Instructions for use

hyplex® SuperBug ID

Multiplex-PCR for the detection of carbapenemases producing bacteria

in vitro diagnosticum

1. Introduction

The **hyplex® SuperBug ID test system** is a qualitative *in vitro* diagnostic tool for the detection of bacteria that are capable, from a genetic stand point, of producing carbapenemases of various types.
hyplex® SuperBug ID detects all described variants of metallo-ß-lactamases of the **VIM-** , **IMP-** and **NDM**-type as well the **OXA-48** gene and all variants of **KPC**.

With the **hyplex® SuperBug ID PCR Module (Cat. No. 3900),** amplification products are generated that can be visualised with the **hyplex® SuperBug ID Hybridisation Modules (Cat. Nos. 3901 to 3905)** through reversible hybridisation with specific oligonucleotide probes.

2. Carbapenemases

Background:

The increase in antibiotic resistance among gram-negative bacteria is a notable example of how bacteria can procure, maintain, and express new genetic information that can confer resistance to one or several antibiotics. Reports of resistance vary, but a general consensus appears to prevail that quinolone and broad-spectrum ß-lactam resistance is increasing in members of the family *Enterobacteriaceae* and *Acinetobacter* spp. and that treatment regimes for the eradication of *Pseudomonas aeruginosa* infections are becoming increasingly limited. While the advent of carbapenems in the 1980s heralded a new treatment option for serious bacterial infections, carbapenem resistance can now be observed in *Enterobacteriaceae* and *Acinetobacter* spp. and is becoming commonplace in *P. aeruginosa*. The common form of resistance is either through lack of drug penetration (i.e., outer membrane protein (OMP) mutations and efflux pumps), hyperproduction of an AmpC-type ß-lactamase, and/or carbapenem-

hydrolyzing ß-lactamases. Two types of carbapenem-hydrolyzing enzymes have been described: serine enzymes possessing a serine moiety at the active site, and metallo-ß-lactamases (MBLs), requiring divalent cations, usually zinc, as metal cofactors for enzyme activity. The serine carbapenemases are invariably derivatives of class A or class D enzymes and usually mediate carbapenem resistance in *Enterobacteriaceae* or *Acinetobacter* spp. Despite the avidity of these enzymes for carbapenems, they do not always mediate high-level resistance and not all are inhibited by clavulanic acid.
MBLs, like all ß-lactamases, can be divided into those that are normally chromosomally mediated and those that are encoded by transferable genes. However, in the past 5 years many new transferable types of MBLs have been studied and appear to have rapidly spread. In some countries, *P. aeruginosa* possessing MBLs constitute nearly 20 % of all nosocomial isolates, whereas in other countries the number is still comparatively small. In recent years MBL genes have spread from *P. aeruginosa* to *Enterobacteriaceae*, and a clinical scenario appears to be developing that could simulate the global spread of extended-spectrum ß-lactamases. Moreover, given that MBLs will hydrolyze virtually all classes of ß-lactams and that we are several years away from the implementation of a therapeutic inhibitor, their continued spread would be a clinical catastrophe.

Metallo-ß-lactamases (MBLs):

All MBLs hydrolyze imipenem, but their ability to achieve this varies considerably and the rate of hydrolysis may or may not correlate with the bacterium's level of resistance to carbapenems. However, these enzymes possess the characteristic hallmark of being universally inhibited by EDTA as well as other chelating agents of divalent cations, a quintessential feature of MBLs that correlates with their mechanistic function.

Acquired metallo-ß-lactamases (MBLs) are emerging resistance determinants in *Pseudomonas aeruginosa* and other gram-negative pathogens. These enzymes can hydrolyze most ß-lactams, including carbapenems, and can confer a broad-spectrum ß-lactam resistance phenotype to the bacterial host, which is not reversible by conventional ß-lactamase inhibitors. MBLs are commonly encoded by genes carried on mobile elements (integron-borne gene cassettes) that can spread horizontally among different replicons and strains.
Originally thought to be uncommon and restricted to some geographical areas, acquired MBLs are presently known to be widespread, and at least four different types of these enzymes, IMP, VIM, SPM, and GIM, have been identified, with the **IMP**- and the **VIM**-types being prevalent.

The VIM-type MBLs were originally detected in Europe, where these enzymes apparently prevail over IMP-type enzymes. Subsequently, VIM-type enzymes were also reported in Asia and America. MBLs of the VIM type are more common among nonfermenting gram-negative bacteria. However, during the last few years, studies have reported on the dissemination of VIM-type MBLs in members of the family *Enterobacteriaceae*, suggesting the ongoing spread of these resistance determinants among pathogens with higher infectivities.

The IMP-type enzymes were originally reported in Japan in the early 1990s. In that area, IMP-type enzymes apparently represent the most common type of acquired MBL

among gram-negative nosocomial pathogens, and IMP producers have been involved in nosocomial outbreaks. In Europe, although a number of IMP-type variants have been detected since the late 1990s, these enzymes appear to be considerably less common than VIM-type enzymes.

NDM-1 was first identified in December 2009 and was named after New Delhi, as it was first described by Yong et al. in a Swedish national who fell ill with an antibiotic-resistant bacterial infection that he acquired in India. The infection was unsuccessfully treated in a New Delhi hospital and after the patient's repatriation to Sweden; a carbapenem-resistant Klebsiella pneumoniae strain bearing the novel gene was identified. It was later detected in bacteria in India, Pakistan, the United Kingdom, the United States, Canada, Japan and several European countries. The most common bacteria that make this enzyme are *Escherichia coli* and *Klebsiella pneumoniae*, but the gene for NDM-1 can spread from one strain of bacteria to another by horizontal gene transfer and was found *in Morganella morganii*, *Providencia* spp., *Citrobacter freundii* and *Acinetobacter baumannii*, too.

Klebsiella pneumonia carbapenemase (KPC):

The initial report of a KPC β-lactamase which is capable of hydrolysing carbapenems, penicillins, cephalosporins and aztreonam, was from a carbapenem-resistant *K. pneumoniae* strain isolated in USA, and subsequently KPC producers have also been detected in other regions all over the world. There are currently nine recognized variants, with KPC-2 and KPC-3 being more commonly isolated.

Recently, isolates producing KPC-2 /-3 enzyme have been detected in European countries such as Greece, the UK, France and Germany. In addition, the spread of KPC producers may be underestimated, as the detection of KPC- and also MBL-producing *K. pneumoniae* may be unsuccessful because these enzymes do not always confer obvious carbapenem resistance.

OXA-48 carbapenemase:

The OXA-48-carbapenemase was first described in 2004 in a *K.-pneumoniae*-isolate from Turkey. Recently, isolates producing such carbapenemase were detected in various countries like Belgium, Israel, India, UK and Greece. In Germany it was found the first time 2008 in the south-western part of the country. Like in KPC-producing bacteria, the phenotypic detection of this resistence type seems to be difficult and unreliable.

3. Structure of the hyplex® SuperBug ID test system

hyplex® SuperBug ID PCR module (Cat. No. 3900):
Manufacture of specific PCR amplifications products from sample material, sufficient for a maximum of ten hybridisations per PCR reaction.
Applicable Hyb-modules: Cat. Nos. 3901 to 3905

hyplex® SuperBug ID Hybridisation modules (Cat. Nos. 3901 to 3905):
Single wells with immobilized oligonucleotide probes for the specific detection of the three different carbapenemases coding genes (sales unit: 96 wells per probe) and reagents to conduct 96 reverse hybridisations.
Sample material: PCR products formed using the **hyplex® SuperBug ID** PCR module.

The table below lists the specific probes (hybridisation modules) available separately for the **hyplex® SuperBug ID** test system.

Hybridisation modules for the **hyplex® SuperBug ID** test system

Symbols	Specificity	Well colour	Cat. No.:
MTS BR VIM	VIM (alle variants VIM-1 to VIM-13)	green	**3901**
MTS BR IMP	IMP (all variants IMP-1 to IMP-22)	white	**3902**
MTS BR KPC	KPC (all variants KPC-1 bis KPC-10)	black	**3903**
MTS BR O48	OXA-48	yellow	**3904**
MTS BR NDM	NDM-1	burgundy	**3905**

One PCR amplification may be used to perform ten hybridisations. Thus one sample can be tested for the five parameters mentioned and the various probes may be selected as required.

4. Principle of the test

The **hyplex® SuperBug ID** PCR module contains labelled oligonucleotide primers that provide the means for the simultaneous, specific amplification of different DNA regions in a single PCR reaction.
In the presence of the relevant DNA, specific amplification products (of all variants) of the genes **VIM, IMP, NDM-1, KPC** and **OXA-48** synthesised and are subsequently visualised with the **hyplex® SuperBug ID** hybridisation modules.
For that amplification products from one PCR with the **hyplex® SuperBug ID** PCR modules are added heat-denatured to single-stranded, specific probes which are immobilized on the polystyrene surfaces of microtiter plates. Using a hybridisation buffer, hybridisation of complementary sequences occurs which can then be detected using the ELISA principle. After several stringent wash steps, a peroxidase (POD) conjugate is added. This binds highly specifically to the labelling of the single strand of the PCR product bound to the oligonucleotide probe. After further wash steps, TMB substrate solution is added which produces a blue colour when converted by POD. This reaction is stopped using a stop solution. A colour change to yellow is observed. Extinction of the various wells is then measured in a photometer at a wavelength of 450 nm.

Positive signals indicate specific amplification of certain DNA sequences during the **hyplex® SuperBug ID** PCR and thus the presence of the corresponding genes in the sample to be investigated.

5. Reagents

5.1 Contents of PCR modules:

The reagents contained in one kit are sufficient for 96 determinations.
Each reagent set contains:

PRIMER	Primer Mix (green cap, ready for use) Contains labelled oligonucleotide primer	240 µl
dNTP	Nucleotide Mix (yellow cap, ready for use) Contains dATP, dCTP, dGTP, dTTP	120 µl
CONTROL | +	Positive control (red cap, ready for use)	125 µl
CONTROL | -	Negative control (blue cap, ready for use)	100 µl
MTS | BR | CONTROL | +	Positive control probes, breakable (colour coding: blue)	16 wells

5.2 Contents of Hybridisation modules:

The reagents contained in one kit are sufficient for 96 determinations.
Each reagent set contains:

MTS | BR | BLANK	Reagent control, breakable	16 wells
MTS | BR | XX	Colour-coded wells with specific oligonucleotide probe, breakable	96 wells
HYBBUF	Hybridisation buffer (ready for use) Contains standard saline citrate buffer (SSC), sodium dodecyl sulphate (SDS) and N-lauroylsarcosine	10 ml
STRGWASH	Stringent washing solution (ready for use) Contains SSC and SDS	100 ml
WASHBUF | 20 X	Washing buffer **(concentrated 20 times)** Contains phosphate buffer, NaCl and detergent, preservatives: methylisothiazolone and oxypyrion	12 ml
CONJ | 100 X	Conjugate (**concentrated 100 times**, transparent cap) Preservatives: methylisothiazolone, dimethyaminoantipyrine and chloracetamide	120 µl
SUBS | TMB	TMB (tetramethylbenzidine) substrate solution (ready for use)	12 ml
STOP | H_3PO_4 | 25%	Stop solution (25 % phosphoric acid, ready for use)	12 ml

5.3 Additional reagents and accessories required

Reagents

- **UltraStart Tth - DNA polymerase incl. reaction buffer (Cat. No. 3955)**
- sterile, double-distilled H_2O
- **hyplex® lysis buffer (Cat. No. 3950)**

Accessories

- Heating block
- PCR thermocycler
- PCR reaction vessels (recommended: 0.2 ml)
- Pipettes with sterile disposable tips
- Incubator (50 °C ± 1 °C)
- Microtiter plate photometer for measuring extinction at 450 nm and 620 nm

6. Warnings and precautions

- All reagents and materials which come into contact with potentially infectious samples must be treated with suitable disinfectant or autoclaved.
- Suitable disposable gloves must be worn during the entire test.
- The conjugate, the TMB substrate solution and the washing buffer contain preservatives. Avoid contact with the skin or mucous membranes. If this does happen, rinse affected areas with plenty of water.
- Phosphoric acid is an irritating substance. Avoid contact with the skin and mucous membranes. If this does happen, rinse affected areas with plenty of water.

7. Handling notes

With the exception of the positive control probes (2...8 °C), the reagents of the **hyplex® SuperBug ID** PCR modules should be stored at **- 20 °C** after receiving them. Avoid frequent thawing and freezing of the reagents. After using for the first time, the reagents may be stored at 2...8 °C to avoid thawing and freezing steps but in this case the reagents should be used within three months.
The reagents of the **hyplex® SuperBug ID** hybridisation modules should be stored at **2...8 °C**.
Before beginning the test, the washing buffer, TMB substrate solution, the stop solution as well as the closed snap-seal bag with the microtitre wells should be kept at room temperature (18...25 °C) for at least 30 minutes. The stringent washing solution must be preheated to 50 °C. Always keep the hybridisation buffer and the conjugate cool.
The kits have an expiry date. Quality cannot be guaranteed after this date.
To minimize the probability of nonspecific product formation, it is recommended that the amplification reactions be pipetted on ice and that DNA polymerase be the last reagent to be added. Due to the danger of contamination, suitable (non-powdered) disposable gloves and pipette tips with filter insert should be used when preparing for the test.

8. Sample material

The sample material may be isolated DNA, pure bacterial cells or potentially infected patient specimens.
If cells are used as sample material, a single bacterial colony is suspended in 300 µl **hyplex® Lysis Buffer (Cat. No. 3950)**. The cell suspension is incubated in a thermal block (99° C) for 10 min. After a centrifugation step (2 min at 10,000 x g), 5 µl of the supernatant is used for the amplification reaction.
For sample preparation, isolation and purification of genomic DNA from blood cultures the usage of **Filtration vials (Cat. No. 3957)** and **hyplex® Prep Module (Cat. No. 3951)** or **hyplex® QickPrep (Cat. No. 3980)** is recommended.
To directly test swab specimens without inhibition in this system: A swab that was transported in medium (e.g. Amies medium) should be pressed out in 300 µl **hyplex® Lysis Buffer (Cat. No. 3950)**. A dry swab without transport medium should be pressed out in 500 µl **hyplex® Lysis Buffer (Cat. No. 3950)**.
In both cases, the suspension is then incubated in a heating block (99° C) for 10 min. After a centrifugation step (2 min at 10,000 x g), 5 µl of the supernatant is used for the amplification reaction.

Type of sample	DNA preparation	Sample volume to be used
Nose swab	Simple cell lysis with lysis buffer	5 µl lysate supernatant
Throat swab	Simple cell lysis with lysis buffer	5 µl lysate supernatant
Skin swab	Simple cell lysis with lysis buffer	5 µl lysate supernatant
Wound swab	Simple cell lysis with lysis buffer	5 µl lysate supernatant
(Peri-) Anal swab	DNA isolation using a commercially available system	1 - 5 µl DNA eluate
Tracheal secretion	DNA isolation using a commercially available system	1 - 5 µl DNA eluate
Sputum, puncture specimen, urine	DNA isolation using a commercially available system	1 - 5 µl DNA eluate
Blood cultures	DNA isolation using hyplex® PrepModule	5 µl DNA eluate

For the isolation of genomic DNA from patient specimens such as anal swabs, tracheal secretion, puncture specimens or extremely purulent or sanguineous wound swabs, commercially available systems should be used, such as:

- MagNA Pure LC DNA Isolation Kit I (Roche Diagnostics GmbH)
- **hyplex® Prep Module (Cat. No. 3951)**
- **hyplex® QickPrep (Cat. No. 3980)**

Repeated freezing and thawing of isolated DNA should be avoided.

9. Test procedure

9.1 Preparing the amplification reaction

The reactions are prepared using the buffer supplied by the DNA polymerase manufacturer and using the following batch:

x µl	Sample material (e.g. **5 µl** sample lysate and **5 µl** negative / positive control)
1 µl	Nucleotide mix (yellow cap)
2 µl	Primer mix (green cap)
5 µl	10x PCR buffer
1 µl	UltraStart Tth - DNA polymerase (1 U)
to 50 µl	double-distilled H_2O

If genomic DNA is used as sample material, the amplification reaction should be added between 1 and 100 ng DNA.

Caution! Too much DNA or cell material may considerably reduce the effectiveness of a PCR and lead to false negative results.

9.2 Amplification reaction

The PCR thermocycler should be programmed as follows:

Cycle	Temperature [° C]	Time	Reaction
Once	94	5 min	Initial denaturation of the DNA
35 times	94	25 sec	Denaturation of the DNA
	52	25 sec	Binding of the primers
	72	20 sec + 1 sec/cycle **or** 45 sec	3´-OH extension of the primers
Once	72	3 min	Final extension

After completing the PCR, the reaction mixture should be stored at -20 ° C until reverse hybridisation is conducted with the hyplex® hybridisation modules.

9.3 Reverse hybridisation

The desired **hyplex® SuperBug ID** hybridisation modules are required to perform reverse hybridisation. The amounts mentioned below each refer to the processing of an **individual** microtiter plate well.
The **hyplex® SuperBug ID** test system offers the user the option of designing the test individually to suit his needs and interests. When using several different probes at the same time, the indicated amounts must be multiplied by the number of wells needed.

Example: If 4 samples should be tested for the presence of carbapenemases-coding genes with the **VIM, IMP, NDM-1, OXA-48** and **KPC** probes, the quantity data must be multiplied by a factor of 27 (4 x 5 = 20 wells for the samples, 5 wells for the negative control, 1 well for the positive control and 1 well for the reagent control).

Before use, the stringent washing solution must be preheated to 50 °C and the washing buffer, TMB substrate solution, stop solution and the required microtiter plates should be brought to room temperature (18...25 °C).

9.3.1 Test plates

In plastic frames, the different probes in their colour-coded wells may be combined with each other as required. To do this, the wells are broken away individually from the delivered strips and inserted firmly into the frame. Wells which are not required are stored again at 2...8 °C in the snap-seal bag together with the desiccant.
One of the reagent controls contained in the hyplex Hyb-module must be used for every test batch to determine the reagent background signal. After initial addition of 50 µl hybridisation buffer, they are processed in the same way as the samples. There must also be a positive and a negative control for each test batch.
The PCR batch of the positive control is diluted in accordance with Section 9.3.2 and 50 µl of the resulting solution is inserted into a positive control well and further treated in accordance with the protocol (see below).
The PCR batch of the negative control is diluted in accordance with Section 9.3.2 and 50 µl of the resulting solution is pipetted into the used specific probe wells and further treated in accordance with the protocol (see below).

9.3.2 Preparation of the PCR sample and hybridisation procedure

The 50 µl reaction mix of the **hyplex® SuperBug ID** PCR carried out is denatured in the reaction vessel for 10 min at 95 °C. It is recommended that the procedure is carried out in a thermal cycler with a heated lid.
5 µl of the PCR batch per well is then immediately added to **50 µl** cool (2...8 °C) hybridisation solution, is well mixed, and **50 µl** of this is then quickly transferred by pipette to the corresponding well. The microtitre plate is incubated for **30 min** at **50 °C**. To prevent high evaporation loss of the hybridisation solution, the microtitre plate should be covered.

9.3.3 Stringent washing

The wells are completely emptied and washed **briefly three times** with **200 µl** of the preheated stringent wash solution on each occasion.
It should be ensured that the stringent wash solution is removed between each washing step. After the last washing step, the plate is tapped onto a paper towel to remove any last residue of fluid from the wells.

9.3.4 Manufacture of the washing buffer

The washing buffer concentrate is diluted **1+19** with deionized H_2O. Per well, 0.05 ml concentrate and 0.95 ml deionized H_2O are mixed. The wash buffer can also be manufactured in greater amounts and stored for later use for a week at room temperature.

9.3.5 Washing
The wells are washed once briefly at **room temperature** with **200 µl** washing buffer.

9.3.6 Incubation with peroxidase conjugate
The conjugate solution must always be prepared afresh.
Per well, 1 µl conjugate concentrate (transparent cap) is added to 100 µl washing buffer in a clean vessel and mixed well (dilution **1+100**). **100 µl** are pipetted into each well and the microtiter plate is incubated for **30 min** at **room temperature**.

9.3.7 Washing
The wells are emptied and each well is washed **three times** briefly with **200 µl** washing buffer at **room temperature**. Please ensure that the washing solution is removed completely between the washing steps.

9.3.8 Substrate reaction
The TMB substrate solution is ready for use. Pipette **100 µl** per well. Incubate the microtiter plate for **15 min** at **room temperature** away from direct sunlight. The time is calculated from pipetting of the first well.

9.3.9 Stopping the reaction
To stop the reaction, pipette **100 µl** of stop solution into each well. Observe the same pipetting procedure as for pipetting of the TMB substrate solution.

9.3.10 Extinction measurement
Measure the extinction of the individual wells in a microtiter plate photometer at 450 nm and a reference length of 620 - 650 nm. Zero comparison is made against air. Measurement should take place within 60 minutes of stopping the reaction.

10. Brief instructions on test procedure

Dilutions:

• Washing buffer dilution	1+19 with deion. H_2O	0.05 ml + 0.95 ml per well
• Conjugate dilution	1+100 with diluted washing buffer	1 µl + 100 µl per well

Test steps:

• Denaturation	PCR reaction batch	10 min at 95 °C
• Sample dilution:	Add 5 µl PCR reaction batch to 50 µl hybridisation solution	
• Hybridisation:	50 µl per well	30 min at 50 °C ± 1 °C
• Stringent washing:	200 µl per well	3 times briefly
• Wash step:	200 µl per well	Once briefly
• Conjugate incubation:	100 µl per well	30 min at room temperature
• Wash step:	200 µl per well	3 times briefly
• Substrate incubation:	100 µl per well	15 min at room temperature
• Stop:	100 µl per well with stop solution	
• Photometric measurement	450 / 620 nm	

11. Evaluation

The test can be evaluated under the following conditions:

- Extinction of reagent control **≤ 0.200**
- Extinction of negative control (NCE) **≤ 0.200**
- Extinction of positive control **≥ 1.500**

When the signal strength of the positive control is low, weakly positive samples may be unintentionally evaluated as negative. The hybridisation temperature or the temperature of stringent wash solution may have been too high (see also 14).

Analytical criteria with use of cellular material from a pure culture or from DNA isolated from it:

- Samples with extinction values of greater than 0.400 are treated as **positive**
- Samples with extinction values of between 0.200 and 0.400 are **borderline**. They should be retested.
- Samples with extinction values of less than 0.200 are treated as **negative**

Analytical criteria with directly use of patient material or DNA isolated from it:

- Samples with extinction values of greater than 0.300 are treated as **positive**
- Samples with extinction values of greater than 3 x NCE and less than 0.300 are **borderline**. They should be retested.
- Samples with extinction values of less than 3 x NCE or less than 0.150 are treated as **negative**

12. Interpretation of the test results

The **hyplex® SuperBug ID** system is a rapid, universally applicable screening test for the detection of genes encoding the most common medically relevant metallo-ß-lactamases **VIM**, **IMP** and **NDM-1** as well the carbapenemases of the **OXA-48**- and **KPC**-type.

Positive results of the **hyplex® SuperBug ID** system demonstrate the presence of carbapenemases-associated genes in a sample. This does not mean that the genes are also actually expressed. The pathogens found in the tested sample, however, possess the potential to express the genes. In addition, no conclusions on, for example, the minimal inhibiting concentration of certain β-lactam antibiotics, and in special carbapenems, for the pathogen can be made based on the results of this test system.
The results obtained from the **hyplex® SuperBug ID** system are dependent upon the quantity and quality of the DNA in the sample. Through the **hyplex® SuperBug ID** PCR Module, low copy numbers of relevant DNA (corresponding to 9.3 x 10^3 genome copies / ml) are amplified to such an extent that positive signals are observed.
A negative test result from the **hyplex® SuperBug ID** system cannot completely exclude the possibility of the presence of carbapenemases producing bacteria in the sample material.

For example, further enzymes with the capability of hydrolysing carbapenems do exist, confering resistence to that kind of antibiotics, but do not belong to the three types of carbapenemases which are detected with this test system. These other enzymes (like GIM and SPM) are only found in sporadic cases and have no significant clinical importance until now or are detected by the **hyplex® CarbOxa ID** in the case of OXA-type enzymes.
Addtionally, the efficiency of the PCR can be drastically reduced by inhibitory substances in the sample material, which in turn would lead to lower signals. Under certain conditions, DNA isolated from a sample can be fragmented or not be of the required purity (presence of PCR inhibitors).
Therefore, using commercially available DNA isolation and purification kits and, in case of doubt, performing an amplification control reaction (see 13) are recommended.
If in an otherwise valid test, the sample absorbances are in the marginal range (see 11), it is probable that the sample is positive, but the amplification of the DNA may have been reduced by less than optimal conditions. In this case, repeating the test with freshly isolated DNA or after an enrichment step is recommended.

13. Control results of the hyplex® SuperBug ID Test System

After performing the positive control test, an amplification product is generated that, after reversible hybridisation with the specific positive control probe, yields a positive signal with an $OD_{450\ nm} > 1.5$.
In case of questionable negative results, the positive control can also be used as an amplification control. The presence of PCR inhibiting substances in a sample can be checked by adding the positive control included in the **hyplex® SuperBug ID** PCR module to the PCR reaction mixture of the sample to be tested.
If no signal is obtained from the positive control, the PCR was inhibited and a conclusion regarding the existence of relevant DNA in the sample cannot be made. Repeating the test with freshly purified DNA is recommended.
If the positive control produces a positive signal, a systematic error in the PCR and the hybridisation can be excluded; the test sample is, therefore, negative.
The negative control should not yield any amplification product and, hence, a hybridisation signal should not be observed with any of the probes.

14. Troubleshooting

Constantly weak signals or no signals at all (incl. positive control)
- Temperature of the stringent washing solution clearly above 50 °C
- PCR products not (sufficiently) denatured or renatured again prior to hybridisation
- Substrate not equilibrated to room temperature
- Wrong amount of conjugate and/or substrate used

Weak signals or no signals at all with the exception of the positive control
- Quality and/or quantity of the isolated DNA do not allow effective amplification. Check PCR products in 2% agarose gel. If necessary, repeat DNA isolation and amplification, possibly use another method of DNA isolation
- Insufficient thermal lysis (too short; temperature not 99 °C) when using the hyplex lysis buffer
- When using cell or patient material, use the positive control to check for the presence of PCR-inhibiting substances (see Section 13) or perform DNA isolation

Generally high (background) signals

- The use of insufficiently heated stringent washing solution or an incubation temperature which is too low may lead to nonspecific signals. Specific, positive signals are potentiated to the same degree, thus maintaining the contrast between nonspecific and specific signals. If there is doubt as to the evaluability of the test run (see Section **Fout! Verwijzingsbron niet gevonden.**), repetition with the correct parameters is recommended.

15. Performance data

15.1 Specificity and sensitivity:

96 carbapenemases-negative strains from the following species were used to test the specificity of the **hyplex® SuperBug ID** system.

Organism	
Citrobacter brackii	*Klebsiella oxytoca*
Citrobacter freundii	*Klebsiella pneumoniae*
Enterobacter aerogenes	*Legionella pneumophila*
Enterobacter amnigenus	*Morganella morganii*
Enterobacter cloacae	*Mycoplasma genitalium*
Enterobacter gergoviae	*Mycoplasma hominis*
Enterobacter intermedius	*Pseudomonas aeruginosa*
Enterobacter kobei	*Pseudomonas alcaligenes*
Enterococcus asini	*Pseudomonas fluorescens*
Enterococcus avium	*Serratia liquefaciens*
Enterococcus casseliflavus	*Serratia marcescens*
Enterococcus cecorum	*Staphylococcus arlettae*
Enterococcus columbae	*Staphylococcus aureus*
Enterococcus dispar	*Staphylococcus capitis*
Enterococcus durans	*Staphylococcus caprae*
Enterococcus faecalis	*Staphylococcus epidermidis*
Enterococcus faecium	*Staphylococcus haemolyticus*
Enterococcus flavescens	*Staphylococcus hominis*
Enterococcus gallinarum	*Staphylococcus saprophyticus*
Enterococcus hirae	*Staphylococcus simulans*
Enterococcus malodoratus	*Staphylococcus warneri*
Enterococcus mundtii	*Streptococcus agalacticae*
Enterococcus pseudoavium	*Streptococcus dysgalacticae*
Enterococcus raffinosus	*Streptococcus mutans*
Enterococcus saccharolyticus	*Streptococcus oralis*
Enterococcus seriolicida	*Streptococcus pyogenes*
Enterococcus solitarius	*Streptococcus salivarius*
Enterococcus sulfreus	*Streptococcus sanguinis*
Escherichia coli	*Ureaplasma urealyticum*

Results:
No positive signals in any of the tested reference strains were observed.

Specificity based on the tested reference strains: 100 %

26 carbapenem resistant, genetically characterized strains were used to test the sensitivity of the **hyplex® SuperBug ID** system.

Species	Strain-Nr.	Source	Gene variant	NDM-1	OXA-48	KPC	VIM	IMP
Pseudomonas aeruginosa	VR-143/97	Siena[a]	VIM-1	-	-	-	+++	-
Pseudomonas aeruginosa	AV-65	Siena[a]	IMP-13	-	-	-	-	+++
Pseudomonas putida	VA-758/00	Siena[a]	IMP-12	-	-	-	-	++
Pseudomonas aeruginosa	101/1477	Siena[a]	IMP-1	-	-	-	-	+++
Pseudomonas aeruginosa	VR-193/98	Siena[a]	VIM-2	-	-	-	+++	-
Acinetobacter baumannii	ACX 54/97	Siena[a]	IMP-2	-	-	-	-	+++
Pseudomonas aeruginosa	2611/04	Warschau[b]	VIM-4	-	-	-	+++	-
Pseudomonas aeruginosa	1956/01	Warschau[b]	VIM-2	-	-	-	+++	-
Pseudomonas aeruginosa	1264/01	Warschau[b]	VIM-4	-	-	-	+++	-
Pseudomonas aeruginosa	1266/01	Warschau[b]	VIM-2	-	-	-	+++	-
Pseudomonas aeruginosa	060907-3147	München[c]	VIM-2	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 6/100	Athen[d]	VIM-1	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 5866	Athen[d]	VIM-1	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 54/163	Athen[d]	VIM-1	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 12/227	Athen[d]	VIM-1	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 55/265	Athen[d]	VIM-1	-	-	-	+++	-
Klebsiella pneumoniae	Kpn 848	Athen[d]	VIM-1	-	-	-	+++	-
Pseudomonas aeruginosa	K34-7	Tromsø[e]	VIM-2	-	-	-	+++	-
Pseudomonas aeruginosa	K34-73	Tromsø[e]	VIM-1	-	-	-	+++	-
Pseudomonas aeruginosa	K34-74	Tromsø[e]	VIM-1	-	-	-	+++	-
Acinetobacter baumannii	65FFC	Coimbra[f]	IMP-5	-	-	-	-	+++
Klebsiella pneumoniae	Kp1239	Westmed[h]	IMP-4	-	-	-	-	+++
Salmonella cubana	AM04707	Athen[d]	KPC-2	-	-	+++	-	-
Klebsiella pneumoniae	375/08	Wernigerode[g]	KPC-3	-	-	+++	-	-
Klebsiella pneumoniae	238/09	Wernigerode[g]	OXA-48	-	+++	-	-	-
Escherichia coli	02/10	Wernigerode[g]	NDM-1	+++	-	-	-	-

[a] kindly provided by Prof. Rossolini, University of Siena
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[c] kindly provided by Prof. Bauernfeind, MICOER, Munich
[d] kindly provided by Dr. Miriagou, Hellenic Pasteur Institute, Athens
[e] kindly provided by Dr. Samuelsen, University Hospital of North Norway, Tromsø
[f] kindly provided by Dr. da Silva, University of Coimbra
[g] kindly provided by Dr. Pfeiffer, RKI Wernigerode
[h] kindly provided by Prof. Dr. Irdell, Westmead Hospital, Australia

Result:
Sensitivity based on the tested reference strains: 100 %

15.2 Clinical evaluation:

A) During a study at the „Institut für Medizinische Mikrobiologie des Universitätsklinikum Jena“ kindly supported by the „Robert-Koch-Institut, Zweigstelle Wernigerode, FG13 Nosokomiale Infektionen“, 75 different strains (19 *E. coli*, 37 *K. pneumoniae*, 7 *K. oxytoca*, 4 *E. cloacae*, 7 *Ps. aeruginosa* und 1 *A. baumannii*) were examined with the **hyplex® SuperBug ID.** In comparison to reference molecularbiological examinations of the two institutes following data were obtained:

	hyplex® SuperBug ID						**Sum**
Reference PCRs	**VIM pos.**	**IMP pos.**	**KPC pos.**	**OXA-48 pos.**	**NDM-1 pos.**	**negative**	
VIM positive	**10**	0	0	0	0	0	10
IMP positive	0	**2**	0	0	0	0	2
KPC positive	0	0	**5**	0	0	0	5
OXA-48 positive	0	0	0	**4**	0	0	4
NDM-1 positive	0	0	0	0	**1**	0	1
negative	0	0	0	0	0	**53**	53
Sum	10	2	5	4	1	53	**75**

Result:
Sensitivity and specificity based on the tested reference strains: 100 %

B) During a study conducted by three hospitals in Athens, Greece (Evangelismos, Geniko Kratiko, Laiko) coordinated by Dr. V. Miriagou (Hellenic Pasteur Institute) a total of 90 positive blood culture bottles as well as 236 further clinical samples (60 Urine, 91 Wound swabs and 85 Tracheal secretions) were microbiological examinated. Following results, in comparison to the **hyplex® MBL ID** system, were obtained:

Urine samples, Wound swabs and Tracheal secretions		**hyplex® MBL ID positive (VIM)**	**hyplex® MBL ID negative**	Total
Microbiological result	**positive**	53	1	54
Microbiological result	**negative**	5	177	182
	Total	58	178	**236**
Blood cultures				Total
Microbiological result	**positive**	19	0	19
Microbiological result	**negative**	0	71	71
	Total	19	71	**90**

Following performance data in comparison to the microbiology as “standard” were obtained:

Blood cultures:

Sensitivity:	**100 %**
Specificity:	**100 %**
PPV:	**100 %**
NPV:	**100 %**

Urine samples, Wound swabs and Tracheal secretions:

Sensitivity:	**98,1 %**
Specificity:	**97,3 %**
PPV:	**91,4 %**
NPV:	**99,4 %**

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