MDR Direct Flow Chip Kit

Detection of multidrug-resistant pathogens by multiplex PCR and reverse hybridization.

for manual and automatic hybriSpot platforms

Compatible with hybriSoft HSHS versions 2.02.00.R09.01 and later.
For compatibility with other versions, please contact the manufacturer / supplier.

REF **Ref. MAD-003946M-HS12**
Ref. MAD-003946M-HS

Σ **24 determinations**
24 determinations

For in vitro diagnostic use only
Directive 98/79/EC and ISO 18113-2

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IVD

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1 INTENDED USE

"Multidrug Resistance (MDR) Direct Flow Chip is an in vitro diagnostic kit that allows rapid qualitative detection of multidrug-resistant bacteria. It is based on multiplex PCR and includes the detection of 5 bacterial species *(S. aureus, K. pneumoniae, P. aeruginosa, E. coli and A. baumannii*) and a total of 55 resistance markers including the main "enzymatic" type mechanisms described for nine different antibiotic classes: β-lactams, glycopeptides, oxazolidiones, macrolides, aminoglycosides, sulfonamides, fluoroquinolones, polymyxins, chloramphenicol, as well as point mutations most frequently detected in fluoroquinolone-resistant strains of *E. coli* and *P. aeruginosa*. Among these, the kit detects fifteen genes that offer resistance to carbapenem (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 tests is performed directly on rectal exudates, nasopharyngeal exudates/aspirates and/or blood cultures in infected patients or patients at risk for colonization as a measure to help in the control and prevention of this type of infections. The method is based on amplification of DNA targets through multiplex PCR reaction, and subsequent reverse dot-blot hybridization on a membrane that contains specific probes.

Genetic determinant	Associated antibiotic resistance
aac (6´)-Ib	Aminoglycosides
armA	
rmtB	
rmtC	
rmtF	
blaCMY	B-lactam antibiotics
blaDHA	
blaCTX	Cephalosporins
blaSHV-SK	
blaSHV-S	
blaSHV	
ges	Cephalosporins /Carbapenems
gim	Carbapenems
imp_like	
kpc	
ndm	

nmc/imi	
oxa23_like	
oxa24_like	
oxa48_like	
oxa51_like	
oxa58_like	
sim	B-lactam antibiotics
sme	Carbapenems
spm	
vim	
catB3	Chloramphenicol
mcr1	Colistin
mcr2	
gyrE-S83L	Quinolones
gyrE-S83L-D87G	
gyrE-S83L-D87G, parES80I	
gyrE-S83L-D87N	
gyrE-S83W-D87G	
gyrP-T83I	
gyrP-T83I-D87G	
gyrP-T83I-D87N	
parE-S80I	
cfr	Macrolides/lincosamide/streptogramin
ermA	Macrolides
ermB	
ermC	
mefA/E	
msrA	
mecA	B-lactam antibiotics
mecC	
oqxA	Phenicol/quinolone
oqxB	
qnrA	Quinolones
qnrB	
qnrS	
sul1	Sulfonamides
sul2	
sul3	
vanA	Vancomycin
vanB	
Target	**Organism**
nuc	*Staphylococcus aureus*
ecfX	*Pseudomonas aeruginosa*

ompA	*Acinetobacter baumannii*
gyrA	*Escherichia coli*
khe	*Klebsiella pneumoniae*

Table 1: Target genes used for amplification of antibiotic and bacterial resistance markers in MDR Direct Flow Chip kit.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The MDR Direct Flow Chip kit is based on a methodology consisting of simultaneous amplification of *S. aureus, K. pneumoniae, P. aeruginosa, E. coli* and *A. baumannii* and 56 resistance markers by multiplex PCR directly from cell extracts, bacterial colonies and/or blood culture, followed by membrane hybridization with specific DNA probes using DNA-Flow technology for hybriSpot platforms, both automated and manual. The biotinylated amplicons generated after the PCR are hybridized in membranes containing an array of specific probes for each target as well as amplification and hybridization control probes. The DNA-Flow technology allows a very 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 by an immunoenzymatic colorimetric reaction with Streptavidin−Phosphatase and a chromogen (NBT-BCIP) generating insoluble precipitates in the membrane in those positions in which there has been hybridization. The results are analyzed automatically with the hybriSoft™ software.

3 COMPONENTS

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

KIT/COMPONENTS	FORMAT	REFERENCES
MDR Direct Flow Chip kit (Manual)	24 tests	**MAD-003946M-HS12**
1. MDR Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003946M-P**
MDR PCR Mix 1	3 strips × 8 tubes (green)	MAD-003946M-MIX1
MDR PCR Mix 2	3 strips × 8 tubes (yellow)	MAD-003946M-MIX2
2. MDR Chips	**24 tests**	**MAD-003946M-CH-HS**
3. Flow Chip Hybridization Reagents Type I (Manual)	**24 tests**	**MAD-003925M-HS12**
Hybridization Solution (Reagent A)	40ml	MAD-003930MA-HS12-24
Blocking Solution (Reagent B)	10ml	MAD-003930MB-HS12-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10ml	MAD-003930MC-HS12-24
Washing Buffer I (Reagent D)	35ml	MAD-003930MD-HS12-24
Reagent E	10ml	MAD-003930ME-HS12-24
Washing Buffer II (Reagent F)	18ml	MAD-003930MF-HS12-24

Table 2: Reagents provided in the MDR Direct Flow Chip kit (Manual) format.

KIT/COMPONENTS	FORMAT	REFERENCES
MDR Direct Flow Chip kit (Auto)	**24 tests**	**MAD-003946M-HS12**
1. MDR Direct Flow Chip kit (PCR Reagents)	**24 tests**	**MAD-003946M-P**
MDR PCR Mix 1	3 strips ×	MAD-003946M-MIX1
MDR PCR Mix 2	3 strips × 8 tubes	MAD-003946M-MIX2
2. MDR Chips	**24 tests**	**MAD-003946M-CH-HS**
3. Flow Chip Hybridization Reagents Type I (Auto)	**24 tests**	**MAD-003925M-HS**
Hybridization Solution (Reagent A)	60ml	MAD-003930MA-HS24-24
Blocking Solution (Reagent B)	10ml	MAD-003930MB-HS24-24
Streptavidin-Alkaline Phosphatase (Reagent C)	10ml	MAD-003930MC-HS24-24
Washing Buffer I (Reagent D)	35ml	MAD-003930MD-HS24-24
Reagent E	10ml	MAD-003930ME-HS24

Table 3: Reagents provided in the MDR Direct Flow Chip kit (Auto: hybriSpot 24 and hybriSpot 12 PCR AUTO) format.

- 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.

- **MDR Direct Flow Chip kit (PCR Reagents):** it is commercialized in a format 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 Mix 1 (green) are arranged in a pink lyophilized sphere format with the following components: PCR buffer, polymerase, Uracil DNA glycosylase, dNTPs (U/T), DNase-free water, DNA from an exogenous amplification control and biotinylated primers. The primers included are specific for the amplification of: methicillin resistance gene (mecA, mecC), vancomycin resistance genes (vanA, vanB), class A Carbapenemase resistance genes (kpc, sme, nmc/imi, ges), class B Carbapenemase resistance genes (vim, gim, spm, ndm, sim, imp_like), class D Carbapenemase resistance genes (oxa23_like, oxa24_like, oxa48_like, oxa51_like, oxa58_like), SHV ß-Lactamase gene (blaSHV), extended-spectrum SHV ß-Lactamase genes (blaSHV-S, blaSHV-SK), extended-spectrum CTX-M ß-Lactamase gene (blaCTX), *Staphylococcus aureus* (SA), *Klebsiella pneumoniae* (kpneum), *Pseudomonas aeruginosa* (Paer), *Acinetobacter baumannii* (Abau). In addition, it includes primers to amplify a fragment of human genomic DNA (BG) as an endogenous control, and primers to amplify synthetic DNA (CI-1) added as an exogenous amplification control.

  - The tubes corresponding to Mix 2 (yellow) are arranged in a blue lyophilized sphere format with the following components: PCR buffer, polymerase, Uracil DNA glycosylase, dNTPs (U/T), DNase-free water, DNA from an exogenous amplification control and biotinylated primers. The primers included are those specific for the amplification of: Colistin resistance genes (mcr1, mcr2), sulfonamide resistance genes (sul1, sul2, sul3), β-Lactamase AMPC genes (blaDHA, blaCMY), macrolide resistance genes (msrA, mef, ermA, ermB, ermC), aminoglycoside resistance genes (aac, armA, rmtB, rmtC, rmtF), Escherichia coli gyrase A WT gene (gyrA) for detection of *Escherichia coli* and mutations associated with resistance to fluoroquinolones (gyrE-S83L, gyrE-S83L-D87G, gyrE-S83L-D87N, gyrE-S83W-D87G), Escherichia coli topoisomerase IV gene (parC) for detection of the quinolone resistance-associated mutation parE-S80I, Pseudomonas aeruginosa gyrase A gene (gyrA-Paer) for detection of fluoroquinolone resistance-associated mutations (gyrP-T83I, gyrP-T83I-D87N, gyrP-

T83I-D87G), quinolone or fluoroquinolone resistance genes (qnrA, qnrB, qnrS), olaquindox resistance genes (oqxA, oqxB), linezolid resistance gene (cfr), chloramphenicol resistance gene (catB3). In addition, primers to amplify a fragment of human genomic DNA (RNaseP) as an endogenous control and primers to amplify synthetic DNA (CI-2) added as an exogenous amplification control are included.

- **MDR Chips:** The kit includes a total of 24 Chips or membranes (ref: MAD-003946M-CH-HS) containing an array of specific DNA probes for each of the targets included in the analysis, as well as others corresponding to the amplification controls incorporated in this kit. The position of all them on the Chip can be referred to in the section 10 of this manual (INTERPRETATION OF RESULTS).

- **Flow Chip Hybridization Reagents:** It contains all the reagents necessary for the reverse hybridization process through Flow-Through.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED AND OPTIONAL MATERIAL

4.1 Reagents and materials

A. Common reagents for manual and automatic platforms:

- Disposable gloves.
- DNase/RNase-free filter pipette tips.
- For the manipulation of clinical samples: DNase/RNase-free double distilled water

B. Specific reagents (Auto, ref: MAD-003946M-HS24):

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

4.2 Equipment

A. Common equipment for manual and automatic platforms:

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

B. Specific equipment:

- With MDR Direct Flow Chip kit (Manual) (ref: MAD-003936M-HS12)
    - Thermal cycler.
    - Thermal block to heat PCR tubes (can be substituted by a thermal cycler).
    - Cooling plate (4 °C).
    - Manual equipment for hybridization hybriSpot 12 (VIT-HS12).
    - Thermostatic bath / heater.
- With MDR Direct 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).
    - Thermal cycler (not necessary for hybriSpot 12 PCR AUTO).
    - Thermal block to heat PCR tubes (not necessary for hybriSpot 12 PCR AUTO).

- Cooling plate (4 °C).

4.3 Optional additional material

- In order to manipulate clinical specimens, the product Transport and Dilution Medium (TDM) can be used (Ref: MAD-003930TDM). The working protocol, depending on the type of starting sample, is reflected in section 7. Sample preparation.

5 STORAGE AND STABILITY CONDITIONS

The MDR Direct Flow Chip kit consists of two components that are supplied in separate boxes:

- **MDR Direct Flow Chip kit (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 is opened, store the remaining tubes up to a maximum of one week at 2-8 °C in the original package.**
- **MDR Chips:** Shipped and stored at 2-8 °C*. **Do not freeze**. The hybridization Chips are stable until the expiration date indicated.
- **Hybridization reagents:** Shipped and stored at 2-8 °C*. **Do not freeze.** The hybridization reagents are stable until the expiration date indicated. Previous considerations 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 46 °C before its use.
  - The rest of hybridization reagents must be used at room temperature (15-25 °C).

***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 for 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.
- **MDR Direct Flow Chip kit** uses as a starting material nucleic acids previously extracted and purified or clinical samples that require a previous manipulation for their analysis. Processing protocols are provided for the different types of clinical samples which has been validated with this kit (see section 7.1).
- **General considerations to avoid the contamination with PCR product:**
  The greatest contamination source is normally the same amplified PCR product. Therefore, it is recommended to carry out the handling of the amplified products in a different area than the one the PCR reaction is performed. It is recommended to work on different pre- and post-PCR areas where the handling of the test DNA and preparation of the PCR tubes (pre-PCR) and the handling and hybridization of the amplified products (post-PCR) are performed. These areas must be physically separated and different laboratory material must be used (laboratory coats, pipettes, tips, etc.) to avoid the

contamination of the samples with the amplified DNA, which could lead to false positive diagnoses. The workflow must always go in a single direction, from the pre-PCR area to the post-PCR area and never the opposite way. The material and personal flow from the post-PCR area to the pre-PCR area must be avoided. Furthermore, 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 test samples or amplified products. The hybridization in membrane of this control must be negative, marking only the hybridization control and the amplification exogenous control. This way, it is verified that there is no contamination of DNA of patients and/or amplified DNA in the pre-PCR area.

- **Warning**: the use of ethylene oxide for the preparation of clinical samples and/or the PCR mix could interfere in the correct development of the PCR reaction. It is recommended to avoid the use of this component for these purposes.
- **Waste disposal:** The handling of wastes generated by the use of the products commercialized by Vitro S.A. must be performed according to the applicable law in the country in which these products are being used. As reference, the following table indicates the classification of wastes generated by this kit according to the European Law, specifically according to the *European Commission Decision of December 18 2014* amending decision 2000/532/CE on the list of waste pursuant to Directive 2008/98/EC of the European Parliament and of the Council:

POTENTIAL WASTE 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 platforms HS12 and HS24)	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, this waste must be considered as "waste 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	"Waste 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 containing waste or contaminated by dangerous substances"

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

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

7 SAMPLE PREPARATION

7.1 Rectal exudates

The MDR Direct Flow Chip kit has been validated for its use in **direct PCR from cell suspensions of rectal exudates** without being necessary the extraction of DNA. The recommended protocol for swabs processing is the following:

1. Place the swab in 0.5 ml of DNase/RNase-free double distilled water.
2. Shake the swab within the tube so that the cells spread in the liquid.
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.
4. Add 30 µl of this dilution, previously homogenized, to the two PCR tube with the lyophilized Master Mix (Mix 1 and Mix 2).

In case of processing 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 7.1.3.

NOTE: If some inhibitors remain after diluting 1:50, it is recommended to dilute 1:2 from the dilution 1:50 or purify the DNA from the initial suspension (0.5 ml).

The kit can also be used from **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 system. Therefore, if any other purification system is used, this must be previously verified.

7.2 Nasopharyngeal exudates/aspirates

The MDR Direct Flow Chip kit has been validated for its use in **direct PCR from cell suspensions of nasopharyngeal exudates/aspirates** without being necessary the extraction of DNA. The recommended protocol for processing of the swabs is the following:

1. Place the swab in 0.5 ml of DNase/RNase-free double distilled water.
2. Shake the swab within the tube so that the cells spread in the liquid.
3. Dilute the resulting suspension 1:5 in DNase/RNase-free double distilled water (with this dilution, the concentration of potential inhibitors in this type of samples is reduced): 100 µl sample + 400 µl DNase/RNase-free double distilled water, shake in vortex.
4. Add 30 µl of this dilution, previously homogenized in vortex, to the two PCR tube with the lyophilized Master Mix (Mix 1 and Mix 2).

In case of processing 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 swab from section 7.2.3.

7.3 Rectal exudates and nasal exudates as a single sample

The MDR Direct Flow Chip kit has been validated for its use in **direct PCR from a double sample extraction of rectal exudate and nasal exudate** from the same patient, without being necessary the extraction of DNA. The protocol applied for processing both swabs in parallel and subsequent analysis as a single sample was the following:

1. Place each of the swabs separately in 0.5 ml DNase/RNase-free double distilled water.
2. Shake each swab within the tube so that the cells spread in the liquid.
3. Add 25 µl of each of the suspensions obtained for each swab in a single Eppendorf tube with 450µl of DNase/RNase-free double distilled water, so that each one will be diluted 1:20. Shake in vortex.
4. Add 30 µl of this dilution, previously homogenized, to the PCR tubes with the lyophilized Master Mix (Mix 1 and Mix 2).

In case of processing swabs with transport medium it is recommended to shake the swabs manually or with vortex in the its transport medium for a few seconds and proceed likewise as for dry swabs from section 7.3.3.

NOTE: If some inhibitors remain after diluting 1:20, it is recommended to dilute 1:50 of rectal exudate and 1:20 of nasal exudate. To do this, add 10 µl of the suspension obtained from the rectal exudate and 25 µl of the suspension obtained of the nasal exudate in a single Eppendorf tube with 465µl of DNase/RNase-free double distilled water.

7.4 Blood cultures

The MDR Direct Flow Chip kit has been validated for its use in **direct PCR from blood cultures** without being necessary the extraction of DNA. The recommended protocol for processing blood cultures is the following:

1. Shake well the blood culture container until a homogenized mix is obtained, take a volume of 100 µl and take it to an Eppendorf tube.
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.
3. Add 30 µl of this dilution, previously homogenized, to the PCR tubes with the lyophilized Master Mix (Mix 1 and Mix 2).

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

4. Shake well the blood culture container until a homogenized mix is obtained, take a volume of 100 µl and take it to an Eppendorf tube.
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.
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. Add 30 µl of this dilution, previously homogenized, to the PCR tubes with the lyophilized Master Mix (Mix 1 and Mix 2).

7.5 Bacterial colonies

The MDR Direct Flow Chip kit has been validated for its use from bacterial colonies directly. The recommended protocol is the following:

1. Take a small quantity of the colony with sterile handle.
2. Re-suspend each sample in 500 µl DNase/RNase-free double distilled of water.
3. Shake well in vortex until a homogeneous cell suspension is obtained.
4. Add 30 µl of this dilution, previously homogenized, to the PCR tubes with the lyophilized Master Mix (Mix 1 and Mix 2).

Rectal exudates, nasopharyngeal exudates/aspirates and blood cultures should be treated as potential infectious agents. The guidelines for handling this type of samples can be consulted in the publications of the Center for Disease Control and Prevention (CDC) of the United States. All dangerous or biologically contaminated materials must be disposed safely and properly according to the guidelines of your institution.

7.6 Protocols for sample processing with transport and dilution medium (TDM)

Alternatively, the option Transport & Dilution Medium TDM can be used (Ref: MAD-003930TDM) for processing the different types of clinical samples described in previous sections. A table describing the steps for sample processing with this reagent depending on the type of starting sample is shown below (table 5).

STARTING SAMPLE	FORMAT	PROTOCOL SAMPLE PROCESSING WITH REAGENT TRANSPORT AND DILUTION MEDIUM (TDM) (Ref: MAD-003930TDM)
Rectal exudates	Swab without transport medium	1. Place the swab in one of the vials with 900 µl pre-aliquoted dilution and transport medium (TDM). 2. Shake the swab within the tube so that the cells spread 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 as a template to perform amplification.
	Swab with transport medium	1. Shake the pellet manually or with vortex 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, previously homogenized, for the PCR.
Nasopharyngeal exudates	Swab without transport medium	1. Place the swab in one of the vials with 900 µl of TDM medium. 2. Shake the pellet within the tube so that the cells spread in the liquid. 3. Take a volume of 500 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 as a template to perform amplification.
Rectal exudates and nasopharyngeal exudates as a single sample	Swabs without transport medium	1. Place the rectal swab in one of the vials with 900 µl of TDM and the nasopharyngeal swab in another TDM vial. 2. Shake each swab in the tubes so that the cells spread in the liquid. 3. Take a volume of 100 µl from each of the resulting cell suspensions and add them to a new vial of TDM. 4. Shake the resulting dilution in vortex and use 30 µl of this sample as a template to perform amplification.
Blood cultures	Aerobic medium and anaerobic medium	1. Shake well the blood culture vial until a homogeneous mix is obtained. 2. Take a volume of 10 µl blood culture and add it to one of the vials with 900 µl of TDM. 3. Shake the resulting dilution in vortex and use 30 µl of this sample as a template to perform amplification.
Bacterial colonies	-	1. Take a small quantity of the colony with sterile handle. 2. Re-suspend the sample in 900 µl of TDM. 4. Shake the resulting dilution in vortex and use 30 µl of this sample as a template to perform amplification.

Table 5. Protocols for sample processing with the dilution and transport reagent TDM.

8 ANALYSIS PROCEDURE

8.1 Multiplex DNA amplification reaction

The PCR reaction is carried out in a final volume of 30 µL in two 0.2 mL PCR tube strips containing the lyophilized PCR reaction mix. The pinkish and blue lyophilized spheres correspond to mix 1 and mix 2, respectively, and are supplied in separate strips. Two PCR tubes must be used for each sample, one for each strip.

The process is as follows:

- Take one tube, containing each of the lyophilized PCR mixtures, for each sample to be analyzed.
- Add up to 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 thermal cycler and set the following amplification conditions:

AMPLIFICATION PROGRAM IN THERMOCYCLER

- Whether working with individual PCR tubes or tubes strips, once the purified DNA or cell suspension has been added, place the tubes or strips in the thermal cycler and program the amplification conditions below:

1 cycle		25°C	10 min
1 cycle		95°C	3 min
40 cycles		95°C	15 s
		55°C	45 s
		72°C	1 min
		8°C	∞

Table 6: PCR program.

If you are not going to proceed with the hybridization directly, you can store the tubes or tubes strips with the amplified product in the post-PCR area at a temperature of 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.

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.
Depending on the type of kit with which we are working, we will proceed as follows:

A. For MDR Direct Flow Chip kit (Manual, ref: MAD-003946M-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, perform the following steps:

1. Pre-heat **Reagent A at 46 °C for at least 20 min** in a thermostatic bath.
2. **Mix the PCR products obtained with the mix 1 and mix 2** and aliquot 50 µl of the mix in a new tube, being this the material used in the following steps.

3. **Denature the PCR products** by heating at 95 °C for 10 min (in a thermal cycler or heat block) and cool rapidly, keeping the samples at 4 °C for at least 2 min.
4. Place every MDR Chip in the position indicated in the platform (HS12).

HYBRIDIZATION PROTOCOL

a) Add **300 µl of reagent A (Hybridization Solution)** pre-heated at **46 °C** and incubate for at least **2 min** at **46 °C**.
b) Remove the reagent A by activating the vacuum pump.
c) Add **50 µl** corresponding to the mix of PCR products from Mix 1 and Mix2 (previously denatured and kept on ice) to **230 µl of reagent A (Hybridization Solution)** (46 °C) and dispense the mix onto the corresponding MDR Chip-HS.
d) Incubate at **46 °C** for **8 min**.
e) Activate the pump to remove the PCR products (make sure that the pump is active for at least 30 s).
f) Wash **3x 300 µl** with **reagent A** (46 °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–alkaline phosphatase Conjugate)** and incubate for **5 min** at **29 °C**.
k) Activate the pump to remove the reagent.
l) **Set the temperature at 36°C**.
m) Wash the membranes **4x 300 µl** with **Reagent D (Washing buffer I)**.
n) Develop the membranes by adding **300 µl of reagent E (Developing solution)** and incubate for **10 min** at 36 °C.
o) Activate the pump to remove the 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) Image capture, analysis and results report following the instructions of the HS12 user manual.

B. For MDR Direct Flow Chip kit (Auto, ref: MAD-003946M-HS) in HS24 platform:

The whole hybridization process is performed automatically by hybriSpot. 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, perform the following steps:

1. **Set the instrument by following the instructions from the user's manual (provided with the instrument).**
2. Denature the PCR products by heating them at **95 °C for 10 min** in a thermal cycler or a heating block and cool quickly on 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. Follow the instructions in the manual to place the Mix 1 and 2 PCR tubes, MDR Chips and reagents in their corresponding positions in the 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 platform HS12 PCR AUTO.

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 read carefully the user manual (included in the HS12a platform). Follow the instructions in the manual to place the PCR tube strips, CHIPs and hybridization reagents in the instrument.

Protocol:

1. Take a tube containing each of the lyophilized PCR mixes per sample to be analyzed.
2. Add up to 30 µl of sample in each tube following the recommended protocol 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 described in the manual of the HS12a instrument to place the PCR tube strips, chips and hybridization reagents in the instrument and start the process.

10 QUALITY CONTROL PROCEDURE

The MDR Direct Flow Chip kit contains different internal controls to control the quality of the results.

SPOTS	CONTROL	POSITION	INTERPRETATION
B	Hybridization control	1A-1B-2M-7G-12A	5 positions are correct
CI-1	Exogenous amplification control Mix 1	1C-7H	0, 1 or 2 positions are correct
CI-2	Exogenous amplification control Mix 2	1D-7I	0, 1 or 2 positions are correct
BG	Endogenous amplification control Mix 1	1E-7J	0, 1 or 2 positions are correct
RNaseP	Endogenous amplification control Mix 2	1F-7K	0, 1 or 2 positions are correct

Table 7: Control probes included in the MDR Chip.

- **Hybridization control:** After the development of the membranes, an intense signal must appear in all five hybridization control positions (B), which serve as a quality control. This signal indicates that the hybridization reagents and developing have worked properly. If the signal does not appear, it indicates that an error has occurred during the hybridization process or that a reagent has not been used properly. Furthermore, this signal allows the software to orientate correctly the probe panel to perform the subsequent analysis.

- **Exogenous amplification control (CI-1, CI-2):** probe for the detection of synthetic DNA included in the PCR mix. This DNA will be co-amplified along with the genetic material of the sample. Two positive signals in the exogenous amplification control (CI) will indicate that the PCR reaction has worked correctly. A negative result in this control does not invalidate the result if the endogenous control has correctly amplified and/or the sample has been positive for any of the targets included in the panel.
- **Endogenous amplification control (BG, RNaseP):** probe for the detection of human beta-globin and RNasaP gene DNA amplified during the PCR. All the samples where the test DNA has been amplified correctly will have a positive signal in the Endogenous amplification control (BG, RNaseP). This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of the DNA used for it have been optimal. The absence of signal for this control means that it has been an error during the amplification, a low quality/amount of the DNA used in the amplification or the absence of human DNA in the amplification. The latter case may occur with some samples, taking into account the dilution to which clinical samples are subjected for PCR preparation. This signal will not appear if bacterial colonies are used as the starting sample. However, a negative result in this control does not invalidate the result if the exogenous control has correctly amplified and/or the sample has been positive for any of the targets included in the panel.

The samples that are positive for any of the resistance markers included in the kit must provide signal for some of the specific probes. Moreover, the five hybridization control signals (B), two exogenous amplification control signals (CI), and two endogenous amplification control (BG, RNaseP) signals must appear (as long as the sample contains human DNA). In case signals for the amplification controls do not appear, but they do for the resistance markers, a message of *absence of human DNA/presence of PCR inhibitors* is included in the report. In this case, the user must verify the quality of the samples before validating the results.

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

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

11 INTERPRETATION OF RESULTS

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

	1	2	3	4	5	6	7	8	9	10	11	12	13
A	B	msrA		catB3	ndm			mcr1	rmtB	qnrB	nmc/imi	B	
B	B	mef	gyrE-S83L	mecA	sim	Abau		sul1	rmtC	qnrS	ges	oxa51_like	
C	CI-1	ermA	gyrE-D87G	vanA	imp_like	mcr2		sul2	rmtF	oqxA	vim	oxa58_like	
D	CI-2	ermB	gyrE-S83W	vanB	blaSHV-S	mecC		sul3	Ecoli	oqxB	gim	SA	
E	BG	ermC	gyrE-S83W-D87N	blaSHV	blaSHV-SK	gyrP-D87G		blaDHA		cfr	spm	kpneum	
F	RnaseP	aac	gyrP-T83I	blaCTX	oxa23_like	parE-S80I		blaCMY		catB3	ndm	Paer	
G	mcr1	armA	gyrP-D87N	kpc	oxa24 _like		B	msrA	gyrE-S83L	mecA	sim		
H	sul1	rmtB	qnrA	sme	oxa48_like		CI-1	mef	gyrE-D87G	vanA	imp_like	Abau	
I	sul2	rmtC	qnrB	nmc/imi	oxa51_like		CI-2	ermA	gyrE-S83W	vanB	blaSHV-S	mcr2	
J	sul3	rmtF	qnrS	ges	oxa58_like		BG	ermB	gyrE-S83W-D87N	blaSHV	blaSHV-SK	mecC	
K	blaDHA	Ecoli	oqxA	vim	SA		RnaseP	ermC	gyrP-T83I	blaCTX	oxa23_like	gyrP-D87G	
L	blaCMY		oqxB	gim	kpneum			aac	gyrP-D87N	kpc	oxa24 _like	parE-S80I	
M		B	cfr	spm	Paer			armA	qnrA	sme	oxa48_like		

Figure 1: Scheme of the arrangement of the probes on the array. Specific probes for the pathogens and resistance genes of the study and those probes used as amplification and hybridization controls are included. The coordinates of each of them are also indicated.

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

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 following table shows the type of probes used and the positions in which they have been placed on the MDR Chip. The potential results obtained and the interpretation of results are also included:

Expected results (Organisms/Resistance)	Probe ID	Probe/positions (column-row)					
		Probe	B	CI-1	CI-2	BG	RnaseP
Staphylococcus aureus	**SA**	5K-12D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
METHICILLIN-RESISTANCE GENE mecA	**mecA**	4B-10G	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
VANCOMYCIN-RESISTANCE GENE vanA	**vanA**	4C-10H	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
VANCOMYCIN-RESISTANCE GENE mecB	**vanB**	4D-10I	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS A KPC CARBAPENEMASE	**kpc**	4G-10L	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS A SME CARBAPENEMASE	**sme**	4H-10M	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS A NMC/IMI CARBAPENEMASE	**nmc/imi**	4I-11A	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
SHV ß-LACTAMASE	**blaSHV**	4E-10J	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
SHV EXTENDED-SPECTRUM ß-LACTAMASE (double mutant)	**blaSHV-SK blaSHV-S**	5E-11J 5D-11I	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
SHV EXTENDED-SPECTRUM ß-LACTAMASE (single mutant)	**blaSHV-S**	5D-11I	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CTX-M EXTENDED-SPECTRUM ß-LACTAMASE	**blaCTX**	4F-10K	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS A GES CARBAPENEMASE	**ges**	4J-11B	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B VIM CARBAPENEMASE	**vim**	4K-11C	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B GIM CARBAPENEMASE	**gim**	4L-11D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B SPM CARBAPENEMASE	**spm**	4M-11E	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B NDM CARBAPENEMASE	**ndm**	5A-11F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B SIM CARBAPENEMASE	**sim**	5B-11G	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS B CARBAPENEMASE IMP3, 15, 19_like	**imp _like**	5C-11H	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K

CLASS D OXA48_like CARBAPENEMASE	**oxa48_like**	5H-11M	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS D OXA23_like CARBAPENEMASE	**oxa23_like**	5F-11K	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS D OXA24_like CARBAPENEMASE	**oxa24_like**	5G-11L	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS D OXA51_like CARBAPENEMASE	**oxa51_like**	5I-12B	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
CLASS D OXA58_like CARBAPENEMASE	**oxa58_like**	5J-12C	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Colistin resistance gene (mcr-1)	**mcr1**	1G-8A	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Sulfonamide resistance gene (sul-1)	**sul1**	1H-8B	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Sulfonamide resistance gene (sul-2)	**sul2**	1I-8C	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Sulfonamide resistance gene (sul-3)	**sul3**	1J-8D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
β-lactamase AmpC	**blaDHA**	1K-8E	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
β-lactamase AmpC	**blaCMY**	1L-8F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Macrolide resistance gene (msrA)	**msrA**	2A-8G	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Macrolide resistance gene (mefA/E)	**mef**	2B-8H	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Macrolide resistance gene (ermA)	**ermA**	2C-8I	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Macrolide resistance gene (ermB)	**ermB**	2D-8J	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Macrolide resistance gene (ermC)	**ermC**	2E-8K	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Aminoglycoside resistance gene (aac (6´)-Ib)	**aac**	2F-8L	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Aminoglycoside resistance gene (armA)	**armA**	2G-8M	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Aminoglycoside resistance gene (rmtB)	**rmtB**	2H-9A	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Aminoglycoside resistance gene (rmtC)	**rmtC**	2I-9B	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K

Aminoglycoside resistance gene (rmtF)	**rmtF**	2J-9C	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
E. coli	**Ecoli**	2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Low-level resistance to fluoroquinolones (gyrE-S83L mut.)	**gyrE-S83L Ecoli**	3B-9G 2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-S83L-D87G mut.)	**gyrE-S83L gyrE-D87G Ecoli**	3B-9G 3C-9H 2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-S83L-D87G-parE-S80I mut.)	**gyrE-S83L gyrE-D87G parE-S80I Ecoli**	3B-9G 3C-9H 6F-12L 2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-S83L-D87N mut.)	**gyrE-S83L gyrE-S83W-D87N Ecoli**	3B-9G 3E-9J 2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-S83W-D87G mut.)	**gyrE-S83W gyrE-D87G Ecoli**	3C-9H 3D-9I 2K-9D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-T83I mut.)	**gyrP-T83I Paer**	3F-9K 5M-12F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-T83I-D87N mut.)	**gyrP-T83I gyrP-D87N Paer**	3F-9K 3G-9L 5M-12F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Resistance to fluoroquinolones (gyrE-T83I-D87G mut.)	**gyrP-T83I gyrP-D87G Paer**	3F-9K 6E-12K 5M-12F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Quinolone or fluoroquinolone resistance gene (qnrA)	**qnrA**	3H-9M	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Quinolone or fluoroquinolone resistance gene (qnrB)	**qnrB**	3I-10A	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Quinolone or fluoroquinolone resistance gene (qnrS)	**qnrS**	3J-10B	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Olaquindox resistance gene (oqxA)	**oqxA**	3K-10C	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Olaquindox resistance gene (oqxB)	**oqxB**	3L-10D	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Linezolid resistance gene (cfr)	**cfr**	3M-10E	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Chloramphenicol resistance gene (catB3)	**catB3**	4A-10F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Klebsiella pneumoniae	**kpneum**	5L-12E	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Pseudomonas aeruginosa	**Paer**	5M-12F	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K

Acinetobacter baumannii	**Abau**	6B-12H	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Colistin resistance gene (mcr-2)	**mcr2**	6C-12I	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
Methicillin resistance gene (mecC)	**mecC**	6D-12J	1A-1B-2M-7G-12A	--/1C-7H	--/1D-7I	-- / 1E-7J	-- / 1F-7K
NEGATIVE MDR	--	--	1A-1B-2M-7G-12A	1C-7H	1D-7I	1E-7J	1F-7K
INVALID RESULTS (presence of PCR inhibitors) (Absence of human DNA control)	--	--	1A-1B-2M-7G-12A	--	--	--	--
NEGATIVE MDR (Absence of human DNA control)	--	--	1A-1B-2M-7G-12A	1C-7H	1D-7I	--	--
Image unavailable/Bad image/Hybridization error	--	--	--	--	--	--	--

Table 8: Position of the probes on the MDR Chip and interpretation of results.

Other possible results:

1. In rectal and nasopharyngeal exudates, they often show positivity for the mecA probe due to the presence of methicillin-resistant coagulase negative *Staphylococcus* in the flora. In case nasopharyngeal exudates/aspirates, in which it is sought to identify carriers of SARM, when a sample is positive for *S. aureus* it can also be positive for mecA, and this resistance gene cannot be necessarily attributed to the *S. aureus* strain.
2. Limited spectrum SHV-1 β-lactamase can be found in *K. pneumoniae* strains with a high frequency (between 80- -90%) and is encoded chromosomally. However, there are two possibilities so that this gene provides an extended-spectrum phenotype: one is through the overexpression of the gene (it occurs when its location is plasmid, which is the form found in other Enterobateriaceae, such as *E. coli*) and other is due to the appearance of specific mutations in its sequence. The kits has been designed and validated to detect all SHV variants, the wild strain SHV-1 and all the mutated forms of the enzyme are detected by the generic probe blaSHV, without differentiating them. Considering that *K. pneumoniae* could be found as normal part of the flora, the SHV detection would not indicate necessarily phenotypic evidence of production of extended-spectrum β-lactamase. In case positive is obtained for the mutated version of the SHV, both single and double mutation, it would indicate the production of an extended-spectrum β-lactamase.

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical performance in hybriSpot 12 (HS12)

12.1.1 Repeatability

The repeatability of the method was analyzed by using synthetic DNA for each of the specific targets of the panel. Two different concentrations of synthetic DNA were used and from each one of them at least five replicas were obtained. The test was performed by the same operator, in a single location and using the same reagent lot.

Target	No. copies/reaction	Positive/Tested	% positive
aac (6´)-Ib	2000	5/5	100%
	1000	7/7	100%
Acinetobacter baumannii	20	5/5	100%
	10	7/7	100%
armA	2000	5/5	100%
	1000	7/7	100%
blaCMY	100	5/5	100%
	20	7/7	100%
blaCTX	500	5/5	100%
	100	6/7	85%
blaDHA	250	5/5	100%
	100	7/7	100%
blaSHV	100	5/5	100%
	50	7/7	100%
blaSHV-S	1000	7/7	100%
blaSHV-SK	500	7/7	100%
	250	6/7	85.7%
catB3	20	5/5	100%
	10	7/7	100%
cfr	100	5/5	100%
	20	7/7	100%
ermA	1000	5/5	100%
	500	7/7	100%
ermB	100	5/5	100%
ermC	2000	5/5	100%
	1000	7/7	100%
Escherichia coli	250	5/5	100%
	100	7/7	100%
ges	50	5/5	100%
	10	7/7	100%
gim	100	5/5	100%
	50	7/7	100%
gyrE-S83L	1000	5/5	100%
	100	7/7	100%
gyrE-S83W	1000	5/5	100%
	100	7/7	100%
gyrE-S83W-D87N	1000	5/5	100%
	100	7/7	100%
gyrE-D87G	1000	5/5	100%
	100	7/7	100%
gyrP-T83I	1000	5/5	100%
	100	7/7	100%
gyrP-D87G	1000	5/5	100%
	100	7/7	100%
gyrP-D87N	1000	5/5	100%

imp	500	5/5	100%
	250	7/7	100%
Klebsiella pneumoniae	20	5/5	100%
	10	7/7	100%
kpc	500	7/7	100%
mcr1	100	5/5	100%
	50	7/7	100%
mcr2	20	5/5	100%
	10	7/7	100%
mecA	50	5/5	100%
	10	7/7	100%
mecC	50	5/5	100%
	10	7/7	100%
mef	2000	5/5	100%
	1000	7/7	100%
msrA	2000	5/5	100%
	1000	2/7	28%
ndm	500	7/7	100%
nmc/imi	50	5/5	100%
	10	7/7	100%
oqxA	50	5/5	100%
	10	7/7	100%
oqxB	100	5/5	100%
	10	7/7	100%
oxa23	100	5/5	100%
	50	7/7	100%
oxa24	50	5/5	100%
	10	7/7	100%
oxa48	100	5/5	100%
	50	7/7	100%
oxa51	250	7/7	100%
	50	5/7	71%
oxa58	100	7/7	100%
	50	4/5	80%
parE-S80I	1000	5/5	100%
	100	7/7	100%
Pseudomonas aeruginosa	20	5/5	100%
	10	7/7	100%
qnrA	500	5/5	100%
	100	7/7	100%
qnrB	20	5/5	100%
	10	7/7	100%
qnrS	20	5/5	100%
	10	7/7	100%
rmtB	1000	5/5	100%
	500	7/7	100%
rmtC	100	5/5	100%

rmtF	20	7/7	100%
	10	5/5	100%
sim	50	5/5	100%
	10	6/7	86%
sme	50	5/5	100%
	10	7/7	100%
spm	50	5/5	100%
	10	7/7	100%
Staphylococcus aureus	100	5/5	100%
	10	7/7	100%
sul1	20000	5/5	100%
	1000	7/7	100%
sul2	100	5/5	100%
sul3	1000	5/5	100%
	500	7/7	100%
vanA	50	5/5	100%
	10	7/7	100%
vanB	500	5/5	100%
	250	7/7	100%
	100	2/5	40%
vim	50	5/5	100%
	10	7/7	100%

Table 9: Repeatability test for each of the genes included in the panel.

12.1.2 Analytical specificity

For the test, 10^6 copies/reaction of synthetic DNA designed for each marker were used. No cross-reactions were observed between the genes included in the test, with the exception of the parE-S80I mutation whose specificity is greater than 92%.

Target	Specificity
Staphylococcus aureus	100%
Klebsiella pneumoniae	100%
Pseudomonas aeruginosa	100%
Escherichia coli	100%
Acinetobacter baumannii	100%
aac (6´)-Ib	100%
armA	100%
rmtB	100%
rmtC	100%
rmtF	100%
blaCMY	100%
blaDHA	100%
blaCTX	100%
blaSHV-SK	100%
blaSHV-S	100%
blaSHV	100%
ges	100%
gim	100%
imp_like	100%

kpc	100%
ndm	100%
nmc/imi	100%
oxa23_like	100%
oxa24_like	100%
oxa48_like	100%
oxa51_like	100%
oxa58_like	100%
sim	100%
sme	100%
spm	100%
vim	100%
catB3	100%
mcr1	100%
mcr2	100%
gyrE-S83L	100%
gyrE-S83W	100%
gyrE-D87G	100%
gyrE-S83W-D87N	100%
gyrP-T83I	100%
gyrP-T83I-D87G	100%
gyrP-T83I-D87N	100%
parE-S80I	92.31%
cfr	100%
ermA	100%
ermB	100%
ermC	100%
mef	100%
msrA	100%
mecA	100%
mecC	100%
oqxA	100%
oqxB	100%
qnrA	100%
qnrB	100%
qnrS	100%
sul1	100%
sul2	100%
sul3	100%
vanA	100%
vanB	100%
vim	100%

Table 10: Specificity of MDR Direct Flow Chip.

12.1.3 Analytical sensitivity

The limit of detection (LoD) of the kit was calculated for each of the analyzed genes. The determination of the minimum number of copies detected was performed through serial dilutions of the synthetic DNA of each one of the genes included in the panel with 5 ng of human genomic DNA. Each sample was analyzed between 6 and 14 times.

All the PCRs were hybridized by using the hybriSpot 12 platform. The results were analyzed with hybriSoft and the established value to consider the positive signals was 4 (gray intensity).

Target	Probe	No. copies/ reaction	Positive/ tested	Sensitivity	CI 95%	Specificity	CI 95%
aac (6´)-Ib	aac	1000	6/6	100%	51.56-100%	100%	99-100%
Acinetobacter baumannii	Abau	20	10/10	100%	65.54-100%	100%	99.08-100%
		10	14/14	100%	73.24-100%	100%	99.07-100%
armA	armA	1000	6/6	100%	51.56-100%	100%	99-100%
blaCMY	blaCMY	100	6/6	100%	51.56-100%	100%	99-100%
	blaCMY	20	6/6	100%	51.56-100%	100%	99-100%
blaCTX	blaCTX	500	10/10	100%	65.54-100%	100%	99.08-100%
	blaCTX	250	14/14	100%	73.24-100%	100%	99.07-100%
	blaCTX	100	8/10	80%	44.22-96.46%	100%	99.08-100%
blaDHA	blaDHA	100	6/6	100%	51.56-100%	100%	99-100%
blaSHV	blaSHV	100	10/10	100%	65.54-100%	100%	99.08-100%
	blaSHV	50	14/14	100%	73.24-100%	100%	99.07-100%
blaSHV-S	blaSHV-S	1000	14/14	100%	73.24-100%	100%	99.07-100%
	blaSHV-S	250	3/7	42.8%	20.14-79.86%	100%	99.08-100%
blaSHV-SK	blaSHV-SK	500	12/12	100%	65.54-100%	100%	99.07-100%
	blaSHV-SK	250	6/7	85.7%	50.88-97.06%	100%	99.08-100%
catB3	catB3	20	6/6	100%	51.56-100%	100%	99-100%
	catB3	10	6/6	100%	51.56-100%	100%	99-100%
cfr	cfr	100	6/6	100%	51.56-100%	100%	99-100%
	cfr	20	6/6	100%	51.56-100%	100%	99-100%
ermA	ermA	1000	6/6	100%	51.56-100%	100%	99-100%
	ermA	500	6/6	100%	51.56-100%	100%	99-100%
ermB	ermB	100	6/6	100%	51.56-100%	100%	99-100%
	ermB	20	0/6	0%	0-48.31%	100%	99-100%
ermC	ermC	1000	6/6	100%	51.56-100%	100%	99-100%
Escherichia coli	Ecoli	250	6/6	100%	51.56-100%	100%	99-100%
		100	6/6	100%	51.56-100%	100%	99-100%
ges	ges	50	10/10	100%	65.54-100%	100%	99.08-100%
	ges	10	14/14	100%	73.24-100%	100%	99.07-100%
gim	gim	100	10/10	100%	65.54-100%	100%	99.08-100%
	gim	50	14/14	100%	73.24-100%	100%	99.07-100%
gyrE-S83L	gyrE-S83L	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrE-S83L	100	6/6	100%	51.56-100%	100%	99-100%
gyrE-S83W	gyrE-S83W	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrE-S83W	100	6/6	100%	51.56-100%	100%	99-100%
gyrE-D87G	gyrE-D87G	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrE-D87G	100	6/6	100%	51.56-100%	100%	99-100%
gyrE-S83W-D87N	gyrE-S83W-D87N	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrE-S83W-D87N	100	6/6	100%	51.56-100%	100%	99-100%
gyrP-T83I	gyrP-T83I	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrP-T83I	100	6/6	100%	51.56-100%	100%	99-100%
gyrP-D87G	gyrP-T83I-D87G	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrP-T83I-D87G	100	6/6	100%	51.56-100%	100%	99-100%
gyrP-D87N	gyrP-T83I-D87N	1000	6/6	100%	51.56-100%	100%	99-100%
	gyrP-T83I-D87N	100	0/6	0%	0-48.31%	100%	99-100%
imp	imp_like	500	10/10	100%	65.54-100%	100%	99.08-100%

	imp_like	250	14/14	100%	73.24-100%	100%	99.07-100%
Klebsiella pneumoniae	Kpneum	20	10/10	100%	65.54-100%	100%	99.08-100%
	Kpneum	10	14/14	100%	73.24-100%	100%	99.07-100%
kpc	kpc	500	10/10	100%	65.54-100%	100%	99.08-100%
	kpc	100	14/14	100%	73.24-100%	100%	99.07-100%
mcr1	mcr1	100	6/6	100%	51.56-100%	100%	99-100%
		50	6/6	100%	51.56-100%	100%	99-100%
mcr2	mcr2	10	6/6	100%	51.56-100%	100%	99-100%
		20	6/6	100%	51.56-100%	100%	99-100%
mecA	mecA	50	10/10	100%	65.54-100%	98.43%	96.81-99.27%
	mecA	10	14/14	100%	73.24-100%	98.43%	96.81-99.27%
mecC	mecC	50	6/6	100%	51.56-100%	100%	99-100%
		10	6/6	100%	51.56-100%	100%	99-100%
mef	mef	2000	6/6	100%	51.56-100%	100%	99-100%
	mef	1000	6/6	100%	51.56-100%	100%	99-100%
msrA	msrA	2000	6/6	100%	51.56-100%	100%	99-100%
	msrA	1000	2/6	33%	6-75.9%	100%	99-100%
ndm	ndm	1000	10/10	100%	65.54-100%	100%	99.08-100%
	ndm	500	14/14	100%	73.24-100%	100%	99.07-100%
nmc/imi	nmc/imi	50	10/10	100%	65.54-100%	100%	99.08-100%
	nmc/imi	10	14/14	100%	73.24-100%	100%	99.07-100%
oqxA	oqxA	50	6/6	100%	51.56-100%	100%	99-100%
		10	4/6	66%	24.1-94%	100%	99-100%
oqxB	oqxB	100	6/6	100%	51.56-100%	100%	99-100%
		10	6/6	100%	51.56-100%	100%	99-100%
oxa23	oxa23_like	100	10/10	100%	65.54-100%	100%	99.08-100%
	oxa23_like	50	14/14	100%	73.24-100%	100%	99.07-100%
oxa24	oxa24_like	50	10/10	100%	65.54-100%	100%	99.08-100%
	oxa24_like	10	14/14	100%	73.24-100%	100%	99.07-100%
oxa48	oxa48_like	100	10/10	100%	65.54-100%	100%	99.08-100%
	oxa48_like	50	14/14	100%	73.24-100%	100%	99.07-100%
oxa51	oxa51_like	500	10/10	100%	65.54-100%	100%	99.08-100%
	oxa51_like	250	14/14	100%	73.24-100%	100%	99.07-100%
	oxa51_like	100	6/10	60%	27.37-86.31%	100%	99.08-100%
oxa58	oxa58_like	100	8/8	100%	59.77-100%	100%	99.08-100%
	oxa58_like	50	5/10	50%	20.14-79.86%	100%	99.08-100%
parE-S80I	parE-S80I	1000	6/6	100%	51.56-100%	100%	99-100%
	parE-S80I	100	6/6	100%	51.56-100%	100%	99-100%
Pseudomonas aeruginosa	Paer	20	10/10	100%	65.54-100%	100%	99.08-100%
	Paer	10	14/14	100%	73.24-100%	100%	99-100%
qnrA	qnrA	500	6/6	100%	51.56-100%	100%	99-100%
	qnrA	100	6/6	100%	51.56-100%	100%	99-100%
qnrB	qnrB	20	6/6	100%	51.56-100%	100%	99-100%
	qnrB	10	6/6	100%	51.56-100%	100%	99-100%
qnrS	qnrS	20	6/6	100%	51.56-100%	100%	99-100%
	qnrS	10	6/6	100%	51.56-100%	100%	99-100%
rmtB	rmtB	1000	6/6	100%	51.56-100%	100%	99-100%
	rmtB	500	6/6	100%	51.56-100%	100%	99-100%
rmtC	rmtC	100	6/6	100%	51.56-100%	100%	99-100%
rmtF	rmtF	20	6/6	100%	51.56-100%	100%	99-100%
	rmtF	10	6/6	100%	51.56-100%	100%	99-100%
sim	sim	50	10/10	100%	65.54-100%	100%	99.08-100%
	sim	10	10/12	83.3%	50.88-97.06%	100%	99.07-100%
sme	sme	50	10/10	100%	65.54-100%	100%	99.08-100%
	sme	10	14/14	100%	73.24-100%	100%	99.07-100%
spm	spm	50	10/10	100%	65.54-100%	100%	99.08-100%
	spm	10	14/14	100%	73.24-100%	100%	99.07-100%
	SA	100	10/10	100%	65.54-100%	100%	99.08-100%

Staphylococcus aureus	SA	10	14/14	100%	73.24-100%	100%	99.07-100%
sul1	sul1	1000	6/6	100%	51.56-100%	100%	99-100%
sul2	sul2	100	6/6	100%	51.56-100%	100%	99-100%
sul3	sul3	1000	6/6	100%	51.56-100%	100%	99-100%
	sul3	500	6/6	100%	51.56-100%	100%	99-100%
vanA	vanA	50	10/10	100%	65.54-100%	100%	99.08-100%
	vanA	10	14/14	100%	73.24-100%	100%	99.07-100%
vanB	vanB	500	10/10	100%	65.54-100%	100%	99.08-100%
	vanB	250	14/14	100%	73.24-100%	100%	99.07-100%
	vanB	100	4/10	40%	13.7-72.63%	100%	99.08-100%
vim	vim	50	10/10	100%	65.54-100%	100%	99.08-100%
	vim	10	14/14	100%	73.24-100%	100%	99.07-100%

Table 11: Analytical sensitivity (LoD): genomic DNA copies of each target resulting in 100% of positive cases of the replicas, analyzed with the hybriSoft software and a positivity cut-off point value of 4.

*The *ges* gene provides resistance to ampicillin and shows a certain degree of homology with the ampicillin-resistant gene *bla*TEM. The latter is used as a selection marker in the construction of plasmid to obtain the recombinant protein Taq DNA polymerase in *E. coli*. The specificity of 97.3% for *ges* can be due to the presence of trace quantities of *E. coli* with this enzyme marker.
** The *mec*A gene provides resistance to methicillin and is frequently associated to *Staphylococcus epidermidis*, characteristic bacteria of the skin flora. The specificity of 98.4% for *mec*A could be due to contamination of the samples during handling.

12.2 Analytical performance in hybriSpot 24 (HS24)

The performance and robustness of the MDR Direct Flow Chip kit in the automated instrument HS24 was validated by analyzing quantified limit copies of synthetic fragments from four genotypes included in the panel. 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.

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

Replicas of a positive sample containing a limit number of *kpc* DNA copies (500 copies) were made. These replicas were placed in different positions of the reaction chamber in the HS24 instrument and four 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 analyzed automatically with hybriSoft and were compared with the ones obtained in HS12 (Control). Differences between the different positions of the reaction chamber nor the used protocol were not detected.

12.2.2 Reproducibility of results in different hybridization positions in HS24

Eight replicas of a limit dilution of three resistant genes of the panel were made, to which 5ng of human genomic DNA as added. The replicas were mixed in a pool and were used as follows:

-4 replicas were analyzed in the HS24 distributed in the first and last positions of both chambers of the platform in order to cover the best and worst conditions of the protocol.

In each series, 3 genotypes were analyzed according to the distribution below:

- Genotype 1: positions 1, 10, 13, 22
- Genotype 2: positions 2, 11, 14, 23
- Genotype 3: positions 3, 12, 15, 24

In parallel, two replicas of each genotype were used as a control on the HS12.

The results were analyzed automatically with hybriSoft and were compared with the ones obtained in the HS12 platform (Control). In all cases, the samples were detected as positive. The differences of intensity observed between positions are acceptable.

Target	No.	Positive/tested	Difference between
*bla*CTX-M16	500	4/4	No
ndm	500	4/4	No
*oxa*51	250	4/4	No

Table 12: Reproducibility of MDR Direct Flow Chip in HS24. The results were analyzed automatically with hybriSoft establishing a positivity cut-off value of 4.

12.3 Analytical performance in hybriSpot 12 PCR AUTO (HS12a)

The performance and sturdiness of MDR Direct Flow Chip were validated in the automatic platform HS12a. For this, 3 replicas of each resistance marker and pathogen of the panel to limit dilutions were made and were amplified and hybridized in different positions of the equipment. The whole process was performed automatically in the HS12a, and the results were analyzed with hybriSoft.

Target	Probe	No. copies/ reaction	Positive/ tested
aac (6´)-Ib	aac	1000	3/3
Acinetobacter baumannii	Abau	10	3/3
armA	armA	1000	3/3
blaCMY	blaCMY	20	3/3
blaCTX	blaCTX	250	3/3
blaDHA	blaDHA	100	3/3
blaSHV	blaSHV	50	3/3
blaSHV-S	blaSHV-S	1000	3/3
blaSHV-SK	blaSHV-SK	500	3/3
catB3	catB3	10	3/3
cfr	cfr	20	3/3
ermA	ermA	500	3/3
ermB	ermB	100	3/3
ermC	ermC	1000	3/3
Escherichia coli	Ecoli	100	3/3
ges	ges	10	3/3

gim	gim	50	3/3
gyrE-S83L	gyrE-S83L	100	3/3
gyrE-S83W	gyrE-S83W	100	3/3
gyrE-D87G	gyrE-D87G	100	3/3
gyrE-S83W-D87N	gyrE-S83W-D87N	100	3/3
gyrP-T83I	gyrP-T83I	100	3/3
gyrP-D87G	gyrP-T83I-D87G	100	3/3
gyrP-D87N	gyrP-T83I-D87N	1000	3/3
imp	imp_like	250	3/3
Klebsiella pneumoniae	Kpneum	10	3/3
kpc	kpc	100	3/3
mcr1	mcr1	50	3/3
mcr2	mcr2	10	3/3
mecA	mecA	10	3/3
mecC	mecC	10	3/3
mef	mef	1000	3/3
msrA	msrA	2000	3/3
ndm	ndm	500	3/3
nmc/imi	nmc/imi	10	3/3
oqxA	oqxA	50	3/3
oqxB	oqxB	10	3/3
oxa23	oxa23_like	50	3/3
oxa24	oxa24_like	10	3/3
oxa48	oxa48_like	50	3/3
oxa51	oxa51_like	500	3/3
oxa58	oxa58_like	250	3/3
parE-S80I	parE-S80I	100	3/3
Pseudomonas aeruginosa	Paer	10	3/3
qnrA	qnrA	100	3/3
qnrB	qnrB	10	3/3
qnrS	qnrS	10	3/3
rmtB	rmtB	500	3/3
rmtC	rmtC	100	3/3
rmtF	rmtF	10	3/3
sim	sim	50	3/3
sme	sme	10	3/3
spm	spm	10	3/3
Staphylococcus aureus	SA	10	3/3
sul1	sul1	1000	3/3
sul2	sul2	100	3/3
sul3	sul3	500	3/3
vanA	vanA	10	3/3
vanB	vanB	250	3/3

vim	vim	10	3/3

Table 13: Reproducibility of MDR Direct Flow Chip in HS12a. The results were analyzed automatically with hybriSoft establishing a positivity cut-off value of 4.

12.4 Clinical

12.4.1 Identification of resistance mechanisms with MDR Direct Flow CHIP

For the clinical validation of the MDR Direct Flow Chip kit, a retrospective study was carried out with a total of 70 clinical isolates (purified DNA) and 24 clinical samples (direct PCR) from the Hospital General Universitario de Elche, which included microorganisms multidrug resistant to different classes of antibiotics. In addition, a total of 44 bacterial colonies carrying multiple resistance genes from the Hospital Universitario Virgen del Rocío (Seville) were included. The following table shows the resistance genes detected in the samples and the correlation with the antibiotic susceptibility profiles (AST) obtained for each one.

Resistance markers detected on the MDR chip	Isolated/genes of genes	Phenotypical antibiotic resistance
8 mecA 2 mecC 6 CMY 5 DHA 14 CTX 3 SHV-SK 1 SHV-S 6 SHV	1 *S. epidermidis*, 7 *S. aureus* 2 *S. aureus* 3 *K. pneumoniae*, 3 *E. coli* 2 *K. pneumoniae*, 2 *E. coli*, 1 *Enterobacter* 6 *K. pneumoniae*, 5 *E. coli*, 1 *P. aeruginosa*, 1 *Enterobacter*, 1 *Proteus* 1 *K. pneumoniae*, 1 *E.* coli, 1 *Providencia* 1 *K. pneumoniae* 5 *K. pneumoniae*, *1 Enterobacter*	Penicillins/Cephalosporins
2 GES 3 IMP 6 VIM 8 oxa48 5 NDM 5 KPC	2 *P. aeruginosa* 2 *P. aeruginosa*, *1 A. guillouae* 3 *P. aeruginosa*, 3 *K. pneumoniae* 5 *K. pneumoniae*, 1 *Enterobacter*, *2 E. coli* 1 *K. oxytoca,* 3 *K. pneumoniae,* 1 *Enterobacter* 5 *K. oxytoca*	Carbapenems
2 mef 3 ermA 2 ermB 2 ermC 6 msrA	1 *S. maltophilia*, 1 *Providencia* 3 *S. aureus* 2 *Enterococcus* *1 S. aureus*, *1 A. guillouae* 6 *S. aureus*	Macrolides
23 aac (6')-Ib 1 armA 1 rmtC	5 *P. aeruginosa*, 9 *K. pneumoniae*, 5 *E. coli*, *3 Enterobacter*, 1 *K. oxytoca* 1 *K. pneumoniae* 1 Enterobacter	Aminoglycosides
32 sul1 20 sul2 6 sul3	16 *E. coli*, 11 P. *aeruginosa,* 2 *Enterobacter*, 3 *K. pneumoniae* 1 *P. aeruginosa*, 8 *E. coli*, 1 *Proteus*, 9 *K. pneumoniae*, 1 *Enterobacter* 6 *E. coli*	Sulfonamides
8 qnrS 20 qnrB	4 *K. pneumoniae*, 3 *E. coli*, 1 *P. aeruginosa* 7 *K. pneumoniae*, 7 *E. coli*, 3 *P. aeruginosa*, 1 *S. aureus*, 1 *S. epidermidis*, 1 *K. oxytoca* *2 E. coli*	Quinolones

2 gyrA-S83L 15 gyrA-S83LD87N 13 gyrA-T83I 5 gyrA-D87N 5 oqxA/B	15 *E. coli* 13 *P. aeruginosa* 5 *P. aeruginosa* 5 *K. pneumoniae*	
3 mcr-1 1 mcr-2	3 *E. coli* *1 E. coli*	Colistin
1 vanA 1 vanB	1 *E. faecium* 1 *E. faecium*	Vancomycin
3 CatB3	2 *K. pneumoniae*, *1 E. coli*	Chloramphenicol

Table 14: Antibiotic resistance genes identified with the MDR Direct Flow Chip kit.

The resistance markers detected can explain the phenotypic profile of antibiotic-resistant bacteria in 90% of the cases. In addition, preliminary results have been obtained showing a correct performance of the test in direct PCR from clinical samples without the need to extract DNA, such as blood cultures and rectal and/or nasal exudates.

13 LIMITATIONS

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

14 TROUBLESHOOTING

Problem	Potential	Solutions
No signal is observed/ There is no hybridization signal	Error in the hybridization protocol	Check that all the hybridization reagents have been added in the correct order (manual platform). Check the functioning of hybriSpot (automatic platform). Repeat the test.
	The hybridization reagents have expired, or they have not been stored properly	Check the expiration date and the storage conditions of the reagents and the Chips. Repeat the test.
	Possible degradation of the DNA at 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 resistance in negative control	Problems of contamination in the pre-PCR or post-PCR zones.	Decontaminate (1% bleach) the working areas and repeat the test.
No signal of exogenous amplification control	Problems in the amplification by PCR.	Check that the program of the thermal cycler is the appropriate, that the PCR master mix has been prepared properly and that the PCR reagents are stored correctly. Repeat the test.

	Presence of PCR inhibitors in the test sample.	If the starting sample correspond to a dilution 1:100 of a suspension of rectal exudate, purify the DNA with any of the validated extraction systems (see sample preparation in section 7).
No signal of endogenous amplification control	Not enough amount of human DNA in the test sample. Presence of PCR inhibitors in the test sample.	Repeat the PCR by increasing the amount of starting sample or reducing the initial dilution of the sample. In any case, for rectal exudates, do not use dilutions less than 1:40 dilution. Check that the extraction system of genetic material used works properly , including an extraction control.
Weak signals in the hybridization	PCR reagents and/or expired or stored improperly. Error in the hybridization protocol. The PCR product was not denatured correctly before the hybridization. Incorrect sample volume used to re-suspend the lyophilized product. Partial inhibition of the PCR.	Check the expiration date of the reagents, the storage of the PCR mix and reagents. Check the hybridization temperatures and times and verify the functioning of the hybriSoft equipment. Verify that the denaturation has been performed correctly. Repeat the test. Repeat the test by using the correct sample volume Dilute the sample during processing or DNA extraction. Verify the correct functioning of the extraction system of nucleic acids used.

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16 LABEL AND BOX SYMBOLS

IVD	In vitro diagnostic medical device		Expiration date
REF	Catalog number		Temperature limit
LOT	Lot code		Manufacturer
	Refer to the instructions for use		Sufficient content for <n> assays
	Safety data sheet		

17 GLOSSARY

DNA: deoxyribonucleic acid
PCR: polymerase chain reaction
HS12: hybriSpot 12 (manual platform)
HS24: hybriSpot 24 (automatic platform)
NBT-BCIP: nitro-blue tetrazolium and 5-bromo-4-chloro-3-indolyphosphate
$MgCl_2$: magnesium chloride
dNTPs: deoxynucleotide driphosphates
DNases: deoxyribonucleases
RNases: ribonucleases
dUTP: deoxyuridine triphosphate
CDC: Center for Disease Control and Prevention of USA

18 CHANGELOG

Date	Description
2021/08/03	Creation of the document.
2022/01/07	Room temperature is modified in Section 5.
2022/02/17	Reference MAD-DDW is included in Section 3 “Components”.
2022/03/31	- Section 1 “Intended use” is modified (reference is made to qualitative detection and that the kit is based on multiplex PCR). - Specificity for blaDHA is included in Table 11. - Specificity and 95% CI are modified for ges in Table 11.
2022/06/23	A typo has been corrected in Table 8.