

Note: For C-terminal tagging, use the primer C sequence given in Figure 2B.5 regardless of the epitope tagging sequence you add to primer D.

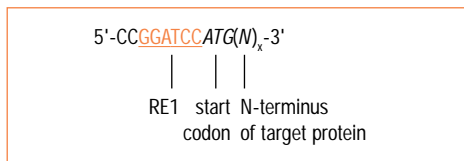


Figure 2B.5: Upstream primer for tagging the carboxy-terminal end of a gene. The restriction site (RE 1) shown in this example is a *Bam* H1 site, but any suitable restriction site may be used. *N* = A, T, G, or C; *x* = number of nucleotides from the part of the gene that encodes the N-terminus of the target protein. Typically, *x* would be 18–28.

► **Downstream Primer D** (Figure 2B.6): This downstream (3' end) primer includes a restriction enzyme site (RE 2, which should not be identical to RE 1 in Figure 2B.5 above), a stop codon, the epitope tag sequence, and the DNA sequence for the carboxy-terminus of the target protein.

Note: The primer shown in Figure 2B.6 is for the insertion of the *c-myc* epitope. For insertion of HA or VSV-G epitopes, substitute the appropriate tag sequence from Table 2B.3 in place of the *c-myc* sequence.



Figure 2B.6: Downstream primer for tagging the carboxy-terminal end of a gene with the *c-myc* epitope. For tagging with the HA or VSV-G epitope, replace the *c-myc* sequence with the appropriate sequence listed in Table 2B.3. The restriction site (RE 2) shown in this example is an *Xba* 1 site, but any suitable restriction site may be used. *M* = A, T, G, or C; *y* = number of nucleotides from the part of the gene that encodes the C-terminus of the target protein. Typically, *y* would be 18–28.

If you want to tag the protein with	Then substitute this sequence for the <i>c-myc</i> sequence in upstream primer A (Figure 2B.3)
HA	A Y D P V D Y P Y 5'-AGCGTAGTCTGGGACGTCGTATGGGTA-3'
VSV-G	K G L R N M E I D T Y 5'-CITACCCAGGCGGTTTCATTTCGATATCAGTGTA-3'

Table 2B.3: Tag coding sequences for substituting HA or VSV-G epitope tags into downstream primer D

Getting started: Procedures for epitope tagging by PCR

Caution: These procedures must be optimized empirically for each individual experimental system (epitope tag sequence, tagged protein, cell in which protein is expressed). Use the parameters listed in these procedures (cycling times and temperatures, reagent concentrations, and so forth) only for initial experiments or for determining optimal ranges for each parameter. Pay particular attention to those procedural steps marked with a **Note** or **Caution** statement, since these are critical steps that must be optimized if the procedure is to produce meaningful results.

Note: The products indicated in colored type are available from Boehringer Mannheim. For detailed ordering information on these and related products, see the Boehringer Mannheim Product Ordering Guide, Section 6B of this manual.

I. Attaching the tag by PCR

1 Set the primer-template annealing temperature 5°C below the T_m of the primer pair. Ideally the annealing temperature should be between 50°C and 72°C.

Note: PCR annealing temperatures that are set too low may lead to false priming while annealing temperature set too high may cause inefficient priming.

2 In a sterile thin-walled reaction tube on ice, prepare a 100 µl PCR mix that contains the following:

- Reaction buffer, pH 8.3–8.9 (depending on polymerase used)
- Template DNA (<1 µg/100 µl)
- 1–3 mM MgCl₂
- 0.2 mM of each deoxynucleotide triphosphate (dNTP)
- 0.1–1.0 µM upstream primer
- 0.1–1.0 µM downstream primer
- Stabilizers and cofactors for polymerase (if needed)
- Approximately 2.5 units of a thermostable DNA polymerase (for example, *Taq*, *Tth*, or *Pwo* DNA polymerase²), added just before the reaction starts

Note: For optimal accuracy and yield, use the Expand™ High Fidelity system² in place of a single DNA polymerase.

² These products are sold under licensing arrangements with Roche Molecular Systems and the Perkin-Elmer Corporation. Purchase of these products are accompanied by a license to use them in the Polymerase Chain Reaction (PCR) process in conjunction with an Authorized Thermal Cycler. For complete license disclaimer, see page iv of this manual.

Caution: Optimal reaction conditions must be determined empirically for each template-primer system. For a detailed discussion of PCR, see the [Boehringer Mannheim PCR Applications Manual](#).

- 3 Gently vortex the reaction mixture and then centrifuge briefly to collect the sample at the bottom of the tube.
- 4 Overlay the reaction mix with 100 μ l of mineral oil.
- 5 Place reaction mix in an Authorized Thermal Cycler and start PCR. Follow the manufacturers guidelines included with the thermostable polymerase to set up the amplification profile. For instance, a general amplification profile that will work with *Taq*, *Tth*, or *Pwo* polymerase and primers with a T_m between 55°C and 70°C would be:

	Temperature	Time	Number of cycles
Initial denaturation	95°C	1 min	1
Denaturation	95°C	30 s	
Annealing*	50–65°C	1 min	25–30
Elongation	72°C	0.75–3.0 min	
Final elongation	72°C	7 min	

*Depends on primer design. See Step 1 above.

- 6 Hold the finished product at 4°C until it is used.
- 7 Purify the PCR product (DNA sequence for tagged protein) by standard methods (for example as detailed in the [Boehringer Mannheim PCR Applications Manual](#)).

II. Cloning the tagged protein sequence into an expression vector

- 1 Digest the purified PCR product with RE 1 and RE 2.
Note: RE 1 and RE 2 are restriction enzymes that recognize the RE 1 and RE 2 restriction sites added to the PCR primers under “Examples: PCR primer sequences for adding the *c-myc*, *HA*, and *VSV-G* epitopes” in this section.
- 2 Purify the restriction product by gel electrophoresis.
- 3 Digest a suitable vector (for instance, commercially available prokaryotic expression vectors such as pTrcHisB from Invitrogen) with RE 1 and RE 2.

- 4 Dephosphorylate the linearized vector with **alkaline phosphatase**.
- 5 Ligate the purified restriction product (from Step 2) into the dephosphorylated vector with T4 ligase.
- 6 Transform appropriate competent *E. coli* host cells with ligated vector DNA under standard transformation conditions (Sambrook, Fritsch and Maniatis, 1989; p. 1.74–1.84).
- 7 Isolate transformants with the appropriate selection marker (such as ampicillin resistance).
- 8 Screen for the presence of the epitope-tagged gene by doing one of the following:
 - Analyze whole cell DNA from the isolated colonies by direct PCR with the appropriate oligonucleotide primers (that is, the primers used to produce the original epitope-tagged gene, as described above). Also amplify the expression plasmid containing the epitope-tagged gene, as a control.

If the colony contains the epitope-tagged gene, PCR will produce a band the size of the tagged gene. If the colony does not contain the epitope-tagged gene, PCR will not produce the band.

- Prepare plasmid minipreps (Sambrook, Fritsch, and Maniatis, 1989; p. 1.25–1.32) from the isolated colonies and digest each plasmid with RE 1 and RE 2. Also digest the expression plasmid containing the epitope-tagged gene (positive control) and the expression plasmid without the gene (negative control).

If the colony contains the epitope-tagged gene, the digest will produce a band the size of the tagged gene. If the colony does not contain the epitope-tagged gene, the digest will not contain a band of the proper size.

Note: If you added an internal restriction site (RE 3) to one of the PCR primers to help in screening, you can confirm the presence of the epitope-tagged gene in a plasmid by digesting the plasmid with RE 3 and either RE 1 or RE 2. The band containing the epitope-tagged gene will be smaller after that digest than it is after the RE 1 + RE 2 digest.