

PCRmax Ltd TM qPCR test

Corynebacterium diphtheriae A and B

C.diphtheriae Toxin sub-units A &
B

150 tests

PCRmax 

For general laboratory and research use only



Quantification of Corynebacterium diphtheriae A and B genomes.
Advanced kit handbook HB10.07.08
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Introduction to *Corynebacterium diphtheriae* A and B

Corynebacterium diphtheriae is a rod-shaped, non-motile, Gram-positive bacterium of the *Corynebacteriaceae* family. This bacterium ranges in size from 1-8 μ m in length a genome of around 2.5M nucleotides arranged in a single circular formation. This bacterium is pathogenic to humans and causes the disease diphtheria.

C. diphtheriae resides in the nose, mouth, throat and skin of humans and causes infections that can be classified as two different forms of Diphtheria: respiratory and cutaneous, with cutaneous diphtheria resulting in sores and ulcers on the skin and the respiratory disease causing an infection of the upper respiratory tract by adhering to the pharyngeal cells and colonizing them. This bacterial adhesion results in a pseudomembrane forming over the cells which develops into lesions. *C. diphtheriae* infections may become toxigenic when the bacteria produce Diphtheria toxin although to produce the exotoxin extracellular levels of iron must be low and the bacteria must be infected by a bacteriophage which encodes the *tox* gene.

Diphtheria toxin consists of 2 domains, an active domain and a binding domain. The toxin binds to receptors on the host cell and is internalized by receptor mediated endocytosis. Once within the endosome, the toxin undergoes a conformational change initiated by the acidity of the endosome that allows the active domain to insert into the endosome membrane. The binding domain is then cleaved allowing the active domain to be released into the cytoplasm where it interferes with protein synthesis by deactivating Elongation factor II. This ultimately leads to cell death.

Cutaneous diphtheria infection shows symptoms after an incubation period of around 7 days. The respiratory condition has a shorter incubation period of up to 5 days and usually presents with sore throat, fatigue and fever. In around 10% of cases, the infection can lead to a swollen neck that causes breathing difficulties or asphyxiation. More generalized symptoms may be listlessness or a fast heart rate which may lead to cardiomyopathy or peripheral neuropathy. Transmission of this bacterium is mainly via aerosol secretions of infected individuals and so the disease is very contagious but the infection can be treated with antibiotics.

Specificity

The PCRmax qPCR Kit for *Corynebacterium diphtheriae* A and B (*C.diphtheriae* A and B) genomes is designed for the in vitro quantification of *C.diphtheriae* A and B genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the *C.diphtheriae* A and B genome.

The primers and probe sequences in this kit have 100% homology with a broad range of *C.diphtheriae* A and B sequences based on a comprehensive bioinformatics analysis.

The target sequences (toxin subunits A and B) have previously been shown to be a good genetic marker for *C. diphtheriae* in other real time PCR based studies (Mothershed EA et.al 2002). The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

If you require further information, or have a specific question about the detection profile of this kit then please send an e.mail to help@pcrmax.com and our bioinformatics team will answer your question.

Kit Contents

- **Corynebacterium diphtheriae A primer/probe mix (150 reactions BROWN)**
FAM labelled
- **Corynebacterium diphtheriae B primer/probe mix (150 reactions BROWN)**
FAM labelled
- **Corynebacterium diphtheriae A positive control template (for Standard curve RED)**
- **Corynebacterium diphtheriae B positive control template (for Standard curve RED)**
- **Internal extraction control primer/probe mix (150 reactions BROWN)**
VIC labelled as standard
- **Internal extraction control DNA (150 reactions BLUE)**
- **Endogenous control primer/probe mix (150 reactions BROWN)**
FAM labelled
- **RNAse/DNAse free water (WHITE)**
for resuspension of primer/probe mixes and internal extraction control DNA
- **Template preparation buffer (YELLOW)**
for resuspension of positive control templates and standard curve preparation

Reagents and equipment to be supplied by the user

Real-Time PCR Instrument

DNA extraction kit

This kit is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

Lyophilised 2x qPCR Mastermix

This kit is designed to be compatible with all commercially available Mastermixes that run with standard cycling conditions.

Pipettors and Tips

Vortex and centrifuge

Thin walled 1.5 ml PCR reaction tubes

Kit storage and stability

This kit is stable at room temperature but should be stored at -20°C on arrival. Once the lyophilised components have been resuspended they should not be exposed to temperatures above -20°C for longer than 30 minutes and unnecessary repeated freeze/thawing should be avoided. The kit is stable for six months from the date of resuspension under these circumstances.

If a standard curve dilution series is prepared this can be stored frozen for an extended period. If you see any degradation in this serial dilution a fresh standard curve can be prepared from the positive control.

PCRmax does not recommend using the kit after the expiry date stated on the pack.

Suitable sample material

All kinds of sample material suited for PCR amplification can be used. Please ensure the samples are suitable in terms of purity, concentration, and DNA integrity (An internal PCR control is supplied to test for non specific PCR inhibitors). Always run at least one negative control with the samples. To prepare a negative-control, replace the template DNA sample with RNase/DNase free water.

Dynamic range of test

Under optimal PCR conditions PCRmax C.diphtheriae A and B detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

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Principles of the test

The kit contains two primer and probe sets. One set for the detection of *C.diphtheriae* Toxin sub-unit A a second for *C.diphtheriae* Toxin sub-unit B. Thus the kit provides a flexible solution for the precise detection and analysis of *Corynebacterium diphtheriae* in clinical samples.

Real-time PCR

C.diphtheriae A and *C.diphtheriae* B specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mixes provided exploit the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the *C.diphtheriae* A and B DNA. A fluorogenic probe is included in the same reaction mixture which consists of a DNA probe labeled with a 5`-dye and a 3`-quencher. During PCR amplification, the probe is cleaved and the reporter dye and quencher are separated. The resulting increase in fluorescence can be detected on a range of real-time PCR platforms.

Positive control

For copy number determination and as a positive control for the PCR set up, the kit contains a positive control template. This can be used to generate a standard curve of *C. diphtheriae* A or *C.diphtheriae* B copy number / Cq value. Alternatively the positive control can be used at a single dilution where full quantitative analysis of the samples is not required. Each time the kit is used, at least one positive control reaction must be included in the run. A positive result indicates that the primers and probes for detecting the target *C. diphtheriae* A and B gene worked properly in that particular experimental scenario. If a negative result is obtained the test results are invalid and must be repeated. Care should be taken to ensure that the positive control does not contaminate any other kit component which would lead to false-positive results. This can be achieved by handling this component in a Post PCR environment. Care should also be taken to avoid cross-contamination of other samples when adding the positive control to the run. This can be avoided by sealing all other samples and negative controls before pipetting the positive control into the positive control well.

Negative control

To validate any positive findings a negative control reaction should be included every time the kit is used. For this reaction the RNase/DNase free water should be used instead of template. A negative result indicates that the reagents have not become contaminated while setting up the run.

Internal DNA extraction control

When performing DNA extraction, it is often advantageous to have an exogenous source of DNA template that is spiked into the lysis buffer. This control DNA is then co-purified with the sample DNA and can be detected as a positive control for the extraction process. Successful co-purification and real-time PCR for the control DNA also indicates that PCR inhibitors are not present at a high concentration.

A separate primer and probe mix are supplied with this kit to detect the exogenous DNA using real-time PCR. The primers are present at PCR limiting concentrations which allows multiplexing with the target sequence primers. Amplification of the control DNA does not interfere with detection of the *C.diphtheriae* A or *C.diphtheriae* B target DNA even when present at low copy number. The Internal control is detected through the VIC channel and gives a Cq value of 28+/-3.

Endogenous control

To confirm extraction of a valid biological template, a primer and probe mix is included to detect an endogenous gene. Detection of the endogenous control is through the FAM channel and it is NOT therefore possible to perform a multiplex with the *C.diphtheriae* A or *C.diphtheriae* B primers. A poor endogenous control signal may indicate that the sample did not contain sufficient biological material.

Carry-over prevention using UNG (optional)

Carry over contamination between PCR reactions can be prevented by including uracil-N-glycosylase (UNG) in the reaction mix. Some commercial mastermix preparations contain UNG or alternatively it can be added as a separate component. UNG can only prevent carry over from PCR reactions that include deoxyuridine triphosphate (dUTP) in the original PCR reaction. PCRmax recommend the application of 0.2U UNG per assay with a 15 minute incubation step at 37°C prior to amplification. The heat-labile UNG is then inactivated during the Taq polymerase activation step.

Reconstitution Protocol

To minimize the risk of contamination with foreign DNA, we recommend that all pipetting be performed in a PCR clean environment. Ideally this would be a designated PCR lab or PCR cabinet. Filter tips are recommended for all pipetting steps.

- 1. Pulse-spin each tube in a centrifuge before opening.**
This will ensure lyophilised primer and probe mix is in the base of the tube and is not spilt upon opening the tube.
- 2. Reconstitute the kit components in the RNase/DNase free water supplied, according to the table below:**
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in water	Volume
Pre-PCR pack	
C.diphtheriae A primer/probe mix (BROWN)	165 µl
C.diphtheriae B primer/probe mix (BROWN)	165 µl
Internal extraction control primer/probe mix (BROWN)	165 µl
Endogenous control primer/probe mix (BROWN)	165 µl
Pre-PCR heat-sealed foil	
Internal extraction control DNA (BLUE)	600 µl

- 3. Reconstitute the positive control templates in the template preparation buffer supplied, according to the table below:**
To ensure complete resuspension, vortex each tube thoroughly.

Component - resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
C.diphtheriae A Positive Control Template (RED) *	500 µl
C.diphtheriae B Positive Control Template (RED) *	500 µl

* This component contains high copy number template and is a VERY significant contamination risk. It must be opened and handled in a separate laboratory environment, away from the other components.

DNA extraction

The internal extraction control DNA can be added either to the DNA lysis/extraction buffer or to the DNA sample once it has been resuspended in lysis buffer.

DO NOT add the internal extraction control DNA directly to the unprocessed biological sample as this will lead to degradation and a loss in signal.

- 1. Add 4µl of the Internal extraction control DNA (BLUE) to each sample in DNA lysis/extraction buffer per sample.**
- 2. Complete DNA extraction according to the manufacturers protocols.**

Real-time PCR detection protocol

- 1. For each DNA sample prepare a reaction mix according to the table below:**
Include sufficient reactions for positive and negative controls.

Component	Volume
Lyophilised 2x qPCR Mastermix	10 μ l
C.diphtheriae A or C.diphtheriae B primer/probe mix (BROWN)	1 μ l
Internal extraction control primer/probe mix (BROWN)	1 μ l
RNAse/DNAse free water (WHITE)	3 μ l
Final Volume	15 μl

- 2. For each DNA sample prepare an endogenous control reaction according to the table below (Optional):**
This control reaction will provide crucial information regarding the quality of the biological sample.

Component	Volume
Lyophilised 2x qPCR Mastermix	10 μ l
Endogenous control primer/probe mix (BROWN)	1 μ l
RNAse/DNAse free water (WHITE)	4 μ l
Final Volume	15 μl

- 3. Pipette 15 μ l of each mix into individual wells according to your real-time PCR experimental plate set up.**
- 4. Prepare sample DNA templates for each of your samples.**
- 5. Pipette 5 μ l of DNA template into each well, according to your experimental plate set up.**
For negative control wells use 5 μ l of RNAse/DNAse free water. The final volume in each well is 20 μ l.

- 6. If a standard curve is included for quantitative analysis prepare a reaction mix according to the table below:**

Component	Volume
Lyophilised 2x qPCR Mastermix	10 μ l
C.diphtheriae A or C.diphtheriae B primer/probe mix (BROWN)	1 μ l
RNAse/DNAse free water (WHITE)	4 μ l
Final Volume	15 μl

7. Preparation of standard curve dilution series.

- 1) Pipette 90µl of template preparation buffer into 5 tubes and label 2-6
- 2) Pipette 10µl of Positive Control Template (RED) into tube 2
- 3) Vortex thoroughly
- 4) Change pipette tip and pipette 10µl from tube 2 into tube 3
- 5) Vortex thoroughly

Repeat steps 4 and 5 to complete the dilution series

Standard Curve	Copy Number
Tube 1 Positive control (RED)	2 x 10 ⁵ per µl
Tube 2	2 x 10 ⁴ per µl
Tube 3	2 x 10 ³ per µl
Tube 4	2 x 10 ² per µl
Tube 5	20 per µl
Tube 6	2 per µl

8. **Pipette 5µl of standard template into each well for the standard curve according to your experimental plate set up.**
The final volume in each well is 20µl.

Amplification Protocol

Amplification conditions using Lyophilised 2x qPCR Mastermix.

	Step	Time	Temp
	UNG treatment (if required) **	15 mins	37 °C
	Enzyme activation	2 mins	95 °C
Cycling x50	Denaturation	10 secs	95 °C
	DATA COLLECTION *	60 secs	60 °C

* Fluorogenic data should be collected during this step through the FAM and VIC channels

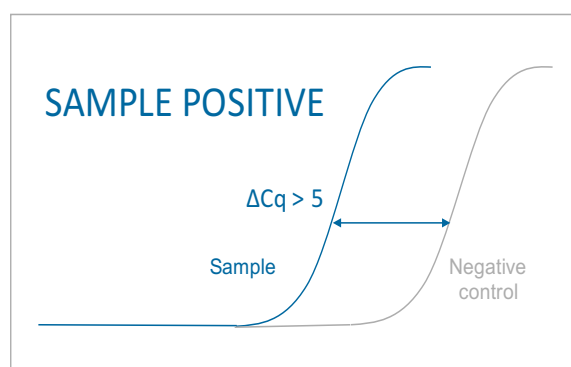
** Required if your Mastermix includes UNG to prevent PCR carryover contamination

Interpretation of Results

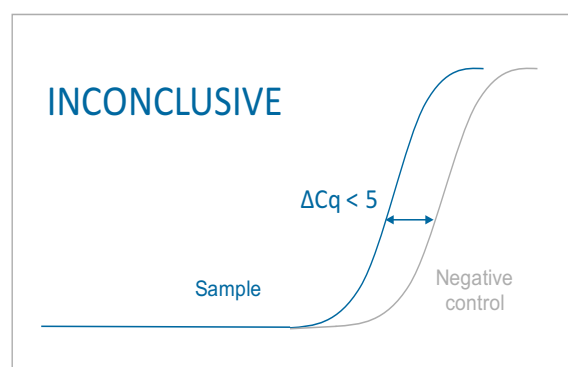
Target (FAM)	Internal control (VIC)	Positive control	Negative control	Interpretation
≤ 30	+ / -	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	+	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
> 30	-	+	-	POSITIVE QUALITATIVE RESULT do not report copy number as this may be due to poor sample extraction
-	+	+	-	NEGATIVE RESULT
+ / -	+ / -	+	≤ 35	EXPERIMENT FAILED due to test contamination
+ / -	+ / -	+	> 35	*
-	-	+	-	SAMPLE PREPARATION FAILED
+ / -	+ / -	-	+ / -	EXPERIMENT FAILED

Positive control template (RED) is expected to amplify between Cq 16 and 23. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.

*Where the test sample is positive and the negative control is positive with a Cq > 35, the sample must be reinterpreted based on the relative signal strength of the two results:



If the sample amplifies > 5 Cq earlier than the negative control then the sample should be reinterpreted (via the table above) with the negative control verified as negative.



If the sample amplifies < 5 Cq earlier than the negative control then the positive sample result is invalidated and the result should be determined inconclusive due to test contamination. The test for this sample should be repeated.

Internal PCR control

The Cq value obtained with the internal control will vary significantly depending on the extraction efficiency, the quantity of DNA added to the PCR reaction and the individual machine settings. Cq values of 28 ± 3 are within the normal range. When amplifying a *C. diphtheriae* A and B sample with a high genome copy number, the internal extraction control may not produce an amplification plot. This does not invalidate the test and should be interpreted as a positive experimental result.

Endogenous control

The signal obtained from the endogenous control primer and probe set will vary according to the amount of biological material present in a given sample. An early signal indicates the presence of a good yield of biological material. A late signal suggests that little biological material is present in the sample.