390003-AF
July 2007

GenomeLab™ Sequencing Chemistry Protocol

CEQ™ / GenomeLab™ Series

Genetic Analysis Systems

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4300 N. Harbor Blvd., Fullerton, CA 92834-3100

Printed in U.S.A.

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Table of Contents

Section 1 GenomeLab Sequencing Kits

GenomeLab Methods Development Kit (PN 608000) 1
DNA Polymerase 1
Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP) 1
dNTP Mix Solutions 1
Sequencing Reaction Buffer 1
pUC18 Control Template 2
-47 Sequencing Primer 2
Glycogen 2
Sample Loading Solution 2
Mineral Oil 2
GenomeLab DTCS - Quick Start Kit (PN 608120) 3
GenomeLab DTCS - Quick Start Master Mix 3
Required Materials Not Provided by Beckman Coulter 3
Sterile Water 3
95% Ethanol/Water, 70% Ethanol/Water 3
3M Sodium Acetate pH 5.2 3
100 mM Na_2EDTA pH 8.0 4
Sterile Tubes - 0.5 mL Microfuge Tubes 4
0.2 mL Thin Wall Thermal Cycling Tubes or Plates 4
Thermal Cycler with Heated Lid 4

Section 2 Preparation of the DNA Sequencing Reaction

GenomeLab Methods Development Kit 5
Template Pre-Heat Treatment 6
GenomeLab DTCS - Quick Start Kit 7
Template Pre-Heat Treatment 8
Thermal Cycling Program 8
Recommendations for Obtaining Optimal Results 8
Primer Design 9
Template 9
Thermal Cycling Conditions 9
Optional SAP Treatment for dGTP Samples 10
Incubation Conditions for SAP Treatment 10

Section 3 Post Sequencing Reaction Purification

Ethanol Precipitation in a 0.5 mL Microfuge Tube 11
Ethanol Plate Precipitation in a Sample Plate (Beckman Coulter PN 609801) 12
Sample Preparation for Loading into the System 14

Section 4 Template Preparation

DNA Template Preparation 15

DNA Template Amount 15
Template Pre-Heat Treatment 17

Appendix A

Technical Bulletins 19
Additional Technical Support 19
In the United States: 19
Worldwide: 19

GenomeLab Sequencing Kits

1.1 GenomeLab Methods Development Kit (PN 608000)

The kit should arrive frozen and be transferred to a NON-FROST-FREE FREEZER set at -20°C for storage. The important components that require -20°C storage are the DNA polymerase, the dye labeled terminators, and the Sample Loading Solution.

NOTE The enzyme is in glycerol and WILL NOT freeze solid.

DNA Polymerase

The DNA polymerase supplied in the kit is a proprietary polymerase.

Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)

Beckman Coulter, Inc. manufactures the four dye-terminators, each one containing a different fluor whose excitation and emission spectrum is in the near infrared region. These dyes are NOT compatible with the ABI or Amersham systems and likewise, the Amersham and ABI dyes are NOT compatible with the Beckman Coulter system. For optimal performance, thaw the dye-terminators on ice.

dNTP Mix Solutions

These solutions contain the four dNTPs required for chain extension during the polymerization reaction. The ratios of the dNTPs to the terminators have been carefully optimized to give the best performance of the kit. Changing the amounts of the dNTPs or terminators from the recommended amounts will cause differences in the frequencies of chain termination during the cycle sequencing reactions and will affect the performance of the kit. In addition, the dNTP(I) Mix contains dITP, a dGTP analog used to reduce "compressions." Because dITP is used along with the DNA Polymerase, the extension temperature of 60°C must be maintained. The dNTP(G) mix contains the standard deoxynucleotides including dGTP which can help sequence through some difficult secondary structure and G-C rich regions. This dNTP(G) mix will demonstrate band compressions that should be verified with the dITP chemistry.

Sequencing Reaction Buffer

The buffer is 10X concentrated and optimized for use with the DNA Polymerase. When thawing, make sure that no precipitate is visible in the tube. If a precipitate is present, you can warm the buffer to 65°C for a few minutes to redissolve the precipitate. If the precipitate is not redissolved before use, the efficiency of the sequencing reactions will be adversely affected, causing low signal on the system.

pUC18 Control Template

The control template is a highly purified sample of pUC18 DNA. The DNA is a double stranded plasmid of 2685 base pairs in length. The sequence of the pUC18 used in the GenomeLab Methods Development kit differs from that published in GenBank by one base; therefore, comparisons should be made only to the sequence provided by Beckman Coulter, Inc. (pUC18dG).

NOTE The pUC18 control template is different from the GenomeLab Sequencing Test Sample. The GenomeLab Sequencing Test Sample is the product of sequencing pUC18 control template. It contains the sequencing fragments and residual template that is purified along with the sequencing fragments.

-47 Sequencing Primer

The -47 Sequencing Primer is 24 bases long, with a 62.5% GC content and a T_m (dissociation temperature as determined by Oligo 5.0 software nearest neighbor method: NBI/Genovus) of 79.8°C and has the following sequence:

5'-d(CGCCAGGGTTTTCCCAGTCACGAC)-3'

The primer has been HPLC purified and diluted to a concentration of 1.6 pMol/µL (or 1.6 µM). The -47 Sequencing Primer does not form internal structures and is an excellent sequencing primer. You can also use other primers such as T3, T7, SP6, and some M13 forward and reverse primers, although they tend to be less efficient since they are shorter and have lower melting temperatures. The cycling conditions stated in the protocol were not optimized for these other primers; however, further modifications to the standard protocol could enhance the sequencing data obtained using these primers. It is likely that changes to the thermal cycling parameters may be needed to optimize certain template/primer combinations. (See "Thermal Cycling Program" on page 8.)

Glycogen

Glycogen is used to help precipitate the sequencing fragments following the sequencing reactions. Omitting glycogen leads to poor precipitations and low fluorescence signal when performing the separation on the system.

Sample Loading Solution

Sample Loading Solution (SLS) is used for the resuspension of the sequencing reaction products following post reaction clean up. Once the Sample Loading Solution is initially thawed, it should be aliquoted in smaller volumes, frozen at -20°C, and not thawed more than one additional time. Improper storage or excessive freeze thawing leads to low, or complete loss of, fluorescence signal.

Mineral Oil

Mineral oil is used to overlay the samples after their final resuspension in Sample Loading Solution (SLS) in the sample plate. The oil protects the dyes and increases their stability. In general, samples can be reinjected two to three days following their initial injection, if left at room temperature. For longer term storage, samples should be kept at -20°C. If additional Mineral Oil is required, use the same oil as supplied in the GenomeLab Methods Development kit (PN 608000). Using other Mineral Oil is not recommended as it can result in loss of fluorescence signal on the system.

1.2 GenomeLab DTCS - Quick Start Kit (PN 608120)

The GenomeLab DTCS - Quick Start Kit provides a convenient alternative to sequencing reaction preparation. The Reaction Buffer, dNTPs, dye labeled terminators and DNA polymerase are provided as a “master mix” solution in a single tube. The kit should arrive frozen and be transferred to a NON-FROST-FREE FREEZER set at -20°C for storage. The important components that require -20°C storage are the dye labeled terminators and the DNA Polymerase, contained in the GenomeLab DTCS Quick Start Master Mix, and the Sample Loading Solution.

GenomeLab DTCS - Quick Start Master Mix

This solution contains, in a single tube, the DNA polymerase, reaction buffer, deoxynucleotide triphosphates, and all four dye labeled dideoxynucleotide triphosphate terminators. All information for the individual components remain the same as for the GenomeLab Methods Development Kit above, except for the reaction buffer. The reaction buffer is less concentrated in the master mix and should not precipitate out of solution. The GenomeLab DTCS - Quick Start Master Mix should be thawed on ice.

The following list identifies the remaining components, which are the same as those provided in the GenomeLab Methods Development Kit.

- pUC18 Control Template (See "pUC18 Control Template" on page 2.)
- -47 Sequencing Primer (See "-47 Sequencing Primer" on page 2.)
- Glycogen (See "Glycogen" on page 2.)
- Sample Loading Solution (See “Sample Loading Solution” on page 2.)
- Mineral Oil (See "Mineral Oil" on page 2.)

1.3 Required Materials Not Provided by Beckman Coulter

Sterile Water

Use deionized and sterilized water that has NOT been treated with DEPC (diethyl pyrocarbonate). DEPC is a reagent commonly used to treat water to inhibit the activity of RNase enzymes. Residual DEPC interferes with sequencing enzymatic reactions and can cause low signal and current instability.

95% Ethanol/Water, 70% Ethanol/Water

These reagents are used to precipitate the reactions following cycle sequencing. The ethanol should be absolute (200 proof), 100% (molecular biology grade) and the solution should be stored at -20°C and used at that temperature. Use sterile water as described above to dilute the ethanol to the required concentration.

3M Sodium Acetate pH 5.2

The sodium acetate is used as part of the Stop solution. It is mixed 1:1 with the 100 mM Na_2EDTA just prior to use in post reaction clean up. Because the pH is critical, make sure the sodium acetate used is the correct pH; otherwise, the unincorporated dye terminators may not be efficiently removed. A 3M Sodium Acetate, pH 5.2 solution is available from Sigma (100 or 500 mL, cat # S7899).

100 mM Na_2EDTA pH 8.0

This is the second component of the Stop solution required for post reaction clean up. You should make up the Stop solution fresh from the Sodium Acetate and EDTA stocks; otherwise, precipitation of salts out of this solution may occur, which leads to inefficient removal of unincorporated dye terminators. A 0.5M Na_2EDTA (pH 8.0) solution is available from Sigma (100 mL, cat # E7889).

Sterile Tubes - 0.5 mL Microfuge Tubes

Use 0.5 mL microfuge tubes for post reaction clean up. To prevent degradation of the DNA fragments, you must use sterile tubes.

0.2 mL Thin Wall Thermal Cycling Tubes or Plates

The exact tubes or plates used depend on the thermal cycler. In general, you should use the tubes or plates recommended by the thermal cycler manufacturer. The sample plate (Beckman Coulter PN 609801) is compatible with certain thermal cyclers, and can be used for that purpose. The walls of the sample plate are thicker than other plates and therefore, may require adjustments to compensate for slightly slower temperature ramping times.

Thermal Cycler with Heated Lid

Thermal cyclers with heated lids do not require an oil overlay. Working with oil and removing samples from under oil is difficult and can lead to sample loss, which decreases fluorescence signals. All thermal cyclers are slightly different in their heating/cooling/ramping capabilities. In certain cases, you may need to alter the cycling conditions slightly to compensate for this. The conditions listed in the protocol work well for MJ Research PTC 200 and PTC 100 and the Perkin Elmer PE 2400, PE 9600, and PE 9700 thermal cyclers.

Preparation of the DNA Sequencing Reaction

2.1 GenomeLab Methods Development Kit

Prepare sequencing reactions in a 0.2 mL thin wall thermal cycling tube or microplate well. Keep all reagents on ice while preparing the sequencing reactions and add them in the order listed in the following table.

Be sure to thaw and mix the solutions thoroughly before use. Notes for most of the reaction components are listed below.

Reaction Component	dITP Chemistry	dGTP Chemistry	See Note
Sterile H_2O (to adjust total volume to 20 µL)	x.x µL	x.x µL	
DNA Template* (see "DNA Template Preparation" on page 15.)	0.5 - 7.0 µL	0.5 - 5.0 µL	
Customer supplied primer OR -47 sequencing primer	(1.6 pMol/µL) 2.0 µL	(1.6 pMol/µL) 2.0 µL	1
10X sequencing reaction Buffer	2.0 µL	2.0 µL	2
dNTP(I) or dNTP(G) Mix	1.0 µL dNTP(I) Mix	1.0 µL dNTP(G) Mix	3
ddUTP Dye Terminator	2.0 µL	2.0 µL	4
ddGTP Dye Terminator	**1.0 µL**	**3.0 µL**	4
ddCTP Dye Terminator	2.0 µL	2.0 µL	4
ddATP Dye Terminator	2.0 µL	2.0 µL	4
DNA Polymerase Enzyme	1.0 µL	1.0 µL	5
Total Reaction Volume	20.0 µL	20.0 µL	

* Use 0.5 µL for pUC18 control template

Mix the reaction components thoroughly. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before thermal cycling.

Although mixing occurs as the temperature cycles during the thermal cycling steps, it is not as effective as thorough mixing prior to cycling. In addition, the entire solution needs to be consolidated so the temperature changes are uniform for the entire mix.

NOTE The ***water*** should be deionized, sterile water but not DEPC treated. The exact amount added depends on the template volume (see table above).

NOTE The ***DNA template*** should be in water or 10 mM Tris-HCl pH 8.5. EDTA should not be present as it can inhibit the sequencing reaction.

1. Primers should be purified by “trityl-on HPLC” or OCP columns and not acrylamide gel purified. Impurities in the primer can load contaminants on the system causing current failures and low signals. Desalted primers MAY be acceptable, but if there is any question, use HPLC or OCP columns for primer purification.

If the cycling conditions or bad primer design are an issue, then the current will be normal but little or no signal will be observed.

The template to primer ratio is also important. If not enough primer is present, insufficient extension products will be generated during the sequencing reactions. This leads to little or no fluorescence signal on the system. The optimum ratio for dsDNA is greater than or equal to 40:1 of primer to template. Higher ratios may improve the quality of the sequencing results in some cases. For PCR products, where generally only small amounts of template are used, ratios greater than 600:1 of primer to template have been shown to work.

2. Make certain that there is no precipitate in the solution before use. Dissolve any precipitate present by warming the solution.
3. The dNTP(I) Mix contains dITP, a dGTP analog used to reduce “compressions.” Because dITP is used the extension temperature of 60°C must be maintained instead of 72°C normally used with thermal stable DNA polymerases.

   The dNTP(G) Mix contains the standard deoxynucleotides including dGTP. Due to band compressions, we do not recommend using dGTP chemistry for routine sequencing. The dGTP chemistry is recommended only for sequencing through difficult regions that may include polymerase hard stops, secondary structures and GC rich regions. Use the dITP chemistry to confirm all band compression regions and the regions adjacent to band compressions.
4. The terminators are in methanol and will therefore freeze at a lower temperature than the other components. Do not warm the dye labeled terminator solutions at 37°C, as the methanol will eventually be lost, resulting in dye degradation.
5. The enzyme is in a glycerol buffer and will not freeze, even at -20°C. Make sure to centrifuge the tube briefly to consolidate the liquid before use. Glycerol is difficult to pipette due to its viscosity; therefore, take extra care when adding this component.

Template Pre-Heat Treatment

For certain plasmid DNA templates, a pre-heat treatment improves both signal strength and current stability. Perform this treatment only on the DNA template and water. Do not add any other sequencing reaction components to the template before performing this pre-heat treatment. This treatment is not recommended for PCR product sequencing templates. For details on this treatment, see "Template Pre-Heat Treatment" on page 17.

For longer sequencing read lengths and the control template, heat the temp at 65°C for 5 minutes in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing reaction components. For heavily supercoiled templates, heat the template at 96°C for 1 minute. If the raw data signal declines steeply when using this treatment, reduce the heating temperature or time conditions. If the current is low or unstable following this treatment, the treatment can be increased to 96°C for 3 minutes.

2.2 GenomeLab DTCS - Quick Start Kit

The GenomeLab DTCS - Quick Start Kit eliminates multiple pipetting steps and possible reagent omission errors while preparing sequencing reactions. The volume of Master Mix added to the sequencing reaction is only 8 µL leaving more volume for DNA template and primer. The reactions can be performed in a 0.2 mL thin wall tube or microplate well. Keep all reagents on ice while preparing the sequencing reactions and add them in the order listed below.

Be sure to thaw and mix the solutions thoroughly before use. Notes for the different components are listed below.

Reaction Component	Amount	See note
H_2O (to adjust total volume to 20 µL)	x.x µL	
DNA Template* (see "DNA Template Preparation" on page 15)	0.5 - 10.0 µL	
Customer supplied primer OR -47 sequencing primer	(1.6 pMol/µL) 2.0 µL	6
GenomeLab DTCS - Quick Start Master Mix	8.0 µL	7
Total Reaction Volume	20.0 µL	

* Use 0.5 µL for pUC18 control template

Mix the reaction components thoroughly. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before thermal cycling.

Although mixing will occur as the temperature cycles during the thermal cycling steps, it is not as effective as thorough mixing prior to cycling. In addition, the entire solution needs to be consolidated so that the temperature changes are uniform for the entire mix.

NOTE The water should be deionized, sterile water but not DEPC treated. The exact amount added depends on the template volume (see table above).

NOTE The DNA should be in water or 10 mM Tris-HCl pH 8.5. EDTA should not be present as it can inhibit the sequencing reaction.

1. Primers should be purified by “trityl-on HPLC” or OCP columns and not acrylamide gel purified. Impurities in the primer may load contaminants on the system causing current failures and low signals. Desalted primers MAY be acceptable, but if there is any question, use HPLC or OCP columns for primer purification.

   If the cycling conditions or bad primer design are an issue, then the current will be normal but little or no signal will be observed.

   The template to primer ratio is also important. If not enough primer is present, insufficient extension products will be generated during the sequencing reactions. This leads to little or no fluorescence signal on the system. The optimum ratio for dsDNA is greater than or equal to 40:1 of primer to template. Higher ratios may improve the quality of the sequencing results in some cases. For PCR products, where generally only small amounts of template are used, ratios greater than 600:1 of primer to template have been shown to work.

2. The GenomeLab DTCS - Quick Start Master Mix contains the DNA polymerase, dNTPs (including dITP), dye labeled terminators, and reaction buffer components. Since dITP, a dGTP analog, is used to reduce “compressions,” the thermal cycling extension temperature of 60°C must be maintained instead of 72°C normally used with thermal stable DNA polymerases. This master mix should be thawed and kept on ice. Improper handling may lead to little or no fluorescent signal on the system.

Template Pre-Heat Treatment

For certain plasmid DNA templates, a pre-heat treatment improves both signal strength and current stability. Perform this treatment only on the DNA template and water. Do not add any other sequencing reaction components to the template before performing this pre-heat treatment. This treatment is not recommended for PCR product sequencing templates. For further details on this treatment see "Template Pre-Heat Treatment" on page 17.

For longer sequencing read lengths and the control template, heat the temp at 65°C for 5 minutes in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing reaction components. For heavily supercoiled templates, heat the template at 96°C for 1 minute. If the raw data signal declines steeply when using this treatment, reduce the heating temperature or time conditions. If the current is low or unstable following this treatment, the treatment can be increased to 96°C for 3 minutes.

2.3 Thermal Cycling Program

dITP Chemistry		dGTP Chemistry	
96°C	20 sec.	96°C	20 sec.
50°C	20 sec.	50-68°C	20 sec.
60°C	4 min.	68-72°C	2 min.

Complete 30 cycles of the above three temperatures followed by holding at 4°C.

The dITP chemistry cycling parameters are optimized for the control template and primer, but should work in most other cases. For the dGTP chemistry, an annealing temperature of 58°C and an extension temperature of 68°C is suitable for the supplied M13-47 primer and most templates, and the pUC18 control. It may be necessary to modify the thermal cycling parameters for other primer and template combinations.

For the annealing step, a temperature based on the primer melting temperature (T_m) minus 3 to 5°C is recommended as a starting point. The higher extension temperature of 72°C may be helpful with highly G-C rich templates.

Recommendations for Obtaining Optimal Results

The following topics discuss cases that may require changes to the primer design, template, and thermal cycling conditions.

Primer Design

Primer design is critical to good sequencing. The conditions for thermal cycling listed in the protocol work well for the -47 primer and other "universal" primers. Some primers may require changes in the cycling conditions for optimal performance. Since other primers (e.g. SP6, T3, T7, -21 primer) are shorter and have lower melting temperatures, sequencing protocols using these primers often employ lower annealing temperatures.

Primers should be at least 18 bases long to ensure good hybridization. The best primers for cycle sequencing are between 23-26 bases in length (for example, the primer supplied in the GenomeLab Methods Development and Quick Start kits is 24 bases long). In addition, primers should have a melting temperature (or T_m) of greater than 55°C, and the optimum T_m is above 60°C for the thermal cycling conditions listed above. The GC content of primers should be around 50%. Again, if this is not possible, a longer primer should be designed to bring the T_m up to the recommended range.

Primers should not self anneal (form internal secondary structures) or hybridize within themselves (form "primer dimer") because this reduces priming and generates fewer extension products. Software programs such as Oligo 5.0 (NBI/Genovus) are available to help with primer design. Oligo 6.0 is available from Molecular Biology Insight, Inc. (800-747-4362).

As with PCR, some new primers require optimization of the cycling conditions. The conditions given in the protocol are good for a well designed primer. In cases where the T_m is low, changes may need to be made. In general, this requires altering the annealing temperature. Changing the extension temperature and time or denaturation temperature and time do not normally enhance the sequencing results with dITP chemistry, but may be required with the dGTP chemistry.

Template

Templates high in GC content and large templates can be more difficult to sequence. In some cases increasing the denaturation time will help. There is a limit to how long the time can be extended as the enzyme degrades more quickly the longer it is kept at elevated temperatures. The enzyme is more labile at increased temperatures, therefore increasing the denaturation temperature above 96°C is not recommended. In these cases, it may help to use the dGTP chemistry to sequence through the difficult regions.

Thermal Cycling Conditions

For the dITP or DTCS Quick Start Chemistry, a 4-minute extension time has been selected to facilitate the sequencing of templates that are not as pure as the control template. Since the DNA polymerase does not incorporate dITP as readily as dGTP, a longer extension time is typically required. A two minute extension time is sufficient for the dGTP chemistry due to the use of the standard deoxynucleotides. The extension time may be reduced for very clean plasmid templates or short PCR products that yield sufficiently high fluorescence signal on the system. We recommend that the extension time not be reduced to less than one minute.

The annealing temperature should be dictated by the primer's T_m (see above) and logically cannot exceed the extension temperature. In the case of the dGTP chemistry, the primer T_m and appropriate annealing temperature and time can be critical. Inappropriate annealing temperatures may lead to mis-priming and undesirable secondary sequencing products. A two-step cycle, which combines the annealing and extension steps, has been shown to work for certain primer-template combinations. The two-step cycling procedure is only applicable when the T_m for the primer is at least 5°C higher than the extension temperature (for example, if the extension temperature is 60°C, the T_m for the primer would have to be at least 65°C). For highly G-C rich templates, raising the extension temperature to 72°C has also been shown as beneficial in reducing undesirable secondary sequencing products.

The number of cycles is optimized to give the best value for time spent cycling versus amount of signal generated. Decreasing the number of cycles will decrease the number of extension products generated and therefore decrease the signal. The combination of increased primer and increased cycle number may help in certain cases where low signal is a problem.

Optional SAP Treatment for dGTP Samples

After completing thermal cycling, you may perform an optional Shrimp Alkaline Phosphatase (SAP) treatment to remove free dye terminator peaks as needed.

Add the following to each 20 µL of sequencing reaction:

2 µL	10x SAP Reaction Buffer*
1 µL	SAP (1 unit/µL)

Mix thoroughly by pipetting up and down. Consolidate the liquid to the bottom of the tube or well by briefly centrifuging before incubation.

*If 10x SAP reaction buffer is not available, replace the 2 µL of 10x SAP reaction buffer with 2 µL of 100 mM $MgCl_2$.

Incubation Conditions for SAP Treatment

37°C	30 min.

Followed by holding at 4°C

Continue to the ethanol precipitation step.

NOTE If a SAP treatment is performed, modify the “Delay” setting on the Initial Data Detection tab of the Sequencing Analysis Parameters Editor in the sequencing analysis module to 0.1 minute. Alternatively, if a SAP treatment is not performed, but exclusion of free dye terminator peaks is desired, modify the “Delay” setting on the Initial Data Detection tab of the Sequencing Analysis Parameters Editor in the sequencing analysis module to 1.4 minutes.

Post Sequencing Reaction Purification

3.1 Ethanol Precipitation in a 0.5 mL Microfuge Tube

1. Prepare a labeled sterile 0.5 mL microfuge tube for each sample.
2. Prepare fresh Stop Solution/Glycogen mixture of 2 µL of 3 M NaOAc (pH 5.2), 2 µL of 100 mM Na_2-EDTA (pH 8.0) and 1 µL of 20 mg/mL of Glycogen (supplied with the kit).

   To each of the wells, add 5 µL of the Stop Solution/Glycogen mixture.

NOTE A stop solution "premix" which includes the glycogen may alternatively be made just prior to starting the sample cleanup as follows (amounts below are per row of 8 samples):

- 20 µL 3 M NaOAc pH 5.2
- 20 µL 100 mM EDTA pH 8.0
- 10 µL 20 mg/mL Glycogen

NOTE Pipette 5 µL of this stop solution premix into each tube before transferring the sequencing reactions into them.

Prepare the stop solution immediately before use and keep it at room temperature. If the stop solution is chilled or left at room temperature for too long, the EDTA will precipitate. The glycogen should be stored at -20°C with the kit.

The salt and glycogen facilitate precipitation of the DNA from the sequencing reactions. Precipitation is very inefficient without them and leads to low signal and potential color imbalance (low A signal may be observed) when run on the system.

3. Transfer the sequencing reactions to the appropriately well and mix thoroughly. All components must be mixed for efficient precipitation.
4. Add 60 µL cold 95% ethanol/water (v/v) from -20°C freezer and mix thoroughly. Immediately centrifuge at 14,000 rpm at 4°C for 15 minutes. Carefully remove the supernatant with a micropipette (the pellet should be visible).

NOTE For multiple samples, always add the cold ethanol/water immediately before centrifugation.

Thorough mixing is very important at this stage. Again, if it is not done correctly, the precipitation will be less than optimal and could lead to low signal from the system. Do not leave the samples for extended periods of time before centrifuging because this will precipitate some of the salt and dyes that the ethanol precipitation is designed to eliminate. Leaving samples at -20°C following addition of the ethanol may also have a detrimental effect. Ideally, the samples should be centrifuged immediately following addition of the ethanol.

5. Rinse the pellet **two times** with 200 µL 70% ethanol/water (v/v) from -20°C freezer. For each rinse, centrifuge immediately at 14,000 rpm at 4°C for a minimum of 2 minutes. After centrifugation, carefully remove all of the supernatant with a micropipette.

   The purpose of the washes is to remove the residual salts that remain after removing the supernatant from the precipitation step. Since salts are preferentially loaded by electrokinetic injection on the system, fewer sequencing fragments will be loaded. As the salts are removed, the pellet may become invisible. Do not disturb the pellet when adding the 70% ethanol; this causes the pellet to dislodge and could lead to the entire sample being lost during the processing of the sample. Simply allowing the 70% ethanol to cover the pellet is sufficient to reduce the salt remaining in the sample.

6. Remove as much of the ethanol as possible following centrifugation. Be very careful not to suck the pellet into the pipette tip. The pellet will be fairly loose on the side of the tube and can easily be dislodged.
7. Vacuum dry the samples for 40 minutes using a Speed Vac (without heated drying.)

NOTE If extra ethanol has been left from the previous step, increased drying time may be required. In all cases, make sure that the pellet is COMPLETELY dry before going on to the next step. Ethanol left on the pellet suppresses the signal on the system. **Do NOT use heated drying to remove the ethanol.** Heating the samples while they are drying can lead to severe degradation of signals on the system.

8. Resuspend the sample in 40 µL of Sample Loading Solution (SLS).
9. Make sure that the samples are fully resuspended in the SLS. This will take from 5 - 15 minutes. **Heating the samples to resuspend them is NOT recommended.** The resuspended samples can be stored in closed tubes at -20°C for up to one month.

WARNING DO NOT STORE SAMPLES AS DRY PELLETS!

3.2 Ethanol Plate Precipitation in a Sample Plate (Beckman Coulter PN 609801)

1. Spin down the sample plate after thermal cycling at maximum speed for 30 seconds to ensure that any condensation is at the bottom of the well.
2. To each well, add 4 µL Stop Solution (1.5 M NaOAc + 50 mM EDTA prepared daily from stock solutions previously listed) and 1 µL of 20 mg/mL glycogen (supplied with the kit). **Mix Thoroughly!**

NOTE A stop solution "premix" which includes the glycogen may alternatively be made just prior to starting the sample cleanup as follows (amounts below are per row of 8 samples):

- 20 µL 3 M NaOAc pH 5.2
- 20 µL 100 mM EDTA pH 8.0
- 10 µL 20 mg/mL Glycogen

NOTE Pipette 5 µL of this stop solution premix into each well before transferring the sequencing reactions into them.

3. Prepare the stop solution immediately before use and keep it at room temperature. If the stop solution is chilled or is left at room temperature for too long the EDTA will precipitate. The glycogen should be stored at -20°C with the kit.

   The salt and glycogen facilitate precipitation of the DNA from the sequencing reactions. Precipitation is inefficient without them and leads to low signal and potential color imbalance (low A signal may be observed) when run on the system.
4. Add 60 µL cold 95% ethanol/water (v/v) from -20°C freezer and cover plate with Seal and Sample Aluminum Foil Lids (Beckman Coulter, Inc. PN 538619).
5. Invert the plate 5-10 times vigorously or vortex plate.

NOTE For multiple samples, always add the cold ethanol/water immediately before centrifugation.

Thorough mixing is very important at this stage; otherwise, precipitation will be less than optimal and could lead to low signal on the system. Do not leave the samples for extended periods of time before centrifuging because this will precipitate some of the salt and dye terminators that the ethanol precipitation is designed to eliminate. Leaving samples at -20°C following the addition of the ethanol may also have a detrimental effect. Ideally, the samples should be centrifuged immediately following addition of the ethanol.

6. Immediately centrifuge the plate according to the following chart:

Centrifuge	Rotor	G	RPM	Time
Beckman Coulter Avanti J-E	JS-5.3	6,130	5,300	2 minutes
Beckman Coulter Allegra 25R	S5700	5,957	5,700	2 minutes
Beckman Coulter Allegra 25R	Microplate Carrier	3,007	4,100	10 minutes
Beckman Coulter GS-15R	S2096	1,107	3,000	30 minutes

7. After centrifugation remove the plate and place 3-4 folds of paper towels on the centrifuge plate holder. Carefully remove the foil lid and gently invert the plate to remove the supernatant. Do not turn the plate right side up before spinning as this may disrupt the DNA pellets.
8. Place the inverted plate onto the paper towel lined plate holder and spin at 300 rpm for 20 seconds. For the Avanti J-E centrifuge, carefully watch the speed indicator since this centrifuge accelerates very quickly. Once at speed let it run for 10 seconds then press the Stop button.
9. Rinse the DNA pellet with 200 µL 70% ethanol/water (v/v) from -20°C freezer. DO NOT mix or vortex the plate.
10. The purpose of the rinses is to remove the residual salts that remain after removing the supernatant from the precipitation step. Since salts are preferentially loaded by electrokinetic injection on the system, fewer sequencing fragments will be loaded. As the salts are removed, the pellet may become invisible. Do not disturb the pellets when adding the 70% ethanol; this dislodges the pellets and could lead to the entire sample being lost during the processing of the sample. Simply allowing the 70% ethanol to cover the pellets is sufficient to reduce the salt remaining in the sample.
11. After the addition of 70% ethanol/water (v/v), centrifuge the plate immediately, according to the following chart:

Centrifuge	Rotor	G	RPM	Time
Beckman Coulter Avanti J-E	JS-5.3	6,130	5,300	2 minutes
Beckman Coulter Allegra 25R	S5700	5,957	5,700	2 minutes
Beckman Coulter Allegra 25R	Microplate Carrier	3,007	4,100	3 minutes
Beckman GS-15R	S2096	1,107	3,000	5 minutes

12. After centrifugation, gently invert the plate to remove the supernatant. Place the inverted plate on the paper towel lined plate holder and spin at 300 rpm for 20 seconds.

NOTE For the Avanti J-E centrifuge, carefully watch the speed indicator since this centrifuge accelerates very quickly. Once at speed let it run for 10 seconds then press the Stop button.

13. Repeat the rinse (steps 9-12).
14. Vacuum dry the samples for 10 minutes. If you are not using a speed vac with a plate rotor to dry the DNA pellets, be careful to apply and release the vacuum slowly.
15. Resuspend the pellets in 40 µL of Sample Loading Solution (SLS).

Make sure the samples are fully resuspended in the SLS. This takes from 5-15 minutes.

WARNING Do NOT heat the samples in order to resuspend them.

The resuspended samples can be stored in the sealed plate at -20°C for up to one month.

WARNING DO NOT STORE SAMPLES AS DRY PELLETS!

3.3 Sample Preparation for Loading into the System

1. If tube precipitation was performed, transfer the resuspended samples to the appropriate wells of the sample plate (PN 609801). If plate precipitation was performed then skip to step b.

WARNING Use of any other plate WILL NOT WORK and WILL RESULT IN DAMAGE to the capillary array.

2. Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit).
3. The mineral oil acts as a protectant for the samples. This means that samples from a plate can be placed on the system and then reinjected later if desired. If samples are prepared and are to be stored in the sample plate prior to running, it is recommended that they are resuspended in SLS, overlaid with mineral oil, sealed with Beckman Coulter Seal and Sample Aluminum foil lids (PN 538619) and stored at -20°C until used. Samples can be stored this way for up to one month.

WARNING DO NOT STORE SAMPLES AS DRY PELLETS!

4. Load the sample plate into the system and start the desired method. The plate is "notched" at one corner and fits into the plate holder in only one direction. Take care when loading the sample plate onto the system and do not damage the exposed capillary tips.
5. When sequencing with the dGTP chemistry, the capillary temperature of the separation method used on the system may be increased to reduce the appearance of some band compressions.

Template Preparation

4.1 DNA Template Preparation

Prepare sufficient template to allow for its accurate quantitation and purity verification. Quality of the DNA template depends on the procedure and the source of the DNA utilized. The following are recommended protocols:

- QIAGEN Qiawell and QIAprep DNA isolation protocols (dsDNA and ssDNA)
- QIAGEN Qiaquick PCR purification protocol (PCR products)
- Promega SV Prep

NOTE The quality and quantity of template DNA should be determined by agarose gel electrophoresis. MDQ capillary electrophoresis is strongly recommended for this purpose.

Template preparation is probably the most critical factor in obtaining good sequence data from the system. The kits listed above have been used successfully for a number of different templates. Other protocols and kits from other manufacturers have not yet proven reliable enough for us to make a recommendation for their use. In some cases, CsCl DNA preps have been shown to cause low signal and current instability problems. If current instabilities and/or low signal occur we recommend using Template Pre-Heat Treatment (see page 17).

Low signals can be caused by impure template preparations inhibiting the sequencing reactions or by these impurities being co-injected into the capillary.

Current instabilities and current failures are often associated with impurities in template preparations. These abnormal currents can lead to variable peak spacing and retardation of the migration of the sequencing fragments through the capillary that leads to shorter read lengths.

We strongly recommend using agarose gels to check the quantity and purity of DNA before starting the sequencing reactions. Use a marker DNA, such as a known quantity of lambda DNA digested with HindIII, for quantitation.

4.2 DNA Template Amount

The amount of template DNA to use in the sequencing reaction depends on the form of the DNA (dsDNA plasmid, ssDNA M13, PCR product, etc.). It is important to quantitate the amount (Moles) of DNA when performing the DNA sequencing reaction (see formula and plots below for details). This is important because the molar amount of template needs to be known so that the minimum molar ratio of primer to template is achieved (primer to template ratio of ~40:1). In addition, knowing the amount of template is important so that too much template is not used in the sequencing reaction. Listed below are the recommended amounts of DNA:

dsDNA	50 - 100 fmol
ssDNA	25 - 50 fmol
Purified PCR products	25 - 100 fmol

Template amount is critical. There are numerous methods available to estimate the amount of DNA in a sample; however, most of them are VERY inaccurate. The most commonly used, and probably the least accurate, is measuring the absorbance at 260 nm. Even good quality DNA samples will contain material other than DNA that absorbs at 260 nm. This leads to an erroneously high reading and too little DNA added to the reactions. Some relatively new fluorescent methods using dyes such as PicoGreen (Molecular Probes) are better, but care must be taken in setting up standard curves. The standards must be of similar type of DNA to the sample being quantitated (i.e. use supercoiled plasmid when quantitating plasmids and lambda DNA when quantitating phage clones, etc.).

Agarose gels are the best way of assessing the quality and quantity of the DNA in a sample. The technique is common, easy to do and is also useful for assessing quality of preparations. Quantitation of the amount of DNA in an unknown sample can be made by comparison to a known amount of a standard DNA such as HindIII digested Lambda DNA. In 1 µg of Lambda HindIII digest, for example, each of the DNA fragments is 32 fmoles. Therefore, if an unknown sample of approximately 6-7 kb has the same intensity as the 6.5 kb Lambda marker band (using 1 µg of the Lambda DNA), then the unknown is approximately 32 fmoles. However, the amount of each fragment (in ng) is proportional to its size. Therefore, the Lambda HindIII fragment that is 2.0 kb contains 2.0/48.5 x 1 µg = 0.041 µg or 41 ng, while the 6.5 kb fragment contains 6.5/48.5 x 1 µg = 0.134 µg or 134 ng. Using the size of the unknown and the estimated ng of the DNA, the fmoles of the unknown DNA can be estimated (see table below).

Another way to determine the amount of DNA to use in the sequencing reaction is to use the calculations in the GenomeLab Methods Development or Quick Start kit protocols. The calculations are thoroughly explained in each protocol (see also the ng to fmol conversion in the table below). A quick way to calculate amounts is to start with the control (pUC18) template at about 3 kb and 142 fmol/ µL or 250 ng/µL. For the control template 125 ng are used in the reaction. For example, if a 6 kb plasmid is used then twice the amount of DNA (in ng) is required to get the same number of fmol; therefore, 250 ng of template would be added.

Gel analysis can become a roadblock as more and more samples are run, but initially, we strongly recommend that all samples be quantitated using this method. Once confidence has been gained in the prep method and a correlation done between gels and a substitute quantitative measure, then the gels can be eliminated.

In general, the more template put in the reactions the more fragments that will be produced. However, increased template in the samples can lead to current failures as the template can interfere with the injection of the sequencing fragments, so this is often not the correct method to increase signal. In some cases, we have seen increased signal by lowering the amount of template. Presumably, this is because there is still sufficient template to generate fragments, but there is not enough to interfere with the loading of the samples.

For large plasmids, 15 kb for example, a relatively large amount of template will be needed, about 1.00 µg. This can potentially lead to current problems as there is a potential for interference with loading of the fragments. If this happens, this could be a case where decreasing the template would help. Increasing the primer and number of cycles may also help to generate more fragments from less template.

NOTE The Template Pre-Heat Treatment should be performed first for large supercoiled plasmids (See Section 4.3).

Shown below is a table correlating length and amounts for dsDNA.

Size (kilobase pairs)	ng for 25 fmol	ng for 50 fmol	ng for 100 fmol
0.2	3.3	6.5	13
0.3	4.9	9.8	20
0.4	6.5	13	26
0.5	8.1	16	33
1.0	16	33	65
2.0	33	65	130
3.0	50	100	195
4.0	65	130	260
5.0	80	165	325
6.0	100	195	390
8.0	130	260	520
10.0	165	325	650
12.0	195	390	780
14.0	230	455	910
16.0	260	520	1040
18.0	295	585	1170
20.0	325	650	1300
48.5	790	1500*	1500*

For ssDNA, the values (ng) should be divided by 2.
*Use no more than 1.5 µg of template DNA.

4.3 Template Pre-Heat Treatment

For certain plasmid DNA templates, the following pre-heat treatment improves both signal strength and current stability.

For longer sequencing read lengths and the control template, heat the template at 65°C for 5 minutes in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing reaction components. For heavily supercoiled templates, heat the template at 96°C for 1 minute. If the raw data signal declines steeply when using this treatment, change the heating conditions to 86°C for 5 minutes. If the current is low or unstable following this treatment, increase the treatment to 96°C for 3 minutes.

Pre-heating the template in water prior to adding the sample to the sequencing reactions causes nicking of the DNA. This helps produce a more efficient cycle sequencing reaction.

In certain cases, the treatment causes too much nicking and the template degrades rapidly during the subsequent cycling. If this happens, the raw data signal declines rapidly since longer fragments are not produced from a degraded template. If this happens, reduce the heat treatment to 86°C for 5 minutes. In some cases the treatment may require even lower

temperature and time conditions to produce a good raw data signal and profile. In certain other cases no pre-heat treatment is required to obtain good sequencing data.

Conversely, there are samples that exhibit unstable currents after treatment at 96°C for 1 minute. In these cases, you should treat the sample for 2 or 3 minutes at 96°C.

The treatment serves to increase signal and stabilize currents when sequencing plasmids. These effects are generated by nicking the plasmid DNA. For best results the template alone should be pre-heated.

Certain sequencing protocols use an initial high temperature step to pre-denature plasmids prior to thermal cycling. This step is carried out in the presence of all sequencing reaction components. Using the pre-heat treatment in water eliminates the need for such steps in the cycling protocol.

Although we do not recommend using this pre-heat treatment with PCR products, this treatment has been shown to work with the sample plate (Beckman Coulter PN 609801). The plates were sealed with the appropriate caps (Corning Science Products PN 6556) or cap mats (Corning Science Products Reusable Sealing Mat-96. Cat # 6555). Other tubes and plates may differ in their thermal characteristics and the pre-heat treatment may have to be adjusted accordingly.

Appendix A

Technical Bulletins

An up-to-date list of Sequencing publications can be found on our website: http://www.beckmancoulter.com.

Use the **Resource Center** drop-down menu and choose **Literature**.

Additional Technical Support

In the United States:

Contact Beckman Coulter Technical Support

1.800.742.2345

Worldwide:

Contact your Beckman Coulter Technical Support Associate.

Appendix A
Technical Bulletins