Tick-Borne Bacteria Flow Chip Kit

Detection of bacterial arthropod-borne pathogens through multiplex PCR and reverse hybridization

For all hybriSpot platforms

Compatible with version 2.2.0R05 of hybriSoft HSHS.
For compatibility with other versions, please contact the manufacturer / supplier

REF	**MAD-003940M-HS12-24**	Σ	**24 determinations**
REF	**MAD-003940M-HS24-24**	Σ	**24 determinations**

For in vitro diagnostic use only
According to Directive 98/79/CE and ISO Standard 18113-2

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Contents

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1 INTENDED USE

Tick-Borne Bacteria Flow Chip is an in vitro diagnostic kit for the identification of bacterial arthropod-borne pathogens. The Tick-Borne Bacteria Flow Chip system allows the simultaneous qualitative detection of the main bacterial species belonging to the genera *Anaplasma, Ehrlichia, Borrelia, Bartonella, Coxiella, Rickettsia* and *Francisella* through the bacterial DNA amplification with two multiplex PCRs and subsequent reverse hybridization ("reverse dot blot") on a membrane containing specific probes for each genus to be detected using the technology based on the "DNA-Flow" technology, which increases the sensitivity of the technique and saves time significantly.

Bacterium	Target
Anaplasma spp.	16S rRNA
A. phagocytophilum	MSP2
Ehrlichia chaffeensis, *E. ewingii* and *Candidatus Neoehrlichia mikurensis*	16S rRNA
Bartonella spp.	16S
Bartonella spp.	gltA
Borrelia spp.	16S rRNA
Coxiella burnetii	Transposase IS1111
Francisella spp.	17 kDa TUL4
Rickettsia spp.	23S-5S
Rickettsia typhus group	23S-5S
Rickettsia spotted fever group	23S-5S

Table 1: Amplified genomic regions for each bacterial genus.

The differential diagnosis of this group of pathogens implies serious problems today due, on the one hand, to the fact that clinical manifestations are non-specific and, in many cases, usual. Thus, they do not allow a quick identification of the causing agent and implies performing several determinations of the different agents under suspicion. On the other hand, there are no commercialized methods for direct detection. Tick-Borne Bacteria Flow Chip is a quick and sensitive method that allows the simultaneous detection of bacterial arthropod-borne pathogens in an easy way.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

Tick-Borne Bacteria Flow Chip kit is based on a methodology that consists on the simultaneous amplification of specific fragments of each genus of study by multiplex PCR (Polymerase Chain Reaction), followed by hybridization in specific DNA probes through the automatic DNA-Flow technology, both manually and automatically. The primers and probes included in the kit were designed to cover very preserved regions of each target gene for each of the different genera. This design allows the kit to be able to detect a wide range of pathogens for each of the included genera apart from possible variants that may arise in the future.

The biotinylated amplicons generated after the PCR are hybridized in membranes containing specific probes for each bacterial genus, as well as DNA extraction control probes, amplification and hybridization probes. The DNA-Flow technology allows a very fast binding of the PCR product with its specific probe in a three-dimensional porous environment, as compared to the hybridization in a conventional surface. Once the binding between the specific amplicons and their corresponding probes has occurred, the signal is visualized by an immunoenzymatic colorimetric reaction with

Streptavidin–Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization. The results are analyzed automatically with the hybriSoft software.

3 COMPONENTS

The **Tick-Borne Bacteria Flow Chip** kit is commercialized in two main formats depending on the type of hybridization platform to be used for the analysis of clinical samples. Both formats provide the necessary reagents for the amplification by multiplex PCR and subsequent hybridization of 24 clinical samples. Each kit format contains the following components and references:

A. **Manual Platform (HS12)**

Kit/Components	Format	References
Tick-Borne Bacteria Flow Chip kit (Manual)	**24 tests**	**MAD-003940M-HS12-24**
1. Tick-Borne Bacteria Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003940M-P-HS-24**
PCR Mix 1	1 vial x 900 µl	MAD-003940M-MIX1-HS
PCR Mix 2	1 vial x 900 µl	MAD-003940M-MIX2-HS
Hot Start II DNA Polymerase	1 vial x 50 µl	MAD-F122-5
2. Tick-Borne Bacteria Chip	**24 units**	**MAD-003940M-CH-HS-24**
3. Flow Chip Hybridization Reagents (Manual)	**24 tests**	**MAD-003940M-H-HS12-24**
Hybridization Solution (Reagent A)	40 ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10 ml	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS12-24
Reagent E	10 ml	MAD-003930ME
Washing Buffer II (Reagent F)	18 ml	MAD-003930MF-HS12-24

Table 2: Reagents provided in the Tick-Borne Bacteria Flow Chip kit (Manual) format.

B. **Automatic platforms: Auto HS12 and HS24**

Kit/Components	Format	References
Tick-Borne Bacteria Flow Chip kit (Auto HS12 and HS24)	**24 tests**	**MAD-003940M-HS24-24**
1. Tick-Borne Bacteria Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003940M-P-HS-24**
PCR Mix 1	1 vial x 900 µl	MAD-003940M-MIX1-HS
PCR Mix 2	1 vial x 900 µl	MAD-003940M-MIX2-HS
Hot Start II DNA Polymerase	1 vial x 50 µl	MAD-F122-5
2. Tick-Borne Bacteria Chip	**24 units**	**MAD-003940M-CH-HS-24**
3. Flow Chip Hybridization Reagents (Auto HS24)	**24 tests**	**MAD-003940M-H-HS24-24**
Hybridization Solution (Reagent A)	60 ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10 ml	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10 ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35 ml	MAD-003930MD-HS24-24
Reagent E	10ml	MAD-003930ME-HS24

Table 3: Reagents provided in the Tick-Borne Bacteria Flow Chip kit (Auto) format.

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- **Tick-Borne Bacteria Flow Chip kit (PCR Reagents):**
  - **PCR Mix1 and PCR Mix 2:** contain PCR buffer, MgCl2, dNTPs, DNase-free water and biotinylated primers. Both PCR mixes include the primers for the specific amplification of the seven bacterial genera, Anaplasma, Ehrlichia, Borrelia, Bartonella, Coxiella, Rickettsia and Francisella. Besides, they include primers to amplify two internal controls; a fragment of human genomic DNA (endogenous control) and a synthetic DNA fragment (amplification exogenous control), included in the same PCR mix for the detection of potential PCR inhibitors.
  - ***Hot Start II DNA Polymerase***
- **TICK-Borne Bacteria Chips:** The kit includes 24 Chips in total (ref: MAD-003940M-CH-HS-24) that contain an array of DNA probes specific to each of the pathogens included in the analysis, as well as others corresponding to the internal controls included in this kit. The position of all them on the Chip can be referred to in section 10 of this manual (ANALYSIS AND INTERPRETATION OF RESULTS).
- **Flow Chip Hybridization Reagents:** It contains all the reagents necessary for the reverse Flow-Through hybridization process.

Tick-Borne Bacteria Flow Chip kit is protected by a license under patent (ES 2 264642 B1, EP1 895 015 B1, ES 2327593 B1 and US7989170B2).

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and materials

A. Common reagents and materials for manual and automatic platforms:

- Disposable gloves.
- DNase/RNase-free tubes of 0.2/0.5 ml
- DNase/RNase-free filter pipette tips.
- DNase/RNase-free double distilled water.
- Reagents for the extraction of human genomic DNA from blood samples, cerebrospinal fluid, exudates, fresh/frozen tissue section biopsies. For DNA extraction, we recommend the “DNA Mini Kit, Qiagen, Ref.: 51304”.

B. Specific reagents and materials (ref.: MAD-003940M-HS24-24):

- Wash Reagent (ref: MAD-003930WSH).
- PCR tube strips with pierceable cap (ref: MAD-003900ST-HS)

4.2 Equipment

A. Common equipment for manual and automatic platforms:

- Microcentrifuge.
- Automatic micropipettes: P1000, P200, P20 and P2.
- HybriSoft software.
- Cold plate (4 °C).

B. Specific equipment

- With Tick-Borne Bacteria Flow Chip kit (HS12 Manual) (ref: MAD-003940M-HS12-24)
    - Thermocycler.
    - Thermal block to heat PCR tubes, which can be replaced by a thermal cycler.
    - Cold plate (4ºC)
    - Thermostatic bath / heater
    - Manual equipment for hybridization hybriSpot 12 (VIT-HS12)

- With Tick-Borne Bacteria Flow Chip kit (Auto: hybriSpot 24 and hybriSpot 12 PCR AUTO) (ref: MAD-003940M-HS24-24)
    - Thermal cycler (not necessary for hybriSpot 12 PCR AUTO).
      Thermal block to heat PCR tubes (can be replaced with athermal cycler (not necessary for hybriSpot 12 PCR AUTO).
    - Automatic platform for hybridization hybriSpot 24 (VIT-HS24) or automatic platform for hybridization hybriSpot 12 PCR AUTO (VIT-HS12a).

5 STORAGE AND STABILITY CONDITIONS

Tick-Borne Bacteria Flow Chip kit consists of three components that are supplied in two different packages; in one package, Tick-Borne Bacteria Flow Chip kit (PCR Reagents), and in the second Tick-Borne Bacteria Chip along with Flow Chip Hybridization Reagents:

- **Tick-Borne Bacteria Flow Chip kit (PCR Reagents):** Shipped at 2-8°C* and that must be stored at -10°C to -30°C upon receipt. Thaw on ice before use. The reagents are stable until the expiration date indicated on the label. These reagents must be stored away from any source of DNA contamination (for example, PCR products). Avoid more than 5 cycles of freezing/thawing of the reagents to preserve the stability of the PCR reagents.
- **Tick-Borne Bacteria Flow Chips and Hybridization reagents:** Shipped and stored at 2-8°C*. **Do not freeze**. Both the chips and the hybridization reagents are stable until the expiration date indicated on the label.

  Previous considerations on the hybridization reagents:

  - The hybridization reagent A must be pre-heated in a thermostatic bath or heater (only before use in the platform HS12) at 41°C before its use.
  - The rest of hybridization reagents must be used at room temperature (15-25°C).

Previous considerations on the chips:

- Once the package containing the chips is opened, keep the sponge and the desiccant inside until end use to guarantee a correct preservation of the chips.

***Note:** a temperature indicator is included in the package to control the conditions during shipment. In case the cold chain is interrupted, it is recommended to contact the manufacturer before using the reagents.

6 WARNINGS AND PRECAUTIONS

- **Read the instructions of use before using this product.**
- **The safety and disposal precautions are described in the Safety Data Sheet of this product.** This product is only intended for professional laboratory purposes, and it is not intended for pharmacological, home or any other type of use. The current version of the Safety Data Sheet of this product can be downloaded in the web page www.vitro.bio or requested at regulatory@vitro.bio.
- **General considerations to avoid the contamination with PCR product:**

  The greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and hybridization of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. It is recommended to include negative amplification controls containing all the reagents handled in the kit, from the extraction to the amplification, except for the DNA sample, in order to detect and control any possible contamination of the reagents with test samples or amplified products. The hybridization in membrane of this control must be negative, marking only the hybridization control and the amplification exogenous control. This way, it is verified that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.
- **Warning**: the use of ethylene oxide for the preparation of clinical samples and/or the PCR mix could interfere in the correct development of the PCR reaction. It is recommended to avoid the use of this component for these purposes.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the *European Commission Decision of December 18 2014* amending decision 2000/532/CE on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	ELW CODE*	TYPE OF WASTE ACCORDING TO ELW
1. Rubbish/Waste generated from hybridization reagents 2. Disposal of Liquid Wastes ("Wastes" in the manual and automatic platforms)	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, these wastes must be considered as "wastes whose storage and disposal is subjected to special requirements in order to prevent infection"
POTENTIAL WASTES GENERATED AFTER USING THIS PRODUCT	**ELW CODE***	**TYPE OF WASTE ACCORDING TO ELW**
3. Chips used 4. Perishable material (tubes, tips, aluminum foil, etc.) 5. Any element that has been in contact with DNA	180103	"Wastes whose collection and disposal is subject to special requirements in order to prevent infection"
6. Container for reagents used classified as dangerous (according to the Safety Data Sheet)	150110	"Containers having residues of or contaminated by dangerous substances"

Table 4: Classification of wastes generated by this kit according to the European Legislation. *ELW: European Legislation of Waste

Note: This classification is included as a general guideline of action, being under the final responsibility of the user the accomplishment of all the local, regional and national regulations on the disposal of this type of materials.

7 SAMPLE PREPARATION

Tick-Borne Bacteria Flow Chip Kits has been validated from different types of samples:

- Clinical samples from patients (purified DNA from serum, biopsies, blood and cerebrospinal fluid).
- Total DNA from arthropods that carry the bacterium of interest.
- Samples from animal reservoirs.

The table below shows the type of samples indicated for the diagnosis of infections transmitted by the pathogens included in the kit.

Tick-Borne Bacteria Flow Chip Kit: Type of clinical sample indicated for PCR							
Bacterium	**Fresh tissue**	**Ganglionic aspiration**	**Exudate**	**Cerebrospinal fluid (CSF)**	**Blood**	**Serum**	**Synovial fluid**
Anaplasma	----	----	----	----	YES	Only for serology	----
Bartonella	Biopsy: ganglionic, skin, vascular lesion.	YES	Adenopathy, pharynx, skin lesion (cat scratch).	Encephalitis cases	YES	Only for serology	----
Borrelia	Skin biopsy (in case of skin lesion), organs affected.	----	----	YES (low sensitivity)	YES (in cases of recurrent fever). Little	Only for serology	YES

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					efficient for Lyme disease.		
Coxiella	Biopsy: ganglionic, hepatic, vascular lesion, bone (osteomyelitis), heart valve.	----	Sputum in pneumonias (rare).	Encephalitis (low sensitivity)	YES	Only for serology	----
Francisella	Ganglionic biopsy	YES	Adenopathy, connective, skin lesion, sputum (in respiratory infection)	----	----	Only for serology	----
Rickettsia	Skin biopsy	----	Content in papules/macules	YES	YES	Only for serology	----

Table 5: Type of sample indicated for the detection of the pathogens included in the Tick-borne Bacteria flow Chip

It is recommended to use between 200-400 ng DNA per PCR mix. This system has been validated with the DNA Mini Kit, Qiagen, Ref.: 51304. The use of any other extraction system has not been validated and it must be previously verified.

8 ANALYSIS PROCEDURE

8.1. Amplification reaction by multiplex PCR

The following thermal cyclers have been validated with Tick-Borne Bacteria Flow Chip:

- Veriti 96 (Life Technologies)
- GeneAMP® PCR System 9.700 Thermal Cycler (Applied Biosystems)
- Mastercycler® personal (Eppendorf)

Important notes before starting:

- ***It is important that the whole process is performed on an ice sheet to avoid the degradation of the enzymes in the kit and to avoid unspecific bindings between the primers.***
- ***It is very important to use exclusively the tube strips with pierceable caps supplied by Vitro S.A. when working with the automatic platforms HS24/HS12 Auto (Ref: MAD-003900ST-HS).***

Depending on the type of hybridization platform we are working with, we will proceed as follows:

A. For Tick-Borne Bacteria Flow Chip kit (Manual, ref: MAD-003940M-HS12-24):

The PCR reaction is carried out in a final volume of **40 µl** in PCR tubes of 0.2 ml not supplied with the kit.

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For each test DNA sample to analyze, 2 PCR mixes (PCR Mix 1 and PCR Mix 2 separately) will be amplified. Thaw the PCR Mix 1 and PCR Mix 2 and keep in ice. For each reaction, a reaction mix is prepared following the instructions in table 6.

In case of processing several samples at the same time, a stock solution must be prepared and aliquoted before adding the test sample. In order to adjust the volume and avoid errors during pipetting, it is recommended to prepare the stock solution including an extra reaction to the samples to be analyzed. For instance, if 4 samples are going to be processed, prepare one stock solution for 5 samples. The necessary volumes for 1 and 5 reactions are shown in table 6.

Component	1 reaction	5 reactions
PCR Mix 1/2	31.2 µl	156 µl
Hot Start II DNA Polymerase	0.8 µl	4 µl
Final volume before adding DNA	32 µl	160 µl
DNA	1-8 µl (200-400ng) per PCR mix (*)	
DNase-free water	complete the remaining volume up to 40 µl if necessary	

Table 6: Preparation of the reaction mixes

(*) It is recommended to use between 200-400 ng DNA per PCR mix. To do so, 1-8 µl DNA will be added according to the starting concentration (ng/µl). Some examples of volume of DNA that must be added according to the starting concentration are shown in the table below:

Starting Concentration (ng/µl)	V (µl) of DNA (final 200 ng)	V (µl) of DNA (final 250 ng)	V (µl) of DNA (final 300 ng)	V (µl) of DNA (final 350 ng)	V (µl) of DNA (final 400 ng)
25	8	-	-	-	-
50	4	5	6	7	8
100	2	2.5	3	3.5	4
150	1.33	1.67	2	2.33	2.7
200	1	1.25	1.2	1.75	2

Table 7: Example of Volume of DNA (µl) to add to the PCR according to the starting concentration to reach the amount of DNA recommended in the PCR (200-400 ng)

Mix well each PCR tube (PCR Mix 1 and PCR Mix 2) by reverse and centrifuge 5 seconds in microcentrifuge before aliquoting.

Once the PCR Mix 1 and PCR Mix 2 are aliquoted, add the volume of test sample indicated in table 6 to each tube.

***Note**: It is important to keep the tubes in ice up to the moment of placing them in the thermal cycler in order to avoid non-specific binding of primers.*

Place the PCR tubes in the thermal cycler and program the amplification conditions listed below:

PCR Program		
1 cycle	98°C	5 min
43 cycles	98°C	5 s
	60°C	5 s

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	72°C	10 s
1 cycle	72°C	1 min
	8°C	∞

Table 8: PCR Program

If the samples are not going to be processed at that moment, they can be stored in the post-PCR zone at 2-8 °C for 1-2 days. For a longer storage, it is recommended to freeze them at -20° C.

B. For Tick-Borne Bacteria Flow Chip kit (Automatic, ref: MAD-003940M-HS24-24):

The PCR reaction is carried out in a final volume of **40 µl** in 8-strip tubes not supplied with the kit (*).
(*) Important note: It is very important to use exclusively the tube strips with pierceable caps supplied by Vitro S.A. when working with the automatic platforms HS24/HS12 Auto (Ref: MAD-003900ST-HS).
If the number of samples to be analyzed is lower than 8, the necessary tubes can be separated from the strip with no need for using complete strips.

For each test DNA sample to analyze, 2 PCR mixes (PCR Mix 1 and PCR Mix 2 independently) will be amplified. Thaw the PCR Mix 1 and PCR Mix 2 and keep in ice. For each reaction, a reaction mix is prepared following the proportions in table 6.

In case of processing several samples at the same time, a stock solution must be prepared and aliquoted before adding the test sample. In order to adjust the volume and avoid errors during pipetting, it is recommended to prepare the stock solution including an extra reaction to the samples to be analyzed. For instance, if 4 samples are going to be processed, prepare one stock solution for 5 samples. The necessary volumes for 1 and 5 reactions are shown in table 6.

Mix well each PCR tube (PCR Mix 1 and PCR Mix 2) by reverse and centrifuge 5 seconds in microcentrifuge before aliquoting.

You can choose the positions in which the PCR Mix 1 and PCR Mix 2 are aliquoted:

Alternate mode: Mix 1 in odd positions (1, 3, 5, 7) and Mix 2 in even positions (2, 4, 6, 8)

Thus, for every PCR strip, a maximum of 4 samples will be processed and will be distributed as shown in the image below:

	Distribution of samples per PCR strip in alternate mode							
Strip 1	Test sample 1		Test sample 2		Test sample 3		Test sample 4	
	Well 1	Well 2	Well 3	Well 4	Well 5	Well 6	Well 7	Well 8
	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2

Table 9: Distribution scheme of the PCR Mix 1 and PCR Mix 2 per PCR strip in alternate mode

- Mix1: positions 1-3-5-7
- Mix2: positions 2-4-6-8

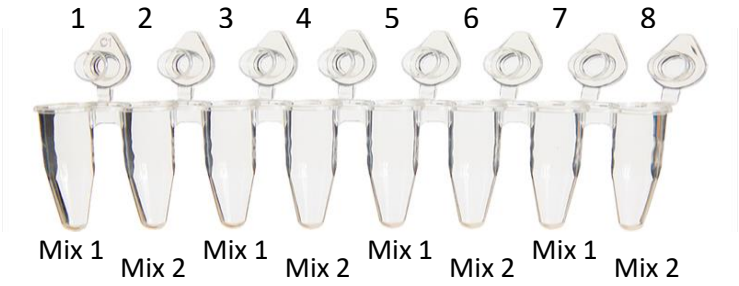

Figure 1: Position of the PCR strip of each PCR mix in alternate mode

Once the PCR Mix 1 and PCR Mix 2 are aliquoted, add the volume of test sample indicated in table 6 to each tube in the positions indicated in table 9.

Contiguous mode: Mix 1 and Mix 2 in contiguous positions of separate strips

The samples will be distributed as shown in the table and image below:

	Distribution of samples per PCR strip in contiguous mode							
Strip 1	Test sample 1		Test sample 2		Test sample 3		Test sample 4	
	Well 1-Strip 1	Well 1-Strip 2	Well 2-Strip 1	Well 2-Strip 2	Well 3-Strip 1	Well 3-Strip 2	Well 3-Strip 1	Well 3-Strip 2
	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2	PCR Mix 1	PCR Mix 2

Table 10: Distribution scheme of the PCR Mix 1 and PCR Mix 2 in two separate strips

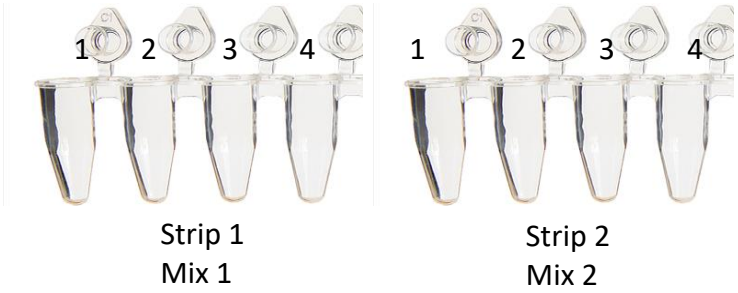

Figure 2: Position in 2 PCR strips of each PCR mix in contiguous mode

Once the PCR Mix 1 and PCR Mix 2 are aliquoted, add the volume of test sample indicated in table 6 to each tube in the positions indicated in table 10.

Place the PCR strips in the thermal cycler and program the amplification conditions indicated in table 8.

Very important: Consult the the user manual of the hybridization platform to place the tubes in the platform properly and to set the instrument according to the aliquoting mode of the PCR mixes.

***Note**: It is important to keep the tubes in ice up to the moment of placing them in the thermal cycler in order to avoid non-specific binding of primers.*

If the samples are not going to be processed at that moment, they can be stored in the post-PCR zone at 2-8 °C for 1-2 days. For a longer storage, it is recommended to freeze them at -20° C.

8.2. Flow-through reverse hybridization

All the reagents are provided in a “ready-to-use” format.

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The Chips are single-use. They must be handled with gloves and away from any contamination source. Depending on the type of kit with which we are working, we will proceed as follows:

A. For Tick-Borne Bacteria Flow Chip kit (Manual, ref: MAD-003940M-HS12-24):

The hybridization process is performed manually in hybriSpot (HS12) following the instructions provided by the wizard of the system. The management of the samples, the capture of images, the analysis and report of the results are performed by the hybriSoft software.

Note: Configure the instrument by following the instructions of the user manual (provided with the instrument).

Before starting the hybridization process, perform the following steps:

1. Pre-heat **Reagent A (Hybridization Solution)** at **41°C** for at least 20 min in a thermostatic bath.
2. Turn on the HS12 instrument and set the temperature at **41°C**
3. Denature the PCR products (PCR Mix 1 and PCR Mix 2) by heating at **95 °C for 10 min** (in thermal cycler) and **cool quickly in ice** for at least **2 min**.
4. **Mix the PCR product (product of PCR Mix 1 and PCR Mix 2)** processed per test sample **in only a vial** and keep in ice until use.
5. Place a Tick-Borne Bacteria Chip per each samples to analyze in the reaction chamber of the HS12 platform.
6. Follow the instructions provided in the manual of the HS12 instrument to carry out the entry of the samples data, the image capture and the results analysis.

MANUAL HYBRIDIZATION PROTOCOL:

1. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated at **41 °C** and incubate for at least **2 min at 41°C**.
2. Remove **Reagent A (Hybridization Solution)** by activating the vacuum pump.
3. Mix **50 µl** of each PCR sample (previously denatured and kept in ice) with **230 µl** of **Reagent A (Hybridization Solution)** (**41°C**) and dispense the mix on the corresponding **Tick-Borne Bacteria Chip**.
4. Incubate at **41°C** for **20 min**.
5. Activate the pump for 30 seconds to remove the PCR products.
6. Wash **3 times with 300 µl** with **Reagent A (Hybridization Solution)** (**41°C**)**.**
7. **Set the temperature at 29 °C**.
8. Block the membranes for **5 min** with **300 µl** of **Reagent B (Blocking Solution).**
9. Activate the pump to remove the Reagent B.
10. Add **300 µl** of **Reagent C (Streptavidin-Alkaline Phosphatase)** and incubate for **8 min** at **29°C**.
11. Activate the pump to remove the Reagent C.
12. Set the temperature at **36°C.**
13. Wash the membranes **4 times with 300 µl** with **Reagent D (Washing Buffer I).**
14. Develop the membranes by adding **300 µl** of **Reagent E (Developer solution)** and incubate **10 min** at **36°C.**
15. Activate the pump to remove the reagent E
16. Wash the membranes **2 times with 300 µl** with **Reagent F (Washing Buffer II).**
17. Image capture, analysis and results report through the hybriSoft software, following the instructions of the HS12's user manual.

B. For Tick-Borne Bacteria Flow Chip kit (HS12 Auto, ref: MAD-003940M-HS24-24):

The PCR reaction and hybridization is carried out automatically on hybriSpot 12 Auto (HS12 Auto). The management of the samples, the capture of images, the analysis and report of the results are performed by the hybriSoft software.

Before starting the amplification and hybridization process, perform the following steps:

1. **Set the instrument by following the instructions from the user's manual (provided with the instrument).**
2. Follow the instructions of section 8.1 subsection B for preparing the PCR mixes.
3. Follow the instructions in the manual to place the tube strips, chips and hybridization reagents in the instrument and start the process.

C. For Tick-Borne Bacteria Flow Chip kit (HS24, ref: MAD-003940M-HS24-24):

The whole hybridization process is performed automatically on hybriSpot 24 (HS24). The management of the samples, the capture of images, the analysis and report of the results are performed by the hybriSoft software.

1. **Before starting the hybridization process, perform the following steps.** Set the instrument by following the instructions from the user's manual (provided **with the instrument).**
2. Denature the PCR products by heating them at 95 °C for 8-10 min in a thermal cycler or a heating block and cool quickly in ice for at least 2 min. **It is not necessary to mix the PCR product (product of PCR Mix 1 and PCR Mix2) processed for each test sample in only a vial. The instrument will perform it automatically**
3. Follow the instructions provided in the HS24's user manual to carry out the samples data entry and their processing. Select the corresponding working protocol in the system.
4. Place the tube strips/plates containing the PCR product in the sample rack of the HS24 instrument in the positions the management software, hybriSoft, indicates. **Consult the user manual to place the tubes and configure the instrument according to the mode in which the PCR Mix has been aliquoted (alternate/contiguous).**
5. Dispense the Tick-Borne Bacteria Chips and the hybridization reagents in their corresponding positions in the hybriSpot 24.
6. Once all the hybridization reagents, samples and chips have been correctly placed in the instrument, press the start button in the hS Control window to start the protocol.

9 QUALITY CONTROL PROCEDURE

Tick Borne Bacteria Flow Chip kit contains different internal controls to control the quality of the results.

PROBE	CONTROL
B	Hybridization control
CI:	Exogenous amplification control
BG	Endogenous amplification control

Table 11: Control probes included in Tick-Borne Bacteria Chip.

Hybridization control: After the development of the membranes, an intense signal must appear in all five hybridization control positions (B), which serve as a quality control. This signal indicates that the

hybridization reagents and developing have worked properly. If the signal does not appear, it indicates that an error has occurred during the hybridization process or that a reagent has not been used properly. Furthermore, this signal allows the software to orientate correctly the probe panel to perform the subsequent analysis.

Exogenous amplification control (CI): probe for the detection of synthetic DNA included in the PCR mix. This DNA will be co-amplified along with the genetic material of the sample. Two positive signals in the exogenous amplification control (CI) will indicate that the PCR reaction has worked correctly. A negative result in this control does not invalidate the result if the endogenous control has correctly amplified and/or the sample has been positive for any of the targets included in the panel.

Endogenous amplification control (BG): probe for the detection of human beta-globin gene DNA amplified during the PCR. All the samples where the test DNA has been amplified correctly will have a positive signal in the Endogenous amplification control (BG). This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of the DNA used for it have been optimal. The absence of signal for this control means that it has been an error during the amplification, a low quality/amount of the DNA used in the amplification or the absence of human DNA in the amplification (in those cases in which a human sample is not being processed, this signal will not appear). However, a negative result in this control does not invalidate the result if the exogenous control has correctly amplified and/or the sample has been positive for any of the targets included in the panel.

The samples that are positive for any of the bacterial genera included in the kit will show two signals for some of the specific probes. Moreover, the five hybridization control signals (B), two exogenous amplification control signals (CI), and two endogenous amplification control (BG) signals must appear (as long as the sample contains human DNA).

When a sample is positive for any of the bacterial genera included in the kit, with a negative result for the exogenous and endogenous amplification controls, the report from the automatic analysis of the results with hybriSoft software shows a warning saying "*no human DNA/ no exogenous control*" for the user to perform the appropriate verifications before validating the result.

When the samples are negative for all the bacterial genera included in the kit, they will show the five positive signals for the hybridization control (B), and two signals for the Exogenous amplification control (CI). Furthermore, the signals of the Endogenous amplification control (BG) will also appear if the analyzed sample contains human DNA.

The user is responsible for determining the appropriate quality control procedures for their laboratory and comply with the applicable legislation.

10 INTERPRETATION OF RESULTS

The interpretation of results is done automatically using hybriSoft analysis software.
The following scheme shows the arrangement of the probes on the Tick-Borne Bacteria Chip:

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B			FR				B	
B	GR				EH		BOR	
CI	TG		BAR		AN			
BG	SFG						COX	
			BAR-2	B				FR
				CI				
	EH		BOR	BG	GR			BAR
	AN				TG			BAR-2
	B		COX		SFG			

Figure 3: Scheme of the arrangement of the probes on the array. Specific probes for the pathogens and those probes used as amplification and hybridization controls are included.

"B": Hybridation control
"CI": Exogenous amplification control
"BG": Endogenous amplification control (fragment human ß-Globin)
"X": Specific probes for each pathogen
All the probes are duplicated to guarantee the reliability in the automatic analysis of the results. The hybridization control (B) is repeated in 5 positions and allows the software to orientate correctly the probe panel for its analysis afterwards.

The distribution of the different probes included in the Tick-Borne Bacteria Flow Chip, as well as the possible expected results are shown below (tables 12 and 13).

	1	2	3	4	5	6	7	8	9
A	B			FR				B	
B	B	GR				EH		BOR	
C	CI:	TG		BAR		AN			
D	BG	SFG						COX	
E				BAR-2	B				FR
F					CI:				
G		EH		BOR	BG	GR			BAR
H		AN				TG			BAR-2
I		B		COX		SFG			

Table 12: Position of the probes included in the Tick-Borne Bacteria Chip. The coordinates of each of them are also indicated.

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Expected result	Probe /Position			
	Bacterium	B	CI	BG
Positive *Anaplasma phagocytophilum, A. bovis* or *A. equi.*	2H-6C (AN)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Ehrlichia chaffeensis, E. ewingii* or *Candidatus Neoehrlichia mikurensis.*	2G-6B (EH)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Bartonella spp.*	4C-9G (BAR) + 4E-9H (BAR-2)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Borrelia spp.*	4G-8B (BOR)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Coxiella burnetti*	4I-8D (COX)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Francisella spp.*	4A-9E (FR)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Rickettsia spp.*	2B-6G (GR)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Rickettsia typhus group*	2B-6G (GR) + 2C-6H (TG)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive *Rickettsia spotted fever* or *R. bellii group*	2B-6G (GR) + 2D-6I (SFG)	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Negative sample	--	1A-1B-2I-5E-8A	1C-5F	-- / 1D-5G
Negative sample	--	1A-1B-2I-5E-8A	-- / 1C-5F	1D-5G
Invalid sample (inhibited PCR)	--	1A-1B-2I-5E-8A	--	--
Hybridization error	--	--	--	--

Table 13: Position of the probes included in Tick-Borne Bacteria Chip and interpretation of results.

The different spotted probes on the Tick-Borne Bacteria Chip as well as the species detected with each of them are shown in the table below:

Probes	Bacterial Species
AN (16S rRNA and MSP2)	Mix of generic and specific probes to identify the *Anaplasma phagocytophilum, A. bovis* and *A. equi.*
EH (16S rRNA)	Mix of specific probes for *Ehrlichia chaffeensis, E. ewingii* and *Candidatus Neoehrlichia mikurensis.*
BAR (16S) and BAR-2 (gltA) (*)	Generic probes for the following species of Bartonella: *B. alsatica, B. bacilliformis, B. birtlesii, B. bovis, B. capreoli, B. clarridgeiae, B. elizabethae, B. doshiae, B. grahamii, B. quintana, B. taylorii, B. tribocorum, B. vinsonii arupensis, B. vinsonii berkhofii, B. vinsonii , B. koehlerae, B. rattimassiliensis, B. phoceensis, B. henselae, B. queenslandensis* and *B. rattaustaliani.*
BOR (16S rRNA)	Generic probe for the detection of all the species belonging to the genus Borrelia.
FR (TUL4)	Generic probe for the detection of any subspecies of *Francisella tularensis,* including *F. tularensis* subsp. *tularensis, F. tularensis* subsp. *holarctica, F. tularensis* subsp. *novicida, F. hispaniensis* and the so-called endosymbiont of different species of ixodida and argasidae.
GR (23S-5S) (**)	Generic probe for the detection of any species of the genus Rickettsia, including those that are not currently characterized.
SFG (23S-5S)	Generic probe for the detection of any species belonging to the spotted fever and/or *R. bellii* group
TG (23S-5S)	Generic probe for the detection of any species of Rickettsia belonging to the typhus group: *R. prowazekii* and *R. typhi (R. mooserii).*
COX (Transposase IS1111)	Specific probe for *Coxiella burnetii*

Table 14: Bacterial species detected with each of the probes included in the Tick-Borne Bacteria Chip.

(*) In order to consider a sample as positive for Bartonella, the positivity signal must appear for each of the two generic probes of Bartonella (BAR and BAR-2).

(**) When a sample is positive for any species of the genus Rickettsia, two signals will appear:

- One signal for the GR probe and other for the SFG probe if the species detected belongs to the spotted fever and/or R. bellii group.
- One signal for the GR probe and other for the TG probe if the species detected belongs to the typhus group.

In case only the signal of the GR probe is detected, it is recommended to repeat the test and confirm the result by sequencing.

An example of a report in which the analyzed case has been positive for *Rickettsia prowazekii* is shown below.

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Tick-Borne Bacteria Flow Chip Kit

LOTS

PCR:	ZOO-056	12/30/2019
Chips:	ZOOE029.3	10/30/2020
Reagent:	HPV066-3L	2/14/2020

SAMPLE DETAILS

ID SAMPLE: Sample-04 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

ZOO POSITIVE

Positive sample for:

Rickettsia spp., Rickettsia typhus group

The sample is negative for the rest of pathogens included in the Tick Borne bacteria flow chip test.

PROTOCOL

Simultaneous detection of DNA from tick-borne bacterial species that cause zoonosis, from genera Anaplasma, Ehrlichia, Borrelia, Bartonella, Coxiella, Rickettsia and Francisella by multiplex PCR and reverse dot blot.

Sample preparation for PCR:

- DNA extraction: DNA Mini Kit, Qiagen, Ref: 51304).
- Use suspension of extracted DNA in each PCR mix (200-400 ng of DNA).

PCR Protocol: 1x 98°C 5min, 43x98-60-72°C (5-5-10), 1x72°C 1min.

REVERSE-DOT BLOT protocol:

- Hybridization of the biotinilated PCR product to the Tick-borne bacteria CHIP.
- Post-hybridization washing.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.

Automatic analysis

NOTES

FACULTATIVE:	Default Doctor, doctor	**Validated:**	7/3/2019
Performed by:	Default Tech, tech	**Processed:**	7/3/2019

Instr. : Mock **Serial Nº:** 000001 **hybriSoft:** HSHS 2.2.0.R03 / HSHS IPL 1.0.1.R0000

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Tick-Borne Bacteria Flow Chip Kit

LOTS

PCR:	ZOO-056	12/30/2019
Chips:	ZOOE029.3	10/30/2020
Reagent:	HPV066-3L	2/14/2020

SAMPLE DETAILS

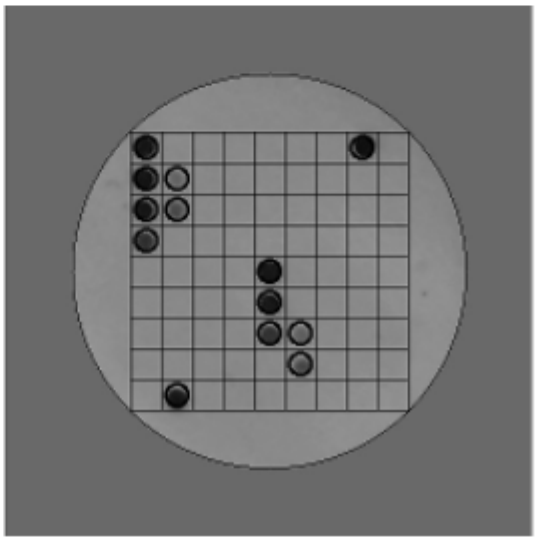
ID SAMPLE: Sample-04 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

SEX: - **BIRTHDATE:** **AGE:**

REPORT

B			FR				B	
B	GR				EH		BOR	
CI	TG		BAR		AN			
BG	SFG						COX	
			BAR-2	B				FR
				CI				
	EH		BOR	BG	GR			BAR
	AN				TG			BAR-2
	B		COX		SFG			



- Spot B: Hybridization control (5 signals to orientate the CHIP)
- Spot CI: Amplification control
- Spot BG: DNA Control (Genomic human DNA probe)
- Spot #:Pathogen specific probes

All the spots are printed in duplicate.

ANALYSIS INFORMATION

Threshold: 4

FACULTATIVE:	Default Doctor, doctor	**Validated:**	7/3/2019
Performed by:	Default Tech, tech	**Processed:**	7/3/2019

Instr. : Mock **Serial Nº:** 000001 **hybriSoft:** HSHS 2.2.0.R03 / HSHS IPL 1.0.1.R0000

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11 PERFORMANCE CHARACTERISTICS

11.1 Analytical functioning in hybriSpot 12 (HS12)

11.1.1 Repeatability

The repeatability of the method was analyzed by testing a minimum of eight times for seven bacterial genera included in the panel at two different concentrations. The test was performed by the same operator in a single location, on the same day and using the same lot of reagents.

Bacterium/Probe	Concentration (copies/ul)	Positive/tested	% positive
Rickettsia prowazekii/GR-TG	20	8/8	100%
	50	8/8	100%
Candidatus Neoehrlichia mikurensis/EH	10	8/8	100%
	50	8/8	100%
A.phagocytophilum/AN	10	8/8	100%
	20	8/8	100%
Francisella turalensis/FRA	50	8/8	100%
	100	8/8	100%
Bartonella henselae/BAR and BAR-2	10	8/8	100%
	20	8/8	100%
Borrelia burgdorferi/BOR	10	8/8	100%
	20	8/8	100%
Coxiella burnetii/COX	10	8/8	100%
	20	8/8	100%

Table 5: Repeatability test for each of the bacterial genera included in the panel.

11.1.2 Reproducibility

The accuracy of the method was analyzed by varying two factors that may contribute to the inaccuracy of the method: different lots of Tick-Borne Bacteria Flow Chip kit and different operators. Eight of the bacteria that are included in the panel were tested in duplicate and at two different concentrations, using two different lots of the Tick-Borne Bacteria Flow Chip kit. The samples were tested with the hybriSpot12 platform and were analyzed with the hybriSoft software. All the valid results were included to calculate the percentage of the positive results. No false positives were obtained. The percentage of the positive results are indicated in table 16.

The two lots tested result in the same percentage of positives, except for *Rickettsia prowazekii* 50 copies, *Candidatus Neoehrlichia mikurensis* 10 copies and *Bartonella henselae* 20 and 10 copies with which the lot 009 obtains less percentage of positivity. As for the variability factor “operator”, the operator number 1 tends to obtain a greater number of positive cases than the operator number 2.

Bacterium	No. copies of recombinant plasmid	Batch No.			No of operator		
		No	Positive/valid	%	No	Positive/valid	%
Rickettsia prowazekii	50 copies	009	5/8	62.5	1	7/8	87.5
		010	8/8	100	2	6/8	75
	20 copies	009	7/8	87.5	1	8/8	100
		010	7/8	87.5	2	6/8	75
Candidatus Neoehrlichia mikurensis	20 copies	009	8/8	100	1	8/8	100
		010	8/8	100	2	8/8	100
	10 copies	009	7/8	87.5	1	7/8	87.5
		010	8/8	100	2	8/8	100
A.phagocytophilum	20 copies	009	8/8	100	1	8/8	100
		010	8/8	100	2	8/8	100
	10 copies	009	8/8	100	1	8/8	100
		010	8/8	100	2	8/8	100
Francisella tularensis	100 copies	009	8/8	100	1	8/8	100
		010	8/8	100	2	8/8	100
	50 copies	009	8/8	100	1	8/8	100
		010	8/8	100	2	8/8	100
Bartonella henselae	20 copies	009	7/8	87.5	1	7/8	87.5
		010	8/8	100	2	8/8	100
	10 copies	009	6/8	75	1	6/8	75
		010	7/8	87.5	2	7/8	87.5

Table 6: Reproducibility test for eight of the bacterial species included in the panel.

11.1.3 Analytical specificity

No cross-reactivities were observed between the bacterial genera included in the kit. For the test, 10^6 copies/reaction of synthetic DNA designed for each bacterial genera were used:

Bacterium	Specificity
Rickettsia typhus group	100%
Candidatus Neoehrlichia mikurensis	100%
A.phagocytophilum	100%
Francisella spp	100%
Bartonella spp.	100%
Borrelia spp.	100%
Coxiella burnetti	100%

Table 7: Intra-panel specificity of Tick-Borne Bacteria Flow Chip.

No cross-reactivities were observed with any of the phylogenetically-related bacterial species evaluated. 10^6 EG of each strain were used for the test.

Organism
Orientia tsutsugamushi
Mycoplasma pneumoniae
Chlamydia pneumoniae
Legionella pneumophila
Brucella mellitensis
Ochrobactrum anthropi
Leptospira interrogans
Treponema pallidum
Mesorhizobium spp.

Table 8: Specificity of Tick-Borne Bacteria Flow Chip

11.1.4 Analytical sensitivity

The limit of detection (LoD) of the kit was calculated for each of the analyzed genera. The minimum number of bacterial copies detected was established through serial dilutions of clonal DNA fragments of each of the bacterial species detected by the method and 5 ng of human genomic DNA, amplified following the protocol of the kit. Each sample was repeated 7-16 times in order to calculate sensitivity, specificity and confidence intervals. All the PCRs were hybridized by using the hybriSpot 12 platform. The results were analyzed with hybriSoft v2.0 and the established value to consider the positive signals was 4 (gray intensity).

Bacterium	Nº of copies of recombinant plasmid	Positive detected/ Total positives	Sensitivity	Confidence interval 95%	Specificity	Confidence interval
Rickettsia prowazekii (GR probe)	20 copies	8/8	100%	67.6-100%	100%	98.1-100%
	10 copies	6/7	86%	48.7-97.4%	100%	98.1-100%
Rickettsia prowazekii (TG probe)	20 copies	8/8	100%	67.6-100%	99%	97.2-99.9%
	10 copies	6/7	86%	48.7-97.4%	99%	97.2-99.9%
Candidatus Neoehrlichia mikurensis (EH probe)	10 copies	8/8	100%	75.8-100%	99%	97.1-99.9%
	5 copies	3/8	38%	13.7-69.4%	99%	97.1-99.9%
A.phagocytophilum (AN probe)	10 copies	8/8	100%	67.6%-100%	100%	97.7%-100%
	5 copies	8/8	100%	67.6%-100%	100%	97.7%-100%
Francisella tularensis (FRA probe)	50 copies	16/16	100%	80.7-100%	99%	96.6-99.9%
	20 copies	10/16	63%	38.6-81.5%	99%	96.6-99.9%
Bartonella henselae (BAR 1 and BAR2 probes)	20 copies	8/8	100%	67.6-100%	98%	94.9-99.2
	10 copies	8/8	100%	67.6-100%	96%	92.1-97.9
Borrelia burgdorferi (BOR probe)	10 copies	8/8	100%	67.6-100%	100%	97.2-99.9%
	5 copies	8/8	100%	67.6-100%	100%	98.1-100%
Coxiella burnetii (COX probe)	20 copies	8/8	100%	67.6-100%	100%	98.1-100%
	10 copies	8/8	100%	67.6-100%	100%	98.1-100%

Table 19. Analytical sensitivity (LoD): number of copies of each gene resulting in 100% of positive cases of the replicas, analyzed with the hybriSoft software and a positivity cut-off point value of 4.

11.2 Analytical functioning in hybriSpot 24 (HS24)

The functioning and robustness of the Tick-borne bacteria Flow Chip was validated in the automatic equipment HS24 by analyzing limit concentrations of recombinant plasmid DNA of all the bacteria included in the panel (5 copies for *B. burgdorferi,* 10 copies for *C. burnetti, B. henselae, A. phagocytophilum* and Candidatus *Neoehrlichia mikurensis*, 20 copies for *Rickettsia* prowazekii and 50 copies for *Francisella tularensis*). This validation proves the reproducibility of the results between the positions 1 and 24 of the HS24 platform and the reproducibility of the results with different programs for a different number of samples.

11.2.1 Reproducibility of results in programs for a different number of samples

Replicas for a positive sample containing a limit number of copies of Rickettsia spp. (20 GE) were made. These replicas were placed in different positions of the reaction chamber of the HS24 system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 replicas)
- Protocol for 15 samples (3 replicas)
- Protocol for 24 samples (4 replicas)

The results were automatically analyzed with hybriSoft and no differences between the different positions of the reaction chamber nor the used protocol were detected.

11.2.2 Reproducibility of results in different hybridization positions in HS24

Four replicas for each pathogen were made, placed in different positions of the two reaction chambers of the HS24 and using the protocol for 24 samples. The results were automatically analyzed with hybriSoft, proving a 100% of reproducibility for all the analyzed bacteria in different positions.

Organism	Nº GE/reaction	Positive/tested	Difference between positions
Rickettsia prowazekii	20	4/4	No
Candidatus Neoehrlichia mikurensis	10	4/4	No
A.phagocytophilum	10	4/4	No
Francisella tularensis	50	4/4	No
Bartonella henselae	10	4/4	No
Borrelia burgdorferi	5	4/4	No
Coxiella burnetii	10	4/4	No

Table 9: Reproducibility of the Tick-borne Bacteria Flow Chip kit in HS24. The positivity is analyzed with the hybriSoft software with a cut-off value of 4.

11.3 Analytical functioning in hybriSpot 12 PCR auto (HS12a)

The functioning and robustness of the Tick-borne bacteria Flow Chip kit associated with the hybridization platform *HybriSpot 12 PCR AUTO* (HS12a) was validated through functional tests in which the following parameters were evaluated:

- Verification of the sensitivity limit (LoD) for each pathogen
- Reproducibility of results in programs for a different number of samples

11.3.1 Verification of the sensitivity limit (LoD) for each pathogen of the panel

Replicas of samples containing each of the synthetic fragments corresponding to each of the pathogens along with 5 ng of human genomic DNA were amplified. The amplification and amplification was performed in two automatic systems HS12a.

Pathogen	Probe	No. Copies/reaction	Positive/Tested
Rickettsia typhi	GR	80	3/3
Rickettsia typhi	TG	80	3/3
Rickettsia conorii	SFG	100	3/3
Candidatus Neoehrlichia mikurensis	EH	40	3/3
A.phagocytophilum	AN	40	3/3
Francisella tularensis	FR	400	3/3
Bartonella henselae	BAR+BAR2	200	3/3
Borrelia burgdorferi	BOR	200	3/3
Coxiella burnetii	COX	80	3/3

Table 10: Reproducibility of the Tick-borne Bacteria Flow Chip kit in HS12a. The results were analyzed automatically with hybriSoft, establishing a positivity cut-off value of 4.

11.3.2 Reproducibility of results in programs for a different number of samples

Replicas of synthetic DNA from two different pathogens *Francisella tularensis* and *Candidatus Neoehrlichia mikurensis* were amplified with 400 and 200 copies/reaction, respectively. Both synthetic DNA were added 5 ng of human genomic DNA. Once the PCR is finished, the replicas of each positive sample were mixed and a same volume of PCR product was dispensed in different positions of the hybridization chambers of the Hs12a, where two different protocols were evaluated:

- Protocol 1 for 2 samples: 2 replicas of each synthetic DNA in positions 1 and 2 both pathogens.
- Protocol 2 for 12 samples: 3 replicas of each synthetic DNA, positions 1, 6 and 11 for *Francisella tularensis* and positions 2, 7 and 12 for *Candidatus Neoehrlichia mikurensis*.

The results obtained were analyzed automatically with the *HybriSoft software*. In all cases, the synthetic DNAs of the two pathogens used were detected as positive. The differences of intensity between positions are acceptable.

11.4 Clinical

11.4.1 Identification of arthropod-borne bacterial pathogens with Tick-Borne Bacteria Flow CHIP in samples from patients

The assessment of the functioning of the Tick-Borne Bacteria Flow CHIP (TBFC) was performed by the National Center for Microbiology, Carlos III Health Institute (Majadahonda-Madrid-Spain) from clinical samples (n=215) of the same center. These samples were previously analyzed through simple PCR and “reverse line blot” (RLB) with chemiluminescent developing. All the samples were from diagnosed cases before 2007, and were processed according to the Spanish law and the Declaration of Helsinki regarding the individual privacy of the patients.

Different types of clinical samples were analyzed: Purified DNA from serum, fresh tissue, blood or cerebrospinal fluid.

These cases corresponded to positive samples for each pathogen (*Ehrlichia ssp* was not included in the study), as well as negative samples.

All the results obtained were analyzed with hybriSoft v2.0 with a positivity cut-off point of 4.
117/215 positive cases (54.42%) were detected, with a percentage of global concordance between both methods of 97.21% (Kappa=0.943). When analyzing the concordance between the two methods for each of the pathogens individually, the data obtained are summarized in the table 21.

	Detected positivity with each method.			
Bacterium	**RLB +TBFC**	**RLB**	**TBFC**	**Concordance percentage between methods and kappa coefficient.**
Anaplasma phagocytophilum	1	1	1	**100 % (k=1)**
Bartonella	32	34	32	**99 ,07% (k=0.964)**
Borrelia	17	17	18	**99.53 % (k=0,969)**
Rickettsia	27	27	28	**99.53 % (k=0.979)**
Coxiella	24	24	24	**100% (k=1)**
Francisella	18	20	18	**99 ,07% (k=0.942)**
Total				
Positive	117	121	119	**97.21% (k=0,943)**

Table 22: Number of positive cases detected for both methods (RLB+TBFC), number of positive cases detected with each method for each pathogen and concordance percentage between both methods.

11.4.2 Clinical specificity and sensitivity

The specificity and the clinical sensitivity for the Tick-Borne Bacteria Flow Chip was calculated from the results obtained from the samples analyzed by the Centro Nacional de Microbiología, Instituto de Salud Carlos III (Majadahonda-Madrid-Spain) as it has been described in the section above. The diagnostic specificity is expressed as a percentage (numeric fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided into the sum of the number of true negative values (TN) plus the number of false positive values (FP), or 100 x TN/(TN + FP). The diagnostic sensitivity is expressed as a percentage (numeric fraction multiplied by 100), calculated as 100 x the number of true positive values (TP) divided into the sum of the number of true positive values (TP) plus the number of false negative values (FN), or 100 x TP/(TP + FN).

Bacterium	TN	FP	TP	FN	Clinical specificity	Clinical sensitivity
A. phagocytophilum	214	0	1	0	100%	100%
Bartonella	183	0	32	0	100%	100%
Borrelia	197	1	17	0	99%	100%
Rickettsia	197	1	27	0	99%	100%
Coxiella	193	0	22	0	100%	100%
Francisella	195	0	18	2	100%	90%

Ehrlichia	NT	NT	NT	NT	NT	NT

Table 23: Specificity and diagnostic sensitivity of Tick-Borne Bacteria Flow Chip on patients' samples. NT: Not tested

11.4.3 Specificity and clinical sensitivity in patient's vectors

Tick-Borne Bacteria Flow Chip kit was assessed in a group of ticks (n=79) isolated in the veterinary practice. They were analyzed in parallel through simple PCR and "reverse line blot" (RLB) with chemiluminescent developing. The results were confirmed through Sanger sequencing.

All the results obtained with the Tick-Borne Bacteria Flow Chip were analyzed with the hybriSoft v2.0 with a positivity cut-off point established at 4.

Bacterium	TN	FP	TP	FN	Clinical specificity	Clinical sensitivity
Rickettsia	77	0	2	0	100%	100%
Ehrlichia	76	0	3	0	100%	100%
Anaplasma	54	0	25	0	100%	100%
Francisella	NT	NT	NT	NT	NT	NT
Bartonella	78	0	1	1	100%	50%
Borrelia	67	0	10	2	100%	83.30%
Coxiella	NT	NT	NT	NT	NT	NT

Table 24: Specificity and diagnostic sensitivity of Tick-Borne Bacteria Flow Chip kit in tick's samples. NT: Not tested

12 LIMITATIONS

Use of unsuitable samples: the method has been validated with purified gene material from serum, blood, biopsies, cerebrospinal fluid, arthropods and animal reservoirs.
The analysis of any other type of specimen not indicated can lead to wrong or inconclusive results due to PCR reaction inhibition by inhibiting chemical agents.

13 TROUBLESHOOTING

Problem	Causes	Solutions
No signal is observed/ There is no hybridization signal	Error in the hybridization protocol	Check that all the hybridization reagents have been added in the correct order (HS12). Check the operation of hybriSpot (HS24). Repeat the test.
	The hybridization reagents have expired or they have not been stored properly	Check the expiration date and the storage conditions of the reagents and the Chips. Repeat the test.
	Possible degradation of the DNA of the Chips during the decontamination process of the surfaces and the material.	Clean with a lot of distilled water the reaction chambers. Repeat the test.
Presence of bacterial species in negative control	Problems of contamination in the pre-PCR or post-PCR zones.	Decontaminate (1% bleach) the working areas and repeat the test.
Absence of exogenous amplification control.	Problems in the amplification by PCR.	Check that the program of the thermocycler is the appropriate, that the stock PCR solution has been prepared properly and that the PCR reagents are

	Presence of PCR inhibitors in the test sample.	stored correctly. Repeat the test. Verify the correct functioning of the extraction system of nucleic acids used. Repeat the test
Absence of endogenous amplification control.	Not enough amount of human DNA in the test sample. Presence of PCR inhibitors in the test sample. Sample of non-human origin	Verify that the extraction system of genetic material work properly , including an extraction control. In case a non-human sample is being processed, this signal will not appear
Weak signals in the hybridization	PCR reagents and/or expired or stored improperly. Error in the hybridization protocol. The PCR product was not denatured correctly before the hybridization. Low quality/quantity of the DNA used.	Check the expiration date of the reagents, the storage of the PCR mix and reagents. Check the hybridization temperatures and times and verify the functioning of the hybriSoft equipment. Verify that the denaturation has been performed correctly. Repeat the test. Increase the amount of sample or starting DNA. Verify the correct functioning of the extraction system of nucleic acids used.

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15 LABEL AND BOX SYMBOLS

IVD	Health product for in vitro diagnosis.		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions of use		Sufficient content for <n> assays
	Material safety data sheet		

16 GLOSSARY

DNA: deoxyribonucleic acid
RNA: ribonucleic acid
DNases: deoxyribonucleases
dNTPs: deoxyribonucleotide triphosphate
ELISA: Enzyme-Linked Immunosorbent Assay
FN: false negatives
FP: false positives
HS12: hybriSpot 12
LoD: Limit of detection
MgCl2: magnesium chloride
Min: minute
NBT-BCIP: nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate
Ng: nanograms
NT: Not tested
PCR: polymerase chain reaction
RLB: reverse line blot
RNases: ribonucleases
rRNA: ribosomal RNA
Sec: seconds
Spp.: species

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µl: microliters
TN: true negatives
TP: true positives

17 CHANGELOG

Date	Description
2022-01-04	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Storage temperature and room temperature are modified in Section 5