INSTRUCTIONS FOR USE

Pathogens xB instructions for use E 2023-10-24

IVD CE

October 2023.

IPC	**REF / UDI-DI**	*09120127730169*
GINA 500	**REF / UDI-DI**	*09120127730244*
GINA 500 + DNA Purification	**REF / UDI-DI**	*09120127730145*

Kit for enriching bacterial and fungal DNA from human blood (+ DNA purification) including an Internal Process Control (IPC)

LINA	**REF / UDI-DI**	*09120127730152*

A modulation buffer for extraction-free testing of Bronchoalveolar Lavage (BAL) and Blood Culture (BC)

PCR-Box Bacteria / Resistance / Fungi / IPC	**REF / UDI-DI**	*09120127730084*
	REF / UDI-DI	*09120127730107*
	REF / UDI-DI	*09120127730091*
	REF / UDI-DI	*09120127730114*
hybcell Bacteria / Fungi / Pathogens DNA xB	**REF / UDI-DI**	*09120127730053*
	REF / UDI-DI	*09120127730060*
	REF / UDI-DI	*09120127730077*

Multiplex DNA tests for detection of bacterial 16S DNA and bacterial antibiotic resistance marker genes from human samples with an indication of homologies to known bacterial type strains and detection of fungal 28S DNA from human samples with an indication of homologies to known fungal type strains.

Cube Dx GmbH, Westbahnstraße 55, A-4300 St. Valentin / Austria, info@cubedx.com, www.cubedx.com

Content

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1. Explanation of symbols

Symbol	Explanation
CE IVD	CE mark. In vitro diagnostic medical device.
	Manufacturer.
EXP	Expiry date.
REF	Catalog number, UDI-DI.
SN	Serial number.
	Reference to the instructions for use.
	Only use it once. Do not reuse.
	Use by date.
	Temperature limit for storage.
	Sufficient for <n> tests.
CONTROL	Control material.
H225	Highly flammable liquid and vapour.
H301	Toxic if swallowed.
H315	Causes skin irritation.
H318	Causes serious eye damage.
H319	Causes serious eye irritation.
H371	May cause damage to organs.
H412	Harmful to aquatic life with long lasting effects.
P210	Keep away from heat, hot surfaces, sparks, open flames and other ignition sources. No smoking.
P233	Keep container tightly closed
P260	Do not breathe dust/fume/gas/mist/vapours/spray.
P273	Avoid release to the environment.
P280	Wear protective gloves/protective clothing/eye protection/face protection.
P301+P310	IF SWALLOWED: Immediately call a POISON CENTER/doctor.
P305+P351+P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present, and easy to do. Continue rinsing.
P337+P313	If eye irritation persists: Get medical advice/attention.
P370+P378	In case of fire: Use sand, carbon dioxide or powder extinguisher to extinguish.
P403+P235	Store in a well-ventilated place. Keep cool.
P501	Dispose of contents/container in accordance with local/regional/national/international regulations.

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2. Introduction and intended use

Intended Use

The products described in this instructions for use are combined to detect (present / not present) and identify (species, genera) bacteria, antibiotic resistance genes and fungi in human samples based on the analysis of short DNA sequences of the microbial genome.

The products are designed to detect even very low concentrations of bacterial and / or fungal DNA directly from samples like EDTA whole blood. As a consequence, no culturing of the sample is necessary for diagnosing an infection and the time to result is reduced significantly in comparison to conventional microbiological culturing methods.

The following samples – require different sample treatments (either the GINA or LINA procedure) – define different intended uses:

1. **EDTA (whole blood) samples: Assessment of sepsis, bacteremia, candidosis.**
2. **Bronchoalveolar lavage (BAL): Assessment of pneumonia.**
3. **Positive blood cultures: Identification of bacteria and fungi after blood culturing.**

The products are not intended to provide information about the quantity of microorganisms in the sample.

The tests must only be conducted by professional users who have been trained in its usage in an adequate laboratory environment.

General Information

Identifying the causative microorganism directly from whole blood enables early targeted antimicrobial therapy. A targeted antimicrobial therapy is the precondition for successful treatment of the infection and the limitation of often severe adverse effects, that might ultimately lead to the death of the patient. Conventional culturing methods might fail to deliver early results, especially if the causative microorganism is a fastidious bacterium (e.g., *Bordetella pertussis)* that requires unique growth conditions, a slow-growing bacteria (e.g. *Helicobacter pylori* which needs up to 7 days to grow in culture) or fungi (e.g. *Candida glabrata*). Especially in such cases, Cube Dx's early identification of microorganisms has the potential to be beneficial for patients.

However, the products are designed for complementary use with blood culturing. It is by no means intended to replace blood culture techniques. Results obtained from the direct blood test should be interpreted in conjunction with other relevant clinical and laboratory findings, to aid in the provision of targeted therapy for patients suspected of sepsis.

Contradictory results to blood culture may occasionally occur: for example, a negative result may be presented by the products while a blood culture result is positive and vice versa. Such discrepancies may be a result of a very low number of microorganisms in the patient's blood, as only 0.5ml sample volume is taken for the test (in comparison to 2x10ml for blood culturing). Another reason might be the occurrence of rare type strains that have not been considered during the test design or the fundamental differences in the underlying technologies for the read-out of results (genetic information based on selected type strains vs. protein patterns used by MALDI-TOF).

Different points in time when the samples are taken may also result in discrepant results. We strongly recommend collecting EDTA samples at the same time when blood culture samples are collected – if still possible before the administration of antimicrobials.

This test is to be carried out as suggested in this manual, interruptions of the workflow for example, by freezing the eluate for some days, may alter results as well.

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IPC, GINA 500, GINA 500 + DNA Purification

GINA pathogen enrichment (and DNA purification) kits remove the vast majority of human (blood) cells and cellular debris from whole blood and other human samples. The procedure is intended to drastically increase the percentage of pathogenic (bacterial and fungal) DNA of intact microorganisms relative to human DNA in the resulting solution and to provide better conditions for downstream PCR reactions. Intact microorganisms are those that are still viable (active or attenuated (= inhibited in their growth for example by administration of certain antibiotics)). On the contrary, damaged microorganisms and free DNA are removed during the procedure. The test offers results without any bias related to the characteristics of different microorganisms.

As a consequence, only microorganisms that can still do harm to the patient are relevant for the follow-up processes (compact sequencing). Some antimicrobials focus on preventing growth but do not neutralize microbes. In such cases, the microorganisms are not removed, as they are still intact. These microorganisms pose a risk to the patient, once the antimicrobial treatment stops.

The second fundamental feature of the *GINA* pathogen enrichment is its highly effective and efficient lysis of bacterial and fungal cells (after enrichment).

Quality assurance concepts for such highly sensitive molecular pathogen identification from human samples must ensure that negative results are only caused by negative samples - and not by any flaws during the processing of the sample. Therefore, stringent process control has to undergo the same procedures as the sample itself – without setting off sensitivities of the tests. Cube Dx's Internal Process Control (IPC) consists of frozen biological material dissolved within the human sample before the enrichment process starts. The IPC undergoes the same extraction procedures as the sample itself. Both the follow-up PCR and hybcell test (if amplicon is transferred) confirm the presence of IPC DNA and therefore the validity of the results.

For processing *GINA* kits*, a table-top centrifuge with a rotor for 2mL tubes can apply* 11.000g (e.g., Eppendorf, Hermle, etc.) and a conventional heating block (e.g., Analytic Jena, Coyote Bioscience) capable to heat to 100°C are needed.

LINA

The identification of pathogens and antibiotic-resistance genes should be simple and fast. The LINA transfer and modulation buffer shorten the time for molecular identification as it eliminates the RNA/DNA extraction processes and enables direct PCR.

This buffer is designed for use with samples containing an abundance of microorganisms, for example, Broncho Alveolar Lavage (BAL) in the diagnosis of pneumonia and for positive human blood cultures.

Together with Cube Dx's PCR products (Bacteria, Fungi, Resistance), pathogen identification hybcells, microorganisms, and resistance genes can be determined in less than 2 hours!

The procedure must be carried out in an environment suitable for molecular biological testing. This includes DNA- and DNase-free pipets, separated rooms for DNA isolation and amplification/detection, and the possibility of UV decontamination.

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PCR-Box Bacteria / Resistance / Fungi / IPC
hybcell Bacteria / Fungi / Pathogens DNA xB

The *PCR-Box Bacteria*, *PCR-Box Fungi*, *PCR-Box Resistance,* and qualitative tests *hybcell Bacteria DNA xB*, *hybcell Fungi DNA xB,* and *hybcell Pathogens DNA xB* are in-vitro tests for the detection and identification of bacteria, antibiotic resistance mechanisms and fungi from human samples based on homologies of bacterial 16S DNA, resistance genes and fungal 28S DNA. The *PCR-Box IPC* amplifies the DNA of the IPC (Internal Process Control) added to the initial EDTA blood sample processed with *GINA*. The test might support therapeutic decisions for suspected (severe) bacterial and/or fungal infections in combination with other clinical information.

Bacteria and antibiotic resistance genes potentially presented by *hybcell Bacteria DNA xB* and by *hybcell Pathogens DNA xB*:

Blood Culture Sepsis Pneumonia

Genus	Species	Profile
Abiothrophia	*Abiotrophia defectiva*	
Acinetobacter	*Acinetobacter baumannii*	
	Acinetobacter calcoaceticus complex	
Actinobacillus	*Actinobacillus pleuropneumoniae*	
Anaerococcus		
Bacteriodes	*Bacteroides fragilis*	
Bordetella	*Bordetella pertussis*	
Borreliella		
	Borreliella burgdorferi	
Brucella		
Burkholderia	*Burkholderia cepacia complex*	
	Burkholderia pseudomallei	
Campylobacter		
Citrobacter	*Citrobacter koseri*	
	Citrobacter freundii complex	
Corynebacterium		
	Corynebacterium diphtheriae	
	Corynebacterium jeikeium	
	Corynebacterium ulcerans	
Enterobacter	*Enterobacter cloacae*	
	Enterobacter cloacae complex	
Enterococcus	*Enterococcus faecalis*	
	Enterococcus faecium	
Escherichia	*Escherichia coli*	
Finegoldia	*Finegoldia magna*	
Fusobacterium		
	Fusobacterium nucleatum	
	Fusobacterium necrophorum	
Granulicatella	*Granulicatella adiacens*	

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Haemophilus	*Haemophilus haemolyticus*			
	Haemophilus influenzae			
Helicobacter	*Helicobacter pylori*			
Klebsiella	*Klebsiella aerogenes*			
	Klebsiella oxytoca			
	Klebsiella pneumoniae			
Legionella	*Legionella pneumophila*			
Listeria				
Moraxella	*Moraxella catarrhalis*			
Morganella	*Morganella morganii*			
Neisseria	*Neisseria meningitidis*			
Pasteurella	*Pasteurella multocida*			
Prevotella	*Prevotella buccae*			
	Prevotella intermedia			
Propionibacterium				
	Propionibacterium acnes			
Proteus				
	Proteus mirabilis			
Providencia	*Providencia stuartii*			
Pseudomonas	*Pseudomonas aeruginosa*			
	Pseudomonas non-aeruginosa			
Salmonella	*Salmonella enterica*			
Serratia	*Serratia marcescens*			
Staphylococcus				
	Staphylococcus aureus			
	Staphylococcus non-aureus			
Stenotrophomonas	*Stenotrophomonas maltophilia group*			
Streptococcus				
	Streptococcus anginosus group			
	Streptococcus agalactiae			
	Streptococcus dysgalactiae			
	Streptococcus gordonii			
	Streptococcus mitis group			
	Streptococcus pneumoniae			
	Streptococcus pyogenes			
	Streptococcus salivarius group			
Yersinia	*Yersinia enterocolitica*			
	Yersinia pseudotuberculosis complex			

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Gram	Resistance	Resistance genes	Profile
Positive	Vancomycin resistances	*vanA*	
		vanB	
	Methicillin resistances	*mecA*	
		mecC	
Negative	Betalactamase/ Carpabenemase	*CTX m1/m3*	
		IMP	
		KPC	
		NDM	
		OXA48	

Fungi potentially presented by *hybcell Fungi DNA xB* and by *hybcell Pathogens DNA xB*:

Genus	Species	Profile
Aspergillus		
	Aspergillus clavatus	
	Aspergillus flavus	
	Aspergillus fumigatus	
	Aspergillus niger	
	Aspergillus terreus	
Candida		
	Candida albicans	
	Candida dubliniensis	
	Candida parapsilosis	
	Candida tropicalis	
Nakaseomyces	*Candida glabrata*	
Clavispora	*Candida auris*	
Cladosporium		
Filobasidiella	*Cryptococcus neoformans*	
	Cryptococcus gattii	
Fusarium	*Fusarium oxysporum species complex*	
	Fusarium solani species complex	
Pichia	*Pichia kudriavzevii*	
Pneumocystis	*Pneumocystis jirovecii*	
	Pneumocystis murina	
Saccharomyces		
	Saccharomyces cerevisiae	
Scedosporium		

The test may be used for different diagnostic applications but not all bacterial and fungal targets are relevant for all uses. Therefore, it is possible to narrow the scope of results of a report within the hyborg software by defining a profile (by selecting the targets which should be considered for the report).

The test must be carried out in an environment suitable for molecular biological testing. This includes DNA-and DNase-free pipets, separated rooms for DNA isolation and amplification/detection, and the possibility of UV de-contamination.

The necessary equipment includes a freezer (-15 to -25°C) as well as a DNA workbench. The sample materials are solutions containing DNA that was extracted with an appropriate DNA extraction product/procedure.

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For processing *PCR-Box Bacteria, PCR-Box Resistances, PCR-Box Fungi,* and *PCR-Box IPC* either a qPCR device (either Rotor-Gene from Qiagen; CFX96 from Biorad or Quantstudio from Thermo) or a thermal cycler (TPersonal from Analytic Jena) is needed.

For processing *hybcell Bacteria DNA xB, hybcell Fungi DNA xB,* or *hybcell Pathogens DNA xB,* a *hyborg Dx RED2* device with preinstalled hyborg Software (Cube Dx) is required.

The test results should be evaluated in the context of the patient's medical record, his/her clinical status, and other findings.

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3. Technical description

The course of sepsis or other severe infections and especially the chances of recovery and survival are dependent on early identification of the causing pathogen(s).

The chances of survival and recovery after suffering from sepsis and other severe infections may be increased by early identification and targeted treatment of the causing pathogen(s).

IPC, GINA 500, GINA 500 + DNA Purification

Cube Dx' Internal Process Control (IPC) consists of frozen biological material, which is dissolved in the sample before enrichment. This biological material is similar to pathogenic microorganisms causing sepsis or other severe infections.

The kit *GINA 500 (for 500µl of sample liquid, with or without DNA purification)* is designed for clinical routine application for enriching pathogenic (bacterial, fungal) DNA. After enrichment, the solution is purified and the eluate may be used in PCR reactions (e.g., bacterial DNA, fungal DNA, resistance marker genes). In case PCR products have been amplified in a sample, the respective pathogen can be identified straight-forward by Cube Dx's *compact sequencing*.

The kit is based on the following process steps:

- Lysis and removal of human cells: LE solution is added to the sample, and most human (and compromised pathogen) cells are lysed and removed after centrifugation.
- Lysis of pathogen cells: NA solution is added and incubated. Pelleted pathogen cells are lysed.
- Neutralization: The lysate is transferred into the T solution to stop the process of lysis and neutralize the resulting solution.
- *Including DNA purification*: spin column technology is used to purify DNA from the *GINA* solution.

The result may be falsified due to the nature of the sample, errors during the procedure (low amount of DNA, contamination with environmental microorganisms / DNA), other influences (degraded DNA, contamination with chemicals), or technical errors.

The following circumstances deteriorate results for a sample:

- The time between drawing the (blood) sample and the start of sample preparation is more than 4 hours.
- The storage of the sample between drawing and the start of sample preparation is not according to the specifications (specified: store dry and between 4°C and 8°C, refer to the storage and shelf-life chapter).

LINA

LINA is 8mL of buffer filled in a single ready-to-use tube. The buffer dilutes any PCR inhibitors in the sample, so these are no longer effective. The sample buffer mixture is directly transferred into the PCR reactions (without any further extraction process). The short and simple protocol reduces the time to result drastically.

The result may be falsified due to the nature of the sample or errors during the procedure (e.g., a low number of microorganisms in the sample or technical errors).

The following circumstances deteriorate results for a sample:

- Use of a larger sample volume than specified (increases inhibitors).

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PCR-Box Bacteria / Resistance / Fungi / IPC
hybcell Bacteria / Fungi / Pathogens DNA xB

The tests *hybcell Bacteria DNA xB, hybcell Fungi DNA xB,* and *hybcell Pathogens DNA xB* and their related PCR reaction mix – *PCR-Box Bacteria, PCR-Box Resistance, PCR-Box Fungi,* and *PCR-Box IPC* – are designed for clinical routine application to detect and identify pathogenic bacteria and their antibiotic resistance marker genes as well as pathogenic fungi by using DNA extracted from samples like whole blood or positive blood cultures. *PCR-Box IPC* amplifies DNA from the *IPC* to confirm the validity of the test procedure by a positive *IPC* result on the hybcell.

The test is especially useful for patients in need of immediate and specific antimicrobial treatment (e.g., sepsis), for patients having already undergone treatment with antibiotics/antimycotics (as culturing might then be inhibited), or if the causative pathogens are difficult to culture.

The test is based on the following process steps/test principles:

- Amplification of DNA – detection of bacteria/fungi/resistance marker genes: Isolated DNA is amplified by polymerase chain reaction (PCR). Target regions are 16S rDNA of bacteria, 28S rDNA of fungi, and respective resistance marker genes. During amplification, single DNA strands are labeled with a fluorescent dye. If using a qPCR device, the presence of bacteria, fungi, or resistance marker genes might be derived from the resulting amplification curves.
- Identification: Qualitative analysis is performed by applying *compact sequencing*. Amplicons bind to their complementary, immobilized probes which are elongated by a highly specific DNA polymerase in case of a perfect match (primer extension). Unspecific amplicons and non-elongated primers are removed during stringent washing steps. The *hyborg* (an instrument for analysis) scans and analyzes the specific fluorescence signals.

The result may be falsified due to errors during sample preparation (low amount of DNA, contamination with environmental pathogens / DNA) or other influences during preparation (degraded DNA, contamination with chemicals), technical errors, or errors during amplification or identification. If there is suspicion that a result is incorrect or deteriorated, the results should not be taken into account. Even if internal controls should single out the most erroneous results, some of these results may remain uncovered.

The following circumstances deteriorate the results of a sample:

- The time between drawing the sample and the start of sample preparation is more than 4 hours
- Storage of the sample between drawing the sample and the start of sample preparation is not according to the specifications (specified: store dry and between 4°C and 8°C, refer to the storage and shelf-life chapter).

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4. Product components

Internal Process Control (IPC):

- *IPC* (REF / UDI-DI 09120127730169): store **frozen** at **-15 to -25°C**
  - 25 x 20 µL IPC
    (25 x separately packed 0,5mL microtubes with biological material (IPC, each 20µL))

To enrich pathogens (bacteria and fungi) from a 500µl (or less) sample, the following specific products are required:

- *GINA 500* (REF / UDI-DI 09120127730244): store at **room temperature (8 to 25°C)**
  - 2 x 25 *LE solution* (1400µl); (2 x 25 x 2mL tubes with yellow cap)
  - 1 x 12mL *NA solution* (red mark on bottle and cap)
  - 1 x 25mL *T solution* (green mark on bottle and cap)

To enrich pathogens (bacteria and fungi) and purify RNA/DNA from 500µl (or less) of the sample, the following specific products are required:

- *GINA 500 + DNA Purification* (REF / UDI-DI 09120127730145)*:* store at **room temperature (8 to 25°C)**
  - 2 x 25 *LE solution* (1400µl); (2 x 25 x 2mL tubes with yellow cap)
  - 1 x 12mL *NA solution* (red mark on the bottle and cap)
  - 1 x 25mL *T solution* (green mark on the bottle and cap)
  - 1 x 30mL *Wash Buffer BW* (bottle)
  - 1 x 60mL *Wash Buffer B5* (bottle)
  - 1 x 13mL *Elution Buffer BE* (bottle)
  - 50 x *Column*
  - 50 x *Collection Tube*
  - 50 x *Elution Tube*

To directly test samples with an abundance of microorganisms (positive blood cultures, BAL), the following specific product is required:

- *LINA* (REF / UDI-DI 09120127730152): store at **room temperature (8 to 25°C)**
  - 50 x *LINA* (8ml)

To detect bacteria, the following specific products are required:

- *PCR-Box Bacteria* (REF / UDI-DI 09120127730084): store **frozen** at **-15 to -25°C**
  - 12 x 20 µL PCR master mixes Bacteria Rev.2
    (12 x separately packed 0,2mL PCR tubes with PCR master mixes Bacteria (each 20µL))

To detect resistance marker genes, the following specific products are required:

- *PCR-Box Resistance* (REF / UDI-DI 09120127730107): store **frozen** at **-15 to -25°C**
  - 12 x 20 µL PCR master mixes Resistance Rev.2
    (12 x separately packed 0,2mL PCR tubes with PCR master mixes Resistance (each 20µL))

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To detect fungi, the following specific products are required:

- *PCR-Box Fungi* (REF / UDI-DI 091201277300921): store **frozen** at **-15 to -25°C**
  - 12 x 20 µL PCR master mixes Fungi Rev.2
    (12 x separately packed 0,2mL PCR tubes with PCR master mixes Fungi (each 20µL))

To detect IPC DNA, the following specific products are required:

- *PCR-Box IPC* (REF / UDI-DI 09120127730114): store **frozen** at **-15 to -25°C**
  - 12 x 20 µL PCR master mixes IPC Rev.2
    (12 x separately packed 0,2mL PCR tubes with PCR master mixes IPC (each 20µL))

To identify bacteria and resistance marker genes, the following specific products are required (apart from general buffers for the hyborg device):

- *hybcell Bacteria DNA xB* Kit (REF / UDI-DI 09120127730053): store at **room temperature (8 to 25°C)**
  - 24 x hybcell Bacteria DNA xB Rev.2
    (24 x separately packed hybcells Bacteria DNA xB)
  - 24 x Lid
  - 1x PPE-Additive (900µl)

To identify fungi, the following specific products are required (apart from general buffers for the hyborg device):

- *hybcell Fungi DNA xB* Kit (REF / UDI-DI 09120127730060): store at **room temperature (8 to 25°C)**
  - 24 x hybcell Fungi DNA xB Rev.2
    (24 x separately packed hybcells Fungi DNA xB)
  - 24 x Lid
  - 1x PPE-Additive (900µl)

To identify bacteria, fungi, and resistance marker genes, the following specific products are required (apart from general buffers for the hyborg device):

- *hybcell Pathogens DNA xB* Kit (REF / UDI-DI 09120127730077): store at **room temperature (8 to 25°C)**
  - 24 x hybcell Pathogens DNA xB Rev.2
    (24 x separately packed hybcells Pathogens DNA xB)
  - 24 x Lid
  - 1x PPE-Additive (900µl)

Pay attention not to mix up components of different lots!

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5. Storage and shelf life

Products

The minimum shelf life of the products is only guaranteed if the required temperature and humidity conditions are safeguarded during transportation and storage. The expiry date of the products is printed on the product labels.

- *GINA 500, GINA 500 + DNA Purification, LINA, hybcells (Bacteria DNA xB / Fungi DNA xB / Pathogens DNA xB) and their PPE-Additive* are delivered at room temperature and must be stored at **8 to 25°C**.
- *IPC, PCR-Box Bacteria*, *PCR-Box Resistance, PCR-Box Fungi,* and *PCR-Box IPC* are delivered frozen and must be stored at **-15 to -25°C**.

If the protective sealing of hybcells or any other packaging (e.g., any tubes) is damaged / or the minimum shelf life has expired, the product/component must not be used. hybcells have to be used immediately after opening the protective sealing. Repeated freezing-and unfreezing cycles (> 2x) of PCR-Boxes should be avoided. Repeated thawing and freezing destroy IPC and have to be avoided. IPC has to be used immediately after opening the tube.

Samples

Blood

- Store cool (between 4°C and 8°C) and dry for a maximum of 4 hours for optimal results. Storing samples under these conditions is accepted for a maximum of 48 hours.
- Do not freeze blood!

BAL

- Store cool (between 4°C and 8°C) and dry for a maximum of 4 hours for optimal results. Storing samples under these conditions is accepted for a maximum of 48 hours.
- Avoid freezing. If freezing is necessary keep the samples between -15°C and -25°C.

Blood Culture

- Store cool (between 4°C and 8°C) and dry for up to 48 hours.
- Store frozen (between -15°C and -25°C).

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6. Required equipment

The following equipment is required for conducting the test:

Required Accessories / Infrastructure		REF / UDI-DI
Mini-centrifuge (0,2 mL rotor)	Thermo[1]: MySpin	
Mini Vortex Mixer	Fisher Scientific[2]	
Freezer (-20°C)		
DNA workbench	Starlab[3] (example): Laminar Flow PCR workbench with UV-light PEQLAB[4] (example): PCR-working station	
Pipettes: ▪ 20 – 200 µL ▪ 100 – 1000µl	GILSON[5]: PIPETMAN P200N PIPETMAN P1000N	
Standard table centrifuge (With rotor for 2 mL tubes)	Eppendorf[6]: Centrifuge 5430	
Standard heating block	Coyote Bioscience[7] H2O3-H	
qPCR device or thermal cycler	Qiagen[8]: Rotor-Gene Biorad[9]: CFX96 Thermo[10] Quantstudio 3 / 5 Analytic Jena[11]: TPersonal Thermocycler (Biometra)	
System Liquid	Cube Dx: 1l, sufficient for 8 weeks	09120127730022
PE-Buffer	Cube Dx: 1l, sufficient for 96 hybcells	09120127730138
Hyborg	Cube Dx: hyborg Dx RED2	09120127730015

Required accessories.

1 www.thermofisher.com / order / catalog / product / 75004081
2 www.fishersci.com / shop / products / variable-speed-mini-vortex-mix / 14955163
3 www.starlab.de
4 www.peqlab.de
5 www.gilson.com
6 www.eppendorf.com
7 www.coyotebio.com
8 www.qiagen.com
9 www.bio-rad.com
10 www.thermofisher.com
11 www.biometra.com

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7. Test procedure

! Before beginning the test procedure. Assure that the hyborg is ready for operation!

- Check if the hyborg is switched on (check the screen of the device – refer to the hyborg Dx manual for further details).
- Check if the hyborg is equipped with sufficient System Liquid and PE-Buffer. If not, refill these liquids.
- Empty the waste container if necessary (position W).
- Check if the necessary protocol is available (if not, load the protocol, refer to the hyborg Dx manual for further details).

Note, that some steps of the procedure require the preparation of equipment or reagents. As these tasks may be associated with waiting times, read the entire chapter of the procedure before starting.

During processing the samples, a laboratory coat, latex gloves, sleeve guards, hair (and beard) net, and a surgical mask must be worn to avoid contamination of the test reagents. Pathogen enrichment (see steps 2.-8. below, in red) must be done under a DNA workbench.

In the following sections, the workflow is described based on the following 3 steps;

1. Sample Preparation: with GINA/LINA
2. Detection: PCR/qPCR
3. Identification: compact sequencing

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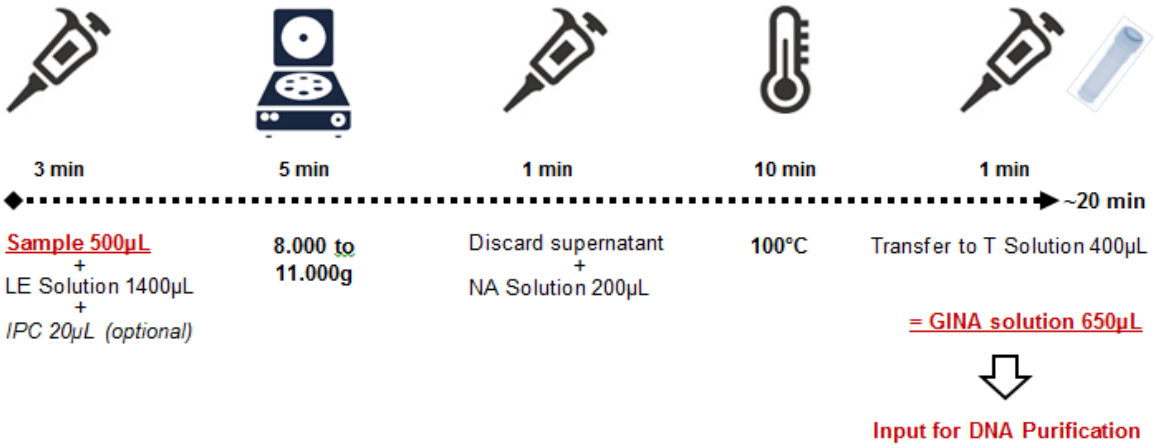
1 Sample prep: GINA/LINA

IPC + GINA: Enrichment (and Purification) procedure

Whole blood samples can be collected in K3E K3EDTA or K2E K2EDTA Vacuette Tubes.

The procedure starts with a native sample of EDTA-whole blood. Vortex the sample before use! If you are using IPC, pipette 500µl of the blood sample into the IPC first.



1. Make sure the equipment and all kit components are ready for use. Briefly spin down the needed tubes with *LE solution, IPC, or EPC* to avoid carry-over of liquids potentially present in the screw caps, when opening the vials. Turn on the heating block to 100°C.

2. Prepare *LE solution* and sample. **Do not shake or agitate the *LE solution* tube (yellow cap) to avoid the build-up of foam**! Transfer 500µl (or less) of EDTA blood (or other diluted samples) into the *LE solution* (yellow cap) and pipet up and down to mix.

3. Optional: Pipette 500µl of the blood sample into the IPC (and/or EPC if used) and thereafter pipette the mixture into the LE buffer.

4. Close the tube, mark it, and vortex vigorously for 5 seconds or invert the tubes several times. Incubate for ~2 min at room temperature (18°C to 25°C).

5. Centrifuge for 5 minutes between 9.000 and 11.000g (preferably with 11.000g). If available, use a soft ramping of the centrifugation speed.

6. Remove the supernatant carefully by **decanting** and add 200 µL *NA solution* (red cap) into the tube with the yellow cap. Close the screw cap tightly.

    - Remark: Some sample liquid (~50 µL) may stay on top of the pellet after decanting. **Whole blood samples should turn greenish at this point.**

7. Vortex vigorously for 5 seconds. Make sure that the tubes are still tightly closed.

8. Incubate at 100°C for 10 minutes (+ / - 1 minute), using a heating block.

9. Add 400µl *T solution* (green cap) into the tube with the yellow cap to neutralize.

    - Remark: **Whole blood samples should turn from greenish to dark reddish.**

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10. Purify DNA, using common DNA extraction products (in the case of *GINA 500 + DNA purification*: Machery Nagel Nucleo Spin reagents are included in the kit. Otherwise: follow the manufacturer's instructions, and skip steps 11-17).

11. For each sample, place one *Column* into a *Collection Tube* and mark the *Collection Tube* with the sample ID. Transfer the whole *GINA* solution (600 to 650 µL) to the column. Discard the tube with the yellow cap.

12. Centrifuge for 1 min between 9.000 and 11.000g. Remove the *Column*, decant the flow-through liquid, and insert the *Column* again.

13. Add 500µl *Wash Buffer BW* and centrifuge for 1 minute at between 9.000 and 11.000g. Remove the *Column*, decant the flow-through liquid, and insert the *Column* again.

14. Add 600µl *Wash Buffer B5* and centrifuge for 1 minute at between 9.000 and 11.000g. Remove the *Column*, decant the flow-through liquid, and insert the *Column* again.

15. Centrifuge for 1 minute at between 9.000 and 11.000g to dry the silica membrane. Check if some liquid remains at the bottom of the *Column*. If yes, repeat this step.

16. Place the *Column* into an *Elution Tube* and mark the *Elution Tube* with the sample ID. Add 100 to 150µl *Elution Buffer BE*. Incubate at room temperature for 1 min. Centrifuge for 1 minute at between 9.000g to 11.000g. Check the elution volume. If the volume appears to be too low, repeat centrifugation. Discard the *Column*.

17. Open the *Elution Tube* and incubate at 100°C for 3 minutes in the heating block.

18. The collected liquid containing the DNA (eluate) might be used for PCR-based applications or stored at -20°C for later processing. Before using the eluate, **vortex** the *Elution Tube* firmly.

LINA: Modulation procedure

The procedure starts with either a (positive) blood culture or a BAL sample.

Note, that some steps of the procedure require the preparation of equipment or reagents. As these tasks may be associated with waiting times, read the entire chapter of the procedure before starting.

During processing the samples, a laboratory coat, latex gloves, sleeve guards, hair (and beard) net, and a surgical mask must be worn to avoid contamination of the test reagents.

1. Make sure the equipment and all kit components are ready for use.
2. Optional: Pipette 20µl IPC (one reaction) into the LINA tube.
3. Pipette the sample into the LINA tube:
    - (Positive) blood culture: 2µL
    - BAL: 20µL

    Remark:

    Different institutes have varying methods of collecting BAL samples. Therefore, the amount of BAL used may have to be adapted. However, too high volumes of BAL may result in inhibition. So, using IPC is recommended to indicate inhibition.
4. Close the tube and shake or vortex firmly.

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2 Detection: PCR/qPCR

PCR-Box Bacteria / Resistance / Fungi / IPC
hybcell Bacteria / Fungi / Pathogens DNA xB

Note, that some steps of the test procedure require the preparation of equipment or the thawing of reagents. As these tasks are associated with waiting times, read the entire chapter of the test procedure before starting.

During test preparation and processing a laboratory coat, latex gloves, sleeve guards, hair (and beard) net, and a surgical mask must be worn to avoid contamination of the test reagents. Preparation of PCR (see step 2. below, in red) must be done under a DNA workbench.

The test procedure starts with the solution resulting from GINA pathogen enrichment and DNA purification or the LINA modulation buffer (e.g., with positive blood culture).

1. Make sure the equipment and all kit components are ready for use.
2. (q)PCR reaction:

- Program the qPCR device or PCR-thermocycler and save the program as “Patho_1”:

1	95°C for 2:00
2	95°C for 0:10
3	56°C for 0:10
5	72°C for 0:30
	+Plate Read
	GO TO 2, 44 more times
6	75°C for 1:00
7	Melt curve 75°C to 95°C in increments of 0,3°C for 0:10
	+Plate Read
8	25°C for hold

Fluorophore / channel: SYBR Green

Remark:

Thermal cyclers may differ in their thermal characteristics, therefore the optimization of the temperatures stated in the protocol may be recommended (for validated devices only) if the results and undesirable.

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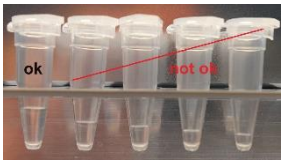
- Unpack and thaw single 0,2 ml tubes with the needed master mixes of Bacteria (red dot), master mixes Resistance (yellow dot), Fungi (green dot), and IPC (blue dot). Homogenize (vortex) and spin down briefly the solution in each tube.
- Check, if the volume of the PCR-mix is approximately 20µl (see picture, the left tube is filled with 20µl). Do not use PCR-mixes that have been improperly filled.



- Add 20 µL sample DNA solution (or 20 µL DNA-free water as NTC) to the PCR master mixes.
- Close PCR tubes (if you don't use a rotating thermocycler homogenize and spin down liquids before starting PCR).
- Start (q)PCR program "Patho_1" (programmed before).

The amplified DNA is either used immediately for the compact sequencing reaction or it can be stored at 4°C to 8°C overnight or stored frozen at -15°C to -25°C for longer periods.

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3 Identification: compact sequencing

1. Assure that the hyborg is ready for operation.
2. Open the packaging of the hybcell (rip the sealing at the notch), and place the hybcell into the rack (positions A-H).
3. Combine up to 3 of the desired amplicons from the same sample maximum – for example, 30µl of *PCR-Box Bacteria*, *PCR-Box Fungi, and PCR-Box Resistance* – into one of the amplicon tubes (e.g., *PCR-Box Bacteria)*. Thereafter pipette 30µl of the PPE-Additive (found in the hybcell box) into the tube with the amplicon mixture.

   Attention!

   Introducing only 1 or 2 PCR amplicons into the hybcell results in the best performance regarding the signal background. Up to 3 PCR amplicons can be introduced into the hybcell. By introducing all 4 amplicons the risk of heightened signal background increases and may lead to a reduced specificity and sensitivity of the test.

   Remember, that the validity of the Internal Process Control (IPC) can already be derived from the PCR amplification curves (compare results in Chapter 9)

   Different examples of amplicon combinations suitable for the loading of the hybcell

Results of PCR				Amplicons transferred into hybcell
Bacteria	**Fungi**	**Resistance**	**IPC**	
pos.	pos.	pos.	pos.	Bacteria + Fungi + Resistance
pos.	pos.	neg.	pos.	Bacteria + Fungi
pos.	neg.	pos.	pos.	Bacteria + Resistance
neg.	pos.	neg.	pos.	Fungi + IPC

4. Pipette up and down to mix all the constituents in the tube (**a pH indicator is present in the additive; the color of the solution may therefore change upon introducing the amplicons. This does not influence the performance of the product**). Avoid bubbles!
5. Pipette the entire volume from the tube (~ 120 µL maximum) into the hybcell (through the central channel) at once. The final volume is dependent on the presence of suitable amplicons after the qPCR or on the used PCR products.

   Use a 200 µL pipette with appropriate filter tips! Do not block the hybcell central channel (sample inflow) with the pipette tip while introducing the amplicon mix! Only insert the tip as deep as needed into the hybcell central channel, make sure to allow a loose fit.
6. Cover the hybcell using the provided lid.

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7. Start processing the samples after entering the sample and hybcell ID (see hyborg Dx RED2 manual for further details). Load the device with the prepared rack.

   Insert rack correctly (hybcell barcodes/labels have to face the inside of the device)! Pay attention that all hybcells are in the correct position.

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8. Results

PCR Analysis

PCR results are the key determinant of whether a sample is positive or negative for bacterial, fungal, IPC DNA, or resistance genes. A positive PCR result would direct the user into the next step which is the identification of the pathogen by the hybcell. A negative result means there is no pathogen DNA or resistance gene present, therefore, there is no need for identification by the hybcell.

To determine if a result is positive or negative both the amplification curve and the melting curve have to be analyzed. All PCR-mixes use the same fluorescence dye *SYBR Green*.

Attention!

Be aware that every PCR setup is slightly different in its characteristics and that Ct-values and melting peaks vary from site to site. Therefore, the below parameters are recommendations based on experience, on where to set the thresholds for Ct-value and melting temperature. Both criteria must be met to define a PCR result as positive.

Every lab must verify these recommendations and if necessary, adjust to its own thresholds. The final positive result is established by the hybcell test. So, consider including all samples in your hybcell runs where there might be the slightest doubt that the PCR is negative. Especially during setting up the test in your lab and in the early phase of using it, be more generous with including samples in your hybcell testing.

	Ct-Value	Melting Temperature
Bacteria	< 34	80℃ to 90℃
Fungi	< 40	80℃ to 94℃
Resistance	< 34	80℃ to 90℃
IPC	< 34	86℃ ± 1℃

Note that the melting temperature is dependent on the species or resistance gene which is amplified and therefore varies for the pan-bacterial, pan-fungal, and multiplex resistance gene PCR.

Below is an example of a blood sample spiked with an External Process Control (EPC) of Candida albicans and Internal Process Control (IPC).

	Ct-Value	Melting Temperature	Derived Result
Bacteria	32.8	74.92°C	**Negative**
Fungi	25	88.54°C	**Positive**
IPC	24	86.51°C	**Positive**

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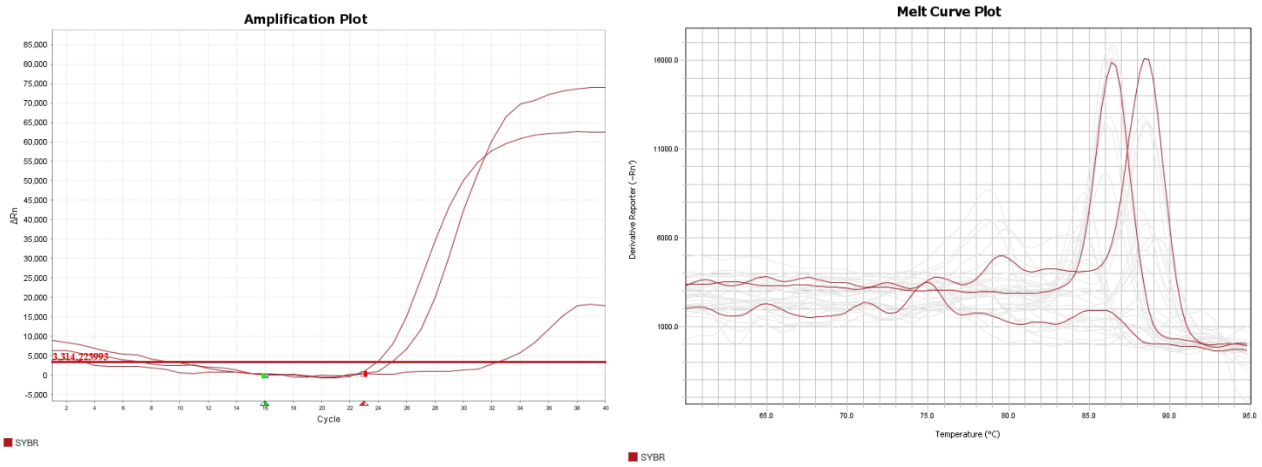

Some reasons for variations in Ct-values and melting temperatures:

- The threshold for the Ct-Value calculation is set by the user
- Different PCR devices offer different software with different characteristics. For example, auto-scale, threshold settings, and so forth which influence the Ct values and the visual presentation of the curves.
- The PCR-Box Resistances comprise several primers (resistance genes). Therefore, primer dimers are more likely to occur as with the single-plex PCR for bacteria or fungi.
- Bacterial or fungal contaminations acquired during sample taking or the test procedure lower the Ct value. Possible reasons for contamination are described in our brief instructions “Pathogens xB_brief instructions Contamination Prevention Guide_24_10_2023”. Here you will get information on sample taking, sample processing, required protective gear, disinfection of surfaces, etc.
- The salt concentration and other conditions of the eluates might vary due to variances in the composition of samples and usage of different sample collection products.
- Finally, the amplified microorganism itself may influence the Ct value (and even more the melting temperature).

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Internal Process Control (IPC)

The Internal Process Control (IPC) confirms the validity of negative results. It is a positive process control that enables the user to differentiate between negative results and invalid results – results that most probably have been hampered by flaws in the process.

The validity of IPC is either confirmed by the PCR result (when using Realtime PCR instruments, see above) or with the hybcell test (see below).

IPC and therefore the test is valid if the below criteria for the IPC-PCR are met. In that case, adding the amplicon to the hybcell test is not necessary (see above, limitation of PCR amplicons).

	Ct-Value	Melting Temperature
IPC	< 34	86℃ ± 1℃

Attention!

The Internal Process Control (IPC) is designed to confirm negative results. In case of a positive amplification of the PCR-mix for bacteria or the PCR-mix for fungi, the amplification results for IPC can be ignored and the hybcell test should be performed. Analogous to that, the result for IPC can be ignored in case of any identification of bacteria or fungi with hybcell, if the IPC result of the test is "NOT DETECTED" where it is expected to be "DETECTED".

hybcell Controls

hybcell tests feature several internal controls to ensure proper results. If all internal controls are passed, the result for 'Controls' is 'PASSED' (and shown as such on the report). If one or more controls fail, the controls are marked as 'FAILED' on the report. If any control fails, the results are invalid and the test has to be repeated.

- **Process Control**: Checks the processing of the hybcell.
- **Surface Control:** Checks the hybcell type, sufficient fluorescence, and the scanning process.
- **Background Noise Control**: Checks unspecific binding, and basic features of the hyborg software.

Check for PCR-mixes

As the user chooses to use all or just selected PCR mixes, the usage of PCR mixes is indicated with probes on the hybcell surface. If a PCR mix is added, the result on the report for this mix is 'ADDED'; otherwise, it is 'MISSING'.

- **Bacteria PCR mix**: Checks if the *PCR-Box Bacteria* was used.
- **Fungi PCR mix**: Checks if the *PCR-Box Fungi* was used.

Test specific Controls

The tests feature a test-specific control. If the control is passed, the result is 'PASSED'. Otherwise, the result is 'FAILED'. Even if it fails, the test is analyzed and results are presented. However, these controls help to judge the plausibility of the results.

- **Specificity Control**: Checks if the process of compact sequencing suffered major flaws.

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- **Internal Process Control (IPC)**

The Internal Process Control is a test-specific control that is examined with every hybcell test. Only if the (positive) PCR-amplicon of IPC is added into the hybcell and corresponding probes on the hybcell reach the signal threshold, the result is 'DETECTED'. Otherwise – if either no positive amplicon is added or the signal threshold is not passed – the result is 'NOT DETECTED'.

General nomenclature

- **Bacteria species** are positive if a species 16S rDNA was amplified and corresponding primer extension took place (e.g., *Staphylococcus aureus*).
- **Bacteria genus** is positive if a species 16S rDNA was amplified and if the primer extension pattern matches a genus (e.g., *Staphylococcus*), but not necessarily a specific species of the tested panel.
- **Bacteria pan** is positive if amplified bacterial DNA is present.
- **Fungal species** is positive if a species 28S rDNA was amplified and corresponding primer extension took place (e.g., *Candida albicans*).
- **Fungal genus** is positive if a species 28S rDNA was amplified and if the primer extension pattern matches a genus (e.g., *Candida*), but not necessarily a specific species of the tested panel.
- **Fungi pan** is positive if amplified fungal 28S rDNA is present.

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hyborg Reports

CubeDx GmbH
Westbahnstr. 55
4300 St. Valentin
Austria

cube dx
hybCell technology

Sample	633248 BR	**Test**	hybcell Pathogen DNA xB (7)
Date	06.10.2023 21:42	**Profile**	-
Remark		**hybcell**	2509A510038
Liquids			

Controls		
Controls	PASSED	
Bacteria PCR Mix	ADDED	
Fungi PCR Mix	MISSING	

Parameters	Result	Representation
Specificity Control	PASSED	
Internal Process Control	DETECTED	
BACTERIA		
Bacteria Pan	Positive	50 10000
Gram pos	Positive	50 10000
Staphylococcus sp.	Positive	50 10000
Staphylococcus aureus	Positive	50 10000
RESISTANCES		
Methicillin	Positive	50 10000
Methicillin Type A	Positive	50 10000

Negative Parameters

Abiotrophia defectiva, Acinetobacter baumannii, Acinetobacter calcoaceticus complex, Actinobacillus pleuropneumoniae, Anaerococcus sp., Aspergillus clavatus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus sp., Aspergillus terreus, Bacteroides fragilis, Bordetella pertussis, Borreliella burgdorferi, Borreliella sp., Brucella sp., Burkholderia cepacia complex, Burkholderia pseudomallei, Campylobacter sp., Candida albicans, Candida auris, Candida dubliniensis, Candida glabrata, Candida parapsilosis, Candida sp., Candida tropicalis, Carbapenemases, Citrobacter freundii complex, Citrobacter koseri, Cladosporium, Corynebacterium diphtheriae, Corynebacterium jeikeium, Corynebacterium sp., Corynebacterium ulcerans, Cryptococcus gattii, Cryptococcus neoformans, CTX-m1/m3, Enterobacter cloacae, Enterobacter cloacae complex, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Extended Spectrum beta Lactamases, Finegoldia magna, Fungi Pan, Fusarium oxysporum species complex, Fusarium solani species complex, Fusobacterium necrophorum, Fusobacterium nucleatum, Fusobacterium sp., Gram neg, Granulicatella adiacens, Haemophilus haemolyticus, Haemophilus influenzae, Helicobacter pylori, IMP, Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, KPC, Legionella pneumophila, Listeria sp., Methicillin Type C, Moraxella catarrhalis, Morganella morganii, NDM, Neisseria meningitidis, OXA-48, Pasteurella multocida, Pichia kudriavzevii, Pneumocystis jirovecii, Pneumocystis murina, Prevotella buccae, Prevotella intermedia, Propionibacterium acnes, Propionibacterium sp., Proteus mirabilis, Proteus sp., Providencia stuartii, Pseudomonas aeruginosa, Pseudomonas non-aeruginosa, Saccharomyces cerevisiae, Saccharomyces sp., Salmonella enterica, Scedosporium, Serratia marcescens, Staphylococcus non-aureus, Stenotrophomonas maltophilia group, Streptococcus agalactiae, Streptococcus anginosus group, Streptococcus dysgalactiae, Streptococcus gordonii, Streptococcus mitis group, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius group, Streptococcus sp., Vancomycin, Vancomycin Type A, Vancomycin Type B, Yersinia enterocolitica, Yersinia pseudotuberculosis complex

An example of a PDF report for a sample positive for bacteria and its respective resistance.

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Westbahnstr. 55
4300 St. Valentin
Austria

cube dx
hybCell technology

Sample	65181 F	**Test**	hybcell Pathogen DNA xB (7)
Date	06.10.2023 21:39	**Profile**	-
Remark		**hybcell**	2509A510038
Liquids			

Controls		
Controls	PASSED	
Bacteria PCR Mix	ADDED	
Fungi PCR Mix	ADDED	

Parameters	Result	Representation
Specificity Control	PASSED	
Internal Process Control	DETECTED	
BACTERIA		
Negative		
RESISTANCES		
Negative		
FUNGI		
Fungi Pan	Positive	50 10000
Pichia kudriavzevii	Positive	50 10000

Negative Parameters

Abiotrophia defectiva, Acinetobacter baumannii, Acinetobacter calcoaceticus complex, Actinobacillus pleuropneumoniae, Anaerococcus sp., Aspergillus clavatus, Aspergillus flavus, Aspergillus fumigatus, Aspergillus niger, Aspergillus sp., Aspergillus terreus, Bacteria Pan, Bacteroides fragilis, Bordetella pertussis, Borreliella burgdorferi, Borreliella sp., Brucella sp., Burkholderia cepacia complex, Burkholderia pseudomallei, Campylobacter sp., Candida albicans, Candida auris, Candida dubliniensis, Candida glabrata, Candida parapsilosis, Candida sp., Candida tropicalis, Carbapenemases, Citrobacter freundii complex, Citrobacter koseri, Cladosporium, Corynebacterium diphtheriae, Corynebacterium jeikeium, Corynebacterium sp., Corynebacterium ulcerans, Cryptococcus gattii, Cryptococcus neoformans, CTX-m1/m3, Enterobacter cloacae, Enterobacter cloacae complex, Enterococcus faecalis, Enterococcus faecium, Escherichia coli, Extended Spectrum beta Lactamases, Finegoldia magna, Fusarium oxysporum species complex, Fusarium solani species complex, Fusobacterium necrophorum, Fusobacterium nucleatum, Fusobacterium sp., Gram neg, Gram pos, Granulicatella adiacens, Haemophilus haemolyticus, Haemophilus influenzae, Helicobacter pylori, IMP, Klebsiella aerogenes, Klebsiella oxytoca, Klebsiella pneumoniae, KPC, Legionella pneumophila, Listeria sp., Methicillin, Methicillin Type A, Methicillin Type C, Moraxella catarrhalis, Morganella morganii, NDM, Neisseria meningitidis, OXA-48, Pasteurella multocida, Pneumocystis jirovecii, Pneumocystis murina, Prevotella buccae, Prevotella intermedia, Propionibacterium acnes, Propionibacterium sp., Proteus mirabilis, Proteus sp., Providencia stuartii, Pseudomonas aeruginosa, Pseudomonas non-aeruginosa, Saccharomyces cerevisiae, Saccharomyces sp., Salmonella enterica, Scedosporium, Serratia marcescens, Staphylococcus aureus, Staphylococcus non-aureus, Staphylococcus sp., Stenotrophomonas maltophilia group, Streptococcus agalactiae, Streptococcus anginosus group, Streptococcus dysgalactiae, Streptococcus gordonii, Streptococcus mitis group, Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus salivarius group, Streptococcus sp., Vancomycin, Vancomycin Type A, Vancomycin Type B, Yersinia enterocolitica, Yersinia pseudotuberculosis complex

An example of a PDF report for a sample positive for fungi.

Protocol (.hyb)

Calibration curves and pattern recognition were done for all microorganisms and genes (identified bacterial 16S rDNA / identified fungal 28S rDNA / identified resistance marker DNA) and are part of the hyborg protocol (XML-

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file with the extension .hyb). Calibration is independent of the hyborg device (unit use). However, it is a precondition that the hyborg operates in the specified environmental conditions (e.g., liquid delivery, heating, laser power, etc.).

Specific protocols are imported into the hyborg software before the first use of a new lot. Up-to-date protocols are provided on the Cube Dx homepage (https://www.cubedx.com/support/protocols) or by your local distributor. Protocols may also be updated automatically.

Off-profile Parameters

According to the intended purpose, clinically relevant results are indicated. The protocol for the lot (fixed for the CE-IVD test kits) defines clinically relevant bacteria, resistance gene markers, and fungi. The results outside this scope are labelled as “off-profile parameters”. Such results may be interpreted by infectious disease specialists.

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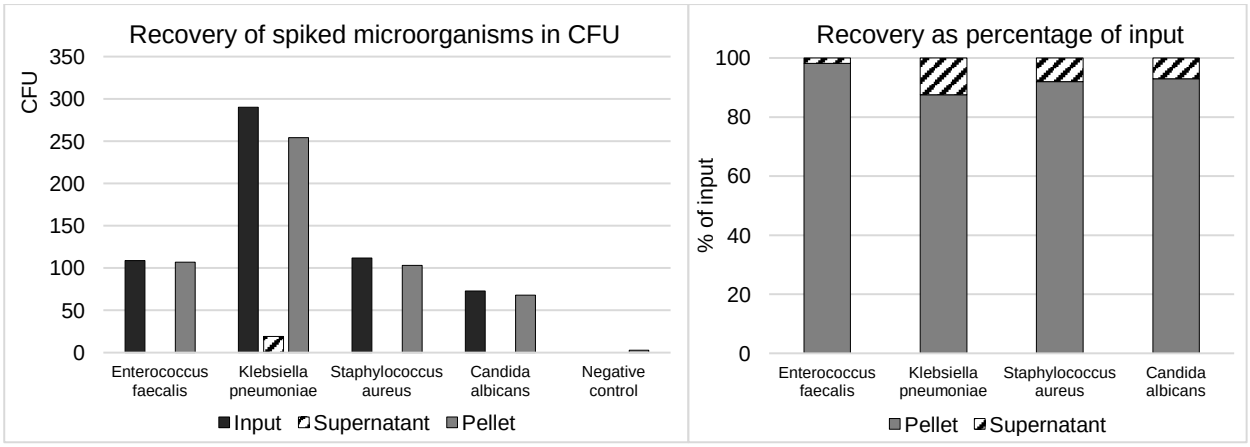
9. Analytical Performance

IPC, GINA 500, GINA 500 + DNA Purification

Recovery of pathogens: Living microorganisms (*Staphylococcus aureus Candida albicans*, *Enterococcus faecalis, Klebsiella pneumoniae*) were spiked into EDTA whole blood samples of healthy probands. These samples were homogenized (vortexed). The empty growth medium was spiked as a negative control. The first step of the *GINA 500* protocol was executed (*LE solution* + centrifugation). The resulting pellets were resuspended in 100µl EDTA whole blood and plated out on LB agar. After centrifugation, 100µl of the supernatant was also plated out to determine the number of living microorganisms that were not bound in the pellet (= loss). Colonies were counted and documented after 24 to 48 hours of incubation.



The rate of recovery lies between 88% (*Klebsiella pneumoniae*) and 98% (*Enterococcus faecalis*).

Bacteria

The limit of detection (LOD) was determined by diluting cultures of *Staphylococcus aureus*, *Klebsiella pneumoniae,* and *Pseudomonas aeroginosa* and processing these with the *GINA 500 + DNA purification* product and protocol. To determine the corresponding CFUs aliquots of the dilutions were plated out and colonies were counted after 24 / 48 hours of incubation.

For all three targets, the **LOD was determined between 10 to 20 CFU / mL**.

Selectivity was tested with referenced DNA samples from ATCC (American Type Culture Collection) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen).

Acinetobacter baumannii c.	DSM30007	*Actinobacter pleuropneumoniae*	DSM13472
Borreliella burgdorferi	DSM4680	*Burkholderia cepacia complex*	DSM7288
Brucella sp.	DSM103976	*Campylobacter jejunii*	DSM4688
Citrobacter freundii compl.	DSM30039	*Citrobacter koseri*	DSM4596
Corynebacterium diphtheriae	ATCC 700971D-5	*Corynebacterium jeikeium*	DSM7113
Corynebacterium ulcerans	DSM46325	*Enterobacter aerogenes*	DSM30053
Enterobacter cloacae compl.	DSM30054	*Enterococcus faecium*	DSM20477
Enterococcus faecalis	DSM20478	*Escherichia coli*	DSM30083
Finegoldia magna	DSM20470	*Fusobacterium necrophorum*	DSM20698
Fusobacterium nucleatum	DSM15643	*Haemophilus influenzae*	DSM4690
Helicobacter pylori	DSM21031	*Klebsiella oxytoca*	DSM5175
Klebsiella pneumoniae	DSM30104	*Legionella pneumophila*	DSM25213
Listeria monocytogenes	DSM15675	*Moraxella catarrhalis*	DSM9143
Morganella morganii	DSM30117	*Neisseria meningitidis*	DSM10036
Prevotella intermedia	DSM20706	*Propionibacterium granulosum*	ATCC 25746D-5

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Proteus mirabilis	DSM4479	*Pseudomonas aeroginosa*	DSM50070
Pseudomonas syringae	DSM50274	*Salmonella enterica*	DSM554
Serratia marcescens	DSM30121	*Staphylococcus aureus*	DSM20774
Staphylococcus epidermidis	DSM20044	*Staphylococcus haemolyticus*	DSM20263
Stenotrophomonas maltophilia	DSM21257	*Streptococcus agalactiae*	DSM2134
Streptococcus anginosus gr.	DSM20563	*Streptococcus dysgalactiae*	DSM20662
Streptococcus pneumoniae	DSM20566	*Streptococcus pyogenes*	DSM20565
Yersinia enterocolitica	DSM11067	*Yersinia pseudotuberculosis*	DSM8992

For each experiment DNA of two different species was mixed and tested.

Each tested bacterial DNA did show the **expected result** on the hybcell report.

No unspecific results or cross-reactivities have been observed.

Repeatability was determined by amplifying different dilutions of *Staphylococcus aureus* DNA several times each.

- **PCR-Box Bacteria**, calculated CV at a mean Cq-value of 23,4: CV = 1,3 %.

Fungi

The limit of detection (LOD) was determined by diluting cultures of Candida albicans and processing them with the *GINA 500 + DNA purification* product and protocol. To determine the corresponding CFUs aliquots of the dilutions were plated out and colonies were counted after 24 / 48 hours of incubation.

The **LOD is ~ 2 CFU / mL**.

Selectivity was mainly tested with referenced DNA samples from ATCC (American Type Culture Collection) and DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen):

Aspergillus clavatus	ATCC 1007D-2	*Aspergillus flavus*	ATCC
Aspergillus fumigatus	ATCC 1022	*Aspergillus niger*	DSM1957
Candida albicans	ATCC 11006	*Candida dubliniensis*	DSM28723
Candida glabrata	ATCC	*Candida parapsilosis*	ATCC 22019D-5
Candida tropicalis	ATCC MYA-3404D-5	*Cladosporium sp.*	DSM19653
Cryptococcus neoformans	ATCC MAY-565	*Pichia kudriavzevii*	ATCC
Saccharomyces cerevisiae	Molzym P1		

For each experiment, DNA of a bacterial species and a fungal species was mixed and tested.

Each tested fungal DNA did show the **expected result** on the hybcell report.

The following unspecific results could be observed:

Testing ***Aspergillus clavatus*** showed positive results for *Aspergillus* ***clavatus*** + *Aspergillus* ***fumigatus***.

Repeatability was determined by amplifying different dilutions of *Candida albicans* DNA several times each.

- **PCR-Box Fungi**, calculated CV at a mean Cq-value of 35,4: CV = 2,4 %.

IPC

Repeatability was tested with 16 different EDTA whole blood samples: IPC (20 µL) was added, and the samples were processed according to the *GINA 500* protocol (including DNA purification). The IPC-PCR (see graphs below) was run, and the results were verified by running *hybcell Pathogens DNA xB* as well as by sequencing (Sanger) the PCR products.

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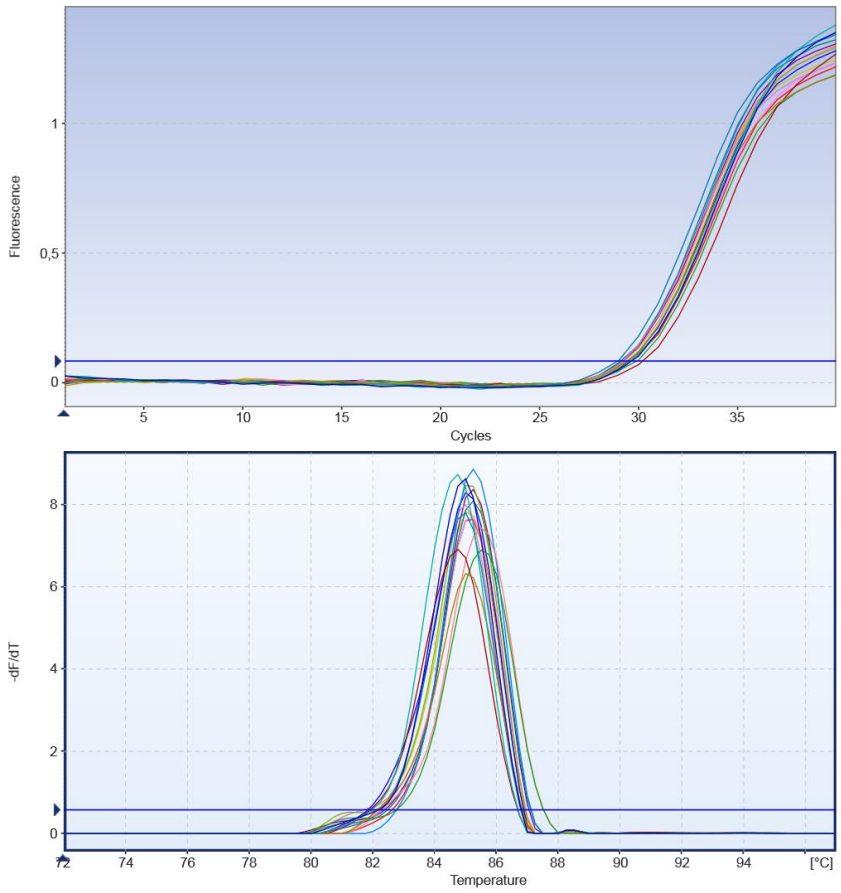

The analysis of quantification cycles (Cq) resulted in (all values rounded):

Average:	Cq 29,6
Standard Deviation:	+/- 0,3
Coefficient of Variation (CV):	1,1%

The **threshold for the Cq** of the IPC is set to **30 +/- 2** (28 to 32). This threshold can slightly vary between different PCR machines.

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10. Clinical Performance

GINA – whole blood

Performance evaluation presented during ECCMID 2021 (01833 SMARTDIAGNOS – next-generation molecular sepsis diagnosis):

Results / Conclusions

In total 352 samples were tested with *GINA and compact sequencing* and compared to blood culturing with MALDI-TOF identification or clinical evaluation. Sensitivity was 74% and specificity 98%. 96% of the samples were correctly classified by the *GINA and compact sequencing* system. The system performs well in detecting pathogens directly in blood and covers at least 80-85% of the microorganisms causing severe infections in Europe. The system is easy to use with a 3-4h response time for a single sample.

		Blood Culture		Overall Correctness	Sensitivity	Specificity
		Positive	Negative			
Cube Dx	Positive	28	5	96%	74%	98%
	Negative	10	309			
Total		352				

LINA – (positive) blood culture

Performance evaluation presented during ECCMID 2020 (Abstract 6917 – Molecular pathogen identification and resistance gene detection from positive blood culture):

Results / Conclusion

In total 277 samples were tested with *LINA and compact sequencing* and compared to blood culturing with MALDI-TOF identification or clinical evaluation. Results for positive BC samples with *LINA compact sequencing* were obtained within 2-3 hours. *LINA* detected almost all positive blood cultures concordantly with currently established methods resulting in a sensitivity of 98%. In addition, several mixed infections and slow-growing bacteria were identified that were missed by culturing and MALDI-TOF identification, including *Acinetobacter species* which are highly relevant carriers of antibiotic resistance genes.

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		Blood Culture		Overall Correctness	Sensitivity	Specificity
		Positive	Negative			
Cube Dx	Positive	166	13	94%	98%	88%
	Negative	4	94			
	Total	277				

LINA – BAL

Performance evaluation performed in cooperation with a German University hospital (unpublished):

Results / Conclusion

BAL samples from 79 patients (Institute for Medical Microbiology, University Hospital Essen / Germany) were analyzed by the state-of-the-art blood culture method the Unyvero system, and Cube Dx's *LINA and compact sequencing technology*. The matching results of at least two of the reference methods were considered "true". One sample was excluded from further analysis because the reference methods could not determine a consistent result. Therefore, the total number of samples was reduced to 78. For 31 samples the result was correctly classified as positive, for 32 samples the result was correctly classified as negative. From 9 false-positive results, 5 showed *Haemophilus influenzae*. Of 6 false-negative results, 3 did not indicate *Staphylococcus aureus*.

		Concession-Results		Overall Correctness	Sensitivity	Specificity
		Positive	Negative			
Cube Dx	Positive	31	9	81%	84%	78%
	Negative	6	32			
	Total	78				

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11. Changes in analytical performance and disposal

Changes in analytical performance

To verify the functionality of the test and implementation, a weekly examination with a reference standard (e.g., Cube Dx's External Process Controls (EPCs)) is recommended. A functionality test every month is mandatory.

To verify the functionality of the EPCs, run several tests and check the outcome. If the outcome is not as expected, use EPCs from another lot and repeat the tests.

Additionally, run a control hybcell once every month to control the basic functionalities of hyborg Dx RED2. These functionalities are liquid transport, optics and scanning, temperature control, and hybcell handling.

If you have doubts about the analytical performance do some additional tests with control material (= well-described material, for example, Cube Dx' EPCs) to confirm the analytical performance.

In case of changing analytical performance refer to the section *Troubleshooting* (below) of this manual.

In the event that the shortcomings cannot be resolved, please contact Cube Dx or respective distribution partners.

Disposal

All single-use materials (PCR tubes, hybcells, pipette tips, etc.) can be disposed of without any special procedures. The usual precautions for potentially infectious material have to be applied.

Patient sample containers (e.g., EDTA tubes) and LE-solution tubes (GINA 500 Kit, yellow cap) potentially contain infectious material and have to be disposed of according to your organization's rules for the disposal of infectious material.

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12. Troubleshooting

Sample Preparation

Problem	Possible causes	Measure / Precaution
Loss of the pellet	▪ Pipetted away	▪ Start with decanting the supernatant and thereafter pipette away the remaining solution ▪ Repeat the extraction step
Contamination	▪ Contamination during the sample preparation step	▪ Use the recommended safety gear ▪ Clean surfaces with 1% hypochlorite, followed by 80% EtOH

Detection by PCR

Problem	Possible causes	Measure / Precaution
Odd-looking amplification curves	▪ Spread out of the eluate in the PCR tube ▪ Uneven distribution of the sample-PCR mix solution ▪ Bubbles at the bottom of the PCR tube	▪ Spin down the PCR tubes before introducing them into the device
PCR inhibition	▪ Dilution of the PCR mix ▪ Using too high sample volumes, especially with BAL samples ▪ Ethanol residues present in the eluate	▪ Use the recommended eluate amount for the PCR reaction ▪ use a dilution series when unsure what volume of BAL is suitable ▪ check the column for EtOH residue before elution, and follow the protocol's 3-minute heating step after elution.

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Identification by the hybcell

Problem	Possible causes	Measure / Precaution
Unspecific hybcell signals	▪ unprocessed hybcells (containing the amplicons) are not processed for too long (1-2 days)	▪ Transfer the amplicons into the hybcell only when they can be processed immediately; IF NOT; store the amplicons as instructed in the manual.
	▪ expiration of opened buffers	▪ Check the lifetime of the buffers after opening the bottles
	▪ forceful introduction of the pipette tip into the hybcell	▪ Gently introduce the pipette tip into the hybcell without sealing its central channel
	▪ Liquids are empty or the liquid handling of the device is erroneous.	▪ Check the filling levels of all liquids. If necessary, refill liquids.
	▪ Insufficient washing procedure.	
	• Using expired/spoilt hybcell	
Grid	▪ Using the wrong hybcell	▪ Check the hybcell type and used protocol.
	▪ Using the “wrong” protocol.	
	▪ Using expired/spoilt products (for example due to damaged package, etc.)	▪ Check the expiry dates of products.
	▪ Software error.	▪ Check the functionality of the hyborg, by using *hybcell Control xC*. Repeat the test.
	▪ Device error.	
Specificity Control	▪ Using expired products.	▪ Check the functionality of the hyborg.
	▪ Insufficient / no PCR-product pipetted into hybcell.	▪ Repeat the test.
	▪ Spoilt PCR.	▪ Check the filling levels of all liquids. If necessary, refill liquids.
	▪ No or insufficient PE-Buffer used.	

Troubleshooting

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In case of problems with the device or the test, please contact:

- Cube Dx GmbH
  Westbahnstraße 55, 4300 St. Valentin, Austria
  Contact information: www.cubedx.com

For additional information about device and software usage see the hyborg Dx RED2 manual.

Serious Incidents / Vigilance

Make sure to immediately report serious incidents related to the use of the device to Cube Dx or respective distribution partners and the national competent authority. Please note your national legislation about reporting serious incidents!

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