Bacteria Hemorrhagic Septicemia

aerolysin (aerA)

Standard Kit

150 tests

For general laboratory and research use only

Introduction to Bacteria Hemorrhagic Septicemia

Aeromonas hydrophila is a heterotrophic, gram-negative bacterium of the Aeromonadaceae family. This bacterium has a rod shape with rounded ends reaching 1-3µm in length. Polar flagella allow motility. 3 enterotoxins encoded by the 5Mbp genome are thought to be responsible for making this bacterium pathogenic. These entereotoxins are namely: cytotoxic (act); heat-labile cytotonic (alt) and heat-stable cytotonic (ast) of which the former is thought to contribute most to toxicity. A. hydrophila can be considered as an opportunistic pathogen which is linked to infections in fish and amphibians as well as humans, but may not be the cause.

Infection most usually occurs when an organism is dealing with environmental change. This could be a change in temperature or when infected with a different bacterium. In fish, the bacteria mainly infect the kidneys and liver resulting in disease that is thought to be the result of stress. In amphibians, the infection can cause internal heamorrhaging. Infection in humans can be caused by ingestion of infected food such as fish or meat. Once ingested the bacterium progresses to an organ via the bloodstream. Alternatively the bacterium can infect open wounds. The bacteria have pili which they utilise in attachment to host organism and invasion of the cells or organs. Colonisation occurs by asexual reproduction. Upon cell invasion, the enterotoxins are activated causing cell damage.
Transmission in fish occurs when infected fish are moved into new fisheries without sanitation. The bacteria are largely antibiotic resistant, can survive refrigeration and are also able to grow at temperatures in a wide range up to 37°C. However, a sodium/calcium hypochlorite solution can kill this bacterium.

Infection in fish may cause diseases including tail and fin rot, heamorrahagic septicemia and ulcer disease. In amphibians this bacterium is linked to a disease called red leg which is a result of internal heamorrhaging. Infection in humans can lead to a number of diseases including septicemia, meningitis, pneumonia and gastroenteritis. The bacteria can also infect open wounds causing cellulitis, myonecrosis and ecthyma gangrenosum. The latter two diseases occur more commonly in the immunocompromised, although infections do also occur in immunocompetent people.

Specificity

The Kit for Bacteria Hemorrhagic Septicemia (A.hydrophila) genomes is designed for the in vitro quantification of A.hydrophila genomes. The kit is designed to have the broadest detection profile possible whilst remaining specific to the A. hydrophila genome.

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

The primers have 100% homology with >95% of sequenced Aeromonas hydrophila isolates in the NCBI database. Due to the high sequence homology, the primers also have a high level of homology to other closely related Aeromonas sp. The primers therefore may also detect A. salmonicida, A. caviae and A. sobria.

Kit Contents

- **A.hydrophila specific primer/probe mix (150 reactions BROWN)**
  FAM labelled
- **A.hydrophila 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

DNA extraction kit

This kit is recommended for use with 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 PrecisionPLUS™ 2 x qPCR Mastermix

This kit is designed to work well with all commercially available Mastermixes. However, we recommend the use of oasig™ or PrecisionPLUS™ 2x qPCR Mastermix.

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 re-suspended 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.

We do 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 A.hydrophila 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 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 A.hydrophila 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 A.hydrophila 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 A.hydrophila 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 A.hydrophila 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.

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. We 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	
A.hydrophila primer/probe mix (BROWN)	165 µl

3. **Reconstitute 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	
A.hydrophila 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.

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
oasig™ or PrecisionPLUS™ 2x qPCR Mastermix	10 µl
A.hydrophila 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 real-time PCR 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 Mastermix	10 µl
A.hydrophila 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.

Amplification Protocol

Amplification conditions using oasig™ or PrecisionPLUS™ 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

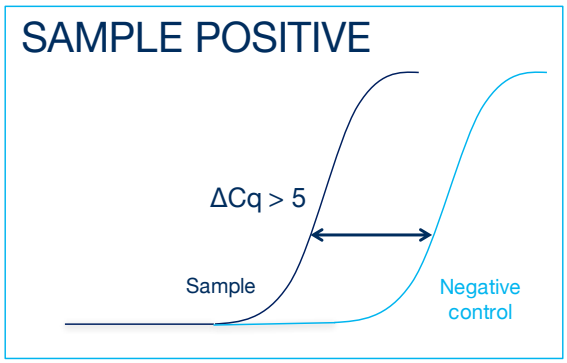
* Fluorogenic data should be collected during this step through the FAM channel
** Required if your Mastermix includes UNG to prevent PCR carryover contamination

Interpretation of Results

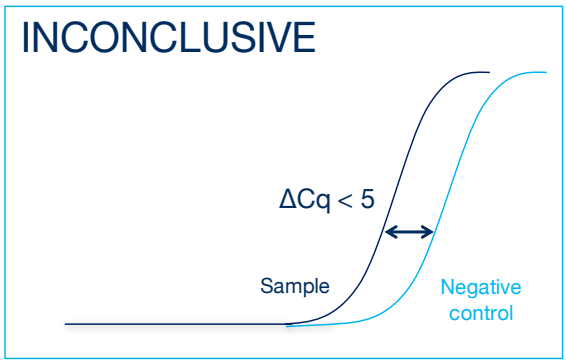
Target	Positive control	Negative control	Interpretation
+	+	-	**POSITIVE QUANTITATIVE RESULT** calculate copy number
-	+	-	**NEGATIVE RESULT**
+ / -	+	≤ 35	**EXPERIMENT FAILED** due to test contamination
+ / -	+	> 35	*
+ / -	-	+ / -	**EXPERIMENT FAILED**

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.