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The Bcc qPCR NAD assay for the specific rapid quantitative detection of all Bcc species V.1

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We use this protocol and it's working

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Abstract

The Bcc qPCR NAD assay presented is an internally controlled duplex assay (incorporating an IAC), targeting a region of the *smpB* gene for the specific rapid quantitative detection of all Bcc species. This Bcc qPCR NAD assay was validated with equivalence to ISO/TS 12869:2019 with high specificity (100% when tested on an extensive panel of target and non-target microorganisms with no cross-reactivity observed) and high analytical sensitivity (relatively low LOD and LOQ at 3 GE/reaction with ≥90% probability and 20 GE/reaction, respectively). The high performance of the calibration function of the Bcc qPCR NAD assay in terms of accuracy, qPCR efficiency and broad dynamic calibration range (20 GE-10^7GE/reaction) will allow for the absolute quantification of the starting Bcc DNA concentration in contaminated samples. The incorporation of the IAC into the Bcc qPCR NAD assay ensures the robustness and fidelity of the results generated.

The development and validation of this Bcc qPCR NAD assay has been published in:
Duong HT, Fullbrook S, Reddington K, Minogue E, Barry T. Design, Development and Validation of a Culture-Independent Nucleic Acid Diagnostics Method for the Rapid Detection and Quantification of the *Burkholderia cepacia* Complex in Water with an Equivalence to ISO/TS 12869:2019. PDA Journal of Pharmaceutical Science and Technology. 2023. doi:10.5731/pdajpst.2021.012728

Guidelines

- The laboratory workspace and equipment were sterilized by UV-light, DNase solution, and 70% ethanol.
- Due to the sensitivity of the assays, extra care must be paid to avoid sample contamination during analysis, and as such Good Laboratory Practice must be followed. Wear laboratory coats and gloves at all times.
- Filter pipet tips were used in all steps of the laboratory work.
- The material to be used (tubes, tips, water, etc.) must be free of nucleases.

Materials

- Nuclease-free sterile water (Invitrogen)
- Microcentrifuge tubes (Sterile and Nuclease free)
- p1000, p200, p20, p10 pipettes
- p1000, p200, p20, p10 filter pipet tips
- Large centrifuge (for spinning down 96 well plate)
- Tabletop centrifuge (for spinning down samples and reagents)
- 2x LightCycler 480 probes master kit (Roche Diagnostics)
- LightCycler® 480 machine (Roche Diagnostics)
- Dimethyl sulfoxide (for molecular biology, Nuclease-free) (Sigma-Alrich)
- 96-well PCR white plate
- Adhesive sealing film optical for qPCR
- UV sterilisation hoods
- qPCR oligonucleotide primers and Taqman hydrolysis probes

	A	B	C	D
	Name	**Gene target**	**Function**	**DNA sequence (5′-3′)**
	Bcc F	*smpB*	Bcc forward primer	CRATCCHTTCATGAGCATCA
	Bcc R	*smpB*	Bcc reverse primer	TTGACYTCCCAGCCYTC
	Bcc P1	*smpB*	Bcc specificity hydrolysis probe 1	5HEX/ACAACAGGA/ZEN/ARGCGCACTTCG/3IABkFQ
	Bcc P2	*smpB*	Bcc specificity hydrolysis probe 2	5HEX/CAACAGGAA/ZEN/AGCGCGCTTCG/3IABkFQ
	SIAC F	Synthetic construct	IAC forward primer	ATGCCAGTCAGCATAAGGA
	SIAC R	Synthetic construct	IAC reverse primer	CAGACCTCTGGTAGGATGTAC
	SIAC P	Synthetic construct	IAC specific hydrolysis probe	5Cy5/TCGGCACTA/TAO/CCGACACGAAC/3IAbRQSp

/5HEX/ = 5' HEX (6-hexachlorofluorescein) fluorophore; /ZEN/ = ZEN™ internal quencher; /3IABkFQ/ = 3' Iowa Black® FQ terminal quencher; /5Cy5/ = 5' Cy5 (cyanine5) fluorophore; /TAO/ = TAO™internal quencher; /3IAbRQSp/ = 3' Iowa Black® RQ-Sp terminal quencher

- Non-competitive internal amplification control which is a synthetic construct sequence that was previously developed (Barry, T.; Reddington, K.; Minogue, E. A Method for the Detection of Legionella. WO 2018/065497 AI, April 12, 2018.)

Note

IAC is incorporated into the primary Bcc qPCR NAD assay. This is carried out to eliminate false negative interpretation of NAD diagnostic data, which may result from the presence of inhibitory substances in the sample matrix, thermocycler malfunction or problems with the Bcc qPCR NAD assay reagents.

- Positive control DNA template (Purified gDNA from *B. cepacia* DSM7288 at 10^4 GE/reaction)

Troubleshooting

Safety warnings

- Handling high concentration of positive controls is performed in a post-PCR room, which is physically separated from the pre-PCR room to avoid contamination.
- Always add the samples first and seal them before adding positive control at the end.

Prepare a qPCR master mix (in a DNA-free preparation hood)

1 Thaw qPCR reagents and samples on the bench, flick to mix and briefly spin down on a tabletop centrifuge.

2 Prepare a master mix for the number of qPCR reactions, plus negative controls, positive controls (written per reaction):

- 10 µL 2x LightCycler 480 probes master kit
- 3% v/v Dimethyl sulfoxide (DMSO)
- 1 µM Bcc forward primer and 1 µM Bcc reverse primer
- 0.2 µM HEX-labelled Bcc specific hydrolysis probe 1 and 0.2 µM HEX-labelled Bcc specific hydrolysis probe 2
- 0.3 µM IAC forward primer and 0.3 µM IAC reverse primer
- 0.2 µM Cy5-labelled IAC specific hydrolysis probe
- IAC synthetic construct 10^3 GE/reaction
- The volume is adjusted to 15 µl with the addition of nuclease-free distilled H_2O (dH_2O).

Note

- A qPCR master mix is a solution that has all of the components for a qPCR reaction except the template or control DNA.
- Prepare the master mix in the biosafety hood.
- All the reagents and master mix should be kept on ice during the preparation.
- Make sure to account for pipetting errors and make slightly more master mix than needed (add extra 1-2 reactions).

3 Gently mix by inversion and briefly centrifuge to collect the liquid at the bottom of the tube.

Set up a qPCR plate (in a PCR template addition hood)

4 Aliquot 15 µL of master mix to each well of a 96-well PCR white plate.

Note

Mix master mix frequently while pipetting, and ensure each well's contents are mixed properly.

5 Add 5 µL of each DNA sample, or 5 µL of gDNA of *B. cepacia* DSM7288 10^4 GE/reaction (for positive control), or 5 µL of nuclease-free grade water (for negative controls) into appropriate wells of the 96-well PCR white plate.

Note

- Samples should be run in at least duplicate, but preferably triplicate.
- Keep a detailed plate map of wells containing DNA samples and positive and negative controls.

6 Seal the plate thoroughly with optical plate sealing film, and centrifuge briefly at low speed to ensure the contents of the wells are at the bottom. Try to remove any bubbles by gently tapping the plate against the bench and spinning again.

The Bcc qPCR NAD assay

7 Turn on the LightCycler® 480 machine and the attached computer. Login to the computer and the designated software (LightCycler 480 software) using the login details on the computer.

8 Transfer the plate into the thermal cycler after setting it up appropriately and carry out qPCR reaction at the following conditions:

1. 95 °C for 00:10:00
2. 95 °C for 00:00:10
3. 62 °C for 00:00:30
4. Repeat steps 2-3 50cycles (Fluorescent products were detected once every cycle, at the end of the extension phase)
5. 40 °C for 00:00:10

9 **Data analysis:**
Once qCPR experiment is complete, analyse the result using the LightCycler 480 software to obtain threshold cycle (Ct) signals from each sample.

Note

Check that the correct filter combination has been chosen to correspond with the probe used in the experiment, e.g. HEX (for Bcc assay), or CY5 (for IAC assay).

To determine the quantity of Bcc GE detected in each qPCR reaction, each positive result is quantified from the obtained Ct values and compared to the established Bcc qPCR NAD calibration curve:

y=-3.224x + 39.46
where x is the $\log_{10}$ of the starting quantity and y is the Ct value.

Note

- Positive control, 10^4 GE/reaction, is expected to amplify between Ct 26 and 27. Failure to satisfy this quality control criterion is a strong indication that the experiment has been compromised.
- IAC signal should be detected in all samples except for the non-template control sample. IAC Ct values of 31-33 are within the normal range.

Protocol references

1. Duong HT, Fullbrook S, Reddington K, Minogue E, Barry T. Design, Development and Validation of a Culture-Independent Nucleic Acid Diagnostics Method for the Rapid Detection and Quantification of the *Burkholderia cepacia* Complex in Water with an Equivalence to ISO/TS 12869:2019. PDA Journal of Pharmaceutical Science and Technology. 2023. doi:10.5731/pdajpst.2021.012728

2. Barry, T.; Reddington, K.; Minogue, E. A Method for the Detection of Legionella. WO 2018/065497 AI, April 12, 2018.

Citations

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