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Identification of Corynebacteria of the *diphtheriae* Species Complex and detection of the diphtheria toxin gene by Multiplex Real-Time PCR V.1

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We use this protocol and it's working

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Abstract

This protocol describes a quadruplex real-time PCR (Dip4plex), performed with the Qiagen QuantiNova™ Multiplex PCR Kit, for simultaneous identification of Corynebacteria of the *diphtheriae* species complex and for the detection of the diphtheria toxin (*tox*) gene.

Guidelines

Work under biosafety level 2+/P2+ conditions:

- Perform the experiment in a class II biosafety cabinet.
- Wear appropriate PPE: lab coat, gloves, and eye protection.
- Decontaminate surfaces and equipment (e.g., pipettes) with Surfa'Safe premium™, then DNA Away™.

To prevent cross-contamination, the experiment should be performed in separate rooms for the following steps:

1. DNA extraction in a L2+ laboratory
2. A "clean" room for PCR mix preparation
3. A third room for adding extracted DNAs to the PCR mix (post-PCR setup).

Materials

- QuantiNova™ Multiplex PCR Kit (Qiagen) (stored at –30°C to –15°C until expiration date). *NB: After thawing, the Master Mix 4× is stable for 6 months at +2 °C to +8 °C, based on the kit's expiration date.*
- Rotor-Gene Q thermocycler (Qiagen)
- 1.5 mL DNA/RNA-free Eppendorf tubes
- Pipettes: P1000, P200, P20, P10
- Filtered pipette tips
- Primers and probes (Tib Molbiol or equivalent—see Annex 6). *NB: Primers must be of ultra-pure quality. Before usage, primers are lyophilised and stored at +2°C to +8°C for up to 1 year; stocks (100 µM) are in DNase/RNase-free water at –30°C to –15°C up to 1 year. Probes are light-sensitive—handle in low light.*
- DNase/RNase-free water (Promega)
- DNA Away™ (Dutscher) and Surfa'Safe premium™ (Anios) for decontamination
- Positive control DNAs (10 pg/µL) extracted from reference strains:

1. *C. diphtheriae tox*+ (NCTC 10648);
2. *C. belfantii tox*– (NCTC 10356);
3. *C. ulcerans tox*– (NCTC 12077); and
4. *C. pseudotuberculosis tox*– (CIP 102968).

NB: Positive controls should be aliquoted and stored at –30°C to –15°C; thawed aliquots may be stored at +2°C to +8°C for 6 months. The choice of storage temperatures and length is based on our experience in the laboratory.

OBJECTIVES

1 **Dip4plex targets:**

- *rpoB* fragments specific for either *C. diphtheriae/C. belfantii/C. rouxii,* or for *C. ulcerans/C. pseudotuberculosis/C. ramonii*
- the catalytic fragment A of the *tox* gene
- a 16S rRNA housekeeping gene fragment (universal bacterial control)

Detection

The detection is achieved by hydrolysis probes labelled FAM, HEX, Rox and LC640:

	A	B	C	D	E
	Probe	**Fluorophore/Quencher Mix**	**Fluorescence Channel**	**Excitation (nm)**	**Emission (nm)**
	C. ulcerans, C. pseudotuberculosis	FAM/BHQ-1	Green	470±10	510±5
	C. diphtheriae, C. belfantii, C. rouxii	HEX/BHQ-1	Yellow	530±5	557±5
	Tox gene	Rox/BHQ-2	Orange	585±5	610±5
	Universal 16S rRNA gene	LC640/BHQ-2	Red	625±5	660±10

DNA Extraction

DNA is extracted by boiling bacterial cultures or clinical samples in 0.5 mL ultrapure water at 100°C for 15 min; the supernatant is used directly for real-time PCR.

PROCEDURE

2 **DNA-matrix extraction (boil prep):**

Prepare a **negative extraction control** by processing 0.5 mL DNase/RNase-free water in a 1.5 mL Eppendorf tube in parallel with the samples.

2.1 **From a cultured isolate:**

1. In a 1.5 mL Eppendorf tube, resuspend ~1 µL bacterial culture loop in 0.5 mL sterile water. Vortex thoroughly.
2. Incubate at 100 °C ± 2 °C for 15 min.
3. Vortex, then centrifuge 1 min at 13000×g, room temperature.
4. Transfer the supernatant to a clean 1.5 mL Eppendorf tube.

The PCR is carried out on the supernatant, on two tubes: a tube with 5 µL of non-diluted supernatant, and a 5 µL tube of supernatant diluted 1:100. The 1:100 dilution is carried out with two serial dilutions of 1:10 (5 µL qsp50 µL) in DNase/RNase-free water.

2.2 **From a swab:**

Culture:
Inoculate the swab on Tinsdale and Columbia 5% sheep blood (COS) agar and streak the plates with 1 µL loops; place a Fosfomycin disc on the COS plate. Incubate at 35°C ± 2°C for 20h ± 4h if needed.

DNA extraction:
1. In a 1.5 mL Eppendorf tube, submerge the swab tip in 0.5 mL sterile water (DNase/RNase free).
2. Cut the shaft to the cap and vortex.
3. Incubate at 100°C ± 2°C for 15 min.
4. Remove the swab with sterile tweezers.
5. Centrifuge 1 min at 13000×g at room temperature.
6. Transfer the supernatant to a clean 1.5 mL Eppendorf tube.

The PCR is carried out on the supernatant, on two tubes: a tube of non-diluted supernatant, and a tube of supernatant diluted 1:100. The 1:100 dilution is carried out with two serial dilutions of 1:10 (5 µL qsp50 µL) in DNase/RNase-free water.

2.3 **From a pseudo-membrane or connective tissue:**

Culture:
Inoculate the pseudo-membrane fragment or connective tissue on Tinsdale and Columbia 5% sheep blood (COS) agar and streak the plates with 1 µL loops; place a Fosfomycin disc on the COS plate. Incubate at 35°C ± 2°C for 20h ± 4h if needed.

DNA extraction:
Using sterile, single-use scissors and tweezers, cut off a small piece of pseudo-membrane or
connective tissue, then:
1. Grind the tissue in a sterile mini-homogenizer until a homogeneous suspension is obtained.
2. Transfer the homogenate into a 1.5 mL Eppendorf tube containing 0.5 mL sterile DNase/RNase-free water.
3. Vortex vigorously.
4. Incubate the tube in a heating block at 10°C ± 2 °C for 15 min.
5. Centrifuge at 13000×g for 1 min at room temperature.
6. Transfer the supernatant to a clean 1.5 mL Eppendorf tube.

The PCR is carried out on the supernatant, on two tubes: a tube of non-diluted supernatant, and a tube of supernatant diluted 1:100. The 1:100 dilution is carried out with two serial dilutions of 1:10 (5 µL qsp50 µL) in DNase/RNase-free water.

2.4 Preparation of the 20× Dip4plex PCR mix (clean room)

Combine stock primers/probes (100 pmol/µL) as follows for final volumes of 100, 150 or 200 µL of 20× mix:

	A	B	C	D
	Primer/Probes (stock solutions at 100pmol/ µL)	**Mix 20× 100 µL**	**Mix 20× 150 µL**	**Mix 20× 200 µL**
	Dip rpob-F	5 µL	7,5 µL	10 µL
	Dip rpob-R	5 µL	7,5 µL	10 µL
	DIP-HEX probe	2 µL	3 µL	4 µL
	Ulc rpob-F	5 µL	7,5 µL	10 µL
	Ulc rpob-R	5 µL	7,5 µL	10 µL
	ULC-FAM probe	2 µL	3 µL	4 µL
	Dip_tox-A-F	5 µL	7,5 µL	10 µL
	Dip_tox-A-R	5 µL	7,5 µL	10 µL
	TOX_A_ROX probe	2 µL	3 µL	4 µL
	16S_qPCR-F	5 µL	7,5 µL	10 µL
	16S_qPCR-R	5 µL	7,5 µL	10 µL
	16S_qPCR probe	2 µL	3 µL	4 µL
	TE buffer 1× pH 8,0	52 µL	78 µL	104 µL

	A	B	C	D
	Final volume	**100µL**	**150µL**	**200µL**

2.5 **Validation of the 20× mix**

We advise to test each new lot with negative and positive controls. Record results and assign a lot number (date). Aliquot the 20× mix with 20 µL per tube; store at –30°C to –15°C, protected from light, up to 2 months.

2.6 **Preparation of the reaction mix for Dip4plex qPCR (clean room):**

The QuantiNova™ Multiplex PCR Kit can be used at laboratory temperature, but we advise to use a refrigerated block dedicated to the clean room, taken from the freezer (-20°C) 10 minutes before usage.

The volume of reaction mix needed depends on the number of samples tested:

	A	B	C
	Reagents (refrigerated block)	**Volume (1 tube)**	**Volume Example (21 tubes)**
	PCR-grade H2O	9 µL	189 µL
	Mix Dip4plex 20×	1 µL	21 µL
	4× QuantiNova™ Multiplex PCR Kit	5 µL	105 µL
	Final volume of reaction mix	**15 µL**	**315 µL**

Distribute 15 µL / tube of 0,2 mL qPCR Qiagen after gentle homogenisation.

	A	B
	Extracted DNAs / Positive and negative controls	5 µL/tube
	Reaction mix	15 µL/tube

	A	B
	Final volume	**20 µL/tube**

For each experiment, we advise to include **internal quality controls**. We suggest the followings:

- Negative extraction control (NF water boil prep)
- Negative amplification control (NF water PCR)
- Positive control for amplification of the *tox* gene and for *C. diphtheriae* (NCTC 10648: *C. diphtheriae tox*+)
- Positive control for amplification of *C. diphtheriae/belfantii/rouxii* (NCTC 10356: *C. belfantii*)
- Positive control for amplification of *C. ulcerans/C. ramonii* (NCTC 12077)
- Positive control for amplification of *C. pseudotuberculosis* (CIP 102968)

Following steps:

1. Switch in the Rotor-Gene Q thermocycler
2. Remove the rotor from its spindle and take off the locking ring
3. Load the microtubes into the rotor, making sure each tube's number matches its designated position
4. Fill any empty slots with empty tubes
5. Reinstall the locking ring and insert the rotor assembly into the Rotor-Gene Q thermocycler
6. Confirm that both the rotor and locking ring are firmly locked before closing the Rotor-Gene Q lid
7. Open the Q-Rex software and launch the Dip4plex real-time PCR program
8. Start the run, then record and save all run details (sample names, tube positions and sample types)

2.7 Dip4plex Real-Time PCR program (Rotor-Gene Q)

	A	B	C	D
	Stage	**Temperature**	**Length**	**Cycles**
	Initial inactivation	95°C	5 minutes	1
	Denaturation	95°C	10 seconds	45
	Annealing/extension	60°C	20 seconds	45

2.8 **Data Analysis**

In “Basic” mode, set threshold to 0.05 and threshold start cycle to 1 for each channel. Perform colour compensation to eliminate spectral bleed-through among FAM, HEX, Rox and LC640.

Fluorescence channels and detection ranges:

	A	B
	FAM	(470 ± 10 – 510 ± 5)
	HEX	(530 ± 5 – 557 ± 5)
	Rox	(585 ± 5 – 610 ± 5)
	LC640	(625 ± 5 – 660 ± 10)

VALIDATION CRITERIA

3 **For extraction and PCR amplification negative controls:** no amplification curves in the FAM, HEX or Rox channels. The channel LC640 (16S) may show some amplification curves which correspond to residual bacterial DNA present in the Master mix of the Qiagen kit. This DNA originates from bacteria used to produce the Taq polymerase, and its concentration can vary from one kit lot to the next, generating variable negative control curves in the LC640 channel (i.e., it is impossible to establish a standard Ct value for the amplification of the negative control 16S gene fragment). It is necessary to compare the Ct of negative controls with those of the positive controls in the LC640 channel. If the Ct of the negative controls are superior to the Ct of the positive controls, then the negative controls are validated.

For the positive controls: amplification curves must show lag, exponential and plateau phases.

Acceptable Ct values for the quality controls of the real-time PCR:

	A	B	C	D	E	F
	C. diphtheriae	***Tox gene***	***C. ulcerans***	***C. pseudotuberculosis***	**NF water PCR amplification**	**NF water extraction**
	24 ± 2	21 ± 2	21 ± 2	26 ± 2	> to positive controls of the same channel	> to positive controls of the same channel

If the results for the extraction and PCR amplification negative controls and for the positive controls are correct, the results can be interpreted as below:

	A	B	C	D	E
		FAM 465-510 nm filter	**HEX 533-580 nm filter**	**Rox 533-610 nm filter**	**LC640 498-640 nm filter**
	Negative control extraction	No signal (-)	No signal (-)	No signal (-)	Ct > to the positive control Ct values in the same channel
	Negative control PCR amplification	No signal (-)	No signal (-)	No signal (-)	Ct > to the positive control Ct values in the same channel
	Positive control *C. diphtheriae tox+*	No signal (-)	Classical curve with Ct of 24±2	Classical curve with Ct of 21±2	Ct < to the negative control Ct values in the same channel
	Positive control *C. belfantii tox-*	No signal (-)	Classical curve with Ct of 24±2	No signal (-)	Ct < to the negative control Ct values in the same channel
	Positive control *C. ulcerans tox-*	Classical curve with Ct of 21±2	No signal (-)	No signal (-)	Ct < to the negative control Ct values in the same channel
	Positive control *C.*	Classical curve with	No signal (-)	No signal (-)	Ct < to the negative

	A	B	C	D	E
	pseudotuberculosis tox-	Ct of 29±2			control Ct values in the same channel

QUALITATIVE INTERPRETATION

4 A sample is negative if there is no signal in FAM/HEX/Rox channels and a 16S signal (LC640) with Ct lower than negative controls in that same channel.

A sample is positive if there is a classical amplification curve in any of FAM, HEX or Rox channels and a valid 16S Ct (LC640 with a Ct lower than negative controls).

LIMITATIONS

5 Dip4plex cannot distinguish *C. ulcerans* from *C. pseudotuberculosis* or *C. ramonii*, nor *C. diphtheriae* from *C. rouxii* or *C. belfantii*.

ANNEX

6 See primer and probe sequences.

Oligonucleotides sequences and expected amplicon sizes of the four gene targets

Target gene	Oligonucleotide name	Sequence (5' — 3')	Amplicon Fragment size (bp)	Reference
*C. diphtheriae rpoB**	dip_rpobF	CGT TCG CAA AGA TTA CGG AAC CA		
	dip_rpobR	CAC TCA GGC GTA CCA ATC AAC	97 bp	De Zoysa *et al* (2016)
	Cdip HP	HEX§-AGG TTC CGG GGC TTC TCG ATA TTC A-BHQ‖1		
C. ulcerans rpoB	ulc_rpobF	TTC GCA TGG CTC ATT GGC AC		
	ulc_rpobR	TCC AGG ATG TCT TCC AGT CC	98 bp	De Zoysa *et al* (2016)
	CulcHP	FAM-CCA GCA GGA GGA GCT GGG TGA A-BHQ1		
tox†	toxAF	CTT TTC TTC GTA CCA CGG GAC TAA		
	toxAR	CTA TAA AAC CCT TTC CAA TCA TCG TC	117 bp	De Zoysa *et al* (2016)
	diptoxHP	ROX¶-AAG GTA TAC AAA AGC CAA AAT CTG GTA CACA AGG-BHQ2		
Universal 16S rRNA	16S_u_F	TGT CGT CAG CTC GTG TCG TG		
	16S_u_R	ACG TCA TCC CCA CCT TCC TC	136 bp	This study
	16S_u_HP	LC640-TCC CGC AAC GAG CGC AAC CCT T-BHQ2		

*RNA polymerase β-subunit-encoding gene.
†Diphtheria toxin gene.
§Hexachlorofluorescein.
‖Black-hole quencher.
¶6-Carboxyl-X-rhodamine.

SOURCE INFORMATION

7 The protocol was adapted from:

- an initial triplex PCR developed by De Zoysa A, Efstratiou A, Mann G, Harrison TG, Fry NK. Development, validation and implementation of a quadruplex real-time PCR assay for identification of potentially toxigenic corynebacteria. J Med Microbiol 2016;65:1521-1527.
- A modification of the method by Badell E, Guillot S, Tulliez M, Pascal M, Panunzi LG, Rose S, Litt D, Fry NK, Brisse S. Improved quadruplex real-time PCR assay for the diagnosis of diphtheria. J Med Microbiol 2019;68:1455—1465 (in which the 16S rRNA target was added as internal control).