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TECHNICAL NOTE

Evaluation of a decreased cycle number (28 cycles) and decreased injection time (5 seconds) when the GlobalFiler™ PCR Amplification Kit is run on a 3130xl Genetic Analyzer

The performance of the GlobalFiler PCR Amplification Kit (PN 4476135 and 4482815) run on the 3130xl Genetic Analyzer was recently reassessed. Internal testing shows that, depending on instrument sensitivity, samples amplified with the GlobalFiler kit and run under default HID injection conditions on the 3130xl can demonstrate off-scale peak heights for homozygous peaks when greater than or equal to 1 ng of DNA is targeted. This elevated peak height and the resulting spectral artifacts may impact data interpretation. In order to produce data that is within the linear dynamic range of the 3130xl instrument in this situation, a decreased PCR cycle number and a decreased injection time were evaluated and compared to the default PCR cycle number and injection time conditions.

The following summarizes the internal studies executed at Thermo Fisher Scientific to verify the performance of the GlobalFiler kit at 28 and 29 cycles and injected on the 3130xl at 3 kV for 10 and 5 seconds.

Summary of Internal Studies

- Three DNA samples (2 male, 1 female), positive control DNA 007, and GlobalFiler Allelic Ladder were processed on a single 3130xl Genetic Analyzer with Data Collection v4.0 with 6-Dye Module v1.
- Amplification was performed at 28 and 29 PCR cycles with the GlobalFiler kit. A sensitivity series was prepared and total DNA inputs of 2, 1, 0.5, 0.25, 0.125, and 0.063 ng were targeted. The data from the 1ng and the 0.125 ng samples are presented.
- The injection of the 28 and 29 PCR cycle data was performed at 3kV for 10 and 5 seconds.
- The Peak Amplitude Threshold (PAT) was set at 50 RFU for all studies.
- Performance was evaluated based on the following criteria:
    - Intra-color balance (ICB) ≥ 40%
    - Allele peak height ratio or intra-locus balance (ILB) ≥ 70%
    - No drop-out for the 0.125 ng DNA input samples

Experiments Performed

A. **Effect of injection time on signal intensity**: Ten replicate runs of the GlobalFiler Allelic Ladder were run at 3kV for 10 and 5 second injection times on the 3130xl.

Summary of Results – Effect of injection time on signal intensity

The channel-average peak height intensities of the GlobalFiler allelic ladder runs at 10 second and 5 second injection times are shown in Figure 1 and Table 1. Signal intensities decreased by an average of 43% when the injection time was decreased from 10 seconds to 5 seconds.

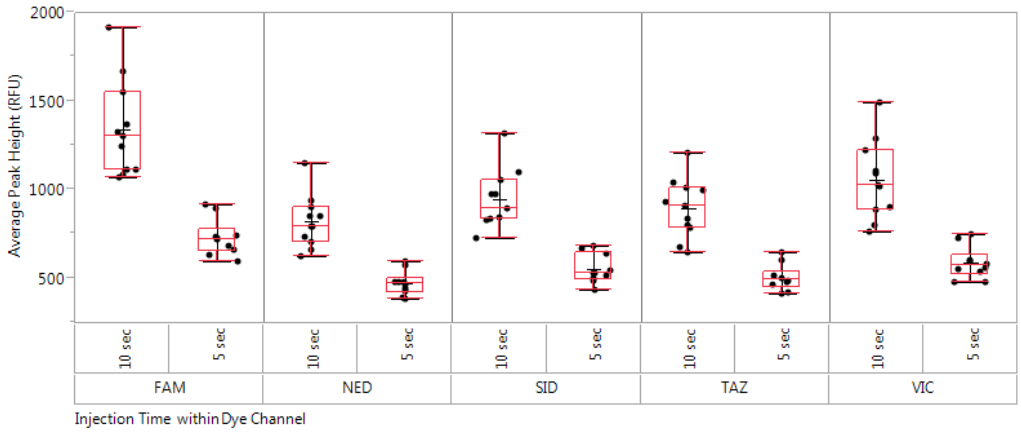


Figure 1. Channel-average allelic ladder peak heights from 10 replicate runs each at 10-second and 5-second injection times.

Table 1. Change in the channel-average signal intensity of the GlobalFiler allelic ladder upon decrease of the injection time from 10 seconds to 5 seconds. The % change is relative to the signal at 10 seconds.

Channel	Average peak height				% change
	10-seconds	σ_{N-1}	5-seconds	σ_{N-1}	
FAM	1345	272	734	104	45%
VIC	1059	220	591	89	44%
NED	824	148	475	69	42%
TAZ	899	167	507	74	44%
SID	949	167	557	83	41%
Average	1015	203	573	101	43%

B. Effect of changing the number of PCR cycles on signal intensity: The effect of changing the number of PCR cycles on signal intensity was evaluated using DNA samples F1, M1, and positive control at 1 ng/reaction in GlobalFiler STR assays with 28 and 29 PCR cycles. The injection time was set to 10 seconds for all three samples. Three replicates of samples F1 and M1 were run at both PCR cycling conditions. There were 6 replicate runs of the positive control at 28 cycles, and 9 replicate runs at 29 cycles.

Summary of Results – Effect of PCR cycles on signal intensity

The channel-average peak height intensities of the three DNA samples are shown in Table 2. There is DNA-to-DNA differences in the relative change; F1 and M1 were more impacted by reduction in the number of cycles than the positive control, showing an average relative decrease in the average peak height of 33% and 38% respectively, while the positive control showed an average relative decrease of 26%.

Table 2. Change in the channel-average signal intensity of PCR fragments from the GlobalFiler assay of three DNA samples at 28 and 29 cycles; the injection time was kept constant at 10 seconds. The % change is relative to the signal at 29 cycles.

Sample	Channel	Average peak height				% change
		28 cycles	σ_{N-1}	29 cycles	σ_{N-1}	
F1	FAM	2408	502	3544	692	-32%
	VIC	3257	516	4684	292	-30%
	NED	1987	250	3095	125	-36%
	TAZ	2641	276	3933	293	-33%
	SID	2145	281	3205	489	-33%
	Average	2488	498	3692	644	-33%
M1	FAM	2051	485	3346	599	-39%
	VIC	2321	346	3598	370	-35%
	NED	2109	207	3872	553	-46%
	TAZ	2597	590	4095	635	-37%
	SID	2003	377	2956	325	-32%
	Average	2216	245	3573	446	-38%
POS	FAM	3315	382	4472	632	-26%
	VIC	2902	382	3986	551	-27%
	NED	1873	183	2871	244	-35%
	TAZ	3817	485	4851	667	-21%
	SID	2530	376	3310	495	-24%
	Average	2887	742	3898	813	-26%

C. **Comparison of the ICB and ILB at 28 and 29 PCR cycles, and 10 and 5 second injection times**: Three reference DNA samples (2 male, 1 female) with known genotypes and the positive control 007 DNA were tested in this study.

Assay performance was tested at 1 ng of input DNA for all samples and 0.125 ng of input DNA for the three reference DNA samples.

The ICB is calculated as the ratio of the minimum average peak height within a dye channel to the maximum average peak height within the same dye channel. It provides an indication of the marker-to-marker color balance within a dye channel. The target specification is an ICB ≥ 40%.

For a particular dye channel,

$$\textbf{ICB} = \frac{\text{MIN (Average peak height)}}{\text{MAX(Average peak height)}} X100$$

Average peak height at a marker is genotype dependent and calculated according to the formulas below.

Heterozygous allele:

$$\text{Average peak height} = \frac{\text{Peak height(allele1)} + \text{Peak height(allele2)}}{2}$$

Homozygous allele:

$$\text{Average peak height} = \frac{\text{Peak height(allele1)}}{2}$$

The ILB (also referred to as peak height ratio) provides a measure of the balance between alleles within a locus. The target specification is the peak height ratio between 2 alleles of the same marker must be ≥ 70%. The ILB is calculated as the ratio of the minimum peak height to maximum peak height between two heterozygous alleles, as shown in the calculation below.

$$\textbf{ILB for each marker} = \frac{\text{MIN (Allele 1, Allele 2)}}{\text{MAX(Allele 1, Allele 2)}} X100$$

In the example shown in Table 2, the ILB values were calculated from positive control data in the VIC dye channel.

Table 3. Calculation of the ILB for the VIC channel from DNA positive control data at 1 ng load, 29 cycles PCR, and 10-second injection time.

Marker	Allele 1	Allele 2	Peak Height 1 (RFU)	Peak Height 2 (RFU)	Average peak height (RFU)	ILB
Yindel	2	2	4212	4212	2106	100%
AMEL	X	Y	5331	4728	5030	89%
D8S1179	12	13	4443	4525	4484	98%
D21S11	28	31	3827	3760	3794	98%
D18S51	12	15	4353	3984	4169	92%
DYS391	11	11	5249	5249	2625	100%

The ICB for the VIC dye channel is the ratio of the minimum to the maximum average peak heights of AMEL, D8S1179, D21S11, and D18S51. Using the data in Table 2,

ICB = 3794/5030 = 0.754 (75%)

In the calculation of the VIC dye channel ICB, the Y-indel and DYS391 peak heights are excluded because these markers are haploid markers, and will typically give lower average signal intensities relative to other markers in the VIC dye channel.

Summary of results – Intra-color balance (ICB) and intra-locus balance (ILB) at 28 and 29 PCR cycles, and 10 and 5 second injection times:

At 1 ng of total input DNA, the ICB does not change significantly between 28 and 29 cycles, at 5 and 10 second injection times in all the DNA samples tested (Figures 2 – 5).

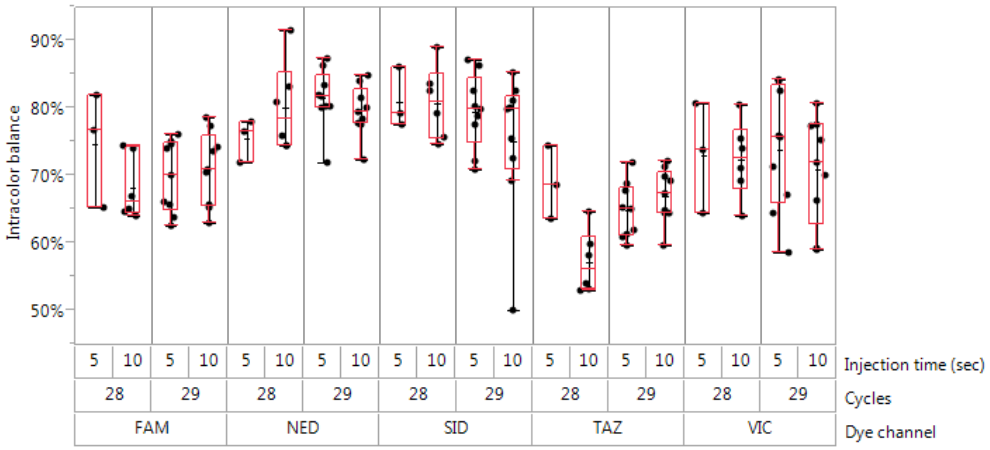


Figure 2. ICB of the positive control DNA sample at 1 ng load. The data includes 28 and 29 cycles and 5 and 10 second injection times. The ICB at all dye channels meets the target specification, and there is no significant difference between these values at all conditions tested.

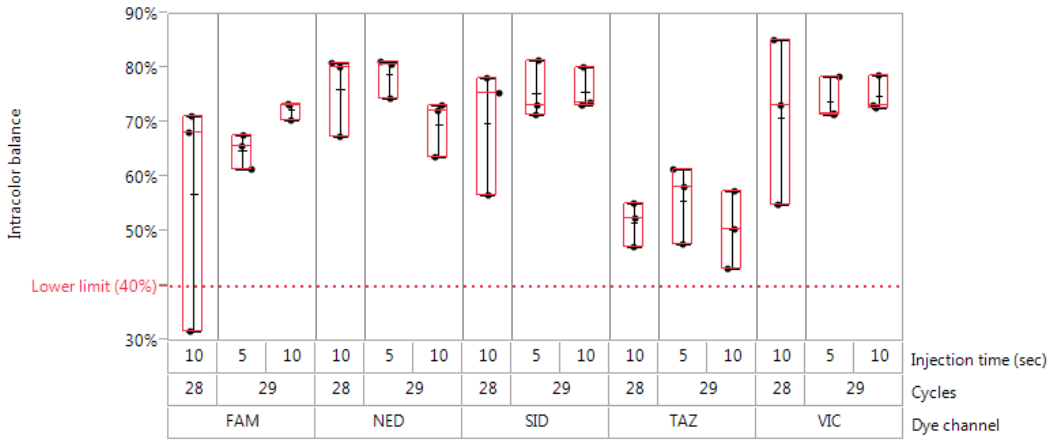


Figure 3. ICB of the male sample M1 at 1 ng load. The data includes 28 cycles/10 second injection time and 29 cycles/5 and 10 second injection times. There is one outlier at 28 cycles/10 seconds in the FAM™ dye channel; however the average of the three replicates is greater than the target specification.

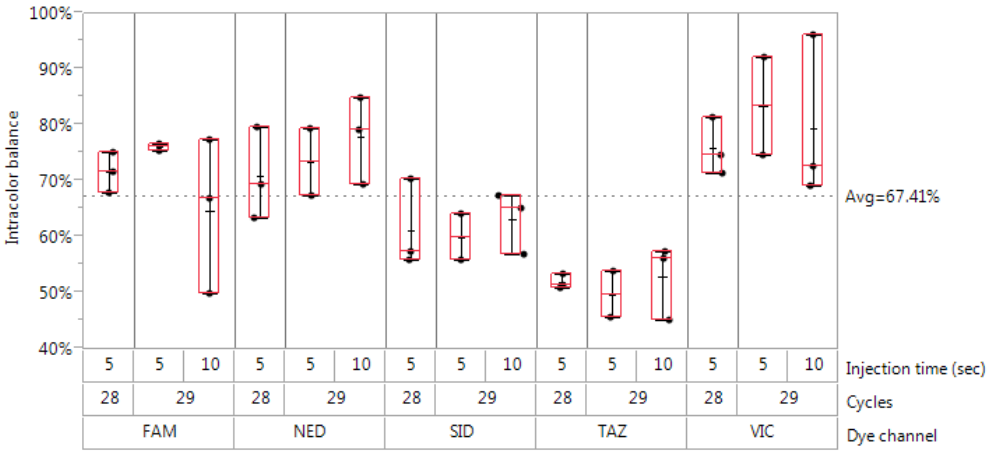


Figure 4. ICB of the male sample M2 at 1 ng load. The data includes 28 cycles/5 second injection time and 29 cycles/5 and 10 second injection times. The ICB at all dye channels meets the target specification, and there is no significant difference between these values at all conditions tested.

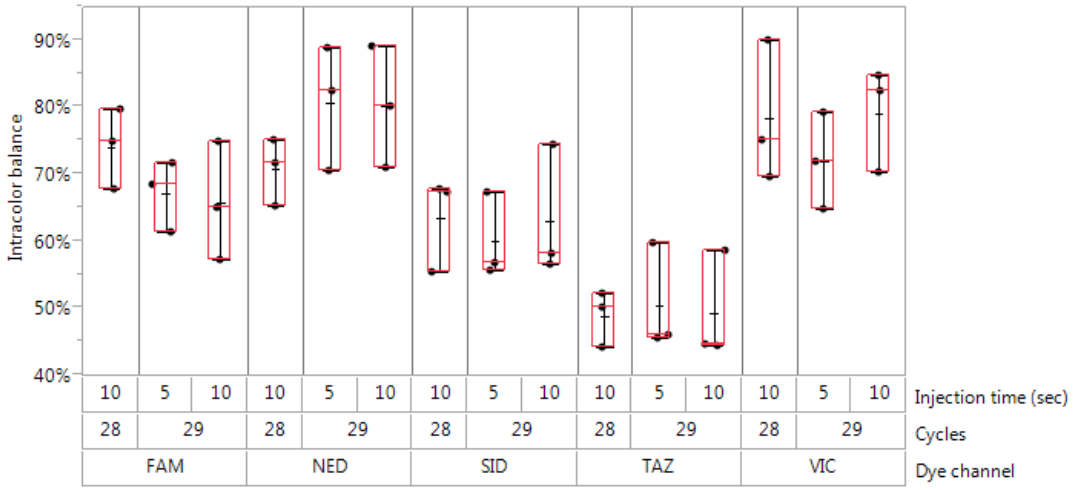


Figure 5. ICB of the female sample F1 at 1 ng load. The data includes 28 cycles/10 second injection time and 29 cycles/5 and 10 second injection times. The ICB at all dye channels meets the target specification, and there is no significant difference between these values at all conditions tested.

Figures 6 – 9 show the ILB for the four DNA samples tested in this study. At 1 ng of total input DNA, the ILB does not change significantly when reducing the number of cycles from 29 to 28 or the injection time from 10 to 5 seconds.

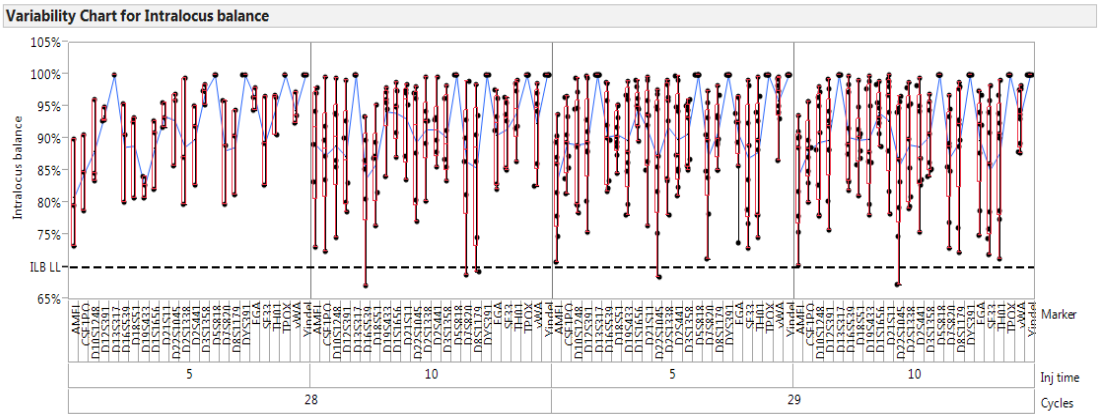


Figure 6. ILB of the positive control 007 DNA at 1 ng input. Some replicates have outlier points with ILB <70%. However, the average ILB is > 70% and met the performance criteria specified. Similar observations were made for the two male samples (M1, M2) and the female sample (F1) in this study.

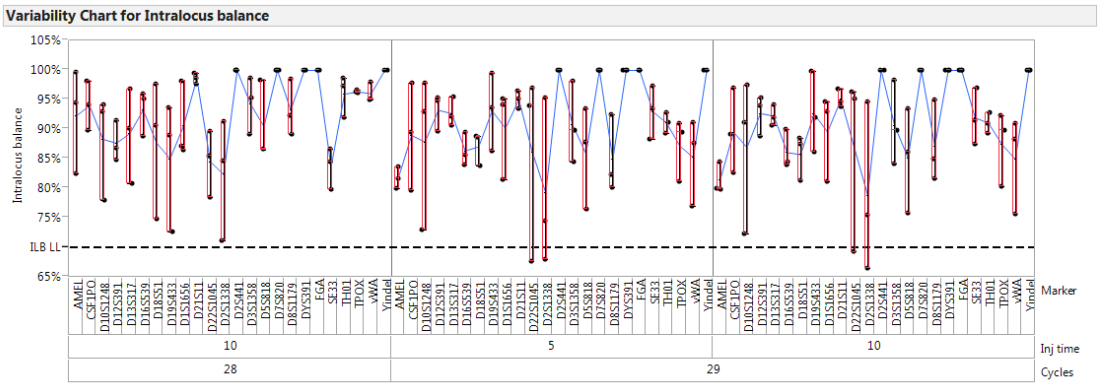


Figure 7. ILB of the male sample M1 at 1 ng input. The average ILB is > 70%

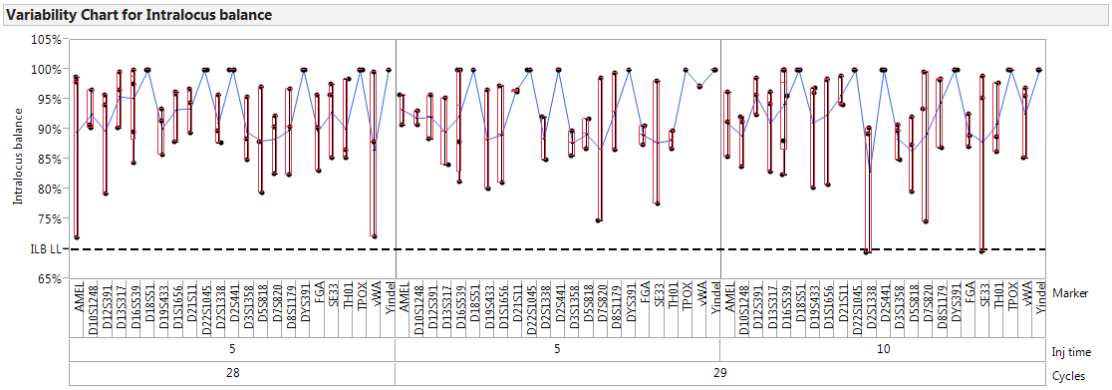


Figure 8. ILB of the male sample M2 at 1 ng input. The average ILB is > 70%.

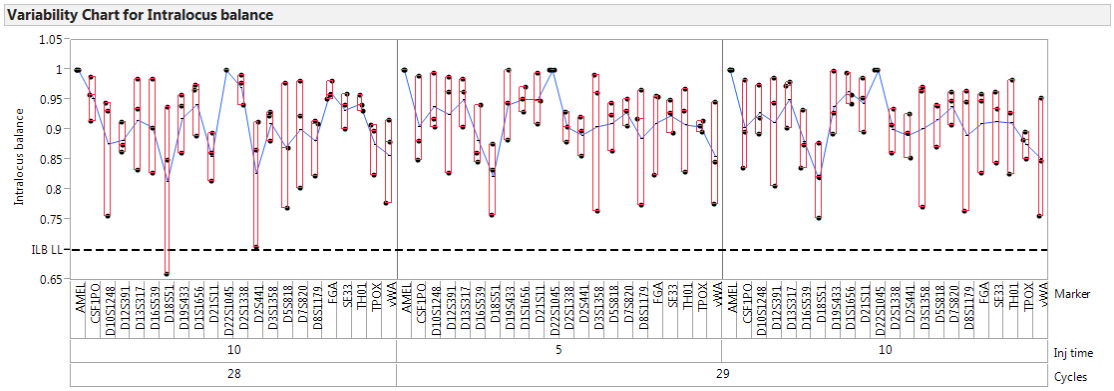
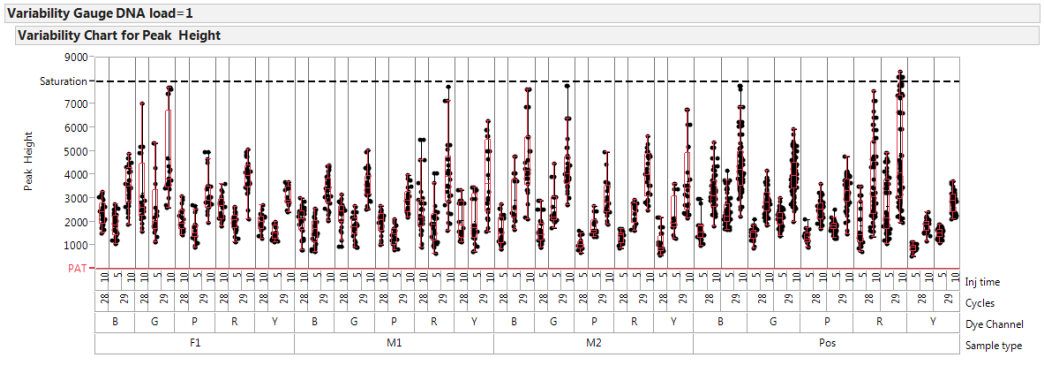


Figure 9. ILB of the female sample F1 at 1 ng input. The average ILB is > 70%.

D. **Combined effect of changing the number of cycles and injection time on CE signal intensity:** Data from the entire sensitivity series (2, 1, 0.5, 0.25, 0.125, and 0.063 ng of DNA) were evaluated for the three reference samples at 28 and 29 PCR cycles and with 10 and 5 second injection times. With 2 ng DNA input, the GlobalFiler assay showed high signal intensities under all conditions tested such that the peak heights of several markers were off-scale. At 0.063 ng of DNA, some of the replicates showed partial profiles (PAT = 50 RFU) at both 28 and 29 cycles and at both 5 and 10 second injection times. The data analysis described below focuses on the 1 ng and 0.125 ng DNA input samples.

Summary of Results – Combined effect of changing PCR cycle number and inject time on CE signal intensity

A variability gauge analysis was performed in this study. This is a method of data analysis designed to evaluate the relative contributions of changes in the assay parameters (cycle number and injection time) to the total variation in the results. All four of the DNA samples (3 reference and positive control 007) were included for the 1ng DNA input at 28 and 29 PCR cycles and 10 and 5 second injection times (Figure 10). The same analysis was done for the three reference samples at 0.125ng DNA input, 28 and 29 PCR cycles, and 10 and 5 second injection times (Figure 11). As indicated below, in both DNA template concentrations, a 5 second reduction in injection time has a greater contribution to signal decrease than reducing the number of PCR cycles by one. In other words, there is a more significant decrease in the signal intensity by decreasing the injection time to 5 seconds while keeping 29 PCR cycles, compared to decreasing the number of PCR cycles to 28, while keeping the injection time at 10 seconds.



Data was unbalanced, so a REML Fit was performed.

Variance Components

Component	Var Component	% of Total	20 40 60 80	Sqrt(Var Comp)
Sample type	86601.2	2.8		294.3
Dye Channel	192306.9	6.2		438.5
Cycles	517418.5	16.7		719.3
Inj time	1401349.7	45.1		1183.8
Within	909903.0	29.3		953.9
Total	3107579.3	100.0		1762.8

Figure 10. Variability gauge analysis at 1ng DNA input for all four DNA samples. Injection time has a more significant effect on signal intensity (45%) than changing the number of PCR cycles from 29 to 28 (17%). Some of the positive control peaks are off-scale at 29 PCR cycles/10 second injection. The peak amplitude threshold (PAT) was set to 50 RFU.

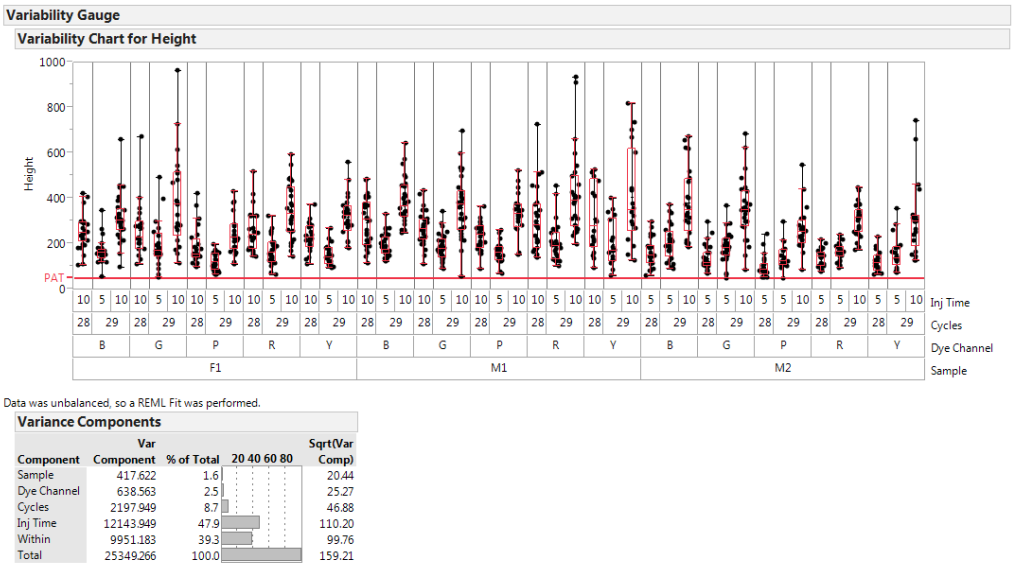


Figure 11. Variability gauge analysis at 0.125ng DNA for the three reference samples. The results are very similar to that obtained at 1ng DNA load. The injection time has a more significant effect on signal intensity (48%) than changing the number of PCR cycles from 29 to 28 (9%). The peak amplitude threshold (PAT) was set to 50 RFU.

The correct allele calls were made for all four DNA samples at 1ng DNA load. 27 runs were generated from the three samples using a 0.125ng DNA input. Within this sample set there were two instances, of allele dropout where the signal intensities were less than 50 RFU. These instances both occurred at 28 cycles with a 5 second injection time (one was SE33 in the female sample, and the other D16S539 in the second male sample),

Conclusions

For 1 ng and 0.125 ng DNA input amounts, this study showed that there is a more significant decrease in the signal intensity by changing the injection time on the 3130xl Genetic Analyzer from 10 seconds to 5 seconds rather than decreasing the number of PCR cycles from 29 to 28.

In the four DNA samples tested, there is no apparent negative impact of reducing the number of cycles from 29 to 28 or the injection time of the 3130xl Genetic Analyzer from 10 seconds to 5 seconds on the ICB or the ILB of the 1 ng DNA template samples.

Instrument-to-instrument variability exists and some laboratories will have capillary electrophoresis instruments with higher sensitivity. Changing the injection time to reduce the signal intensity may be necessary in order to avoid signal saturation. Furthermore, laboratories can incrementally evaluate a range of injection times to optimize peak heights. Laboratories should perform internal validation studies in accordance with internal standard operating procedures to ensure that modifications to the default conditions are delivering sensitivity required to generate usable and reliable information. If changing the injection time is not sufficient in reducing signal saturation, reducing the number of cycles from 29 to 28 is another technique that may be investigated.

Applicability to Other Chemistries and Instruments

The content of this technical note and the data generated was specific for the GlobalFiler chemistry run on a 3130xl Genetic Analyzer. Given that the mechanisms of PCR and CE across STR kits and CE instrumentation are the same, the results obtained and conclusions generated are highly likely to be applicable to other HID STR chemistries and capillary electrophoresis instruments. As noted previously, laboratories should perform the necessary internal validation studies to ensure that the selected PCR and injection parameters perform as expected in the laboratory system.