

User manual

Check-Direct CPE

Real-time PCR kit for the detection of carbapenemase-producing *Enterobacteriaceae*

Version 2.0

Date of issue: 07.10.2019

 18-0084
  48
  084-03

For use on: ABI-7500
LC480 I & II
CFX96
RotorGene-Q

For Research Use Only (RUO)
 Not for use in diagnostic procedures

Contents

Introduction.....	2
Principle of the method.....	2
Kit contents (for 48 reactions)	2
Materials required but not supplied with the kit.....	3
Storage, handling, and stability	3
Good laboratory practices	4
Specimen collection and DNA extraction	5
Real-time PCR assay and cyclor operation.....	5
Results interpretation	6
Frequently asked questions (FAQ) & Troubleshooting.....	8
Key to symbols used.....	9
Limitations.....	9
Technical assistance	10
Appendix 1a: ABI 7500 program	11
Appendix 1b: LC480 program.....	13
Appendix 1c: CFX96 program.....	15
Appendix 1d: RotorGene-Q program	17

Introduction

The worldwide emergence and dissemination of carbapenem resistance among *Enterobacteriaceae* is a serious threat to public health. These organisms are associated with high mortality rates and have the potential to spread widely. The most common cause of carbapenem resistance in *Enterobacteriaceae* is the expression of carbapenemases, *i.e.* Carbapenemase-Producing *Enterobacteriaceae* or CPE. CPE have elevated or complete resistance to carbapenems and most other β -lactam antibiotics. Presently, the vast majority of CPE are associated with the presence of one of the following plasmid-encoded carbapenemases: KPC (*Klebsiella pneumoniae* carbapenemase), VIM (Verona integron–encoded metallo- β -lactamase), NDM (New Delhi metallo- β -lactamase) or OXA-48 (Oxacillinase-48). Moreover, CPE often have other non- β -lactam resistance determinants resulting in multidrug- and pandrug-resistant isolates.

Principle of the method

Check-Direct CPE assay is based on specific recognition and amplification of target sequences by PCR, and the simultaneous detection of the accumulation of PCR amplification products by fluorescent DNA probes. A control DNA molecule, the internal control, is added to the clinical specimen prior to DNA extraction to monitor that DNA extraction and PCR amplification were successful. Five molecular beacon probes, labeled with 4 different dyes are used to detect the various carbapenemases and the control DNA. Check-Direct CPE discriminates between KPC, NDM/VIM, and OXA-48. For each of the 4 carbapenemase genes, KPC, OXA-48, NDM and VIM, many gene variants exist. PCR primers and fluorescent probes of Check-Direct CPE are selected to target homologous gene segments of these carbapenemase genes, and in this way gene variants are reliably detected.

Kit contents (for 48 reactions)

Components (Mat. No.)	Description	Storage conditions
CPE PCR Mastermix (9-0080)	1 transparent tube and cap 660 μ l	+ 2-8 °C
CPE solution (9-0071)	1 brown tube (purple inlay ●) 140 μ l	2-8 °C, store in the dark
Internal control (9-0077)	1 tube (red inlay ●) 600 μ l	
Negative control (9-0070)	1 tube (white inlay ○) 100 μ l	
KPC positive control (9-0073)	1 tube (green inlay ●) 100 μ l	
NDM positive control (9-0075)	1 tube (gold inlay ●) 100 μ l	
VIM positive control (9-0074)	1 tube (yellow inlay ●) 100 μ l	
OXA-48 positive control (9-0076)	1 tube (orange inlay ●) 100 μ l	
User manual (9-0082)	Leaflet – download from website	-

Materials required but not supplied with the kit

		Supplies	Equipment
General		<ul style="list-style-type: none"> Disposable laboratory (powder-free) gloves Lab coat Pipettes & disposable filter tips for volumes of 1 to 1000 µl 1.5 ml tubes ("Eppendorf tubes") 	<ul style="list-style-type: none"> Vortex mixer Mini-centrifuge Thermo Block for 1,5 tubes (for DNA extraction from cells)
Real-time PCR instrument	ABI 7500	<ul style="list-style-type: none"> 96-well PCR clear plate (REF N8010560) PCR plate seal 	<ul style="list-style-type: none"> ABI 7500 (Applied Biosystems, US) Plate centrifuge
	LC® 480	<ul style="list-style-type: none"> LightCycler® 480 multiwell plate 96 (Product no. 04729692001) 4-Color Compensation Set (Ref: 18-0070, Check-Points Health B.V., NL) PCR plate seal 	<ul style="list-style-type: none"> LightCycler®480 I/II (Roche, CH) Plate centrifuge
	CFX96™	<ul style="list-style-type: none"> 96-well PCR white plate PCR plate seal 	<ul style="list-style-type: none"> CFX96™ (Bio-Rad, US) Plate centrifuge
	Rotor-Gene Q	<ul style="list-style-type: none"> PCR strip tubes and caps 0.1ml (QIAGEN, Germany) 	<ul style="list-style-type: none"> Rotor-Gene Q 5plex 72- well rotor and locking ring Loading block 72x0.1ml tubes
Specimen	Bacterial culture	<ul style="list-style-type: none"> PCR-grade water (Milli-Q or aqua bidest) Eppendorf tubes (safe lock) Check-Direct Quick Extraction kit (Check-Points) 	<ul style="list-style-type: none"> Densitometer for bacterial suspensions Centrifuge for Eppendorf tubes Heating block for Eppendorf tubes

Storage, handling, and stability

Check-Direct CPE reagents are shipped cooled. All reagents should be stored at 2-8°C upon receipt. Please visually inspect the box upon initial opening to ensure that its contents are intact. Please contact the Check-Points office at support@check-points.com if you have any further questions. Store kit reagents at 2-8°C until expiration date.

Good laboratory practices

Recommendations for best results

- The test must be performed by adequately trained personnel.
- Do not use reagents after their expiration date.
- Before use, vortex reagents briefly to obtain a homogeneous solution. After vortexing briefly, spin down the solution to avoid contamination when opening the cap.
- Protect reagents from light to avoid photo-bleaching of the dyes.
- Periodically, verify the accuracy and precision of pipettes, as well as correct functioning and calibration of the instruments.

Prevention of contaminations

Use separate rooms: a microbiology room, a pre-PCR room and a PCR room.

- Bacterial cell suspensions are prepared in the microbiology room.
- Crude DNA extraction from the bacterial cell suspension is carried out in the microbiology room.
- Preparation of the amplification reactions is carried out in the pre-PCR room.
- Incubation in the real-time PCR thermocycler is carried out in the PCR room.
- Never transfer items from the PCR room to the pre-PCR room.

To keep laboratory free of PCR product contamination:

- Use pipettes with hydrophobic filter tips.
- Make sure to always use a new pipette tip when adding solutions, test samples, and controls to wells of a 96-well plate.
- Follow proper pipette-dispensing techniques to prevent aerosols.
- Wear clean disposable gloves and clean lab coats for the different steps of the test.
- Change gloves whenever you suspect that they are contaminated.
- Keep the tubes of all kit components and samples closed as much as possible.
- Clean the lab benches and all equipment regularly with a 0.5% sodium hypochlorite solution.

Users are strongly advised to read the full protocol before starting the test.

Check-Direct CPE is a very sensitive assay detecting down to 1 cfu per test. Care should be taken to avoid contamination particularly when working with bacterial cell suspensions.

Specimen collection and DNA extraction

Crude DNA extraction from bacterial cell suspensions

Important points before starting: DNA extraction is carried out in the microbiology room.

Use Milli-Q or aqua bidest; Do not use the “saline water” routinely used for preparing cell suspensions.

Procedure:

1. Inoculate nutrient agar plates with the clinical samples or the bacterial strains to be tested and incubate overnight at 37°C. Typical growth media include blood agar, MacConkey agar and Tryptic Soy agar.
2. Prepare a bacterial cell suspension of McFarland 0,5 – 1,0 or OD₆₀₀ 0,08 – 0,15 using PCR-grade water (Milli-Q water or aqua bidest).
3. For each cell suspension, transfer 200 µl to a 1.5 ml Eppendorf tube (preferably safe lock) and add 10 µl of the internal control solution (IC solution, ●). Mix briefly.
4. Heat the tubes at 98°C for 10 minutes. After incubation vortex the tubes vigorously for 30 seconds.
5. Centrifuge the tubes in an Eppendorf centrifuge for 2 minutes at maximum speed.
6. Use the supernatant immediately or store at +4°C and use within 24 hours. Alternatively, store at -20°C for longer periods.

Positive and negative control preparation

To validate the run, perform positive and negative control reactions for each Check-Direct CPE PCR run. The positive and negative controls are supplied with the kit.

- **Positive control(s)**

One positive control per target is provided with the kit. Each positive control contains the internal control. These controls may be used individually or combined. Refer to step 2.4 of the *Real-Time PCR preparation* for further details.

- **Negative control(s)**

Use the negative control (○) provided in the kit as a sample to validate the run. The negative control contains the internal control. We also recommend performing a DNA extraction as specified earlier, with the internal control solution using a sample known to be negative for the test in use (*i.e.*, carbapenemase negative sample, or elution buffer).

Real-time PCR assay and cycler operation

1. Multiplex real-time PCR setup

Table 1 presents the multiplex real-time PCR setup with the targets detected in each detector channel of the various cyclers. Details for each real-time PCR cycler are described in Appendix 1.

Table 1: Multiplex real-time PCR setup for the various cyclers.

Detector	FAM/Green	VIC/Yellow	Texas Red/610	Cy5/670
Channel	1	2	3	4
Target	KPC	VIM/NDM	OXA-48-like	I.C.*

*I.C: Internal Control

When the test is performed for the first time create the PCR test program “Check-Direct CPE” as described in Appendix 1.

2. Real-time PCR preparations

- 2.1 Calculate the number of reactions. Mix reagents well, spin down.
- 2.2 Prepare the real-time PCR (qPCR) mix as described in Table 2. Multiply the CPE solution and the CPE PCR Mastermix by the right number of samples and include 10% surplus to ensure that you have enough qPCR reaction mix for all the calculated reactions. For ABI 7500, LC®480 and CFX96™ 96-well PCR plates are used, for Rotor-Gene Q PCR-strip tubes.

- 2.3 Pipette 15 µl of qPCR mix to the wells or tubes.
- 2.4 Pipette 10 µl of test sample or control sample to each pre-filled well or tube. (The 4 positive controls may also be combined into a single “mixed positive control” by adding equal volumes of each control up to a volume of 10 µl per reaction, i.e. 4 x 2.5 µl of each control). See Table 2.
- 2.5 Seal the 96-well plate, or close the PCR-strip tubes. Mix the plate by tapping it on the bench and spin down briefly.
- 2.6 Transfer the plate or tubes to the PCR room.

Table 2: real-time PCR reaction mix setup.

Component	qPCR mix	Reaction
CPE PCR Mastermix	12,5 µl	
CPE Solution (●)	2,5 µl	
qPCR mix	15,0 µl →	15 µl
Sample		10 µl
Reaction volume		25 µl

3. Cyclor Operation

- 3.1 Insert the plates or tubes into the real-time PCR instrument.
- 3.2 Specify the run parameters for each cyclor as described in Appendix 1.
- 3.3 Enter the sample I.D.'s if required. For most systems this may also be done after the run.
- 3.4 Click the “Start Run” button.
- 3.5 When the run is completed, discard the plates or tubes according to local regulations.

Results interpretation

1. The Internal control should yield an amplification plot passing the threshold in the Cy5 channel.
2. Positive controls should yield an amplification plot passing the threshold and negative controls should yield no amplification plot. Examples of typical results for each real-time instrument are shown in appendices 1a through 1d. For use with other instruments, customer needs to perform an in-house validation.
3. Table 3 below shows the positions of the carbapenemase target genes in the various channels. VIM and NDM are detected in the same channel.

Table 3: Carbapenemase genes and the channels in which they are detected.

Detector	FAM/Green	VIC/Yellow	Texas Red/Orange	Cy5
Channel	1	2	3	4
Target	KPC	VIM & NDM	OXA-48-like	I.C.*

*I.C: Internal Control

4. Run validation: Table 4

Check the positive and negative control amplification curves. Valid run reports:

- No instruments system failures during the run.
- Negative control with a C_T value of < 34.5 in the Cy 5 detector channel and no C_T value in the other detector channel.
- Positive control C_T values as expected in **Table 4**. The exact C_T values of the positive controls vary depending on the qPCR instruments used and the threshold settings.

Table 4: Criteria for a valid run with Check-Direct CPE test*.

Sample Type / Expected C_T values	KPC FAM – green	NDM VIC - yellow	VIM VIC - yellow	OXA-48 Red(TXR) – orange	IC Cy5 – red
Positive control	< 31	< 32.5	< 29	< 27.5	< 34.5
Negative sample (extracted with IC)	N/A	N/A	N/A	N/A	< 34.5

*N.B. Combined positive controls in a single reaction will show a higher C_T -value. This deviation may be up to 4 C_T 's.

5. Results interpretation: Table 5

- If the run is valid, interpret results as positive, negative or invalid using the C_T values specified in **Table 5**.
- Positive carbapenemase samples will show a C_T value in the FAM, VIC and/or Red(TXR) channel.
- Negative carbapenemase samples will show no C_T value in the FAM, VIC, and Red(TXR) Channel. In the Cy5 Channel, a C_T value is expected at < 34.5 .
- Samples with a negative C_T -value (N/A or undetermined) for FAM, VIC or Red(TXR) and with a negative or ≥ 34.5 C_T -value for Cy5 (IC) indicate that the reaction is inhibited and therefore that the sample is invalid. See **Table 5** and “FAQ and Troubleshooting”).
- For cell suspensions the bacterial load is well defined. Therefore, the C_T -value will be within a specific range. C_T -values out of this range cannot be trusted and the test should be repeated. A typical error is a high C_T -value due to contamination when working with cell suspensions. Please check the maximum C_T -value for a true positive sample in Table 5. Positive carbapenemase samples will also show a C_T value in the Cy5 channel if the target has not out competed the internal control (IC) during the reaction.
- Always visually inspect the amplification plots to verify the results.

Table 5: Data interpretation guidelines for crude DNA extracts from bacterial cells*.

KPC, NDM/VIM, OXA-48 C_T values	IC C_T values	Interpretation
≤ 31	YES or N/A**	Positive sample
N/A	< 34.5	Negative sample
> 31	YES or N/A	Invalid
N/A	N/A or ≥ 34.5	Invalid

* If observed C_T values vary significantly from expected C_T values, see FAQ and Troubleshooting section.

** N/A represents a negative test result. The specific denotation may vary between different systems.

Frequently asked questions (FAQ) & Troubleshooting

1. The real-time results show no $C_T/C_P/C_q$ values or interpretation indicating that the sample is invalid.

Possible causes and troubleshooting:

- The sample DNA was not added to the assay.
- The sample DNA tested with Check-Direct CPE is negative and the internal control was not added prior to DNA extraction. Please repeat the DNA extraction.
- The DNA extraction failed since the internal control was not detected. Please repeat the DNA extraction.
- The sample DNA contains contaminants inhibiting the reactions. Please repeat the DNA extraction.
- CPE Solution or CPE PCR Mastermix was not added to the assay. Please repeat the test.
- Reagent solutions are degraded or may have expired.

2. The real-time results show no $C_T/C_P/C_q$ values for the positive control. Possible causes and troubleshooting:

- The positive control solution was not added. Repeat the test.
- CPE Solution or CPE PCR Mastermix was not added to the assay. Please repeat the test.
- Reagent solutions are degraded or may have expired.

3. Troubleshooting for invalid result.

Repeat the test by preparing a new DNA extract from the original specimen. Alternatively, repeat the test with a new DNA extraction from a newly collected specimen.

4. Real-time results show very low fluorescent signals in all samples and detector channels, including the internal control signal.

Possible causes and troubleshooting:

- The CPE solution containing the fluorescent probes and primers is degraded. Please check expiration date and if the kit was stored correctly.
- The real-time PCR system may be responsible for these results. Please refer to instrument User's manual or contact your real-time PCR instrument local representative.

5. $C_T/C_P/C_q$ values troubleshooting.

- ***Samples with very low C_T values and no amplification curves.*** The manual threshold is too low. Samples showing a C_T value <15 and no amplification curve are considered negative. The observed C_T value is an artifact of the software analysis: the threshold crosses the background noise of the curve.
- ***Invalid Run: C_T values expected for the controls in Table 4 do not match the C_T values observed in the experiment.*** Check that these differences are not due to the threshold being too high or too low. If changing the threshold does not improve the results, go to FAQ 2. to 5.

6. The real-time PCR instrument gives an error message.

Refer to the real-time PCR instrument user manual or contact the local technical support of the real-time PCR instrument company.

















7. Duplicate samples tested with Check-Direct CPE test did not yield identical results.

C_T (C_P/C_q) values of identical samples may vary between individual reactions. Large variations, > 2 C_T values, suggest pipetting errors or other differences between the duplicate samples.

8. Data Analysis and Interpretation.

If you encounter difficulties with the data analysis and interpretation, please contact Check-Points Technical Support at support@check-points.com.

Key to symbols used

Symbol	Definition	Symbol	Definition
	Catalog number		Negative control
	Batch code		KPC positive control
	IFU number		VIM positive control
	Use before YYYY-MM		NDM positive control
	Consult instructions for use		OXA-48 positive control
	Manufacturer		Internal control
	Temperature limitation		CPE solution
	Contains sufficient for < n > tests		CPE PCR Mastermix

Limitations

Check-Direct CPE is a DNA-based real-time PCR assay to detect the presence of the carbapenemase genes KPC, NDM, VIM, and OXA-48 in *Enterobacteriaceae*. The test detects the following carbapenemase gene variants: bla_{VIM1-6;8-34};bla_{OXA-48-162-163-181-232-244-245};bla_{NDM1-8} and bla_{KPC1-15}. KPC, NDM, VIM, and OXA-48 represent the clinically most prevalent carbapenemases in *Enterobacteriaceae* in most parts of the world. However, other rare carbapenemases may also be responsible for carbapenemase production in *Enterobacteriaceae* and these are not detected by Check-Direct CPE. Carbapenem resistance is caused by carbapenemase production, but also by various other mechanisms. A negative result with Check-Direct CPE does not imply that the bacterium is not carbapenem resistant; it implies that the bacterium is not likely to carry any of the carbapenemase gene variants of KPC, NDM, VIM and OXA-48 detected by Check-Direct CPE. Therefore, the test result of Check-Direct CPE should never be used as guidance for therapy.

Crude DNA extraction from bacterial cells may be used following the protocol specified in this manual. Other DNA extraction systems have not been approved for use with Check-Direct CPE yet.

The assay has been tested extensively with samples containing various gram-negative bacteria, such as *Escherichia*, *Salmonella*, *Klebsiella*, *Enterobacter*, *Citrobacter* and *Pseudomonas*, with excellent results. However, it may never be excluded that other Gram-negative bacteria or certain strains of the above species will yield poor results. Check-Direct CPE cannot and does not make any representation or warranty that it is capable of correctly detecting KPC, NDM, VIM, and OXA48 in all gram-negative species, subspecies or types or in all clinical sample sources. Results may need to be confirmed by additional methodologies in specific cases (*e.g.* for regulatory samples). Due to the high variability of bacterial genomes it is possible that certain subtypes might not be detected. The test reflects the state of knowledge of Check-Points Health B.V.

Technical assistance

support@check-points.com

+31 317 453 908

Despite the utmost care in the development and preparation of the protocol Check-Points cannot take any responsibility for errors, omissions and/or future changes herein.

Literature Citation: When describing a procedure for publication using this product, please refer to it as the *Check-Direct CPE*.

Notice to Purchaser:

This product is sold under license from PHRI Properties and may be used under PHRI Properties patent rights only for research use.

Trademarks

ABI, FAM, VIC, ROX are registered trademarks of Applied Biosystems Corporation.

Cy5 is a registered trademark of Amersham Biosciences Ltd.

Texas Red® is a registered trademark of Molecular Probes, Inc.

LightCycler® is a registered trademark of Roche Diagnostics.

Check-Points Health BV Tel: +31 317 453 908
Binnenhaven 5 info@check-points.com
6709 PD Wageningen www.check-points.com
The Netherlands



Appendix 1a: ABI 7500 program

Important:

- Please refer to the ABI7500 instruction manual for a detailed description on how to operate the real-time PCR instrument and how to analyze the data.
- Always visually inspect the amplification plot for each sample tested versus C_T values obtained with the software.

ABI 7500 cyler program

Check-Direct CPE detects 4 carbapenemase genes and an internal DNA control target. Table A1 displays in which detector channel each gene or target is detected.

Table A1: Multiplex real-time PCR setup for the ABI7500 cyler.

Detector	FAM	VIC	Texas Red	Cy5
Channel	1	2	3	4
Target	KPC	VIM & NDM	OXA-48-like	I.C.*

*I.C: Internal Control

The ABI7500 cyler needs to be programmed as outlined below:

- Run mode: Standard 7500 (96 wells)
- Experiment: Quantitation – Standard Curve
- TaqMan® Reagents
- Standard ramp speed
- Reaction volume: 25 μ l
- ROX™ passive reference dye: Included in the qPCR Buffer
- Targets: Reporter Dyes setup, see Table A. Quencher standard (NFQ-MGB).

Table B1: Real-time protocol parameters for ABI7500.

Step	Temperature	Time	Cycles	Data Collection	Ramp Rate Mode
1:	50°C	2 min	1	OFF	Standard
2:	95°C	3 min*	1	OFF	Standard
3:	95°C	15 sec	45	Plate read Optics on	Standard
4:	60°C	60 sec			

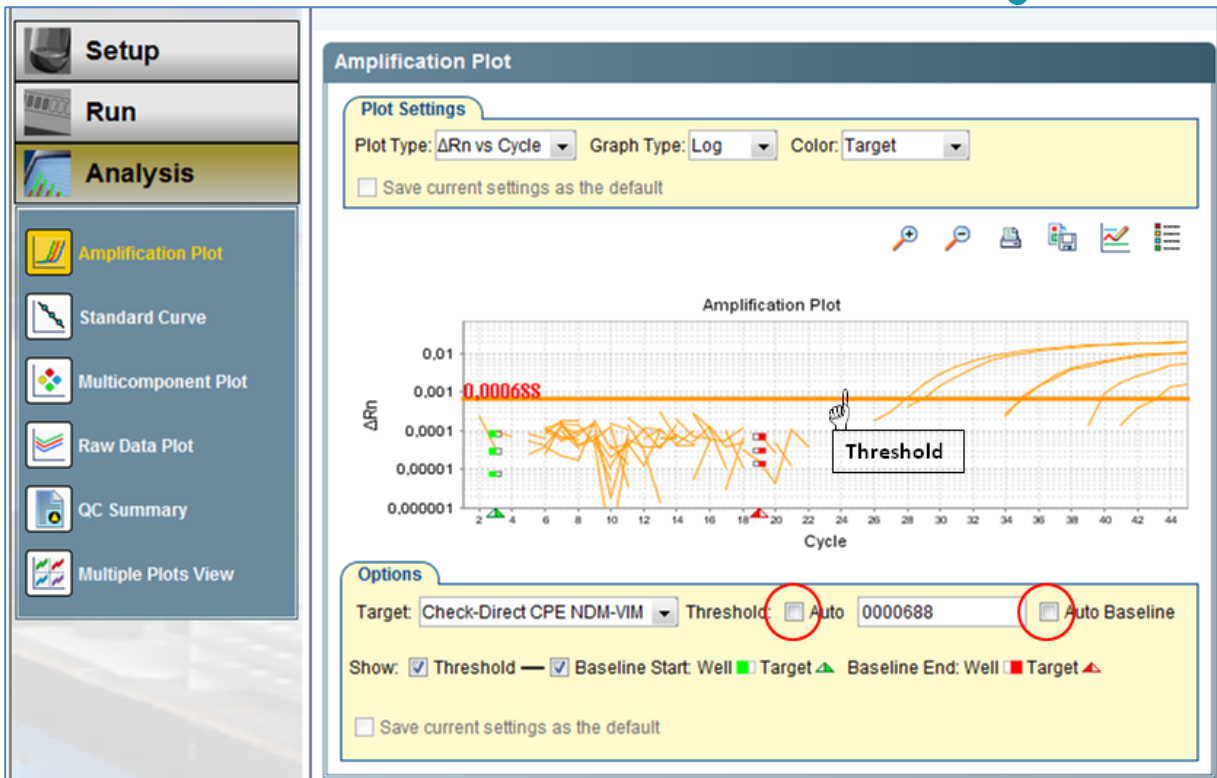
*Denaturation time may be up to 10 minutes

Data analysis

1. Analyze data using ROX as a passive reference.
2. In the **Options** setting deselect the box for auto threshold and auto baseline for each **Target** as shown in the **Analysis** Tab depicted below and select **Reanalyze**.
3. Set the threshold values in the analysis settings window for each detector channel to the values specified in Table C1.
4. Check amplification plots versus C_T values calculated by the software for each target.

Table C1: Threshold values

Detector	Target	Threshold
FAM - green	KPC	0.002
VIC - yellow	NDM- VIM	0.001
TXR - orange	OXA-48	0.006
Cy5 - red	IC	0.003



Analysis Tab of a typical analytical window on the ABI7500 with ABI7500 software v2.0.6
 Amplification plot of positive and negative samples for the Check-Direct CPE test (logarithmic scale); Red circle: deselect the box of auto Threshold/Auto baseline.

Appendix 1b: LC480 program

Important:

- Please refer to the LC480 instruction manual for a detailed description on how to operate the real-time PCR instrument and how to analyze the data.
- Always visually inspect the amplification plot for each sample tested versus C_p values obtained with the software.

LC480 cyclor program

Check-Direct CPE detects 4 carbapenemase genes and an internal DNA control target. Table A2 displays in which detector channel each gene or target is detected.

Table A2: Multiplex real-time PCR setup for the LC480 cyclor system I & II.

Detector	FAM	VIC	Red	Cy5
Channel	1	2	3	4
Target	KPC	VIM & NDM	OXA-48-like	I.C.*

*I.C: Internal Control

To use the Check-Direct CPE real-time PCR kit on the LightCycler®480 system I & II, a 4-color compensation object is required and generated by using the 4-Color Compensation Set supplied by Check-Points Health B.V. (Ref: 18-0070).

- Detection format **Check-Points 4-color set** (see 4-Color Compensation Set, User Manual V. 1.1 or higher)
- Reaction Volume: 25 μ l
- For the 45-cycle amplification step: select “Quantification” in the “Analysis Mode” tab. In the “Acquisition mode” tab select “none” for 95°C and “single” for 60°C.
- Color compensation object or file required

Table B2: Real-time protocol parameters for LC480.

Step	Temperature	Time	Cycles	Data Collection	Ramp Rate Mode
					LC®480 I & II
1:	50°C	2 min	1	OFF	4.4°C/s
2:	95°C	3 min*	1	OFF	4.4°C/s
3:	95°C	15 sec	45	Plate read Optics on	4.4°C/s 2.2°C/s
4:	60°C	60 sec			

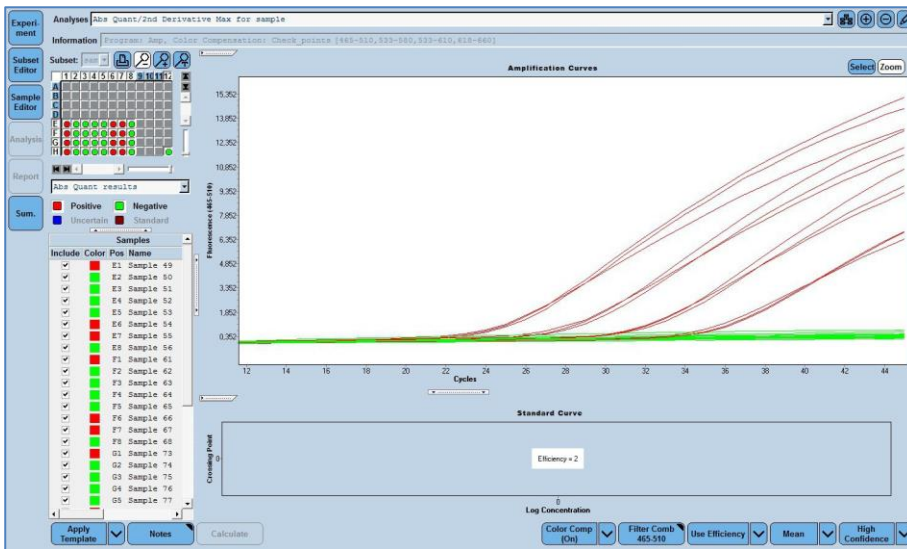
*Denaturation time may be up to 10 minutes

Data analysis

1. Select **Abs Quant/2nd Derivative Max** in the **Analysis** tab.
2. Analyze the data using the **color compensation object** application-specific to the Check-Direct CPE assay previously created. Select **Color Comp (On) – In database** select the CC object (Refer to Check-Points User Manual for the 4-Color Compensation set, Ref: 18-0070).
3. Select the **High Confidence** option and **Calculate** C_p values for each filter combination.
4. For each filter combination, always check amplification plot versus C_p values.
5. In the results table, check C_p values for each targets versus the *Status* given by the software (red= positive; green= negative; blue= call uncertain). Validate the *Status* of the sample using the software by visually inspecting amplification curves (See Figure).

Table C2: Recommended threshold settings.

Detector	Target	LC®480 I&II
FAM	KPC	Auto-calculated
VIC	NDM- VIM	Auto-calculated
RED	OXA-48	Auto-calculated
Cy5	IC	Auto-calculated



Screen shot of a typical analytical window on the LC[®]480 system I with the LC[®]480 software v1.5

Amplification plot of positive and negative samples for the Check-Direct CPE test (linear scale); threshold is automatically calculated by the software (Table C2).

Appendix 1c: CFX96 program

Important:

- Please refer to the CFX96 instruction manual for a detailed description on how to operate the real-time PCR instrument and how to analyze the data.
- Always visually inspect the amplification plot for each sample tested versus C_T values obtained with the software.

CFX96 cyclers program

Check-Direct CPE detects 4 carbapenemase genes and an internal DNA control target. Table A3 displays in which detector channel each gene or target is detected.

Table A3: Multiplex real-time PCR setup for the CFX96.

Detector	FAM	VIC	Texas Red	Cy5
Channel	1	2	3	4
Target	KPC	VIM & NDM	OXA-48-like	I.C.*

*I.C: Internal Control

The CFX96 cycler needs to be programmed as outlined below:

- Sample Volume: 25 μ l
- Temperature Control Mode: Calculated
- Scan Mode: All channel
- Plate type: BR white
- Plate Setup: View/Edit Plate and Select Fluorophore as indicated in Table A3.

Table B3: Real-time protocol parameters for CFX96.

Step	Temperature	Time	Cycles	Data Collection	Ramp Rate Mode
1:	50°C	2 min	1	OFF	Standard
2:	95°C	3 min*	1	OFF	Standard
3:	95°C	15 sec	45	Plate read Optics on	Standard
4:	60°C	60 sec			

*Denaturation time may be up to 10 minutes

Data analysis

1. Open the *Data file* for *Data Analysis*. In the **Analysis Settings** use the following parameters:
 - Analysis Mode: Fluorophore
 - Baseline Setting: Baseline Subtracted Curve Fit; Apply Fluorescent Drift Correction
 - C_q Determination: Single Threshold
 - Baseline Method: Auto Calculated
 - Cycle to Analyze: 10-45
 - Baseline Threshold: User defined (to be set per fluorophore)
2. Set the threshold manually for each detector channel using the threshold values recommended in Table C3.
3. Check amplification plots (in the *Quantification* Tab; See Figure below) versus C_q values calculated by the software for all targets in the results table.

Table C3: Threshold values.

Detector	Target	Threshold
FAM	KPC	60 - 150
VIC	NDM- VIM	200 - 250
TXR	OXA-48	400 - 800
Cy5	IC	40 - 100

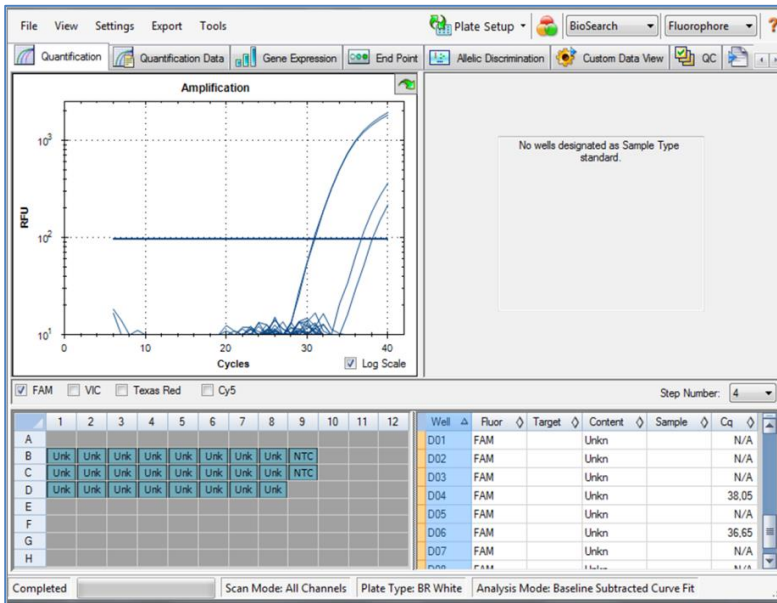


Figure: Screen shot of the typical analytical window on the CFX96™ with the CFX96™ software v2.0

Amplification plot of positive and negative samples for the Check-Direct CPE test (logarithmic scale); Adjust threshold manually using the threshold bar on the amplification plot; (Blue: FAM signal).

Appendix 1d: RotorGene-Q program

Important:

- Please refer to the RotorGene-Q instruction manual for a detailed description on how to operate the real-time PCR instrument and how to analyze the data.
- Always visually inspect the amplification plot for each sample tested versus C_T values obtained with the software.

RotorGene-Q program

Check-Direct CPE detects 4 carbapenemase genes and an internal DNA control target. Table A4 displays in which detector channel each gene or target is detected.

Table A4: Multiplex real-time PCR setup for the RotorGene-Q cyclers.

Detector	Green	Yellow	Orange	Red
Channel	1	2	3	4
Target	KPC	VIM & NDM	OXA-48-like	I.C.*

*I.C: Internal Control

The RotorGene-Q needs to be programmed as outlined below:

- Reaction Volume: 25 μ l
- 72-well rotor selected
- Gain settings: Green 6 / Yellow 4 / Orange 3 / Red 8.

Table B4: Real-time protocol parameters for RotorGene-Q.

Step	Temperature	Time	Cycles	Data Collection	Ramp Rate Mode
1:	50°C	2 min	1	OFF	Standard
2:	95°C	3 min*	1	OFF	Standard
3:	95°C	15 sec	45	Plate read Optics on	Standard
4:	60°C	60 sec			

*Denaturation time may be up to 10 minutes

Data analysis

1. Open the data file. Open the raw channel page for each detector. Select **Options** and **Crop start cycles**. In the pop-up window **Remove data before cycle**, enter **10** and select **OK**.
2. Select **Analysis** and the proper channel (from 10) and press **Show**.
3. Then, use the parameters indicated in **Table D** to analyze each detector channel.
4. Set the threshold manually for each detector channel using the value recommended in **Table C4**.
5. Check amplification plots versus C_T values calculated by the software for each target in the *Quant. Results* table (See Figure below).

Table C4: Threshold values.

Detector	Target	Threshold
Green	KPC	0.02
Yellow	NDM- VIM	0.015
Orange	OXA-48	0.03
Red	IC	0.06

Table D4: Rotor-Gene Q analytical parameters.

Detector / TARGET	Dynamic Tube	Slope Correct	Outliers Removal	Eliminate Cycles
Green / KPC	ON	ON	10%	10
Yellow / NDM-VIM	ON	ON	6%	10
Orange / OXA-48	ON	ON	10%	10
Red / IC	ON	ON	15%	10

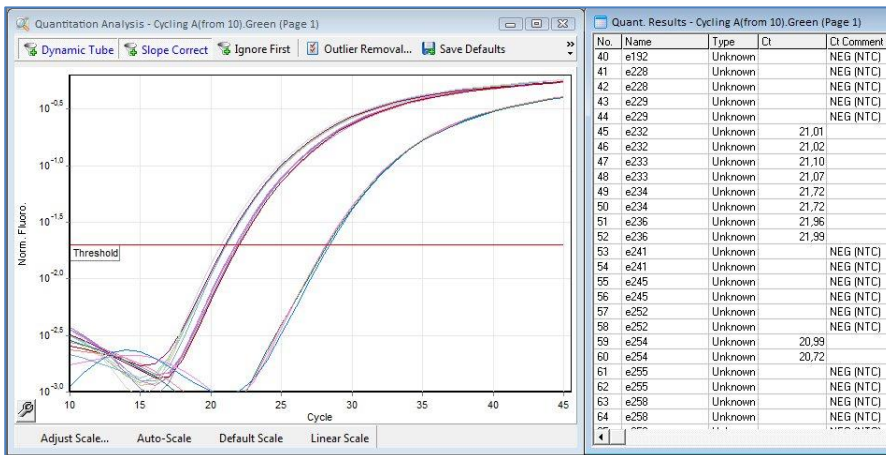


Figure: Screen shot of typical analytical window with the Rotor-Gene Q software v2.1.0 (Build 9) Amplification plot of positive and negative samples for the Check-Direct CPE test (logarithmic scale) in the Green channel.