Enzyme-Immunoassay for *in-vitro* detection of IgM antibodies against virus capsid antigen (VCA) p23/p18 of Epstein-Barr Virus (EBV) in human serum or plasma
1- INTENDED USE
The Anti-EBV VCA IgM ELISA is an in-vitro diagnostic device for the detection of IgM antibodies against the VCA antigens p23 and p18 of EBV. Results obtained with this test, in conjunction with other clinical and patient data obtained in assays for other Epstein-Barr virus specific antibodies such as anti-EA IgG/IgM, anti-VCA IgG and anti-EBNA-1 IgG, assist in serological diagnosis of EBV infection. Primary infection with EBV can result in infectious mononucleosis (IM = morbus Pfeiffer) (1,2). The illness predominantly occurs among older adolescents and young adults. The acute disease can show the following symptoms: fever, pharyngitis, tonsillitis, lymphadenopathy, malaise, headache, myalgia, spleno- and hepatomegaly, rash, and leucocytosis (2). Other pathogenic infectious agents such as cytomegalovirus, Toxoplasma gondii, rubella virus, hepatitis viruses, human immunodeficiency virus (HIV) may cause similar symptoms. The Anti-EBV VCA IgM ELISA can be used for the identification of an EBV infection.

2- PRINCIPLES OF THE PROCEDURE
The Anti-EBV VCA IgM ELISA is a highly sensitive IgM (μ-chain) specific capture enzyme immunosorbent assay (ELISA) for the detection of EBV specific antibodies in serum or plasma (3). During the first incubation step, IgM antibodies of the sample will bind to the microplate. Other immunoglobulin types will be removed by washing. During a second incubation, captured VCA p23-18-specific IgM antibodies will be detected. This is performed by the addition of an antigen-enzyme conjugate. The recombinant (rec) VCA p23-18 (3) is directly and covalently labelled with horseradish peroxidase (HRP). Non-specifically bound conjugate is removed by another washing step. For the last incubation, the substrate solution (TMB, 3,3’,5,5´-Tetramethylbenzidine) is filled into the wells. The enzyme reaction is stopped by adding sulphuric acid (colour change from blue to yellow) and the optical density is measured with a spectrophotometer at 450 nm and a reference wavelength of 615-690 nm.
## 3- REAGENTS

Supplied quantities of reagents have been calculated to allow 96 tests. All reagents are exclusively for in vitro diagnostic use.

<table>
<thead>
<tr>
<th>Label</th>
<th>Description</th>
<th>Presentation</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>Microplate: 12 single strips with 8 wells each, coated with polyclonal anti-human IgM antibody (concentration: &gt; 0.5 μg/ml)</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R2</td>
<td>EBV Concentrated Washing Solution (500x): Preservative: 0.01% 2-bromo-2-nitro-1,3-propanediol</td>
<td>To be diluted</td>
</tr>
<tr>
<td>R3</td>
<td>EBV VCA IgM Negative Control: Negative human serum for EBV-VCA IgM antibodies, and negative for HBs antigen, anti-HIV1, anti-HIV2 and anti-HCV Preservative: 0.005 % gentamycin, 0.05 % streptomycin, 0.05 % penicillin V</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R5</td>
<td>EBV VCA IgM Positive Control: Human serum reactive for EBV-VCA IgM antibodies, and negative for HBs antigen, anti-HIV1, anti-HIV2 and anti-HCV Preservative: 0.005 % gentamycin, 0.05 % streptomycin, 0.05 % penicillin V</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R6</td>
<td>Rec. p23-18 Fusion Protein Conjugated: Preservative: 0.025% penicillin V, 0.025% streptomycinsulfat, &lt; 1.5% ProClin™ 300</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R7</td>
<td>Sample Diluent: Preservative: 0.01% neomycinsulfat, 0.03% chloramphenicol</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R9</td>
<td>Chromogen TMB: 3, 3’, 5, 5’ tetramethylbenzidine (TMB) solution &lt; 0.05 % in H₂O. See Warnings and Precautions</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>R10</td>
<td>Stopping Solution: Sulfuric acid &lt; 1N H₂SO₄</td>
<td>Ready-to-use</td>
</tr>
<tr>
<td>Storage Bag</td>
<td>Polyethylene bag for storing remaining microplate strips.</td>
<td>1</td>
</tr>
</tbody>
</table>
Preservatives: total concentration < 0.11%

Storage and handling requirements
The reagents are stable up to the stated expiry dates on the individual labels when stored at 2-8°C. After opening reagents have to be used within 30 days. For repeatedly testing, store the reagents immediately after usage at 2-8°C. The microplate sealed in an aluminum bag with a desiccant must be at room temperature before opening. Return unused strips with the desiccant to the storage bag and store in this way at 2-8°C. Do not touch the upper rim or the bottom of the wells with fingers.

4- WARNING AND PRECAUTIONS
Do not ingest reagents. Avoid contact with eyes and skin. All samples and materials used for the test must be treated as being potentially infectious and appropriate safety precautions taken. The controls are negative for anti-HIV 1/2, anti-HCV, HBsAg, anti-lues and elevated transaminases. Do not pipet with mouth. According to good laboratory practice wear gloves, laboratory coat and safety glasses. Liquids and non-combustible materials should be decontaminated with sodium hypochlorite (final concentration: 3%, activity time at least 30 minutes). Liquid waste which contains acids must be neutralized before disposal. Used microplates and all materials that are to be re-used must be autoclaved for 1 hour at 121°C. The Chromogen TMB (R9) is sensitive of light and has to be protected from light. The test must be performed by well-trained and authorized laboratory technicians. Testing is performed under aseptic and microbiologically controlled conditions. Inform the manufacturer if the original test kit is damaged.

CAUTION: Some of the reagents contain ProClin™ 300 < 1.5%
For risks and security recommendations refer to the table at the end of the package insert.

5- SPECIMENS
Fresh serum or plasma samples, free from haemolysis should be used. Highly lipaemic, icteric or microbiologically contaminated sera or plasma samples and concentrated immunoglobulin preparations can lead to unreliable test results. Avoid repeated freezing and thawing of the samples. If samples are to be transported, they must be packed in accordance with legal requirements for the transportation of infectious materials. The samples should not be inactivated, as unspecific reactions may otherwise occur.
6- MATERIALS REQUIRED NOT PROVIDED
Micropipettes, spectral photometer (450 nm, reference wavelength 615-690 nm), microplate washer (with bottom wash) and incubator (37°C) for microplates.

7- INSTRUCTIONS FOR USE

Reagents preparation
Dilute the Concentrated Washing Solution (R2) with demineralized or deionized water (1:501). The Washing Solution prepared is stable for 1 week when stored at 2-8°C. All other test components are ready for use. All reagents are lot specific and can not be used with kits of other lots. Do not use reagents of other manufacturers.

Specimen preparation
The protocol (see Pipetting Procedure) has to be followed strictly.

• Sample dilution 1:21 with pre-dilution in tubes:
  Dilute Negative Control (R3), Positive Control (R5) and samples 1:21 in a tube (e.g. 25 μl control or sample + 500 μl of Diluent (R7)). Mix well.

• Sample dilution 1:21 with dilution directly in plate:
  Pipet 200 μl of Diluent (R7) into every well. The dilution directly in the microtest plate is particularly suitable for the use of automatic pipetting devices. If the dilution directly in the plate is performed manually it is important to avoid non-specific protein binding by observing the following steps: Pipet first 200 μl of Diluent (R7) into the well and add 10 μl of sample or controls subsequently. Mix 5 up to 7 times when adding 10 μl sample or control.

Washing procedure
The wash procedure is critical. Insufficient washing will result in poor precision and unspecific reactions.
Wash five times with wash buffer. For that remove the liquid in the well and dispense with 300μl washing buffer. This washing procedure is repeated 5 times. Tap out the plate briefly after washing. Do not allow the plate to dry out.
Pipetting procedure of qualitative IgM determination (dilution tube)

Allow all reagents to reach room temperature before use.
The controls and the blank should be pipetted last. After pipetting the controls and samples immediately begin with incubation of the plate.

### Step 1

<table>
<thead>
<tr>
<th>Well [µl]</th>
<th>A1/B1</th>
<th>C1/D1</th>
<th>E1/F1</th>
<th>G1…</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>200 µl R7</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Negative Control (R3) in duplicate</td>
<td>-</td>
<td>200 µl R3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Positive Control (R5) in duplicate</td>
<td>-</td>
<td>-</td>
<td>200 µl R5</td>
<td>-</td>
</tr>
<tr>
<td>Sample 1:21</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>200 µl Sample</td>
</tr>
</tbody>
</table>

Seal microplate using self-adhesive foils (not required in an ELISA* processor).

**Incubation 60 ± 2 min., 37 ± 1°C**

<table>
<thead>
<tr>
<th>Processor*: 60 ± 2 min., 37 ± 1°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 washes</td>
</tr>
<tr>
<td>Diluted Washing Solution (R2)</td>
</tr>
</tbody>
</table>

### Step 2

<table>
<thead>
<tr>
<th>Well [µl]</th>
<th>Conjugate (R6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 µl</td>
</tr>
</tbody>
</table>

Seal microplate using self-adhesive foils (not required in an ELISA* processor).

**Incubation 30 ± 1 min., 37 ± 1°C**

<table>
<thead>
<tr>
<th>Processor*: 30 ± 1 min., 37 ± 1°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 washes</td>
</tr>
<tr>
<td>Diluted Washing Solution (R2)</td>
</tr>
</tbody>
</table>

### Step 3

<table>
<thead>
<tr>
<th>Well [µl]</th>
<th>Chromogen TMB (R9)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100 µl</td>
</tr>
</tbody>
</table>

**Incubation 30 ± 1 min., at room temperature in the dark**

Processor*: 15 ± 1 min., at room temperature in the dark

| Stopping Solution (R10) | 100 µl | 100 µl | 100 µl | 100 µl |

* If an ELISA processor is used the operator has to validate the test under his own reliability.

Measure the extinction immediately or **within 15 min.** after stop at **450 nm** using a spectral photometer (reference wavelength: 615 - 690 nm).
8- QUALITY CONTROL
All manufactured reagents are prepared according to our Quality System, starting from reception of raw material to commercialization of the final product. Each lot is submitted to quality control assessments and is released to the market only after conforming to pre-defined acceptance criteria. The records related to production and controls of each single lot are kept within Bio-Rad.

9- INTERPRETATION OF RESULTS
Samples with an extinction value below the grey zone are considered to be negative. If a sample has an extinction value equal to or greater than the grey zone, it is considered to be positive for EBV VCA specific IgM antibodies. If the OD-value of the retested sample is within the grey area (questionable result), we recommend to request for a follow up sample.

Calculation of the cut-off-value and grey area
The cut-off value is calculated from the mean OD value of the Negative Control (R3x) plus 0.200:

- Cut-off value = R3x + 0.200

The grey area extends between cut-off-value and cut-off-value minus 10%.

Test validation criteria for qualitative determination
After measuring the extinction values at 450 nm in all wells (reference filter: 615-690 nm), the mean value of the blanks is subtracted from the extinction values of the controls and samples:

- Mean extinction of blanks ≤ 0.150 OD

After subtraction of the blank, the control values must meet the following criteria of validity:

- Mean OD-value of R3 ≤ 0.200
- Mean OD-value of R5 ≥ 0.400
Interpretation of anti-EBV VCA IgM positive results

<table>
<thead>
<tr>
<th>Infection Status</th>
<th>VCA IgM</th>
<th>VCA IgG</th>
<th>EBNA IgG</th>
<th>Information</th>
</tr>
</thead>
<tbody>
<tr>
<td>Seronegative</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1</td>
</tr>
<tr>
<td><strong>Primary infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early phase</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Acute phase</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Late phase</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>2</td>
</tr>
<tr>
<td><strong>Past-infection</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Past-infection</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Persisting IgM</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
</tr>
<tr>
<td>Reactivation</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>4</td>
</tr>
<tr>
<td><strong>Not defined</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Implausible</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
<tr>
<td>Implausible</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>5</td>
</tr>
</tbody>
</table>

1. In the very early phase of a primary infection, the serology can still be negative. It is recommended that in the case of a VCA IgM result within the grey zone, the sample be treated as a "possible early phase of a primary infection" and to confirm or rule out this result by analysis of a follow up sample.

2. There is an exception in the case of an immunodeficient or immune suppressed patient, where this can be a secondary pattern caused by the loss of EBNA IgG. In this situation, it is recommended that EBNA IgG values within the grey zone be evaluated as positive and the sample reported as a "past-infection".

3. IgM persists in many cases for longer than 6 months and can at this point in time coincide with a positive EBNA IgG result. However, a "late phase of a primary infection" should be reported only in the case of very weak EBNA (OD < 0.500). The diagnosis is otherwise "past-infection".

4. A reactivation cannot be defined using VCA serology. Quantification of the VCA IgG can help. Values which extend to well above the normal range (> 2,500 RU/ml) are suspect. The classic marker for the serological definition of EBV reactivation are IgG antibodies against early antigen (EA) (e.g. anti-EBV EA IgG ELISA, Art. No. 807016). Anti-EA IgG should be determined in addition if an EBV reactivation is suspected.
5. The implausible reactivity patterns are extremely unlikely constellations which have not been found in clinical studies. VCA IgG values within the grey zone should be defined as positive. The interpretation in this case is "past-infection". For VCA IgG values below the grey zone, the EBV serology should be repeated completely to rule out any errors in the test procedure. If the implausible result is obtained again, it is recommended that a new sample be requested. If the result is reconfirmed, a "past-infection" must be reported.

10- TEST LIMITATIONS

A negative test result in the Anti-EBV VCA IgM ELISA does not completely exclude an EBV infection. The test results should be used in conjunction with information available from the patient clinical evaluation and other available diagnostic procedures. Test results of specimens from immunosuppressed patients may be difficult to interpret. Positive test results may not be valid in persons who have received blood transfusions or other blood products within the past several months. The Anti-EBV VCA IgM ELISA was analyzed with the following potential cross reactive samples: anti-rheumatoid sera (23), Anti-Hepatitis B Virus acute positive (6), Anti-Hepatitis C Virus acute positive (6), Anti-Varicella Virus acute positive (3), Anti-Cytomegalovirus-acute positive (3) and Anti-Toxoplasma acute positive (4). One HCV positive sample gave a positive result in the Anti-EBV VCA IgM ELISA.

11- PERFORMANCES CHARACTERISTICS

Sensitivity and specificity

Results obtained with the Anti EBV VCA IgM test in conjunction with further assays such as anti-VCA IgG and anti-EBNA-1 IgG assist in serological diagnosis of EBV infection. From 69 sera of patients which were primary infected with EBV, 67 (97.1%) were positive (4). All 46 seronegative samples were tested negative (specificity = 100%) with the Anti EBV VCA IgM ELISA (4).

Precision study

The intra-assay variability of the Anti-EBV VCA IgM ELISA was evaluated by pipetting a negative, a weak reactive and a strong reactive sample within one test run repeatedly in 6 wells each. We obtained the following coefficient of variation respectively (CV): 11.7%, 2.3% and 4.4%.

The inter-assay variability was evaluated by testing 3 samples of negative, weak and strong reactivity in 6 wells each and in 3 subsequent runs. We obtained the following coefficient (CV): 7.4%, 3.5% and 4.8%.
12- REFERENCES

13- TROUBLE SHOOTING GUIDE
1. Unexpected high rate of reactive results:
   a. Samples and controls were pipetted prior to pipetting of Diluent (R7).
   b. Mixing was insufficient.
2. Mean blank value higher than criteria of validity (≥ 0.150 OD):
   a. Chromogen TMB (R9) turned blue due to oxidation or contamination.
   b. Washing fault: Perform 5x wash cycles/washing step. If using a manual washing device, perform 7x wash cycles/washing step. Use Bio-Rad Washing Solution (R2) contained in the kit.
   c. Incubation fault: Temperature too high, incubation time was exceeded or plate was not incubated directly after finishing of pipetting.
   d. Wavelength fault: Measurement without reference filter will increase OD values approximately + 0.120 OD.
3. Yellow coloration in all wells (see 2a, 2b):
   a. Washing Solution (R2) contamination. Prepare a new Washing Solution (R2).
   b. Diluent (R7) or Conjugate (R6) contamination; Repeat test with reagents from unopened vials. Use reagents under less microbial conditions.
4. Mean value of Positive Control (R5) ≤ 0.400 OD:
   a. Exceed of expire date.
   b. Temperature too low or fall below incubation time.
   c. Washing fault: Too intensive washing or mechanic contact of manifold and solid phase of the well.
   d. Contamination of Control (R5) or 3b.
5. Mean value of Negative Control (R3) ≥ 0.200 OD (see 1 and 2a-d):
   a. Negative Control (R3) was not pipetted subsequent to pipetting of samples; Pipet all samples prior to pipetting of blanks and controls.
   b. Contamination with the lid of the Positive Control (R5).
R43:

(GB) • May cause sensitisation by skin contact.
(FR) • Peut entraîner une sensibilisation par contact avec la peau.
(ES) • Posibilidad de sensibilización en contacto con la piel.
(IT) • Può provocare sensibilizzazione per contatto con la pelle.
(DE) • Sensibilisierung durch Hautkontakt möglich.
(PT) • Pode causar sensibilização em contacto com a pele.
(SE) • Kan ge allergi vid hudkontakt.
(DK) • Kan give overfølsomhed ved kontakt med huden.
(GR) • Μπορεί να προκαλέσει αλλεργική αντιδράση στην επικοινωνία με το δέρμα.
(PL) • Może powodować uczulenie w kontakcie ze skórą.
(LT) • Gali sukelti alergiją susiūlius su oda.
(HU) • Bőrrel érintkezve túlérzékenyedés okozhat (szenzibilizáló hatását lehet).
(EE) • Koikkuupuutel nahaga võib põhjustada üllitundlikkust.
(SK) • Môže spôsobiť senzibilizáciu pri kontakte s pokožkou.
(CZ) • Může vyvolat senzibilizaci při styku s kůží.
(RO) • Poate provoca o sensibilizare în contact cu pielea.
(BG) • Възможна е сенсибилизация при контакт с кожата.
(LV) • Sasaroties ar ādu, var izraisīt paaugažinātu jutīgumu.
(MT) • Jista’ jikkaġuna sensitizzzjoni meta’ jmiss il-ģikda.
(NL) • Kan overgevoeligheid veroorzaken bij contact met de huid.
(SI) • Stik s kožo lahko povzroči preobčutljivost.
(FI) • Ihokosketus voi aiheuttaa herkistymistä.

S24-37-60:

(GB) • Avoid contact with skin. Wear suitable gloves. This material and its container must be disposed of as hazardous waste.
(FR) • Évitez le contact avec la peau. Porter des gants appropriés. Éliminer le produit et son récipient comme un déchet dangereux.
(ES) • Evítense el contacto con la piel. Úsense guantes adecuados. Elimínense el producto y su recipiente como residuos peligrosos.
(IT) • Evitare il contatto con la pelle. Usare guanti adatti. Questo materiale e il suo contenitore devono essere smaltiti come rifiuti pericolosi.
(PT) • Evitar o contacto com a pele. Usar luvas adequadas. Este produto e o seu recipiente devem ser eliminados como resíduos perigosos.
(SE) • Undvik kontakt med huden. Använd lämpliga skyddshandskar. Detta material och dess behållare skall tas om hand som farligt avfall.
(DK) • Undgå kontakt med huden. Brug egnede beskyttelseshandsker under arbejdet. Dette materiale og dets beholder skal bortskaffes som farligt affald.
(GR) • Να φερότε κατάλληλα γάντια. Το υλικό και το περιθώριο του να θεωρηθούν κατά τη διάρκεια της επικοινωνίας αποτρέψτε.
(PL) • Unikać zanieczyszczenia skóry. Nosić odpowiednie rękawice ochronne. Produkt i opakowanie usuwać jako odpad niebezpieczny.
(LT) • Vengti patekimo ant odos. Mūsų tinkamas pirštines. Šios medžiagos atliekos ir jos pakutė turi būti šalinamos kaip pavojingos atliekos.
(HU) • A bőrrel való érintkezés kерülendő. Megfelelő védőkesztyűt kell viselni. Az anyagot és/vagy ecényzetét veszélyes hulladékként kell ártalmatlanítani.
(SK) • Zabráňte kontaktu s pokožkou. Noste vhodné rukavice, Tento materiál a príslušná nádoba musia byť zlikvidované ako nebezpečný odpad. Tento materiál a príslušná nádoba musia byť zlikvidované ako nebezpečný odpad.
(CZ) • Zamezte styku s kůží. Použivejte vhodné ochranné rukavice. Tento materiál a jeho obal musí být zneškodněny jako nebezpečný odpad.
(RO) • A se evita contactul cu pielea. A se purta mănuși corespunzătoare. Acest produs și ambalajul său se vor depozita ca un deșeu periculos.
(BG) • Да се избива контакт с кожата. Да се носят подходящи ръкавици. Този материал и неговата опаковка да се третират като опасен отпадък.
(LV) • Nepielaut nokļūšanu uz ādas. Strādāt aizsargājot un tās iepakojumu kā bīstamos atkritumus.

(MT) • Evita l-kuntatt mal-giżda. Ilbes ingwanti adatt. Dan il-materjal u l-kontenitur tiegħu għandhom jintremew ma' skart perikoluż.

(NL) • Aanraking met de huid vermijden. Draag geschikte handschoenen. Deze stof en de verpakking als gevaarlijk afval afvoeren.

(SI) • Preprečiti stik z kožo. Nosili primerne zaščitne rokavice. Snov/pripravek in embalažo odstraniti kot nevarni odpadek.

(FI) • Varottava kemikaalin joutumista iholle. Käytettävä sopivia suoja- ja käytetytä otelmaan on käsittävä ongelmajätteenä.