CE 0318*

STD Direct Flow Chip Kit

Detection of pathogens responsible for sexually transmitted diseases (STD) through multiplex PCR and reverse hybridization

For all hybriSpot platforms

Compatible with version 2.2.0.R11.of hybriSoft HSHS.
For compatibility with other versions, please contact the manufacturer / supplier.

REF	**Ref. MAD-003938M-HS12**	Σ	**24 determinations**
	Ref. MAD-003938M-HS		**24 determinations**

For in vitro diagnostic use only*
Directive 98/79/EC

**The Notified Body 0318 only intervenes in the evaluation of the compliance of the test for Chlamydia trachomatis Biovars A-K in urine and semen samples; urethral, endocervical and anal swabs. The rest of pathogens have the self-certified CE marking.*

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IVD

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1 INTENDED USE

STD Direct Flow Chip is an *in vitro* diagnostic kit for the detection of pathogenic organisms causing sexually transmitted diseases (STD) in humans. The organisms causing these infections are often difficult to detect and include viruses, bacteria or parasites, usually occurring co-infections. The methods that are currently used for their diagnosis are very laborious and not always show a 100% specificity. The *STD Direct Flow Chip* allows the simultaneous detection of 11 pathogens: *Chlamydia trachomatis*[1*], *Haemophilus ducreyi*, Herpes simplex virus 1 and Herpes simplex virus 2, *Mycoplasma genitalium*, *Mycoplasma hominis, Neisseria gonorrhoeae, Treponema pallidum*, *Trichomonas vaginalis* and *Ureaplasma*[2] *(urealyticum/parvum)*. The *STD Direct Flow Chip kit* allows the direct detection of these infectious agents from different types of clinical samples (urine, semen, liquid-based endocervical cytologies; and urethral, endocervical, vaginal, anal and throat swabs) without previous extraction of DNA, as well as from purified DNA of these type of clinical samples.

[1]Biovars and Serovars of the *Chlamydia trachomatis* species detected with the *STD Direct Flow Chip* kit:

- *Chlamydia trachomatis* Biovar Trachoma: Serovars A-K.
- *Chamydia trachomatis* Biovar LGV: Serovars L1-L3.

** The Notified Body 0318 only intervenes in the evaluation of the compliance of the test for Chlamydia trachomatis Biovars A-K in urine and semen samples; urethral, endocervical and anal swabs. The detection of Chlamydia trachomatis* Biovars L1-L3*, as well as the detection of the* Biovars A-K *in endocervical pharyngeal and cytological samples are out of the scope of the certification of the Notified Body 0318.*

[2]Ureaplasmas detected with the *STD Direct Flow Chip* kit:

- *Ureaplasma urealyticum*: UUR2, UUR4, UUR5, UUR7, UUR8, UUR9, UUR10, UUR11, UUR12, UUR13.
- *Ureaplasma parvum*: UPA1, UPA3, UPA6, UPA14.

Note: In the literature, it is common to use the terms Biovar or Serovar indistinctly to refer to Serovars A-K of the Trachoma Biovar and L1-L3 of the Biovar LGV of the *C. trachomatis* species.

Microbiological status: Non-sterile product.

2 PRINCIPLE OF THE METHOD

The *STD Direct Flow Chip* kit is based on a methodology consisting of the simultaneous amplification of bacterial, viral and protozoal DNA by multiplex PCR in only one step and directly from cellular extracts, followed by hybridization on a membrane with specific DNA probes using the DNA-Flow technology for HybriSpot platforms, both automated and manual. The biotinylated amplicons generated after the multiplex PCR are hybridized in membranes containing an array of specific probes for each human pathogen, as well as amplification and hybridization control probes. Unlike conventional hybridization on surfaces, the *DNA-Flow* technology allows a very fast binding of the PCR product and its specific probe in a three-dimensional porous environment. Once the binding has occurred between the specific amplicons and their corresponding probes, the signal is visualized by a colorimetric immunoenzymatic reaction with Streptavidin-Phosphatase and a chromogen (NBT-BCIP), which generates insoluble precipitates on the membrane in the positions in which there has been hybridization. The results are analyzed automatically with the *HybriSoft software*.

3 COMPONENTS

The **STD Direct Flow Chip** kit is commercialized in two formats according to 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
STD Direct Flow Chip kit (Manual)	**24 tests**	**MAD-003938M-HS12**
1. STD Direct Flow Chip kit (PCR	**24 tests**	**MAD-003938M-P**
STD Flow Chip kit (PCR Mix)	3 strips x 8 tubes (blue)	MAD-003938M-MIX
2. STD Chips	**24 tests**	**MAD-003938M-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
Washing Buffer II (Reagent F)	18ml	MAD-003930MF-HS12-24

Table 1. Reagents provided in the *STD Direct Flow Chip kit* (Manual) format.

KIT/COMPONENTS	FORMAT	REFERENCES
STD Direct Flow Chip kit (Auto)	**24 tests**	**MAD-003938M-HS**
1. STD Direct Flow Chip kit (PCR	**24 tests**	**MAD-003938M-P**
STD Flow Chip kit (PCR Mix)	3 strips x 8 tubes (blue)	MAD-003938M-MIX
2. STD Chips	**24 tests**	**MAD-003938M-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 2. Reagents provided in the *STD Direct Flow Chip kit* (Auto) format.

- Both presentations include 60mL of DNase/RNase-free double distilled water for the handling of clinical samples: RNASE/DNASE-FREE DISTILLED WATER; Ref: MAD-DDW.

- **STD Flow Chip kit (PCR Mix)** contains the lyophilized PCR mix made up of PCR buffer, dNTPs (U/T), DNase/RNase-free water, biotinylated primers, Hot Start Polymerase and Uracil DNA Glycosylase. The primers included are specific for the amplification of the following species of pathogenic organisms: *Chlamydia trachomatis, Haemophilus ducreyi*, Herpes simplex virus 1, Herpes simplex virus 2, *Mycoplasma genitalium, Mycoplasma hominis, Neisseria gonorrhoeae, Treponema pallidum,*

Trichomonas vaginalis and *Ureaplasma urealyticum/parvum*. Furthermore, the PCR mix includes primers for the amplification of a human genomic DNA fragment used as an internal control, and primers along with DNA of an exogenous control of amplification.

- **STD Chips:** The kit includes a total of 24 Chips or membranes (ref: MAD-003938M-CH-HS) that contain an array of DNA probes specific to each of the pathogens included in the analysis, as well as others corresponding to the internal controls included in this kit. The position of all them on the Chip can be referred to in the section 10 of this manual (INTERPRETATION OF RESULTS).
- **Flow Chip Hybridization Reagents:** It contains all the reagents necessary for the reverse Flow-Through hybridization process.

4 ADDITIONAL REQUIRED MATERIAL NOT SUPPLIED

4.1. Reagents and materials

A. Common reagents to manual and automatic platforms:

- Disposable gloves.
- DNase/RNase-free tubes of 0.2/0.5 ml
- Pipette tips with DNase/RNase-free filters.

- For the manipulation of clinical samples of semen: *Paraffin Tissue Processing Kit* (MAD-003952M).

B. Specific reagents (Auto, ref: MAD-003938M-HS):

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

4.2. Equipment

A. Common equipment for the manual and automatic platforms:

- Microcentrifuge.
- Automatic micropipettes: P1000, P200, P20 and P2.
- For enzymatic lysis of semen samples, it is recommended to use a dry-heat block for 1.5 mL tubes with a shaking program.
- *HybriSoft software.*

B. Specific equipment:

- *STD Direct Flow Chip kit* (Manual) (Ref: MAD-003938M-HS12):
  - Manual equipment for hybridization hybriSpot 12 (VIT-HS12).
  - Thermal cycler
  - Thermal block to heat PCR tubes (can be substituted by a thermal cycler)
  - Cooling plate (4°C)
  - Thermostatic bath / heater.
- *STD Direct Flow Chip kit* (Auto) (Ref: MAD-003938M-HS):
  - 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).

5 STORAGE AND STABILITY CONDITIONS

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

STD 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 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.**

- **STD Chips:** Shipped and stored at 2-8°C*. **Do not freeze**. The Chips are stable until their indicated expiration date.
- **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 preheated at 41 °C in a thermostatic bath or heater (only for manual format) before use.
- The rest of hybridization reagents must be used at room temperature (15-25°C).

Previous considerations on the chips:

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

*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 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.
- **STD 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. Manipulation protocols are provided in the different types of clinical samples, whose processing 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* (Cod-UNG), which degrades the PCR products containing dUTP, is included in the kit. It is recommended to include negative amplification controls containing all the reagents handled in the kit, from the extraction to the amplification, except for the DNA 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 manual and automatic platforms)	161001	"Aqueous liquid wastes containing dangerous substances" after adding 10% of the total volume of a disinfectant agent. If the disinfection is not carried out, 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 3. 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 PREPARATION OF THE CLINICAL SAMPLE FOR ANALYSIS

The STD Direct Flow Chip kit has been validated for its use in direct PCR, without previous DNA extraction, from different types of clinical samples. According to the type of starting clinical sample, the processing

protocol is the following:

7.1. Clinical samples of urine

The *STD Direct Flow Chip kit* has been validated for its use in **direct PCR from clinical urine samples** without being necessary the extraction of DNA. The recommended protocol for the processing of this type of sample is the following:

- Homogenize the urine sample by using a vortex. Take a volume of 400 µl of the homogenized mix and transfer it to a suitable microcentrifuge tube.
- Centrifuge the sample for 3 min at 12,000 rpm. After the centrifugation, dispose carefully the supernatant. It is recommended to use a Pasteur pipette or micropipette of 1 mL.
- Wash the cell pellet with 400 µL **DNase/RNase-free double distilled water.** Centrifuge the sample for 3 min at 12,000 rpm. Remove carefully the supernatant.
- Resuspend the cell button in 400 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.
- Shake the sample with a vortex and use 30 ul of the cell suspension as DNA template for the PCR reaction. If the processing analysis is not going to be performed, after that, store at -20°C for a maximum period of 2 months.

7.2. Clinical samples of semen

The *STD Direct Flow Chip* kit has been validated for its use in **direct PCR from clinical semen samples** without being necessary the extraction of DNA. The recommended protocol for the processing of this type of sample is the following:

- Homogenize the sperm sample by using a pipette. Take a volume of 400 µl of the homogenized one and transfer it to a suitable microcentrifuge tube of the same volume.
- Centrifuge the sample for 3 min at 12,000 rpm. After the centrifugation, dispose carefully the supernatant. It is recommended to use a Pasteur pipette or micropipette of 1 mL.
- The clinical samples of semen require an enzymatic treatment for a correct cellular lysis. For this, it is necessary to use the reagents *DNA Release* and *Extraction Buffer* included in the *Paraffin Tissue Processing Kit* (ref: MAD-003952M, property of VITRO S.A) as follows: add 100 µL of lysis solution to each sperm sample [dilution 1:50 of *DNA Release* in *Extraction Buffer* (2 µL of *DNA Release* solution in 98 µL of *Extraction Buffer*)].
- Add 100 µL of lysis solution to the cellular sediment and homogenize.
- Incubate the homogenized product at 55°C for 30 minutes. If possible, incubate the samples with shaking at 1,000 rpm.
- Incubate the cellular lysis at 95°C for 10 min and after that centrifuge at 2,000 rpm for 1 min to precipitate/sediment the cell debris. Transfer the supernatant to a new tube. Add 27 µl of DNase/RNase-free double distilled water and 3 µl of supernatant as DNA template for the PCR per lyophilized PCR tube. If the processing analysis is not going to be performed, after that, store at -20°C for a maximum period of 2 months.

7.3. Urethral, cervical, anal and throat clinical samples

The *STD Direct Flow Chip* kit has been validated for its use in **direct PCR from clinical urethral, cervical, vaginal, anal and throat samples** without being necessary the extraction of DNA. All the samples included in this section share the use of pellets to take them. The recommended protocol for the processing of this type of sample is the following:

- Shake the swab in 400 µl **DNase/RNase-free double distilled water** in a tube of 1.5-2 ml.
- Shake the sample with a vortex and use 30 µl of the cell suspension as DNA template for the PCR reaction.
- If the pellet already contains a transport medium (PBS or the like), it is recommended to follow the protocol below:
    - Shake in vortex to scatter the material in the solution. Transfer the homogenized sample (cell suspension) to a suitable microcentrifuge tube.
    - Centrifuge for 3 min at a speed of 12,000 rpm.
    - After the centrifugation, dispose carefully most of the supernatant with a Pasteur pipette or micropipette of 1 mL.
    - Resuspend the cell button in 400 µl **DNase/RNase-free double distilled water** to obtain a homogeneous cell suspension.
    - Shake the sample with vortex and use 30 µL of the cell suspension as DNA template for the PCR reaction.
- Note: If the processed sample is not going to be analyzed immediately, it must be stored at 4°C for a maximum period of 1 week or at -20°C for a maximum period of 2 months.
- **Note: For anal clinical samples, an additional wash with DNase/RNase-free double distilled water might be necessary depending on the turbidity caused by the organic matter in order to minimize the inhibitory effect on the PCR by certain agents contained in this type of samples.**

7.4. Liquid-based endocervical samples

- Take 400 µl of homogenized sample with vortex and put in a tube of 1.5-2 ml.
- Centrifuge for 3 min at a speed of 12,000 rpm.
- After the centrifugation, dispose carefully most of the supernatant with a Pasteur pipette or micropipette of 1 mL.
- Resuspend the resulting cell button in 400 ul **DNase/RNase-free double distilled water.**
- Centrifuge for 3 min at a speed of 12,000 rpm. Remove carefully most of the supernatant with a Pasteur pipette or micropipette of 1 mL.
- Resuspend the cell button in 400 µl **DNase/RNase-free double distilled water** to obtain a homogeneous suspension of cells.
- Shake the sample with a vortex and use 30 ul of the cell suspension as DNA template for the PCR reaction.

7.5. Considerations for a correct manipulation of clinical samples

Apart from following carefully the indications given in the processing protocols of each of the types of clinical samples for which this kit has been validated, a set of usual incidences that can occur when processing any

type of clinical sample and how to avoid them in order to make the most of it in the direct analysis with the *STD Direct Flow Chip* kit is described below:

INDICENTS	CONSEQUENCES	HOW TO AVOID IT
Use a different aqueous solution to DNase/RNase-free double distilled water	PCR inhibition	Use DNase/RNase-free double distilled water only
Cells decant on the bottom of the tube.	When pipetting the sample to be added to the PCR, there is a risk of not collecting enough sample.	Shake the tube containing the sample to resuspend the cells before aspirating them with the pipette to add them to the PCR tube. The user must confirm it visually.
The sample contains a lot of cells that form lumps.	When pipetting a lump, the tip can get clogged and no sample can be aspirated to be added to the PCR tube.	The user must confirm visually that the sample is aspirated properly.
The sample contains few cells.	If the cell pellet is resuspended in a large volume of double-distilled water, it may give false-negative results or "Blank" samples due to insufficient material.	Resuspend the cell pellet with less volume of double-distilled water.
The sample was added to the PCR tube, but it was not placed in the thermal cycler afterwards.	The cells begin to lyse and release proteases that can destroy the polymerase giving "blank" sample results.	Once the samples have been added to the PCR tubes, amplify immediately.
The samples have been stored in double-distilled water for more than 1 week at 4°C.	The cells begin to lyse and release proteases that can inhibit the polymerase giving "blank" sample results.	Use the resuspended samples in double-distilled water after obtaining them. Freeze them if they are not going to be used right after.

Table 4. List of incidents that may occur during the processing of clinical samples, consequences, and solutions offered to obtain an optimal analysis performance.

IMPORTANT: If the clinical samples are not to be processed immediately upon receipt, it is recommended to store them at 4°C for a maximum period of 1 week or at -20°C for a maximum period of 2 months.

STD Direct Flow Chip kit has also been validated with DNA as the starting material, obtained using the following DNA purification kits and extraction instruments*:

EXTRACTION KITS	EXTRACTION EQUIPMENT
MagNA Pure Compact Nucleic Acid Isolation kit I (Roche Diagnostic's)	MagNA Pure Compact Instrument. Version 1.1.2 (Roche Diagnostic's)
EZ1 DSP Virus Kit (Qiagen)	Qiagen EZ1 Advanced XL (Qiagen)

Table 5. Extraction kits and instruments used for the purification of DNA from clinical samples.

***Note: The system has not been validated with other DNA extraction systems. Therefore, the reliability of the results obtained cannot be guaranteed if a different purification system is used.**

8 ANALYSIS PROCEDURE FOR HS12 AND HS24 PLATFORMS

8.1. Multiplex DNA amplification reaction

The following thermal cyclers have been validated with *STD Direct Flow Chip:*

- *Veriti 96 Well Thermal Cycler* (Life Technologies)
- *SimpliAmp Thermal Cycler* (Applied Biosystem)
- *TProfessional ThermoCycler* (Biometra)

The PCR reaction is carried out in a final volume of 30 µl in tubes containing the lyophilized PCR reaction mix.

Procedure:

- Take a tube containing the lyophilized PCR mix per sample to be analyzed.
- Add up to 30 µl of direct sample in each tube following the recommended protocol in section 7.
- If it involves a semen sample, add **27 µl DNase/RNase-free double distilled water** and **3 µl** of the processed clinical sample per reaction tube according to the guidelines given 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 8, the necessary tubes can be separated from the strip with no need for using complete strips. The rest of the tube strip with the lyophilized PCR mix 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:

PCR PROGRAM		
25°C	10 min	1 cycle
95°C	3 min	1 cycle
95°C	30 sec	40 cycles
55°C	45 sec	
72°C	30 sec	
72°C	5 min	1 cycle
8°C	∞	

Table 6. PCR program.

Keep the tubes refrigerated at 8-10 °C when the reaction is finished. If the samples are not going to be processed at 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 tube.

8.2. Flow-through reverse hybridization

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

The membranes are single-use and must be handled with gloves.
Depending on the type of kit with which we are working, we will proceed as follows:

A. For STD Direct Flow Chip kit Manual, (ref: MAD-003938M-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. Denature the PCR products by heating at **95 °C during 10 min** in a thermal cycler and **cool quickly in ice** during at least **2 min**.
2. Preheat the **Reagent A** at 41 °C.
3. Place every **STD Chip** in the position indicated in the platform (HS12).

Manual hybridization protocol:

a) Set the temperature of the equipment at 41°C. Add **300 µl** of **Reagent A (Hybridization Solution)** preheated for at least 20 minutes at 41°C for every Chip and incubate for at least **2 min at 41°C**.
b) Remove the **reagent A (Hybridization Solution)** by activating the vacuum pump.
c) Mix **30 µl** of each PCR sample (previously denatured and kept in ice) with **270 µl** of **Reagent A (Hybridization Solution)** (41°C) and dispense the mix on the corresponding **STD Chip**.
d) Incubate at **41 °C** for **8 min**.
e) Activate the pump for at least 30 s to remove the PCR products.
f) Wash **3** times with **300 µl** with **Reagent A (Hybridization Solution)** (41 ºC).
g) Set the temperature at **29 °C**.
h) Add **300 µl** of **Reagent B (Blocking Solution)** and incubate for 5 min.
i) Activate the pump to remove the reagent B.
j) When the temperature reaches **29°C**, add **300 µL** of **Reagent C (Streptavidin-Alkaline Phosphatase)** to each chip.
k) Incubate for **5 min at 29 °C**.
l) Activate the pump to remove the reagent.
m) Set the temperature at **36°C**.
n) Wash the membranes **4** times with **300 µl** with **reagent D (Washing buffer I).**
o) When the temperature reaches **36°C**, add **300 µl** of **Reagent E (development solution)** to every Chip. Incubate for **10 min at 36 °C**.
p) Activate the pump to remove the reagent.
q) Wash the membranes **2** times with **300 µl** with **reagent F (Washing buffer II).**
r) Activate the pump to remove the reagent.
s) Perform the image capture, analysis and result report following the instructions of the equipment's user manual.

B. For STD Direct Flow Chip kit Auto, (ref: MAD-003938M-HS):

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

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

Before starting the hybridization process:

1. Denature the PCR products by heating at **95 °C during 10 min** in a thermal cycler and **cool quickly in ice** during at least **2 min**.
2. Place the PCR tubes, the STD Chips and the reagents in their corresponding positions of hybriSpot 24.
3. Select the corresponding protocol in the equipment to start the automatic process.

9 ANALYSIS PROCEDURE FOR HS12a

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

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

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

Procedure:

- Take a tube containing the lyophilized PCR mix per sample to be analyzed.
- Add the DNA samples to a PCR tube following the instructions described in section 8.1.
- Homogenize the mix by pipetting and centrifuge for a few seconds.
- 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.
- Follow the instructions in the manual to place the tube strips, chips and hybridization reagents in the instrument and start the process.

10 QUALITY CONTROL PROCEDURE

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

SPOTS	CONTROL	POSITION (see Figure 1)
B	Hybridization control	1A-1B-2I-5E-8A
CI	Exogenous amplification control	1C-5F
BG	Endogenous amplification control	1D-5G

Table 7. Control probes included in *STD Chip.*

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

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

Endogenous amplification control (BG): probe for the detection of human beta-globin gene DNA amplified during the PCR. All the samples where the test DNA has been amplified correctly will have a positive signal in the Endogenous amplification control (BG). This signal shows the quality/quantity of the DNA used in the amplification. A positive signal indicates that the amplification has worked correctly and that the quality and quantity of the DNA used for it have been optimal. The lack of signal for this control indicates errors during the amplification, due to low quality/quantity of the DNA used in the amplification or lack of human DNA in the amplification. 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 organisms included in the panel. The latter case is likely to occur with clinical specimen types containing a lower number of human cells.

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

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 the analysis software *HybriSoft*. The following scheme shows the arrangement of the probes on the *STD Chip*:

	1	2	3	4	5	6	7	8	9
A	B	MG				CT-S1		B	
B	B		MH				CT-S2		
C	CI	TV		UU-P		MG		UU-P	
D	BG		HD				MH		
E		HSV-1 / HSV-2		NG	B	TV		NG	
F			HSV-1		CI		HD		
G		CT-S1		TP	BG	HSV-1 / HSV-2		TP	
H			CT-S2				HSV-1		
I		B		pcCT				pcCT	

Figure 1: Scheme of the arrangement of the probes on the array.

"B": Hybridization control
"CI": Exogenous amplification control
"BG": Endogenous amplification control (fragment human ß-Globin)
"X": Specific probes for each pathogen

All the probes are duplicated to guarantee the reliability in the automatic analysis of the results. The hybridization control (B) is repeated in 5 positions and allows the software to orientate correctly the probe panel for its analysis afterwards.

The distribution of the different probes included in the **STD Chip** as well as the possible expected results are shown below:

EXPECTED RESULTS (DETECTED PATHOGENS)	PROBE ID	PROBE/POSITION			
		PROBE	B	CI	BG
Mycoplasma genitalium	**MG**	2A-6C	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Mycoplasma hominis	**MH**	3B-7D	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Trichomonas vaginalis	**TV**	2C-6E	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Ureaplasma urealyticum/parvum	**UU-P**	4C-8C	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Haemophilus ducreyi	**HD**	3D-7F	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Herpes simplex virus 1*	**HSV-1/HSV-2 + HSV-1**	2E-6G-3F-7H	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Herpes simplex virus 2	**HSV-1/HSV-2**	2E-6G	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Treponema pallidum	**TP**	4G-8G	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Neisseria gonorrhoeae	**NG**	4E-8E	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G

*Chlamydia trachomatis***	**pcCT**	4I-8I	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Chlamydia trachomatis (Serovars A-K)	**pcCT + CT-S1 + CT-S2**	4I-8I-2G-6A-3H-7B	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Chlamydia trachomatis (Serovars L1-L3)	**pcCT + CT-S2**	4I-8I-3H-7B	1A-1B-2I-5E-8A	--/1C-5F	-- /1D-5G
Negative sample	--	--	1A-1B-2I-5E-8A	--/1C-5F	1D-5G
Negative sample	--	--	1A-1B-2I-5E-8A	1C-5F	-- /1D-5G
Target	--	--	1A-1B-2I-5E-8A	1C-5F	--
Invalid results	--	--	1A-1B-2I-5E-8A	--	--
Hybridization error	--	--	--	--	--

Table 8. Position of the probes on the *STD Chip* and interpretation of results.

***Note: The positive identification of the Herpes simplex virus 1 is obtained when, simultaneously, the hybridization signals on the STD Chip from the probe mix HSV-1/HSV-2 (positions 2E-6G) and the probe signal of HSV-1 (positions 3F-7H) occur. When only the hybridization signal of the probe mix HSV-1/HSV-2 (positions 2E-6G) occurs, then it corresponds to the Herpes simplex virus 2. Co-infections of both types of Herpes simplex viruses 1 and 2 show the same patter as an infection by the Herpes simplex virus 1 (2E-6G-3F-7H). Real situations in which only the probe signal HSV-1 on the STD Chip are not considered.**

****Note: At least one positive signal for the pcCT probe (positions 4I-8I) must appear in a CT-positive sample. When only positivity for the pcCT probe (positions 4I-8I) is detected, the serovar of *Chlamydia trachomatis* cannot be discriminated. Positive identification of *Chlamydia trachomatis* species Serovars A-K is obtained when there is positivity for the three probes pcCT (positions 4I-8I), CT-S1 (positions 2G-6A) and CT-S2 (3H-7B) in Chip STD. When only the signals corresponding to the pcCT (positions 4I-8I) and CT-S2 (positions 3H-7B) probes are present, the identification corresponds to Serovars L1-L3 of the same species. Real situations in which the pcCT (positions 4I-8I) and CT-S1 probes are positive on the STD Chip are not contemplated.**

Below, an example of a report in which the analyzed sample has been positive for *Chlamydia trachomatis* (CT) pathogen is shown.

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STD Direct Flow Chip Kit

LOTES

PCR:	STD_I+D_001L	07/03/2021
Chips:	pcCT002	08/03/2024
Reactivo:	Rg-ID-214	15/05/2021

DETALLES DE LA MUESTRA

ID MUESTRA: 020420_MClio-pcCT-rectal-19276331-05-2 **TIPO DE MUESTRA:**

ID PACIENTE: **PACIENTE:**

SEXO: - **FECHA NAC.:** **EDAD:**

INFORME

STD POSITIVO

Muestra positiva para:

Chlamydia trachomatis (Serotipos A-K)

Muestra negativa para el resto de patógenos incluidos en el test STD flow chip.

PROTOCOLO

Detección de patógenos mediante PCR en un solo paso e hibridación reversa automatizada:

- Herpes simplex virus-1,Herpes simplex virus-2, Neisseria gonorrhoeae, Chlamydia trachomatis (Serovares A-K), Chlamydia trachomatis (Serovares L1-L3 =Linfogranuloma venéreo),Mycoplasma genitalium, Mycoplasma hominis, Thricomonas vaginalis, Ureaplasmas (urealyticum/parvum), Haemophilus ducreyi y Treponema pallidum.

Preparación de la muestra/extracción del ADN

- Usar la muestra procesada/ DNA purificado para amplificar por PCR.

Protocolo PCR STD Direct Flow CHIP: 1x [25°C, 10 min]; 1x [95°C, 3 min]; 40x [95°C, 30 s -55°C, 45 s - 72°C, 30 s]; 1x [72°C, 5min]; 1x [8°C, ∞].

Protocolo HIBRIDACIÓN REVERSA:

- Hibridación del producto de PCR biotinilado con STD Direct Flow CHIP
- Lavados post-hibridación
- Incubación con enzima Estreptavidina-Fosfatasa
- Revelado con NBT-BCIP

Análisis automático de resultados

NOTAS

FACULTATIVO:	Default Doctor, doctor	**Validado:**	19/11/2020
Realizado por:	Default Tech, tech	**Procesado:**	19/11/2020

Instr. : Mock **Serial Nº:** 001 **hybriSoft:** HSHS 2.2.0.R05 / HSHS IPL 1.0.1.R0000

vitro master diagnóstica®

STD Direct Flow Chip Kit

LOTES

PCR:	STD_I+D_001L	07/03/2021
Chips:	pcCT002	08/03/2024
Reactivo:	Rg-ID-214	15/05/2021

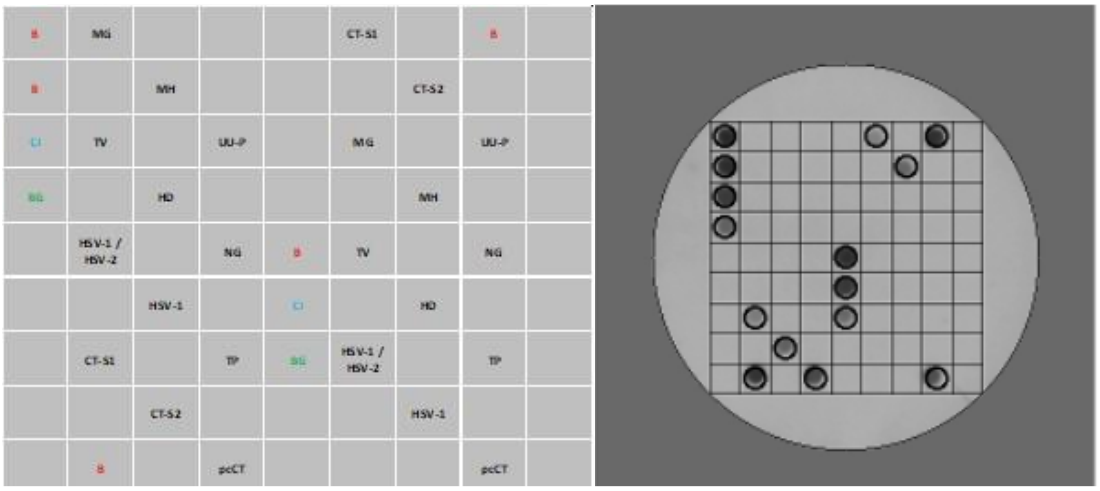
DETALLES DE LA MUESTRA

ID MUESTRA: 020420_MClio-pcCT-rectal-19276331-05-2 **TIPO DE MUESTRA:**

ID PACIENTE: **PACIENTE:**

SEXO: - **FECHA NAC.:** **EDAD:**

INFORME



- Spot B: Control de hibridación (5 puntos para orientar correctamente el CHIP)
- Spot CI: Control de amplificación
- Spot BG: Control de DNA (Sonda de DNA genómico humano)
- Spot #: Sondas específicas para cada patógeno

Todos los puntos están impresos por duplicado.

INFORMACIÓN DEL ANÁLISIS

Umbral: 4

FACULTATIVO:	Default Doctor, doctor	**Validado:**	19/11/2020
Realizado por:	Default Tech, tech	**Procesado:**	19/11/2020

Instr. : Mock **Serial Nº:** 001 **hybriSoft:** HSHS 2.2.0.R05 / HSHS IPL 1.0.1.R0000

Figure 2. Example of a report of a positive case for *Chlamydia trachomatis* (CT) pathogen.

12 PERFORMANCE CHARACTERISTICS

12.1 Analytical functioning on a manual platform

12.1.1 *Repeatability*

The repeatability of the method was analyzed by using synthetic DNA fragment from each of the specific targets of the pathogens of the panel. Two different concentrations of synthetic DNA were used and from each one of them at least 6 copies were obtained. The test was performed by the same operator, in a single location and using the same reagent lot.

TARGET	PROBES	No. COPIES / REACTION	POSITIVE / TESTED	% POSITIVE
M. genitalium	MG	50	6/6	100%
M. hominis	MH	10	6/6	100%
U. urealyticum/parvum	UU-P	10	6/6	100%
N. gonorrhoeae	NG	10	6/6	100%
T. vaginalis	TV	10	6/6	100%
HSV-1	HSV-1/HSV-2 + HSV-1	10	7/7	100%
HSV-2	HSV-1/HSV-2	10	7/7	100%
T. pallidum	TP	10	7/7	100%
H. ducreyi	HD	10	6/6	100%
***C. trachomatis* (Serovars L1-L3)**	CT-S2	10	6/6	100%
***C. trachomatis* (serovars A-K)**	CT-S1 + CT-S2	10	6/6	100%
Chlamydia trachomatis	pcCT	10	6/6	100%

Table 9. Repeatability assay for each of the pathogens included in the panel.

12.1.2 *Reproducibility*

The method precision was analyzed by simulating the inter-laboratory variability by changing the operator, the PCR mix lot used and the equipment used. 15 clinical cases (10 positive and 5 negative clinical cases) from semen, endocervical and urethral origin were analyzed in parallel. For all the clinical cases, the analysis were performed with the direct method (without previous DNA purification).

	LABORATORY 1		
LABORATORY 2	Positive	negative	Total
positive	**15**	**0**	15
negative	**0**	**5**	5
Total	15	5	**20**

Table 10. Reproducibility assay using clinical specimens.

Concordance was calculated for both conditions, obtaining a kappa index of 1,000, a standard error of zero and a 95% CI of 1,000-1,000. In both conditions, the statistical significance of the reproducibility tests with the *STD Direct Flow Chip* kit were demonstrated.

12.1.3 Analytical specificity

Experiments to determine potential cases of cross non-specificity between members of the panel were performed using a specific number of copies of each of the synthetic oligos ($1x10^6$ copies) representing each pathogen, with no cross non-specificity observed between members of the panel:

ORGANISM	SPECIFICITY
M. genitalium	100%
M. hominis	100%
U. urealyticum/parvum	100%
N. gonorrhoeae	100%
T. vaginalis	100%
HSV-1	100%
HSV-2	100%
T. pallidum	100%
H. ducreyi	100%
C. trachomatis	100%

Table 11. *STD Direct Flow Chip* kit intra-panel specificity.

There was also no non-specificity with other pathogens that are either phylogenetically related to the panel members or are associated with other microorganisms that coexist in the same flora:

TESTED MICROORGANISMS ($1x10^4$ TOTAL COPIES)	
Aeromonas hydrophila	*Listeria monocytogenes*
Acinetobacter baumannii	*Morganella morganii*
Campylobacter jejuni	*Proteus penneri*
Candida albicans	*Providencia rettgeri*
Citrobacter freundii	*Providencia stuartii*
Citrobacter koseri	*Salmonella enterica*
Citrobacter spp	*Salmonella enteritidis*
Cytomegalovirus	*Serratia marcescens*
Cryptococcus neoformans	*Serratia urealytica*
Enterobacter aerogenes	*Staphylococcus aureus*
Enterobacter cloacae	*Stenotrophomonas maltophilia*
Enterococcus faecalis	*Streptococcus agalactiae*
Epstein Barr virus	*Streptococcus pneumoniae*
Escherichia coli	*Proteus mirabilis*
Haemophilus influenzae	*Pseudomonas aeruginosa*
Klebsiella pneumoniae	*Human Papillomavirus (6, 11, 16, 18)*
Kluyvera ascorbata	*Yersinia enterocolitica*
Neisseria meningitidis	

Table 12. List of pathogens included in the "inter-panel" Specificity tests by the *STD Direct Flow Chip* kit.

12.1.4 *Analytical sensitivity*

In order to analytically verify the proper performance of the system designed, synthetic double-stranded DNA fragments mimicking the different target regions of the pathogens included in the panel were used. The kit's limit of detection (LoD) was calculated for each one 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 pathogens included in the panel with 5 ng of human genomic DNA. In order to calculate sensitivity, each case was repeated 12 times. All PCRs were hybridized by using the manual platform. The results (next page) were analyzed with *HybriSoft software* and the value established for a positive signal was 4 (gray intensity).

ORGANISM	PROBE	No. COPIES/ REACTION	POSITIVE/ TESTED	SENSITIVITY	95% CONFIDENCE INTERVAL	SPECIFICITY	95% CONFIDENCE INTERVAL
M. genitalium	MG	50	12/12	100%	69.87-100%	100%	98.10-100%
M. hominis	MH	10	12/12	100%	69.87-100%	100%	98.10-100%
U. urealyticum/ U. parvum	UU-P	10	12/12	100%	69.87-100%	100%	98.10-100%
N. gonorrhoeae	NG	10	12/12	100%	69.87-100%	100%	98.10-100%
T. vaginalis	TV	10	12/12	100%	69.87-100%	100%	98.10-100%
HSV-1	HSV-1/HSV-2 + HSV-1	10	12/12	100%	69.87-100%	100%	98.10-100%
HSV-2	HSV-1/HSV-2	10	12/12	100%	69.87-100%	100%	98.10-100%
T. pallidum	TP	10	12/12	100%	69.87-100%	100%	98.10-100%
H. ducreyi	HD	10	12/12	100%	69.87-100%	100%	98.10-100%
C. trachomatis (serovars A-K)	CT-S1+CT-S2	10	12/12	100%	69.87-100%	100%	98.10-100%
C. trachomatis (Serovars L1-L3)	CT-S2	10	12/12	100%	69.87-100%	100%	98.10-100%
C. trachomatis	pcCT	10	12/12	100%	69.87-100%	100%	98.10-100%

Table 13. Analytical sensitivity and specificity test using different synthetic DNA copy numbers corresponding to the pathogens included in the panel, establishing a positivity cut-off value of 4.

12.2 Analytical functioning on automatic platform Hybrispot 24

The performance and robustness of the *STD Direct Flow Chip* kit in the automatic platform was validated by analyzing a number of limit copies of synthetic DNA fragments from four pathogens included in the panel. The reproducibility of the results obtained by the automatic platform was evaluated by comparing the results obtained in the manual platform. Two types of test were performed:

12.2.1 Reproducibility of results in programs for different number of samples

Replicates of a positive sample containing a limit number of *Chlamydia trachomatis* DNA copies (10 copies) were analyzed. These replicates were placed in different positions of the reaction chamber in the equipment and four different protocols were assessed:

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

The results were automatically analyzed with *HybriSoft* and 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 the automatic platform

Four replicates for three pathogens from the panel were prepared and located in different positions of the two reaction chambers of the equipment, using the protocol for 24 samples. The results were analyzed automatically with *HybriSoft*, showing a percentage of reproducibility for all the analyzed pathogens in different positions.

ORGANISM	No. COPIES/REACTION	POSITIVE/TESTED	DIFFERENCES BETWEEN POSITIONS
Chlamydia trachomatis	10	4/4	No
Trichomonas vaginalis	10	4/4	No
Mycoplasma hominis	10	4/4	No

Table 14. Reproducibility results obtained with the automatic platform and the *STD Direct Flow Chip* kit. The results were analyzed automatically with *HybriSoft* establishing a positivity cut-off value of 4.

This validation proves the reproducibility of the results between the positions 1 and 24 of the equipment and the reproducibility of the results with different programs for a different number of samples.

12.3 Analytical functioning in the automatic platform Hybrispot 12 PCR AUTO

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

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

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

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

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

12.3.2 Verification of sensitivity limit

The functioning and the robustness of the STD Direct Flow Chip was validated in the automatic equipment HS12a by analyzing limit concentrations of synthetic DNA fragments of all the pathogens included in the panel.
3 replicas of each positive sample were made. The whole process was performed automatically in two different HS12a instruments, and the results were analyzed with hybriSoft.

Organism	No. copies/ reaction	Positive/ Tested
M. genitalium	50	3/3
M. hominis	10	3/3
U. urealyticum/ U. parvum	10	3/3
N. gonorrhoeae	10	3/3
T. vaginalis	10	3/3
HSV-1	10	3/3
HSV-2	10	3/3
T. pallidum	10	3/3
H. ducreyi	10	3/3
C. trachomatis (Serovars A-K)	10	3/3
C. trachomatis (Serovars L1-L3)	10	3/3
C. trachomatis (target pcCT)	10	3/3

Table 15: Verification of sensitivity limit of STD Direct Flow Chip kit in HS12a. The positivity was analyzed with the hybriSoft software by establishing as a cut-off point a value of 4. NT: not tested

12.3.3 Clinical performance

The clinical performance of the STD Direct Flow Chip kit was validated using DNA purified by means of any of the extraction methods mentioned, as well as using as the starting material for analysis the clinical sample without previous genomic DNA extraction (direct PCR); both are detailed in section 7 of this manual. The diagnostic capacity of the *STD Direct Flow Chip* was evaluated by studying its diagnostic sensitivity and specificity. These two parameters are defined and calculated as follows:

- The **diagnostic specificity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 x the number of true negative values (TN) divided by the sum of true negative values (TN) plus the number of false positive (FP) values, or 100 × TN/ (TN + FP).

- The **diagnostic sensitivity** is expressed as a percentage (numerical fraction multiplied by 100), calculated as 100 × the number of true positive values (TP) divided by the sum of true positive values (TP) plus the number of false negative values (FN), or 100 × TP/ (TP + FN).

12.3.3.1 Diagnostic sensitivity and specificity using purified DNA:

Purified DNA from 165 clinical specimens was retrospectively analyzed with *STD Direct Flow Chip* kit. The reference system considered was the standard version, not lyophilized, of the kit

ORGANISM	TN	FP	TP	FN	DIAGNOSTIC SPECIFICITY	DIAGNOSTIC SENSITIVITY
N. gonorrhoeae	140	1	24	0	99.3%	100%
T. vaginalis	163	1	1	0	99.4%	NT
M. genitalium	158	0	7	0	100%	100%
Ureaplasma urealyticum/parvum	103	0	62	3	100%	95.38%
C. trachomatis (serovars A-K)*	87	0	78	0	100%	100%
C. trachomatis (serovars L1-L3)	165	0	0	0	100%	NT
M. hominis	128	0	37	1	100%	97.36%
HSV-1	147	2	16	0	98.65%	100%
HSV-2	161	0	4	0	100%	100%
T. pallidum	163	1	1	0	99.4%	NT
H. ducreyi*	164	1	0	0	99.4%	NT

Table 16. Diagnostic sensitivity and specificity results obtained with the *STD Direct Flow Chip* kit from the analysis of purified DNA. NT: Not tested.

Note: The NB 0318 is only involved in the conformity assessment of the Chlamydia trachomatis Biovars A-K assay. The detection of Chlamydia trachomatis Biovars L1-L3, as well as the detection of Biovars A-K in pharyngeal and endocervical cytology samples in liquid medium are outside the scope of certification by NB0318.

****Note: Clinical sensitivity for T. *vaginalis, T. pallidum and H. ducreyi* could not be evaluated due to the lack of positive clinical samples.**

12.3.3.2 Diagnostic sensitivity and specificity using clinical samples:

The diagnostic specificity and sensitivity of the direct method using clinical samples were determined retrospectively with 226 clinical specimens from different origin, using the standard version of the kit, not lyophilized, as the reference system.

ORGANISM	TN	FP	TP	FN	DIAGNOSTIC SPECIFICITY	DIAGNOSTIC SENSITIVITY
N. gonorrhoeae	205	0	21	0	100%	100%
T. vaginalis	207	2	17	0	99.04%	100%
M. genitalium	216	1	8	1	99.54%	88.88%
Ureaplasma urealyticum/parvum	161	0	64	1	100%	98.46%
C. trachomatis (serovars A-K)*	111	0	115	0	100%	100%
C. trachomatis (serovars L1-L3)	222	0	4	0	100%	NT
M. hominis	181	1	44	0	97.77%	100%
HSV-1	224	0	2	0	100%	NT
HSV-2	219	0	7	0	100%	100%

T. pallidum	225	0	1	0	100%	NT
H. ducreyi*	224	2	0	0	99.11%	NT

Table 17. Diagnostic sensitivity and specificity results obtained with the *STD Direct Flow Chip* kit from the analysis of clinical samples. NT: Not tested.

Note: The NB 0318 is only involved in the conformity assessment of the Chlamydia trachomatis Biovars A-K assay. The detection of Chlamydia trachomatis Biovars L1-L3, as well as the detection of Biovars A-K in pharyngeal and endocervical cytology samples in liquid medium are outside the scope of certification by NB0318.
****Note: Clinical sensitivity for HSV-1, *T. pallidum and H. ducreyi* could not be evaluated due to the lack of positive clinical samples.**

13 LIMITATIONS

Use of inappropriate samples: the method has been validated using clinical specimens directly, or purified genetic material from such samples. The clinical specimen types that have been validated are: urine; sperm; and urethral, endocervical, vaginal, perianal, and throat swabs. 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	Causes	Solutions
No signal is observed/ there is no hybridization signal	Failure in the hybridization protocol.	Check that all the reagents have been correctly added during the hybridization process.
		Check the correct functioning of hybriSpot 12/12a/24. Repeat the test.
	PCR reagents and/or expired or not stored properly.	Check the expiration date and the storage conditions of the reagents and the Chips. Repeat the test.
	Chip probes destroyed by rests of decontamination reagents (e.g. Bleach) in the wells.	Clean with plenty of distilled water and repeat the experiment.
No signals in the endogenous amplification control.	Not enough amount of human DNA in the clinical sample.	Repeat the PCR by increasing the amount of starting sample. Repeat the test.
	Presence of PCR inhibitors.	Purify the DNA of the sample and repeat the test.

Problem	Causes	Solutions
Presence of chromogen precipitates in the Chip after finishing the hybridization protocol.	High cell and/or blood content of the sample.	Repeat the PCR by diluting the starting sample.
Weak hybridization signals.	PCR reagents and/or expired or stored improperly.	Check the expiration date of all the reagents and the storage conditions. Repeat the test.
	Sample volume used to re-suspend the erroneous lyophilized product.	Repeat the test by using the correct sample volume
	Failure in the hybridization protocol.	Check the correct functioning of hybriSpot HS12/12a/24 and the hybridization protocol. Repeat the test.
	Low quality/quantity of the DNA in the sample.	Concentrate the sample during its processing by adding less water volume.
Absence of exogenous amplification control.	Problems in the amplification by PCR.	Check that the program of the thermal cycler is the appropriate, that the mother PCR mix has been prepared properly and that the reagents are stored correctly. Repeat the test.
	Presence of PCR inhibitors in the test sample.	Verify the correct functioning of the extraction system of nucleic acids used. Repeat the test.

Table 18. Possible incidents, causes and solutions against the problems that can arise during the analysis.

15 BIBLIOGRAPHY

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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 for use		Sufficient content for <n> assays
	Safety data sheet		

17 GLOSSARY

DNA: Deoxyribonucleic acid.
CT: *Chlamydia trachomatis*.
Cod UNG: Cod Uracil-DNA Glycosylase.
DNase: Deoxyribonuclease.
dUTP: Deoxyuridine Triphosphate.
STD: Sexually transmitted disease.
FN: False negative. *False negative results.*
FP: False positive. False positive results
HD: *Haemophilus ducreyi.*
HS12: *HybriSpot 12* equipment.
HS12a: *HybriSpot 12 PCR AUTO* equipment.
HS24: *HybriSpot 24* equipment.
L1-L3: Chlamydia trachomatis serovars L1-L3.
MG: *Mycoplasma genitalium.*
MH: *Mycoplasma hominis.*
NBT-BCIP: Nitroblue Tetrazolium Chloride- 5-Bromo-4-Chloro-3-Indolyl phosphate.
NG: *Neisseria gonorrhoeae.*
PCR: Polymerase Chain Reaction.
RNase: Ribonuclease.
TN: True negative. *True negative results.*
TP: *Treponema pallidum.*
TP: True positive. True positive results
TV: *Trichomonas vaginalis.*
UP: *Ureaplasma parvum.*
UU: *Ureaplasma urealyticum.*
HSV-1: Herpes simplex virus 1.
HSV-2: Herpes simplex virus 2.

18 CHANGELOG

Date	Description
2020-11-19	• Inclusion of the section changelog • Inclusion of the explanation of the pictogram of the Safety Sheet • Incorporation of a new pCCT target to improve sensitivity of *Chlamydia trachomatis*
2022-01-25	• Room temperature is modified in Section 5