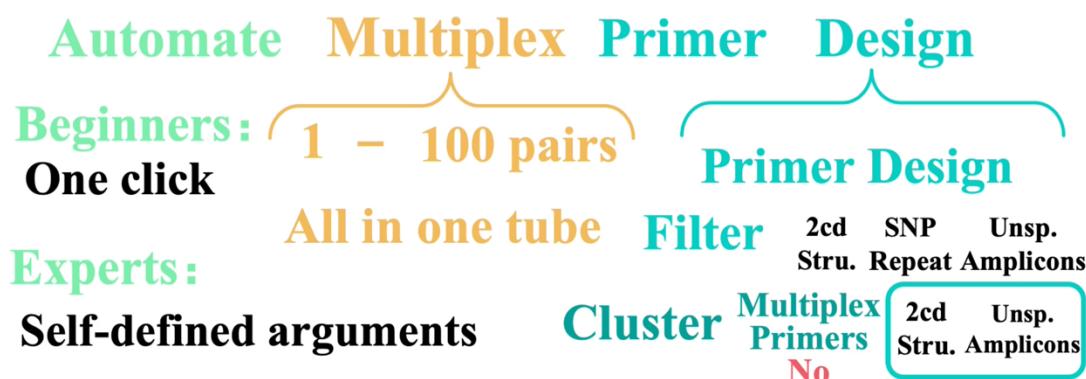


Ultiplex multiplex PCR primer design website

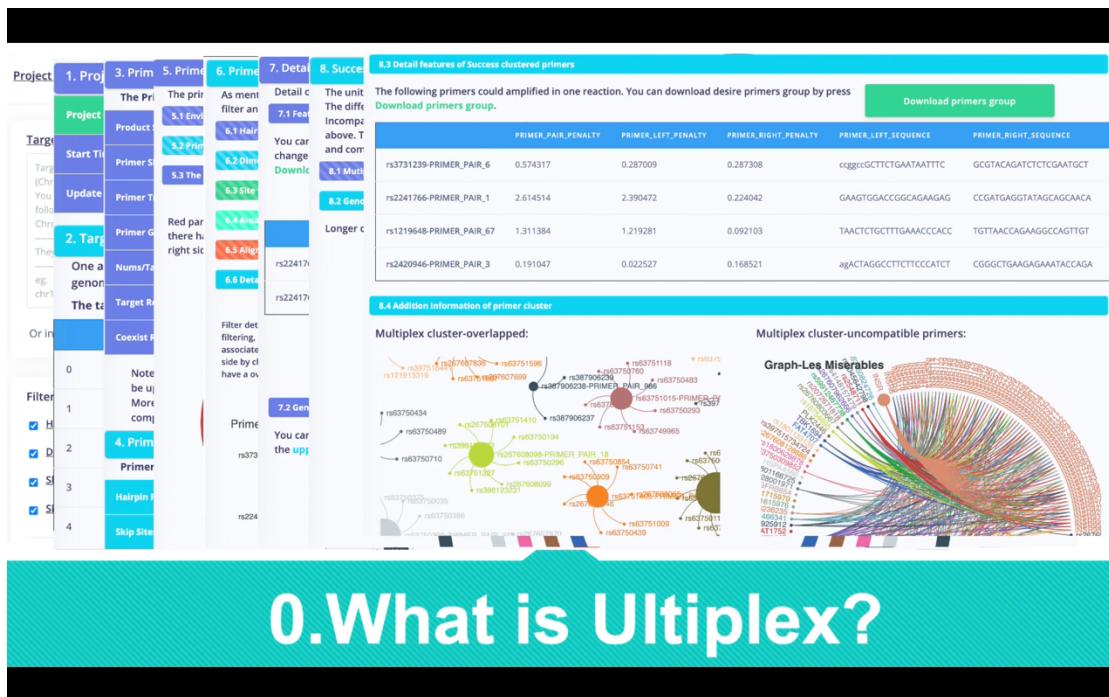
Ultiplex is a multiplex PCR primer design website, and it is automated. Ultiplex is a multiplex PCR primer design website, it could design primers for 1 to 100 targets. And all primers are reacted in one tube. Ultiplex is a PCR primer design website, it could design primers, filter primers, delete primers which contain harmful second structures, such as hairpin and dimer. Delete primers which contain SNP or located in repeat sequence. Delete primers which have nonspecific amplicons. Ultiplex also can cluster primer pairs, guarantee there are no dimers or nonspecific amplicons between primer pairs of different targets in one group.



0.What is Ultiplex?

Ultiplex also is an automated primer design software. For beginners, you only need one click to finish primer design. For experts, you can define parameters by yourself. Ultiplex provide user-friendly web-based interface and informative results. You can adjust your input parameters to improve your design results.

Generated primers have good quality. There are no unspecific amplicons in the experiments of 108 single output primer pairs. Each pair only have one clear amplicon. After 2cd round PCR, products are at the adequate range for NGS and could be sequenced directly.

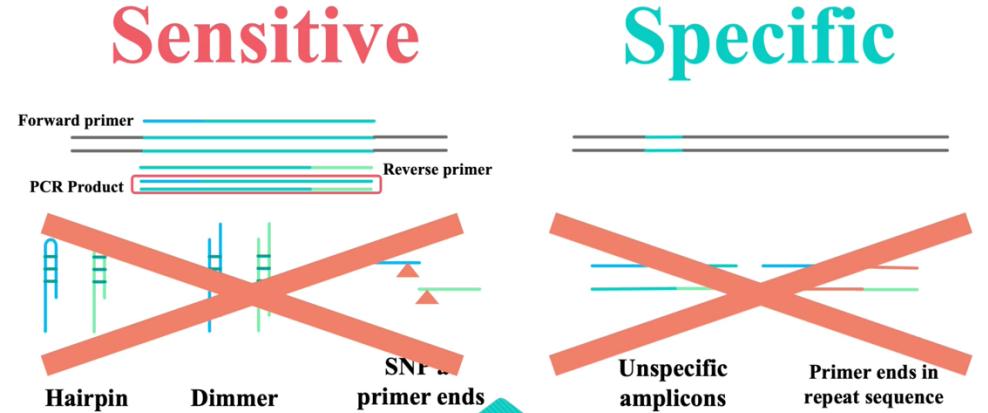


0.What is Ultiplex?

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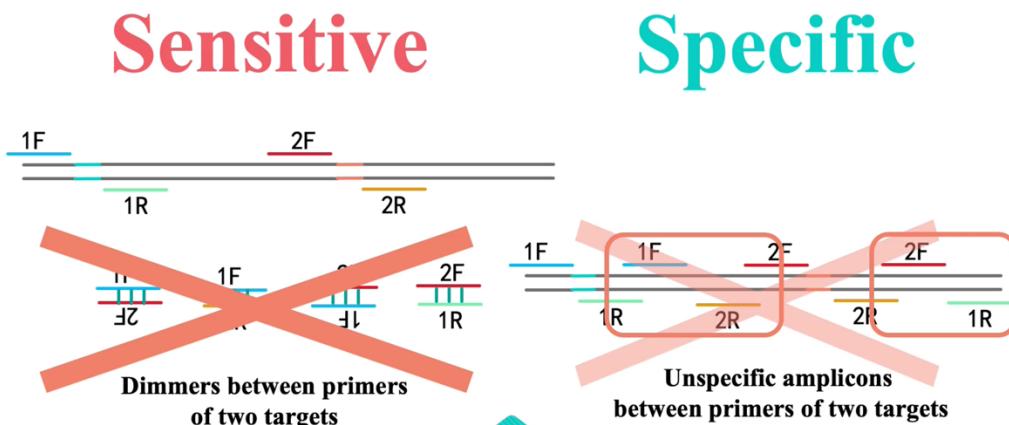
1. Background knowledge

How to design primers for one target? Ideal primers should be sensitive and specific in PCR reaction. sensitive means primers could amplify products in high efficiency. Factors affected primer efficiency, such as hairpin, dimer and snp in the ends of primers, should be eliminate. Specific means primers could generate nonspecifical amplicons. Those primers and those primers located in repeat sequence should be eliminate.



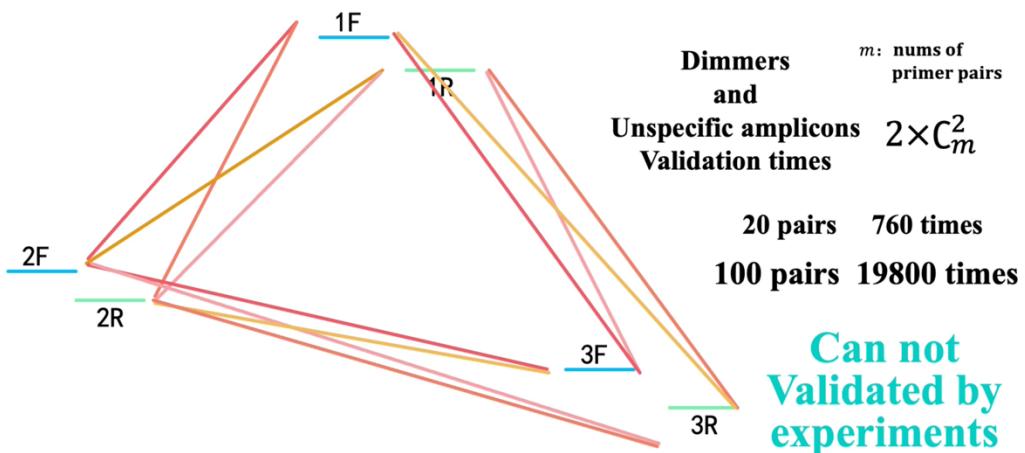
1.1 How to design primers for one target

How to design primers for two targets? In addition of demands for each target, dimers between primer pairs of two targets should be eliminated, and also those nonspecifical amplicons between four primers on genome should be eliminated.



1.2 How to design primers for two targets

How to design primers for more targets. In addition of demands for each target, dimers and nonspecifical amplicons between all primers should be prevent. If there are 20 targets in one reaction, 760 times validations will be needed to check the dimers and nonspecifical amplicons. When the number of targets raise to 100, 19800 times validations will be needed. Experiments could not finish these validations in short times. Ultiplex provide automated multiplex PCR primer design functions and could finish large number target primer design.



1.2 How to design primers for more targets

2. Introduction of layouts

New project layouts are showed as followed. At first, Input your project name. Please make sure your target name not in our database. then you can input your targets information. This software design primers based on the genome coordinates of your targets. Target's information should be in four columns, include chromosome name, start coordinate, end coordinate, and your target name. Columns can be separated by space or tab. you can paste your bed formatted strings in the input box or upload by files upload box.

2.1.1 Input your project name (Must Be Unique)

2.1.2 Target information

You can paste your bed formatted strings in the input box or upload by files upload box.

Targets:
Targets: chr1 45332446 45332446 rs200495564
chr2 47429942 47429942 rs267607950

Chromosome Start End Target name

Or Must Upload upload

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR 选择文件 未选择任何文件 upload
- Coexist Ref.: Null OR 选择文件 未选择任何文件 upload

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Delete Unspecific Amplicon: 500 bp
- Skip Sites: SITES SNP_hg38 OR 选择文件 未选择任何文件 Attach eg: chr10 72098085 72098086
- Skip Area: AREAS Repeat(hg38) OR 选择文件 未选择任何文件 Attach eg: chr10 72098085 72098186

5' Tags:

- Add Forward primers' 5' tag GTCTCGTGGGCTCGGA
- Add Reverse primers' 5' tag TCGTCGGCAGCGTCAG

2.1 New project layouts

After filled information, please make sure upload your target information. Website will check the format of your input information. After your upload, website will show total number of your uploaded targets.

2.1.1 Input your project name (Must Be Unique)

2.1.2 Target information

You can paste your bed formatted strings in the input box or upload by files upload box.

You uploaded 17 targets successfully!

	CHR	CHRSTART	CHREND	NAME
0	chr1	45331556	45331556	rs36053993
1	chr1	45332088	45332088	rs77542170
2	chr1	45332445	45332445	rs140342925
3	chr1	45332446	45332446	rs200495564

Or input from file 选择文件 未选择任何文件 upload

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR 选择文件 未选择任何文件 upload
- Coexist Ref.: Null OR 选择文件 未选择任何文件 upload

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Delete Unspecific Amplicon: 500 bp
- Skip Sites: SITES SNP_hg38 OR 选择文件 未选择任何文件 Attach eg: chr10 72098085 72098086
- Skip Area: AREAS Repeat(hg38) OR 选择文件 未选择任何文件 Attach eg: chr10 72098085 72098186

5' Tags:

- Add Forward primers' 5' tag GTCTCGTGGGCTCGGA
- Add Reverse primers' 5' tag TCGTCGGCAGCGTCAG

2.1 New project layouts

You can input parameters for primer design, include Product Size, Primer Size, Primer TM Value, Primer numbers for each target, bigger numbers will cause slower design speed, Target Reference, Coexist Reference.

2.1.2 Target information

You can paste your bed formatted strings in the input box or upload by files upload box.

2.1.1 Input your project name (Must Be Unique)

You uploaded 17 targets successfully!			
CHR	CHRSTART	CHREND	NAME
0	chr1	45331556	45331556 rs36053993
1	chr1	45332088	45332088 rs77542170
2	chr1	45332445	45332445 rs140342925
3	chr1	45332446	45332446 rs200495564

Or input from file 未选择任何文件

2.1.3 Parameters for primer design

Primer Size
Product Size: MIN 200 MAX 300
Primer Size: MIN 15 OPT 22 MAX 25
Primer Tm: MIN 57 OPT 60 MAX 63
Primer GC%: MIN 20 MAX 80
Nums/Target: 20
GC content

Primer numbers for each target
Target Ref.: hg38 OR 选择文件 未选择任何文件
Coexist Ref.: Null OR 选择文件 未选择任何文件

Filter Parameters:

<input checked="" type="checkbox"/> Hairpin Filter: TM 45 °C	<input checked="" type="checkbox"/> Dimer Filter: TM 40 °C
<input checked="" type="checkbox"/> Delete Unspecific Amplicons: 500 bp	<input checked="" type="checkbox"/> Unspecific Alignment Energy: DELTA DG 10000 kcal
<input checked="" type="checkbox"/> Skip Sites: SITES SNP_hg38 OR 选择文件 未选择任何文件 <input type="button" value="Attach"/> eg: chr10 72098085 72098086	
<input checked="" type="checkbox"/> Skip Area: AREAS Repeat(hg38) OR 选择文件 未选择任何文件 <input type="button" value="Attach"/> eg: chr10 72098085 72098186	

5' Tags:
 Add Forward primers' 5' tag Add Reverse primers' 5' tag

2.1 New project layouts

Input primer filter parameters, to filter primers Contained Hairpin, to filter primers Contained Dimers, to filter primers contained nonspecific amplicons, to filter primers contained SNP, to Filtered primers contained repeat, you can choose whether add 2cd round PCR tags, after that, press start design.

2.1.2 Target information

You can paste your bed formatted strings in the input box or upload by files upload box.

2.1.1 Input your project name (Must Be Unique)

You uploaded 17 targets successfully!			
CHR	CHRSTART	CHREND	NAME
0	chr1	45331556	45331556 rs36053993
1	chr1	45332088	45332088 rs77542170
2	chr1	45332445	45332445 rs140342925
3	chr1	45332446	45332446 rs200495564

Or input from file 未选择任何文件

2.1.3 Parameters for primer design

Primer Size
Product Size: MIN 200 MAX 300
Primer Size: MIN 15 OPT 22 MAX 25
Primer Tm: MIN 57 OPT 60 MAX 63
Primer GC%: MIN 20 MAX 80
Nums/Target: 20
GC content

Primer numbers for each target
Target Ref.: hg38 OR 选择文件 未选择任何文件
Coexist Ref.: Null OR 选择文件 未选择任何文件

2.1.4 Filter parameters

Filtered primers contained Hairpins	Filtered primers contained Dimers
<input checked="" type="checkbox"/> Hairpin Filter: TM 45 °C	<input checked="" type="checkbox"/> Dimer Filter: TM 40 °C
<input checked="" type="checkbox"/> Delete Unspecific.Amplicons: 500 bp	<input checked="" type="checkbox"/> Unspecific.Alignment Energy: DELTA DG 10000 kcal
<input checked="" type="checkbox"/> Skip.Sites: SITES SNP_hg38 OR 选择文件 未选择任何文件 <input type="button" value="Attach"/> eg: chr10 72098085 72098086 Filtered primers contained SNP	
<input checked="" type="checkbox"/> Skip.Area: AREAS Repeat(hg38) OR 选择文件 未选择任何文件 <input type="button" value="Attach"/> eg: chr10 72098085 72098186 Filtered primers contained repeat	

5' Tags:
 Add Forward primers' 5' tag Add Reverse primers' 5' tag

2.1 New project layouts

After your submission, it will be redirect to results interface. there is a Results link on the top of the page. This is the only way to check your primer design results in 7 days. Please write down this link on your notebook or your computer.

1. Project Information:

Project Name	hg38_test	User	127.0.0.1_2021-02-26-17-42-23_61
Start Time	2021-02-26 17:42:23	Process	Multiplex
Update Time	2021-02-26 18:03:47:491358	Finish Time	None

2. Target Information:

One advantage of ultiplex is the unspecific alignment checking around genome. To design primers and check the specificity in the genome background, the genome coordinates of target areas are needed, and they must be input in BED format with an additional column-target ID.

The target information of this primer design project are as below.

CHR	CHIRESTART	CHIREEND	NAME	
0	chr2	47463154	47463554	rs63751600
1	chr2	47466809	47466809	rs267807969
2	chr2	47800042	47800042	rs63750075
3	chr2	47806534	47806534	rs267808130
4	chr3	36993591	36993591	rs63751131

2.2.1 Results links

2.2.2 Update and progress information

2.2.3 Target information

2.2 Result layouts

Followed with Update and progress information, Target information, Parameters for primer design, Filter parameters.

3. Primer Design Parameters:

The Primer Design Parameters of this primer design project are as below.

Product Size(bp)	Min	200	Max	300		
Primer Size(bp)	Min	15	Opt	22	Max	25
Primer Tm(°C)	Min	57	Opt	60	Max	63
Primer GC(%)	Min	20	Max	80		
Nums/Target	20					
Target Ref	hg38					
Coeexist Ref	Null					

Note: 1)The design parameters are similar to those of Primer3Plus, include PCR product length, primer size and so on. 2)Genome references also need to be uploaded in FASTA format. Human genome references hg19 and hg38 were uploaded as defaults. Researchers can self-define their own reference. More conveniently, researchers can define coexisting species references to eliminate the nonspecific amplification of the genomes of other species in a complex environment.

4. Primer Filter Parameters:

Primer Filter Parameters of this primer design project are as below.

Hairpin Filter	45°C	Dimer Filter	40°C	Unspecific Amplicons	500bp	Unspecific Enzyme	Repeat(hg38)	10000cycles
Skip Sites	SNP_hg38							
Forward primers' 5' tag	GTCTCGTGGGCTCGAGATGTATAAGACACAG							
Reverse primers' 5' tag	TCGTCGGCAGGTCAGATGTATAAGACACAG							

2.2.4 Parameters for primer design

2.2.5 Filter parameters

2.2 Result layouts

Summary of primer design results will show the success rate of primer design, as well as success targets' number and failed targets' number. In the drop-down menu, you can select one failed target to check the detail failure reason, include GC content failed, too low or high TM, long poly-x seq, Unacceptable product size. For example, in this

project and for this target, there are 225 forward primers, 301 reverse primers, paired primer pairs 67725, but all primer pairs have unacceptable product size. As we dig deeper, we could find, this target cover 400bp in the uploaded bed information. however, primer design parameters require products smaller than 300bp. You can adjust your input information to improve your design results.

5. Primer Design Report:

The primer design phase are formed by two part, environment generation and primer design.

5.1 Environment Generated: 100% complete

5.2 Primer design: 100% complete

5.3 The detail report of Primer design

5.3.1 Summary of primer design results

Red parts show the number of successful design targets and failed targets. If there have target could not get primers, the fail reason will show at the right side.

5.3.2 Reason for the failure targets

The design failure reason will show as below, such as GC content failed, low tm, high tm, long poly-x seq and so on. Based on this information, you can justify your primer design.

2.2.6 Summary of primer design results.
Success rate of primer design

2.2.7 Reason for the failure targets

2.2.7.1 Choose one target
2.2.7.2 Failure reason:
GC content failed, low/high TM, long poly-x seq, Unacceptable product size

2.2.7.3 In this project, 225 forward primers, 301 reverse primers, paired primer pairs 67725, but all primer pairs have unacceptable product size.

400 bp
chr2 47463154 47463554 rs63751600
Product Size: MIN 200 MAX 300

2.2 Result layouts

The Process bar show the real-time progress of your design project. Finished results in each phrase will be showed on this page, and unfinished results will be showed after finished.

6. Primer Filter Report:

As mentioned at part 4, to guaranteed the efficiency and specificity of primers, several filter steps were processed, such as Haipin filter, Dimer filter, site filter, area filter and unspecific alignment filter.

6.1 Haipin Filter: 100% complete

6.2 Dimer Filter: 100% complete

6.3 Site Filter: 100% complete

6.4 Area Filter: 100% complete

6.5 Alignment Filter: 100% complete

6.6 Detail result of primer filter

2.2.8 Process bar

2.2.9 Summary of primer filtration

6.6.1 Summary of successful filtered primers

Filter details of targets which possess at least one primer pair after serials of primer filtering. There are some filters which have been processed, such as Haipin filter, dimer filter, site filter, area filter and unspecific alignment filter. For more detail information, you can adjust scrollbars at right side by change the **zoom factor** of the scrollbars. You can also **scroll** the scrollbars to have a overview of all successful targets.

	area	site	dimer	blast	haipin	success	
Primers	0	20	40	60	80	100	120
rs0371460	0	0	0	0	0	0	0
rs03751131	7	9	10	0	0	0	0
r13785456	0	0	0	0	0	0	0
s037906237	0	0	0	0	0	0	0
rs03749965	0	0	0	0	0	0	0
rs03750071	0	0	0	0	0	0	0

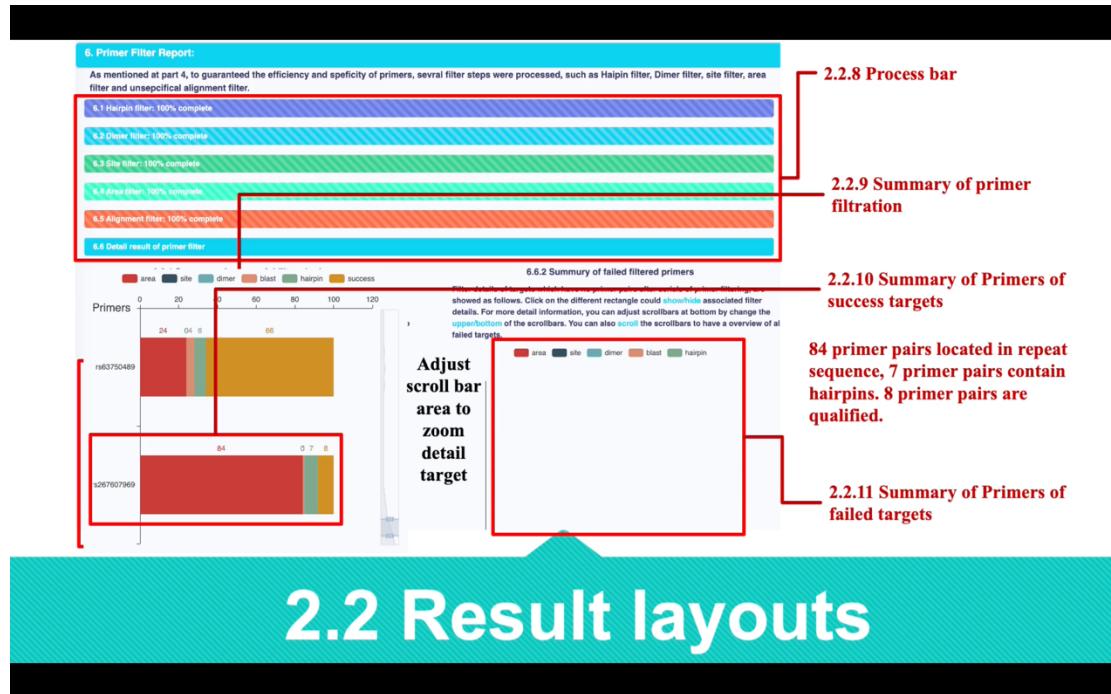
6.6.2 Summary of failed filtered primers

Filter details of targets which have no primer pairs after serials of primer filtering. There are some filters which have been processed, such as Haipin filter, dimer filter, site filter, area filter and unspecific alignment filter. For more detail information, you can adjust scrollbars at bottom by change the **zoom factor** of the scrollbars. You can also **scroll** the scrollbars to have an overview of all failed targets.

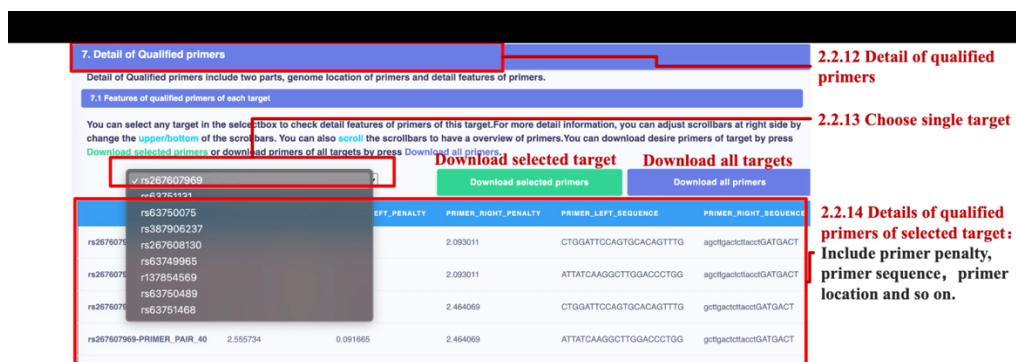
	area	site	dimer	blast	haipin		
Primers	0	20	40	60	80	100	120
rs0371460	0	0	0	0	0	0	0
rs03751131	7	9	10	0	0	0	0
r13785456	0	0	0	0	0	0	0
s037906237	0	0	0	0	0	0	0
rs03749965	0	0	0	0	0	0	0
rs03750071	0	0	0	0	0	0	0

2.2 Result layouts

Summary of primer filtration will show numbers of primers which have hairpin or dimers, numbers of primers which located in repeat sequence or SNP, numbers of primers which have nonspecific amplicons, and numbers of final qualified primers.



you can check all targets through scroll bar, you can adjust bar area to zoom pictures. For example, this target has 84 primer pairs located in repeat sequence, 7 primer pairs contained hairpins. And 8 primer pairs are qualified. If one target has no qualified primers, the results will emphatically appear in the summary of primers of failed targets. according this summary, you can adjust your input information to improve your results.



2.2 Result layouts

7. Detail of Qualified primers

Detail of Qualified primers include two parts, genome location of primers and detail features of primers.

7.1 Features of qualified primers of each target

You can select any target in the selectbox to check detail features of primers of this target. For more detail information, you can adjust scrollbars at right side by change the **upperbottom** of the scrollbars. You can also scroll the scrollbars to have a overview of primers. You can download desire primers of target by press **Download selected primers** or download primers of all targets by press **Download all primers**.

Download selected target Download all targets

7.2 Genome location of qualified primers of each target

You can select any target in the selectbox to check genome location of this target. For more detail information, you can adjust scrollbars at right side by change the **upperbottom** of the scrollbars. You can also scroll the scrollbars to have a overview of primers.

Forward primers Reverse primers

2.2.12 Detail of qualified primers

2.2.13 Choose single target

2.2.14 Details of qualified primers of selected target:
Include primer penalty, primer sequence, primer location and so on.

2.2.15 chromosome locations of primers of selected target

2.2 Result layouts

In the Detail results of qualified primers, you can Choose single target to download selected target information, you can also download all targets primers. You can also check Details of qualified primers of selected target include primer penalty, primer sequence, primer location and so on. You can also check chromosome locations of primers of selected target.

8. Success clustered primers:

The unity and incompatibility between different pairs are tested for each pair, and compatible pairs are clustered. Unity refers to product length and Tm unity. The difference in the length of the two primer pairs should be less than 150 bp, and the difference in Tm between the two primer pairs should be less than 5°C. Incompatibility refers to dimers and nonspecific alignments generated between different pairs. The checks for dimers and nonspecific alignment are described above. The only difference is that these tests are conducted between different pairs. As the relationships between pairs are deduced, the list of maxim unifiable and compatible primers is generated.

2.2.16 Success clustered primers

8.1 Multiplex cluster: 100% complete

8.2 Genome location of successful primers

2.2.15 Genome location of successful primers

2.2.16 Detail features of success clusted primers

Include primer penalty, primer sequence, primer location and so on.

8.3 Detail Features of Success clustered primers

The following primers could amplified in one reaction. You can download desire primers group by press **Download primers group**.

Download primers group Download Whole multiplex primer set

PRIMER_PAIR_ID	PRIMER_PAIR_NAME	PRIMER_LEFT_SEQUENCE	PRIMER_RIGHT_SEQUENCE
rs03731131-PRIMER_PAIR_6	rs03731131-PRIMER_PAIR_6	ACTTCGTTGAGCATCTAGCG	CCTGGTTC
rs037900337-PRIMER_PAIR_73	rs037900337-PRIMER_PAIR_73	ACAGCCTGATTCATGCGACA	GTTTCA
rs03750075-PRIMER_PAIR_29	rs03750075-PRIMER_PAIR_29	CCCCAGGTGCTTAAGGTATGA	AGCCACCA
rs137854569-PRIMER_PAIR_64	rs137854569-PRIMER_PAIR_64	CCATTCTCGAGTTTAACTGCTCA	GCTGTTC

2.2 Result layouts

The unity and incompatibility between different pairs are tested for each pair, and compatible pairs are clustered. The list of maxim unifiable and compatible primers is generated. the Overview of Genome location of compatible primer set will be showed

as a circos map. You can also check Details of primers of compatible primer set include primer penalty, primer sequence , primer location and so on. Download Whole multiplex primer set. You can synthesize primers with downloaded files.

3. Example.

3.1 Primer design with human hg38 reference (One click).

Input your project name (Must Be Unique) first. Then paste your target information.

Upload. Keep other parameters as default. Add 5'tags. You can Start your primer design.

Project Name: hg38_test 2.4.1.1 Input your project name (Must Be Unique)

Targets:
You uploaded 10 targets successfully!

ID	CHROM	CHRSTART	CHREND	NAME
0	chr2	47403154	47403554	rs63751600
1	chr2	47498869	47498869	rs267607969
2	chr2	47800042	47800042	rs3750075
3	chr2	47806534	47806534	rs267608130

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR Choose File No file chosen upload
- Coexist Ref.: Null OR Choose File No file chosen upload

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Delete Unspecific Amplicons: 500 bp
- Skip Sites: SITES SNP_hg38 OR Choose File No file chosen Attach eg: chr10 72098085 72098086
- Skip Area: AREAS Repeat(hg38) OR Choose File No file chosen Attach eg: chr10 72098085 72098166

3.1.2 Paste your target information 3.1.3 Upload 3.1.4 keep other parameters as default 3.1.5 add 5' tags

3.1.6 Start design Start multiplex primer designing

3.1. Primer design with human hg38 reference

3.2 Primer design with human hg19 reference

Input your project name (Must Be Unique) first. Then Paste your target information.

Upload. Select “hg19” in “target reference” drop-down menu. Select “SNP_hg19” in “skip sites” drop-down menu. Select “Repeat(hg19)” in “skip area” drop-down menu.

Add 5'tags. Keep other parameters as default. You can Start your primer design.

Project Name: hg19_test — **3.2.1 Input your project name (Must Be Unique)**

Targets:
You uploaded 10 targets successfully!

ID	CHROM	CHROStart	CHREnd	NAME
0	4	113625548	113625548	rs10023113
1	4	101816093	101816093	rs10028805
2	8	126999952	126999952	rs1008908
3	8	128531703	128531703	rs10089218

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR Choose File No file chosen upload
- Coexist Ref.: Null OR Choose File No file chosen upload

3.2.2 Paste your target information

3.2.3 Upload

3.2.6 Select “Repeat(hg19)” in “skip area” drop-down menu

3.2.8 Start design

Start multiplex primer designing

3.2. Primer design with human hg19 reference

3.3 Skip self-defined sites and hg38 SNPs

There are sites (SNPs and other small variances), researchers need to skip for primer design. In this System, human SNPs are stored previously. If you want to skip sites for your own purpose, you can use this function. You can choose whether involved system human SNPs.

Project Name: hg38_test — **3.3.1 Input your project name (Must Be Unique)**

Targets:
You uploaded 10 targets successfully!

ID	CHROM	CHROStart	CHREnd	NAME
0	chr2	47463154	47463554	rs83751600
1	chr2	47468609	47468609	rs267657969
2	chr2	47800042	47800042	rs37300075
3	chr2	47806534	47806534	rs267608130

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR Choose File No file chosen upload
- Coexist Ref.: Null OR Choose File No file chosen upload

3.3.2 Paste your target information

3.3.3 Upload

3.3.6 Select “SNP_hg38” in “skip sites” drop-down menu

3.3.7 Add 5' tags

S' Tags:

Add Forward primers' 5' tag ATCTCGTGGCTCGT

Add Reverse primers' 5' tag TGATCCACACGCTC

3.3 Skip self-defined sites and hg38 SNPs

Input your project name (Must Be Unique) first. Then Paste your target information. Upload. Select “SNP_hg38” in “skip sites” drop-down menu. Click on the choose file

button, choose your own 3 column bed files. Click on open button. Click on attach button.

3.3.2 Paste your target information

3.3.3 Upload

3.3.1 Input your project name (Must Be Unique)

3.3 Skip self-defined sites and hg38 SNPs

Now you finished your own sites file uploading. In the drop-down menu, the name is your own sites file name and hg38 snp name. Keep other parameters as default. you can Start your primer design.

3.3.2 Paste your target information

3.3.3 Upload

3.3.1 Input your project name (Must Be Unique)

3.3.4 Upload

3.3.6 Start design

3.3.5 add 5' tags

3.3 Skip self-defined sites and hg38 SNPs

3.4 Only skip self-defined sites

There are sites (SNPs and other small variances), researchers need to skip for primer design. In this system, human SNPs are stored previously. If you want to skip sites for your own purpose, you can use this function. You can choose whether involved system human SNPs.

The screenshot shows the 'Filter Parameters' section of a software interface. Under the 'Skip Sites' heading, there is a dropdown menu with 'SITES' selected. The dropdown contains three options: 'SNP_hg38', 'SNP_hg19', and 'Null'. The 'Null' option is highlighted with a red arrow pointing to it. To the right of the dropdown, there is an 'OR' button followed by a 'Choose File' button and a 'No file chosen' message. Below this, another 'OR' button is followed by a 'Choose File' button and a 'No file chosen' message. On the far right, there are two 'Attach' buttons with example file paths: 'eg: chr10 72098085 72098086' and 'eg: chr10 72098085 72098186'.

3.4 Only skip self-defined sites

Select “Null” in “skip sites” drop-down menu. Click on the choose file button, choose your own 3 column bed files.

The screenshot shows the same 'Filter Parameters' interface as before, but now with a file dialog window open. The dialog shows a file named 'skipsites.bed' with the following content:

chr1	101	102
chr1	200	202
chr1	150	151
chr1	900	901

To the right of the dialog, a red note reads:

Uploaded file
Must be “bed”
format and smaller
than 3M. if your file
is bigger than 3M,
please contact with
admin.

3.4 Only skip self-defined sites

Click on open button. Click on attach button. Now you finished your own sites file uploading. In the drop-down menu, the name is your own sites file name. keep other parameters as default. you can Start your primer design.

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Dimer Filter: TM 40 °C
- Delete Unspecific Amplicons: 500 bp
- Unspecific Alignment Energy: DELTA DG 10000 kcal
- Skip Sites: SITES skipsites&Null OR Choose File skipsites.bed Attach eg: chr10 72098085 72098086
- Skip Area: AREAS Repeat(hg38) OR Choose File No file chosen Attach eg: chr10 72098085 72098186

Uploaded file
Must be “bed”
format and smaller
than 3M. if your file
is bigger than 3M,
please contact with
admin.

3.4 Only skip self-defined sites

3.5 Skip self-defined areas and hg38 repeat sequences

There are areas (repeats/tandem repeats/indels), researchers need to skip for primer design. In this system, human repeat sequence is stored previously. If you want to skip areas for your own purpose, you can use this function. You can choose whether involved system human repeat sequence.

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Dimer Filter: TM 40 °C
- Delete Unspecific Amplicons: 500 bp
- Unspecific Alignment Energy: DELTA DG 10000 kcal
- Skip Sites: SITES SNP_hg38 OR Choose File skipsites.bed Attach eg: chr10 72098085 72098086
- Skip Area: AREAS ✓ Repeat(hg38)
Repeat(hg19)
Null OR Choose File No file chosen Attach eg: chr10 72098085 72098186

3.5 Skip self-defined areas and hg38 repeat sequences

Select “Reapet(hg38)” in “skip areas” drop-down menu. Click on the choose file button, choose your own 3 column bed files.

The screenshot shows the software's filter parameters section. Under "Skip Area:", "AREAS" is selected, and the dropdown menu shows "Repeat(hg38)" as the chosen option. A file selection dialog is open, showing two files: "skiparea.bed" and "skipsites.bed". The "skiparea.bed" file is selected and its contents are displayed:

```

chr1 1 100
chr1 100 200
chr1 150 500
chr1 900 950

```

A red note on the right side of the dialog states: "Uploaded file Must be ‘bed’ format and smaller than 3M. if your file is bigger than 3M, please contact with admin."

3.5 Skip self-defined areas and hg38 repeat sequences

Click on open button. Click on attach button. Now you finished your own areas file uploading. In the drop-down menu, the name is your own areas file name and hg38 repeat name. Keep other parameters as default. you can Start your primer design.

The screenshot shows the software's filter parameters section. Under "Skip Area:", "AREAS" is selected, and the dropdown menu shows "skiparea&Repeat(hg38)" as the chosen option. A file selection dialog is open, showing two files: "skiparea.bed" and "skipsites.bed". The "skiparea.bed" file is selected and its contents are displayed:

```

chr1 1 100
chr1 100 200
chr1 150 500
chr1 900 950

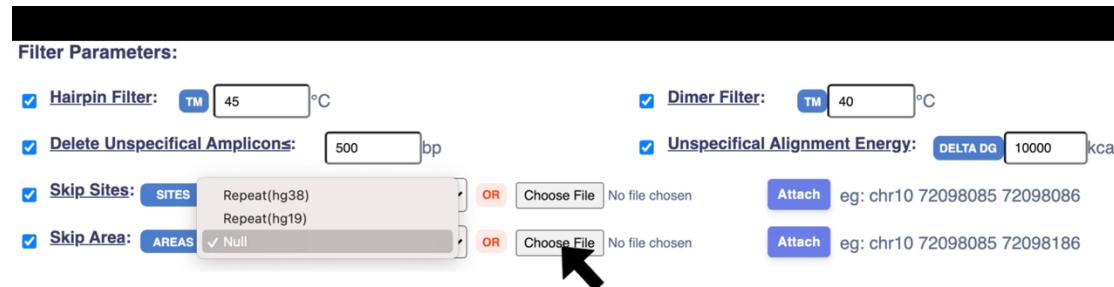
```

A red note on the right side of the dialog states: "Uploaded file Must be ‘bed’ format and smaller than 3M. if your file is bigger than 3M, please contact with admin."

3.5 Skip self-defined areas and hg38 repeat sequences

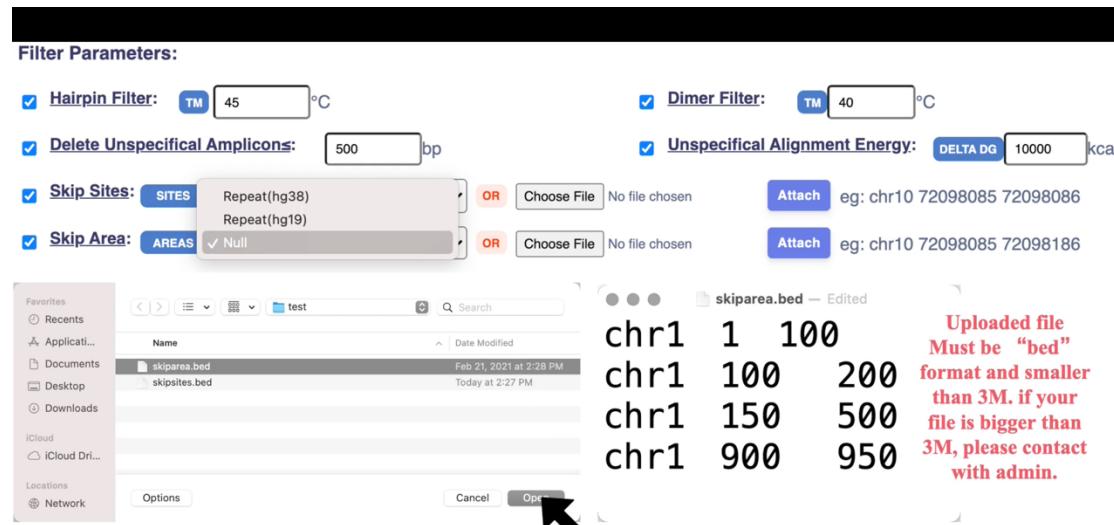
3.6 Only skip self-defined areas

There are areas (repeats/tandem repeats/indels), researchers need to skip for primer design. In this system, human repeat sequence is stored previously. If you want to skip areas for your own purpose, you can use this function. You can choose whether involved system human repeat sequence.



3.6 Only skip self-defined areas

Select “Null” in “skip areas” drop-down menu. Click on the choose file button, choose your own 3 column bed files.



3.6 Only skip self-defined areas

Click on open button. Click on attach button. Now you finished your own areas file uploading. In the drop-down menu, the name is your own areas file name. Keep other parameters as default. you can Start your primer design.

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Dimer Filter: TM 40 °C
- Delete Unspecifical Amplicons: 500 bp
- Unspecifical Alignment Energy: DELTA DG 10000 kcal
- Skip Sites: SITES SNP_hg38 OR Choose File No file chosen
- Attach eg: chr10 72098085 72098086
- Skip Area: AREAS skiparea&Null OR Choose File skiparea.bed
- Attach eg: chr10 72098085 72098186

Uploaded file
Must be "bed"
format and smaller
than 3M. if your
file is bigger than
3M, please contact
with admin.

3.6 Only skip self-defined areas

3.7 Self-defined genome reference (not human)

This website only uploaded human references previously. If you want design primers for plants, other animals and microbe, your reference sequence needs to upload first.

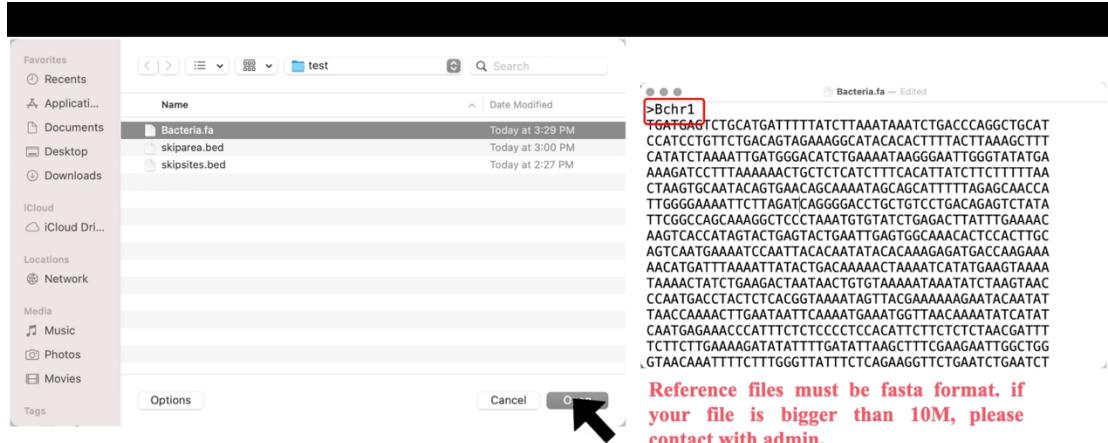
Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20
- Target Ref.: hg38 OR Choose File No fil...osen upload
- Coexist Ref.: Null OR Choose File No fil...osen upload

3.7. Self-defined genome reference

click on the choose file button of target reference, choose your own fasta formate file.

Click on open button. Click on upload button. Reference files must be fasta format. If your file is bigger than 10M, please contact with admin. Please make sure the chromosome names in Targets input box are in uploaded reference files. Now you finished your reference file uploading. Keep other parameters as default. you can Start your primer design.



3.7. Self-defined genome reference

Parameters:

<u>Product Size:</u>	<input type="text" value="MIN"/> 200	<input type="text" value="MAX"/> 300	
<u>Primer Size:</u>	<input type="text" value="MIN"/> 15	<input type="text" value="OPT"/> 22	<input type="text" value="MAX"/> 25
<u>Primer Tm:</u>	<input type="text" value="MIN"/> 57	<input type="text" value="OPT"/> 60	<input type="text" value="MAX"/> 63
<u>Primer GC%:</u>	<input type="text" value="MIN"/> 20	<input type="text" value="MAX"/> 80	
<u>Nums/Target:</u>	<input type="text" value="20"/>		

Target Ref.: Bacteria Choose File Bacteria.fa

Coexist Ref.: Null Choose File No fil...osen

Or input from file No file chosen

Targets:

```
>Bchr1
>Bchr1 1 100 Bsites01
>Bchr1 100 200 Bsites02
>Bchr1 150 500 Bsites03
>Bchr1 900 950 Bsites04
```

Please make sure the chromosome names in Targets input box are in uploaded reference files.

3.7. Self-defined genome reference

3.8 Self-defined coexist reference (Human gut microbiota primer design)

Researchers may need to design primers for several species in complex samples. There are similar sequences in different species genome. Unspecific amplicons and dimer between primers of different species need to be eliminated. You can use this function to define coexist reference.

Select desire reference in “coexist reference” drop-down menu or upload yours self-defined coexist reference file. Input other parameters according to your needs. And then you can Start your primer design.

The screenshot shows a software interface for primer design. On the left, a table displays four targets (Bsites01-04) with 'Bchr1' as the chromosome and coordinates ranging from 1 to 950. A red box highlights the 'Bchr1' column. Below the table are input fields for 'Choose File' and 'upload'. On the right, there are several parameter input fields with ranges and dropdown menus. A red box highlights the 'Coexist Ref.' dropdown menu, which lists 'hg38', 'hg19', 'Null', and 'Bacteria'. A large red box also highlights the text instructions: 'Select desire reference in "coexist reference" drop-down menu or upload yours self-defined coexist reference file.' At the bottom, a large teal banner displays the section title '3.8. Self-defined coexist reference'.

	CHR	CHRESTART	CHREND	NAME
0	Bchr1	1	100	Bsites01
1	Bchr1	100	200	Bsites02
2	Bchr1	150	500	Bsites03
3	Bchr1	900	950	Bsites04

Parameters:

- Product Size: MIN 200 MAX 300
- Primer Size: MIN 15 OPT 22 MAX 25
- Primer Tm: MIN 57 OPT 60 MAX 63
- Primer GC%: MIN 20 MAX 80
- Nums/Target: 20

Target Ref.: Bacteria OR Choose File No fil...osen upload

Coexist Ref.: hg38 hg19 Null Bacteria

Select desire reference in "coexist reference" drop-down menu or upload yours self-defined coexist reference file.

Filter Parameters:

- Hairpin Filter: TM 45 °C
- Dimer Filter: TM 40 °C
- Delete Unspecifical Amplicons: 500 bp
- Unspecifical Alignment Energy: DELTA DG 10000 kcal
- Skip Sites: SITES SNP_hg38 OR Choose File No file chosen Attach eg: chr10 72098085 72098086
- Skip Area: AREAS Repeat(hg38) OR Choose File No file chosen Attach eg: chr10 72098085 72098186

3.8. Self-defined coexist reference