Martina uses pRK 2013 Plasmid for triparental conjugation.

She gave us her Strains (#4-71,72,73) + the original strain from which she got it (#4-60) on plate, so we can use them. They're both DH5a strains, but her's are called Top10 Δ dapA, so they just grow with 300uM DAP.

We did the first step (inoculating the HB101 in LB) for electrocompetent E.coli so that we can transform the plasmid (PilN? to restore natural competence) in HB101 as our donor strain for triparental conjugation.

15.04.19: Inoculation etc. did not work as planned in the last days. Inoculating HB101 again: 11:50 OD600= 0,094; 9,6ml Culture on 500ml LB.
<table>
<thead>
<tr>
<th>Autor: Michael Burgis</th>
<th>erstellt: 15.04.2019 15:54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eintrag 2/16: Noch kein Eintragstitel</td>
<td>aktualisiert: 15.04.2019 15:56</td>
</tr>
<tr>
<td>In Projekt: Natural competence</td>
<td></td>
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<tr>
<td>Keine Tags verwendet</td>
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</tbody>
</table>
Eintrag 3/16: Chemokempotent HB101
In Projekt: Natural competence
Mit Tags: HB101, Kompis
<table>
<thead>
<tr>
<th>Autor: Vinca Seiler</th>
<th>erstellt: 13.05.2019 11:39</th>
</tr>
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<tbody>
<tr>
<td>In Projekt: Natural competence</td>
<td></td>
</tr>
<tr>
<td>Keine Tags verwendet</td>
<td></td>
</tr>
</tbody>
</table>
Planing the cloning for Cpf1

→ ordering prmers

**o_iGEM_1_612_TargPilN_f**

Sequence (5’ to 3’): AGATGTCTGCTATCCTCACCGACC

Type: Primer
Length: 24
% GC: 54.2
Hairpin Tm: None
Self Dimer Tm: None
Tm: 63.6
created by: primer3

**o_iGEM_1_613_TargPilN_r**

Sequence (5’ to 3’): AGACGGTCGGTGAGGATAGCAGAC

Type: Primer
Length: 24
% GC: 58.3
Hairpin Tm: None
Self Dimer Tm: 1.9
Tm: 65.6
created by: primer3
o_iGEM_1_614_Rep.pilN_f
Sequence (5' to 3'): CATTCTTTTGTCTAGCTTTAATGCGGTAGTTGGTACCCCTGCTCGTGCGACTC

Type: Primer
Length: 54
% GC: 70.6
Hairpin Tm: 45.4
Self Dimer Tm: None
Tm: 60.2
created by: primer3

o_iGEM_1_615_Rep.pilN_r
Sequence (5' to 3'): GCCCGGATTACAGATCCTCTAGAGTCGACGGTACCCGTCAGCTTTGGGGCTG

Type: Primer
Length: 52
% GC: 70.6
Hairpin Tm: 54.8
Self Dimer Tm: 9.8
Tm: 60.4
created by: primer3
Making Preculture of pSL2680 for cloning of Cpf1-Plasmid

- 20 ml LB + kan
→ inoculate with Culture from Addgene
- Plasmidisolation of 2x 10 ml Preculture of pSL 2680

- Via Kit: from MN

- Eluation in 50 l prewarmed water

- DNA-concentration measured with NanoDrop

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>pSL 2680</td>
<td>50 ul</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pSL 2680</td>
<td>50 ul</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Restriction with AarI

Digest 50l of miniprep with aarI (Thermo fisher) using the following reaction mix:

10l aarI buffer
50l plasmid prep
2l of 50X aarI oligo
4l aarI enzyme
34l water

Incubate at 37C over night
Put all of the Restriction of pSL 2680 with AarI an Agarose Gel

Gel-electrophoresis

Prepare Agarose Gel:
- Pour prepare Agarose + TEA in chamber
  [1% [w/v] Agarose in 1x TAE-Puffer (20 mM Eisessig, 40 mM Tris, 1 mM EDTA; pH 8)]
- Add 50 l/l Ethidiumbromid
  2-3 drops for small gel

Let solidify (ca.15-20 min)

Prepare Probe:
- Mix probe with (6X) Loading-Dye
- Apply DNA ladder & probes at gel
- Let Gel run at 135 V
- Document Gel on UV-Table

→ run for 30 min
Gel extract the digest

Excise largest band (~12kb)

Elute gel extraction with 20l water.

NanoDrop:
5.7 ng/ul (1.74 / 0.03)
Set up Annealing reaction in 1.5 mL microcentrifuge tube
use o_iGEM_1_612_TargPilN_f and o_iGEM_1_613_TargPilN_r

<table>
<thead>
<tr>
<th>Volume (ul)</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.5 ul</td>
<td>10 uM Primer f</td>
</tr>
<tr>
<td>1.5 ul</td>
<td>10 uM Primer r</td>
</tr>
<tr>
<td>5 ul</td>
<td>T4 ligase buffer</td>
</tr>
<tr>
<td>42 ul</td>
<td>ddH₂O</td>
</tr>
</tbody>
</table>

Incubate in heatblock for 10 min at 85°C. Turn off heatblock and allow samples to remain in the heatblock for slow cooling to room temperature. Proceed with next step or freeze annealed oligos for long term storage.

**Ligation of Annealed Oligos with AarI digest pSL 2680**

→ of TargPilN (o_iGEM_1_612_TargPilN_f and o_iGEM_1_613_TargPilN_r)
→ Burgis (KO...)
→ Burgi (gRNA...)

Ligate diluted oligos into pSL2680 digest from the previous day with the following reaction mix:

- 8.0 l pSL2680 gel extraction
- 0.5l of annealed oligos
- 1 l ligase buffer
- 0.5 l ligase

Incubate overnight at room temp. for 24h
### Transformation

- Use 100 l competent E.coli cells from -80 °C freezer on ice!!!!
- Add 2 ul Plasmid
- Incubate together for 30 min on ice
- Heatshock: 45 - 60 sec at 42°C
- Incubate 5 min on ice
- Add 500 l LB-Medium
- Incubate at 37°C for 2 h
- Plate on LB-Agar-plates with kan
- Incubate plates at 37°C over night

<table>
<thead>
<tr>
<th>Competent cells</th>
<th>Plasmid name</th>
<th>Antibiotic used</th>
<th>Amount plated A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top10</td>
<td>AarI dij. pSL 2680 + TargPilN</td>
<td>kan</td>
<td>100 ul</td>
</tr>
<tr>
<td>Top10</td>
<td>AarI dij. pSL 2680 + Burgis</td>
<td>kan</td>
<td>100 ul</td>
</tr>
<tr>
<td>Top10</td>
<td>AarI dij. pSL 2680 + Burgi (gRNA...)</td>
<td>kan</td>
<td>100 ul</td>
</tr>
<tr>
<td>DH5a</td>
<td>AarI dij. pSL 2680 + TargPilN</td>
<td>kan</td>
<td>100 ul</td>
</tr>
<tr>
<td>DH5a</td>
<td>AarI dij. pSL 2680 + Burgi (KO...)</td>
<td>kan</td>
<td>100 ul</td>
</tr>
</tbody>
</table>
Testing with PCR if pilN of pilN_NSII_cm has integrated in Neutral-Site-II

- of Cultures:
  → UTEX WT
  → Single Culture CB
  → CAa
  → CAü
  → CBa
  → CBü

- with Primer:
  → o_1_001_pilN-seqA_fw + o_iGEM_1_023_US-US_r
  → o_1_002_pilN-seqA_rev + o_iGEM_1_024_DS-DS_f
  → o_iGEM_1_023_US-US_r + o_iGEM_1_024_DS-DS_f

Colonie PCR:
→ Pick some culture and dilute in H2O
→ heat up to 100°C for 10min
→ centrifuge and use 2 ul of the water on top of the cellfragments
PCR-Conditions:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>2 min</td>
</tr>
<tr>
<td>95°C</td>
<td>30 sec x 30</td>
</tr>
<tr>
<td>58°C</td>
<td>30 sec</td>
</tr>
<tr>
<td>72°C</td>
<td>2.5 min (1+23 2+24) / 5min (23+24)</td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
</tr>
<tr>
<td>12°C</td>
<td>infinity</td>
</tr>
</tbody>
</table>

Gel-electrophoresis

Prepare Agarose Gel:

- Pour prepare Agarose + TEA in chamber
  [1% [w/v] Agarose in 1x TAE-Puffer (20 mM Eisessig, 40 mM Tris, 1 mM EDTA; pH 8)]
- Add 50 l/l Ethidiumbromid
  2-3 drops for small gel

Let solidify (ca.15-20 min)

Prepare Probe:

- Mix 10 ul probe with 2ul (6X) Loading-Dye
- Apply DNA ladder & probes at gel
- Let Gel run at135 V
- Document Gel on UV-Table
PCR didn't funktion
→ repeat!!!!
Inoculate UTEX with (?) pilN-NSII-cm
- Use Plates CAa CAü CBa CBü
- Use 20 ml liquid BG11 with 5 ul cam

→ Pick colonies and solve in BG11

Restreak UTEX with (?) pilN-NSII-cm
- Use Plates CAa CAü CBa CBü
- Pick colonies and solve in 1 ml BG11
→ plate 100ul on BG11-cam plates

→ put in Incubator with 41 °C + 300 uE + 5% CO₂
- Plates from Transformation 25.5.19
  → just Colonies on Transformations of Top10

- Precultures in LB with kan of E.Coli from these Plates
  → 4 from each Transformation
- Ordering Primers for Controlling the lost of lac-Genes in pSL2680

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>Kontroll-PCR für Seq - Integrationsbereich gRNA / Template - &gt; Universell</th>
</tr>
</thead>
<tbody>
<tr>
<td>o_iGEM_1_616_Klon-Cpf1-kontr_f</td>
<td>GCAGAATAGGAATAACTAAGAATTC</td>
<td>Vinca / Burgy</td>
</tr>
<tr>
<td>o_iGEM_1_617_Klon-Cpf1-kontr_r</td>
<td>GATTCCATTTTACTGATGAATG</td>
<td>Vinca / Burgy</td>
</tr>
</tbody>
</table>
- PCR to check if pilN is integrated in NSII
- Template: colonie PCR of
  → UTEX WT
  → Colonies form plates of Triparental Konj. (CAa, CAü, CBa, CBü)
  → PCC WT (2 different plates)
  ⇒ 1x 2ul Template diluted in BG11 direct to PCR
1x Template diluted in H₂O, heated up to 100°C for 10 min, spin down, and use 2ul of the supernatant

- PCR with 3 different Oligocombinations
  → o_iGEM_1_001:
  → o_iGEM_1_002:
  → o_iGEM_1_023:
  → o_iGEM_1_024:
  ⇒ 1_023 + 1_024 ; 1_001 + 1_023 ;1_002 + 1_024

- PCR with Q5
After successful cloning of the pSL2680 plasmid with the gRNA and repair template for the piLN gene with one SNP in order to restore the natural competence of our strain, this plasmid has to be introduced via conjugation.

After picking 20 colonies, we identified 2 viable plasmids that were cloned correctly - I & J - and continue work with pSL2680-piLN-I.

27.09.19

First try with pRK2013 as helper plasmid and HB101 harbouring our plasmid of interest.

preparations for conjugation:

- inoculation of DH5α Strain harbouring pRK2013 plasmid for conjugation
- inoculation of one colony (I) of HB101 w/ pSL2680-piLN-I
- preparation of BG11+5%LB plates (1,5% agar)
  - 950ml BG11 + 50ml LB + 15g agar
- preparation of normal LB plates w/ Kan50

28.09.19

conjugation:

1. overnight cultures of our UDAR strain & 4787- (plasmid cured) strain were inoculated at OD730=0,1
2. at OD730=-0,25 overnight cultures of DH5α w/ pRK2013 & HB101 w/ pSL2680-piLN-I were reinoculated at OD600=0,1
3. when UDAR & 4787- reached OD730=~0,6 and the E.coli strains OD600=~0,5, 2ml of each E.coli and 1ml of each S.elongatus culture was spinned down at 4000g for 2min
   1. pellets were washed two times in 2ml/1ml LB/BGM respectively and in the end resuspended in 200µl LB/BGM respectively
4. 100µl of each E.coli strain was mixed w/200µl of the S.elongatus UDAR or 4787- strain
5. both mixtures (DH5α w/ pRK2013 + HB101 w/ pSL2680-piLN-I + S.elongatus UDAR & DH5α w/ pRK2013 + HB101 w/ pSL2680-piLN-I + S.elongatus 4787-) were inoculated at 37°C & 200µE for 30min.
6. the mixtures were blotted on Nuclepore Track-Etched membranes on BG11+5%LB plates and incubated at 37°C & 200µE for 24h

29.09.19

conjugation:
7. filters were transferred onto BG11 plates w/ Kan50

02.10.19

after 3 days of incubation the filters were again transferred on fresh BG11 plates w/ Kan50

04.10.19

Cells were washed from the filter w/ BG11 and restreaked on a new BG11+Kan50 plate

Second try

To make sure to get results, another conjugation approach was started.

This time two different plasmids were used: pRL443 and pRL623

02.10.

In order to conjugate our plasmid of interest it has to be harboured by a HB101 strain that also has the pRL623 plasmid. We previously had transformed HB101 cells w/ pRL623, so just pSL2680-piLN-I had to be introduced.

To fasten the process, electroporation was used instead of making competent cells first and then transforming them.

Electroporation was performed following this protocol: Quick electroporation protocol for E.coli - entry #14 in project 'Generell_Strain Engineering' (Jonas Freudigmann, 03.10.2019)

03.10.

Three colonies were picked and inoculated in 10ml LB + kan50 + cam34
Autor: Jonas Freudigmann
Eintrag 16/16: Noch kein Eintragstitel
In Projekt: Natural competence
Keine Tags verwendet