Cloning of Codon Optimized Subtilisin E in pTEF-MF

Introduction

As the test expression of the Subtilisin E gene from *Bacillus subtilis* was not successful, we used a codon optimized variant of the Subtilisin E gene. As a host we chose the *S. cerevisiae* strand CENPK2-1D again as it has the deficiencies we need for selection. For vector backbone we tried the constitutive pTEF-MF vector again. A scheme of the cloning can be seen below:

![Diagram of cloning](https://via.placeholder.com/150)

*Figure 1: Cloning of codon optimized Subtilisin E into pTEF-MF*

For all agarose gels we used 1% agarose and 1 kb Thermo scientific ladder 08:

*Figure 2: 1kb Thermo scientific ladder*
Day 1

Codon Optimization
We used the IDT codon optimization tool to convert the *E. coli* optimized Subtilisin E gene into one that *S. cerevisiae* can translate better.
The sequence of the codon optimized construct can be seen as part BBa_K2020023.

Day 7

Dilution
After the gBlock from IDT arrived we diluted the it as described in their instructions.

PCR Amplification of gBlock
Just restricting the gBlock and then clone it into a vector does not work without prior amplification.
That’s why we first amplified it with the primers YP0028 and YP0029 according to the PCR protocol.

<table>
<thead>
<tr>
<th>ID</th>
<th>92°C</th>
<th>1 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>92°C</td>
<td>20 sec</td>
</tr>
<tr>
<td>A</td>
<td>60-70°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>E</td>
<td>72°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>FE</td>
<td>72°C</td>
<td>3 min</td>
</tr>
<tr>
<td>S</td>
<td>4°C</td>
<td>storage</td>
</tr>
</tbody>
</table>

Agarose Gel
We prepared an agarose gel to see if the PCR product is present and has the right size of about 1100 bp.

![Agarose Gel](image)

*Figure 3; Agarose gel; 1: PCR product after amplification; 0: ladder*

In both samples PCR product is visible on agarose gel at the right size.
PCR Clean Up
We did a PCR clean-up of the amplified codon optimized Subtilisin E.

Nanodrop
We did a nanodrop of the cleaned up samples of codon optimized Subtilisin E to determine DNA concentration.
1. 244.7 ng/µl
2. 220.9 ng/µl

Restriction
We double digested the codon optimized Subtilisin E and the pTEF-MF backbone with EcoRI and Xhol.

Dephosphorylation
We dephosphorylated the double digested backbone pTEF-MF.

Ligation
The amplified codon optimized Subtilisin E was ligated with the dephosphorylated pTEF-MF backbone and as control we made a self-ligation of the dephosphorylated backbone.

Pipetting scheme:
1. 1:9 ratio regarding the concentration in ng/µl
2. 1:9 ratio regarding DNA molecules
We ligated half of each sample for 3h at 16°C and the other half overnight in case this was not efficient enough.

Transformation in E. coli Dh5 alpha
We transformed 2 and 5 µl of the 3 h ligation into DH5alpha respectively.

Day 9

Transformation Results
The transformation was successful, but we only had one colony.

Colony PCR
We made a colony PCR of the picked clone with primers YP0016 and YP0017. As the ideal annealing temperature was not yet known, we used a gradient with 11 different temperatures.

<table>
<thead>
<tr>
<th>Cycles</th>
<th>98°C</th>
<th>58-65°C</th>
<th>72°C</th>
<th>4°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ID</td>
<td>30 sec</td>
<td>30 sec</td>
<td>90 sec</td>
<td>storage</td>
</tr>
<tr>
<td>D</td>
<td>10 sec</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>10 sec</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td>90 sec</td>
<td></td>
</tr>
<tr>
<td>FE</td>
<td></td>
<td></td>
<td>10 sec</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Agarose Gel
After performing the colony PCR, we loaded the samples on an agarose gel. The expected size was about 1400 bp.

![Agarose Gel Image]

**Figure 4; Agarose gel; 00: PCR product of colony 00; 1: PCR product of colony 1; 2: PCR product of colony 2; 3: PCR product of colony 3; 4: PCR product of colony 4; 5: PCR product of colony 5; 6: PCR product of colony 6; 7: PCR product of colony 7; 8: PCR product of colony 8; 9: PCR product of colony 9; 10: PCR product of colony 10**

It is visible that every temperature created PCR product at the right size and all temperatures worked for our primers.

Day 10

**Plasmid Isolation**
We isolated the plasmid of the overnight pTEF-MF with codon optimized Subtilisin E we made during the colony PCR.

**Sequencing**
We sent the isolated plasmid in for sequencing with primers YP0019 and YP0030.
Day 11

Sequencing Results
The sequencing results of clone 2 were perfect.

Transformation in Yeast
We transformed the clone 2 of pTEF-MF with codon optimized Subtilisin E into CENPK2-1D.

Results
After day 12 we had the expression system with confirmed sequence our host organism. After the transformation in CENPK2-1D we tested the expression of Subtilisin E.