



# OligoArchitect™ Online

## Glossary of Parameters

### SYBR® Green

#### Template Structure Search Parameters

**Monovalent Ion Concentration:** This is the concentration of all monovalent ions, e.g.  $K^+$ , present in the reaction mixture.  $K^+$  encourages annealing by reducing charge repulsion between the negatively charged primer and template backbones.

The default value = 100.0 mM.

**Free  $Mg^{++}$  Ion Concentration:** This is the concentration of  $Mg^{2+}$  (magnesium ion) in the reaction mixture.  $Mg^{2+}$  is a cofactor required by thermostable DNA polymerases.

The default value = 5.0 mM.

**Folding Temperature:** This is the temperature at which the algorithm searches for secondary structures along the template. Ideally, the temperature should be set to that used for the extension phase of the amplification reaction.

The default value = 25.0 °C.

**Search Range:** This is the length of the template that is searched for secondary structures, which might form during the extension phase of the amplification reaction. The minimum searchable length is 50 bases.

The default value = 1 – 1200 bases (maximum range).

#### Oligo Search Range

**Sense Primer Search Range:** Provides control as to where the sense primer is designed along the template, e.g. if the template is 1500 bp and the sense primer search range is set to 1 – 200, the sense primer will be designed in that range. The antisense primer will be designed elsewhere along the template, avoiding the first 200 bp.

**Anti-Sense Primer Search Range:** Provides control as to where the antisense primer is designed along the template, e.g. if the template is 1500 bp and the antisense primer search range is set to 1300 – 1500, the antisense primer will be designed in that range. The sense primer will be designed elsewhere along the template, avoiding the last 200 bp.

**Amplicon Search Range:** This is the default setting. Provides control as to which part of the template undergoes amplification. The 5' ends of each primer are the ends of the amplicon.

The default value = length of the template.

#### Primer Parameters

**$T_m$ :** The melting temperature ( $T_m$ ) is the temperature at which 50% of the primer/template dissociates to become single-stranded and therefore is a measure of duplex stability. Primers with a  $T_m$  in the range of 50 – 60 °C typically produce the best results.



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The default value =  $55.0 \pm 5.0$  °C. OligoArchitect Online will identify primers for all sequences as close as possible to the specified value of the  $T_m$ . When a value for  $T_m$  is manually selected, the option to adjust the default annealing temperature ( $T_a$ ) is eliminated.  $T_m$  values are calculated using the nearest neighbor formula with SantaLucia values (*PNAS*, Vol. 95, pp. 1460 – 1465, February 1998).

**$T_a$ :** The annealing temperature ( $T_a$ ) chosen for a particular PCR protocol depends on the length and GC content of the primers. As a rule of thumb, the  $T_a$  is 5 °C below the lowest  $T_m$  of the pair of primers. If the  $T_a$  is too low, secondary annealing will cause mismatches, leading to nonspecific amplification. If the  $T_a$  is too high, reduced annealing will lead to low amplification. The formula is as follows:

$$T_a = [0.3 \times T_m(\text{primer})] + [0.7 \times T_m(\text{product})] - 14.9$$

$T_m(\text{primer}) = T_m$  of the less stable primer-template pair

$T_m(\text{product}) = T_m$  of the PCR amplicon

The default value =  $55.0 \pm 5.0$  °C. The tool will identify primers for all sequences as close as possible to the specified template  $T_a$ . The option to adjust the primer melting temperature ( $T_m$ ) is not available when the default  $T_a$  remains selected.

**Length:** Since there are primarily four bases (A, C, G, T) in any natural DNA template sequence, an 18-base sequence will statistically be present only once in every  $4^{18}$  bases or approximately 69 billion. Therefore, a primer of 18 bases should be complex enough to minimize secondary annealing. Likewise, primers longer than approximately 24 bases generally do not show higher specificity.

The default value = 18 – 24 bp.

**Amplicon Length:** Typically, chosen lengths to be amplified are > 500 bp for traditional PCR and 100 – 200 bp for qPCR. The formula is as follows:

$$\text{Amplicon Length} = (\text{position of antisense primer} - \text{position of sense primer}) + 1$$

The default value = 75 - 200 bp.

**Alternate Primer Pairs:** While the best primers are displayed on the main results screen, up to 50 alternative designs are available.

The default value = 5 primer pairs.

## Advanced Primer Parameters

**Hairpin Maximum  $\Delta G$  (3' End):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of the most stable hairpin that is acceptable at the 3' end.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate stable, undesirable hairpins that can adversely affect the reaction.  $\Delta G = \geq -3$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -3.0 kcal mol<sup>-1</sup>.

**Hairpin Maximum  $\Delta G$  (Internal):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of the most stable hairpin in the internal region of a primer.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate stable, undesirable hairpins that can adversely affect the reaction.  $\Delta G = \geq -5$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -5.0 kcal mol<sup>-1</sup>.

**3' End Maximum  $\Delta G$ :** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of the most stable duplex between the last 5 bases from the 3' end and its exact complement in the template.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for false priming as the 3' end can initiate polymerization even if the remainder of the primer does not bind well. Primers with a  $\Delta G$  more negative than the specified value are not considered. Primers with a  $\Delta G$  between -4 and the specified value are rated lower.

The default value = -12.0 kcal mol<sup>-1</sup>.

**Self Dimer Maximum  $\Delta G$  (3' End):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the 3' ends of two same-sense primers.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for identical primers to hybridize to each other rather than to the template.  $\Delta G = \geq -5$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -5.0 kcal mol<sup>-1</sup>.

**Self Dimer Maximum  $\Delta G$  (Internal):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the internal regions of two same-sense primers.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for identical primers to hybridize to each other rather than to the template.  $\Delta G = \geq -6$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -6.0 kcal mol<sup>-1</sup>.



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**Run/Repeat (dinucleotide) maximum length:** Primers with long runs of the same base or repeats of a consecutive dinucleotide should be avoided as they lead to mispriming. Runs/repeats of 4 bp are usually well tolerated.

The default value = 4 bp.

**GC clamp – Consecutive G/C's at 3' end:** The presence of consecutive G and C bases within the last 5 bases of the 3' end help promote specific binding due to the greater number of hydrogen bonds present. For example, the sequence:

GCTTGCTGCGTTCACACTGC

contains 1 GC clamp. More than 3 G's or C's should be avoided, as it will make the 3' end maximum  $\Delta G$  more negative and therefore unfavorable. Primers with less than the specified number of G's or C's are rated lower. If the specified value is set to 0, the tool will attempt to design such primers. However, if no such primers can be designed, primers with GC clamps will be reported and rated lower.

The default value = 1.

**GC%:** It is an empirical observation that the optimal range for SYBR Green I primers is 30.0 – 80.0%.

The default value = 30.0 – 80.0%.

### Advanced Primer Pair Parameters

**Maximum Primer Pair  $T_m$  Mismatch:** This is the maximum difference between the  $T_m$  values of the two primers. A difference of  $\geq 5$  °C can prevent amplification.

The default value = 4.0 °C.

**Cross Dimer Maximum  $\Delta G$  (3' End):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the 3' ends of the sense and antisense primers.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for the sense and antisense primers to hybridize to each other rather than to the template.  $\Delta G = \geq -5$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -5.0 kcal mol<sup>-1</sup>.

**Cross Dimer Maximum  $\Delta G$  (Internal):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the internal regions of the sense and antisense primers.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for the sense and antisense primers to hybridize to each other rather than to the template.  $\Delta G = \geq -6$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -6.0 kcal mol<sup>-1</sup>.

### Reactions Conditions

**Primer Concentration:** The default value = 100.0 nM.

**Monovalent Ion Concentration:** This is the concentration of all monovalent ions, e.g. K<sup>+</sup>, present in the reaction mixture. K<sup>+</sup> encourages annealing by reducing charge repulsion between the negatively charged primer and template backbones.

The default value = 100.0 mM.

**Free Mg<sup>++</sup> Ion Concentration:** This is the concentration of Mg<sup>2+</sup> (magnesium ion) in the reaction mixture. Mg<sup>2+</sup> is a cofactor required by thermostable DNA polymerases.

The default value = 3.0 mM.

**Total Na<sup>+</sup> Equivalent:** The sodium equivalent is calculated as follows:

$[Na^+] = \text{Monovalent ion concentration} + 4 \times (\text{Free Mg}^{2+})^{1/2}$   
(all in molarity)

**Temperature for Free Energy Calculation:** This is used to calculate the Gibbs free energy ( $\Delta G$ ) in the formula:  $\Delta G = \Delta H - T \Delta S$ .

The default value = 25.0 °C (298K).

## Dual-Labeled Probe

### Template Structure Search Parameters

**Monovalent Ion Concentration:** This is the concentration of all monovalent ions, e.g. K<sup>+</sup>, present in the reaction mixture. K<sup>+</sup> encourages annealing by reducing charge repulsion between the negatively charged primer and template backbones.

The default value = 100.0 mM.

**Free Mg<sup>++</sup> Ion Concentration:** This is the concentration of Mg<sup>2+</sup> (magnesium ion) in the reaction mixture. Mg<sup>2+</sup> is a cofactor required by thermostable DNA polymerases.

The default value = 5.0 mM.

**Folding Temperature:** This is the temperature at which the algorithm searches for secondary structures along the template. Ideally, the temperature should be set to that used for the extension phase of the amplification reaction.

The default value = 25.0 °C.



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**Search Range:** This is the length of the template that is searched for secondary structures, which might form during the extension phase of the amplification reaction. The minimum searchable length is 50 bases.

The default value = 1 – 1200 bases (maximum range).

### Oligo Search Range

**Sense Primer Search Range:** Provides control as to where the sense primer is designed along the template, e.g. if the template is 1500 bp and the sense primer search range is set to 1 – 200, the sense primer will be designed in that range. The antisense primer will be designed elsewhere along the template, avoiding the first 200 bp.

**Anti-Sense Primer Search Range:** Provides control as to where the antisense primer is designed along the template, e.g. if the template is 1500 bp and the antisense primer search range is set to 1300 – 1500, the antisense primer will be designed in that range. The sense primer will be designed elsewhere along the template, avoiding the last 200 bp.

**Dual Labeled Probe Search Range:** Provides control as to where the probe is designed along the template, e.g. if the template is 1500 bp and the probe search range is set to 650 – 850, the probe will be designed in that range. The sense and antisense primers will be designed upstream and downstream, respectively, elsewhere along the template, avoiding the middle 200 bp.

**Amplicon Search Range:** This is the default setting. Provides control as to which part of the template undergoes amplification. The 5' ends of each primer are the ends of the amplicon.

The default value = length of the template.

### Primer Parameters

**Length:** Since there are primarily four bases (A, C, G, T) in any natural DNA sequence, an 18-base sequence will statistically be present only once in every  $4^{18}$  bases or approximately 69 billion. Therefore, a primer of 18 bases should be complex enough to minimize secondary annealing. Likewise, primers longer than approximately 25 bases generally do not show higher specificity.

The default value = 18 – 25 bp.

**T<sub>m</sub>:** The melting temperature (T<sub>m</sub>) is the temperature at which 50% of the primer / template dissociates to become single-stranded and therefore is a measure of duplex stability. Primers with a T<sub>m</sub> in the range of 54 – 64 °C typically produce the best results. Primers with T<sub>m</sub> > 65 °C often undergo secondary annealing.

The default value = 59.0 ± 5.0 °C. The tool will find primers for all sequences as close as possible to the specified value of the T<sub>m</sub>. The values are calculated using the nearest neighbor formula with SantaLucia values (*PNAS*, Vol. 95, pp. 1460 – 1465, February 1998).

**Amplicon Length:** Typically, chosen lengths to be amplified are 100 – 150 bp for qPCR. The formula is as follows:

Amplicon Length = (position of antisense primer – position of sense primer) + 1

The default value = 70 - 200 bp.

**Alternate Assays:** While the best primer / probe set is displayed on the main results screen, up to 50 alternative assays are available.

The default value = 5 assays.

### Probe Parameters

**Length:** Non-modified probes shorter than 15 bases often lack specificity. Probes longer than 30 bases (canonical B-DNA has a rise—bp length along the helical axis—of 3.4 Å) may produce a poor signal to noise ratio since FRET between a reporter and quencher typically occurs at a distance of no greater than 100 Å. Ideal probe lengths are typically 15 – 40 bases.

The default value = 20 – 27 bp.

**T<sub>m</sub>:** The melting temperature (T<sub>m</sub>) is the temperature at which 50% of the probe / template dissociate to become single-stranded and therefore is a measure of duplex stability. The probe T<sub>m</sub> should be approximately 10 °C higher than the T<sub>m</sub> of the primers to ensure that the probe is fully hybridized during primer extension.

The default value = Primer T<sub>m</sub> + 10.0 °C ± 5.0 °C.

### Output Options

**Design Sense Probe / Design Anti-sense Probe:** This option defines the sense of the probe and is only available when the sequence has a SNP.

The default value = Design Sense Probe.



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**Alternate Assays:** While the best primer / probe set is displayed on the main results screen, up to 50 alternative assays are available.

The default value = 5 assays.

### Advanced Design Parameters

**Hairpin Maximum  $\Delta G$ :** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of the most stable hairpin in the internal region of a probe.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate stable, undesirable hairpins that can adversely affect the reaction.  $\Delta G = \geq -6$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -6.0 kcal mol<sup>-1</sup>.

**Self Dimer Maximum  $\Delta G$ :** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the internal regions of two probes.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for identical probes to hybridize to each other rather than to the template.  $\Delta G = \geq -10$  kcal mol<sup>-1</sup> is usually well tolerated.

The default value = -10.0 kcal mol<sup>-1</sup>.

**Run/Repeat (dinucleotide) Maximum Length:** Probes with long runs of the same base or repeats of a consecutive dinucleotide should be avoided as they lead to misbinding. Runs/repeats of 4 bp are usually well tolerated.

The default value = 4 bp.

**Maximum Bases Between Primer & Probe:** This is the maximum acceptable distance between primer and probe. The probe should be designed to within 10 – 20 base pairs of the primer that anneals to the same strand as the probe, without any overlaps.

The default value = 20 bp.

**GC%:** The ideal range for Dual-Labeled Probes in the 5' nuclease assay is 30.0 – 80.0%.

The default value = 30.0 – 80.0%.

**Probe must not have more G than C bases:** It is an empirical observation that probes with more G than C bases will often produce reduced normalized fluorescence values.

The default value = checked.

**Avoid G base at 5'end:** Guanine is an effective quencher of fluorescence and should not be adjacent to the reporter dye.

The default value = checked.

### Advanced Primer Parameters

**3' End Maximum  $\Delta G$ :** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of the most stable duplex between the last 5 bases from the 3' end and its exact complement in the template.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for false priming as the 3' end can initiate polymerization even if the remainder of the primer does not bind well. Primers with a  $\Delta G$  more negative than the specified value are not considered. Primers with a  $\Delta G$  between -4 and the specified value are rated lower.

The default value = -12.0 kcal mol<sup>-1</sup>.

**G/C Clamp – Consecutive G/C's at 3' End:** The presence of consecutive G and C bases within the last 5 bases of the 3' end helps promote specific binding due to the greater number of hydrogen bonds present. For example, the sequence:

GCTTGCTGCGTTCACACTGC

contains 1 GC clamp. More than 3 G's or C's should be avoided, as it would make the 3' end maximum  $\Delta G$  more negative and therefore unfavorable. Primers with less than the specified number of G's or C's are rated lower. If the specified value is set to 0, the program will attempt to design such primers. However, if no such primers can be designed, primers with GC clamps will be reported and rated lower.

The default value = 1.

**GC count in first 5 bases at 5'end >:** This means that probes with more than two G's and C's in the first five bases at the 5' end are considered.

The default value = 2.

**Avoid T base at 3' end:** It is an empirical observation that thymidine tends to misprime more readily than other bases and therefore should be avoided.

The default value = checked.

### Advanced Primer Pair Parameters

**Maximum Primer Pair  $T_m$  Mismatch:** This is the maximum difference between the  $T_m$  values of the two primers. A difference of  $\geq 5$  °C can prevent amplification.

The default value = 3.0 °C.



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**Cross Dimer Maximum  $\Delta G$  (3' End):** The Gibbs free energy change ( $\Delta G$ ) is a measure of the spontaneity of formation of a dimer between the 3' ends of the sense and antisense primers.  $\Delta G$  is the energy required to break the secondary structure, and larger negative values indicate a higher propensity for the sense and antisense primers to hybridize to each other rather than to the template.  $\Delta G = \geq -9 \text{ kcal mol}^{-1}$  is usually well tolerated.

The default value =  $-9.0 \text{ kcal mol}^{-1}$ .

### Reaction Conditions

**Primer Concentration:** The default value = 250.0 nM.

**Probe Concentration:** The default value = 100.0 nM.

**Monovalent Ion Concentration:** This is the concentration of all monovalent ions, e.g.  $\text{K}^+$  (potassium ion), present in the reaction mixture.  $\text{K}^+$  encourages annealing by reducing charge repulsion between the negatively charged primer and template backbones.

The default value = 100.0 mM.

**Free  $\text{Mg}^{2+}$  Ion Concentration:** This is the concentration of  $\text{Mg}^{2+}$  (magnesium ion) in the reaction mixture.  $\text{Mg}^{2+}$  is a cofactor required by thermostable DNA polymerases.

The default value = 5.0 mM.

**Total  $\text{Na}^{+}$  Equivalent:** The sodium equivalent is calculated as follows:

$[\text{Na}^{+}] = \text{Monovalent ion concentration} + 4 \times (\text{Free } \text{Mg}^{2+})^{1/2}$   
(all in molarity)

**Temperature for Free Energy Calculation:** This is used to calculate the Gibbs free energy ( $\Delta G$ ) in the formula:  $\Delta G = \Delta H - T \Delta S$ .

The default value = 25.0 °C (298K).

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