ADIAVET™ BRACHY REAL TIME

TEST FOR THE DETECTION OF *BRACHYSPIRA HYODYSENTERIAE* AND *BRACHYSPIRA PILOSICOLI* BY REAL-TIME ENZYMATIC GENE AMPLIFICATION (PCR TEST)

Reference:
418021 (100 reactions)

VET

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English version
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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
2014/09	NE063-01	N/A	First publication
2016/06	NE063-02	Administrative	Changing logos
2016/06	NE063-02	Administrative	Biosearch legal mention
2016/06	NE063-02	Administrative	Addition of table "Analysis options according to the specimen" §I.3
2016/06	NE063-02	Correction	Modification of protocole §IV.2. kit NucleoSpin Tissue

I. General informations

1. Purpose of the test

ADIAVET™ BRACHY REAL TIME kit is intended to detect *Brachyspira hyodysenteriae* and *Brachyspira pilosicoli* using real-time Polymerase Chain Reaction (PCR) technology from swab, tissue and faeces specimens of pig.

2. *Brachyspira*

Bacteria of *Brachyspira* genus are involved in different digestive diseases of swine. *Brachyspira hyodysenteriae* (serpulina) is the etiological agent of swine dysentery, a mucohemorrhagic diarrheal disease in which lesions are confined to the large intestine of pigs (Harris and Glock, 1986). *B. hyodysenteriae* has a strong beta-hemolytic activity and is enteropathogenic for swine. *B. pilosicoli* causes a relatively mild intestinal infection, which has been named porcine intestinal spirochaetosis. *B. intermedia* has also been described as probable causes of mild colitis. *B. innocens* is described as non pathogenic for pigs (Stanton *et al.*, 1997). Strong complete hemolysis is well-established characteristic differentiating the highly pathogenic *B. hyodysenteriae* from others *Brachyspira* groups. Weak - haemolytic strains (*B. pilosicoli, innocens* and *intermedia*) are more difficult to identify. *B. pilosicoli* strains can be differentiated from others by their hippurate activity (Fellström *et al.*, 1997). Nevertheless, *Brachyspira* species are difficult to cultivate and their identifications are time consuming. The PCR technique allows to detect all *Brashyspira* species and to differentiate *B. hyodysenteriae* and *B. pilosicoli.*

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™ BRACHY REAL TIME kit enables the simultaneous detection of:

- *Brachyspira hyodysentariae* (probe labelled in FAM),
- *Brachyspira pilosicoli* (probe labelled in Cy5),
- 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 (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 (rectal…)	☑	3
Tissue (intestinal mucosa…)	☑	☒
Faeces	☑	3

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

II. Material & reagents

1. Reagents provided with the kit

Designation	Reagents	418021 (100R)
A5	Amplification solution	2 x 1000 µl green tubes
B.hyo CTL+	Positive control *Brachyspira hyodysenteriae*	1 purple tube
B.pilo CTL+	Positive control *Brachyspira pilosicoli*	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 B.hyo CTL+

B.hyo CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free water to the B.hyo 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 B.hyo CTL+ in a well.

4. Use of B.pilo CTL+

B.pilo CTL+ is a positive control of amplification.
Add **200 µl** of Nuclease-free water to the B.pilo CTL+ tube and mix by vortexing 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 B.pilo CTL+ in a well.

5. 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
- 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, 10 or 15 ml
- Latex or nitrile powder-free gloves
- Scalpel blades
- 96-100% ethanol solution
- Nuclease-free water
- Sterile saline water (NaCl 8.5 g/l)

- 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)

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 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 amplification step, for all the types of samples, is 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 CTL+ validates the amplification of the both 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 *B. hyodysentariae* and/or *B. pilosicoli*. It could come from a positive sample available in the laboratory or from a negative sample spiked with a solution of *B. hyodysentariae* and/or *B. pilosicoli*. 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 using QIAamp® DNA Mini kit

All the centrifugations are performed at room temperature.

	Intestinal mucosa (ileon)	Feaces	Rectal swabs
Preparation of the sample	Put **5mm²** or **0.1g** of **tissue** in a microtube.	*Pool analysis is possible, up to 3.* **n x 1g** of **feaces** **+** **n x 5 ml** of **saline water** Vortex **~30 secondes** Take ***50 µl*** *of the* ***mix.***	Put **1 swab** in a microtube (prefer 2 ml-microtube). *Pool analysis is possible, up to 3.* *Succefully vortex each* ***swab*** *in* ***500 µl*** of **saline water**. Take ***50 µl*** *of the* ***mix.***
Lysis	Add **180 µl** of **ATL buffer**, **20 µl** of **proteinase K**. Vortex. Incubate **1** to **3 hours** at **+55°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.		

2. Extraction using NucleoSpin® Tissue kit

All the centrifugations are performed at room temperature.

	Intestinal mucosa (ileon)	**Feaces**	**Rectal swabs**
Preparation of the sample	Put **5mm²** or **0.1g** of **tissue** in a microtube.	*Pool analysis is possible, up to 3.* **n x 1g** of **feaces** **+** **n x 5 ml** of **saline water** Vortex **~30 secondes** Take ***50 µl*** *of the* ***mix.***	Put **1 swab** in a microtube (prefer 2 ml-microtube). *Pool analysis is possible, up to 3.* *Succefully vortex each* ***swab*** *in* ***500 µl*** of **saline water**. Take ***50 µl*** *of the* ***mix.***
Lysis	Add **180 µl** of **T1 buffer**, **25 µl** of **proteinase K**. Vortex. Incubate **1** to **3 hours** at **+55°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.		

3. Extraction from bacterial culture

Put 500 µl of physiological water in a microtube.
Transfer 1 or several colonies in the microtube.
NB: transfer too much sample could be inhibitor for the PCR
Incubate 15 minutes at 100°C.
Let the solution cooling.
Store at +2/8°C several hours then at <-15°C several months.

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 or § II-4 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 *Brachyspira hyodysentariae* target is read in FAM. The *Brachyspira pilosicoli* 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.

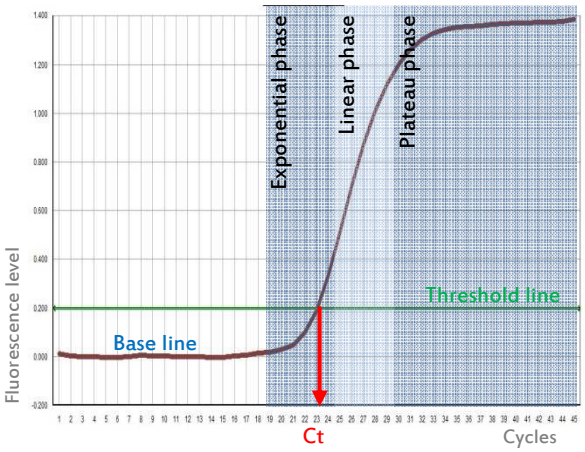
The **« Characteristic amplification curve »** qualifies a fluorescence curve with an exponential phase, a linear phase and a plateau phase.

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.

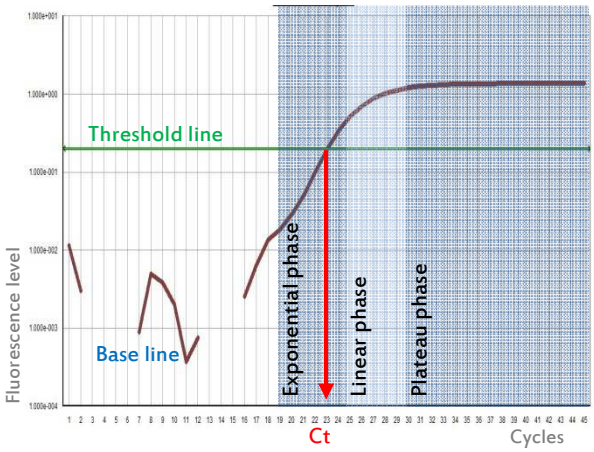
The **« threshold cycle » (Ct)** of a well corresponds, for each detected fluorophore, at the crossing point of the threshold line with the fluorescent curve. The Ct value expressed by the machine for each well depends on the threshold position and on the quantity of target sequences initially present 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 and Cy5 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 (B.hyo CTL+)	Amplification positive control (B.pilo CTL+)	Extraction negative control	Extraction positive control *
FAM amplification	no	yes	no	no	yes
Cy5 amplification	no	no	yes	no	yes
VIC/HEX amplification	yes	yes	yes	yes	yes
Validation of	Absence of contamination for amplification	Amplification of the target *B. hyodysentariae*	Amplification of the target *B. pilosicoli*	Absence of contamination for extraction	Extraction and amplification steps

* Optional

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

B. Result interpretation

DNA extraction and amplification for each sample are **valid** if at least a characteristic amplification curve is observed for *B. hyodysentariae* (FAM), for *B. pilosicoli* (Cy5) or for the internal control (VIC/HEX).

Example	A	B	C	D	E
FAM amplification	No	Yes	No	Yes	No
Cy5 amplification	No	No	Yes	Yes	No
VIC/HEX amplification	Yes	Yes/No	Yes/No	Yes/No	No
Results	Negative	Positive for *B. hyodysentariae*	Positive for *B. pilosicoli*	Positive for *B. hyodysentariae* and *B. pilosicoli*	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 (examples B and D) and/or in Cy5 (examples C and D). Internal control can be co-amplified.

A total absence of characteristic amplification curve for a sample (example E) 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.

VII. Literature references

Harris, D. J and Glock R. D (1986). Swine dysentery and spirocheatal disease, A.D. Leeman, B. Straw, R.D. Glock, W.L Mengeling, R.H.C. Penny and E.scholl (Eds), diseases of swine-6 th ed.Iowa State University Press. Ames.

Stanton, T. B. Fournié-Amazouz, E. Postic, D. Trott, D. J. Grimont, P. Baranton, G. Hampson, D.J and I. Saint-Girons. (1997). Recognition of two species of intestinal spirochaetes: Serpulina intermedia sp.nov. and Serpulina murdochii sp. nov. Int. J. Syst. Bacteriol. 47, 1007-1012.

Fellström C, Pettersson B, Thomson J, Gunnarsson A, Persson M & Johansson K-A (1997). Identification of Serpulina species associated with porcine colitis by biochemical analysis and PCR. Journal of Clinical Microbiology. 1997, 35, 462-467

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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