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Manual

rapidSTRIPE Babesia Assay

Order No.:

845-IS-1009010	10 reactions
845-IS-1009025	25 reactions
845-IS-1009050	50 reactions

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Manufacturer:
AJ Innuscreen GmbH
Robert-Rössle-Straße 10
13125 Berlin
Made in Germany!

Distribution/Publisher:
Analytik Jena AG
Konrad-Zuse-Straße 1
07745 Jena/Germany

Phone +49 (0) 36 41 / 77-94 00
Fax +49 (0) 36 41 / 77-76 77 76
www.bio.analytik-jena.com
lifescience@analytik-jena.com

Content

1 Introduction

The arising warm climate in middle Europe will lead to a dramatic increasing of ticks. Referring to this fact, also the risk of a tick bite will be increased. Ticks are carrier of infectious pathogens and induce different diseases (zoonoses).

Babesia are parasitizing protozoa in the erythrocytes of vertebrates, which are transferred by different types of Ixodidae in dependence on the geographical region. Babesia are pathogens, which cause Babesiosis in human and animals. Host animals are mice, bovine, horses and dogs. In exceptional cases also humans can be used as host.

B. divergens and *B. microti*, are spread in e.g. Europe and North America and cause an influenza-like clinical pattern with fever, shivering, arthralgia, thrombopenia, haemolytic anemia and hemoglobinuria. Lethal processes are particularly described after splenectomy.

Ticks, which are biting infected animals, are assimilating again *Babesia* in combination with the erythrocytes. Inside the intestine sack of the ticks, the *Babesia* are set free from the erythrocytes. They proliferate inside the intestine of the ticks. In the salivary the *Babesia* devide itself into thousand of sporozoites, which are transferred to a new host during a blood meal of the tick.

2 Test description and principle

The rapidSTRIPE Babesia Assay is a molecular diagnostic test system to determine *Babesia* genus in ticks.

The rapidSTRIPE Babesia Assay detects the nucleic acids of the pathogens directly inside the sample material (ticks). The test contains 3 modules, which functions are optimized to each other.

2.1 Module nucleic acid purification

Module nucleic acid purification is used for the isolation of nucleic acids from the sample material (tick tissue). Thereby it is possible to extract only DNA (blackPREP Tick DNA Kit) or to process a simultaneously isolation of DNA and RNA from the tick tissue (blackPREP Tick DNA/RNA Kit).
The blackPREP Tick DNA Kit is used to isolate DNA form ticks and is applicable for the detection of bacteria and protozoa inside the tick tissue.
The blackPREP Tick DNA/RNA Kit is used for the simultaneous isolation of DNA and RNA directly from ticks. This is of especially interest, if next to the analysis of bacterial pathogens, also e.g. RNA viruses have to be determined (e.g. detection of TBE).
In both extraction kits, first the tick has to be mechanical homogenized using e.g. SpeedMill (Analytik Jena AG) or other commercial available homogenizers on the basis of beads.
After the mechanical homogenization, the lysis or denaturation of the sample is followed. Consecutively the released nucleic acids are specific bound onto a spin filter surface, washed and finally eluted. Now the nucleic acids are ready for any further down stream application.

2.2 Module PCR amplification / hybridization

Module PCR amplification / hybridization is used for the detection of the *Babesia* specific DNA. The isolated DNA is used for a specific *Babesia* amplification reaction. The amplification is in the following combined with a hybridization reaction using a *Babesia* specific probe within the same well of the PCR plastic. This reaction formats allows thus a specific determination of the *Babesia* DNA and avoids false negative results because of a mispriming.
The amplification protocols are optimally adapted to the unique rapidPCR technology using Low Profile Rapid (LPR) blocks or Standard Profile Rapid (SPR) blocks (Analytik Jena AG), as well as optimized to the usage of standard PCR thermal cyclers, e.g. FlexCycler (Analytik Jena AG). Thereby the advantage of the rapidPCR technology is given by a duration of the test performance in less than 1 hour. Each kit contains special PCR microplates or strips for the rapidPCR and 0.2 ml 8 well strips for standard PCR.

2.3 Module detection

Module detection is used to visualize and to analyse the amplification – hybridization results by an user-friendly Lateral Flow Strip. To visualize the reaction the amplification mix will be transferred onto a Lateral Flow Strip. A positive PCR/hybridization result will be confirmed by a visible test line.

Attention

Results from ticks may not be adducted as exclusive basis for further therapies!

3 Performance assessment, spectrum of application and specificity

This test was used for the analysis of about 400 ticks to determine the occurrence of *Babesia* infections. Positive test results were sequenced consecutively to verify the results and to classify *Babesia*. The following tick species were analysed:

- Dermacentor reticulatus
- Ixodes hexagonus
- Haemaphysalis concinna
- Ixodes ricinus

The following table gives an overview of the results, which were determined in this study.

Tick species	**Number of ticks**	
	Complete	***Babesia* positive**
Dermacentor reticulatus	36	-
Ixodes hexagonus	1	-
Haemaphysalis concinna	5	-
Ixodes ricinus	358	18
Complete	**400**	**18**

with this test the following types of *Babesia* can be determined definitely.

- Babesia canis vogeli
- Babesia canis canis
- Babesia sp. Xinjiang 1648
- Babesia gibsoni
- Babesia sp. 'Oklahoma dog
- Babesia canis rossi
- Babesia orientalis
- Babesia divergens
- Babesia capreoli
- Babesia odocoilei isolate Wisconsin 1
- Babesia caballi isolate Spain
- Babesia bigemina
- Babesia bennetti
- Babesia major
- Babesia motasi
- Babesia crassa
- Babesia ovata
- Babesia sp. EU1

4 Kit components, storage and stability

Important!

The master mix has to be prepared freshly before each application. Storage of a ready-to-use master mix could lead to false positive results due to the formation of primer – probe – dimers.

Note!

Only for Module PCR amplification / hybridization and Module detection and the usage of 36, 96 well microplates LP, 8 well strips LP or 8 well strips 0.2 ml.

Kit components and volumes or amounts are listed in the component table below. All components are ready to use and stable until expiry date mentioned on the kit packaging, if stored as specified in the following.

Module PCR amplification / hybridization

Component	Content per reactions			Storage
	10	25	50	
8 well strip LP with sealing foil	3	5	10	Packed within **Module detection**
8 well strip 0.2 ml with lid	2	4	7	
Babesia positive control	7.5 µl	15 µl	25 µl	- 20 °C
Primer 1 BA	25 µl	60 µl	100 µl	- 20 °C
Primer 2 BA	25 µl	60 µl	100 µl	- 20 °C
Probe BA	25 µl	60 µl	100 µl	- 20 °C
dNTP mix	10 µl	20 µl	40 µl	- 20 °C
10x SpeedAmp PCR Buffer	50 µl	100 µl	200 µl	- 20 °C
10x PCR Buffer	50 µl	100 µl	200 µl	- 20 °C
PCR-grade H_20	250 µl	500 µl	1000 µl	- 20 °C
innuTaq HOT DNA Polymerase	10 µl	15 µl	20 µl	- 20 °C work on ice

Module detection

Components	Content per reactions			Storage
	10	25	50	
Lateral Flow Strips	10	25	2x 25	4 °C close airproof
Running buffer	2 ml	5 ml	10 ml	4 °C
Sample Tubes (2.0 ml)	10	25	50	Room temperature

5 Necessary laboratory equipment and additives

- SpeedMill (Analytik Jena AG) or other commercial available homogenizers on the basis of beads
- rapidPCR thermal cycler with a Low Profile Rapid (LPR) block / Standard Profile Rapid (SPR) block (Analytik Jena AG) or a standard PCR thermal cycler with heated lid and 0.2 ml wells (e.g. FlexCycler, Analytik Jena AG)
- Microcentrifuge
- Vortexer
- Variable pipettes for 10 µl, 100 µl and 1.000 µl (use separate pipettes for extraction, amplification and detection)
- Sterile pipette tips with protection against contamination (filter tips)

6 Remarks and safety precautions

All reagents in this kit only have to be used for the intention mentioned inside the user manual. The application may only be exercised by authorized personal.

During the operation, the described protocol has to be followed strictly. Furthermore the regularities to operate quality controls within medical laboratories have to be considered.

The reagents should be stored inside the original vessels at the mentioned temperatures. Single components of different charges and consumables may not be exchanged. The mentioned expiry dates have to be considered.

The material to be determined has to be categorized as potential infectious. The accordant precautions have to be noticed.

For the exposure to the kit reagents and the sample material, the accordant regulations to prevent accidents for the medical service have to be observed. Particularly the following precautions have to be considered:

- Don’t eat, drink or smoke!
- Always wear protective clothing and gloves!

The reagent vessels could be disposed with the normal laboratory waste.

Performance of the test

7 Performance of the test

Important notes!

- Do not exchange the components of different kits or kit charges
- Open and close the vessels of single components always separately
- Change contaminated gloves immediately
- Spatial separation of the amplification and detection area
- Perform the procedure in the order of the following steps:

    1. Sample preparation / nucleic acid extraction
    2. Amplification and hybridization
    3. Detection

- Do not open PCR plastics, which contain amplified samples in the area of sample preparation (NA isolation) or preparation of amplification
- Amplified samples and controls are potential sources of contamination
- Use separate pipettes with sterile filter tips for the preparation of the PCR reaction master mixes
- Open the reaction vessels carefully to avoid the generation of aerosols

7.1 Nucleic acid isolation

The isolation of the nucleic acids has to be done using the blackPREP Tick DNA Kit or the blackPREP Tick DNA/RNA Kit. The protocols inside the accordant user manual have to be followed exactly.

Note: The operation of the test was optimized by using nucleic acids, which were isolated by the above mentioned extraction kits. Alternatively, also nucleic acids, which were isolated by other methods could be used.

7.2 PCR amplification / hybridization

Note

- Only for application of Module PCR amplification / hybridization
- For usage of 36, 96 well microplates LP, 8 well strips LP or 8 well strip 0.2 ml

The performance of the amplification and the hybridization of the PCR product could be done either using a rapidPCR thermal cycler, as well as using a standard PCR thermal cycler (including a heated lid). The thermal cycler also needs a sample protection system (SPS) that cools samples to the set temperature (105ºC – 120ºC) while the lid is heating in order to prevent primer/probe dimer formation, non-specific annealing and early elongation.

7.2.1 Initial steps

1. Divide the DNA eluates and controls to the accordant PCR plastic

	8 Well Strip LP	8 Well Strip 0,2 ml
Sample (extracted DNA)	1.5 µl	2.5 µl
Positive (positive control)	1.5 µl	2.5 µl
Negative (PCR-grade H_2O)	1.5 µl	2.5 µl

2. The prepared plastic has to be stored on the cooling block until the amplification is started

7.2.2 Preparation of the PCR reaction mix

! **Important!**
The master mix has to be prepared freshly before each application. Storage of a ready-to-use master mix could lead to false positive results due to the formation of primer – probe – dimers.

1. Thaw all reagents of Module PCR amplification / hybridization, vortex, spin down and store the components on ice during the preparation
2. The preparation of the master mix for one sample is described in the following table. The preparation of the master mix has to be done for the number of used samples (including positive and negative controls)

Mastermix	**rapidPCR**		**standard PCR**
Plastic	8 well strip LP (20 µl)	8 well strip (0.2 ml)	8 well strip (0.2 ml)
10x SpeedAmp PCR Buffer	1.5 µl	2.5 µl	-
10x PCR Buffer	-	-	2.5 µl
Primer 1 BA	1.0 µl	1.5 µl	1.5 µl
Primer 2 BA	1.0 µl	1.5 µl	1.5 µl
Sonde BA	1.0 µl	1.5 µl	1.5 µl
dNTP Mix	0.3 µl	0.5 µl	0.5 µl
innuTaq Hot DNA Polymerase	0.15 µl	0.25 µl	0.25 µl
PCR-grade H_2O	8.55 µl	14.75 µl	14.75 µl
Final PCR volume	15 µl/reaction	25 µl/ reaction	25 µl/ reaction

3. The master mix has to be added to the wells, which still contain the pre-pipetted DNA samples (positive and negative controls respectively) as described in the following

	rapidPCR		standard PCR
Plastic	8 well strip LP (20 µl)	8 well strip (0.2 ml)	8 well strip (0.2 ml)
Master mix	13.5 µl	22.5 µl	22.5 µl
Finale PCR volume	15 µl/reaction	25 µl/ reaction	25 µl/ reaction

4. Seal the PCR plastic with the accordant foil (PP), put it into the thermal cycler and close the lid
5. Start the PCR time and temperature protocol

7.2.3 Amplification and hybridization

The PCR protocol contains two steps:

Step 1: Amplification and labelling of the *Babesia* specific DNA fragment

Step 2: Hybridization of the amplified DNA sequence using the *Babesia* specific Probe.

! **Attention!**
The following PCR protocols are adapted to the accordant PCR thermal cycler.

rapidPCR thermal cycler with LPR or SPR block:

Amplification and hybridization

Step	Cycle	Profile	Temperature	Holding time	Ramp rate
1	1	Initial denaturation	95 °C	120 sec	max
2	42	Denaturation	95 °C	4 sec	max
		Annealing	58 °C	4 sec	max
		Elongation	72 °C	20 sec	max
3	1	Denaturation	95 °C	300 sec	max
		Hybridization	49 °C	650 sec	max

Standby: 18 °C **Time:** approx. 51 min

Standard PCR thermal cycler:

Amplification and hybridization

Step	Cycle	Profile	Temperature	Holding time	Ramp rate
1	1	Initial denaturation	95 °C	120 sec	max
2	42	Denaturation	95 °C	30 sec	max
		Annealing	58 °C	30 sec	max
		Elongation	72 °C	60 sec	max
3	1	Denaturation	95 °C	300 sec	max
		Hybridization	49 °C	650 sec	max

Standby: 18 °C **Time:** depending on thermal cycler

7.3 Detection (Module 3)

7.3.1 Introduction

The determination of the combined amplification / hybridization reaction is done by visualization on a Lateral Flow Strip (fig. 1). The Lateral Flow Strip consists of the following areas:

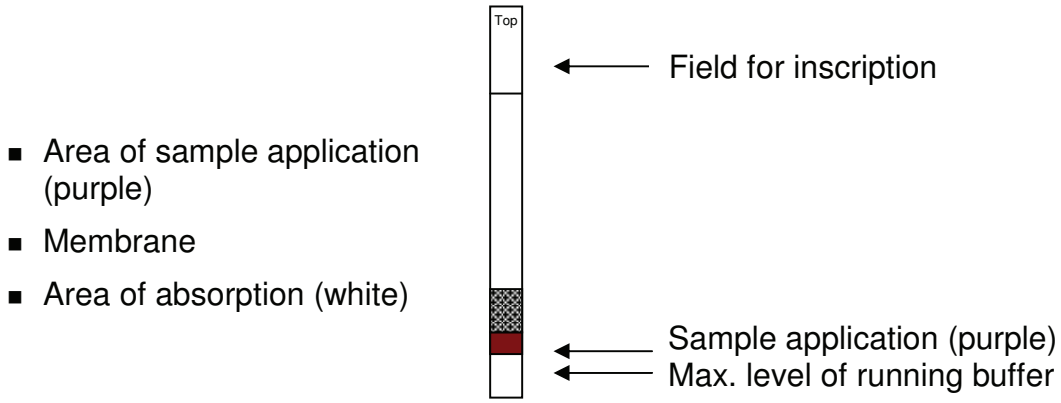

Fig. 1: Design of the Lateral Flow Strip

The whole Lateral Flow Strip, besides the lower part of the sample application area, is covered with a foil and can be touched on this foil. The foil above the absorption area can be used for any inscriptions. After the test is finished, the Lateral Flow Strips can be archived in a progress report.

7.3.2 Performance

1. Take the needed number of Lateral Flow Strips out of its package, inscribe it and place it ready

   Note: Only areas, which are covered with a foil can be touched and inscribed. Store the residual Lateral Flow Strips closed under adequate conditions.

2. Apply 10 µl of the PCR / hybridization reaction on the head of the sample application area (fig. 1, purple) at the border of the foil and incubate for at least 1 minute at room temperature.

   Note: Thereby the occurrence of a smear is normal.

3. Add **150 µl Running Buffer** to each single 2.0 ml Sample Tube
4. Place the Lateral Flow Strips with the membrane into the prepared 2.0 ml Sample Tubes and incubate until the area of sample application is discolored (approx. 20 min).

8 Analysis

The test is valid, if for each determined sample (positive control, negative control and sample) a colored control line is visible (fig. 2).

For each current test performance, the accordant positive and negative controls have to be correct. In case of the PCR negative control the test line has to be invisible (fig. 2B). If the test line of these samples is visible, the analysis for all tested samples has to be repeated.

1. Two red lines are visible: (Test and control line) — The sample is **positive** (fig. 2A).

Attention

Also a light colored test line has to be valued as positive. Compare with the negative control. If necessary repeat the whole test to confirm the result. The intensity of the control line has no influence on the result validation, because the control line is always more intensive in comparison to the test line.

Positive results can be visible before the incubation is finished.

2. Only one red line (level of the control line) **is visible:** — The sample is **negative** (fig. 2B).

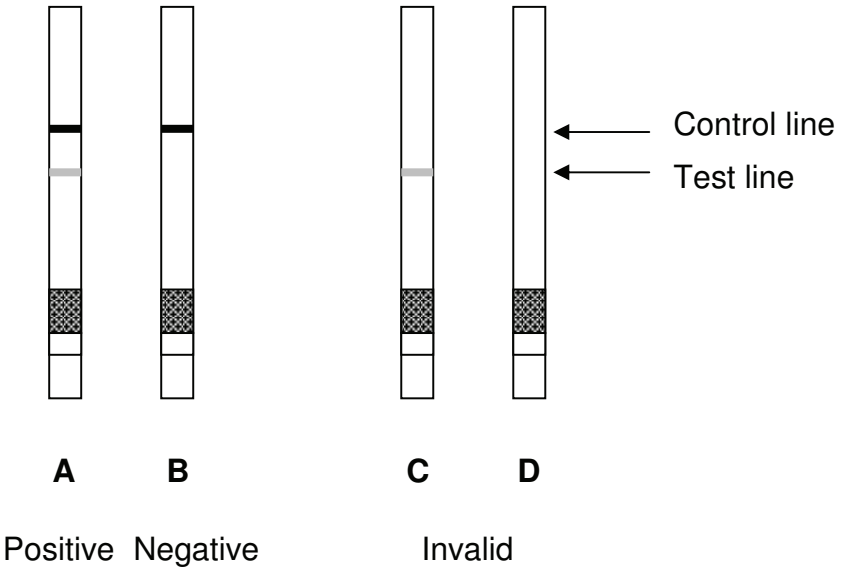

Fig. 2: Analysis of the reaction on the Lateral Flow Strip

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Analytik Jena AG
Life Science
Konrad-Zuse-Strasse 1
07745 Jena / Germany

Phone +49 (0) 36 41 77-94 00
Fax +49 (0) 36 41 77-76 77 76

lifescience@analytik-jena.com
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