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BrilliantDye™ Terminator (v3.1) Cycle Sequencing Kit

BioLink SKU	Reactions	RR seq. Premix	5X Seq. Buffer	pGem control*	M13(-21) primer*
SQRD3-024	24	1 x 192µL	1 x 0.65mL	10µL	10µL
SQRD3-100	100	1 x 800µL	1 x 2.0mL	10µL	10µL
SQRD3-1000	1,000	10 x 800µL	8 x 2.0mL	50µL	50µL
SQRD3-5000	5,000	2 x 20mL	2 x 28mL	50µL	50µL
SQRD3-25K	25,000	10 x 20mL	10 x 28mL	50µL	50µL

*Omitted from trial kit

Protocol

The BrilliantDye v3.1 Terminator Cycle Sequencing Kit contains all required reagent components for the sequencing reaction in a ready reaction, pre-mixed format. These reagents are suitable for performing fluorescence-based cycle sequencing reactions on single-stranded or double-stranded DNA templates, including PCR fragments and plasmids.

Diluting: The kit includes BrilliantDye Terminator Sequencing Buffer (5X), which has been optimized for use with the reaction mix. This buffer should be used for any reaction optimization (page 3).

Purification of PCR templates: For optimum results, purify the PCR product before sequencing by removing dNTPs and primers. We recommend NimaGen's AmpliClean™ Magnetic bead based PCR Cleanup kit (AP-005, AP-050 or AP-500) or ExS-Pure™ enzymatic PCR cleanup kit (EXS-100, EXS-500 or EXS-5000).

Template Quality/Quantity: A common cause of poor Sequencing results is the quality or the quantity of the template used for the sequencing reaction. The template should be as much as possible free from proteins, RNA, chromosomal DNA, PCR primers, dNTPs, enzymes, buffer components, salts, organic chemicals and residual detergents.

For setting up the cycle sequencing reaction, use the following guidelines in template quantity.

Too low template results in weak signals and elevated signal-to-noise (S/N) ratios. Too much template results in short reads with overloaded signals.

PCR 100-200 bp	1-3 ng
PCR 200-500 bp	3-10 ng
PCR 500-1000 bp	5-20 ng
PCR 1000-2000 bp	10-40 ng
>2000 bp	20-50 ng
Plasmid DNA	150-300 ng

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Primer Quality/Quantity: Always use high quality primers for Cycle Sequencing, as well as for generating PCR template. Most common cause of primer issues is the so-called N-1 artifact, caused by primer solutions that contain partially non full-length product, causing the typical "n-1 stutter peaks". We recommend to store Sequencing primers in a concentration of 3.2 - 5 µM (pMol/µL) at -20°C and avoid many freeze-thaw cycles. Use 3-5 pMol sequencing primer per reaction.

Reaction Setup: The 2.5x concentrated BrilliantDye v3.1 ready-reaction premix can be diluted, using the provided 5x Sequencing Buffer. Always make sure that the end concentration is 1x. Be aware that the Premix has an intrinsic buffer concentration of 2.5x, → a standard reaction should contain 8 µL of the premix in an end volume of 20 µL. However we do not recommend to use full reactions, in order to prevent overloaded signals and to save material. General rule for using the 5x Sequencing Buffer in combination with the 2.5x rr Premix:

$$V_B = \left(\frac{V_T/2.5 - V_M}{2}\right)$$

V_B=Volume of 5x Sequencing Buffer in the reaction
V_T=Total Sequencing Reaction Volume
V_M=Volume of BrilliantDye v3.1 Seq. Mix in the reaction

Example:

▪ 1 µL BrilliantDye v3.1 rr Premix	(V_M)
▪ 1.5 µL 5x Sequencing Buffer	(V_B)
▪ 1 µL Template	
▪ 1 µL primer (3.2 - 5 pMol)	
▪ 5.5 µL Water	
10 µL	(V_T)

Thermal Cycling: For the Cycle Sequencing reaction we recommend any brand of High Quality thermal cycler with the following features:

- 96 well (0.2 mL standard format)
- Heated lid (105 °C)
- Thermal ramp of appr. 1°C / sec.
- Fully programmable in multiple stages
- Capability to cool down to 4°C at the end of the program

Example: 9700 or Veriti® PCR System from Applied Biosystems.

Protocol for Thermal Cycling:

Initial Denaturation	96°C	45 sec
25 cycles	96°C	10 sec
	50°C	5 sec
	60°C	4 min.
	4°C	∞

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Purification of the Extension Product: Before capillary electrophoresis the Cycle Sequencing products need to be purified to remove unincorporated fluorescent ddNTPs and salts. Several methods can be used for this purpose, including Ethanol Precipitation, Sephadex based filtration (Edge Biosystems) or magnetic bead based purification. We recommend to use NimaGen's D-Pure™ DyeTerminator Cleanup kit as a cost-effective, high quality purification method, in combination with an Alpaqua 96-well Ring Magnet, also available via NimaGen.

Instrument platforms: The purified extension products can be analyzed by capillary electrophoresis on one of the following platforms:

- Applied Biosystems 310 DNA sequencer
- Applied Biosystems / Hitachi 3100 (Avant) Genetic Analyzer
- Applied Biosystems / Hitachi 3130 (XL) Genetic Analyzer
- Applied Biosystems / Hitachi 3500 (xL) Genetic Analyzer
- Applied Biosystems / Hitachi 3730 (XL) DNA Analyzer
- Applied Biosystems SeqStudio Genetic Analyzer
- Promega Spectrum Compact CE system

Dye Set / Matrix File / Spectral Calibration: The kit is optimized to run with Filterset Z for BigDyeTerminator v3.1. Refer to your instrument manual how to calibrate with this Dye Set. Calibration can be performed using the pGEM control included in the kit.

Data Analysis: For primary base calling, easiest option is to use Sequencing Analysis Software, provided with the automated sequencer. We recommend to use the KB Base Caller, in combination with a DyeSet/Primer file, suitable for BigDye v3.1. For improved basecalling with longer read lengths, NimaGen recommends PeakTrace (https://www.nucleics.com/peaktrace/).

Control provided in the kit: All BrilliantDye kits contain control DNA template (pGEM) and control primer (-21M13). Use 1 µL of this template and 1 µL of the primer in a Sequencing Reaction to verify the performance of your total workflow and troubleshoot issues, correlated to your templates and/or primer. The sequence of the first part of the pGEM control:

TGTAAAACGACGGCCAGT (-21 M13 primer) –
GAATTGTAATACGACTCACTATAGGGCGAATTCGAGCTCGGTACCCGGGGATCCTCTAGAGTCGACCTGCAGGCA
TGCAAGCTTGAGTATTCTATAGTGTCACCTAAATAGCTTGGCGTAATCATGGTCATAGCTGTTTCCTGTGTGAAA
TTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCATAAAGTGTAAAGCCTGGGGTGCCTAATGAGT
GAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTA
ATGAATCGGCCAACGCGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCT
GCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGG
GGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGC
GTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGAC
AGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTAC
CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTC
GGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGG
TAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAG
CAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGT
ATTTGGTATCTGCGCTCTGCTGAAG

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