Proof of Concept for Recombinant Cellular Controls in Quantitative Molecular Pathogen Detection^{∇†}

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In this study, we present the concept of internal sample process controls (ISPCs) to monitor the efficiency of an analytical chain using sample preparation and quantitative PCR (qPCR). A recombinant *Listeria monocytogenes* $\Delta prfA$ (targeted deletion) strain containing a competitive artificial single-copy genomic target was applied to naturally contaminated samples to demonstrate its analytical suitability as an ISPC.

Since its first implementation, PCR has been refined to a method that provides many opportunities for improved analysis of microorganisms (9). However, for its routine use in diagnosis, standardized validation protocols and guidelines for publication of newly developed assays, as well as supporting analytical elements (such as sample preparation), are necessary prerequisites (3, 4, 11, 19). The latter requirement, in particular, leads to a modular characteristic of the analytical chain necessary to reduce the sample size and exclude inhibitory effects (22–25). These preliminary methodical steps play a key role for processing the food sample matrix. Therefore, quality assurance for the modular analytical chain requires the extension of control concepts to total-analytical-chain approaches (3). This can be expected from using internal sample process controls (ISPCs) rather than internal amplification controls (IACs). The latter are non-target DNA sequences present in the same sample tube and are coamplified simultaneously with the target sequence (11, 12, 18). However, this is true only for the enzymatic amplification reaction. In contrast, ISPCs are passaged through all analytical steps, thereby making all effects of the methodical steps on the final quantitative PCR (qPCR) result accessible and interpretable (Fig. 1A).

In practice, the most suitable ISPC is a recombinant bacterial organism with a single-copy artificial DNA target for coamplification. It should be closely related to the actual pathogen, to provide the highest degree of similarity to the target organism under analysis. It can be added at several stages of the protocol to provide information about the overall performance or the individual efficiency of the several methodical steps (Fig. 1). An ISPC also provides the

potential for quantitative monitoring of the efficiency of the individual steps of the protocol (Fig. 1).

A few related types of sample controls have been presented in the past. Internal controls have been reported recently for detection of food-borne and waterborne RNA viruses (7, 8, 14). Murphy et al. (17) presented a recombinant *Escherichia coli* strain, including fragments of the green fluorescence gene and the *iroB* gene of *Salmonella enterica*. The control was not used for quantitative purposes, and the underlying Gram-negative *E. coli* strain exhibits characteristics during sample preparation different from those of *Listeria monocytogenes*. The ISPC presented in this study is *L. monocytogenes* strain EGDe, containing a targeted-deletion PCR sequence with an artificial DNA sequence (IAC) comprising the same primer sequences as the wild-type strain and containing an artificial non-*Listeria* probe binding sequence within its genome (*L. monocytogenes* ISPC).

Detailed information about the materials and methods used in this study is given in the supplemental material. The recombinant IAC⁺ *L. monocytogenes* EGDe $\Delta prfA$ strain (serotype 1/2a) used as the *L. monocytogenes* ISPC has been deposited in the DSMZ (no. DSM23639). The genome of this strain has a deleted *prfA* locus and contains a single-copy insertion of the pPL2 phage insertion vector, according to Lauer et al., including an IAC with a previously published format (2, 6, 13, 20). qPCR detection of the wild-type *L. monocytogenes* strain EGDe was performed by targeting a 274-bp fragment of the *prfA* gene according to Rossmanith et al. with a 6-carboxyfluorescein (FAM)-labeled Lip probe and detection of the 100-bp artificial IAC inserted in the *L. monocytogenes* ISPC by using the HEX-labeled probe pLucLm5 (5, 20). The qPCR result was expressed as bacterial cell equivalents (BCE).

Single-copy insertion of the IAC into the genome of the *L. monocytogenes* EGDe $\Delta prfA$ strain was demonstrated by comparison of the *L. monocytogenes* ISPC genomic DNA and *L. monocytogenes* wild-type data. The resulting threshold cycle (C_T) values match those presented in Table S2 in

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FIG. 1. The concept of internal sample process controls (ISPCs). (A) Operating range of internal amplification controls (IACs) and ISPCs within the analytical chain. The calculation of target loss for the whole analytical chain (B) and the methodical step of DNA isolation (C) are schematically illustrated, based on the use of an ISPC.

the supplemental material, with an average deviation of $\leq 1\%$, thus indicating single-copy insertion of the pPL2-IAC vector.

The ISPC was applied to naturally contaminated samples of Quargel cheese, which originated from a recent *L. monocytogenes* outbreak in Austria (10). Two individually packed samples from two different production lots were processed in duplicate, resulting in eight individual samples. Additionally, 1.4×10^3 CFU (relative standard deviation [RSD], 29.0%) of the *L. monocytogenes* ISPC were added per sample. The samples were processed in two ways. A direct quantification of the value of the *L. monocytogenes* wild type was obtained by a sample preparation method called “matrix lysis,” as previously published and with subsequent qPCR (15, 21). A solution of 1-ethyl-3-methylimidazolium-thiocyanate was used as a detergent/chaotrope. Additionally, the protocol of International Organization for Standardization standard ISO-11290-2 was performed for comparison (1). Furthermore, samples, taken from lot 2, were processed by a DNA isolation protocol omitting the proteinase K step. This artificially biases the digestion performance and correspondingly the efficiency of the whole protocol, thus mimicking a possible erroneous influence during the procedure.

The *L. monocytogenes* wild-type contamination values of the samples directly obtained after qPCR reached averages of 1.4×10^6 BCE (RSD, 5.9%) and 1.1×10^8 BCE (RSD, 8.9%) for the two respective Quargel cheese lots. These values have been corrected according to the rate of efficiency for each sample, as obtained by comparing the values of each replicate to the overall loss of *L. monocytogenes* ISPC cells from the initial level of 1.4×10^3 CFU (RSD, 29.0%) to 6.9×10^2 BCE (RSD, 5.9%) after qPCR (Fig. 2). This resulted in 2.32×10^6 BCE/g (RSD, 36.0%) and $5.0 \times$

10^8 BCE/g (RSD, 9.9%) for the two cheese lots compared with 2.07×10^6 CFU/g (RSD, 91.1%) and 4.9×10^8 CFU/g (RSD, 73.2%), as obtained by the ISO-11290-2 protocol (Fig. 2). The samples processed by artificially biased DNA isolation averaged in basic values 4.9×10^5 BCE (RSD, 6.3%) and 3.7×10^7 BCE (RSD, 2.9%) for the respective cheese lots. After correction, as described above, the respective *L. monocytogenes* wild-type contamination levels were 1.8×10^6 BCE (RSD, 47.0%) and 4.6×10^8 BCE (RSD, 10.1%) (Fig. 2).

In summary, analysis of the data provided by the control showed a total average loss for the sample preparation procedure of 43% and a calculated average loss of 51% during sample preparation and DNA isolation/purification (Fig. 2D). Based on these values, the resulting qPCR data reproducing the *L. monocytogenes* wild-type contamination of the two examined lots of Quargel cheese have been analyzed and corrected. The resulting data, after correction, correlated with the values provided by direct quantification, according to ISO-11290-2 ($P > 0.05$) (Fig. 2A and B).

In addition, the resulting data from the lot, which have been investigated by a DNA isolation protocol with biased performance, also correlated after correction with the values provided by direct quantification, according to ISO-11290-2 ($P > 0.05$) (Fig. 2C).

In conclusion, we present the first application of a recombinant ISPC for diagnostic use in pathogen detection. This ISPC enables the estimation of negative results and the efficiency of the whole detection procedure for each sample. Implementation of such an ISPC warrants the excellent quantitative properties of qPCR for pathogen detection. More effort must be made in future to develop similar controls for alternative PCR assays covering different target sequences in *L. monocytogenes*

FIG. 2. Results from examination of naturally contaminated Quargel cheese (A and B) illustrate examination of two lots of Quargel cheese, according to ISO-11290-2 and by matrix lysis sample preparation and subsequent DNA isolation and real-time PCR. Panel C illustrates the Quargel cheese samples with artificially biased recovery, by omission of the proteinase K step of the protocol during DNA isolation. The uncorrected average bacterial cell equivalent (BCE) value of these samples reflected reduced recovery. The corrected values of the two lots and the biased samples correlate with the values of *L. monocytogenes* contamination, as obtained by direct quantification according to ISO-11290-2 ($P > 0.05$). (D) Recovery rates of the several methodical steps included in the detection of *L. monocytogenes*. Shown is a comparison of the initial amount of process control cells (100%; diagonal gray bar) with the BCE value (92%; white bar), as obtained after DNA isolation and real-time PCR without sample preparation (matrix lysis), together with the BCE value (49%; black bar) after the whole protocol, including matrix lysis.

and for other relevant bacterial pathogens in clinical and food diagnosis (16).

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