608019 AY
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GenomeLab Methods Development Kit Dye Terminator Cycle Sequencing

The GenomeLab Methods Development Kit (MDK) offers multiple sequencing chemistries for performing DNA sequencing. **It consists of a set of core reagents plus two dNTP solutions: dNTP(I) Mix containing dITP and dNTP(G) Mix containing dGTP.** Separate cycling conditions are also used for these two different chemistries. The dITP chemistry offers the full capabilities of the previous CEQ DTCS kit, and is recommended for routine sequencing. The dGTP chemistry is recommended when customers cannot sequence through some difficult templates using dITP-based sequencing chemistries: Quick Start Kit and previous CEQ DTCS kit.

Note: 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. The dITP chemistry should be used to confirm all band compression regions and the regions adjacent to band compression. The quality values and quality scores available for analyzed data are tuned for the dITP chemistry, and may not accurately estimate the data quality of the dGTP chemistry.

Material Required

Materials provided by Beckman Coulter:
Methods Development Kit (P/N 608000):
- DNA Polymerase
- Dye Terminators (ddUTP, ddGTP, ddCTP, ddATP)
- dNTP(I) Mix Solution
- dNTP(G) Mix Solution
- Sequencing Reaction Buffer
- pUC18 Control Template (0.25 µg/µL)
- M13 -47 Sequencing Primer (1.6 pmol/µL or 1.6 µM)
- Glycogen (20 mg/mL)
- Mineral Oil
- Sample Loading Solution (SLS)

Required materials not provided by Beckman Coulter:
- Molecular Biology Grade: Sterile dH_2O, 95% (v/v) ethanol/dH_2O, 70% (v/v) ethanol/ dH_2O
- 3M Sodium Acetate pH 5.2 - Sigma, Cat # 430771
- 100 mM Na_2-EDTA pH 8.0 (diluted from 0.5M Na_2-EDTA pH 8.0 - Sigma, Cat # 7889)
- Sterile tubes, 0.5 mL microfuge, 0.2 mL thin-wall thermal cycling tubes or plates
- Thermal cycler with heated lid

CAUTION

DNA polymerase is in a 50% glycerol solution. Pipet this solution slowly and carefully. The viscosity of the glycerol in the enzyme solution can lead to pipetting errors.

Preparation and Storage

Preparation and Storage of the Kit:

Storage of the Methods Development Kit must be in a -20°C non-frost-free freezer.

Preparation and Storage of the Premix:

1. Prepare each Premix in a sterile 1.5 mL microfuge tube:

Component	dITP Chemistry	dGTP Chemistry
10X Sequencing Reaction Buffer	200 µL	200 µL
dNTP Mix	100 µL	100 µL
ddUTP Dye Terminator	200 µL	200 µL
ddGTP Dye Terminator	**100 µL**	**300 µL**
ddCTP Dye Terminator	200 µL	200 µL
ddATP Dye Terminator	200 µL	200 µL
Polymerase Enzyme	100 µL	100 µL
Total Volume	1100 µL	1300 µL

2. Mix and aliquot the Premix into sterile 0.5 mL microfuge tubes:

Component	dITP Chemistry	dGTP Chemistry
16-Sample Premix Aliquot	180 µL	215 µL

Each aliquot is enough for 16 samples.

3. Store the aliquots in a -20°C non-frost-free freezer. Minimize freezing and thawing of the aliquoted Premix.

Preparation of the DNA sequencing reaction*:

Prepare the 20 µL sequencing reaction in a 0.2 mL thin-wall tube or microplate well. Keep all reagents on ice while preparing the sequencing reactions and add components in the order listed below.

Component	dITP Chemistry	dGTP Chemistry
H_2O (to adjust total volume to 20 µL)	x.x µL	x.x µL
DNA Template† (See Template Preparation)	0.5 - 7.0 µL	0.5 - 5.0 µL
Customer supplied or -47 Sequencing Primer (1.6 pmol/µL or 1.6 µM)	2.0 µL	2.0 µL
Premix	11.0 µL	13.0 µL
Total Volume	20.0 µL	20.0 µL

†Use 0.5 µL for pUC18 control template.

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

Thermal cycling programs:

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.**

for 30 cycles followed by holding at 4°C

**Note: For the supplied M13 -47 primer, an annealing temperature of 58°C and an extension temperature of 68°C is suitable for most templates and the control PUC 18. The thermal cycling parameters may need to be modified 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.*

***Note: The higher extension temperature of 72°C has been shown to be helpful with highly G-C rich templates.*

Ethanol precipitation:

1. Prepare a labeled, sterile 0.5 mL microfuge tube for each sample.
2. Prepare fresh Stop Solution/Glycogen mixture as follows (per sequencing reaction): 2 µL of 3 M Sodium Acetate (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 labeled tubes, add 5 µL of the Stop Solution/Glycogen mixture. Transfer the sequencing reaction to the appropriately labeled 0.5 mL tube and mix thoroughly.
3. Add 60 µL cold 95% (v/v) ethanol/dH_2O 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/dH_2O immediately before centrifugation.
4. Rinse the pellet 2 times with 200 µL 70% (v/v) ethanol/dH_2O 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.
5. Vacuum dry for 10 minutes (or until dry).
6. Resuspend the sample in 40 µL of the Sample Loading Solution (provided in the kit). See Appendix C for handling and storage of the Sample Loading Solution.

   Note: For plate precipitation instructions, refer to the Applications Information Bulletin (A1903A), A Rapid and Efficient Method for the Post-Reaction Clean Up of Labeled Dye Terminator Sequencing Products.

Sample preparation for loading into the instrument:

1. Transfer the resuspended samples to the appropriate wells of the polypropylene sample plate recommended for the instrument.
2. Overlay each of the resuspended samples with one drop of light mineral oil (provided in the kit).
3. Load the sample plate into the instrument and start the desired method.

   Note: When sequencing with dGTP chemistry, the capillary temperature of the separation method used on the CEQ Genetic Analysis System may be increased to reduce some band compressions.

Appendix

Appendix A

Sequencing of PCR products

All PCR products must be homogeneous in size as judged by gel electrophoresis.

Purified PCR products

- Remove unincorporated primers and dNTPs using QIAGEN QIAquick™ PCR purification system. Alternatively, unincorporated primers and dNTPs can be removed by Exo-SAP digestion using USB ExoSAP-IT®, followed by ethanol precipitation.
- Use 25-100 fmoles of purified PCR product and 3.2 pmoles of primer.

Unpurified PCR products

- For the original purified PCR product amplification, the primer concentration should be 0.2 µM or less, while the dNTP concentration should be 50 µM or less.
- The amplification should be sufficient to produce a concentration of amplified fragment that is >10 fmoles/µL.
- Dilute this amplified fragment approximately 10 fold to result in a concentration of >1 fmol/µL.
- Use 5-15 fmoles of this diluted, unpurified PCR product and 3.2 pmoles of primer.

Appendix B

Sequencing of Large Templates

Adding 50-100 fmol for large templates such as BACs, cosmids and PACs is impractical. The following procedure should be used when sequencing large templates.

1. Use 1.5 µg of the template in 6 µL of deionized water.
2. Pre-heat the template at 96°C for 1 minute. See Template Pre-Heat Treatment for details.
3. Add the sequencing reaction components as described in the standard protocol.
4. Cycle for 50 cycles using the appropriate cycling conditions for the primer being used.
5. Precipitate with ethanol.

Appendix C

- Store the Sample Loading Solution in 350 µL aliquots at -20°C in a non-frost-free freezer.
- Use each aliquot only once. Do not freeze/thaw the Sample Loading Solution.

Appendix D

Optional SAP Treatment for dGTP samples

After thermal cycling is complete, an optional Shrimp Alkaline Phosphatase (SAP) treatment can be performed for removal of 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 ethanol precipitation step.

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.

Template Preparation

DNA Template Preparation:

Prepare sufficient template to allow for accurate quantitation and purity testing. Quality of the DNA template will depend upon the procedure and the source of the DNA used. The following are the recommended protocols:

- QIAGEN QIAwell™ and QIAprep™ DNA isolation protocols (dsDNA and ssDNA)
- QIAGEN QIAquick™ PCR purification protocol (PCR products) *

**Note: Determine the quality and quantity of template DNA by agarose gel electrophoresis.*

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 accurately quantitate the amount (moles) of DNA when performing the DNA sequencing reaction (see formula and table below for details). The molar ratio of primer to template must be ≥ 40:1. Listed below are the recommended amounts of DNA:

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

The following table can be used to estimate DNA concentrations.

Table for estimating the dsDNA concentration.**

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.
† Do not use more than 1.5 µg of template DNA.

Template Pre-Heat Treatment

For certain plasmid DNA templates including the control template, a pre-heat treatment can improve both signal strength and current stability.

Dilute the template DNA in the appropriate amount of water for the final sequencing reaction. 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. Do not add any other sequencing-reaction components to the plasmid template before carrying out this pre-heat treatment. 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 continues to be unstable following this treatment, the treatment can be increased to 96°C for 3 minutes.

See the detailed Dye Terminator Cycle Sequencing Chemistry Protocol (P/N 718119) or (P/N 390003) for more information.

Handling Precautions

Please be aware of the handling precautions listed below. For detailed information, see 67-548-EEC (Directive on Dangerous Substances), 88-379-EEC (Dangerous Preparations Directive) and 21 CFR 1910.1200 (USA OSHA Hazard Communications).

Sample Loading Solution:
Toxic. Contains Formamide. R61 May cause harm to unborn child. R36/37 Irritating to eyes and respiratory system. S24/25 Avoid contact with skin and eyes. S37 Wear suitable gloves. S45 In case of accident, or if you feel unwell, seek medical advice immediately. S53 Avoid exposure-obtain special instructions before use.

Dye Terminators:
Contains <20% Methanol. R20/21/22 Harmful by inhalation, in contact with skin and if swallowed. R23/24/25 Toxic by inhalation, in contact with skin and if swallowed. R39 Danger of very serious irreversible effects. S36/37 Wear suitable protective clothing and gloves. S45 In case of accident or if you feel unwell, seek medical advice immediately. S60 This material and/or its container must be disposed of as hazardous waste. S7/9 Keep container tightly closed and in a well-ventilated place.

GHS HAZARD CLASSIFICATION

Sequencing Reaction Buffer — WARNING

H316 Causes mild skin irritation.
P332+P313 If skin irritation occurs: Get medical advice/attention.
Tris(hydroxymethyl)–aminomethane <10%

ddUTP Dye-Terminator — DANGER

H226 Flammable liquid and vapour.
H302 Harmful if swallowed.
H312 Harmful in contact with skin.
H332 Harmful if inhaled.
H370 Causes damage to organs.
P210 Keep away from heat, hot surfaces, and sparks. No smoking.
P233 Keep container tightly closed.
P240 Ground container and receiving equipment.
P241 Use explosion-proof electrical equipment.
P242 Use non-sparking tools.
P243 Take action to prevent static discharge.
P261 Avoid breathing vapours.
P270 Do no eat, drink or smoke when using this product.
P271 Use only outdoors or in a well-ventilated area.
P280 Wear protective gloves, protective clothing and eye/face protection.
P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P302+P352 IF ON SKIN: Wash with plenty of soap and water.
P303+P361+P353 IF ON SKIN (or hair): Rinse skin with water.
P304+P340 IF INHALED: Remove person to fresh air and keep at rest in a position comfortable for breathing.
P308+P311 If exposed or concerned: Call a doctor/physician.
P312 Call a POISON CENTER or doctor/physician if you feel unwell.
P330 Rinse mouth.
P362+P364 Take off contaminated clothing and wash it before use.
P370+P378 In case of fire: Use water spray for extinction.
P403+P235 Store in a well-ventilated place. Keep cool.
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/national regulations
Methanol 20-25%

ddGTP Dye-Terminator — DANGER

H226 Flammable liquid and vapour.
H302 Harmful if swallowed.
H313 May be harmful in contact with skin
H370 Causes damage to organs.
P210 Keep away from heat, hot surfaces, and sparks. No smoking.
P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P303+P361+P353 IF ON SKIN (or hair): Rinse skin with water.
P308+P311 If exposed or concerned: Call a doctor/physician.
P312 Call a POISON CENTER or doctor/physician if you feel unwell.
P330 Rinse mouth.
P370+P378 In case of fire: Use water spray for extinction.
P403+P235 Store in a well-ventilated place. Keep cool.
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/national regulations
Methanol 6-9%

ddCTP Dye-Terminator — DANGER

H226 Flammable liquid and vapour.
H302 Harmful if swallowed.
H313 May be harmful in contact with skin
H370 Causes damage to organs.
P210 Keep away from heat, hot surfaces, and sparks. No smoking.
P233 Keep container tightly closed.
P240 Ground container and receiving equipment.
P241 Use explosion-proof electrical equipment.
P242 Use non-sparking tools.
P243 Take action to prevent static discharge.
P270 Do no eat, drink or smoke when using this product.
P280 Wear protective gloves, protective clothing and eye/face protection.
P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P303+P361+P353 IF ON SKIN (or hair): Rinse skin with water.
P308+P311 If exposed or concerned: Call a doctor/physician.
P312 Call a POISON CENTER or doctor/physician if you feel unwell.
P330 Rinse mouth.
P370+P378 In case of fire: Use water spray for extinction.
P403+P235 Store in a well-ventilated place. Keep cool.
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/national regulations
Methanol 6-9%

ddATP Dye-Terminator — DANGER

H226 Flammable liquid and vapour.
H302 Harmful if swallowed.
H313 May be harmful in contact with skin
H370 Causes damage to organs.
P210 Keep away from heat, hot surfaces, and sparks. No smoking.
P233 Keep container tightly closed.
P240 Ground container and receiving equipment.
P241 Use explosion-proof electrical equipment.
P242 Use non-sparking tools.
P243 Take action to prevent static discharge.
P270 Do no eat, drink or smoke when using this product.
P280 Wear protective gloves, protective clothing and eye/face protection.
P301+P312 IF SWALLOWED: Call a POISON CENTER or doctor/physician if you feel unwell.
P303+P361+P353 IF ON SKIN (or hair): Rinse skin with water.
P308+P311 If exposed or concerned: Call a doctor/physician.
P312 Call a POISON CENTER or doctor/physician if you feel unwell.
P330 Rinse mouth.
P370+P378 In case of fire: Use water spray for extinction.
P403+P235 Store in a well-ventilated place. Keep cool.
P405 Store locked up.
P501 Dispose of contents/container in accordance with local/national regulations
Methanol 10-15%

CEQ Sample Loading Solution — DANGER

H360 May damage fertility or the unborn child.
P201 Obtain special instructions before use.
P280 Wear protective gloves, protective clothing and eye/face protection.
P308+P313 IF exposed or concerned: Get medical advice/attention.
Formamide >90%

EUROPEAN HAZARD CLASSIFICATION

ddUTP Dye-Terminator — T;R20/21/22-39/23/24/25

R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
R39/23/24/25 Toxic: danger of very serious irreversible effects through inhalation, in contact with skin and if swallowed.
S36/37 Wear suitable protective clothing and gloves.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

ddGTP Dye-Terminator — Xn;R20/21/22-68/20/21/22

R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
R68/20/21/22 Harmful: possible risk of irreversible effects through inhalation, in contact with skin and if swallowed.
S36/37 Wear suitable protective clothing and gloves.

ddCTP Dye-Terminator — Xn;R20/21/22-68/20/21/22

R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
R68/20/21/22 Harmful: possible risk of irreversible effects through inhalation, in contact with skin and if swallowed.
S36/37 Wear suitable protective clothing and gloves.

ddATP Dye-Terminator — T;R20/21/22-39/23/24/25

R20/21/22 Harmful by inhalation, in contact with skin and if swallowed.
R39/23/24/25 Toxic: danger of very serious irreversible effects through inhalation,in contact with skin and if swallowed.
S36/37 Wear suitable protective clothing and gloves.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).

CEQ Sample Loading Solution — T;R61

R61 May cause harm to unborn child.
S36 Wear suitable protective clothing.
S38 In case of insufficient ventilation, wear suitable respiratory equipment.
S45 In case of accident or if you feel unwell, seek medical advice immediately (show the label where possible).
S53 Avoid exposure - obtain special instructions before use.

SDS Safety Data Sheet is available at techdocs.beckmancoulter.com.

Notes

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