

Primerdesign™ Ltd

Corynebacterium diphtheriae A and B

C.diphtheriae Toxin sub-units A & B

genesig® Standard Kit

150 tests



GENESIG

Kits by Primerdesign

For general laboratory and research use only

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 1-8µm 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. diphtheria 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. diphtheria* 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 Primerdesign genesig 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 enquiry@primerdesign.co.uk 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)**
- **RNase/DNase free water (WHITE)**
for resuspension of primer/probe mixes
- **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 recommended for use with genesig Easy DNA/RNA extraction kit. However, it is designed to work well with all processes that yield high quality DNA with minimal PCR inhibitors.

oasig™ lyophilised or Precision®PLUS 2X qPCR Master Mix

This kit is intended for use with oasig or PrecisionPLUS2X qPCR Master Mix.

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 at a time 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.

Primerdesign 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 genesig 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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This product is developed, designed and sold for research purposes only. It is not intended for human diagnostic or drug purposes or to be administered to humans unless clearly expressed for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. During the warranty period Primerdesign genesig detection kits allow precise and reproducible data recovery combined with excellent sensitivity. For data obtained by violation to the general GLP guidelines and the manufacturer's recommendations the right to claim under guarantee is expired. PCR is a proprietary technology covered by several US and foreign patents. These patents are owned by Roche Molecular Systems Inc. and have been sub-licensed by PE Corporation in certain fields. Depending on your specific application you may need a license from Roche or PE to practice PCR. Additional information on purchasing licenses to practice the PCR process may be obtained by contacting the Director of Licensing at Roche Molecular Systems, 1145 Atlantic Avenue, Alameda, CA 94501 or Applied Biosystems business group of the Applied Biosystems Corporation, 850 Lincoln Centre Drive, Foster City, CA 94404. In addition, the 5' nuclease assay and other homogeneous amplification methods used in connection with the PCR process may be covered by U.S. Patents 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc, and by U.S. Patent 5,538,848, owned by The Perkin-Elmer Corporation.

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The PCR process is covered by US Patents 4,683,195, and 4,683,202 and foreign equivalents owned by Hoffmann-La Roche AG. BI, ABI PRISM®, GeneAmp® and MicroAmp® are registered trademarks of the Applied Biosystems (Applied Biosystems Corporation). BIOMEK® is a registered trademark of Beckman Instruments, Inc.; iCycler™ is a registered trademark of Bio-Rad Laboratories, Rotor-Gene is a trademark of Corbett Research. LightCycler™ is a registered trademark of the Idaho Technology Inc. GeneAmp®, TaqMan® and AmpliTaqGold® are registered trademarks of Roche Molecular Systems, Inc., The purchase of the Primerdesign reagents cannot be construed as an authorization or implicit license to practice PCR under any patents held by Hoffmann-La Roche Inc.

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 mixes are provided and these 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. Fluorogenic probes are included in the reaction mixtures 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 qPCR 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 and 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.

Resuspension 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. Resuspend 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

3. Resuspend 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.

qPCR 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
oasig or PrecisionPLUS 2X qPCR Master Mix	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

2. **Pipette 15 μ l of this mix into each well according to your qPCR experimental plate set up.**
3. **Prepare DNA templates for each of your samples.**
4. **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.
5. **If a standard curve is included for quantitative analysis, prepare a reaction mix according to the table below:**

Component	Volume
oasig or PrecisionPLUS 2X qPCR Master Mix	10 μ l
C.diphtheriae A and C.diphtheriae B primer/probe mix (BROWN)	1 μ l
RNase/DNase free water (WHITE)	4 μ l
Final Volume	15 μl

6. Preparation of a 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)	2×10^5 per µl
Tube 2	2×10^4 per µl
Tube 3	2×10^3 per µl
Tube 4	2×10^2 per µl
Tube 5	20 per µl
Tube 6	2 per µl

7. 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 oasis or PrecisionPLUS 2X qPCR Master Mix.

	Step	Time	Temp
	Enzyme activation	2 min	95 °C
Cycling x50	Denaturation	10 s	95 °C
	DATA COLLECTION *	60 s	60 °C

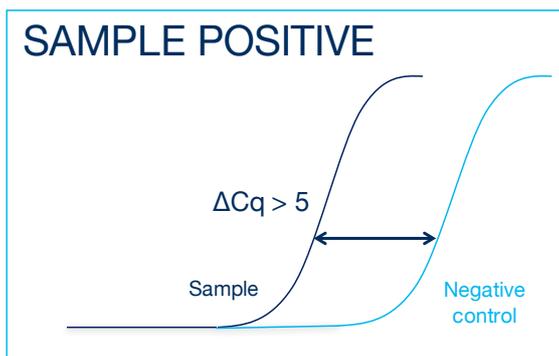
* Fluorogenic data should be collected during this step through the FAM channel

Interpretation of results

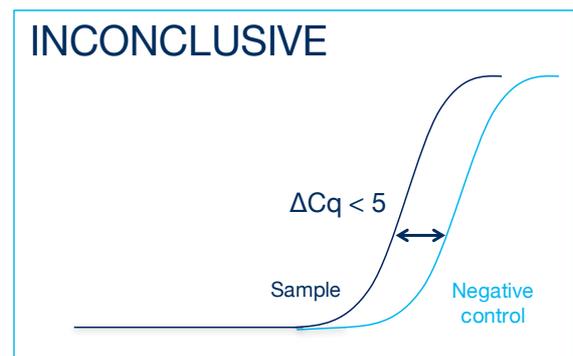
Target	Positive control	Negative control	Interpretation
+	+	-	POSITIVE QUANTITATIVE RESULT calculate copy number
-	+	-	NEGATIVE RESULT
+ / -	+	≤ 35	EXPERIMENT FAILED due to test contamination
+ / -	+	> 35	*
+ / -	-	+ / -	EXPERIMENT FAILED

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.