APPLICATION INFORMATION

Genetic Analysis

SEQUENCING OF DIFFICULT DNA TEMPLATES WITH THE GENOMELAB METHODS DEVELOPMENT KIT

Doni Clark, Jane Luo, Rene Oda

Introduction

Routine automated cycle sequencing of DNA templates has typically been performed utilizing deoxyguanosine triphosphate (dGTP) analogs to limit the occurrence of band compressions when the sequence products are separated. The typically utilized dGTP analog is deoxyinosine triphosphate (dITP). This dGTP analog has long standing proof of its ability to produce high-quality sequence data for routine sequencing applications. dITP is not without its own limitations, though. Thermostable DNA polymerases do not efficiently incorporate dITP above 60°C even though their optimal activity is at 72°C. When certain high G-C content or secondary structure regions are encountered, the DNA polymerase will stall and stop polymerization in the presence of dITP. In such cases where problematic sequence regions are encountered, the natural substrate, dGTP, is often more capable of proceeding through the difficult sequence.

The new GenomeLab™ Methods Development Kit for research use provides both dITP- and dGTP-based sequencing chemistries for use with CEQ™ Genetic Analysis Systems. The Methods Development Kit provides the same dITP chemistry previously available in the CEQ DTCS Kit via the renamed dNTP(I) nucleotide mix, and now has the added benefit of a dNTP(G) nucleotide mix. In this report, the capabilities of the dNTP(G) mix to produce sequence data through difficult G-C and polymerase hard stop regions is demonstrated.

Method

Plasmid DNA Purification

Plasmid DNA was purified from cell cultures grown overnight in rich bacterial media with 100 µg/mL ampicillin. Isolation of the plasmid DNA was performed by means of Qiagen Qiaprep* Plasmid DNA Purification Kits. The plasmid DNA quality was evaluated by agarose gel electrophoresis and concentration measured by optical density.

Sequencing Reaction Preparation

All tested plasmids were sequenced using the existing dITP based chemistries from the CEQ Quick Start Kit and the GenomeLab Methods Development Kit (MDK), as well as the new dGTP-based chemistry from the Methods Development Kit. M13 universal forward or reverse primers were used during this study. Master mixes of the dITP and dGTP chemistries from the MDK were prepared according to Table 1.

The different nucleotide chemistries should be prepared in separate thermal cycling plates or individual tubes due to the use of different thermal cycling parameters. Approximately 50 fmol of plasmid DNA was used per sequencing reaction. The appropriate amount of additional sterile water was calculated for each plasmid and added to the sample plate wells, followed by addition of the plasmid DNA (Table 2). A pre-heat treatment at 65°C for 5 minutes was performed for all DNAs prior to the addition of sequencing reaction master mix.

Table 1.

Component	*1 x MDK dITP*	*50 x MDK dITP*	*1 x MDK dGTP*	*50 x MDK dGTP*
10x Sequencing Buffer	2 µL	100 µL	2 µL	100 µL
dNTP(I) Mix	1 µL	50 µL	-	-
dNTP(G) Mix	-	-	1 µL	50 µL
ddUTP Dye Terminator	2 µL	100 µL	2 µL	100 µL
ddGTP Dye Terminator**	**1 µL**	**50 µL**	**4 µL**	**200 µL**
ddCTP Dye Terminator	2 µL	100 µL	2 µL	100 µL
ddATP Dye Terminator	2 µL	100 µL	2 µL	100 µL
DNA Polymerase	1 µL	50 µL	1 µL	50 µL
Total Master Mix Per Well	11 µL	11 µL	14 µL	14 µL

******Note**: The amount of ddGTP added per reaction has changed in the GenomeLab™ Methods Development Kit as compared to the CEQ™ DTCS Kit. Use of the original CEQ DTCS Kit quantity of 2 µL of ddGTP Dye Terminator per dITP sequencing reaction will still result in satisfactory data in most cases, but will reduce the total number of possible dGTP reactions from this new kit.*

Table 2.

Component	*CEQ Quick Start Kit*	*MDK dITP Chemistry*	*MDK dGTP Chemistry*
Sterile Water (to bring reaction to 20 µL)	x.x µL	x.x µL	x.x µL
DNA Template	0.5 µL - 10 µL	0.5 µL - 7 µL	0.5 µL - 4 µL
Primer (1.6 pmole/µL)	2 µL	2 µL	2 µL
Master Mix	8 µL	11 µL	14 µL
Total Volume	20 µL	20 µL	20 µL

Thermal Cycling

It has been well established that the use of dITP in cycle-sequencing reactions limits the extension step in the thermal cycling parameters to 60°C due to the incorporation efficiency of this dGTP analog by the thermostable DNA Polymerase. The use of the natural nucleotide substrate, dGTP, does allow the extension step to be performed at higher temperatures, though the Tm of the primer must also be considered. With the dGTP chemistry, the annealing step is the most critical step. Improper annealing temperatures can result in mispriming events. It is highly recommended with this chemistry that primers have melting temperatures greater than 60°C.

In multiple studies with the dGTP chemistry it was determined that an extension temperature of 68°C was suitable for most primers with Tms of greater than 60°C. For lower melting temperature primers the extension temperature may need to be decreased to yield suitable results. The annealing temperature should be set at 3° to 5°C under the melting temperature for the primer. The thermal cycling parameters in Table 3 were used in this study and represent the standard dITP chemistry parameters and a dGTP cycling parameter set suitable for the supplied M13-47 universal primer and the M13-48 reverse universal primer.

Table 3.

dITP Chemistry (MDK or Quick Start)	*MDK dGTP Chemistry*
96°C 20 seconds	96°C 20 seconds
50°C 20 seconds	58°C 20 seconds
60°C 4 minutes	68°C 2 minutes

For 30 cycles followed by holding at 4°C.

Post Reaction Purification

Purification of the sequencing reaction products was performed using the plate precipitation technique detailed in application bulletin A-1903A available on the Beckman Coulter, Inc. website. After drying the plates, the sequencing products were re-suspended in 40 µL of Sample Loading Solution and overlaid with mineral oil.

Separation

Samples were loaded on a CEQ™ Genetic Analysis System and separated using the Seq-Test separation method.

Results and Discussion

Three DNA plasmid samples were sequenced with the CEQ Quick Start and GenomeLab™ Methods Development kit dITP chemistries and each demonstrated failure to produce sequence fragments beyond a polymerase drop-off region. **Plasmids 1 and 2 could not be sequenced, even with other competitive chemistries.** With all three plasmids, the use of the dGTP nucleotide mix from the Methods Development Kit was successful in producing sequence data through the difficult sequence region.

The first plasmid exhibits a problematic local G-C rich region early in the sequence run, where the polymerase stalls in the presence of dITP, and is incapable of successfully continuing through the challenging region (Figures 1A and 1B). In the presence of the natural nucleotide, dGTP, the polymerase can polymerize through this early difficult local G-C rich sequence (Figure 1C).

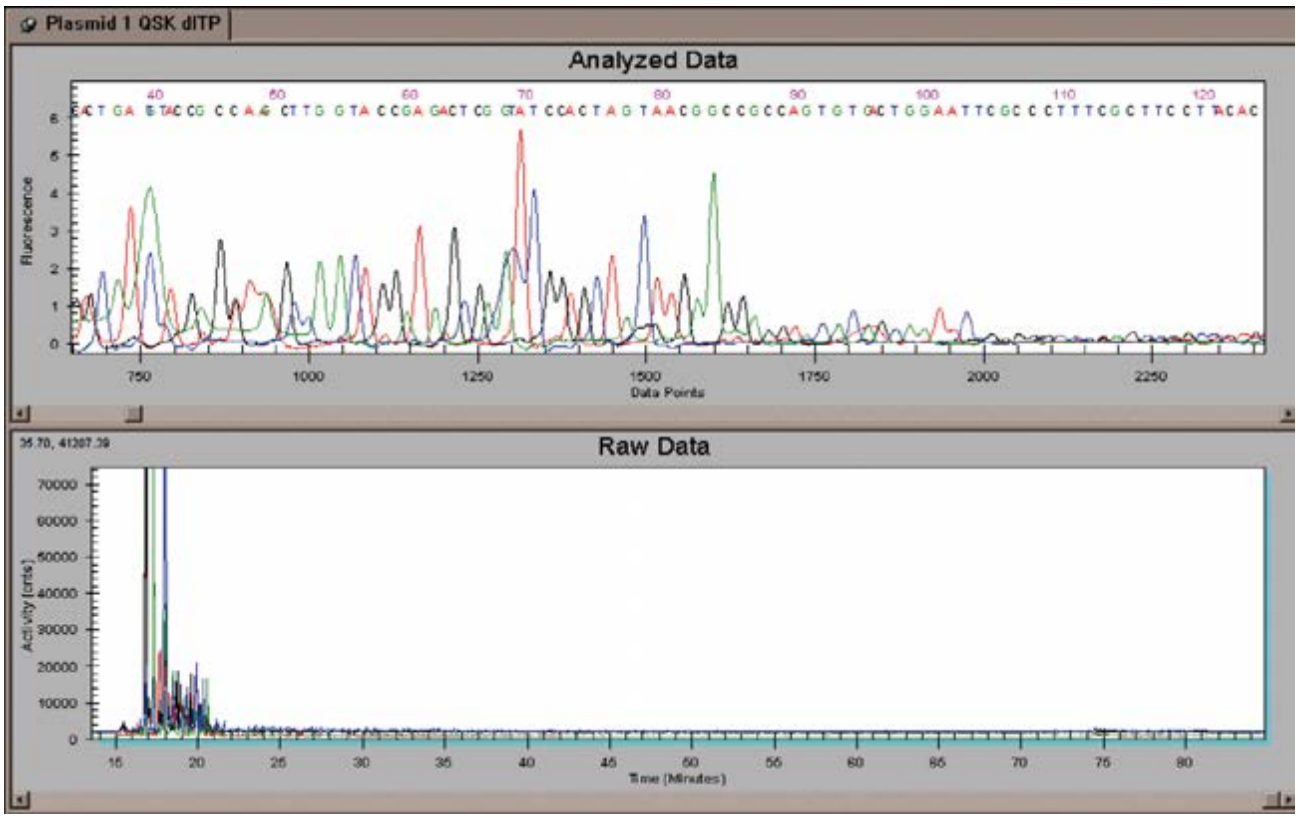

Figure 1A: *Plasmid 1 sequenced with the Quick Start Kit dITP chemistry.*

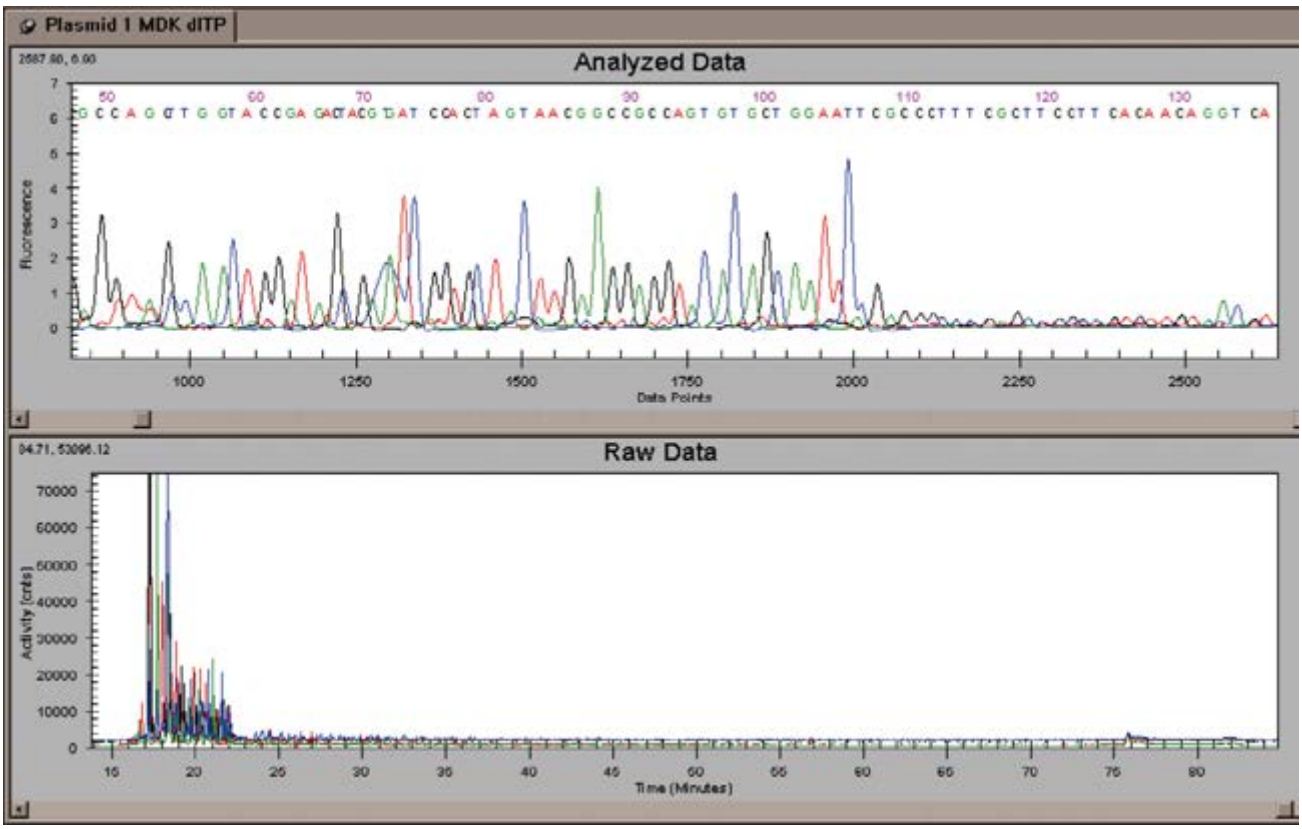

Figure 1B: *Plasmid 1 sequenced with the GenomeLab™ Methods Development Kit dNTP(I) nucleotide mix.*

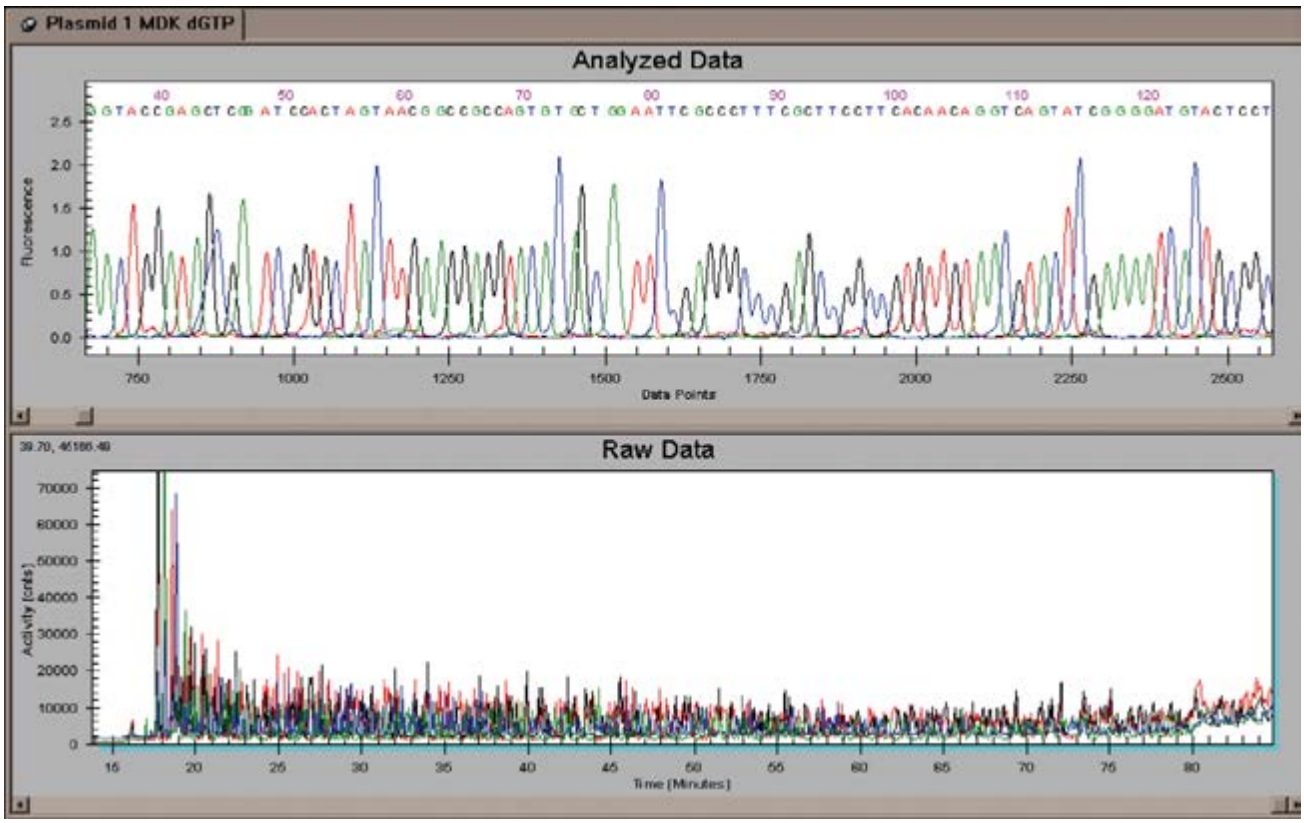

Figure 1C: *Plasmid 1 sequenced with the GenomeLab Methods Development Kit dNTP(G) nucleotide mix.*

Plasmid 2 demonstrates polymerase stalling and drop off in a repeat sequence. Again the dITP-based chemistries have difficulty continuing through this sequence (Figures 2A and 2B), whereas the dGTP based sequencing chemistry successfully provides data through the hard stop (Figure 2C).

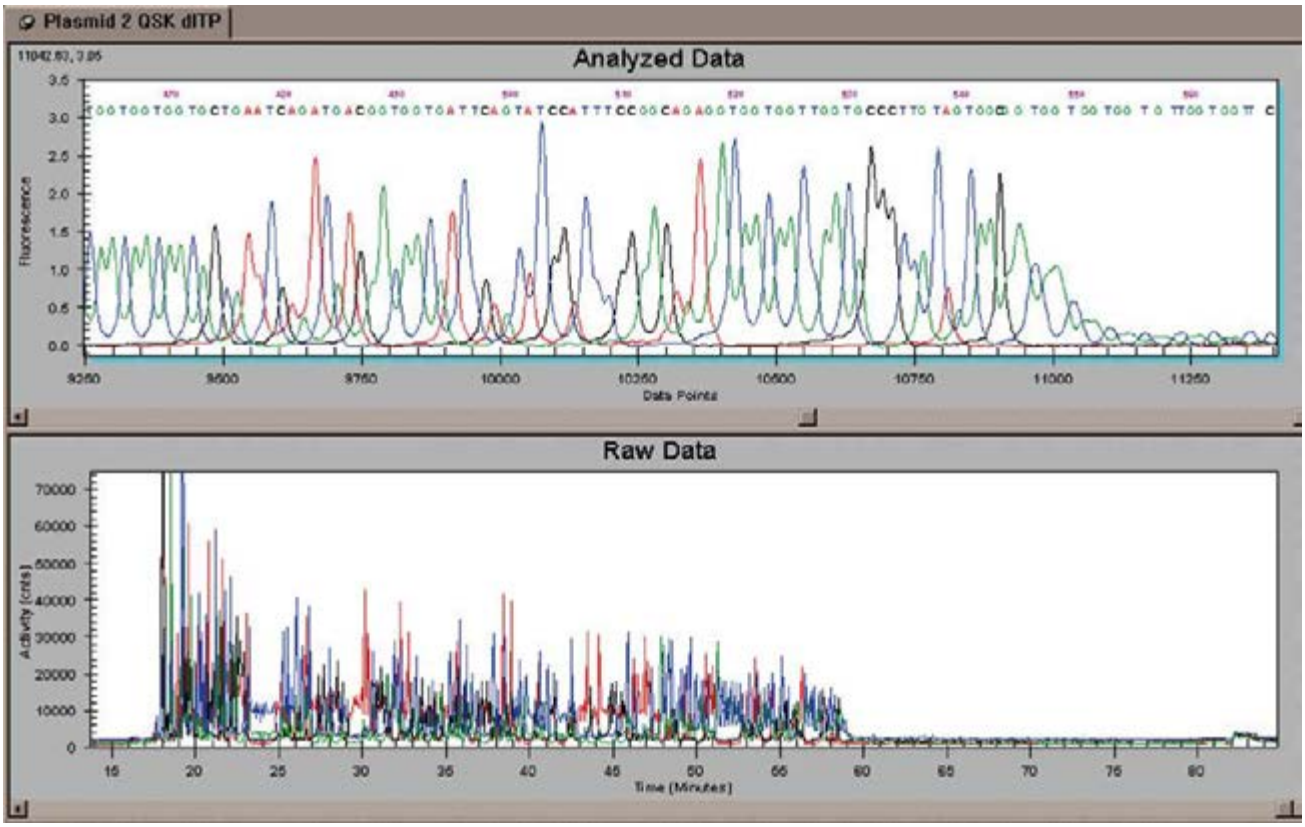

Figure 2A: *Plasmid 2 sequenced with the Quick Start Kit.*

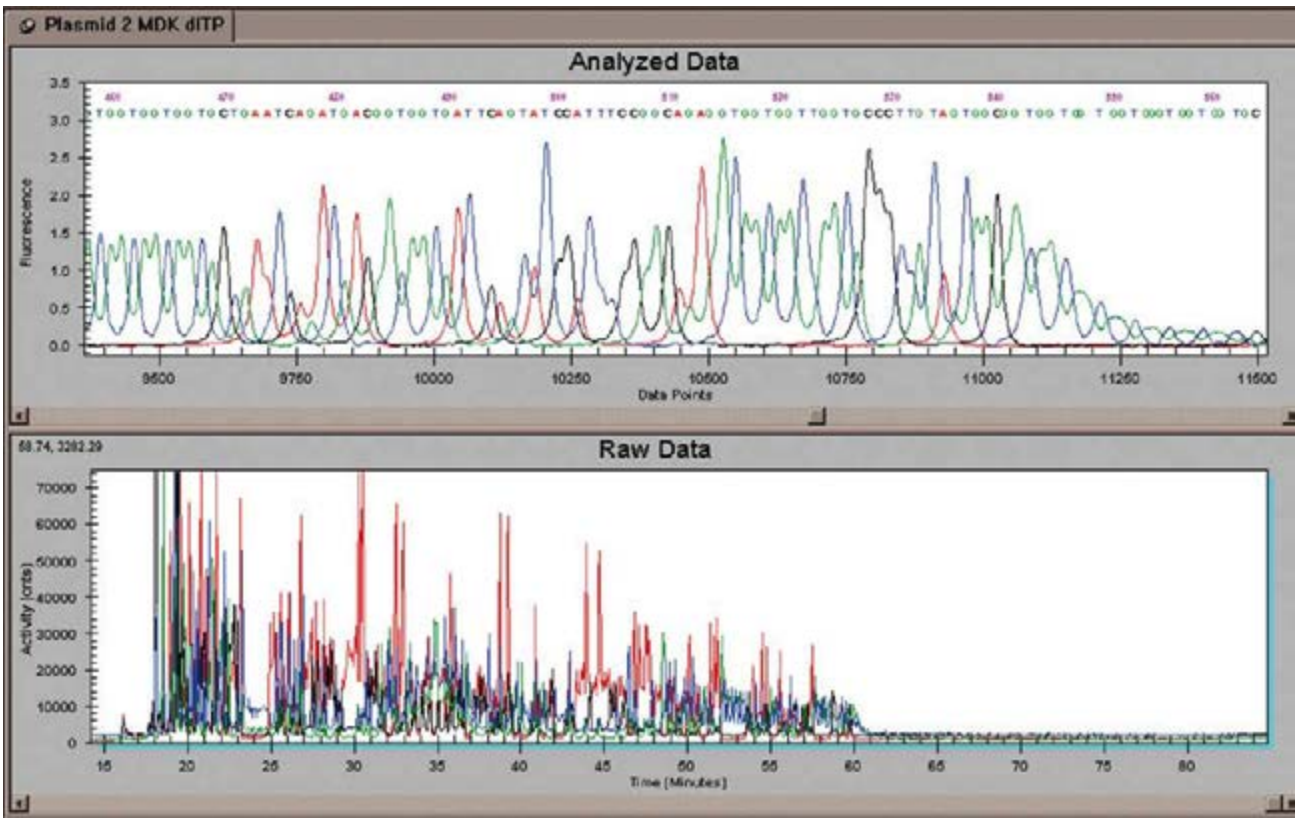

Figure 2B: *Plasmid 2 sequenced with the GenomeLab™ Methods Development Kit dNTP(I) nucleotide mix.*

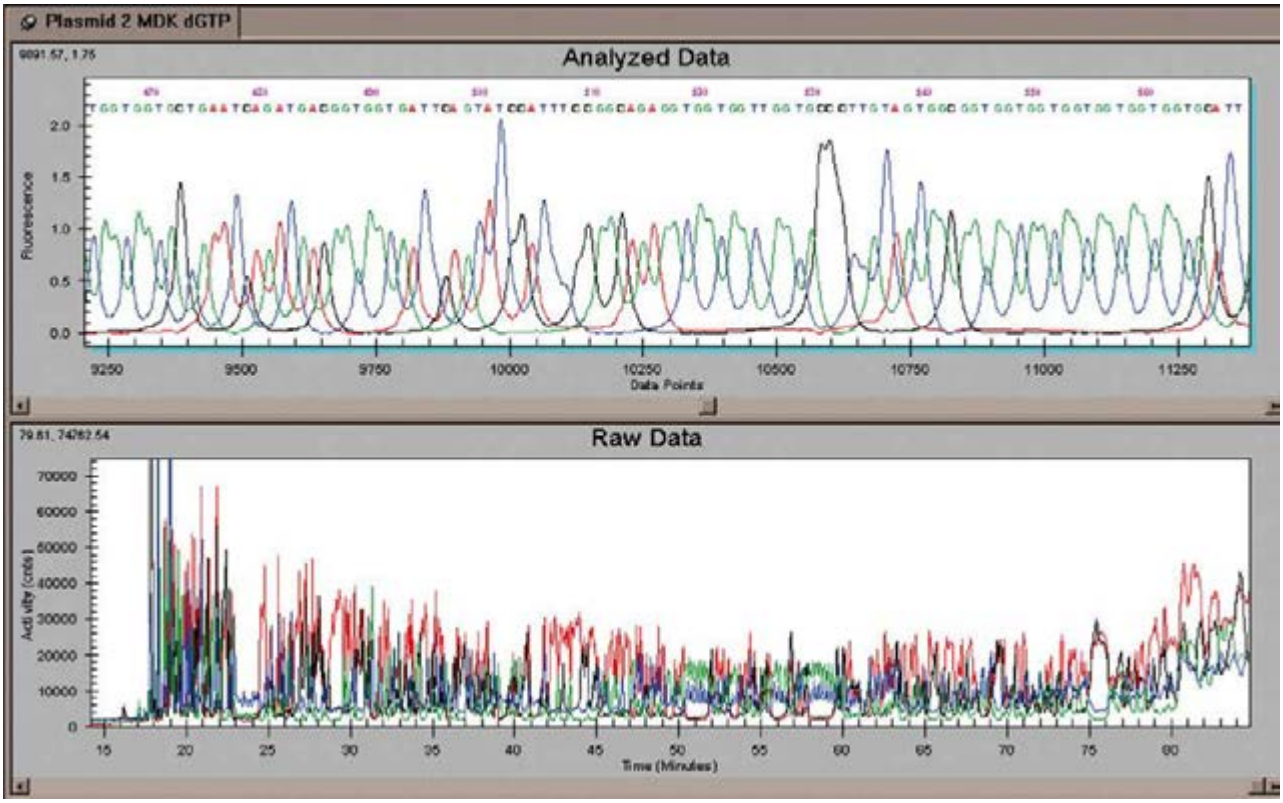

Figure 2C: *Plasmid 2 sequenced with the GenomeLab™ Methods Development Kit dNTP(G) nucleotide mix.*

Plasmid 3 is an example of a highly G-C rich DNA sequence that shows another polymerase stall and drop off location. Although this plasmid can be sequenced with the dITP chemistries when a higher quantity of template is present, in the presence of lower template quantities, the stalling of the polymerase presents a problem (Figures 3A and 3B). The dGTP chemistry is again successful in sequencing through the problematic region (Figure 3C).

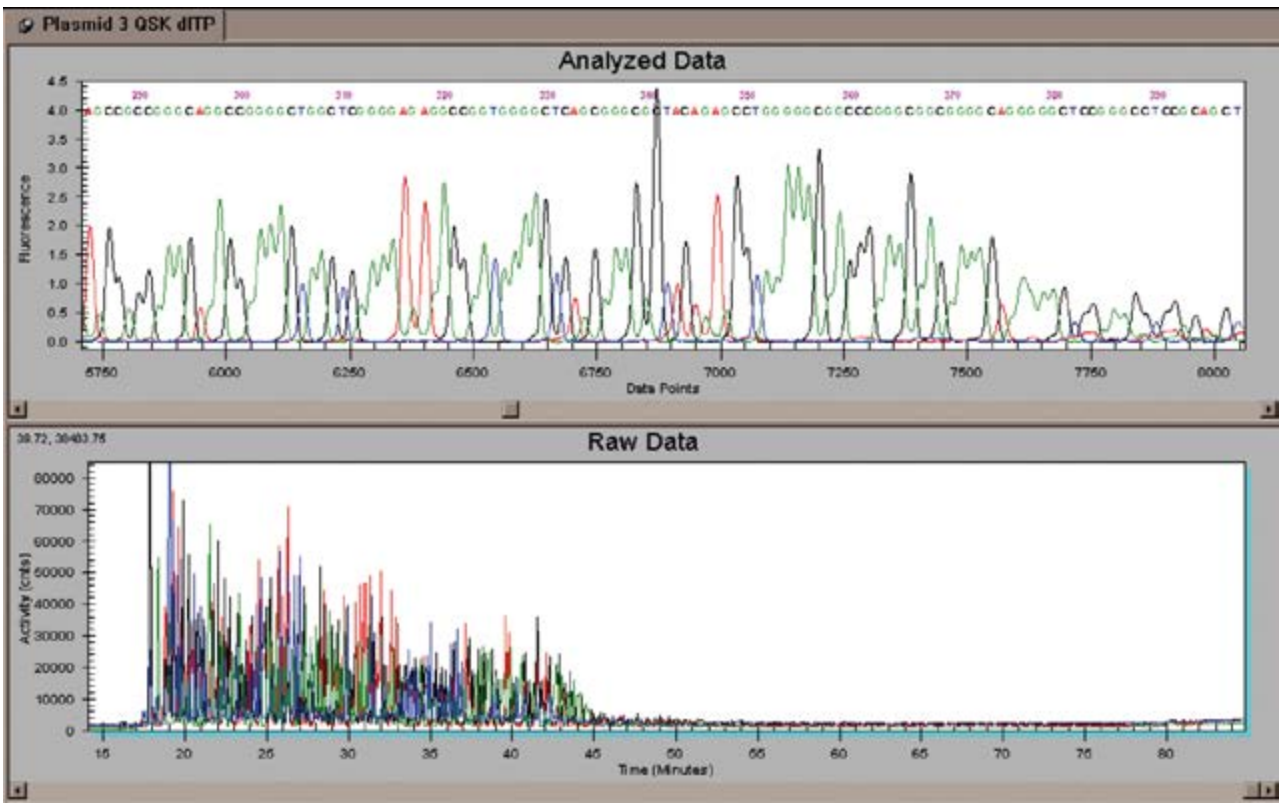

Figure 3A: *Plasmid 3 sequenced with the Quick Start Kit.*

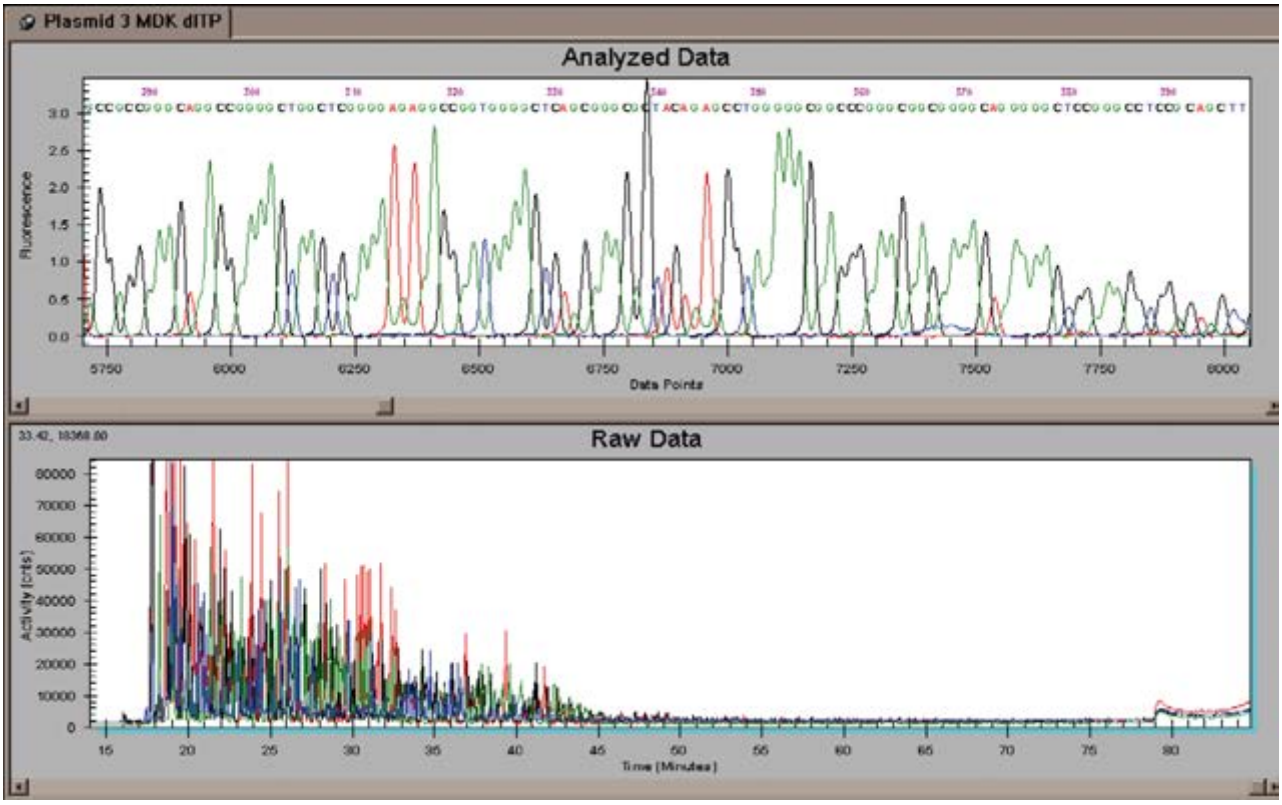

Figure 3B: *Plasmid 3 sequenced with the GenomeLab™ Methods Development Kit dNTP(I) nucleotide mix.*

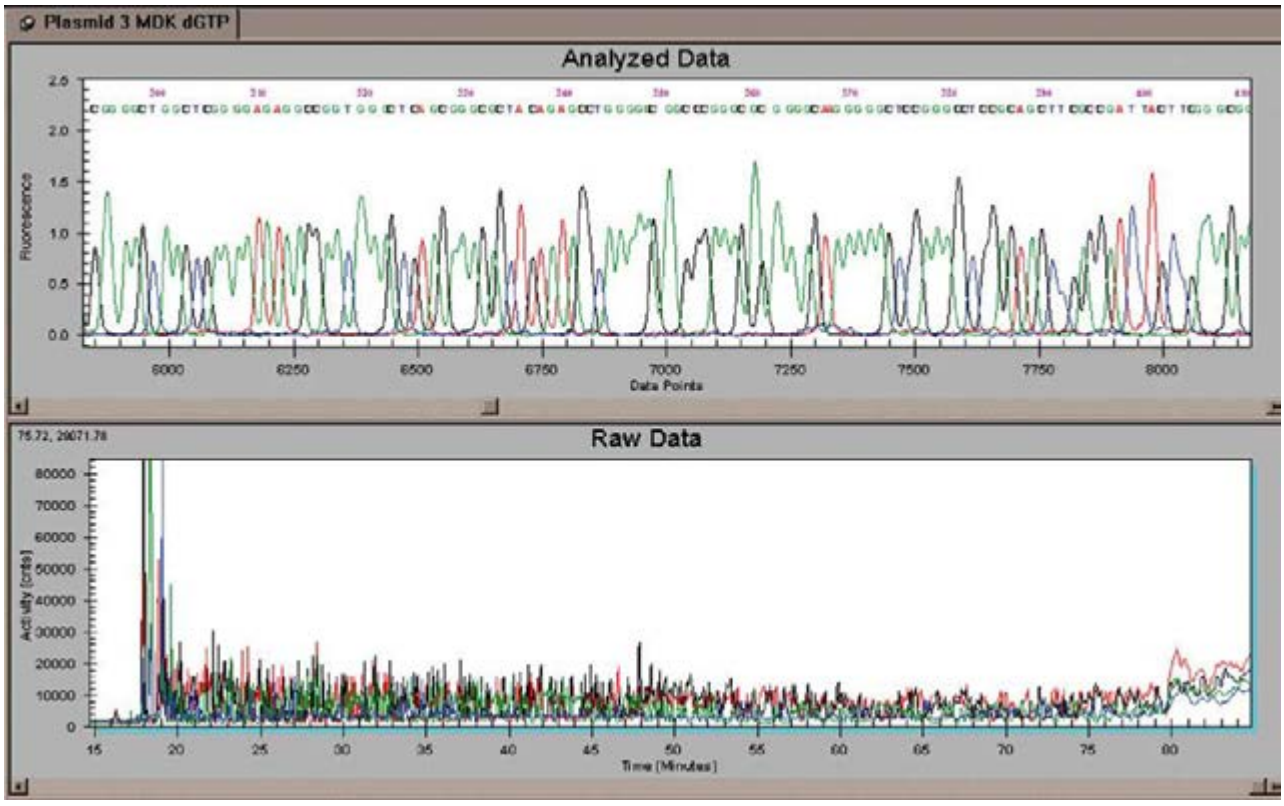

Figure 3C: *Plasmid 3 sequenced with the GenomeLab Methods Development Kit dNTP(G) nucleotide mix.*

Another attribute of the dGTP chemistry is the presence of band compressions. Figure 4 demonstrates the presence of band compressions that dGTP chemistry is known to exhibit. A region of band compressions from dGTP chemistry-derived sequence data of the plasmid Lunatic Fringe is outlined between red lines in the top panel of Figure 4. The bottom panel shows the same region of sequence data from the dITP chemistry.

Due to the tendency of the dGTP sequencing chemistry to exhibit these compressions, **it is not recommended that the dGTP chemistry be used for routine sequencing applications.** Rather, where dGTP should be used is in sequencing through difficult G-C rich, polymerase hard stop, or secondary structure regions through which the dITP chemistry is incapable of continuing. A reduction in the severity of some band compressions can be accomplished by increasing the capillary separation temperature of the run method used.

Another characteristic of this dGTP chemistry is the presence of larger free dye terminator peaks that can cause early errors in the sequence data. Setting the "Delay" to 1.4 minutes on the "Initial Data Detection" tab of the "Sequence Analysis Parameters Editor" (Figure 5) will exclude the majority of the free dye terminators if desired.

If reading close to the primer is desired, removal of free dye terminators can be accomplished by performing a Shrimp Alkaline Phosphatase (SAP) treatment of the sequencing products (Figure 6). In this case, the "Delay" setting can be set to 0.1 minutes to include peaks that are no longer obstructed by free terminator peaks. After the sequencing reaction has completed cycling, 2 µL of 10 x SAP reaction buffer and 1 µL of SAP (1 unit/µL) is added to the sample tube or well, and the mixture incubated for 30 minutes at 37°C.

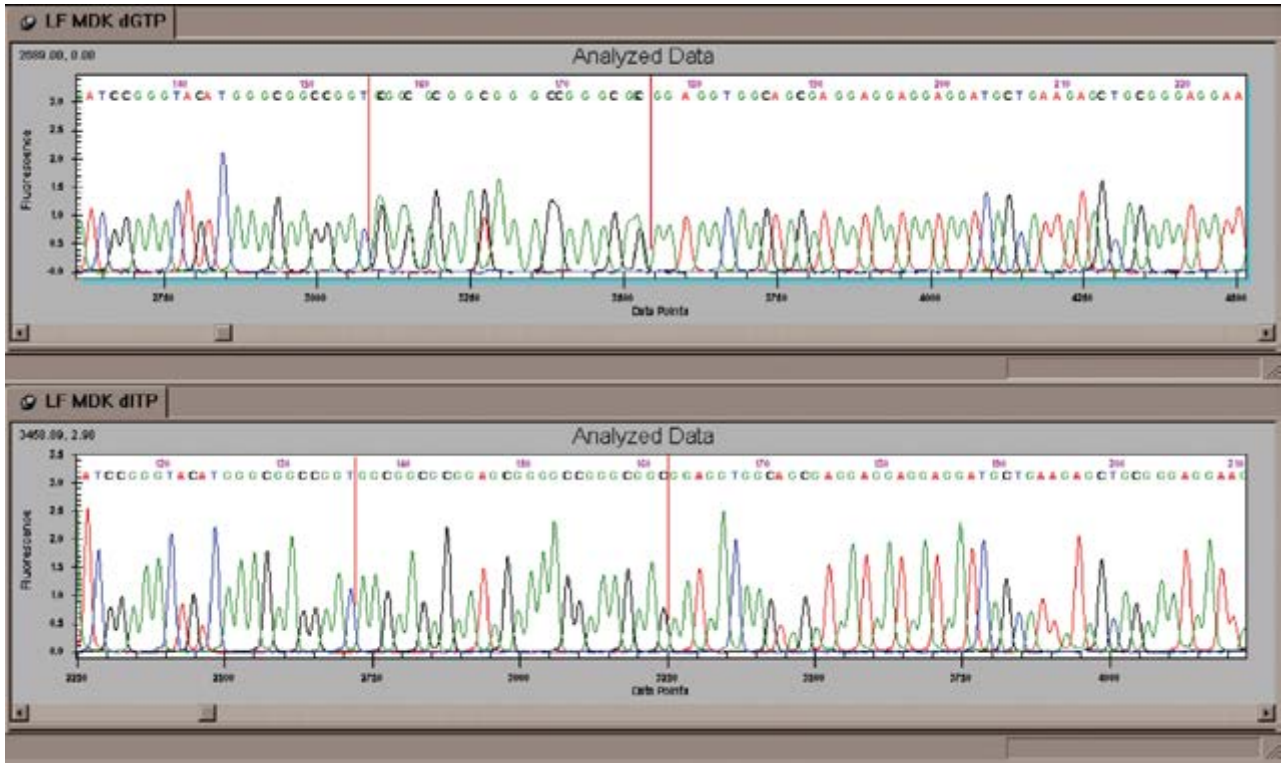

Figure 4: *Band compressions in Lunatic Fringe derived from dGTP sequencing chemistry.*

Enzyme deactivation at 65°C is not strictly necessary since the sequencing reaction products will be purified afterward. Care should be taken to make certain that fresh SAP is used due to the tendency of this treatment to cause internal sequence dye blobs due to incomplete SAP digestion of the free dye terminators. It is also recommended that careful and thorough washing of the purified sequence products be performed to limit the occurrence of any potential internal dye blobs if this treatment is utilized.

Summary

The new GenomeLab™ Methods Development Kit allows users to obtain sequence data from difficult DNA regions that previously could not be sequenced with the standard dITP-based chemistries. While the Methods Development Kit still maintains the dITP chemistry of the previous CEQ DTCS Kit, it now offers flexibility by providing a choice of nucleotide mixtures. It is now possible to use one sequencing kit for routine sequencing applications via the dNTP(I) nucleotide mix, and when required, the dNTP(G) nucleotide mix for those occasions where problematic G-C, polymerase hard stop, and secondary structure regions are encountered.

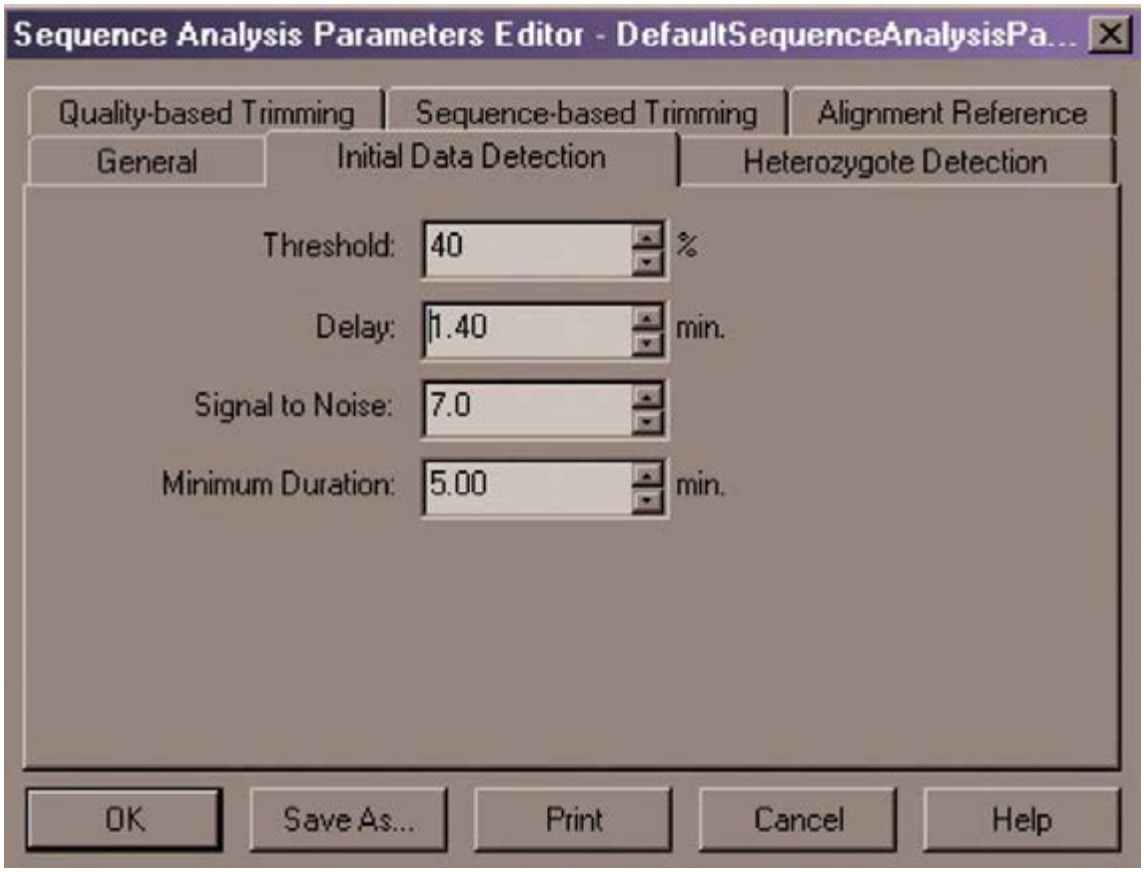

Figure 5: *Delay setting for removal of free dye terminator peaks from analyzed data.*

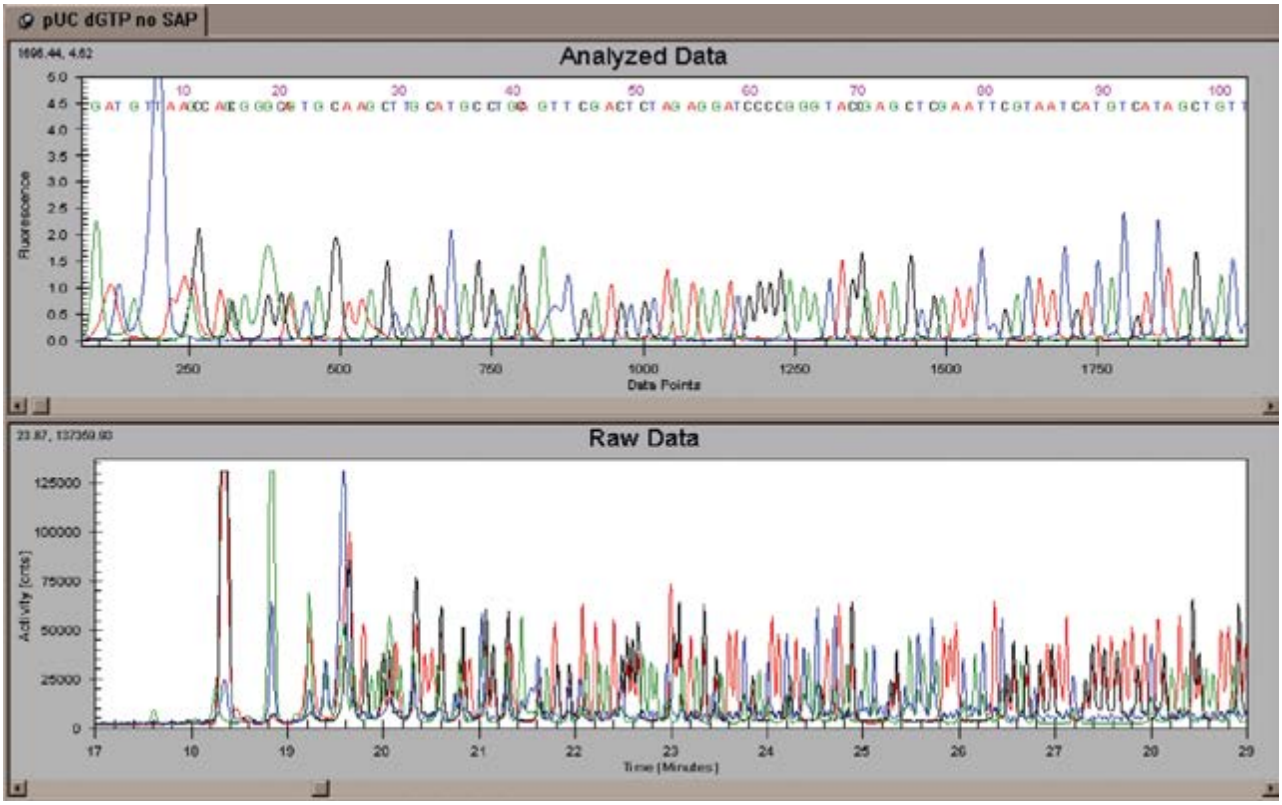

Figure 6A: *pUC18 without SAP treatment and default analysis parameters.*

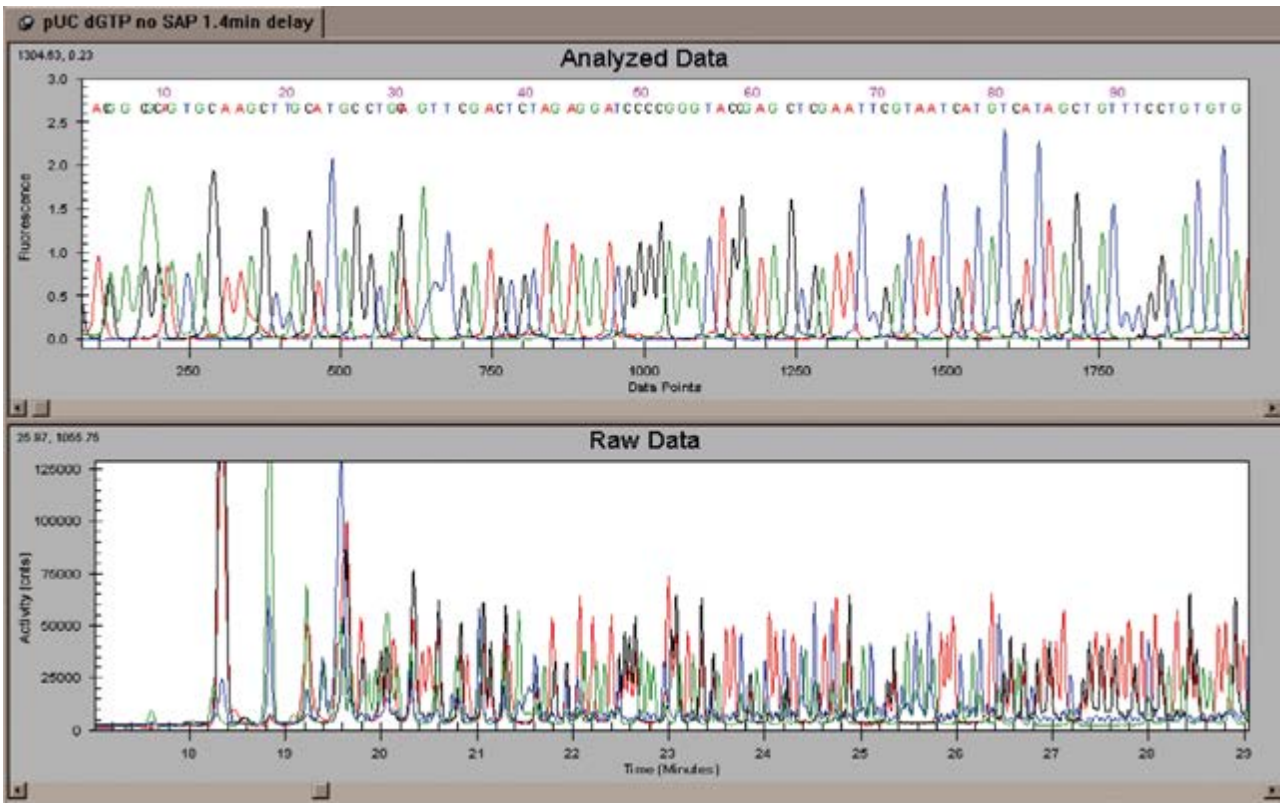

Figure 6B: *pUC18 without SAP and "Delay" set to 1.4 minutes. Large free dye terminator peak is excluded.*

References

1. Vos, P.,Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., Zabeau, M. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res*, *23*, 4407-4414 (1995)
2. Kim, D.H., Heber, D., and Still, D.W. Genetic diversity of *Echinacea* species based upon amplified fragment length polymorphism markers. *Genome*, *47*, 102-111 (2004)

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