Methods for quantification of circulating DNA in human plasma

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The analysis of human plasma for circulating DNA is a rapidly developing field in both diagnosis and disease management. One potential application is non-invasive genetic testing of the developing foetus at an early stage in pregnancy. A universal positive control for the detection of foetal material is central to the wider application of the method for reliable routine diagnostic use, and the optimisation and modification of existing Q-PCR techniques can be used to significantly improve the reliability and quality of nucleic acid based measurements.

Analysis of circulating DNA extracted from human plasma using real-time quantitative PCR (Q-PCR) is a rapidly developing field. Current applications include monitoring disease progression in cancer patients [1], predicting the outcome for victims of trauma [2], and in the case of pregnant women, providing a potential means for non-invasive genetic testing of the developing foetus [3]. As with many expanding areas of research, the development of standardised methodology for performing analyses lags behind the rate at which new data is generated. This therefore limits both the comparability of results between different laboratories and the identification of best practices. Details relating to specific methodologies such as how DNA calibrators are prepared, the way that standard curves are constructed and how samples are quantified, can be limited in the literature.

Since the discovery that small amounts of foetal DNA circulate in maternal blood, there has been great interest in the application of nucleic acid based tests on this non-invasively obtained material. A significant issue is the very low amount of foetal material available for detection, particularly during the first trimester of pregnancy when testing is generally performed. Accurate quantification and reliable detection of foetal material are technically challenging, however, there are certain modifications to routine Q-PCR analysis that can improve the quality of results. The main issues to address fall into two categories: assay methodology and data analysis.

Methodology: preparation of DNA solutions for calibration curves

We have investigated a range of different DNA solution preparation methods for use in Q-PCR calibration curves. The effect of each method on the DNA detection rate and data repeatability was observed with particular focus on low concentration solutions where variation is generally greater. A fluorogenic 5’ exonuclease (TaqMan) assay was performed to detect the male specific SRY target in human genomic DNA. Male DNA was quantified using PicoGreen and diluted to a range of 0.5-1000 target copies. The DNA standards were diluted in water, a carrier DNA solution (herring sperm DNA), and a stabilising solution, using both regular laboratory microtubes and ‘low retention’ microtubes designed to reduce adhesion of molecules onto the walls of the tubes. Each reaction was performed in duplicate and repeated in three separate PCR runs on the ABI PRISM 7700 Sequence Detection System. The results demonstrated that the choice of diluent did not significantly improve detection at low concentrations [in terms of increased numbers of positive replicates and lower Ct (Cycle threshold)], when compared to the control group. However, there was a noticeable improvement in detection at very low DNA concentrations when using the low retention microtubes compared to the standard tubes. Figure 1 displays the mean Ct for six replicates at each concentration level. Above 5 copies the choice of microtube does not influence the mean Ct. However, below 5 copies the use of low retention plastics improves detection, as demonstrated by the lower Ct, indicating a higher DNA concentration. The higher apparent analyte concentration in the low retention plastic ware reflects less sample loss through adhesion onto the microtube walls, compared to regular tubes. Similar observations have been made by other researchers focussing on low-level DNA detection [4].

Q-PCR assay performance characteristics for different target sequences

When performing non-invasive prenatal studies for the detection of specific targets, the plasma taken from the mother contains both her DNA and small amounts of foetal DNA. The distinction between maternal and foetal DNA levels can be made by detecting two different target sequences. Measuring a housekeeping gene such as GAPDH is representative of total DNA levels, the majority of which is maternal in origin. Foetal DNA can be detected by targeting a male specific gene, commonly SRY. The current lack of a specific positive control for all foetal DNA excludes female foetuses from such analysis, and development of appropriate controls is now a focus of many research groups.

Q-PCR measurement of the two targets, GAPDH and SRY, has identified differences in the precision of data produced at various concentration levels by the two assays. Figure 2 highlights the dramatic increase in variation of results for DNA detection below 5 target copies, which is more marked in the SRY than the GAPDH assay (36 replicates performed at each level). This variation at low levels is of particular significance for the SRY assay because it occurs in a concentration range within which the majority of first trimester foetal samples fall, and is of clinical interest. Total DNA levels are at much higher concentrations, and therefore are outside the zone of high variability. The high variability at low levels reflects the inconsistency of sampling from low-density solutions. To address this variability and improve the precision of results, the number of replicate analyses performed can be increased [5]. However, for non-invasive prenatal monitoring this can be difficult with the limited amount of extracted DNA available, and the capacity of the instrument may also limit the number of replicate analyses that can be performed. An alternative approach recently reported by other researchers in the field is the use of multi-copy target genes such as DYS14, rather than single copy targets like SRY, which improves the ratio of target sequence per genome, thus increasing both assay precision and the probability of detection [6].

During the early stages of pregnancy, the concentration of foetal DNA per mL of maternal blood is extremely low, and may be as few as 1 or 2 copies. Performing three or more replicate analyses of such
a sample is likely to produce a proportion of results with a negative signal, which is due to sampling variation. The convention in Q-PCR analysis is to report amplification results in terms of the point in the reaction at which the fluorescent signal increases above a threshold level (the cycle threshold, Ct). This means that ‘non-amplification’ or negative results are either assigned ‘no value’ or a Ct equal to the number of PCR cycles performed. A reaction given ‘no value’ is a problem as it effectively excludes the data from analysis and this information is lost. Being assigned a Ct equal to the number of cycles performed is equally problematic when averaging the replicate reactions, as this value is essentially arbitrary and so can skew the results significantly. A more useful interpretation of the data for low-level samples is to assign the ‘non-amplification’ sample replicates a concentration of ‘0’, and convert all other replicates with assigned Ct values into a quantitative measurement via interpolation from the standard curve. A final mean value for the sample can then be calculated using the results of all of the replicates in calculating concentration, and through the construction of calibration curves using only concentration points for which a full set of data, unbiased by drop outs, is available.

Construction of standard curves
When plotting calibration curves over a range of DNA concentrations, there is variation in the standard deviation of data at each level, as seen in Figure 2. We have found that the poorer repeatability of replicate calibration reactions at lower concentrations can affect the quality of the standard curve. At low target levels of approximately 10 copies or less, the distribution of replicate calibration points begins to widen. One approach to reduce the influence of this variation is to apply a weighted regression to the data set, which anchors the calibration line most heavily to the points with the smallest standard deviation. An alternative approach that we have found effective is to remove any concentration levels from the curve completely which contain any data points that ‘drop out’. These dropouts are at the lower end of the curve, so the line based on the higher concentrations is then extrapolated back to 0. This has the effect of eliminating the data for which there is a high degree of uncertainty associated.

Conclusions
The potential for non-invasive genetic testing of foetal material at an early stage in pregnancy is both exciting and extensive. Development of a universal positive control for the detection of foetal material is central to wider application of the method for reliable routine diagnostic use. In addition, optimisation and modification of existing Q-PCR techniques can be used to significantly improve the reliability and quality of nucleic acid-based measurements. In practical terms, using low retention plastic ware and performing a sufficient number of replicate analyses on each sample can increase the likelihood of successful detection of low concentrations of foetal material. Quantification may also be improved by including information from all sample replicates in calculating concentration, and through the construction of calibration curves using only concentration points for which a full set of data, unbiased by drop outs, is available.

References

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