

PCR bias in multiplex real-time quantitative PCR

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Abstract:

We recently conducted a study using a novel multiplex real-time quantitative PCR assay for the diagnosis of trisomies of the chromosomes 18 and 21, where the difference in copy number is only 50%. To calculate the ratio of the two chromosomes under investigation, we implemented the efficiencies based $\Delta\Delta C_T$ -method. The amplification efficiencies of the two sequences amplified were determined by dilution curve and the fluorescence increase method and shown to be equal and close to optimal. However, the ratios between the two chromosomes, as calculated by the formula were shifted towards 1.00. The multiplex amplification suffers from decreased ΔC_T -values when the targets are present in similar numbers. We propose a modification for the ratio determination based on an empirically determined "relative" amplification efficiency. We reanalysed the whole data-set acquired in the aneuploidy study, applying an efficiency of 2.2, instead of the measured efficiencies between 1.8 and 2.0. This procedure eliminates the discrepancy between calculated and actual ratio: The ratios calculated in this manner are close to 0.67 for trisomy 18 samples, and 1.50 for trisomy 21 samples, which is representative of the real situation. A bias against the more abundant locus towards the PCR product ratio of 1:1 was already observed in a previous pilot study and had been reported for the later cycles of PCR. Also another multiplex PCR based method for the detection of trisomies recently described a decreased, non-linear relationship between chromosome dosage and the mean peak ratios of PCR products, as observed after capillary electrophoresis, with unclear cause. Suggested reason is that the rate of formation of the more abundant PCR product declines faster than that one of the less abundant due to self-hybridisation of PCR products, thus preventing primer annealing to a fraction of the template. The higher self-inhibition during all PCR cycles for the more abundant product results in a successive decrease of the original difference of concentrations between the targets.

Introduction

In the past decade, quantitative fluorescent PCR of short tandem repeats has emerged as the method of choice for the rapid detection of chromosomal aberrations in amniotic fluids. Recently we presented an alternative PCR approach for the detection of trisomy 21, based on the real-time detection of the simultaneous amplification of a sequence in the Down's syndrome critical region of chromosome 21 and a control sequence from the GAPDH gene on chromosome 12 using TaqMan probes. Trisomy 21 samples could be segregated from normal karyotype according to the resulting ΔC_T . This represented the first report using real-time PCR with a sensitivity that demonstrated a 3:2 difference between gene loci. We modified our initial assay to quantify chromosome 21 in relation to chromosome 18 and thus test for the two most prevalent aneuploidies. With the large data set of a blinded study we developed a new interpretation to the model for relative quantification. Our assay is an ideal tool to examine the validity of efficiency determination and quantification models. We could observe that multiplex amplification of targets present in similar numbers is subject to slight PCR bias. We introduce a correction factor and a relative amplification efficiency for real-time qPCR experiments that allows relative quantification of high accuracy.

Methods

- The multiplex real-time qPCR analysis for sequences on chromosomes 18 and 21 was performed in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Switzerland). The reactions were prepared on ice and cycling conditions were as follows: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 1 min at 60°C and 15 s at 95°C.
- Concentration:** Reactions require template amounts of 700 to 10'000 genome equivalents, which corresponds to C_T -values (at threshold 0.2) in the range of 25.00 to 29.00 cycles, to guarantee optimal assay performance.
- To ensure that both sequences amplify equally efficiently, 4 thresholds along the linear phase of the amplification plot are analyzed for the karyotype determination.
- The use of a single reference sample is not reliable and may result in an erroneous diagnostic call. We based the calculations on the control measurements of several experiments to increase the accuracy of the ΔC_T (calibrator) value.

Efficiency determination

The efficiencies of the PCRs were determined by standard curve. Serial dilutions of a DNA template with known DNA concentration were amplified and the C_T -values plotted to the logarithm of the concentration. The slope can be converted into the reaction efficiency by the following calculation:

$$\text{Efficiency} = E = 10^{-1/(\text{slope})}$$

The standard curve results were confirmed by calculating efficiencies from the fluorescence increase of the amplification curves, with a linear regression of the Log(flourescence) during the observable exponential phase plotted against the cycle number, using the LinRegPCR program.

Results

Determination of PCR efficiency

Standard curves demonstrate that the two multiplexed amplifications proceed with nearly identical and optimal efficiencies for all 4 fluorescence intensities used as thresholds. The concentrations of the standard dilutions were set in order to cover the whole range for which the assay is reported to be valid. Using the replicates of the dilution curve for the determination by the fluorescence increase method with the LinRegPCR program resulted in stable efficiencies for each amplification, irrespective of the input DNA concentration. They ranged between 1.8 and 2.0, with three and four fluorescence readings included in the calculation.

| | Dilution curve (threshold 0.2) | Dilution curve (threshold 0.3) | Dilution curve (threshold 0.4) | Dilution curve (threshold 0.67) | LinReg 3 data points | LinReg 4 data points |
|---------------|--------------------------------|--------------------------------|--------------------------------|---------------------------------|----------------------|----------------------|
| Chromosome 18 | 1.98 | 1.97 | 1.97 | 1.95 | 2.03 (1.99-2.08) | 1.92 (1.89-1.95) |
| Chromosome 21 | 1.98 | 1.96 | 1.96 | 1.93 | 1.92 (1.85-1.94) | 1.82 (1.79-1.87) |

Table 1: Efficiencies determined by standard curve and fluorescence increase methods. The fluorescence signals of the two probes do not increase in parallel but the slope of the amplification curve of chromosome 21 is marginally flatter and thus the efficiencies determined by the fluorescence increase are lower. Analysing several experiments from the study, each >90 replicates, resulted in average reaction efficiencies that were around 0.10 greater for the chromosome 18 amplification, with p-values <0.000 using the paired samples t-test.

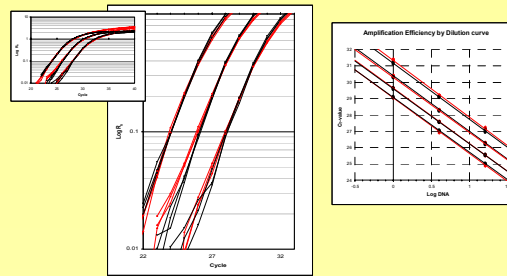


Fig. 1: Amplification plots of diluted standard DNA and the standard curves generated for the four thresholds. Red curves are from chromosome 21 amplification black curves from chromosome 18.

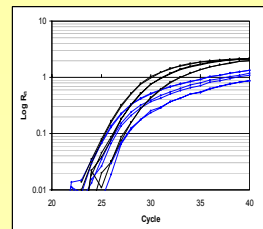


Fig. 2: Evidence, that the efficiency determined by fluorescence increase is not necessarily the true efficiency: The chromosome 21 MGB probe was replaced by a FAM labelled probe with a black hole quencher (BHQ). While the mean amplification efficiency of 1.95 for chromosome 18 with the MGB probe (black curves) is comparable to the main experiments, the mean efficiency calculated from the BHQ probe signal (blue curves) is only 1.63. At the same time, both standard curves generated are perfectly parallel.

New Model for the determination of Target Sequence ratio in multiplex experiments

Based on the equal and optimal efficiencies, the ratio between the two chromosomes can be calculated by the following formula and no normalisation is required:

$$[1] \quad \text{Chromosome 21 / Chromosome 18} = 2^{-\Delta C_T}$$

In theory the ΔC_T for a normal karyotype sample equals 0.00 at any threshold. However, the fluorescent signals generated by the two multiplexed reactions are not absolutely identical. As a consequence ratios determined by equation [1] are shifted and need to be corrected. Applying the calibrator-value already used in the blinded analysis has the result that the calculated ratios for normal karyotype samples are averaging 1.00 at every threshold.

$$[2] \quad \text{Chr21/Chr18} = 2^{(\Delta C_T \cdot \text{sample} - \Delta C_T \cdot \text{calibrator})}$$

The ratios calculated by this formula for the samples with trisomy are shifted towards 1.00. We empirically determined that the efficiency corrected model with an efficiency of 2.20 corrects this discrepancy.

$$[3] \quad \text{Chr21/Chr18} = E_{\text{rel}}^{(\Delta C_T \cdot 18 - \Delta C_T \cdot 21 - \Delta C_T \cdot \text{calibrator})}$$

$$E_{\text{rel}} = \text{relative Efficiency} = 2.20$$

| karyotype | replicates | ΔC_T (threshold 0.2) | ΔC_T (threshold 0.3) | ΔC_T (threshold 0.4) | ΔC_T (threshold 0.67) | Average Ratio 2 ΔC_T | Average Ratio 2.2 ΔC_T |
|------------|------------|------------------------------|------------------------------|------------------------------|-------------------------------|------------------------------|--------------------------------|
| Trisomy 18 | 62 | -0.53 | -0.59 | -0.68 | -0.78 | 0.69 | 0.66 |
| balanced | 358 | -0.01 | -0.06 | -0.12 | -0.19 | 1.00 | 1.01 |
| Trisomy 21 | 103 | 0.45 | 0.40 | 0.37 | 0.33 | 1.40 | 1.47 |

Table 2: The ratios determined by using equations [2] and [3] and the averaged ΔC_T values of all measurements, which are used as the ΔC_T (calibrator).

The application of this "relative" efficiency of 2.2 instead of 1.8 to 2.0, as determined by standard curves, results in calculated ratios close to 0.67 for trisomy 18 samples, and 1.50 for trisomy 21 samples, at all four thresholds. We reanalysed the whole data set acquired in the blinded study using equation [3] to calculate the chromosomal ratios of the samples. The average ratios for the trisomy samples are clearly more representative of the reality than when using efficiencies of 2.0 or lower.

Correction factor

We introduce a calibration method for real-time qPCR experiments based on multiple measurements that allows relative quantification of high accuracy. This approach can also be used for the comparison between different experimental conditions. A ΔC_T (correction) value is calculated from several samples that are measured in both experiments:

$$\Delta C_T (\text{correction}) = [\text{avg. } C_T \text{ chr 18} - \text{avg. } C_T \text{ chr 21}] \cdot \exp A - [\text{ditto}] \cdot \exp B$$

$$\Delta C_T (\text{sample, experiment B}) = \Delta C_T (\text{sample, experiment A}) - \Delta C_T (\text{correction})$$

By applying ΔC_T (correction) of the identical samples measured under experimental conditions A and B these two experiments become directly comparable.

For Example as a result of old probe aliquots in one experiment the ΔC_T -values are 0.2 smaller. These shifted values would have caused 1 false and 5 non-determined calls of totally 11 samples. The application of ΔC_T (correction) solves this problem: all samples from the experiment with the old probes can now be scored according to the established cut-off regions.

The method can be useful for compensation for different performance parameters of real-time qPCR and enables comparison of data acquired under similar but different experimental conditions: Basically the value of the correction constant depends on all parameters of the experiment: probe properties, the machine and plasticware used for the real-time qPCR and the threshold, even differences between batches of probes exist. ΔC_T (correction) can be established by running parallel standard curves. At the same time the standard curves ascertain that no differences in PCR efficiencies occur.

It is also possible to compensate for different efficiencies.

Conclusions

Determination of efficiency by fluorescence increase with the LinReg program is not 100% straightforward in TaqMan experiments: The signals are influenced by different characteristics of the probes, such as dyes, the sequence, coupling efficiency and purity. These have varying impacts on the rate of cleavage by the polymerase, quenching, levels of background fluorescence and signal strength.

Efficiencies of probe based real-time PCR determined by fluorescence increase are not necessarily the true efficiency. However, the LinReg program is an excellent tool to compare efficiencies between amplifications with the same detector.

Effects that can falsify the efficiencies determined by dilution curve are aggravated in multiplex amplifications: genomic DNA can be too concentrated for optimal efficiency. At low concentrations, variability is increased.

With the large set of data acquired we were able to develop a new interpretation to the model for relative quantification, introducing a correction factor for different detection conditions and a relative efficiency to compensate for the PCR bias.

PCR bias

We observed that multiplex amplification suffers from ΔC_T -shifting when the targets are present in similar numbers and proposed a modification for the ratio determination based on an empirically determined "relative" amplification efficiency.

We have already seen this bias against the more abundant PCR product, towards the ratio of 1:1, in a previous pilot study and it has been reported for the later PCR cycles. Another multiplex PCR based method for the detection of trisomies recently described a decreased, non-linear relationship between chromosome dosage and the mean peak ratios of PCR products.

We suggest that the rate of the abundant PCR product declines faster than that of the less abundant one is the self-hybridisation of PCR products, thus preventing primer annealing to a fraction of the template. The higher self-inhibition over all PCR cycles for the more abundant product results in a successive decrease of the original difference of the copy number.

For real-time PCR this effect has not been described before. Data analysis in real-time PCR is during the exponential phase, when the rehybridisation is not considered a limiting factor for amplification. The small extend of the effect is accompanied by a lack of reports on gene copy number measurements that have targets with such precisely defined template ratios as reported here, where increased sensitivity would be required as to demonstrate 3:2 dosage differences.