INSTRUCTION MANUAL

ADIAVET™ ORT REAL TIME

TEST FOR THE DETECTION OF *ORNITHOBACTERIUM RHINOTRACHEALE* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
416443 (100 reactions)

VET

NOTE
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English version
NE262-05
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ADIAVET™ ORT REAL TIME

I. Revision history

N/A	Not Applicable (first publication)
Correction	Correction of document anomalies
Technical change	Addition, revision and/or removal of information related to the product
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
2014/02	NE262-03	Correction	Removal of signal word in page 5, §III.1.
2014/12	NE262-04	Technical change	Removal of reference 416442 (50 reactions)
2016/07	NE262-05	Administrative	Changing logos
2016/07	NE262-05	Administrative	Biosearch legal mention
2016/07	NE262-05	Administrative	Addition of table "Analysis options according to the specimen" §I.3.

II. General information

1. Purpose of the test

ADIAVET™ ORT REAL TIME kit is intended to detect *Ornithobacterium rhinotracheale* using real-time Polymerase Chain Reaction (PCR) technology from swab of chicken and turkey, as well as from environmental specimen.

2. Pathogen

Ornithobacterium rhinotracheale is a gram negative bacillus, unique registered specie of *Ornithobacterium* genus. It causes respiratory troubles by several bird species, especially by chickens and turkeys. Clinical signs are nasal discharge, sneezing, coughing and sinusitis.
Clinical diagnosis is of poor interest because it is not specific. Serological diagnosis is made using ELISA tests. However, these tests don't allow diagnosing an acute infection because antibodies appear late. Differential diagnosis (especially with *Riemerella anatipestifer*, *Haemophilus paragallinarum*, *Pasteurella multocida* and *Coenonia anatina*) is currently achieved on bacteriological criteria.

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™ ORT REAL TIME kit enables the simultaneous detection of:

- ORT (probe labelled in FAM),
- GAPDH, an internal control of extraction and amplification steps specific from an endogenous DNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

Adiagène recommends using this test with DNA purification kits (Adiagène, 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	Pool of sample is possible*, up to
Swab on live animals (palate slit, tracheal…)	☑	6
Swab on dead animals (joint, injured organ…)	☑	6
Environmental specimen	☑	☒

***** It depends on the epidemiological case and on the quality of the specimen.

III. Material and reagents

1. Reagents provided with the kit

Designation	Reagents	416443 (100R)
A5	Amplification solution	2 x 1000 µl green tubes
ORT CTL+	Positive control *Ornithobacterium rhinotracheale*	1 purple tube

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 defrost 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 ORT CTL+

Add **200 µl** of Nuclease-free water to the ORT 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 ORT CTL+ in a well.

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
- 2 heating baths or block heaters for microtubes
- Instrument for homogenous mixing of tubes
- 1-10 µl pipette, 20-200 µl pipette and 200-1000 µl pipette
- Nuclease-free filter tips
- Nuclease-free microtubes: 1.5 ml and 2 ml
- Powder-free nitrile or latex gloves
- 96-100% ethanol solution
- Sterile swabs
- Buffered peptone water
- Sterile distilled water
- Sterile saline water (NaCl 8.5 g/l)
- PBS 1X 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 or 250 tests: ref. 740952.250)

or

- DNA extraction kit (plate columns)

- ADIAPURE™ PURIFICATION (Bio-X Diagnotics, 192 tests: ref. ADIADP001-192 or 480 tests: ref. ADIADP001-480)

IV. Recommendation 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 Adiagène, Qiagen and Machery-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 handled with gloves and into a chemical cabinet.

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

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. Samples recommendations

Use swabs moistened in 2 % peptone water or swabs in charcoal. Dry drag swabs can be used for environmental samples.

A. On live animals

Take a swab and sample the trachea, or the palate slit of young birds. Sample only one swab per animal. Replace the swab in its case and send it at +2/8°C to the laboratory as soon as possible.

B. On dead animals

Sample the necrotic organs (trachea, lungs, articulations, air sacs...). Replace the swab in its case and send it at +2/8°C to the laboratory as soon as possible.

C. In buildings

Sample the surfaces to be tested with a dry grag swab. Put the drag swab in a hermetically sealed bag and send it to the laboratory as soon as possible.

4. Controls to include

The use of controls allows verifying 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 all the types of samples, are validated with the association of the controls included in the kit.

- The internal endogenous control (GAPDH) naturally found in the samples allows verifying the extraction and amplification steps of each sample.
- The ORT CTL+ allows validating the amplification of the target.

Other controls must or could be added:

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

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 dilutions.

- **Control of extraction (recommended)**

A positive control could be added in each trial. The control is a sample including ORT. It could come from a positive sample available in the laboratory or from a negative sample spiked with a bacterial solution of ORT. 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 ADIAPURE™ PURIFICATION kit

See the NE001 user manual available of the web site mentioned on the certificate of analysis included in the used ADIAPURE™ kit.

2. Using QIAamp® DNA Mini kit

Before starting the test, heat water baths or incubators, one to +56°C and the other one to +70°C.
*For each sample analysed, put **180 µl** of buffer **ATL** and **20 µl** of **proteinase K** (QIAamp® DNA mini kit) in a sterile microtube. Clearly identify each microtube.*

A. Sample preparation

a) From swabs

Put the swab in the microtube containing buffer **ATL** (**180 µl**) and **proteinase K** solution (**20 µl**) and gently homogenize 5 seconds. Put one swab in one microtube or homogenize successively three swabs in the same microtube to make a pool. If the swabs haven't been dipped in 2% buffered peptone water, they may absorb all the solution. In this case, the amount of buffer **ATL** (**360 µl**) and **proteinase K** (**40 µl**) solutions should be increased. Press the swab against the rim of the microtube to collect as much buffer as possible.

Remark: In case of very soiled swabs, prefer to sample individual.

b) For solid culture

Scrape the medium with a swab (dipped in 2% buffered peptone water), place the swab in the microtube containing **ATL** (**180 µl**) and **proteinase K** (**20 µl**) and homogenize 5 seconds. Press the swab against the rim of the microtube to collect as much buffer as possible.

c) For broth culture

Centrifuge **100** to **500 µl** of **culture** in a microtube (10 000 g for 20 minutes). Discard the supernatant and add **180 µl** of buffer **ATL** and **20 µl** of **proteinase K** solution to the pellet. Homogenize.

d) From drag swabs

Add **30 ml** of **peptone water** in the bag containing the drag swab. Homogenize by mixing the bag. If the drag swab has absorbed all the liquid, add 10 ml more peptone water and mix the bag again.
Take **200 µl** of the **obtained liquid** with a 2ml-pipette and put it in a sterile 1.5 ml-microtube. Add **180 µl** of buffer **ATL** and **20 µl** of **proteinase K**.

B. DNA purification

All the centrifugations are performed at room temperature.

Before the beginning of extraction, identify the microtubes and columns required for the DNA purification.

Lysis	Incubate **15 minutes** at **+56°C**. Mix now and then during incubation. Briefly centrifuge microtubes to remove drops inside the lid. Add **200 µl** of **buffer AL**. Mix. Incubate **10 minutes** at **+70°C**. Briefly centrifuge microtubes to remove drops inside the lid.
Binding preparation	Add **210 µl** of **ethanol 100%**. Mix. Briefly centrifuge microtubes to remove drops inside the lid.
Transfer to columns and binding to the membrane	Apply the **whole** obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g.
1st wash	Change the collection tube and add **500 µl** of **buffer AW1** to the column. Centrifuge 1 minute at 10 000 g.
2nd wash	Change the collection tube and add **500 µl** of **buffer AW2** to the column. Centrifuge 1 minute at 10 000 g.
Column dry	Change the collection tube. Centrifuge 3 minutes at 10 000 g.
Elution	Transfer the column to a microtube. Add **100 µl** of **buffer AE**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.
Storage	Close the tubes and store them at +2/8°C if using immediately, or at <-15°C.

3. Using NucleoSpin® Tissue kit

Before starting the test, heat water baths or incubators, one to +56°C and the other one to +70°C.
*For each sample analysed, put **180 µl** of buffer **T1** and **25 µl** of **proteinase K** (NucleoSpin® Tissue kit) in a sterile microtube. Clearly identify each microtube.*

A. Sample preparation

a) From swabs

Put the swab in the microtube containing buffer **T1** (**180 µl**) and **proteinase K** solution (**25 µl**) and gently homogenize 5 seconds. Put one swab in one microtube or homogenize successively three swabs in the same microtube to make a pool. If the swabs haven't been dipped in 2% buffered peptone water, they may absorb all the solution. In this case, the amount of buffer **T1** (**360 µl**) and **proteinase K** (**50 µl**) solutions should be increased. Press the swab against the rim of the microtube to collect as much buffer as possible.

Remark: In case of very soiled swabs, prefer to sample individual.

b) For solid culture

Scrape the medium with a swab (dipped in 2% buffered peptone water), place the swab in the microtube containing **T1** (**180 µl**) and **proteinase K** (**25 µl**) and homogenize 5 seconds. Press the swab against the rim of the microtube to collect as much buffer as possible.

c) For broth culture

Centrifuge **100** to **500 µl** of culture in a microtube (10 000 g for 20 minutes). Discard the supernatant and add **180 µl** of buffer **T1** and **25 µl** of **proteinase K** solution to the pellet. Homogenize.

d) From drag swabs

Add **30 ml** of **peptone water** in the bag containing the drag swab. Homogenize by mixing the bag. If the drag swab has absorbed all the liquid, add 10 ml more peptone water and mix the bag again.
Take **200 µl** of the **obtained liquid** with a 2ml-pipette and put it in a sterile 1.5 ml-microtube. Add **180 µl** of buffer **T1** and **25 µl** of **proteinase K**.

B. DNA purification

All the centrifugations are performed at room temperature.

Before the beginning of extraction, identify the microtubes and columns required for the DNA purification.

Lysis	Incubate **15 minutes** at **+56°C**. Mix now and then during incubation. Briefly centrifuge microtubes to remove drops inside the lid. Add **200 µl** of **buffer B3**. Mix. Incubate **10 minutes** at **+70°C**. Briefly centrifuge microtubes to remove drops inside the lid.
Binding preparation	Add **210 µl** of **ethanol 100%**. Mix. Briefly centrifuge microtubes to remove drops inside the lid.
Transfer to columns and binding to the membrane	Apply the **whole** obtained solution to the corresponding column and centrifuge 1 minute at 10 000 g.
1st wash	Change the collection tube and add **500 µl** of **buffer BW** to the column. Centrifuge 1 minute at 10 000 g.
2nd wash	Change the collection tube and add **500 µl** of **buffer B5** to the column. Centrifuge 1 minute at 10 000 g.
Column dry	Change the collection tube. Centrifuge 3 minutes at 10 000 g.
Elution	Transfer the column to a microtube. Add **100 µl** of **buffer BE**. Incubate ~1 minute at room temperature and centrifuge 1 minute at 10 000 g.
Storage	Close the tubes and store them at +2/8°C if using immediately, or at <-15°C.

VI. Amplification

a - Determine the number of analysed samples including the controls (e.g. positive and negative extraction controls, positive control of amplification (ORT CTL+) and No Template Control (NTC)).

b - Defrost the A5 solution at room temperature. Homogenize. Dispense **20 µl** of the 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 extraction negative control (obligatory) and the extraction 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.
For the No Template Control (NTC), nothing is added to the 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 the real-time PCR amplification.

The ORT 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

The thermal program of the kits ADI013 can also be used.

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.

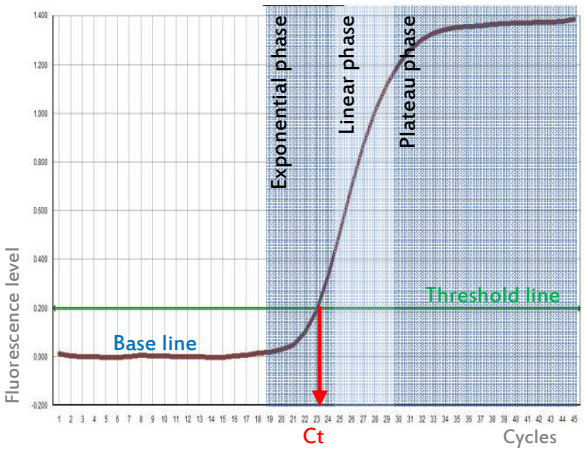
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

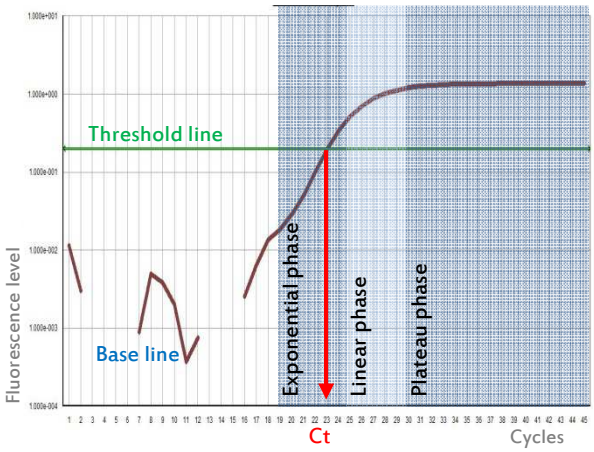
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present 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	No Template Control (NTC)	ORT CTL+	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	no	no/yes	no	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 ORT 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 ORT (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 (example A).

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

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 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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