Dx EBV ASSAY

96 REF 37021

Direct Quantitative detection of Epstein-Barr virus DNA by Real-Time PCR

IVD CE

EBV_rB0414

1-INTENDED USE

The Dx EBV Assay is intended for the *in vitro* Quantification of the Epstein-Barr virus genome DNA in human plasma and whole blood samples with a simultaneous control of the extraction/amplification reaction thanks to an Internal Control (IC).

The Dx EBV Assay is standardized against the World Health Organization (WHO) First International Standard for EBV (NIBSC code 09/260) to express the viral loads in International Unit (IU/ml).

The Dx EBV Assay is validated for its use in combination with the Dx Real-Time System (Bio-Rad) and its Dx Real-Time Software only.

Thanks to the common Nucleic Acid Extraction and Amplification procedures applied, the quantification of the EBV with the Dx EBV Assay can be performed simultaneously with the other quantitative assays of the Dx Assays immunocompromised panel.

2. SUMMARY AND EXPLANATION OF THE TEST

Epstein-Barr virus (EBV) is a common herpesvirus that infects more than 90% of the world's population, leaving the majority of individuals with a lifelong silent infection. Although most primary EBV infections are asymptomatic, the virus, mainly in immune-compromised individuals such as transplant recipients and AIDS patients, is the major predisposing factor for the development of a wide range of B-cell lympho-proliferative disorders (Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's and non-Hodgkin's lymphomas).

The EBV genome is a linear, double stranded DNA molecule of about 175 kb. It encoded a series of products interacting with or exhibiting homology to a wide variety of anti-apoptotic molecules, cytokines, and signal transducers, hence promoting EBV infection, immortalization, and transformation.

Early detection of EBV is crucial for effective treatment and immunosuppressive drug therapy amendment which may result in proliferative disease regression.

Treatment strategies for EBV-associated post-transplant lympho-proliferative disorder include reduction of immunosuppression, which is often successful in solid organ transplant, use of anti-B-cell monoclonal antibody, conventional chemotherapy and radiation. Current therapies for EBV-associated non-Hodgkin's lymphomas include chemotherapy and radiation therapy.

Molecular based assay such as real time PCR assays were demonstrated to be a useful tool for diagnosis of primary EBV infection because of high sensitivity, specificity, easy of use and quick method.

Quantitative EBV DNA measurement is essential for differentiating the low-level infection of healthy carriers from the high levels characteristic of EBV-related disease

3. PRINCIPLES OF THE PROCEDURE

The Dx EBV Assay test in based on two major steps: sample preparation and amplification/detection of the target DNA by real-time PCR. The Real Time PCR phase allows the amplification and the detection of the EBV DNA, recovered from the biological sample under investigation through an extraction step. During the Real-Time PCR, amplification products are detected and quantified against a standard curve using fluorescent dyes.

An Internal Control (IC) is systematically extracted, amplified and detected with each sample thus verifying the extraction procedure and presence in the sample of potential PCR inhibitors.

Thanks to the common IC used in the extraction step, the same purified sample matrix (plasma matrix or whole blood matrix) can be investigated also with the other quantitative assays of the Dx Assays immunocompromised panel.

Sample Preparation

Automated and manual DNA extraction are performed on all sample types listed in the section "Collection and Handling of Specimens", using respectively the bioMérieux NucliSens® easyMAG® System and the Qiagen QIAamp® DNA mini kit according to the Manufacturer Instruction Manuals (bioMérieux, QIAGEN).

A negative control (NC) is taken through the entire sample preparation procedure with the other specimens.

The Internal Control (IC) is added to each specimen and to the negative control at the beginning of the sample preparation procedure.

Real Time PCR Amplification and Detection

The Dx EBV Assay is validated for its use in combination with the Dx Real-Time System (Bio-Rad).

After extraction, extracted DNA and master mix are dispensed manually in the Dx 24-Well PCR Strips. In real-time PCR, specific fluorescent oligonucleotide probes are used to detect the DNA during amplification, by hybridizing to the amplicons. In the Dx EBV Assay two different types of oligonucleotide probes are used: [1] EBV probe targeting a fragment of the virus genome, and [2] Internal Control probe controlling the extraction procedure and the presence of PCR inhibitors.

In the absence of target DNA for a given oligonucleotide probe, the corresponding fluorescence will not be emitted so that no signal will be detected. When the target DNA is present, fluorescence intensity increases as the amount of amplicons increases with amplification cycles. At each elongation step, the Dx Real-Time System optical module measures the fluorescence obtained from each fluorophore, and the associated Dx Real-Time Software plots the fluorescence intensity *versus* the number of cycles. At the end of the assay, the Dx Real-Time Software automatically analyzes results for all samples, quantification standard (QS) and controls.

4. REAGENTS

4.1. Description

There are sufficient reagents provided in the kit to perform 96 tests. All reagents are intended for *in vitro* diagnostic use only.

Label		Description	PRESENTATION
AM	**Amplification Mix**	**Concentrated amplification mix (blue)** DNA Polymerase in a PCR buffer containing primers, specific fluorescent probes, dNTPs, $MgCl_2$ Preservative: 0.03% ProClin™ 300	**2 x 150 µl** To be diluted by adding DIL
DIL	**Amplification Mix Diluent**	**Amplification mix diluent (white)** PCR buffer containing $MgCl_2$ Preservative: 0.03% ProClin™ 300	**2 x 645 µl** Ready to use
IC	**Internal Control**	**Internal Control (yellow)** Non-infectious DNA in Tris-HCl-EDTA buffer. Preservative: 0.03% ProClin™ 300	**1 x 1600 µl** Ready to use
NC	**Negative Control**	**Negative Control (green)** Tris-HCl-EDTA buffer. Preservative: 0.03% ProClin™ 300	**2 x 1100 µl** Ready to use
PC	**Positive Control**	**Positive Control (red)** Non-infectious DNA containing EBV sequence in Tris-HCl-EDTA buffer. Preservative: 0.03% ProClin™ 300	**1 x 600 µl** Ready to use
QS1	**Quantification Standard**	**Quantification Standard (brown)** Non-infectious DNA containing EBV sequence in Tris-HCl-EDTA buffer. Concentration: 100000 copies/µl Preservative: 0.03% ProClin™ 300	**1 x 600 µl** Ready to use
QS2	**Quantification Standard**	**Quantification Standard (brown)** Non-infectious DNA containing EBV sequence in Tris-HCl-EDTA buffer. Concentration: 1000 copies/µl Preservative: 0.03% ProClin™ 300	**1 x 600 µl** Ready to use
QS3	**Quantification Standard**	**Quantification Standard and Reference for Curve adjustment (orange)** Non-infectious DNA containing EBV sequence in Tris-HCl-EDTA buffer. Concentration: 100 copies/µl Preservative: 0.03% ProClin™ 300	**2 x 600 µl** Ready to use
QS4	**Quantification Standard**	**Quantification Standard (brown)** Non-infectious DNA containing EBV sequence in Tris-HCl-EDTA buffer. Concentration: 10 copies/µl Preservative: 0.03% ProClin™ 300	**1 x 600 µl** Ready to use

4.2. Storage and handling requirements

The Dx EBV Assay kit must be stored frozen at -18°C/-22°C. When correctly stored at this temperature, all the reagents can be used until the expiry date shown on the label.

Identification	Storage after opening
Negative Control **(NC)**	2 months at -18°C/-22°C. Each vial can be used twice. If contamination occurs in one of these reagents, discard it
Positive Control **(PC)**	4 months at -18°C/-22°C. Each vial can be used up to 4 times. If contamination occurs in one of these reagents, discard it.
Internal Control **(IC)**	4 months at -18°C/-22°C. Each vial can be used up to 4 times. If contamination occurs in one of these reagents, discard it.
Quantitative Standards **(QS1, QS2, QS3, QS4)**	2 months at -18°C/-22°C. Each vial can be used twice. If contamination occurs in one of these reagents, discard it.
Reconstituted Mix **(AM+DIL)**	2 months at -18°C/-22°C. Each vial can be used twice. If frozen, the reconstituted mix can be thawed only once.

5. WARNINGS AND PRECAUTIONS

For *in vitro* diagnostic use only.

5.1. Health and Safety precautions

- Wear disposable gloves, laboratory coats and protective eyewear when handling reagents and samples.
- Thoroughly wash your hands after handling reagents and samples. Do not eat, drink or smoke in designated work areas.
- Handle all samples as potentially infectious, in accordance with Good Laboratory Practices.
- Chemicals must be handled and disposed in accordance with Good Laboratory Practices.
- The Safety Data Sheet is available upon request to your local Bio-Rad agent.

5.2. Precautions related to the procedure

- This kit is validated for use with the Dx Real-Time System (Bio-Rad Cat. # 94000) and its Dx Real-Time Software only.
- DNA extraction must be performed, using respectively the bioMérieux NucliSens® easyMAG® or the Qiagen QIAamp® DNA mini kit according to the Manufacturer Instruction Manuals.
- Do not use expired reagents.
- With the exception of the Internal Control (IC) do not interchange, mix or combine reagents from kits with different lot numbers.
- Do not change the assay procedure.
- Carefully avoid cross-contamination during specimen handling steps: ensure that specimen containers do not contact one another; if gloves come in contact with specimen, change them immediately.
- Use a new filter tip for each sample.
- Use DNase and RNase-free microtubes and filter tips.
- Carefully reconstitute the Amplification Mix avoiding any contamination.
- Check the pipettes and other equipment for accuracy and correct operation.
- Avoid spilling samples or solutions containing samples. Spills must be rinsed with bleach diluted at 10%. The material used for cleaning must be discarded in a contaminated residue container.
- Work surfaces, pipettes and other equipment must be decontaminated on a regular basis with bleach diluted at 1%.
- **Work areas**: within the laboratory, two dedicated areas must be unidirectionally used to perform sample preparation and amplification/ detection steps:
    1. **The Pre-amplification area** is dedicated to: (a) the preparation of samples and controls and (b) the PCR setup (pipetting of Amplification mix, controls and samples in the Dx 24-Well PCR Strips). These two steps must be done in two distinct zones of the Pre-amplification area.
    2. **The Amplification area** is dedicated to the Real-Time PCR reaction. All reagents, equipment and laboratory coats used in one area must remain in this area and never be moved in the other area. Never transfer amplification products or Amplification area equipment in the Pre-amplification area.

6. COLLECTION AND HANDLING OF SPECIMENS

Samples must be collected and transported following the instructions of the Laboratory and the local legislation for hazardous and infectious material transport.

Samples must be collected and transported in the shortest possible time referring at the specific final use.

Samples have to be clearly identified with codes or names in order to avoid misinterpretation of results.

Plasma samples:

The Whole Blood must be collected in EDTA containing tubes according to the Laboratory guidelines

Store collected whole blood at room temperature and separate plasma from cells within 2 hours of collection by centrifugation (about 1200g for at least 10 minutes at Room Temperature). Plasma not directly treated upon arrival must be stored at +2°C/+8°C for one week maximum. For a longer period, store the plasma at -18°C/-22°C.

Plasma must be sent to the Laboratory preferably at room temperature (+18°C/+25°C) or +2°C/+8°C.

When using frozen samples, thaw the samples just before the extraction step in order to avoid the nucleic acid degradation.

Whole blood samples:

The Whole Blood must be collected in EDTA containing tubes according to the Laboratory guidelines.

Store collected whole blood at room temperature (+18°C/+25°C) or +2°C/+8°C no longer than 6 hours. For a longer period, store the whole blood at -18°C/-22°C.

Whole Blood must be sent to the Laboratory at room temperature (+18°C/+25°C) or +2°C/+8°C.

When using frozen samples, thaw the samples just before the extraction step in order to avoid the nucleic acid degradation.

7. PROCEDURE

7.1. MATERIALS REQUIRED

7.1.1 Materials provided

Dx EBV Assay (cat. # 37021)

7.1.2. Materials required sold separately

- Dx Real-Time System and accessories (Bio-Rad, cat.# 94000)
- Dx Strip Cap Tool (provided in the Dx Real-Time System package Bio-Rad cat.#94000)
- Dx 24-Well PCR Strips (cat. # 94020)

7.1.3. Materials required but not provided

For NucliSens® easyMAG® system extraction

- NucliSens® easyMAG® instrument, reagents and consumables from bioMérieux: easyMAG® Extraction Buffers 1, 2 and 3 (cat. # 280130, 280131, 280132), easyMAG® Magnetic Silica (cat. # 280133), easyMAG® Lysis Buffer (cat. # 280134) easyMAG® Disposables (cat. # 280135), Biohit tips (cat. # 280146) and (optional) strip 8 wells (cat. # 278303).
- Desktop centrifuge for 1.5 ml and 2 ml microtubes
- Vortex mixer
- 1.5 or 2 ml hermetic screw-cap microtubes with round bottom shape
- Adjustable calibrated pipettes p10 or p20, p100 ul or p200 ul and p1000 ul
- Sterile pipette tips with filter
- Molecular grade water
- Optional: Phosphate-buffered saline (PBS)
- Disposable powder-free gloves

For QIAamp® DNA mini kit extraction

QiAamp® DNA Mini Kit, 250(Cat.# 51306), extra reagents and consumables from Qiagen: Proteinase K (2 x 2 ml cat.# 19131 or 1x10 ml cat.# 19133), buffer AL (1 x 216 ml cat.# 19075) and collection tubes 2 ml (cat.# 19201)

- Desktop centrifuge for 1.5 ml and 2 ml microtubes
- Vortex mixer
- Ethanol 96-100%
- Optional: Phosphate-buffered saline (PBS)
- Heating block able to reach 56°C
- Adjustable calibrated pipettes p10 or p20, p100 or p200 ul and p1000. Sterile pipette tips with filters
- 1.5 or 2 ml hermetic screw-cap microtubes with round bottom shape
- Disposable powder-free gloves

7.2. REAGENT PREPARATION AND STORAGE

7.2.1 When using NucliSens® easyMAG® system extraction

The NucliSens® easyMAG® platform is intended for the automated isolation of total nucleic acid (DNA/RNA) from biological specimens.

Storage of Lysis buffer at 2-8°C may give rise to the appearance of crystal due to high salt concentration. The crystal has to be dissolved before use.

Ensure that NucliSens® easyMag® Buffer 2 and 3 are at room temperature before starting the procedure.

Once loaded in the reagent area of the Instrument all buffers are stable up to 1 month under ambient conditions.

Once opened, Magnetic Silica can be stored for a maximum of 14 days at 2-8°C.

For plasma samples extraction the Magnetic Silica must be diluted with molecular grade water as reported in section 7.3.2 at point 9 and must be used immediately.

For more details please refer to the NucliSens® easyMAG™ user manual

7.2.2 When using Qiagen® extraction

The QIAamp® DNA Mini Kit is intended for the purification of total DNA from body fluid using a microcentrifuge.

Buffer AW1: Before using for the first time add ethanol (96-100%) as indicated on the bottle. Prepared Buffer AW1 is stable for 1 year when stored closed at room temperature.

Buffer AW2: Before using for the first time add ethanol (96-100%) as indicated on the bottle. Prepared Buffer AW2 is stable for 1 year when stored closed at room temperature.

For more details please refer to the Qiagen QIAamp® DNA Mini Kit user manual.

7.2.3 Reconstitution of Amplification Mix

Before reconstitution, AM (Amplification Mix) and DIL (Amplification Mix Diluent) vials must be thawed, vortexed for 5 seconds then centrifuged for 15 seconds.

Add 645 µl of Amplification Mix Diluent (DIL) into one vial of concentrated Amplification Mix (AM). Vortex for 10 seconds then centrifuge briefly.

One vial of reconstituted Amplification Mix (AM+DIL) is sufficient for 48 real-time PCR reactions. Prepare as many vials of reconstituted Amplification mix (AM+DIL) as necessary (for example, 2 vials if 96 tests are to be processed).

The reconstituted Amplification Mix (AM+DIL) can be used immediately or stored at –20°C for up to 2 months. Each vial can be used twice. If frozen, the reconstituted mix can be thawed only once.

AM (Amplification Mix) and the reconstituted Amplification Mix (AM+DIL) are light sensitive. Protect it from strong light exposition

7.3. INSTRUCTIONS FOR USE

Follow strictly these instructions and apply Good Laboratory Practices.

7.3.1. Automated DNA extraction with NucliSENS® easyMAG® (bioMérieux)

1. Before using it for the first time, follow the Manual Instruction of producer for the preparation of reagents and instrumentation.
2. Determine the number of samples to be tested and take one screw-cap microtube per sample plus one more for the Negative Control (NC). Identify each microtube.
3. Equilibrate samples to room temperature

Procedure for Plasma Samples

4. Set the instrument as **General Protocol.**

Note: Use Specific B Protocol if plasma and whole blood are extracted together

5. Sample plasma volume = 500µl
6. Elution Volume = 55µl
7. Sample type = Primary, with Lysis incubation.
8. Add 500µl of sample into suitable disposable rack (8 samples/each).
9. Insert the disposable rack in the instrument proper place.
10. Start with automatic lysis phase dispensing with incubation on-board (10 min).

Note: when plasma and whole blood samples will be extracted together (Specific B protocol) the 10 minutes incubation of plasma samples will be performed off-board.

11. During lysis phase proceed to the preparation of the PreMix as follow:

Reagent (for plasma extraction)	1 test	12 tests	24 tests
Magnetic Silica	50µl	600 µl	1200 µl
Molecular grade water	50µl	600 µl	1200 µl
Internal Control (IC)	6µl	72 µl	144 µl

The PreMix must be used immediately. The remaining prepared PreMix must be discarded.

12. Add 100µl of PreMix to each sample. Mix sample with the Biohit Electronic Pipet using the Program 3.

13. Restart the instrument with automatic phase (40 min).

Carefully transfer the purified sample into a clean 1.5 ml screw-cap microtube, avoiding the contact with the silica, within 30 min by the end of the automated extraction.

The transferred extracted DNA can be kept at room temperature for 2 hours or stored at 2-8°C for up to 4 days. For longer storage, extracted DNA must be stored at -20°C for up to 6 months.

Procedure for Whole Blood Samples

1. Set the instrument as **Specific B Protocol**
2. Sample whole blood volume = 200µl
3. Elution Volume = 40µl
4. Sample type = Primary, without Lysis incubation
5. Insert the disposable rack in the instrument proper place.
6. Start with the Lysis Buffer dispensing
7. During Lysis Buffer dispensing phase proceed to the preparation of the PreMix as follow:

Reagent (for whole blood extraction)	1 test	12 tests	24 tests
Magnetic Silica	100µl	1200 µl	2400 µl
Lysis Buffer	600µl	7200 µl	14400 µl
Internal Control (IC)	6µl	72 µl	144 µl

8. Add 200µl of sample to the lysis buffer into suitable disposable rack (8 samples/each) and mix by pipetting.
9. Add 700 µl of PreMix to each sample. Mix sample with the Biohit Electronic Pipet using the Program 3.
10. Restart the instrument with automatic phase (60 min).

Carefully transfer the purified sample into a clean 1.5 ml screw-cap microtube, avoiding the contact with the silica , within 30 min by the end of the automated extraction.

The transferred extracted DNA can be kept at room temperature for 2 hours or stored at 2-8°C for up to 4 days. For longer storage, extracted DNA must be stored at -20°C for up to 6 months.

Procedure for simultaneous extraction of Whole Blood and Plasma Samples

Follow the correct procedure as reported below:

INSTRUMENT SET-UP		
Specific B protocol		
Sample type = Primary		
Sample volume depending on the sample matrix (see specific sections above)		
Elution volume depending on the sample matrix (see specific sections above)		
Run without incubation step (lysis incubation off-board for plasma)		
OFF-BOARD		
	Whole blood	**Plasma**
step1	-	Add 500µl of plasma sample into suitable disposable rack in the right position
ON-BOARD		
step2	Insert the disposable rack in the instrument proper place	

step3	Start the Lysis Buffer dispensing	
OFF-BOARD		
step4	During the step 3 (on-board) proceed to the preparation of the pre-mix as reported in the specific sections above	
step5	At the end of the step 3 take-off the suitable disposable rack	
step6	Add 200µl of WB sample in the right position of the suitable disposable rack and mix by pipetting	Lysis incubation at room temperature (10 min)
step7	Add the specific Pre-mix (700µl) to the mixture "sample + lysis buffer"	Add the specific Pre-mix (100µl) to the mixture "sample + lysis buffer"
ON-BOARD		
step8	Insert the disposable rack in the instrument proper place	
step9	Mix samples using Biohit Electronic Pipet Program 3	
step10	Start Instrument Run (60min)	

Carefully transfer the purified sample into a clean 1.5 ml screw-cap microtube, avoiding the contact with the silica, within 30 min by the end of the automated extraction.

The transferred extracted DNA can be kept at room temperature for 2 hours or stored at 2-8°C for up to 4 days. For longer storage, extracted DNA must be stored at -20°C for up to 6 months.

7.3.2. Manual DNA extraction with QIAamp® DNA Mini Kit (Qiagen)

1. Before using for the first time, follow the Manual Instruction of producer for the preparation of reagents.
2. Determine the number of samples to be tested and take one screw-cap microtube per sample plus one more for the Negative Control (NC). Identify each microtube.
3. Equilibrate samples to room temperature
4. Warm the heating block to 56°C.
5. If a precipitate has formed in Buffer AL, dissolve by incubation at 56°C.

Procedure for Plasma Samples

6. Add 40µl of Proteinase K (PK) into the bottom of each 1.5 ml screw-cap microtube.

Note : Do not add proteinase K directly to buffer AL

7. Add 400µl sample to the 1.5 ml screw-cap microtube.
8. Add 400µl Buffer AL to the sample. Mix by vortexing briefly for 15 sec.
9. Add 6µl Internal Control (IC) supplied with Dx EBV Assay.
10. Incubate at 56°C for 10 min.
11. Spin to remove drops from the inside of the lid.
12. Add 400µl ethanol 96-100% to the sample, mix by vortexing briefly for 15 seconds. Spin briefly to remove drops from the inside of the lid.
13. Carefully transfer 620µl of the mixture to the QIAamp spin column without wetting the rim. Close the cap and centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate.
14. Repeat step 13 by transferring the remaining mixture volume.
15. Carefully add 500µl buffer AW1 to the spin column without wetting the rim. Close the cap and centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate.
16. Carefully add 500µl buffer AW2 to the spin column without wetting the rim. Close the cap and centrifuge at 20000g (14000rpm) for 3 min.
17. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate. Centrifuge at full speed for 1 min.
18. Place the QIAamp spin column in a clean 1.5 ml screw-cap microtube and discard the tube containing the filtrate.
19. Open the QIAamp spin column and add 50µl Buffer AE. Incubate at room temperature (18-25°C) for 1min and centrifuge at 6000g (8000rpm) for 1 min.
20. Discard the QIAamp spin column.

Extracted DNA can be stored at 2-8°C for up to 4 days or stored at -20°C for up to 6 months.

Procedure for Whole Blood Samples

6. Add 20µl of Proteinase K (PK) into the bottom of each 1.5 ml screw-cap microtube.

Note : Do not add proteinase K directly to buffer AL

7. Add 200µl sample to the 1.5 ml screw-cap microtube.
8. Add 200µl Buffer AL to the sample. Mix by vortexing briefly for 15 sec.
9. Add 6µl Internal Control (IC) supplied with Dx EBV Assay.
10. Incubate at 56°C for 10 min.
11. Spin to remove drops from the inside of the lid.
12. Add 200µl ethanol 96-100% to the sample, mix by vortexing briefly for 15 seconds. Spin briefly to remove drops from the inside of the lid.
13. Carefully transfer the mixture to the QIAamp spin column without wetting the rim. Close the cap and centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate.
14. Carefully add 500µl buffer AW1 to the spin column without wetting the rim. Close the cap and centrifuge at 6000g (8000rpm) for 1 min. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate.
15. Carefully add 500µl buffer AW2 to the spin column without wetting the rim. Close the cap and centrifuge at 20000g (14000rpm) for 3 min.
16. Place the QIAamp spin column in a clean collection tube and discard the tube containing the filtrate. Centrifuge at full speed for 1 min.
17. Place the QIAamp spin column in a clean 1.5 ml screw-cap microtube and discard the tube containing the filtrate.
18. Open the QIAamp spin column and add 100µl Buffer AE. Incubate at room temperature (18-25°C) for 1min and centrifuge at 6000g (8000rpm) for 1 min.
19. Discard the QIAamp spin column.

Extracted DNA can be stored at 2-8°C for up to 4 days or stored at -20°C for up to 6 months.

7.3.3 Standard curve

Quantification Standard (QS1 to QS4), provided in the Dx EBV Assay, must be included in every first run of a new lot to generate a Standard Curve in the Dx Real Time Software.

The stored Standard Curve (Slope, R2) is valid for up to 6 months and the Quantification Standard (QS3), coming from the same kit lot, has to be used in the following runs as calibrator.

NOTE: Make sure the QS3 storage conditions are strictly applied as described in section 4.2.

If the QS3 Ct is in the acceptability criteria, the stored standard curve will be translated to fit the QS3 value.

7.3.4. Real-time PCR Amplification/Detection

Please refer to the Dx Real-Time System User Manual for more detailed instructions.

Each assay run must include one negative control and one positive control and the necessary quantification standards.

In the Pre-amplification area:

1. Reconstitute Amplification Mix vials as necessary following the procedure on section 7.2.3 (test number = n samples + 1 Positive Control + 1 Negative Control and the necessary Quantification Standards).
2. Take the required number of Dx 24-Well PCR Strips, place them on a holder and dispense carefully, with the repeat pipettor, 15µl of reconstituted Amplification mix (AM+DIL) into each well.
3. Add 10µl of extracted DNA **or** extracted Negative Control (NC) **or** non-extracted Positive Control (PC) and the necessary Quantification Standard (QS1 to QS4 or QS3 only) into the corresponding wells, following the established plate map.
4. Place the Dx 8-Cap Strips on the Dx 24-Well PCR Strips and close them firmly.
5. Centrifuge the Dx 24-Well PCR Strips for 30 seconds at 400 g in order to avoid any bubble in the wells.

In the Amplification area:

1. Switch on the computer then the Dx Real-Time System. Open the Dx Real-Time Software. Enter your user name and login and click on «Setup».
2. Click on “create a new plate” and select the PCR kit name in “PCR kit”. Please refer to your local support for more information about the PCR kit name.
3. Click on the “Run” button then click on “Open Lid”.
4. Load the Dx 24-Well PCR Strips in the Dx Real-Time System and use the Dx Strip Cap Tool for seating the Dx 8-Cap Strips.
5. Click on the “Close lid” button then on the “Start run” button.
6. Click on “Results” to have a view on the results.

After completion of the real-time PCR reaction, remove the Dx 24-Well PCR Strips from the Dx Real-Time System, place them in a sealable plastic bag and dispose according to Good Laboratory Practices.

8. CALIBRATION

During each PCR cycle, at the elongation step, the Dx Real-Time System optical module measures the fluorescence obtained from each fluorophore, and the associated Dx Real-Time Software plots the fluorescence intensity versus number of cycles.

After the plate has been run, the Dx Real-Time Software automatically analyzes the collected fluorescence data and interprets the batch results based on the assay interpretation rules. Analysis of the Dx EBV Assay test results is based on the **Ct value**.

Ct is defined as the fractional PCR cycle number at which the background subtracted fluorescence crosses a threshold set empirically for each nucleic acid sequence detected by the assay. Within the assay quantification range, the Ct value is theoretically inversely proportional to the logarithm of the copy number of the target sequence in the sample before amplification. The higher the Ct value is, the lower the initial copy number is.

For each sample, the values of the mathematical parameter of interest are calculated for its PCR curves, and are displayed next to the interpreted result and flag(s). If a PCR curve shows no significant amplification, a "*" is displayed in place of a numeric value.

9. QUALITY CONTROL

Positive and Negative Controls

A Negative and a Positive control must be included in each assay run in order to detect potential failure in specimen processing, amplification or detection steps.

If Ct values of the controls are out of their expected range, the Dx Real-Time Software invalidates the whole assay run and displays one of the following flags:

* Negative Control:

Flag	Signification
Invalid_IC	Invalid Internal Control
EBV_conta	contamination by EBV DNA

* Positive Control:

Flag	Signification
EBV_out	EBV value out of range

If one of these flags appears, the run must then be reprocessed

Standard Curve

For the samples quantification all the Quantification Standard (QS) provided in the Dx EBV Assay kit must be included in the run to create a calibration standard curve (first run).

* Standard Curve (QS1 to QS4):

	Acceptability Criteria
Slope	-3.9 < slope < -3.1
Correlation (R2)	R2 > 0.98

If the values are out of the acceptances criteria, the entire PCR assay must be than reprocessed starting with new PCR assay run.

* Quantification Standard (QS3):

In the following runs only Quantification Standard (QS3) should be included to validate the standard curve performed previously. If Ct value of the Quantification Standard (QS3) is out of its expected range, the Dx Real-Time Software invalidates the whole assay run and displays the following flag:

Flag	Signification
QS_out	Quantification standard value out of range

If the value is out of the range, the entire PCR assay must be than reprocessed starting with new PCR assay run.

Detection of Inhibition: Internal Control

The Internal Control (IC) is added to each sample and to the Negative Control at the beginning of DNA extraction step, and is detected with a specific probe during the real-time PCR reaction.

Specimens whose Internal Control's Ct value is above the expected value (meaning possibly inhibited) are interpreted by the Dx Real-Time Software as follows:

- Any sample negative is reported as "Failed" for this target.
- Any sample with a detectable target (< LLOQ or within the range of quantification of the target) is reported as "EBV_POS" for this given target but not quantifiable (invalid_IC).

- Any sample with a viral load above the upper limit of quantification (ULOQ) of the target is reported as “EBV_POS” with the flag “> ULOQ”.

If the Internal Control Ct value is out of the acceptability criteria and it is therefore declared “Invalid”, repeat the analysis extracting and testing the sample under examination properly diluted in PBS, following the common laboratory practice for the Real Time PCR molecular diagnostics.

The Sample dilution factor (e.g.: 1/2, 1/10, 1/100) must be carefully chosen on the basis of the sample Ct value displayed on the Dx Software and should be inversely proportional to the sample Ct value.

10. INTERPRETATION OF RESULTS

10.1. Test validation criteria

The assay run is valid if:

- The Positive Control’s Ct value is within the expected range for the target
- The Negative Control has no Ct value for the target and internal control Ct value is within the expected range
- The Standard Curve values satisfy the acceptability criteria

  or

- The Quantification standard 3 (QS3) value is within the expected range for the target

If the assay run is valid, the Dx Real-Time Software reports the run status as “Passed”. If not, the Dx Real-Time Software reports the run status as “Failed”

If the run status is “Failed”, all test results in that run are reported as “Invalid” and all corresponding samples and controls must be reprocessed as reported in section 9.

10.2. Calculation / Results interpretation

Dx EBV assay was standardized against the 1st WHO International Standard for EBV (NIBSC code 09/260) to express samples concentration also in International Unit (IU/ml).

Results calculation is performed by the Dx Real-Time Software, which automatically determines Ct values for EBV and for the Internal Control in samples and controls.

If the test is valid, any sample (inhibited or not) with a detectable EBV DNA is reported as “EBV_POS” for this given target by the Dx Real-Time Software. However a positive sample with the flag “Invalid_IC” cannot be quantified. The corresponding sample should therefore be reprocessed starting from the DNA extraction step of the sample properly diluted in PBS as described in section 9.

The following table summarizes the different situations for interpreting EBV test, with the associated messages and their meaning:

Criteria	Interpretation “Result”	Signification	Message “Flag”
VALID Internal Control	Negative Sample “EBV_neg”	EBV DNA not detected	None
	Positive Sample no titer “EBV_POS”	EBV DNA below the Lower limit of Quantification (LLOQ)	< LLOQ
	Positive sample Titer in Copies/ml or UI/ml “EBV_POS”	EBV DNA equal or greater than the Lower Limit of Quantification (LLOQ) and less than or equal to the Upper Limit of Quantification (ULOQ)	None
	Positive Sample no titer ”EBV_POS”	EBV DNA greater than the Upper Limit of Quantification (ULOQ)	> ULOQ
INVALID Internal Control	“Failed”	EBV DNA not detected	Invalid_IC
	Positive Sample no titer ”EBV_POS”	EBV DNA detectable but not quantificable	Invalid_IC
		EBV DNA greater than the Upper Limit of Quantification (ULOQ)	> ULOQ

11. TEST LIMITATIONS

- Optimal performances of this test depend directly on the quality of specimens. It is therefore important to comply with indications given in the chapter “Collection and handling of specimens”.
- The assay should be performed only on indicated sample types. Other sample types have not been validated.
- Use of this assay is limited to personnel who have been trained on the use of the Dx EBV Assay, Dx Real-Time System and Dx Real-Time Software.
- A negative result does not exclude the possibility of infection because results are dependent on several variables. An improper specimen collection or handling, the presence of inhibitors or a technical error can lead to a false result.
- As with any diagnostic test, results from the Dx EBV Assay should be interpreted in conjunction with other laboratory and clinical findings.
- Samples containing residues of fibrin or heavy particles or microbial filaments and bodies should be discarded as they could give rise to false results.
- Haemolysed (red) and hyperlipemic (“milky”) plasma samples have to be discarded as they could generate false results.

- Plasma samples, which has been collected in tubes containing heparin as an anticoagulant should not be used because heparin (>10 IU/ml) affects the PCR reactions.
- The described performances of the kit can only be guaranteed for the recommended extraction systems and PCR instrument.

12. PERFORMANCE CHARACTERISTICS

12.1. PRECISION MEASUREMENT

12.1.1 Reproducibility and Repeatability measurement

A sample panel constituted of negative samples and EBV positive samples at different levels (low, medium and high) was created and tested for precision study in 3 extraction replicates.

This study was done with 2 different Dx EBV assay lots (Lot1, Lot2) on 2 different Dx Real-Time System on a period of 10 days with 3 runs.

EBV target: Total precision results – Ct value of positive samples

*Reproducibility

EBV Panel		Total				Within run			
Sample	EBV lot	N	Mean Ct	SD	CV%	N	Mean Ct	SD	CV%
Low (3xLOD)	**Lot 1 & Lot 2**	66	36.75	1.040	**2.83**	22	36.31	0.478	**1.32**
Medium	**Lot 1 & Lot 2**	66	31.35	0.309	**0.99**	22	31.60	0.291	**0.92**
High	**Lot 1 & Lot 2**	66	20.98	0.349	**1.66**	22	21.27	0.111	**0.52**

*Repeatability

EBV Panel		Total				Within run			
Sample	EBV lot	N	Mean Ct	SD	CV%	N	Mean Ct	SD	CV%
Low (3xLOD)	**Lot 1**	33	36.13	0.519	**1.44**	22	36.06	0.538	**1.49**
	Lot 2	33	37.36	1.070	**2.86**	22	37.87	0.900	**2.38**
Medium	**Lot 1**	33	31.36	0.255	**0.81**	22	31.31	0.231	**0.74**
	Lot 2	33	31.33	0.360	**1.15**	22	31.11	0.175	**0.56**
High	**Lot 1**	33	21.14	0.130	**0.61**	22	21.08	0.091	**0.43**
	Lot 2	33	20.82	0.422	**2.03**	22	20.59	0.306	**1.49**

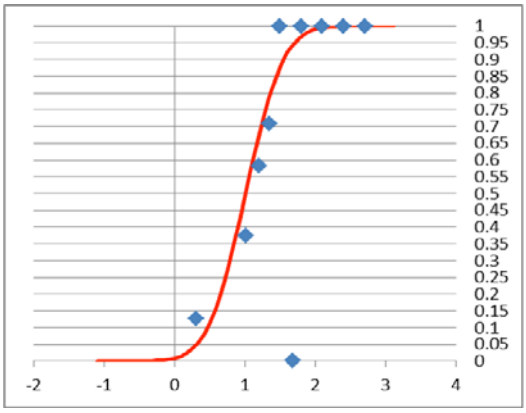
12.2. ANALYTICAL PERFORMANCE

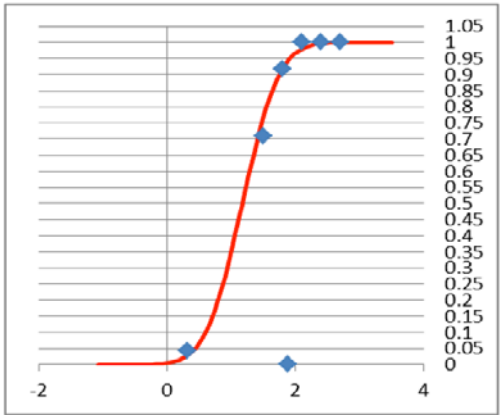
12.2.1 Limit of Detection (LOD)

The limit of detection (LOD) has been determined, considering the extraction method, by testing EBV WHO (NIBSC code 09/260) several dilution series into borderline concentration and using the probit analysis to retain the dilution corresponding to a positive level of 95%.

The Statistical analysis (Probit) has been applied using the results obtained from 24 replicates of 6 dilution points tested in three different runs (8 replicates x 3 runs).

NucliSens® easyMAG® Automatic Extraction QIAamp® DNA Mini Kit Manual Extraction





LOD (95%) = 47.92 IU/ml = 79.07 copies/ml LOD (95%) = 77.71 IU/ml = 247.8 copies/ml

12.2.2 Analytical Specificity

The analytical specificity of the Dx EBV Assay was evaluated on a panel of samples coming from patients suffering infections caused by potential interfering organisms obtained from a Reference Clinical Centre.

Cross reactivity pathogens

Family	Target	Concentration (copies/ml)	Result
Herpes virus	CMV	10000	No Cross Reaction
	VZV	10000	No Cross Reaction
	HHV6	10000	No Cross Reaction
	HHV8	1000	No Cross Reaction
	HSV1	100000	No Cross Reaction
	HSV2	100000	No Cross Reaction
Polyomavirus	JCV	10000	No Cross Reaction
	BKV	100000	No Cross Reaction
Hepatitis virus	HBV	1000000	No Cross Reaction
	HCV	1000000	No Cross Reaction

No cross reaction were observed with the panel tested

12.3. CLINICAL PERFORMANCE

12.3.1 Results expressed in International Units

A Conversion Factor (copies to International Unit) has been determined for the Dx EBV Assay testing serial dilutions of the 1st WHO International Standard for EBV (NIBSC code 09/260) in pooled negative EDTA plasma and whole blood..The results obtained in copies/ml can be converted into WHO International Units/ml with the Conversion Factor (CF), specifically determined for each extraction method used in combination with the kit, as described here below:

	NucliSens® easyMag® Extraction	Qiagen® Extraction
PLASMA - Conversion factor (CF)	1.65	3.19
WHOLE BLOOD - Conversion factor (CF)	2.05	1.47

The Formula to convert the copies/ml into International Units/ml is the following:

Results (IU/ml) = value copies/ml / Conversion Factor

12.3.2 Dynamic Range

The diagnostic dynamic range has been determined for the Dx EBV Assay testing serial dilutions of the 1st WHO International Standard for EBV (NIBSC code 09/260) and of a Plasmid, carrying the EBV target sequence, in pooled negative EDTA plasma and whole blood. On the basis of the results obtained from this study using different extraction methods, the Dynamic Range for the Dx EBV Assay is the following:

Extraction Method	Dynamic Range for Plasma samples	Dynamic Range for Whole Blood samples
NucliSENS® easyMAG®	250 IU/ml ≤ Dynamic Range ≤ 6 666 667 IU/ml 413 copies/ml ≤ Dynamic Range ≤ 11 000 000 copies/ml	244 IU/ml ≤ Dynamic Range≤ 9 760 000 IU/ml 500 copies/ml ≤ Dynamic Range≤ 20 000 000 copies/ml
QIAamp® DNA mini kit	250 IU/ml ≤ Dynamic Range ≤ 3 918 495 IU/ml 798 copies/ml ≤ Dynamic Range ≤ 12 500 000 copies/ml	206 IU/ml ≤ Dynamic Range≤ 10 300 000 IU/ml 303 copies/ml ≤ Dynamic Range≤ 15 200 000 copies/ml

12.3.3 Diagnostic sensitivity

The diagnostic sensitivity of the Dx EBV Assay was evaluated on QCMD panel 2012 also used to confirm the viral load in IU/ml.

All the samples were extracted with NucliSens® easyMAG® automatic extraction (bioMérieux) and QIAamp® DNA Mini Kit (QIAGEN).

QCMD 2012	Qiagen® Extraction		NucliSens® easyMag® Extraction		Reference Results (Copies/ml)		
Sample n°	Results (copies/ml)	Log Results	Results (copies/ml)	Log Results	Copies/ml	Reported Log Range	
EBV12-01	1570	**3.196**	POS (<LOQ)		571	**2.297**	**3.217**
EBV12-02	13000	**4.114**	6520	**3.814**	6310	**3.392**	**4.208**
EBV12-03	327000	**5.515**	179000	**5.253**	161000	**4.84**	**5.576**
EBV12-04	129000	**5.111**	79400	**4.9**	65800	**4.417**	**5.219**
EBV12-05	46700	**4.669**	23600	**4.373**	21400	**3.953**	**4.709**
EBV12-06	69000	**4.839**	37600	**4.575**	30700	**4.105**	**4.869**
EBV12-07	136000	**5.134**	67200	**4.827**	62100	**4.409**	**5.177**
EBV12-08	7070	**3.849**	3370	**3.528**	3180	**3.092**	**3.914**
EBV12-09	5210	**3.717**	2820	**3.45**	2110	**2.908**	**3.74**
EBV12-10	NEG		NEG		NEG		

QCMD 2012	Qiagen® Extraction		NucliSens® easyMag® Extraction		Reference Results (IU/ml)		
Sample n°	Results (IU/ml)	Log Results	Results (IU/ml)	Log Results	IU/ml	Reported Log Range	
EBV12-01	492	2.692	POS (<LOQ)		234	1.862	2.876
EBV12-02	4080	**3.61**	3950	**3.597**	4240	**3.305**	**3.949**
EBV12-03	103000	**5.011**	108000	**5.035**	86500	**4.684**	**5.19**
EBV12-04	40400	**4.607**	48100	**4.682**	41000	**4.375**	**4.851**
EBV12-05	14600	**4.166**	14300	**4.155**	14200	**3.9**	**4.406**
EBV12-06	21600	**4.335**	22800	**4.358**	18800	**4.017**	**4.533**
EBV12-07	42600	**4.63**	40700	**4.61**	30600	**3.982**	**4.99**
EBV12-08	2220	**3.346**	2040	**3.31**	2400	**2.845**	**3.917**
EBV12-09	1630	**3.213**	1710	**3.233**	1120	**2.571**	**3.531**
EBV12-10	NEG		NEG		NEG		

12.3.4 Clinical Study

PLASMA

A retrospective clinical study was carried out on plasma samples of patients transplanted and characterized by a CE marked Real-Time PCR commercial kit. The same plasma samples extracts (NucliSens® easyMAG® automatic extraction) were analyzed with the Dx EBV Assay and with another available CE marked commercial kit.

Qualitative analysis:

		Reference CE marked commercial kit		
NucliSens® easyMAG®		Pos	Neg	
Dx EBV Assay	Pos	24	0	24
	Neg	0	30	30
Total		24	30	54

Concordance = 100%

		CE marked commercial kit		
NucliSens® easyMAG®		Pos	Neg	
Dx EBV Assay	Pos	24	0	24
	Neg	1	29	30
Total		25	29	54

Concordance = 98%

The sample with discordant result had viral load below the limit of detection (LOD).

The same plasma samples were also extracted with the manual extraction system and tested with Dx EBV assay and the CE marked Real-Time PCR commercial kit validated with the same extraction.

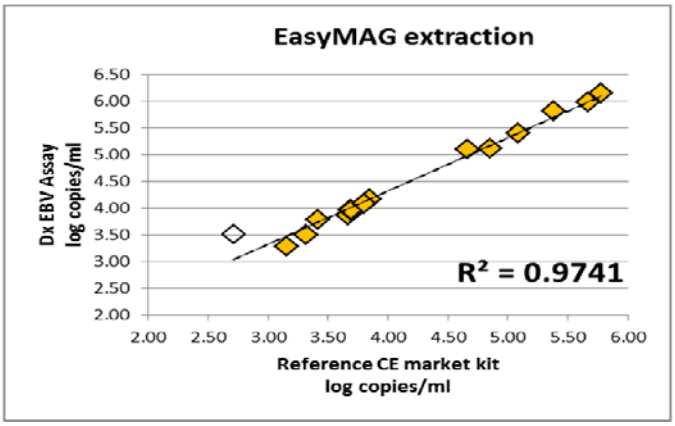
		CE marked commercial kit		
Qiagen® DNA mini kit		Pos	Neg	
Dx EBV Assay	Pos	24	0	24
	Neg	1	29	30
Total		25	29	54

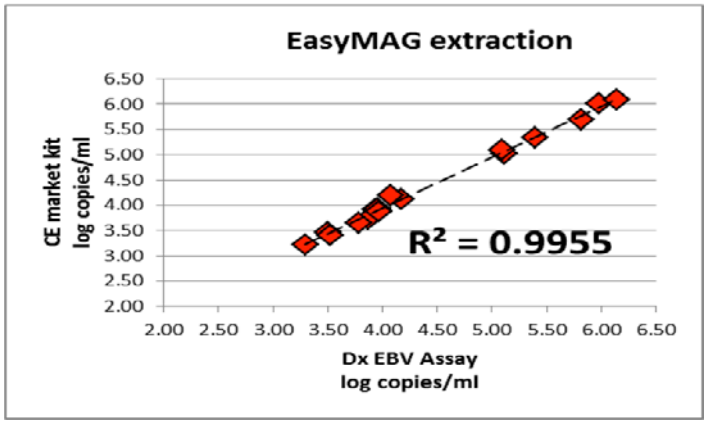
Concordance = 98%

The sample with discordant result had viral load below the limit of detection (LOD).

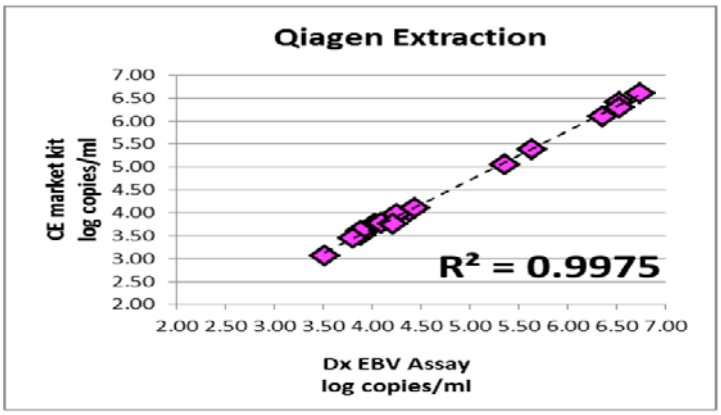
Quantitative analysis:

On the same samples were performed a correlation analysis comparing the viral load (copies/ml) obtained with the Dx EBV Assay and two CE market kits, considering also the extraction method used.





◈ Δlog > 0.5



The correlation was always > 0.95%

WHOLE BLOOD

A retrospective clinical study was carried out on whole blood samples of patients transplanted and analyzed with the Dx EBV Assay. Positive and negative whole blood samples were extracted with NucliSens® easyMAG® automatic extraction and were also characterized by CE marked Real-Time PCR commercial kit.

Qualitative analysis:

		Reference CE marked commercial kit (1)		
NucliSens® easyMAG®		Pos	Neg	
Dx EBV Assay	Pos	25	1	26
	Neg	1	23	24
Total		26	24	50

Concordance = 98%

All samples with discordant results had viral loads below the limit of detection (LOD).

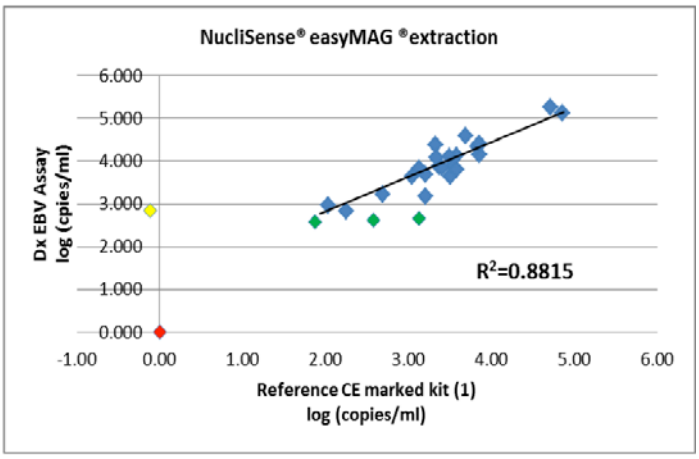
The same positive whole blood samples were also extracted with the manual extraction system and tested with Dx EBV assay and with a CE marked Real-Time PCR commercial kit validated with the same extraction.

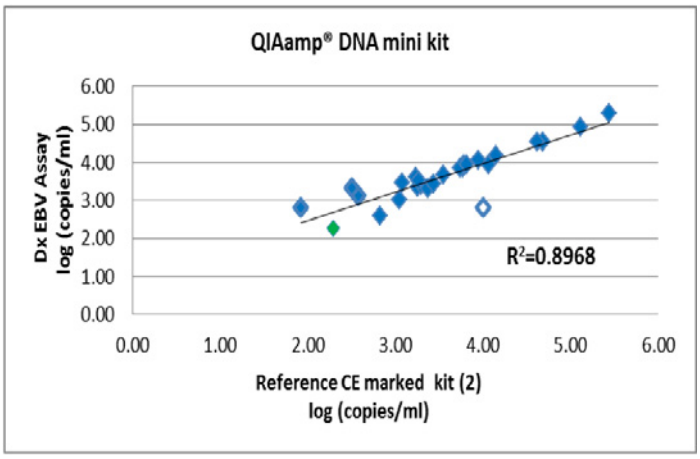
		Reference CE marked commercial kit (2)		
Qiagen® DNA mini kit		Pos	Neg	
Dx EBV Assay	Pos	26	0	26
	Neg	0	0	0
Total		26	0	26

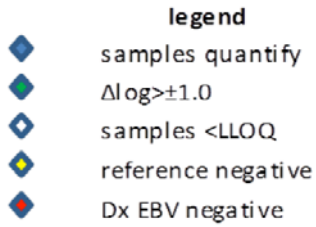
Concordance = 100%

Quantitative analysis:

On the same samples were performed a correlation analysis comparing the viral load (copies/ml) obtained with the Dx EBV Assay and the CE marked kits, considering also the extraction method used.







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