Published in final edited form as:

Anal Biochem. 2009 May 15; 388(2): 353–355. doi:10.1016/j.ab.2009.03.015.

pTOM, a vector for expressing two proteins in mammalian cells

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Abstract

We developed a pTOM construct that can express two proteins in a cell. Using pTOM ensures that two polypeptide-encoding nucleotide sequences are simultaneously transfected into the same cell. The ability to simultaneously express two separate polypeptide-encoding nucleotide sequences from the same vector in the same cell allows the user to determine the relationship between the two proteins. Another advantage is that one of the proteins can be used as a transfection marker/selector. The vector contains multiple cloning sites for insertions of polypeptide-encoding nucleotide sequences. Positive clones can be easily selected when performing cloning using ampicillin. Overall, this vector provides a convenient way to express dual proteins in a single mammalian cell.

The transfection of a vector encoding a polypeptide nucleotide sequence of a protein is the most common technique employed in modern molecular biology to investigate protein functions. The most common way to introduce two genes into a cell system is to cotransfect two different constructs into the cell. The separate addition of two vectors, each encoding a different polypeptide-encoding nucleotide sequence, by transfection can result in a variance in the expression levels of each protein. Furthermore, the efficiency of cotransfection is largely dependent on the ratio of the plasmids used and the different promoters that drive expression of the polypeptide-encoding nucleotide sequences. Assuming that the two plasmids are well mixed prior to transfection, the cell should take up both proteins and coexpress them proportionately. In practice, expression results in mammalian cells often show that some cells apparently express solely one of the two genes when using fluorescent proteins as tags to facilitate observations. Instead of coexpressing two separate constructs, we have developed a construct, named pTOM,¹ which expresses two proteins of interest in mammalian cells. By using its common multiple cloning sites (MCSs) and ampicillin-resistant selection, it is easy to construct the demanded plasmid. This ensures that two genes are simultaneously transfected into the same cell and are expressed. Therefore, this also facilitates the study of the relationship between the two proteins.

The backbone of pTOM was built on both pEBG and pEGFP vectors (BD Biosciences Clontech). A fragment of approximately 6 kb nucleotides of vector pEBG was synthesized using a high-fidelity polymerase chain reaction (PCR) kit. Subsequently, the PCR product from pEBG was engineered to anneal with the pEGFP vector using restriction enzymes. The

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¹Abbreviations used: pTOM, two proteins in mammalian cells; MCS, multiple cloning site; PCR, polymerase chain reaction; CMV, cytomegalovirus; hEF1 α , human elongation factor-1 α ; GFP, green fluorescent protein; GST, glutathione S-transferase; LB, Luria-Bertani; DAP4, death-associated protein-4; RFP, red fluorescent protein; siRNA, small interference RNA; FACS, fluorescence-activated cell sorting; IRES, internal ribosome entry site.

newly synthesized plasmid was named pTOM and was sequenced. A vector map with unique restriction enzyme sites and MCSs is shown in Fig. 1. The vector contains two promoters, cytomegalovirus (CMV) and human elongation factor-1 α (hEF1 α), which drive the high expression levels of green fluorescent protein (GFP) and glutathione *S*-transferase (GST) fusion proteins, respectively. The ampicillin-resistant gene allows much more convenient selection that is suitable for Luria–Bertani (LB) agar broth/plate preparation in most current laboratories.

To test the efficiency of the expressions of the two proteins larger than 50 kDa, a plasmid expressing both GFP and GST–MST1 [1] was constructed using *Bam*HI and *Not*I sites at the 5' and 3' ends, respectively, and this was then transfected into HEK 293 cells. The results of fluorescence microscopy are shown in Fig. 2A. Further verification of the expressions of both proteins using GST and GFP antibodies in a Western blot analysis is shown in Fig. 2B (left panel). In another test, a gene encoding the death-associated protein-4 (DAP4) [1] was also constructed in this vector and transfected into HEK 293 cells. The expressions of both GFP and GST–DAP4 are shown in Fig. 2B (right panel).

To examine whether the two proteins were simultaneously expressed in a single cell, a red fluorescent protein (RFP) gene was inserted into pTOM fused with GST and transfected into HEK 293 cells. As shown in Fig. 2C, more than 95% of cells expressed both GFP and GST–RFP in any field examined. It is noteworthy that the GST–RFP was distributed as a cytosolic protein, whereas GFP expression was more homogeneous in the cell. This rules out the possibility that the red color seen here resulted from leakage of the green filter in the fluorescent microscope.

The pTOM construct contains three promoters: the CMV, hEF1 α , and β -lactamase promoters. The CMV promoter is one of the most commonly used promoters for expressing transgenes in eukaryotic cells [2,3]. The hEF1 α promoter was shown to be another strong promoter in driving high levels of transgene expressions in human cell lines [4]. Proteins driven by this promoter are consistent from a temporal viewpoint and are independent of the cell type. A combination of both promoters in the same plasmid ensures that both proteins can be highly expressed. As seen in Fig. 2C, more than 95% of cells expressing the pTOM GFP and GST–RFP constructs showed both GFP and RFP fusion proteins. The β -lactamase promoter, which drives ampicillin-resistant gene expression, conveys the antibiotic resistance feature for selecting transformed bacteria.

With the backbone of pTOM, potential modifications and applications have multiple aspects. Upstream of both GFP and GST, there is no single cut for restriction enzyme sites, whereas there are two sites for *Age*I in this vector; one is between the CMV promoter and the GFP sequence, and the other is between the ampicillin-resistant gene and the hEF1 α promoter. Likewise, there are three cuts of *Eco*RI sites in this vector; one of them is between the hEF1 α promoter and the GST sequence. These sites can sequentially be changed so that one can finally replace these sequences with relevant tags to fulfill particular purposes. First, the GST tag located downstream of the hEF1 α promoter can be changed to fulfill the needs of experimental purposes. For instance, if the protein intended to be fused with the tag (GST) is smaller than the tag, the entire fusion protein might be unable to mimic the function of the endogenous protein *in vivo* because the tag protein may produce an interference effect. In that case, the GST protein can be replaced with a short tag, the FLAG peptide (MDYKDDDDK), so that the fusion protein can function more accurately as an endogenous one as well as being correctly folded. Alternatively, the same region can be modified to be a U6 promoter with a looped DNA structure that carries small interference RNA (siRNA). Accordingly, transfected cells with siRNA can be sorted as GFP-positive cells. Second, the GFP tag located downstream of the CMV promoter can be replaced with

other proteins to serve as an alternative marker. For instance, this region can be engineered to express receptors so that the respective ligands can be used to purify transfected cells as an alternative to using fluorescence-activated cell sorting (FACS) analysis to perform the sorting. Finally, both tags in pTOM can be replaced by proteins that can be used in a mammalian two-hybrid system to simplify the methodology. Currently, there are multiple systems available to perform screening in mammalian cells, and in each method bait and prey genes are inserted into separate plasmids [5,6]. Subsequently, some of these methods require cotransfection of three plasmids into the same cell in those systems. By substituting GFP-MCS1 and GST-MCS2 in pTOM with bait and library genes, respectively, bait and screened proteins are more likely to simultaneously be expressed in the same cell. This will allow researchers to investigate interactions between mammalian proteins that might not fold correctly in yeast or that require post-translational modification or external stimulation that is not present in yeast.

We developed pTOM independent of the existing product, pBudCE4 (Invitrogen), which also expresses two proteins in one vector. However, having compared these two vectors, we found that pTOM has some advantages over pBudCE4. The fusion proteins differ; pTOM is constructed with both GFP and GST at the N terminus, whereas pBudCE4 uses both mycHis and V5His at the C terminus. In addition, the use of Zeocin in pBudCE4 as a selectable marker is not as common as the use of ampicillin in pTOM. Finally, MCSs in pTOM are more common and convenient for producing the desired constructs. Another system for dual protein expression that is often adopted is one that uses internal ribosome entry sites (IRESs) to drive two different proteins in the same vector [7]. Nevertheless, most people have often experienced one protein being brightly expressed while the other protein is dimly expressed. In contrast to what people have seen and as shown in Fig. 2C, using fluorescent proteins expressed by pTOM constructs not only demonstrates that two proteins can be expressed in the same cell but also shows that the expression levels of the two proteins are quite comparable.

In conclusion, we have constructed a pTOM vector to simultaneously express two proteins in mammalian cells and serve as a suitable marker for selection/sorting as well. The common MCS and ampicillin-resistant features make it convenient to work with pTOM. Furthermore, potential applications in the proteomics era are both encouraging and promising.

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Fig. 1.

Map of pTOM and the MCSs. (A) The vector contains two promoters, cytomegalovirus (CMV) and human elongation factor-1 α (hEF1 α), which drive the high expression levels of the green fluorescent protein (GFP) and glutathione *S*-transferase (GST) fusion proteins, respectively. It has a Simian virus 40 (SV40) polyadenylation (SV40 Poly-A) signal downstream of MCS1 and a human colony-stimulating factor-3 (CSF-3) polyadenylation (CSF-3 Poly-A) signal downstream of MCS2. The restriction enzyme sites indicated on the map are unique sites in this vector. It also contains an ampicillin-resistant (Amp^r) gene for LB agar broth/plate selection. (B) The two MCSs shown are located immediately downstream of the GFP and GST genes. They contain the most commonly used restriction enzyme sites.

Fig. 2.

Expression of constructs containing the GFP and GST–MST1 genes or GFP and GST–DAP4 genes. (A) HEK 293 cells were transfected with pTOM containing GFP and GST–MST1 using Lipofectamine 2000 according to the manufacturer's instructions. Next, 24 h after transfection, cells were observed and directly imaged under a fluorescence microscope. A representative field is shown. (B) HEK 293 cells expressing GFP and GST–MST1 (left panel) or GFP and GST–DAP4 (right panel) using the pTOM vector were harvested at 24 h after transfection. An aliquot of cell lysates was subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). The transferred membranes were cut into upper and lower parts and blotted with anti-GST and anti-GFP antibodies, respectively. (C) Coexpression of the GFP and GST–RFP genes in each cell using the pTOM construct. HEK 293 cells were transfected with pTOM that contained both the GFP and GST–RFP genes. Next, 24 h after transfection, cells were fixed with 3.7% formaldehyde for 10 min at room temperature. Cells in different fields were examined and imaged under a fluorescence microscope.