CEQ™2000 Dye Terminator Cycle Sequencing Chemistry Protocol

A Step by Step Guide to Dye Terminator Cycle Sequencing on the CEQ 2000

718119AB
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Materials provided by Beckman Coulter:

CEQ Dye terminator Cycle Sequencing Kit (P/N 608000)

The kit should arrive frozen and be stored in a NON FROST FREE FREEZER at -20°C. The important components that require -20°C storage are the enzyme and the dyes. Note that the enzyme is in glycerol and WILL NOT be frozen solid.

DNA Polymerase

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

CEQ Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)

Beckman Coulter manufactures the four dye-terminators, each one contains 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 CEQ.

dNTP Mix solution

This solution contains 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 mix contains dITP, a dGTP analog used to reduce “compressions.” Because dITP is used along with the DNA Polymerase, 60°C must be used for the extension temperature instead of 72°C used in other systems.

Sequencing Reaction Buffer

The buffer is 10X concentrated and optimized for use with the DNA Polymerase. When thawing out make sure that no precipitate is visible in the tube. If a precipitate is present, the buffer can be warmed 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 CEQ.

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 CEQ kit differs from that published in GenBank by one base, so comparisons should only be made to the sequence provided by BCI (pUC18dG).

Note that the pUC18 control template is different from the CEQ Test Sample. The CEQ 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 Tm (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 1.6pMol/µL (or 1.6µM). The -47 Sequencing Primer does not form internal structures and is an excellent sequencing primer. Other primers such as T3, T7, SP6 some M13 forward and M13 reverse primers can also be used, 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 later under Thermal Cycling Program.)

Glycogen

Glycogen is used to help precipitate the sequencing fragments following the sequencing reactions. Omission of glycogen can lead to poor precipitations and hence low signal from the CEQ.

Mineral Oil

Mineral oil is used to overlay the CEQ samples after their final resuspension in formamide in the sequencing plate. The oil protects the dyes and hence increases their stability. In general, CEQ 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 DTCS kit (Sigma part # M5904). The use of other mineral oil is not recommended as it can result in loss of signal on the CEQ 2000.

Required materials not provided by Beckman Coulter

Sterile Water

The water is used in the CEQ sequencing reactions. The water should be deionized and sterilized. It should NOT be 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 enzymatic reactions and can cause low signal and current instability.

95% Ethanol / water, 70% ethanol / water

These reagents are used to precipitate the CEQ reactions following cycle sequencing. The ethanol should be absolute, 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 100mM Na_2EDTA just prior to use in post reaction clean up. pH is critical. Be sure that 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 500mL, cat # S7899).

100mM Na_2EDTA pH 8.0

This is the second component of the Stop solution required for post reaction clean up. In addition, the Stop solution should be made up fresh from the stocks, otherwise precipitation of salts out of this solution may occur. This will lead to inefficient removal of unincorporated dye terminators. A 0.5M Na_2EDTA (~pH 8-8.5) solution is available from Sigma (100mL, cat # E7889).

Formamide, Ultra Pure Bioreagent - J.T. Baker Cat # 4028-00

The ultra pure grade formamide ***MUST*** be deionized using the mixed bed resin (below) before use. Impurities present in the formamide can cause current failures and decreased injection of the DNA sequencing fragments on the CEQ giving poor separations and / or low signals. The formamide should be stored in small aliquots and frozen at -20°C until use. Aliquots should be used only once and the remainder discarded after use. A convenient aliquot is 350µL, which is enough for a row of samples and overage (40µL x 8 = 320µL plus ~10% overage).

Mixed Bed Resin AG501-X8(D) - BioRad Cat # 143-6425

The resin is used to deionize the formamide before use on the CEQ. Make sure that the protocol below is followed exactly. Short cuts in the time the formamide is deionized and improper storage will lead to low signal and current problems on the CEQ.

The correct procedure to use is as follows:

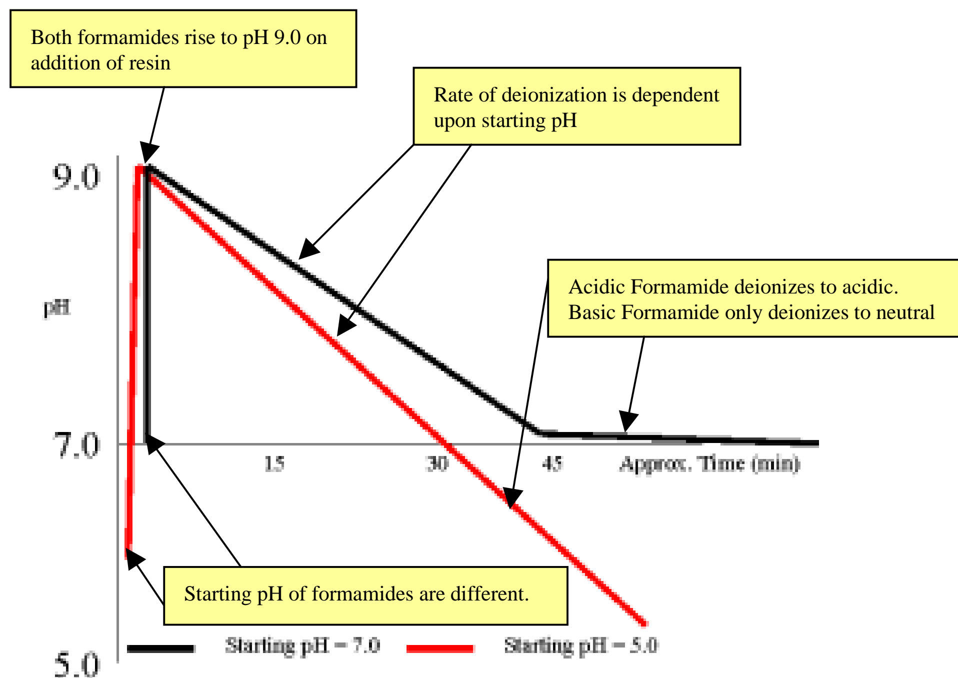
1) Transfer 100 mL of formamide (J.T. Baker, cat # 4028-00) into a 250 mL Erlenmeyer flask. Check the pH using pH paper (VWR Scientific Product, cat # EM-9590-3).
2) Add 5.0 gm of mixed-bed resin (Bio-Rad, cat # 143-6425) and stir with a magnetic stir bar. If the initial pH of the formamide is less than 7.0, stir the resin for 45 min. If the initial pH is greater than 7.0, stir the resin for 60 min. Keep the flask covered while stirring.
3) Check the pH of the formamide after stirring. If the pH is greater than 7.0 proceed to the next step, if pH less than 7.0, discard the formamide and start again with a fresh bottle of formamide.
4) Filter the sample through a 0.2µm nylon filtration unit (Nalgene, cat # 153-0020 or 151-5020 or Corning, cat # 430771 or 430773).
5) Store the deionized formamide in 350µL aliquots at -20°C in a non frost free freezer.

Use each aliquot only once - do not freeze / thaw the deionized formamide.

The following points are important for producing good quality formamide:

- The pH of the formamide plays an important role. Slightly alkaline pH is good, acidic pH produces data with low signal.
- Deionization needs to be controlled closely. Different bottles of formamide will have different pH's - even though they may have been purchased form the same vendor. The pH should be checked first ***BEFORE*** the addition of the resin. Once the resin is added, the pH will IMMEDIATELY increase to about pH 9.0. As the resin is stirred with the formamide, the pH will begin to drop. The rate at which this pH drop occurs depends upon the starting pH of the formamide. The pH of slightly acidic formamide will drop faster than the pH of neutral to slightly

alkaline formamide. Thus the protocol calls for longer times for deionization when the pH of the starting formamide is higher to begin with. This is illustrated below:



Note that longer deionization than recommended will not help and may in fact cause more problems as the pH may fall below pH 7.0 again.

- Formamide with an acidic pH will continue to drop in pH as deionization proceeds, higher starting pH formamide will remain about neutral even after longer deionization.
- Aeration of the formamide can cause problems. The stirring should be controlled so that aeration is kept to a minimum and the flask should be covered.
- It is possible that some bottles of formamide can never be deionized sufficiently to produce good data on the CEQ. In this case, the formamide should be discarded and deionization started again using a fresh bottle.

0.2 micron Nylon Filter

Use of other filters such as cellulose nitrate or cellulose acetate may introduce contaminants that could interfere with the CE separation. The following filtration units work well: Nalgene 0.22µm Nylon filtration unit (250mL size - cat # 153-0020 or 500mL size - cat # 151-4020) or Corning 0.22µm Nylon filtration unit (200mL size - cat # 430771 or 500mL size - cat # 430773).

Sterile tube - 0.5mL microfuge

The 0.5mL tubes are used for post reaction clean up. Sterile tubes should be used to prevent degradation of the DNA fragments.

0.2mL thin wall thermal cycling tubes or plates

The exact tubes or plates to be used will depend upon the thermal cycler in use. In general, whichever tubes or plates are recommended by the thermal cycler manufacturer should be used. The CEQ sample plate is compatible with certain thermal cyclers, and can be used for that purpose. The walls of the CEQ plate are thicker than other plates and therefore, adjustments MAY be required to compensate for slightly slower temperature ramping times.

Thermal Cycler with Heated Lid

Thermal cyclers with heated lids do not require the use of an oil overlay. Working with oil and removing samples from under oil is difficult and can lead to sample loss and therefore lower signals. All thermal cyclers are slightly different in their heating / cooling / ramping capabilities. The cycling conditions may have to be altered slightly in certain cases to compensate for this. The conditions listed in the protocol work well for MJ Research PTC 200 and MJ Research PTC 100 and the PE 2400, PE9600, and PE9700 cyclers.

Preparation of the DNA Sequencing Reaction

Prepare sequencing reactions in a 0.2mL thin wall tube or microplate well. Note all reagents should be kept on ice while preparing the sequencing reactions and added in the order listed below:

Be sure to thaw and mix the solutions thoroughly before use.

H_2O (to adjust total volume to 20µL)	xxµL

The water should be deionized, sterile water but not DEPC treated. The exact amount added will depend upon the template concentration (see later).

10X sequencing reaction Buffer	2.0µL

Make sure that there is no precipitate in the solution before use. Dissolve any precipitate present by warming the solution.

dNTP mix	1.0µL
ddUTP Dye Terminator	2.0µL
ddGTP Dye Terminator	2.0µL
ddCTP Dye Terminator	2.0µL
ddATP Dye Terminator	2.0µL

The terminators are in methanol and will therefore freeze at a lower temperature than the other components. Do not warm the dye solutions at 37°C, as the methanol will eventually be lost, resulting in dye degradation.

DNA Polymerase Enzyme	1.0µL

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 a little difficult to pipette due to its viscosity, therefore take extra care when adding this component.

DNA Template* (see template preparation)	0.5 - 6.0µL

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

Customer supplied primer OR -47 sequencing primer	(1.6pMol/µL) 2.0µL

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

* Use 0.5 µL for pUC18 control template

Impure primers will lead to current problems and low signals on the CEQ. 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 will lead to little or no signal on the CEQ. The optimum ratio for dsDNA is greater than or equal to 40:1 primer: 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.

Total Reaction Volume	20.0µL

Mix 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 mixing by hand prior to cycling. In addition, the entire solution needs to be consolidated so that the temperature changes are uniform for the entire mix.

Thermal Cycling Program

96°C	20 sec.
50°C	20 sec.
60°C	4 min.

for 30 cycles followed by holding at 4°C

These cycling parameters are optimized for the control template and primer, but should work in most other cases.

Cases where changes to these conditions may be required include:

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 T_ms, 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 CEQ DTCS kit 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 will reduce priming and fewer extension products will be generated. 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 will 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 have to be made. In general, this will mean altering the annealing temperature. Changing the extension temperature and time or denaturation temperature and time will not normally affect the sequencing results.

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 will degrade 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.

Thermal Cycling Conditions

A 4-minute extension time has been selected to facilitate the sequencing of templates that are not as pure as the control template. The extension time may be reduced for very clean plasmid templates or short PCR products that yield sufficiently high signal on the CEQ 2000. We recommend that the extension not be reduced to less than one minute.

The annealing temperature should be dictated by the primer's T_m (see above) and logically can not exceed the extension temperature. 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 (i.e. Since the extension temperature is 60°C, the T_m for the primer would have to be at least 65°C).

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.

Ethanol Precipitation

a) Prepare a labeled sterile 0.5mL microfuge tube for each sample.

b) To each labeled tube, add 4µL Stop Solution (1.5M NaOAc + 50mM EDTA **prepared daily from stock solutions previously listed**) and 1µL of 20mg/mL glycogen (supplied with the kit).

Make sure that the stop solution is prepared immediately before use and kept 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 will be very inefficient without them and will lead to low signal and potential color imbalance (low A signal may be observed) when run on the CEQ.

c) Transfer the sequencing reactions to the appropriately labeled tube and mix thoroughly.

All components must be mixed for efficient precipitation.

d) Add 60µL cold 95% (v/v) ethanol / water from -20°C freezer and mix thoroughly. Immediately centrifuge at 14,000rpm at 4°C for 15 minutes. Carefully remove the supernatant with a micropipette (the pellet should be visible).

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 CEQ. 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.

e) Rinse the pellet two times with 200µL 70% ethanol / water (v/v) from -20°C freezer. For each rinse, centrifuge immediately at 14,000rpm 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 CEQ, 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 will cause 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.

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.

f) Vacuum dry for 40 minutes.

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 will cause suppression of signal on the CEQ. Do not use heated drying to remove the ethanol. Heating the samples while they are dry can lead to severe degradation of signals on the CEQ.

g) Resuspend the sample in 40µL of deionized formamide (JT Baker cat # 4028-00, no water added). (Deionize the formamide using BioRad Mixed bed resin AG501-X8(D) cat # 143-6425 following the protocol detailed in the section “Mixed Bed Resin AG501-X8(D) - BioRad cat # 143-6425” on page 5.)

Using formamide that has not been deionized, or has been improperly deionized will lead to current or signal problems on the CEQ. Formamide degrades into formic acid and ammonia. The mixed bed resin removes the formic acid and may remove other contaminants from the formamide.

Make sure that the samples are fully resuspended in the formamide. 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. DO NOT STORE SAMPLES AS DRY PELLETS!

Sample Preparation for loading into the CEQ

a) Transfer the resuspended samples to the appropriate wells of the CEQ sample plate.

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

b) Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit).

   The mineral oil acts as a protectant for the samples. This means that samples from a plate can be placed on the CEQ 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 formamide, overlaid with oil, sealed with Beckman Coulter Seal and Sample Aluminum foil lids (part # 538619) and stored at -20°C until used. Samples can be stored this way for up to one month. DO NOT STORE SAMPLES AS DRY PELLETS!

c) Load the sample plate into the CEQ and start the desired method.

   The plate is “notched” at one corner and fits into the plate holder in only one way. Care needs to be taken when loading the sample plate onto the CEQ 2000 as not to damage the exposed capillary tips.

Template Preparation

DNA template preparation

Prepare sufficient template to allow for its accurate quantitation and purity verification. Quality of the DNA template will depend upon 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)

The quality and quantity of template DNA should be determined by agarose gel electrophoresis.

Template preparation is probably the most critical factor in obtaining good sequence data from the CEQ 2000. The kits listed above have been used successfully for a number of different templates. The Qiawell kit uses a two step purification procedure using ion exchange followed by silica binding. 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 16).

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 the use of 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.

DNA Template Amount

The amount of template DNA to use in the sequencing reaction is dependent 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 - 100fmol
ssDNA	25 - 50fmol
Purified PCR product	10 - 50fmol

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 260nm. Even good quality DNA samples will contain material other than DNA that absorbs at 260nm. This will lead to an erroneously high reading and too little DNA will be 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 1µg 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 1µg 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 1µg = 0.041µg or 41ng, while the 6.5kbp fragment contains 6.5/48.5 x 1µg = 0.134µg or 134ng. 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 DTCS kit one-page protocol. The calculations are thoroughly explained in the DTCS kit protocol (see also the ng to fmol conversion table shown below). A quick way to calculate amounts is to start with the control (pUC18) template at about 3Kbp and 142fmol/µL or 250ng/µL. For the control template 125ng are used in the reaction. For example, if a 6kb plasmid is used then twice the amount of DNA (in ng) will be required to get the same number of fmol. Therefore 250ng of that 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, 15kb for example, a relatively large amount of template will be needed, about 1.00µg. 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.

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.			

Template Pre-Heat Treatment

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

Heat the template at 96°C for 1 min in a thermal cycler and then cool to room temperature before adding the remainder of the sequencing reaction components. If the raw data signal declines steeply when using this treatment, change the heating conditions to 86°C for 5 min. If the current is low or unstable following this treatment, increase the treatment to 96°C for 3 min.

Pre heating the sample 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 will decline rapidly since longer fragments will not be produced from a degraded template. If this happens, the heat treatment should be reduced to 86°C for 5 min. In some cases treatment at 76°C for 5 min is sufficient to produce a good raw data signal and profile. In certain other cases, for example, the pUC control template, no pre-heat treatment is required in order to obtain good sequencing data.

Conversely, there are samples that will exhibit unstable currents after treatment at 96°C for 1 minute. In these cases the sample should be treated 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.

We do not recommend using this pre-heat treatment with PCR products.

This pre-heat treatment has been shown to work with the CEQ sample plate Beckman Coulter Part # 609801). The plates were sealed with the appropriate caps (Corning Science Products part # 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.

Thermal Cycling and Plate Precipitation in the CEQ 2000 Sample Plate

This procedure has only been done using an MJ Research PTC 200 Thermal Cycler. Optimization may be required for use of any other thermal cyclers.

1) For pre heat treatment of template:
   a) Add appropriate amount of template in water (not to exceed 6 µL total volume) to the appropriate well in the sample plate.
   b) Cover the plate with either strip caps (Corning Science Products, cat # 6556) or a reusable sealing mat (Corning Science Products, cat # 6555), and place into thermal cycler at 96°C for 1 minute (see pre heat treatment TIB for any further instructions). If strip caps are used, make sure caps are correctly seated on the plate by using the end of a pen or Sharpie to push the caps onto the plate.
   c) Add 2 µL of primer (3.2 pMol) and 12 µL master mix to each well. Mix using a multi-channel pipetter or vortex plate.
   d) Cover the plate with either strip caps or a reusable sealing mat (see above for cat #). Place plate into thermal cycler and run the following program:

      96°C 20 sec.

      50°C 20 sec.

      60°C 4 min.

      cycle a minimum of 30 cycles

2) Plate precipitation:
   a) Spin down the solution and add 5 µL of stop solution:

      1.5M NaOAc pH-5.2

      50mM Na_2EDTA pH 8.0

      4 µg glycogen

   b) Add 60 µL 95% EtOH, cover plate with Seal and Sample Aluminum Foil Lids (Beckman Coulter cat # 538619). Invert the plate 5-10 times vigorously and place in 20°C freezer for 10 minutes. Centrifuge at 1,400g or higher for 30 minutes at 4°C. After centrifugation remove the plates and place 3-4 golds 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 as this may disrupt the DNA pellet.

   Place the inverted plate onto the plate holders (containing folded paper towels) in the centrifuge and spin at 300rpm for 20 seconds.

   c) Rinse the pellet 2 times with 200 µL ice cold 70% EtOH/water (v/v). After each rinse centrifuge immediately at 1,400g for 5 minutes at 4°C. After centrifugation, gently invert the plates to remove the supernatant. As before, do not turn the plates

right side up. Place the inverted plate into the centrifuge plate holder containing 3-4 folds of paper towels and spin at 300 rpm for 20 seconds.

d) Vacuum dry the samples for 40 minutes. Be careful to apply and release vacuum slowly so as not to lose pellets. Resuspend the samples in 40 µL of deionized formamide (see instructions in the DTCS Detailed Guide, pg. 5).

e) Overlay each of the resuspended samples with one drop of light mineral oil, and load the sample plate into the CEQ 2000.

Stock Solutions

3M Sodium Acetate pH 5.2 (0.2 µm nylon filter sterilized) store at room temperature.

100 mM Na_2EDTA pH 8.0 (0.2 µm nylon filter sterilized) store at room temperature.

Stop Solution:

To prepare 1.5 M NaOAc/50 mM Na_2EDTA, add equal volumes of 3M NaOAc and 100 mM Na_2EDTA. Add a ratio of 4 volumes of 1.5M NaOAc/50mM Na_2EDTA to 1 volume of 20 mg/mL glycogen.

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