Primer1: Primer Design Web Service for Tetra-Primer ARMS-PCR

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Abstract: Tetra-primer ARMS-PCR is used extensively as a low cost, single PCR assay requiring no post-PCR manipulation. The design of successful primers depends on a number of variables such as melting temperatures, GC content, complementarity and selection of mismatch bases. The optimal selection of primers can be achieved in an automated way using a program which evaluates candidate primers for a given sequence. The Primer1 software was developed originally for use in the context of restriction fragment length polymorphism analysis using gel electrophoresis. However, recent applications have been more diverse, reviewed here, and we present an overview of the Primer1 software for primer design and web-service. We have updated the Primer1 program, and provide more complete details of the implementation. We also provide test data and output. The program is now available on a new, efficient, LAMP web service for users at: http://primer1.soton.ac.uk/primer1.html

Keywords: Javascript, mismatch base, primer design, single nucleotide polymorphism, tetra-primer ARMS-PCR, web service.

INTRODUCTION

The Amplification Refractory Mutation System (ARMS) [1] is a rapid and reliable method for analysis of point mutations or small deletions. ARMS was originally developed for allele specific amplification avoiding the use of restriction enzymes and radioisotopes. ARMS-PCR enables the identification of specific genotypes in a single step PCR, without the need for costly and often difficult post-PCR manipulation.

Standard ARMS employs two complementary reactions involving three primers. One primer is specific for the wild-type DNA sequence in both reactions. Allele specificity is conferred by differences in the 3' terminal bases of the other primers corresponding to either the wild type DNA sequence or mutated sequence (one per reaction). Central to the success of this method is the finding that oligonucleotides with a mismatched 3' residue do not function as PCR primers under appropriate conditions. Thus, for the allele specific primers, the normal primer is refractory to PCR on mutant DNA and the mutant specific primer is similarly non-functional on normal DNA. Newton et al. [1] noted that, in some cases, having only the single 3' mismatched base was insufficient to prevent undesired amplification so they deliberately introduced additional mismatched bases near the 3' end.

Ye et al. [2] were the first to describe tetra-primer PCR for which allele-specific amplification is achieved in a single PCR reaction using four primers. This approach introduced a mismatched base in the middle of allele-specific primers. Ye et al. [3] combined tetra-primer PCR with ARMS to form the tetra-primer ARMS-PCR or T-ARMS technique. T-ARMS combines two inner SNP-specific primers and two outer primers in a single reaction and encompasses deliberate mismatches at position -2 from the 3' end of inner primers. Other single-tube four-primer approaches were described by Hamajima et al. [4] and Hamajima [5] as PCR-CTPP (Confronting Two-Pair Primers) and Hersberger et al. [6]. Kawase et al. [7] successfully introduced triplex PCR-CTPP to simultaneously genotype three polymorphisms.

Software for T-arms primer design was developed and outlined in 2001 by Ye et al. [3]. Other tools developed subsequently that undertake T-arms primer design include the BatchPrimer3 program [8]. The Ye et al. program was first described in the context of microplate array diagonal gel electrophoresis (MADGE). The T-ARMS methodology has subsequently, however, been applied much more extensively for a wide diversity of applications. We present here more complete details of the updated Primer1 program. We also provide example input and output data, and detail a revised web service which will enable long term availability.

SOME RECENT APPLICATIONS

The advantages of the single PCR amplification with no post-PCR manipulation have been recognized and underpin numerous recent applications. Poe et al. [9] combined multiplex T-ARMS with microfluidics hardware as an inexpensive and rapid strategy to detect three single nucleotide polymorphisms (SNPs) underlying warfarin sensitivity. Lajin et al. [10] employed T-ARMS for the simultaneous detection of three polymorphisms involved in...
the folate-homocysteine metabolic pathway. This approach was extremely fast, economical and simple, in contrast to alternative methods. Etlik et al. [11] established that there are substantial advantages in terms of time, cost and reliability for “one-tube” evaluation of wild type and mutant alleles with a consensus gene-specific internal control in a clinical laboratory setting. Piccioli et al. [12, 13] developed hexaprimmer T-ARMS, for two closely located polymorphisms, and capable of providing direct information about haplotype structure. Lajim et al. [14] have recently described a powerful quadruplex T-arms application to simultaneously detect four polymorphisms in the apoptotic pathway using 13 primers.

**PRIMER1 IMPLEMENTATION AND USAGE**

The Primer1 program is implemented in Java and uses Java threads and locks to coordinate different processes. User input is processed in Javascript ahead of submission to the program Common Gateway Interface (CGI). The Primer1 program operates through web interface input of a target DNA sequence. The required sequence size is of up to 1000 bases which is presented 5′ to 3′. Whitespace and all numerical digits within the sequence are ignored. The sequence location of the polymorphic site is input along with the bases for the reference and the alternative alleles. Also input are some criteria for primer design: the desired minimum, maximum and average melting temperatures for a primer oligo (Tm); the millimolar concentration of salt (KCl) and the nanomolar concentration of annealing oligos in the PCR (this is used in the calculation of Tm); the %GC content (the minimum and maximum allowable for any primer); the primer lengths and the minimum and maximum complementarity and product sizes. The algorithm used for calculating complementarity is similar to Rozen and Skaltsky [15]. Briefly, for the tests of self-complementarity, a primer sequence in the 5′ to 3′ orientation is compared with the same sequence 3′ to 5′. For the tests of complementarity between two different primers, one of the primer sequences 5′ to 3′ is compared to the other sequence 3′ to 5′. The maximum complementarity (default 8.0) is a limit to reduce the possibility of a single primer self-annealing and the possibility of annealing between left and right primers. A total primer-primer annealing score of zero reflects no local alignment between two oligonucleotides. Annealing scores are summed across bases which are scored as: 1.0 for complementary bases; -0.25 for a match with an ‘N’; -1.0 for a mismatch; -2.0 for a single base gap. The maximum 3′ complementarity (for which the default is 3.0) follows the same scoring system and measures the maximum allowed 3′-anchored global alignment score.

The program follows the Little [16] rules for selecting the additional mismatch base. Under this scheme ‘strong’ mismatches at the 3′ terminus (G/A or C/T mismatches) are optimally paired with ‘weak’ second mismatches (C/A or G/T) and vice versa with ‘medium’ strength combinations (A/A, C/C, G/G or T/T) as an alternative. Tm is calculated using the nearest neighbor parameters [17] and the formula given by Rychlik et al. [18].

Subject to the user input settings, the program identifies primer sets in sequence: see Fig. (6) of Ye et al. [3]. Firstly, all of the possible inner forward and inner reverse primers are identified. For this set an ‘optimal’ inner primer pair is identified which has the closest match to the input Tm and also the minimum Tm difference between the two primers. Outer reverse and outer forward primers are then selected, matching the mean Tm of the two inner primers.

**WEB SERVICE**

Ye et al. [3] outline a T-ARMS primer design program which we have now updated (April 2012) to enable continuing availability and functionality. The updated program has been relocated to an efficient web server. As part of the update we have improved the error handling in the original software and updated the html scripts for use on the current web server. We also now provide a test input sequence and program output in the Appendix. This input sequence can, with Primer1 default settings, be used to design primers for the IL6 -174 G/C promoter polymorphism. The primer1 program is available on the LAMP web server at: http://primer1.soton.ac.uk/primer1.html.

**CONFLICT OF INTEREST**

The authors confirm that this article content has no conflicts of interest.

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Declared none.

**APPENDICES**

**Test Sequence**

The following DNA sequence (with genebank ID: AF048692) is from the interleukin 6 (IL6) promoter region and can be used ith the primer1 default settings to design primers for the C-174G polymorphism (position 477 in this sequence).

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1 ggagtcacag actccacagt gagacgctt gaagtaacgt cagaaatttt gaggagttc
61 aggagcctca caacagcggc tcacagggag agccagaaca cagaagacttt ttagcttatt
121 gtagttttatt ctcttttatt ctcttttatt ctcttt tctcttttatt ctcttt tctcttttatt
181 agtttgctct atctttttt ttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt
241 gttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt
301 tttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt
361 aagtcttttt tcacagtttt ctaaattaat gtaaacttta caagacttta acatcttatt aacaagatcctt ttagctttatt
421 tegaagtta ctagatttatct tcttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt
481 cttaagagag ctagtttttt tcttttttttttt tttttttttttt tttttttttttt tttttttttttt tttttttttttt
541 cccctccccccc accttcctctttaaagttttt aaagttttttttt tttttttttttt tttttttttttt tttttttttttt
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601 attagagtct caacccccaa taaatatagg actggagatg tctgaggctc attgagccct
661 cagccacccg ggaagaaag agaagctcta ttcctcttc cagagccacg ctatgaactc
721 ctcttctaca agtaagtgca ggaatctctt agecccttgaac ctcagacgcccc atctcctccc
781 ttacgaggg ggggtgtggt gcccagggat gcggggcgcc ggcagcagag gcaggctccc
841 atctggtgtg tcagtcac

WITH DEFAULT SETTINGS THE FOLLOWING PRIMER SETS ARE OUTPUT:

**********************OUTPUT 1**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
350 CAAGACATGCCAAAGTGCTGAGTCACTA 377 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 155
Product size of two outer primers: 306

**********************OUTPUT 2**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
320 TTCGTGCATGACTTCAGCTTTCTCTTTG 348 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 155
Product size of two outer primers: 306

**********************OUTPUT 3**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
333 TCAGCTTTACTCTTTGTAAGACATGCCA 361 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 172
Product size of two outer primers: 323

**********************OUTPUT 5**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGAGTGTCCTTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
334 CAGCTTTACTCTTTGTAAGACATGCCA 361 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 155
Product size of two outer primers: 323

**********************OUTPUT 5**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGAGTGTCCTTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
339 TTACTCTTTGTAAGACATGCCA 364 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 166
Product size of two outer primers: 317

**********************OUTPUT 6**********************

Forward inner primer (C allele): Melting temperature
451 CACTTTTCCCCCCTAGTTGTCTTCCCC 477 68
Reverse inner primer (G allele):
504 ATTGTGCAATGTGAGTGTCCTTTTAGCTTC 477 68
Forward outer primer (5' - 3'):
346 TTGTCAAGAGGTCCTCTTATGATGC 372 68
Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCACAGACATCTCCAGTCTC 628 68
Product size for C allele: 206
Product size for G allele: 159
Product size of two outer primers: 310
**OUTPUT 7**

Forward inner primer (C allele): Melting temperature
451 CACTTTCCCCCTTAGTGTGCTTCCC 477 68

Reverse inner primer (G allele):
504 ATTGTGCAATGTGACGTCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):
350 CAAGACATGCCAAAGTGCTGAGTCACT 376 68

Reverse outer primer (5' - 3'):
655 AGAATGAGCCTCAGACATCTCCAGTCCT 628 68

Product size for C allele: 206
Product size for G allele: 155

Product size of two outer primers: 306

**REFERENCES**


