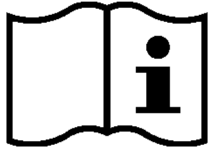
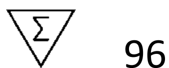


Userś Manual

Rabies Virus Antigen ELISA

A monoclonal-based ELISA testkit to detect nucleoproteins of Rabies Virus in antigen suspensions
(according to WHO levels)

 D1006-AG02

Σ 96

December 2020

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.

Inhoud / Table of Contents / Inhaltsverzeichnis / Tabella die Contenuti / Tabla de Contenidos

1. Introduction 3
2. Intended use of the test kit 3
3. Principle of the test kit 3
4. Contents 4
5. Handling and storage of specimens 4
6. Wash protocol 5
7. Preparations 5
8. Test protocol **qualitative** 6
   Before starting this test read “**preparations**” 6
9. Test protocol Quantitative 8
   Before starting this test read “**preparations**” 8
10. Precautions 9
11. Validation of the test 10
12. Interpretation of the test results 10
13. Symbols used with EVL ASSAYS 11

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

1. Introduction

Rabies vaccines for use in man or animal consist of inactivated antigen suspensions of in vitro cultured rabies virus. The potency virus test for inactivated rabies vaccines as recommended by the World Health Organization is the NIH mouse protection test (NIH test, Seligmann, 1973). This NIH test suffers from several drawbacks (e.g. animal distress, time consuming, poor reproducibility), there for the introduction of alternative testing methods is highly recommended. Enzyme immunoassays (ELISA) are being developed to quantify assay the nucleoprotein content of rabies vaccines. ELISAs are rapid and highly reproducible, and the use of standardized test kits enables the user to determine the nucleoprotein contents of inactivated antigen preparations according to the WHO standard. For this purpose the test kit includes a positive standard extensively tested in the NIH test.

2. Intended use of the test kit

This diagnostic test is intended for the detection of rabies virus nucleoprotein in inactivated antigen samples. This standardized ELISA is based on monoclonal antibodies, with high sensitivity and specificity for the nucleoprotein of rabies virus. This testkit was used in the collaborative study organized by the National Institute of Health and Environmental Protection (RIV, The Netherlands).

3. Principle of the test kit

The test is based on the reaction of rabies nucleoproteins with biotinylated monoclonal antibodies. To this end rabies nucleoproteins have been coated to a 96-well microtiter strip plate.
The antigen samples are added to the wells of a pre-incubation plate. Immediately the same volume of a biotinylated monoclonal antibody solution (MAb conjugate) is added.
After pre-incubation the antigen/conjugate mixture is transferred to the coated microtiter plate to bind un-complexed MAb conjugate. After washing, the bound MAb conjugate is detected by Avidin HRPO conjugate.

- **Qualitative**
  The antigen sample is added (diluted 1:8) to the wells of the coated plate.
- **Quantitative**
  The antigen sample also can be titrated using a 2-step dilution, starting with 1:2 (→ 1:4 → 1:8 → 1:16 → 1:32 → 1:64 → 1:128→ 1:256).

After developing with substrate, the color reaction in the wells is reversed related to the concentration of rabies virus nucleoprotein in the sample.

4. Contents

- 12 x 8 Microtiter strips
- 1 x Strip holder
- 1 x 22 ml ELISA buffer
- 1 x 7 ml Biotinylated monoclonal antibody (MAb biotin conjugate)
- 1 x 13 ml Streptavidin conjugate
- 1 x 0,5 ml Inactivated rabies positive standard (1,6 IU) (freeze-dried)
- 1 x 1,0 ml Inactivated rabies negative control sample (freeze-dried)
- 1 x 20 ml Wash solution (200x concentrated), **dilute in de-ionized water before use!**
- 1 x 8 ml Substrate A
- 1 x 8 ml Substrate B
- 1 x 8 ml Stop solution
- 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.

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.

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

8. Test protocol qualitative

Before starting this test read "**preparations**"

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

4. Add for dilution of the **positive standard 60µl ELISA buffer** (green cap) to the wells **1B to 1H** of a round-bottomed plate.
5. Add for dilution of the **negative control 60µl ELISA buffer** (green cap) to the **wells 2B, 2C, 2D** of a round-bottomed plate.
6. Make a 2-step dilution with a final volume of 60µl of the **positive standard** (purple cap) in ELISA buffer (green cap) **starting undiluted → 1:2 → 1:4 → 1:8 → 1:16 → 1:32 → 1:64 → 1:128** in a round-bottomed microtiter plate (not supplied).
   Example: - Add 120µl positive control to the **well 1A** of the microtiter strip.
   - Mix well and transfer 60 µl to the well **1B**
   - Mix well and transfer 60µl to the well **1C**
   - Mix well and transfer 60µl to the well **1D**
   - Mix well and transfer 60µl to the well **1E**
   - Mix well and transfer 60µl to the well **1F**
   - Mix well and transfer 60µl to the well **1G**
   - Mix well and transfer 60µl to the well **1H**
   - Mix well and discard 60µl.
7. Make a 2-step dilution with a final volume of 60µl of the **negative control** (silver cap) **in ELISA buffer** (green cap) **starting undiluted → 1:2 → 1:4 → 1:8** in a round-bottomed microtiter plate (not supplied).
   Example: - Add 120µl negative control to the **well 2A** of the microtiter strip.
   - Mix well and transfer 60 µl 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.
8. Dilute the **sample 1:8 in ELISA buffer** (green cap) in a round-bottomed plate (not supplied). Make sure to make to obtain a final volume of 60ul.
   Example: - Add 52,5µl ELISA buffer to **well 2E**, add 7,5µl of the sample to the **well 2E** and mix well.
9. Take 2 wells as **substrate controls** add only **60µl ELISA buffer** (green cap) to these wells.

10. Add immediately **60µl MAB Biotin conjugate** to all the wells containing samples/buffer.
11. Seal and incubate for 60 min at 37°C.

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. 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) 5x with washing solution, according to washing protocol.

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

13. Transfer **100µl of the incubated antigen/conjugate mixture** to the pre-coated ELISA plate.
14. Seal and incubate for 60 min at 37°C.

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

16. Add **100µl Streptavidin conjugate** to all wells.
17. Seal and incubate for 25 min at 37°C.

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

19. 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.**

20. Add **100µl substrate solution** to each well.
21. Incubate 15-25 min. in the dark (e.g. cover the wells with a sheet of paper) at room temperature (21°C.). Make sure the negative does not become too dark.

22. Add **50µl stop solution** to each well; mix well.
23. Read the absorbency values immediately (within 10 min!) at 450 nm using 620nm as reference on the ELISA reader. **Use the substrate controls as blank.**

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

9. Test protocol Quantitative

Before starting this test read “**preparations**”

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

4. Add for dilution of the **positive standard 60µl ELISA buffer** (green cap) to the wells **1B, 1C, 1D** of a round-bottom microtiter plate.
5. Add for dilution of the **negative control 60µl ELISA buffer** (green cap) to the wells **1F, 1G, 1H** of a round-bottom microtiter plate.
6. Add for dilution of the **samples 120µl ELISA buffer** to the other **wells 2A and/or 2E.** And 60µl to the **wells 2B, 2C, 2D and/or 2F, 2G, 2H** (depending on the number of samples) of a round-bottom microtiter plate.

7. Make a 2-step dilution with a final volume of 60µl of the **positive standard** (purple cap) **in ELISA buffer** (green cap) starting **undiluted → 1:2 → 1:4 → 1:8** in a round-bottomed microtiter plate (not supplied).
   Example: - Add 120µl positive control to the **well 1A** of the microtiter strip.
   - Mix well and transfer 60µl to the well **1B**
   - Mix well and transfer 60µl to the well **1C**
   - Mix well and transfer 60µl to the well **1D**
   - Mix well and discard 60µl.
8. Make a 2-step dilution with a final volume of 60µl of the **negative control** (silver cap) **in ELISA buffer** (green cap) starting **undiluted → 1:2 → 1:4 → 1:8** in a round-bottomed microtiter plate (not supplied).
   Example: - Add 120µl negative control to the **well 1E** of the microtiter strip.
   - Mix well and transfer 60µl to the well **1F**
   - Mix well and transfer 60µl to the well **1G**
   - Mix well and transfer 60µl to the well **1H**
   - Mix well and discard 60µl.
9. Make a 2-step dilution with a final volume of 60µl of each antigen **sample in ELISA buffer** (green cap) **starting 1:2 → 1:4 → 1:8 → 1:16** in a round-bottomed microtiter plate (not supplied).
   Example: - Add 60µl of each sample to the well **2A and/or 2E** of the microtiter strip.
   - Mix well and transfer 60µl to the well **2B and/or 2F**
   - Mix well and transfer 60µl to the well **2C and/or 2G**
   - Mix well and transfer 60µl to the well **2D and/or 2H**
   - Mix well and discard 60µl.
10. Take 2 wells as **substrate controls** add only **60µl ELISA buffer** (green cap) to these wells.
11. Add immediately **60µl MAB Biotin conjugate** to all the wells containing samples/buffer.
12. Seal and incubate for 60 min at 37°C.

13. 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) 5x with washing solution, according to washing protocol.

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

14. Transfer **100µl of the incubated antigen/conjugate mixture** to the pre-coated ELISA plate.
15. Seal and incubate for 60 min at 37°C.

16. Wash the strips according to the wash protocols [see sub 6].
17. Add **100µl Streptavidin conjugate** to all wells.
18. Seal and incubate for 25 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 15-25 min. in the dark (e.g. cover the wells with a sheet of paper) at room temperature (21°C.). Make sure the negative 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 using 620nm as reference on the 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.

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

11. Validation of the test

Qualitative:

- The results are valid if the following criteria are met:
  - The mean value (MV) of the measured OD value for the Positive Control (PC), undiluted, must be ≤0.500.
  - The MV of the measured OD value for the Negative Control (NC), undiluted, must be ≥1.100.

  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/N) of sample OD to mean OD of the negative control is calculated according to the following equation:

  $$S/N = \frac{OD_{sample}}{MV\ OD_{NC}}$$

Quantitatief:

- In order to confirm appropriate test conditions the OD of the positive control, undiluted, should be ≤0.500 OD units (450nm) and give an endpoint titer of ≥8.
  The negative control, undiluted, should be ≥1.100 OD units (450nm) and give an endpoint titer of ≤2 .

12. Interpretation of the test results

This test can be used in 2 ways.

Qualitative: Positive – Negative

- A sample with the S/N ratio >0.7 is negative.
  - Specific antigen of Rabies could not be detected.
- A sample with the S/N ratio ≤0.6 is positive.
  - Specific antigen of Rabies was detected.
- A sample with the S/N ratio between 0.61 and 0.7 is doubtful.

Quantitative: End point titre

- The ELISA titre can be calculated by constructing a curve and using cut-off line (dilution 1:2 -4 – 8 - 16 – 32- 64 etc. total 8 dilutions of 2 steps) **OD** on Y-as and **titre** on X-as
  Elisa titres can be calculated using as cut-off the OD value of the undiluted negative control multiplied by the factor 0.68.

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

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
Σ	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

The entire risk as to the performance of these products is assumed by the purchaser. EVL shall not be liable for indirect, special or consequential damages of any kind resulting from use of the products In case of problems or questions contact EVL.

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