TECH TIP #77

Thermo SCIENTIFIC

Cloning guide for Pierce qIP Protein Interaction Kits

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Introduction

Thermo Scientific™ Pierce™ qIP Protein Interaction Reagents comprise a platform for quantitative immunoprecipitation (qIP) to measure the interaction of two proteins that are transiently co-expressed in mammalian cells as epitope-tagged and luciferase-tagged fusions, respectively. The specific luciferase required is Thermo Scientific™ TurboLuc™ (Tluc) Luciferase, which is especially small and bright. Protein interactions are quantified by measuring Tluc Luciferase activity following pull-down of epitope-tagged proteins with anti-epitope agarose or magnetic beads. Therefore, the assay system requires that the genes for proteins-of-interest X and Y be cloned as fusions with epitope (e.g., HA or c-Myc) and Tluc tags, respectively. This cloning guide provides specific and practical information about proposed primer sequences, cloning sites, and subcloning strategies for this qIP assay system.

General Considerations and Overview

The first step in cloning genes of interest into appropriate epitope-tagged or Tluc-tagged Pierce qIP Vectors is to PCR-amplify the gene sequence using two short oligonucleotide primers. Important considerations for primer design include:

- Selecting and including compatible restriction sites for either directional or non-directional cloning
- Choosing either N- or C-terminal tag for genes of interest X and Y
- Ensuring that the proper reading-frame is preserved
- Including Kozak sequence for proper translational initiation when a gene is cloned into a C-terminal tag vector

All Pierce qIP Vectors contain the same multiple cloning site (MCS) sequences (Figures 1, 2 and 5). Therefore, once a gene of interest is successfully cloned into one qIP vector with the proper reading frame, the gene can be moved to another qIP vector without additional PCR amplification (Figure 3). This feature provides the convenient and flexible option to subclone a gene from an N-terminal tag vector to C-terminal tag vector.

Pierce qIP Kits include prepared control vectors that contain genes for BAD, Bcl-xL and RFP proteins:

- pCMV BAD C-tag (HA or Myc)
- pCMV RFP C-tag (HA or Myc)
- pCMV Bcl-xL N-Tluc tag

In each case, the specific genes are cloned into the nominal MCS Pierce qIP Vectors using cloning sites and PCR primers illustrated in Figure 4. As such, these control vectors can be used as expression vectors for cloning or subcloning a gene of interest X or Y by simply dropping out BAD or Bcl-xL genes via NotI/BglII restriction enzyme digestion or dropping out the RFP gene via NotI digestion.

Figure 5 provides MCS sequence information for all six MCS qIP Vectors; complete sequences are available on our website.

Pierce Biotechnology 3747 N. Meridian Road | PO Box 117 Rockford, IL 61105 USA | (815) 968-0747 (815) 968-7316 fax | www.thermoscientific.com/pierce

Cloning a gene of interest into epitope-tagged qIP vectors

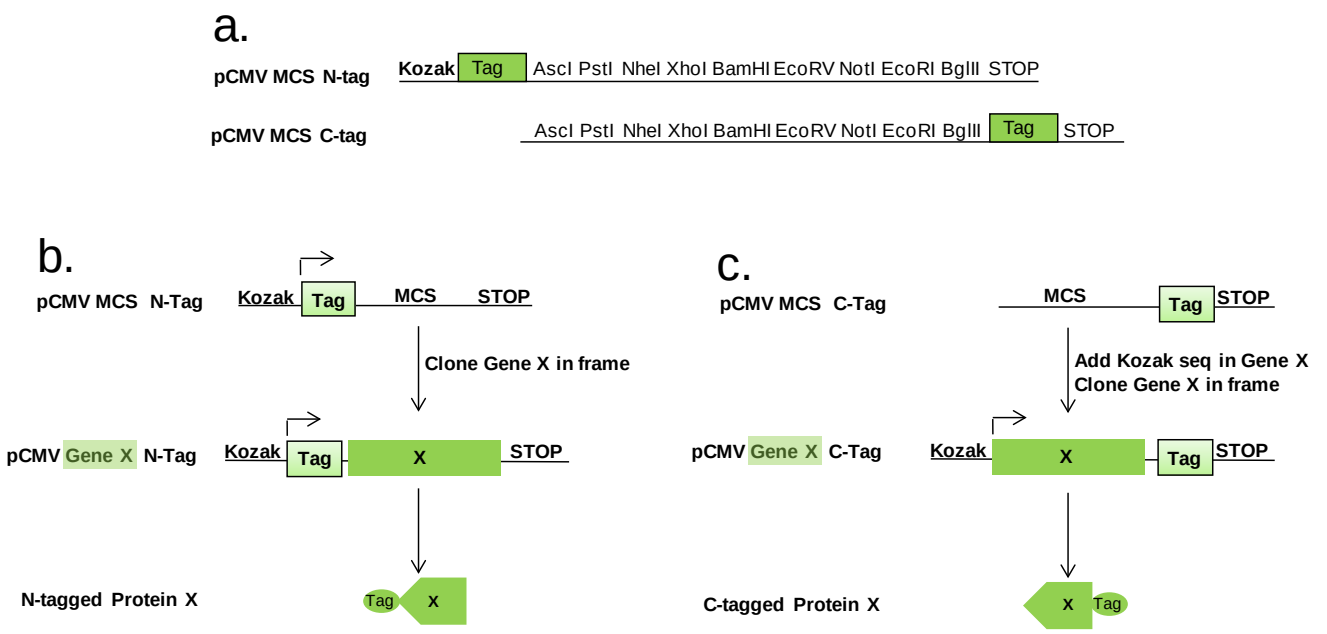


Figure1. Schematic of cloning strategy for epitope-tagged vector. (a) multiple cloning site (MCS) information, (b) cloning a gene of interest X into N-terminal tag vector, or (c) into C-terminal tag vector . Entire vector sequences are available on our website, thermoscientific.com/pierce, and detail MCS sequence information is also available in Figure 5.

To clone a gene of interest (Gene X) into an N-terminal epitope-tag vector (Figure 1b), amplify the gene by PCR with two short oligo-nucleotide primers. There are two important parameters that must be considered when designing these primers. The first is the reading frame relative to N-terminal tag because Gene X protein must be expressed in frame with an N-terminal tag. For example, the proposed 5' forward PCR primer (below) includes two nucleotides (TT, underlined) after the NotI site in order to keep the Gene X in frame with N-terminal epitope-tag. The second important parameter of primer design is orientation of the cloning. Below is an example of two primer sequences with NotI and BglII sites for directional cloning. If directional cloning is not an option because a NotI or BglII restriction site occurs within the gene of interest, then non-directional cloning can be done using a single NotI or BglII restriction site, but then the orientation of the gene must be determined. Design the 3' reverse complementary PCR primer to include the gene codon just upstream of the stop codon.

Design of PCR Primers for cloning a gene in qIP epitope-tagged vectors.

PCR Primer	Composition	Sequence
5'-Forward	NotI -TT-{Gene X forward}	5'-GATACTCGCGGCCGCTT-{Gene X forward sequence}
3'-Reverse	BglII-{Gene X reverse}	5'-GGGGTAAGATCT-{Gene X reverse complement sequence}

To clone a gene of interest into a C-terminal tag vector (Figure 1c), design the 5' forward primer to include a Kozak sequence with an ATG start codon for proper initiation of translation (Kozak 1991). The Kozak sequence used in the Pierce qIP Vectors is ACCATGG (start codon underlined). Other sequences are possible based on the Kozak consensus sequence, such as (A/G)NNATG**G** (Kozak, 1987,1990). However, the G shown in bold at +4 position is critical, while either A or G at -3 position is allowed.

Cloning a gene of interest into Tluc-tagged qIP vectors

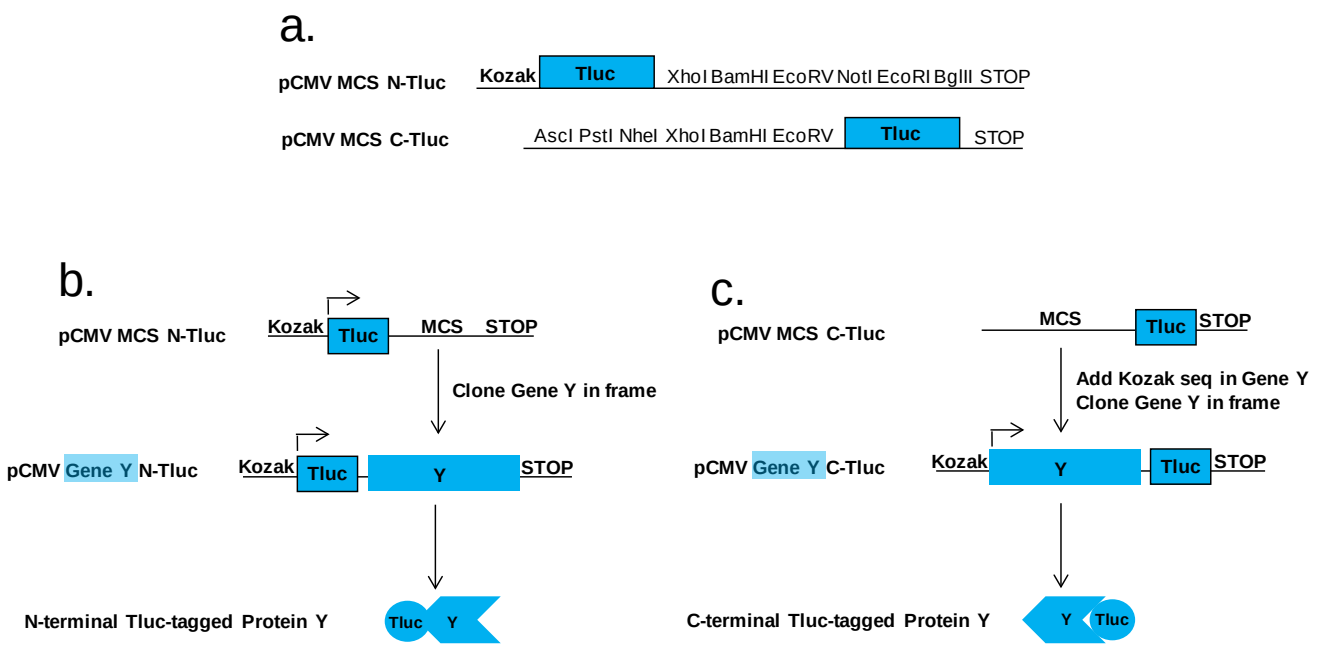


Figure 2. Schematic of cloning strategy for Tluc-tagged vector. (a) multiple cloning site (MCS) information, (b) cloning a gene of interest Y into N-terminal tag vector, or (c) into C-terminal tag vector . Entire vector sequences are available on our website, thermoscientific.com/pierce, and detail MCS sequence information is also available later section of this instruction manual.

The strategy to clone a gene of interest (Gene Y) into the N-terminal TurboLuc (Tluc) luciferase tag vector (Figure 2b) is the same as the strategy described in Figure 1b to clone Gene X into an N-terminal epitope-tag vector. PCR-amplify Gene Y with two short oligonucleotide primers (below). For directional cloning (recommended), primers must contain different unique restriction sites; for non-directional cloning, primers with one restriction site can be used. The reading frame relative to N-terminal Tluc tag must be preserved. In the proposed 5'-forward primer listed below, this is accomplished by including two nucleotides (TT, underlined) next to the NotI site. Design the 3' reverse complementary PCR primer to include the gene codon just upstream of the stop codon.

Design of PCR Primers for cloning a gene into qIP Tluc-tagged vectors.

PCR Primer	Composition	Sequence
5'-Forward	NotI -TT-{Gene Y forward}	5'-GATACTCGCGGCCGCTT-{Gene Y forward sequence}
3'-Reverse	BglII-{Gene Y reverse}	5'-GGGGTAAGATCT-{Gene Y reverse complement sequence}

To clone a gene of interest into a C-terminal tag vector (Figure 2c), design the 5' forward primer to include a Kozak sequence with an ATG start codon for proper initiation of translation (Kozak 1991). The Kozak sequence used in the Pierce qIP Vectors is ACCATGG (start codon underlined). Other sequences are possible based on the Kozak consensus sequence, such as (A/G)NNATG**G** (Kozak, 1987,1990). However, the G shown in bold at +4 position is critical, while either A or G at -3 position is allowed.

Subcloning a gene of interest from one qIP vector to another

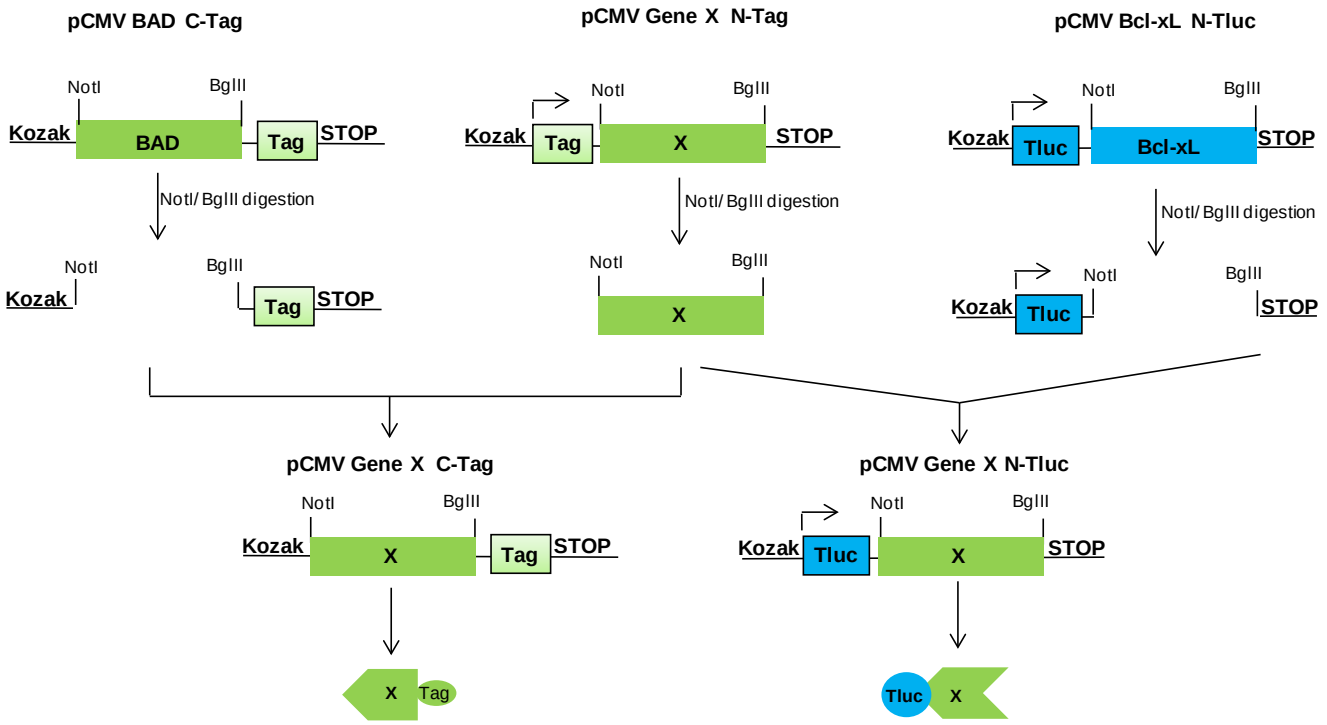


Figure 3. Schematic of convenient subcloning strategy to move a gene X from one expression vector to another vector without performing additional PCR gene amplification. The Gene X fragment released form pCMV Gene X N-tag vector can be subcloned into the positive control vector digested with NotI/BglII. This subcloning strategy provides easy and convenient way to move a gene of interest from one expression vector to another.

Once a gene of interest (Gene X) has been cloned in-frame into an N-terminal epitope-tag vector via NotI/BglII sites (pCMV Gene X N-tag; Figure 3, top center), it then can be subcloned into a C-terminal epitope-tag vector without additional PCR amplification of Gene X with Kozak sequence.

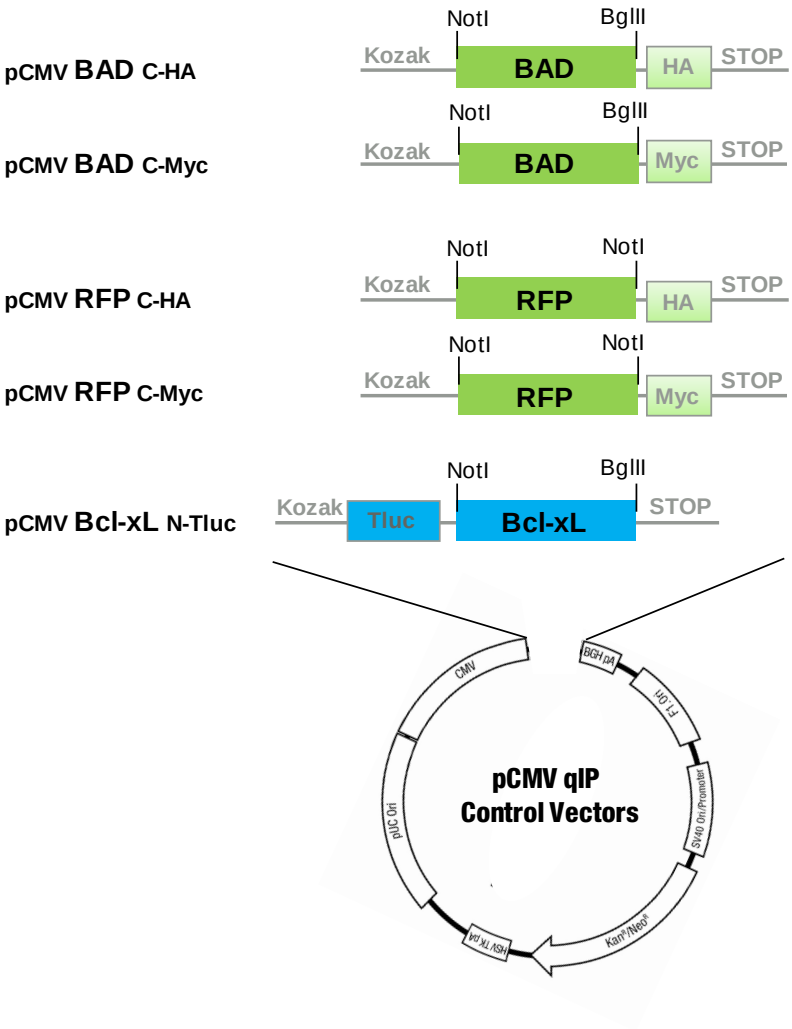
For example (Figure 3, left side), Gene X can be exchanged with the BAD gene of the pCMV BAD C-tag control vector (Part Nos. 82028, 82029; see Figure 4, next page). Gene X and BAD are excised from their respective vectors by NotI/BglII digestion, then Gene X is ligated with the vacated pCMV BAD C-tag vector to create the pCMV Gene X C-tag vector. The pCMV BAD C-tag vector already contains Kozak sequence at the N-terminus.

Alternatively, if Gene X had been cloned in-frame into an N-terminal epitope-tag vector via only the NotI site (non-directional cloning), then it can be subcloned into the C-terminal epitope-tag vector by exchanging it with the RFP gene of the pCMV RFP C-tag control vector. The orientation of Gene X must be determined after cloning.

Finally, the Gene X fragment can be moved from the epitope-tag vector to the Tluc vector by ligating into the pCMV Bcl-xL N-Tluc vector that has been digested with NotI/BglII to remove the Bcl-xL gene. The ligation will create pCMV Gene X N-Tluc expression vector. Moving a gene from an epitope-tag vector to the Tluc vector changes its role in the qIP assay; the protein it expresses changes from being the primary IP target protein to being the co-IP/reporter protein.

Positive and negative control qIP vectors

a.



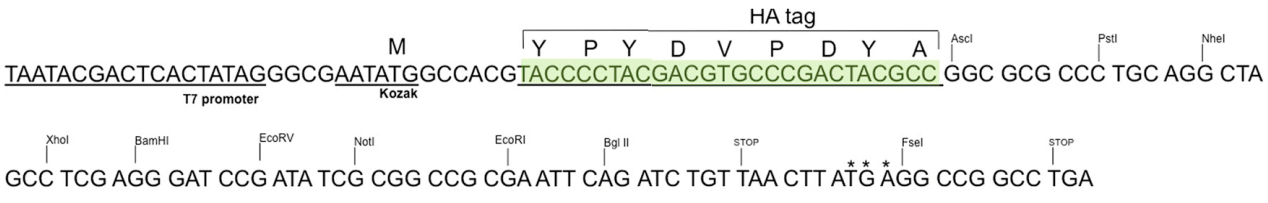
b.

5'-NotI-TT-BAD:	GATATCGCGGCCGC**TT**TTCCAGATCCCAGAGTTTGAG
3'-BglII-BAD:	GGGGTAAGATCTCTGGGAGGGGGCGGAGCTTCC
5'-NotI-TT-RFP:	GATATCGCGGCCGC**TT**CTTTCCAAACAAGTTTTGCCTCATG
3'-NotI-G-RFP:	ATTCAAGCGGCCGC**G**AGTTTCCTTAACATCTACTGTGAAGT
5'-NotI-TT-Bcl-xL:	GATATCGCGGCCGC**TT**TCTCAGAGCAACCGGGAGCTG
3'-BglII-Bcl-xL:	GGGGTAAGATCTTTTCCGACTGAAGAGTGAGCC

Figure 4. Control vectors for qIP. (a) Positive and negative control vector maps with cloning sites. (b) Primer sequences used to clone BAD, Bcl-xL,and RFP gene. The nucleotides underlined are added to the primer sequence to keep the gene in frame with the N- or C-terminal tag. Entire vector sequences are available on our website, and detail MCS sequence information appear below in Figure 5.

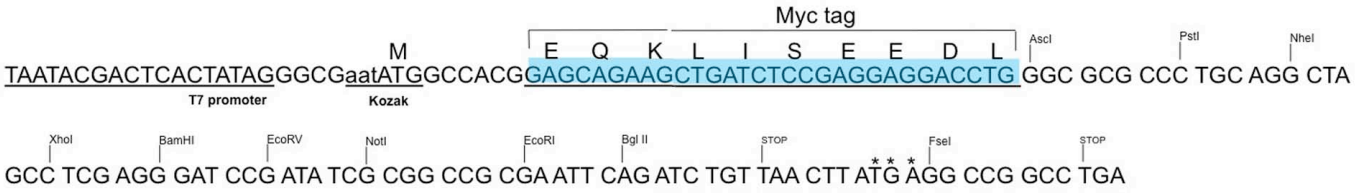
Multiple cloning sites (MCS) sequences of qIP vectors

1. pCMV MCS N-HA *** Stop codon in different reading frame.

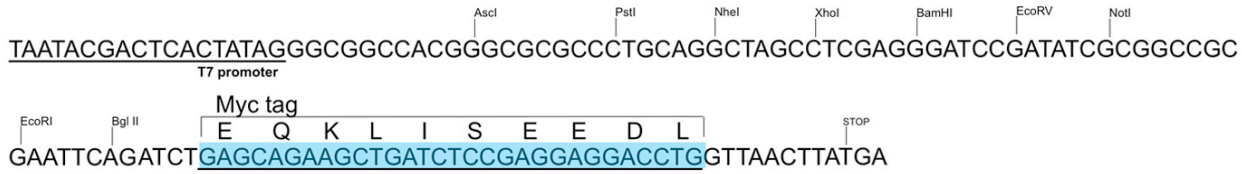


2. pCMV MCS C-HA *** Stop codon in different reading frame.

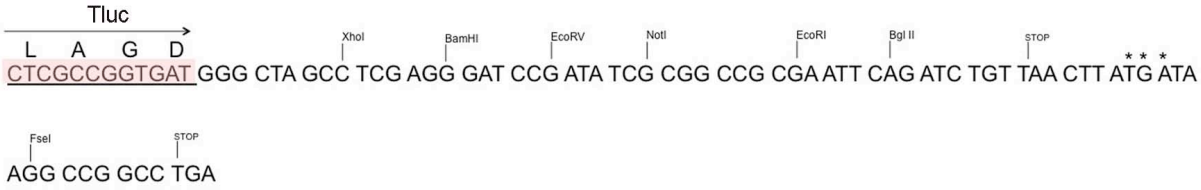
3. pCMV MCS N-Myc *** Stop codon in different reading frame.



4. pCMV MCS C-Myc *** Stop codon in different reading frame.



5. pCMV MCS N-Tluc *** Stop codon in different reading frame.



6. pCMV MCS C-Tluc *** Stop codon in different reading frame.

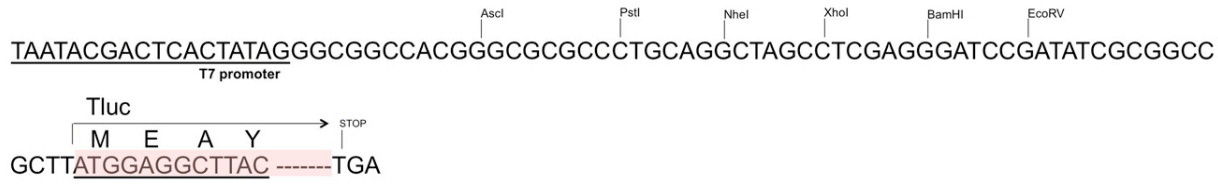


Figure 5. Detail DNA sequence information of qIP mammalian expression vector with multiple cloning sites (MCS).

Related Thermo Scientific Products

82013	**Pierce qIP Protein Interaction Buffer L (1X),** 140mL, sufficient for 100 qIP reactions
82014	**Pierce qIP Protein Interaction Buffer D (10X),** 50mL, sufficient for 100 qIP reactions
82015	**Pierce qIP Protein Interaction Tluc Assay Reagents,** 150 luciferase assay reactions
82016	**Pierce qIP Protein Interaction Tluc Assay Reagents,** 1500 luciferase assay reactions
82017	**pCMV MCS N-HA** (N-terminus HA tag expression vector)
82018	**pCMV MCS C-HA** (C-terminus HA tag expression vector)
82019	**pCMV MCS N-Myc** (N-terminus Myc tag expression vector)
82020	**pCMV MCS C-Myc** (C-terminus Myc tag expression vector)
82023	**pCMV MCS N-Tluc** (N-terminus Tluc tag expression vector)
82024	**pCMV MCS C-Tluc** (C-terminus Tluc tag expression vector)
82025	**pCMV RFP C-HA** (C-terminus HA tag negative control vector)
82026	**pCMV RFP C-Myc** (C-terminus Myc tag negative control vector)
82028	**pCMV BAD C-HA** (C-terminus HA tag positive control vector)
82029	**pCMV BAD C-Myc** (C-terminus Myc tag positive control vector)
82031	**pCMV Bcl-xL N-Tluc** (N-terminus Tluc tag positive control vector)
82032	**Pierce Agarose qIP Protein Interaction Kit, Tluc and HA Tags,** 25 qIP reactions
82033	**Pierce Agarose qIP Protein Interaction Kit, Tluc and Myc Tags,** 25 qIP reactions
82035	**Pierce Magnetic qIP Protein Interaction Kit, Tluc and HA Tags,** 25 qIP reactions
82036	**Pierce Magnetic qIP Protein Interaction Kit, Tluc and Myc Tags,** 25 qIP reactions
R0533	**TurboFect™ Transfection Reagent**
78437	**Halt™ Protease Inhibitor Cocktail, EDTA-free**
28374	**Dulbecco's Phosphate-Buffered Saline (DPBS)**
26181	**Pierce Anti-HA Agarose,** 2mL
88836	**Pierce Anti-HA Magnetic Beads,** 1mL
20168	**Pierce Anti-c-Myc Agarose,** 2mL
88842	**Pierce Anti-c-Myc Magnetic Beads,** 1mL
69705	**Pierce Spin Columns – Screw Cap,** 25 columns
5250030	**Varioskan™ Flash with top reading**
5250040	**Varioskan Flash with top and bottom reading**
5250500	**Varioskan LumiSens Option, factory fitted** (also enabling luminometric spectral scanning)
5250510	**Dispenser option, with 1 mL syringe,** factory fitted

General References

1. Kozak, M. (1987). An analysis of 5´-noncoding sequences from 699 vertebrate messenger RNAs. Nucleic Acids Res. 15, 8125-48.
2. Kozak, M. (1991). An analysis of vertebrate mRNA sequences: Intimations of translational control. J. Cell Biology 115, 887-903.
3. Kozak, M. (1990). Downstream secondary structure facilitates recognition of initiator codons by eukaryotic ribosomes. PNAS USA 87, 8301-5.
4. Michael R. Green, Joseph Sambrook, Molecular Cloning: A Laboratory Manual (Fourth Edition).

Current product instructions are available at *www.thermoscientific.com/pierce*. For a faxed copy, call 800-874-3723 or contact your local distributor.