**STEP-BY-STEP MANUAL: GATE KIT FOR EASY TAL EFFECTOR ASSEMBLY**

**Introduction:**
Transactivator-like (TAL) Effectors are fusion proteins of DNA-binding and effector domains: While the DNA-binding domain anchors the TALE at a locus in a genome, the effector domain (e.g. a nuclease) executes its particular function (e.g. inducing double strand breaks). Besides being more efficient\(^1\) and showing less cytotoxicity\(^2\) than other genome engineering tools, their modular mode of DNA recognition makes it relatively easy to produce TALEs that bind to any desired locus. The DNA-binding domain consists of an array of almost similar repeats, each of which interacts with one single nucleotide in the target sequence. Thus, by changing the order of these repeats, researchers can target novel sequences of DNA. In the following step-by-step manual, we show you how to assemble six pairs of gene fragments of these repeats (six so called direpeats) directly in the expression vector in one single reaction via Golden Gate cloning. The GATE kit has been developed by the student team from Freiburg University participating in the 2012 iGEM competition. For further details, please visit their website: http://2012.igem.org/Team:Freiburg.

1. **Materials:**
   - GATE assembly Kit (including 96 direpeat plasmids and the expression plasmids pTALEN, pTAL-TF and pTAL-KRAB for expression on eukaryotic cells)
   - T4 Ligase
   - T4 Ligase Buffer
   - BsmBI (NEB) or Esp3I (Fermentas)
   - Distilled water (dH\(_2\)O)
   - Transformation competent Bacteria (note: bacteria need to be susceptible to the ccdB kill cassette, ordinary DH10B or Top10 work fine)
   - LB-Buffer (without antibiotics)
   - Mini prep Kit

2. **Equipment:**
   - 2µl/20µl/1000µl pipette plus tips
   - PCR-Tubes
   - Thermo cycler
   - LB Agar plate with Ampicillin (100 µg/ml)
   - Thermo block
   - 37°C incubator

**Step 1: Choose your TAL Effector**
We provide three different TALEs: A TAL Effector Nuclease (TALEN) for efficient knock-outs or knock-ins, a TAL protein coupled to the strong transcriptional activator VP64 (TAL-TF) and to the transcriptional repressor KRAB (TAL-KRAB). The genes of these three effectors are already in the according expression vectors for mammalian cells.

**TALEN:**
TALENS cut dsDNA as dimers. That is why, two TALEs that bind to opposite strands of DNA need to be assembled. In the TALEN constructs we provide\(^3\), the spacer inbetween the two TALEN binding sites should be 16bp long.
TAL-TF/KRAB:

Example: We choose to assemble a TAL-KRAB construct.

Step 2: Choose your DNA target sequence.
Target sequences generally need to be 14 bp long. Besides, it is required that they start and end with a thymine base. The following website provides valuable help for finding the right target sequence:
https://boglab.plp.iastate.edu/node/add/talen (for TALENs)
https://boglab.plp.iastate.edu/node/add/single-tale (for TAL-TFs and TAL-KRABs)
Example: We target the following sequence:
TGATTCGCATGATT

Step 3: Find the right di-repeats:
Remove the flanking thymine bases from your target sequence, split it into six pairs of nucleotides and number the repeats from one to six. You can find the di-repeat gene fragments that bind to your pairs of nucleotides in the 96 well plate of our GATE kit. The Di-Repeats are provided as DNA in leophylized form. To get the needed concentration of 60ng/µl please resuspend the DNA in 10µl H₂O
Example:
TGATTCGCATGATT
Remove flanking Ts:
GATTCGCATGAT
Split into pairs of nucleotides:
GA-TT-CG-CA-TG-AT
Number the pairs
1.GA, 2.TT, 3.CG, 4.CA, 5.TG, 6.AT

**Step 4: Insert the di-repeats into the expression vector.**
Mix the following components in a PCR tube. Also prepare a negative control lacking the di-repeats.

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 di-repeat plasmids (60ng)</td>
<td>1 each</td>
</tr>
<tr>
<td>Expression vector (170ng)</td>
<td>1</td>
</tr>
<tr>
<td>(pTALEN/pTAL-TF/pTAL-KRAB)</td>
<td></td>
</tr>
<tr>
<td>T4 Ligase Buffer (10x)</td>
<td>2</td>
</tr>
<tr>
<td>T4 Ligase (60 U)</td>
<td>1</td>
</tr>
<tr>
<td>BsmBI (15 U)</td>
<td>1,5</td>
</tr>
<tr>
<td>dH₂O</td>
<td>8,5</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

Note: You might need to dilute the T4 ligase.

Put the PCR-tubes in a thermo cycler and run the following program:

<table>
<thead>
<tr>
<th>Cycle Number</th>
<th>Temperature, Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-13</td>
<td>37 °C, 5 minutes</td>
</tr>
<tr>
<td></td>
<td>20 °C, 5 minutes</td>
</tr>
<tr>
<td>14</td>
<td>50 °C, 10 minutes</td>
</tr>
<tr>
<td>15</td>
<td>80 °C, 10 minutes</td>
</tr>
</tbody>
</table>

**Step 5: Transform bacteria:**
Transform the products (from step 4) into a competent *E. coli* strain according to the manufacturer’s instructions. DH10B and Top10 bacteria work fine. Note that selection of the right clones will not be successful with ccdB-resistant *E.coli* strains.
Add 5 μl of the ligation product (from step 4) to 50 μl of competent bacteria, incubate on ice for 30 min, perform heat shock at 42 °C for 1 min, then incubate on ice for 5 min.
Add 500 μl of pre-warmed LB medium, incubate at 37 °C for 1 h on a shaking thermo block, plate on an agar plate containing ampicillin and incubate at 37°C overnight.

**Step 6: Sequence validation:**
On the next day, you should find numerous colonies on the agar plates and 0-2 colonies on the negative control plate. Use a sterile pipette tip to carefully distribute a single colony on a new LB agar plate with ampicillin. Incubate for 16 hours. Scrape the bacterial lawn off the plates and perform mini prep according to the manufacturer’s instructions. Since the GATE assembly protocol is very reliable (95% of the 40 clones we tested displayed the right sequence), colony PCR or test digest are not necessary. Instead, sequence one colony using the following primer:
Seq Primer: **GGAGCACCCCTCAACCT**
As a reference sequence, string together the sequences of your di-repeats.

**Step 7: Transfection:**
You have assembled the repeat array already in the expression plasmid. Thus, you can directly move on transfecting your cells.

**References**