

Quantitative Detection of Epstein-Barr Virus DNA in Clinical Specimens by Rapid Real-Time PCR Targeting a Highly Conserved Region of EBNA-1

Servi J. C. Stevens, Sandra A. W. M. Verkuijlen,
and Jaap M. Middeldorp

Summary

Here we describe a LightCycler-based real-time PCR for quantitative detection of EBV DNA in clinical samples such as unfractionated whole blood, serum, or plasma. This assay is based on amplification of a highly conserved 213-bp region of the *EBNA-1* gene, a single-copy gene of EBV required for maintenance of the EBV genome within the infected host cell. For real-time detection of amplicons, two internal hybridization probes are added, labeled with the fluoregenic dyes fluorescein and LCRed640, respectively. Simultaneous hybridization of these probes to the amplification products brings them in close proximity. Subsequent excitation of the fluorescein label by filtered excitation light from a light source in the LightCycler device will lead to fluorescence energy transfer (FRET) from the fluorescein label to the LCRed640 label. The light emitted from the LCRed640 label is then measured and correlates to the amount of product generated. The cycle at which the fluorescence exceeds the background is designated the *threshold cycle*. By comparing the threshold cycle of a clinical specimen with those of standard curve samples, the amount of EBV DNA in clinical samples can be determined. This real-time PCR approach is extremely rapid owing to efficient heat conduction by using glass capillaries, small reaction volumes, and air as heating medium. The “closed-tube” system eliminates the risk of PCR contamination by product carryover and also the need for post-PCR detection.

Key Words: Epstein-Barr virus; herpesviruses; DNA quantification; real-time PCR; viral load; EBNA-1; LightCycler.

1. Introduction

Epstein-Barr virus (EBV) is a human γ -herpesvirus infecting more than 90% of the population worldwide. In adolescents, EBV is the causative agent of infectious mononucleosis. Furthermore, EBV is associated with a still increas-

ing number of malignant proliferative disorders of both lymphoid and epithelial origin, including Burkitt's and Hodgkin's lymphoma, B- and T-cell non-Hodgkin's lymphoma, and nasopharyngeal and gastric carcinoma (1,2). In immunocompromised individuals such as transplant recipients and AIDS patients, EBV is the driving force behind lymphoproliferative disorders, originating from infected B cells, which initially are benign and polyclonal but may progress to malignant lymphoma if left untreated (1).

In healthy carriers of EBV, the number of EBV-infected B cells remains stable throughout life, reflecting the tightly controlled balance between EBV-driven B-cell proliferation and the host immune response. This is also characterized by persistent high levels of anti-EBV T cells and serum antibodies to latent and lytic EBV antigens (3,4). Disturbance of this balance, e.g., by iatrogenic or natural immunosuppression, leads to increased numbers of EBV-infected B cells and EBV DNA loads in blood (reviewed in **ref. 5**). Consequently, in defined high-risk populations such as solid organ and hematopoietic stem cell transplant recipients, monitoring of EBV DNA load in peripheral blood is a suitable and widely used diagnostic tool for predicting posttransplant lymphoproliferative disease (PTLD) and guidance of pre-emptive therapeutic intervention (5). Introduction of EBV DNA load monitoring in the routine diagnostic patient care of most transplant centers in the late 1990s has greatly increased transplant success and decreased EBV-related morbidity and mortality in transplant settings.

In immunocompetent patients with EBV-linked malignancies, such as nasopharyngeal carcinoma (NPC), monitoring of EBV DNA loads in peripheral blood may be useful in predicting the efficacy of therapeutic interventions (6–8).

Although at present EBV DNA load monitoring is widely applied, basic interlaboratory standardization of clinical specimen type, polymerase chain reaction (PCR) technique, unit of measurement and clinically relevant cutoff values has not yet been established. Thus, a variety of clinical specimens are being used for determination of EBV DNA loads in the circulation, including whole blood, plasma, serum, and isolated leukocyte cell fractions (reviewed in **ref. 5**; see also **Note 5**). Concerning PCR target sequences, the *Bam*HI W-repeat region, which is often used for sensitive qualitative detection of the virus, is unsuited for quantification purposes because the number of *Bam*HI W repeats differs between clinical isolates of EBV (9). Consequently most, but not all, studies use a single-copy EBV gene target in PCR. However, for some of these genes virtually nothing is known about nucleotide polymorphism in clinical EBV isolates. Furthermore, variation in amplicon size may influence quantification owing to differences in amplification efficiency. Real-time PCR is currently the most widely used technique for EBV load determination, a technique that depends on the use of external

standard dilution series. It is proposed that the Namalwa cell line, which stably contains two integrated copies of the EBV genome, is most suited for standardization (9).

This chapter describes a reproducible LightCycler-based real-time PCR assay for quantitative detection of EBV DNA with an analytical sensitivity of 10 copies of viral DNA or <1 EBV-positive lymphoblastoid cell line in whole-blood DNA background (10). This method allows rapid quantification of the EBV DNA load in clinical specimens owing to use of small reaction volumes in thin borosilicate glass capillaries for efficient heat conduction and air for heating and cooling (11). Using this assay, EBV DNA load can be determined in less than 2.5 h including DNA isolation, LightCycler reaction setup, EBV DNA amplification, and data analysis. The closed-tube format of real-time PCR strongly reduces the risk of PCR contamination by product carryover. Real-time quantification of PCR products by double-probe hybridization provides high specificity, and fluorescence detection eliminates the need for post-PCR detection procedures and allows quantification in the log-linear phase of amplification over a wide dynamic range. These features strongly favor the use of real-time PCR-based methods in routine molecular diagnostic settings over conventional semiquantitative, limiting dilution or quantitative competitive PCR methods.

The EBV real-time PCR described in this chapter is based on amplification of a 213-bp region of Epstein-Barr nuclear antigen-1 (*EBNA-1*), a single-copy gene of EBV. *EBNA-1* protein is expressed in all EBV-carrying cells and is essential for maintenance of the viral genome and establishment of latency by anchoring the viral episome to the host chromosome (12). Mutational hot spots within *EBNA-1* have been mapped extensively in clinical isolates of EBV (13–15), permitting primer selection in a highly conserved region (16). This *EBNA-1* region of the EBV genome allowed reliable EBV DNA load quantification in samples from patients with various EBV-associated diseases worldwide (16,17). To increase assay specificity and allow quantification, two internal oligonucleotide hybridization probes, labeled with fluorescein and LCRed640, respectively (10), are added to the PCR. During PCR amplicon formation, simultaneous annealing of the two probes to the *EBNA-1* PCR product brings them into close proximity. Excitation of the fluorescein label by filtered excitation light from a light source in the LightCycler device will lead to fluorescence energy transfer from the fluorescein label to the LCRed640 label. The light emitted from this LCRed640 label correlates with the amount of PCR product generated. The cycle at which the fluorescence exceeds the background is designated the *threshold cycle* (C_t). By relating the C_t of a clinical specimen to a defined series of C_t values in a standard curve, the initial amount of EBV DNA in the specimen can be determined.

2. Materials

1. LightCycler device and computer with LightCycler software (Roche Diagnostics, Basel, Switzerland).
2. LightCycler accessories sample carousel, centrifuge, capillary adapters and capillary cooling block and Capillaries and stoppers.
3. LightCycler-FastStart DNA Master Hybridization Probes kit containing 10X stock LC Fast Start Reaction Mix Hybridization Probes, LC Fast Start Enzyme, MgCl₂, and water (Roche Diagnostics).
4. Forward primer: EBV F QP1L (5'-gccggtgtgttcgtatatgg-3') (*see Note 1*).
5. Reverse primer: EBV R QP2 L (5'-caaacctcagcaaatatag-3') (*see Note 1*).
6. Hybridization probe 1: *EBNA-1* FLN (5'-tctcccttggaatggcccctg-fluorescein) (*see Note 2*).
7. Hybridization probe 2: *EBNA-1* LCN (5'-LCRed640-acccggcccaaacctg-3') (*see Note 2*).
8. For real-time PCR standard curve:
EBV-positive Burkitt's lymphoma cell line Namalwa (American Type Culture Collection [ATCC] CRL-1432; *see Note 3*) or purified and spectrophotometrically quantified plasmid DNA containing the *EBNA-1* gene, e.g., pBR322 containing the *Bam*HI-K fragment of the EBV genome (18); (*see Note 4*).
9. 0.1 M HCl.

3. Methods

1. Isolate DNA from the clinical specimen of choice (*see Notes 5 and 6*).
2. Prepare the FastStart DNA Master Hybridization Probes mix stock solution (containing enzyme and buffer) by adding 60 μ L of the LC Fast Start Reaction Mix Hybridization Probes (tube 1b from the kit mentioned in **Subheading 2., item 3**) to the LC Fast Start Enzyme (tube 1a; *see Note 7*). Mix gently by pipeting up and down. Do not vortex.
3. Prepare PCR master mix (*see Notes 8 and 9*) by combining the following reagents in a sterile precooled plastic Eppendorf tube (volumes given per reaction; multiply volumes by the amount of samples to be amplified): 7.6 μ L sterile water, PCR grade (colorless cap); 2.4 μ L MgCl₂ (25 mM stock solution, blue cap; *see Note 10*); 0.5 μ L EBV F QP1L forward primer (20 pmol/ μ L); 0.5 μ L EBV R QP2L reverse primer (20 pmol/ μ L); 1 μ L hybridization probe 1 EBNA-1 FLN (4 pmol/ μ L); 1 μ L hybridization probe 2 EBNA-1 LCN (4 pmol/ μ L); and 2 μ L FastStart DNA Master Hybridization Probes mix.
4. Pipet 15 μ L of PCR master mix to a precooled capillary placed in an adapter in the cooling block (*see Note 11*).
5. Add 5 μ L of isolated DNA to the 15 μ L PCR mix in the capillary.
6. For the standard curve, add, for example, 10, 10², 10³, 10⁴, and 10⁵ copies of plasmid DNA or the DNA equivalent of 5, 50, 500, 5000, and 50,000 Namalwa cells in a volume of 5 μ L to five separate reactions of 15 μ L PCR mix (*see Note 12*).
7. Seal each capillary with a stopper and place capillaries in the carousel in LightCycler centrifuge. Close centrifuge, press *start*, and open the lid when the centrifuge is finished.

- 8. Remove the carousel, and place it in LightCycler device.
- 9. The PCR consists of three programs (preincubation, amplification, and cooling). Use the following amplification conditions.

a. Program 1: preincubation (“activation” of *Taq* polymerase and denaturation of template DNA).

Cycle program data	Value
No. of cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	95
Incubation time (min:s)	10:00
Temperature transition rate (°C/s)	20.0
Secondary target temperature (°C)	0
Step size (°C)	0.0
Step delay (cycles)	0
Acquisition mode	None

b. Program 2: amplification.

Cycle program data	Value		
Cycles	45		
Analysis mode	Quantification		
Temperature targets	Segment 1	Segment 2	Segment 3
Target temperature (°C)	95	55	72
Incubation time (s)	10	10	10
Temperature transition rate (°C/s)	20.0	20.0	20.0
Secondary target temperature (°C)	0	0	0
Step size (°C)	0.0	0.0	0.0
Step delay (cycles)	0	0	0
Acquisition mode	None	Single	None

c. Program 3: cooling.

Cycle program data	Value
Cycles	1
Analysis mode	None
Temperature targets	Segment 1
Target temperature (°C)	40
Incubation time (s)	30
Temperature transition rate (°C/s)	20.0
Secondary target temperature (°C)	0
Step size (°C)	0.0
Step delay (cycles)	0
Acquisition mode	None

- 10. Set the fluorescence gains as follows: channel F1 gain = 1; channel F2 gain = 15; channel F3 gain = 30 (see **Note 13**).
- 11. Define the name of the samples and sample type (*positive, negative, standard, or unknown*) in the software field *Run* under the heading *edit samples*. For standards, give known concentration (for example in *copies/reaction*). When completed, select *Done* and then *Save* and provide the *settings file* with an appropriate name. Then select *Run* and then again *Save* and provide *data file* with an appropriate name. Select *Done*. The LightCycler run is initiated.
- 12. After completion of the run, perform data analysis using the channel setting F2/F1, baseline adjustment to *arithmetic*, and Fit Points setting at 2. First determine the baseline in *Step 1: Baseline* in the *LightCycler Data Analysis* mode of the software. Set noise band in the log-linear phase of the amplification in *Step 2: Noise Band* in the *LightCycler Data Analysis* mode of the software. Then, in *Step 3: Analysis*, the software generates a standard curve ($y = ax + b$) by plotting *cycle number* against *log concentration (copies per reaction)* for standard samples (with known copy numbers; see **Note 14**). The log amount of EBV (x) in an “unknown sample” can be calculated from its threshold cycle (y). The threshold cycle is determined by the software as the cycle in which the fluorescence signal exceeds the mean background fluorescence at baseline by 3 SD.
- 13. The software automatically calculates the number of EBV DNA copies present in each reaction. This can be seen in the LightCycler Quantification Report. Recalculate this to copies/mL for whole blood, plasma, or serum samples (see **Note 15** and **16**).
- 14. (Optional). In addition to the quantification using the standard curve approach, an optional melting curve analysis can be included after the last cycle of amplification to determine the specificity of the amplified region hybridizing with the probes. For this, before starting a run include the following program directly after after program 2 (**step 9**), and omit program 3.
 - a. Melting curve program.

Cycle program data		Value		
Cycles		1		
Analysis mode		Melting curve		
Temperature targets	Segment 1	Segment 2	Segment 3	
Target temperature (°C)	95	40	95	
Incubation time (s)	0	60	0	
Temperature transition rate (°C/s)	20.0	20.0	0.1	
Secondary target temperature (°C)	0	0	0	
Step size (°C)	0.0	0.0	0.0	
Step delay (cycles)	0	0	0	
Acquisition mode	None	None	Continuous	

- 15. When the run is completed, analyze as described above (see **steps 12** and **13**). For melting curve analysis, select the melting curve region of the PCR by placing vertical cursors at the beginning (40°C) and the end (95°C) of the temperature profile in the analysis front screen.

16. Use the fluorescence settings F2/F1 and select the *temperature* option for the x-axis.
17. Analyze data in the *melting curve* field, in which $-d(F2/F1)/dT$ is plotted against the temperature increase. In step 1 (*Melting peaks*), set method to *linear with background correction*. Place “end cursors” (blue) into the flat part of the melting curve after the end of the melting process. Place “beginning cursors” (green) into a region before the product melting begins for all samples. Do not include regions of the curve that still rise.
18. Determine T_m (for each sample individually or collectively for all samples in case of a homogeneous pattern) in the menu *Extra: manual Tm* by placing vertical cursors at the points where $-d(F2/F1)/dT$ reaches its maximum (and the decrease in fluorescence per temperature unit is at the highest). The T_m of a perfectly matching product–probe combination (e.g., for the EBV prototype B95-8 strain) is approx 58.1°C. Mismatches will yield lower T_m .

4. Notes

1. Primers should be high-performance liquid chromatography (HPLC)-purified, and for each batch of primers, PCR products should always first be tested on standard agarose gel electrophoresis for a discrete 213-bp band after PCR.
2. Hybridization probes with the fluoregenic labels fluorescein and LCR640 can be ordered, for example, from TIB Molbiol (Berlin, Germany) at www.tib-molbiol.de and should be dissolved and stored according to the manufacturer’s instructions.
3. The EBV-positive Burkitt’s lymphoma cell line Namalwa contains two copies of EBV per cell, integrated into chromosome 1 (19). Cells can be counted microscopically using a Burker-T rk chamber or by an automated cell counting device. The Namalwa cell line can be obtained from the ATCC under number CRL 1432.
4. The concentration (C) of purified plasmid DNA can be quantified spectrophotometrically by measuring absorption at 260 nm (A_{260}) and using **Eq. 1**:

$$C \text{ (ng/}\mu\text{L)} = A_{260} \times \text{dilution factor} \times 50 \quad (1)$$

where 1 A_{260} U of double-stranded DNA corresponds to a concentration of 50 ng/ μ L.

The number of plasmid copies (no. plasmid) can be calculated using **Eq. 2**:

$$\text{no. plasmid (copies/}\mu\text{L)} = (C \times N_{\text{avogadro}}) / (660 \times \text{plasmid size} \times 10^9) \quad (2)$$

where C is given in ng/ μ L, $N_{\text{avogadro}} = 6.022 \times 10^{23}$ /mol, 660 Daltons is the average molecular mass of a DNA bp, plasmid size is given in bp, and 10^9 is the conversion factor from nanograms to grams.

5. For quantification of circulating EBV DNA loads in clinical settings, we recommend the use of unfractionated whole blood because it combines all blood compartments, including both cell-associated and cell-free EBV DNA, and it gives a standardized and absolute value for the EBV DNA load (EBV DNA copies/mL total circulatory compartment) (20). Some studies report plasma and serum as suitable clinical specimens in hematopoietic stem cell transplant recipients (21), but we were unable to

detect elevated EBV DNA loads in serum of solid organ transplant recipients or AIDS patients, despite extremely elevated (cell-associated) EBV DNA loads in unfractionated whole blood (17,22). In addition, nonstandardized blood clotting and plasma isolation conditions may introduce uncontrollable variables, such as DNA release from apoptosed or fragile cells. At present little is known about the physical characteristics and origin of elevated EBV DNA loads in most populations, and the load may represent virion-derived EBV DNA, cell-associated EBV DNA, or cell-free EBV DNA released from in vivo or in vitro lysed cells. Detection of a certain form of EBV DNA may have additional clinical value in a given population. Because this value is currently undefined, it must be determined for each population independently. Isolated peripheral blood mononuclear cells or B cells as clinical specimens, and calculation of the EBV DNA load per 10^6 cells or per μg cellular DNA is not recommended because cell counts may vary considerably in immunosuppressed patients, which would influence the relative amount of EBV DNA obtained from these clinical materials, whereas absolute EBV load may not vary.

6. DNA purity is of vital importance for quantitative real-time PCR analysis, as remaining PCR inhibiting substances such as heparin, ethylene adraminetetraacetic acid (EDTA), lipids, and hemoglobin may lead to lower quantification or negative results. Extraction-based DNA isolation methods, e.g., by the MagNA Pure LC Instrument (Roche Diagnostics) or NucliSens silica-based DNA extraction (BioMerieux) are preferable, as they remove interfering substances more effectively in particular from peripheral whole blood samples (23,24).
7. Avoid repeated freezing and thawing of LC Fast Start Reaction Mix Hybridization Probes (tube 1b and LC Fast Start Enzyme (tube 1a). The Fast Start DNA Master Hybridization Probes mix stock solution should be stored at $2-8^{\circ}\text{C}$ and used within 1 wk. Prepare the PCR master mix in tubes placed in the precooled LC cooling block, and keep all reagents in this block after thawing. The cooling block should be stored at $2-8^{\circ}\text{C}$ and can be used for up to 4 h outside the refrigerator.
8. To decrease the risk of DNA contamination and false positivity of PCR, clean the laboratory bench and pipets with 0.1 M HCl or 10% bleach and subsequently water before starting the experiment. During the experiment, open tubes only when necessary. Wear gloves when pipeting. Use filter tips for all pipeting. Use separate laboratories for preparation of PCR mixes, isolating DNA and amplification. Aliquot all PCR reagents (25).
9. To decrease further the risk of contamination owing to PCR product carryover, heat-labile uracil-DNA-glycosylase (UNG; Roche Diagnostics) can be optionally added to LightCycler reactions. This technique relies on incorporation of deoxyuridine triphosphate during the LightCycler reaction instead of dTTP. UNG cleaves DNA at any site where a deoxyuridylate residue has been incorporated. Subsequently, at high temperatures the abasic sites are hydrolyzed, and the UNG enzyme is inactivated. Native (template) DNA does not contain uracil and is therefore not degraded.
10. The final MgCl_2 concentration in the PCR is 4 mM, as the Fast Start DNA Master Hybridization Probes mix already contains 1 mM, and separate MgCl_2 (2.4 μL /reaction) is added to achieve the final 4 mM concentration.

11. Do not touch the surface of the glass capillaries, and always wear gloves when handling them. Handle capillaries very careful, as they are extremely fragile. When needed, samples can be analyzed by standard agarose gel electrophoresis after real-time PCR. For this, capillaries (without stoppers) can be placed upside down in an Eppendorf reagent tube and centrifuged in a benchtop centrifuge for 10 s at low speed, to collect the PCR product.
12. Once the number of plasmid DNA copies per μL standard curve DNA stock is calculated (see **Note 4**), dilute in steps of 10-fold dilutions to the required concentrations. To decrease pipeting errors, pipet volumes of at least 5 μL in all dilution steps. Always keep plasmid and Namalwa DNA dilutions on ice because the diluted standard curve samples contain extremely small amounts of DNA, which may easily be degraded. Avoid repeated freezing and thawing of DNA stocks, and aliquot quantified DNA standards at relatively high concentrations or add carrier DNA such as herring sperm DNA.
13. With the LightCycler software version 3.5 or higher, no fluorescence gain setting is required.
14. The efficiency (E) of the LightCycler PCR can be calculated from the slope of the standard curve: $E = 10^{-1/\text{slope}}$. Thus, a theoretical efficiency of 2 ($= 100\%$) will yield a slope of -3.3 . For accurate quantification, it is crucial for clinical samples to have the same amplification efficiency as the standard curve samples. This can be judged from the graph in which log fluorescence ($F2/F1$) is plotted against the cycle number. The slope of the amplification curves of clinical samples and standard samples should be parallel. If not, it may be necessary to perform additional DNA purifications or dilute the sample.
15. The clinically relevant cutoff value of EBV DNA load monitoring should be determined for each population individually. It should be based, for example, on EBV DNA loads in healthy carriers or matched patient controls without EBV-associated diseases.
16. For LightCycler-negative clinical samples, the presence of amplifiable DNA in the sample can be checked by performing PCR for a cellular target, e.g., β -globin. The presence of substances interfering with PCR can be checked by spiking the sample with a low amount (e.g., 100 copies) of EBV plasmid or cell line DNA and subsequent reamplification. Spiked samples should be accurately quantified within a pre-defined range of assay variation (**10**).

References

1. International Agency for Research on Cancer (1997) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, vol. 70: *Epstein-Barr Virus and Kaposi's Sarcoma Herpesvirus/Human Herpesvirus 8*. WHO, Lyon, France.
2. Middeldorp, J. M., Brink, A. A. T. P., van den Brule, A. J. C., and Meijer, C. J. L. M. (2003) Pathogenic roles for Epstein-Barr virus (EBV) gene products in EBV-associated proliferative disorders. *Crit. Rev. Oncol. Hematol.* **45**, 1–36.
3. Thorley-Lawson, D. (2001) Epstein-Barr virus: exploiting the immune system. *Nat. Rev. Immunol.* **1**, 75–82.

4. Yao, Q. Y., Rickinson, A. B., and Epstein, M. A. (1985) A re-examination of the Epstein-Barr virus carrier state in healthy seropositive individuals. *Int. J. Cancer*. **35**, 35–42.
5. Stevens, S. J. C., Verschuuren, E. A. M., Verkuijlen, S. A. W. M., van den Brule, A. J. C., Meijer, C. J. L. M., and Middeldorp, J. M. (2002) Role of Epstein-Barr virus DNA load monitoring in prevention and early detection of post-transplant lymphoproliferative disease. *Leuk. Lymphoma* **43**, 831–840.
6. Chan, A. T., Lo, Y. M., Zee, B., et al. (2002) Plasma Epstein-Barr virus DNA and residual disease after radiotherapy for undifferentiated nasopharyngeal carcinoma. *J. Natl. Cancer Inst.* **94**, 1614–1619.
7. Lo, Y. M. (2001) Quantitative analysis of Epstein-Barr virus DNA in plasma and serum: applications to tumor detection and monitoring. *Ann. NY Acad. Sci.* **945**, 68–72.
8. Lo, Y. M., Chan, A. T., Chan, L. Y., et al. (2000) Molecular prognostication of nasopharyngeal carcinoma by quantitative analysis of circulating Epstein-Barr virus DNA. *Cancer Res.* **60**, 6878–6881.
9. Rowe, D. T., Webber, S., Schauer, E. M., Reyes, J., and Green, M. (2001) Epstein-Barr virus load monitoring: its role in the prevention and management of post-transplant lymphoproliferative disease. *Transplant. Infect. Dis.* **3**, 79–87.
10. Stevens, S. J. C., Verkuijlen, S. A. W. M., van den Brule, A. J. C., and Middeldorp, J. M. (2002) Comparison of quantitative competitive PCR with LightCycler-based PCR for measuring Epstein-Barr virus DNA load in clinical specimens. *J. Clin. Microbiol.* **40**, 3986–3992.
11. Wittwer, C. T., Ririe, K. M., Andrew, R. V., David, D. A., Gundry, R. A., and Balis, U. J. (1997) The LightCycler: a microvolume multisample fluorimeter with rapid temperature control. *Biotechniques* **22**, 176–181.
12. Leight, E. R. and Sugden, B. (2000) EBNA-1: a protein pivotal to latent infection by Epstein-Barr virus. *Rev. Med. Virol.* **10**, 83–100.
13. Bhatia, K., Raj, A., Guitierrez, M. I., et al. (1996) Variation in the sequence of Epstein Barr virus nuclear antigen 1 in normal peripheral blood lymphocytes and in Burkitt's lymphomas. *Oncogene* **13**, 177–181.
14. Snudden, D. K., Smith, P. R., Lai, D., Ng, M. H., and Griffin, B. E. (1995) Alterations in the structure of the EBV nuclear antigen, EBNA1, in epithelial cell tumours. *Oncogene* **10**, 1545–1552.
15. Wraitham, M. N., Stewart, J. P., Janjua, N. J., et al. (1995) Antigenic and sequence variation in the C-terminal unique domain of the Epstein-Barr virus nuclear antigen EBNA-1. *Virology* **208**, 521–530.
16. Stevens, S. J. C., Vervoort, M. B. H. J., van den Brule, A. J. C., Meenhorst, P. L., Meijer, C. J., and Middeldorp, J. M. (1999) Monitoring of Epstein-Barr virus DNA load in peripheral blood by quantitative competitive PCR. *J. Clin. Microbiol.* **37**, 2852–2857.
17. Stevens, S. J. C., Verschuuren, E. A. M., Pronk, I., et al. (2001) Frequent monitoring of Epstein-Barr virus DNA load in unfractionated whole blood is essential for early detection of posttransplant lymphoproliferative disease in high-risk patients. *Blood* **97**, 1165–1171.

18. Arrand, J. R., Rymo, L., Walsh, J. E., Bjorck, E., Lindahl, T., and Griffin, B. E. (1981) Molecular cloning of the complete Epstein-Barr virus genome as a set of overlapping restriction endonuclease fragments. *Nucleic Acids Res.* **9**, 2999–3014.
19. Lawrence, J. B., Villnave, C. A., and Singer, R. H. (1988) Sensitive high resolution chromatin and chromosome mapping in situ: presence and orientation of two closely integrated copies of EBV in a lymphoma line. *Cell* **42**, 51–61.
20. Stevens, S. J. C., Pronk, I., and Middeldorp, J. M. (2001) Toward standardization of Epstein-Barr virus DNA load monitoring: unfractionated whole blood as preferred clinical specimen. *J. Clin. Microbiol.* **39**, 1211–1216.
21. van Esser, J. W., Niesters, H. G., Thijsen, S. F., et al. (2001) Molecular quantification of viral load in plasma allows for fast and accurate prediction of response to therapy of Epstein-Barr virus-associated lymphoproliferative disease after allogeneic stem cell transplantation. *Br. J. Haematol.* **113**, 814–821.
22. Stevens, S. J. C., Blank, B. S., Smits, P. H., Meenhorst, P. L., and Middeldorp, J. M. (2002) High Epstein-Barr virus (EBV) DNA loads in HIV-infected patients: correlation with antiretroviral therapy and quantitative EBV serology. *AIDS* **16**, 993–1001.
23. Boom R, Sol CJ, Salimans MM, Jansen CL, Wertheim-van Dillen PM, van der Noordaa, J. (1990) Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503.
24. Witt, D. J. and Kemper, M. (1999) Techniques for the evaluation of nucleic acid amplification technology performance with specimens containing interfering substances: efficacy of Boom methodology for extraction of HIV-1 RNA. *J. Virol. Methods* **79**, 97–111.
25. Kwok, S. and Higuchi, R. (1989) Avoiding false positives with PCR. *Nature* **339**, 237–238.



<http://www.springer.com/978-1-58829-353-4>

DNA Viruses

Methods and Protocols

Lieberman, P.M. (Ed.)

2005, XIV, 498 p., Hardcover

ISBN: 978-1-58829-353-4

A product of Humana Press