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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

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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 hexaprimer T-ARMS, for two closely located polymorphisms, and capable of providing direct information about haplotype structure. Lajin *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 complementary is similar to Rozen and Skaletsky [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, 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/primer-1.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).

1 ggagtcacac actccacctg gagacgcctt gaagtaactg cacgaaattt gaggatggcc

61 aggcagtcta caacagccgc tcacagggag agccagaaca cagaagaact cagatgactg

121 gtagtattac cttcttcata atccaggctt ggggggctgc gatggagtca gaggaaactc

181 agttcagaac atctttggtt tttacaaata caaattaact ggaacgctaa attctagcct

241 gttaatctgg tcactgaaaa aaaaattttt ttttttcaaa aaacatagct ttagcttatt

301 ttttttctct ttgtaaaact tcgtgcatga cttcagcttt actctttgtc aagacatgcc

361 aaagtgctga gtcactaata aaagaaaaaa agaaagtaaa ggaagagtgg ttctgcttct

421 tagcgctagc ctcaatgacg acctaagctg cacttttccc cctagttgtg tcttgcgatg

481 ctaaaggacg tcacattgca caatcttaat aaggtttcca atcagcccca cccgctctgg

541 ccccaccctc accctccaac aaagatttat caaatgtggg attttcccat gagtctcaat

601 attagagtct caacccccaa taaatatagg actggagatg tctgaggctc attctgccct

661 cgagccaccg ggaacgaaag agaagctcta tctcccctcc aggagcccag ctatgaactc

721 cttctccaca agtaagtgca ggaaatcctt agccctggaa ctgccagccg gtcgagccct

781 gtgtgaggga ggggtgtgtg gcccagggat gcggggcgcc agcagcagag gcaggctccc

841 agctgtgctg tcagtcac

WITH DEFAULT SETTINGS THE FOLLOWING PRIMER SETS ARE OUTPUT:

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

Forward inner primer (C allele): Melting temperature

451 CACTTTTCCCCCTAGTTGTGTCTTCCC 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 AGAATGAGCCTCAGACATCTCCAGTCCT 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 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):

320 TTCGTGCATGACTTCAGCTTTACTCTTTG 348 68

Reverse outer primer (5' - 3'):

655 AGAATGAGCCTCAGACATCTCCAGTCCT 628 68

Product size for C allele: 206

Product size for G allele: 185

Product size of two outer primers: 336

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

Forward inner primer (C allele): Melting temperature

451 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):

333 TCAGCTTTACTCTTTGTCAAGACATGCCA 361 68

Reverse outer primer (5' - 3'):

655 AGAATGAGCCTCAGACATCTCCAGTCCT 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 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):

334 CAGCTTTACTCTTTGTCAAGACATGCCAA 362 68

Reverse outer primer (5' - 3'):

655 AGAATGAGCCTCAGACATCTCCAGTCCT 628 68

Product size for C allele: 206

Product size for G allele: 171

Product size of two outer primers: 322

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

Forward inner primer (C allele): Melting temperature

451 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):

339 TTACTCTTTGTCAAGACATGCCAAAGTGC 367 68

Reverse outer primer (5' - 3'):

655 AGAATGAGCCTCAGACATCTCCAGTCCT 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 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 477 68

Forward outer primer (5' - 3'):

346 TTGTCAAGACATGCCAAAGTGCTGAGT 372 68

Reverse outer primer (5' - 3'):

655 AGAATGAGCCTCAGACATCTCCAGTCCT 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 CACTTTTCCCCCTAGTTGTGTCTTCCC 477 68

Reverse inner primer (G allele):

504 ATTGTGCAATGTGACGTCCTTTAGCTTC 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

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