TECHNICAL INFORMATION

Genetic Analysis

METHOD FOR LONG SEQUENCING READ LENGTHS ON THE CEQ™ GENETIC ANALYSIS SYSTEM

Doni Clark, Jim Thorn, and Keith Roby
Beckman Coulter, Inc.

Introduction

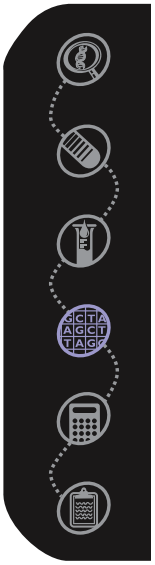
Automated DNA sequence analyzers have increased the throughput and ease of use of an application that has become a core technique for many areas of genetic analysis. For large de novo sequencing projects, automated DNA sequence analyzers have obviously shown their advantage and strength. While most DNA sequencing is confirmatory in nature, with required read lengths of 700 bases or less, some researchers need to maximize base read lengths to economize on laboratory resources.

Although the CEQ™ Genetic Analysis System produces sequence read lengths greater than 700 bases using the standard sequencing methods provided with the instrument, longer sequencing read lengths are possible by modifying the separation parameters. This bulletin presents a strategy for increasing base read lengths by 20% or more by modifying the separation voltage, duration, capillary temperature, and injection time parameters as compared to the existing CEQ LFR1 sequencing separation method. Applying this new separation method can achieve average 98% accuracy base read length cutoffs greater than 900 bases with some individual samples obtaining 98% accuracy cutoffs of 1000+ bases.

Method

DNA Purification

Overnight cultures of DH5α cells containing pUC18 as a control plasmid and pUC19 vector containing a 1.2 kB Glucouronidase gene insert (pGus) were grown overnight in rich bacterial media with 100 µg/mL ampicillin to an optical density of 4-5. The cultures were transferred to deep square-well plates at 1 mL per well. Plasmid DNA preparation was performed on a Biomek® FX Laboratory Automation Workstation from Beckman Coulter using both the Promega Wizard® SV 96 Plasmid DNA Purification System and the Qiagen QIAprep® 96 Turbo Miniprep plasmid DNA purification kit. The Biomek FX program for the Promega Wizard has been described previously in Beckman Coulter Application Bulletin A-1907A.[1] This Biomek FX program was modified for use with the Qiagen QIAprep DNA purification kit.



DNA Sequence Reaction

Since many DNA prep technologies yield a large quantity of supercoiled plasmid which can affect the performance of sequence fragment separations, appropriate preheat treatment of plasmid DNA for any particular cell type and purification method combination should be determined by performing a temperature-versus-time matrix.[2] In addition, for obtaining longer read length separations, the preheat treatment should be as gentle as possible in order to relax the supercoiled plasmids while not nicking them so much that producing longer fragments during the cycle sequencing reactions becomes problematic. Both of the DH5α-grown plasmids in this study were preheat treatment optimized using a matrix of three temperatures (65°C, 76°C, and 86°C) and four time

lengths (1 to 4 minutes). The final preheat treatment condition for both of these purification methods that maintained appropriate signal strength for the longer sequencing fragments was 3 minutes at 86°C.

Sequencing reactions were performed with both the pGus plasmid and the pUC18 control plasmid using the CEQ™ Dye Terminator Cycle Sequencing Kit chemistry (CEQ DTCS Kit, Beckman Coulter, Inc., PN 608000). All reactions used 70 fmol of plasmid DNA template which was thermal cycled as suggested in the CEQ DTCS kit protocol. While this quantity of DNA template worked well in our studies, many researchers may need to adjust this template quantity for their specific needs based on template type and size. Another means of increasing the overall signal and hence the amount of longer fragment signal is to increase the number of cycles in the thermal cycling parameters. This can be combined with resuspension of the final sequencing products in less Sample Loading Solution (30 µL instead of 40 µL). It should be noted that incrementally increasing signal strength by increasing the effective concentration of the sequence fragments in the sample causes more sample to be injected onto the capillary which negatively affects the resolution of the longer fragments. Long fragment signal strength and resolution are both critical factors for achieving long base read lengths; therefore, a balance must be achieved that provides adequate signal strength for detection but not so much as to negatively impact resolution. Purification of the sequencing reaction products was performed by the plate precipitation technique detailed in Application Bulletin A-1903A.[3] After drying the plates, the sequencing products were resuspended in 40 µL Sample Loading Solution, overlaid with mineral oil, and loaded onto the CEQ 8000 Genetic Analysis System. Note: This method was not tested on the dual-plated CEQ 8800 System.

Results and Discussion

To obtain longer sequencing read lengths, the separation method parameters were modified to reduce the voltage and increase the data collection duration over a range of capillary temperatures. After performing a series of voltage versus temperature experiments, it was found that the following separation method yielded the most consistent results (Figure 1).

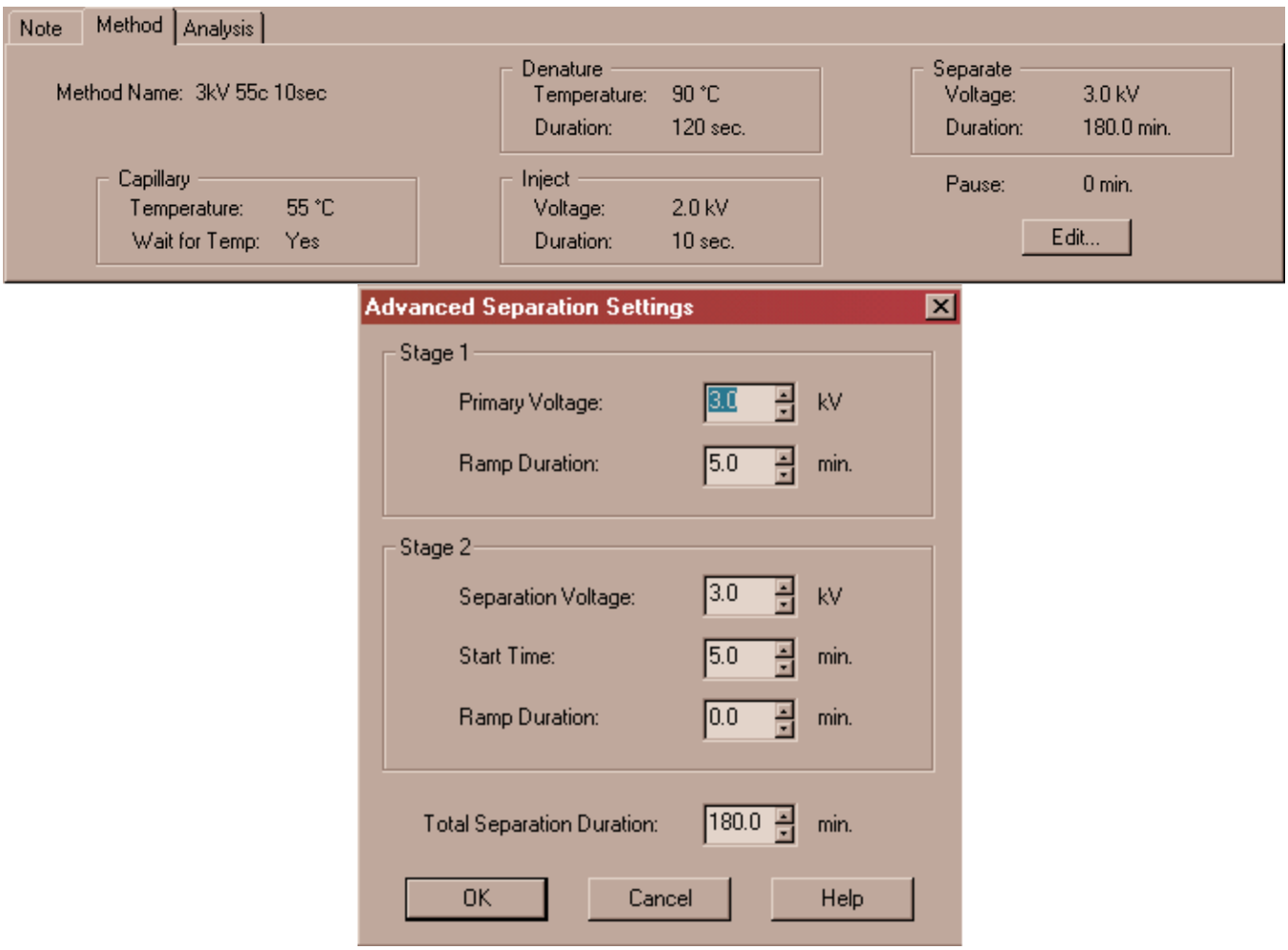


Figure 1. *Sample Setup module Method tab.*

Using the new separation method parameters, a comparison was performed against the default LFR-1 separation method supplied with the CEQ 8000 Genetic Analysis System. For the pUC18 DNA prepared with the Promega kit, 24 individual samples were run. All other sample sets for both the control pUC18 and pGUS DNAs represent a total of 48 individual samples per DNA purification chemistry and separation method. Comparison of peak resolution is performed in Figure 2. Visually, peak resolution is similar between the standard LFR1 separation method and the new longer read method until approximately base 400. Beyond that point, the new longer read separation method clearly demonstrates improved peak resolution.

Note: Capillary lifetime studies were not tested with the new, longer-read separation method.

Table 1A shows representative base read length 98% accuracy cutoffs for each plasmid, separation method, and purification chemistry combination. The sample names are coded as such: QpUC18 and QpGUS represent the template DNAs purified by the Qiagen chemistry, while pUC18 and pGUS from Table 1B are the DNA templates purified by the Promega chemistry. 86c 3min indicates the preheat treatment used for all samples, and LFR1 or 3kV 55c indicate, respectively, the standard CEQ Genetic Analysis System sequencing method or an abbreviation of the new optimized separation method.

The 98% accuracy cutoff results were averaged and compared for each set of templates (Table 2). In each combination of DNA purification chemistry and plasmid template, the new separation method demonstrated an increase of greater than 20% for the base read length 98% accuracy cutoff values.

Use of this separation method increases the overall base sequence yield from a single reaction by approximately 20% and thereby maximizes the amount of sequence information obtained from a single reaction. In some instances where DNA templates may not yield sufficient fluorescent signal, it may be helpful to increase either the number of cycles during the sequencing reaction thermal cycling, or to increase the injection time duration in the separation method. Be aware, though that adding too much DNA template to the sequencing reaction to boost signal strength by making more product or increasing the injection time too much will greatly impact the resolution of the longer sequencing fragments and should be avoided. This is an important point to consider since, in order to achieve the longest possible sequencing read lengths, it is absolutely critical to maintain resolution quality.

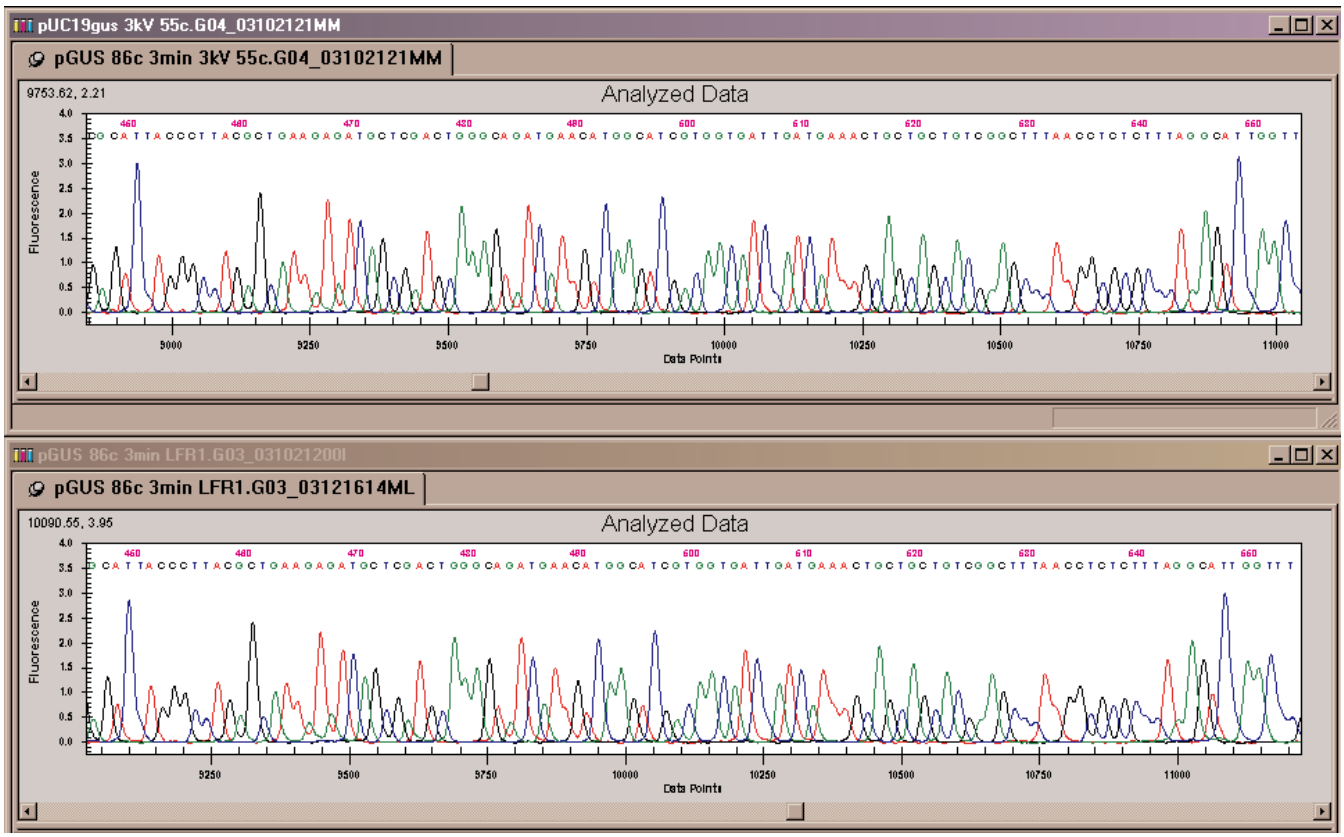


Figure 2. *Comparison of separation methods for pGUS demonstrating improvement in resolution of longer read separation method at bases 510 to 600.*

Table 1A. Qiagen QIAprep Base Read Length 98% Accuracy Cutoff					
Qiagen QIAprep 96 Turbo Miniprep Results Name	**Total Read Length**	**98.00% Cutoff Base #**	**Qiagen QIAprep 96 Turbo Miniprep Results Name**	**Total Read Length**	**98.00% Cutoff Base #**
QpUC18 86c 3min 3kV 55c.A07_03110103KY	1042	972	QpGUS 86c 3min 3kV 55c.A09_03110608V9	1037	906
QpUC18 86c 3min 3kV 55c.B07_03110103KY	1083	996	QpGUS 86c 3min 3kV 55c.B09_03110608V9	905	905
QpUC18 86c 3min 3kV 55c.C07_03110103KY	1055	974	QpGUS 86c 3min 3kV 55c.C09_03110608V9	900	900
QpUC18 86c 3min 3kV 55c.D07_03110103KY	1061	1005	QpGUS 86c 3min 3kV 55c.D09_03110608V9	1063	961
QpUC18 86c 3min 3kV 55c.E07_03110103KY	1271	1032	QpGUS 86c 3min 3kV 55c.E09_03110608V9	1012	911
QpUC18 86c 3min 3kV 55c.F07_03110103KY	1050	1020	QpGUS 86c 3min 3kV 55c.F09_03110608V9	1033	906
QpUC18 86c 3min 3kV 55c.G07_03110103KY	1115	1026	QpGUS 86c 3min 3kV 55c.G09_03110608V9	969	950
QpUC18 86c 3min 3kV 55c.H07_03110103KY	997	936	QpGUS 86c 3min 3kV 55c.H09_03110608V9	1022	912
QpUC18 86c 3min LFR1.A10_03111209JW	788	788	QpGUS 86c 3min LFR1.A12_03110720XN	770	768
QpUC18 86c 3min LFR1.B10_03111209JY	803	803	QpGUS 86c 3min LFR1.B12_03110720XN	790	761
QpUC18 86c 3min LFR1.C10_03111209K0	798	796	QpGUS 86c 3min LFR1.C12_03110720XN	788	767
QpUC18 86c 3min LFR1.D10_03111209K1	807	807	QpGUS 86c 3min LFR1.D12_03110720XN	778	778
QpUC18 86c 3min LFR1.E10_03111209K3	803	796	QpGUS 86c 3min LFR1.E12_03110720XN	744	744
QpUC18 86c 3min LFR1.F10_03111209K5	804	801	QpGUS 86c 3min LFR1.F12_03110720XN	791	766
QpUC18 86c 3min LFR1.G10_03111209K7	806	795	QpGUS 86c 3min LFR1.G12_03110720XN	798	761
QpUC18 86c 3min LFR1.H10_03111209K9	810	810	QpGUS 86c 3min LFR1.H12_03110720XN	711	711

Table 1B. Promega SV96 Base Read Length 98% Accuracy Cutoff					
Promega Wizard SV 96 Plasmid DNA Results Name	**Total Read Length**	**98.00% Cutoff Base #**	**Promega Wizard SV 96 Plasmid DNA Results Name**	**Total Read Length**	**98.00% Cutoff Base #**
pUC18 86c 3min 3kV 55c.H05_03103001P3	1059	1025	pUC19gus 3kV 55c.A04_03102121MM	1169	959
pUC18 86c 3min 3kV 55c.B04_03102922FY	1075	961	pUC19gus 3kV 55c.B04_03102121MM	1029	943
pUC18 86c 3min 3kV 55c.C04_03102922FY	1025	1004	pUC19gus 3kV 55c.C04_03102121MM	1060	977
pUC18 86c 3min 3kV 55c.D04_03102922FY	1029	1023	pUC19gus 3kV 55c.D04_03102121MM	1100	985
pUC18 86c 3min 3kV 55c.E04_03102922FY	1054	996	pUC19gus 3kV 55c.E04_03102121MM	1015	971
pUC18 86c 3min 3kV 55c.F04_03102922FY	1049	1022	pUC19gus 3kV 55c.F04_03102121MM	1077	981
pUC18 86c 3min 3kV 55c.G04_03102922FY	1124	982	pUC19gus 3kV 55c.G04_03102121MM	1104	1017
pUC18 86c 3min 3kV 55c.H04_03102922FY	1108	1030	pUC19gus 3kV 55c.H04_03102121MM	1071	1044
pUC18 86c 3min LFR1.A03_03102920TH	742	730	Gus 8643 40 µL LFR1.A05_031018054A	769	769
pUC18 86c 3min LFR1.B03_03102920TH	823	769	Gus 8643 40 µL LFR1.B05_031018054A	833	812
pUC18 86c 3min LFR1.C03_03102920TH	830	801	Gus 8643 40 µL LFR1.C05_031018054A	811	811
pUC18 86c 3min LFR1.D03_03102920TH	826	800	Gus 8643 40 µL LFR1.D05_031018054A	847	837
pUC18 86c 3min LFR1.E03_03102920TH	802	789	Gus 8643 40 µL LFR1.E05_031018054A	833	809
pUC18 86c 3min LFR1.F03_03102920TH	818	801	Gus 8643 40 µL LFR1.F05_031018054A	821	821
pUC18 86c 3min LFR1.G03_03102920TH	807	786	Gus 8643 40 µL LFR1.G05_031018054A	799	789
pUC18 86c 3min LFR1.H03_03102920TH	803	783	Gus 8643 40 µL LFR1.H05_031018054A	796	796

Table 2. Increase in Base Read Length 98% Accuracy Cutoff			
	Average 98% Cutoff		*% Increase of 98% Cutoff*
DNA prep/Plasmid	*LFR1*	*3kV 55C*	
Promega pUC18	767	958	25
Promega pUC19gus	798	964	21
Qiagen pUC18	786	977	24
Qiagen pUC19gus	759	930	23

References

1. Quick Start Guide to Purifying Plasmids on the Biomek® FX Using Beckman Coulter Wizard SV 96 Reagents. Beckman Coulter *Application Information Bulletin Quick Start Guide A-1907-QS* (2001).
2. Improved Sequencing of Plasmids on the CEQ 2000 by a Simple Template Pre-Heating Procedure. Beckman Coulter *Application Information Bulletin A-1872A* (1999).
3. Roby, K., Gull, H. A Rapid and Efficient Method for the Post-Reaction Clean Up of Labeled Dye Terminator Sequencing Products. Beckman Coulter *Application Information Bulletin A-1903A* (2001).

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