ADIAVET™ CEMO KLEB/PSEUDO REAL TIME

TEST FOR THE DETECTION OF *KLEBSIELLA PNEUMONIAE* AND *PSEUDOMONAS AERUGINOSA* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

References:
ADI511-100 (100 reactions)
ADI511-500 (500 reactions)

Kits developed and validated in collaboration with

LABÉO
Pôle d'analyses et de recherche de Normandie

VET

English version
NE511-04
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ADIAVET™ CEMO KLEB/PSEUDO REAL TIME

Revision historic

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 historic

Release Date	Part Number	Change type	Change summaru
2016/05	NE511-01	N/A	First publication
2017/03	NE511-02	Technical change	Change PBS by PBS 1X ; § IV-1-a and II-4
2017/10	NE511-03	Technical change	Addition of a reference 500 reactions kit (ADI511-500) Addition of magnetic beads extractin protocol Validation and interpretation of results §VI.2-B
2017/12	NE511-04	Technical change	Modification of §VI.2-A Validation of run

I. General informations

1. Purpose of the test

ADIAVET™ CEMO KLEB/PSEUDO REAL TIME kit is intended to detect *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* using real-time Polymerase Chain Reaction (PCR) technology from swab and semen specimen of equine and bacterial culture.

2. *K. pneumoniae* and *P. aeruginosa*

Contagious equine metritis (CEM) is an inflammatory disease of the proximal and distal reproductive tract of the mare mainly caused by *Taylorella equigenitalis*. Others pathogens encountered in metritis, *Taylorella asinigenitalis*, *K. pneumoniae* and *P. aeruginosa* are found (OIE 2012).

K. pneumoniae and *P. aeruginosa* are Gram-negative bacillus classified in the *Enterobacteriaceae* family.
K. pneumoniae is non-motile and facultative anaerobic. *P. aeruginosa* is motile and aerobic.
They are ubiquitous bacteria found in human and in various animal species as well as in aquatic habitats and in the soil.

CEM diagnostic is standardized (e.g. OIE Terrestrial manual 2012 Chapter 2.5.2 and U47-108). Whereas *K. pneumoniae* and *P. aeruginosa* are not OIE notified bacteria, the standard sampling process is often apply for their detection. Briefly, swabbing of genital tractus of mare and stallion is performed. Swab is sending to laboratory for bacterial diagnostic. Bacterial isolation is performed onto selective agar media. Serology method can be alternative way but interpretation is often delicate, becoming increasingly obsolete outside epidemiological context.

A variety of discriminatory real time PCR assays for rapid, sensitive and specific detection for *K. pneumoniae* and *P. aeruginosa* directly from genital swabs without a need for additional bacterial isolation have been developed. PCR addresses most of the associated short-comings of bacteriology with fewer false negatives due to enhanced sensitivity and discriminatory specificity in detecting bacterial presence despite factors including lower bacterial concentrations and heavily contaminated, damaged or delayed samples.
This PCR can be realized at the same time with the ADIAVET™ CEMO TAYLORELLA REAL TIME PCR kit for the detection of *T. equigenitalis* and *T. asinigenitalis*.

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™ CEMO KLEB/PSEUDO REAL TIME kit enables the simultaneous detection of:

- *K. pneumoniae* (FAM labeled probe)
- *P. aeruginosa* (Cy5 labeled probe)
- an internal control of amplification step specific from an exogenous DNA (probe labelled with a fluorochrome read in the same spectra as VIC and HEX).

Adiagene recommends using this test with DNA purification kits (Adiagene, 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
Swab	☑
Semen	☑
Bacterial culture	☑

II. Material & reagents

1. Reagents provided with the kit

REF **ADI511-100**	Pack of 100 tests
REF **ADI511-500**	Pack of 500 tests

REF ADI511-100

A5 .....amplification solution	2 x 1000 µl tubes with green cap (a ready-to-use reagent)
KLE/PSE CTL+ ... positive control *K. pneumoniae* and *P. aeruginosa*	1 tube with purple cap (to reconstitute)

Package insert downloadable from www.biox.com

REF ADI511-500

A5 .....amplification solution	10 x 1000 µl tubes with green cap (a ready-to-use reagent)
KLE/PSE CTL+ ... positive control *K. pneumoniae* and *P. aeruginosa*	1 tube with purple cap (to reconstitute)

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 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 KLE/PSE CTL+

KLE/PSE CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free water to the KLE/PSE CTL+ tube and vortex at least 20 seconds.
Aliquot this solution by 6 or 12 µl and store them to <-15°C.
For each analysis, use 5 µl of KLE/PSE 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, 96-wells plate
- Etuve, heating baths or block heaters
- Vortex
- 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
- Sterile tube of 5 ml
- 96-wells plate (capability > 200 µl)
- Latex or nitrile powder-free gloves
- 96-100% ethanol solution
- Nuclease-free water
- PBS 1X buffer pH 7.4 (recommended composition, NaCl 150 mM, Na_2HPO_4 5 mM, KH_2PO_4 1.7mM, without Ca^{2+}, without K^+ - another composition could be used after a validation made by the user)

- DNA extraction kit (individual silica 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)

-Automated DNA/RNA extraction method using magnetic bead

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

III. Recommendations before the analysis of samples

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

1. Precautions

Adiagene has elaborated this PCR test with the use of Adiagene, 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 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 24 hours, then at <-15°C.

3. Samples preparation

See § IV for the extraction and purification of DNA.

4. Controls preparation

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).

The amplification step, for all the types of samples, are validated with the association of the controls included in the kit.

- The internal control included in A5 reagent verifies the amplification steps of each sample.
- The KLE/PSE CTL+ valides 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.

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

A positive control could be added in each trial. The control is a sample including *K. pneumoniae* and *P. aeruginosa*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *K. pneumoniae* and *P. aeruginosa*. 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.

IV. Extraction and Purification

1. Extraction without purification from swab

A. Place **500 µl** of PBS 1X buffer in a tube of 5 ml.
B. Add swab.
C. Mix.
D. Replace swab in its transport tube.

E. Transfer **100 µl** of the obtained suspension in well-plate or microtube.
F. Close (e.g. with adhesive film for plate).
G. Incubate **10 minutes at 95°C** (e.g. in block heater or thermal cycler)

H. To ensure the accuracy of subsequent pipetting, allow the samples to cool (e.g., 15-30 minutes at room temperature or 5-20 minutes at 2-8°C, according to the number of samples per assay)

Note 1: In case of a new analysis, each individual supernatant (nucleic acid extract) can be store at 2-8°C for 24 hours, then store them at a temperature below -15°C.

2. Extraction from bacterial culture

Put 100 µl of Nuclease-free water in a microtube.
Transfer 1 or several colonies in the microtube.
NB: transfer too much sample could be inhibitor for the PCR
Incubate **10 minutes at 95°C**.
Let the solution cooling.
Store at +2/8°C several hours then at <-15°C several months.

3. Extraction from RNA/DNA magnetic beads

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

4. Extraction using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

	Swab	Semen
Preparation of the sample	Put 1 swab in a tube. Add **500 µL** of 1X PBS buffer. Mix. Transfer **200 µL** of the supernatant to a microtube.	Put **200 µL** in a microtube.
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Vortex. Incubate **30 minutes** at **+70°C** (or a night at +56°C).	
	Add **200 µl** of **AL buffer**. Vortex. Incubate **10 minutes** at **+70°C**.	
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortex (~15 secondes).	
Transfer to columns and binding to the membrane	Identify columns, apply the **whole** obtained solution to the corresponding column. Centrifuge 1 minute at 10 000 g. *If the whole sample has not been loaded once, apply the residual volume onto the column and centrifuge 1 minute at 10 000 g.*	
1st wash	Change the collection tube and add **500 µl** of **AW1 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **500 µl** of **AW2 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	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.	
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.	

5. Extraction using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	Swab	Semen
Preparation of the sample	Put 1 swab in a tube. Add **500 µL** of 1X PBS buffer. Mix. Transfer **200 µL** of the supernatant to a microtube.	Put **200 µL** in a microtube.
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Vortex. Incubate **30 minutes** at **+70°C** (or a night at +56°C).	
	Add **200 µl** of **B3 buffer**. Vortex. Incubate **10 minutes** at **+70°C**.	
Binding preparation	Add **200 µl** of **ethanol 100%**. Homogenise the mixture by pipeting (~10 times) or by vortexing (~15 secondes).	
Transfer to columns and binding to the membrane	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.*	
1st wash	Change the collection tube and add **500 µl** of **BW buffer** to the column. Centrifuge 1 minute at 10 000 g.	
2nd wash	Change the collection tube and add **600 µl** of **B5 buffer** to the column. Centrifuge 1 minute at 10 000 g.	
Column dry step	Change the collection tube. Centrifuge 3 minutes at 10 000 g.	
Elution	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.	
Storage	Close the tubes, identify and store at +2/8°C for 24 hours, then at <-15°C.	

V. Amplification

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

b - Defrost the A5 solution at room temperature. Vortex. 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 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 CTL+, 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 *K. pneumoniae* target is read in FAM. The *P. aeruginosa* target is read in Cy5. 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.

VI. 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.

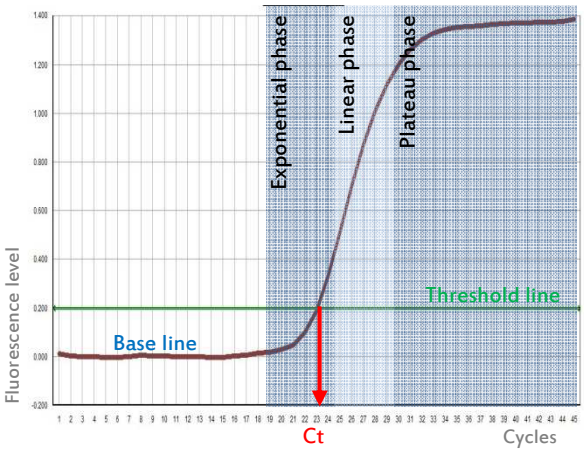
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

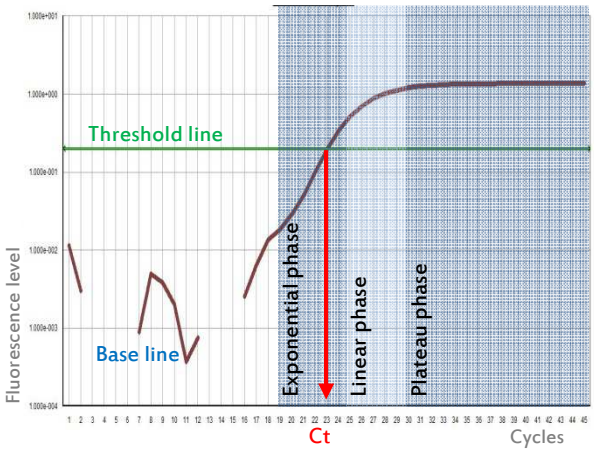
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present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

A. Validation of the run

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

Controls	No Template Control (NTC)	Amplification positive control (CTL+)	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	yes
VIC/HEX amplification	yes	no/yes	yes	no/yes
Cy5 amplification	no	yes	no	yes
Validation of	Absence of contamination for amplification	Amplification of the targets	Absence of contamination for extraction	Extraction and amplification steps

* Optional

The indicative Ct values (FAM, Cy5 and VIC/HEX dyes) of the positive control (CTL+) are indicated in the certificate of analysis of the kit.

B. Result interpretation

Nucleic acids extraction and amplification are valid if at least one characteristic amplification curve is observed per sample in FAM, in Cy5 **and/or** in HEX or its equivalent.

A positive sample displays a characteristic amplification curve and a Ct value in FAM (*K. pneumoniae*) and/or Cy5 (*P. aeruginosa*).

Note: Some *Klebsiella variicola* strains can be detected in FAM

Sample status

			Status	
Ct Value in HEX or tis equivalent (Internal control)	**Ct value in FAM *(K. pneumoniae)***	**Ct value in Cy5 *(P. aeruginosa)***	***K. pneumoniae***	***P. aeruginosa***
>36	**≤45**	**≤45**	Inhibited or potentially inhibited	Inhibited or potentially inhibited
≤36	**≤36**	**≤36**	Detected	Detected
		>40		No detected
		36 < Ct ≤ 40		Detected, no reproducible
	>40	**≤36**	No detected	Detected
		>40		No detected
		36 < Ct ≤ 40		Detected, no reproducible
	36 < Ct ≤ 40	**≤36**	Detected, no reproducible	Detected
		>40		No detected
		36 < Ct ≤ 40		Detected, no reproducible

"Inhibited or potentially inhibited":

- A competition can also occur if the sample is very heavily loaded with the *K. pneumoniae* and / or *P. aeruginosa* targets (Note: if it's a competition, I'is not necessary to repeat the analysis of the sample, the sample is considered positive).

- In the case of an absence of characteristic amplification curve and/or Ct value of internal control greater than 36. Possible causes are either a defective PCR (presence of inhibitors, set up error (refer to the "validation of the run" section), absence of sample or degraded sample) or a deficiency in the extraction of nucleic acids (loss or destruction of nucleic acids).

Recommendations:

1- Proceed to a new PCR assay from native nucleic acids extracts, pure and tenfold diluted in Nuclease-free water.
2- If assay is inconclusive, extract nucleic acids again or ask again for another sampling.

"+, no reproducible": sample over limit of detection, a new PCR assay might or might not confirm the positive status.

VII. Literature references

Norme AFNOR NF U47-600-2 (février 2015), Méthodes d'analyse en santé animale – PCR (réaction de polymérisation en chaîne) - Partie 2 : exigences et recommandations pour le développement et la validation de la PCR en santé animale.

OIE Terrestrial Manual – 2012 – Contagious equine metritis – Chapter 2.5.2

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

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