User's Manual

Rabies Virus

Antibody ELISA

A peptide based ELISA to detect IgM antibodies against proteins of Rabies Virus in serum or plasma samples

REF D1006-AB02

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Gebruik alleen de juiste versie van het protocol dat meegestuurd wordt met de kit.
Please use only the valid version of the package insert provided with the kit.
Verwenden Sie nur die jeweils gültige, im Testkit enthaltene, Arbeitsanleitung.
Si prega di usare la versione valida dell'inserto del pacco a disposizione con il kit.
Por favor, se usa solo la version valida de la metodico técnico incluido aqui en el kit.

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1. Introduction

Rabies virus can infect all warm-blooded species and in many species the disease can present itself in two different forms. Furious rabies, in which predominantly the brain is infected and paralytic rabies in which predominantly the spinal cord is involved. When cells of the limbic system are infected the first changes in behavior characteristic of rabies may be observed. It has been suggested that the phase before infecting cells of the nervous system may take a considerable length of time, causing a variable incubation period from 10 days to several years. Hence the virus is present in the saliva, which favors the most natural way of transmission by biting in the various stages of the disease, also sporadic cased of aerosol infections have been documented. Carnivores, especially domestic dogs and cats, and also rodent and recently bats, are usually involved in transmission of infections to dogs and man. Infections of dogs with rabies virus seem to be invariably fatal. Persistent in apparent infection accompanied by virus shedding has been documented in several human and animal species including cats and raccoons. This standardized ELISA test system based on synthetic peptides is intended to use as a rapid screening test for the detection of rabies antibodies in serum or plasma samples of dogs.

2. Intended use of the test kit

This diagnostic test-system for the establishment of Rabies infection is intended to identify antibodies against neutralizing epitopes of rabies virus, in serum samples. In contrast to other test systems this standardized ELISA based on synthetic peptides, has a very high sensitivity and specificity.

3. Principle of the test kit

The test is based on the reaction of synthetic peptides with polyclonal mouse dog antibodies.
To this end synthetic peptides have been coated to a 96-well microtiter strip plate. The dog serum is added to the wells of the coated plate.

➢ **Qualitative**
The sample is added (diluted 1:30) to the wells of the coated plate.
➢ **Quantitative**
The sample also can be titrated using a 3-step dilution, starting with a dilution 1:30 ($\rightarrow$ 1:90 $\rightarrow$ 1:270 $\rightarrow$ 1:810).

After washing, the bound dog antibodies are detected by HRPO conjugated anti-species conjugate. The color reaction in the wells is directly related to the concentration of rabies virus antibodies in the serum or plasma sample.

4. Contents

- 12 x 8 Microtiter strips
- 1 x Strip holder
- 1 x 18 ml ELISA buffer (green cap)
- 1 x 12 ml HRPO conjugated anti-species antibodies (red cap)
- 1 x 0,5 ml Positive control (freeze dried) (purple cap)
- 1 x 1,0 ml Negative control (freeze dried) (silver cap)
- 1 x 20 ml Wash solution (200x concentrated) (black cap), **dilute in de-ionized water before use!**
- 1 x 8 ml Substrate A (white cap)
- 1 x 8 ml Substrate B (blue cap)
- 1 x 8 ml Stop solution (yellow cap)
- 1 x Plastic cover seal
- 1 x User's manual

Supplies needed (not included)

- Round-bottomed microtiter plate
- Validated precision pipettes
- Pipette tips and clean containers/tubes (EVL)
- ELISA plate reader

5. Handling and storage of specimens

The kit should be stored at 4°C.

An open packet should be used within 10 days.

Samples may be used fresh or may be kept frozen below -20°C before use.

After first use ready-to-use controls and/or reconstituted controls should be aliquoted immediately and stored at -20°C.

Avoid repeated freezing and thawing as this increases non-specific reactivity.

BV European Veterinary Laboratory   Tel   0348-412549
Postbus 198   Fax   0348-414626
3440 AD Woerden   Web   www.evlonline.org
The Netherlands   Email   info@evlonline.eu

6. Wash protocol

In ELISA's, un-complexed components must be removed efficiently between each incubation step. This is accomplished by appropriate washing. It should be stressed that each washing step must be carried out with care to guarantee reproducible inter- and intra-assay results. It is essential to follow the washing procedures outlined below. Washing may be done manually or with automatic equipment. Automatic washing equipment usually gives better result.

Manual washing
1. Empty each well by turning the microtiter plate upside down, followed by a firm vertical downward movement to remove the buffer.
2. Fill all the wells with 250 µl wash solution.
3. This washing cycle (step 1 and 2) should be carried out at least 5 times.
4. Turn the plate upside down and empty the wells with a firm vertical movement.
5. Place the inverted plate on absorbent paper towels and tap the plate firmly to remove any residual wash solution in the wells.
6. Take care that none of the wells dry out before the next reagent is added.

Washing with automatic equipment
When automatic plate washing equipment is used, check that all wells are aspirated completely and that the wash solution is correctly added, reaching the rim of each well during each rinsing cycle. The washer should be programmed to execute at least 5 washing cycles.

7. Preparations

- Before using the reagents needed, take them out of the kit and place them on the table for ± 15 min. at room temperature (± 21°C) without exposing them to direct sunlight or (other) heat sources.
- Buffer, controls, standards and conjugates need to be shaken gently before use, in order to dissolve/ mix any components that may have precipitated. Gently tap the vials onto the table, so any fluid still retained in the cap falls back into the solution.
- If fluids need to be mixed into the test well, gently shake by tapping the wells with the fingers or re-suspend with the last pipette tip used for that particular well. Avoid contamination through spattering and prevent any fluid to enter inside the pipette itself.
- Place the reagents back at 4-8°C immediately after use.

8. Test protocol qualitative

Before starting this test read "preparations"

1. Open the packet of strips and take out the strips to be used. Cover the remaining strips with a part of the provided seal and store them at +4°C. and use them within 10 days.

   Wash microtiter strip(s) with washing solution, according to washing protocol.

   The washing solutions provided must be diluted 200x in aquabidest (5 mega Ohm) water !
   Use validated precision pipettes and use a clean pipette tip **before** pipetting the buffer, control, samples, conjugate and substrate.

2. Reconstitute directly before use the **positive control** (purple cap) in **0,5ml aquabidest** (5 mega Ohm water), divide into aliquots, and store immediately at -20 °C until use, avoid freeze and thaw cycles.

3. Reconstitute directly before use the **negative control** (silver cap) in **1,0ml aquabidest** (5 mega Ohm water), divide into aliquots, and store immediately at -20 °C until use, avoid freeze and thaw cycles.

4. Dilute the **positive control** (purple cap) **starting 1:30 → 1:60 → 1:180 → 1:540 in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - A **pre-dilution** is needed:
       - Add 80µl ELISA buffer to **row 1A**, add 20µl of the positive control to the **well 1A** and mix well.
       - Add 150µl ELISA buffer to **row 2A**,
       - And 120µl to **2B, 2C, 2D**
       - Add 30µl of pre-dilution **well 1A** in the well **2A** and mix well
       - Mix well and transfer 60µl to the well **2B**
       - Mix well and transfer 60µl to the well **2C**
       - Mix well and transfer 60µl to the well **2D**
       - Mix well and discard 60µl.

5. Dilute the **negative control** (silver cap) **1:30 in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - Add 145µl ELISA buffer to **well 2E**, add 5µl of the negative control to the **well 2E** and mix well.

6. Dilute the **sample 1:30 in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - Add 145µl ELISA buffer to **row 2F**, add 5µl of the sample to the **well 2F** and mix well.

7. Take 2 wells as **substrate controls** add only **120µl ELISA buffer** (green cap) to these wells.

8. Transfer 100µl of all dilutions of **row 2** to the virus-coated microtiter strips, including the substrate controls.

9. Seal and incubate for 60 min at 37°C.

10. Wash the strips according to the wash protocol [see sub 6].

11. Add **100µl HRPO conjugated anti-species antibodies** to all wells.

12. Seal and incubate for 60 min at 37°C.

13. Wash the strips according to the wash protocol ^{see sub 6}.

14. Mix equal parts of substrate A (white cap) and substrate B (blue cap) with gentle shaking. **Prepare immediately before use! Only prepare amount needed. Substrate can only be used for 1-2 hours after being mixed.**

15. Add **100μl substrate solution** to each well.

16. Incubate 10-20 min. in the dark (e.g. cover the wells with a sheet of paper). at room temperature (21°C.). Make sure the negative control does not become too dark.

17. Add **50μl stop solution** to each well; mix well.

18. Read the absorbency values immediately (within 10 min!) at 450 Nm by using an ELISA reader. **Use the substrate controls as blank.**

NB: if you pipet directly into the coated ELISA plate with only a small number of samples (<6), make sure the first dilution is done in round bottom microtiter plate second step can be done directly in the coated Elisa plate.

9. Test protocol quantitative

Before starting this test read "preparations"

1. Open the packet of strips and take out the strips to be used. Cover the remaining strips with a part of the provided seal and store them at +4°C. and use them within 10 days.

   Wash microtiter strip(s) with washing solution, according to washing protocol.

   The washing solutions provided must be diluted 200x in aquabidest (5 mega Ohm) water!

   Use validated precision pipettes and use a clean pipette tip **before** pipetting the buffer, control, samples, conjugate and substrate.

2. Reconstitute directly before use the **positive control** (purple cap) **in 0,5ml aquabidest** (5 mega Ohm water), divide into aliquots, and store immediately at -20 °C until use, avoid freeze and thaw cycles.

3. Reconstitute directly before use the **negative control** (silver cap) **in 1,0ml aquabidest** (5 mega Ohm water), divide into aliquots, and store immediately at -20 °C until use, avoid freeze and thaw cycles.

4. Make a pre-dilution of the **positive control** (purple cap) **in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - Add 80µl ELISA buffer to **well 1A** and add 20µl of the positive control to the well **1A.**

5. Make a pre-dilution of the **negative control** (silver cap) **in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - Add 80µl ELISA buffer to **well 1B** and add 20µl of the negative control to the well **1B.**

6. Make a **pre-dilution** of **each sample in ELISA buffer** (green cap) in a round-bottomed plate (not supplied).
   Example: - Add 80µl ELISA buffer to **well 1C** and add 20µl of the sample to the **well 1C.**

7. Take 2 wells as **substrate controls** add only **140µl ELISA buffer** (green cap) to these wells.

8. Add for dilution of the **positive control 125µl ELISA buffer** to **well 1A.**
   And 100µl to **1B, 1C, 1D** of the coated microtiter strip.

9. Add for dilution of the **negative control 125µl ELISA buffer** to **well 1E.**
   And 100µl to **1F, 1G, 1H** of the coated microtiter strip.

10. Add for dilution of the **samples 125µl ELISA buffer** to the other **well 2A and 2E.**
    And 100µl to **well 2B, 2C, 2D and 2F, 2G, 2H** (depending on the number of samples) of the coated microtiter strip.

11. Make a 3-step dilution of the **positive control** in the coated microtiter strip, **starting 1:30 → 1:90 → 1:270 → 1:810**.
    Example: - Add 25µl positive control from step 4 to the **well 1A** of the microtiter strip.
    - Mix well and transfer 50 µl to the well **1B**
    - Mix well and transfer 50µl to the well **1C**
    - Mix well and transfer 50µl to the well **1D**
    - Mix well and discard 50µl.

12. Make a 3-step dilution of the **negative control** in the coated microtiter strip, **starting 1:30 → 1:90 → 1:270 → 1:810**.
    Example: - Add 25µl negative control from step 5 to the **well 1E** of the microtiter strip.
    - Mix well and transfer 50µl to the next well **1F**
    - Mix well transfer 50µl to the next well **1G**
    - Mix well and transfer 50µl to the well **1H**
    - Mix well and discard 50µl.
13. Make 3-step dilution of **each sample** in the coated microtiter strip, **starting 1:30 → 1:90 → 1:270 → 1:810**.
    Example: - Add 25µl of each sample from step 6 to the well **2A and/or 2E** of the microtiter strip.
    - Mix well and transfer 50 µl to the well **2B and/or 2F**
    - Mix well and transfer 50µl to the well **2C and/or 2G**
    - Mix well and transfer 50µl to the well **2D and/or 2H**
    - Mix well and discard 50µl.
14. Add **100µl** of the **substrate control** of step 7 to the last 2 wells of the microtiter strip.
15. Seal and incubate for 60 min at 37°C.

16. Wash the strips according to the wash protocol ^{see sub 6}.
17. Add **100µl HRPO conjugated anti-species antibodies** to all wells.
18. Seal and incubate for 60 min at 37°C.

19. Wash the strips according to the wash protocol ^{see sub 6}.
20. Mix equal parts of substrate A (white cap) and substrate B (blue cap) with gentle shaking. **Prepare immediately before use! Only prepare amount needed. Substrate can only be used for 1-2 hours after being mixed.**

21. Add **100µl substrate solution** to each well.
22. Incubate 10-20 min. in the dark (e.g. cover the wells with a sheet of paper). at room temperature (21°C.). Make sure the negative control does not become too dark.

23. Add **50µl stop solution** to each well; mix well.
24. Read the absorbency values immediately (within 10 min!) at 450 Nm by using an ELISA reader. **Use the substrate controls as blank.**

10.   Precautions

➢ Handle all biological material as though capable of transmitting infectious diseases.
➢ Do not pipette by mouth.
➢ Do not eat, drink, smoke or prepare foods, or apply cosmetics within the designated working area.
➢ TMB substrate (buffer B) is toxic by inhalation, through contact with skin or when swallowed; observe care when handling substrate.
➢ Do not use components past the expiry date and do not mix components from different serial lots.
➢ Optimal, results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.
➢ Each well is ultimately used as an optical cuvette. Therefore, do not touch the under-surface of the microtiter plate and protect it from damage and dirt.

11.   Validation of the test

Qualitative:

➢ The results are valid if the following criteria are met:
  o The mean value (MV) of the measured OD value for the Positive Control (PC), diluted 1:30, must be ≥0.500.
  o The MV of the measured OD value for the Negative Control (NC), diluted 1:30, must be ≤0.200.

In case of invalid assays the test should be repeated after a thorough review of the instructions for use.

Calculation
Calculate the mean values (MV) of the measured OD for the Negative Control (NC) and the Positive Control (PC).

The ratio (S/P) of sample OD to mean OD of the positive control is calculated according to the following equation:

S/P= $\dfrac{OD_{sample} - MV\ OD_{NC}}{MV\ OD_{PC} - MV\ OD_{NC}}$

Quantitative:
In order to confirm appropriate test conditions the OD of the positive control, diluted 1:30, should be ≥0.500 OD units (450nm) and give an endpoint titer of ≥90.
The negative control, diluted 1:30, should be ≤0.200 OD units (450nm) and give an endpoint titer of ≤30.

BV European Veterinary Laboratory
Postbus 198
3440 AD Woerden
The Netherlands

Tel   0348-412549
Fax   0348-414626
Web   www.evlonline.org
Email info@evlonline.eu

12.　　Interpretation of the test results

This test can be used in 2 ways.

Qualitative: Positive – Negative
 ➢ A sample with the S/P ratio <0.4 is negative.
   o Specific antibodies to Rabies could not be detected.
 ➢ A sample with the S/P ratio ≥0.4 is positive.
   o Specific antibodies to Rabies were detected.

Quantitative: End point titre
 ➢ The ELISA titre can be calculated by constructing a curve and using cut-off line (dilution 1:30 – 90 - 270 – 810 - 2430 - 7290 etc. total 8 dilutions of 3 steps) OD on Y-as and titre on X-as
 Elisa titres can be calculated using as cut-off 2,5 times OD value of negative control at 1:30.

13.　　Symbols used with EVL ASSAYS

Symbol	English	Deutsch	Français	Español	Italiano
	Consult instructions for use	Gebrauchsanweisung beachten	Consulter les instructions d'utilisation	Consulte las instrucciones de uso	Consultare le istruzioni per l'uso
CE	European Conformity	CE-Konfirmitäts-kennzeichnung	Conformité aux normes européennes	Conformidad europea	Conformità europea
IVD	In vitro diagnostic device	In-vitro-Diagnostikum	Usage Diagnostic in vitro	Para uso Diagnóstico in vitro	Per uso Diagnostica in vitro
RUO	For research use only	Nur für Forschungszwecke	Seulement dans le cadre de recherches	Sólo para uso en investigación	Solo a scopo di ricerca
REF	Catalogue number	Katalog-Nr.	Numéro de catalogue	Número de catálogo	Numero di Catalogo
LOT	Lot. No. / Batch code	Chargen-Nr.	Numéro de lot	Número de lote	Numero di lotto
∑	Contains sufficient for <n> tests/	Ausreichend für "n" Ansätze	Contenu suffisant pour "n" tests	Contenido suficiente para <n> ensayos	Contenuto sufficiente per "n" saggi
	Storage Temperature	Lagerungstemperatur	Température de conservation	Temperatura de conservación	Temperatura di conservazione
	Expiration Date	Mindesthaltbarkeits-datum	Date limite d'utilisation	Fecha de caducidad	Data di scadenza
	Legal Manufacturer	Hersteller	Fabricant	Fabricante	Fabbricante
Distributed by	Distributor	Vertreiber	Distributeur	Distribuidor	Distributore
Content	Content	Inhalt	Conditionnement	Contenido	Contenuto
Volume/No.	Volume / No.	Volumen/Anzahl	Volume/Quantité	Volumen/Número	Volume/Quantità

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