Bacterial CNS Flow Chip Kit

Detection of bacteria causing meningitis/encephalitis in humans through multiplex PCR and reverse hybridization

For all hybriSpot platforms

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

REF **MAD-003935M-HS12-24**	**24 tests**
REF **MAD-003935M-HS24-24**	**24 tests**

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

Vitro S.A.
Calle Luís Fuentes Bejarano 60 Ed. Nudo Norte Local 3 41020 Sevilla (Spain)
Tel: +34 954 933 200. vitro@vitro.bio; www.vitro.bio

Rev: 2021/12/30

CONTENTS

1 INTENDED USE

Bacterial CNS Flow Chip is an in vitro diagnostic kit of bacteria and fungi that cause meningitis and encephalitis in humans. The methods that are currently used for their diagnosis are very laborious and not always show a 100% specificity. The Bacterial CNS Flow Chip system allows the simultaneous detection of nine bacterial species (*Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes, Haemophilus influenzae, Mycobacterium tuberculosis complex, Treponema pallidum, Coxiella burnetti,* and *Borrelia burgdorferi*) and a fungus (*Cryptococcus neoformans*) through the amplification of the bacterial DNA by multiplex PCR and reverse hybridization on a membrane that contains specific DNA probes for every species.

Organism	Target gene
Neisseria meningitidis	ctrA
Haemophilus influenzae	gyrB
Streptococcus pneumoniae	ply
Streptococcus agalactiae	cfb
Listeria monocytogenes	hly
Cryptococcus neoformans	18S
Treponema pallidum	PolA
Mycobacterium tuberculosis complex	IS6110
Coxiella burnetti	trans
Borrelia burgdorferi	osp

Table 1: Target genes used for the amplification of bacteria and fungi.

Microbiological status: Product not sterile

2 PRINCIPLE OF THE METHOD

Bacterial CNS Flow Chip is based on a methodology that consists on the simultaneous amplification of 9 bacteria and a fungus through multiplex PCR, followed by the hybridization in membranes with specific DNA probes through the DNA-Flow for both automatic and manual hybriSpot platforms. The biotinylated amplicons generated after the RT-PCR are hybridized in membranes containing an array of specific probes for each bacterium, as well as amplification and hybridization control probes. The DNA-Flow technology allows the fast binding of the PCR product and 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 through an immunoenzymatic colorimetric reaction with Streptavidin−Alkaline 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 kit **Bacterial CNS Flow Chip** is marketed in two main formats according to the type of hybridization platform to be used for the analysis of clinical samples. Both formats provide the necessary reagents for the amplification through multiplex PCR and subsequent hybridization of 24 clinical samples. Each kit format contains the following components and references:

3.1 Reagents for multiplex PCR

- 24 tests for the manual platform HS12 (MAD-003935M-P-HS12-24):

Name	Format	Reference
Bacterial CNS PCR Mix	1 vial x 1000 µl	MAD-003935M-MIX-HS12
Hot Start DNA Polymerase	1 vial x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	1 vial x 24 µl	MAD-UNG-4

Table 2: Reagents provided in the HS12 format (manual platform).

- 24 tests for the automatic platform HS24 (MAD-003935M-P-HS24-24):

Name	Format	Reference
Bacterial CNS PCR Mix	1 vial x 1200 µl	MAD-003935M-MIX-HS24
Hot Start DNA Polymerase	1 vial x 15 µl	MAD-POL-3
Uracil-DNA Glycosylase	1 vial x 28 µl	MAD-UNG-3

Table 3: Reagents provided in the HS24 and HS12a formats (automatic platforms).

Bacterial CNS PCR mix contains the PCR buffer, $MgCl_2$, dNTPs (U/T), DNase/RNase-free water and biotinylated primers. The primers included are specific to the amplification of nine species of bacteria (*Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes, Haemophilus influenzae, Mycobacterium tuberculosis complex, Treponema pallidum, Coxiella burnetti,* and *Borrelia burgdorferi*) and the fungus *Cryptococcus neoformans*. There are also included primers for the amplification of a fragment of human genomic DNA (beta-globin as internal control) and primers and DNA of synthetic exogenous control of amplification.

3.2 Reagents for reverse hybridization

- 24 tests for the manual platform HS12 (MAD-003935M-H-HS12-24):

Name	Format	Reference
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
Bacterial CNS Chip	1X 24 units	MAD-003935M-CH-HS-24

Table 4: Reagents provided in the HS12 format (manual platform).

- 24 tests for the automatic platform HS24 (MAD-003935M-H-HS24-24):

Name	Format	Reference
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	10 ml	MAD-003930ME-HS24
Bacterial CNS Chip	1X 24 units	MAD-003935M-CH-HS-24

Table 5: Reagents provided in the HS24 and HS12a formats (automatic platforms).

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1 Reagents and Materials

A. Common reagents to manual and automatic platforms:

- Reagents to purify bacterial DNA of cerebrospinal fluid (CSF)
- Disposable gloves
- DNase/RNase-free Eppendorf tubes of 0.2/0.5/1.5 ml
- Pipette tips with DNase/RNase-free filters

B. Specific reagents for automatic platforms (Ref: MAD-003935M-HS24):

- Pierceable PCR Strip tubes with attached caps (Ref: MAD-003900ST-HS).
- Washing Reagent (Ref: MAD-003930WSH).

4.2 Equipment

A. Common equipment for manual and automatic platforms:

- Microcentrifuge
- Automatic micropipettes: P1000, P200, P20 and P2
- Thermocycler
- HybriSoft software

B. Specific equipment:

- With Bacterial CNS Flow Chip kit for automatic platform HS12 (Ref: MAD-003935M-HS12)
  - Manual equipment for hybridization hybriSpot 12 (VIT-HS12).
  - Thermocycler
  - Thermal block to heat PCR tubes (can be substituted by a thermocycler)
  - Cold plate (4°C)
  - Thermostatic bath / heater.
- With Bacterial CNS Flow Chip kit for automatic platform (Ref: MAD-003935M-HS24)
  - Automatic equipment for hybridization hybriSpot 24 (VIT-HS24) or hybriSpot 12 PCR AUTO (VIT-HS12a).
  - Thermocycler (not necessary for hybriSpot 12 PCR AUTO).
  - Thermal block to heat PCR tubes (not necessary for hybriSpot 12 PCR AUTO).
  - Cold plate (4°C).

5 STORAGE AND STABILITY CONDITIONS

Bacterial CNS Flow Chip consists of two components that are supplied in separate boxes:

PCR reagents: Shipment between 2 and 8 °C*. Once received, it must be kept stored at -10 °C to -30 °C . It will be stable until the specified the expiration date. The PCR reagents must be stored in areas free of DNA or PCR products contamination. Avoid multiple cycles of freezing and thawing.

Hybridization reagents. Shipment and storage at 2-8°C*. The reagents as well as the Bacterial CNS Chips are stable until the specified expiration date. Do not freeze. Previous recommendations on the hybridization reagents:

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

Previous chip considerations:

- Once the packaging containing the chips has been opened, keep the cylindrical foam and sorbent packet inside until end of use to ensure the adequate preservation of the membranes.

***Note: Inside each box, there is a band indicating the time and temperature to control the conditions during shipment. It is recommended to contact the manufacturer before using the reagents included in the box if the cold chain has been interrupted.**

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.
- **Bacterial CNS Flow Chip** kit uses as starting materials nucleic acids previously extracted and purified. It is the client's responsibility to include the necessary controls to verify that the system of extraction of the used genetic material works properly.
- **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 diagnosis. 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. Furthermore, in order to avoid the contamination with previous PCR

products, the enzyme Cod-UNG, which degrades the PCR products containing dUTP, is included in the kit.

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/RNA 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.

- **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 equipments)	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"
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 6: Classification of wastes generated by this kit according to the European Legislation. *ELW: English acronym for *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

7.1 Sample taking

Bacterial CNS Flow Chip has been validated with purified genetic material from cerebrospinal fluid (CSF). The CSF must be collected from a sterile container and must be transported at 2-8 °C according to the Center for Disease Control and Prevention (CDC) guidelines. This material must be stored at 2-8 °C during a maximum of 3 days or at -70 °C for longer periods of time in order to preserve the viral viability.

7.2 Extraction of nucleic acids from the CSF

THE CSF must be treated as a potential infectious agent. The recommendations for the handling of this type of samples can be found in the publications of the CDC. All the material contaminated with biological and dangerous agents must be handled properly according to the recommendations of the workplace.
Bacterial CNS Flow Chip has been evaluated with purified genetic material from human cerebrospinal fluid. This kit has been validated with starting DNA obtained following the next extraction system:

- NucliSENS® easyMag® (bioMérieux S.A.)
- MagNA Pure 96 System (Roche Diagnostics)

Note: The system has not been validated with other DNA extraction systems. Therefore, if an alternative purification system is used, it must be previously validated.

8 ANALYSIS PROCEDURE FOR HS12 AND HS24 PLATFORMS

8.1 Reaction of amplification through multiplex PCR

The following thermocyclers have been validated with Bacterial CNS Flow Chip:

- Veriti 96 (Life Technologies)
- GeneAmp PCR System 7900 (Applied Biosystems)
- TProfessional Thermocycler (Biometra)

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 important that, while working with the HS24 or HS12a, you must only use the tube strips provided by Vitro S.A., pierceable PCR Strip tubes with attached caps (Ref: MAD-003900ST-HS).**

8.1.1 Amplification reaction using Bacterial Flow Chip Kit (Manual, Ref: MAD-003935M-HS12)

- In order to avoid the freezing and thawing cycles, it is recommended to aliquot the PCR mix the first time it is used:
  - Defrost **Bacterial CNS PCR Mix** on ice.
  - Mix thoroughly by turning upside down the vial several times.
  - Add to the vial of PCR mix all the volume of Hot Start DNA Polymerase and Uracil-DNA Glycosylase, mix thoroughly by turning the vial upside down several times and centrifuge for a few seconds.
  - Aliquot **36 µl** of the new mix in 24 tubes of PCR and store at -20ºC (stable for 12 months).
- Defrost a tube of PCR per sample and add **4 µl** of the purified DNA.
- If the mix is prepared when it is going to be used, mix according to the following instructions:

Component	Volume per reaction
Bacterial CNS PCR mix	34.8 µl
Hot Start DNA Polymerase	0.4 µl
Uracil-DNA Glycosylase	0.8 µl
Purified DNA	4 µl

Table 7: Reagents and necessary volumes to perform the PCR reaction.

- Place the PCR tubes in the thermocycler and set the conditions of amplification that are detailed below:

1 cycle	25°C	10 min
1 cycle	94°C	3 min
40 cycles	94°C	30 s
	50°C	30 s
	72°C	30 s
1 cycle	72°C	5 min
	8°C	∞

Table 8: Multiplex PCR program

If the samples are not going to be processed in that moment, they can be stored in the post-PCR zone at 4ºC for 1-2 days. For a longer storage, it is recommended to freeze them at -20 ° C.

8.1.2 Amplification reaction using Bacterial CNS Flow Chip kit (Auto, Ref: MAD-003935M-HS24)

- In order to avoid the freezing and thawing cycles, it is recommended to aliquot the PCR mix the first time it is used:
  - Defrost **Bacterial CNS PCR Mix** on ice.
  - Mix thoroughly by turning upside down the vial several times.
  - Add to the vial of PCR mix all the volume of Hot Start DNA Polymerase and Uracil-DNA Glycosylase, mix thoroughly by turning the vial upside down several times and centrifuge for a few seconds.
  - Aliquot **45 µl** of the new mix in tube strips (Ref: MAD-003900ST-HS) and store at -20°C (stable for 12 months).
- Defrost a tube of PCR per sample and add **5 µl** of the purified DNA.

- If the mix is prepared when it is going to be used, mix according to the following instructions:

Component	Volume per reaction
Bacterial CNS PCR mix	43.5 µl
Hot Start DNA Polymerase	0.5 µl
Uracil-DNA Glycosylase	1 µl
Purified DNA	5 µl

Table 9: Reagents and necessary volumes to perform the PCR reaction.

- Close the tube strips and place them in the thermocycler.
- Set the amplification conditions as follows:

1 cycle	25°C	10 min
1 cycle	94°C	3 min
40 cycles	94°C	30 s
	50°C	30 s
	72°C	30 s
1 cycle	72°C	5 min
	8°C	∞

Table 10: Multiplex PCR program

If the samples are not going to be processed in that moment, they can be stored in the post-PCR zone at 4ºC 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.
The Chips are single-use. They must be handled with gloves and away from any contamination source.

8.2.1 Hybridization process using Bacterial Flow Chip Kit (Manual, Ref: MAD-003935M-HS12)

The hybridization process is performed semiautomatically in hybriSpot (HS12). The introduction of the samples, the capture of images and the analysis of the results are managed through the hybriSoft software.

Before starting the hybridization process (HS12):

1. Denature the PCR products by heating at **95 °C during 10 min** in a thermocycler and **cool quickly in ice** during at least **2 min**.
2. Preheat the **Reagent A** (Reagent A) at 41 °C.
3. Place every **Bacterial CNS Chip** in the indicated position in the platform (HS12)

Manual hybridization protocol:

1. Set the temperature of the equipment at 41 °C. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated for at least 20 minutes at 41 °C for every Chip and incubate for at least **2 min at 41 °C**.
2. Remove the **reagent A (Hybridization Solution)** by activating the vacuum pump.
3. Mix **30 µl** of every sample of PCR (previously denatured and stored in ice) with **270 µl** of **Reagent A (Hybridization Solution)** (41 °C) and pour the mix onto the corresponding **Bacterial CNS Chip**.
4. Incubate at **41 °C** for **8 min**.
5. Activate the pump for at least 30 s 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. Add **300 µl** of **Reagent B (Blocking Solution)** and incubate for 5 min.
9. Activate the pump to remove the reagent B.
10. When the temperature has reached **29 °C**, add **300 µl** of **Reagent C (Streptavidin-Alkaline Phosphatase)** to every Chip
11. Incubate for **5 min at 29 °C**.
12. Activate the pump to remove the reagent.
13. Set the temperature at **36 °C**.
14. Wash the membranes **4** times with **300 µl** with **reagent D (Washing buffer I).**
15. When the temperature has reached **36 °C**, add **300 µl** of **Reagent E (developer solution)** to every Chip. Incubate for **10 min at 36 °C**.
16. Activate the pump to remove the reagent.
17. Wash the membranes **2** times with **300 µl** with **reagent F (Washing buffer II).**
18. Activate the pump to remove the reagent.
19. Perform the image capture, analysis and results report following the instructions of the HS12 user manual.

8.2.2 Hybridization process using Bacterial Flow Chip Kit (Auto, Ref: MAD-003935M-HS24)

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

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

Before starting the hybridization process:

1. Denature the PCR products by heating at **95 °C during 10 min** in a thermocycler and **cool quickly in ice** during at least **2 min**.
2. Put the amplified samples, Bacterial CNS Chips and reagents in the designated place of the HS24 and select the corresponding protocol of the instrument to start the process automatically

9 ANALYSIS PROCEDURE FOR HS12a

The amplification through PCR and hybridization processes are performed automatically in the platform HS12a.

The processing of the samples, the capture of images and the results analysis are performed by the hybriSoft software.

Before starting the process, it is recommended to carefully read the user manual (included in the HS12a equipment) and follow the instructions to place the tube strips, chips and hybridization reagents in the instrument.

Procedure:

- Defrost **Bacterial CNS PCR Mix** on ice.
- Mix thoroughly by turning upside down the vial several times.

- Add to the vial of PCR mix all the volume of Hot Start DNA Polymerase and Uracil-DNA Glycosylase, mix thoroughly by turning the vial upside down several times and centrifuge for a few seconds.
- Aliquot **45 µl** of the new mix in tube strips (Ref: MAD-003900ST-HS) and store at -20°C (stable for 12 months).
- Defrost a tube of PCR per sample and add **5 µl** of the purified DNA.
- Follow the instructions in the manual to place the tube strips, chips and hybridization reagents in the instrument and start the process.

10 QUALITY CONTROL PROCEDURE

Bacterial CNS Flow Chip Kit contains several internal controls to ensure the quality of the results.

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

Table 13: Control probes included in Bacterial CNS Chip.

Hybridization control: After the developing of the membranes, an intense signal must appear in the five positions of the hybridization control (B) that serves as 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 to detect the synthetic DNA included in the PCR mix. This DNA will be co-amplified along with the genetic material of the sample. The two positions of the Exogenous amplification control (CI) will indicate that the reaction of PCR has worked properly.

Endogenous amplification control (BG): Probe to detect the gen of the human beta-globin in the test sample, that is co-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 shows that the amplification has worked correctly and that the quality and quantity of starting DNA has 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 sample. This last case is possible when the cerebrospinal fluid does not contain human cells, and a warning of *"absence of human DNA control"* is displayed in the automatic analysis with the hybriSoft software. If a signal for this endogenous amplification control is not observed, the user has to verify that the process of DNA extraction from the starting sample has been performed correctly, adding an exogenous human DNA to the sample of cerebrospinal fluid before the extraction (e. g. 200-500 ng of human genomic DNA depending of the final volume of elution of the method).

When a sample is positive for any of the bacteria included in the kit, with negative result for the exogenous and endogenous amplification controls, in the automatic analysis report with the hybriSoft software, a warning "*absence of exogenous control/absence of control of human DNA"* appears so that the user performs the appropriate verifications before validating the results.

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

11 INTERPRETATION OF RESULTS

In the following diagram, the distribution of the probes in the Bacterial CNS Chip is shown:

B							B	
B							MTB	
CI	NEISS	AGAL	TPA		LIS	CRYP	BOR	
BG								
	PNEU	HINF	COX	B	NEISS	AGAL	TPA	
				CI				
	LIS	CRYP	BOR	BG	PNEU	HINF	COX	
	MTB							
	B							

"B": hybridization control
"CI": Exogenous amplification control
"BG": Endogenous amplification control (fragment human ß-Globin)
"X": Specific probes for every bacterium/fungus
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 **Bacterial CNS Chip** as well as the possible expected results are shown below.

	1	2	3	4	5	6	7	8
A	B							B
B	B							MTB
C	CI	NEISS	AGAL	TPA		LIS	CRYP	BOR
D	BG							
E		PNEU	HINF	COX	B	NEISS	AGAL	TPA
F					CI			
G		LIS	CRYP	BOR	BG	PNEU	HINF	COX
H		MTB						
I		B						

Table 14a: Position of the probes included in Bacterial CNS Chip.

Expected results	Probe/Position (column-row)			
	Organism	B	CI	BG
Positive Neisseria meningitidis	2C-6E	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Streptococcus pneumoniae	2E-6G	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Listeria monocytogenes	2G-6C	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Mycobacterium tuberculosis complex	2H-8B	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Streptococcus agalactiae	3C-7E	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Haemophilus influenzae	3E-7G	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Cryptococcus neoformans	3G-7C	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Treponema pallidum	4C-8E	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Coxiella burnetti	4E-8G	1A-1B-2I-5E-8A	-- / 1C-5F	-- / 1D-5G
Positive Borrelia burgdorferi	4G-8C	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 results	--	1A-1B-2I-5E-8A	--	--
Hybridization error	--	--	--	--

Table 14b: Position of the probes included in Bacterial CNS Chip and interpretation of the results.

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

Bacterial CNS Flow Chip Kit

LOTS

PCR:	PCRBNS-001	9/30/2017
Chips:	CHIPBNS-001	10/31/2017
Reagents:	REAG-001	8/31/2017

SAMPLE DETAILS

ID SAMPLE: Sample-BNS

ID PATIENT:

PATIENT:

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

SAMPLE TYPE:

REPORT

BNS POSITIVE

SAMPLE POSITIVE FOR:

Streptococcus pneumoniae

PROTOCOL

Detection of pathogens associated with meningitis and encephalitis by multiplex-PCR and Automatic Reverse Dot Blot:
Bacteria: Neisseria meningitidis, Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes, Haemophilus influenzae, Mycobacterium tuberculosis complex, Treponema pallidum, Coxiella burnetti, Borrelia burgdorferi
Fungus: Cryptococcus neoformans

- Sample preparation/DNA purification
- Add suspension of CSF/purified DNA for PCR amplification:
- PCR protocol: 1x 25° 10 min; 1x 94° 3 min; 40x (94° 30 s-50° 30 s-72°C 30 s); 1x 72° 5 min.
- REVERSE-DOT BLOT protocol:
- Hybridization of the biotinylated PCR products to the Bacterial CNS CHIP
- Post-hybridization washes.
- Streptavidin-Alkaline Phosphatase incubation.
- NBT-BCIP development.
- Automatic analysis of results

NOTES

FACULTATIVE: Default Doctor, doctor **Validated:** 6/14/2017

Performed by: Default Tech, tech **Processed:** 6/14/2017

vitro master diagnóstica®

CE

vitro

Bacterial CNS Flow Chip Kit

LOTS

PCR:	PCRBNS-001	9/30/2017
Chips:	CHIPBNS-001	10/31/2017
Reagents:	REAG-001	8/31/2017

SAMPLE DETAILS

ID SAMPLE: Sample-BNS

ID PATIENT:

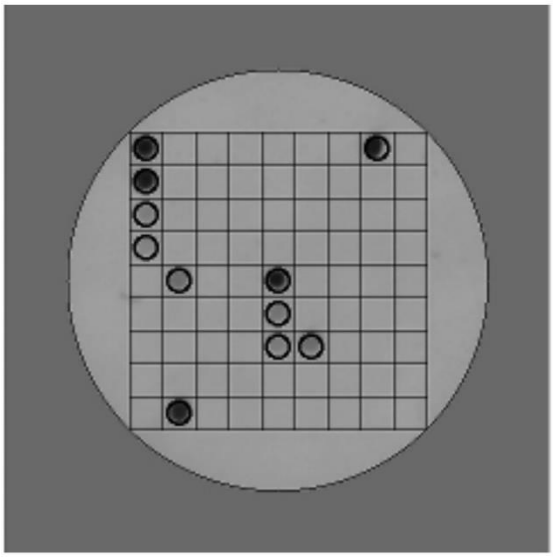
PATIENT:

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

SAMPLE TYPE:

REPORT

B							B	
B							MTB	
CI	NEIS	SAGAL	TPA		LIST	CRYPT	BOR	
BG								
	SPNEU	HINF	COX	B	NEIS	SAGAL	TPA	
				CI				
	LIST	CRYPT	BOR	BG	SPNEU	HINF	COX	
	MTB							
	B							



- 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:**	6/14/2017
Performed by:	Default Tech, tech	**Processed:**	6/14/2017

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical functioning in hybriSpot 12 (HS12)

12.1.1 Repeatability

The repeatability of the kit was analyzed by testing the method at least six times for each bacterium/fungus 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.

Organism	Concentration (copies/reaction)	Positive/tested	% positive
Neisseria meningitidis	100	6/6	100%
	10	6/6	100%
Streptococcus pneumoniae	100	6/6	100%
	10	6/6	100%
Listeria monocytogenes	100	6/6	100%
	10	6/6	100%
Mycobacterium tuberculosis complex	100	6/6	100%
	10	6/6	100%
Streptococcus agalactiae	100	6/6	100%
	50	6/6	100%
Haemophilus influenzae	100	6/6	100%
	10	6/6	100%
Cryptococcus neoformans	100	6/6	100%
	10	6/6	100%
Treponema pallidum	100	6/6	100%
	50	6/6	100%
Coxiella burnetti	100	6/6	100%
	50	3/6	50%
Borrelia burgdorferi	100	6/6	100%
	50	4/6	67%

Table 15: Repeatability assay for each of the bacterium/fungus included in the panel.

12.1.2 Reproducibility

The accuracy of the method was analyzed by varying two factors that could contribute to the variability: thermocycler and operator. Three species included in the panel were tested in triplicate using two different concentrations, two different operators and two different thermocyclers. False positives were not obtained. The operator 1 obtained a greater percentage of positive cases than the operator 2.

Bacterium	GE/reaction	No of thermocycler			No of operator		
		No	Positive/valid	%	No	Positive/valid	%
Neisseria meningitidis	100 copies	014	6/6	100	1	6/6	100
		021	6/6	100	2	6/6	100
	10 copies	014	6/6	100	1	5/6	83
		021	6/6	100	2	5/6	83
Streptococcus pneumoniae	100 copies	014	6/6	100	1	6/6	100
		021	6/6	100	2	6/6	100
	10 copies	014	6/6	100	1	5/6	83
		021	6/6	100	2	5/6	83
Haemophilus influenzae	100 copies	014	6/6	100	1	6/6	100
		021	5/6	83	2	5/6	83
	10 copies	014	6/6	100	1	6/6	100
		021	5/6	83	2	5/6	83

Table 16: Reproducibility of Bacterial CNS Flow Chip Kit.

12.1.3 Analytical specificity

Cross-reactivities were not observed between the organisms included in the test.

Organism	Specificity
Neisseria meningitidis	100%
Streptococcus pneumoniae	100%
Listeria monocytogenes	100%
Mycobacterium tuberculosis complex	100%
Streptococcus agalactiae	100%
Haemophilus influenzae	100%
Cryptococcus neoformans	100%
Treponema pallidum	100%
Coxiella burnetti	100%
Borrelia burgdorferi	100%

Table 17: Specificity of Bacterial CNS Flow Chip .

Cross-reactivities are not observed with other viruses and bacteria:

Bacterium	Virus
Staphylococcus aureus	Herpes simplex-1
Mycobacterium abcessus	Herpes simplex-2
Enterococcus faecalis	Epstein Barr virus
Klebsiella.pneumoniae	Cytomegalovirus
Proteus mirabilis	Varicella Zoster virus
Escherichia coli	
Pseudomonas aeruginosa	
Enterobacter cloacae	

Table 18: Specificity of Bacterial CNS Flow Chip .

12.1.4 Analytical sensitivity

The limit of detection for each pathogen was calculated. The analytical sensitivity was performed using several dilutions of genomic DNA from each pathogen included in the panel. Each sample was tested a minimum of 6 times in order to calculate sensitivity, specificity and confidence intervals. The results were analyzed with the hybriSpot platform and hybriSoft software. A value of 4 was established as a threshold for the positivity of the sample.

Organism	GE/reaction	Detected positives/total positives	Sensitivity	Confidence interval 95%	Specificity	Confidence interval 95%
Neisseria meningitidis	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Streptococcus pneumoniae	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Listeria monocytogenes	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Mycobacterium tuberculosis complex	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Streptococcus agalactiae	50 copies	12/12	100%	(75.8%-100%)	100%	(97.8%-100%)
	30 copies	3/6	50%	(18.8%-81.3%)	100%	(97.8%-100%)
Haemophilus influenzae	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Cryptococcus neoformans	10 copies	12/12	100%	(75.8%-100%)	100%	(98%-100%)
Treponema pallidum	50 copies	12/12	100%	(75.8%-100%)	100%	(97.8%-100%)
	10 copies	3/6	50%	(18.8%-81.3%)	100%	(97.8%-100%)
Coxiella burnetti	100 copies	12/12	100%	(75.8%-100%)	100%	(97.9%-100%)
Borrelia burgdorferi	100 copies	12/12	100%	(75.8%-100%)	100%	(97.9%-100%)
	50 copies	8/12	67%	(39.1%-86.2%)	100%	(97.9%-100%)

Table 19: Analytical sensitivity (LoD): genomic DNA copies of each pathogen included in the PCR reaction with a positive result in 100% of the cases analyzed with hybriSoft software, establishing a positivity cut-off point at a value of 4.

12.2 Analytical functioning in hybriSpot 24 (HS24)

The functioning and sturdiness of the Bacterial CNS Flow Chip was validated in the automated platform HS24 by analyzing limit concentrations of genomic DNA from all the bacteria and fungus included in the panel (10 copies for *N. meningitidis*, *L. monocytogenes*, *Mycobacterium tuberculosis complex*, *C. neoformans*, *S. pneumoniae* and *H. influenzae*, 50 copies for *T. pallidum* and *S. agalactiae* and 100 copies for *B. burgdorferi* and *C. burnetti*). 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.

- **Reproducibility of results in program for a different number of samples**

Replicas of a positive sample that contained a limit concentration of *S. agalactiae* (50 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.

- **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 genotypes in different positions.

Organism	No GE/reaction	Positive/tested	Difference between positions
Neisseria meningitidis	10	4/4	No
Streptococcus pneumoniae	10	4/4	No
Listeria monocytogenes	10	4/4	No
Mycobacterium tuberculosis complex	10	4/4	No
Streptococcus agalactiae	50	4/4	No
Haemophilus influenzae	10	4/4	No
Cryptococcus neoformans	10	4/4	No
Treponema pallidum	50	4/4	No
Coxiella burnetti	100	4/4	No
Borrelia burgdorferi	100	4/4	No

Table 20: Reproducibility of Bacterial CNS Flow Chip Kit in HS24. The positivity was analyzed with hybriSoft by establishing as a cut-off point a value of 4.

12.3 Analytical functioning in hybriSpot 12 PCR AUTO (HS12a)

The functioning and the robustness of the Bacterial CNS Flow Chip was validated in the automatic platform HS12a by analyzing limit concentrations of synthetic DNA fragments of all the pathogens included in the panel. This validation also proves the reproducibility of the results with different programs for different number of samples.

11.3.1. Reproducibility of results in program for a different number of samples

Replicates of a positive sample containing a limit number of copies of *Neisseria meningitidis'* DNA (10 copies) were analyzed. These replicas were placed in different positions of the reaction chamber of the HS12a system and different protocols were evaluated:

- Protocol for 2 samples (2 replicas)
- Protocol for 12 samples (3 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.

11.3.2. Verification of sensitivity limit

The functioning and the robustness of the Bacterial CNS Flow Chip was validated in the automatic platform HS12a by analyzing limit concentrations of synthetic DNA fragments of all the pathogens included in the panel.

Six replicas of each positive sample were made. The whole process was performed automatically in two different HS12a systems, and the results were analyzed with hybriSoft.

Organism	No. copies/ reaction	Positive/ Tested
Neisseria meningitidis	10	6/6
Streptococcus pneumoniae	10	6/6
Listeria monocytogenes	10	6/6
Mycobacterium tuberculosis	10	6/6
Streptococcus agalactiae	50	6/6
Haemophilus influenzae	10	6/6
Cryptococcus neoformans	10	6/6
Treponema pallidum	50	6/6
Coxiella burnetti	100	6/6
Borrelia burgdorferi	100	6/6

Table 21: Verification of sensitivity limit of Bacterial CNS Flow Chip kit in HS12a. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 4. NT: not tested

12.4 Clinical performance

The assessment of the functioning of the kit was performed with a total of 402 clinical cases retrospectively and prospectively previously analyzed with a reference method, the bacterial culture.

12.4.1 Diagnostic Specificity

The diagnostic specificity is expressed as a percentage (numeric fraction multiplied by 100), calculated as 11 x the number of true negative values (TN, true negative, negative result obtained by the reference kit used and the evaluated kit) divided into the sum of the number of true negative values (TN) plus the number of false positive values (FP, false positive, negative result obtained by the reference kit used and positive by the evaluated kit), or 100 x TN/(TN + FP).

Organism	TN	FP	Diagnostic specificity
Neisseria meningitidis	397	0	100%
Streptococcus pneumoniae	383	0	100%
Listeria monocytogenes	395	0	100%
Mycobacterium tuberculosis complex	392	0	100%
Streptococcus agalactiae	398	0	100%
Haemophilus influenzae	401	0	100%
Cryptococcus neoformans	401	0	100%
Treponema pallidum	402	0	100%
Coxiella burnetti	401	0	100%
Borrelia burgdorferi	402	0	100%

Table 22: Diagnostic specificity of Bacterial CNS Flow Chip Kit.

1.1.1 Diagnostic Sensitivity

The diagnostic sensitivity is expressed as a percentage (numeric fraction multiplied by 100), calculated as 100 x the number of true positive values (TP, true positive, positive result obtained by the reference kit used and the evaluated kit) divided into the sum of the number of true positive values (TP) plus the number of false negative values (FN, false negative, positive result obtained by the reference kit used and negative by the evaluated kit), or 100 x TP/(TP + FN).

Organism	TP	FN	Diagnostic sensitivity
Neisseria meningitidis	5	0	100%
Streptococcus pneumoniae	19	0	100%
Listeria monocytogenes	7*	0	100%
Mycobacterium tuberculosis complex	10	0	100%
Streptococcus agalactiae	4**	0	100%
Haemophilus influenzae	1	0	100%
Cryptococcus neoformans	1	0	100%
Treponema pallidum	NT	NT	NT
Coxiella burnetti	1***	0	100%
Borrelia burgdorferi	NT	NT	NT

Table 23: Diagnostic Sensitivity of Bacterial CNS Flow Chip Kit. NT: not tested * A case of positive *Listeria monocytogenes* according to the reference method and negative with the Bacterial CNS Flow Chip kit. It was not confirmed with the 2nd method used, Sepsis Flow Chip kit. ** A case of positive *Streptococcus agalactiae* according to the reference method and negative with Bacterial CNS Flow Chip kit. It was not confirmed with the 2nd used method, Sepsis Flow Chip kit.*** A negative case according to the reference method and positive with the Bacterial CNS Flow Chip kit. It was not confirmed with the 2nd method used, Tick-borne Flow Chip kit.

13 LIMITATIONS

Use of inappropriate samples: the method has been validated with purified DNA material from CSF. The analysis of any other type of sample can give wrong or non-conclusive results for suppression of the PCR reaction through suppressive chemical agents.

14 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 properly. Verify the functioning of the hybriSoft equipment. Repeat the test.
	Hybridization reagents expired or not stored appropriately.	Verify the expiration date as well as the proper storage of the hybridization reagents and 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 pathogen 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 mother PCR mix has been prepared properly and that the reagents are stored correctly. Repeat the test.
	Presence of PCR inhibitors in the test sample.	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.	Verify that the extraction system of genetic material work properly , including an extraction control.
	Presence of PCR inhibitors in the test sample.	
Weak signals in the hybridization	PCR reagents and/or expired or stored improperly.	Check the expiration date of the reagents, the storage of the PCR mix and reagents,
	Error in the hybridization protocol.	Check the hybridization temperatures and times and verify the functioning of the hybriSoft equipment.
	The PCR product was not denatured correctly before the hybridization.	Verify that the denaturation has been performed correctly. Repeat the test.
	Low quality/quantity of the DNA used.	Increase the amount of sample or starting DNA. Verify the correct functioning of the extraction system of nucleic acids used.

16 BIBLIOGRAPHY

- Durand ML, Calderwood SB, Weber DJ, *et al*. Acute bacterial meningitis in adults: a review of 493 episodes. N Engl J Med 1993; 328:21–8.
- Loring KE. In: Tintinalli JE, Kelen GD, Stapczynski JS. CNS infections. Emergency medicine: a comprehensive study guide. 6th edition. New York: McGraw-Hill; 2004. p. 1431–7
- Lavoie FW, Caucier JR. In: Marx JA, Hockberger RS,Walls RM, *et al*. Central nervous system infections. 6th edition.Philadelphia: Mosby Elsevier; 2006. p. 1710–25
- Pickering LK, Baker CJ, Long SS, *et al*. Haemophilus influenzae infections. Report of the Committee on Infectious Diseases. 27th edition. Elk Grove Village (IL): American Academy of Pediatrics; 2006. p. 310–8
- Pickering LK, Baker CJ, Long SS, *et al*. Pneumococcal infections. 27th edition. Elk Grove Village (IL): American Academy of Pediatrics; 2006. p. 525-37
- Bøving MK1, Pedersen LN, Møller JK. Eight-plex PCR and liquid-array detection of bacterial and viral pathogens in cerebrospinal fluid from patients with suspected meningitis. J Clin Microbiol. 2009 Apr;47(4):908-13
- Matthijs C. Brouwer, Allan R. Tunkel and Diederik van de Beek. Epidemiology, Diagnosis, and Antimicrobial Treatment of Acute Bacterial Meningitis. *Clin. Microbiol. Rev.* 2010, 23(3):467
- Abdeldaim GM, Strålin K, Korsgaard J, Blomberg J, Welinder-Olsson C, Herrmann B. Multiplex quantitative PCR for detection of lower respiratory tract infection and meningitis caused by Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis. BMC Microbiol. 2010 Dec 3;10:310
- Wang X, Theodore MJ, Mair R, Trujillo-Lopez E, du Plessis M, Wolter N, Baughman AL, Hatcher C, Vuong J, Lott L, von Gottberg A, Sacchi C, McDonald JM, Messonnier NE, Mayer LW. Clinical validation of multiplex real-time PCR assays for detection of bacterial meningitis pathogens. J Clin Microbiol. 2012 Mar;50(3):702-8
- Zhu H, Wang Q, Wen L, Xu J, Shao Z, Chen M, Chen M, Reeves PR, Cao B, Wang L. Development of a multiplex PCR assay for detection and genogrouping of Neisseria meningitidis. J Clin Microbiol. 2012 Jan;50(1):46-51
- Wang Y, Guo G, Wang H, Yang X, Shao F, Yang C, Gao W, Shao Z, Zhang J, Luo J, Yang Y, Kong F, Zhu B. Comparative study of bacteriological culture and real-time fluorescence quantitative PCR (RT-PCR) and multiplex PCR-based reverse line blot (mPCR/RLB) hybridization assay in the diagnosis of bacterial neonatal meningitis. BMC Pediatr. 2014 Sep 8;14:224

17 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		

18 GLOSSARY

CNS: central nervous system
CSF: cerebrospinal fluid
DNA: deoxyribonucleic acid
RNA: ribonucleic acid
PCR: polymerase chain reaction
HS12: hybriSpot 12
HS12a: hybriSpot 12 PCR AUTO
HS24: hybriSpot 24
GE: genome equivalents
NBT-BCIP: nitroblue tetrazolium chloride- 5-Bromo-4-chloro-3-indolyl phosphate
Cod UNG: Cod Uracil-DNA Glycosylase
$MgCl_2$: magnesium chloride
dNTPs: Deoxynucleotide triphosphates
DNases: deoxyribonuclease
RNases: ribonucleases
dUTP: Deoxyuridine Triphosphate
CDC: Centers for disease control and prevention
CFU: Colony forming units

19 CHANGELOG

Date	Description
2021-12-30	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Storage temperature is modified in Section 5.