Sepsis Flow Chip Kit

Detection of bacteria, fungi and antibiotic resistance markers 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	**Ref. MAD-003936M-HS12-24**	**24 tests**
	Ref. MAD-003936M-HS12-48	**48 tests**
	Ref. MAD-003936M-HS24-24	**24 tests**
	Ref. MAD-003936M-HS24-48	**48 tests**

For in vitro diagnostic use only
Guideline 98/79/CE and ISO 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

Content

1 INTENDED USE

Sepsis Flow Chip is an in vitro diagnostic kit for human nosocomial infections based on multiplex PCR and reverse dot blot hybridization for simultaneous detection of bacteria, fungi, and major antibiotic resistance genes in a single assay. Sepsis Flow Chip system allows simultaneous detection of over 36 bacteria species (*Coagulase-Negative Staphylococci, Staphylococcus aureus, Streptococcus spp., Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Listeria monocytogenes, Enterococcus spp., Pseudomonas aeruginosa, Acinetobacter baumannii, Neisseria meningitidis, Stenotrophomonas maltophilia, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Enterobacteriaceae species, and Proteus/Morganella spp.*), fungi species (*Candida albicans and Candida spp*.) and twenty antibiotic resistance markers. Regarding the antibiotic resistance markers, the kit detects one gene for methicillin resistance (mecA), two genes for vancomycin resistance (vanA and vanB), two for β-lactam antibiotic resistance (blaSHV and extended-spectrum blaCTX- M), and fifteen genes for carbapenems resistance (kpc allele: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22 and 23, sme allele: 1, 2, 3, 4 and 5, nmc/imi allele: 1, 2, 3, 4, 5, 6, 7, 8 and 9, ges allele: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 and 26, vim allele: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45 and 46, gim allele: 1 and 2, spm, ndm allele: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15 and 16, sim, imp3, 15, 19_like allele: 1, 2, 3, 5, 6, 8, 9, 10, 11, 15, 19, 20, 21, 24, 25, 28, 29, 30, 40, 41, 42 and 47, oxa23_like allele: 23, 27, 49, 73, 133, 146, 165, 166, 167, 168, 169, 170, 171 and 225, oxa24_like allele: 24, 25, 26, 40, 72, 139 and 160, oxa48_like allele: 48, 162, 163 and 181, oxa51_like allele: 51, 60, 65, 66, 67, 68, 69, 70, 75, 76, 77, 78, 79, 80, 82, 83, 84, 88, 89, 90, 91, 92, 93, 94, 95, 98, 99, 106, 107, 108, 109, 110, 111, 112, 113, 115, 116, 117, 128, 130, 131, 132, 138, 144, 148, 149, 150, 172, 173, 174, 175, 176, 177, 178, 179, 180, 195, 196, 197, 194, 200, 201, 202, 203, 206, 208 and 223, oxa58_like allele: 58, 96, 97 and 164). The method is based on amplifying the target DNAs with two multiplex PCR reactions and subsequently hybridizing the biotinylated amplicons to specific DNA probes.

Organism	Target
Staphylococcus Coagulase-Negative	16S rDNA
Staphylococcus aureus	nuc
Streptococcus spp.	16S rDNA
Streptococcus pneumoniae	cpsA
Streptococcus agalactiae	16S rDNA
Streptococcus pyogenes	16S rDNA
Listeria monocytogenes	16S rDNA
Enterococcus spp.	16S rDNA
Pseudomonas aeruginosa	ecfX
Acinetobacter baumannii	16S rDNA
Neisseria meningitidis	16S rDNA
Stenotrophomonas maltophilia	16S rDNA
Escherichia coli	16S rDNA
Klebsiella pneumoniae	khe
Serratia marcescens	16S rDNA
Enterobacteriaceae	16S rDNA
Proteus spp./Morganella	16S rDNA
Candida spp.	18S-5.8S ITS rDNA
Candida albicans	18S-5.8S ITS rDNA

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

Microbiological status: Product not sterile.

2 TEST PRINCIPLE

The Sepsis Flow Chip is based on a methodology which involves the simultaneous amplification of at least 36 bacterial species and several fungal species plus twenty resistance markers by multiplex PCR followed by the hybridization in membranes with DNA specific probes through the DNA-Flow technology for hybriSpot platforms, both automatic and manual. The biotynilated amplicons generated after the PCR are hybridized in membranes containing an array of specific probes for each pathogen and resistance marker, 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-Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization. The results are analysed automatically with software hybriSoft.

3 COMPONENTS

The **Sepsis Flow Chip** kit is presented 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 multiplex PCR amplification and subsequent hybridization of 24 or 48 clinical samples. Each format of the kit contains the following components and references:

3.1 Reagents for multiplex PCR

- 24 tests (MAD-003936M-P-HS-24):

Name	Format	Tube color	Mix color	Reference
Mix 1 Multiplex PCR	3 strips x 8 tubes	Clear	Pink	MAD-003936M-MIX1-HS
Mix 2 Multiplex PCR	3 strips x 8 tubes	Clear	White	MAD-003936M-MIX2-HS

Table 2: Reagents provided in kits of 24 tests to performe the multiplex PCR (Manual and Auto).

- 48 tests (MAD-003936M-P-HS-48):

Name	Format	Tube color	Mix color	Reference
Mix 1 Multiplex PCR	6 strips x 8 tubes	Clear	Pink	MAD-003936M-MIX1-HS
Mix 2 Multiplex PCR	6 strips x 8 tubes	Clear	White	MAD-003936M-MIX2-HS

Table 3: Reagents provided in kits of 48 tests to perform the multiplex PCR (Manual and Auto).

- Both presentations include DNase/RNase-free double distilled water for the handling of clinical samples: RNASE/DNASE-FREE DISTILLED WATER; Ref: MAD-DDW; Vol 60mL. It is included 1 vial for 24 tests and 2 vials for 48 tests.

- **Sepsis Flow Chip kit (PCR Reagents):** it is commercialized in a format of strips of 8 tubes of 0.2 ml containing the lyophilized reagents corresponding to two PCR mixes – mix 1 and mix 2.
  - The tubes corresponding to the Mix 1 (clear) are disposed in a lyophilized sphere format in pink whose components are: PCR buffer, $MgCl_2$, dNTPs (U/T), DNase/RNase-free water, biotinylated and non-biotinylated primers, Hot Start Taq DNA Polymerase, Uracil DNA Glycosylase, and Amaranth dye. Primers included are specific for the amplification of at least 36 bacterial species (*Coagulase-Negative*

Staphylococci, Staphylococcus aureus, Streptococcus spp., Streptococcus pneumoniae, Streptococcus agalactiae, Streptococcus pyogenes, Listeria monocytogenes, Enterococcus spp., Pseudomonas aeruginosa, Acinetobacter baumannii, Neisseria meningitidis, Stenotrophomonas maltophilia, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Enterobacteriaceae species, and Proteus/Morganella spp.), several fungal species (*Candida albicans and Candida spp.),* one methicillin resistance gen (mecA), two vancomycin resistance genes (vanA and vanB), and two β-lactam resistance genes (blaSHV, blaCTX-M). Furthermore, it includes primers for amplifying a fragment of human genomic DNA (endogenous control).

- The tubes corresponding to the Mix 2 (clear) are disposed in a lyophilized sphere format in white whose components are: PCR buffer, $MgCl_2$, dNTPs (U/T), DNase/RNase-free water, biotinylated and non-biotinylated primers, Hot Start Taq DNA Polymerase, and Uracil DNA Glycosylase. Primers included are specific for the amplification of fifteen genes for carbapenems resistance (kpc, sme, nmc/imi, ges, vim, gim, spm, ndm, sim, imp, oxa23_like, oxa24_like, oxa48_like, oxa51_like, and oxa58_like). Furthermore, it includes an exogenous synthetic DNA, used as amplification exogenous control, and specific primers to amplify it.

3.2 Reagents for reverse hybridization

- 24 tests:
  - (MAD-003936M-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
Sepsis Chip (HS)	24 units	MAD-003936M-CH-HS-24

Table 4: Reagents provided in kits of 24 tests to perform the hybridization (compatible with the hybriSpot 12 platform).

  - (MAD-003936M-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
Sepsis Chip (HS)	24 units	MAD-003936M-CH-HS-24

Table 5: Reagents provided in kits of 24 tests to perform the hybridization (compatible with the hybriSpot 24 and hybriSpot 12 PCR AUTO platforms).

- 48 tests:
  - (MAD-003936M-H-HS12-48):

Name	Format	Reference
Hybridization Solution (Reagent A)	80 ml	MAD-003930MA-HS12-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS12-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS12-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS12-48
Reagent E	18 ml	MAD-003930ME-HS12-48
Washing Buffer II (Reagent F)	35 ml	MAD-003930MF-HS12-48
Sepsis Chip (HS)	2 x 24 units	MAD-003936M-CH-HS-24

Table 6: Reagents provided in kits of 48 tests to perform the hybridization (compatible with the hybriSpot 12 platform).

➢ (MAD-003936M-H-HS24-48):

Name	Format	Reference
Hybridization Solution (Reagent A)	115 ml	MAD-003930MA-HS24-48
Blocking Solution (Reagent B)	18 ml	MAD-003930MB-HS24-48
Streptavidin-Alkaline Phosphatase (Reagent C)	18 ml	MAD-003930MC-HS24-48
Washing Buffer I (Reagent D)	70 ml	MAD-003930MD-HS24-48
Reagent E	18 ml	MAD-003930ME-HS24-48
Sepsis Chip (HS)	2 x 24 units	MAD-003936M-CH-HS-24

Table 7: Reagents provided in kits of 48 tests to perform the hybridization (compatible with the hybriSpot 24 and hybriSpot 12 PCR AUTO platforms).

- **Sepsis Chip**: The kit includes a total of 24, 48 Chips or membranes (ref: MAD-003936M-CH-HS-24) containing an array of specific DNA probes for each one of the targets included in the analysis, as well as the ones corresponding to the amplification controls incorporated in this kit. The disposition of all of them on the Chip can be checked in the section 11 of this manual (INTERPRETATION OF THE RESULTS).
- **Flow Chip Hybridization Reagents:** contains all the necessary reagents for the reverse hybridization process through Flow-Through.

4 ADDITIONAL REQUIRED MATIERAL BUT NOT SUPPLIED AND OPTIONAL MATERIAL

4.1 Reagents and materials

A. Common reagents to platforms HS12, HS12a and HS24:

- Disposable gloves.
- DNasa/RNasa-free tubes of 0.2/0.5/1.5 ml.
- Pipette tips with DNasa/RNasa-free filters.

B. Specific Reagents to platforms HS12a and HS24:

- Washing Reagent (ref: MAD-003930WSH).

4.2 Equipment

A. Common equipment to platforms HS12, HS12a and HS24:

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

B. Specific Equipment:

- With Sepsis Flow Chip kit (Manual) (ref: MAD-003936M-HS12)
  - Manual Equipment for hybridization hybriSpot 12 (VIT-HS12).
  - Thermocycler
  - Thermal block to heat PCR tubes (can be substituted by a thermocycler)
  - Cold plate (4ºC)
  - Thermostatic bath / heater.
- With Sepsis Flow Chip kit (Auto: hybriSpot 24 and hybriSpot 12 PCR AUTO) (ref: MAD-003936M-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 (4ºC).

4.3 Additional and optional material

- Optionally, for clinical sample handling is possible to use the Transport and Dilution Medium (TDM) (Ref: MAD-003930TDM). Protocols for sample processing using this reagent are described in section 7. Sample preparation.

5 STORAGE AND STABILITY CONDITIONS

Sepsis Flow Chip kit consists of 2 components that are supplied in separate boxes:

- Multiplex PCR Reagents: Shipment at 2-8 °C*. Upon receipt, they must be stored at 2-8 °C. They will be stable until the specified expiration date. The PCR reagents must be stored in areas free of DNA or PCR products contamination. **Once the package containing the tubes strip with the lyophilized PCR mix is opened, store the remaining tubes up to a maximum of one week at 2-8 °C in the original package.**
- Hybridization Reagents: Shipped and stored at 2-8°C*. **Do not freeze**. Reagents and Chips are stable until expiry date. Previous considerations on the hybridization reagents:
  - The hybridization reagent A must be pre-heated in a thermostatized bath or heater (only before using in manual equipment) at 51°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.

*A temperature indicator is included in the package to control the conditions during the 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 the product.**
- **The safety and disposal of wastes recommendations are described in the Safety Data Sheet of this product.** This product is exclusively targeted at a professional use in a laboratory, and not as a drug, for domestic use or other purposes. The current version of the safety data sheet of this product can be downloaded from the website www.vitro.bio or requested at regulatory@vitro.bio.
- **Sepsis Flow Chip kit** uses as starting materials nucleic acids previously extracted and purified, bacterial colonies, or clinical samples requiring from a previous manipulation for its analysis. Protocols are provided for manipulation of the different types of clinical specimens whose processing has been validated with this kit (see section 7).
- **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 where the PCR reaction is performed. It is recommended to work on differnt pre- and post-PCR areas where the handling of the problema DNA and preparation of the PCR tubes (pre-PCR) and

the handling and hybridization of the amplified products (post-PCR) are carried out. These areas must be physically separated, and different laboratory material must be used (laboratory coats, pipettes, 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 and the pre-PCR area must be avoided. Also, in order to avoid the contamination with previous PCR products, the enzyme uracil-DNA glycosylase, 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 sample, in order to detect and control any possible contamination of the reagents with problema 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, we verify that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.

- **Precaution**: the use of ethylene oxide for the preparation of clinical samples and/or the PCR mix could interfere in the right development of the PCR reaction. It is recommended to avoid using this compound for such purposes.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by Vitro S.A, S.L., 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 18 December 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 reagets 2. Disposal of Liquid Wastes ("Wastes" in the manual and automatic equipments).	161001	"Aquose liquid wastes containing dangeours 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. Used Chips 4. Perishable material (tubes, tips, aluminium 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 dangerouss (according to the Safety Data Sheet)	150110	"Packaging containing residues of or contaminated by dangerous substances"

Table 8: Classification of wastes generated by this kit according to the European Legilastion*ELW: English acronym for *European Legislation of Waste*.

Note: This classification is included as general pattern 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 Blood cultures

The Sepsis Flow Chip has been designed and validated for its use in **direct PCR with diluted samples of blood cultures**. For processing blood cultures from adults, we recommend doing a 1:100 dilution routinely.
Procedure:

7.1.1 Shake the blood culture thoroughly until you obtain a homogeneous mixture, take a volume of 100 µl and transfer it to an Eppendorf tube.

7.1.2 Dilute the blood culture 1:100 in DNase/RNase-free double distilled water in a final volume of 1 ml:

- 1:100: 10 µl blood culture + 990 µl **DNase/RNase-free double distilled water**, shake in vortex.

7.1.3 Add 30 µl of this dilution, previously homogenized, to the PCR tube with the lyophilized Master Mix 1 and 2.

If there is PCR inhibition, we recommend preparing a 1:1000 dilution from the previously ditluted blood culture (100 µl blood culture 1:100 + 900 µl **DNase/RNase-free double distilled water**, shake in vortex).

Add 30 µl of this dilution, previously homogenized, to the PCR tube with the lyophilized Master Mix1 and 2.

If pediatric samples are analyzed, the dilution to be applied to the blood culture is 1:1000.

7.1.4 Shake the blood culture thoroughly until you obtain a homogeneous mixture, take a volume of 100 µl and take it to an Eppendorf tube.

7.1.5 Dilute the blood culture 1:100 in **DNase/RNase-free double distilled water** in a final volume of 1 ml:

- 1:100: 10 µl blood culture + 990 µl **DNase/RNase-free double distilled water**, shake in vortex.

7.1.6 Dilute 1:10 the blood culture previously diluted 1:100 in **DNase/RNase-free double distilled water** in a final volume of 1 ml:

- 1:10: 100 µl blood culture 1:100 + 900 µl **DNase/RNase-free double distilled water**, shake in vortex.

7.1.7 Add 30 µl of this dilution, previously homogenized, to the PCR tube with the lyophilized Master Mix 1 and 2.

NOTE: If the blood cultures are not going to be analysed in the moment, it is possible to store an aliquot of the dilution 1:100 at 4 °C for a maximun of two days or frozen at -20 °C for at least three months. After thawing the aliquot, it is recommended to shake it to homogenize the sample.

The following Thermocyclers have been validated with the Sepsis Flow Chip kit:

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

The Sepsis Flow Chip kit works properly with the following blood culture media:

- BD BACTEC™ Plus Aerobic/F y Plus Anaerobic/F Medium (Becton Dickinson)
- BD BACTEC Peds Plus™/F Medium Bactec (Becton Dickinson)
- BacT/ALERT® FA Plus Aerobic and FN Plus Anaerobic (bioMérieux)
- BacT/ALERT® PF Plus (bioMérieux)

7.2 Rectal exudates

Sepsis Flow Chip has been validated for its use in **direct PCR starting from suspensions of rectal exudates** with no need of DNA extraction. The recommended protocol for the swab processing is as follows:

7.2.1 Place the swab in 0.5 ml D**Nase/RNase-free double distilled water**.

7.2.2 Shake the swab into the tube so that the cells spread in the liquid.

7.2.3 Dilute the resulting suspension 1:50 in **DNase/RNase-free double distilled water** (with this dilution, the concentration of potential inhibitors in this type of samples is reduced): 10 µl sample + 490 µl **DNase/RNase-free double distilled water**, shake in vortex.

7.2.4 Add 30 µl of this dilution, previously homogenized, to the PCR tube with the lyophilized Master Mix 1 and 2.

*NOTE: If the swabs are not going to be analysed in that moment, it is possible to store them frozen at -20 °C for at least three months. After freezing them, add the 0.5 mL of **DNase/RNase-free double distilled water** and homogenize the sample before diluting it. Diluted samples can be stored at 4 °C for a maximun of two days or frozen at -20 °C for at least three months. If after diluting 1:50 there are inhibitors left in the sample, it is recommended to dilute 1:2 from the 1:50 dilution or purify the DNA from the initial suspension (0.5 ml).*

In case of working with swabs with transport medium, it is recommended to shake the swab manually or with vortex in its transport medium for a few seconds and proceed likewise as for dry swabs from section 7.2.3.

The kit can also be used with **DNA purified from rectal exudates**. It has been validated with the following extraction systems:

- NucliSENS® easyMag® (bioMérieux S.A.)
- MagNa Pure (Roche)
- Chelex® (Bio-Rad)

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.

7.3 Bacterial colonies

The Sepsis Flow Chip kit has been validated for its use in **direct PCR starting from bacterial colonies**. The recommended protocol for it is the following:

7.3.1 Take a small quantity of the colony with sterile handle.

7.3.2 Resuspend each sample in 500 µl of **DNase/RNase-free double distilled water**.

7.3.3 Mix in vortex vigorously until obtaining a homogeneous cell suspension.

7.3.4 Add 30 µl of this dilution, previously homogenized, to the PCR tube with the lyophilized Master Mix 1 and 2.

7.3.5 Amplify following the instructions described in the section 8.1.

The boold cultures, rectal exudates and bacterial colonies must be treated as potential infectious agents. Guidelines for handling this type of specimens are available from the US Centers for Disease Control and Prevention (CDC). All hazardous or biologically contaminated materials should be disposed of in a safely and acceptable manner according to your institution's guidelines.

7.4 Protocols for sample processing using the Transport and Dilution Medium (TDM)

Optionally, the Transport & Dilution Medium TDM (Ref: MAD-003930TDM) may be used for processing the

different types of samples mentioned above. The steps to be followed for the processing with this reagent the different type of samples are described in table 9:

STARTING SAMPLE	FORMAT	PROTOCOLS FOR SAMPLE PROCESSING USING THE TRANSPORT AND DILUTION MEDIUM (TDM) (Ref: MAD-003930TDM)
Blood cultures	Aerobic and anaerobic medium	1. Shake the blood culture bottle gently until obtaining a homogenous sample. 2. Take a volume of 10 µl from the blood culture and add it to one of the vials with 900 µl of TDM. 3. Shake the resulting dilution in vortex and add 30 µl of this sample to the Mix1 of PCR and 30 µL to the Mix2 of PCR as template to perform the amplification.
Rectal exudates	Swabs without transport medium	1. Place the swab in one of the vials with 900 µl pre-aliquoted transport and dilution medium (TDM). 2. Shake the swab into the vial to produce the dispersion of cells in the liquid. 3. Take a volume of 35 µl from this sample and add to a new vial of TDM. 4. Shake the resulting dilution in vortex and use 30 µl of this sample for the Mix1 of PCR and other 30 µL for the Mix2 of PCR as template to perform the amplification.
	Swabs with transport medium	1. Shake manually or vortex the swab in its transport medium for a few seconds. 2. Add 18 µl of sample to one of the vials with 900 µl of TDM. 3. Use 30 µl of this dilution for the Mix1 of PCR and other 30 µL for the Mix2 of PCR as template, previously homogenized, to perform the amplification.

Table 9. Protocols for sample processing with the Transport and Dilution Medium (TDM).

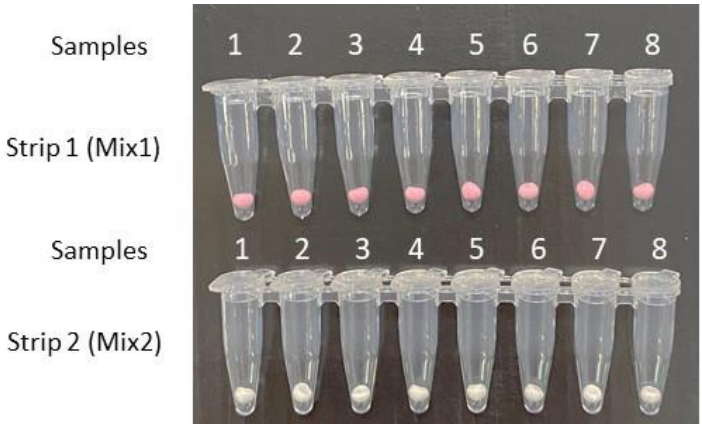
8 ANALYSIS PROCEDURE FOR HS12 AND HS24 PLATFORMS

8.1 Multiplex DNA amplification reaction

The PCR reaction is carried out in a final volume of 30 µl in PCR strip tubes of 0.2 mL containing the lyophilized PCR reaction Mix1 and Mix2. The pink and white lyophilized spheres correspond to the Mix 1 and Mix 2, respectively and are supplied in separated strips. Two PCR tubes must be used for each sample, one from each strip.

If the number of samples to be analyzed is lower or higher than 8, the necessary tubes can be separated from each strip with no need for using complete strips.

Once the strip is open, **the rest of the lyophilized tubes that are not going to be used at that moment must be stored for maximum of 1 week at 4°C in their original package.**
The diagram below shows an example of the distribution of samples/strips:



The process is as follows:

- Take one Mix 1 and Mix2 Multiplex PCR tube containing the lyophilized PCR mix per each sample to be analyzed.
- Add 30 µl of sample in each tube following the recommended protocol in section 7.
- Homogenize the mix by pipetting and centrifuge for a few seconds.
- If the number of samples to be analyzed is lower or higher than eight, the necessary tubes can be separated from the strip with no need for using complete strips. The rest of the lyophilized tube strip that is not going to be used at that moment must be stored for maximum of 1 week at 4 °C in its original package.
- Place the tubes in the thermocycler and set the following amplification conditions:

AMPLIFICATION PROGRAM IN THERMOCYCLER

1 cycle	25°C	10 min
1 cycle	94°C	5 min
40 cycles	94°C	30 s
	55°C	45 s
	72°C	1 min
1 cycle	72°C	7 min
	8°C	∞

Table 10: PCR program.

Keep the tubes refrigerated at 8-10 °C when the reaction is finished. If the samples are not going to be processed in that moment, they can be stored in the post-PCR zone at 8-10 °C for 1-2 days. To store them for a longer period of time, it is recommended to do so at -20 °C.

Important note: If purified DNA is used for PCR, 30 µl of this DNA can be added directly to the lyophilized PCR tubes.

8.2 Flow-through Reverse Hybridization

All the reagents are provided in a "ready-to-use" format.
The Chips are of a single use. They must be handled with gloves and away from any contamination source.

According to the type of kit we are working with, we will proceed as follows:

A. For Sepsis Flow Chip kit (Manual, ref: MAD-003936M-HS12):

The full hybridization process is performed semi-automatically in hybriSpot (HS12) following the instructions provided by the wizard of the system. The management of the samples, the capture of images and 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:

1. Pre-heat the Reagent A at 51° C (Hybridization Solution) for at least 20 min in a thermostatically controlled bath.
2. For each sample mix the PCR product of the Mix1 with the PCR product of the Mix2. Denature the PCR products heating them at 95°C for 8-10 min (in thermocycler or a heating block) and cool down quickly on ice for at least 2 min.
3. Place a Sepsis Chip for each sample to be tested on the pedestals included in the reaction chamber of the HS12 equipment.
4. Follow the instructions provided in the HS12 equipment manual to carry out the introduction of the samples' data, the image capture and the result analysis.

HYBRIDIZATION PROTOCOL:

a) Add 300 µl of Reagent A (Hybridization Solution) pre-heated at 51°C for at least 20 min. Incubate for at least 2 min at 51°C.
b) Remove the reagent A by activating the vacuum.
c) Add 50 µl corresponding to the mixture of PCR product from Mix1 and Mix2 (previously denatured and maintained on ice) to 230 µl of reagent A (51°C) and dispense the mix on the corresponding Sepsis Chip-HS.
d) Incubate at 51°C for 8 min.
e) Activate the pump to remove the PCR products (make sure that the pump is active for at least 30 seconds).
f) Wash 3x 300 µl with reagent A (51°C).
g) Set the temperature at 29°C.
h) Block the membranes for at least 5 min with 300 µl of reagent B (Blocking solution).
i) When the temperature reaches 29°C activate the pump to remove reagent B.
j) Add 300 µl of reagent C (streptavidin-phosphatase alkaline complex) and incubate for 5 min at 29°C.
k) Activate the pump to remove reagent C.
l) Set the temperature at 36°C.
m) Wash the membranes 4x 300 µl with reagent D (Washing solution I).
n) Develop the membranes adding 300 µl of reagent E (Developing Solution) and incubate for 10 min at 36°C.
o) Activate the pump to remove reagent E.
p) Wash the membranes with 2x 300 µl with reagent F (Washing solution II).
q) Activate the pump to remove the reagent.
r) Capture the membrane image and analyse result following instructions of the HS12 user manual.

B. For Sepsis Flow Chip kit (Auto, ref: MAD-003936M-HS24) in HS24 platform:

The whole hybridization process is performed automatically in hybriSpot 24platform (HS24). The sample management, image capture, result analysis and report are performed using the hybriSoft software.

Before starting the hybridization process:

1. **Configure the instrument following the user manual instructions (provided with the equipment).**
2. Denature the PCR products by heating them at **95 °C for 8-10 min** in a thermocycler or a heating block and cool quickly in ice for at least 2 min.
3. Follow the instructions provided in the instrument's user manual to carry out the samples data entry.
4. Place the previously denatured amplified samples, the Sepsis Chips and the reagents in their corresponding positions in hybriSpot 24.
5. 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 ANALYSIS PROCEDURE FOR THE PLATFORM HS12 PCR AUTO

The amplification by PCR and hybridization processes are performed automatically in the HS12 PCR AUTO platform.

The samples processing, images capture, 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:

1. Take a tube of Mix1 and Mix2 Multiplex PCR containing the lyophilized PCR mix per each sample to be analyzed.
2. Add the DNA samples to a PCR tube following the instructions described in section 8.1.
3. Homogenize the mix by pipetting and centrifuge for a few seconds.
4. If the number of samples to be analyzed is lower or higher than 8, the necessary tubes can be separated from the strip with no need for using complete strips. The rest of the lyophilized tube strip that is not going to be used at that moment must be stored for maximum of 1 week at 4 °C in its original package.
5. 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 POCEDURE

The Sepsis Flow Chip kit has several controls to monitor the quality of the results.

SPOTS	CONTROL	POSITION	INTERPRETATION
B	Hybridization control	1A-1B-2K-6F-10A	5 positions are right
CI	Exogenous Amplification Control	1C-6G	0, 1 or 2 positions are right
BG	Endogenous Amplification Control	1D-6H	0, 1 or 2 positions are right

Table 11: Control probes included in the Sepsis Chip.

Hybridization control: After the hybridization, an intense signal must appear in the five positions of the hybridization control (B), indicating that the hybridization process has worked properly. This signal

indicates that the hybridization and developing reagents have worked properly. If no signal appears, it means that there has been an error during the hybridization process or that the hybridization reagents have not been used properly. Moreover, these signals allow to the software to orient the probe panel to insure correct analysis.

Exogenous amplification control (CI): probe for detection of the synthetic DNA included in the PCR reaction. This DNA is co-amplified along with the genetic material of the sample. Two positive signals in the control of exogenous amplification (CI) indicate that the PCR has worked properly. A negative result in this control does not invalidate the result of the technique 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 DNA of the human beta-globin gene that is co-amplified during the PCR. All samples where the template DNA has been amplified correctly will have a positive signal in the endogenous amplification Control (BG). This signal is indicative of the DNA quality/quantity 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 lack of signal for this control indicates errors during the amplification, low quality/quantity of the DNA used in the amplification or lack of human DNA in the amplification. This last case can occur when the blood volume in the blood cultures is too low and taking into account the dilution that is made to the sample for the PCR. Nonetheless, a negative result for this control does not invalidate the result of the technique if the exogenous control has amplified correctly and/or the sample has been positive for any of the targets included in the panel.

The samples positive for some of the pathogens/resistance markers included in the kit must give signal for some of the specific probes. Moreover, the five hybridization controls (B) signals, two exogenous amplification control (CI) signals and two endogenous amplification control (BG) signals, must appear (as long as the sample contains human DNA). If no signal for the amplification controls appears, but it does appear for the pathogens/resistance markers, a message of *human DNA absence / PCR inhibitors presence* is included in the report. In that case, the user should verify the quality of the samples before validating the result.

When the samples are negative for all pathogens/resistance markers included in the kit, they will have the five positive signals for the hybridization control (B), and two signals for the exogenous amplification Control (CI). The endogenous amplification Control (BG) signals will also appear if the analysed sample contains human DNA.

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

11 INTERPRETATION OF RESULTS

Interpretation of results is done automatically through the analysis software hybriSoft. The following drawing shows the probes position in the Sepsis Chip:

	1	2	3	4	5	6	7	8	9	10
A	B		LIS	kpc	spm		ECOLI	vanB		B
B	B	ABAU	ENTEROC	sme	ndm		ENTEROB	vanA	ges	oxa23
C	CI	SMAR/ KLEB	PAER	nmc/ imi	sim			mecA	vim	oxa24
D	BG	SAGAL	KLEB	SPYOG	imp	SMALTO	CALB		gim	oxa48
E		STAPHYL	STREP	blaSHV		CAND		PROT/ MOR	kpc	oxa51
F	SPNEU	SA	NEIS	blaCTX		B	ABAU	LIS	spm	oxa58
G		ECOLI	PROT/ MOR	ges	oxa23	CI	SMAR/ KLEB	ENTEROC	sme	ndm
H	SMALTO	ENTEROB		vim	oxa24	BG	SAGAL	PAER	nmc/ imi	sim
I	CAND		mecA	gim	oxa48		STAPHYL	KLEB	SPYOG	imp
J		CALB	vanA		oxa51	SPNEU	SA	STREP	blaSHV	
K		B	vanB		oxa58			NEIS	blaCTX	

Image 1: Drawing of the probes disposition on the array. The specific probes for the study of pathogens and resistance genes and those probes used as amplification and hybridization controls are included. The coordinates of each of them are also included.

"B": hybridization control
"CI": Exogenous amplification Control
"BG": Endogenous amplification Control (fragment human ß-Globin)
"X": Specific probes for each bacteria, fungi and resistance marker

All probes are duplicated in order to guarantee the reliability in the automatic analysis of results. The hybridization control (B) is repeated in 5 positions and guides the software to correctly orient the probes panel for its subsequent analysis.

The following table (Table 12) shows the types of probes used and the positions in which these have been spotted on the Sepsis Chip. Likewise, the possible results obtained and their interpretation are indicated:

Expected Results (Organisms/Resistance)	Probe ID	Probe/positions (column-row)			
		Probe	B	CI	BG
Streptococcus pneumoniae	SPNEU	1F-6J	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Streptococcus pyogenes	SPYOG	4D-9I	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Stenotrophomonas maltophilia	SMALTO	1H-6D	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Candida spp.	CAND	1I-6E	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Acinetobacter baumannii	ABAU	2B-7F	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Serratia marcescens	SMAR/KLEB	2C-7G	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Klebsiella pneumoniae	SMAR/KLEB	2C-7G-3D-8I	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Klebsiella pneumoniae	KLEB	3D-8I	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Streptococcus agalactiae	SAGAL	2D-7H	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Coagulase-negative staphylococci	STAPHYL	2E-7I	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Staphylococcus aureus	SA	2F-7J	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Escherichia coli[1]	ECOLI	2G-7A	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Enterobacteria	ENTEROB	2H-7B	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Candida albicans	CALB	2J-7D	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Listeria monocytogenes	LIS	3A-8F	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Enterococcus	ENTEROC	3B-8G	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Pseudomonas aeruginosa	PAER	3C-8h	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Streptococcus spp.	STREP	3E-8J	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Neisseria meningitidis	NEIS	3F-8K	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Proteus spp.	PROT/MOR	3G-8E	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
Morganella morganii	PROT/MOR	3G-8E-2H-7B	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
RESISTANCE GENE METHICILLIN mecA	mecA	3I-8C	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
RESISTANCE GENE VANCOMYCIN vanA	vanA	3J-8B	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
RESISTANCE GENE VANCOMYCIN vanB	vanB	3K-8A	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS A CARBAPENEMASE KPC	kpc	4A-9E	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS A CARBAPENEMASE SME	sme	4B-9G	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS A CARBAPENEMASE NMC/IMI	nmc/imi	4C-9H	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
SHV ß-LACTAMASE	blaSHV	4E-9J	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CTX-M EXTENDED-SPECTRUM ß-LACTAMASE	blaCTX	4F-9K	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS A CARBAPENEMASE GES	ges	4G-9B	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE VIM	vim	4H-9C	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE GIM	gim	4I-9D	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE SPM	spm	5A-9F	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE NDM	ndm	5B-10G	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE SIM	sim	5C-10H	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS B CARBAPENEMASE IMP3, 15, 19_like	imp3	5D-10I	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS D CARBAPENEMASE OXA23_like	oxa23	5G-10B	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS D CARBAPENEMASE OXA24_like	oxa24	5H-10C	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H

CLASS D CARBAPENEMASE OXA48_like	oxa48	5I-10D	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS D CARBAPENEMASE OXA51_like	oxa51	5J-10E	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
CLASS D CARBAPENEMASE OXA58_like	oxa58	5K-10F	1A-1B-2K-6F-10A	-- / 1C-6G	-- / 1D-6H
NOT VALID RESULTS (Note: Lack of Human DNA control. Lack of exogenous control)	--	--	1A-1B-2K-6F-10A	--	--
Negative SEP (Note: Lack of human DNA control)	--	--	1A-1B-2K-6F-10A	1C-6G	--
Image not available/Defective image/Hybridization error	--	--	--	--	--

Table 12: Position of the probes in the Sepsis Chip and results interpretation.

[1] *Sepsis Flow CHIP kit* will not distinguish *Escherichia coli* from *Shigella spp.* When the patient is under clinical suspicion and we obtain a positive result for *E. coli,* the possibility of *Shigella* infection should be assessed.

Other possible results:

1. When a sample is positive for *S. pneumoniae* two positive probes can appear in the Chip, SPNEU: specific probe for *S. pneumoniae* and STREP: generic probe for species of the *Streptococcus genus*. Nonetheless, in these cases, we cannot discard the fact that in the sample there may be a co-infection of *S. pneumoniae* with others *Streptococcus spp*.
2. When a sample is positive for *S. agalactiae* two positive probes can appear in the Chip, SAGAL: specific probe for *S. agalactiae* and STREP: generic probe for species of the *Streptococcus genus*. Nonetheless, in these cases, we cannot discard the fact that in the sample there may be a co-infection of *S. agalactiae* with others *Streptococcus spp*.
3. When a sample is positive for *S. pyogenes* two positive probes can appear in the Chip, SPYOG: specific probe for *S. pyogenes* and STREP: generic probe for species of the *Streptococcus genus*. Nonetheless, in these cases, we cannot discard the fact that in the sample there may be a co-infection of *S. pyogenes* with others *Streptococcus spp*.
4. When a sample is positive for *S. aureus* two positive probes can appear in the Chip, SA: specific probe for *S. aureus* and STAPHYL: generic probe for species of the *Staphylococcus* genus. Nonetheless, in these cases we cannot discard the fact that in the sample there may be a co-infection of *S. aureus* with others *Staphylococcus spp*.
5. When a positive signal for the probe STAPHYL alone, mecA alone or both probes, appear in the Chip, the most probable interpretation is Coagulase-negative *Staphylococcus*.
6. Oxa51 resistance gene has been detected until now only in: *A. baumannii*, *E. coli and P. aeruginosa.* In *Acinetobacter baumannii* this gene is chromosomally encoded while in *E. coli* and *P. aeruginosa* the resistance gene is found in plasmids. When a sample is positive for *A. baumannii*, two different positive probes can appear in the Chip. ABAU: specific probe for *A.* baumannii and oxa51: specific probe for oxa51. Some mutations have been described in the region of the 16S in which the specific ABAU probe has been designed. Therefore, if only a positive signal for oxa51 is observed in the Chip, with no signals for any of A. baumannii, E. coli or P. aeruginosa specific probes; it could correspond to an Acinetobacter baumannii strain wearing a mutation in this 16S region. In this case it is recommended to identify the pathogen by another method.
7. When a sample is positive for K. pneumoniae, E. coli, S. marcescens or Morganella morganii two different probes will appear in the Sepsis Chip: i) the specific probe for each bacteria (KLEB, ECOLI, SMAR/KLEB, PROT/MOR) and ii) the generic probe for Enterobacteriaceae (ENTEROB). Since Enterobacteriaceae probe has been validated to detect other Enterobacteria like *Citrobacter*,

Salmonella, *K. oxytoca* and *Enterobacter*, we cannot discard that, in a sample positive for *K. pneumoniae, E. coli, S. marcescens* or *Morganella morganii*, there may be a possible co-infection with another Enterobacteria that is recognized by the probe.

8. PROT/MOR probe can detect both *Proteus mirabilis* and *Morganella morganii*. The way to distinguish a pathogen from the other is that in a sample with a single infection with any of these two pathogens, *Morganella morganii* will also give a positive singal for the ENTEROB probe while *Proteus mirabilis* will not. However, we cannot distinguish a positive sample for *Morganella* morganii from another sample that presents a co-infection with Proteus and other Enterobacteria recognized by the ENTEROB probe.
9. SMAR/KLEB probe can detect both K. pneumoniae and S. marcescens. In a sample with a single infection with any of these two pathogens, the distinction is possible because K. pneumoniae also will give a positive signal for K. pneumoniae (KLEB) specific probe, while S. marcescens will only give the signal for SMAR/KLEB probe. However, we cannot distinguish K. pneumoniae from a sample that presents a co-infection with K. pneumoniae and S. marcescens.
10. The narrow-spectrum β-lactamase SHV-1 is found at a higher frequency (up to 80 to 90%) in strains of Klebsiella pneumoniae. For this reason, when a sample is positive for *K. pneumoniae*, it is also positive for the gen *shv*. In that case, the detection of SHV would not indicate necessarily a phenotypical evidence of production of extended-spectrum β-lactamase.
11. The existence of trace for microbial DNA in the Taq DNA polymerases has been described. Due to the fact that the detection method presents a high sensibility, sometimes, we could observe weak signals in the Chip of the generic probe for Enterobacteriaceae and the probe for *P. aeruginosa* (PAER). There could also be weak signals for *Staphylococcus spp.* and *Streptococcus spp*. probably caused by contamination of samples, materials or regents with those bacteria during its handling.

Bacteremias are normally caused by a single pathogen. Sometimes, it is possible to detect two or three microorganisms in blood culture samples, in these cases, one of them would be the causative agent of the infection, and the other/s would be associated with possible contaminations during the blood sample manipulation.

These generic probes have been tested with the following species:

- STAPHYL probe has been validated for detection of:
  - *S. epidermidis*
  - *S. haemolyticus*
  - *S. capitis*
  - *S. hominis-hominis*
  - *S. intermedius*

- ENTEROC probe has been validated for the detection of:
  - *E. faecalis*
  - *E. faecium*
- STREP probe has been validated for the detection of:
  - *S. pasteurianus*
  - *S. dysgalactiae*
  - *S. gallolyticus*
  - *S. macedonicus*

  - *S. mitis/oralis*
  - *S. salivarius*
  - *S. infantarium*
  - *S. pyogenes*
  - *S. intermedius*
- Other species of *Streptococcus* tested and NOT DETECTED with STREP probe:
  - *S. viridans*
  - *S. anginosus*
  - *S. parasanguinis*
- ENTEROB probe has been validated for the detection of:
  - *E. aerogenes*
  - *E. cloacae*
  - *K. oxytoca*
  - *K. pneumoniae*
  - *Morganella morganii*
  - *E. coli*
  - *S. marcescens*
  - *Citrobacter*
  - *Salmonella enterica*
- CAND probe has been validated for the detection of:
  - *C. tropicalis*
  - *C. parapsilosis*
  - *C. krusei*

Below, an example in which the analysed case has been positive for *Klebsiella pneumoniae* is shown.

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Sepsis Flow Chip Kit

LOTS

PCR:	SEP002L	10/10/2020
Chips:	E035-13 HS	10/10/2020
Reagent:	SEPH066-2	10/10/2020

SAMPLE DETAILS

ID SAMPLE: Muestra123 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

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

REPORT

SEP POSITIVE

SAMPLE POSITIVE FOR:

PATHOGENS:

Enterobacteriaceae, Klebsiella pneumoniae

RESISTANCE GENES:

Extended-spectrum ß-lactamase SHV

The sample is negative for the rest of pathogens and antibiotic resistance included in the SEPSIS flow chip test.

PROTOCOL

Detection of a panel of bacteria, fungi, and antibiotic resistance markers by multiplex-PCR and Automatic Reverse Dot Blot that includes:

- Gram positive bacteria: Coagulase negative Staphylococcus , Staphylococcus aureus, Enterococcus spp., Streptococcus spp., Streptococcus pneumoniae, Streptococcus agalactiae, Listeria monocytogenes.
- Gram negative bacteria: Pseudomonas aeruginosa, Acinetobacter baumannii, Stenotrophomonas maltophilia, Escherichia coli, Klebsiella pneumoniae, Serratia marcescens, Enterobacteriaceae, Proteus spp., Morganella morganii, Neisseria meningitidis
- Fungi: Candida spp., C.albicans
- Resistance markers: mecA, vanA, vanB, blaSHV, blaCTX-M, KPC, SME, NMC-IMI, GES, VIM, GIM, SPM, NDM, SIM, IMP, OXA23, OXA24, OXA48, OXA51, OXA58.

- Sample preparation/DNA purification:
- Add suspension of DNA (prepared according manufacturer´s instructions) for PCR amplification
- PCR protocol: 1x 25° 10 min; 1x 94° 5 min; 40x (94° 30 s-55° 45 s-72° 60 s); 1x 72° 7 min.
- REVERSE-DOT BLOT protocol:

Hybridization of the biotinylated PCR products to the Sepsis CHIP, Post-hybridization washes, Streptavidin-Alkaline Phosphatase incubation, NBT-BCIP development and Automatic analysis of results

ANTIBIOTIC SUSCEPTIBILITY PROFILE

Possible resistance to: Penicillins, and 1st generation of cephalosporins (Note: Mutated versions of SHV could confer resistance to penicillins and 1st, 2nd and 3rd generation of cephalosporins).

NOTES

FACULTATIVE:	Default Doctor, doctor	**Validated:**	5/3/2019
Performed by:	Default Tech, tech	**Processed:**	5/3/2019

Instr. : Mock **Serial Nº:** 000024 **hybriSoft:** HSHS 2.2.0.R00 / HSHS IPL 1.0.0.R05

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Sepsis Flow Chip Kit

LOTS

PCR:	SEP002L	10/10/2020
Chips:	E035-13 HS	10/10/2020
Reagent:	SEPH066-2	10/10/2020

SAMPLE DETAILS

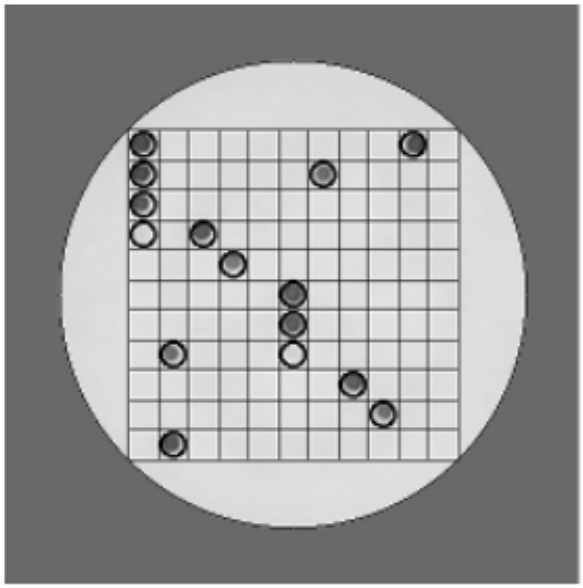
ID SAMPLE: Muestra123 **SAMPLE TYPE:**

ID PATIENT: **PATIENT:**

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

REPORT

B		LIS	kpc	spm		ECOLI	vanB		B	
B	ABAU	ENTEROC	sme	ndm		ENTEROB	vanA	ges	oxa23	
CI	SMAR/KLEB	PAER	nmc/ imi	sim			mecA	vim	oxa24	
BG	SAGAL	KLEB	SPYOG	img	SMALTO	CALB		gim	oxa48	
	STAPHYL	STREP	blaSHV		CAND		PROT/MOR	kpc	oxa51	
SPNEU	SA	MEIS	blaCTX		B	ABAU	LIS	spm	oxa58	
	ECOLI	PROT/MOR	ges	oxa23	CI	SMAR/KLEB	ENTEROC	sme	ndm	
SMALTO	ENTEROB		vim	oxa24	BG	SAGAL	PAER	nmc/ imi	sim	
CAND		mecA	gim	oxa48		STAPHYL	KLEB	SPYOG	img	
	CALB	vanA		oxa51	SPNEU	SA	STREP	blaSHV		
	B	vanB		oxa58			MEIS	blaCTX		



- 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:** 5/3/2019

Performed by: Default Tech, tech **Processed:** 5/3/2019

Instr. : Mock **Serial Nº:** 000024 **hybriSoft:** HSHS 2.2.0.R00 / HSHS IPL 1.0.0.R05

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical performance in hybriSpot 12 (HS12)

12.1.1 Repeatability

The repeatability of the method was analyzed by testing the method at least seven times for each pathogen included in the panel at two different concentrations. The test was performed by the same operator at a single location, on the same day, and using the same batch of reagents.

Organism	Equivalents genome/reaction	Number of positives/tested	% positive
Staphylococcus epidermidis	100	7/7	100%
	10	6/7	86%
Staphylococcus aureus	100	7/7	100%
	10	7/7	100%
Streptococcus pneumoniae	100	7/7	100%
	10	7/7	100%
Streptococcus agalactiae	100	7/7	100%
	50	7/7	100%
Streptococcus pyogenes	100	7/7	100%
	10	7/7	100%
Listeria monocytogenes	100	7/7	100%
	10	7/7	100%
Enterococcus faecalis	100	7/7	100%
	10	4/7	57%
Enterococcus faecium	100	7/7	100%
	10	5/7	71%
Pseudomonas aeruginosa	100	7/7	100%
	10	7/7	100%
Acinetobacter baumannii	100	7/7	100%
	10	7/7	100%
Neisseria meningitidis	500	7/7	100%
	100	5/7	71%
Stenotrophomonas maltophilia	100	7/7	100%
	10	7/7	100%
Escherichia coli	100	7/7	100%
	10	7/7	100%
Klebsiella pneumoniae	100	7/7	100%
	10	6/7	86%
Serratia marcescens	100	7/7	100%
	10	7/7	100%
Enterobacter cloacae	100	7/7	100%
	10	7/7	100%
Proteus mirabilis/Morganella	100	7/7	100%
	10	7/7	100%
Candida albicans	100	7/7	100%
	10	7/7	100%

Table 13: Repeatability test for each one of the pathogens included in the panel.

12.1.2 **Reproducibility**

The precision of the assay was tested simulating the inter-laboratory variability varying both the operator (1 and 2), as well as the lot of PCR mix used (SE005 and SE008) and the thermocycler (Veriti TC-13 and Biometra TC-21) for each condition. Nine of the pathogens included in the panel were tested 8 times and at two different concentrations using purified genomic DNA from clinical isolates and 24 negative samples. All the valid results were included to calculate the percentage of positive results. No false positive results were obtained. The percentages of positive results are indicated in table 14. The concordance for both conditions is very good, kappa index of 0.93, SD of 0.07 and CI 95% of 0.8-1.07.

Organism	GE /reaction	Condition		
		Laboratory	Positive/Valid	% positivity
E. coli	10	1	6/8	75
		2	6/8	75
	50	1	8/8	100
		2	8/8	100
P. mirabilis/Morganella	10	1	8/8	100
		2	8/8	100
	50	1	8/8	100
		2	8/8	100
S. pneumoniae	10	1	6/8	75
		2	7/8	87.5
	50	1	8/8	100
		2	8/8	100
S. pyogenes	10	1	7/8	87.5
		2	7/8	87.5
	50	1	8/8	100
		2	8/8	100
L. monocytogenes	10	1	7/8	87.5
		2	6/8	75
	50	1	7/8	87.5
		2	8/8	100
S. maltophilia	10	1	6/8	75
		2	7/8	87.5
	50	1	8/8	100
		2	8/8	100
P. aeruginosa	10	1	8/8	100
		2	7/8	87.5
	50	1	8/8	100
		2	8/8	100
Staphylococcus Coagulase-Negative	100	1	8/8	100
		2	8/8	100
S. aureus	10	1	8/8	100
		2	8/8	100
	50	1	8/8	100
		2	8/8	100
C. albicans	10	1	8/8	100
		2	8/8	100

Table 14: Reproducibility test for bacteria included int he SEPSIS panel.

12.1.3 **Analytical Specificity**

No cross-reactions between the organisms included in the test were observed, when analyzed starting from 10^6 GE of each strain:

Organism	Specificity
Coagulase-Negative *Staphylococci*	100%
Staphylococcus aureus	100%
Streptococcus spp.	100%
Streptococcus pneumoniae	100%
Streptococcus agalactiae	100%
Streptococcus pyogenes	100%
Listeria monocytogenes	100%
Enterococcus spp.	100%
Pseudomonas aeruginosa	100%
Acinetobacter baumannii	100%
Neisseria meningitidis	100%
Stenotrophomonas maltophilia	100%
Escherichia coli	100%
Klebsiella pneumoniae	100%
Serratia marcescens	100%
Enterobacteriaceae	100%
Proteus spp./Morganella	100%
Candida spp.	100%
Candida albicans	100%

Table 15: Specificity of Sepsis Flow Chip.

No cross-reactions with other bacteria, viruses and fungi that could be present in the hospital environment were detected. For the assay, we started from 10^6 GE/reaction of genomic DNA for each organism.

Organism		
Bacterium	**Virus**	**Fungi**
Haemophilus influenzae	Herpes simplex-1	*Cryptococcus neoformans*
Mycobacterium tuberculosis	Herpes simplex-2	
Coxiella burnetti	Epstein Barr virus	
Borrelia burgdorferi	Varicella Zoster virus	
Treponema pallidum		

Table 16: Specificity of Sepsis Flow Chip.

12.1.4 **Analytical Sensitivity**

The limit of detection was calculated for each one of the analysed pathogens. The determination of the minimum number of copies detected was performed through serial dilutions of the genomic DNA of each one of the strains included in the panel with 5 ng of human genomic DNA. In order to calculate the sensitivity and CI, each sample was repeated between 5 and 14 times. All the PCRs were hybridised using the platform hybriSpot 12. The results were analysed with hybriSoft and the established value to consider the positive signals was of 4 (grey intensity).

Organism	Probe	EG/ reaction	Positive/ Tested	Sensitivity	Confidence Interval 95%	Specificity	Confidence Interval 95%
S. epidermidis	mecA	10	14/14	100%	78.5%-100%	100%	98.1%-99.9%
	mecA	100	14/14	100%	78.5%-100%	100%	98.1%-99.9%
	STAPHYL	10	10/14	71%	45.4%-88.3%	100%	98.7%-100%
	STAPHYL	100	14/14	100%	78.5%-100%	100%	98.7%-100%
S. aureus	SA	10	14/14	100%	78.5%-100%	100%	98.8%-100%
	SA	100	6/6	100%	61%-100%	100%	98.8%-100%
	STAPHYL	10	2/14	14%	4%-39.9%	100%	98.7%-100%
	STAPHYL	100	6/6	100%	61%-100%	100%	98.7%-100%
S. pneumoniae	SPNE	10	14/14	100%	78.5%-100%	100%	98.8%-100%
	SPNE	100	6/6	100%	61%-100%	100%	98.8%-100%
	STREP	10	13/14	93%	68.5%-98.7%	96%*	93.1%-97.7%
	STREP	100	6/6	100%	61%-100%	96%*	93.1%-97.7%
S. agalactiae	SAGAL	50	14/14	100%	78.5%-100%	100%	98.8%-100%
	SAGAL	100	6/6	100%	61%-100%	100%	98.8%-100%
S. pyogenes	SPYOG	10	14/14	100%	78.5%-100%	100%	98.8%-100%
	SPYOG	100	6/6	100%	61%-100%	100%	98.8%-100%
L. monocytogenes	LIS	10	14/14	100%	78.5%-100%	100%	98.6%-100%
	LIS	100	6/6	100%	61%-100%	100%	98.6%-100%
E. faecalis	ENTEROC	10	2/14	14%	4%-39.9%	100%	98.6%-100%
	ENTEROC	100	14/14	100%	78.5%-100%	100%	98.6%-100%
E. faecium	ENTEROC	50	4/6	67%	30%-90.4%	100%	98.6%-100%
	ENTEROC	100	14/14	100%	78.5%-100%	100%	98.6%-100%
P. aeruginosa	PAER	10	14/14	100%	78.5%-100%	97%**	94.3%-98.3%
	PAER	100	6/6	100%	61%-100%	97%**	94.3%-98.3%
A. baumannii	ABAU	10	14/14	100%	78.5%-100%	100%	98.9%-100%
	ABAU	100	6/6	100%	61%-100%	100%	98.9%-100%
N. meningitidis	NEIS	100	8/10	80%	49%-94.3%	100%	98.8%-100%
	NEIS	500	10/10	100%	74.2%-100%	100%	98.8%-100%
S. maltophilia	SMALTO	10	14/14	100%	78.5%-100%	100%	98.8%-100%
	SMALTO	100	5/5	100%	56.5%-100%	100%	98.8%-100%
E. coli	ECOLI	10	14/14	100%	78.5%-100%	100%	98.6%-100%
	ECOLI	100	6/6	100%	61%-100%	100%	98.6%-100%
K. pneumoniae	KLEB	10	12/14	86%	60.1%-96%	100%	98.6%-100%
	KLEB	100	6/6	100%	61%-100%	100%	98.6%-100%
S. marcescens	SMAR/KLEB	10	8/8	100%	67.6%-100%	100%	98.8%-100%
	SMAR/KLEB	100	14/14	100%	78.5%-100%	100%	98.8%-100%
E. cloacae	ENTEROB	10	14/14	100%	78.5%-100%	97%**	94.3%-98.5%
	ENTEROB	100	6/6	100%	61%-100%	97%**	94.3%-98.5%
***P. mirabilis*/Morganella**	PROT/MOR	10	17/17	100%	81.6%-100%	100%	98.8%-100%
	PROT/MOR	100	6/6	100%	61%-100%	100%	98.8%-100%
C. albicans	CALB	10	14/14	100%	78.5%-100%	100%	98.9%-100%
	CALB	100	6/6	100%	61%-100%	100%	98.9%-100%
	CAND	10	14/14	100%	78.5%-100%	100%	98.9%-100%
	CAND	100	6/6	100%	61%-100%	100%	98.9%-100%

Table 17: Analytical sensitivity (LoD): number of gemonic equivalents giving positive results in 100% of the replicates, analysing with hybriSoft software and a cut-off point of positivity of 4.

* The probe of *Streptococcus spp.* shows a 96% of specificity by contamination with minimal quantities of *Streptococcus* spp. during the handling of the samples, reagents or plastics.

**The probes PAER and ENTEROB show a 97% of specificity due to the presence of trace quantities for microbial DNA in the commercial thermostable polymerases. The bacterial contamination source is thought to be any step of the purification process or any reagent added to the enzyme. After carrying out alignments of of three Taq polymerases, it has been observed that the contaminant DNA presents homology with species of *Pseudomonas* and other phytobacteria, *Escherichia coli, Salmonella* and *Shigella*. (Spangler et al 2009. PLoS ONE, 4(9): e7010).

12.2 Analytical Performance in hybriSpot 24

The performance and robustness of the Sepsis Flow Chip kit in the automatic equipment HS24 was validated by analysing limit concentrations of synthetic fragments of the main pathogens causative of human nosocomial infections included in the panel. This validation shows the reproducibility of the results between the positions 1 and 24 of the equipment HS24 and the reproducibility of the results with different programs for different number of samples.

12.2.1 Reproducibility of results in programs for different number of samples

Replicates of a positive sample containing a number of limit copies of *E. coli* (10 GE/reaction) were performed. These replicates were placed in different positions of the reaction chamber in the HS24 equipment and four different protocols were assessed:

- Protocol for 2 samples (2 replicates)
- Protocol for 6 samples (2 replicates)
- Protocol for 12 samples (3 replicates)
- Protocol for 15 samples (4 replicates)
- Protocol for 24 samples (5 replicates)

The results were analysed automatically with hybriSoft and no differences between the different positions of the reaction chamber or between the protocols used were detected.

12.2.2 Reproducibility of results in different positions of hybridization in HS24

Twelve to twenty-two replicates were tested for different pathogens, placed in different positions of the two reaction chambers of the HS24, in several runs and with the protocol for 24 samples. The results were automatically analyzed with hybriSoft, showing a percentage of reproducibility of 100% for all the analyzed samples in different positions except for one replica of *Klebsiella pneumoniae*, for which the reproducibility was 95.5%.

Bacterium	GE/reaction	Positive/tested	Differences between positions
S. aureus	10	12/12	No
S. pneumoniae	10	12/12	No
S. agalactiae	50	12/12	No
S. pyogenes	10	12/12	No
A. baumannii	10	12/12	No
K. pneumoniae	100	21/22	Slight
P. mirabilis/Morganella	10	12/12	No
C. albicans	10	12/12	No
P. aeruginosa	10	12/12	No
S. epidermidis	100	12/12	No
E. coli	10	12/12	No

Table 18: Reproducibility of Sepsis Flow Chip in HS24. The results were analysed automatically with hybriSoft and a cut-off point of positivity of 4.

12.3 Analytical performance in hybriSpot 12 PCR AUTO (HS12a)

The performance and robustness of Sepsis Flow Chip were 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 a different number of samples.

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

Replicates of positive samples that containing several resistance markers at limit concentrations were made. 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 differences of intensity between the different positions of the reaction chamber or the used protocol are acceptable.

- **Verification of analytical sensitivity**

For this assay, 3 replicas of each pathogen of the panel at a limit concentration were made. The amplification and hybridization process were made in different positions of the equipment for the different targets.
The whole process was performed automatically in in two different HS12a instruments, and the results were analyzed with hybriSoft.

Organism	Probe	EG/ reaction	Positive/ Tested
S. epidermidis	mecA	100	3/3
	STAPHYL	100	3/3
S. aureus	SA	10	3/3
S. pneumoniae	SPNE	10	3/3
S. agalactiae	SAGAL	50	3/3
S. pyogenes	SPYOG	10	3/3
L. monocytogenes	LIS	10	3/3
E. faecalis	ENTEROC	100	3/3
E. faecium	ENTEROC	100	3/3
P. aeruginosa	PAER	10	3/3
A. baumannii	ABAU	10	3/3
N. meningitidis	NEIS	500	3/3
S. maltophilia	SMALTO	10	3/3
E. coli	ECOLI	10	3/3
K. pneumoniae	KLEB	100	3/3
S. marcescens	SMAR/KLEB	10	3/3

E. cloacae	ENTEROB	10	3/3
***P. mirabilis*/Morganella**	PROT/MOR	10	3/3
C. albicans	CALB	10	3/3
	CAND	10	3/3

Table 19: Reproducibility of Sepsis Flow Chip in HS12a. The results were analyzed automatically with hybriSoft establishing a positivity cut-off value of 4.

12.4 Clinical performance

12.4.1 Clinical Specificity and Sensitivity in Blood Cultures

One hundred and ninety-six samples of blood cultures (168 positive and 28 negative) that have been previously analysed with a phenotypical reference method, were analysed with the Sepsis Flow Chip kit in a retrospective study. The diagnostic specificity is expressed as a percentage (numerical fraction multiplied by a hundredfold), calculated as 100 x the number of true negative values (TN) divided by the sum of the number of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP). The diagnostic sensibility is expressed as a percentage (numerical fraction multiplied by a hundredfold), calculated as 100 × the number of true positive values (TP) divided by the sum of the number of true positive values (TP) plus the number of false negative (FN), or 100 × TP/ (TP + FN).

Organism	TN	FP	TP	FN	Diagnostic Specificity	Diagnostic Sensitivity
***Staphylococcus* Coagulase-Negative**	144	0	51	1	100%	98%
Staphylococcus aureus	185	0	11	0	100%	100%
***mec*A**	156	0	40	0	100%	100%
Streptococcus spp.	190	0	6	0	100%	100%
Streptococcus pneumoniae	192	0	4	0	100%	100%
Streptococcus agalactiae	196	0	0	0	100%	NT
Streptococcus pyogenes	194	0	2	0	100%	100%
Listeria monocytogenes	196	0	0	0	100%	NT
Enterococcus spp.	184	0	12	0	100%	100%
Pseudomonas aeruginosa	192	0	4	0	100%	100%
Acinetobacter baumannii	190	0	6	0	100%	100%
Neisseria meningitidis	196	0	0	0	100%	NT
Stenotrophomonas maltophilia	195	0	1	0	100%	100%
Escherichia coli	145	0	51	0	100%	100%
Klebsiella pneumoniae	189	0	7	0	100%	100%
***bla*CTX-M**	191	0	5	0	100%	100%
***bla*SHV**	188	0	8	0	100%	100%
Serratia marcescens	194	0	2	0	100%	100%
Enterobacteriaceae	185	0	11	0	100%	100%
Proteus mirabilis	193	0	3	0	100%	100%
Morganella morganii	194	1	2	0	99.5%	100%
Candida spp.	196	0	0	0	100%	NT
Candida albicans	190	0	6	0	100%	100%

Table 20: Diagnostic specificity and sensitivity of Sepsis Flow CHIP in blood cultures samples. NT: Not tested.

12.4.2 **Identification of resistance mechanisms with Sepsis Flow CHIP**

To assess the detection of the totality of the resistance genes included in the panel, a collection of 217 clinical isolates, previously characterized with phenotypic and molecular standard methods, was analysed. These were carriers of all the antibiotic resistance markers included in the detection panel of the Sepsis Flow Chip kit. The clinical isolates had been collected between 2011 and 2015, and the origin of the isolation corresponded to rectal carriers or blood cultures. Different strains of the ATCC (n=6) were included as negative controls.

From the total of 217 samples, the kit correctly identified all the bacterial strains. Regarding the resistances, it correctly detected 31 out of 31 Gram positive strains with different resistance markers and 28 out of 28 Gram negative strains carriers of *bla*CTX-M and/or *bla*SHV. From the rest of Gram negative strains carbapenemases' carriers, the kit correctly detected 157 from a total of 158 (table 21). The only one that was not detected was a strain of *Klebsiella pneumoniae* with IMP gene. When sequencing this gene, we verified that it was the imp-4 allele, which is not included in the detection panel of the kit. No false positive results were obtained. The Sepsis Flow Chip method showed a sensitivity and specificity of 100% in a collection of clinical isolates containing all the antibiotic resistance markers covered by the kit.

Tested resistance markers	Results with SFC kit
Tested gram positive strains	
vanA	*Enterococcus spp.* vanA (1)
vanB	*Enterococcus spp.* vanB (2)
mecA	*S. aureus* mecA (3), *Staphylococcus* CoNS (25)
Tested gram negative strains	
CTX-M	*E. coli* (20)
SHV	*K. pneumoniae* (5), *E. coli* (2)
CTX-M + SHV	*E. coli* (1)
KPC + SHV	*K. pneumoniae* (1)
SME	*S. marcescens* (2)
NMC	*E. asburiae* (1), *E. cloacae* (1)
GES	*A. baumannii* (2), *E. coli* (1)
IMP + OXA-51	A. baumannii (1)
IMP + SHV	*K. pneumoniae* (2)
IMP	*K. oxytoca* (1), *P. aeruginosa* (1)
VIM + CTX-M	*E. cloacae* (1)
VIM	*K. pneumoniae* VIM (13), *E. coli* VIM (2), *P. aeruginosa* VIM (63), *E. cloacae* (9), *K. oxytoca* (5)
SPM	*P. aeruginosa* (2)
SIM	*A. baumannii* (1)
NDM	*E. coli* (2)
OXA-23 + OXA-51	*A. baumannii* (1)
OXA-24 + OXA-51	*A. baumannii* (2)
OXA-48	*K. pneumoniae* (21), *E. coli* (2)

OXA-51	*A. baumannii* (9)
OXA-58	*A. baumannii* (1)
OXA-58 + OXA-51	*A. baumannii* (11)
Negative Control Samples	
Lack of antibiotic-resistance genes	ATCC 12401, ATCC 29213, ATCC 35659, ATCC 49619, ATCC BAA-751 and ATCC 25922

Table 21: Antibiotic resistance genes identified with the Sepsis Flow Chip.

12.4.3 **Clinical Specificity and Sensitivity in Rectal Exudates**

A total of 73 rectal exudates (34 positive and 39 negative), which had been previously analysed with phenotypical and molecular reference methods for the detection of extended spectrum betalactamases and carbapenemases, were analysed in a retrospective study.

The clinical specificity and sensitivity of the Sepsis Flow Chip kit was determined according to the formulae described in section 11.3.1.

	Gold Standard Methods		
Sepsis Flow Chip kit	Absent	Present	Total
Positive Test	**2**	**34**	36
Negative Test	**39**	**0**	39
Total	41	34	75

	Estimated value	Confidence Interval 95%	
		Inferior Limit	Superior Limit
Sensitivity	**1**	0.873	1
Specificity *	**0.951***	0.822	0.991

Table 22: Diagnostic Specificity and Sensitivity of Sepsis Flow Chip in rectal exudates samples.

*The clinical specificity is 95.1% due to the detection of two false positive for wide-spectrum SHV corresponding to the chromosomally encoded gene in strains of *K. pneumoniae* that does not confer resistance to extended-spectrum betalactamics.

12.4.4 **Validation of Sepsis Flow Chip kit for its use in direct PCR from colonies**

The Sepsis Flow Chip kit has been validated for its use starting directly from cell suspesions. For that, a total of 40 colonies of clinical isolates were tested coming from 7 Spanish hospitals (H. Carlos Haya, H. Valle Hebrón, H. Virgen del Rocío, H. Donostia, H. Virgen de las Nieves, HUCA and H. San Pedro). The different microorganisms tested either did not contain antibiotic resistance markers or were carriers of one or various resistance genes. Different lots of kits were used in the different hospitals for validation: Lot SEP011, Lot SEP012, Lot SEP014, Lot SEP015 and Lot SEP016. The thermocyclers used were those that are routinely used in the hospital, mainly different models of Applied Biosystems (Veriti, GeneAMP® PCR System).

Following the protocol of direct PCR from colony (section 7.3), the Sepsis Flow chip kit correctly detected 100% of the bacterial genus found in all the clinical isolates (n=40). Regarding the antibiotic resistance

genes, the kit correctly detected 58 our 59 genes. The gene that was not detected was blaTEM, the reason is that it is a marker that is not included in the detection panel of the kit.

Tested strains and resistance markers	Results with SFC kit
Enterococcus spp. (1)	*Enterococcus spp.* (1)
Streptococcus pneumoniae (1)	*Streptococcus spp., Streptococcus pneumoniae* (1)
Candida albicans (1)	*Candida spp., Candida albicans* (1)
Klebsiella pneumoniae, SHV (3)	*Klebsiella pneumoniae*, SHV (2) *Klebsiella pneumoniae*, Enterobacteriaceae, SHV (1)
Klebsiella pneumoniae, SHV, CTX-M (6)	*Klebsiella pneumoniae*, SHV, CTX-M (6)
Klebsiella pneumoniae, SHV, CTX-M, Oxa48 (1)	*Klebsiella pneumoniae*, SHV, CTX-M, Oxa48 (1)
Klebsiella pneumoniae, SHV, Oxa48 (2)	*Klebsiella pneumoniae*, Enterobacteriaceae, SHV, Oxa48 (2)
Klebsiella pneumoniae, SHV, VIM (3)	*Klebsiella pneumoniae*, Enterobacteriaceae, SHV, VIM, GES (1) *Klebsiella pneumoniae*, Enterobacteriaceae, SHV, VIM (2)
Klebsiella pneumoniae, SHV, CTX-M, VIM (1)	*Klebsiella pneumoniae*, SHV, CTX-M, VIM (1)
Klebsiella pneumoniae, SHV, KPC (2)	*Klebsiella pneumoniae,* SHV, KPC (2)
Klebsiella pneumoniae, BLEE (1)	Enterobacteriaceae, IMP19 (1)
Enterobacteria, CTX-M (1)	Morganella morganii, CTX-M (1)
P. aeruginosa (1)	*P. aeruginosa* (1)
P. aeruginosa, VIM (1)	*P. aeruginosa*, VIM (1)
Proteus mirabilis, CTX-M-32 (1)	Proteus, CTX-M (1)
Salmonella enterica, CTX-M-10 (1)	Enterobacteriaceae, CTX-M (1)
Salmonella enterica, SHV-12 (1)	Enterobacteriaceae, SHV (1)
Enterobacter cloacae, CTX-M-15 (1)	Enterobacteriaceae, CTX-M (1)
Enterobacter cloacae, Oxa48 (1)	Enterobacteriaceae, Oxa48 (1)
Enterobacter cloacae, CTX-M-15, Oxa48 (1)	Enterobacteriaceae, CTX-M, Oxa48, GES (1)
E. coli, TEM (1)	*E. coli*, Enterobacteriaceae (1)
E. coli, Oxa48 (2)	*E. coli*, Oxa48 (2)
E. coli, CTX-M (2)	*E. coli*, Enterobacteriaceae, CTX-M (2)
E. coli, CTX-M, Oxa48 (1)	*E. coli*, Enterobacteriaceae, CTX-M, Oxa48 (1)
E. coli, SIM, Oxa48 (1)	*E. coli*, SIM, Oxa48 (1)
E. coli, SHV, KPC, VIM (1)	*E. coli*, Enterobacteriaceae, SHV, KPC, VIM (1)
Acinetobacter, Oxa23, oxa51 (1)	*Acinetobacter*, Oxa23, oxa51 (1)

Table 23: Clinical isolates identified with Sepsis Flow Chip kit in direct PCR from colonies.

13 LIMITATIONS

Use of inappropriate samples: the method has been validated with diluted samples of blood cultures, purified genetic material from rectal exudates, diluted samples of rectal exudates and colonies (see section 7). The analysis of any other type of sample not indicated can lead to wrong or inconclusive results due to PCR inhibition by inhibiting chemical agents.

14 PROBLEMS AND SOLUTIONS

Problem	Causes	Solutions
No signal is observed/ There is no hybridization signal	Error in the hybridization protocol	Check that all hybridization reagents have been added in the right order (manual platform). Check the performance of equipment (automatic platform). Repeat the test.
	The hybridization reagents have expired or have not been stored properly	Check the expiration date and the storage conditions of the reagents and the chips. Repeat the test.
	Possible Chips DNA degradation during the decontamination process of surface and material.	Clean the reaction chambers with abundant distilled water. Repeat the test.
Presence of resistances in negative control	Contamination problems in the pre-PCR or post-PCR zones.	Decontaminate (1% bleach) the working areas and repeat the test.
There is no signal of amplification exogenous control	Problems in the amplifications by PCR.	Check that the thermocycler program is the right one, that the PCR masterMix has been prepared properly and that the PCR reagents are correctly stored. Repeat the test.
	Presence of PCR inhibitors in the test sample.	If the starting sample corresponds to a 1:50 dilution of a rectal exudate suspension, it is recommended to dilute 1:2 from the 1:50 dilution or purify the DNA from the initial suspension (0.5 ml) with any of the validated extraction systems (see Sample Preparation in section 7). If we start from purified DNA, check that the extraction system of the genetic material used correctly works including an extraction control.
There is no signal of amplification endogenous control	Insufficient quantity of human DNA in the test sample.	Repeat the PCR increasing the quantity of the starting sample or decreasing the initial dilution of the sample. In any case, when it comes to rectal exudates samples, do not use dilutions lower than the 1:50 dilutions.
	Presence of PCR inhibitors in the test sample.	
Weak signals in the hybridization	PCR reagents and/or hybridisation expired or stored incorrectly.	Check the reagents' expiration date, the storage of PCR mix and reagents.
	Error in the hybridisation protocol.	Check the temperatures and times of hybridization and check the performance of the hybriSpot equipment.
	The PCR product was not correctly denatured before the hybridisation.	Check that the denaturation has been done correctly. Repeat the test.
	Low quality/quantity of the DNA used.	Increase the quantity of sample of starting DNA. Check the right performance of the nucleic acids extraction system used.

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16 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	Σ	Sufficient content for <n> assays
	Material safety data sheet		

17 GLOSSARY

DNA: deoxyribonucleic acid
PCR: Polymerase Chain Reaction
HS12: hybriSpot 12 (manual platform)
HS24: hybriSpot 24 (automatic platform)
HS12a: hybriSpot 12 PCR AUTO (automatic platform)
NBT-BCIP: Nitro blue tetrazolium 5-bromo-4-chloro-3-indolyl-phosphate chloride
$MgCl_2$: magnesium chloride
dNTPs: deoxynucleotide triphosphate
DNases: Deoxyribonuclease
RNases: ribonucleases
dUTP: deoxyuridine triphosphate
CDC: U.S. Centres for Disease Control and Prevention
GE: Genome equivalents
TP: true positives
TN: true negatives
FP: false positives
FN: false negatives
ATCC: American Type Culture Collection
SFC: Sepsis Flow Chip

18 CHANGELOG

Date	Description
2022-01-04	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Room temperature is modified in Section 5