

## **VIROTECH Liquor/CSF Standards**

**Borrelia + VlsE IgG Liquor/CSF Standards Order-No.: EC022L60**  
**Borrelia IgM Liquor/CSF Standards Order-No.: EC022L80**  
**CMV IgG Liquor/CSF Standards Order-No.: EC113L60\***  
**EBV IgG Liquor/CSF Standards Order-No.: EC102L60**  
**FSME/TBE IgG Liquor/CSF Standards Order-No.: EC117L60**  
**FSME/TBE IgM Liquor/CSF Standards Order-No.: EC117L80**  
**HSV 1 (gG1) IgG Liquor/CSF Standards Order-No.: EC130L60**  
**HSV 2 (gG2) IgG Liquor/CSF Standards Order-No.: EC131L60**  
**HSV Screen IgG Liquor/CSF Standards Order-No.: EC108L60**  
**Masern/Measles IgG Liquor/CSF Standards Order-No.: EC105L60**  
**Masern/Measles IgM Liquor/CSF Standards Order-No.: EC105L80**  
**Mumps IgG Liquor/CSF Standards Order-No.: EC106L60**  
**Rubella IgG Liquor/CSF Standards Order-No.: EC109L60**  
**VZV IgG Liquor/CSF Standards Order-No.: EC110L60**  
**VZV IgM Liquor/CSF Standards Order-No.: EC110L80**  
**VZV IgA Liquor/CSF Standards Order-No.: EC110L40**

Please also refer to our CSF diagnostic test, with separate instructions for  
Rubella IgG EC109L00

**FOR IN-VITRO DIAGNOSIS ONLY**

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## 1. Intended Use

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The CSF-Standards are intended for drawing up of a calibration curve which is used for detection of CNS-own antibody synthesis by parallel examination of Serum-CSF-pairs. The pathogen-specific quotient of CSF and serum is calculated. The ratio between this pathogen-specific antibody quotient and the total immunoglobulin quotient is called antibody-index (AI).

## 2. Diagnostic Relevance

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During acute infections of the central nervous system (CNS) and also during chronic inflammatory processes (e.g. multiple sclerosis) pathogen-specific antibodies are developed in the CNS. In the first case those are antibodies against the causative pathogen, in the second case a poly-specific intrathecal immune response against possibly several pathogens without actual pathogen presence is possible (1, 2).

Bacterial infections of the CNS are mainly distinguished by highly pathologic and quite characteristic CSF-results. The diagnostic detection of viral CNS infections in cerebral-spinal fluid (CSF) is, depending on stage of infection and individual status of immunity, possible in two ways: by direct antigen detection or by detection of pathogen-specific antibodies. It is well known that culture of viral pathogens – unlike bacterial pathogens - is complicated, alternative pathogen-specific testings are bound to high methodical efforts. The pathogen-specific antibody detection usually takes effect earliest 6 days after onset of disease, but is routinely used in CSF-diagnosis by now (3).

The antibodies detected in the CSF may either diffuse out of the plasma into the CSF-area or be result of a local synthesis (intrathecal antibody production). The specific antibody-index (AI), describing the relation between the specific antibody quotient and the total immunoglobulin quotient serves for the clarification of a CNS infection. A local antibody-synthesis is present, if the pathogen-specific antibody quotient of a certain antibody-class is bigger than the corresponding total-immunoglobulin-quotient ( $Q_{\text{total}}$ ). (Please refer to our CSF diagnostic booklet for further information).

## 3. Test Principle

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The antibody searched for in the human serum and CSF 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.

The extinction (OD) of the color solution is directly proportional to the concentration of the analysed pathogen-specific IgG-, IgM- respectively IgA-antibody in Serum and CSF. For the detection of CNS-own antibody-synthesis it is necessary to proceed a quantification of the antibody concentrations that are available in extinctions initially. For this, the arrays of standard sera with graded pathogen-specific antibody-concentration are provided. Those standard sera serve for the creation of a reference curve, that can be made manually or by using a suitable programme and allows the conversion of the detected OD-values into arbitrary defined non-dimensional measurement units (wME). By allocating of the obtained measurement units (wME) with the nephelometrical measured Sera- and CSF-Total-IgG-, IgM- respectively IgA-concentrations the so called antibody index (AI) is detected (refer to the calculation of the AI in point 8.3). This antibody index mentions the searched pathogen-specific antibody quotient as a multiple respectively as a fraction of the respective Total-Immunoglobuline-Quotient. The value is therefore independent from the condition of the individual cerebral barrier-function. The antibody-index allows the conclusion to the presence and dimension of a CNS-own synthesis of pathogen-specific antibodies.

This method is not valid in case of a poly-specific intrathecal immunoglobuline-synthesis, as then the total-IgX-quotient is no longer suitable as barrier parameter and has to be replaced by the so called Limes value (refer to calculation of the Limes Quotient in point 8.3.4 B).

## 4. Package Contents

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Standards for the quantification of pathogen-specific antibody concentrations, 4 vials à 1000µl, human serum with protein-stabilizer and preservative, ready to use, 100wME; 25wME; 6,2wME; 1,5wME (wME = arbitrary measurement units)

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

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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  |
| RF-SorboTech               | 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  |
| PBS-Dilution Buffer (blue) | After Opening                 | +2 to +8°C                                                 | 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 standards. Nevertheless, samples, diluted samples, standards, conjugate 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. Test Procedure

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

### 7.1 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.

#### Consider the following for the serum samples:

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).

#### Consider the following for the CSF samples

Always prepare patient-dilution freshly.

If the CSF samples are to be stored for an extended period, it is best to aliquot them and then to freeze them at -80°C. This avoids repeated thawing.

1. Vein- and lumbar puncture should always be performed at approx. the same time.
2. Only optically clear and uncelled and not inactivated CSF may be used.
3. Do not use haemolytic or microbiologically contaminated or turbid CSF.
4. The use of deepfrozen CSF is possible if after thawing the conditions of items 2 and 3 are fulfilled.

## 7.2 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 standards are **parameter specific** and only to use with the plate lots they are related to. Please refer to Quality Control Certificate of the serum kit for possible combinations of plate lots and standard lots.

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. IgM-Diagnostic: Pre-absorbance with RF-SorboTech

High IgG-titer or rheumatoid factors may disturb the specific detection of IgM-antibodies and may lead to false positive resp. false negative results. **Hence for correct IgM determination it is necessary to pre-treat the sera and fluids with RF-SorboTech** (VIROTECH-adsorbent). Please make sure: to use the green dilution buffer in the case of VZV IgM. Pre-absorption is not necessary for the IgM controls and the standards.

## 7.3 VIROTECH ELISA Test Procedure

- CSF/serumpairs are principally to be analysed next to each other in the same determination row on one test plate
  - We recommend a double insertion for blank, controls, standard sera, patient sera and CSF-samples.
  - To minimize matrix effects as much as possible, a working-dilution of 1:2 for CSF and 1:404 for serum is used.  
It is recommended for IgM Diagnostics in general that a start be made with a dilution of 1:101 followed, if necessary, ( - the 100wME measurement point exceeded) by a 1:404 dilution. In the case of IgG, IgM and IgA diagnostic testing, the use of two dilutions is generally recommended for CSF and serum, e.g. CSF 1:2 and 1:4; serum 1:101 and 1:404, in order to rule out testing with antibody excess.
  - Please carry out the pre-treatment with RF-SorboTech for IgM diagnostics. (Please make sure: to use the green dilution buffer in the case of VZV IgM).
1. For each test run, pipette **100µl** each of the **dilution buffer** (blank), the **ready-to-use standard sera**, the **ready-to-use AI controls** (if available) or **serum quality controls** and the **diluted CSF and serum samples**.  
Working dilutions of the serum samples:  
IgG: 1:404 (e.g. 5µl serum + 500µl dilution buffer (1:101 dilution), then dilution 1:4, e.g. 100µl of 1:101 dilution + 300µl dilution buffer).  
IgM: 1:101 (e.g. 10µl serum + 1ml dilution buffer/RF-SorboTech).  
IgA: 1:404 (e.g. 5µl serum + 500µl dilution buffer (1:101 dilution), then dilution 1:4, e.g. 100µl of 1:101 dilution + 300µl dilution buffer).  
Working dilution of **CSF: 1:2**; e.g. **150µl CSF + 150µl 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 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

## 7.4 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.

## 8. Test Evaluation

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### 8.1 Test function control

To guarantee the optimal function of the test kit, the OD values of the 100 arbitrary units IgG, IgM and. IgA antibody standard serums must be above the minimal values given in the quality control certificates. When using the AI controls, keep to the ranges given in the control certificate.

If there are no AI controls, the validity of the test run must be checked with the serum quality controls.

a) OD values

The OD of the blank should be  $<0.15$ .

The OD values of the negative controls should be under the OD values given in the quality control certificate. The OD values of the positive controls and of the cut-off controls should be above the OD values given 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 given in the quality control certificate.

If the requirements (OD values, VE) are not fulfilled, the test must be repeated.

### 8.2 Interpretation

For CSF diagnostic testing, calculation through the cut-off control - as in serology - is **not** possible!

To quantify the pathogen-specific antibody content of serum-CSF pairs, a reference curve is prepared, either manually or instrumentally, with the help of the IgG-, IgM- or IgA antibody standard sera. The OD values of the standard sera are plotted on the y-axis and the antibody concentrations in wME on the x-axis. The reference curve, created by hand or by instrument (100wME, 25wME, 6.2wME, 1.5wME) shall have a sufficiently steep curve, a curve origin near the zero coordination point and acceptable deviation of all curve points from the extrapolished curve-course.

The OD values of the serum-CSF pairs can now be expressed in wME by reading off from the curve and after multiplication by the appropriate dilution factor they correspond to the concentrations of the pathogen-specific IgG, IgM or IgA antibody in serum and CSF.. To obtain plausible antibody indices, OD-values below 0,05 and wME-values below 1,5 or above 100 shall not be considered for the calculation. When the OD values measured lead to values greater than 100wME then greater serum dilutions than 1:101 / 1:404, or a greater CSF dilution than 1:2 can be used, while taking the changed dilution ratios into account.

VIROTECH provides user friendly CSF software solutions to simplify the AI calculation.

### 8.3 Calculation of the antibody index AI (with example)

Abbreviations:

$IgX_{total}$  = Total IgX (IgG, IgM or IgA, mg/l)

$IgX_{spec.}$  = pathogen-specific IgX (IgG, IgM or IgA)

Q = Quotient

$Q_{alb}$  = Quotient resulting from the albumin content of CSF and albumin content of the serum (mg/l)/only necessary for calculation of Limes value!

#### 8.3.1 $QIgX_{spec}$ (pathogen-specific antibody quotient)

**Serum**

- OD-values read: 0,700

- thus established concentration from the reference curve: 3,5 wME
- dilution: 1:400

#### CSF

- OD-values read: 0,500
- thus established concentration from the reference curve: 2,5 wME
- dilution: 1:2

$$Q_{\lg X_{\text{spec.}}} = \frac{\lg X_{\text{spec. CSF (wME)}} \times \text{dilution}}{\lg X_{\text{spec. Serum (wME)}} \times \text{dilution}} = \frac{2,5 \text{ wME} \times 2}{3,5 \text{ wME} \times 400} = 3,6 \times 10^{-3}$$

### 8.3.2 $Q_{\lg X}$ (Total Immunoglobulin Value: Value of the clinical chemistry)

- $\lg X_{\text{CSF}} = 33 \text{ mg/l}$
- $\lg X_{\text{Serum}} = 10000 \text{ mg/l}$

$$Q_{\lg X_{\text{total}}} = \frac{\lg X_{\text{total CSF}}}{\lg X_{\text{total Serum}}} = \frac{33 \text{ mg/l}}{10.000 \text{ mg/l}} = 3,3 \times 10^{-3}$$

### 8.3.3 Calculation of the Limes Quotient ( $Q_{\text{LIM}}$ )

In case of an additional poly-specific intrathecal immunoglobulin synthesis, the Total-IgX-Quotient for the AI-determination is no longer suitable. The so called  $Q_{\text{LIM}}$  has to be used instead of the Total-IgX-Quotient. Therefore it is necessary to determine the albumin quotient additionally (value of the clinical chemistry).

Calculation of the LIMES-Value (according to Reiber):

$$\begin{aligned} Q_{\text{LIM-IgG}} &= 0,93 \times \sqrt{Q_{\text{alb}}^2 + 6 \times 10^{-6}} - 1,7 \times 10^{-3} \\ Q_{\text{LIM-IgM}} &= 0,67 \times \sqrt{Q_{\text{alb}}^2 + 120 \times 10^{-6}} - 7,1 \times 10^{-3} \\ Q_{\text{LIM-IgA}} &= 0,77 \times \sqrt{Q_{\text{alb}}^2 + 23 \times 10^{-6}} - 3,1 \times 10^{-3} \end{aligned}$$

### 8.3.4 Calculation of the Antibody Index (AI)

#### A. $Q_{\lg X} < Q_{\text{LIM}}$

The antibody index (AI) states the relation between the pathogen-specific antibody quotient ( $Q_{\text{spec.}}$ ) and the total-immunoglobulin-quotient ( $Q_{\text{total}}$ ). Thus, a pathogen-specific antibody-synthesis can be detected and quantified. In this case, the total-immunoglobulin-quotient is used as barrier-parameter.

$$AI = \frac{Q_{\lg X_{\text{spec.}}}}{Q_{\lg X_{\text{total}}}} = \frac{\frac{\lg X_{\text{spec. CSF}} \times \text{dilution}}{\lg X_{\text{spec. Serum}} \times \text{dilution}}}{\frac{\lg X_{\text{total CSF}}}{\lg X_{\text{total Serum}}}} = \frac{3,6 \times 10^{-3}}{3,3 \times 10^{-3}} = 11$$

#### B. $Q_{\lg X} > Q_{\text{LIM}}$

In case an additional poly-specific intrathecal immunoglobulin-synthesis is present the measured  $Q_{\text{total}}$  must no longer be used for the AI-calculation as an antibody synthesis searched for or eventually present at the same time may be falsified in its extent or even get totally unrecognizable. In this cases the so called Limes value of the immunoglobulin quotient is calculated (see formula) or graphically determined by using the albumin quotient which has to be calculated additionally. This Limes value is used instead of the immunoglobulin quotient for calculation of AI-value.

$$AI = \frac{Q_{\lg X_{\text{spec.}}}}{Q_{\text{Lim}}}$$

## 8.4 Interpretation

### AI –Evaluations (4):

|               |               |                                                                                                                                   |
|---------------|---------------|-----------------------------------------------------------------------------------------------------------------------------------|
| AI: < 0.6     | undetectable  | Should not theoretically occur. Occasionally found in routine work. No pathological significance. Desirable to search for errors. |
| AI: 0.6 – 1.3 | normal:       | Intrathecale antibody production is improbable.                                                                                   |
| AI: 1.4 – 1.5 | borderline:   | It is recommended that the sample should be retested, or that a later serum-CSF pair should be tested.                            |
| AI: >1.5      | pathological: | Indicates intrathecal antibody production.                                                                                        |

1. Since a minimum of four different results (pathogen-specific CSF- and serum-antibody measurement-units, total serum- and CSF-IgG-, IgM- or IgA-value, CSF- and serum-albumine in mg/l) are considered for the calculation of the diagnostical relevant AI-value, all methodic and coincidental errors add up here. In the most unfavourable case, a continuing mistake in the same sense is possible; a double determination or better the measuring of two different sample dilutions are the best way to recognize this. For this reason, a clinical relevant limited AI value of 1,5 has established as note for a local synthesis of pathogen-specific antibodies in the CSF.
2. Normally for virus-specific antibodies of the IgG-, IgM- or IgA-class there is the same ratio between CSF and serum as it is found for the summarised IgG-, IgM- or IgA-fraction. The theoretically expected AI-value is therefore 1,0. Corresponding tests have shown, that for all virus-specific antibodies a reference-range of 0,6 – 1,3 is valid. AI values between 1.4 – 1.5 are classified as borderline. AI-values above 1,5 may be considered as pathologic in case of sufficient analytical quality of all incoming single values and may be characterized by a CNS-own synthesis of the corresponding virus-specific antibodies.
3. AI-values below 0,6 are theoretically impossible and do normally point out an analytical mistake.
4. Only high AI-values without corresponding clinical reference do not allow a definite conclusion for an acute stage of an infectious CNS-disease. Long-time persisting and poly-specific CNS-own antibody-synthesis in particular of the IgG-class but also of the IgM-class are possible. IgM-AI-increases are usually considered as prove for florid CNS-infections. In case of doubt, the significant change of the AI-value from a second determination, similar to titer motion, is of advantage for the judgement of an infection of the central nervous system. Such a control is mandatorily bound to a further CSF-withdrawel, taken in an adequate time interval. However, for its indication only the clinical aspects are decisive, as a rule.

## 8.5 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. In case of very high pathogen-specific antibody concentrations in the cerebrospinal fluid or in the serum, a risk that the antigen-concentration in the wells is insufficient to fulfill the optimum conditions for a quantitative antibody detection is present. If an antibody-excess is suspected (please consider also Heidelberg curve and total CSF-result) a second determination with higher dilution of serum respectively CSF has to follow.

**Notice the detailed performance data (sensitivity and specificity) for Borrelia – and CMV – CSF diagnostic in the regular instruction sheet in addition to the serology.**

## 9. Literature

1. Zimmermann K. , Liquordiagnostik, MTA 11 (1996)4 ; 258 - 260
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4. Peterleit, Sindern, Wick (2007): Leitlinien der Liquordiagnostik und Methodenkatalog der Deutschen Gesellschaft für Liquordiagnostik und Klinische Neurochemie, Springer Verlag, ISBN 978-3-540-39017-6

## Preparation of the Patient Samples and Washing Solution

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

▼ **IgG/IgA-Samples – Dilution**  
1:404

▼ **CSF-Dilution**  
1:2

e.g.:

1:101: 5 µl serum/plasma + 500 µl dilution buffer

1:404: 100 µl diluted serum 1:101 + 300 µl dilution buffer

150 µl CSF-sample + 150 µl Dilution Buffer

▼ **IgM-Samples – Dilution**  
1:101/1:404

▼ **CSF-Dilution**  
1:2

**Rheumatoid factor-absorption with RF-Sorbo-Tech**

e.g.:

1:101: 5 µl serum/plasma + 450 µl dilution buffer + 1 drop RF-SorboTech

Incubate at RT for 15 min

1:404: 100 µl serum/VP/RF-SorboTech mixture + 300 µl dilution buffer

50 µl RF-SorboTech + 200 µl Dilution Buffer

225 µl RF-SorboTech-buffer mixture + 225 µl CSF-sample, incubate for 15 min at room temperature

## Test procedure

Samples Incubation

30 Minutes at 37°C

**100 µl Patient Samples**

blank (Dilution Buffer) and Standards

Wash 4 times

**400 µl Washing Solution**

Remove Residues on a Cellulose Pad

Conjugate Incubation

30 Minutes at 37°C

**100 µl Conjugate**

IgG, IgM, IgA

Wash 4 times

**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)