9.7 Perform the following protocol in the instrument:

Temperature/Time	# of cycles
50^{0}C / 2 min†	1
95^{0}C/2 min	1
95^{0}C/5 sec	45
60^{0}C/30sec (data collection*)	

† Required for optimal uracil-N glycosylase (UNG) activity to increase the specificity of the reaction and to eliminate PCR carryover contamination

* KPC/OXA/NDM mix 1 - FAM (495-520) for the internal control; HEX (535-556) for the *bla$_{NDM}$* gene; Texas Red (586-610) for the *bla$_{KPC}$* gene; Cy5 (646-662) for the *bla$_{OXA}$* gene.

* VIM / IMP mix 2 - FAM (495-520) for the internal control; HEX (535-556) for the *bla$_{VIM}$* gene; Cy5 (646-662) for the *bla$_{IMP}$* gene.

DATA ANALYSIS AND INTERPRETATION

The results are interpreted by the real time PCR instrument software. The following table shows the possible results and their interpretation:

Ct value	Results analysis	comments
1. Signal detected (Ct<38) in **Cy5** filter	The sample contains OXA or IMP DNA, the result is positive*	No need to check the internal control since high initial concentrations of KPC, OXA , NDM, VIM and IMP DNA can lead to a reduced or absent fluorescence signal of the internal control(due to competition).
2. Signal detected (Ct<38) in **TexasRed** filter	The sample contains KPC DNA, the result is positive*	
3. Signal detected (Ct<38) in **HEX** filter	The sample contains NDM or VIM DNA, the result is positive	
4. Signal detected (Ct 38-40) in **TexasRed/Cy5/HEX** filter, signal detected in **FAM** filter	Re-test. If it still Ct 38-40 report as in #6	Overload bacterial mass may lead to nonspecific results such or false positive signals (>Ct 35) in all channels or PCR inhibition
5. No signal in **TexasRed/Cy5/HEX** or Ct>40, signal detected in **FAM** filter	The KPC, OXA, NDM, VIM and IMP DNA in the sample is not detectable	Signal from FAM filter pair rules out the possibility of PCR inhibition
6. No signal in **TexasRed, Cy5**, **HEX** and **FAM** filter	Diagnostic statement cannot be made	No signal in FAM points out to PCR inhibition either from the sample or a problem in DNA isolation such as too much starting material. Repeat using diluted DNA or pick less from the colonies.

Threshold setting: adjust above the maximum level of the Negative control**.**

Quality control: Negative control, positive control and internal control must be performed correctly; otherwise the sample result is invalid**.**

*A typical positive signal generated from direct colony amplification is characterized by high Ct values (< 35)

- Claims for merchandise damaged in transit must be submitted to the carrier.
- For further information, please contact our Technical Department (lab1@hylabs.co.il)

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Hy-CRE

KPC/OXA/NDM/VIM/IMP Detection Real Time PCR kit

Cat. No. KI-5058/25

For use with Biorad CFX96™ Real-Time PCR Detection System, ABI Prism® 7000/7300/7500 and Roche LightCycler®480 real time PCR systems

Manufactured by Hy Laboratories Ltd. Park Tamar, Rehovot 76326 Israel. Tel. (972) 89366475 Fax (972) 8 9366474 www.hylabs.co.il

STORAGE AND HANDLING REQUIREMENTS

All reaction tubes and controls: -20^{0}C (constant temp. freezer, no frost-free). Repeated thawing and freezing (>3x) should be avoided, as this may reduce the sensitivity of the assay. When in use, always keep the Reaction Mix & Control tubes cooled or **on ice**. Manufacturing date and expiry date are indicated. Dispose of unused reagents and waste in accordance with local regulations. Reaction Mix should be stored in the dark.

WARNINGS AND PRECAUTIONS

Rules to prevent contamination in amplification assays:
Use filter tips to avoid aerosol formation. All materials should be sterile. Perform negative controls in parallel. Different steps of the whole procedure should be performed in especially dedicated areas (DNA purification area, Amplification area, Post-Amplification area, etc.). Work flow between these areas must proceed in a unidirectional manner. Gloves must be worn in each area and must be changed before leaving that area. Use of dedicated micropipettes in each area is highly recommended. Discard any reagents that may be suspect for their purity. Follow general instructions for laboratory safety.

INTENDED USE

KPC/OXA/NDM/VIM/IMP real time (RT) PCR kit detects the presence of *Klebsiella Pneumoniae* Carbapenemase (**KPC**), Oxacillinase group of β-lactamase (**OXA**), New Delhi Metallo-beta-lactamase (**NDM**), Verona Integron-encoded Metallo-β-lactamase (**VIM**) and IMP-type carbapenemases (**IMP**) resistant genes in swabs, bacterial colonies or DNA. It is based

on specific amplification of the *bla*$_{KPC}$, *bla*$_{OXA}$, *bla*$_{NDM}$, *bla*$_{VIM}$ and *bla*$_{IMP}$ genes by real time (rt) PCR and by monitoring the fluorescence intensities during time. The PCR cycle at which an increase in the fluorescence signal is detected initially (Ct) is proportional to the amount of the specific PCR product. It allows the detection of the accumulating product without having to re-open the reaction tube after the amplification. The kit also contains an **internal control (IC)** for monitoring false negatives reactions due to PCR inhibition and uracil-N glycosylase (UNG) to eliminate PCR carryover contamination.

KIT CONTENTS

KPC/OXA/NDM/VIM/IMP RT-PCR Kit includes 2 tubes of UNG PCR premix which contain all reagents (enzyme, buffer, probes and primers) besides tested DNA. The premix contains uracil-N glycosylase (UNG) to increase the specificity of the reaction and to eliminate PCR carryover contamination. The reaction mixture is prepared by the addition of DNA sample to the PCR premix.

The kit contains:

- **Mix 1**: Reaction mix (1 tube) for detection of KPC/OXA/NDM+IC (*KPC/OXA/NDM Rxn mix*)
- **Mix 2**: Reaction mix (1 tube) for detection of VIM/IMP+IC (*VIM/IMP Rxn mix*)
- **KPC/OXA/NDM Positive control** DNA(1 tube) contains 2x10^{7}copies/µl of each target, non-infective (*KPC OXA NDM Positive control*)
- **VIM/IMP Positive control** DNA (1 tube) contains 2x10^{7}copies/µl of each target, non-infective (*VIM IMP Positive control*)
- **PCR grade water**, sterile (1 tube).

Store all tubes at –20^{0}C. Thaw and handle on ice or cooling blok.

PRINCIPLES OF THE PROCEDURE

The amplification system of this kit is based on a one-stage real time PCR that amplifies the target genes which are monitored by accumulation of fluorescence dyes. One should include a negative control and a positive control in each test. Add 5µl of the positive control sample to each of your positive control tubes.

MATERIALS REQUIRED BUT NOT PROVIDED

NOTE: *For all instrumentation, regular maintenance and calibration is necessary.*

- Dedicated micropipettes
- Consumables: Filter tips, gloves, 2 ml tubes etc.
- Heating block
- Real time PCR system and compatible reaction tubes/plates
- Disposable gloves, powderless
- Biohazard waste container
- Centrifuge with appropriate rotors

INSTRUCTIONS FOR USE

NOTE: The test is performed directly from bacterial colonies but can also use DNA isolated from swabs, bacterial colonies or broth. Purified DNA must be in sufficient quantity in order to add to the reaction tube 5-10ng DNA in 5 µl.

PRE-PCR SAMPLE PREPARATION

1. Boiling method for bacterial colonies:

1.1. Add 100µl of TE pH 8 (or sterile PCR grade water) to a clean 1.8ml tube (not included).

1.2. Gently, touch 2-3 isolated colonies (or pick from 3 locations in case of smear) using a sterile microbiology needle. Do not penetrate the agar growth substrate or smear the colony as this may have adverse effects on the subsequent PCR reaction. Place the tip into the tube, swirl gently and close the tube.

1.3. Place the tubes in a heating block at 100^{0}C for 5-10 min. Let cool and centrifuge 1 min at 12,000 rpm. Transfer 5µl from the supernatant (without touching the pellet) to the rtPCR reaction mix. Dispose the used tips in the biohazard bag.

2. Rapid method:

2.1. Gently, touch 2-3 colonies on an agar plate using a sterile microbiological needle. Use precaution as in step1.2.

2.2. Place the needle into the rtPCR reaction mix tube, swirl gently and close the tube. Dispose of used tips into a biohazard bag.

2.3. Add 5µl sterile PCR grade water to reach final volume of 20µl

PCR AMPLIFICATION

1. Establish number of reaction mixture tubes/strips needed for analysis. 1 negative control tube and 1 positive control tube should be included in each run.
2. Add 15µl from each of the 2 Reaction Mix into each rtPCR tubes/strips.
3. Add 5µl from the PCR grade water tube to negative control reaction tubes. Close the plate/tubes to avoid contamination.
4. Then, separately add 5µl DNA sample supernatant to the different reaction plate/tubes. Immediately close the plate/tubes to avoid contamination (when using the direct method, add 5µl PCR-grade water before adding the colonies)
5. The Positive control DNA can be used to generate a standard curve of the desired geneaccording to copy number/Ctvalue by serial dilution (in water) of the positive control (1:10, 1:100, and 1:1000). Alternatively, the positive control can be used at a single dilution as a positive control for the PCR set up. Dilute the Positive control 1:100 in water to final concentration of 2x105copies/µl. Add 5µl from the diluted positive control to final concentration of 10^6 copies of each gene. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. To prevent contamination in amplification assays Care should also be taken as described in section 2.
6. Close tubes and place them in a RT-PCR instrument.