

MeltCalc Professional Edition Version 2.x for commercial use

A Microsoft® Excel® add-in for thermodynamic DNA stability calculations and automatic design of hybridization probes

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Disclaimer

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The following **references** describe the MeltCalc program and its first application to hybridization assays on the LightCycler. **The first reference should be cited whenever publications report results obtained with MeltCalc. Both references should be cited when the application was on the LightCycler:**

(1) **Schütz,E. and N.von Ahsen.** 1999. Spreadsheet software for thermodynamic melting point prediction of oligonucleotide hybridization with and without mismatches. *Biotechniques* 27:1218-1224.

(2) **von Ahsen,N., M.Oellerich, V.W.Armstrong and E.Schütz.** 1999. Application of a thermodynamic nearest-neighbor model to estimate nucleic acid stability and optimize probe design: Prediction of melting points of different mutations of apolipoprotein B 3500 and factor V Leiden with a hybridization probe genotyping assay on the LightCycler. *Clin Chem* 45:2094-2101.

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MeltCalc may not be distributed as part of or for use with any commercial product without express written permission from the authors. The program may not be resold, modified for resale, or used to manufacture commercial products without written approval of the authors.

Limitations and Precautions

The output calculated with this program is based on the most precise estimation of nearest-neighbor parameters for DNA with and without mismatches by SantaLucia's group (3-9). However, every model-based approach has its limitations that lie in the model itself. For example single stranded oligonucleotides can have an enthalpically order that can significantly contribute to the duplex formation as shown by Vesnaver and Breslauer (10). For general reading about the nearest neighbor model, its history, derivation, application and validation refer to the references (9;11-13). Some sequences may show non two-state melting behavior. This will reduce the accuracy of predictions

for such oligonucleotides and is a reason why for longer DNA strands the use of the nearest-neighbor model is no more necessary. MeltCalc switches to a simpler formula (14;15) when the input sequence is longer than 256 bp.

We ask every user to treat results of these calculations with great caution. For different applications the accuracy of the predictions may vary greatly. Our experimental validation was only done with LightCycler (2) hybridization probe assays with limited variation in magnesium concentrations, probe length and probe composition.

The thermodynamic calculations assume that the annealing occurs at pH 7.0. This is not the case in standard PCR buffer systems, but the resulting error seems to be small. More important is that terminal mismatches and penultimate mismatches are not properly reflected by the parameters (3;8). The thermodynamic data set for tandem mismatches (i.e., immediately adjacent mismatches) is still incomplete. Only for some tandem mismatches is a reliable prediction possible (3). The influence of the salt concentration on oligonucleotide stability is also still under investigation (16;17). Nevertheless, the methods presented here (17;18) will allow a reliable estimate of hybridization probe T_m on the LightCycler but may still require correction of the sodium equivalents according to the local PCR conditions.

Licensing

Please refer to the separate licensing agreement. The text is also included at the end of this document. When you order an activation key you accept the terms of the license agreement that comes with this software. You accept the software's limitations mentioned in the "Limitations and Precautions" section above.

Support

The best way to get support is via the email address info@meltcalc.de. Please visit the MeltCalc homepage for further information at: <http://www.meltcalc.de>.

We are not in the oligonucleotide synthesis business nor are we affiliated with it. For questions regarding LC-Red dyes please contact your Roche representative. LightCycler hybridization probes are available from Genset (<http://www.gensetoligos.com>), Synthegen (<http://www.synthegen.com>) or TIB-Molbiol (<http://www.tib-molbiol.de>).

What is new in version 2.0x?

New features in this version are:

- Calculation of T_m is now including "dangling end" parameters (if applicable, see below for explanation). This is important for the optimization of hybridization probes; every probe has two dangling ends, since the DNA template is usually longer than the probes!
- Maximal possible sequence length increased from 256bp to 1755bp for all features
- A formula for calculation of T_m for long sequences is added
- Visual crosshybridization control in a separate table

Probe design:

- Probe stability is calculated using the dangling end parameters to provide you with most accurate T_m prediction.
- Space between anchor and probes for LightCycler™ assays can be chosen (1 to 5 base pairs)
- “Insert mutations” function helps the user to incorporate base exchanges at certain positions in the mismatched sequence string

Minor modifications:

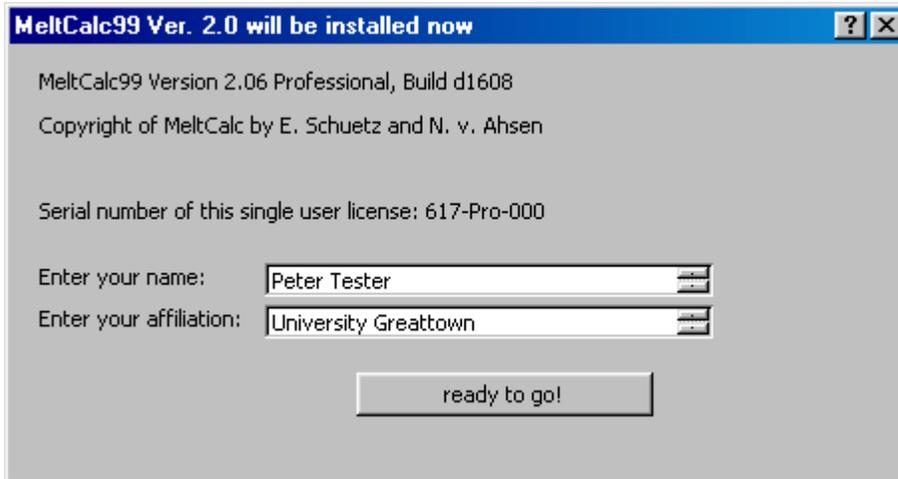
- Variable input fields that size with your input string
- Mismatches are highlighted automatically
- Calculation of sodium equivalents has been modified
- SYBR green-I™ influence on T_m is estimated, based on empirical data

I. Installing and uninstalling of MeltCalc for Windows PCs

Installing

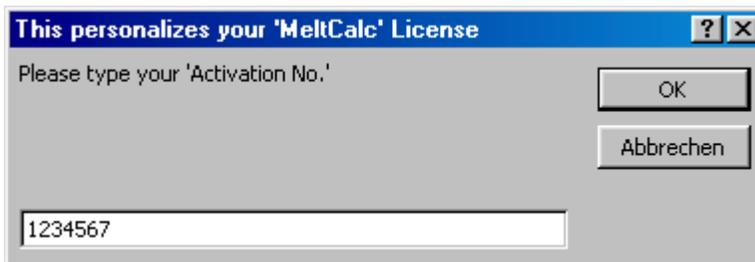
1. Create a new folder and copy all the files on the disk into that folder. Alternatively you may use drag and drop from the explorer to copy the whole “MeltCalc” folder from the disk to your favorite drive.
2. Open that respective folder in the explorer and double click on the Meltcalc99_2.06ProC.xla file. The filename is subject to change according to version numbers but it will always be the only file with the .xla extension in the folder. In case your windows explorer is configured to suppress files extensions just double click on the correct file name. The explorer will typically show a tiny  symbol to indicate that a file type is registered as Excel add-in. Excel will start up and the macro be executed. Recent Excel versions will ask if activation of macros is appropriate. You have to agree to execute MeltCalc. The file is released free of viruses or trojans to the best of our knowledge.
3. Every time the program starts you have to acknowledge by clicking on the “OK” button that you agree to the license agreement.
4. Please consider that you are only licensed for a single computer. So consider carefully on which computer you install MeltCalc. **The program will only be functional on this single computer.**

If this is your first time start-up of MeltCalc the program generates a serial-number which is printed in an input window. Write down this number and input the name of the license holder in this field. Type the affiliation of the license holder in the input field and click on “ready to go!”.



The MeltCalc program now generates a text file called “Meltreg.txt” in the folder from where the file has been executed. The program ends here. Send this “Meltreg.txt” as an email attachment or copied into an email message to support@meltcalc.de or by fax to +31(208)714855 or by mail to one of the authors. We will immediately generate your activation code and send it back to you.

5. After you got your activation key restart MeltCalc. You will be prompted to enter your activation key.



Enter it in the field and click “OK”. MeltCalc will now start up. **Keep a copy of your activation number with your files.** You will need this number again if you need to reinstall the program on the same computer again.

6. Certain hardware changes may terminate the license. If this happens to you, just start a new installation on the new system. You will need a new activation code, so please contact us as described above. Problems with your internal clock as well as manipulations on the MeltCalc program itself may terminate the program. If this happens to you, delete the MeltCalc folder on your hard disk and reinstall the software. You will have to type in the activation code that you already have for the program on your computer. MeltCalc will be functional again or please contact us.

Uninstalling

MeltCalc leaves no traces in the registry or other directories on your PC. If you want to uninstall MeltCalc simply delete the program folder.

II. Introduction and overview

MeltCalc is a Microsoft Excel add-in for the calculation of melting temperatures (T_m) of matched and mismatched DNA-strands. Calculations are based on the thermodynamic nearest neighbor model. Furthermore it can automatically select probe and anchor set suitable for LightCycler product quantification or genotyping with hybridization probes. MeltCalc does not support primer design. For this purpose we recommend the software “primer3” which is released under the GNU public license. A web interface for primer3 and further information is available via:

http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi. This software uses up to date algorithms for T_m prediction and has a good parameterization so that you really get the primers you want. A summary of other primer design software available via the internet is at http://www.bioinformatik.de/cgi-bin/browse/Catalog/PCR/Primer_Design.

The latest significant improvement for T_m prediction of hybridization probes was the report of dangling end parameters for DNA sequences (4). Whenever an oligonucleotide probe hybridizes to a long DNA target strand a 5' and a 3' dangling end interaction results. The dangling end interaction refers to a contribution from the next unpaired base on the strand to which the probe hybridizes. Dangling ends are stabilizing in 24 of the 32 possible cases, only three dangling ends cause destabilization. Stability contributions from dangling ends can be as high as an additional Watson-Crick base pair and must be considered for probe design. 5' dangling ends are equally or more stabilizing than their 3' counterparts. Therefore the position of the dangling end must be defined before proper calculations can be carried out.

Throughout these instructions when talking about mutation detection, the hybridization probe sited above the mutation will simply be called “probe” and the longer hybridization probe annealing next to the probe is called “anchor”. Usually the probe is 3' labeled with fluorescein and the anchor is 5' labeled with an appropriate dye (e.g. LC-Red640TM, LC-Red705TM or Cy5.5TM) and 3' phosphorylated.

For long double stranded DNA a polymer formula will be used for calculation of T_m and an estimate of the effect of destabilizing mutations will be given. This function may be helpful for an initial estimate of the acquisition temperature for quantitative PCR in the SYBR green format. SYBR green has a dose dependent stabilizing effect on DNA strands. MeltCalc is now able to account for different SYBR green concentration when T_m s are calculated.

III. Input fields

The calculations of the program are based on user input in certain fields. All input fields are colored in light brown whereas fields where the calculated results are output are colored in darker brown.

Oligoconc. [μM]	0.1	DMSO [%]	5
Na eq. [mM]	220	SYBR-I [x]	0
Length [bp]	64	T_m [$^{\circ}\text{C}$]	ΔT_m [$^{\circ}\text{C}$]
Sequence (perfect match)	tgcacatgactgagcgtactGacgtcagtgtaactgactgtgactg actgtgcatgcatgcatg	78.5	1.3
Sequence (mismatch)	tgcacatgactgagcgtactAacgtcagtgtaactgactgtgactg actgtgcatgcatgcatg	77.3	
Reverse sequence (pm)	catgcatgcatgacacagtcagtcacagtcagtcacactgacgtCagt acgctcagtcatgatgca	78.3	1.3
Reverse sequence (mm)	catgcatgcatgacacagtcagtcacagtcagtcacactgacgtTagt acgctcagtcatgatgca	77.0	

The oligoconcentration field refers to the concentration in μM of annealing oligonucleotide probe in the assay. This is usually in the range of 0.1-0.3 μM for LightCycler applications. The Na-eq. field refers to the sodium equivalent concentration in the PCR reaction. We routinely use 2.5 mM magnesium in standard PCR buffer. This results in approx. 220 mM Na-eq. For other conditions a sodium equivalent calculator is included within Meltcalc (“*Calculate [Na] equivalents*”). If dimethylsulfoxide (DMSO) is present in the PCR reaction its concentration in percent must be input into the DMSO field. DMSO decreases the T_m by approx. 0.6°C per percent (18). We routinely include 5% DMSO in our PCR reactions. If SYBR green I (SYBR-I) is present in the PCR reaction its x-fold concentration must be input into the SYBR-I field. SYBR green I is supplied as a 10,000x stock solutions and increases the T_m concentration dependent. We routinely include 0.5x SYBR green I in reactions for quantitative PCR in the SYBR green I format. The sequence (perfect match) field holds the input of the sense strand wildtype sequence of the oligonucleotide being investigated. The maximum length of the string in the input field can be 1755 bp. All sequence fields will only accept input of a/c/g/t or A/C/G/T but no other characters. Capital letters indicate mismatched positions between both input sequences. If you copy and paste data e.g. from GenBank with intervening numbers and spaces the “*Clean up your input*” function can help you to clean you data. In the sequence (mismatch) field either the same sequence should be input again (if data for an oligonucleotide without mismatches should be calculated) or the sequence were mismatches of interest are present.

Input examples for these fields are:

Calculation for a 17mer oligonucleotide without mismatches:

Sequence (perfect match)	actgactgatcgggccc
Sequence (mismatch)	actgactgatcgggccc

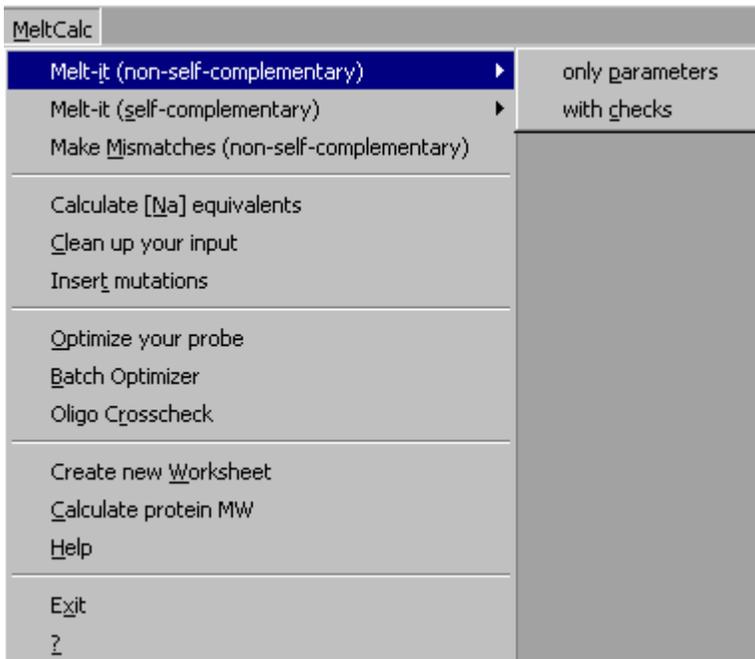
Calculation for a 17mer oligonucleotide were a G→A transition at position four is

destabilizing the hybridization. Note that mismatches in the sequences are indicated by capital letters.

Sequence (perfect match)	actGactgatcgggccc
Sequence (mismatch)	actAactgatcgggccc

IV. Functions of Meltcalc

Whenever MeltCalc is active within Excel its menu will be integrated into the standard pull-down menu row. By clicking on MeltCalc you will see the following choice of functions:



A) “Melt-it (non-self-complementary)”

Oligonucleotides

The function “Melt-it (non-self-complementary)” will calculate thermodynamic data for the input oligonucleotide sequence assuming the sequence is non-self-complementary. For the best possible prediction dangling end effects must be considered. You can of course leave these fields empty. The resulting T_m estimates will then usually be within 0.5°C higher to 1.5°C lower than resulting from the exact calculation including the dangling end parameters. Automatic probe design with MeltCalc always automatically includes the dangling end parameters!

Please look at the following example to understand how data are input in these fields:

actgactgatcgggccc is your probe with the sequence of the sense strand. Say the sense strands sequence isgatcgg**actgactgatcgggccc**gatgca... with the probe position shown in bold letters.

Hybridization of this probe will be as follows:



All dangling ends must be input in sense orientated notation. For the example there are two dangling end interactions, $_{CT}^A$ and $_{CT}^G$. According to the MeltCalc notation you have to input the sense bases of the dangling ends which must be considered. The next box shows the right input strings for the example. You need to tell the software *where* the dangling end is and *what type of base* causes the dangling end. For the *where* definition you enter the strings in the lower two cells (the probe has sense orientation, it hybridizes with the antisense strand which is represented by the lower cells in 3'→5' direction). For the *what type of base* definition remember that MeltCalc uses sense notation. The example probe has an unpaired adenosin at its 5' end. The 1 bp upstream cytosin on the antisense strand causes the dangling end contribution. The sense orientated equivalent of the cytosin is of course guanosin, therefore enter "g" in the left lower dangling ends box.

Dangling ends			
5'			3'
3'	g	a	5'

Considerations for the probe's 3' "g" base are likewise.

The output will be as follows:

The length of the oligonucleotide and the reverse sequence of both, perfect match and mismatch sequence are printed in the respective fields.

Length [bp]	17
Sequence (perfect match)	actGactgatcgggagg
Sequence (mismatch)	actAactgatcgggagg
Reverse sequence (pm)	ccgcccgatcagtcagtc
Reverse sequence (mm)	ccgcccgatcagtcagt

The resulting thermodynamic data look like this:

T _m [°C]	ΔT _m [°C]	ΔG-5 (°)	ΔG-5 (°)	GC [%]	ΔH	ΔS1	ΔSmod	self-align	hair-pin
65.2	6.9	-5.5	-9.0	64.7	-133.4	-352.4	-359.5	1.8	0.0
58.2		-1.5	-9.0	58.8	-118.1	-314.5	-321.6	0.0	0.0
66.2	8.8	-9.2	-6.1	64.7	-137.0	-361.9	-369.0	0.0	0.0
57.4		-9.2	-0.2	58.8	-109.3	-288.8	-295.9	0.0	0.0

1. The first column shows the T_m for the different hybridization situations: The perfectly matched oligonucleotide's T_m (here: 65.2°C), the T_m if the oligonucleotide probe would hybridize on the given mismatch (here: 58.2°C, the g->a mutation in the example results

into a g-t mismatch). The same output is made for the reverse sequence which is equal to the hybridization on the antisense strand. Note the higher destabilization if the probe is sited on the antisense strand. A ΔT_m of 8.8°C results from the c-a mismatch. The resulting T_m s of probes with the reverse sequence (in cell B6) will not be equal to those in the B4 cell if dangling ends are considered. This is caused by the different resulting dangling ends.

2. The second column shows the ΔT_m which would result from this mismatched hybridization. Note the favorable higher ΔT_m if this mutation would be detected on the antisense strand.

3. The third and fourth column show the calculated 5' and 3' free pentamer energy ($\Delta G-5$), respectively. This may be helpful in evaluating primer performance. The pentamer stability is thought to be useful for the estimation of primer performance, since it has been shown that primers with high priming efficiency are somewhat more stable at their 3'-end (19).

4. The fifth column shows the percentage of GC bases in the oligonucleotide sequences.

5. The sixth column shows the calculated standard thermodynamic enthalpy (ΔH) of the oligonucleotide sequence.

6. The seventh column shows the calculated standard thermodynamic entropy (ΔS) of the oligonucleotide sequence for standard conditions, i.e. 1M NaCl.

7. The eighth column shows the thermodynamic entropy (ΔS_{mod}) of the oligonucleotide sequence for the sodium equivalent concentration as given in the “*Na-eq.*” field.

8. The next two columns will only be calculated if the “*Melt-it (non-self-complementary)-with checks*” function was chosen.

9. The ninth column shows the T_m resulting from oligonucleotide self-align. A value is only displayed if the resulting T_m is $>0^\circ\text{C}$. High self-align temperatures indicate the oligonucleotide may prime itself and easily produce dimers.

10. The tenth and final column shows the T_m resulting from hairpin formation of the oligonucleotide. A value is only displayed if the resulting T_m is $>0^\circ\text{C}$. High hairpin temperatures indicate the oligonucleotide has strong secondary structures and may not be an ideal candidate for hybridization to a target.

Polymer DNA / SYBR green application

Whenever the input string gets longer than 255 bp a thermodynamic calculation is no longer necessary for an estimation of the double strands melting point. MeltCalc then switches to a simpler formula (14;15). To acknowledge this the on-screen output is titled “Polymer DNA formula” and fields for thermodynamic data are left empty.

You can calculate the melting point of a PCR amplicon this way. The influence of SYBR green I on the T_m is included with an empirical formula based on our data. Input your SYBR green I concentrations in the D2 cell of the MeltCalc input screen. SYBR green I is supplied as a 10,000x stock solutions and increases the T_m in a concentration dependent manner. We routinely include 0.5x SYBR green I in reactions for quantitative PCR in the SYBR green I format. The ready made SYBR green master mix from Roche uses a unknown concentration of SYBR green. You are probably well of if you try values from 0.5 – 1.0x as concentration with this mixture. As an initial estimate for quantitative

PCR with SYBR green you may try an acquisition temperature 3°C below the calculated T_m . However, you will probably have to adjust this temperature according to exactly measured T_m s resulting from your actual experiment.

If mismatches are present in the long (>255 bp) input strands MeltCalc also estimates the resulting ΔT_m of the mismatched strand. This is a very rough estimate assuming a destabilization of 1.25°C per percent mismatch. Output is only generated in columns 1,2 and 5 according to the figure shown on the page before. Note again, there are no thermodynamic calculations performed for such long strands. Also the destabilizing effect of mismatches is calculated without consideration of neighboring bases.

B) “Melt-it (self-complementary)”

The function “*Melt-it (self-complementary)*” will calculate thermodynamic data for the input oligonucleotide sequence assuming the sequence is self-complementary. We do not recommend to choose self-complementary oligonucleotides. They will form dimers and perform bad in applications. However, for completion of the thermodynamic calculations we have included this special case. The program will not check if the sequence really is self-complementary.

C) “Make mismatches (non-self-complementary)”

The function “*Make mismatches (non-self-complementary)*” will calculate thermodynamic data for all possible mismatches under the input oligonucleotide. The output will be placed in a separate table called “output”. It is available by clicking on the field at the lower left end of the window.



The output shows the following:

Mismatch	Sequence	Reverse Sequence	ΔG_{37}	T_{Mm}	ΔT_m
Wildtype	actgactgatogggogg	ccgccogatcagtcagtc	-23,21	59,8	
c5	actgCctgatogggogg	ccgccogatcagGcagtc	-21,24	57,6	2,2
c9	actgactgCtogggogg	ccgccogaGcagtcagtc	-21,26	57,3	2,5

In the top column the wildtype sequence and its T_m is given. Then, ordered by their ΔT_m all possible mismatches under the respective probe and the resulting T_m s are output. This is useful for checking if either a) other mutations may destabilize the strand to the same extent as the mutation being investigated or b) mutations may stay undetected because they don't destabilize the strand enough. For a detailed discussion of these issues we suggest reading reference (2). a) is important for probe specificity. You will find that for probably every probe other mismatches are possible that cause the same shift in ΔT_m as the mismatch being investigated into does. b) is important because a rather stable mismatch (e.g. GAC/CGG) under a stable (GC rich) DNA probe may stay undetected. In this situation it is advisable to site the probe on the antisense strand. This changes the given example mismatch to CTG/GCC, an unstable mismatch which is easily detected. In

the example the minimum calculated ΔT_m is 2.2°C. It is therefore likely that mismatches will be detected by the LightCycler. The use of a properly designed probe should allow the detection of every possible mismatch under it. Use the function “*Optimize your probe*” for an automatic probe selection algorithm to find the best probe for a certain mismatch.

D) “Calculate [Na] equivalents”

The function “*Calculate [Na] equivalents*” starts the calculator. Input the requested data and it will estimate the sodium equivalent calculation for a PCR buffer with a known magnesium, potassium and dNTP concentration. We routinely use 2.5 mM magnesium 0.8 mM dNTPs in standard PCR buffer. This results in approx. 220 mM Na-eq(17). The dNTP concentration is now considered for the Na-eq calculation because dNTPs bind magnesium ions. You may need to fine tune you Na-eq. within a certain range to gain better agreement between you measured T_m and calculated results. For optimal accuracy melting curves should always be recorded with the lowest possible ramp rate (0.1°C/sec). Na-eq and continuous fluorescence acquisition. For magnesium concentrations higher than 8 mM can not be calculated. The estimation becomes increasingly inaccurate with higher magnesium concentrations.

E) “Clean up your input”

The function “*Clean up your input*” removes every non a/c/g/t or A/C/G/T character from the upper oligonucleotide input field. If you copy and paste data e.g. from GenBank (available via <http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html>) with intervening numbers and spaces the “*Clean up your input*” function can easily clean your data.

F) “Insert mutations”

It sometimes a difficult task to “*Insert Mutations*” at the right positions if the strand is long. This function assists you provided the base you want to change is one of the outer three positions. First choose this function from the MeltCalc pull down menu.

Do you want to insert mutations?

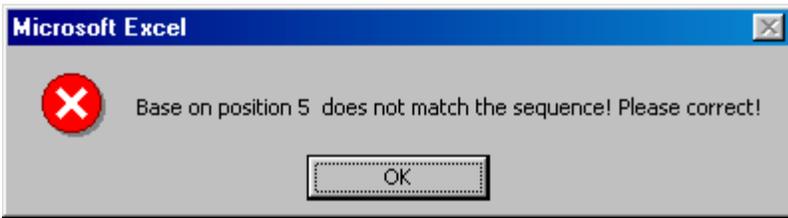
<input checked="" type="checkbox"/>	Position of mutation #1:	123	change:	A	to:	G
<input checked="" type="checkbox"/>	Position of mutation #2:		change:	A	to:	A
<input type="checkbox"/>	Position of mutation #3:		change:	A	to:	A
<input type="checkbox"/>	Position of mutation #4:		change:	A	to:	A

Exit ready to go!

Then activate the tickbox left of “Position of Mutation” by clicking on it. You have to define the mismatch you want to introduce into the strand by indicating the base position and the base exchange you want to introduce. The latter is a safety feature to assure that you are really about to change the correct base. The mutation is then introduced into the mismatch sequence and highlighted by a capital letter.

MeltCalc will automatically change the input box if you defined a position which is different from the sequence. Example:

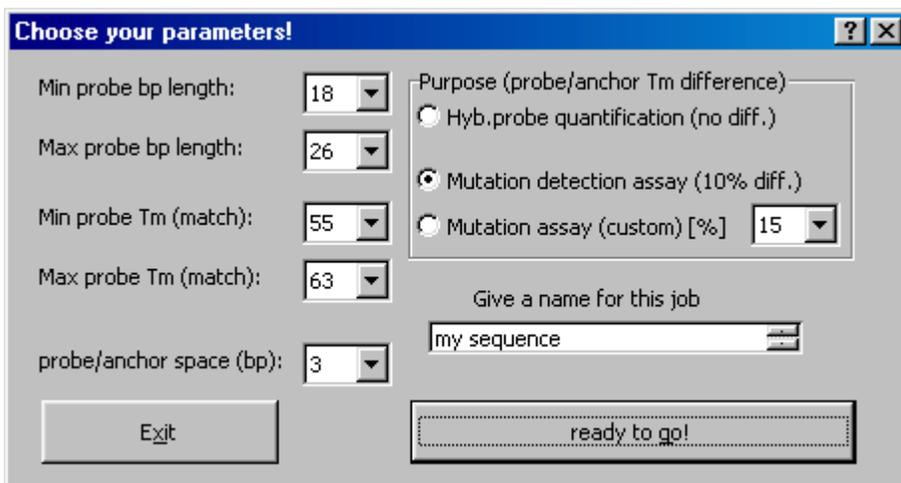
act **g**ac tga tgc ggc is the sequence where you want to introduce an a→g mutation at position 5. To do it right you input position 5, change a to g. If you happen to input position 6, change a to g the software will alert with the box:



However, if you then use the “Insert Mutations” function again you will find that MeltCalc has changed your input into position 6, change c to g because it has realized that correct base at the position you have indicated is a c.

G) “Optimize your probe”

The function “Optimize your probe” is probably the most useful and powerful function of MeltCalc. It starts an automatic hybridization probe designer that will automatically choose probe and anchor sets for you based on your specifications and appropriate thermodynamic calculations. We are not aware of any other software which has this feature. All T_m s are calculated using dangling end parameters. The dangling ends are derived from the input sequence.



You can choose a range for probe length and probe melting temperature and probe purpose (see a) and b) below). You can also input a probe/anchor space in the range of 1 to 5 bp as recommended by Roche for hybridization probes.

Results are output in a separate table called “probes” which is activated automatically after the calculations are finished.



Quantification hybridization probes

- a) Quantification hybridization probes are designed in such a way that probe and anchor have nearly identical melting points. The input sequence should lie between the amplification primers and a length of 1755 bp must not be exceeded. The input of a 100 bp or longer sequence is recommended because this gives the program a wider choice where hybridization probes can be put. The strategy is to calculate a matrix function representing the hybridization strength all along the input sequence. Only where a tight binding without false priming sites on the same amplicon results will the program suggest probes. Calculation can take significant amounts of time dependent on the computer CPU as well as input sequence length. The progress is shown by a counter in the left lower end of the window.

The output is as follows (left half):

5' - probe - 3'	5'	3'	bp	T _m	T _m mis	ΔT _m	orientation
gtcaggcgtcgtcatgatg	50	68	19	59,6	0,0	0,1	FOR
atgatgctaogacgtaoct	63	81	19	57,4	0,0	0,1	FOR
tgatgctaogacgtaoctg	64	82	19	58,3	0,0	0,1	FOR
X-gatgctaogacgtaoctgt	65	83	19	58,3	0,0	0,1	FOR
cgatcatactcaactca	46	28	19	56,1	0,0	0,1	REV
atcatactcaactcagc	44	26	19	56,4	0,0	0,1	REV
catactcaactcagcaa	42	24	19	56,9	0,0	0,1	REV

The chosen probe is printed in 5' to 3' direction ready e.g. for copy and paste into an oligonucleotide order form. Columns two to four indicate the start and end position of the hybridization site with respect to the input sequence and the probe length. Column five shows the calculated T_m. Columns six and seven show nothing useful because no mismatch is present. Column eight indicates from where the probe sequence was derived to gain optimal function. REV means the probe sequence can be found on the antisense strand, so the probe will hybridize with the sense strand. FOR means the probe sequence can be found on the sense strand, so the probe will hybridize with the antisense strand. X means that a significant self-alignment of the probe is present.

Continued results (right half):

5'anchor3'-DYE	T _m	DYE-5'anchor3'	T _m	align
selfalign	60	selfalign	60,5	0
crossalign	58,5	atgtgattcgoggtattatctg	57,4	0
selfalign	60	tgtgattcgoggtattatctgc	59,8	0
cgacgtcaggcgtcgtc	61,5	gtgattcgoggtattatctgca	59,8	21
atcatgacgacgcctga	56,5	acgtcatactaccagctaata	57,5	0
tgacgacgcctgacg	56,8	gtcatactaccagctaatagacg	57,1	0
selfalign	58,2	catactaccagctaatagacgt	55,9	0

Anchor probes flanking the first probe on the 5' and 3' ends are shown. If you order the first probe with 3' fluorescein as usual, the corresponding anchor would be in the column "DYE-5'anchor3'" and should be 3' phosphorylated as well. If, for some reason, you want it the other way around, order the first probe with 5' fluorescein and 3' phosphorylated. The corresponding anchor is then shown in the "5'anchor3'-DYE" column. The distance between both fluorophores is always 3 bp. Note that some anchor probes are rejected because of significant self- and/or crossalign. The last column shows the T_m resulting from the first probe self-align. A value is only displayed if the resulting T_m is >0°C. High self-align temperatures indicate the oligonucleotide may prime itself and easily produce dimers.

Hybridization probes for mutation detection

- b) Hybridization probes for mutation detection are designed in such a way that the probe ΔT_m is maximized to ensure good discrimination of heterozygotes. Only one mismatch can be considered and optimized at a time. This mismatch to optimize for is found automatically by comparing the two sequences in the input fields. The input sequence should lie between the amplification primers and a length of 256 bp must not be exceeded. However, the input of at least 50 bp upstream and 50 bp downstream of the polymorphism site is recommended because this gives the program a wider choice where the hybridization probes can be put. Calculation can take significant amounts of time dependent on the computer CPU as well as input sequence length. The progress is shown in the lower window.

The results are as follows (left half):

5' - probe - 3'	5'	3'	bp	T _m	T _m mis	ΔT_m	orientation
gcatcatgaCgacgcc	69	54	16	56,6	45,4	11,2	REV-wt
atgaCgacgcctgacg	64	49	16	57,5	46,4	11,1	REV-wt
atcatgaCgacgcctga	67	51	17	56,5	45,5	11,0	REV-wt
tgaCgacgcctgacgt	63	48	16	59,2	48,2	10,9	REV-wt
gaCgacgcctgacgtc	62	47	16	58,3	47,6	10,7	REV-wt

The chosen probe is displayed in the first column in 5' to 3' direction ready e.g. for copy and paste into an oligonucleotide order form. Columns two to four indicate the start and end position of the hybridization site with respect to the input sequence and the probe length. Columns five to seven show the calculated T_m , the T_m for the mismatched probe and the resulting ΔT_m ordered by their ΔT_m . Column eight indicates from where the probe sequence was derived to gain optimal function. REV means the probe sequence can be found on the antisense strand, so the probe will hybridize with the sense strand. FOR means the probe sequence can be found on the sense strand, so the probe will hybridize with the antisense strand. "wt" indicates that the probe is wildtype compatible so that mutated strands have a lower T_m . Do not forget that the program may also choose mutation compatible probes (indicated by "mut"). These probes will of course show a lower T_m when hybridized with the wildtype strand!

Continued results (right half):

5'anchor3'-DYE	T_m	DYE-5'anchor3'	T_m	align
gcgaatcacatgacacgtacgtc	63,1	cgtcgtatcatacgtcaacgtcagc	64,8	0
tcacatgacacgtacgtcgtagc	63,6	atcatacgtcaacgtcagcaacgt	64	0
cgaatcacatgacacgtacgtcgt	64,2	cgtatcatcgtcaacgtcagcaa	62,4	0
atcacatgacacgtacgtcgtagca	65,2	tcatacgtcaacgtcagcaacgtcat	65,9	0
tcacatgacacgtacgtcgtagcat	65,2	catacgtcaacgtcagcaacgtca	64,7	12,4

Anchor probes flanking the detection probe on the 5' and 3' ends are shown. If you order the detection probe with 3' fluorescein as usual, the corresponding anchor would be in the column "DYE-5'anchor3'" and should be 3' phosphorylated as well. If, for some reason, you want is the other way around, order the detection probe with 5' fluorescein and 3' phosphorylated. The corresponding anchor is then shown in the "5'anchor3'-DYE" column. The distance between both fluorophores is always 3 bp. The last column shows the T_m resulting from detection probe self-align. A value is only displayed if the resulting T_m is $>0^\circ\text{C}$. High self-align temperatures indicate the oligonucleotide may prime itself and easily produce dimers.

H) "BatchOptimizer"

This powerful function is only available in the MeltCalc professional edition for commercial use. It allows for the fully automatic processing of different sequences entered into an Excel file. Files are recognized by the filename. Every filename beginning with "batch" (not case sensitive) will be processed. You may enter a blank line (see below, row 6). Batch processing is stopped at this position. Sequences must be entered according to the enclosed "Batch_Sample.xls" file.

Details of this file are as follows (left half):

	A	B	C	D	E	F	G
1	email						
2	Job name	Wildtype sequence	Mutation sequence	min bp	maxbp	min temp	max temp
3	TPMT3B (Ex-7)	catcttctaaagattgattttctcccaaaaa	catcttctaaagattgattttctccca	23	25	55	60
4	TPMT4 (110/Ex-10)	aacatgttactcttctgtttcaggtaaaaatg	aacatgttactcttctgtttcaggtaaaa	19	30	51	55
5	TPMT3C (Ex10)	aacatgttactcttctgtttcaggtaaaaatg	aacatgttactcttctgtttcaggtaaaa	19	40	54	60
6							
7	TPMT3D (Ex5)	ttgaaaacataaaitaagtgtaaatgatgattt	ttgaaaacataaaitaagtgtaaatgatg	19	30	55	60
8	TPMT2 (Ex5)	ttgaaaacataaaitaagtgtaaatgatgattt	ttgaaaacataaaitaagtgtaaatgatg	19	30	55	60
9	TPMT5 (Ex4)	atgaaaagtgttcacctaccatacaattgtcct	atgaaaagtgttcacctaccatacaattg	19	30	55	60
10	TPMT6 (Ex8)	cctgtgtagagaaatgtaacaaataccctcttg	cctgtgtagagaaatgtaacaaataccct	19	30	55	60
11	TPMT7 (Ex10)	aacatgttactcttctgtttcaggtaaaaatg	aacatgttactcttctgtttcaggtaaaa	19	30	55	60

You can type any comments into the B1 field.

1. Enter a job name for each sequence. The job name will be used to create a separate table where the results from calculations are output (see below). Every name that Excel would accept for naming a table is allowed, other names are changed accordingly by the software.

2. and 3. If the automatic design of hybridization probes for mutation detection (see G) "Optimize your probe") is wanted you must input a wildtype and a mutation sequence in the respective fields. Input sequence length must not exceed 1755 bp.

4. and 5. Here the minimum and maximum probe length must be entered.

6. and 7. Here the minimum and maximum probe T_m must be entered.

Input continued (right half):

H	I	J	K	L	M	N
Oligoconc	Sodium	DMSO [%]	Primer1	P-1 Tm	Primer2	P-2 Tm
0,1	300	5				
0,1	300	5				
0,1	300	5				
0,1	300	5				
0,1	300	5				
0,1	300	5				
0,1	300	5				
0,1	300	5				

8. Enter the oligonucleotide concentration in μM into this field.

9. Enter the sodium equivalent concentration in the assay into this field. See also D) "Calculate [Na] equivalents".

10. If DMSO is present in the assay enter its concentration here so that the DMSO influence on the melting temperature can be considered.

11. to 14. You may either enter a primer sequence or a primer T_m into the respective fields. For LightCycler online PCR it is necessary that probes are hybridized at the primer annealing temperature. This function takes care of this by either calculating the thermodynamic T_m of your entered primer sequences and only considering probes with a higher T_m or by using the T_m that you entered for these calculations. These fields may be left empty and probes will be calculated according to parameters 4-7.

The results will be presented in an output equal to that of the “Optimize your probe” function. Results for every job name will be output into a separate table.

I) “Oligo Crosscheck”

The function “Oligo Crosscheck” will calculate the maximum melting temperature resulting from crosshybridization of the oligonucleotides put in the separate field on the main window. This is useful to examine for possible unfavorable interactions between amplification primers and probes used in the same assay.

	Oligo Cross-Hybridizer Input Table	Oligo vs	1	2	3	4	5	6	7	8
Oligo No. 1	actgtacgtacgtgacgtgacgtactgg	1		2.2	8.0	39.1	0.0	0.0	10.0	0.0
Oligo No. 2	cgtctatgcatgcatgcattgcatgcag	2			0.0	0.0	19.5	15.1	0.0	0.0
Oligo No. 3	gcgtactgactgtgactgtgac	3								
Oligo No. 4	gacgtcatgtgacgtacgtgtacgt	4			15.8				0.0	0.0
Oligo No. 5	catgacgtactggtcacgtcatgctg	5			0.0	1.6		10.5	0.0	0.0
Oligo No. 6	cgttgcacgtcacttgcacgtgactac	6			0.0	0.0			0.0	0.0
Oligo No. 7	gtcacagctctcagtcagtcagggtc	7				57.9				0.0
Oligo No. 8	gtcacgctctcagtcagtcagggtc	8			45.3					

Up to eight oligonucleotides can be processed in a single run. The output is in a checkerboard style as seen on the right half of the figure above. A value is only displayed if the resulting T_m is $\geq 0^\circ\text{C}$. High cross-align temperatures (shown in red color) indicate that the oligonucleotides may significantly cross-hybridize, prime each other and produce dimers. As you can see in the example some oligonucleotides have cross-dimer melting temperatures $>50^\circ\text{C}$. If this happens to your PCR try to use different amplification primers with less interaction. You can also try to site the probe at an alternative position or on the other DNA strand.



By clicking on the “cross” tab at the bottom of the spreadsheet you can switch to a graphical representation of the oligonucleotide interaction.

N) “?”

The function “?” shows your MeltCalc licensing information.

References

The following **references** describe the MeltCalc program and its first application to hybridization assays on the LightCycler. **Reference (1) should be cited whenever publications report results obtained with MeltCalc. References (1) and (2) should be cited when the application was on the LightCycler.**

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