Relative Fluorescent Quantitation on Capillary Electrophoresis Systems:

Screening for Loss of Heterozygosity in Tumor Samples on the Applied Biosystems 3130 Series Genetic Analyzers with GeneMapper® Software v3.7

Introduction

A variety of capillary electrophoresis-based fragment analysis applications require peak-height comparisons across samples as a relative quantitation method. Some of these applications include screening for loss of heterozygosity using microsatellites or Single Nucleotide Polymorphisms (SNPs), aneuploidy assays, and detection of large chromosomal deletions. The success of relative fluorescent quantitation depends on having optimized assays, a robust and sensitive capillary electrophoresis (CE) platform, and accurate analysis software.

In this Application Note, Loss of Heterozygosity (LOH) screening with microsatellite markers will be used to highlight the 3130 Series Genetic Analyzers, in combination with GeneMapper® Software v3.7, as a complete solution for relative fluorescent quantitation.

The LOH Assay

In the two-hit model used to describe inactivation of tumor suppressor genes, the first mutation or "hit" results in a heterozygous state for the tumor suppressor gene with one wild-type allele and one mutant allele. If a second hit (deletion) follows, the result is the loss of the wild-type allele, also known as LOH.[1] LOH has been shown to occur in different chromosomal regions, and LOH analysis can be useful for tumor screening, and for the detection of regions that contain tumor supressor genes.[2]

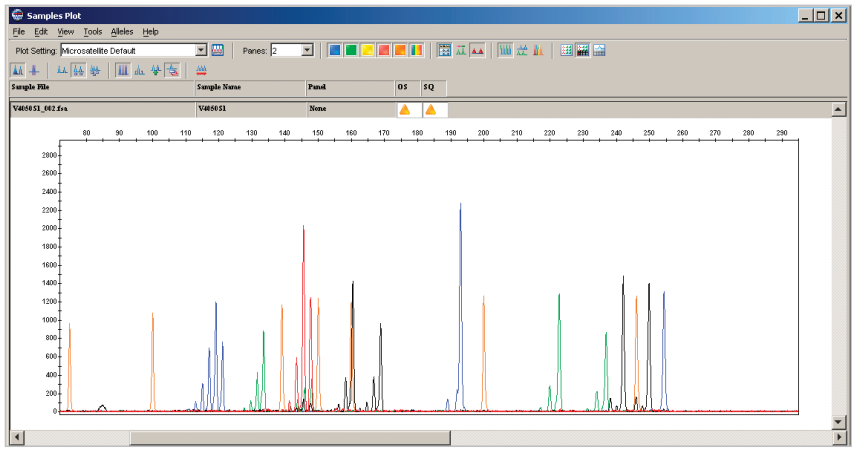

Figure 1. Five-dye electropherogram of microsatellite markers run on the 3130 Series System.

Since LOH can be caused by deletion of genomic DNA regions containing the normal copy of tumor suppressor genes, researchers can use microsatellite markers to screen tumor samples for LOH. After the appropriate markers have been identified for the region of interest, fluorescently labeled primers can be used in multiplex PCR-based microsatellite assays and on genomic DNA that has been isolated from healthy and tumor samples (Figure 1). Custom primers labeled with appropriate fluorophores can be purchased from Applied Biosystems for use in microsatellite assays.

The 3130 Series Genetic Analyzers

The second step in LOH screening involves the separation and detection of PCR products. The 3130 Series Systems are fully automated, high-performance, fluorescence-based, multi-capillary systems that can process multiple samples simultaneously. The 3130 Genetic Analyzer can process 4 samples, and the 3130*xl* Genetic Analyzer can process 16 samples simultaneously. Sample analysis on these instruments is fully automated from the moment each 96- or 384-well plate is placed on the instrument and the run is initiated.

The system provides continuous, unattended operation from sample loading, automated polymer loading, and sample injection, to separation, detection, and

data generation. Because the scarcity of clinical research samples is a critical factor that can affect the success of an LOH assay, it is important to use a robust, reliable, and sensitive platform, such as the 3130 Series Systems.

The 3130 Series Systems also contain several features, such as an Automated Polymer Delivery System that significantly reduces set-up time, easy-to-use wizards for instrument operation and maintenance, and enabling 3130 POP-7™ Polymer. The use of 3130 POP-7 Polymer and a detection cell heater that facilitates better thermal control offer additional advantages such as high-resolution peaks, superior sizing precision (Figure 2), and decreased run time, which, in turn results in faster turnaround times.

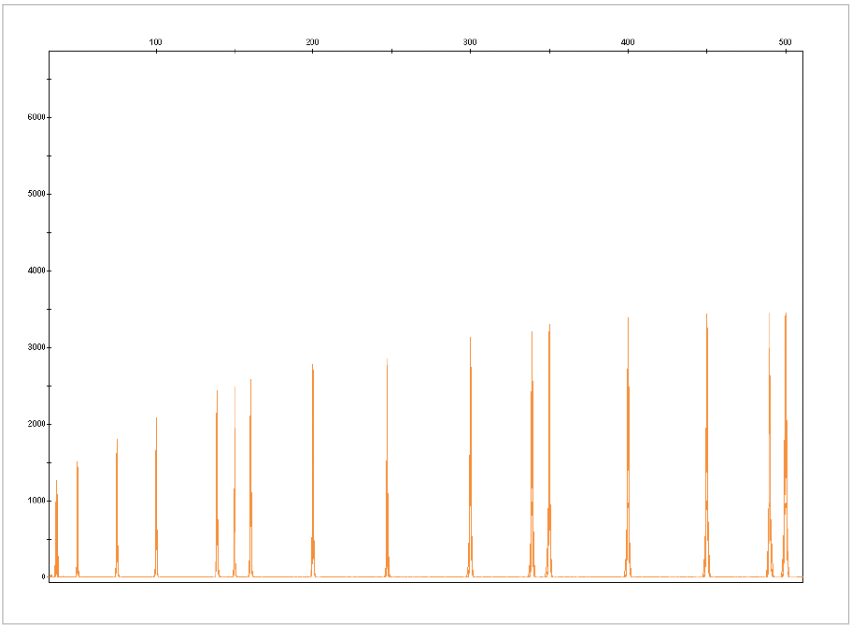

Figure 2. Overlay of 16 GeneScan™-500 LIZ® Size Standard electropherograms run on a 3130*xl* Genetic Analyzer using 3130 POP-7 Polymer.

Data Analysis Using GeneMapper® Software v3.7

Analysis software that can accurately and automatically score samples for LOH is critical for the successful and efficient completion of the assay. In addition to advanced algorithms that recognize and filter amplification chemistry artifacts, such as "Plus A" and stutter peaks, GeneMapper software now contains a convenient, new Report Manager feature that can be used to perform calculations for relative fluorescent quantitation using peak heights across samples (Figure 3).

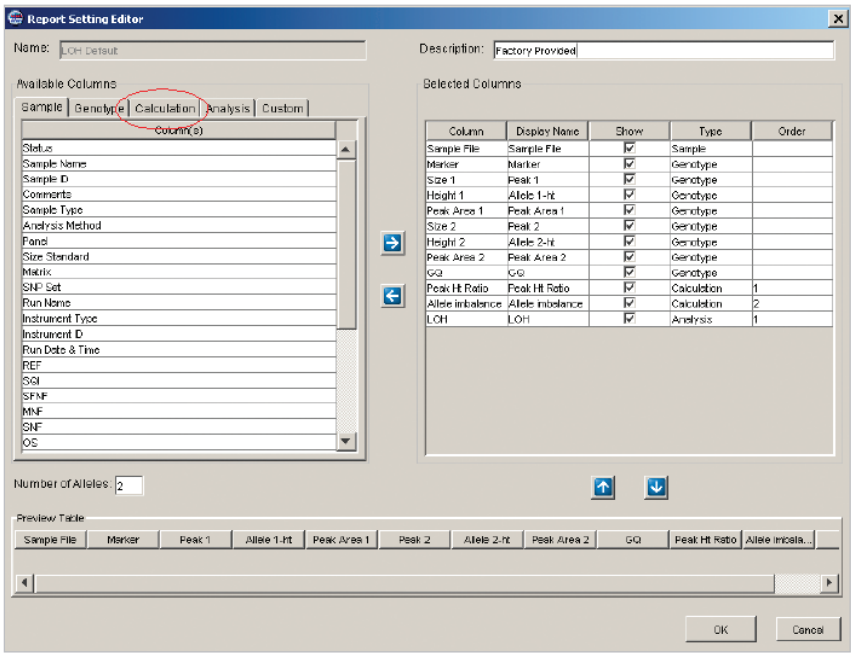

Figure 3. The Report Manager in GeneMapper® Software v3.7 lets you perform custom calculations and analysis for relative fluorescent quantitation.

In a typical LOH assay, samples from healthy tissue are compared to samples from tumor tissue for every marker. In almost any micro-dissection procedure used for tumor-sample isolation, contaminating healthy cells are present, and, thus, some wild-type DNA is also present in the tumor specimen. As a result, some of the wild type allele will be amplified from the healthy cells. Researchers can set a peak-height ratio threshold in the Report Manager feature, based on the observed level of wild-type DNA contamination in their tumor samples.

The Report Manager feature can be then used to specify the typical multi-step calculations that are necessary to determine values for peak-height ratios, and perform a final analysis to identify LOH candidates. Any

	Sample Name	Analysis Me...	Size Standard	Allele 1	Height 1	Allele 2	Height 2	Peak Height ...	LOH	LOH candid...
	LM1	Microsatellite...	GS500(-250)...	162	687	171	483	1.4223603		
	UM1	Microsatellite...	GS500(-250)...	162	454	171	186	2.4408603	1.7160633	Candidate

Figure 4. Report generated by GeneMapper Software v3.7. Candidate LOH samples can be flagged for further review.

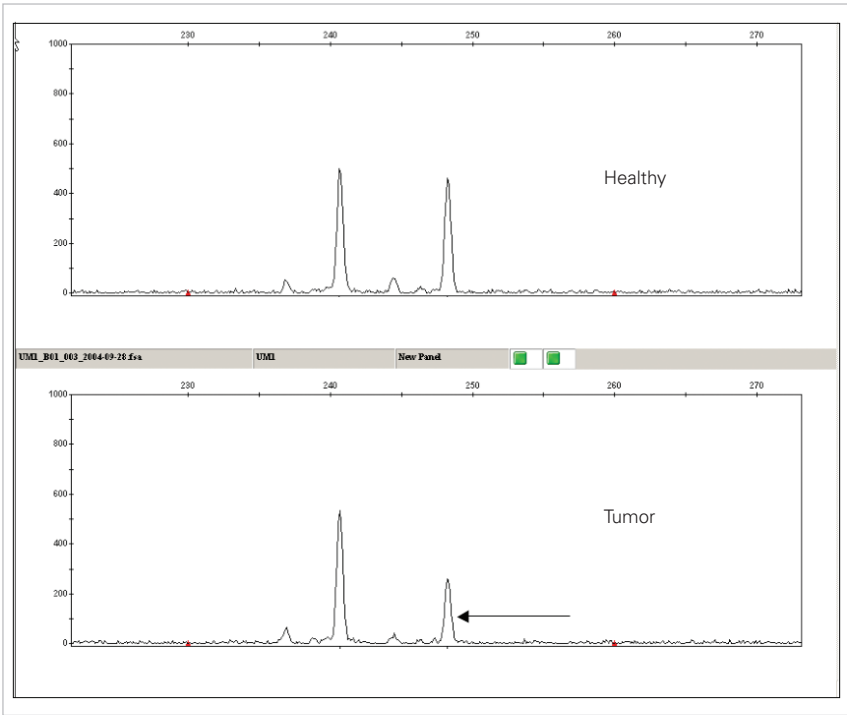

Figure 5. Electropherogram of a microsatellite marker in DNA from healthy (top panel) and tumor sample #2 (bottom panel).

sample above the threshold can be flagged as an LOH candidate and further reviewed in GeneMapper software. Customized calculations can be specified for LOH or any type of relative fluorescent quantitation, and when these have been specified, the calculation will be automatically applied across an entire sample set. After the analysis is complete, an easy-to-read results report can be printed. If necessary, the report can be exported for further analysis.

Superior Data Quality

To demonstrate an LOH assay on the 3130 Series Systems, genomic DNA, isolated from paired healthy and tumor tissue samples, for LOH using microsatellite markers. The microsatellite markers were co-amplified using fluorescently labeled forward primers and unlabeled reverse primers. After completion of the assay, the products were combined with the GeneScan™-500 LIZ® Size Standard

Step 1 Calculate the peak height ratio for the two alleles for both normal and tumor sample in the Report Manager (Figure 7) using the following formula:

$$\text{Peak Height ratio} = \frac{\text{Peak Height of allele 1}}{\text{Peak Height of allele 2}}$$

Step 2 Calculate the value for LOH using the values in Step 1 and the formula shown below. You can specify the row in which the control healthy sample is present as shown in Figure 8.

$$\text{LOH candidate} = \frac{\left\{\dfrac{\text{Peak Height of allele 1 in tumor sample}}{\text{Peak Height of allele 2 in tumor sample}}\right\}}{\left\{\dfrac{\text{Peak Height of allele 1 in healthy sample}}{\text{Peak Height of allele 2 in healthy sample}}\right\}}$$

Step 3 Flag the samples as LOH candidates using the threshold value you specify. For example, if you determine that samples with LOH values greater than 1.5 should be flagged, specify it in the Report Manager as shown in Figure 9. GeneMapper software can now use these calculations to generate a Final Results report as shown in Figure 4.

Figure 6. Multi-step, peak-height calculations shown step-by-step.

and electrophoresed on the 3130 Genetic Analyzer, using a 36 cm capillary array and 3130 POP-7™ Polymer. The protocol used was the FragmentAnalysis36_POP-7 run module in combination with the G5 dye set.

The data shown in Figure 1 highlights the well-resolved peaks that were generated for all markers on the 3130 systems. For each of the markers shown, the Report Manager feature was used to calculate peak-height ratios as well as LOH values, and a final report was generated as shown in Figure 4. Sample 2 was flagged as an LOH candidate in the Report Manager. Manual review of the electropherogram confirmed a reduced peak height for allele 2 in the tumor sample (Figure 5).

Using the Report Manager to Specify Calculations for Peak-Height Ratio

Described in Figure 6 is a step-by-step procedure (Figure 7, 8, and 9) for using the Report Manager to specify customized multi-step, peak-height calculations that can be used to perform relative fluorescent quantitation and generate reports as shown in Figure 4. It should be noted that these calculations need to be specified only once, as they can be saved and applied automatically to data generated in subsequent assays.

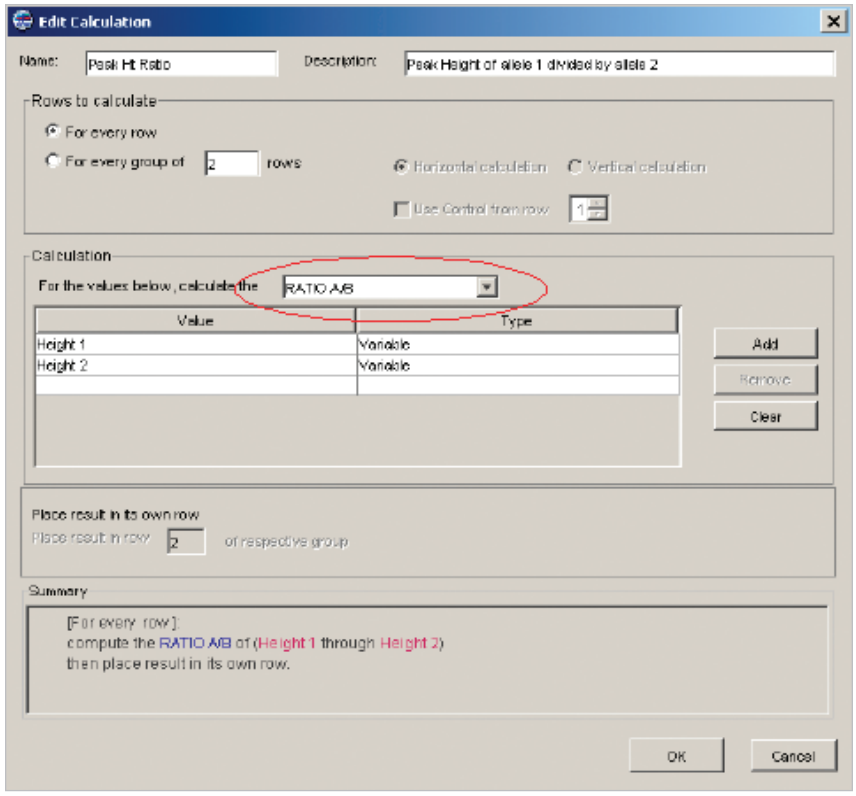

Figure 7. The Calculations option lets you specify custom calculations, such as ratios, averages, and sums.

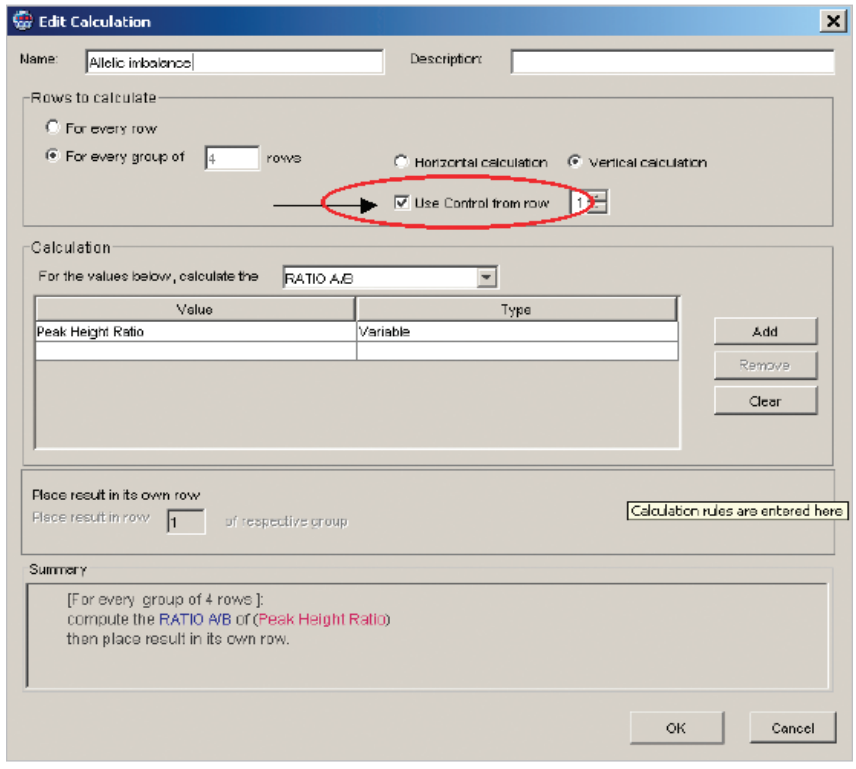

Figure 8. Values for LOH candidates can be determined using the Calculations tab.

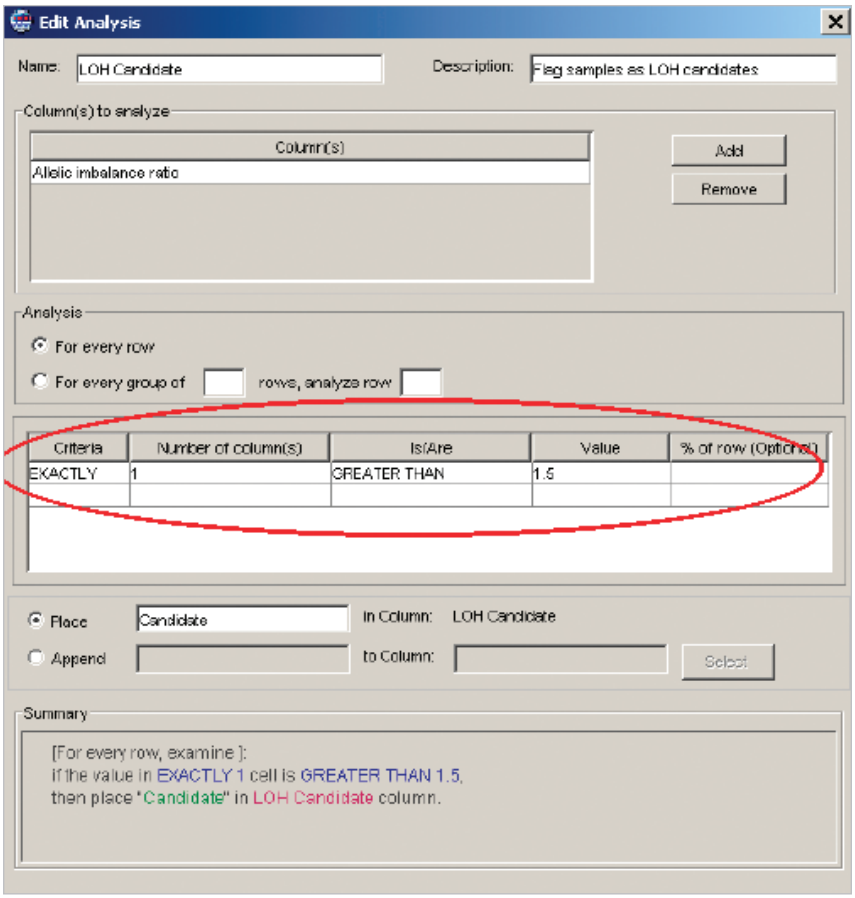

Figure 9. Final analysis can be performed to identify LOH candidate samples.

Conclusion

The Applied Biosystems 3130 Series Genetic Analyzers, in conjunction with GeneMapper Software v3.7, provide an optimal solution for performing routine relative fluorescent quantitation assays. These instruments can be used for both small- and large-scale studies. The integration of these instruments with GeneMapper software provides a complete system for electrophoresis, data collection, fragment size calling, and the final scoring of samples for LOH. Together, these features enable the generation of abundant amounts of high-quality data with minimal hands-on time and streamlined data review.

Acknowledgement

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References

[1] Grundei, T., Vogelsang, H., Ott, K., Mueller, J., Scholz, M., Becker, K., Fink, U., Siewert, J.R., Hofler, H., and Keller, G. 2000. Loss of heterozygosity and microsatellite instability as predictive markers for neoadjuvant treatment in gastric carcinoma. *Clinical cancer research* 6:4782-4788.

[2] Canzian, F., Salovaara, R., Hemminki, A., Kristo, P., Chadwick, R.B., Aaltonen, L.A., and de la Chapelle, A. 1996. Semiautomated assessment of loss of heterozygosity and replication error in tumors. *Cancer Research* 56:3331–3337.

Ordering Information

Description	P/N
3130*xl* and 3100 Capillary Array (36 cm)	4315931
3130 and 3100-*Avant* Capillary Array (36 cm)	4333464
3130 POP-7™ Polymer	4352759
10X Genetic Analyzer Buffer with EDTA	402824
Hi-Di™ Formamide	4311320
GeneScan®-500 LIZ® Size Standard	4322682
Matrix Standard Set DS-33	4323016
Custom Primers	www.appliedbiosystems.com*
GeneMapper® Software v3.7	www.appliedbiosystems.com*

**Or contact your sales representative*

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