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Fiona M.F. Lun[2,3]
Yoyo Y. Jin[2,3]
Hao Sun[2,3]
Tak Y. Leung[4]
Tze K. Lau[4]
Rossa W.K. Chiu[2,3]
Y.M. Dennis Lo[2,3*]

[2] Centre for Research into Circulating Fetal Nucleic Acids
Li Ka Shing Institute of Health Sciences
[3] Department of Chemical Pathology, and
[4] Department of Obstetrics and Gynaecology
The Chinese University of Hong Kong
Prince of Wales Hospital
Shatin, Hong Kong SAR, China

* Address correspondence to this author at:
Department of Chemical Pathology
Prince of Wales Hospital
30-32 Ngan Shing St.
Shatin, New Territories
Hong Kong SAR, China
Fax +852-2636-5090
E-mail loym@cuhk.edu.hk

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Primer Sequence Disclosure: A Clarification of the MIQE Guidelines

To the Editor:

The publication of the minimum information for the publication of real-time quantitative PCR experiments (MIQE)[1] guidelines *(1)* has turned out to be a defining event in the maturing of quantitative real-time PCR (qPCR) technology. The response from instrument and reagent manufacturers has been universally positive. There has been extensive publicity in print, online, and at scientific meetings, and scientific journals are beginning to take note *(2)*. Citations of the MIQE paper are accelerating, with 63 of the 169 citations (as of the end of January 2011) having appeared since September 2010. There is an enormous amount of good will toward this initiative, with many researchers keen to implement the different parameters within their own experimental protocols.

MIQE was never conceived with the intent of imposing an immutable edict, as in the spirit of a regulatory agency. The aim was to provide commonsense guidelines for enhancing the reproducibility and transparency of qPCR assays. MIQE, however, has become a marketing and selling argument ("MIQE compliance"), and this practice places a responsibility on the authors of the guidelines to assess whether the rapidly evolving technology demands refinement of the guidelines to acknowledge researchers' uncertainty.

Most discussion has concerned the stipulation of primer sequence disclosure. Many commercial qPCR assays are not supplied with the primer/probe sequences because most vendors consider such information commercially sensitive. In addition, there usually are no details provided regarding empirical validation of each individual assay. The increasing use of commercial qPCR assays is creating problems, because it leads to publications that cannot satisfy current MIQE requirements and limits the universal acceptance of MIQE. Consequently, we propose a pragmatic amendment of the original guidelines to require "EITHER primer sequences OR amplicon context sequence." This proposal is based on our assessment that in the absence of full disclosure of primer sequence, it is possible to achieve an adequate level of transparency, but only if there is an appropriate level of background information and disclosure of validation results regarding the qPCR assay:

- Our key concern is that today's reports must remain technically accessible in the medium to long term. For that reason, publications must not report assays without reference to sequence data, with invalid Web site references, or with resources obtained from vendors that no longer exist.
- We continue to affirm that full disclosure of the reagents used and validation of their performance are principal requirements for MIQE "compliance."
- Full primer (and probe) sequence disclosure remains our ideal; however, it may be possible to obtain equivalent results from slightly different assays as long as they target the same region and splice variants and they take single-nucleotide polymorphisms and secondary structures into account.

Consequently, if primer sequences are not disclosed, a MIQE-compliant publication should provide all of the following:

- The assay identification provided by the commercial vendor.
- The specific amplicon context sequence for the qPCR assay. Preferably, this information is obtained by sequencing the target PCR amplicon; alternatively, it could be supplied by the vendor or approximated by the authors (Fig. 1).
- The same validation criteria used for assays reporting primer/probe sequences. Specifically, when a precise -fold change for a transcript is reported, an essential require-

[1] Nonstandard abbreviations: MIQE, minimum information for publication of quantitative real-time PCR experiments; qPCR, quantitative real-time PCR.

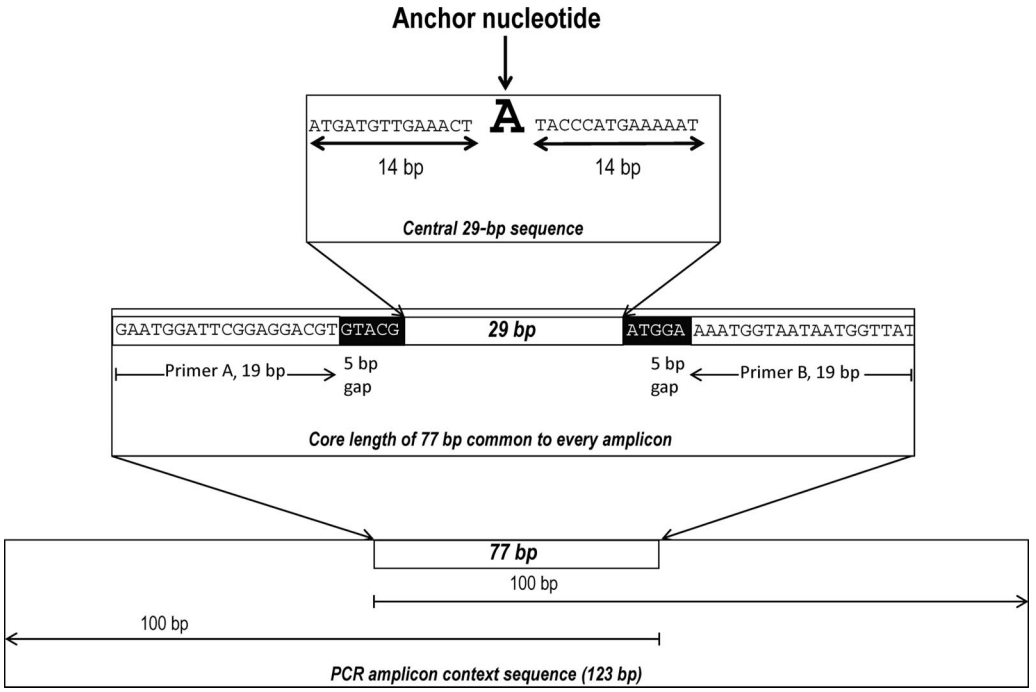

Fig. 1. An example of how to identify a PCR amplicon context sequence.

We introduce the concept of an "anchor nucleotide," which we define as a nucleotide contained anywhere within the probe sequence. The probe and primers are assumed to be 15 bp and 19 bp, respectively, with a 5-bp gap between them. No assumptions are made with respect to optimal or suboptimal primer or probe sequences. The vendor publishes information regarding reference sequence, anchor nucleotide, and amplicon length, which in this example are NM_001145847.1, 2982, and 100 bp, respectively. A central 29-bp sequence centered on the anchor nucleotide delineates the maximum context sequence for the probe. A 77-bp core sequence consists of 24 bp (19 bp for the primer and 5 bp for the gap between the 3′ end of the primer and the 5′ end of the probe) added to either side of the central sequence. The amplicon context is obtained by adding 23 bp (100−77) to either side of the core sequence to demarcate the potential extent of the PCR amplicon. In this example, the 100-bp amplicon would have a 123-bp context sequence, which is what one would submit with the publication. For DNA dye-binding assays, we suggest that vendors provide an amplicon ±20 bp of context sequence.

ment that remains is that the PCR efficiency, analytical sensitivity, and specificity of each individual assay be determined. Investigators should verify this information for the actual assay being reported under the laboratory conditions their personnel used in their laboratory; they should not extrapolate it from commercial assays validated by the vendors.

It is of paramount importance that commercial assay identification can continue to be traced, and it would be helpful to know why any assay was discontinued or replaced. Ideally, users should be able to order/use discontinued/replaced assays, either by the vendor providing them directly or by the vendor releasing primer and probe sequences for those qPCR assays. Vendors must also be more transparent about the bioinformatics efforts they use to validate their assays in silico.

MIQE aims to improve the transparency and hence the reproducibility of published qPCR assays by detailing minimum requirements. Crucially, "minimum" does not mean "ideal." The original stipulation of primer sequence disclosure as "essential" remains our ideal, and is strongly recommended for precise measurements or for situations in which qPCR forms a major part of a published study. Greater transparency in scientific research is always better, and for qPCR that includes primer and probe sequences. Nevertheless, given the commercial reality, we felt it sensible to modify the minimum sequence requirements. We hope these "revised MIQE guidelines"

will enhance their appeal and universality without compromising the importance of MIQE as a set of standards that is beginning to achieve acceptance in the scientific community.

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Stephen A. Bustin[2*]
Vladimir Benes[3]
Jeremy A. Garson[4]
Jan Hellemans[5,6]
Jim Huggett[7,8]
Mikael Kubista[9,10]
Reinhold Mueller[11]
Tania Nolan[12]
Michael W. Pfaffl[13]
Gregory L. Shipley[14]
Jo Vandesompele[5,6]
Carl T. Wittwer[15,16]

[2] *Barts and the London School of Medicine and Dentistry*
Queen Mary University of London
London, UK
[3] *EMBL GeneCore*
Heidelberg, Germany
[4] *Centre for Virology*
Research Department of Infection
University College Medical School
London, UK
[5] *Centre for Medical Genetics Ghent*
Ghent University Hospital
Ghent University
Ghent, Belgium
[6] *Biogazelle*
Zwijnaarde, Belgium
[7] *LGC*
Teddington, UK
[8] *Centre for Infectious Diseases*
University College London
London, UK
[9] *TATAA Biocenter*
Gothenburg, Sweden
[10] *Institute of Biotechnology*
Czech Academy of Sciences
Prague, Czech Republic
[11] *Sequenom*
San Diego, CA
[12] *Sigma-Aldrich*
Haverhill, UK
[13] *Physiology Weihenstephan*
Life Science Center Weihenstephan
TUM
Munich, Germany
[14] *University of Texas Health Science Center at Houston*
Houston, TX
[15] *Department of Pathology*
University of Utah
Salt Lake City, UT
[16] *ARUP Institute for Clinical and Experimental Pathology*
Salt Lake City, UT

*Address correspondence to this author at:
3rd Floor Alexandra Wing
The Royal London Hospital
London E1 1BB, UK
Fax +44-0-20-7377-7283
E-mail s.a.bustin@qmul.ac.uk

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Transferrin Saturation and Mortality

To the Editor:

Ellervik and colleagues *(1)* recently reported a positive association between transferrin saturation (TS) and mortality. Several questions arise from this observation: Is the association due to all causes of iron overload or to hereditary hemochromatosis only? Does the study underestimate the true association? And, is mortality due to variation in iron, transferrin, or both?

We have relevant data from population-based studies of twins and families of European descent living in Australia *(2, 3)*. TS values (calculated from serum transferrin and iron) and *HFE* (hemochromatosis) genotypes for C282Y (rs1800562, genotyped) and H63D (rs1799945, imputed) are available for 8096 adults (3151 men and 4945 women; mean age, 47 years). Replicate TS measurements are available for 460 participants (178 men and 282 women) from studies in 1993–1996 and 2001–2005. Their mean age at the time of the second study was 50 years (range, 39–72 years).

The Discussion in the Ellervik et al. report implies that the association of TS with mortality is driven by the C282Y variant (which is associated with hemochromatosis) and that TS is acting as a surrogate for this variant. There is a lack of equivalence between TS values >50% and *HFE* variants, however. Table 1 shows the relationships between TS and genotype for the 288 participants for whom TS values