ADIAVET™ MAREK REAL TIME

TEST FOR THE DETECTION OF MAREK VIRUS BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
ADI461-100 (100 reactions)

VET

NOTE
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English version
NE461-04
2020/01

ADIAVET™ MAREK REAL TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change product	Addition, revision and/or removal of information related to the
Administrative	Implementation of non-technical changes noticeable to the user

Note: minor typographical, grammar and formatting changes are not included in the revision history.

Release Date	Part Number	Change type	Change summary
2013/06	NE461-01	N/A	First publication
2014/12	NE461-02	Technical change	Addition of "Index of symbols" section, in page 11.
2014/12	NE461-02	Technical change	Removal of reference ADI461-50 (50 reactions)
2015/06	NE461-03	Technical change	Modification of QIAamp DNA Mini kit protocol Addition of NucleoSpin Tissue protocol
2016/07	NE461-03	Administrative	Changing logos
2016/07	NE461-03	Administrative	Biosearch legal mention
2016/07	NE461-03	Administrative	Addition of table "Analysis options according to the specimen" §I.3.
2020/01	NE461-04	Technical change	Addition of a NF-Water tube in the kit. Addition of protocol from spleen.

II. General information

1. Purpose of the test

ADIAVET™ MAREK REAL TIME kit is intended to detect the Gallid Herpesvirus 2, the Marek Virus (the Marek desease virus) using real-time Polymerase Chain Reaction (PCR) technology from breeding air collected in poultry farm and spleen of poultry.

2. Pathogen

Marek virus is a double strand DNA virus of the *Herpesviridae* family. Three serotypes of Marek virus exist. Only the serotype 1 is pathogenic for poultry. This serotype takes together strains with different levels of virulence among which the new emergent high virulent strain and the Rispens virus which is used as vaccine. The strains of the serotypes 2 and 3 are non pathogens.

The oncogenic viruses are responsible of lymphoma associated to nervous and visceral tumors. Clinic symtoms of the Marek disease can be neurological (paresis, paralysis, hanging wing…), weight loss, anemia, lymphoma (nerve or skin damage…), morbidity rate reaching about 10%.

Two forms of Marek virus can be detected. The cell-associated form is responsible to the tumoral processus but is non infectious. The cell-free form of the virus in sub-epidermic layer of desquamous feather follicules is the infectious form.
Infection is spread horizontally by the air-borne route to other chickens. The virus protected inside follicular cells shows a great resistance in the external environment.

The tumors develop slowly. Therefore, Marek disease mainly affects the animals with long economic life (labeled hen, laying hen, reproductive hen). Chicken is the sensitive specie but turkey can be also infected (in which the turkeys specially breed for Christmas showing long economic life).

The ubiquitous spread of Marek virus leads to vaccinate the animal with long economic life. Adapted hygienic processes allow limiting the early contamination that occurs before the vaccine can be efficient.
To assure an optimal efficiency of the vaccine, the Marek disease virus status of the poultry farms should be control before the entrance of the animals.

3. Description and purpose of the test

This test is based on enzymatic gene amplification or PCR technology.
Amplified products are detected in real-time thanks to a specific labelled hydrolysis probe (5'-exonulease technology).

The ADIAVET™ MAREK REAL TIME kit enables the simultaneous detection of:

- The Marek virus (probe labelled in FAM),
- an exogen control EPC-Ext added during the extraction that allows validating extraction and amplification steps (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

ADIAGENE validated the test using DNA purification kits (Qiagen and Macherey-Nagel). Other purification kits can be used if they have been validated by the user.
Analysis options according to the specimen:

Specimen	Individual analysis
Breeding air (tested by concentration in a liquid)	☑
Spleen	☑

III. Material and reagents

1. Reagents provided with the kit

REF ADI461-100

A5	amplification solution	2 x 1000 µl tubes with green cap (a ready-to-use reagent)
Marek CTL+	positive control Marek Virus	1 tube with purple cap (to reconstitute)
EPC Ext	External control of extraction	2 x 300 µl yellow tubes
NF-Water	Nuclease free Water	1 x 1000 µl tube with white cap (a ready-to-use reagent)

Package insert downloadable from www.biox.com

2. Validity and storage

On receipt, the kit should be stored at **<-15°C**.
It is recommended to make fractions of A5 solution if it should be defrosted more than 3 times.
Do not defreeze reagents more than 3 times.
Realtime reagents are susceptible to light: store them in the darkness.
The A5 reagent is ready to use for PCR reaction.
Do not mix reagents of two different batches.

3. Use of controls

A. Use of Marek CTL+

Add **200 µl** of **NF-Water**to the **Marek CTL+** tube included in the kit. Homogenize tube contents using a mixer such as vortex, at least 20 seconds. Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, we recommend to use **5 µl** of **Marek CTL+** in one of the wells.

B. Use of EPC-Ext

Aliquot this solution and store it to <-15°C.
For each extraction, use **5 µl EPC-Ext per sample**.
Each aliquot should not be defrosted more than 3 times

4. Equipment required but not supplied

Material should be Nuclease-free (e.g. autoclaved 25 minutes twice at +120°C or once 60 minutes at +121°C)

- Thermal cycler with consumables for real-time PCR: 0.2 ml PCR tubes or closed 96-wells PCR plates with optical quality.
- Class II Microbiological Safety Cabinet
- Centrifuge for microtubes, tubes of 10 or 15 ml, 96-wells plate
- Etuves, heating baths or block heaters
- Instrument for homogenous mixing of tubes
- 1 - 10 µl pipette, 20 - 200 µl pipette and 200 - 1000 µl pipette
- Nuclease-free filter tips
- Sterile Nuclease-free microtubes: 1.5 ml and 2 ml
- Powder-free Latex or Nitrile gloves
- 96-100% ethanol solution
- Sterile distilled water/ Sterile saline water (NaCl 8.5 g/l)
- PBS buffer
- **DNA extraction kit (individual columns)**
  - QIAamp® DNA Mini Kit (Qiagen, 50 tests: ref. 51304 or 250 tests: ref. 51306)
  - NucleoSpin® Tissue (Macherey-Nagel, 50 tests : ref.740952.50 ou 250 tests : ref. 740952.250)

IV. Recommandation before the analysis of samples

Before starting the test, read the entire protocol and scrupulously respect it.

1. Precautions

Adiagène has elaborated this PCR test with the use of Qiagen and Macherey Nagel extraction kits. Other extraction kits can be used with a previous validation.

Follow the supplier's instructions for the storage, the preparation and the use of the extraction reagents.

Some kits include and/or need the use of toxic reagents. These reagents should be used with gloves and into a chimical cabinet.

We strongly recommend that only appropriately trained personnel perform this test. The quality of the obtained results depends upon rigorous respect of good laboratory practices. Ensure the accuracy and precision of the micropipettes used.

The PCR generates large amount of amplified DNA. A few molecules of amplified products are sufficient to generate a positive result. It is important to reserve 2 rooms, one for manipulation of samples to be tested, and another one for amplified products analysis. Do not open the PCR tubes after amplification.
Samples for analysis should be handled and disposed of as biological waste. **Take all measures of security and confinement required for the manipulation of the concerned biological agents.**

We recommend using fractions of demineralised and saline water and to autoclave them 25 minutes at +120°C. Take a new fraction for each new manipulation to avoid contamination.

2. Storage of samples and DNA extracts

Samples can be stored a couple of days at +2/8°C. After 2 days, we recommend to store them at <-15°C.
Extracted DNAs are quite sensitive molecules. Crude extracts should be stored at the end of extraction on melting ice or at +2/8°C for 24h hours, then at <-15°C.

3. Controls to include

The use of controls allows to verify the reliability of the results.
The controls are included per trial of analysis. A trial is defined as all the samples treated in the same conditions.
The use of the controls follows the recommendation of the normative requirements and recommendations for the development and the validation of veterinary PCR (NF U47-600).

All the steps of the analysis procedure (extraction+amplification), for the type of sample, are validated with the association of the controls included in the kit.

- The exogen control EPC-Ext added during the extraction allows to verify the extraction and amplification steps of each sample.
- The Marek CTL+ allows to validate the amplification of the target.

Other controls must or could be added.

- **Negative control of extraction (obligatory)**

  To verify the absence of cross-contamination, at least one negative control must be included per trial. The control is a negative sample, for example a buffer used for dilution.

- **Positive control of extraction (recommended)**

  A positif control could be added in each trial. The control is a sample incuding Marek virus. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of Marek virus. This positive control will be closed to the limit of detection of the method. It will inform about the fidelity of the obtained results between different trials.

V. Extraction and purification

1. Using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

<table>
<tr><th></th><th>Coriolis collection liquid</th><th>Spleen</th></tr>
<tr><td>Preparation of the sample</td><td>Place 200 µl of sample in a microtube.</td><td>Place 20 mg of sample in a microtube</td></tr>
<tr><td rowspan="3">Lysis</td><td rowspan="2">Add 180 µl of AL buffer, 20 µl of proteinase K and 5 µl of EPC-Ext. Homogenize.</td><td>Add 180 µl of ATL buffer, 20 µl of proteinase K and 5 µl of EPC-Ext. Homogenize.
Incubate 30 minutes at +70°C</td></tr>
<tr><td>Add 200 µl of AL buffer. Homogenize.</td></tr>
<tr><td colspan="2">Incubate 10 minutes at +70°C.</td></tr>
<tr><td>Binding preparation</td><td colspan="2">Add 200 µl of ethanol 100%.
Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 secondes).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="2">Identify columns, apply the whole obtained solution to the corresponding column.
Centrifuge at 10 000 g/1 minute.
If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>1st wash</td><td colspan="2">Change the collection tube and add 500 µl of AW1 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="2">Change the collection tube and add 500 µl of AW2 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Column dry step</td><td colspan="2">Change the collection tube.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="2">Transfer the column to a microtube. Add 200 µl of AE buffer.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="2">Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.</td></tr>
</table>

2. Using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

<table>
<tr><th></th><th>Coriolis collection liquid</th><th>Spleen</th></tr>
<tr><td>Preparation of the sample</td><td>Place 200 µl of sample in a microtube.</td><td>Place 20 mg of sample in a microtube</td></tr>
<tr><td rowspan="3">Lysis</td><td rowspan="2">Add 180 µl of B3 buffer, 25 µl of proteinase K and 5 µl of EPC-Ext. Homogenize.</td><td>Add 180 µl of T1 buffer, 25 µl of proteinase K and 5 µl of EPC-Ext. Homogenize.
Incubate 30 minutes at +70°C.</td></tr>
<tr><td>Add 200 µl of B3 buffer. Homogenize.</td></tr>
<tr><td colspan="2">Incubate 10 minutes at +70°C.</td></tr>
<tr><td>Binding preparation</td><td colspan="2">Add 200 µl of ethanol 100%.
Homogenize the mixture by pipeting (~10 times) or by using a mixer such as vortex (~15 secondes).</td></tr>
<tr><td>Transfer to columns and binding to the membrane</td><td colspan="2">Identify columns, apply the whole obtained solution to the corresponding column.
Centrifuge at 10 000 g/1 minute.
If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>1st wash</td><td colspan="2">Change the collection tube and add 500 µl of BW buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>2nd wash</td><td colspan="2">Change the collection tube and add 600 µl of B5 buffer to the column.
Centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Column dry step</td><td colspan="2">Change the collection tube.
Centrifuge 3 minutes at 10 000 g.</td></tr>
<tr><td>Elution</td><td colspan="2">Transfer the column to a microtube. Add 200 µl of BE buffer.
Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.</td></tr>
<tr><td>Storage</td><td colspan="2">Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.</td></tr>
</table>

VI. Amplification

a- Determine the number of analysed samples including the control (e.g. positive and negative extraction control, positive control of amplification (positive control) and reagent PCR control (No Template Control or NTC)).

b- Defrost A5 solution at room temperature. Homogenize. Dispense **20 µl** of A5 solution in each PCR tubes or PCR plate wells with a micropipette with a Nuclease-free tip.

c- **Immediately replace the A5 solution tube at <-15°C and in darkness.**

d- For each sample, the negative control (obligatory) and the positive control (recommended) add **5 µl** of purified extract to the 20 µl of A5 solution.
For the positive control, add **5 µl** of the solution obtained in § II-3 to the 20 µl of A5 solution.
Immediately replace purified DNA extracts at +2/8°C or at <**-15°C**. Take care to have no bubbles in the bottom of the wells.

e- Once all the tubes have been prepared, run real-time PCR amplification.

The Marek virus target is read in FAM. The Internal Control is read in VIC or HEX. The Quencher is non fluorescent. The solution contains a passive reference ROX for the ABI machines. Fluorescence is read during the elongation step (1 minute at 60°C).

The following program is defined for **ABI Prism** thermalcyclers (like 7500, StepOne…) from **Applied Biosystems** (check the "emulation 9600" option if available), for the **Rotorgene** from **Qiagen** and for the **Chromo 4** from **Biorad**:
2 minutes 50°C
10 minutes 95°C
15 seconds at 95°C and 1 minute at 60°C during 45 cycles

This program is concerning the **MX3000P** and **MX3005P** of **Stratagene**:
2 minutes 50°C
10 minutes 95°C
30 seconds at 95°C and 1 minute at 60°C during 45 cycles

Roche diagnostic: LightCycler 2*, LightCycler 480*
**** NOTE:*** *The use of LightCycler thermalcyclers requires a calibration manipulation. Adiagene will furnish process chart and reagents required for this calibration.*

Contact us if you wish to use other thermalcyclers.

VII. Interpretation of results

1. Definitions

The **« base line »** corresponds to the background of fluorescence and qualifies the non-characteristic part of the curve observed during the first cycles.

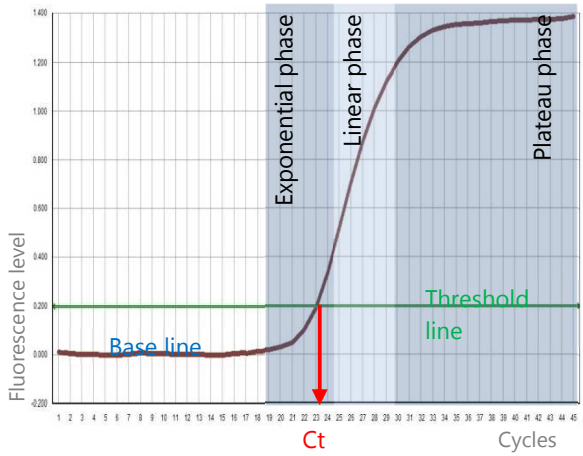
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

The **« threshold line »** has to be placed over the background in the exponential phase of a characteristic amplification curve or a group of characteristic amplification curves.

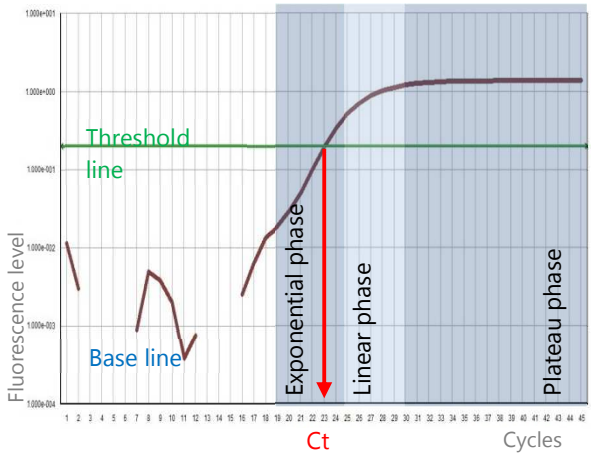
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present in the PCR well.

Example of characteristic amplification curve

Arithmetic scale of Y axis



Logarithmic scale of Y axis



2. Validation and interpretation of results

Display the FAM curves from the plate and set the threshold value as indicated above. Proceed in the same mean for the VIC or HEX curves.

A. Validation of the run

Amplification is considered to be **valid** if the following results are obtained for the controls:

Controls	Reagent control (NTC)	Marek CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	yes	no/yes
Validation of	Absence of contamination for amplification	Amplification of the target	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM and VIC/HEX dyes) of the Marek CTL+ are indicated in the certificate of analysis of the kit.

B. Result interpretation

DNA extraction and amplification for each sample are considered to be **valid** if at least a characteristic amplification curve is observed for Marek virus (FAM) or for the internal control (VIC or HEX).

Example	A	B	C	D
FAM amplification	no	yes	yes	no
VIC/HEX amplification	yes	no	yes	no
Result	negative	positive	positive	undetermined

The sample is considered as **negative** if a characteristic amplification curve is observed in VIC or HEX without any amplification in FAM (A example).

The sample is considered as **positive** if a characteristic amplification curve is observed in FAM (B example). Internal control can be co-amplified (C example).

A total absence of characteristic amplification curve for a sample (example D) shows a defective DNA extraction (lost or destruction of DNA) or a deficient real-time PCR (inhibitors in the sample, program error or no template added). In this case, we recommend first to repeat the test with pure and tenfold diluted DNA in sterile nuclease-free water. Then, if the test is still not valid, a new extraction is recommended.

VIII. Index of symbols

Symbol	Meaning
	Catalogue number
	Manufacturer
	Upper temperature limit
	Use by date
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Keep away from sunlight
	For veterinary in vitro use only – For animal use only

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