Correspondence

A Method to Compensate for Different Amplification Efficiencies

To the Editor-in-Chief:

Meijerink et al¹ developed a novel efficiency compensation control (ECC) for the quantitation of cells with t(14;18) by real-time polymerase chain reaction (PCR). They showed that a decrease in amplification efficiency in a sample caused an increase in the difference in threshold cycles (*Ct*) for a multicopy 36-actin gene and a single copy albumin gene, both of which were co-amplified in the ECC reactions. ECC may be applicable as a general control in real-time quantitative PCR using genomic DNA templates. It would have been useful if the authors had described how they quantify t(14;18) in their samples using the calibrator and the ECC. The authors mentioned that the t(14;18) and the 36-actin reactions had equivalent amplification efficiencies, allowing direct normalization of the t(14;18) PCR to the 36-actin PCR at the *Ct* level. However, PCR efficiencies of independent reactions sometimes differ, making direct normalization inaccurate. They also state that ECC seems useful in identifying patient samples that have PCR inhibitors affecting different reactions to different extents. It would be better to have a way to accurately quantify a target gene in these samples.

Since PCR efficiencies may differ between different samples, different tubes, and different primer pairs, one should consider performing PCR reactions on a genomic reference and a standard in the same tube as a target sequence in quantitative PCR. For this purpose, multiplex PCR on a target sequence, a genomic reference, and internal standards for both the target and the reference sequences has been used.²⁻⁴ An internal standard for a target sequence should have the same primer-binding sites as the target sequence but have an internal insertion/deletion or a different probe-binding site to allow its differentiation from the target by size or probe specificity. The target and its internal standard are amplified in the same reaction tube, so that they have approximately equal amplification efficiency. The genomic reference sequence is used to normalize variation caused by initial DNA input and should also have its own internal standard with the same primer-binding sites, so that the reference and its standard have approximately equal PCR efficiency. Four different PCR reactions on the target, the genomic reference, and their respective internal standards in the same tube allow accurate calculation of the target gene dosage, e.g., *SMN1* copy number determination for spinal muscular atrophy carrier testing.^{3,4} These four reactions can be detected separately by dif-

ferent fluorescence dyes attached to different probes in real-time PCR.

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Author's Reply:

In the accompanying letter, Shuji Ogino suggested a multiplex competitive real-time PCR as an alternative method to quantify residual disease in patient samples with varying content of PCR inhibiting factors that consequently amplify with varying efficiencies. As mentioned by this author, many variable factors contribute to efficiency variation for different PCRs. Although some are inherent to the technique, the effect of primer design on PCR efficiency can easily be tested by real-time PCR for the first time on routine basis through the construction of standard curves. By doing so, we found out that one of the dogmas in PCR technology, i.e., different primer pairs have different PCR efficiencies, may be too exaggerated. When accurately designed with melting temperatures 4 to 6 degrees Celsius higher than the annealing temperature set in the assay, calculated according to the nearest neighbor method using settings that reflect real-time amplification conditions, i.e., 50 mmol/L KCl, 4 mmol/L MgCl₂ (303 mmol/L Na⁺ equivalents) and 300,000 pM of primers, almost any primer pair will amplify with approximately equal efficiencies with a range of about 5%. A similar range will be found for each PCR over multiple experiments, so this range reflects the highest rate of accuracy by the current state of technology. It is therefore that PCRs that do not differ more than 5% in efficiency from each other can be directly compared at the *Ct*-level. In our previous study,¹ we found that different PCRs that

perform with equal efficiencies on ideal template will perform with equal low efficiencies on samples that contain PCR-inhibiting factors. DNA isolated from patient samples will contain varying amounts of PCR-inhibiting factors especially when isolated from blood or bone marrow biopsies. So for molecular diagnostic studies aiming for the quantitation of residual leukemia/lymphoma cells whereby patient sample positivity is compared to a calibrator sample at the Ct-level, one needs to compensate for efficiency differences, otherwise samples with the lowest residual disease content are at highest error. This is why we developed the efficiency compensation control (ECC), so that each sample can be compensated for different amplification efficiency in comparison to the calibrator. Furthermore, using this ECC is also useful in identifying patients samples that contain so many PCR-inhibiting compounds that affect different PCRs to different extents, and it remains to be proven whether any other PCR approach including competitive PCR would be suitable to quantify residual disease in those samples.

The alternative competitive multiplex real-time PCR approach as suggested by Shuji Ogini may be difficult to use for the detection of minimal residual disease (MRD) for several reasons. When screening for minimal residual disease in contrast to gene dosage studies, the difference in initial target gene copies (reflecting the leukemia/lymphoma cell content) in comparison to the initial reference gene copies (reflecting the DNA input) can vary over 5 orders of magnitude in contrast to gene dosage studies where this variation remains within one log. A multiplex setting will therefore not be usable simply due to PCR competition that will occur between the target and reference PCRs when initial copy numbers for both templates differ for more than one order of magnitude. For minimal residual disease studies, these PCRs have to be performed separately.

For a competitive PCR or a competitive real-time PCR approach, the target PCR and its internal competitive construct both amplify with the same primer pair and therefore should have equal efficiencies. Although this approach may therefore seem to be the most accurate and attractive method in that essence, how does it work for samples that contain PCR-inhibiting factors and therefore amplify with lower efficiency? From our previous experience with competitive PCR assays,² it was clear that the copy numbers of the internal competitive con-

struct added to the reaction have to closely match the amount of initial target gene copies (within a 1 log range) otherwise the target would completely inhibit the amplification of the internal competitive construct or *vice versa*. Therefore, it is clear that in a MRD-setting for each patient sample, many reactions with different initial copies of internal competitive constructs are needed for both the target and the reference reaction to bring the internal competitive construct copy numbers within range.

How can one quantify the initial number of target gene or reference gene copies using such a competitive real-time PCR strategy? For this, the exact number of internal competitive construct copies that provide an identical Ct-value in comparison to the target or reference PCR need to be determined. At equal Ct-values is the amount of initial target gene copies equal to the amount of initial internal competitive construct copies added to the reaction. Alternatively, at least two or three reactions having the initial competitive construct and target gene copies within a 1 log range are required to extrapolate the exact number of initial target gene copies. As a second alternative, the Ct-values for target and internal competitive construct PCRs can be used to calculate the initial number of target gene copies but demand the measurement of the exact amplification efficiency for each patient sample that will consequently introduce variation.

So, for routine molecular diagnostic screening, this multiplex competitive real-time PCR approach seems unusable. Besides, for quantitative studies on follicular non-Hodgkin's lymphoma patients having unique t(14;18) chromosomal breakpoints, a new internal competitive construct needs to be designed for each patient.

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