Modification of Cycle Sequencing Conditions for Sequencing Difficult Templates with BigDye® Terminators

GC-rich Templates (primary problem: low signal)

- Increase **amount of template** and **number of cycles**, or
- **16 µl Premix** and **increased denaturation temperature** protocol: 25 x (98°C 20 s, 55°C 15 s, 60°C 4 min), or
- Addition of **1 µl DMSO** to a 20 µl reaction volume (final concentration 5%) or addition of a mixture of 5% DMSO and 5% glycerol, or
- **Preincubation of template for 5 min at 98°C** (prior to the addition of the Ready Reaction premix), or
- Increase **load** (377 instrument) or **injection voltage** (310 instrument)

GT-rich Templates (problems because of dITP and dUTP in Ready-Reaction Mix)

- **"Reverse" cycle sequencing** protocol: 25 x (96°C 5 s, 60°C 90 s, 50°C 90 s), or
- **Reduced extension temperature** protocol: 30 x (96°C 5 s, 50°C 4 min), or
- Increase final concentration of **magnesium chloride** from 2 mM to **3 mM** (1 µl of 20 mM $MgCl_2$ to 20 µl final volume), or
- Use **dRhodamine** terminators (100 Rxn; P/N 403044) or **BigDye® dGTP** terminators (100 Rxn; v1.0, P/N 4307175; v3.0, P/N 4390229)

AT-rich Templates

- **Reduced extension temperature** protocol: 30 x (96°C 5 s, 50°C 4 min), or
- **"Reverse" cycle sequencing** protocol: 25 x (96°C 5 s, 60°C 90 s, 50°C 90 s), or
- **4 µl Premix** in 20 µl reaction volume

Templates with Secondary Structures

- Similar approaches as in **GC-rich DNA** (DMSO, template preincubation or increased denaturation temperature), or
- Generate **ssDNA**, e.g. with magnetic beads, or
- Use **primer** that anneals close to the region of signal loss

Homopolymer Regions (primary problem: base slippage)

- **Reduced extension temperature** protocol: 30 x (96°C 5 s, 50°C 4 min), or
- **Use an anchored primer**, with 25 nucleotides of the homopolymer region and 1 nucleotide at the 3'-end as the anchor, e.g. $T_{25}C$; if nucleotide is unknown, use a mix of 3 anchored primers, e.g: $T_{25}C$, $T_{25}A$, and $T_{25}G$ (5 pmol each), or
- Sequence the **complementary** strand

Repeat-Structures

- Normally not a problem; if **GC-rich,** use similar approaches as for GC-rich DNA, or
- **"Reverse" cycle sequencing** protocol: 25 x (98°C 5 s, 60°C 90 s, 50°C 90 s), or
- **Reduced extension temperature** protocol: 30 x (96°C 5 s, 50°C 4 min)

Low Signal Intensity (with sufficient quantity of template)

- **16 µl Premix** and **1 µl DMSO** in 40 µl total reaction volume
- In some cases of **primer-related problems**, reduce extension temperature to **55°C** instead of 60°C and increase the cycling number form 25 to **30**.