Recombinant DNA Sequencing using GenomeLab GeXP™ Genetic Analysis System

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INTRODUCTION and OVERVIEW

Within the process of manufacturing biologics utilizing recombinant DNA technology, living cells producing recombinant proteins could potentially undergo mutations that alter the properties of a protein with adverse impact to the intended products. Therefore, it is critical to verify the fidelity of the nucleotide sequence encoding the expression product in the Master Cell Bank. For many pharmaceutical companies, verification of coding sequence of the recombinant DNA insert in an expression vector is also conducted at the level of Manufacturers Working Cell Bank (MWCB) and End of Production cells. The GenomeLab GeXP Genetic Analysis System performs Sanger sequencing with high quality results using unique DNA sequencing reagents including linear polyacrylamide gel (LPA), coated capillaries and near-infrared dyes with low background and high signal to noise ratio. In addition, the online thermal denaturation further ensures more uniform sequencing results across the sample plates. In this poster, we present results obtained with the GeXP™ system in sequencing various recombinant DNA in plasmid vectors. The dITP utilized in the GenomeLab Dye Terminator Cycle Sequencing chemistry reduces GC compression, making it ideal for routine sequencing analysis for many recombinant DNA sequences. In cases of difficult templates containing repeat regions or a polymerase hardstop, an alternatively available dGTP chemistry successfully enables accurate reads through these sequences. The GenomeLab GeXP Genetic Analysis System is therefore a valuable tool for verifying coding sequences in the production process of biologics.

METHODS

Plasmid DNA Purification
Plasmid DNA was purified from overnight cultures grown in rich bacterial media with 100 µg/mL ampicillin. Isolation of the plasmid DNA was performed by 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 GenomeLab Quick Start Kit and the GenomeLab Methods Development Kit (MDK), as well as the 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 instructions in the kit insert. The different nucleotide chemistries were 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 1). A pre-heat treatment at 65°C for 5 minutes was performed for all DNAs prior to the addition of sequencing reaction master mix and primer.

Table 1. Sequencing Reaction Preparation.

Component	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

Table 2. Thermal Cycling Conditions.

dITP Chemistry (MDK or Quick Start Kit)	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	For 30 cycles followed by holding at 4°C

Thermal Cycling Conditions: Thermal cycling conditions are listed in Table 2.

Post Reaction Purification
Purification of the sequencing reaction products was performed using the ethanol precipitation technique detailed in application bulletin A-1903A available on the SCIEX website. After drying the DNA pellet, the sequencing products were reconstituted in 40 µL of Sample Loading Solution and overlaid with mineral oil.
With dGTP chemistry, in order to read close to the primer, removal of free dye terminators was accomplished by performing a Shrimp Alkaline Phosphatase (SAP) treatment of the sequencing products prior to ethanol precipitation. After the sequencing reaction completed cycling, 2 µL of 10 x SAP reaction buffer and 1 µL of SAP (1 unit/µL) was added to the sample tube or well, and the mixture was incubated for 30 minutes at 37°C.

Separation
Samples were loaded on a GenomeLab GeXP Genetic Analysis System and separated using the "Seq-Test" method.

Data Analysis
For dITP chemistry, "DefaultSequenceAnalysisParameters" were used. For dGTP chemistry, the "Delay" setting was set to 0.1 minutes in comparison to "DefaultSequenceAnalysisParameters". Analysis was fully automated and was programed to be performed immediately after raw data collection was completed.

RESULTS

The dITP chemistry is suitable for routine sequencing. The deoxyinosine triphosphate (dITP) analog for dGTP is ideal for use in routine sequencing due to the presence of band compressions from the use of native dGTP in sequencing reactions. Figure 1 shows a comparison of sequencing a GC rich template, Lunatic Fringe, with both the dITP chemistry and dGTP chemistry. Band compression was present with the dGTP chemistry, but was absent with the dITP chemistry. Therefore, the dITP chemistry is recommended for routine sequencing. Good sequencing results were obtained with dITP chemistry in sequencing a recombinant template #1 (Figure 2) and recombinant template #2 (Figure 3). As shown in Figure 2, the quality of base-calling is indicated by the "Quality Values".

Figure 1: Band compressions present in dGTP sequencing chemistry was overcome by dITP chemistry.

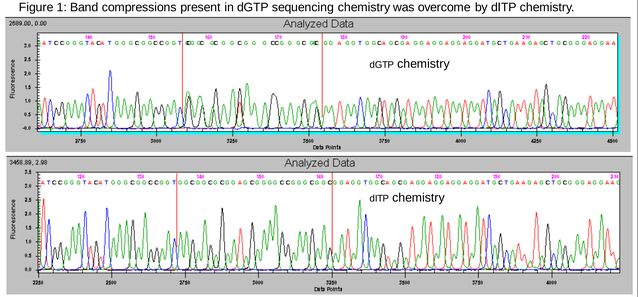


Figure 2. Sequencing results and Quality Values obtained with dITP chemistry using recombinant template #1.

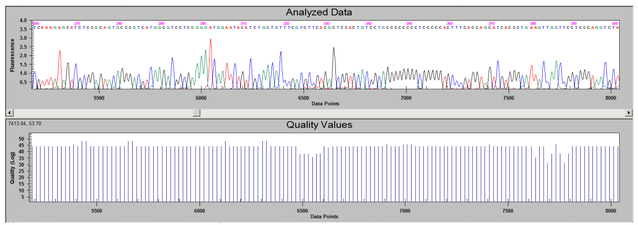
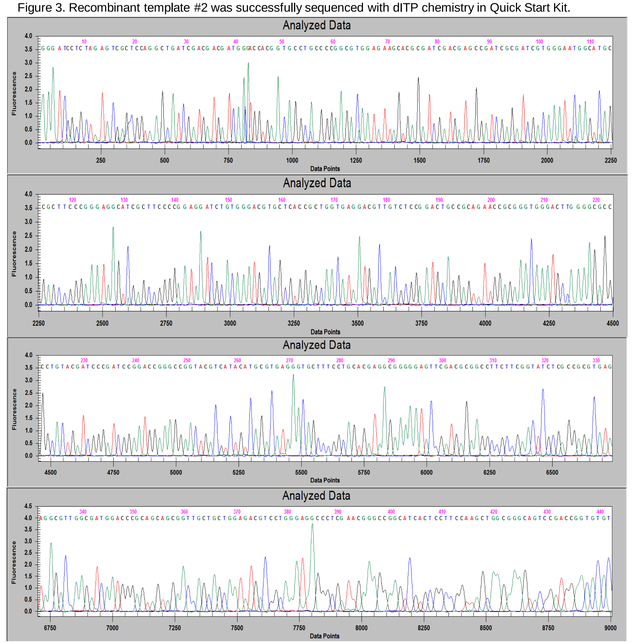


Figure 3. Recombinant template #2 was successfully sequenced with dITP chemistry in Quick Start Kit.



The dGTP chemistry can be used for sequencing difficult templates. Some templates contain highly repetitive sequences, a polymerase hardstop, or extremely GC rich regions that can not be sequenced by dITP chemistry. These templates are considered difficult templates (DTs). In these cases, the dGTP chemistry offered in the MDK kit can be used to sequence through the difficult region. Figure 4 shows a difficult template, DT#1, exhibiting a polymerase hardstop in the presence of dITP (Figure 4A). In the presence of dGTP, the polymerase was able to successfully polymerize through this difficult local region (Figure 4B). In Figure 5, the dGTP chemistry was able to produce excellent results sequencing through a region with 7 Cs, 26 Ts and 6 As (5A) as well as a highly repetitive region (5B) on the difficult template DT#2. Both DT#1 and DT#2 were not successfully sequenced by competitor chemistries. Figure 6 shows that a difficult template, DT#3, with an extremely GC rich region that caused a polymerase stall in the presence of dITP (6A). However, the dGTP chemistry was able to sequence through this difficult template (6B).

Figure 4. DT#1 contains a polymerase hardstop region where the DNA polymerase stalled and disassociated from the template in the presence of dITP (4A). However, DT#1 was successfully sequenced with the dGTP chemistry in GenomeLab Methods Development Kit (4B).

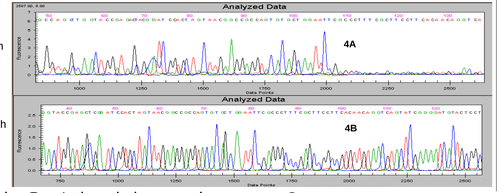
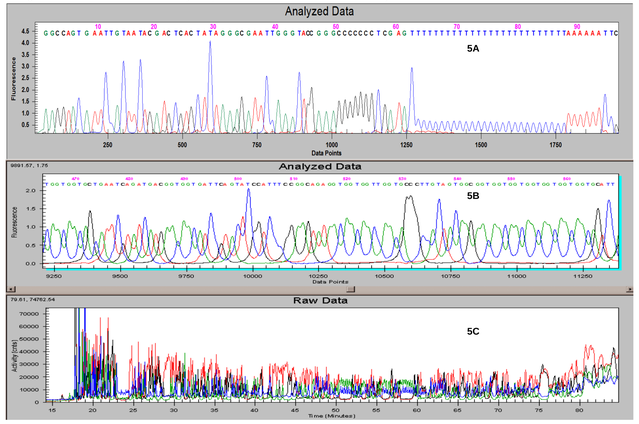


Figure 5: DT#2 was successfully sequenced with the dGTP chemistry in GenomeLab Methods Development Kit.



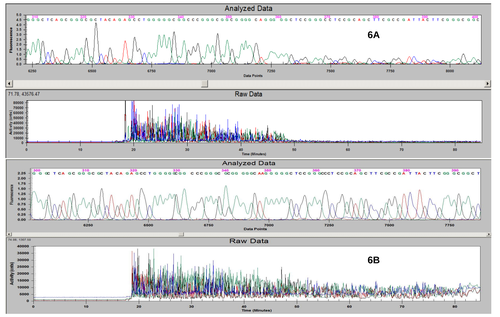


Figure 6. DT#3 contains an extremely GC rich region where the DNA polymerase stalled when dITP chemistry was used (6A). However, DT#3 was successfully sequenced with the dGTP chemistry in GenomeLab Methods Development Kit (6B).

CONCLUSIONS

- The GenomeLab GeXP Genetic Analysis System generates excellent results in sequencing recombinant DNA in plasmid vectors.
- The dITP chemistry is ideal for routine sequencing analysis.
- The dGTP chemistry is useful in sequencing difficult templates with repeat regions, polymerase hardstop or extremely GC rich regions.