MACS molecular

Tips + hints for cloning into MACSelect™ Tag Vectors

Special protocol

Index

1. General hints
2. N-terminal cloning into pMACS K^k.Tag(N) vectors
3. C-terminal cloning into pMACS K^k.Tag(C) vectors
4. Examples
   4.1 N-terminal cloning into pMACS K^k.HA(N)
   4.2 C-terminal cloning into pMACS K^k.HA(C)

1. General hints

A number of critical points have to be considered when cloning a gene into a mammalian expression vector.

- Kozak sequence, the eukaryotic consensus sequence for initiation of translation
- Translational stop codon
- Presence or requirement of signal peptide sequences such as signal peptides for transmembrane proteins or protein secretion
- Correct fusion of gene and tag sequence in respect to reading frame to ensure expression of the full-length protein

Please refer to the individual MACSelect™ Vector Set datasheet or the website at www.miltenyibiotec.com for information on individual MACSelect Tag vectors including vector map, location of features, multiple cloning site, and sequence (website only).

2. N-terminal cloning into pMACS K^k.Tag(N) vectors

Kozak sequence

No Kozak sequence needs to be cloned, as an optimal sequence for initiation of translation is already present at the start of the N-terminal tag coding sequence.

Translation stop sequence

The cloned coding sequence should end with a stop codon to ensure that a protein of the correct length is translated. Stop codon sequences are TGA, TAA, and TAG.

Signal peptide sequences

Secreted proteins and transmembrane proteins normally have an N-terminal signal peptide to ensure correct insertion into the membrane or entry into the secretory pathway. Vectors that introduce tags at the N-terminus of the protein, e.g. MACSelect K^k.Tag(N) vectors, are not suitable for the expression of such proteins, as the tag sequence will be cleaved off the translated product along with the signal peptide. Instead, we recommend tagging proteins containing a signal peptide at the C-terminus using the MACSelect K^k.Tag(C) vectors.
To predict the presence and location of signal peptide cleavage sites in your protein of interest, the SignalP 3.0 software can be used (http://www.cbs.dtu.dk/services/SignalP).

Cloning in-frame

It is essential that the reading frame following the tag sequence is maintained, otherwise the gene of interest will not be expressed. Thus, care should be taken in the design of PCR primers and in the choice of restriction enzymes: The ligated fragments have to be in frame at the 5' end of the cloned sequence, please see examples below.

3. C-terminal cloning into pMACS K^k.Tag(C) vectors

Kozak sequence

To guarantee that the cloned coding sequence will be correctly translated from the expressed mRNA, the Kozak consensus sequence for initiation of translation is required at the initiating ATG codon (bold letters):

$\frac{A}{G}CC\frac{A}{G}CC\mathbf{ATG}G$

Translation stop sequence

No translation stop sequence should be cloned, an optimal sequence for translation termination is already present downstream of the C-terminal tag coding sequence.

▲ **Note:** If your insert sequence contains a stop codon, the C-terminal tag sequence will not be expressed.

Signal peptide sequences

Secreted proteins and transmembrane proteins normally have an N-terminal signal peptide to ensure correct insertion into the membrane or entry into the secretory pathway. For correct expression of a C-terminal tagged sequence, we recommend preserving the native N-terminal sequence of your protein. To predict the presence and location of signal peptide cleavage sites in your protein of interest, the SignalP 3.0 software can be used (http://www.cbs.dtu.dk/services/SignalP).

Cloning in-frame

It is essential that the reading frame is maintained following the cloned sequence, otherwise the tag will not be expressed. Care should be taken in the design of PCR primers and in the choice of restriction enzymes: The ligated fragments have to be in frame at the 3' end of the cloned sequence, please see examples below.

4. Examples

4.1 N-terminal cloning in pMACS K^k.HA(N)

Primers were chosen to amplify a fragment of the human epidermal growth factor receptor (hEGFR) containing only the intracellular domain of the receptor, without the signal peptide and extracellular domains, but including the natural stop signal. The 5' primer contains an overhanging *AgeI* site to facilitate cloning into the pMACS K^k.HA(N) vector. A proofreading polymerase was used for PCR amplification to ensure that the PCR fragment had blunt ends with no overhanging A residues for blunt-end cloning of the

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3’ end. The cytoplasmic domain of hEGFR was amplified by RT-PCR using mRNA isolated from HS294T cells with the µMACS™ mRNA Isolation Kit. The pMACS K^k.HA(N) vector was cleaved with *AgeI* and *EcoRV* restriction enzymes while the PCR fragment was only cleaved with *AgeI*. Both vector and PCR fragments were gel purified. The fragments were ligated and transformed into *E. coli*.

hEGFR-specific primer design

Forward primer sequence

Restriction enzyme site for *AgeI* is underlined.

```
        AgeI
5'- AAC ACC GGT AGG CGC CAC ATC GTT CGG AAG -3'
            T   G   R   R   H   I   V   R   K
```

Location on hEGFR coding sequence

Primer-matching triplets set in bold, corresponding amino acids indicated below.

```
5'- CTC TTC ATG CGA AGG CGC CAC ATC GTT CGG AAG -3'  hEGFR coding sequence
     L   F   M   R   R   R   H   I   V   R   K
```

Reverse primer sequence

```
5'- TCA TGC TCC AAT AAA TTC ACT GC -3'
```

Location on hEGFR coding sequence

Primer-matching triplets set in bold, corresponding amino acids indicated below, * stop codon.

```
hEGFR coding sequence 5'- CAA AGC AGT GAA TTT ATT GGA GCA TGACCACGGAGGA -3'
                           Q   S   S   E   F   I   G   A   *
```

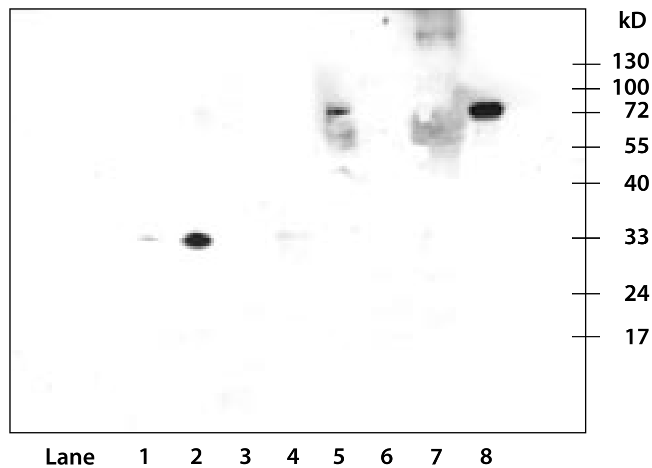

Figure 1: Expression and detection of HA-hEGFR in human Raji cells with and without MACSelect™ cell enrichment. Raji cells were transfected with pMACSK^k.HA(N)-hEGFR. 22 hours later, cells were analyzed either directly (lanes 4–5) or they were first selected with MACSelect™ K^k MicroBeads (lanes 7–8). The lysed cells were analysed by western blot with Anti-HA-HRP antibody (#130-091-972), after SDS-PAGE and transfer to PVDF membrane (lanes 4 and 7; 2×10^5 cells). Alternatively, HA-tagged protein was first immunopurified with µMACS HA Isolation Kit (#130-091-122) before western blot analysis (lanes 5 and 8; 1×10^6 cells). Lane 1–2: 5 ng and 10 ng HA-tagged control protein; 3 and 6: protein size marker (kD).

4.2 C-terminal cloning in pMACS K^k.HA(C)

First, a pair of PCR primers was designed that binds to the region upstream of the human CD4 start site and at the CD4 stop site. Note that these primers surround the natural Kozak sequence at the start site (a non-optimal Kozak sequence is highlighted in bold letters). The amplified fragment excludes the natural stop codon TGA. The primers contain either an overhanging *AgeI* site at the 5‘ end of CD4 sequence or an *EcoRI* site at the 3‘ end of CD4 sequence for cloning into the pMACS K^k.HA(C) vector. Then, the fragment was amplified from the pMACS4.1 plasmid by PCR. Both the PCR fragment and the pMACS K^k.HA(C) vector were cleaved with *AgeI/EcoRI* restriction enzymes and the fragments were gel purified. Finally, the fragments were ligated and transformed into *E. coli*.

CD4-specific primer design

Forward primer sequence

Restriction enzyme site for *AgeI* is underlined. The native, non-optimal Kozak sequence is shown in bold.

```
          AgeI
5'- GAGAAACCGGTCAAGGCCACA ATG AAC CGG GGA G -3"
```

Location on CD4 coding sequence

Primer-matching triplets set in bold, corresponding amino acids indicated below.

```
5'- GGCAAGGCCACA ATG AAC CGG GGA GTC CCT TTT AGG CAC TTG -3'  CD4 coding sequence
                  M   N   R   G   V   P   F   R   H   L...
```

Reverse primer sequence

Restriction enzyme site for *EcoRI* is underlined.

```
      EcoRI
5'- TT GGA ATT CCC GTG CCG GCA CCT GAC ACA GAA G -3'
```

Location on hCD4 coding sequence

Primer-matching triplets set in bold, corresponding amino acids indicated below.

```
5'- ... CTA GGC ATC TTC TTC TGT GTC AGG TGC CGG CAC TGA -3'  CD4 coding sequence
         L   G   I   F   F   C   V   R   C   R   H   *
```

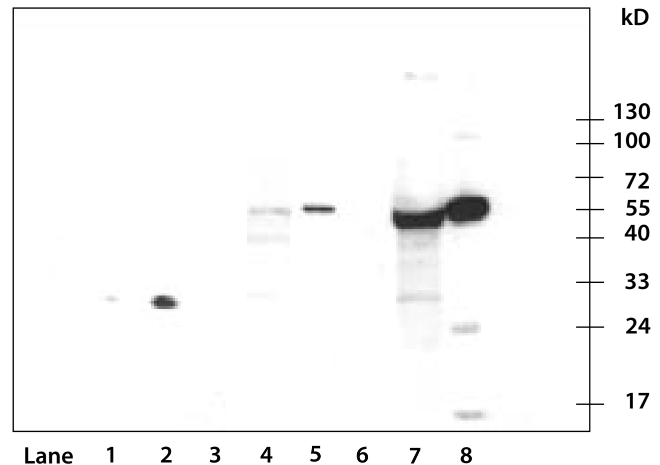

Figure 2: Expression and detection of CD4-HA in human Raji cells with and without MACSelect™ cell enrichment. Raji cells were transfected with pMACSK^k.HA(N)-hCD4. 22 hours later, cells were analyzed either directly (lanes 4–5) or they were first selected with MACSelect™ K^k MicroBeads (lanes 7–8). The lysed cells were analysed by western blot with Anti-HA-HRP antibody (#130-091-972), after SDS-PAGE and transfer to PVDF membrane (lanes 4 and 7; 2×10^5 cells). Alternatively, HA-tagged protein was first immunopurified with µMACS HA Isolation Kit (#130-091-122) before western blot analysis (lanes 5 and 8; 1×10^6 cells). Lane 1–2: 5 ng and 10 ng HA-tagged control protein; 3 and 6: protein size marker (kD).

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