NetPrimer
Manual

PREMIER
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CONTENTS

INTRODUCTION

NetPrimer analyzes primers to optimize PCR, sequencing, and hybridization reactions. Primers are analyzed for amplification related properties including melting temperature (T_m) and secondary structures.

The program has been designed to be easy to use. To analyze a primer, just type in the primer sequence and click the Analyze button. All of the properties of the primer are displayed. To analyze a primer pair, use the sense/anti-sense radio button to enter both primers. Cross dimers are now calculated in addition to the properties for each primer.

A primer report can be printed containing detailed information on the primer pair. Primer name and description areas are provided to aid in identification of the primer from the printout.

PREFERENCES

The preferences used to calculate the results and their importance are described below:

Nucleic acid concentration - Default value = 250 pM.

The value of nucleic acid concentration (C) is the concentration of the target sequence. Since it keeps on changing empirically with the advent of reaction, its value is set virtually based on the experimental data. It is therefore advised not to change this value unless experimental conditions are very uncommon. This value is used for calculating melting temperature of primer.

Monovalent ion concentration - Default value = 50 mM.

This preference should be set to the sum of the concentrations of all the monovalent ions present in the reaction mixture.

Free [Mg^{2+}] ion concentration - Default value = 1.5 mM.

This preference should be set to the concentration of Mg^{2+} ions, used as binders, in a reaction mixture.

Total [Na^{+}] equivalent

This value is calculated using the value of the Monovalent ion concentration and Free [Mg^{2+}] ion concentration. The formula for calculating Total [Na^{+}] equivalent is as follows:

Total [Na^{+}] equivalent = [Monovalent ion concentration] + 4 X sqrt (Free [Mg^{2+}] ion concentration X 1000)

This value is used as salt concentration for calculating the melting temperature of the primer.

DG temperature - Default value = 25 °C.

This preference should be set to the ambient room temperature. It is used to calculate various DG values as given in theories and formulas. DG is calculated using the formula DG = DH - T * DS.

THEORIES AND FORMULAS

All of the formulas used in the program are given below for quick reference:

Rating

The rating of a primer provides a quick way of measuring the predicted efficiency of a primer as well as choosing between closely matched primers. The higher the rating of a primer, the higher its amplification efficiency.

The rating of individual primers is calculated as:

Rating = 100 + (ΔG (Dimer) * 1.8 + ΔG (Hairpin) * 1.4)

Example: Say a primer has worst ΔG (Dimer) as -2.4 and worst ΔG (Hairpin) as -1.0, that primer will have a rating of 94.

Molecular Weight

The molecular weight of primer is the calculated molecular weight determined using the standard values of molecular weight of individual nucleotides. The values used here are:

dG = 329.21 dA = 313.21

dC = 289.19 dT = 304.20

The molecular weight is calculated using the formula:

Molecular Weight = Sum of weights of individual nucleotides + 18.02 (for water).

Example: Say the primer sequence is ATCGATACGTAG. The molecular weight of this primer will be 4 * (Mol. Wt. of A) + 3 * (Mol. Wt. of T) + 2 * (Mol. Wt. of C) + 3 * (Mol. Wt. of G) + 18.02. = 3749.47.

Melting Temperature

The melting temperature is calculated using the formula based on the nearest neighbor thermodynamic theory. It is the temperature at which half of the oligonucleotides are bonded. The formula is from the paper by Freier et. al. These are the latest and most accurate nearest neighbor based T_m calculations.

$T_m = \Delta H/(\Delta S + R * \ln(C/4)) + 16.6 \log ([K^+]/(1 + 0.7\ [K^+])) - 273.15$

ΔH is enthalpy for helix formation.

ΔS is entropy for helix formation.

R is molar gas constant (1.987 cal/°C * mol)

C is the nucleic acid concentration.

$[K^+]$ is salt concentration.

Example: Say the primer sequence is ATCGATACGTAG. The ΔH and ΔS values of this primer will be

-85000 cal/mol and -234.7 cal/°K/mol respectively (as calculated below). After substituting all the values, the T_m value of this primer will be 16.69 °K.

GC%

GC% is the percentage of G and C in the primer. It is calculated by dividing the sum of G and C with the total number of bases present in the primer.

Example: Say the primer sequence is ATCGATACGTAG. The GC% of this primer will be (5/12 * 100) = 41.67.

nmol/A_{260}

This value is the concentration of primer in nanomoles per unit absorbance (OD) at 260 nm. To calculate extinction coefficient of an oligonucleotide say ATGCA use the following formula -

$\varepsilon_{ATGCA} = [2 (\varepsilon_{AT} + \varepsilon_{TG} + \varepsilon_{GC} + \varepsilon_{CA}) - (\varepsilon_{T} + \varepsilon_{G} + \varepsilon_{C})]$

The extinction coefficient so obtained is in A_{260} units/µmol. To obtain nmol/A_{260} multiply the reciprocal of A_{260} units/µmol by 1000. A_{260} units/µmol is calculated from table of extinction coefficients given in Appendix A.

Example: Say the primer sequence is ATCGATACGTAG. The extinction coefficient of this primer as calculated from table of extinction coefficients using above formula is 125.9 A_{260} units/µmol. nmol/ A_{260} for this primer will be 1000/125.9 = 7.94.

µg/A_{260}

This value is the concentration of primer in micrograms per unit absorbance (OD) at 260 nm. To calculate extinction coefficient of an oligonucleotide say ATGCA use the following formula -

$\varepsilon_{ATGCA} = [2 (\varepsilon_{AT} + \varepsilon_{TG} + \varepsilon_{GC} + \varepsilon_{CA}) - (\varepsilon_{T} + \varepsilon_{G} + \varepsilon_{C})]$

This is calculated from table of extinction coefficients given in Appendix A. Then activity in µg/OD is calculated using the following formula:

Activity in µg/OD = Molecular Weight/Extinction Coefficient

Example: Say the primer sequence is ATCGATACGTAG. Its molecular weight will be 3749.47 (as calculated above). Its extinction coefficient will be 125.9. Activity in µg/OD = 3749.47/125.9 = 29.78.

ΔG

This is the free energy of the primer calculated using the nearest neighbor method of Breslauer, K.J. et. al. ΔG is calculated by the formula ΔG = ΔH - TΔS. Here ΔH is the enthalpy of primer, T is the temperature, ΔS is the entropy of primer. T is set by ΔG temp. in the preferences. First the ΔH and ΔS are calculated and then the ΔG is calculated using their values.

Example: Say the primer sequence is ATCGATACGTAG. Its ΔH and ΔS will be -85000 cal/mol and

-234.7 cal/°K/mol respectively (as calculated below). Its ΔG will be -85000 - (298.15 * -234.7) = -15024.195 cal/mol = -15.02 kcal/mol.

3' end stability

The stability of the primer determines its false priming efficiency. An ideal primer has a stable 5' end and an unstable 3' end.

If the primer has a stable 3' end, it will bond to a site which is complementary to it other than the target with its 5' end hanging off the edge. It may then lead to secondary bands.

Primers with low stability at the 3' ends function well because the 3' end bonding to false priming sites are too unstable to extend.

The 3' end stability is the ΔG value of the 5 bases of primer taken from 3' end. The lower this value, numerically, the more liable the primer is to show secondary bands.

Example: Say the primer sequence is ATCGATACGTAG. Its 3' end stability will be ΔG(CGTAG) = ΔH(CGTAG) - 298.15 * ΔS(CGTAG). The ΔH and ΔS will be -32200 cal/mol and -82.8 cal/°K/mol resp. Thus its 3' end stability will be -32200 - (298.15 * -82.8) = -7513.18 cal/mol = -7.51 kcal/mol.

ΔH

This is the enthalpy of the primer as calculated by the nearest neighbor method of Breslauer, K.J. et. al. ΔH for a pentamer is calculated as follows:

$\Delta H_{ATGCA} = \Delta H_{AT} + \Delta H_{TG} + \Delta H_{GC} + \Delta H_{CA}$

The individual values of ΔH for nucleotide pairs are taken from the table given in Appendix A.

Example: Say the primer sequence is ATCGATACGTAG. Its ΔH will be (8600 + 5600 + 11900 + 5600 + 8600 + 6000 + 6500 + 11900 + 6500 + 6000 + 7800) = -85000 cal/mol = -85 kcal/mol.

ΔS

This is the entropy of the primer as calculated by the nearest neighbor method of Breslauer, K.J. et. al. ΔS for a pentamer is calculated as follows:

$\Delta S_{ATGCA} = \Delta S_{AT} + \Delta S_{TG} + \Delta S_{GC} + \Delta S_{CA}$

An initiation value of 15.1 is added to the ΔS calculation. The individual values of ΔS for nucleotide pairs are taken from the table given in Appendix A.

Example: Say the primer sequence is ATCGATACGTAG. Its ΔS will be (23.9 + 13.5 + 27.8 + 13.5 + 23.9 + 16.9 + 17.3 + 27.8 + 17.3 + 16.9 + 20.8) + 15.1 = -234.7 cal/°K/mol = -0.23 kcal/°K/mol.

5' ΔG

Stability of the 5' termini allows for efficient bonding of the primer to the target site. This stable 5' region is called the GC Clamp. It ensures adequate binding of the primer to the template. Use of primers with optimal stability allows for the use of lower annealing temperatures without the production of secondary bands. Notice that the 3' end should not be very stable and the 5' end should

have a strong GC clamp.

The GC Clamp is the ΔG value of the 5 bases of primer taken from 5' end. The lower this value, numerically, the more efficient is the primer.

Example: Say the primer sequence is ATCGATACGTAG. Its 5' ΔG will be ΔG(ATCGA) = ΔH(ATCGA) - 298.15 * ΔS(ATCGA). The ΔH and ΔS will be -31700 cal/mol and -78.7 cal/°K/mol resp. Thus its 3' end stability will be -31700 - (298.15 * -78.7) = -8235.6 cal/mol = -8.23 kcal/mol.

Repeats and runs

Repeats and Runs increase the likelihood of false priming. Primers having 3 or more dinucleotide repeats or 3 or more base runs are reported. For example, if the primer contains ATATAT, which is dinucleotide AT repeated three times, the primer is reported.

Secondary structures of primers

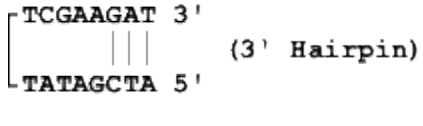
An important factor to consider in the design of a primer is the presence

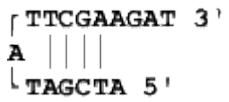
Hairpin

A hairpin loop is formed when primer folds back upon itself and is held in place by intramolecular bonding. Because hairpin loop formation is an intramolecular reaction, it can occur with as few as 3 consecutive homologous bases. To measure the stability of the hairpin loop formed, measure the free energy. The free energy of the loop is based upon the energy of the intramolecular bond and the energy needed to twist the DNA to form the loop. If this free energy is greater than 0, the loop is too unstable to interfere with the reaction. If the free energy is less than 0, the loop could reduce the efficiency of amplification. The Hairpin Report accessed using the:

button on the window allows you to avoid primers containing secondary structure.

Example: Say the primer sequence is ATCGATATTCGAAGAT. It forms two hairpins. One is 3' end hairpin where the primer folds back upon itself and first and last 3 bases bond together and other is internal hairpin where 2nd to 5th and 9th to 12th bases bond together to form hairpin.





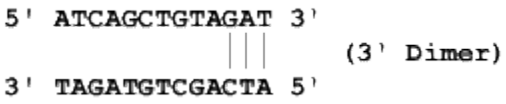
Dimers and Cross Dimers

Dimers occur when a region of homology is present within a primer (self -dimer) or between the sense

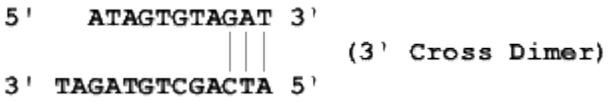
and anti-sense primer (cross dimer). This results in bonding of the two primers, increasing production of the primer dimer artifact and reducing product yields.

Dimers occur within a primer when two copies of the primer bind to each other and cross dimers occur when a primer binds to the other primer in the pair. This is particularly problematic when the homology occurs at the 3' end of either primer. The 3' end will extend readily leading to primer dimer artifact. The dimer and cross dimer reports can be used to test for formation of dimer duplexes.

Example: Say the primer sequence is ATCAGCTGTAGAT. It forms 2 dimers. One is internal dimer where 3rd to 8th bases of primer in 5' to 3' direction (starting from 5' direction) bond with 6th to 11th bases (starting from 3' direction) when primer is placed in reverse direction. The other is 3' end dimer where the last 3 bases (starting from 5' direction) of primer placed in 5' to 3' direction bond with last three base (starting from 3' direction) placed in reverse direction.



Example: Say the sense primer sequence is ATCAGCTGTAGAT and the anti-sense primer sequence is ATAGTGTAGAT, it forms one cross dimer which is a 3' Cross Dimer.



APPENDIX A: Tables

Net Primer uses various standard values in its formulas. Many of them are given below in tabular form.

Table of Extinction Coefficient values (in A_{260} units/µmol):

Pair values -

Second Nucleotide → First Nucleotide ↓	dA	dC	dG	dT
dA	13.7	10.6	12.5	11.4
dC	10.6	7.3	9.0	7.6
dG	12.6	8.8	10.8	10.0
dT	11.7	8.1	9.5	8.4

Individual values -

dA = 15.4 dT = 8.7 dC = 7.4 dG = 11.5

Table of entropy values of a nearest neighbor nucleotide (in -cal/°K/mol):

Second Nucleotide → First Nucleotide ↓	dA	dC	dG	dT
dA	24.0	17.3	20.8	23.9
dC	12.9	26.6	27.8	20.8
dG	13.5	26.7	26.6	17.3
dT	16.9	13.5	12.9	24.0

Table of enthalpy values of a nearest neighbor nucleotide (in -cal/mol):

Second Nucleotide → First Nucleotide ↓	dA	dC	dG	dT
dA	9100	6500	7800	8600
dC	5800	11000	11900	7800

dG	5600	11100	11000	6500
dT	6000	5600	5800	9100

Appendix B: References

We acknowledge all the authors of the references mentioned below whose publication have been helpful during the development of this program.

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2. Breslauer, K.J., Frank, R., Blocker, H., and Markey, L. A. (1986) "Predicting DNA duplex stability from the base sequence", Proc. Natl. Acad. Sci. USA 83, 3746-3750.
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4. Rychlik, W., Spencer, W.J. and Rhoads, R.E. (1990) "Optimization of the annealing temperature for DNA amplification *in vitro*", Nucleic Acids Res. 18, 6409-6412.