

Primer design guidelines

The Xdrop™ enrichment technology relies on carefully designed and highly specific PCR primer pairs. Two sets of non-overlapping PCR primer pairs are required. The first one is called dPCR primer set and is responsible for creating a fluorescent signal used for target selection. The second set of PCR primers, called the qPCR QC primer set, is used to validate the assay and quantify the number of target fragments in the pool of enriched fragments.

If the target spans over a region of 50 kb or longer, a combination of two or more sets of dPCR/qPCR primers are recommended.

Help for designing primers can be found in the online primer design tool at <https://samplix.com/primer>

Click on the button “Design by using FASTA sequence” or “Design by using gene name”, depending on the available information for the design.

```
>Target1 Obtained from custom assembly ABC
GATGGGATTGGGGTTTTCCCTCCCATGTGCTCAAGACTGGCGCTAAAAGTTTTGAGCTT
CTCAAAGCTAGAGCCACGCTCCAGGGAGCAGGTAGCTGCTGGGCTCCGGGGACACTTT
GC GTTCGGGCTGGGAGCGTCTTTCACGACGGTGACACGCTTCCCTGGATTGGGTAAGC
TCCGACTGAACCTGATGAGTCTCTCTGAGTCAAGGCTCTGGGCTCCGCTATTTTCA
GCTCGGGAATCCCTGGGCTGGGGGTGGGCACTGGGCACTAGCGAATTTGGGGGTG
AGTGGGATGGAAGCTTGGCTAGAGGGATCATCATAGGAGTTGCATTGTTGGGAGACTGG
GTGTAGATGATGGGATGTTAGGACCATCCGAACCTAAAGTTGAACGCTAGGACAGAGGA
GTGGAGCTTTGGGGACCTTGAGCCGGCTAAAGCGTACTTCTTGCACATCCACCCGGT
GCTGGGCTAGGGAAATCCCTGAAATAAAGATGCACAAAGCATTGAGGCTGAGACTTTT
```



FASTA input

Design primers using FASTA sequence

For maximum control of your target sequence, you can choose to enter a FASTA sequence as the target for the primer design tool. This will ensure that the primers target the exact sequence of interest.

This option is useful if your target sequence is not accurately described by the reference genome assemblies.



Database search

Design primers using gene name

Find your target of interest by searching the Ensembl reference database. This will allow you to search using gene names, transcript names, and descriptions. This will also allow you to visualize gene splice variants.

This option is useful if your target sequence is accurately described by the reference genome assemblies and their annotations.



Then, you will be asked to log in or create an account.

Log in

Your email:

Password:

By registering on this web page, you are consenting and agreeing to collection and use of that information by Samplix in accordance with its [Privacy Policy](#).

The login allows you to save your design on our server.

If you select the **“Design by using gene name”**, select the genome and search for a gene or another search term. The retrieved entries will be shown below. Identify the one you would like to design primers of, if more than one returned, and click **“Select for design”**. In the example below, Homo sapiens genome (build GRCh38.p12) was selected from the dropdown and BRCA2 was used as search term. The tool identified the BRCA2 gene on the human genome, which was selected for design.

Organism

Search term

For design of primers to enrich for all close homologues, pseudogenes etc. we recommend to use NCBI Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>)

Name	Description	Position	Length	Options
BRCA2	BRCA2%2C DNA repair associated [Source:HGNC Symbol%3BAcc:HGNC:1101]	13 : 32,315,474 - 32,400,266 (forward)	84792	<input type="button" value="Details"/> <input type="button" value="Select for design"/>



My Designs

Details	Name	Source	Submission Time	Status	Options
	BRCA2	Database	Feb 20, 2020 11:35:50	REQUESTED	

The design can be viewed once completed. An email notification is sent upon completion.

Details	Name	Source	Submission Time	Status	Options
	BRCA2	Database	Feb 20, 2020 11:35:50	PROCESSED	View

Note that different primer sets for both dPCR and qPCR QC primer sets are provided by the tool, along with several relevant metrics of the designed primers.

Design result

Name	Organism	Background organism	Design reference
BRCA2	Homo sapiens		32c3e0cd-863e-4c3f-4748-d1e2cb7247db

Solution set: (1)



(Average amplicon distance: 14997 nt)

Type	Name (F/R)	Sequence	Tm °C	Score	Length	GC %	Offtargets
Droplet PCR assays	BRCA2_133F	ACCTCTAAGTACTCAGTCTCGT	57.4	0.62	5832 - 5967 : 136 bp	40 %	Homo sapiens (GRCh38.p12), 13:32,321,305-32,321,440 : 136 bp (On target)
	BRCA2_133R	CCAACCAGAGATGGCTGCT	59.7				
qPCR assays	BRCA2_96F	AGATCACCTCCCTTTGTCCT	57.6	0.79	4813 - 4912 : 100 bp	43 %	Homo sapiens (GRCh38.p12), 13:32,320,286-32,320,385 : 100 bp (On target)
	BRCA2_96R	GCAAGCTGAGCCAAGAGTGT	60.9				
Droplet PCR assays	BRCA2_402F	CTGCAGCATGTCACCCAGTA	60.0	0.80	21086 - 21227 : 142 bp	38 %	Homo sapiens (GRCh38.p12), 13:32,336,559-32,336,700 : 142 bp (On target)
	BRCA2_402R	AGGTTTGACAGAACATCCTTGA	59.9				

General guidelines for primer design

If you would like to use a different tool for designing primers, please take into account the following information regarding amplicon length and melting temperature for dPCR and qPCR QC primers.

	dPCR primers	qPCR QC primers
Amplicon length	120-160 bp	80-120 bp
Melting temperature	~ 60°C	~ 60°C

Note: The qPCR QC primer pair must be different from the enrichment dPCR primer pair and placed within 2 kb distance from it. The qPCR QC and dPCR amplicons must not overlap.

The risk of false-negative enrichment prediction increases if the validation qPCR assay is placed further away from the dPCR assay.

The following guidelines apply to both dPCR and qPCR QC primer pairs:

- Avoid primer pairs with more than 2°C difference in melting temperature between forward and reverse primer.
- Avoid placing primers in low complexity regions.
- Primers need to be specific. Avoid primer pairs that amplify sequences not related to the target sequence.
- Follow the general recommendations for PCR primer design: avoid self-complementarity, stable secondary structures, hairpins etc.

Primer Testing

For every new target and prior to generating droplets, both the dPCR enrichment primers and the qPCR QC primers must be optimized, preferably by qPCR, using your sample DNA and Samplix dPCR reagents ● and qPCR dye ●. For this assay, include a negative control with no template and at least three different concentrations of your template (or a biological replicate) in the range of the amount of input DNA suggested by the online sample calculation tool at samplix.com (see Fig. 1).

Use the Samplix primer test PCR kit (Cat. No. RE10200) that includes Samplix dPCR mix (2X) ● and Samplix qPCR dye ● to verify the primers and reaction efficiency.

If possible, we recommend testing more than one primer set. For each primer set, we recommend constructing a standard curve using at least three different concentrations of input DNA, for example: 50 ng/μl, 10 ng/μl and 2 ng/μl.

The primer test reagents are optimized for the following reaction mix using Samplix Primer test PCR kit (Cat. No. RE10200):

dPCR reaction mix	Final concentration	1 reaction	X reactions
H ₂ O (molecular grade)		7,2 μl	
dPCR mix (2x) ●	1x	10 μl	
Primer Forward 10 μM	0,2 μM	0,4 μl	
Primer Reverse 10 μM	0,2 μM	0,4 μl	
qPCR dye (20x) ●	1x	1 μl	
Total mix		19 μl	
Template DNA		1 μl	
Total volume		20 μl	

And the following qPCR program:

Temperature	Duration	Cycles
30°C	5 sec	1x
94°C	2 min	40x
94°C	3 sec	
Annealing temp.	30 sec	
Data acquisition*	-	
Melting curve 70-90°C		1X

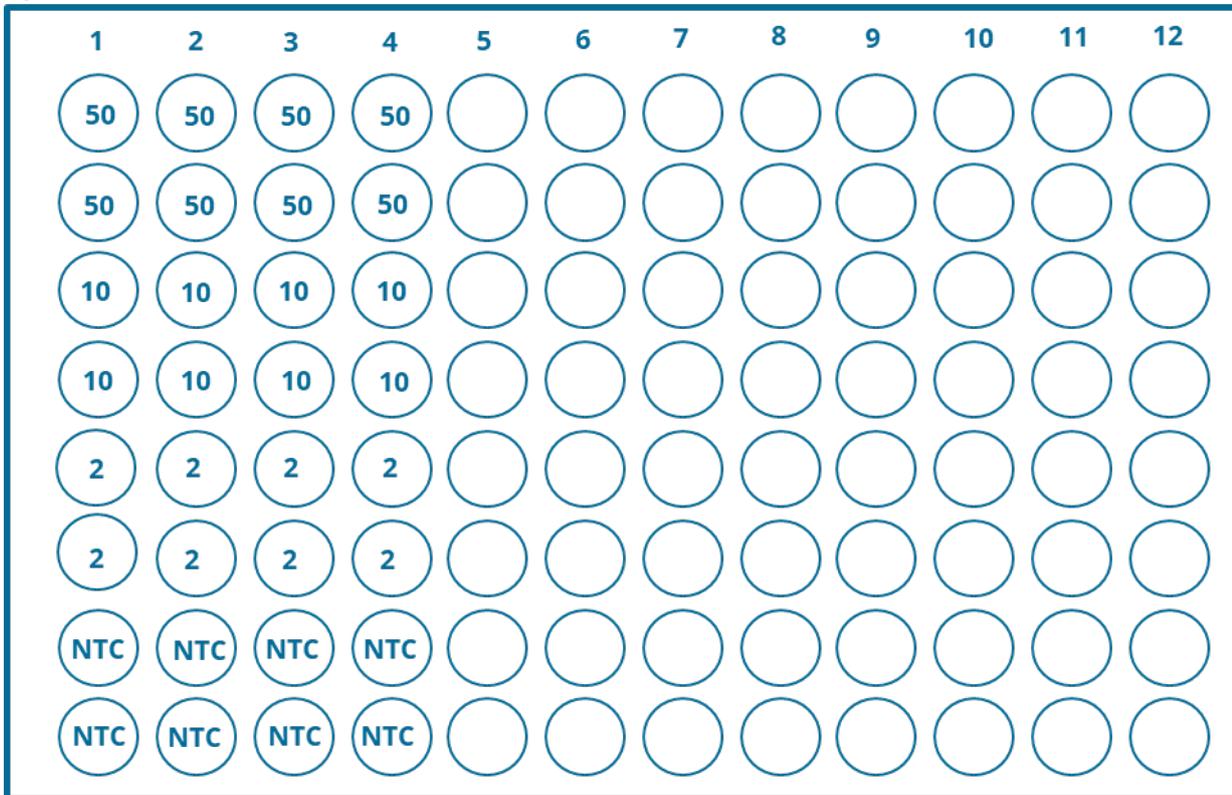
*Follow manufacturer's protocol for data acquisition program.

Note: disable the ROX normalization during setup if your instrument provides this type of functionality.

Note: The annealing temperature needs to be adjusted to the specific primer sets tested (~60 °C).

Note: Samplix dPCR reagents are recommended for qPCR QC primer testing, but different qPCR reagents could also be used.

You can set up a qPCR plate as in the following schematic view, where 4 primer sets (lanes 1-4) and 3 concentrations of input DNA (50 ng/μl, 10 ng/μl and 2 ng/μl) are tested, along with negative controls (NTC), all in duplicates.



Lane	Primer set	Ct value (50 ng)	Ct value (10 ng)	Ct value (2 ng)	Efficiency
1					
2					
3					
4					
5					
6					
7					
8					
9					
10					
11					
12					

We recommend running a melting curve analysis with the template DNA, dPCR primers, and Samplix reagents to check for the presence of alternative amplicons and primer-dimers. Consider running a temperature gradient to determine the optimal annealing temperature.

The supplied qPCR dye (20x) ● maximum values for excitation and emission are approximately 497 nm and 520 nm respectively (SYBR™ green settings). Make sure these values are selected in your qPCR instrument.

Calculate the PCR efficiency using the Ct values as input with the formula: $(10^{(-1/\text{slope})}-1) * 100$

Where the slope refers to the slope of the standard curve (X-axis: Log₁₀ of the DNA concentration; Y-axis: Ct values, see Fig. 1).

Here an example for a primer set called dPCR_set1, tested with three DNA concentrations (50, 10, and 2 ng/μl), tested in duplicate:

Primer set	DNA Concentration (ng/μl)	Log ₁₀ DNA Concentration	Ct replicate 1	Ct replicate 2	Mean Ct
dPCR_set1	50	1,6990	26,51	26,71	26,61
dPCR_set1	10	1,0000	29,18	29,48	29,33
dPCR_set1	2	0,3010	31,17	31,33	31,25

dPCR_set1 Slope = -3,3297

dPCR_set1 Efficiency = 99,7

Make sure that your designed primer pairs have an **efficiency between 90-110 %** and that the suggested DNA input amount yields a Ct value below 30.

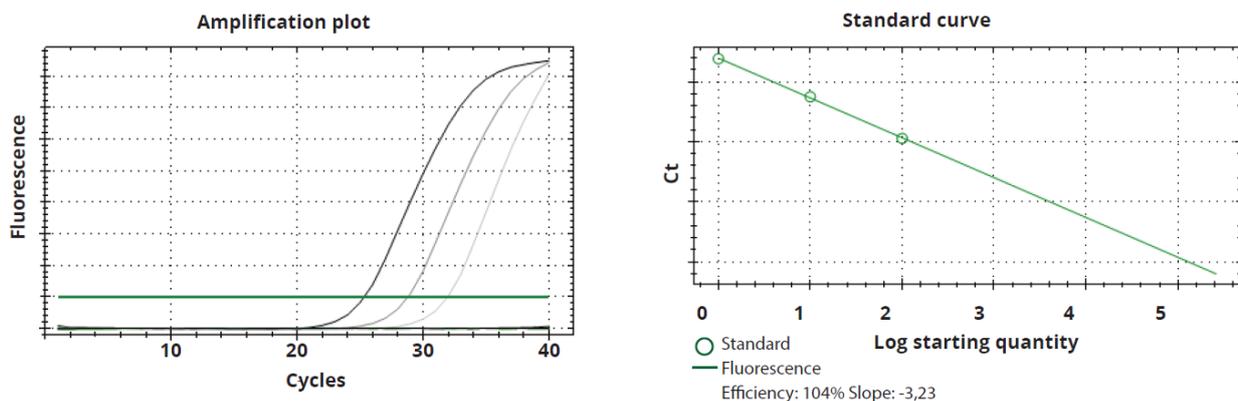


Fig. 1. Calculate PCR efficiency with at least three different concentrations of input DNA using the Samplix Primer test PCR kit (Cat. No. RE10200) and your designed primers. Left: Amplification plot of three DNA concentrations. Right: Standard curve and calculations of PCR efficiency.

Note: We also provide a tool for calculating the optimal amount of input template DNA based on targets per genome or per nanogram. You find both of them under “DNA input calculator” following [this link](#).