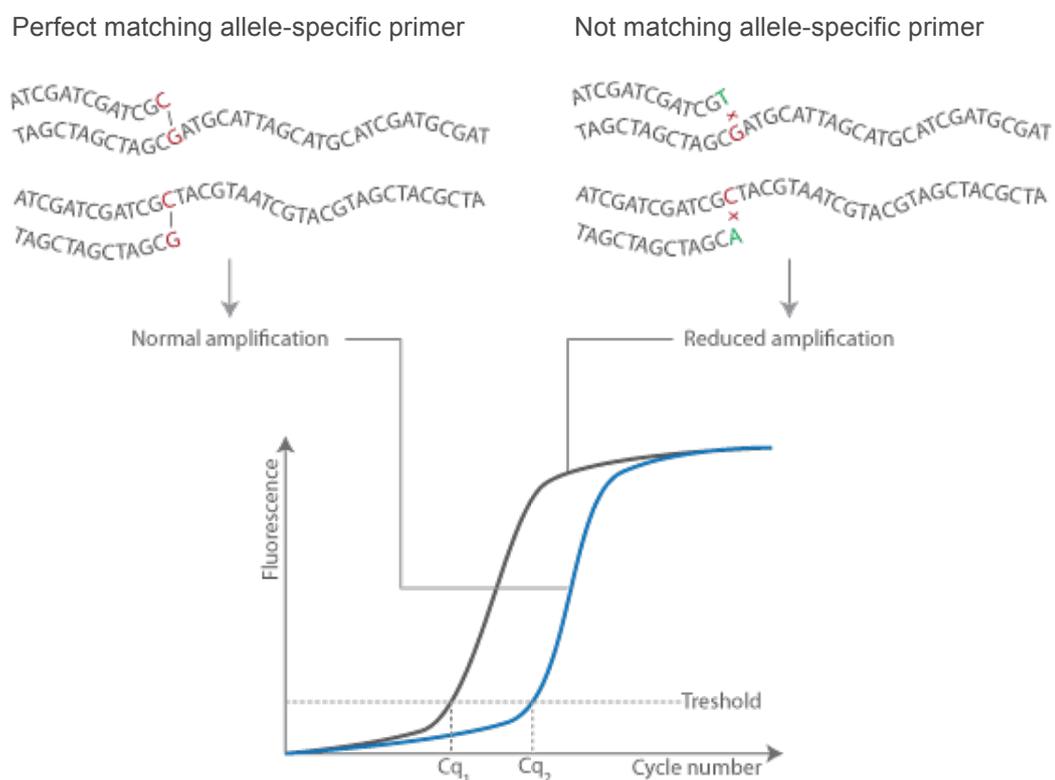


Which genome builds are used? (June 12<sup>th</sup>, 2012)

- Homo sapiens (Ensembl release 67, build 37)
- Danio rerio (Ensembl release 67, build 9)
- Mus musculus (Ensembl release 67, build 37)
- Rattus norvegicus (Ensembl release 67, build 34)
- Arabidopsis thaliana (Gramene release 14, build 67\_10)
- Oryza sativa (Gramene release 14, build 67\_6)

What is a genotyping assay?

A **genotyping assay** is an assay that can be used to assess the **status of a mutation** in a sample. Such an assay consists of a number of **allele-specific primers** and one **constant primer**. For each mutation allele, an allele-specific primer will be designed. To evaluate the mutational status of a sample, different qPCR reactions are performed, each containing the constant primer and one of the allele-specific primers. The set of obtained C<sub>q</sub> values will then give you an indication of the mutational status.



## How to submit a genotyping request?

You have to supply an [email address](#) and [name for the job](#) you will submit. The email address will be used to inform you when your designs are ready and to mail you the results. The job name will, together with your email address, be used to link the results to this job. With the job name and email address, you will also be able to retrieve the results of the corresponding job(s) using the [view designs](#) page.

Give your email address (this will be used to send you the results)

Email address :

Give a project name (you can use this to retrieve your designs)

Project name :

Select an organism



Homo sapiens



Mus musculus



Mus musculus



Oryza sativa



Arabidopsis thaliana

Next you need to select the [organism](#) for which you want to design primers. This is done by clicking on the corresponding icon which will turn blue.

After selecting the organism, an additional input fields will be displayed where you can specify the variant you want to design genotyping primers for. This can be done by either entering the [variant ID](#) or by supplying a [sequence with accompanying mutation annotation](#). Mutations can be annotated in the sequence using one of the following formats (at the location of the mutation) :

Specify SNP ID (rs number)

SNP ID :

[Show design settings](#)

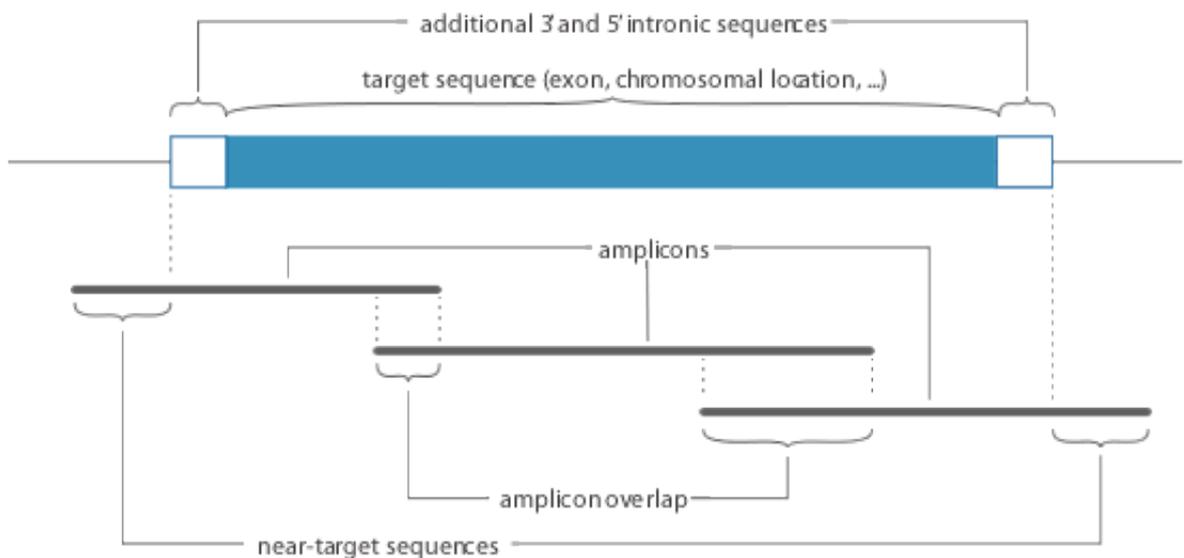
- [substitution](#) : ...[X/Y]... where X is the sequence substituted by Y
- [insertion](#) : ...[-/Y]... where Y is the sequence inserted at the position of the mutation
- [deletion](#) : ...[X/-]... where X is the deleted sequence

By clicking on the "[show design settings](#)" link, more advanced settings for primers, amplicons, and others parameters will be shown. These settings contain default and optimized values but can be customized to your needs. Explanation about these more advanced settings can be found in different topics on this help page.

Finally you click on the  button to add your request to the design queue.

## What is a targeted resequencing assay?

A targeted resequencing assay is a set of one or more overlapping primer pairs covering a region of interest, that can be used as in an [PCR-based target enrichment strategy](#) prior to sequencing. The assays are designed in such a way as to minimize the number of amplicons needed to cover the complete target of interest and to minimize the amount of near- and off-target sequence. Options are available for adding [additional intronic near-on-target sequence](#) (useful when the quality of the sequenced bases is lower at the beginning of a read) and to specify the [minimal overlap](#) between adjacent overlapping amplicons.



## How to submit a targeted resequencing request?

You have to supply an [email address](#) and [name for the job](#) you will submit. The email address will be used to inform you when your designs are ready and to mail you the results. The job name will, together with your email address, be used to link the results to this job. With the job name and email address, you will also be able to retrieve the results of the corresponding job(s) using the [view designs](#) page.

Give your email address (this will be used to send you the results)

Email address :

Give a project name (you can use this to retrieve your designs)

Project name :

Select an organism



Homo sapiens



Mus musculus



Mus musculus



Oryza sativa



Arabidopsis thaliana

Next you need to select the **organism** for which you want to design primers. This is done by clicking on the corresponding icon which will turn blue.

After selecting the organism, and additional input fields will be displayed. The first option presented to the user is whether he/she wants to sequence a cDNA or gDNA sequence. When selecting the cDNA option, a field for supplying the transcript ID of interest will be shown. When selecting the gDNA option, it is possible to design primer pairs covering a **genomic region** by supplying the chromosome name and a chromosome start and end position of the region. When using the gDNA option and supplying an Ensembl gene ID, primer pairs will be designed covering **all coding sequences of all Ensembl transcripts of this gene** (this can be limited to only the exons of protein coding transcripts by checking the **'limit to protein coding transcripts'** box). If primerXL was able to find multiple hits for the gene or transcript ID supplied by the user, an additional page showing all hits will be displayed where the user can select his/her gene or transcript of interest. By clicking on the **"show design settings"** link, more advanced settings for primers, amplicons, and others parameters will be shown. These settings contain default and optimized values but can be customized to your needs. Explanation about these more advanced settings can be found in different topics on this help page.

Specify a chromosomal region or supply an Ensembl gene ID

Ensembl gene ID :

Chromosomal region :

chr  :  -

Optimize design settings for :

- Sanger sequencing
- Roche FLX sequencing
- Illumina sequencing

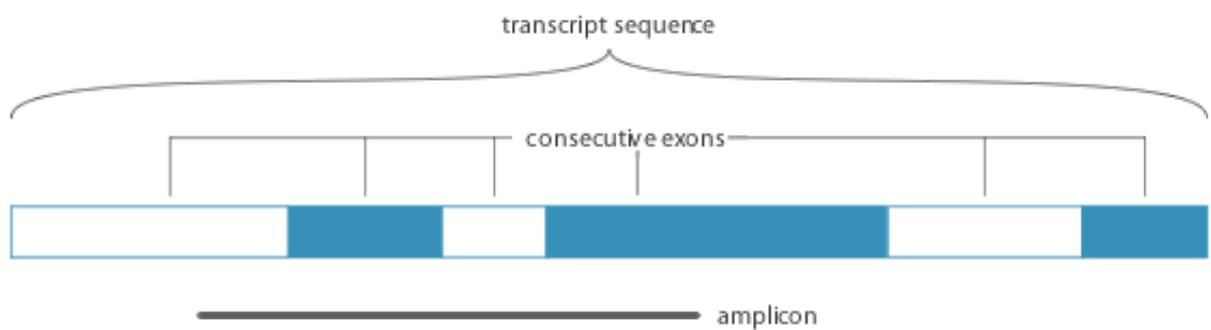
Show design settings

Finally you click on the  button to add your request to the design queue.

## What is meant with a classic qPCR assay?

---

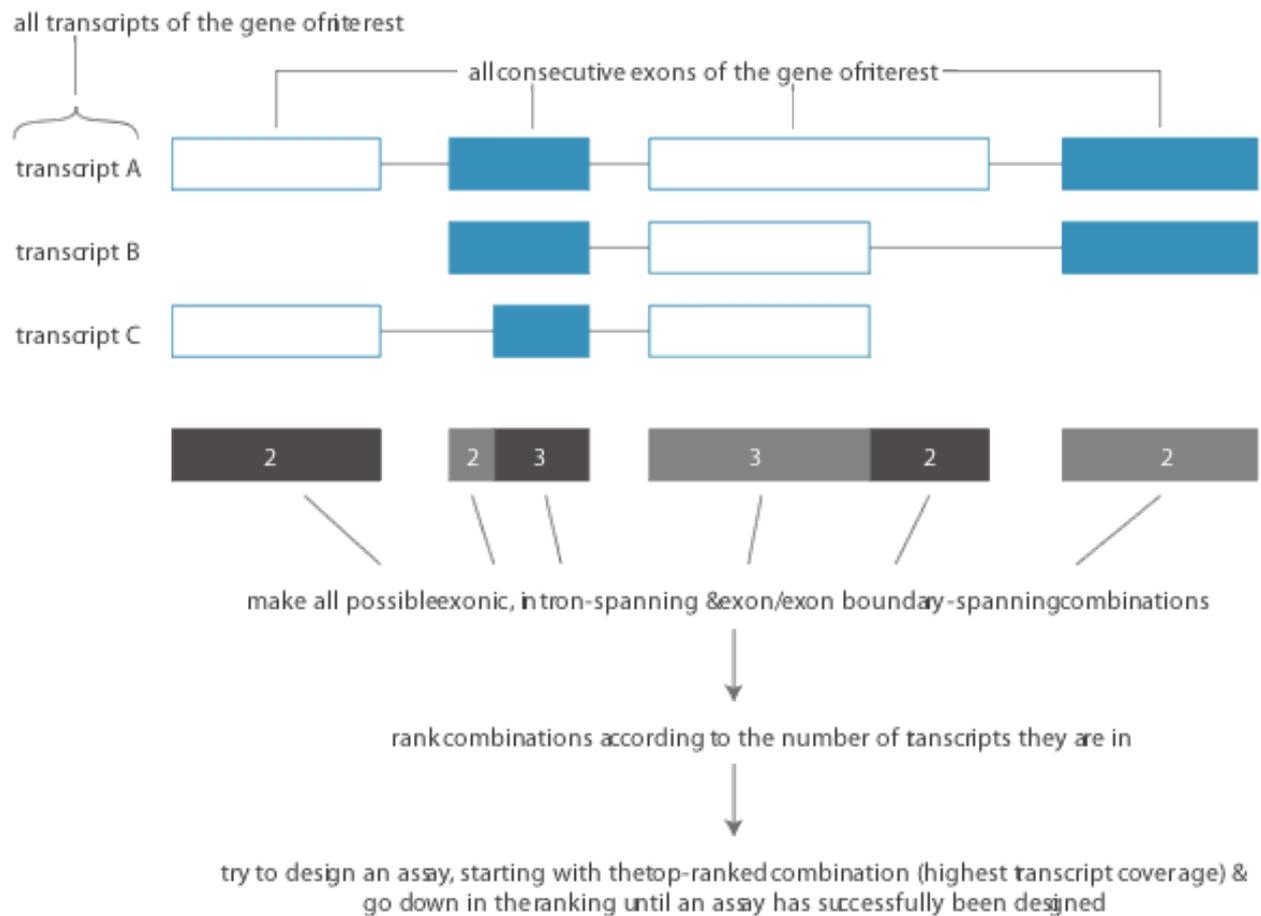
A classic qPCR assay is generated based on the sequence of a [single transcript](#). No additional calculations to determine the transcript coverage of each exon are performed (in contrast to specific or maximal-coverage designs). For this type of design an optional target sequence can be specified which needs to be located inside the amplicon. PrimerXL will then locate this target sequence in the transcript sequence and try to position the primers so that they encompass this target. When no target sequence is supplied, the type of amplicon (exonic, intron-spanning or exon/exon boundary spanning) can be specified.



## What is meant with a single qPCR assay targeting a maximum number of transcripts?

---

For this type of assay, primerXL will determine the [transcript count of each exon](#) (or exon-fragment). [All possible combinations](#) of these exons (exon-fragments) will then be determined (keeping in mind the other user-defined settings such as minimal intron size, amplicon type, ...) and [ranked according to their transcript count](#). The ranking - ascending or descending transcript count - can be set by the user and will result in a single assay, targeting as little transcripts (ranking according to ascending transcript count) or as much transcripts (ranked to descending transcript count) as possible. PrimerXL will then try to design an assay, starting with the highest ranked combination and going down the list, until a successful assay has been found or all combinations have been exhausted. In this way, an assay targeting a maximum number of transcripts of the gene of interest will be generated. An additional option is available to design multiple assays targeting as much transcripts as possible.

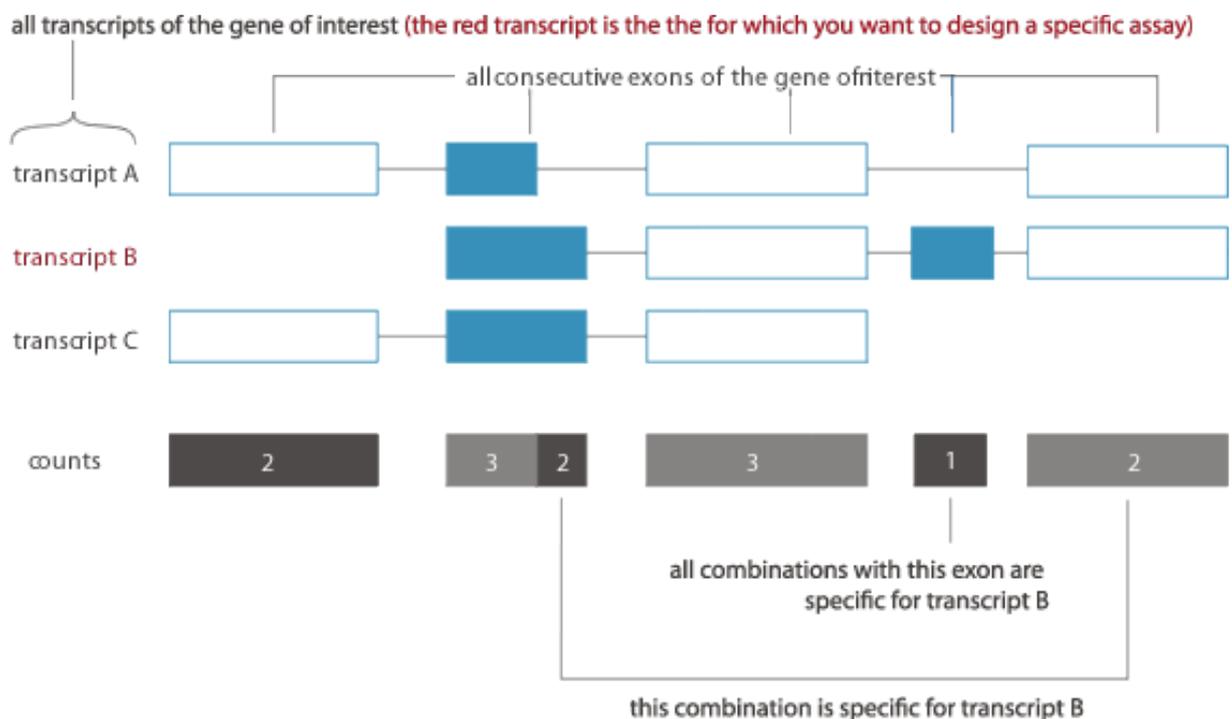


What is meant with a classic qPCR assay?

For this type of assays, primerXL will determine the **transcript count of each exon** (or exon-fragment). **All possible combinations** are then determined and **ranked according to their transcript count**. The ranking - ascending or descending transcript count - can be set by the user and will result in multiple assays, each targeting as little transcripts (ranking according to ascending transcript count) or as much transcripts (ranked to descending transcript count) as possible. PrimerXL will then generate assays, **starting with the highest ranked combination** and going down the list, until all combinations have been exhausted (this in contrast to a single qPCR assay - see above - where the design process is aborted if a successful assay has been found). This type of design will/can generate multiple assays, but an additional option to retain only the '**transcript exclusive**' assays (no overlap in transcript coverage between assays allowed) is available.

### What is meant with a transcript specific qPCR assay?

A transcript specific qPCR assay will **only target the transcript of interest**, no other transcripts (of the same or other genes will be amplified). This is achieved by determining the transcript count of each exon (or exon-fragment), and only taking into account these combinations targeting that single transcript.



### What is meant with a CNV qPCR assay?

A CNV qPCR design will try to generate **an exonic assay for each exon of the transcript of interest**. This type of design can be used to screen for amplified or deleted exons (copy number variation screening).

### What is meant with a CNV qPCR assay?

Here the user will supply a chromosomal location in which primerXL will then try to generate an assay (the amplicon will be located completely inside the user-defined chromosomal location). This type of design can be used for locations outside any of the known genes.



## How to submit a qPCR request?

You have to supply an [email address](#) and [name for the job](#) you will submit. The email address will be used to inform you when your designs are ready and to mail you the results. The job name will, together with your email address, be used to link the results to this job. With the job name and email address, you will also be able to retrieve the results of the corresponding job(s) using the [view designs](#) page.

Select an organism



Homo sapiens



Mus musculus



Mus musculus



Oryza sativa



Arabidopsis thaliana

Next you need to select the [organism](#) for which you want to design primers. This is done by clicking on the corresponding icon which will turn blue.

After selecting the organism, additional options and input fields will be displayed. The "[type of design](#)" parameter lets you select what the developed primer pairs will target.

Different options are available :

Type of design :

- Classic (based on a transcript ID)
- Maximize the number of targeted transcripts of a gene
- Target a specific transcript
- Target all exons of a specific transcript (CNV screening)

Specify and Ensembl gene ID and an Ensembl transcript ID

Ensembl gene ID :

Ensembl transcript ID :



[Show design settings](#)

- [Classic](#) : here the sequence of the specified transcript will be retrieved and a primer pair targeting this transcript will be designed. It's possible that other transcripts of the same gene will be amplified as well.
- [Maximize number of targeted transcripts](#) : using this setting, primerXL will try to design a primer pair targeting as much as possible transcripts of the specified gene.
- [Target a specific transcript](#) : this will make primerXL try to design a primer pair targeting only the specified/desired transcript of supplied gene. Other transcripts of the supplied gene or other genes will not be targeted.
- [Target all exons of a transcript](#) : here for each exon of the specified transcript, primerXL will try to design an exonic primer pair targeting that exon. This option is useful when you want to use qPCR for exon-

deletion/multiplicatin screening.

- [Based on a chromosomal location](#) : here primerXL will try to design a primer pair inside a user-defined chromosomal location.

Depending on the selected design type, different input fields will shown to supply an Ensembl gene or transcript ID, or a chromosomal location. If primerXL was able to find multiple hits for the gene or transcript ID supplied by the user, an additional page showing all hits will be displayed where the user can select his/her gene or transcript of interest. By clicking on the "[show design settings](#)" link, more advances settings for primers, amplicons, and others parameters will be shown. These settings contain default and optimized values but can be customized to your needs. Explanation about these more advanced settings can be found in different topics on this help page.

Finally you click on the  button to add your request to the design queue.

## Explanation of primer parameters

---

### Primer annealing Tm

Minimum, Optimum, and Maximum melting temperatures (Celsius) for a primer oligo. No oligos with temperatures smaller than Min or larger than Max will be picked, and with default conditions will try to pick primers with melting temperatures close to Opt. primerXL uses the oligo melting temperature formula and the table of thermodynamic parameters given in .... The optimal primer annealing Tm is usually best set at 1 °C lower than the temperature at which the PCR reaction will be performed.

### Primer GC content

Minimum, Optimum, and Maximum percentage of Gs and Cs in any primer or oligo.

### Primer length

Minimum, Optimum, and Maximum lengths (in bases) of a primer oligo. No primers shorter than Min or longer than Max will be picked, and with default arguments will attempt to pick primers close with size close to Opt. Min cannot be smaller than 1. Max cannot be larger than 36. Min cannot be greater than Max.

### Primer maximum GC clamp

The number of G/C's in the last 5 base pairs on the 3' end of a primer. Higher numbers will increase annealing of the primer to its target, but can also result in the generation of aspecific amplification products.

### Primer maximum Tm difference

Maximum acceptable (unsigned) difference between the melting temperatures of the left and right primers.

### Primer minimum 3' distance

When returning multiple primer pairs by Primer3, this parameter defines the minimum number of base pairs between the 3' ends of any two left primers or any two right primers.

### Maximum length of poly-X stretches

The maximum allowable length of a mononucleotide repeat, for example AAAAAA

### Maximum primer 3' self-annealing score

The primer 3' self-annealing score of a primer is determined by binding the 3'-end to a identical primer and retaining/scoring the best binding it can find. This is critical for primer quality because it allows primers use itself as a target and amplify a short piece (forming a primer-dimer). These primers are then unable to bind and amplify the target sequence.

This parameter defines the maximum allowable 3'-anchored global alignment score when testing a single primer for self-complementarity, and the maximum allowable 3'-anchored global alignment score when testing for complementarity between left and right primers. The 3'-anchored global alignment score is taken to predict the likelihood of PCR-priming primer-dimers, for example

```

5 ' ATGCCCTAGCTTCCGGATG 3 '
      ||| |||||
3 ' AAGTCCTACATTTAGCCTAGT 5 '

```

or

```

5 ' AGGCTATGGGCCTCGCGA 3 '
      |||||
3 ' AGCGCTCCGGGTATCGGA 5 '

```

In the examples above the scores are 7.00 and 6.00 respectively. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable 3'-anchored global alignment between two oligos.

### Maximum primer overall self-annealing score

This parameter describes the tendency of a primer to bind to itself (interfering with target sequence binding). It will score ANY binding occurring within the entire primer sequence.

It is the maximum allowable local alignment score when testing a single primer for (local) self-complementarity and the maximum allowable local alignment score when testing for complementarity between left and right primers. Local self-complementarity is taken to predict the tendency of primers to anneal to each other without necessarily causing self-priming in the PCR. The scoring system gives 1.00 for complementary bases, -0.25 for a match of any base (or N) with an N, -1.00 for a mismatch, and -2.00 for a gap. Only single-base-pair gaps are allowed. For example, the alignment

```

5 ' ATCGNA 3 '
      || | |
3 ' TA-CGT 5 '

```

is allowed (and yields a score of 1.75), but the alignment

```

5 ' ATCCGNA 3 '
      || | |
3 ' TA--CGT 5 '

```

is not considered. Scores are non-negative, and a score of 0.00 indicates that there is no reasonable local alignment between two oligos.

### Maximum primer 3' cross-annealing score

This parameter is used to define the maximum allowed score of binding between the 3'-end of the left primer to the right primer. It is similar to the [maximum primer 3' self-annealing score](#)

## Explanation of amplicon parameters

---

### Amplicon length

Minimum, Optimum, and Maximum lengths (in bases) of the PCR product. No primers with products shorter than Min or longer than Max will be generated, and with default arguments will attempt to pick primers producing products close to the Optimum length.

### Amplicon type

Indicates the type of amplicon you want. Possibilities are exonic, intron-spanning, exon/exon boundary-spanning and random (= one of the previous 3). A visual explanation of the different types can be found below :

- [exonic](#) : both primers located in the same exon
- [intron-spanning](#) : forward primer located in one exon, reverse primer located in another
- [exon/exon boundary-spanning](#) : 3' end of a primer located in one exon, 5' end of the same primer located in the adjacent exon
- [random](#) : one of the options above

### Minimum intron size

Defines the minimum intron size needed for intron-spanning and exon/exon boundary-spanning designs

### Min/max. overlap of primer with exon/exon boundaries

Only applicable for exon/exon boundary-spanning designs. This parameter can be used to set the size of the region on the primer 3' end that needs to be located in one exon, while the rest of the primer is located in the other (adjacent) exon

### Amplicon location

This option lets you choose the location of the amplicon in the transcript of interest, either randomly located in the transcript or on the 3' or 5' end of the transcript. The 'amplicon location' option is only available when selecting the classic design type

## Explanation of master mix parameters

---

### Mg concentration

The millimolar concentration of divalent salt cations (usually  $\text{MgCl}^{2+}$  in the PCR).

### Na concentration

The millimolar concentration of salt (usually KCl) in the PCR. this argument is used to calculate oligo melting temperatures.

### dNTP concentration

The millimolar concentration of deoxyribonucleotide triphosphate. This argument is considered only if the Mg concentration is specified.

### DNA concentration

The nanomolar concentration of annealing oligos in the PCR. this argument is used to calculate oligo melting temperatures. The value of this parameter is less than the actual concentration of oligos in the reaction because it is the concentration of annealing oligos, which in turn depends on the amount of template (including PCR product) in a given cycle. This concentration increases a great deal during a PCR; fortunately PCR seems quite robust for a variety of oligo melting temperatures.

### PCR reaction temperature

The temperature (in degrees Celcius) at which the PCR reaction will be performed.

## Explanation of tiling parameters (only targeted resequencing requests)

---

### Include minimum # intronic bp

The number of intronic base pairs, up- and downstream of each exon, that need to be included in the target. Useful if the quality scores are lower at the beginning of sequenced reads.

### Minimum overlap between consecutive amplicons

Only applicable for regions/exons that need multiple overlapping assays to be covered completely. In this case, this parameter specifies the minimal overlap between any two adjacent assays.

### Maximum intercalating # bp to join regions

This parameter can be used to specify when two adjacent fragments may be merged into a single, larger one

### Adjust amplicon length for small regions

Only applicable for regions/exons having a length smaller than the minimal set amplicon length. In this case, this parameter will allow primerXL to adjust the amplicon length range dynamically so that it encompasses the length of the region of interest, this minimizing the amount of near-target base pairs

## Explanation of relaxation parameters (only targeted resequencing requests)

---

### Relax SNP analysis

Indicates that the in silico SNP analysis may be relaxed automatically. Relaxation includes :

- disabling SNP masking in the sequence prior to primer design
- reducing the region on the primer 3' end where no SNPs are allowed - see option below)

### Don't allow SNPs in # bp on primer 3' end

Used to specify the region on the primer 3' end where no SNPs allowed to be located in its annealing site. Only applicable in case

SNP masking is disabled, this region will be reduced (down to a minimal region of 5 bp on the primer 3' end) automatically if SNP relaxation is enabled (see above)

### Relax secondary structure analysis

Indicates that the in silico secondary structure analysis may be relaxed automatically. Relaxation includes :

- disabling second masking in the sequence prior to primer design
- reducing the region on the primer 3' end where no secondary structures are allowed - see option below)

### Don't allow sec. structures in # bp on primer 3' end

Used to specify the region on the primer 3' end where no secondary structures allowed to be located in its annealing site. Only applicable in case secondary structure masking is disabled, this region will be reduced (down to a minimal region of 5 bp on the primer 3' end) automatically if secondary structure relaxation is enabled (see above)

### Min. allowed dG of sec. structures in primers

Still to add

### Relax specificity analysis

Indicates that specificity analysis may be relaxed automatically. This is achieved by reducing the tolerance for mismatches in primer annealing sites during primer specificity analysis. The downside of this relaxation is the possibility that the selected assay may be more prone to aspecific product generation

### Relax GC clamp size

Indicates that the size of the GC clamp may be relaxed automatically, up to a maximum of 5. The downside of this relaxation is that the selected assay may be prone to more aspecific product generation

### Relax amplicon length

Indicates that the maximum allowed amplicon length may be relaxed/increased automatically, up to 1.5 times the original maximum set amplicon length

### Relax primer annealing temperature

Indicates that the primer annealing temperature range may be relaxed automatically (by decreasing the lower T<sub>m</sub> range and increasing the upper T<sub>m</sub> range), upto a maximum T<sub>m</sub> range of 5°C

### Relax primer self annealing score

Still to add

### Relax primer GC content

Indicates that the primer GC content may be relaxed automatically (by decreasing the lower GC% value and increasing the upper GC% value), until a maximum range of 0 - 100%

## Explanation of the project overview

	Description/name of the project		Button to download all primer pairs in the project
🟢	test1	1	⬇️
🟡	test2	1	
🟠	test3	9	⬇️
⚪	test4	1	

Status of the project (running/queued/completed)

Number of jobs/genes/transcripts/chromosomal locations present in the project

In the project overview all your projects and their status can be viewed. The first column displays the status of the project :

- ⚪ The design requests are queued, but none of them are completed yet.
- 🟡 Design requests in the project are being processed, but none of the requests are completed yet.
- 🟢 All the design requests in the corresponding project are completed.

-  A number of designs have already been completed (this percentage can be seen by hovering with the cursor over the icon), but none of the requests are being processed at this time (they are queued).

### Explanation of the design overview

Target	Forward	Reverse			
 ■ ENSG00000165704 [ENST00000298556]	TCAAAGACAGCATCTAAGAAGT	GCTGACAAAGATTCAC TGGA		UC	
 ■ ENSG00000165704 [ENST00000298556]	GACTAATTATGGACAGGACTGA	AGCAGGTCAGCAAAGAATTT		UC	
 ■ ENSG00000165704 [ENST00000298556]	GACTAATTATGGACAGGACTGA	AGCAGGTCAGCAAAGAATTT		UC	
 ENSG00000165704 [ENST00000298556]	AGAGTTCAAGTTGAGTTTGGGA	GGCCACAGA ACTAGAACATT		UC	
 ENSG00000165704 [ENST00000298556]	AGAGTTCAAGTTGAGTTTGGGA	GGCCACAGA ACTAGAACATT		UC	
 ■ ENSG00000165704	ACTTCAGGGATTTGAATCATGT	AACTTCATCTTAGGCTTTGT		UC	
 ■ ENSG00000165704	GACTAATTATGGACAGGACTGA	GTTGACTGGTCATTACAATAGC		UC	

Type of design (can be identified using the legend on top of the webpage)

Type of amplicon (can be identified using the legend on the top of the webpage)

Link to UCSC. For exonic or targeted resequencing assays, an in silico PCR will be performed. For other assays, a BLAT search

Link to the webpage displaying all alternative 'good' assays (good assay = assay that passed all in silico evaluations)

Description of the assay (gene ID, transcript ID or chromosomal location)

Forward and reverse primers

'Delete assay' button



Design settings :

type of design      primer settings      master mix settings      amplicon settings

**Assay details ?** **Close**

classic design

Primer length :	16 - 20 - 30 bp	Amplicon length :	80 - 150 bp
Primer Tm :	58.00 - 59.00 - 60.00 °C	Amplicon type :	exon/exon boundary spanning
Primer GC% :	30.00 - 50.00 - 80.00 %	Min. intron size :	500 bp
Primer max. GC clamp :	2	Overlap with exon/exon boundaries :	1 - 15 bp
Primer max. Tm difference :	2.00 °C	Mix Mg concentration :	0.0030 mM
Primer min. 3' distance :	5	Mix Na concentration :	0.0500 mM
Max. poly-X length :	3	Mix dNTP concentration :	1.2000 mM
Max. primer 3' self-annealing score :	3	Mix DNA concentration :	250.00 nM
Max. primer overall self-annealing score :	8	PCR reaction temperature :	60.00 °C
Max. primer 3' cross-annealing score :			

Perform SNP analysis :	yes	Mask SNPs :	yes
Perform secondary structure analysis :	yes	Mask secondary structures :	yes
Perform specificity analysis :	yes	Template :	cDNA

Relax SNP analysis :	yes	No SNPs allowed in :	10
Relax secondary structure analysis :	no	No sec. structures allowed in :	N/A
Relax specificity analysis :	no	Relax primer annealing Tm :	no
Relax GC clamp size :	no	Relax primer self-annealing score :	no
Relax amplicon length :	no	Relax primer GC% :	no

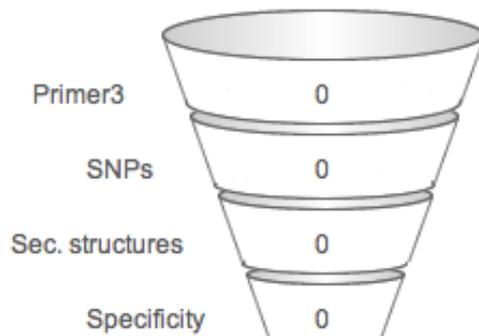
◀ **Display assay details**

relaxation settings      in silico evaluation settings

## Explanation of the failure statistics

Short explanation of the figure on the left and interpretation of the failure statistics. These may help in achieving successful assay design by suggesting which settings to relax

**Failure statistics****Close**



Step	Count
Primer3	0
SNPs	0
Sec. structures	0
Specificity	0

The figure on the right gives an overview of how many designs passed each *in silico* evaluation step.

Based on these numbers, relaxing the [specificity analysis analysis](#) should maximize the change of finding a good assay. This likelihood can also be maximized by relaxing the [primer](#), [amplicon](#) and [master mix](#) parameters. This could increase the number of assays available for downstream *in silico* evaluation.

[Display design settings](#) ▶

Funnel showing how many assays were generated by Primer3 and how many assays passed each *in silico* evaluation. This can give you an indication where the problem resides and help you on deciding which settings to relax (manually)