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MRPrimerW2: an enhanced tool for rapid design of valid high-quality primers with multiple search modes for qPCR experiments

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ABSTRACT

For the best results in quantitative polymerase chain reaction (qPCR) experiments, it is essential to design high-quality primers considering a multitude of constraints and the purpose of experiments. The constraints include many filtering constraints, homology test on a huge number of off-target sequences, the same constraints for batch design of primers, exon spanning, and avoiding single nucleotide polymorphism (SNP) sites. The target sequences are either in database or given as FASTA sequences, and the experiment is for amplifying either each target sequence with each corresponding primer pairs designed under the same constraints or all target sequences with a single pair of primers. Many websites have been proposed, but none of them including our previous MRPrimerW fulfilled all the above features. Here, we describe the MRPrimerW2, the update version of MRPrimerW, which fulfils all the features by maintaining the advantages of MRPrimerW in terms of the kinds and sizes of databases for valid primers and the number of search modes. To achieve it, we exploited GPU computation and a disk-based key-value store using PCIe SSD. The complete set of 3 509 244 680 valid primers of MRPrimerW2 covers 99% of nine important organisms in an exhaustive manner. Free access: http://MRPrimerW2.com

INTRODUCTION

Quantitative polymerase chain reaction (qPCR), also known as real-time PCR, is a widely adopted technique for detecting the mass amplification of specific DNA molecule in real-time. This standard technique is used in a wide range of applications including genetically modified organism (GMO) detection (1), pathogen discovery (2), and validation of changes in expression of interested genes (3). For best results in PCR experiments, optimal primer design is essential. However, it is challenging due to the need to consider both homology test on a huge number of off-target sequences and many filtering constraints. Although many websites are proposed to aid in designing primers, most of them still require an additional step of homology test using BLAST-like tools for the candidate primers satisfying filtering constraints. Moreover, batch design of primers for multi-target qPCR is more complex due to the difficulty of finding a full set of primer pairs that satisfy the same stringent constraints.

There are other four important design features to consider: spanning exon–exon junction, avoiding single nucleotide polymorphism (SNP) sites, supporting input FASTA sequence, and supporting multi-target primer. First, it is useful to design primers across exon–exon junctions such that the designed forward and reverse primers hybridize to different exons which separated by a large intron or intron–exon region to avoid PCR amplification of gDNA (i.e. genomic DNA contamination) as well as to distinguish and quantify splice variants. Second, it is important for the designed primers not to overlie SNP sites because the existence of SNPs within the primers may influence the reaction of PCR experiments (i.e. primer Tm, efficiency of polymerase extension, and target specificity) (4). Third, support-

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ing input FASTA sequence becomes more and more important as the next-generation sequencing (NGS) technology is widely used due to its high accuracy, fast speed and low cost. It can be useful to identify functions and to annotate of new genome sequences obtained by NGS. For example, when a fatal viral infection (e.g. Zika virus) is emerging, primer design for viral sequences with rigorous similarity testing against host sequences in a database can aid in detection and identification of those viruses (5). Fourth, supporting multi-target primer, i.e., designing a pair of primers covering specific multiple target sequences can be useful, in particular, when amplifying multigene family (e.g. olfactory receptors) or many variant genes (e.g. TP53) all at once (23). Multi-target design is different from batch design. The former searches for a pair of primers which amplifies all target sequences, whereas the latter for a set of pairs of primers each of which amplifies each target sequence under the same filtering constraints.

Many websites have been developed to aid in designing primers for qPCR, including Primer3Plus (6,7), BatchPrimer3 (8), Primique (9), QuantPrime (10), Primer-BLAST (11), Oli2go (12) and MRPrimerW (13). There are also many databases containing pre-computed primers, including PrimerBank (14,15) and qPrimerDB (16). Table 1 summarizes a comparison among those websites and databases. Primer3Plus, BatchPrimer3, Primique and QuantPrime focus on supporting user-specific filtering constraints and ranking primer pairs by their penalty scores. BatchPirmer3 and QuantPrime supports batch design of primers with avoiding SNP sites. Primique and QuantPrime perform homology test using a local alignment algorithm in a limited scope, while Primer-BLAST performs homology test using a global alignment algorithm against the entire sequences in a database. Primer-BLAST, however, supports neither batch designing nor multi-target designing. Oli2go supports design of multiplex primers and probes for multiple FASTA sequences. It, however, does not support some important features such as exon spanning, avoiding SNP sites, and ranking. Both PrimerBank and qPrimerDB focus on checking primer specificity. The primers in PrimerBank have been experimentally validated under a uniform condition. The primers in qPrimerDB for 147 livestock and plants have been checked using a thermodynamic-based program, MFPrimer-2.0 (17). Both, however, do not allow users to adjust the filtering constraints and not support the features such as scoring (ranking), TaqMan probes and avoiding SNPs.

MRPrimerW (13) is our previously proposed website which allows users to quickly search for the best valid primer pairs satisfying the same constraints for many target sequences. It extracted a complete set of 341 963 135 valid primers which can amplify only a specific sequence from the human and mouse CCDS databases (https://www.ncbi.nlm.nih.gov/CCDS/) in an exhaustive manner using the large-scale MapReduce program called MRPrimer (18), converted them to a set of indices with annotations, and loaded into the main memory of the website. Then, it performs online processing when users input a set of target sequence IDs and a multitude of filtering constraints as a query. However, MRPrimerW has the major drawbacks that it does not consider sequence variants and SNP sites, does not support other popular model organisms, and does not support designing valid primers for input FASTA sequences against database sequences. Such drawbacks are mainly due to a prohibitive amount of computation and memory space required for supporting the features.

Here, we describe a new website called MRPrimerW2 which solves all the drawbacks of MRPrimerW, but still maintains its advantages such as one-stop searching and high primer specificity. In this update, we changed the template sequence database from CCDS to NCBI Reference Sequence (RefSeq) database (http://www.ncbi.nlm.nih.gov/refseq/) to solve some drawbacks of MRPrimerW, in particular, to support exon spanning and avoiding SNP sites and to provide high-quality primers for other important organisms including human, mouse, rat, zebrafish, cow, pig, thale cress, fruit fly and *Caenorhabditis elegans*. The MapReduce-based offline processing part of the predecessor, MRPrimer (18), may require several tens of days for extracting valid primers from RefSeq databases in an exhaustive manner, since the size of RefSeq database is much larger than that of CCDS. To overcome the computational limitation, we significantly improved the performance of the offline processing part by exploiting GPU computation, in particular, by performing the most time-consuming step, i.e., homology test, using GPUs. We extracted a complete set of 3 509 244 680 valid primers from RefSeq databases within a day. The set of primers include not only single-target primers, but also multi-target ones. Since the size of the entire valid primers was too large to fit in main memory of the web site, we stored them in a disk-based key-value store, LevelDB (http://leveldb.org/) using high-speed PCIe SSD storage. MRPrimerW2 also supports primer design for input FASTA sequences against database sequences. To support fast ad-hoc homology test, we extracted all possible subsequences of 17–27 bp from RefSeq databases (the total number of subsequences is 13 657 773 610) and stored them in LevelDB. Thus, MRPrimerW2 uses a total of 17 167 018 290 primers or subsequences stored in LevelDB for online processing of primer design. As a result, MRPrimerW2 can design the best valid primer pairs for both database sequences and input FASTA sequences with the new features such as exon spanning, avoiding SNP sites and multi-target primer design.

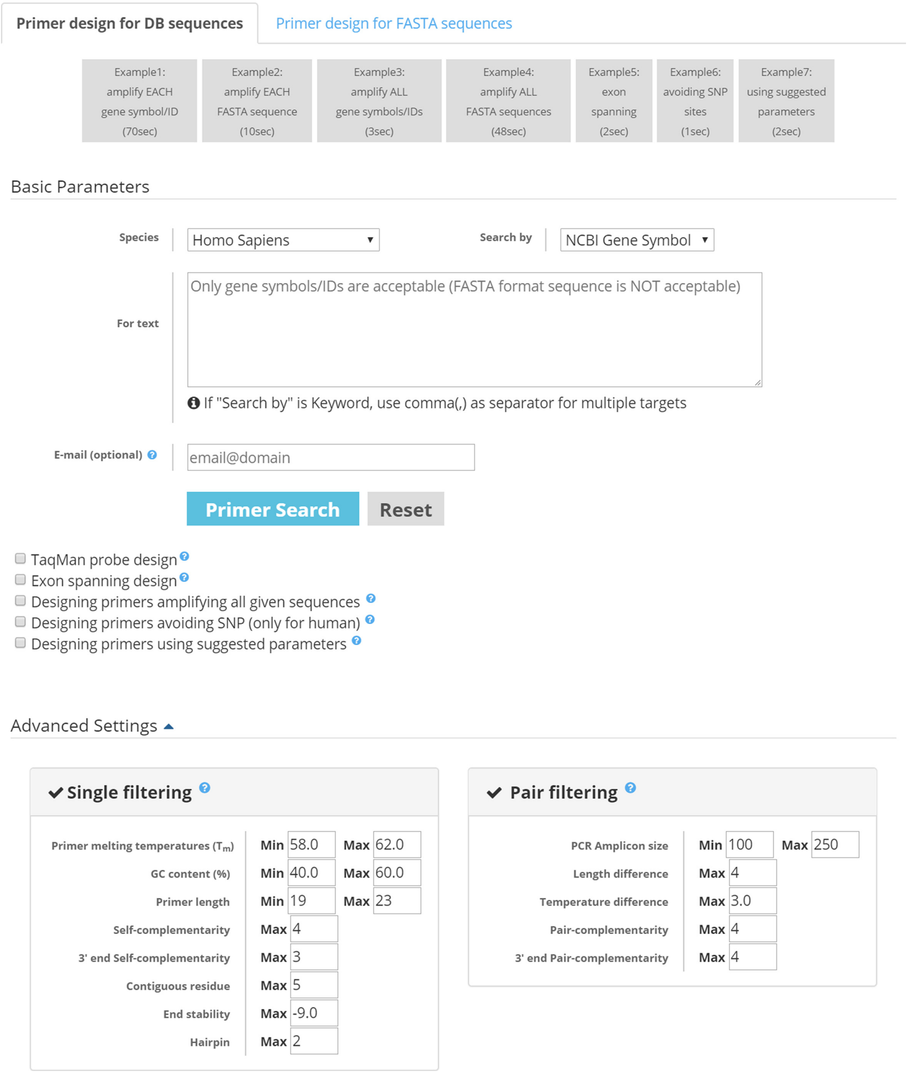
MATERIALS AND METHODS

MRPrimerW2 provides a total of four search modes depending on the source of target sequences and the number of target sequences: single target in DB sequences, multiple targets in DB sequences, single target in FASTA sequences, and multiple targets in FASTA sequences. Figure 1 illustrates the input interface of MRPrimerW2. There are two tabs, 'Primer design for DB sequences' and 'Primer design for FASTA sequences'. The former tab is for the search modes in DB sequences, and the latter tab for the search modes in FASTA sequences. In both tabs, the default search mode is the single-target mode. If users input multiple gene symbols/IDs or FASTA sequences in a certain tab, MRPrimerW2 performs batch design for them, i.e., searches primers for each target sequence under the same filtering constraints. If users want the multi-target mode, they can do

Table 1. Comparison among websites for primer design

Method	Batch designing	Filtering constraints	Homology test	Scoring (ranking)	TaqMan probes	Exon spanning	Avoiding SNP	Input FASTA sequence	Multi-target designing
Primer3Plus	X	O	X	O	O	X	X	O	X
BatchPrimer3	O	O	X	O	O	X	O	O	X
QuantPrime	O	O	△	O	X	O	O	X	X
Primique	O	O	△	O	X	X	X	O	X
Primer-BLAST	X	O	O	△	O	O	O	O	X
Oli2go	O	O	O	X	O	X	X	O	O
PrimerBank	X	X	O	X	X	X	X	X	X
qPrimerDB	O	X	O	X	X	O	X	△	X
MRPrimerW	O	O	O	O	O	X	X	X	X
MRPrimerW2	O	O	O	O	O	O	O	O	O

O: fully supported.
△: partially supported.
X: not supported.


Primer design for DB sequences
Primer design for FASTA sequences
Example1: amplify EACH gene symbol/ID (70sec)
Example2: amplify EACH FASTA sequence (10sec)
Example3: amplify ALL gene symbols/IDs (3sec)
Example4: amplify ALL FASTA sequences (48sec)
Example5: exon spanning (2sec)
Example6: avoiding SNP sites (1sec)
Example7: using suggested parameters (2sec)
Basic Parameters
Primer Search
Reset
Advanced Settings ▲

Species: Homo Sapiens ▾

Search by: NCBI Gene Symbol ▾

For text: Only gene symbols/IDs are acceptable (FASTA format sequence is NOT acceptable)

If "Search by" is Keyword, use comma(,) as separator for multiple targets

E-mail (optional): email@domain

□ TaqMan probe design
□ Exon spanning design
□ Designing primers amplifying all given sequences
□ Designing primers avoiding SNP (only for human)
□ Designing primers using suggested parameters

✓ Single filtering

Primer melting temperatures (T_m)	Min 58.0	Max 62.0
GC content (%)	Min 40.0	Max 60.0
Primer length	Min 19	Max 23
Self-complementarity	Max 4	
3' end Self-complementarity	Max 3	
Contiguous residue	Max 5	
End stability	Max -9.0	
Hairpin	Max 2	

✓ Pair filtering

PCR Amplicon size	Min 100	Max 250
Length difference	Max 4	
Temperature difference	Max 3.0	
Pair-complementarity	Max 4	
3' end Pair-complementarity	Max 4	

Figure 1. Input interface of MRPrimerW2. There are two primer design modes, primer design for DB sequences mode and primer design for FASTA sequence mode. MRPrimerW2 takes as input species, query type (NCBI gene symbol, GenBank accession, NCBI Gene ID, aliases, or keyword), and query (a set of target gene symbol/IDs or FASTA format sequences). Also, a user can input their email address to be sent a link of query results. There are five additional options: TaqMan probe design, exon spanning design, SNP avoid design, amplify multiple gene design, and design primers using suggested parameters. In the Advanced Settings, the user can adjust filtering constraints.

it by checking the option ‘Designing primers amplifying all given sequences’. Batch design and multi-target design are incompatible with each other. Table 2 shows the range of off-target sequences used for homology test in each search mode.

MRPrimerW2 consists of two parts, offline processing (Figure 2A) and online processing (Figure 2B). Offline processing is independent of user queries and builds two kinds of DBs, DB-1 and DB-2, used for online processing. DB-1 is used for the tab ‘Primer design for DB sequences’ and DB-2 for the tab ‘Primer design for FASTA sequences’. Online processing designs the primers for a given query. It has two different pipelines for two different tabs.

Offline processing

Offline processing by MRPrimerW2 takes as input a mRNA sequence database and several filtering constraints and returns all valid primers and probes for building DB-1 that satisfy both homology testing and basic filtering constraints. It performs a total of four steps. During Step 1, we extract all possible subsequences and build DB-2 using them. As a source of input mRNA sequence database, we used the NCBI RefSeq database for human, mouse, rat, zebrafish, cow, pig, thale cress, fruit fly, and C. elegans (Table 3). In this update, we selected the RefSeq database as template because the RefSeq database contains much more organisms than CCDS database and provides whole mRNA sequences including UTRs and exons which allows to design exon spanning primers. We adopted dbSNP (https://www.ncbi.nlm.nih.gov/snp, Pre-build 152, the last update was 21 December 2018) for avoiding SNP sites. The most up-to-date RefSeq databases contain 245 013 mRNA sequences for the nine species, where the prefix of GenBank accession number is NM (the last update was 21 November 2018 for human, and 7 November 2018 for others). The result of offline processing includes 3 509 244 680 valid primers, which cover ∼99.9% of genes in RefSeq. Only ∼0.1% of genes in RefSeq do not have any target gene-specific primers. The total size of current databases, DB-1 and DB2, is about 363GB, which will increase by adding new species later.

Table 4 shows the list of filtering constraints used in offline and online processing. Four parameters (primer length, melting temperature, GC content, and contiguous residue) are applied during Step 2 of offline processing and most of constraints are checked during online processing which user can adjust in web interface. For TaqMan probes, we used different set of filtering constraints as described in previous publication (10). Homology test is done in Steps 3 and 4. The 5′ cross-hybridization filtering step (Step 3) eliminates the candidate primers that has only a few mismatches (up to four mismatches) at the 5′ end against off-target sequences. The general cross-hybridization filtering step (Step 4) eliminates the candidate primers that have only a few mismatches (up to two mismatches) anywhere against off-target sequences. This step is very time-consuming. We implemented the GPU kernel for general cross-hybridization filtering and run the kernel in a single computer with two Intel Xeon 10-core 2.20 GHz CPUs and eight Geforce GTX 1080ti GPUs. Because this step performs homology tests for every candidate primers and probes against the entire DB sequences in an exhaustive manner, the resultant primers and probes are all single-target gene-specific or multi-target gene-specific (i.e., transcript variants).

GPU computation

We performed from Step 1 to Step 3 in Figure 1A using MapReduce as MRPrimerW did, but performed Step 4 using GPUs since it was the most time-consuming step in offline processing. Step 4 checks each candidate primer passed Step 3 whether it occurs in any off-target sequences with only a few mismatches (up to two mismatches). In particular, Step 4 splits not only all candidate primers (output of Step 3) but also all possible subsequences (output of Step 1) into *seed*s and groups them by using *seed*+*offset*+|*P*| as a grouping key, where |*P*| means the length of primer (or subsequence) *P*, and *offset* the position where *seed* occurs in *P*. MRPrimerW (13) performs this grouping using MapReduce shuffle, whereas MRPrimerW2 using hashing in main memory. After grouping, we prepared four arrays for the output of Step 3, P3, P3_offset, SID3, SID3_offset (Supplementary Figure S1A) and four arrays for the output of Step 1, P1, P1_offset, SID1, SID1_offset (Supplementary Figure S1B) in main memory and copied the eight arrays to GPU device memory. Then, we called the GPU kernel with hundreds of thousands GPU threads per GPU where a single thread performs homology test for a single group. Supplementary Figure S1 illustrates two groups of A+0+3 (blue color) and T+1+3 (red color), and so two GPU threads are used. For human RefSeq, a total of 3,309,154 groups are processed when mismatch = 1. To reduce the processing time, we distributed the groups into eight GPUs. We also prepared the output array in GPU memory, which has the same length with P3 array. If a candidate primer in P3 is not similar with any subsequences in the same group of P1 array, the corresponding value in the output array becomes 0 (pass). Otherwise, it becomes 1 (fail). After executing the GPU kernel completely, we copied the output array from GPU memory to main memory.

Building indices

We built two databases using the results of offline processing. Due to the large size of databases, we stored them in a disk-based key-value store LevelDB in this update, while we stored a database in an in-memory database Redis in the predecessor, MRPrimerW. To improve I/O speed to the databases, we used Fusion-io’s PCIe SSD storage of 5.2TB for LevelDB. Supplementary Figure S2 illustrates the set of indices stored in the databases.

DB-1 contains a total of six indices (Supplementary Figure S2A–F): annotation index, valid primer index, probe index, metadata index, top-1 primer pair index and SNP locus index (for human only). Annotation data was downloaded from RefSeq ftp (ftp://ftp.ncbi.nlm.nih.gov/refseq/). Although the schemas of all indices except SNP locus index are the same with those of the predecessor, the contents of the indices are quite different from those of the predecessor. For example, the indices of MRPrimerW2 contains the primers for not only single-target design, but also multi-target design. SNP locus data was downloaded from dbSNP

Table 2. Off-targets in each search mode of MRPrimerW2

	Single target (including batch design)	Multiple targets
DB sequences	Entire DB sequences except each single target	Entire DB sequences except the multiple targets
FASTA sequences	Entire DB sequences	Entire DB sequences

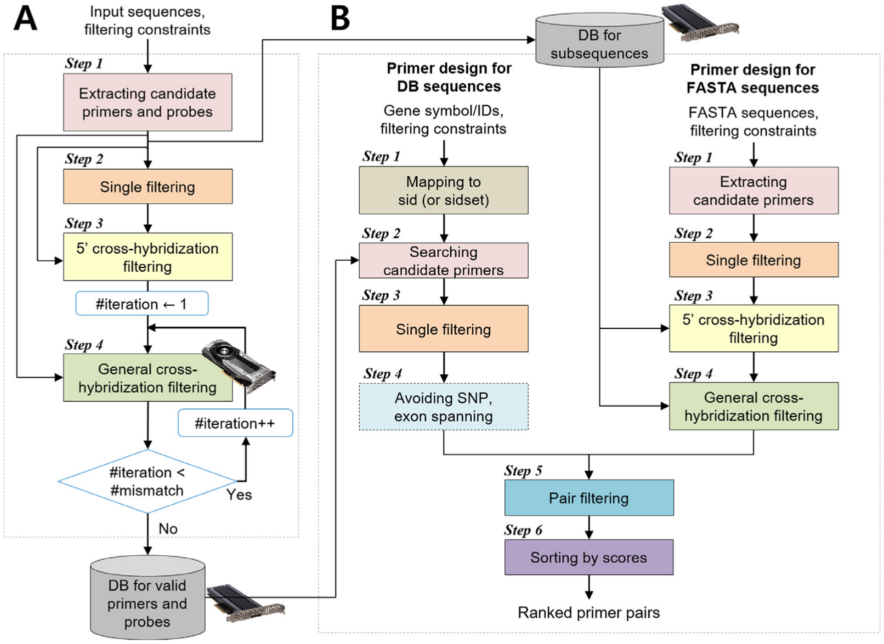

A
Input sequences,
filtering constraints
Step 1
Extracting candidate primers and probes
Step 2
Single filtering
Step 3
5' cross-hybridization filtering
#iteration ← 1
Step 4
General cross-hybridization filtering
#iteration++
#iteration < #mismatch
Yes
No
DB for valid primers and probes
B
DB for subsequences
Primer design for DB sequences
Gene symbol/IDs, filtering constraints
Step 1
Mapping to sid (or sidset)
Step 2
Searching candidate primers
Step 3
Single filtering
Step 4
Avoiding SNP, exon spanning
Primer design for FASTA sequences
FASTA sequences, filtering constraints
Step 1
Extracting candidate primers
Step 2
Single filtering
Step 3
5' cross-hybridization filtering
Step 4
General cross-hybridization filtering
Step 5
Pair filtering
Step 6
Sorting by scores
Ranked primer pairs

Figure 2. Overall flow of the MRPrimerW2. MRPrimerW2 consists of two parts, offline processing (**A**) and online processing (**B**). (A) Offline processing is independent of user queries and builds two kinds of DBs, 'DB for valid primers and probes' and 'DB for subsequences', used for online processing. DB for valid primers and probes is used for the tab 'Primer design for DB sequences' and DB for subsequences for the tab 'Primer design for FASTA sequences'. (B) Online processing consists of six steps to design the primers for a given query. It has two different pipelines for two different tabs. In the tab 'Primer design for DB sequences', MRPrimerW2 retrieves all candidate primers for a given query and checked filtering constraints on the candidate primers. If a user checks the exon spanning option and/or SNP avoiding option, it evaluates the candidate primers with their exon and/or SNP locus information. In the tab 'Primer design for FASTA sequences', MRPrimerW2 extracts all possible subsequences from given FASTA sequence(s) as candidate primers and checks the default filtering constraints for the candidate primers. Then, it performs homology test of the candidate primers using DB for subsequences. Through pair filtering and sorting step, MRPrimerW2 returns the ranked primer pairs.

Table 3. Statistics of MRPrimerW2 primers

	Total number of genes	Number of covered genes (%)	Number of valid primers*	Number of subsequences**	Size of database (MB)
Human (*Homo sapiens*)	51 979	51 976 (99.9%)	863 155 888	4 026 671 584	69 860
Mouse (*Mus musculus*)	35 349	35 323 (99.9%)	574 377 223	2 461 280 404	63 840
Rat (*Rattus norvegicus*)	17 639	17 637 (99.9%)	223 541 821	910 454 326	39 215
Zebrafish (*Danio rerio*)	15 876	15 834 (99.7%)	205 306 695	757 268 626	34 532
Cow (*Bos taurus*)	13 382	13 382 (100%)	152 077 285	676 441 698	31 010
Pig (*Sus scrofa*)	4 180	4 180 (100%)	43 265 923	169 276 012	9 915
Thale cress (*Arabidopsis thaliana*)	48 148	47 953 (99.6%)	597 970 532	1 876 192 010	48 700
Fruit fly (*Drosophila melanogaster*)	30 480	30 471 (99.9%)	532 662 088	1 918 507 800	38 926
***C. elegans* (*Caenorhabditis elegans*)**	28 299	28 257 (99.9%)	316 887 225	861 681 150	27 715
Total	245 332	245 013 (99.9%)	3 509 244 680	13 657 773 610	363 713

*Valid primer refers to the primer passed the homology test.
**Subsequences are in the range of from 17 to 27 bp (in the 'Offline' column in Table 4).

ftp (ftp://ftp.ncbi.nih.gov/snp/). Since SNP occurs very frequently, it is difficult to design primers avoiding all SNP sites. Thus, we used the SNP locus data which has the heterozygosity greater than 0 (i.e. frequent SNP occurrence).

DB-2 contains a total of three indices (Supplementary Figure S2G and H), where there are two indices having the same schema (Supplementary Figure S2H). These indices are used to perform homology test for the candidate primers extracted from input FASTA sequences. The first index (Supplementary Figure S2G) is for the 5′ cross-hybridization filtering step (Step 3 in Figure 2B) where the key contains the suffixes of all possible subsequences by removing a prefix of length four. The second and third indices (Supplementary Figure S2H) are for general cross-

Table 4. List of filtering constraints used in offline and online processing of MRPrimerW2 and qPrimerDB

	Parameter	Value range		
		Offline	Online (default)*	qPrimerDB(16)
each primer	Primer length	17–27 bp	19–23 bp	18–28 bp**
	melting temperature (TM)	56–63°C	58–62°C	58–64°C**
	GC content	30–70%	40–60%	40–60%**
	self-complementarity	-	<5-mer	<15-mer
	3′ self-complementarity	-	<4-mer	<15-mer
	Contiguous residue	<5-mer	<6-mer	<6-mer
	Gibbs free energy (ΔG)	-	≥-9 *kcal/mol*	≥-11 *kcal/mol***
	Hairpin	-	<3-mer	<9-mer
Primer pair	length difference	-	≤5-mer	≤6-mer
	TM difference	-	≤3°C	≤3°C**
	product size	-	100–250 bp	80–300 bp**
	pair-complementarity	-	<5-mer	<14-mer
	3′ pair-complementarity	-	<4-mer	<11-mer

-indicates not applicable.
*The value ranges in this column indicate the default setting on the web server. Users can freely adjust these values.
**These values are specified in the qPrimerDB publication (16). The rest of values are calculated with the best primers for human suggested by qPrimerDB.

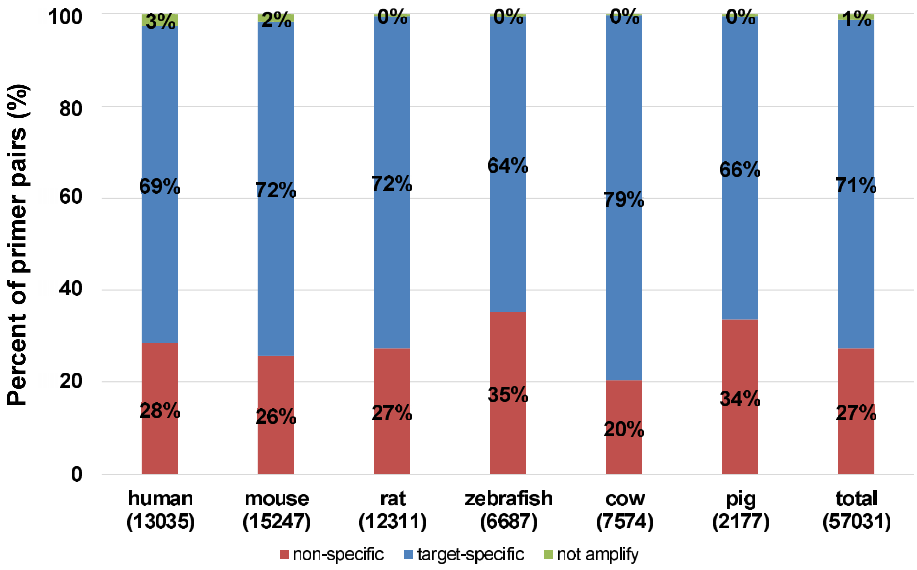


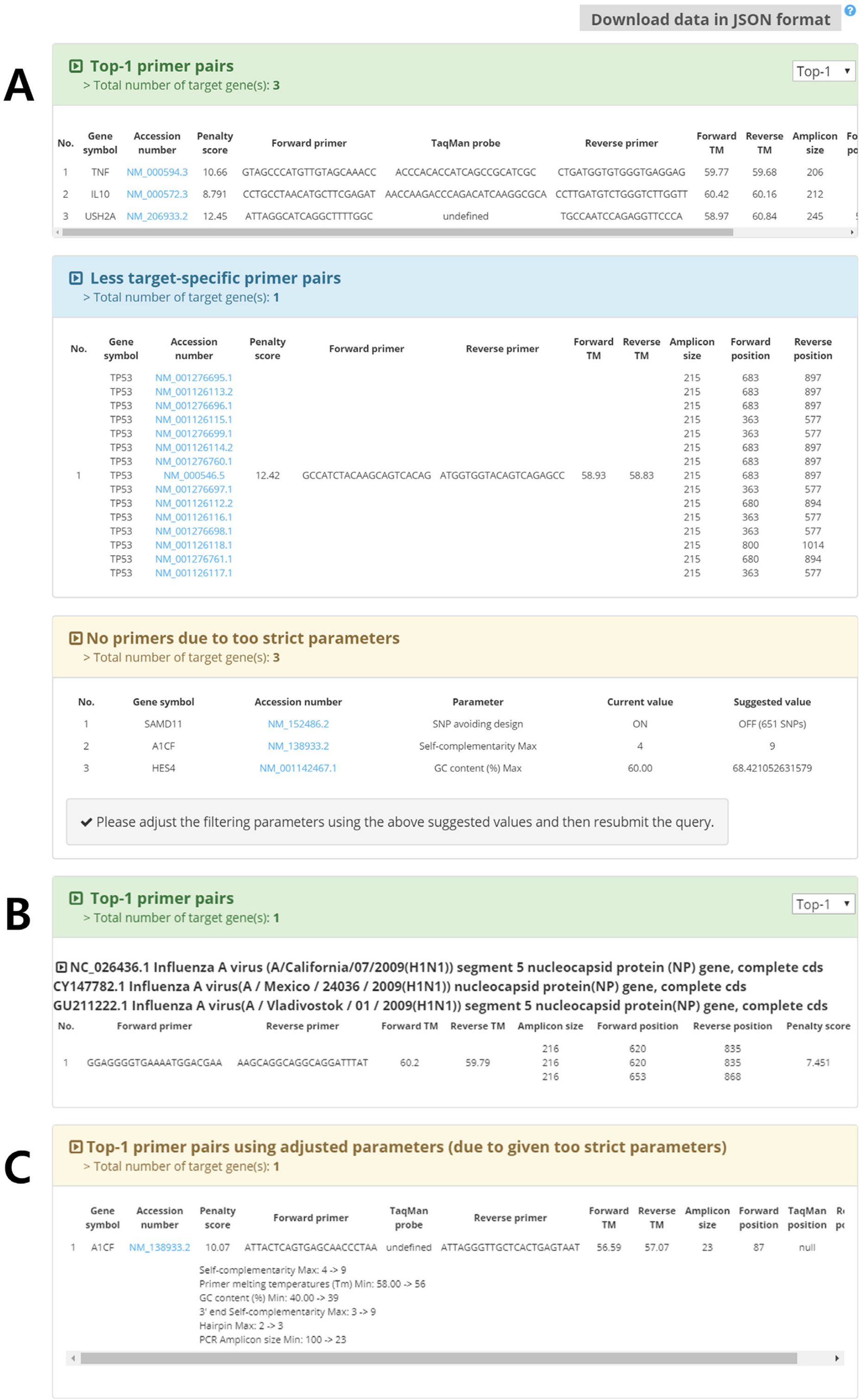
Figure 3. Specificity analysis of qPrimerDB. The distribution of specificity of the collected 124 596 qPrimerDB best primer pairs for six organisms (human, mouse, rat, zebrafish, cow and pig) where the x-axis represents species and the number of primer pairs existed in RefSeq database for each species in parentheses, and the y-axis represents the percent of primer pairs.

hybridization filtering step (Step 4 in Figure 2B). In particular, the second index is for a single mismatch, and the third one for two mismatches. The key of these indices contains a set of *seeds* obtained by splitting each subsequence. A subsequence of length m with at most k mismatches must contain a seed exactly matched of at least $m/(k+1)$ residues (19–21). The value of the indices contains a set of left flank and right flank of each seed within the corresponding subsequence. We illustrate an example of homology test using the indices in Supplementary Figure S3.

Online processing

Online processing consists of a total of six steps (Figure 2B). In the tab 'Primer design for DB sequences', Step 2 retrieves all candidate primers for a given query using the six indices (Supplementary Figure S2A–F), and the remaining Steps 3–6 are performed on the candidate primers. If a user checks the exon spanning option and/or SNP avoiding option, Step 4 evaluates the candidate primers with their exon and/or SNP locus information. Steps 5–6 are basically the same with the corresponding steps of the predecessor. We, however, reimplemented those steps in a more efficient form to process a much larger number of candidate primers compared with the predecessor still quickly.

In the tab 'Primer design for FASTA sequences', Step 1 extracts all possible subsequences of length 19–23 bp from given FASTA sequence(s) as candidate primers. Step 2 checks the default filtering constraints in Table 4 or the constraints adjusted by users for the candidate primers. Steps 3–4 perform homology test of the candidate primers using the three indices (Supplementary Figure S2G and H), that is, homology test against all DB sequences.



Top-1 primer pairs
> Total number of target gene(s): **3**

No.	Gene symbol	Accession number	Penalty score	Forward primer	TaqMan probe	Reverse primer	Forward TM	Reverse TM	Amplicon size	Fo po
1	TNF	NM_000594.3	10.66	GTAGCCCATGTTGTAGCAAACC	ACCCACACCATCAGCCGCATCGC	CTGATGGTGTGGGTGAGGAG	59.77	59.68	206	
2	IL10	NM_000572.3	8.791	CCTGCCTAACATGCTTCGAGAT	AACCAAGACCCAGACATCAAGGCGCA	CCTTGATGTCTGGGTCTTGGTT	60.42	60.16	212	
3	USH2A	NM_206933.2	12.45	ATTAGGCATCAGGCTTTTGGC	undefined	TGCCAATCCAGAGGTTCCCA	58.97	60.84	245	5

Less target-specific primer pairs
> Total number of target gene(s): **1**

No.	Gene symbol	Accession number	Penalty score	Forward primer	Reverse primer	Forward TM	Reverse TM	Amplicon size	Forward position	Reverse position
1	TP53	NM_001276695.1	12.42	GCCATCTACAAGCAGTCACAG	ATGGTGGTACAGTCAGAGCC	58.93	58.83	215	683	897
	TP53	NM_001126113.2						215	683	897
	TP53	NM_001276696.1						215	683	897
	TP53	NM_001126115.1						215	363	577
	TP53	NM_001276699.1						215	363	577
	TP53	NM_001126114.2						215	683	897
	TP53	NM_001276760.1						215	683	897
	TP53	NM_000546.5						215	683	897
	TP53	NM_001276697.1						215	363	577
	TP53	NM_001126112.2						215	680	894
	TP53	NM_001126116.1						215	363	577
	TP53	NM_001276698.1						215	363	577
	TP53	NM_001126118.1						215	800	1014
	TP53	NM_001276761.1						215	680	894
	TP53	NM_001126117.1						215	363	577

No primers due to too strict parameters
> Total number of target gene(s): **3**

No.	Gene symbol	Accession number	Parameter	Current value	Suggested value
1	SAMD11	NM_152486.2	SNP avoiding design	ON	OFF (651 SNPs)
2	A1CF	NM_138933.2	Self-complementarity Max	4	9
3	HES4	NM_001142467.1	GC content (%) Max	60.00	68.421052631579

✓ Please adjust the filtering parameters using the above suggested values and then resubmit the query.

Top-1 primer pairs
> Total number of target gene(s): **1**

NC_026436.1 Influenza A virus (A/California/07/2009(H1N1)) segment 5 nucleocapsid protein (NP) gene, complete cds
CY147782.1 Influenza A virus(A / Mexico / 24036 / 2009(H1N1)) nucleocapsid protein(NP) gene, complete cds
GU211222.1 Influenza A virus(A / Vladivostok / 01 / 2009(H1N1)) segment 5 nucleocapsid protein(NP) gene, complete cds

No.	Forward primer	Reverse primer	Forward TM	Reverse TM	Amplicon size	Forward position	Reverse position	Penalty score
1	GGAGGGGTGAAAATGGACGAA	AAGCAGGCAGGCAGGATTTAT	60.2	59.79	216 216 216	620 620 653	835 835 868	7.451

Top-1 primer pairs using adjusted parameters (due to given too strict parameters)
> Total number of target gene(s): **1**

	Gene symbol	Accession number	Penalty score	Forward primer	TaqMan probe	Reverse primer	Forward TM	Reverse TM	Amplicon size	Forward position	TaqMan position	Re po
1	A1CF	NM_138933.2	10.07	ATTACTCAGTGAGCAACCCTAA	undefined	ATTAGGGTTGCTCACTGAGTAAT	56.59	57.07	23	87	null	

Self-complementarity Max: 4 -> 9
Primer melting temperatures (Tm) Min: 58.00 -> 56
GC content (%) Min: 40.00 -> 39
3' end Self-complementarity Max: 3 -> 9
Hairpin Max: 2 -> 3
PCR Amplicon size Min: 100 -> 23

Figure 4. Output interface of MRPrimerW2. Example result for the tab 'Primer design for DB sequences' (**A**) and (**C**), and for the tab 'Primer design for FASTA sequences' (**B**). (A) In the tab 'Primer design for DB sequences', MRPrimerW2 displays three types of result tables. A user can select the number of the result primer pairs with Top-1, Top-10, and Top-50 option (default is Top-1). Also, the user can download the primer sequences and user-defined filtering constraints using 'Download data in JSON format' button on the top of the tables. The first table shows the feasible and valid primer pairs of each target gene. As the user selected the TaqMan probe design option, the table also shows the sequence and position of the probe. If there is no target-specific primer, MRPrimerW2 returns multi-target specific primers. The input query that does not have any valid primers are shown in the third table suggesting values for relaxing conditions and options. (B) In the tab 'Primer design for FASTA sequences', MRPrimerW2 displays one result table. The table shows the feasible and valid primer pairs of input target sequence(s). (C) If the user selects the option of designing primers using adjusted parameters, MRPrimerW2 adjusts both the filtering constraints and options such as avoiding SNP automatically so as to find the feasible primer pairs and returns them with additional information about how to change given parameters to new ones.

Comparative analysis

To compare the specificity and efficiency of the primers designed by MRPrimerW2 with the recent primer database, qPrimerDB, we collected 124,596 thermodynamic-based best primer pairs for six organisms (human: 17 632, mouse: 20 080, rat: 21 530, zebrafish: 25 052, cow: 19 970 and pig: 20 332) from qPrimerDB (https://biodb.swu.edu.cn/qprimerdb/download-primers). For efficiency test, we evaluated the filtering constraints of the collected 17 632 primer pairs for human ('qPrimerDB' column in Table 4). They specified only four filtering constraints for each primer and two filtering constraints for primer pairs in their publication (16). Comparing with the default constraints of MRPrimerW2, some of the qPrimerDB primer pairs only satisfy much looser filtering constraints especially in terms of self-complementarity, hairpin and pair-complementarity as in in Table 4. In addition, we checked the specificity of qPrimerDB primer pairs using sequence similarity searching based on RefSeq database. Since qPrimerDB uses the Ensembl database, we cannot evaluate 54% of the best primer pairs which do not exist in the RefSeq database. Figure 3 shows the results of the specificity of the remaining 46% of qPrimerDB primer pairs (human: 13 035 primer pairs). For human, 69% are target-specific, but 28% are non-specific (i.e. may amplify multiple off-targets) and the rest 3% do not amplify, which means each of forward and reverse primer is target-specific, but both are located in different genes so that the pairs may not amplify the target. In total, about 71% of the best primer pairs are target-specific, 27% are non-specific, and 1% of the pairs do not amplify.

We also describe the difference between Oli2go (12) and our MRPrimerW2 in terms of multi-target designing of primers. Oli2go is a multiplex oligonucleotide design tool and so returns multiplex primers to users for given multiple target sequences. It depends on using external tools such as BLAST and BWA (22) for homology test and also requires an additional external tool such as MFEprimer (17) for cross dimer check among multiplex primers, i.e., a set of primer pairs covering the target sequences. Multiplex primer design is a well-known NP-complete problem, and so, the resulting primers may not be target-specific enough. In addition, since Oli2go needs to repeat a series of external tools until finding the feasible and valid primers, its run time may be relatively slow. On the contrary, MRPrimerW2 returns a single primer pair instead of multiplex primers for given multiple target sequences. Since MRPrimerW2 calculates all possible primers for all possible combinations of DB sequences and stores them in the indices, it can find a single primer pair even for multiple targets if it exists, and its run time is inherently faster than Oli2go that uses several external tools repeatedly.

WEB SERVER

The MRPrimerW2 web server is connected to LevelDB (http://leveldb.org/) via PHP-LevelDB (https://github.com/reeze/php-leveldb), which is used for communication between LevelDB and PHP. The web server is also communicated with user's web browser via AJAX (Asynchronous JavaScript and XML). In the web browser, HTML with CSS and bootstrap (http://getbootstrap.com/) are used for generating web pages. JavaScript and jQuery are also used for dynamic HTML behaviour. MRPrimerW2 supports major web browsers including Microsoft Internet Explorer, Google Chrome, Mozilla Firefox, Apple Safari, and Opera. MRPrimerW2 provides six example queries corresponding four search modes and two new features.

Figure 4A illustrates the result of the Example1 query for seven target genes (SAMD11, TNF, IL10, TP53, USH2A, A1CF and HES4 in gene symbols), where species is human, and search type is GenBank accession number. A total of three options, TaqMan probe, exon spanning, and avoiding SNP, are selected. In the result, three of target genes, TNF, IL10 and USH2A, have valid primer pairs which are supposed to amplify each target gene, are spanned exons, and do not overlie SNP sites. One target gene TP53 has primer pairs that amplify the variants of the target gene. The remaining three target genes have no valid primer pairs satisfying the given filtering constraints and options. Specifically, MRPrimerW2 suggests turning off the option of avoiding SNP for the target gene SAMD11, and relaxing the filtering constraints for two target genes, A1CF and HES4, to search for valid primer pairs. Figure 4B shows the top-1 primer pair of the Example4 query for three input FASTA sequences, which are some variations of Influenza A virus (H1N1) nucleocapsid protein (NP) genes. Here, the host species is human, and the option of multi-target design is turned on. The resulting primer pairs are supposed to amplify all the input FASTA sequences at once without amplifying any host sequences. Figure 4C shows the top-1 primer pair of the Example 7 query for the target gene A1CF with turning on the option using suggested parameters. A1CF originally has no result in the Example 1 query due to too strict filtering constraints (i.e., parameters). Using the option, however, adjusts the parameters automatically and returns the valid primer pairs if they exist in DB.

CONCLUSIONS

Previously we launched the MRPrimerW web server, a tool for designing high-quality primer pairs for multiple target qPCR experiments. It allows users to quickly search for the best valid primer pairs satisfying the same constraints for many target sequences (i.e. batch design). However, it has several major drawbacks that do not consider sequence variants and SNP sites, not support other popular model organisms, not support designing valid primers for new input FASTA sequences against database sequences, and not support designing multi-target primers. In this update, we developed MRPrimerW2 which solves all the drawbacks of the MRPrimerW, but still maintains the advantages of the predecessor such as one-stop searching considering both filtering constraints and homology test and high primer specificity of primers. MRPrimerW2 provides a total of four search modes, while the predecessor only a single search mode among them. To achieve this, we extracted a complete set of 3 509 244 680 valid primers and 13 657 773 610 subsequences from the human, mouse, rat, zebrafish, cow, pig, thale cress, fruit fly, and *C. elegans* RefSeq databases still in an exhaustive manner. The predecessor cannot extract and utilize such a huge number of primers or subsequences mainly due to a prohibitive amount of computation and memory space required. In MRPrimerW2, we significantly improved the computational performance by exploiting GPUs and also extended the memory space for storing databases by exploiting a disk-based key-value store, LevelDB, using PCIe SSD storage. We believe that MRPrimerW2 will contribute to further increasing the efficiency and specificity of qPCR experiments through supporting input FASTA sequences, supporting multi-target primer design, exon spanning, and avoiding SNP sites.

We consider the follow two directions as future work. The first direction is extending MRPrimerW2 so as to handle more complex cases such as virus family identification and cancer variants amplification. It is usually very difficult to design primers and probes for detecting all variants of a virus or an oncogene at once. In particular, for detecting cancer variants, we need to consider various types of cancer mutations such as SNV, insertion, deletions, and translocations. MRPrimerW2 can be extended to solve the problem by adding a high-level layer on top of the current version and integrating additional DBs such as cancer mutation DBs into primer DBs. The second direction is expanding MRPrimerW2 by integrating it with larger platforms. For doing that, we consider providing the codes that other platforms can easily access and utilizes our DBs as UCSC Genome Browser or Ensembl DB does.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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