VIROTECH EBV EA-D IgG ELISA
(EBV EA-D IgG ELISA)

Order No.: EC202.00 Color Coding: yellow/red

VIROTECH EBV EBNA1 IgG ELISA
(EBV EBNA1 IgG ELISA)

Order No.: EC204.00 Color Coding: yellow/light blue

VIROTECH EBV VCA IgG ELISA
(EBV VCA IgG ELISA)

Order No.: EC205G00 Color Coding: yellow/orange

VIROTECH EBV VCA IgM ELISA
(EBV VCA IgM ELISA)

Order No.: EC203M00 Color Coding: yellow/black

FOR IN VITRO DIAGNOSIS ONLY

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Freigabedatum: 30.03.2020 15:51
REV 21 / VIROTECH EBV EBNA1 & EBV EA-D & EBV VCA ELISA IgG/IgM GB

Contents

1. Intended Use

The VIROTECH EBV EA-D IgG, EBV EBNA1 IgG, EBV VCA IgG and IgM ELISAs are intended for the semiquantitative detection of antibodies against the several markers of Epstein Barr Virus. In combination the kits are intended to differentiate or prove seronegativity, primary infection or past infection.

2. Diagnostic Relevance

The Epstein Barr virus belongs to the family of the Herpesviridae and is transmitted mainly by saliva, when it infects the epithelial cells of the oropharynx initially and then the B-lymphocytes. The virus is the cause of infectious mononucleosis (IM) and chronic active EBV infection. There is also an association between EBV infections and Burkitt's lymphoma and also nasopharyngeal carcinomas in Africa and Asia. According to serological investigations, approx. 95% of adults are seropositive for EBV.

Primary EBV infections are normally asymptomatic but can be the cause of infectious mononucleosis in adolescents and young adults. IM is a self-limiting illness and is characterised by lymphadenopathy, fever, hepatosplenomegaly and leukocytosis with atypical lymphocytes (6).

The purpose of EBV serology is to differentaite or confirm seronegativity, primary infections together with past infections and the differential diagnostic elimination of infections involving diseases with clinically similar symptoms caused by: CMV, rubella virus, mumps virus, HIV, HAV, HBV, HCV and neurotropic viruses as well as brucellosis, listeriosis, leptospirosis, toxoplasmosis and neoplastic diseases such as lymphoma und leukaemia (7).

3. Test Principle

The antibody searched for in the human serum forms an immune complex with the antigen coated on the microtiter-plate. Unbound immunoglobulins are removed by washing processes. The enzyme conjugate attaches to this complex. Unbound conjugate is again removed by washing processes. After adding the substrate solution (TMB), a blue dye is produced by the bound enzyme (peroxidase). The color changes to yellow when the stopping solution is added.

4. Package Contents

4.1 Package contents EBV EA-D IgG ELISA, EBV EBNA1 IgG ELISA, EBV VCA IgG

1. **1 Microtiter-Plate** consisting of 96 with antigen coated, breakable single wells, lyophilised
2. **PBS-Dilution Buffer (blue, ready to use) 2x50ml**, pH 7,2, with preservative and Tween 20
3. **PBS-Washing Solution (20x concentrated) 50ml**, pH 7,2, with preservative and Tween 20
4. **IgG negative Control, 2000µl,** human serum with protein-stabilizer and preservative, ready to use
5. **IgG cut-off Control, 2000µl**, human serum with protein-stabilizer and preservative, ready to use
6. **IgG positive Control, 2000µl,** human serum with protein-stabilizer and preservative, ready to use
7. **IgG-Conjugate (anti-human), 11ml**, (sheep or goat)-horseradish-peroxidase-conjugate with protein-stabilizer and preservative in Tris-Buffer, ready to use
8. **Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11ml**, ready to use
9. **Citrate-Stopping Solution, 6ml**, contains an acid mixture

4.2 Package contents EBV VCA IgM ELISA

1. **1 Microtiter-Plate** consisting of 96 with antigen coated, breakable single wells, lyophilised
2. **PBS-Dilution Buffer (blue, ready to use) 2x50ml**, pH 7,2, with preservative and Tween 20
3. **PBS-Washing Solution (20x concentrated) 50ml**, pH 7,2, with preservative and Tween 20
4. **IgM negative Control, 2000µl,** human serum with protein-stabilizer and preservative, ready to use
5. **IgM cut-off Control, 2000µl**, human serum with protein-stabilizer and preservative, ready to use
6. **IgM positive Control, 2000µl,** human serum with protein-stabilizer and preservative, ready to use
7. **IgM-Conjugate (anti-human), 11ml**, (sheep or goat)-horseradish-peroxidase-conjugate with FCS and preservative in Tris-Buffer, ready to use
8. **Tetramethylbenzidine substrate solution (3,3',5,5'-TMB), 11ml**, ready to use
9. **Citrate-Stopping Solution, 6ml**, contains an acid mixture

5. Storage and Shelflife of the Testkit and the ready to use reagents

Store the testkit at 2-8°C. The shelf life of all components is shown on each respective label; for the kit shelf life please see Quality Control Certificate.

1. Microtiter strips/single wells are to be resealed in package after taking out single wells and stored with desiccant at 2-8°C. Reagents should immediately be returned to storage at 2-8°C after usage.
2. The ready to use conjugate and the TMB-substrate solution are sensitive to light and have to be stored in dark. Should there be a color reaction of the substrate dilution due to incidence of light, it is not useable anymore.
3. Take out only the amount of ready to use conjugate or TMB needed for the test insertion. Additional conjugate or TMB taken out may not be returned but must be dismissed.

Material	Status	Storage	Shelflife
Test Samples	Diluted	+2 to +8°C	max. 6h
	Undiluted	+2 to +8°C	1 week
Controls	After Opening	+2 to +8°C	3 months
Microtitreplate	After Opening	+2 to +8° (storage in the provided bag with desiccant bag)	3 months
Rheumatoid factor - Absorbent	Undiluted, After Opening	+2 to +8°C	3 months
	Diluted	+2 to +8°C	1 week
Conjugate	After Opening	+2 to +8°C (protect from light)	3 months
Tetramethylbenzidine	After Opening	+2 to +8°C (protect from light)	3 months
Stop Solution	After Opening	+2 to +8°C	3 months
Washing Solution	After Opening	+2 to +8°C	3 months
	Final Dilution (ready-to-use)	+2 to +25°C	4 weeks

6. Precautions and Warnings

1. Only sera which have been tested and found to be negative for HIV-1 antibodies, HIV-2 antibodies, HCV antibodies and Hepatitis-B surface-antigen are used as control sera. Nevertheless, samples, diluted samples, controls, conjugates and microtiter strips should be treated as potentially infectious material. Please handle products in accordance with laboratory directions.
2. Those components that contain preservatives, the Citrate Stopping Solution and the TMB have an irritating effect to skin, eyes and mucous. If body parts are contacted, immediately wash them under flowing water and possibly consult a doctor.
3. The disposal of the used materials has to be done according to the country-specific guidelines.

7. Material required but not supplied

1. Aqua dest./demin.
2. Eight-channel pipette 50µl, 100µl
3. Micropipettes: 10µl, 100µl, 1000µl
4. Test tubes
5. Paper towels or absorbent paper
6. Cover for ELISA-plates
7. Disposal box for infectious material
8. ELISA handwasher or automated EIA plate washing device
9. ELISA plate spectrophotometer, wavelength = 450nm, reference length = 620nm (Reference Wavelength 620-690nm)
10. Incubator

8. Test Procedure

Working exactly referring to the VIROTECH Diagnostics user manual is the prerequisite for obtaining correct results.

8.1 Examination Material

Either serum or plasma can be used as test material, even if only serum is mentioned in the instructions. Any type of anticoagulant can be used for plasma.

Always prepare patient-dilution freshly.

For a longer storage the sera must be frozen. Repeated defrosting should be avoided.

1. Only fresh non-inactivated sera should be used.
2. Hyperlipaemic, haemolytic, microbially contaminated and turbid sera should not to be used (false positive/negative results).

8.2 Preparation of Reagents

The VIROTECH Diagnostics System Diagnostica offers a high degree of flexibility regarding the possibility to use the dilution buffer, washing solution, TMB, citrate stopping solution as well as the conjugate for all parameters and for all different lots. The ready to use controls (positive control, negative control, cut-off control) are parameter specific and only to use with the plate lot indicated in the Quality Control Certificate.

1. Set incubator to 37°C and check proper temperature setting before start of incubation.
2. Bring all reagents to room temperature before opening package of microtiter strips.
3. Shake all liquid components well before use.
4. Make up the washing solution concentrate to 1 L with distilled or demineralised water. If crystals have formed in the concentrate, please bring the concentrate to room temperature before use and shake well before use.
5. High IgG-titer or rheumatoid factors may disturb the specific detection of IgM-antibodies and may lead to false positive resp. false negative results. **For a correct IgM-determination it is therefore necessary to pre-treat the sera with VIROTECH RF-SorboTech** (adsorbent). For IgM-controls a pre-absorbent treatment is not necessary.

8.3 VIROTECH ELISA Test Procedure

1. For each test run, pipette 100µl each of ready to use dilution buffer (blank), IgG- and IgM-positive, negative and cut-off controls as well as diluted patient sera. We propose a double insertion (blank, controls and patient sera); for cut-off control a double insertion is absolutely necessary. Working dilution of patient sera: 1+100; e.g. 10µl serum + 1ml dilution buffer.
2. After pipetting start incubation for 30 min. at 37°C (with cover).
3. End incubation period by washing microtiter strips 4 times with 350 – 400µl washing solution per well. Do not leave any washing solution in the wells. Remove residues on a cellulose pad.
4. Pipette 100µl of ready to use conjugate into each well.
5. Incubation of conjugates: 30 min. at 37°C (with cover).
6. Stop conjugate incubation by washing 4 times (pls. refer to point 3 above).
7. Pipette 100µl of ready to use TMB into each well.
8. Incubation of substrate solution: 30 min. at 37°C (with cover, keep in dark).
9. Stopping of substrate reaction: pipette 50µl of citrate stopping solution into each well. Shake plate carefully and thoroughly until liquid is completely mixed and a homogeneous yellow color is visible.
10. Measure extinction (OD) at 450/620nm (Reference Wavelength 620-690nm). Set your photometer in such a way that the blank value is deducted from all other extinctions. Extinctions should be measured within 1 hour after adding the stopping solution!

Pls. refer to last page for Test Procedure Scheme

8.4 Usage of ELISA processors

All VIROTECH Diagnostics ELISAs can be used on ELISA processors. The user is bound to proceed a validation of the devices (processors) on a regular basis.

VIROTECH Diagnostics recommends the following procedure:

1. VIROTECH Diagnostics recommends to proceed the validation of device referring to the instructions of the device manufacturer during the implementation of the ELISA processor respectively after bigger reparations.
2. It is recommended to check the ELISA-processor with the Validationkit (EC250.00) afterwards. A regular check using the Validationkit shall be proceeded minimum once a quarter to test the accuracy of the processor.
3. The release criteria of the Quality Control Certificate of the product must be fulfilled for each testrun.

With this procedure, your ELISA processor will function properly and this will support quality assurance in your laboratory.

9. Test Evaluation

The ready to use controls serve for a semiquantitative determination of specific IgG- and IgM-antibodies. Their concentration can be expressed in VIROTECH units = VE. Fluctuations resulting from the test procedure can be balanced with this calculation method and a high reproducibility is achieved in this way. Use the means of the OD values for calculation of the VE.

9.1 Test function control

a) OD-values

The OD of the blank should be < 0.15.

The OD-values of the negative controls should be lower than the OD-values mentioned in the Quality Control Certificate. The OD-values of the positive controls as well as of the cut-off controls should be above the OD-values mentioned in the Quality Control Certificate.

b) VIROTECH Units (VE)

The VIROTECH Units (VE) of the cut-off controls are defined as 10 VE. The calculated VE of the positive controls should be within the ranges mentioned in the Quality Control Certificate.

If those requirements (OD-values, VE) are not fulfilled, the test has to be repeated.

9.2 Calculation of the VIROTECH Units (VE)

The extinction of the blank value (450/620nm) has to be subtracted from all other extinctions.

$$VE_{(positive\ control)} = \frac{OD_{(positive\ control)}}{OD_{(cut\text{-}off\ control)}} \times 10$$

$$VE_{(patient\ serum)} = \frac{OD_{(patient\ serum)}}{OD_{(cut\text{-}off\ control)}} \times 10$$

9.3 Interpretation Scheme IgG and IgM

Result (VE)	Evaluation
< 9,0	negative
9,0 - 11,0	borderline
> 11,0	positive

1. If the measured values are above the defined borderline range, they are considered to be positive.
2. If the measured VE is within the borderline range, no significant high antibody concentration is present, the samples are considered to be borderline. For the secure detection of an infection it is necessary to determine the antibody concentration of two serum samples. One sample shall be taken directly at the beginning of the infection and a second sample 5 – 10 days later (convalescent serum). The antibody concentration of both samples has to be tested in parallel, that means in one test run. A correct diagnosis based on the evaluation of a single serum sample is not possible.
3. If the measured values are below the defined borderline range, no measurable antigen specific antibodies are present in the samples. The samples are considered to be negative.

9.4 Meaning of the Antigens

Antigen / Description	Meaning of the antigens	Specificity of the antibodies
EBNA1	**E**pstein-**B**arr **N**uclear **A**ntigen, a viral protein, which is expressed in the nucleus of latent infected cells. IgG antibodies against EBNA1 are regarded as reliable marker for a past EBV infection. In rare exceptional cases the IgG immune response against EBNA1 (primary or secondary) may not appear. In immunosuppressed patients IgG antibodytiters against EBNA1 may greatly decrease (secondary EBNA1 loss).	**IgG:** central highly specific marker for past EBV infection
VCA	Several "**V**irus **C**apsid **A**ntigens" have been described. Among them the proteins gp125 and p18 are regarded as immunodominant. As a rule IgM antibodies against VCA-gp125/p18 disappear again several weeks after infection, IgG antibodies against VCA-gp125/p18 are kept lifelong. Sometimes IgM antibodies against VCA-gp125/p18 are built again in reactivations.	**IgG:** highly specific general marker for EBV infections **IgM:** highly specific for an EBV primary infection
EA-D	"**E**arly **A**ntigen-**D**iffuse" belongs to the early antigens, which are synthesised in the viral replication cycle (acute phase of infection). IgG and IgM antibodies against EA-D appear with negative EBNA-IgG at the same time in primary infections typically. IgG antibodies against EA-D decrease during convalescence but may greatly increase again during EBV reactivations. But this antibody increase is no statement about clinical relevance of EBV reactivation.	**IgG:** **1.)** specific for EBV primary infection **2.)** serological marker for EBV reaktivation

9.5 Interpretation Scheme

Evaluation	IgM	IgG		
	VCA	**EBNA1**	**VCA**	**EA-D**
Seronegative	**neg.**	**neg.**	**neg.**	**neg.**
Indication of Primary Infection	**pos.**/neg.	**neg.**	**pos.**/neg.	**pos.**/neg.
Indication of Past Infection	**neg.**	**pos.**/neg.	**pos.**/neg.	**neg.**/pos.

pos./neg.: normally positive
neg./pos.: normally negative

Application of the diagnostic factor:
Under certain circumstances VCA IgM antibodies can persist in spite of an already existing EBNA1 IgG immune response. In such unclear cases when both highly specific markers are positive, ie. EBNA1 IgG for previous infections and VCA IgM for primary infections, the **diagnostic factor of 1.5** is an aid to clear diagnosis. The VU values of EBNA1 IgG and of VCA IgM are compared and the parameter, that is at least a factor of 1.5 greater than than the other is the one used for diagnosis.

If EBNA1 IgG and VCA IgM are both positive then:

- VU value for **EBNA1 IgG** **≥** **1.5x** VU value for **VCA IgM** => **past infection**
- VU value for **VCA IgM** **≥** **1.5x** VU value for **EBNA1 IgG** => **primary infection**

Example: VCA IgM: 25VE and EBNA1 IgG: 15VU

VU value for **VCA IgM: 25VU** **≥** **1.5 x** 15VU-Wert for **EBNA1 IgG: *22.5VU***

VCA IgM: 25VU **≥** **EBNA1 IgG: *22.5VU*** => **primary infection**

Since VCA IgM is at least a factor of 1.5 higher than EBNA1 IgG a clear diagnosis can be made of a primary infection.

9.6 Limits of the Test

1. The interpretation of serological results shall always include the clinical picture, epidemiological data and all further available laboratory results.
2. A negative ELISA result does not completely exclude an EBV infection.
3. Infective agents with similar clinical picture should be considered in differential diagnosis.
4. Crossreactivities of Epstein Barr Virus against the Herpes Virus family are known. With a positive IgM result specially crossreactivities against CMV should be excluded.
5. A negative IgM result does not exclude the possibility of a primary infection, as in some cases no IgM is built during an acute infection (IgM-nonresponder) (7).
6. At the beginning of a primary infection the serology of all parameters may be negative. With clinical suspicion of an EBV infection and a negative serology a second blood sample should be taken.
7. A negative anti-EBNA1 is not necessarily an indication of a primary infection. There can be a scondary anti-EBNA1 loss in immunosuppressed patients and no EBNA1 antibody is formed by 5% of those infected with EBV (EBNA1-non-responders) (7).
8. An accurate interpretation of an EBV infection should be used on the results of the lead antigens VCA IgG, VCA IgM and EBNA1 IgG antibodies, using ELISA, Western Blot or Immunoblot. The EA-D IgG can provide additional information for primary infections and serological reactivations.
9. Antibodies which were passively transmitted shortly before examination may influence the EBV serological result. This might be e.g. by blood transfusion or maternally transmitted antibodies to the infant.
10. The EBV serology does not permit reliable conclusions to be made concerning the clinical relevance of a reactivation (11).

10. Performance Data

10.1 Diagnostical Sensitivity

The following clinically characterized serum collective was investigated to determine the diagnostic sensitivity:
sera from patients with EBV primary (n=51, sera from Dr. Gärtner, Homburg/Saar).
Immunoblots and/or IFA were used as reference tests.

Sera Collective (n=51)		**VIROTECH EBV ELISA IgG + IgM overall result**	
		Primary Infection	**Past Infection**
Clinical Finding	**Primary Infection**	51	0
	Past Infection	0	0

In the overall evaluation the VIROTECH EBV ELISA results are in agreement with the prior clinical evaluations.

10.2 Sensitivity

The following serum collectives were investigated to determine the sensitivity:

1. patients with primary EBV infections (n=123; serum source: Dr. Gärtner, Homburg/Saar, serum panel of a commercial supplier, interlaboratory investigation sera, blood donor sera and routine sera).
2. past EBV infections (n=231; serum source: Dr. Gärtner, Homburg/Saar, interlaboratory investigation sera, serum panel of a commercial supplier, blood donor sera and routine sera).

Immunoblots and/or IFTs were carried out as references.

Serum collective (n=354)		VIROTECH EBV ELISA IgG + IgM overall result	
		Primary infection	**Past infection**
Result	**Primary infection**	120	0
	Past infection	0	221

In the overall evalation there is complete agreement between the VIROTECH EBV ELISA results and the prior results. Unclear results cannot be taken into account for the sensitivity.

10.3 Specificity

The following serum collective was investigated to determine the specificity:
Seronegative (n=22, serum source: Dr. Gärtner, Homburg/Saar, interlaboratory investigation sera), pregnancy sera (n=15), child sera (n=10) and routine sera (n=32).
Immunoblots and/or IFTs were carried out as references.

*Serum collective (n=79)		VIROTECH EBV ELISA IgG + IgM overall result	
		Seronegative	**Past infections**
Results	**Seronegative**	29	0
	Past infections	0	44

In the overall evalation there is complete agreement between the VIROTECH EBV ELISA results and the prior results. Unclear results cannot be taken into account for the specificity.
(*)The unclear results were obtained with one child serum that did not yield an unequivocal serological ELISA result and again 4 sera yielded only a positive VCA IgG result in both ELISA and Immunoblot.

10.4 Diagnostic factor

Six sera yielding unclear results, with a result for both VCA IgM and EBNA1 IgG, were investigated to check the performance of the diagnostic factor.

Serum No.	**VCA IgM**		**EBNA1 IgG**		**Clear diagnosis using diagnostic factor**
	VU valueWert	Assessment	VU value	Assessment	
1	**37.5**	positive	11.7	positive	primary infection
2	**25.0**	positive	14.2	positive	primary infection
3	36.7	positive	**65.6**	positive	past infection
4	20.7	positive	**78.9**	positive	past infection
5	19.1	positive	**54.8**	positive	past infection
6	21.1	positive	**67.0**	positive	past infection

A clear diagnosis could be made with the aid of the diagnostic factor for all the sera investigated.

10.5 Prevalence (Expected Values)

80 sera were tested to determine the prevelance rate of 95% in adults (past EBV infection) as described in literature (8).

Seronegatives	4
Past Infections	76
Primary Infections	0

10.6 Intra-assay-Coefficient of Variation (Repeatability)

In one assay, strips of different plates of one batch have been tested with two sera. The obtained coefficient of variation is for

VCA IgG, EBNA1: < 9%

VCA IgM, EA-D: < 15%

10.7 Inter-assay-Coefficient of Variation (Reproducibility)

Three sera were tested in 10 independent test runs by different persons in different laboratories. The obtained coefficient of variation is for

VCA IgG, VCA IgM, EBNA1, EA-D: < 20%

11. Literature

1. Evans, A.S., J.C. Niedermann, L.C. Cenabre, B. West and V.A. Richards. (1975). A prospective evaluation of heterophile and EBV specific IgM antibody tests in clinical and subclinical infectious mononucleosis. J. Infect. Dis. 132:546-554.
2. Nikoskelainen, J., J. Leikola and E. Klemola. (1974). IgM antibodies specific for EBV in IM without heterophile antibodies. Br. Med. J. Oct 12.4(5936)72-5.
3. Klemola, E., R. von Essen, G. Henle and W. Henle. (1970). Infectious-mononucleosis-like disease with negative heterophil agglutination test. Clinical features in relation to Epstein-Barr virus and cytomegalovirus antibodies. J. Infect. Dis. Jun :121(6) :608-614.
4. Sumaya, C.V. and Y. Ench. (1985). Epstein-Barr virus infectious mononucleosis in children. II. Heterophil antibody and viral-specific responses. Pediatrics Jun: 75(6)1011-9.
5. Schmitz, H., D. Volz, C. Krainick-Riechert and M. Schere. (1972). Acute Epstein-Barr virus infections in children. Med Microbiol Immunol. 158(1):58-63.
6. Inoue, N. et al. (1992). Use of enzyme-linked immunosorbent assays with chimeric fusion proteins to titrate antibodies against Epstein-Barr virus nuclear antigen 1. J Clin Microbiol. Jun;30(6):1442-8.
7. Bauer, G. (2001). Simplicity through complexity: immunoblot with recombinant antigens as the new gold standard in Epstein-Barr virus serology. Clin Lab. 47(5-6):223-30.
8. Modrow, S. und D. Falke (2010). Das Epstein-Barr Virus. S. 572-577. In: Molekulare Virologie, Spektrum Verlag, ISBN: 978-3-8274-1833-3.
9. Gärtner BC et al. (2000). No correlation in Epstein-Barr virus reactivation between serological parameters and viral load. J Clin Microbiol. Jun;38(6): 2458

Preparation of Patient Samples and Washing Solution

▼ **Washing Solution:** Fill up concentrate to 1 liter with aqua dest./demin.

▼ **IgG-Samples – Dilution 1:101**

e.g.:
10 µl serum/plasma + 1000 µl Dilution Buffer
(Serum Dilution Buffer is ready to use)

▼ **IgM-Samples - Dilution 1:101**
Rheumafactor-absorption with RF-SorboTech

e.g.:
5 µl serum/plasma + 450 µl Dilution Buffer +
1 drop RF-SorboTech, incubate for 15 min. at room temperature.

Testprocedure

Samples Incubation	**30 minutes at 37°C**	**100 µl Patient Samples** blank value (Dilution Buffer) and controls
⇩		
Wash 4times		**400 µl Washing Solution** Remove Residues on a Cellulose Pad
⇩		
Conjugate Incubation	**30 minutes at 37°C**	**100 µl Conjugate** IgG, IgM
⇩		
Wash 4times		**400 µl Washing Solution** Remove Residues on a Cellulose Pad
⇩		
Substrate Incubation	**30 minutes at 37°C**	**100 µl Substrate**
⇩		
Stopping		**50 µl Stopping Solution** shake carefully
⇩		
Measure Extinctions		**Photometer at 450/620nm** **(Reference Wavelength 620-690nm)**