PCR Primers Design

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Testing Primer Specificity

- Most important factor affecting PCR: Choosing **appropriate primers**
- To amplify exactly the intended target and avoiding undesired amplification
- Primers should not have **matches** to other targets in certain orientations and within certain distances

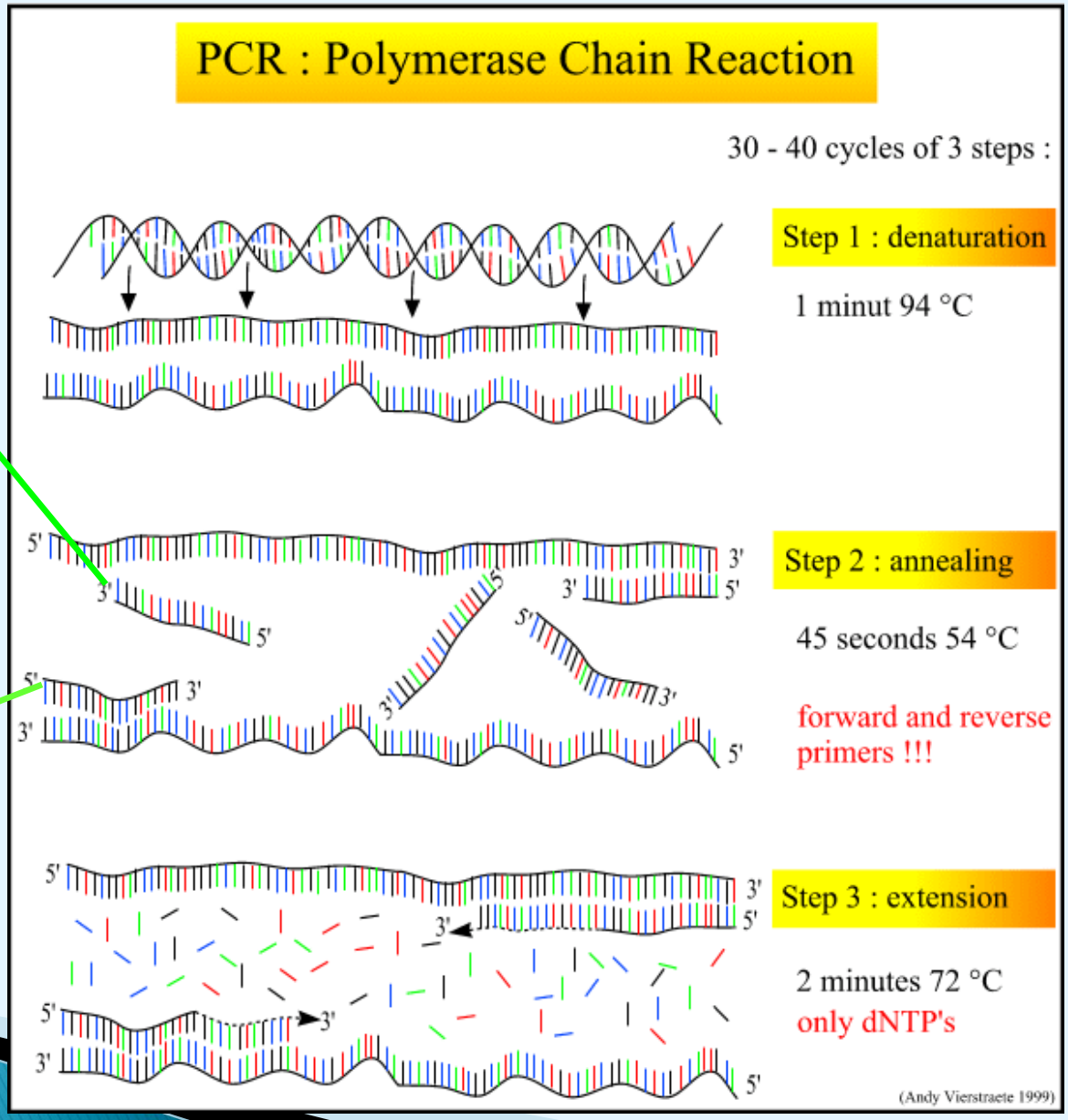
Process of designing specific primers:

- Generating primers flanking the target region (manually or using software tools)
- Searching primers against an appropriate nucleotide sequence database
  - to examine the potential targets
  - to examine many details between primers and targets, such as:
    - number and the positions of matched bases
    - primer orientations and distance between forward and reverse primers
- **Target specificity**: critical primer property

Primers dictate the successfulness of a PCR

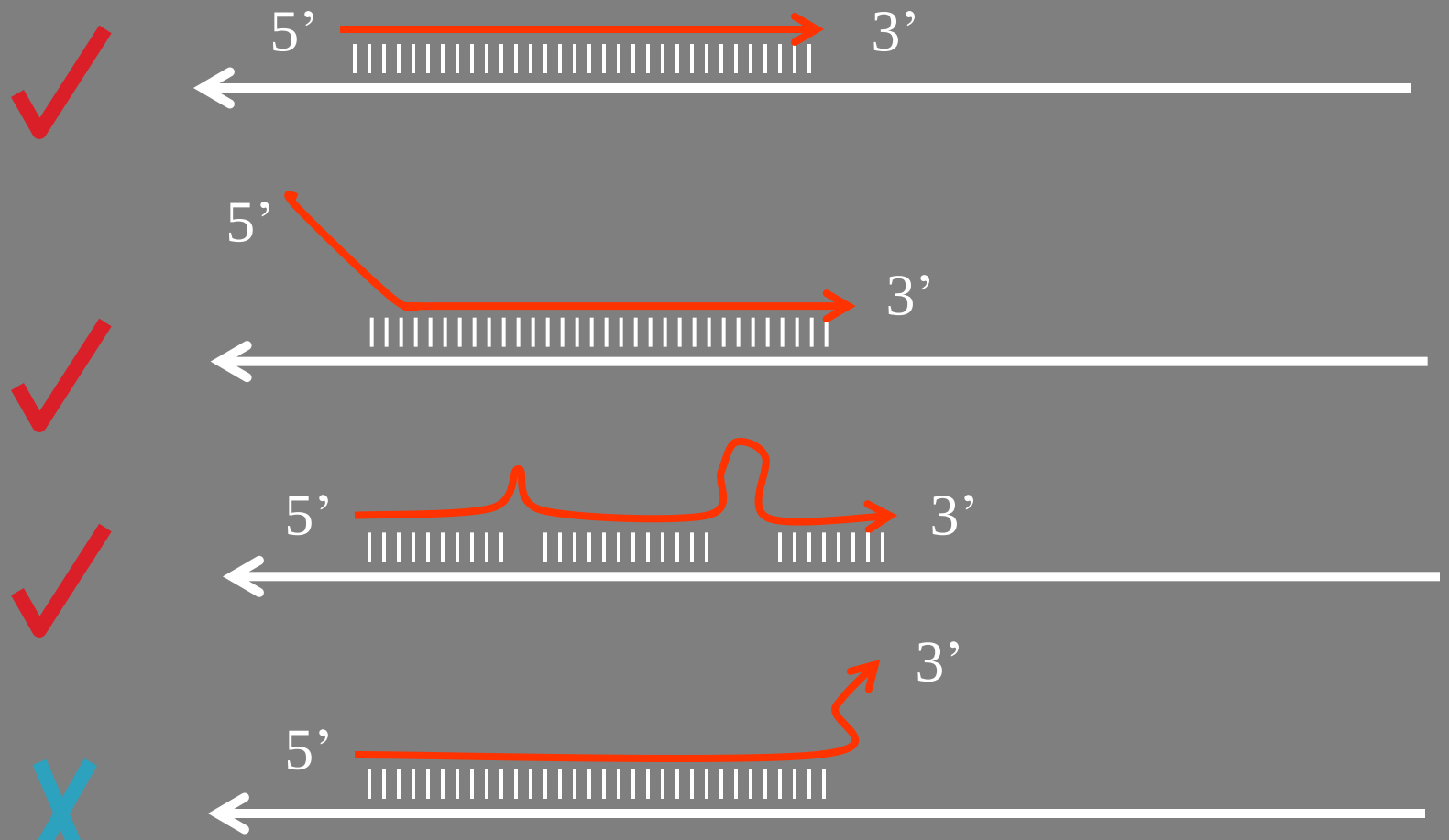
Specificity?

Proper annealing to the template?



- Mismatches towards the 3’ end affect target amplification much more than mismatches towards the 5’ end
- a two base mismatch at the 3’ end generally prevents amplification
- A single base mismatch (even at the very 3’ end), as well as a few mismatches in the middle or toward the 5’ end, still allows amplification, though at a reduced efficiency for some cases

When is a “primer” a primer?



Primer BLAST

Basic Local Alignment Search Tools

- Searching primers against an appropriate nucleotide sequence database
  - potential targets
  - number and the positions of matched bases
  - primer orientations and distance between forward and reverse primers
- time-consuming and very difficult task for users, especially when the primers have a large number of hits
- Similarity Searching using BLAST program has been widely used for primer target detection
  - to compare a query sequence against a sequence database

Now: Primer Blast

- to design target-specific primers for PCR

- **In-Silico PCR** and **Reverse ePCR** **vs** **Primer Blast**:
  - Do not design primers
  - Not sensitive enough
    - to detect targets with a significant number of mismatches (potentially amplifiable)

Primer-BLAST

- incorporates BLAST with a global alignment algorithm
- a full primer-target alignment
- sensitive enough to detect targets that have a significant number of mismatches to primers
- design new target-specific primers in one step
- check the specificity of pre-existing primers
- very sensitive in detecting potential amplification targets
- capability to place primers based on exon/intron boundaries
- and excluding SNP sites in primers

http://www.ncbi.nlm.nih.gov/tools/primer-blast

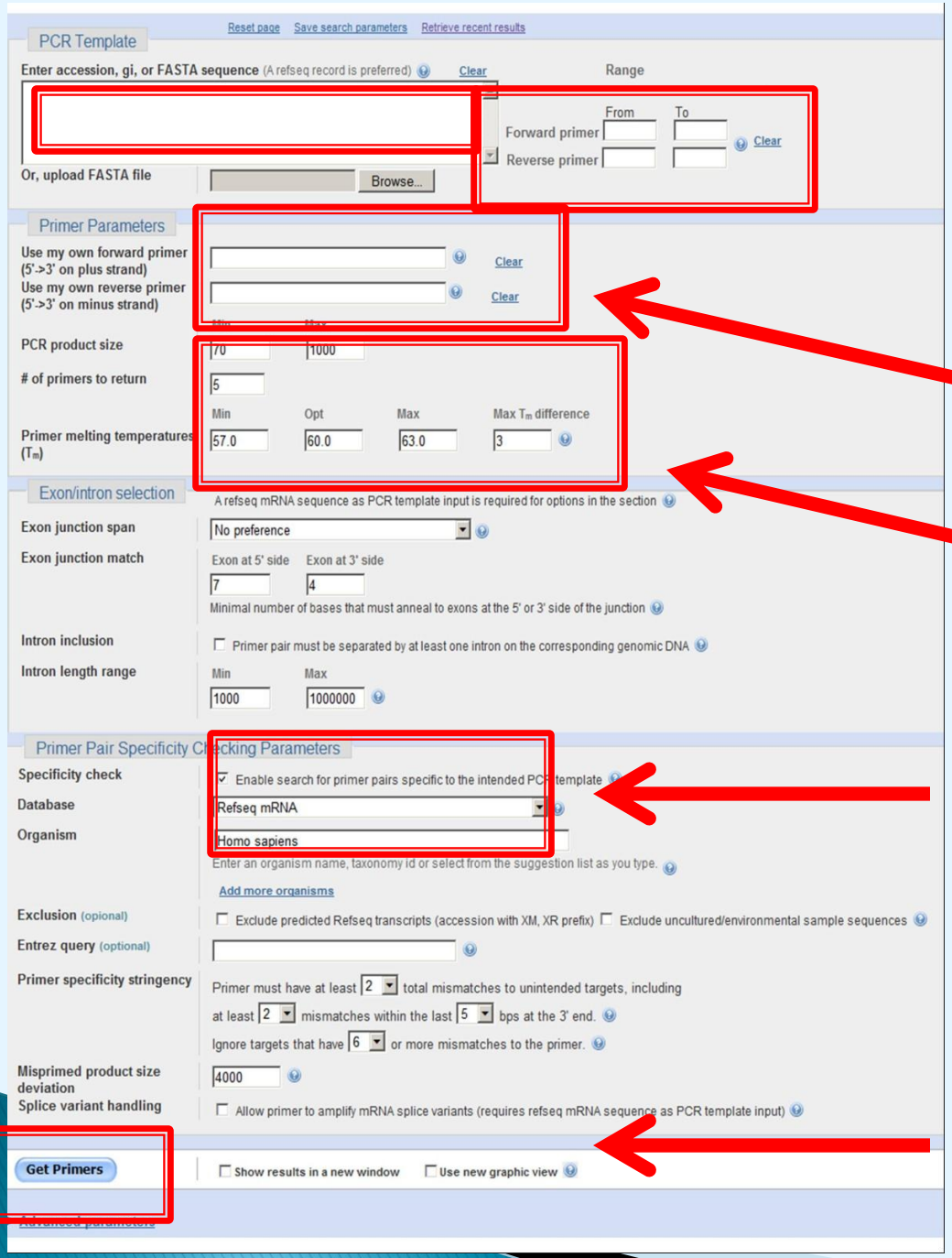
highly sensitive default parameters to find seq potentially amplifiable in PCR

- Detect a target with up to 35% mismatches to the primer sequence
- default BLAST expect value cutoff is 30,000 for the primer-only case (3000 times higher than the standard BLAST program default)
- the higher the expect value cutoff, the more sensitive the search
- word size of seven (standard BLAST uses 11),
- 50,000 for the maximum number of database sequences (standard BLAST uses 250)
- 1 for match reward to mismatch penalty ratio (standard BLAST uses 1.5)

Check primers specificity

- Go to the Primer BLAST submission form.
- Enter one or both primer sequences in the Primer Parameters section of the form
  - If only one primer is available, a template sequence is also required
- In the Primer Pair Specificity Checking Parameters section, select the appropriate source Organism and the smallest Database that is likely to contain the target sequence.
  - For broadest coverage, choose the nr database and do not specify an organism.
- Click the "Get Primers" button to submit the search and retrieve template and specificity information.

Primer BLAST submission form.

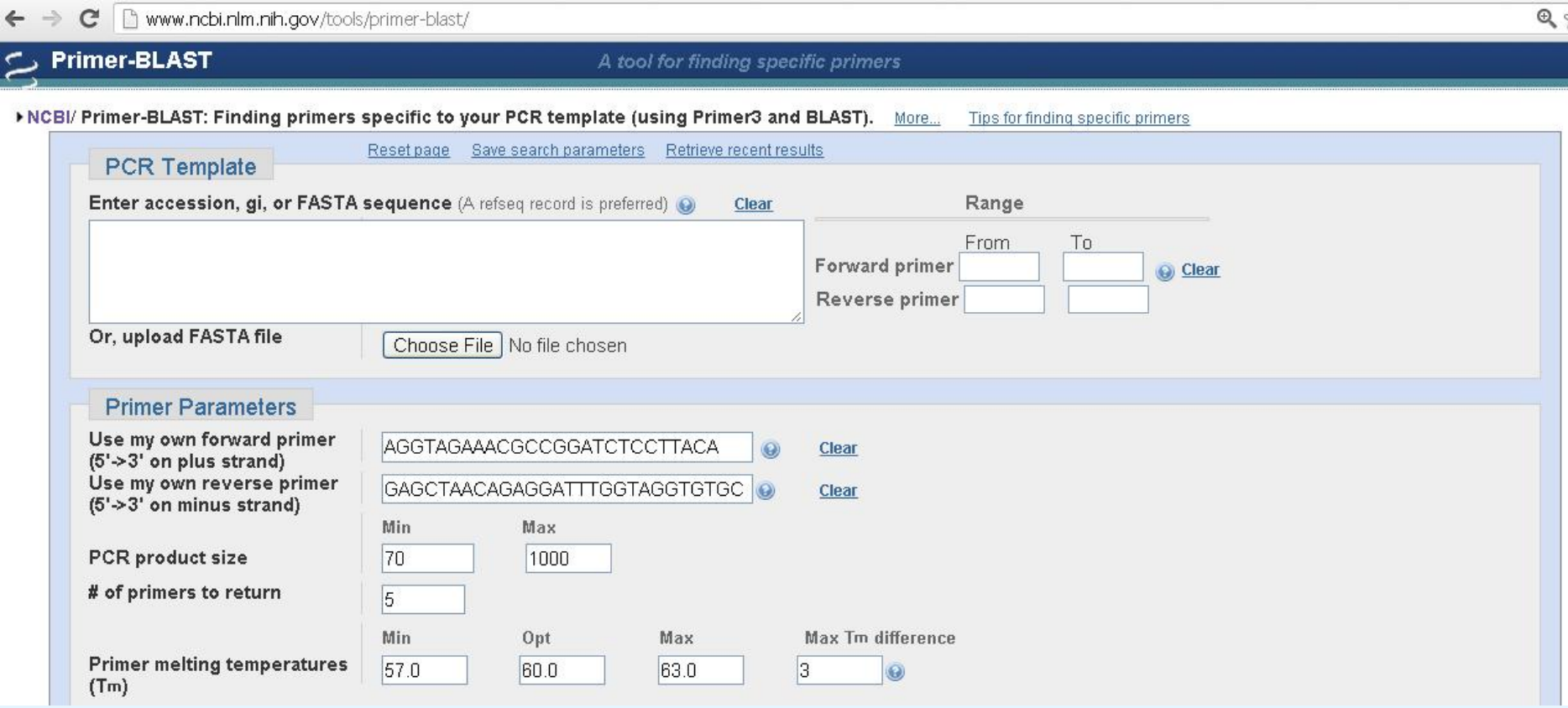


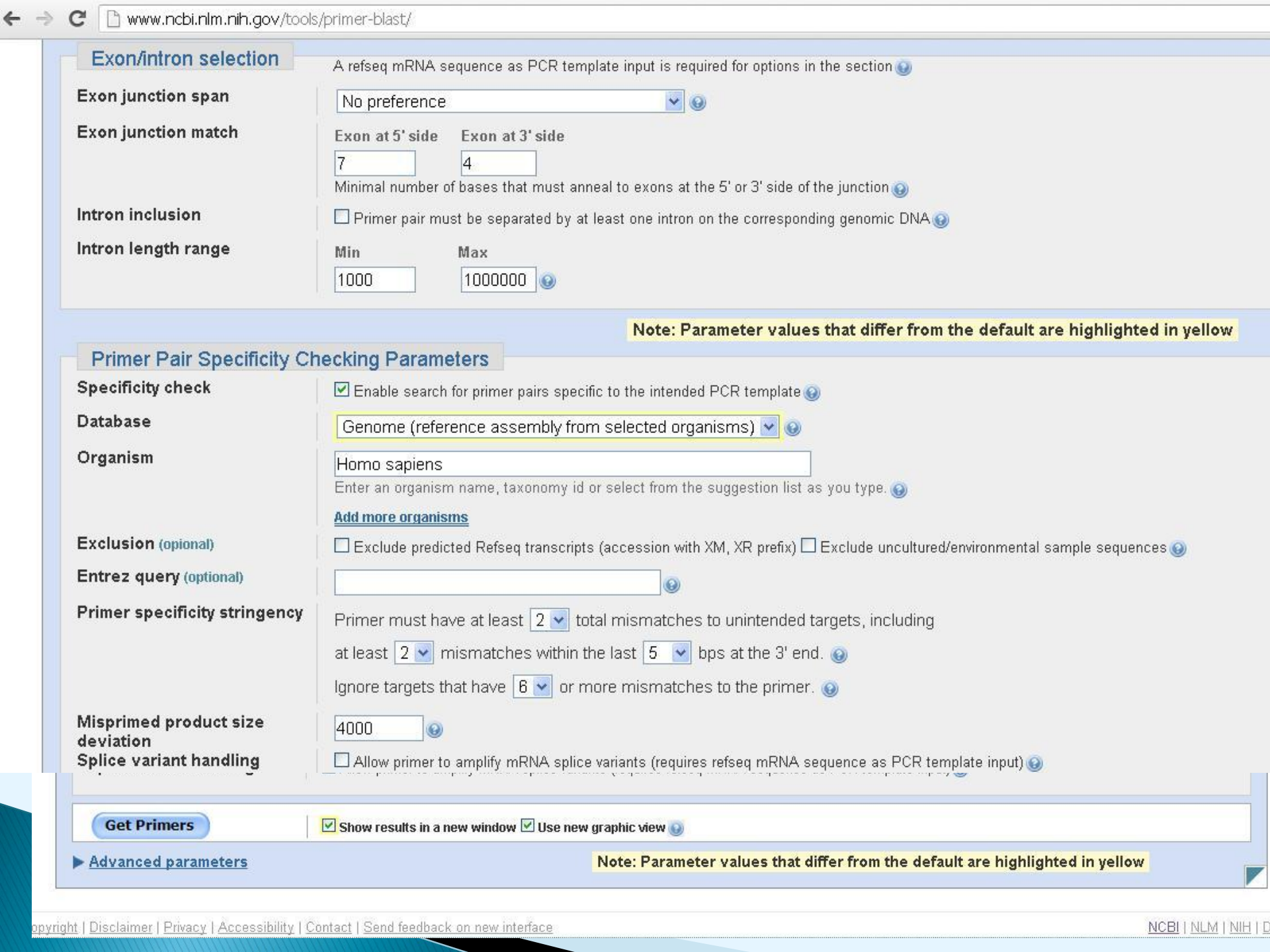
Enter one or both primer sequences in the Primer Parameters section of the form

Primer Pair Specificity Checking Parameters section

Selecting the appropriate source Organism and the smallest Database that is likely to contain the target sequence.

Click the "Get Primers" button to submit the search and retrieve template and specificity information


www.ncbi.nlm.nih.gov/tools/primer-blast/
Primer-BLAST
A tool for finding specific primers
NCBI/ Primer-BLAST: Finding primers specific to your PCR template (using Primer3 and BLAST).
More...
Tips for finding specific primers
Reset page
Save search parameters
Retrieve recent results
PCR Template
Enter accession, gi, or FASTA sequence (A refseq record is preferred)
Clear
Range
From
To
Forward primer
Clear
Reverse primer
Or, upload FASTA file
Choose File
No file chosen
Primer Parameters
Use my own forward primer (5'->3' on plus strand)
AGGTAGAAACGCCGGATCTCCTTACA
Clear
Use my own reverse primer (5'->3' on minus strand)
GAGCTAACAGAGGATTTGGTAGGTGTGC
Clear
Min
Max
PCR product size
70
1000
# of primers to return
5
Min
Opt
Max
Max Tm difference
Primer melting temperatures (Tm)
57.0
60.0
63.0
3


www.ncbi.nlm.nih.gov/tools/primer-blast/
Exon/intron selection
A refseq mRNA sequence as PCR template input is required for options in the section
Exon junction span
No preference
Exon junction match
Exon at 5' side
Exon at 3' side
7
4
Minimal number of bases that must anneal to exons at the 5' or 3' side of the junction
Intron inclusion
Primer pair must be separated by at least one intron on the corresponding genomic DNA
Intron length range
Min
Max
1000
1000000
Note: Parameter values that differ from the default are highlighted in yellow
Primer Pair Specificity Checking Parameters
Specificity check
Enable search for primer pairs specific to the intended PCR template
Database
Genome (reference assembly from selected organisms)
Organism
Homo sapiens
Enter an organism name, taxonomy id or select from the suggestion list as you type.
Add more organisms
Exclusion (opional)
Exclude predicted Refseq transcripts (accession with XM, XR prefix)
Exclude uncultured/environmental sample sequences
Entrez query (optional)
Primer specificity stringency
Primer must have at least 2 total mismatches to unintended targets, including
at least 2 mismatches within the last 5 bps at the 3' end.
Ignore targets that have 6 or more mismatches to the primer.
Misprimed product size deviation
4000
Splice variant handling
Allow primer to amplify mRNA splice variants (requires refseq mRNA sequence as PCR template input)
Get Primers
Show results in a new window
Use new graphic view
Advanced parameters
Note: Parameter values that differ from the default are highlighted in yellow
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NCBI | NLM | NIH | D

Checking the specificity of pre-existing primers

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	GTAGGACTGCTCAGTTCAAACAT	23	52.81	43.48	6.00	2.00
Reverse primer	ACAGTTACTACACCCGTAAGGC	22	53.90	50.00	5.00	2.00

Products on target templates

>NM_001098494.1 Homo sapiens zinc finger protein 419 (ZNF419), transcript variant 5, mRNA

```
product length = 444
Forward primer  1    GTAGGACTGCTCAGTTCAAACAT  23
Template        493  ......................  515

Reverse primer  1    ACAGTTACTACACCCGTAAGGC  22
Template        936  ......................  915


product length = 780
Forward primer  1    GTAGGACTGCTCAGTTCAAACAT  23
Template        493  ......................  515

Reverse primer  1     ACAGTTACTACACCCGTAAGGC  22
Template        1272  .....CG..G...TT.......  1251
```

>NM_001098491.1 Homo sapiens zinc finger protein 419 (ZNF419), transcript variant 1, mRNA

```
product length = 444
Forward primer  1    GTAGGACTGCTCAGTTCAAACAT  23
Template        592  ......................  614

Reverse primer  1     ACAGTTACTACACCCGTAAGGC  22
Template        1035  ......................  1014


product length = 780
Forward primer  1    GTAGGACTGCTCAGTTCAAACAT  23
Template        592  ......................  614

Reverse primer  1     ACAGTTACTACACCCGTAAGGC  22
Template        1371  .....CG..G...TT.......  1350
```

>NM_198542.1 Homo sapiens zinc finger protein 773 (ZNF773), mRNA

```
product length = 444
Forward primer  1    GTAGGACTGCTCAGTTCAAACAT  23
Template        453  ....A.G...CA.........G.  475

Reverse primer  1    ACAGTTACTACACCCGTAAGGC  22
Template        896  .....C.........A......  875
```

- Default specificity parameters
- Perfect matches to the ZNF419 gene transcript variant 5 and would generate a 444 base amplicon.
- Some other potential amplicons:
  - 780 base amplicon: in ZNF419 transcript
  - 444 base amplicon: a different gene (i.e., human zinc finger protein 773, Genbank accession NM_198542.1)
  - However, there are up to 5 mismatches between at least one of the primers and the targets, which is probably sufficient to prevent amplification interference or non-specific amplification
- Users can analyze this result and make judgment based on their own experimental experiences.

Comparison to other primer design tools

- offers the ability to specify the **number of mismatches** that a specific primer pair must have to unintended targets
- custom 3’ end region: number of mismatches must be present
- the specificity of a primer is typically judged by the number of mismatches it has to unintended targets
  - higher number of mismatches offer more specificity
  - the locations of such mismatches (mismatches closer to 3’ end offer more specificity)
- place primers on different exons (i.e., to span an intron) to avoid amplification of genomic DNA
- customization of the number of nucleotide matches on either side of an exon/exon junction
- Primer-BLAST presents detailed alignments between the primers and targets found

- high detection sensitivity
- capable of detecting potential amplification targets that have up to 5 mismatches to a primer
- by using highly sensitive BLAST parameters
- Primer-BLAST (with default parameters) will miss any targets that have 6 or fewer consecutive matches to a primer (since Primer-BLAST uses a word size of 7 by default).
  - For example, if a target has mismatches to a primer of 20 bases at positions 7 and 14 (assuming the 5' end is position one), the target will be missed by Primer-BLAST (with default parameters) even though it has only 2 mismatches.
  - Assuming a random distribution of mismatch locations, it is possible to calculate the number of possible arrangements of 18 matches and 2 mismatches.
  - There are 20*19 different ways to place the 2 mismatches among the 18 matches, but only 2 of these result in a word size shorter than 7, so the probability of missing a target with 2 mismatches to a primer of 20 bases is 2/(20*19) or about 0.5%.

www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1361705656&job_key=JSID_01_88396_130.14.22.21_9003

Primer-BLAST *Primer-Blast results*

NCBI/ Primer-BLAST : results: Job id=JSID_01_88396_130.14.22.21_9003 more...

Input PCR template none

Specificity of primers Target templates were found in selected database: Genome database (reference assembly only) for selected species (Organism limited to Homo sapiens)

Other reports Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGGTAGAAACGCCGGATCTCCTTACA	26	65.17	50.00	7.00	1.00
Reverse primer	GAGCTAACAGAGGATTTGGTAGGTGTGC	28	65.25	50.00	5.00	2.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 241
Features associated with this product:
   cytochrome P450 2C9 precursor

Forward primer  1         AGGTAGAAACGCCGGATCTCCTTACA  26
Template        47506464  ...G.................C..  47506439

Reverse primer  1         GAGCTAACAGAGGATTTGGTAGGTGTGC  28
Template        47506224  ............................  47506251
```

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGGTAGAAACGCCGGATCTCCTTACA	26	65.17	50.00	7.00	1.00
Reverse primer	GAGCTAACAGAGGATTTGGTAGGTGTGC	28	65.25	50.00	5.00	2.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 241
Features associated with this product:
   cytochrome P450 2C9 precursor

Forward primer  1         AGGTAGAAACGCCGGATCTCCTTACA  26
Template        47506464  ...G..................C..  47506439

Reverse primer  1         GAGCTAACAGAGGATTTGGTAGGTGTGC  28
Template        47506224  ............................  47506251


product length = 239
Features associated with this product:
   cytochrome P450 2C19 precursor

Forward primer  1         AGGTAGAAACGCCGGATCTCCTTACA  26
Template        47339662  ...G..................C..  47339637

Reverse primer  1         GAGCTAACAGAGGATTTGGTAGGTGTGC  28
Template        47339424  ............................  47339451
```

www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi

Primer-BLAST *Primer-Blast results*

NCBI/ Primer-BLAST : results: Job id=JSID_01_88405_130.14.22.21_9003 more...

Input PCR template	none
Specificity of primers	Target templates were found in selected database: Genome database (reference assembly only) for selected species (Organism limited to Homo sapiens)
Other reports	▶Search Summary

▼ Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGGTAGAAACGCCGGATCTCCTTACA	26	65.17	50.00	7.00	1.00
Reverse primer	GTGTGTGCCTCTTTGATGGATAAA	24	59.54	41.67	4.00	4.00

Products on target templates

>NT_022778.16 Homo sapiens chromosome 4 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 733
Features flanking this product:
   64708 bp at 5' side: trans-2,3-enoyl-CoA reductase-like
   849323 bp at 3' side: ephrin type-A receptor 5 isoform b precursor

Reverse primer  1        GTGTGTGCCTCTTTGATGGATAAA  24
Template        5551175  ..A.A.....T..A..A.......  5551152

Reverse primer  1        GTGTGTGCCTCTTTGATGGATAAA  24
Template        5550443  ..A.A.....T..A..A.......  5550466
```

NCBI/ Primer-BLAST : results: Job id=JSID_01_88578_130.14.18.128_9003 more...

Input PCR template	none
Specificity of primers	Target templates were found in selected database: Genome database (reference assembly only) for selected species (Organism limited to Homo sapiens)
Other reports	Search Summary

Detailed primer reports

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGAAGGAGCATATAGTGGGCCTAGGT	26	64.38	50.00	6.00	4.00
Reverse primer	GTGTGTGCCTCTTTGATGGATAAA	24	59.54	41.67	4.00	4.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 60
Features flanking this product:
   27127 bp at 5' side: cytochrome P450 2C18 isoform 2 precursor
   76 bp at 3' side: cytochrome P450 2C19 precursor

Forward primer  1         AGAAGGAGCATATAGTGGGCCTAGGT  26
Template        47326791  .........................  47326816

Reverse primer  1         GTGTGTGCCTCTTTGATGGATAAA  24
Template        47326850  ........................  47326827
```

```
product length = 60
Features flanking this product:
   85636 bp at 5' side: cytochrome P450 2C19 precursor
   74 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         AGAAGGAGCATATAGTGGGCCTAGGT  26
Template        47502770  ..................A.......  47502795

Reverse primer  1         GTGTGTGCCTCTTTGATGGATAAA  24
Template        47502829  ........................  47502806
```

>NT_022778.16 Homo sapiens chromosome 4 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 733
Features flanking this product:
   64708 bp at 5' side: trans-2,3-enoyl-CoA reductase-like
   849323 bp at 3' side: ephrin type-A receptor 5 isoform b precursor

Reverse primer  1        GTGTGTGCCTCTTTGATGGATAAA  24
Template        5551175  ..A.A.....T..A..A.......  5551152

Reverse primer  1        GTGTGTGCCTCTTTGATGGATAAA  24
Template        5550443  ..A.A.....T..A..A.......  5550466
```

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	AGAAGGAGCATATAGTGGGCCTAGGT	26	64.38	50.00	6.00	4.00
Reverse primer	TATCTGTAGGATATTTCCAATCACTGGGA	29	61.32	37.93	6.00	5.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 271
Features associated with this product:
   cytochrome P450 2C19 precursor

Forward primer  1         AGAAGGAGCATATAGTGGGCCTAGGT  26
Template        47326791  .........................  47326816

Reverse primer  1         TATCTGTAGGATATTTCCAATCACTGGGA  29
Template        47327061  ............................  47327033


product length = 269
Features associated with this product:
   cytochrome P450 2C9 precursor

Forward primer  1         AGAAGGAGCATATAGTGGGCCTAGGT  26
Template        47502770  ..................A.......  47502795

Reverse primer  1         TATCTGTAGGATATTTCCAATCACTGGGA  29
Template        47503038  ............................  47503010
```

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTTCAAATGGGAAAAGGGAGACCCTGG	27	65.12	48.15	6.00	3.00
Reverse primer	TTCTCATGAGCATCTCTGGGGCTGTTTTCC	30	68.26	50.00	8.00	0.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 329
Features flanking this product:
   26264 bp at 5' side: cytochrome P450 2C18 isoform 2 precursor
   670 bp at 3' side: cytochrome P450 2C19 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47326256  .........................    47326230

Reverse primer  1         TTCTCATGAGCATCTCTGGGGCTGTTTTCC  30
Template        47325928  ............................    47325957


product length = 290
Features flanking this product:
   84809 bp at 5' side: cytochrome P450 2C19 precursor
   671 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47502232  .........................    47502206

Reverse primer  1         TTCTCATGAGCATCTCTGGGGCTGTTTTCC  30
Template        47501943  ...................T.......C..    47501972
```


Primer-BLAST
Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=JSID_01_88610_130.14.18.128_9003
more...
Please correct the following errors: Specified right primer exceeds built-in maximum of 36.

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTTCAAATGGGAAAAGGGAGACCCTGG	27	65.12	48.15	6.00	3.00
Reverse primer	TTCTCATGAGCATCTCTGGG	20	56.70	50.00	8.00	4.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 329
Features flanking this product:
   26264 bp at 5' side: cytochrome P450 2C18 isoform 2 precursor
   670 bp at 3' side: cytochrome P450 2C19 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47326256  .........................    47326230

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        47325928  ....................  47325947


product length = 290
Features flanking this product:
   84809 bp at 5' side: cytochrome P450 2C19 precursor
   671 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47502232  .........................    47502206

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        47501943  ...................T  47501962
```

```
product length = 295
Features flanking this product:
   20855 bp at 5' side: cytochrome P450 2C19 precursor
   64620 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47437989  .................T..G.T....  47438015

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        47438283  .....T.............T  47438264
```

>NT_011512.11 Homo sapiens chromosome 21 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 4870
Features flanking this product:
   195895 bp at 5' side: protein C-ets-2 isoform 1
   151939 bp at 3' side: proteasome assembly chaperone 1 isoform a

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        26057447  C.......G..T...G....  26057428

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        26052578  .G...C.A............  26052597
```

>NT_008183.19 Homo sapiens chromosome 8 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 1041
Features flanking this product:
   17127 bp at 5' side: hydroxyacid-oxoacid transhydrogenase, mitochondrial
   7130 bp at 3' side: uncharacterized protein C8orf46
```

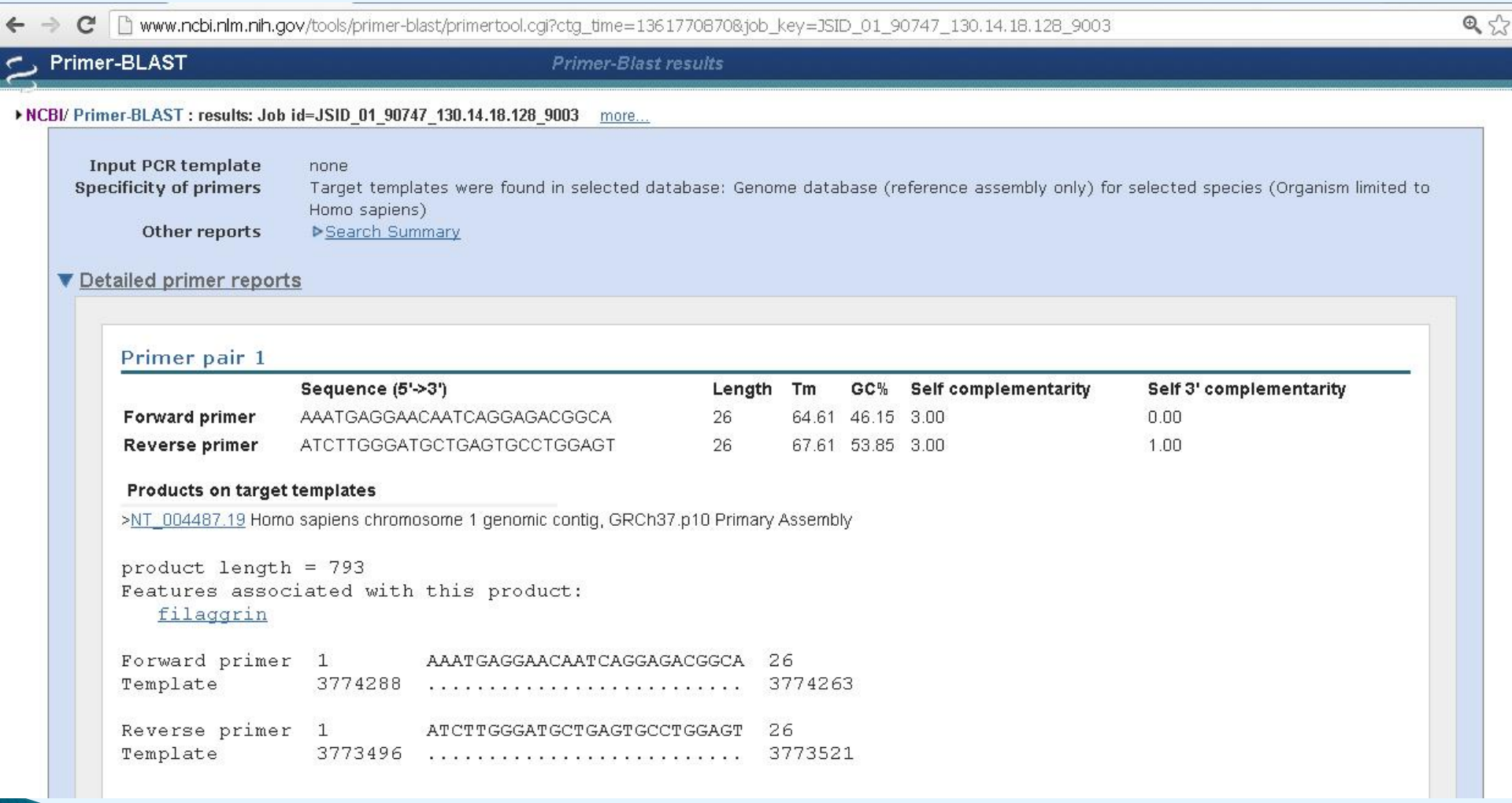
```
Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        47438283  .....T.............T  47438264
```

>NT_011512.11 Homo sapiens chromosome 21 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 4870
Features flanking this product:
   195895 bp at 5' side: protein C-ets-2 isoform 1
   151939 bp at 3' side: proteasome assembly chaperone 1 isoform a

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        26057447  C.......G..T...G....  26057428

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        26052578  .G...C.A............  26052597
```

>NT_008183.19 Homo sapiens chromosome 8 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 1041
Features flanking this product:
   17127 bp at 5' side: hydroxyacid-oxoacid transhydrogenase, mitochondrial
   7130 bp at 3' side: uncharacterized protein C8orf46

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        19263154  GCAG...C............  19263135

Reverse primer  1         TTCTCATGAGCATCTCTGGG  20
Template        19262114  AA.A..........C....T  19262133
```


www.ncbi.nlm.nih.gov/tools/primer-blast/primertool.cgi?ctg_time=1361770870&job_key=JSID_01_90747_130.14.18.128_9003
Primer-BLAST
Primer-Blast results
NCBI/ Primer-BLAST : results: Job id=JSID_01_90747_130.14.18.128_9003 more...
Input PCR template none
Specificity of primers Target templates were found in selected database: Genome database (reference assembly only) for selected species (Organism limited to Homo sapiens)
Other reports Search Summary
Detailed primer reports
Primer pair 1
Sequence (5'->3') Length Tm GC% Self complementarity Self 3' complementarity
Forward primer AAATGAGGAACAATCAGGAGACGGCA 26 64.61 46.15 3.00 0.00
Reverse primer ATCTTGGGATGCTGAGTGCCTGGAGT 26 67.61 53.85 3.00 1.00
Products on target templates
>NT_004487.19 Homo sapiens chromosome 1 genomic contig, GRCh37.p10 Primary Assembly
product length = 793
Features associated with this product:
filaggrin
Forward primer 1 AAATGAGGAACAATCAGGAGACGGCA 26
Template 3774288 3774263
Reverse primer 1 ATCTTGGGATGCTGAGTGCCTGGAGT 26
Template 3773496 3773521

```
product length = 4684
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3774288  .........................  3774263

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3769605  C........................C  3769630


product length = 1765
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3774288  .........................  3774263

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3772524  C........C..............C  3772549


product length = 3709
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3774288  .........................  3774263

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3770580  C........C..............C  3770605
```

```
product length = 1765
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3771369  ..........................T  3771344

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3769605  C........................C  3769630


product length = 3709
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3771369  ..........................T  3771344

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3767661  G........................C  3767686


product length = 790
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3771369  ..........................T  3771344

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3770580  C........C..............C  3770605
```

```
product length = 4681
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3771369  .........................T  3771344

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3766689  G.A.......................C  3766714


product length = 2737
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3771369  .........................T  3771344

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3768633  C........C.T............C  3768658


product length = 790
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3767478  T.........................T  3767453

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3766689  G.A.......................C  3766714
```

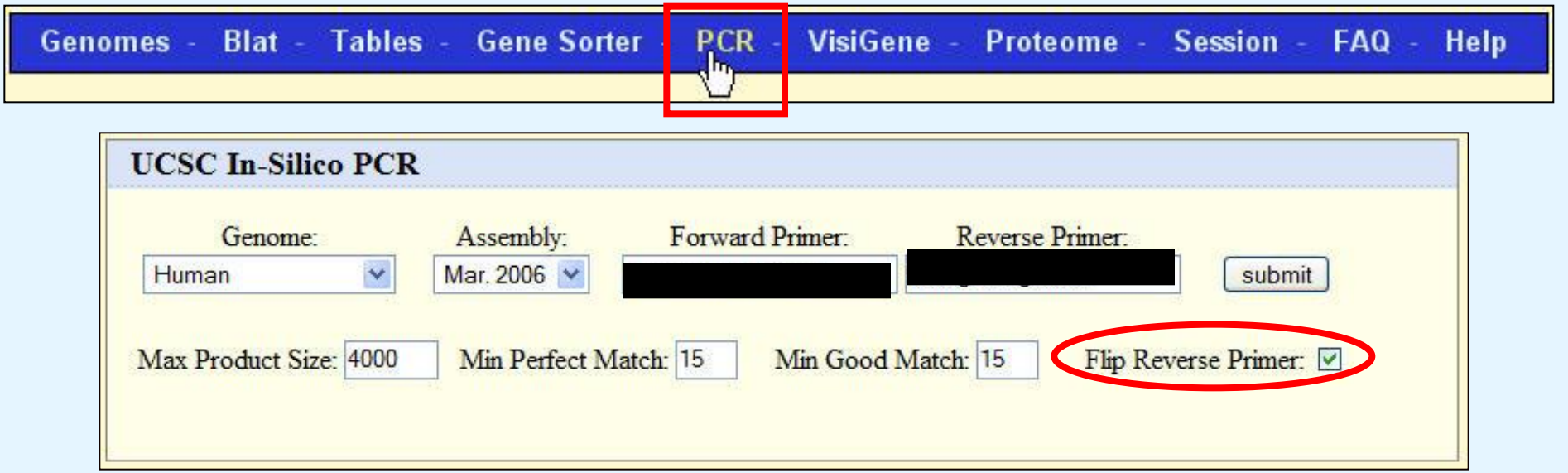
```
product length = 1762
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3767478  T.........................T  3767453

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3765717  C........C..............C  3765742


product length = 2734
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3767478  T.........................T  3767453

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3764745  C........C..............C  3764770


product length = 3709
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3773313  T..............A........T  3773288

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3769605  C.........................C  3769630
```

```
product length = 793
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3770397  T......A.................T  3770372

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3769605  C........................C  3769630


product length = 2737
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3770397  T......A.................T  3770372

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3767661  G........................C  3767686


product length = 790
Features associated with this product:
   filaggrin

Forward primer  1        AAATGAGGAACAATCAGGAGACGGCA  26
Template        3773313  T..............A........T  3773288

Reverse primer  1        ATCTTGGGATGCTGAGTGCCTGGAGT  26
Template        3772524  C........C..............C  3772549
```

UCSC In Silico PCR

Testing Primer Specificity

In-Silico PCR:

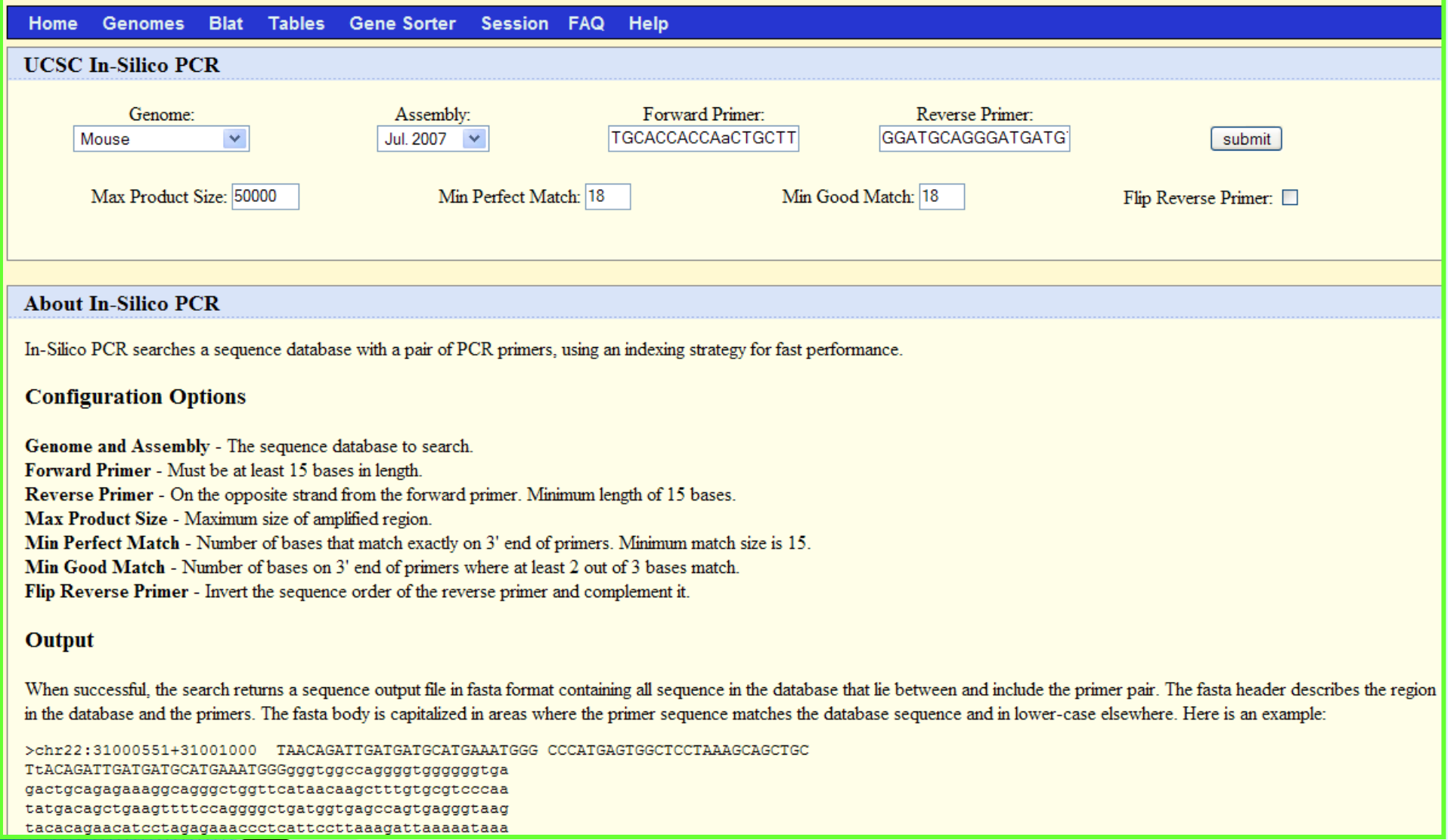


- Select genome
- Enter primers
- Minimum 15 bases
- Flip reverse primer?
- Submit

Configuration Options

Genome and Assembly - The sequence database to search.
Forward Primer - Must be at least 15 bases in length.
Reverse Primer - On the opposite strand from the forward primer. Minimum length of 15 bases.
Max Product Size - Maximum size of amplified region.
Min Perfect Match - Number of bases that match exactly on 3' end of primers. Minimum match size is 15.
Min Good Match - Number of bases on 3' end of primers where at least 2 out of 3 bases match.
Flip Reverse Primer - Invert the sequence order of the reverse primer and complement it.

(note: the tool does not handle ambiguous bases at this time—don't use Ns)



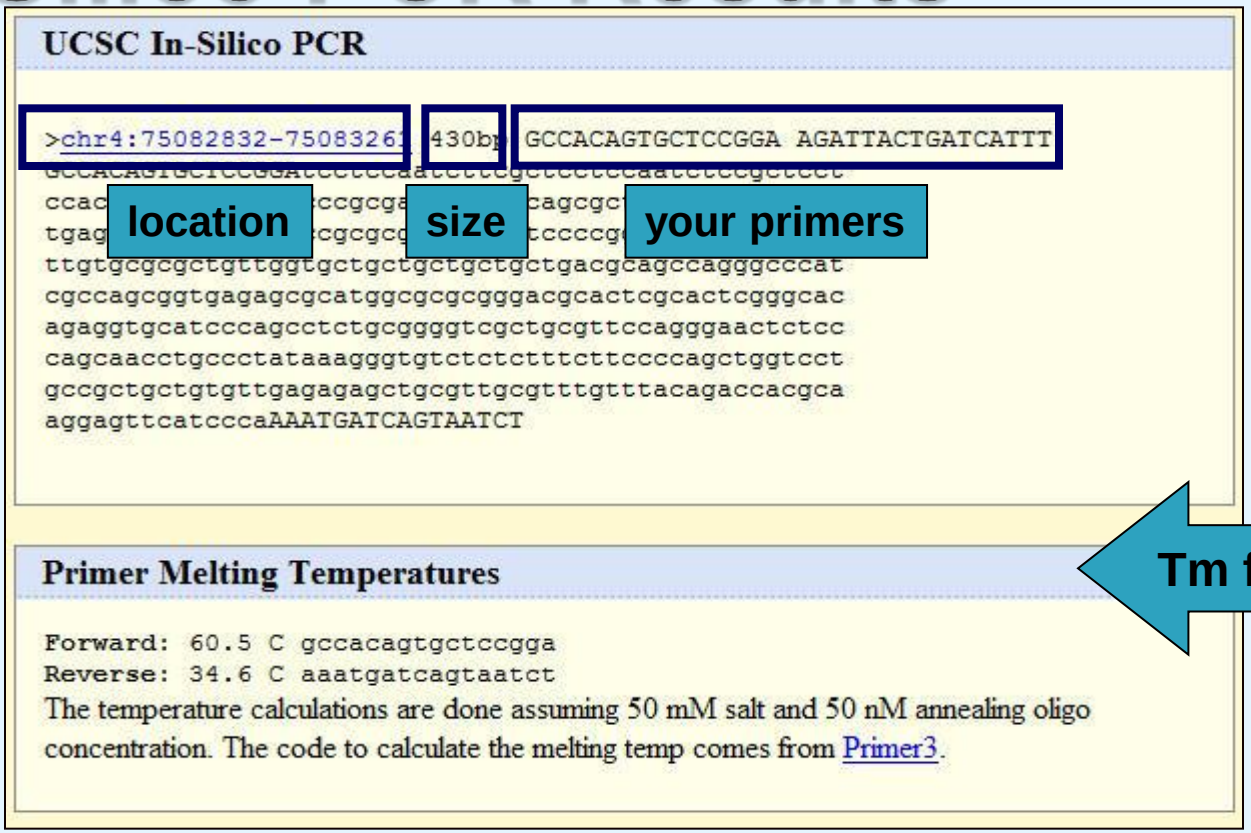
http://genome.ucsc.edu/cgi-bin/hgPcr?db=mm9

About In-Silico PCR

- In-Silico PCR searches a sequence database with a pair of PCR primers, using an indexing strategy for fast performance.
- **Configuration Options**
  - **Genome and Assembly:** The sequence database to search
  - **Forward Primer:** Must be at least 15 bases in length
  - **Reverse Primer:** On the opposite strand from the forward primer. Minimum length of 15 bases.
  - **Max Product Size**
  - **Min Perfect Match:** Number of bases that match exactly on 3' end of primers. Minimum match size is 15.
  - **Min Good Match:** Number of bases on 3' end of primers where at least 2 out of 3 bases match.
  - **Flip Reverse Primer -** Invert the sequence order of the reverse primer and complement it.

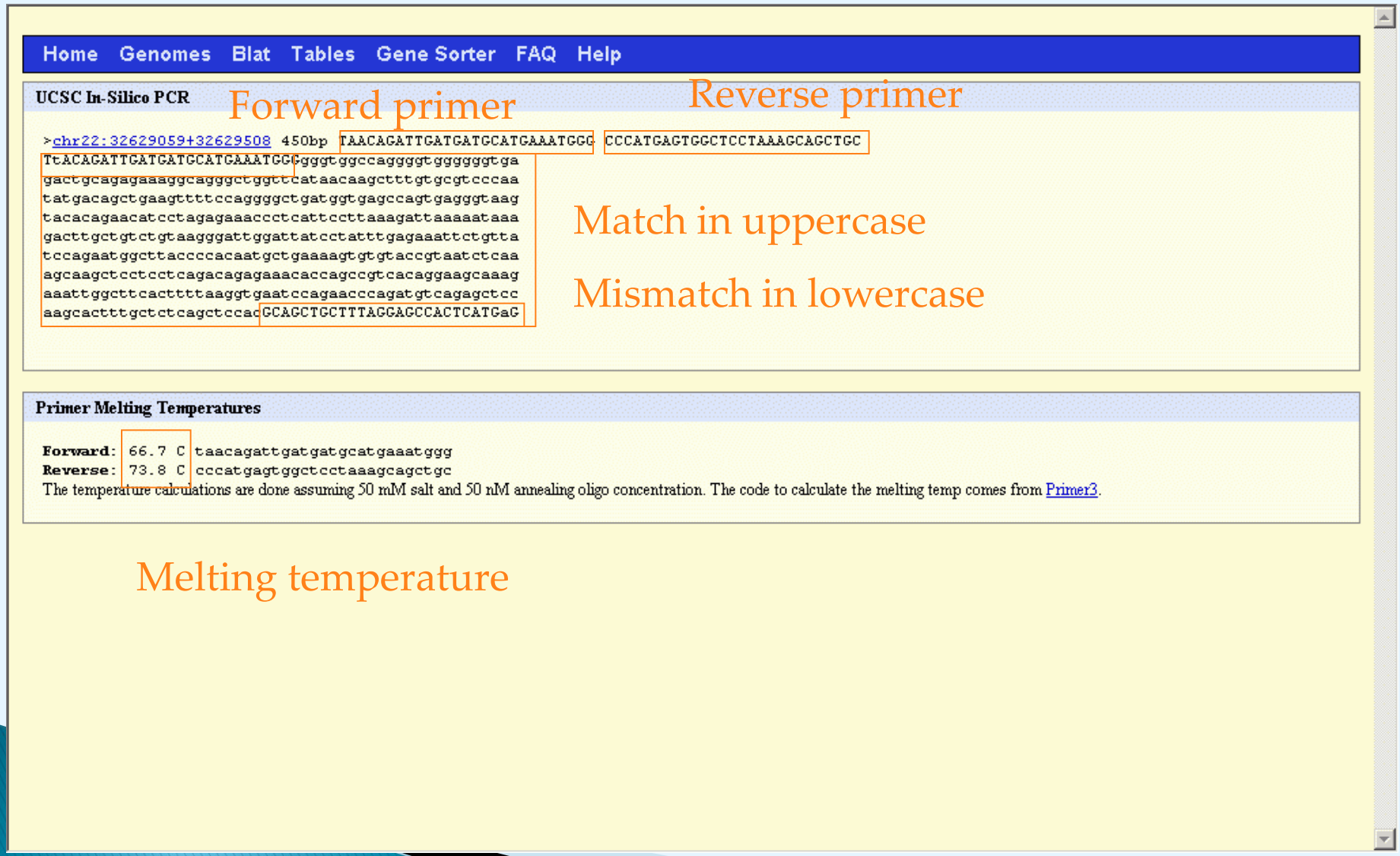
- When successful, the search returns a sequence output file in **fasta format** containing all sequence in the database that lie between and include the primer pair.
- The **fasta header** describes the region in the database and the primers.
- The **fasta body** is capitalized in areas where the primer sequence matches the database sequence and in lower-case elsewhere.
- The + between the coordinates in the fasta header indicates this is on the positive strand.

In-Silico PCR Results



- Genomic location shown, links to Genome Viewer
- Product size shown
- Your primers displayed, flipped if necessary
- Predicted genomic sequence shown
- Primer melting temperatures provided

In Silico PCR Results



UCSC In-Silico PCR

No matches to aggtagaaacgccggatctccttaca aggtagaaacgccggatctccttaca in Human Feb. 2009 (GRCh37/hg19)

Primer Melting Temperatures

Forward: 67.8 C aggtagaaacgccggatctccttaca
Reverse: 67.8 C aggtagaaacgccggatctccttaca
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

UCSC In-Silico PCR

>chr10:96522327+96522386 60bp AGAAGGAGCATATAGTGGGCCTAGGT GTGTGTGCCTCTTTGATGGATAAA
AGAAGGAGCATATAGTGGGCCTAGGTgattggccacTTTATCCATCAAAG
AGGCACACAC

UCSC In-Silico PCR

>chr10:96522327+96522597 271bp AGAAGGAGCATATAGTGGGCCTAGGT TATCTGTAGGATATTTCCAATCACTGGGA
AGAAGGAGCATATAGTGGGCCTAGGTgattggccactttatccatcaaag
aggcacacacacttaattagcatggagtgttataaaaagcttggagtgca
agctcacggttgtcttaacaagaggagaaggcttcaatggatccttttgt
ggtccttgtgctctgtctctcatgtttgcttctcctttcaatctggagac
agagctctgggagaggaaaactccctcctggccctactcctcTCCCAGTG
ATTGGAAATATCCTACAGATA

UCSC In-Silico PCR

>chr10:96521464-96521792 329bp TTTCAAATGGGAAAAGGGAGACCCTGG TTCTCATGAGCATCTCTGGGGCTGTTTTCC
TTTCAAATGGGAAAAGGGAGACCCTGGgagaacaggacacctgttggtgc
cacacagctcatagctggcagaactgggatttgagctgaggtcttctgat
gcccatcgtggcgcattatctcttacatcagagatgctttgagaacagaa
gacacaaatttgaaaaaaaaaatcgtttgctaaaactttgttttagcaaa
acaaaacaacttccaaacattagttattctgaatatataccacattcatc
ctgttcataaaacaggcttcacattaaatagaaccacttatttatctaaG
GAAAACAGCCCCAGAGATGCTCATGAGAA

Primer Melting Temperatures

Forward: 71.8 C tttcaaatgggaaaagggagaccctgg
Reverse: 74.4 C ttctcatgagcatctctggggctgttttcc
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

UCSC In-Silico PCR

No matches to agaaggaatatatagtgggcctaggt tatcaataggccatttccaatgactggg in Human Feb. 2009 (GRCh37/hg19)

Primer Melting Temperatures

Forward: 60.0 C agaaggaatatatagtgggcctaggt
Reverse: 69.0 C tatcaataggccatttccaatgactggg
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

UCSC In-Silico PCR

>chr10:96697479-96697768 290bp TTTCAAATGGGAAAAGGGAGACCCTGG TTCTCATGAGCATCTCTGG
TTTCAAATGGGAAAAGGGAGACCCTGGgagaacaggacacctgttggtgc
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gcccatcgtggtgtattatctcttacaccagagctgccttgagaacaatt
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acatttattctgttcataaaacaggcttcacattaaatagaaccccttat
ttgtctctaagggaaacagcaCCAGAGATGCTCATGAGAA
>chr10:96521464-96521792 329bp TTTCAAATGGGAAAAGGGAGACCCTGG TTCTCATGAGCATCTCTGG
TTTCAAATGGGAAAAGGGAGACCCTGGgagaacaggacacctgttggtgc
cacacagctcatagctggcagaactgggatttgagctgaggtcttctgat
gcccatcgtggcgcattatctcttacatcagagatgctttgagaacagaa
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acaaaacaacttccaaacattagttattctgaatatataccacattcatc
ctgttcataaaacaggcttcacattaaatagaaccacttatttatctaag
gaaaacagccCCAGAGATGCTCATGAGAA

Primer Melting Temperatures

Forward: 71.8 C tttcaaatgggaaaagggagaccctgg
Reverse: 54.7 C ttctcatgagcatctctgg
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

UCSC In-Silico PCR

>chr10:96697464-96697768 305bp TTTCAAATGGGAAAAGGGAGACCCTGG GAGAACTCTTATTTTTTCTCATGAGCATCTCTGG
TTTCAAATGGGAAAAGGGAGACCCTGGgagaacaggacacctgttggtgc
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aTCTC
>chr10:96521449-96521792 344bp TTTCAAATGGGAAAAGGGAGACCCTGG GAGAACTCTTATTTTTTCTCATGAGCATCTCTGG
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cacacagctcatagctggcagaactgggatttgagctgaggtcttctgat
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ctgttcataaaacaggcttcacattaaatagaaccacttatttatctaag
gaaaacagccCCAGAGATGCTCATGAGAAAAAATAAGAGTTCTC

Primer Melting Temperatures

Forward: 71.8 C tttcaaatgggaaaagggagaccctgg
Reverse: 68.4 C gagaactcttatttttctcatgagcatctctgg
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concentration. The code to calculate the melting temp comes from Primer3.

Primer pair 1

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTTCAAATGGGAAAAGGGAGACCCTGG	27	65.12	48.15	6.00	3.00
Reverse primer	GAGAACTCTTATTTTTTCTCATGAGCATCTCTGG	34	64.09	38.24	8.00	2.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 344
Features flanking this product:
   26249 bp at 5' side: cytochrome P450 2C18 isoform 2 precursor
   670 bp at 3' side: cytochrome P450 2C19 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47326256  .........................  47326230

Reverse primer  1         GAGAACTCTTATTTTTTCTCATGAGCATCTCTGG  34
Template        47325913  ................................  47325946


product length = 305
Features flanking this product:
   84794 bp at 5' side: cytochrome P450 2C19 precursor
   671 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47502232  .........................  47502206

Reverse primer  1         GAGAACTCTTATTTTTTCTCATGAGCATCTCTGG  34
Template        47501928  ....T.C.......C..................  47501961
```

	Sequence (5'->3')	Length	Tm	GC%	Self complementarity	Self 3' complementarity
Forward primer	TTTCAAATGGGAAAAGGGAGACCCTGG	27	65.12	48.15	6.00	3.00
Reverse primer	GAGAACTCTTATTTTTTCTCATGAGCATCTCTGGG	35	65.41	40.00	8.00	4.00

Products on target templates

>NT_030059.13 Homo sapiens chromosome 10 genomic contig, GRCh37.p10 Primary Assembly

```
product length = 344
Features flanking this product:
   26249 bp at 5' side: cytochrome P450 2C18 isoform 2 precursor
   670 bp at 3' side: cytochrome P450 2C19 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47326256  .........................    47326230

Reverse primer  1         GAGAACTCTTATTTTTTCTCATGAGCATCTCTGGG  35
Template        47325913  ................................    47325947


product length = 305
Features flanking this product:
   84794 bp at 5' side: cytochrome P450 2C19 precursor
   671 bp at 3' side: cytochrome P450 2C9 precursor

Forward primer  1         TTTCAAATGGGAAAAGGGAGACCCTGG  27
Template        47502232  .........................    47502206

Reverse primer  1         GAGAACTCTTATTTTTTCTCATGAGCATCTCTGGG  35
Template        47501928  ....T.C.......C..................T  47501962
```

UCSC In-Silico PCR

>chr1:152281938-152285646 3709bp AAATGAGGAACAATCAGGAGACGGCA ATCTTGGGATGCTGAGTGCCTGGAG
AAATGAGGAACAATCAGGAGACGGCAccaggcactcagggtcacgtcatc
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genome.ucsc.edu/cgi-bin/hgPcr?hgsid=327396431&org=Human&db=hg19&wp_target=genome&w
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tcccatggatggacagggcccagcactggagtaagacaaggatcccacca
tgagcaggcacgagacaaCTCCAGGCACTCAGCATCCCAAGAT

Primer Melting Temperatures

Forward: 69.7 C aaatgaggaacaatcaggagacggca
Reverse: 70.6 C atcttgggatgctgagtgcctggag
The temperature calculations are done assuming 50 mM salt and 50 nM annealing oligo concer
Primer3.