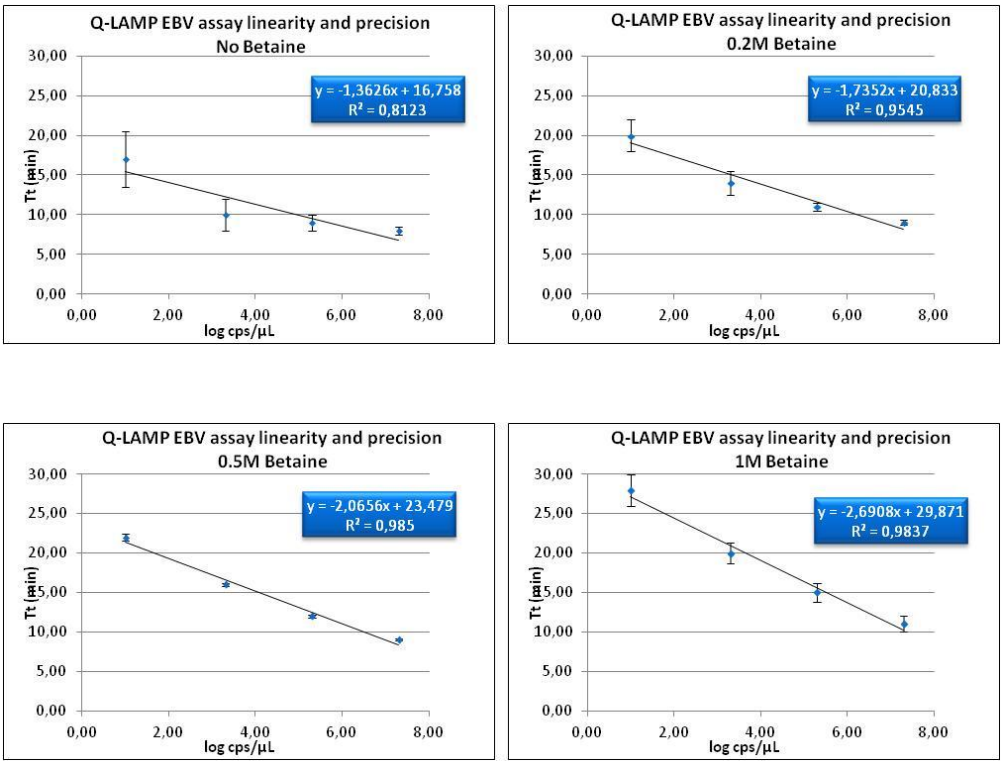


Figure 13: Effect of betaine concentration on the assay performance

Q-LAMP EBV assay has been tested with different concentrations of betaine (0M, 0.2M, 0.5M and 1M) to increase specificity, precision and linearity. Increasing betaine concentration, a higher assay specificity on NTC and negative matrices has been observed, together with a higher precision and linearity using four different EBV plasmid dilutions (2e7cps/uL, 2e5cps/uL, 2e3cps/uL, 1e1cps/uL).

The final reaction conditions of the duplex Q-LAMP EBV assay, obtained at the end of the optimization are the following:

Parameter	Optimized condition
Reaction temperature	64 °C
Reaction Time	60 min
Buffer pH	8.4 pH
dNTPs concentration	0.6 mM
Enzyme concentration	7 U/rx
Primers concentration	0.25 X
$MgSO_4$	4mM
Betaine	0.5M

Table 1: Q-LAMP EBV assay (duplex format) optimized reaction conditions

The table shows the parameters optimized for Q-LAMP EBV assay.

The reaction format is 30µL of reagents (reaction mix) and 20µL of sample. The reaction controls that must to be loaded in each run consists of: positive control (EBV plasmid) and negative control (T4 plasmid).

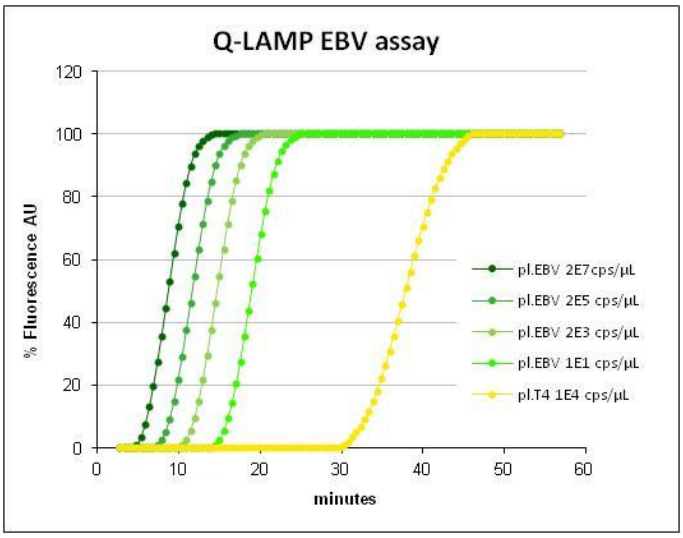


Figure 14: Q-LAMP EBV assay (Duplex format)

Amplification of EBV positive control and T4 negative control duplex assay are shown. Serial dilutions of EBV plasmids have been used to optimize the reactions. T4 plasmid amplification has been used to validate the results.

5. Q-LAMP EBV assay reactions freeze-drying

At the end of the optimization, the final formulation has been freeze-dried in order to have several advantages such as product stability, the easy set-up, the higher reproducibility between experiments and the more standardized production process. The freeze-drying process starts with the freezing of the reaction mix and the subsequent sublimation of the water contained in the mix. This process can alter the characteristics of the reaction mix leading to problems in specificity, sensitivity and precision.

For Q-LAMP EBV assay no changes have been detected.

To evaluate the real time stability of the freeze-dried mix, different pouches containing the lyophils have been incubated at 4°C for 176 days (about 6 months) and have been tested at different time points. In each run has been evaluated High positive Control 2E7cps/µL (HC), Low positive Control 1E1cps/µL (LC) and Negative Control T4 1E4cps/µL (NC). No significant difference has been observed after 176 days in the three samples tested.

5.1. Freeze-drying stability test

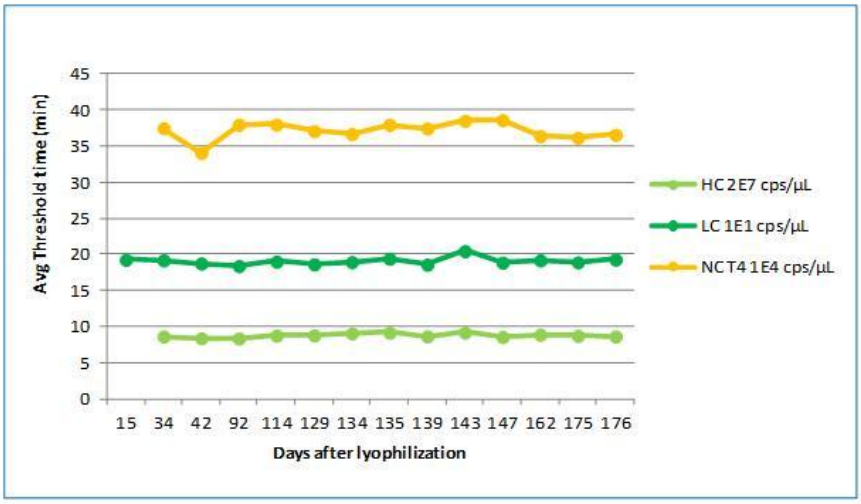


Figure 15: Stability test

Stability evaluation of the Q-LAMP EBV assay lyophil reaction mix. HC 2E7cps/µL, LC 1E1cps/µL and NC T4 1E4cps/µL have been tested. No significant difference has been observed after 176 days in the three samples tested.

6. Extraction protocol evaluation

The extraction method is a key process for the development of this assay as it is able to influence the assay performance.

The extraction method is based on the LIAISON® Ixt platform (developed by DiaSorin). It is an automated, flexible and compact instrument for high quality DNA/RNA extraction and cell separation from a variety of specimen sample types. The LIAISON® Ixt is easy to set up and simple to use, with it's ready to use reagent cartridges which demands minimal technician preparation time, and delivers results in 40-60 minutes. This extraction method is based on unique single use pump-tip device and cartridges containing magnetic beads.

Different protocols and chemistries have been evaluated for DNA extraction, depending on the matrices to be extracted: plasma, serum, Cerebrospinal Fluid) (CSF) and whole blood. The protocols differ in: quantity and size of the magnetic beads, concentration of the buffers, time and temperature of incubation and power used by the piston on pumps.

For plasma, serum and CSF 4 protocols (1-4) have been tested, for whole blood 3 protocols (5-7). Different EBV viral particle (WHO International Standards (IS)) concentrations have been diluted in each of the negative matrix containing the IC T4 bacteriophage and extracted using different protocols. IS have been used as they are expressed in International Unit/mL (IU/ml), that is the measurement units for the viral particles used in all the molecular laboratories.

Q-LAMP EBV assay has been used to test the samples in order to evaluate the most sensitive extraction protocol. For plasma, serum and CSF is the n°4 and for whole blood is the n°7.

Matrix	Extraction Protocol	Viral particle (IU/ml)	% Detected
Plasma	1	5E3	100%
		2E3	74%
	2	2E3	100%
		1E3	94.4%
	3	1E3	100%
		4E2	100%
		2.5E2	86%
	4	4E2	100%
		2.5E2	100%
Serum	1	5E3	95.56%
		2E3	90.2%
	2	2E3	100%
		1E3	57.89%
	3	1E3	100%
		4E2	79%
	4	4E2	100%
		2.5E2	96%
CSF	3	5E2	100%
		4E2	98%
	4	4E2	100%
		3E2	98%
Whole Blood	5	5E3	100%
		3E3	90%
	6	1.3E3	100%
		1E3	80%
	7	1.3E3	100%
		1E3	90%

Table 2: LIAISON® Ixt extraction protocols evaluation

Extraction protocols in different matrices have been evaluated by Q-LAMP EBV assay using different EBV viral particle concentrations extracted in one of the negative matrix within the IC T4 bacteriophage. The more sensitive extraction protocol for plasma, serum and CSF is the n°4,for whole blood is the n°7.

7. Q-LAMP EBV assay analytical performance

7.1. Q-LAMP EBV assay on different matrices

Q-LAMP EBV assay linearity performance has been evaluated using 6 serial dilutions of EBV plasmid (from 1E7cps/µL to 1E1cps/µL) in buffer, whole blood and plasma after extraction. The three curves have a similar behavior that demonstrates the absence of a matrix effect.

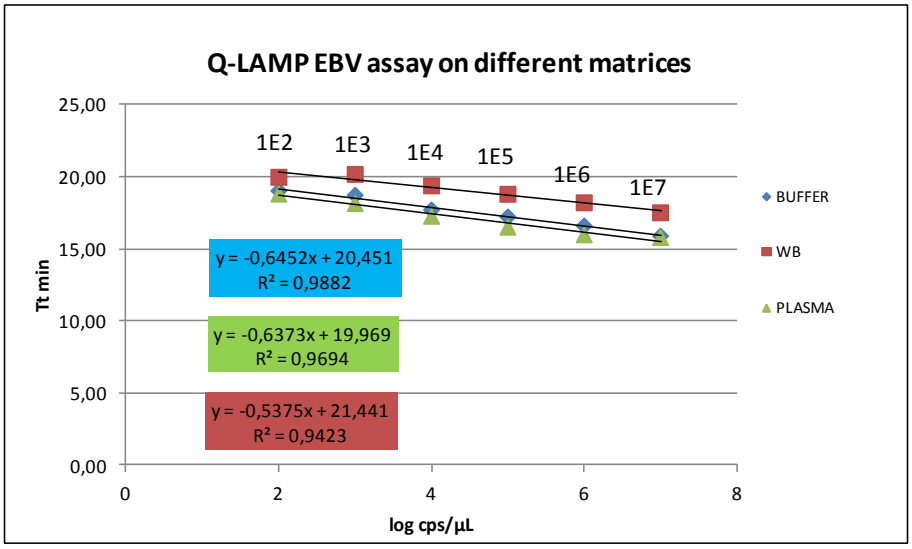


Figure 16: Q-LAMP EBV Assay on different matrices

Matrix effect evaluation by Q-LAMP EBV assay on plasmid dilutions spiked in negative plasma and whole blood versus plasmid dilutions spiked in buffer. The three curves have a similar behavior demonstrating the absence of a matrix effect.

7.2. Q-LAMP EBV assay analytical specificity

Analytical specificity of Q-LAMP EBV assay has been assessed on NTC (H_2O 1134 replicates) and negative control (T4 plasmid diluted in buffer) (385 replicates). The specificity is 100%, no primer dimers have been detected neither in 500nm nor in 530nm channel.

Samples	% (Replicates)
NTC (H_2O)	100 (1134)
Negative Control	100 (385)

Table 3: Q-LAMP EBV assay specificity on NTC and negative control

Evaluation of specificity of the assay on NTC (H_2O) and on negative control. No primer dimers have been detected neither in 500nm nor in 530nm channel.

To evaluate the assay performance associated to the extraction method, the specificity has been also assessed on negative matrices (negative matrices with added IC T4 bacteriophage particles and then extracted): plasma (103 replicates), serum (50 replicates), CSF (36 replicates) and whole blood (90 replicates). The specificity is 100%, only the IC has been detected and no EBV amplification has been observed.

Samples	% (Replicates)
Plasma	100 (103)
Serum	100 (50)
CSF	100 (36)
Whole Blood	100 (90)

Table 4: Q-LAMP EBV assay specificity on matrices

Evaluation of specificity of the assay on different matrices (plasma, serum, CSF and whole blood) extracted with the addition of IC T4 bacteriophage. Only IC has been detected in 100% of the time. No amplification of EBV has been observed.

7.3. Q-LAMP EBV assay analytical sensitivity

Analytical sensitivity is determined by the Limit of Detection (LoD) value, which represents the lowest amount of target that can be detected in at least 95% of replicates, but not necessarily quantified as an exact value. The LoD is a low concentration that is statistically distinguishable from background or negative control, but is not sufficiently precise or accurate to be quantitated. The performance of Q-LAMP EBV assay has been evaluated using low concentration of plasmids (down to 10cps/µl) spiked in extracted negative matrices. The LoD is 1cps/µl detected in more than 95% of replicates thanks to the efficiency of the extraction method.

	% (Replicates)
1 cps/µL in Cal/Ctr Buffer	98.59 (71)
1 cps/µL in Plasma	97.5 (40)
1 cps/µL in Serum	97.5 (40)
1 cps/µL in CSF	95 (30)
1 cps/µL in Whole Blood	96.3 (81)

Table 5: Q-LAMP EBV assay sensitivity on EBV plasmid

To evaluate the sensitivity, the Q-LAMP EBV assay has been performed using EBV plasmids at low concentration (1cps/µl) spiked in matrices extracted within IC. The assay is able to detect 1cps/µl in more than 95% (LOD) of the time in all matrices.

The assay sensitivity has been also associated to the extraction methods based on LIAISON® Ixt platform using low concentration of WHO IS spiked in matrices containing IC following extraction. To calculate the sensitivity in cps/ml the conversion factor determined (see paragraph 7.2) has been used only for the most important and complex matrices (whole blood and plasma). The assay is able to detect 769cps/mL in whole blood and 500cps/mL in plasma in more than 95% of the time. The difference between these two matrices could be due to the different complexity of whole blood compared to plasma.

Samples type	IU/mL	Conversion Factor (IU/cps)	cps/mL	% Replicates
Whole Blood	1000	1.30	769	98 (43)
Plasma	412	0.829	500	95 (40)

Table 6: **Q-LAMP EBV assay sensitivity on IS**

To evaluate the sensitivity of Q-LAMP EBV associated to the extraction methods, low concentration of IS spiked in matrices extracted within IC has been used. To calculate the sensitivity in cps/ml the conversion factor determined (see paragraph 7.2) has been used for the most important and complex matrices.

8. Q-LAMP EBV assay quantitative performance

8.1. Q-LAMP EBV assay precision, linear range and Limit of Quantification (LOQ)

Precision is the variability in the data from replicate determinations of the same homogeneous sample under the normal assay conditions and it is usually expressed as variance, standard deviation or coefficient of variation of a series of measurements. For Q-LAMP EBV assay the standard deviation has been used.

Linear range is the range (between the low and the high limits of target quantitation) where the results are directly proportional to the concentration of the target present in a sample. Classical linearity acceptance criteria require: the correlation coefficient of the linear

regression line (not more than some number close to 1) and the y-intercept (should not differ significantly from zero).

Limit of quantitation (LoQ) is the lowest amount of target in a sample that can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of target in sample matrices (Burd, 2010; Hanneman et al., 2008; Food and Drug Administration, 2001; International Organization for Standardization, 2003).

Minimum acceptable requests of corporate guidelines made in response to the market trend concerning the above parameters are: 1E1cps/µL Lower Quantification Limit (LQL) and 1E4cps/µL Upper Quantification Limit (UQL).

To evaluate these parameters a calibration curve has been performed using different plasmids dilution from 2E7cps/µL (UQL) to 1E1cps/µL (LQL) spiked into negative plasma and whole blood matrices extracted. Three different experiments for each matrix have been performed: 7 different plasmid dilutions and 6 replicates for each dilutions. Precision and linear range are acceptable and meet the requests of corporate guidelines, made in response to the market trend.

Matrix	UQL (cps/µL)	LQL (cps/µL)	SD LAMP reps (log cps/µL) (min-max)
Plasma	$2 \cdot 10^7$	10	0.02 0.27
Whole Blood	$2 \cdot 10^7$	10	0.02 0.20

Table 7: **Q-LAMP EBV assay precision**

Precision has been measured as acceptable standard deviation of the positive matrices tested from the nominal value. Three different experiments for each matrix have been performed: 7 different plasmid dilutions and 6 replicates for each dilutions.

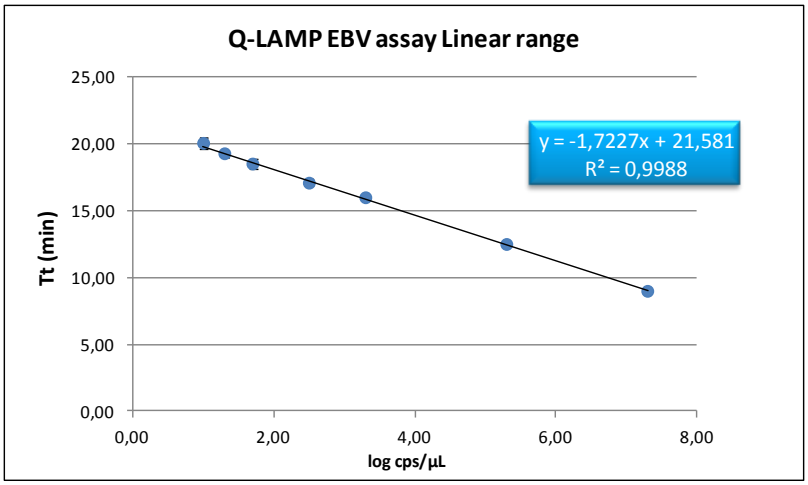


Figure 17: **Q-LAMP EBV assay Linear range**

To evaluate linear range has been performed a calibration curve which consist of different plasmid dilution from 2E7 cps/µL (UQL) to 1E1 cps/µL (LQL) spiked into negative plasma and whole blood matrices extracted.

8.2. Conversion factor determination

Conversion factor (CF) allows comparison of results obtained in different assays. It depends on matrices tested and extraction methods used. The conversion factor has been assessed using different concentration of IS (5E6IU/mL, 5E5IU/mL, 5E4IU/mL, 5E3IU/mL) diluted in negative plasma and whole blood matrices extracted with the IC to generate the EBV calibration curve. Three runs have been performed using each extracted sample in quadruplicate according to Q-LAMP EBV assay and predicate device. The ratio has been calculated for each expected value of diluted IS by dividing with the corresponding determined value in cps/mL. Then the mean has been calculated and it represents the CF.

CONVERSION FACTOR PLASMA	
Q-LAMP EBV assay	0.829 IU/cps
Predicate device	0.29 IU/cps

CONVERSION FACTOR WHOLE BLOOD	
Q-LAMP EBV assay	1.3 IU/cps
Predicate device	0.19 IU/cps

Table 8: **Conversion factor**

A CF for plasma and whole blood has been determined for both assays: Q-LAMP EBV and predicate device.

8.3. Correlation in whole blood

First of the correlation on IS in whole blood all has been performed. Results obtained by Q-LAMP EBV assay have been compared against the nominal value of IS dilutions prepared. 6 different experiments have been performed using 7 different IS dilutions spiked in whole blood and extracted using LIAISON® Ixt platform. 5 replicates for each IS dilutions have been tested for a total of 35 replicates for each experiment. The conversion factor equal to 1.3 IU/cps previously determined has been applied to the results.

Precision (log IU/mL) (min-max)	R^2 (min-max)	Mean IS value reported by Q-LAMP respect to the expected value (log IU/mL) (min-max)
0.01 0.26	0.94 0.99	0.01 0.5

Table 9: Correlation of IS in whole blood

Results obtained by Q-LAMP EBV assay have been correlated against the expected value. 6 different experiments have been performed using 7 different IS dilutions spiked in whole blood. 5 replicates for each dilution have been tested, for a total of 35 replicates for each experiment.

To evaluate the diagnostic performance and the feasibility in clinical practice, the assay has been validated on 30 whole blood positive clinical samples compared with the standard method Real-Time PCR (predicate device) using as extraction method the LIAISON® Ixt platform. The conversion factor of 1.3 IU/cps for Q-LAMP EBV assay and 0.19 IU/cps for

predicate device has been applied to assessed the results in IU/mL. To validate the results in each run have been included 1 HC 2E7cps/µL, 1 LC 1E1cps/µL, 1 NC T4 1E4cps/µL and 1 NTC (water).

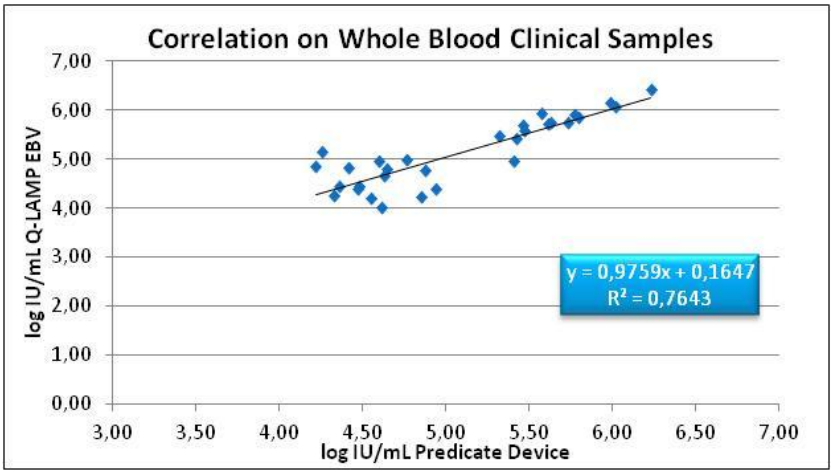


Figure 18: Correlation of 30 clinical samples in whole blood

Results obtained by Q-LAMP EBV assay have been correlated against the predicate device. 30 whole blood clinical positive samples have been extracted using LIAISON® Ixt platform and tested by Q-LAMP EBV assay and predicate device.

Minimum acceptable request corporate guidelines for correlation of IS in whole blood (the most complex matrix) are: precision 0.3 log, correlation R^2= 0.9, mean IS value reported by Q-LAMP +/- 0.5 log of the expected value. For correlation of clinical samples are: R^2= 0.7, Slope of regression line for quantified positive samples 0.8<1.2 and Intercept of regression line for quantified positive samples -0.8<0.8. The results in whole blood are

acceptable and meet the request of corporate guidelines, made in response to the market trend.

8.4. Correlation in plasma

The first step of the correlation in plasma has been the evaluation of WHO IS. Results obtained by Q-LAMP EBV assay have been compared against the nominal value of WHO IS dilutions prepared. 6 different experiments have been performed using 7 different IS dilution spiked in plasma and extracted using LIAISON® Ixt platform. 5 replicates for each IS dilution have been tested for a total of 35 replicates for each experiment. The conversion factor equal to 0.829 IU/cps previously determined has been applied to the results.

Precision (log IU/mL) (min-max)	R^2 (min-max)	Mean IS value reported by Q-LAMP respect to the expected value (log IU/mL) (min-max)
0.03 0.24	0.96 0.98	-0.01 0.41

Table 10: Correlation of IS in plasma

Results obtained by Q-LAMP EBV assay have been correlated against the expected value. 6 different experiments have been performed using 7 different IS dilution spiked in plasma. 5 replicates for each dilution have been tested, for a total of 35 replicates for each experiment.

To evaluate the diagnostic performance and the feasibility in clinical practice, the assay has been validated on 30 plasma positive clinical samples compared with the standard method Real-Time PCR (predicate device) using as extraction method the LIAISON® Ixt platform. The conversion factor of 0.829 IU/cps for Q-LAMP EBV assay and 0.29 IU/cps for predicate device has been applied to assessed the results in IU/mL. To validate the results in each run have been included 1 HC 2E7cps/µL, 1 LC 1E1cps/µL, 1 NC T4 1E4cps/µL and 1 NTC (water).

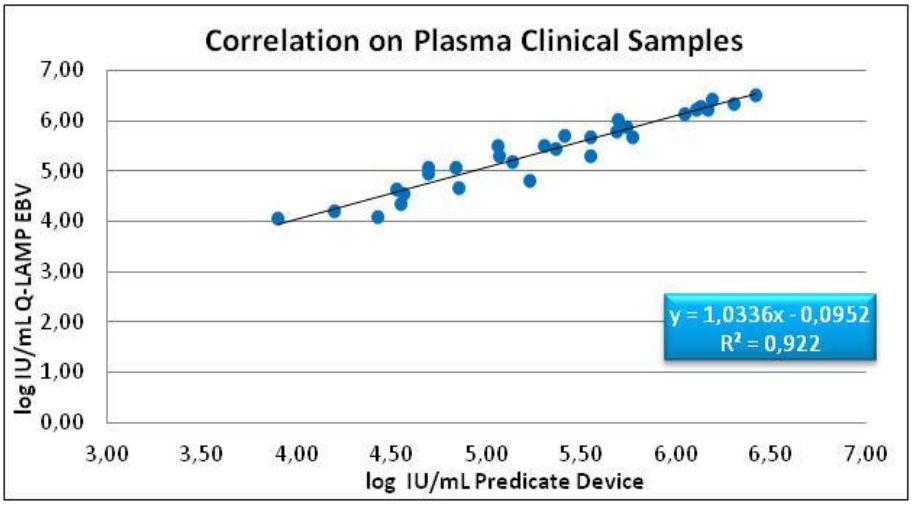


Figure 19: Correlation of 30 clinical samples in plasma

Results obtained by Q-LAMP EBV assay have been correlated against the predicate device. 30 plasma clinical positive samples have been extracted using LIAISON® Ixt platform and tested by Q-LAMP EBV assay and predicate device.

Minimum acceptable request corporate guidelines for correlation of IS in plasma are: precision 0.3 log, correlation R^2= 0.9, mean IS value reported by Q-LAMP +/- 0.5 log of the expected value. For correlation of clinical samples are: R^2= 0.7, Slope of regression line for quantified positive samples 0.8<1.2 and Intercept of regression line for quantified positive samples -0.8<0.8. The results are acceptable and meet the requests of corporate guidelines, made in response to the market trend.

9. Clinical validation

To assess the clinical value of Q-LAMP EBV assay a validation on clinical samples has been performed compared to the predicate device RT-PCR based, using as extraction method the LIAISON® Ixt platform.

9.1. Relative sensitivity

44 positive plasma and 45 positive whole blood clinical samples have been tested by Q-LAMP EBV assay and predicate device. A 100% agreement with RT-PCR method has been demonstrated. To validate the results in each run 1 HC 2E7cps/µL, 1 LC 1E1cps/µL, 1 NC T4 1E4cps/µL and 1 NTC (water) have been included.

		Q-LAMP EBV
PREDICATE DEVICE	Positive Plasma Clinical Samples	44/44
	Positive Whole Blood Clinical Samples	45/45
	% Agreement	100

Table 11: Relative sensitivity in whole blood and plasma

44 positive plasma clinical samples and 45 positive whole blood clinical samples have been tested by Q-LAMP EBV assay and predicate device. 100% agreement with predicate device has been obtained.

9.2. Relative specificity

12 negative plasma and 27 negative whole blood clinical samples have been tested by Q-LAMP EBV assay and predicate device. A 100% agreement with RT-PCR method has been demonstrated, no false negative or false positive results have been detected. To validate the results in each run have been included 1 HC 2E7cps/µL, 1 LC 1E1cps/µL, 1 NC T4 1E4cps/µL and 1 NTC (water).

		Q-LAMP EBV
PREDICATE DEVICE	Negative Plasma Clinical Samples	0/12
	Negative Whole Blood Clinical Samples	0/27
	% Agreement	100

Table 12: Relative specificity in whole blood and plasma

12 negative plasma clinical samples and 27 negative whole blood clinical samples have been tested by Q-LAMP EBV assay and predicate device. 100% agreement with predicate device has been obtained.

DISCUSSION

Following primary infection, which occurs in the vast majority of the world's population, Epstein-Barr virus (EBV) establishes a latent reservoir in resting memory B lymphocytes. Although most people harbor EBV with no long term clinical implications, EBV can be associated with a wide variety of malignant diseases such as lymphomas, post-transplant lymphoproliferative disorders (PTLD), immunodeficiency-related disorders, nasopharyngeal carcinoma (NPC) and gastric carcinoma (Williams et al.,2006; Gärtner et al., 2010; Gulley et al., 2008).

EBV DNA copy number quantification by real-time polymerase chain reaction (qPCR) of blood specimens is often used as a mean of screening for these diseases or assessing treatment response (Kanakry et al., 2016; Meerbach et al., 2008; Van Esser et al., 2001). Laboratory tests for EBV have improved and are increasingly used in diagnosis, prognosis, prediction, and prevention of diseases ranging from infectious mononucleosis to selected subtypes of lymphoma, sarcoma, and carcinoma. Indeed, the presence of EBV is among the most effective tumor markers, supporting clinical management of cancer patients (Gulley et al., 2008).

During the past decades, various forms of PCR such as multiplex PCR and RT-PCR, have been developed to address the need for rapid identification of viruses. Molecular assays display a higher accuracy than the serological assay, which remains the gold standard for the diagnosis of primary

infection, despite the regularly observed false positive and negative results (Gartzonika et al.,2012).

However, all of these PCR-based nucleic acid amplification methods have the intrinsic disadvantage of requiring either a high-precision instrument for amplification or an elaborated method for detection of amplified products. For this reason, there is still a great need for a tool to simplify the cost effective detection of EBV DNA with adequate sensitivity, specificity and precision in a short turnaround time.

Loop-mediated isothermal AMPlification (LAMP) is a powerful nucleic acids amplification technique, which has emerged as an easy-to-perform and rapid tool for molecular diagnostics applications in clinical routine (Notomi et al.,2000). LAMP amplifies DNA or RNA target with high specificity, efficiency and rapidity under isothermal conditions. It is fast and the reaction is usually completed in 60 minutes or less. It is relatively inexpensive thanks to the use of a strand displacement polymerase, avoiding the use of expansive thermocycler instruments. In DiaSorin the technology has been improved giving rise to Q-LAMP, using fluorescent probes which allowed the real time detection and discrimination of multiple targets in the same tube. Initially the Q-LAMP technology has been developed only for qualitative purposes thanks to its peculiarities to be easily applicable in the clinical laboratories by using basic facilities.

This thesis describes the development of quantitative Q-LAMP technology (Loop-Mediated Isothermal Amplification) that has been designed using the

qualitative Q-LAMP properties as the starting point, generating a new model assay for the rapid detection and quantification of the two most clinically relevant subtypes of EBV, Type 1 / A and 2 / B, on four different matrices (whole blood, plasma, serum and CSF) with high sensitivity, specificity and precision.

Q-LAMP EBV assay consists in a duplex primer system: one set is able to detect and quantify simultaneously EBV Type 1 and 2 EBNA-1 gene, and the specific signal is given by a green-labeled probe. The second primer set is specific for the amplification of the Internal Control (IC) using a yellow-labeled probe. The use of the Internal Control in Q-LAMP is very important to control the extraction process and to evaluate the presence of reaction inhibitors allowing to validate negative results. It is made from T4 bacteriophage particles that are added to the sample.

The assay has been developed in a freeze-dried format that further improves the simplicity and rapidity of the technology. The stability test demonstrates no difference using lyophils incubated at 4°C after 176 days in three samples tested: HC 2E7cps/µL (High positive Control), LC 1E1cps/µL (Low positive Control) and NC T4 1E4cps/µL (Negative Control).

An important key process for the development of this assay has been the evaluation of an extraction method, since it has great influence on the sensitivity. The selected extraction method is based on the LIAISON® Ixt platform (developed by DiaSorin). This is based on unique single use pump-tip device and cartridges containing magnetic beads. 7 different protocols and chemistry have been evaluated for DNA extraction starting from

various matrices : plasma, serum, CSF and whole blood, selecting the best performing one for plasma, serum and CFS and one for whole blood.

After a long effort of reaction optimization, also using different reagents and catalysts, the most promising formulation has been selected and freeze-dried. This lyophilized reaction mix has been tested in terms of analytical performance. Q-LAMP EBV assay demonstrated to be highly specific, no primer dimers have been detected on NTC (H_2O, 1134 replicates) and negative control (T4 plasmid diluted in buffer, 385 replicates). Also when associated to the extraction the assay demonstrated a 100% specificity on negative extracted matrices, in which only the IC has been detected and no EBV amplification has been observed.

Excellent results have been obtained for analytical sensitivity. The assay has shown a very high level of detection on EBV plasmid, detecting 1 cps/µl in more than 95% of replicates in all the four matrices. Associated to the extraction step on the LIAISON® Ixt platform, the analytical sensitivity of the assay on viral particles was still good, reaching 769 cps/mL in whole blood and 500 cps/mL in plasma .

The greatest effort in EBV Q-LAMP assay development has been to create a quantitative assay. The intrinsic rapidity of Q-LAMP technology made difficult the development of an assay with a good discrimination capability. A balance between rapidity and precision is extremely important for the successful development of a quantitative assay with adequate performance.

Quantitative assay performance in terms of precision, linear range and LOQ (Limit of Quantification) has been evaluated on WHO International

Standards in whole blood (the most complex matrix) and plasma extracted on the LIAISON® Ixt platform.

Very good results have been obtained on both matrices with a precision lower than 0.3 log, a dose discrimination down to 0.5 log and a coefficient of determination (R^2) always higher than 0.94.

Moreover, a very important need in quantitative assays is to compare results obtained in different laboratories with different assays in order to express the viral DNA load in the same measurement unit: International Unit/mL (IU/mL,). To this aim WHO International Standards (IS) has been used to determined a conversion factor for each matrix, specific for the LIAISON® Ixt platform as extraction method, that allows to convert cps/mL in IU/mL.

The good performance of the assay and the feasibility in clinical diagnostic practice, have been demonstrated testing the correlation of IS and of 30 positive clinical plasma and whole blood samples. The assay meets the market needs, on both matrices whole blood and plasma: R^2 is always higher than 0.75, the slope is $0.8<1.2$ and the intercept is $-0.8<0.8$.

Finally, the clinical value of Q-LAMP EBV assay has been assessed performing a validation on a total of 56 plasma and 72 whole blood positive and negative clinical samples extracted using the LIAISON® Ixt platform and compared to the predicate device Real Time PCR based. High sensitivity and specificity with 100% agreement of Q-LAMP EBV assay has been obtained compared to the predicate device.

In conclusion, Q-LAMP technology can be used as a quantitative method. Q-LAMP EBV assay demonstrated to be suitable for industrialization for a future diagnostic product able to detect and quantifies in 60 minutes simultaneously the two most clinically relevant subtypes of Epstein-Barr virus Type 1 and 2 on whole blood and plasma with high sensitivity, specificity and precision.

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