Primerdesign™ Ltd

Mycobacterium marinum & Mycobacterium ulcerans

Polyphosphate kinase (ppk) gene, partial cds

genesig® Standard Kit

150 tests

GENESIG

Kits by Primerdesign

For general laboratory and research use only

Introduction to Mycobacterium marinum & Mycobacterium ulcerans

Mycobacterium ulcerans is a human pathogen responsible for Buruli ulcer, a necrotizing skin disease most commonly found in West Africa, but outbreaks have also been reported in the Americas, Australia, and Asia.

Mycobacterium marinum (formerly M. balnei) is a free-living bacterium, which causes opportunistic infections in humans. M. marinum sometimes causes a rare disease known as aquarium granuloma, which typically affects individuals who work with fish or keep home aquariums.

M. Ulcerans' genome consists of approx. 5.8Mbp which codes for around 4240 proteins.
The accepted standard for molecular identification of mycobacteria is sequencing analysis of 2 hypervariable regions identified in 16S rRNA gene. M. marinum and M. ulcerans share identical 5´-16S rDNA and 16S-23S rRNA gene spacer sequences Real-Time Polymerase chain reaction (qPCR) methods are based on the 16S rRNA gene, the hsp65 gene or the insertion sequence IS2404

After inoculation into the skin, M. ulcerans proliferates extracellularly and elaborates a toxin, mycolactone, that enters the cells and causes necrosis of the dermis, panniculus, and deep fascia. Early lesions are closed, but as the necrosis spreads, the overlying dermis and epidermis eventually ulcerates. Clumps of extracellular acid-fast bacilli are plentiful and are frequently limited to the base of the ulcer and adjacent necrotic subcutaneous tissue. With healing, there is a granulomatous response, and the ulcerated area is eventually replaced by a depressed scar.
Lesions are usually single and begin as firm, painless, non-tender, movable, subcutaneous nodules 1 to 2 cm in diameter or as small papules. In 1 or 2 months, the nodule may become fluctuant and ulcerates, with an undermined edge that often extends 15 cm or more. The skin adjacent to the lesion, and often that of the entire corresponding limb, may be swollen by edema.

The inhibition of growth of M. marinum at 37°C is related to its ability to infect the cooler parts of the body especially the extremities. Lesions appear after an incubation period of about 2–4 weeks, and after 3–5 weeks they are typically 1-2.5 cm in diameter.

Diagnosis is frequently delayed, probably due to the rarity of the infection and a failure to elicit the usual history of aquatic exposure. Common misdiagnoses include fungal and parasitic infection, cellulitis, skin tuberculosis, rheumatoid arthritis, foreign body reaction, and skin tumor. A high index of suspicion and a detailed history are important in establishing the diagnosis of Mycobacterium infection. Long delays in diagnosis can result in severe, destructive infection. Sometimes, cultures are negative but the diagnosis is still made based on physical signs supported by typical histological findings.

The management of Mycobacterium infections depends on the severity of the infection. A prolonged course of antibiotic therapy is curative in most superficial cases but adjunctive surgical intervention is sometimes indicated in extensive and deep infections.

Specificity

The Primerdesign genesig Kit for Mycobacterium marinum & Mycobacterium ulcerans (M. marinum/M.ulcerans) genomes is designed for the in vitro quantification of M.marinum/M. ulcerans genomes. The kit is designed to have a broad detection profile. Specifically, the primers represent 100% homology with over 95% of the NCBI database reference sequences available at the time of design.

The dynamics of genetic variation means that new sequence information may become available after the initial design. Primerdesign periodically reviews the detection profiles of our kits and when required releases new versions.

The kit is predicted to detect Mycobacterium pseudoshottsii due to sequence information added to the NCBI database since the initial design of the assay.

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

- **M.marinum/M.ulcerans specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **M.marinum/M.ulcerans 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 template and standard curve preparation

Reagents and equipment to be supplied by the user

Real-time PCR Instrument

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 RNA and DNA with minimal PCR inhibitors.

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

This kit is intended for use with oasig or PrecisionPLUS 2X 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 -20ºC 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. 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 M.marinum/M.ulcerans detection kits have very high priming efficiencies of >95% and can detect less than 100 copies of target template.

Notices and disclaimers

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 Applera 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.

Trademarks

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Principles of the test

Real-time PCR

A M.marinum/M.ulcerans specific primer and probe mix is provided and this can be detected through the FAM channel.

The primer and probe mix provided exploits the so-called TaqMan® principle. During PCR amplification, forward and reverse primers hybridize to the M.marinum/M.ulcerans 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 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 M.marinum/M. ulcerans 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 M.marinum/M.ulcerans 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	
M.marinum/M.ulcerans primer/probe mix (BROWN)	165 µl

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

Component - resuspend in template preparation buffer	Volume
Post-PCR heat-sealed foil	
M.marinum/M.ulcerans 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 µl
M.marinum/M.ulcerans primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
Final Volume	**15 µl**

2. **Pipette 15µl 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 5µl of DNA template into each well, according to your experimental plate set up.**
   For negative control wells use 5µl of RNase/DNase free water. The final volume in each well is 20µl.

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 µl
M.marinum/M.ulcerans primer/probe mix (BROWN)	1 µl
RNase/DNase free water (WHITE)	4 µl
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 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

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.

qPCR amplification protocol

Amplification conditions using oasig 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

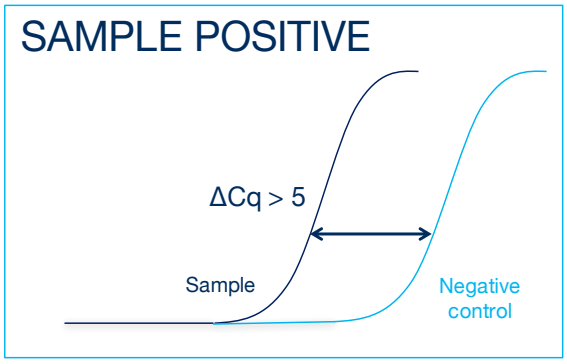
* Fluorogenic data should be collected during this step through the FAM channel

Interpretation of results

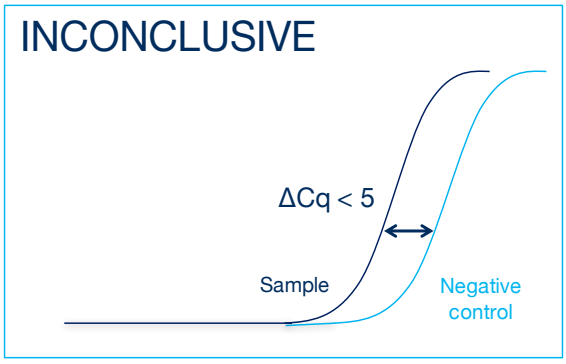
Target	Positive control	Negative control	Interpretation
+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
-	+	-	**NEGATIVE RESULT**
+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+	> 35	*
+ / -	-	+ / -	**EXPERIMENT FAILED**

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.