Primer Design for PCR and Sequencing in High-Throughput Analysis of SNPs

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ABSTRACT

To achieve high-throughput analysis of allele frequencies in human SNPs, we have developed automated methods for designing PCR and DNA sequencing primers. We found we could run the PCR assays at quite stringent, uniform conditions. The design process used freely available databases, including dbSNP, SNPper, and TSC, and publicly available software including RepeatMasker and Primer3. We describe parameters for the software and other considerations that increase experimental success. As anticipated, some assays failed at the design stage due primarily to the genomic locations of repetitive sequences, extreme GC content regions, or lack of sufficient sequence. However, over 23 000 assays, including 96% of those recently analyzed, have been experimentally successful. Similar design methods could be used for PCR assays in any organism with substantial available sequence.

INTRODUCTION

The detection of large numbers of candidate single nucleotide polymorphisms (SNPs) has been an important aspect of human genome investigation. The majority of these SNPs were found by comparing sequences of overlapping genomic clones or by comparison of new sequences to the reference draft sequence (4,10). For brevity, we refer to candidate SNPs simply as SNPs, but it should be noted that for any one candidate, further investigation is required to determine whether or not it is polymorphic in one or more samples. While approximately 66% of SNPs (mainly autosomal) are common (rare allele ≥ 0.2) in at least one sample, about 12% have low levels of variation and 22% have no detectable variation (Reference 4 and unpublished results). While most SNPs are expected to have little or no phenotypic effects, often a particular SNP will be in linkage disequilibrium with many other SNPs (e.g., Reference 9). Some SNPs undoubtedly will be important for health, including susceptibility to various diseases and metabolism of drugs.

To aid in the development of SNPs as research tools, our group has been determining the allele frequencies of a large number of SNPs in three different population samples. To analyze the SNPs, we have developed and performed PCR on each SNP using DNA from a reference individual and the samples of pooled DNA (42 individuals, each from African Americans, Asian Americans, or European Americans), sequenced the PCR products, and estimated the allele frequencies in the samples by determining the contributions of each allele to the electropherogram (3). It was essential that (i) the assays were conducted us-

ing consistent conditions to enable high-throughput analysis, (ii) the assays were conducted under very stringent conditions to minimize spurious products which would obscure DNA sequencing, and (iii) most of the designed assays were experimentally successful. Using publicly available databases and software, we assembled a design process that met these criteria. In our process, one of the PCR primers was also used for cycle sequencing of the PCR product, using fluorescent dye terminators, so only two primers were required; these were relatively inexpensive since they were unlabeled. With slight modifications, the method could be applied to many organisms. In this paper we describe our assay design and give examples of the results.

MATERIALS AND METHODS

The SNPs analyzed in this project were obtained through a variety of means including choosing sets based on spacing along a genome assembly and receiving sets submitted by investigators interested in particular genomic regions. Each analyzed SNP was initially communicated to the project as a unique identifier, an rs number (non-redundant identifier from public database dbSNP: <http://www.ncbi.nlm.nih.gov/SNP/>), (8), an ss number (accession number from dbSNP), or a TSC identifier from The SNP Consortium (TSC: <http://snp.cshl.org/>). The SNP then progressed through a series of steps or pipeline encompassing assay design, experimental analysis, and reporting of results (Table 1).

Obtaining the Sequence

We obtained the sequence surrounding a SNP site from one of three sources. The first source was dbSNP. We submitted lists of rs or ss numbers to this database in batch format through their Web interface, and the surrounding sequence of the longest acquisition was returned. SNP mapping information, updated with each genome assembly build, was downloaded from the ftp site. A second source used was SNPper, <http://bio.chip.org:8080/bio/> (5), where submission of a batch of rs numbers allowed return of up to 1000 bp on either side of the SNP. SNPper determined flanking sequence from a genome assembly, allowing only uniquely mapped candidates to be returned. A third source used was The SNP Consortium, whose personnel kindly provided surrounding sequence for TSC accessions, which have been uniquely mapped. Each of the three resources returned sequence in FASTA format with some variation

Table 1. SNP Characterization Pipeline

Step	Operation
1	Determine SNP Candidates
2	Design Primers
a	Obtain sequence
b	Mark repetitive sequence
c	Choose primers using set parameters
d	Final processing
3	Acquire data
a	Obtain primers
b	PCR
c	Sequence
d	Capillary electrophoresis
4	Analyze data and troubleshoot if necessary
5	Report results

in syntax. For subsequent steps the returned sequences were converted to a consistent format for identification of the SNP within the sequence and a consistent header format designating the rs number and SNP position.

Marking Repetitive Sequences

For specificity of PCR products we attempted to avoid picking primers within repeated regions, including identified high or low copy number repeats. The returned sequences containing the SNP sites were saved for later steps, and then analyzed with the software RepeatMasker, <http://ftp.genome.washington.edu/RM/>. RepeatMasker produced a “masked” version of the sequence, with the bases for identified repeats converted to “N” in FASTA format. The masked sequences and input parameters were then converted to BoulderIO format for submission to the program for choosing primers.

Choosing Primers Using Set Parameters

To choose the primers, we used the software Primer3, release 0.9, Unix version (Steve Rozen, Helen J. Skaletsky, copyright 1996, 1997, 1998; code available at http://www-genome.wi.mit.edu/genome_software/). An online version is also available through the World Wide Web (6). The input parameters we used for Primer3 are shown (Table 2). Since many SNPs were available for analysis, our strategy was to use stringent design criteria to maximize experimental success, expecting a higher design failure rate than may be necessary for some experiments.

In using Primer3 for PCR design, Beasley et al. found that there was a higher experimental failure rate with primers 20 bp or less (or related, if the primer GC content was greater than 50%), and they accordingly adjusted primer size and GC content parameters (1). They used an optimal primer T_m of 62°C, whereas we previously had good success with an optimal T_m of 55°C, and we continued to use the latter value. The first parameter, “TARGET”, was set with a length of 50 bp, beginning 25 bp upstream of the SNP site, thus effectively excluding this region as a source of primers and preventing overlap of the primers with the SNP. Parameters 2–4 set the primer size and were taken from

Table 2. Parameter Values for Primer3

Parameter	Value
1 TARGET	x,y
2 PRIMER_OPT_SIZE	23
3 PRIMER_MAX_SIZE	26
4 PRIMER_MIN_SIZE	20
5 PRIMER_OPT_TM	55
6 PRIMER_MAX_TM	56
7 PRIMER_MIN_TM	54
8 PRIMER_PRODUCT_SIZE_RANGE	150–400
9 PRIMER_PRODUCT_OPT_SIZE	300
10 PRIMER_PAIR_WT_PRODUCT_SIZE_LT	0.20
11 PRIMER_PAIR_WT_PRODUCT_SIZE_GT	0.20
12 PRIMER_MIN_GC	20
13 PRIMER_MAX_GC	50
14 PRIMER_SALT_CONC	50
15 PRIMER_SELF_ANY	8
16 PRIMER_SELF_END	3
17 PRIMER_GC_CLAMP	0
18 PRIMER_MAX_END_STABILITY	8
19 PRIMER_DNA_CONC	40
20 PRIMER_NUM_RETURN	1

x, starting position of target (bp); y, length of target = 50 bp
Units: size (bp), T_m (°C), weight (fraction), GC (%), concentration (mM), return (primer pairs).

Beasley et al., except we found that decreasing the minimal size to 20 bp with our reduced T_m provided an increased rate of success for primer design with no apparent increase of experimental failure. Parameters 5–7 set the T_m to 55±1°C. Parameters 8–11 set the product size range to 150–400 bp. The products were long enough to usually permit an optimal spacing interval of ≥100 bp between the first base of the sequencing primer and the SNP. Compared with longer PCR products, this range and flexibility enabled many assays to be successful, while still permitting a decreased sequencing time and minimizing the occurrence of unknown insertion-deletion events between the sequencing primer and the SNP. Parameters 12–18 were from Beasley et al. For parameter 19, the effective primer concentration, we used a value near the default value. Parameter 20 caused only one pair of primers to be reported, thus simplifying later steps.

Final Processing

After a primer pair was chosen, we did additional analysis to choose the sequencing primer, screening out some situations prone to failures. Long runs of T's, A's, GT's, and CA's decreased the fluorescent intensity of longer products and were likely to be polymorphic for insertion/deletion events. If the primer used for sequencing was less than 100 bp from the candidate site, spurious peaks in the electropherograms could obscure the peaks used for allele frequency estimation. Accordingly, we used a simple algorithm, executed using a Perl script, to check conditions in sequences that were not repeat-masked. The left primer was exam-

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ined to see whether it passed or failed (beginning of the primer sequence less than 100 bp from the candidate or stretches of 8 or more T's or A's, or 10 or more GTs or CA's). The right primer was similarly examined. If both primers failed, the assay was designated as failed. If one primer passed and one failed, the former was designated the sequencing primer. If both passed, the one closest to the candidate site was designated the sequencing primer.

PCR and Sequencing Conditions

To make the assays stringent, we avoided choosing primers within repetitive sequence; we used a hot start DNA polymerase; we used short extension times; and we performed the PCR annealing steps 3°C above the design T_m of 55±1°C. For a particular PCR cycle, the actual T_m for primer and template was expected to be a function of the concentration of both components, with the concentration changing throughout the reactions. PCR amplification using higher annealing temperatures than the nominal T_m have been reported (e.g., 7).

Human genomic DNA (4 ng) from Coriell Institute, Camden, NJ, USA, was amplified in 10-μL reaction mixtures, each containing 0.15 U JumpStart™ Taq DNA polymerase (Sigma-Aldrich, St. Louis, MO, USA), 50 mM KCl, 10 mM Tris-HCl, pH 8.3, 3.5 mM MgCl₂, 100 nM each of the four dNTPs, 1.0 μM primer-1 and 0.1 μM primer-2. We have found addition of extra primer-1 (used for sequencing) at this stage removed the need for later addition of a primer for cycle sequencing (manuscript in preparation). The thermocycling protocol comprised an initial step at 95°C for 2 minutes to activate the polymerase, then 35 cycles of denaturation at 92°C for 10 seconds, annealing at 58°C for 20 seconds, and extension at 68°C for 30 seconds, followed by a final extension at 68°C for 10 minutes.

Cycle DNA sequencing was conducted according to the protocol of the dye manufacturer (Applied Biosystems). Briefly, 2.5 μL of the PCR mixture was added to 6.5 μL of water, 2.0 μL of BigDye version 3 mix, and 1.0 μL of 5× sequencing buffer. The thermocycling protocol comprised an initial step at 96°C for 2 minutes, then 25 cycles of denaturation at 96°C for 15 seconds, annealing at 50°C for 1 second, and extension at 68°C for 4 minutes. Extra dyes were removed from the sequencing reactions according to the manufacturer's protocols, using columns in 96- or 384-well plate format (Princeton Separation, Philadelphia, PA or Genetix, St. James, NY, USA). The samples were electrophoresed on a 3700 DNA Sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

Our SNP characterization pipeline has produced very satisfactory experimental results (e.g., Figure 1). Allele frequencies found using this pipeline have been deposited in the dbSNP and TSC databases. Bulk downloads of our characterization of over 23 000 SNPs are available at <http://snp.cshl.org/pub/SNP/frequency> and <http://snp.wustl.edu>.

To illustrate results from the assay design, two processed groups of SNPs are shown, one utilizing sequences from dbSNP (group A) and the other from SNPper returning 600 bp flanking sequence (group B), which on average provided more flanking sequence than dbSNP (Table 3). As mentioned, our strategy

was to have very consistent, successful assay conditions, even at the expense of failures at the design stage. Group A had a 43% success rate of assay design and group B a 69% success rate, reflecting the advantage of having additional flanking sequence. Analysis with Primer3 tested many primers for each SNP, first the left primers, then the right. If one or more left and right primers were found, the program checked whether any produce the correctly sized products. The largest reason for failure to pick a left or right primer was due to extreme local genomic GC content, followed by long stretches of N's in the sequence (from masking repeats). For example, for all the sequences of group A, 913 006 potential primers were examined. Some 41% of failures were due to GC content, 29% exceeded the maximum T_m , 15% did not meet the minimum T_m , 14% had poly-N's, and about 1% failed for other causes. For group B, 796 191 primers were examined. Some 38% of failures were due to GC content, 34% exceeded the maximum T_m , 17% did not meet the minimum T_m , 9% had poly-N's, and about 2% failed for other causes. While these distributions are similar, clearly there are differences in the samples in GC and repeat content. When the right and left primers were matched into pairs, similar percentages of SNPs failed in assay design for groups A or B because there was no possible product between 150–400 bp, 13% and 15%, respectively. Some additional failures occurred at the post-processing step, 6% and 3% for groups A and B, respectively; the major reason for failure was that neither primer was ≥100 bp from the SNP. Although local genomic context affected assay design results, the additional flanking sequence available for group B clearly increased the percentage of SNPs for which assays could be designed, primarily because Primer3 had a better chance of successfully picking a left and right PCR primer.

In spite of local variations in GC and repeat content in human DNA, the results suggest that the success rate of assay design for our criteria could be increased by obtaining 375 bp of

Figure 1. Electropherogram of human SNP rs1894466. The SNP is located on the X chromosome, and results, which use the assay design described, are from one polymorphism discovery panel DNA. Primers used were p1: 5'-CTTCT-TATTACTGAACTAATGCTGT-3' and p2: 5'-CGGCCAAAAGTACTA-CTAT-3'. The individual was a heterozygote for the predicted [A/C] candidate site. Results of the assay using pooled DNA samples demonstrated the candidate is polymorphic (frequency of C in African Americans, Asians Americans, and European Americans is respectively 0, 0.60, and 0.30; data not shown).

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Table 3. Choosing Primers

Result	Group (%)	
	A	B
Success	43.4	68.9
Failures		
Left and/or right primer	37.3	13.1
Product size	13.3	15.0
Post process	6.1	2.9
Total	100	100
Number attempted	445	206

Table 4. Assay Success

Result	(%)
Success	
Variation	64
No variation	30
Some data missing	2
Total	96
Failure	
No PCR	3
Repeat sequence	1
Total	4
Number attempted	672
Note: X chromosome SNPs	

flanking sequence on either side of the SNP. The 375 bp plus 25 bp from half of the excluded target permits a 400 bp maximum allowed PCR product, reducing failures compared with shorter sequences. A slight improvement in success rate for the post-processing stage could be obtained by increasing the number of primer pairs returned by Primer3, thus increasing the chance for a successful assay. Not all failed assays could be helped by additional flanking sequence and more returned primer pairs, because in some locations the repeats or local GC content preclude picking primers, unless the design criteria are altered. However, many assays can be designed using available sequence and standard criteria. For humans, we have designed and made publicly available 770 042 PCR assays at <http://snp.wustl.edu>.

To estimate the experimental success rate of this design process, we consider 672 assays, all for SNPs located on the X chromosome (Table 4). These were analyzed in a 384-well plate format using liquid handling equipment, which in our experience increased the experimental success rate. In spite of our very stringent PCR conditions, 96% of these assays were successful. Some 3% were failures due to lack of a PCR product, and 1% failed due to apparent amplification of undocumented repeat regions. Incidentally, as we have previously observed, the percentage of SNPs with no observed variation (30%) was increased in this X chromosome group as compared with groups of SNPs primarily from autosomes (e.g., 22%; unpublished results). We have also successfully used similar PCR assay designs for genotyping by single base extension assays using fluorescent polarization (Reference 2 and unpublished results). For any organism having substantial available draft-or-better sequence, adapta-

tions of this method of PCR design and sequencing should allow experimental access to population genomic information.

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REFERENCES

1. Beasley, E.M., R.M. Myers, D.R. Cox, and L.C. Lazzaroni. 1999. Statistical refinement of primer design parameters, p. 55-71. In M.A. Innis, D.M. Gelfand, and J.J. Sninsky (Eds.), *PCR Applications*. Academic Press, San Diego.
2. Hsu, T.M., X. Chen, S. Duan, R.D. Miller, and P.Y. Kwok. 2001. Universal SNP genotyping assay with fluorescence polarization detection. *BioTechniques* 31:560-568.
3. Kwok, P.Y., C. Carlson, T.D. Yager, W. Ankener, and D.A. Nickerson. 1994. Comparative analysis of human DNA variations by fluorescence-based sequencing of PCR products. *Genomics* 23:138-144.
4. Marth, G., R. Yeh, M. Minton, R. Donaldson, Q. Li, S. Duan, R. Davenport, R.D. Miller, and P.Y. Kwok. 2001. Single-nucleotide polymorphisms in the public domain: how useful are they? *Nat. Genet.* 27:371-372.
5. Riva, A.A. and I.S. Kohane. 2001. A web-based tool to retrieve human genome polymorphisms from public databases. *Proc. AMIA Symp.* 2001:558-562.
6. Rozen, S. and H. Skaletsky. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol. Biol.* 132:365-386.
7. Rychlik, W., W.J. Spencer, and R.E. Rhoads. 1990. Optimization of the annealing temperature for DNA amplification in vitro. *Nucleic Acids Res.* 18:6409-6412.
8. Smigielski, E.M., K. Sirotkin, M. Ward, and S.T. Sherry. 2000. dbSNP: a database of single nucleotide polymorphisms. *Nucleic Acids Res.* 28:352-355.
9. Taillon-Miller, P., I. Bauer-Sardina, N.L. Saccone, J. Putzel, T. Laitinen, A. Cao, J. Kere, G. Pilia, et al. 2000. Juxtaposed regions of extensive and minimal linkage disequilibrium in human Xq25 and Xq28. *Nat. Genet.* 25:324-328.
10. The International SNP Map Working Group. 2001. A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms. *Nature* 409:928-933.

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