



### Primer Design and PCR Amplification of ORFs Protocol

The open reading frame (ORF) of the clone must be PCR amplified in order to append cloning sites to the 5' and 3' ends of the sequence. The protocol below provides example for primer design and directions on how to conduct PCR amplification of ORFs.

Primers can be purchased on the OriGene Website

#### Forward primer with Sgf I:

5' GAG**GCGATCGCC**NNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Ns represent the sequence of the ORF beginning with the start codon, ATG. It is important to add the additional "C" base after the Sgf I site to maintain appropriate reading frames with N-terminal tags in some destination vectors.

#### Reverse primer with Mlu I:

5' GCG**ACGCGT**NNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Ns represent the reverse complement of the ORF sequence starting with the stop codon for N-terminally tagged or untagged destination vectors. This ensures that the expressed fusion protein will end at the native C-terminal end of the ORF. For C-terminally tagged vectors, the reverse complement of the ORF sequence should start with the second-to-last codon, as the stop codon must be removed to generate a fusion protein.

If the recognition sites for Sgf I or Mlu I are present internally in the ORF, another rare cutter such as Asc I, Rsr II or Not I can be used in the cloning strategy. In these cases, the sequences of these alternate restriction sites should be used in place of Sgf I and/or Mlu I (examples below). This same primer design strategy described above should be used for the design of other primers. The Ns in the forward primer represent the sequence of the ORF beginning with the start codon, ATG. The Ns in the reverse primers represent the reverse complement of the ORF sequence starting with the stop codon for N-terminally tagged or untagged destination vectors or starting with the second-to-last codon for C-terminally tagged vectors.

#### Forward primer with Asc I:

5' GCC**GCGCGCC**ANNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

An extra nucleotide after Asc I is important to maintain reading frames with N-terminal tags in some destination vectors.

#### Reverse primer with Rsr II:

5' GCGT**CGGTCCG**CTNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Extra nucleotides after Rsr II are important to maintain appropriate reading frames with C-terminal tags in some destination vectors.

Reverse primer with Not I:

5' GCGAC**GCGGCCG**CGTACGCGTNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN 3'

Mlu I is also added for downstream subcloning.

We recommend using a full-length cDNA clone as the template for ORF cloning. The success rate is low when a cDNA pool is used as the template for a PCR cloning reaction. When the GC content of an ORF (or a region of the ORF longer than 100 bp) is above 75%, a special PCR buffer with DMSO or other additive should be used to increase the success rate. The recommended PCR polymerase and buffer are available from New England Biolabs (Phusion™ High-Fidelity PCR Kit, F-553S).

**PCR Reaction Set Up:**

<b>Component</b>	<b>Volume</b>
5X PCR Buffer	4 µl
dNTPs (2.5 mM each)	1.6 µl
Phusion polymerase (2U/ µl)	0.2 µl
Nuclease-free water	11 µl
Forward primer (µM)	0.6 µl
Reverse primer (µM)	0.6 µl
cDNA template	2 µl (50-100ng plasmid)
<b>Total Volume</b>	<b>20 µl</b>

All of the components should be kept on ice. When setting up multiple reactions, a master mix can be prepared without cDNA template or primers. After aliquoting the master mix, the cDNA template and primers can be added individually to each tube.

**PCR cycling conditions:**

The optimum T<sub>m</sub> for annealing should be 55-60°C. The extension time depends upon the length of the ORF. The following program is generally used for ORFs from 500 bp-4000 bp.

1 Cycle of	95°C	1 min
2 Cycles of	95°C	10 sec
	62°C	20 sec
	72°C	4 min
2 Cycles of	95°C	10 sec
	60°C	20 sec
	72°C	4 min
2 cycles of	95°C	10 sec
	58°C	20 sec
	72°C	4 min
15 cycles of	95°C	10 sec
	56°C	20 sec

	72°C	4 min
	72°C	10 min
	4°C	hold

With your final PCR product, you can now move forward with other downstream procedures.

If relevant, view the Entry Vector Cloning Protocol to learn how to clone this amplified ORF into a PCMV6-Entry Vector.