

Primer Design and Quantitation

Overview The choice of primer sequence, method of primer synthesis, and approach to primer purification can have a significant effect on the quality of the sequencing data obtained in dye terminator cycle sequencing reactions. Dye primer cycle sequencing kits include dye-labeled primers that are already optimized and quantitated.

Some of the recommendations given here are based on information that is general knowledge, while others are based on practical experience gained by scientists at Applied Biosystems.

Primer Design The following recommendations are provided to help optimize primer selection:

- ◆ Use Primer Express™ software (P/N 402089) for primer design.
Primer Express software is useful in identifying potential secondary structure problems, calculating melting temperature (T_m) more accurately, and determining if a secondary hybridization site exists on the target DNA.
 - ◆ Primers should be at least 18 bases long to ensure good hybridization.
 - ◆ Avoid runs of an identical nucleotide, especially runs of four or more Gs.
 - ◆ Keep the G-C content in the range 30–80%, preferably 50–55%.
 - ◆ For cycle sequencing, primers with $T_m > 45$ °C produce better results than primers with lower T_m using our recommended thermal cycling parameters.
 - ◆ For primers with a G-C content less than 50%, it may be necessary to extend the primer sequence beyond 18 bases to keep the $T_m > 45$ °C.
 - ◆ Use of primers longer than 18 bases also minimizes the chance of having a secondary hybridization site on the target DNA.
 - ◆ Avoid primers that can hybridize to form dimers.
 - ◆ Avoid palindromes because they can form secondary structures.
 - ◆ The primer should be as pure as possible, preferably purified by HPLC.
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Estimating Melting Temperature The following formula can be used for a rough estimate of melting temperature:

$$T_m = (\text{number of A + T residues}) \times 2 \text{ }^\circ\text{C} + (\text{number of G + C residues}) \times 4 \text{ }^\circ\text{C}$$

Primer Quantitation The following formula, which is derived from Beer's Law, converts A_{260} readings into pmol/ μ L concentrations:

$$C \text{ (pmol/}\mu\text{L or }\mu\text{M)} = (A_{260} \times 100) / (1.54n_A + 0.75n_C + 1.17n_G + 0.92n_T)$$

where:

C = concentration

n_x = number of residues of base x in the oligonucleotide

Oligonucleotide Molecular Weights Molecular weight of a DNA oligonucleotide (sodium salt, $\text{pH} \geq 7$):

$$\text{MW} = (N_A \times 335.2) + (N_C \times 311.2) + (N_G \times 351.2) + (N_T \times 326.2) + P$$

where:

N_x = number of residues of base x in the oligonucleotide

P = -101.0 for dephosphorylated oligonucleotides, 40.0 for phosphorylated oligonucleotides

Primer Problems and Possible Causes

Table 3-2 Primer Problems and Possible Causes

Problems	Possible Causes
Poor priming resulting in weak or no signal	Melting temperature is too low due to low GC content and/or short primer length
	Secondary structure of the primer, particularly at the 3' end
	Secondary structure of the template in the region of hybridization
	Incorrect primer concentration
	Priming site not present
Adequate signal strength with noisy data	Secondary hybridization site, which results in many extra peaks
	Impure primer. You may see a shadow sequence of N-1.

Custom Oligonucleotides You can obtain custom primers from the Applied Biosystems Custom Oligonucleotide Synthesis Service:

- ◆ Phone: (800) 345-5224
 - ◆ E-mail: support@appliedbiosystems.com
 - ◆ Online: www.appliedbiosystems.com/techsupport
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