

Standard Operating Procedure (SOP)

Title: Quantitative Polymerase Chain Reaction (qPCR) for the detection of *Renibacterium salmoninarum*

Number: BACT-7

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| Bacteriology Supervisor: | December 14, 2011 |

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| Reason for Revision: | Update to include internal positive control |
| Area of Application: | Bacteriology |

Purpose:

To detect nucleic acids from *Renibacterium salmoninarum* in DNA extracted from fish tissues or fluids using quantitative PCR

Sections:

- I. Background
- II. Reference
- III. Materials
- IV. Procedure

Disclaimer:

The use of trade, firm, or corporation names in this protocol is for the information and convenience of the reader. Such use does not constitute an official endorsement or approval by the U.S. Department of Interior or the U.S. Geological Survey of any product or service to the exclusion of others that may be suitable.

I. Background

This protocol describes a quantitative PCR assay that is based upon the TaqMan® PCR detection system from Applied Biosystems Inc. The quantitative PCR assay uses sequence specific primers and a fluorescent probe to detect a unique DNA sequence, from the gene encoding the p57 protein (MSA) of *R. salmoninarum*. This protocol uses the *R. salmoninarum* assay published by Chase et al. 2006 but with the probe modified to contain a MGB linker. To obtain absolute quantification of *msa* gene copy number in samples, purified DNA from serial dilutions of *R. salmoninarum* culture is used as a standard. Version 2 of this protocol now includes an internal positive control to exclude false positives.

All PCR-based diagnostic tests are prone to false positives due contamination. Laboratories should have dedicated areas for each stage of the procedure, including:

- **PCR set-up area**: no nucleic acids in this area (PCR reagents only)
- **DNA template area**: area to handle extracted DNA sample

Each area should be equipped with dedicated instruments and supplies (e.g. dedicated pipettors, microcentrifuges, lab jackets, etc.).

II. Reference

Chase, D. M., D. G. Elliott, and R. J. Pascho. 2006. Detection and quantification of *Renibacterium salmoninarum* DNA in salmonid tissues by real-time quantitative polymerase chain reaction analysis. *Journal of Veterinary Diagnostic Investigations* 18:375-380.

III. Materials

Renibacterium salmoninarum absolute standards: Construction of standard curve material for absolute quantification is discussed in Section IV.

Renibacterium salmoninarum primer and probe set: The forward and reverse qPCR primers can be ordered from a variety of vendors and rehydrated with molecular grade water to a stock concentration of 100 μ M. The primers are used at a working concentration of 45 μ M. The MGB probe is proprietary technology of Applied Biosystems Inc[§]. The probes are rehydrated prior to shipment at 100 μ M concentration and used at a working concentration of 6 μ M.

| Primer/Probe | Sequence 5' to 3' |
|---------------------|-------------------------------|
| RS 1238 F | GTGACCAACACCCAGATATCCA |
| RS 1307 R | TCGCCAGACCACCATTACC |
| RS 1262 MGB | 6FAM-CACCAGATGGAGCAAC-NFQ/MGB |

Taqman® Gene Expression Master mix: The Gene Expression Master Mix can be purchased from Applied Biosystems Inc. (part # 4369514). This master mix formulation results in greatly improved sensitivity relative to other master mix formulations tested.

TaqMan® Exogenous Internal Positive Control Reagents (EXO IPC): The exogenous internal positive control (Exo IPC) is purchased from Applied Biosystems Inc. (part # 4308323). The predesigned control consists of the Exo IPC Mix (primers /probe) and the Exo IPC DNA (target DNA); both components are added to the qPCR reactions.

Molecular grade water: DNase- and RNase-free water can be purchased from a variety of vendors. We recommended using a dedicated stock of water for PCR set-up.

Optical plate: 96-well and 384-well optical plates are available from a variety of vendors. Specifications vary depending real-time PCR instrumentation.

Adhesive film for plates: Adhesive film for real-time PCR plates is available from a variety of vendors. Specifications vary depending real-time PCR instrumentation.

[§]**Vendors:** Applied Biosystems Inc. 1 800-327-3002 ; www.appliedbiosystems.com

IV. Procedure

A. Absolute standard for standard curve

Absolute standards can be produced by growing pure cultures of *Renibacterium salmoninarum* in KDM-2 medium at 15°C for 2-4 weeks (medium recipe available in SOP BACT-1). Bacterial cell number is enumerated by MF-FAT (SOP BACT-4) and extracted by standard procedures (SOP BACT-5). DNA is subjected to serial 10-fold dilutions to produce a standard curve. The absolute sensitivity of the qPCR is typically around 5 bacterial cells / PCR reaction (~40 cycle threshold (C_T)). The standard curve dilutions should cover the range between 10^6 to 10^1 cells / reaction.

B. Set up plate map and run file

Use a 96- or 384-well map to organize the samples. If desired, sample names can be entered into the real-time PCR instrument software before the run. Include at least two PCR replicate wells per sample but include three PCR replicate wells for the absolute standards (see Table 1). Run set-up of the real-time instrument varies depending on instrument platform; consult manufacturer for specific instructions. Program the instrument with the thermocycling conditions listed on page 5 (Part C. Step 5). Define the *R. salmoninarum* detector (reporter FAM/ non-fluorescent quencher) and the Exo IPC detector (reporter VIC/ quencher TAMRA).

Table 1. Number of replicate PCR wells recommended for each sample type

| Sample | Number of Replicate Wells | Notes |
|-----------------------------|---------------------------|---|
| Unknown DNA samples | 2 | - |
| Positive Processing Control | 2 | See SOP BACT 5 for more information |
| Negative Processing Control | 2 | See SOP BACT 5 for more information |
| No template control | 2 | Add 5 µl water to each well |
| Absolute standards | 3 | Three replicates of each dilution ranging from 10^6 to 10^1 |

C. qPCR procedure

1. In the PCR set-up area, add PCR reagents except the template DNA into the “Bulk Reaction” tube. See Table 2 for bulk reaction formulation. Make extra master mix to compensate for loss associated with the repeat pipet.
2. Aliquot 19 μL of bulk reaction to each well using a repeat pipet into the 96 or 384-well plate. Add 5 μL of water to no template controls (NTC).
3. Move to DNA template area and add 5 μL of extracted DNA to each well. Add DNA standards and seal plate.
4. Centrifuge plate for 3 minutes at $\sim 1500 \times g$ in the bench-top centrifuge to eliminate air bubbles in wells.
5. Program real-time PCR instrument with an initial incubation of 50°C for 2 min. followed by a 95°C incubation for 10 minutes. Then 40 cycles of the following profile:
 - a. Denaturing at 95°C for 15 seconds.
 - b. Anneal/Extend at 60°C for 60 seconds.

Table 2. Bulk reaction formulation for qPCR reaction

| PCR Reagents | Final Conc. | Stock Conc. | Vol. per Reaction | Vol. for ___ Reactions |
|--------------------------|--------------------|------------------|------------------------------------|------------------------|
| GenEX Master Mix* | 1X | 2X | 12 | |
| Forward Primer | 0.9 μM | 45 μM | 0.48 μL | |
| Reverse Primer | 0.9 μM | 45 μM | 0.48 μL | |
| Taq Man Probe | 0.25 μM | 6 μM | 1.0 μL | |
| Exo IPC Mix [†] | 1X | 10X | 2.4 μL | |
| Exo IPC DNA [†] | 1X | 50X | 0.48 μL | |
| Water | - | - | 2.16 μL | |
| DNA [§] | 10-100 ng | - | 5 μL | - |
| Total | | | 24 μL | |

*Taqman® Gene Expression Master Mix

[†]Taqman ® Exogenous Internal Positive Control[§]Always include a well with only water (no template control)

C. Data interpretation

Analysis of the real-time PCR run varies depending on instrument platform. Consult instrument manufacturer for analysis information. Threshold levels should be applied uniformly across plates. Positive processing controls and standard curve wells should have positive amplification ($<40C_T$) while negative processing controls and no template controls should lack detectable amplification ($>40C_T$). Further discussion of negative and positive processing controls can be found in SOP BACT-5.

Compare the **Exo IPC** C_T values obtained in the NTC wells to the C_T values in the unknown samples. Higher Exo IPC C_T values in unknown samples relative to the NTC wells are indicative of PCR inhibition. A 3 C_T shift upwards indicates an approximate 10-fold reduction in assay sensitivity. For more information see the manufacturer's instructions:

http://www3.appliedbiosystems.com/cms/groups/mcb_support/documents/generaldocuments/cms_041040.pdf

The r^2 of the standard curve should be ≥ 0.95 and the slope should ideally range between -3.0 and -4.0. C_T can be converted into copy number using the slope (m) and y-intercept (b) of the standard curve using the following formula:

- Copy number = $\text{antilog}_{10} ((C_T - b) / m)$

Copy number is typically reported in units of:

- copy # / mL (e.g. ovarian fluid)
- copy # / gram of tissue (e.g. kidney tissue)
- copy # / μg of DNA (e.g. when starting weight or volume is unknown)