IOSR Journal of Biotechnology and Biochemistry (IOSR-JBB)
ISSN: 2455-264X, Volume 2, Issue 6 (Sep. – Oct. 2016), PP 06-11
www.iosrjournals.org

Computation of Melting Temperatures (T_m) of oligonucleotides for high throughput PCR using MS-Excel

Lavanya Kumari.P[1] and Amaravathi.Y[2]
Institute of Frontier Technology, Regional Agricultural Research Station, Acharya NG Ranga Agricultural University, Tirupati, Andhra Pradesh, INDIA.

Abstract: *DNA based molecular markers have enormous potential to improve the efficiency and precision of conventional plant breeding via marker-assisted selection. Most of the molecular markers are individually amplified by oligonucleotide primers using PCR. The efficiency of PCR depends on primer annealing temperature (T_a) which optimize amplification and minimize non-specific binding of primers to the template. In contrast to the majority of the software programs available to calculate melting temperature (T_m) of primers/oligonucleotides which enables us to get T_m for single sequence/ input at a time. Hence, we developed an MS-Excel based programme to calculate T_m for a large set of primers with one click based on two formulae viz., basic and salt adjusted depending on the size of the primers. A wide range of oligonucleotides with size range between 10 to 30mer covering RAPD, AFLP, ISSR SSR and SNP markers were tested which are widely used for marker-assisted selection.*

Keywords: *PCR, DNA based markers, Marker Assisted Selection (MAS,) Melting Temperature (T_m), Annealing Temperature (T_a), MS-Excel*

I Introduction

One of the major concerns of modern agriculture is the identification and utilization of valuable genes of agronomic importance in crop plants. The development and use of DNA based molecular markers to track loci that are tightly linked to important genes have irrevocably changed the fields of genetics and plant breeding. Molecular markers exhibit a high degree of polymorphism and simple inheritance patterns with minimal influence of environment and epistasis. Therefore, in combination with conventional breeding approaches, DNA based molecular markers can be used to monitor the presence or lack of these genes in breeding populations and monitoring the level of gene expression under biotic and abiotic stress situation through Marker Assisted Selection (MAS).

Several techniques have been developed for the past few years for distinguishing the individuals at DNA sequence level. Most of the markers are PCR (Polymerase Chain Reaction) based and are individually amplified by using oligonucleotide primers. If there is no prior sequence information available in the database for a particular species, random markers like Random Amplified polymorphic DNA (RAPD), Amplified Fragment Length Polymorphism (AFLP) and Inter Simple Sequence Repeats (ISSR) can be employed. These are multilocus, mostly dominant genetic markers usually produce multiple DNA fragments (each of which is considered as a locus) allowing the generation of a large number of loci across the genome without prior genome sequence information. On the other hand, if sequence information is available, then species specific markers like microsatellites or Simple Sequence Repeats (SSRs) and Single nucleotide polymorphisms (SNPs) can be used for genotyping.

In all the PCR based markers, PCR will be efficient under optimal conditions like template DNA concentration, salt concentrations and primer annealing temperature (T_a). Keeping other parameters in PCR constant , the successful amplification of a particular locus depends on optimum T_a which in turn depends on primer sequence and GC content, complementarities of forward and reverse primers, reaction volume etc. The primers of different markers vary in length and sequence composition (GC content) which in turn leads to the variation in primer annealing temperature (T_a). Primer annealing is a physical process where the primers will jiggle around and hydrogen bonds will be constantly formed and broken between specific bases in the single stranded primers and the single stranded template DNA. More stable bonds last a little bit longer (primers that fit exactly) and on that little double-stranded piece the DNA polymerase can attach and start copying the template and thereby results in doubling target sequence.

Assuming that all the reagents have been added in proper concentrations, primer annealing temperature is critical for overall success of the PCR experiment. Too high T_a will results in poor reaction efficiency because the likelihood of primer annealing is reduced significantly and there by produce insufficient primer-template hybridization resulting in low or no PCR product yield. Conversely, too low T_a results in annealing of one or both primers to sequences other than the intended target sequence because internal single-base mismatches or partial annealing may be tolerated. This can lead to non-specific PCR amplification and will consequently results in additional amplification products and reduction in the yield of the desired product. Optimal annealing temperatures will result in the highest product yield with the correct amplicon or PCR product. Therefore, there is a need to critically calculate exact annealing temperatures to optimize PCR amplification and to minimize non-specific binding of primers to the template. The optimal T_a is the range of temperatures where efficiency of PCR amplification is maximal with reproducible banding pattern and T_a is a function of melting temperature (T_m) of the primers. T_m is the temperature at which half the double stranded DNA are in the double-helical state and half are in the single stranded random-coil state which in turn positively correlated with length of the primer and GC content. The nucleotides pairing in DNA is specific and G pairs with C by three hydrogen bonds and A pairs with T by two hydrogen bonds and T_m increases with increase in GC content. The success of molecular techniques like PCR depends on the accurate prediction of T_m. This is particularly critical in the case of SSR and SNP markers for whole genome analysis which involves several oligonucleotides simultaneously. Accurate prediction of T_m is also required in case of multiplex PCR, short sequences probes in microarrays and DNA chips for nucleotide hybridization (Pease *et al*., 1994). The annealing temperature for a primer is generally calculated as 5°C lower than the estimated melting temperature.

Inappropriate T_m estimation will lead to the amplification of nonspecific products in case of PCR or an inappropriate hybridization and thereby the efficiency of molecular predictions will be drastically reduced. Therefore, it is important to calculate accurate T_m values for DNA oligos like primers and probes are a critical step which increases the performance of PCR and accuracy of results.

II Methods used to calculate Melting Temperature (T_m)

Calculation of an accurate melting temperature is necessary to establish the annealing temperature at which the PCR reaction is to be performed. A number of methods are available to calculate the T_m of molecular markers and the simplest method described by Marmur and Doty (1962) where the T_m depends only on the GC content. T_m can be estimated using the formula $T_m = (wA+xT)*2 + (yG+zC)*4$ where w, x, y and z are the number of the A, T, G, C nucleotide bases in the sequence respectively (Marmurand Doty1962). This formula holds good for short primers up to 14 nucleotides long whereas for longer primers (>14nucleotides) salt adjusted calculation of melting temperature (T_m) parameters to be taken into consideration (Wetmur, 1991). The T_m of longer primers depends on ionic concentration as DNA is negatively charged molecule and it interacts with ions to compansate the charge. This method was later improved to maximize the accuracy and the modified formula employs the equation $T_m = 100.5 + (41 * (yG+zC)/(wA+xT+yG+zC)) - (820/(wA+xT+yG+zC)) + 16.6*\log_{10}([0.050])$ (Nakano et al, 1999) where w, x, y, z are the number of the bases A, T, G, C in the sequence respectively the term $\log_{10}(0.050)$ adjusts for the salt adjustment at 50 mM Na^+ ion concentration. This equation is accurate for sequences in the 18-25mer range (Howley et al. 1979) and this is employed as most of the SSR and SNP primers length falls in this range.

III Need to develop automatized procedure for T_m calculations

Currently, many online web servers and software programs are available to calculate T_m for oligonucleotides which will enable us to find T_m for a single sequence/ input at a time. Due to this limitation, it will be a cumbersome to feed each and every sequence and noting down the T_m which in turn leads to commit magnitude of errors due to multiple actions. In order to find melting temperatures for 200 sequences this process of pasting the sequence in online web servers needs to repeat obviously 200 times. In general more actions raise the chance of committing errors in any task. Sometimes number of sequences to be considered will be around in thousands. Hence, keeping this in mind authors have planned to develop a procedure using MS-Excel to reduce the complexity as well as time in calculating T_m for several sequences at a time.

IV About MS-Excel

Microsoft Excel is a Spreadsheet program included in the Microsoft Office suite of applications and is available in almost all Personal Computers. Spreadsheets contain rows and columns that can be manipulated mathematically using both basic and complex arithmetic operations and functions. In addition to its standard spreadsheet features, Excel also offers programming support via Microsoft's Visual Basic for Applications (VBA), the ability to access data from external sources via Microsoft's Dynamic Data Exchange (DDE), and extensive graphing and charting capabilities. Inception of its version was started with Excel 2.0 in 1987 and currently Excel 2013 is running but in this study Excel 2010 version was used.

V Functions in MS-Excel

Functions are prewritten formulas and differ from regular formulas in that user supplies the value but not the operators, such as +, -, *, or /. For example, one can use the 'SUM' function to add. Addition can be done using a function=SUM(data range). Function arguments are enclosed within parentheses. Arguments are values (the numbers or cells) on which you want to perform the calculation. For example, arguments specify the numbers or cells you want to add and comma can be used to separate the arguments. The equal sign (=) begins the function. After typing the first letter of a function name, the *Auto complete list* appears. One can double-click on an item in the *Auto complete list* to complete required entry quickly. Excel will complete the function name and enter the first parenthesis.

VI Excel functions used in this study

Table-6.1: MS-Excel functions used in this study

S.No	Function	Description	Type of function
1	SUBSTITUE( )	Substitutes new text for old text in a text string	Text function
2	LEN( )	Returns the number of characters in a text string	Text function
3	LOG10( )	Returns the base-10 logarithm of a number	Maths and Trigonometry functions
4	ROUND( )	Rounds a number to a specified number of digits	Maths and Trigonometry functions
5	MIN( )	Returns the minimum value in a list of arguments	Statistical Functions
6	IFERROR( )	Returns the value of if_error if expression is an error and the value of expression itself otherwise	Logical function

VII T_m calculation using Marmur and Doty (1962)'s formula in MS-Excel (Method1)

Formula(Method1):

$T_m = (wA+xT)*2 + (yG+zC)*4$, where w, x, y and z are the number of the A, T, G, C

Step-1: Open Excel sheet and enter the column headings as shown in table-7.1.

Table-7.1: Descriptions of the column headings to be entered in Excel sheet –Method1

Title	(cell)	Subtitle(cell)	Description of the title	Purpose
S.No	(A1:A2)		Primer number	Data Input
PRIMER	(B1:B2)		Name of the primer	
FORWARD PRIMER	(C1:C2)		Sequence of the Forward Primer	
REVERSE PRIMER	(D1:D2)		Sequence of the Reserve Primer	
Forward Primer (FP)	(E1:I1)	Length (E2)	Total no.of AGCT's in a forward Primer	Details about Forward Primer
		#A (F2)	No.of A's in a given forward primer	
		#G (G2)	No.of G's in a given forward primer	
		#C (H2)	No.of C's in a given forward primer	
		#T(I2)	No.of T's in a given forward primer	
Reverse Primer (RP)	(J1:N1)	Length (J2)	Total no.of AGCT's in a reverse primer	Details about Reverse Primer
		#A (K2)	No.of A's in a given reverse primer	
		#G (L2)	No.of G's in a given reverse primer	
		#C (M2)	No.of C's in a given reverse primer	
		#T (N2)	No.of T's in a given reverse primer	
Melting Temperature (T_m)	(O1:P1)	FP (O2)	Melting Temperature of Forward Primer	Main calculations for Melting Temperature
		RP (P2)	Melting Temperature of Forward Primer	

Note: cell addresses where titles and subtitles to be typed are kept in parenthesis.

Step-2: Enter the data under columns; S.No, PRIMER, FORWARD PRIMER, REVERSE PRIMER the data as shown in fig-7.1.

Fig-7.1: Initial MS-Excel Screen after entering the forward and reverse primers –Method1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	S.NO.	PRIMER	FORWARD PRIMER	REVERSE PRIMER	Forward Primer(FP)					Backward Primer(BP)					Melting Temperature (Tm)	
2					Length	#A	#G	#C	#T	Length	#A	#G	#C	#T	FP	RP
3	1	PPGS SEQ 3A8	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA												
4	2	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG												
5	3	PPGS SEQ 13A10	AACTCGCTTGTACCGGCTAA	AGGAATAATAACAATACCAACAGCA												
6	4	PPGP SEQ 3A1	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG												
7	5	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG												
8	6	PPGP SEQ 1B9	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT												
9	7	PPGP SEQ 2B10	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA												
10	8	PPGS SEQ 10D4	ATCCCTGATTAGTGCAACGC	CGTAGGTGGTTTTAGGAGGG												
11	9	PPGS SEQ 18C5	GGACAGCCGGATGCTATTTA	ACATGAGTCCCTTTTCCCTT												
12	10	PPGS SEQ 15C10	ATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG												
13	11	PPGS SEQ 16C6	TTGCTACTAAGCCGAAAATGAAG	CTTGAAATTAACACATATGCACACA												
14	12	PPGP SEQ 8D9	TGAGTTTCCCCAAAAGGAGA	CAACAACAATACGGCCAACA												
15	13	PPGP SEQ 2D12B	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA												
16	14	PPGP SEQ 3D9	TTCACCCGTACAAACCAGTG	CCTCGGCAGATCTGGAGTAA												
17	15	PPGS SEQ 16G8	CTCAAAAAGCGCTTAGCCAC	CTGCCTACTGCCTACTGCCT												
18	16	PPGS SEQ 17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG												
19	17	PPGS SEQ 14H6	GCAACTAGGGTGTATGCCGT	CAACCCTATACACCGAGGGA												
20	18	PPGP SEQ 2F5	TGACCAAAGTGATGAAGGGA	AAGTTGTTTGTACATCTGTCATCG												
21	19	PPGS SEQ 12F7	TGTCGTTGTAAGACCTCGGA	TTGGTTTCCTTAAGGCTTCG												
22	20	PPGP SEQ 16F1	TGCTTCCATCAGCTTTTCCT	AAATGAGGGCCTCCAAAGTT												

Step-3: Type the formulae in the corresponding cells as mentioned in table-7.2 and click the *enter* key after each entry of formulae. (ensure that formula should start with "=")

Table-7.2: List of the formulae and cell addresses to be entered in MS-Excel – Method1

Title	Subtitle	Cell	Formulae to be typed in cell	Purpose
Forward Primer (FP)	Length	E3	=LEN(C3)	Provides the length of FP
	#A	F3	=LEN(C3)-LEN(SUBSTITUTE(C3,"A",""))	Provides no.of 'A's in FP
	#G	G3	=LEN(C3)-LEN(SUBSTITUTE(C3,"G",""))	Provides no.of 'G's in FP
	#C	H3	=LEN(C3)-LEN(SUBSTITUTE(C3,"C",""))	Provides no.of 'C's in FP
	#T	I3	=LEN(C3)-LEN(SUBSTITUTE(C3,"T",""))	Provides no.of 'T's in FP
Reverse Primer (RP)	Length	J3	=LEN(D3)	Provides the length of RP
	#A	K3	=LEN(D3)-EN(SUBSTITUTE(D3,"A",""))	Provides no.of 'A's in RP
	#G	L3	=LEN(D3)-LEN(SUBSTITUTE(D3,"G",""))	Provides no.of 'G's in RP
	#C	M3	=LEN(D3)-LEN(SUBSTITUTE(D3,"C",""))	Provides no.of 'C's in RP
	#T	N3	=LEN(D3)-LEN(SUBSTITUTE(D3,"T",""))	Provides no.of 'T's in RP
Melting Temperature (T_m)	FP	O3	=(F3+I3)*2+(G3+H3)*4	Calculates T_m for FP
	RP	P3	=(K3+N3)*2+(L3+M3)*4	Calculates T_m for RP

Step-4: After entering all formulae, select cells from E3 to P3 and drag down to get T_m for all the primers at a time. One can perform these calculations for nearly 10,48,576 primers at a time (the number may differ according to versions of MS-Office). The resultant screen will be as shown in fig-7.2.

Fig-7.2: MS-Excel screen after calculating T_m for entered primers using Method1

	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	S.NO.	PRIMER	FORWARD PRIMER	REVERSE PRIMER	Forward Primer(FP)					Backward Primer(BP)					Melting Temperature (Tm)	
2					Length	#A	#G	#C	#T	Length	#A	#G	#C	#T	FP	RP
3	1	PPGS SEQ 3A8	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA	20	5	6	5	4	22	12	3	5	2	62	60
4	2	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	21	11	3	4	3	20	3	3	7	7	56	60
5	3	PPGS SEQ 13A10	AACTCGCTTGTACCGGCTAA	AGGAATAATAACAATACCAACAGCA	20	5	4	6	5	25	14	3	5	3	60	66
6	4	PPGP SEQ 3A1	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG	20	5	8	2	5	21	3	1	7	10	60	58
7	5	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	21	11	3	4	3	20	3	3	7	7	56	60
8	6	PPGP SEQ 1B9	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT	20	1	4	5	10	20	3	3	7	7	58	60
9	7	PPGP SEQ 2B10	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA	20	7	3	5	5	24	10	0	8	6	56	64
10	8	PPGS SEQ 10D4	ATCCCTGATTAGTGCAACGC	CGTAGGTGGTTTTAGGAGGG	20	5	4	6	5	20	3	10	1	6	60	62
11	9	PPGS SEQ 18C5	GGACAGCCGGATGCTATTTA	ACATGAGTCCCTTTTCCCTT	20	5	6	4	5	20	3	2	7	8	60	58
12	10	PPGS SEQ 15C10	ATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG	20	5	3	7	5	20	3	7	3	7	60	60
13	11	PPGS SEQ 16C6	TTGCTACTAAGCCGAAAATGAAG	CTTGAAATTAACACATATGCACACA	23	9	5	4	5	25	11	2	6	6	64	66
14	12	PPGP SEQ 8D9	TGAGTTTCCCCAAAAGGAGA	CAACAACAATACGGCCAACA	20	7	5	4	4	20	10	2	7	1	58	58
15	13	PPGP SEQ 2D12B	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA	20	8	5	5	2	21	7	6	3	5	60	60
16	14	PPGP SEQ 3D9	TTCACCCGTACAAACCAGTG	CCTCGGCAGATCTGGAGTAA	20	6	3	7	4	20	5	6	5	4	60	62
17	15	PPGS SEQ 16G8	CTCAAAAAGCGCTTAGCCAC	CTGCCTACTGCCTACTGCCT	20	7	3	7	3	20	2	3	9	6	60	64
18	16	PPGS SEQ 17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG	20	3	5	5	7	20	5	10	1	4	60	62
19	17	PPGS SEQ 14H6	GCAACTAGGGTGTATGCCGT	CAACCCTATACACCGAGGGA	20	4	7	4	5	20	7	4	7	2	62	62
20	18	PPGP SEQ 2F5	TGACCAAAGTGATGAAGGGA	AAGTTGTTTGTACATCTGTCATCG	20	8	7	2	3	24	5	5	4	10	58	66
21	19	PPGS SEQ 12F7	TGTCGTTGTAAGACCTCGGA	TTGGTTTCCTTAAGGCTTCG	20	4	6	4	6	20	2	5	4	9	60	58
22	20	PPGP SEQ 16F1	TGCTTCCATCAGCTTTTCCT	AAATGAGGGCCTCCAAAGTT	20	2	2	7	9	20	7	5	4	4	58	58

VIII T_m calculation using Nakano et al (1999)'s formula in MS-Excel (Method2)

Formula(Method2):

$$T_m=100.5+(41*(yG+zC)/(wA+xT+yG+zC))-(820/(wA+xT+yG+zC))+ 16.6*\log_{10}([0.050])$$

where w, x, y and z are the number of the A, T, G, C

Step-1: Open a new Excel sheet and enter titles as mentioned in table-8.1.

Table-8.1: Descriptions of the column headings to be entered in Excel sheet

Title (cell)	Subtitle	Description of the title	Purpose
S.No (A1:A2)		No.of primers	Data Input
PRIMER (B1:B2)		Name of the primer	
FORWARD PRIMER (C1:C2)		Sequence of the Forward Primer	
REVERSE PRIMER (D1:D2)		Sequence of the Reserve Primer	
Melting Temperature (T_m) (E1:G1)	T_m- FP (E2)	Melting Temperature of Forward Primer	Main calculations for Melting Temperature
	T_m-RP (F2)	Melting Temperature of Forward Primer	
	Min(F,R) (G2)	Minimum Tm of FP and RP	

Note: cell addresses where titles and subtitles to be typed are kept in parenthesis.

Step-2: Fill up the data under columns; S.No, PRIMER, FORWARD PRIMER, REVERSE PRIMER as shown in Fig-8.1.

Fig-8.1: Initial MS-Excel Screen after entering the Forward and Reverse primers

	A	B	C	D	E	F	G
1	S.NO.	PRIMER	FORWARD PRIMER	REVERSE PRIMER	Melting Temperature (Tm)		
2					Tm-FP	Tm-RP	Min(F,R)
3	1	PPGS SEQ 3A8	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA			
4	2	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG			
5	3	PPGS SEQ 13A10	AACTCGCTTGTACCGGCTAA	AGGAATAATAACAATACCAACAGCA			
6	4	PPGP SEQ 3A1	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG			
7	5	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG			
8	6	PPGP SEQ 1B9	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT			
9	7	PPGP SEQ 2B10	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA			
10	8	PPGS SEQ 10D4	ATCCCTGATTAGTGCAACGC	CGTAGGTGGTTTTAGGAGGG			
11	9	PPGS SEQ 18C5	GGACAGCCGGATGCTATTTA	ACATGAGTCCCTTTTCCCTT			
12	10	PPGS SEQ 15C10	ATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG			
13	11	PPGS SEQ 16C6	TTGCTACTAAGCCGAAAATGAAG	CTTGAAATTAACACATATGCACACA			
14	12	PPGP SEQ 8D9	TGAGTTTCCCCAAAAGGAGA	CAACAACAATACGGCCAACA			
15	13	PPGP SEQ 2D12B	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA			
16	14	PPGP SEQ 3D9	TTCACCCGTACAAACCAGTG	CCTCGGCAGATCTGGAGTAA			
17	15	PPGS SEQ 16G8	CTCAAAAAGCGCTTAGCCAC	CTGCCTACTGCCTACTGCCT			
18	16	PPGS SEQ 17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG			
19	17	PPGS SEQ 14H6	GCAACTAGGGTGTATGCCGT	CAACCCTATACACCGAGGGA			
20	18	PPGP SEQ 2F5	TGACCAAAGTGATGAAGGGA	AAGTTGTTTGTACATCTGTCATCG			
21	19	PPGS SEQ 12F7	TGTCGTTGTAAGACCTCGGA	TTGGTTTCCTTAAGGCTTCG			
22	20	PPGP SEQ 16F1	TGCTTCCATCAGCTTTTCCT	AAATGAGGGCCTCCAAAGTT			

Step-3: Type the formulae in the corresponding cells as mentioned in table-8.2 and click the *enter* key after each entry of formula. (ensure that formula should start with "=")

Table-8.2: List of formulae and cell addresses to be entered in MS-Excel

Title	Subtitle	Cell	Formulae to be typed	Purpose
Melting Temperature (T_m)	T_m -FP	E3	=IFERROR(100.5+(41*(LEN(C3)*2-LEN(SUBSTITUTE(C3,"G",""))-LEN(SUBSTITUTE(C3,"C",""))))/(LEN(C3))-820/LEN(C3)+16.6*(LOG10(0.05))),"")	Calculates T_m for the FP
	T_m -RP	F3	=IFERROR(100.5+(41*(LEN(D3)*2-LEN(SUBSTITUTE(D3,"G",""))-LEN(SUBSTITUTE(D3,"C",""))))/(LEN(D3))-820/LEN(D3)+16.6*(LOG10(0.05))),"")	Calculates T_m for RP
	Min(F,R)	G3	=IF(MIN(E3:F3)=0," ",MIN(E3:F3))	Calculates minimum T_m of FP and RP

Step-4: After entering the formulae in respective cells as per the table 8.2, select cells E3, F3 and drag down to get T_m for all primers at a time. The resultant screen will be as shown in fig-8.2.

Fig-8.2: MS-Excel screen after calculating T_m for entered primers using Method2

	A	B	C	D	E	F	G
1	S.NO.	PRIMER	FORWARD PRIMER	REVERSE PRIMER	Melting Temperature (Tm)		
2					Tm-FP	Tm-RP	Min(F,R)
3	1	PPGS SEQ 3A8	ATACGTGACTTGGGCCAGAC	AGTGAAAAATACACCCAACGAA	60	57	57
4	2	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	54	58	54
5	3	PPGS SEQ 13A10	AACTCGCTTGTACCGGCTAA	AGGAATAATAACAATACCAACAGCA	58	59	58
6	4	PPGP SEQ 3A1	ATCATTGTGCTGAGGGAAGG	CACCATTTTTCTTTTTCACCG	58	55	55
7	5	PPGS SEQ 13A7	AATCCGACGCAATGATAAAAA	TCCCCTTATTGTTCCAGCAG	54	58	54
8	6	PPGP SEQ 1B9	CGTTCTTTGCCGTTGATTCT	AGCACGCTCGTTCTCTCATT	56	58	56
9	7	PPGP SEQ 2B10	AATGCATGAGCTTCCATCAA	AACCCCATCTTAAAATCTTACCAA	54	58	54
10	8	PPGS SEQ 10D4	ATCCCTGATTAGTGCAACGC	CGTAGGTGGTTTTAGGAGGG	58	60	58
11	9	PPGS SEQ 18C5	GGACAGCCGGATGCTATTTA	ACATGAGTCCCTTTTCCCTT	58	56	56
12	10	PPGS SEQ 15C10	ATTCCCATGTCGTCAAGACC	GCGACGGTATTGGCTTTTAG	58	58	58
13	11	PPGS SEQ 16C6	TTGCTACTAAGCCGAAAATGAAG	CTTGAAATTAACACATATGCACACA	59	59	59
14	12	PPGP SEQ 8D9	TGAGTTTCCCCAAAAGGAGA	CAACAACAATACGGCCAACA	56	56	56
15	13	PPGP SEQ 2D12B	AAGCTGAACGAACTCAAGGC	TGCAATGGGTACAATGCTAGA	58	57	57
16	14	PPGP SEQ 3D9	TTCACCCGTACAAACCAGTG	CCTCGGCAGATCTGGAGTAA	58	60	58
17	15	PPGS SEQ 16G8	CTCAAAAAGCGCTTAGCCAC	CTGCCTACTGCCTACTGCCT	58	63	58
18	16	PPGS SEQ 17F6	CGTCGGATTTATCTGCCAGT	AGTAGGGGCAAGGGTTGATG	58	60	58
19	17	PPGS SEQ 14H6	GCAACTAGGGTGTATGCCGT	CAACCCTATACACCGAGGGA	60	60	60
20	18	PPGP SEQ 2F5	TGACCAAAGTGATGAAGGGA	AAGTTGTTTGTACATCTGTCATCG	56	60	56
21	19	PPGS SEQ 12F7	TGTCGTTGTAAGACCTCGGA	TTGGTTTCCTTAAGGCTTCG	58	56	56
22	20	PPGP SEQ 16F1	TGCTTCCATCAGCTTTTCCT	AAATGAGGGCCTCCAAAGTT	56	56	56

IX Conclusion

Molecular markers changed the face of conventional plant breeding and improved the efficiency and precision by identifying the responsible genes for the traits of interest via marker-assisted selections. Most of the molecular markers are PCR based and the annealing temperature is critical in determining the efficient amplification in PCR which inturn depends on base composition of primers. The primer melting temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature. In principle it is kept 3 to 5 degrees lesser than the lowest T_m of the primers. Two methods of calculating T_m are discussed in this paper to speed up the work of the biotechnologist in genotype selection. Step wise procedure explained in this paper will guide the researchers while generating their own MS-Excel templates for T_m calculations for the large set of primers at a time otherwise users can contact any author to get Excel programme sheet.

References

[1] Breslauer, K.J., F. Ronald, H. Blocker, and L.A. Marky. 1986. Predicting DNA duplex stability from the base sequence. *Proc. Natl. Acad. Sci.* 83: 3746-3750.

[2] Freier, S.M., R. Kierzek, J.A. Jaeger, N. Sugimoto, M.H. Caruthers, T. Neilson, and D.H. Turner.1986. Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl.Acad. Sci.* 83: 9373-9377.

[3] Howley,P.M., Israel,M.F., Law,M-F., and Martin,M.A. (1979) *J BiolChem* 254:4876-4883.

[4] Marmur,J., and Doty,P. (1962). Determination of the base composition of deoxyribonucleic acid from its thermal denaturation temperature, *J Mol Biol* **5**:109-118.

[5] Nakano *et al*, (1999) *Proc. Nuclec Acids Res.* **27**:2957-65.

[6] Pease A.C., Solas D., Sullivan E.J., Cronin M.T., Holmes C.P., Fodor S.P.A. (1994) Light-generated oligonucleotide arrays for rapid DNA sequence analysis. *Proc. Natl. Acad. Sci.* **91**:5022–5026.

[7] Singh Phundan, 2007, Plant Breeding: Molecular and new approaches; Ludhiana,pp. 1- 261

[8] Suggs, S.V., T. Hirose, E.H. Myake, M.J. Kawashima, K.I. Johnson, and R.B. Wallace. 1981.ICN-UCLA symposium for developmental biology using purified gene. (ed. D.D. Brown), Vol.23, pp. 683-693. Academic Press, New York.