



qPCR PRIMER DESIGN

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INTRODUCTION

Proper design of primers that will be used in quantitative PCR (qPCR), also called real time PCR (not to be confused with Reverse Transcription PCR [RT-PCR]) is a critical first step in measuring gene expression. There are many tools and guides available online that can assist you with this process. It is highly recommended that you read the principles of qPCR, particularly using SYBR green as a detection method. The purpose of this protocol is to provide a near-foolproof step-by-step guide beginning with selecting a gene of interest to final primer optimization and running your qPCR experiment.

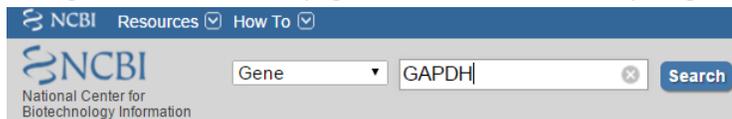
PROTOCOL

Selecting a Gene of Interest

1. First, select a gene that you wish to measure. In this example, we are going to use **Glyceraldehyde 3-phosphate dehydrogenase or GAPDH**. GAPDH has long been used as a 'housekeeping control gene' that in most (but not all) cases serves as a measure of constitutive gene expression.

Some important considerations you will need to remember are;

- What species are you going to be analyzing? Mouse, Human, etc?
 - Are there different splice variants of your gene of interest?
 - Will you have a good positive control to induce this gene?
 - Are there previously published primer sequences available that you can compare against?
 - www.rtprimerdb.org or <https://pga.mgh.harvard.edu/primerbank/>
2. Next, go to the NCBI search page and under "Gene" enter your gene of interest.



3. Your search will likely yield a lot of results, depending on the gene. Narrow the search by selecting your organism on the right side menu.



4. Click on your gene of interest. Review the information to ensure this is the correct gene.

Search results

Items: 1 to 20 of 65

<< First < Prev Page 1 of 4 Next > Last >>

 [See also 579 discontinued or replaced items.](#)

Name/Gene ID	Description	Location	Aliases
<input type="checkbox"/> Gapdh ID: 14433	glyceraldehyde-3-phosphate dehydrogenase [<i>Mus musculus</i> (house mouse)]	Chromosome 6, NC_000072.6 (125161721..125166467, complement)	Gapd

5. Scroll down on the right hand side and open the CCDS sequence.

Related information

- Order cDNA clone
- BioAssay by Target (List)
- BioAssay by Target (Summary)
- BioAssay, by Gene target
- BioAssays, RNAi Target, Tested
- BioProjects
- BioSystems
- CCDS** 
- Conserved Domains
- dbVar
- EST

6. Since you properly reviewed the principles of qPCR you should know that you only want to measure transcription of this gene, not the genomic DNA (gDNA) of this gene. Unless you treat your sample with DNase, there will always be some genomic DNA contamination that may affect your qPCR readouts. Therefore, we want our primers to sit in different exons, with one of the two primers sitting at an exon-exon junction (i.e. in two exons). By doing this, we will ensure that our primers only amplify transcript, because 1.) the product size of a PCR reaction will be too large if there is gDNA contamination and our primers somehow amplify gDNA. 2.) the primer that sits on an exon-exon junction should not anneal, as the transcript will have undergone splicing, such that introns are removed, allowing for the primer to sit down at its appropriate location within the mature transcript.
7. Open the consensus cDNA sequence to view the exons by clicking the red “C”



Report for Gene ID 14433

CCDS	Current	Status	Species	Chrom.	Gene	CCDS Release	NCBI Annotation Release	Ensembl Annotation Release
51913.1		Public	<i>Mus musculus</i>	6	Gapdh	21	106	86

8. Analyze the exons that are alternately colored in blue and black. Therefore an exon-exon junction would be a region where the blue font becomes black. Since your primers are around 20bp, you want **roughly** half in one exon and half in another. You can copy this sequence to design your primers. I suggest copying it into a word document.

CCDS Sequence Data

Blue highlighting indicates alternating exons.

Red highlighting indicates amino acids encoded across a splice junction.

Mouse over the nucleotide or protein sequence below and click on the highlighted codon or residue to select the pair.

Nucleotide Sequence (1002 nt):

```
ATGGTGAAGGTCGGTGTGAACGGATTTGGCCGTATTGGGCGCTGGTACCAGGGCTGCCATTGCAAGTGC
GCAAAGTGGAGATTGTTGCCATCAACGACCCCTTCATTGACCTCACTACATGGTCTACATGTTCCAGTA
TGACTCCACTCACGGCAAATCAACGGCAGCTCAAGGCCGAGAATGGGAAGCTTGTCATCAACGGGAAG
CCCATCACCATCTTCCAGGAGCGAGACCCCACTAACATCAATGGGGTGAAGCCGGTGGTATGTCG
TGGAGTCTACTGGTGTCTTCCACCACATGGAGAAGGCCGGGGCCCACTTGAAGGGTGGAGCCAAAAGGGT
CATCATCTCCGCCCCCTTCTGCCGATGCCCCATGTTTGTGATGGGTGTAACACAGGAAATATGCAAC
TCACTCAAGATTGTCAGCAATGCATCTGCACCACCACTGCTTAGCCCCCTGGCCAGAGGTCATCCATG
ACAACTTTGGCATTGTGGAAAGGCTCATGACCACAGTCCATGCCATCACTGCCACCAGAAGACTGTGGG
TGGCCCTCTGGAAGCTGTGGCTGATGGCGTGGGCTGCCAGAACATCATCCCTGCATCCACTGGT
GCTGCCAAGGCTGTGGCAAGGTCATCCAGAGCTGAACGGGAAGCTCACTGGCATGGCTTCCGGTTC
CTACCCCAATGTGTCCTGCTGGATCTGACGTGCCGCTGGAGAAGCTGCCAAGTATGATGACATCAA
GAAGTGGTGAAGCAGGCTCTGAGGGCCCACTGAAGGGCATCTTGGGCTACAC TGAGGAC CAGGTTGTC
TCTCGCACTTCAACAGCAACTCCCACTTCCACTTTCGATGCCGGGGCTGGCATTGCTCAATGACA
ACTTTGTCAGGCTCATTTCTGATGACAATGAATACGGCTACAGCAACAGGGTGGTGGACCTCATGGC
CTACATGGCCCTCAAGGAGTAA
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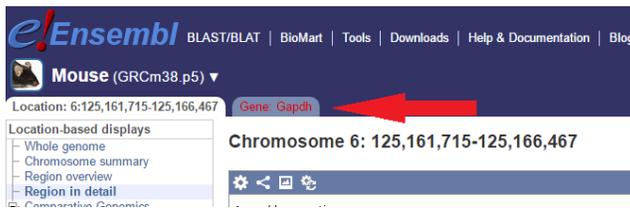
Translation (333 aa):

```
MKVGWVNGFRIGRLVTRAAICSGKVEIVAINDPFDLNYMVMYFQVDSTHGKFNKTKAENGLVINGK
PITIFQERDPTNIXWGEAGAAYVVESTGVFTTHEKAGAHKGGAKRVIISAPSDAPHFVWGVNHEKYDN
SLKTVSHASCTTNCIAPLAKVZHDNFGIVEGLMFTTVAHTTATQKTVDPGSKLWRDGRGAQNIIPASTG
AAKAVKVIPELNGKLTGNAFRVPTFNVSVDLTCRLEKPAKYDDIKKVKQASEGLKGLGYTEDQVV
SCDFNSHSHSFTDAGAGIALNDNFVKLISWYDNEYGVSHRVVDLHAYMASKE
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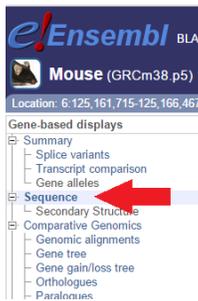
- If your gene of interest only contains a single exon, you will need to do some additional planning. As above, we want our primers to sit in different exons and do not have this option. We are now forced to DNase treat our samples to make sure the genomic DNA is degraded before we reverse transcribe our transcripts into cDNA. In addition to making a primer set that sits within the single exon, we will generate a second primer set where one primer sits in an intron and the other in an exon. This is to make sure there is no residual genomic DNA, we will design a primer pair that will ‘probe’ for genomic DNA in your gene of interest. Then, by keeping an aliquot of your RNA prior to DNase treatment and an aliquot after DNase treatment and probing with this gDNA primer set, you can ensure your sample is gDNA-free.
- On the right hand side of the NCBI gene page, open Ensembl genome browser.



- Click the gene tab in Ensembl to open up the Gene page.



- Click “sequence” on the left hand side to open up the list of introns & exons.



13. Whether you are designing primers for multiple-exon spanning genes, or a gDNA probe set, use your copied sequence with Primer3, which is found at <http://bioinfo.ut.ee/primer3-0.4.0/>
14. As per the Primer3 guidelines, you can now use various brackets () <> [] {} to “force” the program to make your forward and reverse primers to sit within various regions or to avoid certain regions. You will need to adjust these back-and-forth to ensure that one of your primers sits in an exon-exon junction.
15. Most Primer3 parameters are compatible with standard qPCR amplification conditions, such as the Tm range, salt concentrations, maximum self-complimentarity, etc. One parameter that needs to be adjusted is the product size range. You should input a product size range of 100-250 base pairs only, as larger products aren’t required for qPCR. Products smaller than 100bp are not ideal, as they appear at similar sizes on agarose gels as primer dimers. Another parameter to adjust is the “number of returns”. You should ask Primer3 to return around 10-20 primers. Read up on good PCR primer design and pay attention to things like multiple repeated bases (which is bad) and ensuring you have a “GC clamp” (which is good).

[Product Size Ranges](#)

[Number To Return](#)
[Max 3' Stability](#)

[Max Repeat Mispriming](#)
[Pair Max Repeat Mispriming](#)

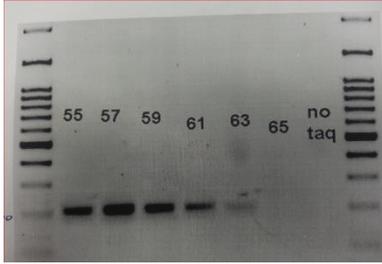
[Max Template Mispriming](#)
[Pair Max Template Mispriming](#)

16. Once you have found primers that you believe are suitable, use the underline or highlight tool on word to highlight the region of the transcript that each primer sits on. Remember, one primer should sit on an exon-exon junction. An easy thing to forget is that you will need to find the reverse complement (http://www.bioinformatics.org/sms/rev_comp.html) of the reverse primer before you can use the CTRL+F function in word to locate where the primer sits. Another easy mistake is to forget to remove “returns” from your template sequence. If your primer sits on the end of one line and into the beginning of another, unless you have removed line breaks your search will not return results. A fast way to remove all line breaks is to
 - CTRL + F
 - Click More >>
 - Click Special
 - Select Manual Line Break
 - Click the replace tab
 - Leave “Replace with” blank and hit Replace All

17. Once you are satisfied with your primer pair for your gene of interest, input them into Primer Blast (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>) to ensure that the primers are specific to your gene of interest. It may return some weak associations where a handful of random bases from your primer aligns with a non-specific gene, this is OK.
 - Remember to change the Organism on this page to your organism. In our case of Mouse GAPDH, the default is Homo Sapiens, so it must be changed to Mus Musculus.
18. Order your primers! Usually we recommend ordering around 2 different primer pairs for each gene, in case one set is sub-optimal. We use IDT as our primer supplier. Simply open the “order custom DNA oligos” page and order the smallest scale (25nMol). Paste the sequences of your primers into the order form. Remember to properly name your primers with “F” and “R” for forward and reverse designations. Primers are delivered to the Mobix facility in the Farncombe office in HSC 3rd floor. They should take 2-3 business days to arrive.

Preparing and testing primers:

1. When your primers arrive, they will contain a paper spec sheet that gives all pertinent information on your primer. It will also include the volume of nuclease-free water required to dissolve your lyophilized primers to a 100 uM concentration. Dissolve your primers and create a “ready mix” of your primers that can be used in PCR or qPCR.
 - Take 10uL of your Forward and 10uL of your reverse primers and add it to 80uL nuclease-free water. This is a 10uM stock that can be used regularly. Store this and your 100uM primer mix at -20°C.
2. Use cDNA that you know is high-quality and of reasonable concentration to test your primers. For example, mouse GAPDH can be tested on any free mouse tissue, cells or cell lines. Use a standard RNA extraction method, such a TriZol to isolate RNA. Convert your RNA to cDNA using a manufacturer’s recommended protocol.
3. Use 1-2 uL of your cDNA with a standard laboratory Taq polymerase in a 25 uL standard PCR reaction. Again, follow manufacturers recommended protocols for the PCR reaction. You should use the manufacturer’s recommended amount of your forward and reverse primer mix (10uM). It is highly advisable to do a gradient PCR using a range of 10°C for your annealing temperatures. This will help find the optimal T_m for your primers. Since most qPCR primers are designed with T_m values around 60°C, I advise having a 55-65°C gradient. It is also important to include a no taq negative control. Remember to reduce the extension time to an appropriate number.
4. After 30-40 cycles, add the appropriate amount of DNA loading dye to your products and run it on a 2% agarose gel with 100V for approximately 1 hour. We use 2% gels for qPCR products because of the better resolution with small PCR products. Your gradient should look similar to this below. You want to have nice sharp bands that are at the correct product size. In this case, based on the gel, we would set our annealing temperature to 57°C for qPCR reactions.



5. If you are testing gDNA probing primers, it is normal to see multiple bands. You should not see multiple bands for your exon-spanning qPCR primers.
6. Sometimes, if your products seem suspicious or you want to ensure 100% product specificity, you can excise the band from the gel and use a cleanup kit to extract the DNA. Then it can be sent for sequencing (using the forward and reverse primers SEPARATELY). You can align this sequence to your target sequence by either entering it into nucleotide BLAST or using an alignment program such as Clustal Omega.
7. If everything checks out, you are now ready to use your primers in a qPCR pilot run. Be sure to include all appropriate positive and negative controls in your plate. You will also want to read up on how to interpret “melt curves” of your primers. Like always, before running your qPCR, be sure you are fully trained and competent at operating the cycler.