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Research paper

Evaluation of microbial qPCR workflows using engineered *Saccharomyces cerevisiae*

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ABSTRACT

Aims: We describe the development and interlaboratory study of modified *Saccharomyces cerevisiae* as a candidate material to evaluate a full detection workflow including DNA extraction and quantitative polymerase chain reaction (qPCR).
Methods and results: S. cerevisiae NE095 was prepared by stable insertion of DNA sequence External RNA Control Consortium-00095 into *S. cerevisiae* BY4739 to convey selectivity. For the interlaboratory study, a binomial regression model was used to select three cell concentrations, high (4×10^7 cells ml^{-1}), intermediate (4×10^5 cells ml^{-1}) and low (4×10^3 cells ml^{-1}), and the number of samples per concentration. Seven participants, including potential end users, had combined rates of positive qPCR detection (quantification cycle <37) of 100%, 40%, and 0% for high, intermediate, and low concentrations, respectively.
Conclusions: The NE095 strain was successfully detected by all participants, with the high concentration indicating a potential target concentration for a reference material.
Significance and impact of the study: The engineered yeast has potential to support measurement assurance for the analytical process of qPCR, encompassing the method, equipment, and operator, to increase confidence in results and better inform decision-making in areas of applied microbiology. This material can also support process assessment for other DNA-based detection technologies.

1. Introduction

Data of high quality is essential in microbial detection, identification, and quantification because of the impact these organisms (pathogenic and beneficial) have on human life in areas including environmental monitoring, food safety, biothreat detection, and clinical outbreaks [1–3]. Despite the importance, analysis of microbial samples remains a practical and technological challenge when it comes to confidence in the measurements, especially for measurements made at the point of need or point of care where results are used to inform critical decision-making. In the biodefense field, for example, there are over 300 technologies marketed for use in biological detection claiming the ability to detect pathogens (biothreats) in suspicious materials [4–6]. However, there remains a scarcity of standards, reference materials, and third-party testing to demonstrate the reliability of these technologies in the hands of end users, despite considerable efforts by the stakeholder community and Federal government. Only a few commercially available biodetection technologies have been submitted to third-party validation, including the Razor^{TM} EX BioDetection System [7], a qPCR-based assay.

qPCR is a well-established technique that provides selectivity and sensitivity in detecting nucleic acid markers [8,9]. However, obtaining reliable data can be challenging because of factors that compromise nucleic acid amplification such as residue from the crude sample (matrix effect) [10]. In addition, measurement inaccuracy as a result of sample collection, processing, and nucleic acid extraction is often observed [10–13]. The absence of method validation brings into question the reliability of the generated data

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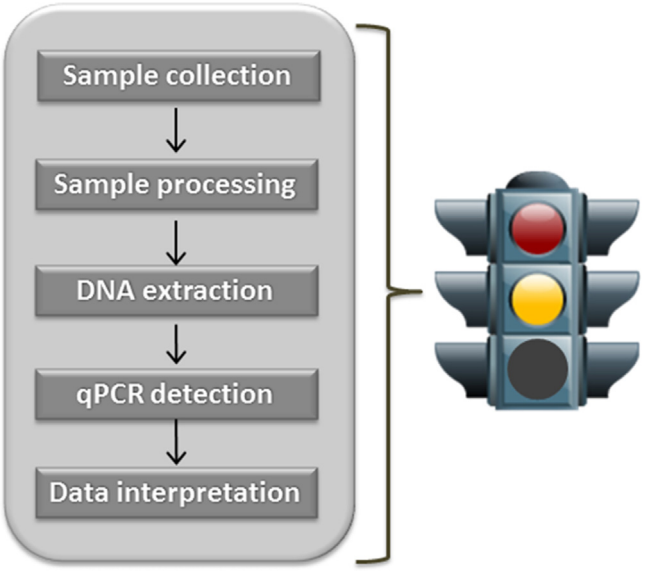

Fig. 1. Workflow steps for a downstream qPCR sample analysis. Analogous to a traffic light, the unsuccessful detection of a reference material provides a red light indicating the analytical process is unsuccessful and potentially helping identify the source of the problem. Successful detection of the material provides a yellow light indicating that the analytical process is working properly, that is the methods are appropriate, the equipment is functioning, and the user is proficient in the required skills. There is no green light since the material cannot be used to validate a specific detection assay for an organism of interest.

[14]. Further, even with the use of a successful detection assay, such as the Biothreat Panel multiplexed PCR-based assay for the detection of *Bacillus anthracis*, *Francisella tularensis* and *Yersinia pestis* or the rapid, high-throughput, culture-based PCR methods to analyze samples for viable spores of *B. anthracis* and its surrogates [15], the final result in the field still depends upon the entire analytical process. The process includes the methods and protocols, the measurement workflow which encompasses all steps applied to obtain the final result, the performance of the equipment at the point of need, operator capabilities and skills, and proficiency testing. Without all of these components in place, results cannot be used with confidence to support decision-making.

Control materials can serve to evaluate or validate analytical processes, establish reliable and comparable analytical results among laboratories or analysts through proficiency testing or competence assessment, and verify accuracy of measurement performance on a daily basis [16]. For example, human DNA Standard Reference Materials (SRMs) are used in the forensics community to reduce variability within and among laboratories [17,18]. Control materials are typically thoroughly characterized using measurement methods with well understood biases and variability. Measurement controls such as reference materials can help provide confidence in the application of qPCR to microbial measurements, with the format of the material enabling performance evaluation at various workflow steps (Fig. 1). For instance, a reference material can be mixed into a matrix of interest to demonstrate a successful DNA extraction step, which is highly susceptible to matrix effects. The use of a reference material to demonstrate a successful qPCR analytical process can increase confidence when the measurement capability is applied in a real-case scenario, such as in a clinical setting, environmental monitoring, or biothreat scenario.

One promising organism to meet these needs for a qPCR reference material is *Saccharomyces cerevisiae* (*S. cerevisiae*), a eukaryotic model system widely used in biology fields such as bioengineering [19]. Yeast can be genetically modified to enable specific detection and offers a low DNA extraction efficiency to challenge extraction protocols [11]. Its physiological resilience under low nutrient conditions and stability under various environmental conditions make yeast suitable for different formats, including a liquid or powder, for broader applicability. Moreover, *S. cerevisiae* has minimal health and security risks and can be handled without special precautions or training. It can therefore serve as a surrogate material for routine training and process evaluation in applications where the true agent of interest is a pathogen or threat agent and not easily used. Further, use of the yeast, and not the agent of interest, can essentially eliminate false positives during real microbial detection situations due to residual material on equipment.

The presence of a non-native target DNA sequence in the yeast genome can eliminate false positives from near-neighbor organisms. For specificity, the target sequence should be rare and not typically found in the environment of interest. NIST SRM 2374 contains a series of nucleic acid sequences selected by the External RNA Control Consortium (ERCC) as control sequences that are rarely (if at all) found in normal environmental conditions (temperature and pressure). One such sequence is ERCC-00095, derived from *Methanocaldococcus jannaschii*, a deep-sea vent archaeon found only in extremely harsh conditions (an extremophile).

The objective of this study was to develop a *S. cerevisiae* strain containing a non-yeast target DNA insert and evaluate the strain via interlaboratory study as a potential material for assessing the qPCR analytical process, in efforts toward a reference material for qPCR. Yeast cells were transformed by inserting the ERCC-00095 DNA sequence into the yeast genome. The modified yeast strain, termed NE095, was prepared at three different cell concentrations and evaluated using qPCR in an interlaboratory pilot study involving five public health laboratories, one mobile laboratory and one in-house laboratory. The NE095 was detectable at the expected concentrations in multiple laboratories and is suitable for a reference material.

2. Materials and methods

2.1. *Preparation and characterization of engineered* S. cerevisiae

2.1.1. *cerevisiae transformation*

The parent organism was a URA3 deficient yeast strain: *Saccharomyces cerevisiae* BY4739 (MATalpha leu2Δ0 lys2Δ0 ura3Δ0) (procured from Open Biosystems) derived from *S. cerevisiae* S288C. The URA3 gene encodes for orotidine-5′-phosphate decarboxylase from *S. cerevisiae* S288C. A target DNA sequence and full-length URA3 gene were inserted via homologous recombination into chromosome IV of the yeast (Fig. 2, Supplemental material: *S. cerevisiae* NE095 transformation, Figs. S1 and S2). The DNA insert was prepared by ligating the target sequence, ERCC-00095 (from NIST SRM 2374, Genbank accession KC702204, without the polyA tail found in the SRM), to the URA3 gene by overlapping PCR. The full-length URA3 gene and URA3 promoter sequence were PCR amplified from a pYES2 vector (Life Technologies, part # V825-20). See Supplemental material for detailed methods describing the transformation process.

2.1.2. *NE095 characterization*

The presence of the insert was demonstrated by PCR, and the sequence of the amplified insert, including the ERCC-00095 and URA3 gene, was confirmed by Sanger sequencing (analyzed by Eurofins MWG Operon, Alabama, USA) (Supplemental material: NE095 insert sequence confirmation, Figs. S3, S4, and S5). Once the insert was demonstrated, the yeast cells were cultured in SD/-Ura broth for subsequent experiments.