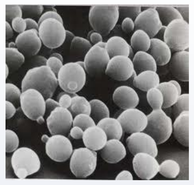
Modifying Commercially Available Vectors for Expression of Standardized iGem BioBricks



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Introduction

The 2012 Georgia State University iGEM Team set out to modify commercially available vectors for standardized expression of iGEM BioBricks. The design and synthesis of a standardized multiple cloning site (sMCS-1) allows any vector to fit the iGEM assembly method. We first began with the glyceraldehyde-3-phosphate dehydrogenase (pGAP) promoter shuttle vector to be used in the methylotrophic yeast *Pichia pastoris*. Creating a standardized expression system within this eukaryotic organism allows the expression of a variety of proteins that can be used for many biological purposes. *P. pastoris*, in comparison to the conventional recombinant host *E. coli*, is an ideal choice for the expression of complex proteins due to its ability to perform post-translational modifications and provide a secretion system for these molecules. Modifying the pGAP vector to be used with the iGEM standard enables Georgia State and other teams to utilize *P. pastoris* as an expression host.

Materials & Methods

Design & Synthesis of sMCS-1

The sMCS-1 DNA fragment was designed to incorporate the following restriction enzyme sites: EcoRI, NotI, XBaI, SpeI, NotI, PstI, and NheI along with random upstream DNA sequences to ensure enzyme activity (Figure 1).

5 ''TCATGATCATGAATTCCATAAGCGGCCGCACCCTCTAGAAAACC
CTTTACTAGTCTTTGGCGGCCGCGAACTGCAGAATCTGA 3'

This segment of DNA was synthesized by Integrated DNA Technologies (IDT, Coraville, IA).

Restriciton Enzyme Digest of pGAPzαA and sMCS-1

pGAPZαA (Invitrogen) was transformed into *E. coli* according to the manufacturer's protocol using a Zeocin resistance marker. Resulting colonies were subjected to a miniprep plasmid extraction (Qiagen) according to the manufacturer's protocol. The plasmid extracts were quantified and digested with EcoRI and XbaI (New England Biolabs) according to the manufacturer's protocol and confirmed using gel electrophoresis (Figure 2).

The sMCS-1 was digested with EcoRI and NheI (New England Biolabs) according to the manufacturer's protocol. Two resulting products were ligated using a T4 DNA ligation kit (Fermentas) according to the manufacturer's protocol and confirmed using gel electrophoresis (Figure 3).

Cloning of RFP BioBrick into Modified pGAPzαA

Red Fluorescent Protein from the 2012 iGEM Kit Plate (BBa_J04450) was digested with EcoRI and PstI (New England Biolabs) according to the manufacturer's protocol and ligated into the modified pGAPZαA multiple cloning site using T4 DNA ligation kit (Fermentas) according to manufacturer's protocol and confirmed using gel electrophoresis (Data not shown).

Standardized Multiple Cloning Site

The sequence in Figure 3 (top sequence) can be modified to fit any multiple cloning site by using primers to add necessary restriction enzyme sites upstream and downstream of the sequence. For example, to swap the MCS of the commercially available pGAPZαA (Invitrogen) with the sMCS-1 an NHE1 restriction enzyme recognition site was added to the 3' end of the top sequence in Figure 3. With the use of PCR, any restriction site can be added to sMCS-1 in order to change the MCS of any commercial vector (Table 1).

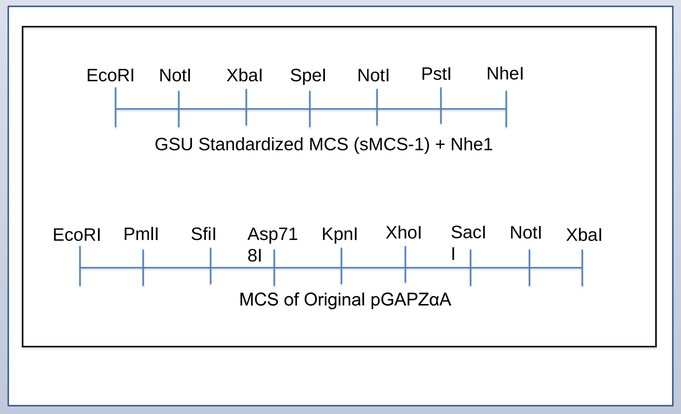


Figure 1: Multiple cloning sites with specific order of restriction enzyme recognition sites.

RE Site	Forward Primer (5' - 3')
BamHI	GACGATGACT**GGATCC**TCATGATCAT
HindIII	GACGATGACT**AAGCTT**TCATGATCAT

RE Site	Reverse Primer (5' - 3')
AvrII	GACGATGACT**CCTAGG**GGCCGCTTA
BglII	GACGATGACT**AGATCT**GGCCGCTTA

Table 1: Possible restriction enzyme sites that can be added to sMCS-1 in order to change the MCS of a commercial vector..

Results

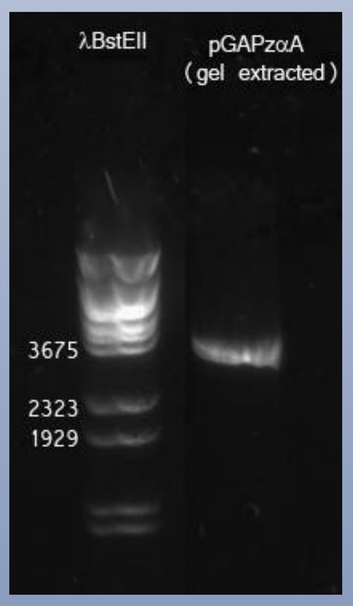
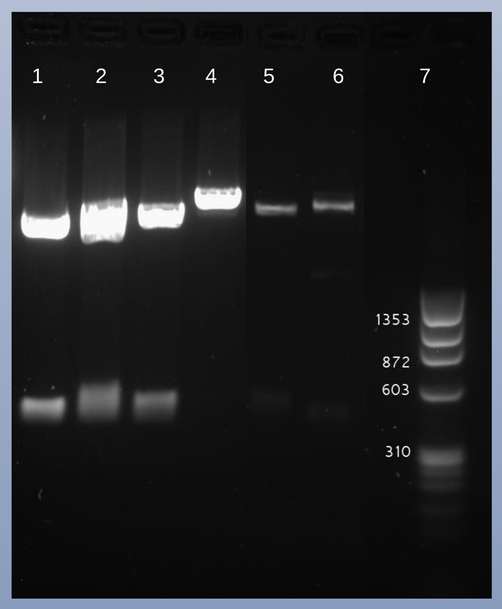


Figure 2: Confirmation of removal of original MCS from pGAPZαA using EcoRI and Xba1.



LANE	SAMPLE
1	pGAPZαB (+): EcoRI / BamHI
2	pGAPZαB (+): PstI / BamHI
3	pGAPZαC (-): EcoRI / BamHI
4	pGAPZαC (-): PstI / BamHI
5	pGAPZαA : EcoRI / BamHI
6	pGAPZαA: PstI / BamHI
7	Marker

Figure 3: Confirmation of insertion of sMCS-1 into pGAPZαA by digest with PstI and BamHI (lane 6, faint band). Original pGAPZαA MCS did not contain PstI site (see Figure 1, lower image)

Discussion

The pGAPZαA was digested with EcoRI and XbaI, removing the native, 67 base pair multiple cloning site and yielding a band at 3033 bps (Figure 2).

The resulting plasmid was then ligated with the Georgia State standardized multiple cloning site (sMCS-1), rendering the pGAPZαA shuttle vector compatible with the iGEM standard. This was confirmed by digest with PstI, found within the sMCS-1 but absent from the original pGAPZαA multiple cloning site (Figure 2). As a positive control, the pGAPZαB vector was digested using PstI (lane 2), and pGAPZαC was digested with the same enzyme as a negative control (lane 4).

Although faint, the banding pattern in lane 6 confirms that the pGAPZαA vector now contains a PstI site (added by the ligation of the sMCS-1), as compared to the positive control in lane 2.

Conclusion

As part of our experimental design, we created a universal multiple cloning site (sMCS-1) that can be used to modify a variety of existing multiple cloning sites to render them iGEM compatible. Having modified pGAPZαA with the sMCS-1 part, we now have an iGEM-compatible shuttle vector that will allow high-level expression of heterologous proteins in *Pichia pastoris*. By inserting any iGEM BioBrick coding sequence into the pGAPzα plasmid, we can produce a secreted protein with appropriate eukaryotic post-translational modifications.

We are currently working on a test of efficacy of the modified pGAPZαA, using the red fluorescence protein (RFP) BioBrick part. We have already ligated the RFP cDNA into the modified shuttle vector and will soon be able to transform *Pichia* and test for protein expression. Our ultimate goal for this branch of the project is to use *Pichia* as a tool to produce commercially and scientifically important proteins such as flu antigens for use in vaccine production. To that end, we are also planning to modify the pGAPZαB and pGAPZαC plasmids using the sMCS-1, which will enable us to express any cDNA in the same reading frame as the α-secretion factor, allowing for easier purification from the growth medium.

The sMCS-1 system that we've designed can be used to modify any vector to allow easy insertion of iGEM BioBricks. Although this project has focused on using the sMCS-1 to alter a shuttle vector for expression in the *Pichia* yeast system, another part of our group has been working to modify the multiple cloning site of the pPZP500 (Dr. Holger Bohlmann & Muhammad Amjad Ali of the University of Natural Resources and Applied Life Sciences/Universität fur Bodenkultör Wien) binary vector, which can be used to express proteins in plants via transformation by *Agrobacterium tumifaciens*.

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