

Simultaneous determination of fetal trisomies 18 and 21 by real-time quantitative PCR

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Abstract

The detection of gross chromosomal abnormalities is a major focus of prenatal diagnostics, of which the most common cytogenetic anomaly in live births is trisomy 21, also known as Down's Syndrome. Currently, prenatal diagnosis of genetic anomalies relies on invasive procedures such as amniocentesis and chorionic villus sampling (CVS), from which the full fetal karyotype is usually determined using cultured cells. The two week of cultivation and subsequent analysis has proven to be associated with considerable parental anxiety. This delay is also associated with considerable medical problems in those situations requiring therapeutic intervention. In order to address these needs, more rapid methods for the prenatal diagnosis of fetal chromosomal aneuploidies have recently been developed and implemented, such as multi-color fluorescence in situ hybridization (FISH) as well as quantitative fluorescent PCR (QF-PCR) analysis of short tandem repeats (STRs). With the advent of real-time PCR it is now possible to measure concentrations of nucleic acid sequences with an accuracy that was not deemed possible only a few years ago. Examples are the analysis of gene expression and gene duplications / losses, where two-fold differences in nucleic acid concentration are routinely determined.

We have investigated whether real-time quantitative PCR (qPCR) could be used for the diagnosis of chromosomal anomalies, in particular the aneuploidies such as trisomy 18 and 21, where the difference in copy number is only 50 %. This approach was first tested in a pilot study, wherein we were able to detect cultured trisomy 21 samples with 100 % specificity and sensitivity. We have now modified this test to permit the simultaneous analysis of trisomies 18 and 21 and have demonstrated that our approach can be used for the highly reproducible and robust detection of only 1.5 fold differences in gene copy number. Our studies have also underscored that several criteria need to be met concerning template preparation and primer purity.

Objective

We developed a real-time qPCR assay for the detection of fetal aneuploidies. By amplifying genetic loci in the Down's region of chromosome 21 and a locus on chromosome 18 simultaneously, we were able to assess the ratio of these loci and hence ploidy.

Material

- Amniotic fluid samples from 97 pregnant women were collected and DNA extracted and analysed by QF-PCR of STRs in London.
- Analysis of STRs showed that 78 samples were of normal karyotype, two were triploid, four were trisomic for chromosome 18 and thirteen were trisomic for chromosome 21.
- Small aliquots of the DNA samples were coded and sent to Basel for blinded real-time qPCR analysis.
- For control purposes, DNA extracted from amniocyte cultures were amplified in parallel with the samples.

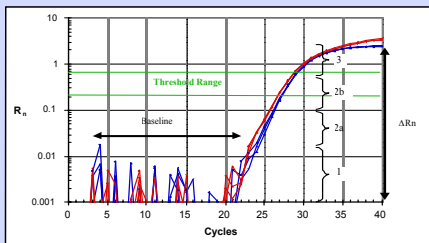


Figure 1: Amplification plot of the real-time PCR data. 1: Phase of observed background fluorescence. 2: Observable exponential phase. 2a: Measurements are close to the detection limit. Due to the high contribution of the background fluorescence to the total fluorescence measured, replicate curves can deviate (as seen in the blue curves). 2b: Influence of background fluorescence minimal. 3: Linear and plateau phases of the amplification with decreasing amplification efficiencies. Displayed are triplicate amplifications of a sample with trisomy 21. The red curves represent data from chromosome 21 (FAM dye), the blue curves from chromosome 18 (VIC dye).

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).
- Primers and probes were designed to amplify a sequence of the amyloid precursor protein gene in the Down's region of chromosome 21 and of the thymidylate synthetase gene on chromosome 18.
- To ascertain equal amplification efficiencies of both target sequences and to compensate deviations in the fluorescence measurements, we examined four separate thresholds along the entire observable exponential phase instead of the usual single threshold.
- Additional safety requirements to obtain reliable results were included: **Concentration and purity** of the DNA sample have to permit an efficient amplification. The final fluorescence of the sample (ΔR_n) is a good measure for the combination of the two: Samples with a final ΔR_n lower than 1.5 were disregarded. Also samples' C_T -values at threshold 0.2 need to fall in the range of 25.00 to 29.00 cycles, which corresponds to 700 to 10'000 genome equivalents as template per reaction.

Results

The ΔC_T -values of the amplified samples cluster in three specific groups depending of their karyotype (Fig.2). These ΔC_T -values were stable over several experiments, as long as the experimental conditions were identical (Fig.3).

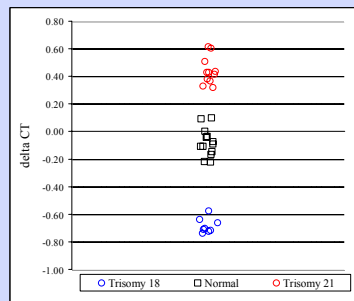


Figure 2: Scatter plot of the ΔC_T -values of the control DNAs for the threshold of 0.45. Depicted are replicate averages per sample. The ΔC_T -values of all samples with normal karyotype group between -0.22 and +0.10, all trisomic samples lie outside of this area. The average of the normal samples is -0.09, this is the ΔC_T (calibrator) for normal karyotype at threshold 0.45.

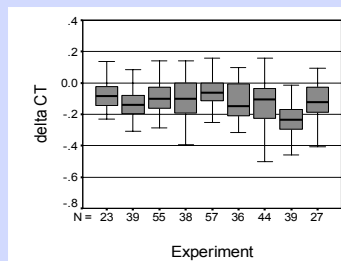


Figure 3: Box plot of ΔC_T -values in 9 experiments of all samples of normal karyotype. Numbers on X-axis indicate number of measurements per experiment.

Accuracy of the blinded karyotype determination using ΔC_T as diagnostic value:

- In the blinded diagnosis, trisomy 21 was correctly determined in eleven, trisomy 18 in four and normal karyotype in 68 cases.
- One triploid sample's chromosomal ratio was correctly determined as balanced, but as is inherent in the test it could not be distinguished from a normal karyotype sample.
- Five samples of normal karyotype were misdiagnosed
- In eight cases the DNA quantity was not sufficient for a diagnosis.
- This means that the correct karyotype was determined in 87 % of the samples, 5 % were falsely diagnosed and 8 % remained undetermined.

Strikingly, the misdiagnosed samples consistently gave the same false result after repeated testing, indicating that the results were representative of the actual chromosomal ratio in these DNA solutions.

This observation led to the hypothesis that the DNA extraction resin had adsorbed a considerable proportion of the DNA. The remaining three resins of misdiagnosed samples were re-suspended in 40 μ l water and the DNA re-solubilised by heating to 95 °C.

The karyotype of these three samples was then correctly determined by real-time qPCR (Fig.4).

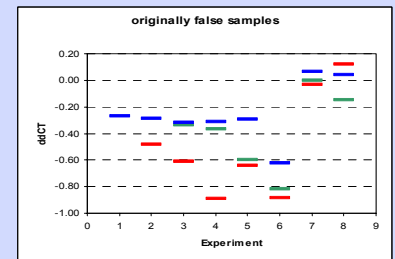


Figure 4: The corrected ΔC_T -values of 3 false samples (-, -, -) that were re-solubilised. Experiments 7 and 8 are the results from the resuspended resins - $\Delta \Delta C_T$ -values between -0.25 and 0.25 indicate that the normal karyotype determined is correct. $\Delta \Delta C_T = \Delta C_T(\text{sample}) - \Delta C_T(\text{calibrator})$

Conclusions

- real-time qPCR can be used for the rapid and precise determination of trisomies.
- It permits the analysis of multiple samples and targets in an automated manner.
- By being a closed system it is less prone to contamination than conventional PCR methods.
- The occurrence of falsely diagnosed samples and the identification of the source of error stress the importance of optimal sample handling for the clinical analysis using PCR based methods.
- In order to achieve the high degree of accuracy required for prenatal diagnosis, optimal reagents have to be used for the amplification: For optimal reproducibility and amplification efficiency, HPLC purified primers and the ABI real-time PCR reaction mix containing Amperase U were used, and in order to generate fluorescent signals of a long observable exponential phase, TaqMan MGB probes are the best choice.