
EpiQ™ Chromatin Analysis Kit

Primer Design and qPCR Optimization Guide



BIO-RAD

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I. Introduction

The EpiQ™ chromatin analysis kit is a research tool used to probe the chromatin structure of a targeted region on the genome through in situ chromatin digestion followed by quantitative PCR (qPCR) analysis. It is essential to design and optimize the qPCR assays for the targeted regions to ensure the accuracy of the EpiQ chromatin analysis kit results.

A typical target for analysis using the EpiQ kit is the proximal promoter region, which can be high in GC content and challenging for efficient qPCR amplification. In addition, the in situ digestion step, which is essential to chromatin structure analysis using the EpiQ kit, is optimized for large target regions (200–350 bp in size) to achieve higher sensitivity in detecting chromatin structure changes. The combination of high GC content and large amplicon size demands careful attention to primer design and to qPCR protocol optimization.

This guide provides detailed instructions on the following key steps:

1. Identifying the proximal promoter region of the targeted gene (Sections A–C)
2. Designing primers to amplify the promoter region (Section D)
3. Verifying selected primer sets in silico (Section E)
4. Optimizing qPCR cycling protocols for selected primer sets (Sections F and G)
5. Determining qPCR efficiency (Section H)
6. Using existing primer sets (for example, chromatin immunoprecipitation [ChIP] primers) for EpiQ analysis (Section I)

Note: This guide contains screenshots from websites as they appeared in September 2010; the appearance of the live Web pages may differ from the screenshots shown in this guide.

Note: To cite University of California–Santa Cruz (UCSC) Genome Bioinformatics, see instructions at <http://genome.ucsc.edu/cite.html>.

To cite Primer3, use the following reference:

Rozen S and Skaletsky HJ (2000). Primer3 on the WWW for general users and for biologist programmers. In: *Bioinformatics Methods and Protocols: Methods in Molecular Biology*, Krawetz S, Misener S (eds). (Totowa, NJ: Humana Press), pp. 365–386.

II. Primer Design

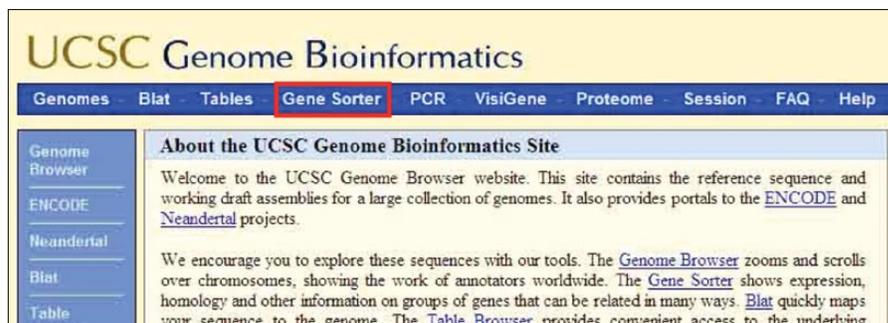
Proper primer design is critical for chromatin structure analyses using the EpiQ™ chromatin analysis kit. In general, primers used in this technique are longer and have a higher melting temperature (T_m) than primers used for typical qPCR-based gene expression analysis. In this section we describe:

- How to use the UCSC genome database to identify the sequence of the proximal promoter of a target gene
- How to use the Primer3 primer design tool, developed by the Massachusetts Institute of Technology (MIT), to design primers to amplify the promoter region
- How to perform in silico verification to assess the specificity of the selected primers

A. Finding the target gene using the UCSC Genome Bioinformatics site

The UCSC Genome Bioinformatics Site is an online resource that contains annotated genome sequence data from a large collection of organisms. All information can be accessed and downloaded free of charge.

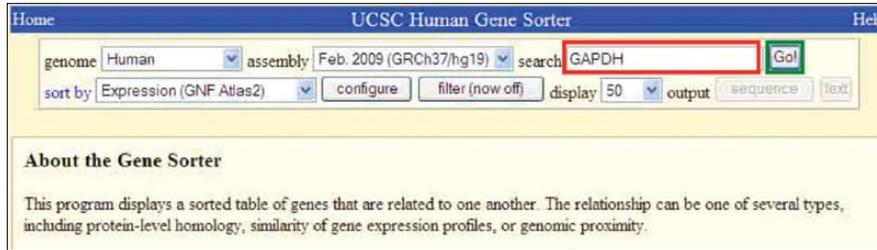
A-1. On the UCSC Genome Bioinformatics website home page (<http://genome.ucsc.edu/>), click Gene Sorter (highlighted in red).



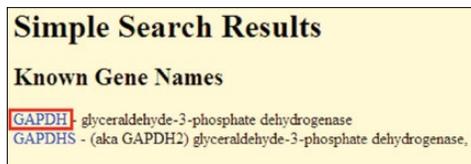
A-2. On the Gene Sorter page:

- (1) Select the genome of interest from the genome dropdown menu.
- (2) Select the latest genome assembly in the assembly dropdown menu.

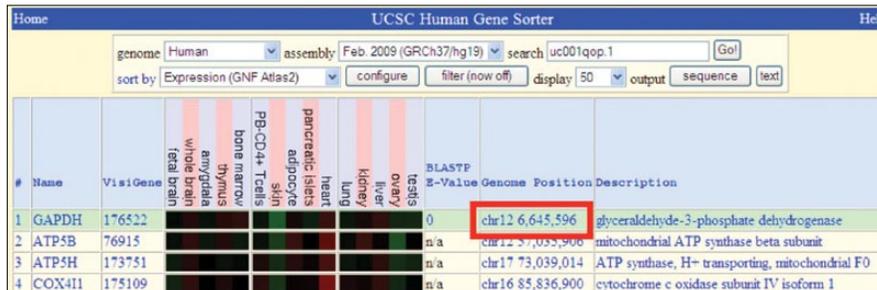
- (3) Enter the name of the gene you want to analyze in the search box (highlighted in red).
- (4) Click Go! (highlighted in green).



A-3. On the Search Results page, click the name of the gene (highlighted in red) that you want to study.

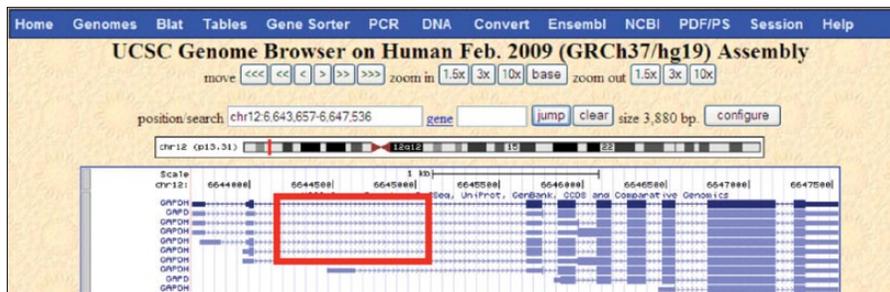


A-4. On the next page (see below) click the Genome Position value (highlighted in red) of your gene of interest.



A-5. The next screen displays the genome region of the selected gene target. It is important to download the sequence of the proximal region in the correct orientation. The orientation of your target gene is indicated by the directionality of the arrows.

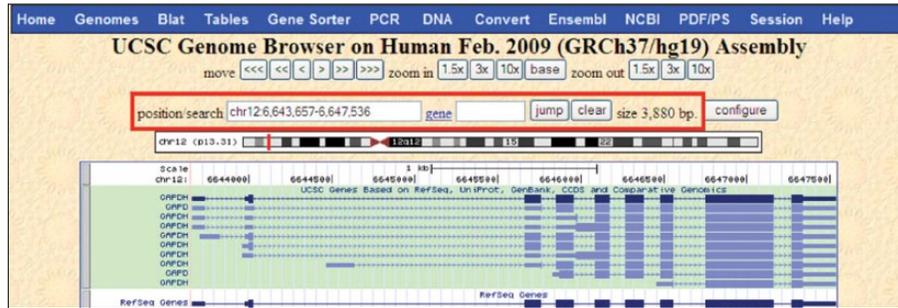
- If the arrows in the region highlighted in red point to the right (>>>>), the target gene is in the forward orientation; continue to Section B
- If the arrows in the region highlighted in red point to the left (<<<<<), the target gene is in the reverse orientation; skip Section B and proceed directly to Section C



B. Identifying and downloading the promoter sequence of a target gene in a forward orientation

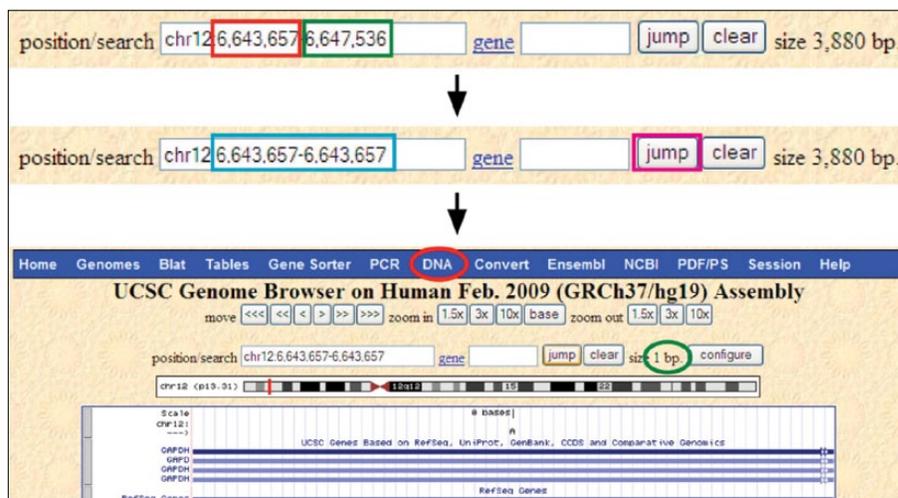
Note: For a gene that is in the forward orientation, the promoter region will be to the left of the coding region.

B-1. The numbers in the position/search field (highlighted in red in the screenshot) define the sequence boundaries of the *GAPDH* transcribed region. We will use this field to identify the transcription start site.



B-2. The first number (highlighted in red in the screenshot) in the position/search field corresponds to the transcription start site; the second number (highlighted in green) corresponds to the transcription end site. To isolate the transcription start site:

- (1) Change the transcription end site number to make it the same as the transcription start site number (highlighted in blue).
- (2) Click jump (highlighted in pink). The screen refreshes to display only the first nucleotide of the transcribed gene sequence.
- (3) Confirm that the size is 1 bp (circled in green).
- (4) Click DNA in the horizontal menu bar (circled in red); you will be taken to the screen shown in B-3.



B-3. To identify the promoter region of the target gene and display the sequence in a usable format:

- (1) Input 300 in the red box to start 300 bp upstream of the current position and 99 in the green box to select an additional 99 bp downstream, as shown in the following screenshot.
- (2) Click extended case/color options (highlighted in blue); you will be taken to the screen shown in B-4.

Home Genomes Genome Browser Blat Tables Gene Sorter PCR Session FAQ Help

Get DNA in Window

Get DNA for

Position

Note: if you would prefer to get DNA for features of a particular track or table, try the [Table Browser](#) using the output format sequence.

Sequence Retrieval Region Options:

Add extra bases upstream (5') and extra downstream (3')

Note: if a feature is close to the beginning or end of a chromosome and upstream/downstream bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.

Sequence Formatting Options:

All upper case.
 All lower case.
 Mask repeats: to lower case to N
 Reverse complement (get '-' strand sequence)

Note: The "Mask repeats" option applies only to "get DNA", not to "extended case/color options".

B-4. To define the format used to display the sequence:

- (1) Change Letters per line (highlighted in red) to 50.
- (2) Check the Toggle Case box (highlighted in green) in the RefSeq Genes row.
- (3) Click submit (highlighted in blue). The sequence of the proximal promoter region of your gene of interest will be displayed on screen as shown in B-5.

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Extended DNA Case/Color

Extended DNA Case/Color Options

Use this page to highlight features in genomic DNA text. DNA covered by a particular track can be highlighted by case, underline, bold, italic, or color. See below for details about color, and for examples. Tracks in "hide" display mode are not shown in the grid below.

Position Reverse complement

Letters per line Default case: Upper Lower

Track Name	Toggle Case	Under-line	Bold	Italic	Red	Green	Blue
Chromosome Band (Ideogram)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
UCSC Genes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
RefSeq Genes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
Human mRNAs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
Spliced ESTs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
SNPs (131)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
RepeatMasker	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>

B-5. The following screenshot shows the DNA sequence of the proximal promoter region. The first 300 nucleotides (uppercase letters, highlighted in red) are the promoter sequence and the last 100 nucleotides (highlighted in blue) are the transcribed sequence. The lowercase letters are the exon 1 sequence; the uppercase letters are the intron 1 sequence.

Home Genomes Genome Browser Blat Tables

Extended DNA Output

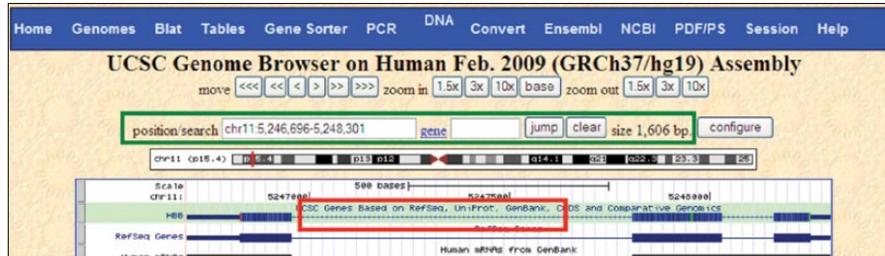
```
>chr12:6643357-6643756
CCGTCCTTGACTCCCTAGTGTCTGCTGCCACAGTCCAGTCCCTGGGAAC
CAGCACCGATCACCTCCCATCGGGCCAATCTCAGTCCCTTCCCCCTACG
TCGGGGCCACACGCTGGGTGCGTGCCAGTGAACCAGGCGGCTGGGA
AAAAAAAAAGCGGGAGAAAGTAGGGCCCGGCTACTAGCGGTTTACGGG
CGCACGTAGCTCAGGCCTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGG
CGGGAGGCGGGTCCGAGTCAACCGCCTGCCGCGCGCCCCGGTTTCTAT
aaattgagcccgagcctcccgttcgctctctgctcctcctggttgaca
gtcagccgatcttcttttgctgctcagGTGAAGACGGGCGAGAGAAA
```

B-6. Copy and paste the sequence into a word processing document for later use. Label the sequence with the species name, gene name, and chromosome region.

C. Identifying and downloading the promoter sequence of a target gene in a reverse orientation

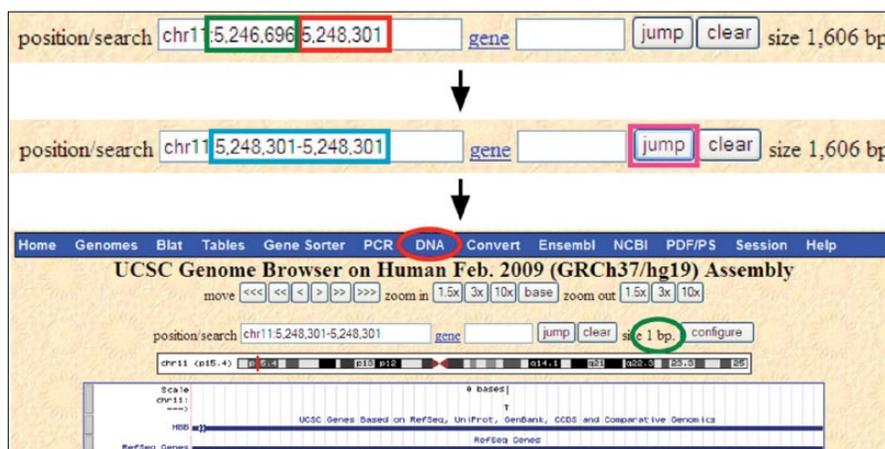
Note: For a gene that is in the reverse orientation, the promoter region will be to the right of the coding sequence.

C-1. The screenshot shows the hemoglobin gene (*HBB*) that is in the reverse orientation. The numbers in the position/search field (highlighted in green) define the sequence boundaries of the *HBB* transcribed region. We will use this field to identify the transcription start site.



C-2. The first number (highlighted in green) in the position/search field corresponds to the transcription end site; the second number (highlighted in red) corresponds to the transcription start site. To isolate the transcription start site:

- (1) Change the transcription end site number to make it the same as the transcription start site number (highlighted in blue).
- (2) Click jump (highlighted in pink). The screen refreshes to display only the first nucleotide of the transcribed gene sequence.
- (3) Confirm that the size is 1 bp (circled in green).
- (4) Click DNA in the horizontal menu bar (circled in red); you will be taken to the screen shown in C-3.



C-3. To define the promoter region of the target gene and display the sequence in a usable format:

- (1) Input 99 in the red box to select 99 bp upstream of the current position and 300 in the green box to select an additional 300 bp downstream, as shown in the following screenshot.
- (2) Click extended case/color options (highlighted in blue); you will be taken to the screen shown in C-4.

Home Genomes Genome Browser Blat Tables Gene Sorter PCR Session FAQ Help

Get DNA in Window

Get DNA for

Position

Note: if you would prefer to get DNA for features of a particular track or table, try the [Table Browser](#) using the output format sequence.

Sequence Retrieval Region Options:

Add extra bases upstream (5') and extra downstream (3')

Note: if a feature is close to the beginning or end of a chromosome and upstream/downstream bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.

Sequence Formatting Options:

All upper case.
 All lower case.
 Mask repeats: to lower case to N
 Reverse complement (get '-' strand sequence)

Note: The "Mask repeats" option applies only to "get DNA", not to "extended case/color options".

C-4. To define the format used to display the sequence:

- (1) Change Letters per line (highlighted in red) to 50.
- (2) Check the Reverse complement box (highlighted in green).
- (3) Check the Toggle Case box (highlighted in blue) in the RefSeq Genes row.
- (4) Click submit (highlighted in pink). The sequence of the proximal promoter region of your gene of interest will be displayed on screen as shown in C-5.

Home Genomes Genome Browser Blat Tables Gene Sorter PCR

Extended DNA Case/Color

Use this page to highlight features in genomic DNA text. DNA covered by a particular track display mode are not shown in the grid below.

Position Reverse complement

Letters per line Default case: Upper Lower

Track Name	Toggle Case	Under-line	Bold	Italic	Red	Green	Blue
Chromosome Band (Ideogram)	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
UCSC Genes	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
RefSeq Genes	<input checked="" type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>
Human mRNAs	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="checkbox"/>	<input type="text" value="0"/>	<input type="text" value="0"/>	<input type="text" value="0"/>

C-5. The following screenshot shows the DNA sequence of the proximal promoter region. The first 300 nucleotides (highlighted in red) are the promoter sequence; the last 100 nucleotides (highlighted in blue) are the transcribed sequence. Exon 1 sequences are indicated in lowercase letters, and intron 1 sequences are shown in uppercase letters (not applicable in this example).

```

Home  Genomes  Genome Browser  Blat  Tables
Extended DNA Output
>chr11:5248202-5248601 (reverse complement)
CAATATGCTTACCAAGCTGTGATTCCAAATATTACGTAAATACACTTGCA
AAGGAGGATGTTTTTAGTAGCAATTTGTACTGATGGTATGGGGCCAAGAG
ATATATCTTAGAGGGAGGGCTGAGGGTTTGAAGTCCCACTCCTAAGCCAG
TGCCAGAAGAGCCAAGGACAGGTACGGCTGTCATCACTTAGACCTCACCC
TGTGGAGCCACCCCTAGGGTTGGCCAATCTACTCCCAGGAGCAGGGAGG
SCAGGAGCCAGGGCTGGGCATAAAAGTCAGGGCAGAGCCATCTATTGCTT
acatttgcttctgacacaaactgtgttcaactagcaacctcaaacagacacc
atgggtgcatctgactcctgaggagaagtctgcccgttactgccctgtgggg

```

C-6. Copy and paste the sequence obtained in Step C-5 into a word processing document for later use. Label the sequence with species name, gene name, and chromosome region.

D. Designing PCR primers to amplify the promoter

Primer3 is a freely accessible primer design tool developed by researchers at MIT. This section describes how to design and select appropriate primer sets for use in qPCR analysis with the EpiQ kit.

D-1. To design primers that amplify the promoter region of a target gene:

- (1) Go to the Primer3 website at <http://frodo.wi.mit.edu/primer3/> (see the following screenshot).
- (2) Paste the DNA sequence that includes the 300 nucleotide promoter region plus the 100 nucleotide gene sequence (Step B-5 or C-5) into the sequence field (highlighted in red). The *GAPDH* promoter sequence is used in this example.
- (3) Check the Pick left primer and Pick right primer boxes (highlighted in green).

Primer3 (v. 0.4.0) Pick primers from a DNA sequence.

[Checks for mispriming in template.](#) [disclaimer](#) [Primer3 Home](#)
[Primer3plus interface](#) [cautions](#) [FAQ/WIKI](#)

Paste source sequence below (5'→3', string of ACGTNacgtn -- other letters treated as N -- numbers and blanks ignored). FASTA format ok. Please N-out undesirable sequence (vector, ALUs, LINEs, etc.) or use a [Mispriming Library \(repeat library\)](#):
 NONE

```

CGGTCCCTGACTCCCTAGTGTCTGCTGCTGCCACAGTCCAGTCCCTGGGAAAC
CAGCACCGATCACCTCCCATCGGGCCAACTCTCAGTCCCTTCCCCCTACG
TCGGGGCCACACGCTCGGTGGTGCCTGACCCAGGGGCTGCGGA
AAAAAAAAAGCGGGAGAAAGTAGGGCCCGGCTACTAGCGGTTTACGGG
CGCACGTAGCTCAGGCTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGG
CGGGAGCGGGTCCGAGTCAACCGCTGCCGCGGCCCGGTTTCTAT
  
```

Pick left primer, or use left primer below: Pick hybridization probe (internal oligo), or use oligo below: Pick right primer, or use right primer below (5' to 3' on opposite strand):

D-2. An amplicon size in the range of 200–350 bp, and ideally 250–300 bp, is optimal for use with the EpiQ chromatin analysis kit. Follow these steps to define the size of the amplicon:

- (1) Scroll down to the Product Size Ranges field (highlighted in red), which lists the default amplicon sizes for PCR amplification.
- (2) Add 250–300, 200–300, 250–350 before the first default entry (150–250) in the Product Size Ranges field.
- (3) Set the Number To Return (highlighted in green) to 10.

Sequence Id: A string to identify your output.
 Targets: E.g. 50,2 requires primers to surround the 2 bases at positions 50 and 51. Or mark the source sequence with [and]: e.g. ...ATCT[CCCC]TCAT.. means that primers must flank the central CCCC.
 Excluded Regions: E.g. 401,7 68,3 forbids selection of primers in the 7 bases starting at 401 and the 3 bases at 68. Or mark the source sequence with < and >: e.g. ...ATCT<CCCC>TCAT.. forbids primers in the central CCCC.

Product Size Range: **250-300 200-300 250-350 150-250 100-300 301-400 401-500 501-600 601-700 701-850 800**

Number To Return: **10** Max 3' Stability: 90

Max Repeat Mispriming: 12.00 Pair Max Repeat Mispriming: 24.00
 Max Template Mispriming: 12.00 Pair Max Template Mispriming: 24.00

D-5. In addition to the recommended primer pair, Primer3 also provides a list of Additional Oligos (highlighted in red) that can be used. Including alternative primer sets for further evaluation can increase the chance of finding an optimal primer set. Select alternative primer sets that are significantly different than the recommended primer set and save them into the same document used in Step D-4.

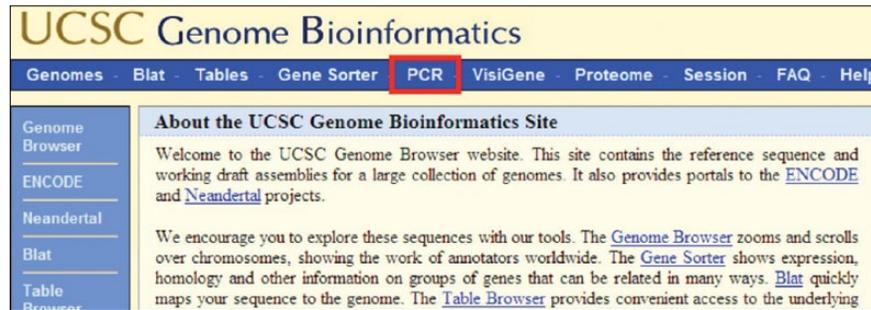
Note: Primers from different sets on the list can be selected and combined as an alternative set as shown (highlighted in green).

ADDITIONAL OLIGOS							
	start	len	tm	gc%	any	3'	seq
1 LEFT PRIMER	59	24	72.02	58.33	5.00	0.00	ATCACCTCCCATCGGGCCAATCTC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 299, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 2.00							
2 LEFT PRIMER	58	24	72.02	58.33	5.00	1.00	GATCACCTCCCATCGGGCCAATCTC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 300, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 1.00							
3 LEFT PRIMER	68	25	73.19	60.00	5.00	0.00	CAICGGGCCAATCTCAGTCCCTTCC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 290, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 2.00							
4 LEFT PRIMER	67	25	73.19	60.00	5.00	0.00	CCATCGGGCCAATCTCAGTCCCTTCC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 291, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 2.00							
5 LEFT PRIMER	58	25	73.23	60.00	5.00	0.00	GATCACCTCCCATCGGGCCAATCTC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 300, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 2.00							
6 LEFT PRIMER	62	24	71.72	58.33	5.00	1.00	ACCTCCCATCGGGCCAATCTCAGT
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 296, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 1.00							
7 LEFT PRIMER	68	25	73.19	60.00	5.00	0.00	CAICGGGCCAATCTCAGTCCCTTCC
RIGHT PRIMER	363	25	72.19	56.00	6.00	3.00	agatgggctgactgtcgaacagga
PRODUCT SIZE: 296, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 3.00							
8 LEFT PRIMER	67	25	73.19	60.00	5.00	0.00	CCATCGGGCCAATCTCAGTCCCTTCC
RIGHT PRIMER	363	25	72.19	56.00	6.00	3.00	agatgggctgactgtcgaacagga
PRODUCT SIZE: 297, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 3.00							
9 LEFT PRIMER	64	24	72.67	62.50	5.00	0.00	CTCCCATCGGGCCAATCTCAGTCC
RIGHT PRIMER	357	25	71.95	60.00	6.00	0.00	ggctgactgtcgaacaggaggagca
PRODUCT SIZE: 294, FAIR ANY COMPL: 5.00, FAIR 3' COMPL: 2.00							

E. Verifying the specificity of primer sets in silico

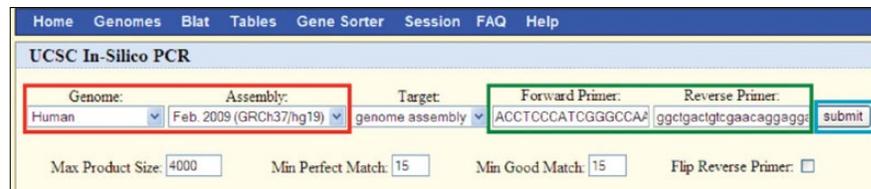
It is important to confirm the specificity of each primer pair to ensure that the primers will not amplify outside of the targeted region. The following section describes how to perform in silico verification.

E-1. Go to the UCSC Genome Bioinformatics website home page (<http://genome.ucsc.edu/>). Click the PCR tab (highlighted in red) to access the In-Silico PCR page (shown in E-2).

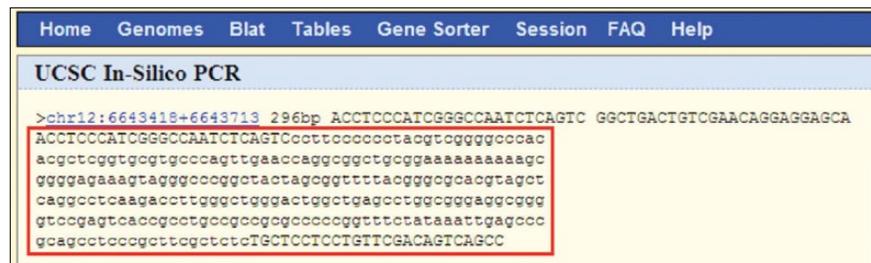


E-2. To assess the specificity of the selected primer sequences:

- (1) Select the appropriate species and the latest genome assembly using the Genome and Assembly dropdown menus (highlighted in red).
- (2) Copy and paste from your word processing document (Steps D-4 and D-5) the selected Left and Right primer sequences into the Forward Primer and Reverse Primer fields (highlighted in green), respectively.
- (3) Click submit (highlighted in blue); you will be taken to a screen displaying all sequences that can potentially be amplified by the pair of primers tested.



E-3. Verify that only one sequence is displayed and that the sequence is from the targeted region. If this is the case, the primer set is likely to be specific for your gene; if not, try another primer pair. A primer set that is verified to be specific by in silico analysis can still lead to nonspecific amplification in qPCR analysis. To ensure optimal qPCR performance, it is recommended that two or three in silico verified primer sets be used for functional verification (see Section F).



III. Optimization of qPCR Conditions

The promoter regions analyzed in the EpiQ™ chromatin assay are usually long (200–350 bp) and GC-rich, which makes qPCR amplification more challenging technically. The EpiQ™ chromatin SYBR® Green supermix is optimized for amplifying difficult genomic DNA regions and is strongly recommended for use with the EpiQ chromatin structure analysis kit. The qPCR protocol for the EpiQ™ chromatin SYBR® Green supermix features a regular annealing/extension step (step 3 in Table 1) followed by a short, high temperature, second extension step (step 4 in Table 1). While the lower temperature annealing/extension step allows efficient hybridization of the primers to the DNA template, the higher temperature of the second extension step allows melting of the local tertiary structure of the template DNA and complete extension of the nascent DNA strand. Use of optimized temperatures at both steps is essential to achieve high amplification efficiency. Sections F and G describe in detail how to determine the optimal amplification protocol for newly designed primer sets.

Table 1. Overview of EpiQ qPCR cycling protocol.

	Number of Cycles	Temperature	Time	Purpose
Step 1	1	96°C	5 min	<ul style="list-style-type: none">• Complete denaturation of template• Activation of hot-start polymerase
Step 2	40	96°C	15 sec	Denaturation of template/amplicon
Step 3	40	Optimized annealing/extension temperature, plate read*	1 min	<ul style="list-style-type: none">• Primer annealing and extension• Data acquisition
Step 4	40	Optimized temperature	30 sec	Second extension step
Step 5		70°C–96°C, 0.2°C/step, 5 sec hold		Melting analysis

* The plate read step is placed prior to the second extension step to avoid loss of SYBR® Green signal at a higher temperature. The signal from the product produced during the second extension step will be captured during the plate read step of the next cycle.

F. Determining the optimal annealing/extension temperature

In this section, we describe how to determine an optimal annealing/extension temperature for a primer set using EpiQ™ chromatin SYBR® Green supermix. A real-time PCR instrument with temperature gradient capability is required to perform the experiment.

F-1. Prepare sufficient qPCR master mix for the replicates needed (see Table 2). The example provided is for a 96-well plate on an instrument that forms a temperature gradient across the eight rows. For a 384-well plate, the volume should be adjusted accordingly, based on 10 μl reaction volume and the number of wells across the temperature gradient.

Table 2. qPCR master mix for gradient analysis.

Reagent	For 8 x 20 μl reactions (gradient across rows)
2x EpiQ™ chromatin SYBR® Green supermix	90 μl
Genomic DNA (1 ng/ μl) (or TE buffer for no template control)	45 μl
Primer mix (2 μM each)	45 μl

F-2. Pipet 20 μl of the qPCR reaction mix into all wells across the temperature gradient. Pipet 20 μl of the no template control (NTC) reaction mix into all wells of the adjacent column (or row).

F-3. Perform qPCR using the cycling protocol:

Step 1. 96°C for 5 min

Step 2. 96°C for 15 sec

Step 3. 63°C–72°C temperature gradient for 1 min; plate read

Step 4. 80°C for 30 sec

Step 5. Go to step 2, 39 more times

Step 6. Melt curve, 70°C to 96°C, 0.2°C increment, 5 sec hold

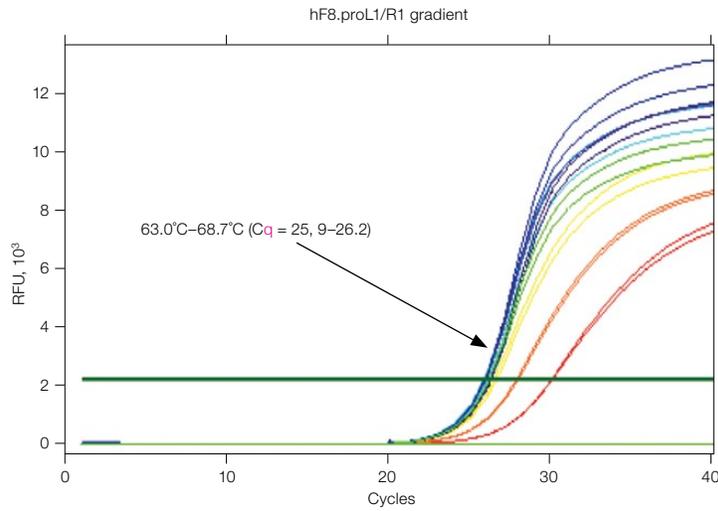
END

F-4. Analyze the results:

- (1) Examine the melting profile. Eliminate any temperatures where an anomalous melt peak is observed. If two melt peaks are observed over several temperatures, the cause could be either a single amplification product with two melt domains or the amplification of two distinct products. To discriminate between these possibilities, analyze the qPCR product by agarose gel electrophoresis. If a single band is observed, proceed to Step F-4(3). If two or more bands are observed, discard the primer set and test a different one.

Note: To avoid cross-contamination, perform gel analysis of qPCR products in a designated location that is separate from the area where qPCR reactions are prepared. Only pipets dedicated for handling qPCR reactions should be used.

- (2) Examine the amplification profile. Eliminate any temperatures where significant nonspecific amplification is observed in the NTC reaction.
- (3) Determine the quantification cycle (C_q) values of the remaining reactions. Any temperature that results in a C_q value within 0.5 cycles of the earliest C_q value can be selected and used as the optimal annealing/extension temperature for subsequent qPCR analysis. The following example shows a gradient experiment varying the annealing/extension temperature from 63.0°C to 72°C, in which 63.0°C–68.7°C produced C_q values differing by no more than 0.3 cycles; thus any temperature within the range of 63°C and 68.7°C can be used as the annealing/extension temperature for qPCR amplification.



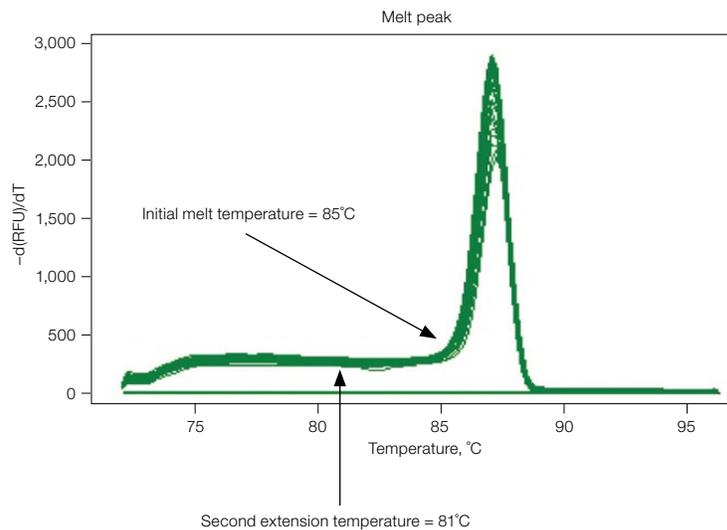
G. Determining the optimal temperature for the second extension step

The optimal temperature of the second extension step can range from 80°C to 90°C and is dependent upon the initial melting temperature of the amplicon. This section describes how to determine the optimal temperature for the second extension step.

G-1. Select a reaction or set of reactions from Step F-4 identified as having an optimal annealing/extension temperature, and display the melt peak profile.

G-2. Determine the optimal temperature of the second extension step by identifying the initial melt temperature (see the following figure).

- If the initial melt temperature is <81°C, the second extension step can be omitted
- If the initial melt temperature is 81°C to 84°C, 80°C should be used for the second extension step
- If the initial melt temperature is >85°C, use a temperature that is 4°C below the initial melt temperature for the second extension step



H. Determining qPCR efficiency

Once the optimal amplification protocol is determined, newly designed primer sets must be verified for amplification efficiency using a standard curve analysis with a serial dilution of genomic DNA. We recommend selecting primer sets that exhibit >80% qPCR efficiency for subsequent chromatin structure analysis. In addition, using the qPCR efficiency correction feature provided in the EpiQ chromatin kit data analysis tool (www.bio-rad.com/epiq) will allow more accurate assessment of chromatin structure.

H-1. Label five 1.5 ml tubes and prepare a 5-fold genomic DNA dilution series in TE buffer from a 1 ng/μl genomic DNA stock as shown in Table 3. Tube 5 contains TE only to serve as an NTC.

Note: Genomic DNA isolated from an undigested EpiQ kit sample (Section C or F of EpiQ chromatin analysis kit manual (www.bio-rad.com/epiq)) or purchased from a commercial source can be used to prepare the genomic DNA dilution series.

Table 3. Genomic DNA dilution for standard curve analysis.

Tubes	Source Genomic DNA	Volume of Source Genomic DNA	Volume of TE Buffer	Resulting DNA Dilution	Input DNA in qPCR (5 μl per 20 μl reaction)
1	1,000 pg/μl	50 μl	0	1,000 pg/μl	5,000 pg
2	1,000 pg/μl (Tube 1)	10 μl	40 μl	200 pg/μl	1,000 pg
3	Tube 2	10 μl	40 μl	40 pg/μl	200 pg
4	Tube 3	10 μl	40 μl	8 pg/μl	40 pg
5	None	None	50 μl	NTC	NTC

H-2. Prepare a qPCR master mix with enough volume to run each dilution in triplicate, as shown in Table 4. Pipet three replicates of each reaction (20 μl) into a 96-well plate.

Table 4. qPCR master mix preparation for triplicates analysis.

Reagent	Volume (for 3.6 x 20 ml reaction)
Genomic DNA dilution	18 ml
Primer mix (2 μM each)	18 ml
EpiQ chromatin SYBR® Green supermix	36 ml

H-3. Run qPCR using the cycling conditions outlined in step F-3, the annealing/extension temperature identified in Step F-4, and the second extension step temperature identified in Step G-2.

H-4. Analyze the results:

- If the NTC reactions exhibit a melt curve that is consistent with the reactions containing template, it indicates that a reagent is contaminated with genomic DNA. If this is the case, change or decontaminate pipets and work surfaces, prepare new genomic DNA dilutions and primer solutions using fresh reagents, and repeat the experiment
- Eliminate any template-containing reactions that exhibit a melt curve that is not consistent with the expected melt-curve profile
- Eliminate any reactions that are obvious outliers (significantly different from other replicates)

H-5. qPCR amplification efficiency is determined from the slope of the plot of log (starting DNA quantity) (y-axis) versus the corresponding Cq value (x-axis) based on the following equation:

$$\text{Efficiency (\%)} = (10^{-1/\text{slope}} - 1) \times 100$$

Note: Most Bio-Rad real-time PCR instrument software programs (except Opticon Monitor™ software) report both slope and qPCR efficiency values from standard curve analyses. Other qPCR analysis software packages may report only the slope value and not qPCR efficiency. In such cases, the qPCR efficiency can be calculated from the slope value using the EpiQ chromatin kit data analysis tool (www.bio-rad.com/epiq).

I. Using existing primer sets with the EpiQ chromatin analysis kit

Preexisting primer sets that amplify genomic DNA (for example, ChIP primers) can be used in the EpiQ chromatin assay. However, amplicon size does affect the EpiQ chromatin analysis results. Small amplicons require more nuclease digestion to achieve optimal results. This section describes how to optimize the chromatin digestion conditions using preexisting primers that amplify smaller genomic regions.

I-1. Use Table 5 to select the reference gene (*RHO*, an epigenetically silenced gene) and control gene (*GAPDH*, a constitutively expressed gene) primer sets that amplify a region similar in size to the target region.

Note: It is important to analyze control gene, reference gene, and target gene amplicons of a similar size to obtain optimal chromatin structure analysis results.

Table 5. Primer pair sequences to amplify amplicons of different sizes for reference and control genes.

Amplicon Size	Species	Reference Gene (<i>RHO</i>)	Control Gene (<i>GAPDH</i>)
300 bp	Human	F AGGTCAC TTTATAAGGGTCTGGGGG R AGTTGATGGGGAAGCCAGCACGAT	F ACCTCCCATCGGGCCAATCTCAGTC R GGCTGACTGTGGAACAGGAGGAGCA
300 bp	Mouse	F AGGTCAC TTTATAAGGGTCTGGGGG R AGTTGATGGGGAAGCCAGCACGAT	F CAGCTCCCCTCCCCCTATCAGTTTCG R ACCAGGGAGGGCTGCAGTCCGTATT
200 bp	Human	F TGGATCCTGAGTACCTCTCCTCCCT R GGCTCCAGCTGGATGACTCTGGGTT	F TCGGGGCCACACGCTCGGTGCGTG R GGCTGACTGTGGAACAGGAGGAGCA
150 bp	Human	F CCTTGGCCCCTTTAGAAGCCAATT R GGCTCCAGCTGGATGACTCTGGGTT	F CGCACGTAGCTCAGGCCTCAAGACC R GGCTGACTGTGGAACAGGAGGAGCA
100 bp	Human	F TTAATATGATTATGAACACCCCAA R GGCTCCAGCTGGATGACTCTGGGTT	F CGGGAGGCGGGTCCGAGTACCCGC R GGCTGACTGTGGAACAGGAGGAGCA
80 bp	Human	F ACACCCCCAATCTCCAGATGCTGA R GGCTCCAGCTGGATGACTCTGGGTT	F CTGCCGCGCGCCCCCGTTTCTAT R GGCTGACTGTGGAACAGGAGGAGCA
70 bp	Human	F TCTCCAGATGCTGATTCAGCCAGG R GGCTCCAGCTGGATGACTCTGGGTT	F CCGGTTTCTATAAATTGAGCCCGCA R GGCTGACTGTGGAACAGGAGGAGCA

Note: The primer sets in bold are provided in the EpiQ chromatin analysis kit.

I-2. Optimize the digestion conditions for the selected target regions by performing a nuclease dose-response experiment by titrating up to 5x the recommended amount of nuclease. In general, shorter amplicons require more nuclease to allow accessible regions of chromatin to be effectively digested. Effective digestion is defined as >95% accessibility of the chromatin structure of the control gene (for example, *GAPDH*).

Note: Increasing digestion time can also influence the results and improve digestion efficiency with small target regions, but the effect is not as significant as that achieved by varying the amount of nuclease used.

I-3. For qPCR analysis, the primer sets listed in Table 5 can be successfully used in the cycling protocol.

Note: For target region primer sets, the qPCR analysis can be run on a separate plate if the optimal cycling protocol differs from the one used for the reference and control genes.

Cycling protocol for reference and control genes:

- 96°C for 5 min
- 40 cycles of
- 96°C for 15 sec
 - 66°C for 1 min
 - Plate read
 - 80°C for 30 sec*

Melt curve, 70°C to 96°C, 0.2°C increments, 5 sec hold

* When small amplicons are used, it is not necessary to use a second extension step.

I-4. The EpiQ chromatin kit data analysis tool (www.bio-rad.com/epiq) provides an easy-to-use module to analyze data from a nuclease dose-response experiment.

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