# Aneuploidy Detection by QF-PCR of STR Markers on the Applied Biosystems 3500xL Genetic Analyzer

## Introduction

Analysis of short tandem repeat (STR) markers using quantitative fluorescence PCR (QF-PCR) is a common strategy employed in clinical research laboratories for the detection of chromosomal aneuploidy. Laboratories that routinely perform these types of analyses demand high-throughput, efficient, and highly automated solutions. In this Application Note, the accuracy, ease of use, and throughput capabilities of the Applied Biosystems 3500xL Genetic Analyzer and GeneMapper® Software v4.1 are demonstrated in aneuploidy analysis of chromosomes 13, 18, 21, and sex chromosomes X and Y.

## Aneuploidy and Its Detection by QF-PCR

Normal human somatic cells are euploid and contain a diploid (2N) set of autosomes and a pair of sex chromosomes. Cells that do not contain an exact diploid set are termed aneuploid and, therefore, either lack or contain additional chromosomes. Common types of aneuploidy are monosomy—for example, the loss of one sex chromosome (e.g., Turner syndrome, 45,X)—and trisomy, three copies of a given chromosome in a diploid cell. Trisomies of autosomes 13 (Patau syndrome), 18 (Edwards syndrome), and 21 (Down syndrome) are possible, as is the presence of extra sex chromosomes such as in Klinefelter syndrome (47,XXY) and Triplo-X syndrome (47,XXX).

In early pregnancy, samples of amniotic fluid, chorionic villi, or umbilical cord blood can be analyzed for aneuploidy using the molecular technique QF-PCR. This analysis is complementary to cytogenetics-based prenatal tests such as full karyotype analysis of metaphase chromosomes and fluorescence in *situ* hybridization (FISH) karyotyping.

Aneuploidy detection by QF-PCR is rapid and informative, employing the amplification and analysis of STR genetic markers. STRs, also known as microsatellites, are polymorphic DNA loci that contain a repeat sequence of 2 to 6 bases. The number of repeats for a given locus may vary, resulting in alleles of differing lengths. Accurate aneuploidy analysis usually involves the relative allele dose quantitation of multiple STRs on a given chromosome, and these markers are typically amplified in addition to the amelogenin gene, used for sex determination. This approach has a number of advantages over traditional cytogenetic analyses, such as the availability of commercial kits, rapid time-to-results, and the requirement for relatively small amounts of input gDNA.

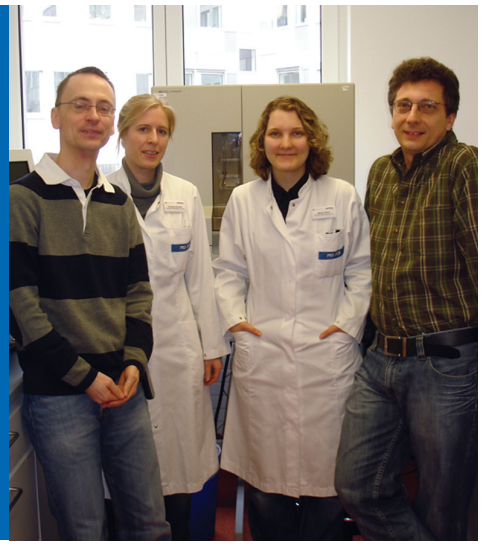
The Applied Biosystems 3500xL Genetic Analyzer provides researchers with powerful multiplexing capabilities for analyzing STR samples, increasing throughput and data accuracy. By labeling different STR amplicons with unique fluorescent dyes, fragments of the same size (or overlapping sizes) can be run in the same capillary. Also, because researchers can design shorter primers and generate smaller amplicons, runs can be completed in less time and with more reproducibility. Finally, by employing both in-lane fragment sizing and signal normalization using the GeneScan™ 600 LIZ® Size Standard v2.0 along with the corresponding instrument run module, researchers can take advantage of the signal normalization feature of the 3500xL Genetic Analyzer (Figure 1). This is of particular benefit in aneuploidy analysis, which relies on detecting small but significant differences in peak height or peak area among a marker's sister alleles to correctly assign ploidy status. Therefore, aneuploidy analysis presents a demonstration case for the Applied Biosystems 3500 Series Genetic Analyzer.

## The QF-PCR Assay for Aneuploidy Detection

To demonstrate a QF-PCR aneuploidy assay on the 3500 Series Systems, genomic DNA samples isolated from amniotic fluid were assessed by amplifying microsatellite markers on chromosomes 13, 18, 21, and X. A 19-plex assay containing 17 microsatellite markers, an STR marker in the amelogenin gene, and a paralogous control marker on chromosomes 3 and X was developed by Dr. Roland Achmann at MVZ genteQ in Hamburg, Germany, and was employed for these experiments. This 19-plex assay uses PCR primers, with one of the two primers for each locus labeled with FAM®, VIC™, NED®, or PET™ dyes (fluorescent dyes used with Applied Biosystems G5 dye set).

**Aneuploidy Analysis in the Laboratory of Dr. Roland Achmann**

Dr. Roland Achmann and colleagues have been carrying out aneuploidy analysis at MVZ genteQ (Hamburg, Germany) for the past several years. The lab provides cytogenetic and molecular genetic support for medical practitioners in private practice and to hospitals throughout Germany, analyzing several thousand samples each year. By incorporating the latest technological and methodological developments, they are continually improving the turnaround time for results and have positioned themselves as an important center for prenatal diagnosis and clinical genetics. Pictured, left to right, are Dr. C. Kähler, C. Schmidt, M. Kraske, and Dr. R. Achmann.



Following the completion of the PCR, aliquots of the resulting dye-labeled amplicons were combined with the GeneScan™ 600 LIZ® Size Standard v2.0. Samples were electrophoresed on the 3500xL Genetic Analyzer using a 50 cm capillary array and 3500 POP-7™ Polymer. The instrument protocol used was the FragmentAnalysis50_POP7 run module in combination with the G5 dye set.

Fluorescently labeled QF-PCR products for a particular microsatellite locus are categorized in Figure 2 as uninformative (homozygotes); normal or diallelic heterozygotes (alleles exhibiting a 1:1 ratio); trisomy with three alleles observed for a single locus (alleles exhibit a 1:1:1 ratio); or trisomy exhibiting unbalanced allelic ratios (e.g., 2:1 allelic ratio). Allelic peak height ratios (A1/A2) are used to determine the copy number of alleles at each locus. For example, "normal" heterozygous diallelic markers fall within a ratio range of 0.8 to 1.4. Trisomy at a given locus is suspected either when three alleles are present or when two alleles are present that give a ratio greater than 1.4. Other conditions are possible such as mosaicism, maternal cell contamination, stutter artifacts, preferential allelic amplification, primer site polymorphisms, and somatic microsatellite mutations. Since any individual locus may yield an uninformative or inconclusive result, QF-PCR panels are designed to amplify multiple loci (in this case 19 loci in the QF-PCR multiplex panel).

## Aneuploidy Data Analysis Using GeneMapper® Software v4.1

Accurate allele scoring, a critical requirement for aneuploidy studies and other QF-PCR experiments, relies on the efficient collection and analysis of large amounts of data from highly automated workflows. GeneMapper® Software v4.1 offers flexible and highly customizable data display and analysis functions for consistent and accurate QF-PCR sample processing. All samples in the aneuploidy study presented here were analyzed using GeneMapper® Software v4.1 and the following settings:

| | |
|---|---|
| Panel | QFPCRv6 |
| Bin Set | Genteq_QFPCR |
| Size Standard | GS600LIZv2 |
| Peak Detection Mode | Advanced |
| Analysis Range | Full range |
| Size Range | All sizes |
| Smoothing | None |
| Size Calling Method | Local Southern method |
| Peak Amplitude Threshold | 500 |
| Baseline Window Size | 51 |
| Minimum Peak Half Width | 2 |
| Polynomial Degree | 3 |
| Peak Window Size | 15 |

In addition to advanced algorithms that recognize and filter amplification chemistry artifacts such as stutter peaks, GeneMapper® software contains a convenient Report Manager feature that can automatically quantitate parameters such as relative fluorescence (based on peak areas or heights from multiple samples, Figure 3). GeneMapper® software also allows the user to specify custom equations for any type of relative fluorescence quantitation (Figures 3 and 4), which can then be automatically applied across an entire sample set and can also be saved and applied to data generated in subsequent assays, if desired. After the analysis is complete, an easy-to-read results report can be printed or exported for further analysis.

The Report Manager feature can be used to specify and automate the typical multistep calculations that are necessary to determine values for peak height or peak area ratios and alert the user to markers that require further investigation. Following analysis of QF-PCR data from the aneuploidy study, the Report Manager feature flagged various markers from chromosome 21 as potential aneuploidy candidates (i.e., those generating three peaks or those generating peak area ratios that fall outside of the normal range, see Figure 5). The data shown in Figure 6 highlight the well-resolved peaks that

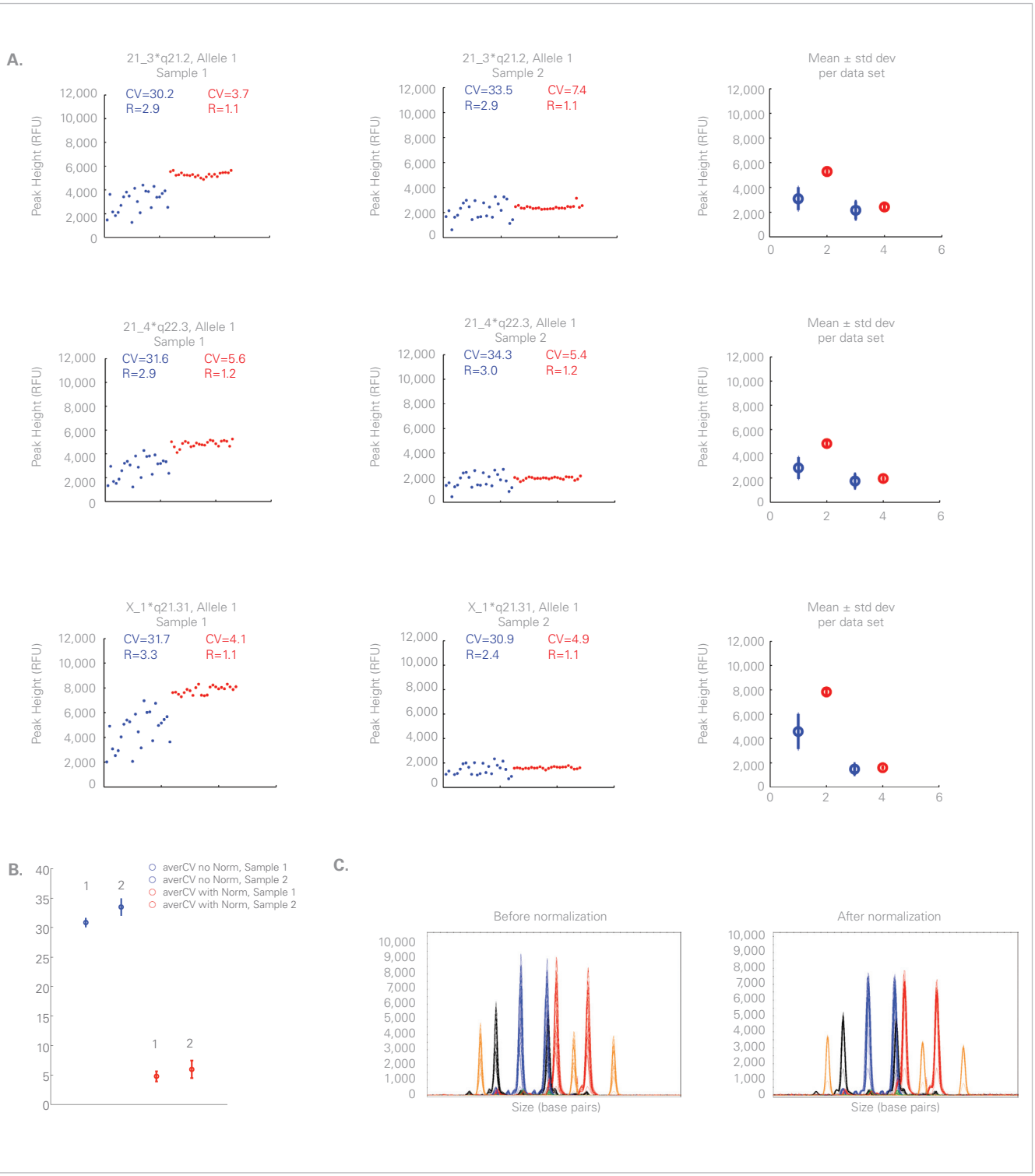



**Figure 1.** 3500 Series Normalization Aided by the GeneScan™ 600 LIZ® Size Standard v2.0. **A.** An example of the effect of normalization on three markers (21_3*q21.2, 21_4*q22.3, X_1*q21.31) before (blue) and after (red) normalization from two separate reactions (sample 1 and sample 2) using different reaction template gDNA. Each reaction sample was replicated in 24 wells on a plate and electrokinetically injected once. The effectiveness of normalization was assessed using the percent coefficient of variation [%CV = (standard deviation/mean) x 100] of the peak height for each allele of a particular marker. The %CV was calculated from 24 replicates for each sample. The variation (R) was calculated using the ratio between the maximum peak height and minimum peak from 90% of the data (to remove outliers) for each marker. The left and center columns show the peak height variation observed for an allele for three different markers for sample 1 and sample 2, respectively; the calculated %CV (CV) and variation (R) for each before normalization (blue) and after normalization (red) are indicated. The right column indicates the peak height mean and standard deviation of an allele for that marker before normalization (blue) and after normalization (red) for samples 1 and 2, respectively. **B.** The average of the %CV across alleles from 19 markers was calculated for samples 1 and 2 before normalization (blue) and after normalization (red). **C.** An electropherogram of selected peaks before normalization is applied (left), and the same peaks after normalization (right).

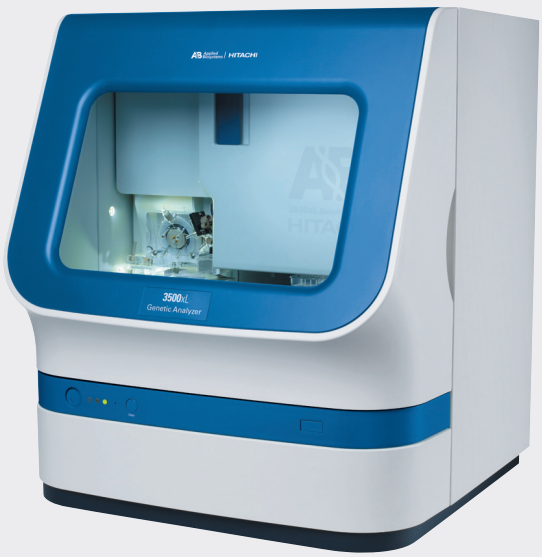
### 3500 Series Genetic Analyzers

3500 and 3500xL Systems deliver consistently reliable results, critical for demanding fragment analysis applications, and offer:

- Optimized instrument run modules and implementation of the GeneScan™ 600 LIZ® Size Standard v2.0 for improved signal normalization, especially when comparing capillary-to-capillary, injection-to-injection, or instrument-to-instrument within an association of investigators who run samples on multiple identical instruments
- New thermal sub-system design for improved temperature stability
- 3500 Data Collection Software with an intuitive workflow that performs size calls and applies quality control flags to alert the user to off-scale data, broad peaks, and interfering artifacts or "pull-up"

The 3500 Series Genetic Analyzers are available in two throughput options: the 8-capillary 3500 system, and the 24-capillary 3500xL system. Sample analysis is fully automated from the moment each 96- or 384-well plate is placed on the instrument and the run is initiated. Easy-to-use wizards for instrument operation and maintenance ensure predictable, hassle-free performance. And, with recent improvements in the instrument run module, the majority of applications can be analyzed on a single configuration of POP-7™ Polymer with a 50 cm capillary array.

The 3500 Series Systems feature simplified and easy-to-install consumables. The Anode and Cathode Buffer Containers are supplied as ready-to-use 1X Genetic




Analysis Buffer formulations. The Automated Polymer Delivery System has been improved with the introduction of polymer pouches, available in 960- and 384-sample sizes, which significantly reduce setup time. RFID (radio frequency identification) tagging of buffers, polymer, and arrays enables automated electronic tracking of lot number, usage, and expiration date information during analysis.

Also, by employing a single-excitation line solid-state laser, the 3500 Series System has a more compact overall footprint than previous genetic analyzers and operates using a standard power supply. The smaller footprint and standard power supply mean that 3500 and 3500xL Genetic Analyzers fit in more places and don't require ducting for heat removal.

were obtained for all aneuploidy markers on the 3500xL Genetic Analyzer, and these electropherogram plot views were vital for the confirmation of the peak area calculations.

### Normalization of Signal Output

When the same sample is analyzed by Capillary Electrophoresis (CE), a certain amount of variation in signal strength may be observed across multiple CE instruments; within a single instrument among different capillaries; or among different injections from the same capillary. For applications that require quantitative analysis, such as QF-PCR, minimal signal variation is desired and data analysis can be aided by reducing signal variation. Applied Biosystems researchers have identified a number of different sources of variation and have elucidated methods to obtain more consistent peak height data on CE instruments from injection-to-injection, capillary-to-capillary, and instrument-to-instrument. Advantages of normalization incorporated into the 3500 Series of Genetic Analyzers, including the use of GeneScan™ 600 LIZ® Size Standard v2.0, would be useful both to single researchers and to groups of investigators with multiple identical instruments who desire consistent and comparable results.

The consistency of QF-PCR data quality following normalization is illustrated in Figure 1. Analysis of alleles from all the markers across the 19-plex aneuploidy assay calculated the average unnormalized %CVs as 30.86 (sample 1) and 33.49 (sample 2). Following normalization, the average %CV dropped to 4.78 and 5.96 for samples 1 and 2, respectively (Figure 1B). The calculation of CV ratios facilitates the comparison of the normalized and unnormalized data from two different samples in


**Figure 2.** Defining Aneuploidy Markers. Detection of fluorescently labeled PCR products allows accurate identification and quantification of uninformative markers (homozygotes); normal heterozygotes (two different alleles with a peak area ratio of 1:1); trisomy samples with three alleles (1:1:1 ratio); and trisomy with unbalanced peaks (ratio of 2:1). The data shown here were generated using the 3500xL Genetic Analyzer.

a meaningful way; the smaller CV of the normalized data indicates the peak heights are less dispersed than the unnormalized data with the larger CV. The dispersed nature of the peak heights in the unnormalized data is evident from the larger standard deviation observed when compared to the normalized data for the three example markers (Figure 1A, right column). This is also apparent by examining the electropherograms before and after normalization (Figure 1C). A further measure of normalization is signal variation (R). This is calculated by taking the ratio of maximum peak height to minimum peak height for an allele of a marker after removing the highest and lowest 5% of peak heights (considered outliers). Analysis of alleles from all the markers across the 19-plex aneuploidy assay resulted in an average signal variation of 2.95x for sample 1 and 2.86x for sample 2. Following normalization, the average signal variation dropped to 1.16x for both samples 1 and 2. This improvement in signal variation is consistent with results obtained by Applied Biosystems when analyzing larger, more complicated data sets. Taken together, these analyses

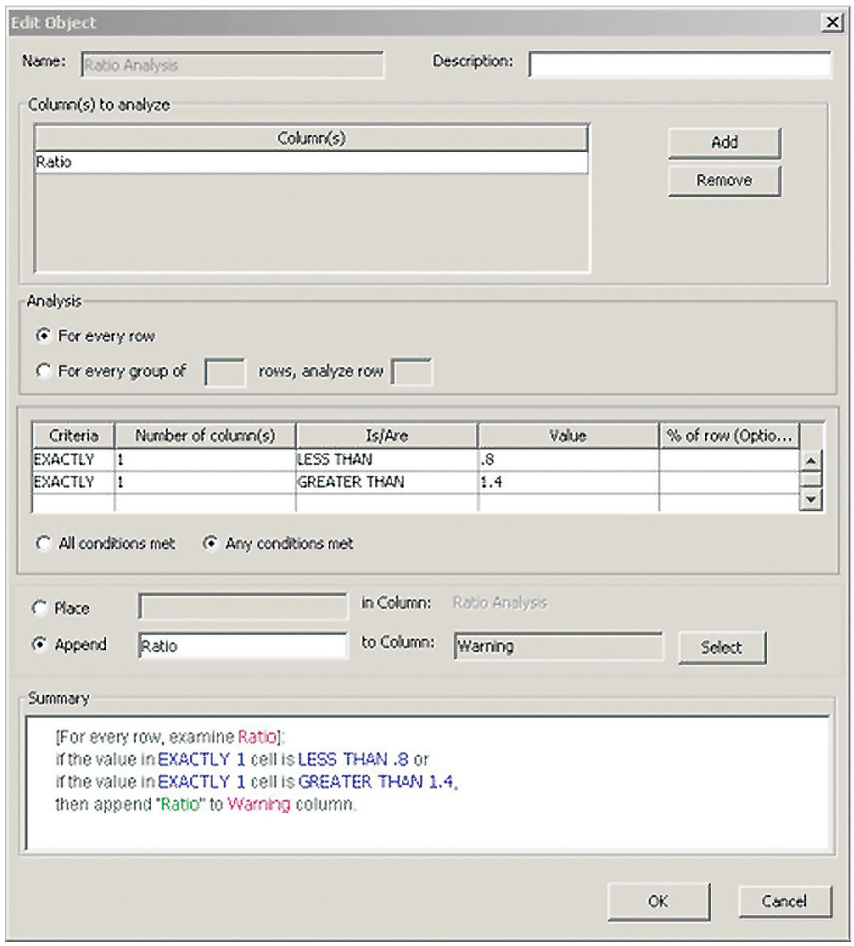



**Figure 3.** GeneMapper® Software Alerts the User to Markers That Require Further Investigation. Final analysis can be performed to identify heterozygous diallelic markers with ratios that fall outside of the determined range, three-allele markers, uninformative single-allele markers, and markers that fail to amplify.

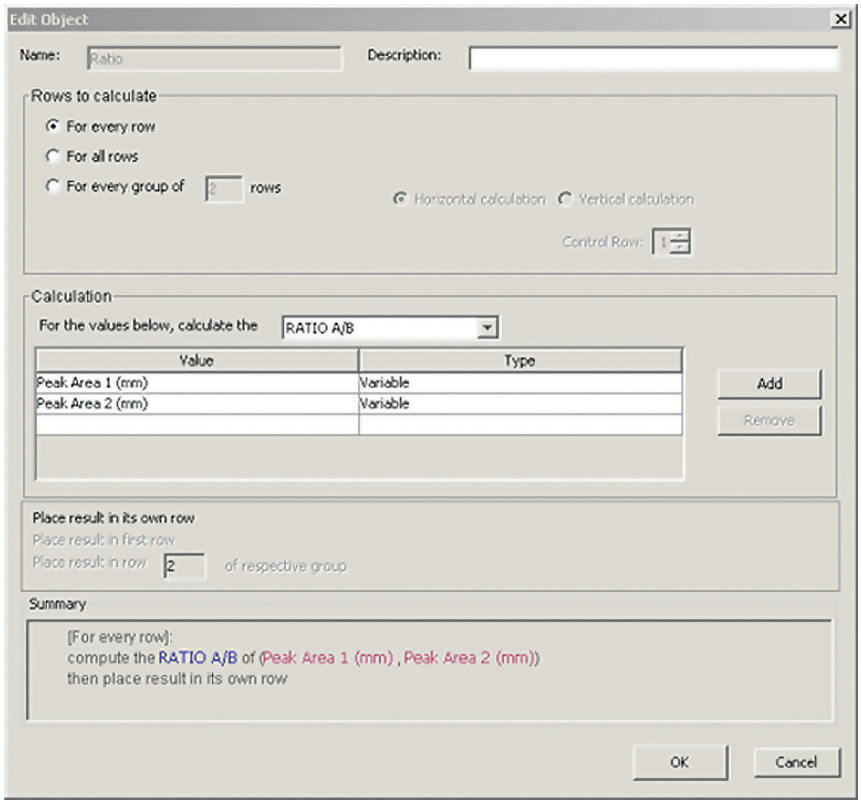
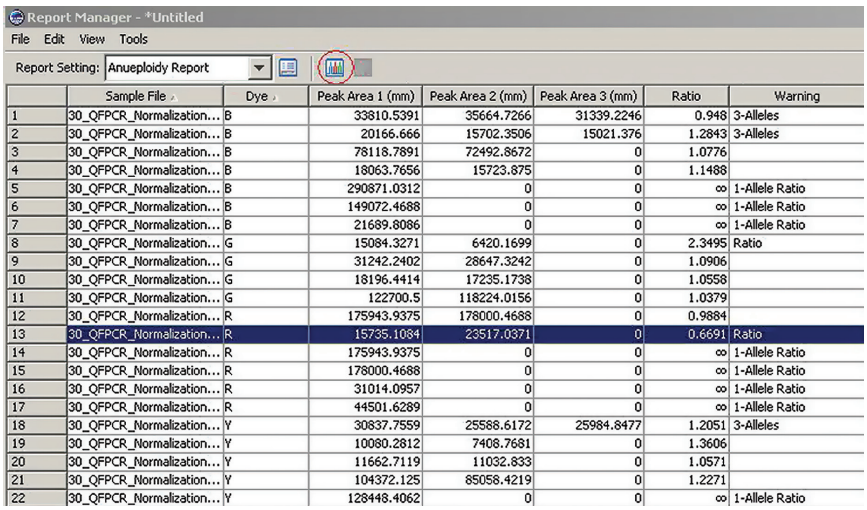



**Figure 4.** GeneMapper® Software Is Customizable. The Calculation option lets you specify custom calculations, such as ratios, averages, and sums.




| | Sample File | Dye | Peak Area 1 (mm) | Peak Area 2 (mm) | Peak Area 3 (mm) | Ratio | Warning |
|---|---|---|---|---|---|---|---|
| 1 | 30_QFPCR_Normalization... | B | 33810.5391 | 35664.7266 | 31339.2246 | 0.948 | 3-Alleles |
| 2 | 30_QFPCR_Normalization... | B | 20166.666 | 15702.3506 | 15021.376 | 1.2843 | 3-Alleles |
| 3 | 30_QFPCR_Normalization... | B | 78118.7891 | 72492.8672 | 0 | 1.0776 | |
| 4 | 30_QFPCR_Normalization... | B | 18063.7656 | 15723.875 | 0 | 1.1488 | |
| 5 | 30_QFPCR_Normalization... | B | 290871.0312 | 0 | 0 | ∞ | 1-Allele Ratio |
| 6 | 30_QFPCR_Normalization... | B | 149072.4688 | 0 | 0 | ∞ | 1-Allele Ratio |
| 7 | 30_QFPCR_Normalization... | B | 21689.8086 | 0 | 0 | ∞ | 1-Allele Ratio |
| 8 | 30_QFPCR_Normalization... | G | 15084.3271 | 6420.1699 | 0 | 2.3495 | Ratio |
| 9 | 30_QFPCR_Normalization... | G | 31242.2402 | 28647.3242 | 0 | 1.0906 | |
| 10 | 30_QFPCR_Normalization... | G | 18196.4414 | 17235.1738 | 0 | 1.0558 | |
| 11 | 30_QFPCR_Normalization... | G | 122700.5 | 118224.0156 | 0 | 1.0379 | |
| 12 | 30_QFPCR_Normalization... | R | 175943.9375 | 178000.4688 | 0 | 0.9884 | |
| 13 | 30_QFPCR_Normalization... | R | 15735.1084 | 23517.0371 | 0 | 0.6691 | Ratio |
| 14 | 30_QFPCR_Normalization... | R | 175943.9375 | 0 | 0 | ∞ | 1-Allele Ratio |
| 15 | 30_QFPCR_Normalization... | R | 178000.4688 | 0 | 0 | ∞ | 1-Allele Ratio |
| 16 | 30_QFPCR_Normalization... | R | 31014.0957 | 0 | 0 | ∞ | 1-Allele Ratio |
| 17 | 30_QFPCR_Normalization... | R | 44501.6289 | 0 | 0 | ∞ | 1-Allele Ratio |
| 18 | 30_QFPCR_Normalization... | Y | 30837.7559 | 25588.6172 | 25984.8477 | 1.2051 | 3-Alleles |
| 19 | 30_QFPCR_Normalization... | Y | 10080.2812 | 7408.7681 | 0 | 1.3606 | |
| 20 | 30_QFPCR_Normalization... | Y | 11662.7119 | 11032.833 | 0 | 1.0571 | |
| 21 | 30_QFPCR_Normalization... | Y | 104372.125 | 85058.4219 | 0 | 1.2271 | |
| 22 | 30_QFPCR_Normalization... | Y | 128448.4062 | 0 | 0 | ∞ | 1-Allele Ratio |

**Figure 5.** Sample Report Generated by GeneMapper® Software v4.1. Candidate markers flagged by warnings can be reviewed by selecting the plot view (indicated with a red circle). Examples of electropherograms in plot view can be seen in Figure 6.

indicate that normalization improves consistency of QF-PCR data and would facilitate the comparison of replicate samples between injections and between capillaries within the same injection.

## Conclusion

The advanced capabilities of the 3500 Series Genetic Analyzers, including new thermal control systems, enhanced optical detection, and new consumables designs, provide an easy-to-use platform for the detection and analysis

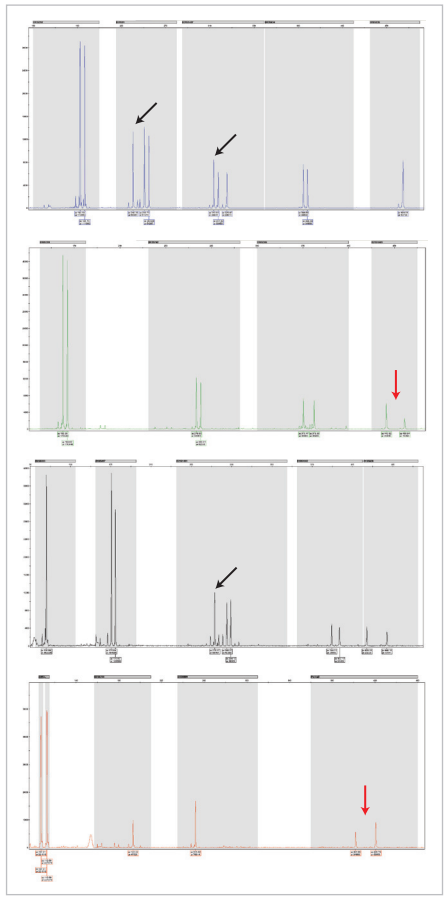


**Figure 6.** Electropherograms of Aneuploidy Markers Run on the 3500xL Genetic Analyzer, Separated by Dye Color. Triallelic loci are indicated by black arrows, and diallelic loci with non-normal peak area ratios are indicated by red arrows.

of multiplexed QF-PCR assays. The optional normalization reagent and compatible run module enable increased precision and accuracy in relative peak area or height determinations, which are particularly important for aneuploidy analysis. In addition, flexible GeneMapper® Software v4.1 can be configured to provide reports and calculations to give user-configured tools for reporting multiplexed QF-PCR assay results.


## Acknowledgment

Applied Biosystems would like to acknowledge the generous contribution of QF-PCR aneuploidy samples from Dr. Roland Achmann, MVZ genteQ, Hamburg, Germany, which were used to generate the data shown in this application note.

## ORDERING INFORMATION

| 3500 Series System Packages | | 3500 | 3500xL |
|---|---|---|---|
| **Package Name** | **Description** | **P/N** | **P/N** |
| 3500 Series Genetic Analyzer for Resequencing & Fragment Analysis | 3500 Series System with Data Collection Software, Sequencing Analysis, Variant Reporter, and GeneMapper® Software packages. System package also includes DNA Sequencing and Fragment Analysis reagent kits for system qualification. | 4440462 | 4440463 |
| 3500 Series Genetic Analyzer for Resequencing & Fragment Analysis With SAE | 3500 Series System with Data Collection Software (includes additional functionality for Security, Audit Trail, and Electronic Signature (SAE) capabilities), Sequencing Analysis, Variant Reporter, and GeneMapper® Software packages. System package also includes DNA Sequencing and Fragment Analysis reagent kits for system qualification. | 4440464 | 4440465 |
| 3500 Series Genetic Analyzer for Resequencing | 3500 Series System with Data Collection Software, Sequencing Analysis, and Variant Reporter Software packages. System package also includes DNA Sequencing reagent kits for system qualification. | 4440466 | 4440467 |
| 3500 Series Genetic Analyzer for Fragment Analysis | 3500 Series System with Data Collection Software and GeneMapper® Software packages. System package also includes DNA Fragment Analysis reagent kits for system qualification. | 4440468 | 4440469 |
| 3500 Series Genetic Analyzer for Sequence Typing & Fragment Analysis | 3500 Series System with Data Collection Software, Sequencing Analysis, SeqScape Software, and GeneMapper® Software packages. System package also includes DNA Sequencing and Fragment analysis reagent kits for system qualification. | 4440470 | 4440471 |

### System Consumables and Reagents

| Description | P/N |
|---|---|
| 3500xL Capillary Array (50 cm) | 4404689 |
| 3500 Capillary Array (50 cm) | 4404685 |
| 3500 POP-7™ Polymer (960 samples) | 4393714 |
| 3500 POP-7™ Polymer (384 samples) | 4393708 |
| Anode Buffer Container (ABC) 3500 Series | 4393927 |
| Cathode Buffer Container (CBC) 3500 Series | 4408256 |
| Septa Cathode Buffer Container 3500 Series | 4410715 |
| Conditioning Reagent 3500 Series | 4393718 |
| Hi-Di™ Formamide (5 mL) 1 bottle | 4401457 |
| BigDye® Terminator v3.1 Cycle Sequencing Kit (1,000 rxns) | 4337456 |
| BigDye® XTerminator™ Purification Kit (1,000 rxns) | 4376487 |

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**FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.**

For those who require IVD-marked devices, the 3500 Dx and 3500xL Dx Genetic Analyzers and system accessories meet the requirements of the In Vitro Diagnostic Medical Devices Directive (98/79/EC). The 3500 Dx and 3500xL Dx systems are for distribution and use in specific European countries only. For more information about the 3500 Dx Series Systems, contact your Applied Biosystems representative.

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Applied Biosystems

**Headquarters**
850 Lincoln Centre Drive | Foster City, CA 94404 USA
Phone 650.638.5800 | Toll Free 800.345.5224
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