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Opinion Article

Mitigation of PCR Inhibition in Molecular Assays for Detection of Toxic Freshwater Cyanobacteria

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Opinion

Freshwater cyanobacteria are already a big ecological concern because they can form harmful algal blooms (HABs) to devastate the influenced aquatic ecosystems. Moreover, production of potent cyanotoxins extends their harm to terrestrial life as animals are poisoned when consuming the contaminated water or plants with toxin residuals. Therefore, a rapid and accurate detection method must be set up for toxic cyanobacteria in case of either acute or chronic animal poisoning incidents. As of today, molecular diagnostic assays have been developed for a wide range of microorganisms including cyanobacteria due to the accuracy, rapidity, and reproducibility. The real-time quantitative polymerase chain reaction (qPCR) technique is an outstanding representative of molecular assays, aiming at marker genes from the microbes of interest. There have been lots of published studies reporting detection of different toxic cyanobacteria by qPCR. The author of this paper has also developed four qPCR assays specific to four common toxic cyanobacteria: Microcystis, Anabaena, Cylindrospermopsis, and Nodularia producing microcystin, anatoxin-a, cylindrospermopsin, and nodularin, respectively. Although qPCR detection has many advantages, it also has several requirements for accurate outcomes of which the nucleic acid purity is of big importance. Because some substances can more or less inhibit PCR amplification, removing them from nucleic acid extracts and hampering their inhibitive impact on PCR becomes critical for qualitative and quantitative performance of the whole assay. For natural water samples, many concomitant substances can play the role of PCR inhibitor such as urea and humic acids. However, it is often hard to eliminate them from isolated nucleic acid simple by a handy lab kit; therefore, an effective extraction method should be found to maximize the elimination of these heterogeneous substances. Moreover, dysfunction of residual inhibitors during the ongoing PCR is a remedial measure if they cannot be completely excluded. As the author has successfully developed qPCR assays for toxic cyanobacteria, conducive knowledge and experience on mitigation of PCR inhibition are introduced in this paper. Herein, only DNA extraction is discussed rather than RNA, though.

First of all, a reliable DNA extraction protocol is suggested in which separation of organic liquids from water by chloroform is conducted. This helps more water-insoluble molecules be expelled to the organic phase and eventually dumped because DNA is dissolved in the watery phase. Preparation of the solution to be separated can be done using sodium dodecyl sulfate (SDS), Cetyltrimethyl Ammonium Bromide (CTAB), etc., and series of mixing and incubation steps. While DNA is usually precipitated from the watery phase by ethanol, the resultant extracts contain carryover of the concomitant impurities despite the massive recovery of total DNA. Therefore, other DNA purification methods can be used, for example, the pervasive lab kits using solid phase columns consisting of silicone. The Zymo Genomic DNA Clean & Concentrator-25 kit is used in the author's studies and recommended due to its efficacy in high-quality DNA purification. Although it is not 100% guaranteed that this combined protocol is the most effective, its DNA extracts have the least heterogeneous impurities, evidenced by spectrophotometric parameters A260/A280 and A260/A230 and capability of PCR amplification of target gene fragments, in comparison to the use of ethanol precipitation instead of the above purification kit or solely a Qiagen DNeasy Blood and Tissue kit following its protocol for gram-negative bacteria.Nevertheless, residuals of PCR inhibitors in DNA extracts can still be discerned by weak or no amplification of target gene segment, even though the best attempts are made. Then dysfunction of the remaining inhibitors is necessary for a successful amplification, particularly in qPCR because even slight inhibition can lead to terribly wrong quantification of templates and mislead diagnostics. Addition of PCR enhancers is a good alternative, but not all of them are able to mitigate the inherent inhibition satisfactorily. For example, the author tried six common PCR enhancers of which only bovine serum albumin (BSA) worked effectively in a qPCR trial with potent inhibition introduced by mixing garden soil into pure cyanobacterial cultures, albeit merely about 10% templates were recovered. However in reality, natural waters generally do not contain that many potential inhibitors, and the full recovery of template quantities by BSA were attained in another trial in which cyanobacterial cells were directly spiked into creek water, and no significant differences were found between spiked samples and non-spiked controls. Yet surprisingly, a few enhancers could even act as inhibitors in control samples without introducing exogenous inhibition, the worst of which template amplification was completely held back by formamide. Therefore in conclusion, BSA can be a good option for post-extraction mitigation of inhibition which directly works in the ongoing PCR.

Accurate and precise quantification is the vital part of qPCR technique, so no deviated estimation from the real and average values of templates is tolerated. While everything is well prepared for an excellent qPCR assay (e.g., primers and probes, master mix, etc.), endogenous inhibition in DNA extracts cannot be easily discerned and can ruin the delicate 'banquet' sneakily. Mitigation of PCR inhibition should thus be taken care of so as to reach a goal of faithful reflection of factual template quantity. In this paper, the author introduces the empirical knowledge on effective mitigation that has been demonstrated in well-designed trials, recommending a useful impurity-eliminating DNA extraction protocol and a well-performed PCR enhancer. Their combined utility will definitely result in free-of-inhibition-concern outcomes of qPCR assays for detecting freshwater cyanobacteria,and employment on more types of samples including other aquatic microalgae as well as marine phytoplankton can be considered.