Prepare for pTPGI_dCas9_VP64 integration

In charge: Yeast group (Cyril, Loïc, Vincent, Axel, Joseph)

Start date: 8/07 - End date: --/-- OR Date: --/--

Aim:

Integrate pTPGI_dCas9_VP64 into yeast genome so that the dCas9_VP64 protein is always expressed by the cells.

Schedule :

- Prepare medium for the yeast
- Make competent yeast.
- Transfect the plasmid in yeast. Control: pTPGI_dCas9_VP64 western blot

The plasmid is ready for integration into yeast genome. Control: western blot.

Results, discussion and comments:

First attempt (02.06.2015):

We used circular pTGI_dCas9_VP64 as a positive transformation control.

We used a plate containing all four URA, LEU, TRP and HIS as a positive control.

We used a TRP- plate as a negative control (plated with untransformed yeast).

We used competent yeast from Bart's lab (so the first steps for the integration were not done).

Results:

The only plate showing some colonies was the positive control, indicating that the medium was not the problem in our experiment.

Plates to prepare:

Yeast (no plasmid): positive control (URA; LEU; HIS; TRP)*2, negative control (TRP-)*2
Linearized: pTGI_dCas9_VP64 : , selection (TRP-) *4,
Transformation control: selection (TRP-)*2
Integration control ?

Plates with all four markers: 2
TRP- plates: 8

Step 1: Medium and plates preparation

Date: --/--

Participants:

Aim:

After the integration, the yeast need to be plated so we will need plates and mediums

Material:

- Amino acids (Histidine, Tryptophan, Uracil, Leucine)
- Amino acid powder
- Yeast nitrogen base
- Ammonium sulfate
- Adenine sulfate
- Agar
- Glucose
- Difco peptone
- Yeast extract
- Water

Procedure:

We will need to make 4 different solutions: YPD (for yeast growth), 40% Glucose, SD medium (for the plates, with selection) and amino acids solution (for the selection). The protocols can be found on EPFL iGEM 2014 Website

YPD:

For 1L of solution, we need to add 20 g of Difco peptone, 10 g Yeast extract, and 20 g of Agar (ONLY if we want to make plates !). We then add 950 ml of water and autoclave the solution. Once the medium is cooled down at approximately 55°C, 2% of dextrose (or glucose) is added (=50 ml of a 40% stock solution). The solution can then be dispensed in 50 ml Falcon tubes, and centrifuged at 1’000 g for 5 minutes.

40% Glucose solution:

For 1L solution, we put 500 ml of water in a beaker. We turn on heat and allow water to warm but not to boil. We then can add 400g of glucose and wait a few minutes (until the solution become colorless). We then add water up to 1L and autoclave the solution.

Amino acids solution:

Using differents amino acids in powder, we made solutions that will be more convenient to use for the plates.

Stock	100 mM His	20 mM Ura	100 mM Leu	40 mM Trp
Quantity for 50 ml	0.418 g	0.0448 g	0.262 g	0.1632 g

SD Medium:

For 2L of medium. We separate the medium in 2 solutions: the agar one and the SD medium one.
For the medium (1L), we have to mix 2.6g amino acid powder, 3.4g yeast nitrogen base, 10g ammonium sulfate, 1g adenine sulfate, and 950 ml water. For the agar (1L), we mix 35g agar and 900 ml water.
After autoclaving the two solutions, we mix them together, cool to 55°C, add 100 ml of 40% glucose, and 16 ml of each amino acid solutions.
We can then pour the plates.

Step 1: Integration preparation

Date: /-

Participants:

Aim:

Before the genome integration, we need to linearize the plasmid and to cultivate the yeast strain.

Material:

- Yeast strain 4271
- Miniprep from pTPGI_dCas9_VP64 (concentration = 0.4905 µg/µl)
- KPNI (20'000 U / ml)
- 10x buffer 1
- ddH2O
- Integration control DNA (concentration = 0.121µg/µl)
- XhoI (20'000 U / ml)
- 10x buffer 3.1

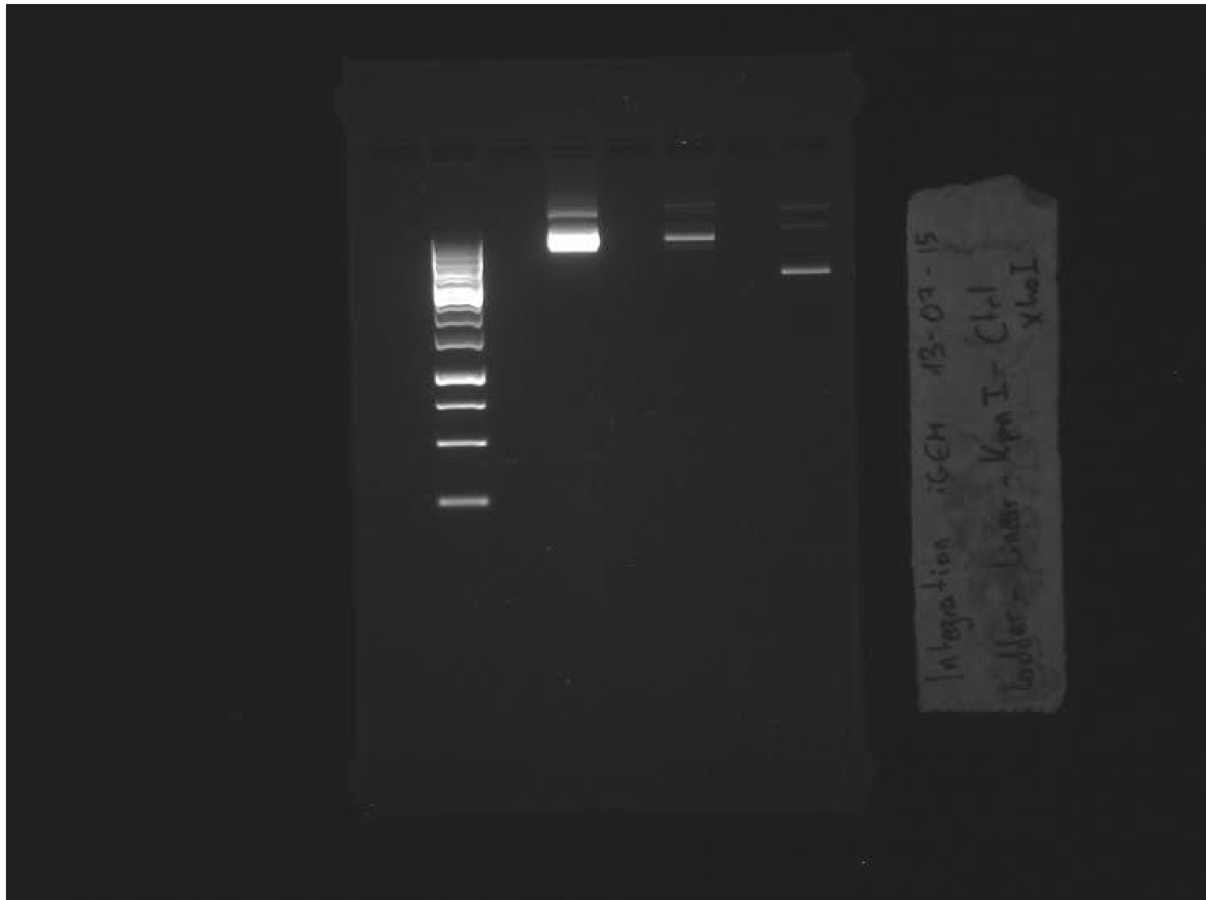
Protocol:

Based on [Openwetware protocol]

- Preparation of Yeast liquid medium
    - mix 20 g Difco peptone with 10 g Yeast extract
    - add water to 950 ml
    - adjust pH if necessary and autoclave
    - when the temperature is cooled to 55°C, add dextrose (or glucose) to 2% (50 ml of a 40% stock)
    - adjust the volume to 1L and transfer the medium in 50 ml tubes and centrifuge at 1000g for 5 minutes at room temperature
- Inoculate the yeast strain into 5 ml of liquid medium (YEPD) and incubate at 37°C overnight
- Linearize at least 3.75 µg plasmid (pTPGI_dCas9_VP64) using KpnI restriction enzyme. To do this:
    - We mix 1 unit of DNA (1µg) for 10 units restriction enzyme (generally 1µl)
    - 50µl total: 4µl KpnI + 4µl 10x buffer 1 + 8.2µl of DNA + 33.8 µl of ddH2O
- Linearize at least 3.75 µg integration control (0.121µg/µl) plasmid using XhoI restriction enzyme. To do this:
    - 10.26 µl total: 1µl XhoI + 1µl 10x buffer 3.1 + 8.26µl DNA

We linearized during 1 hour at 37C and ran a gel to confirm the linearisation.

Results:



Gel to confirm the digestion. Lane 1 : Ladder. Lane 2 : Undigested pTPGI_dCas9_VP64 Lane 3: Digested pTPGI_dCas9_VP64 by KpnI (diluted 5 times compared to lane 2) Lane 4: Digested control plasmid by XhoI

Storage:

Step 2: Integration into Yeast genome

Date: 13/07

Participants: Joseph, Cyril, Loic, Axel

Aim:

As the plasmid is linearized, we can integrate it into the genome of our Yeast strain

Material:

- YPD or the appropriate SD liquid medium
- Sterile 1X TE/1X LiAc
- Sterile 1.5-ml microcentrifuge tubes for the transformation
- Appropriate SD agar plates (100-mm diameter)
- Appropriate DNA template in solution (check amounts required)
- Appropriate yeast reporter strain for making competent cells
- Salmon sperm (carrier DNA)
- Sterile PEG/LiAc solution
- 100% DMSO (Dimethyl sulfoxide; Sigma Cat No. D-8779)
- Sterile 1X TE buffer
- Sterile glass rod, bent Pasteur pipette, or 5-mm glass beads for spreading cells on plates

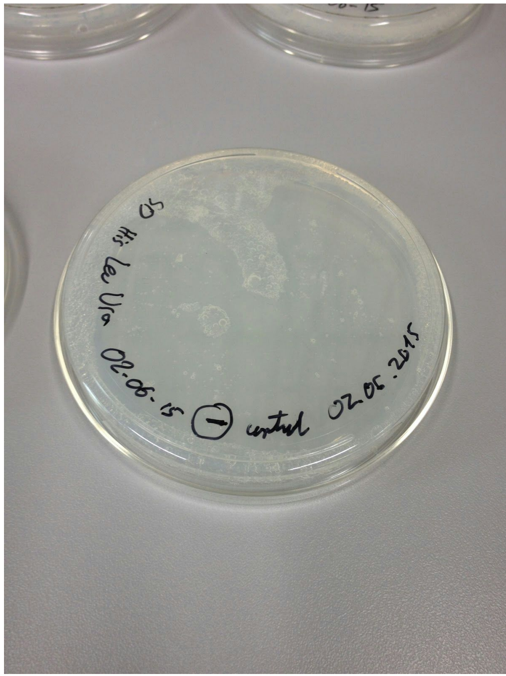
Protocol:

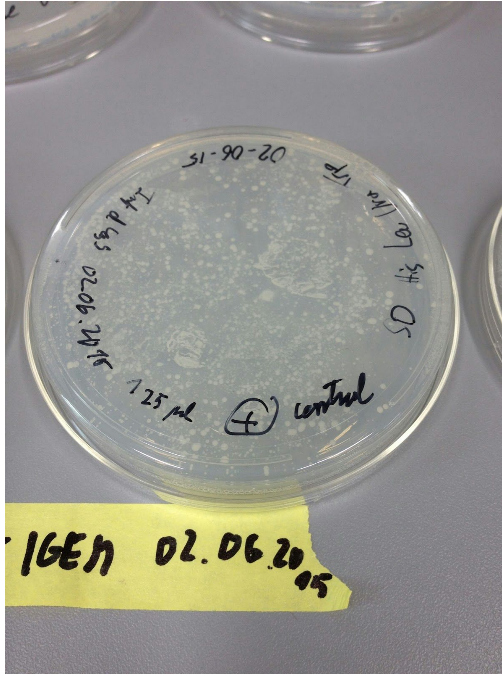
1. Inoculate 1 ml of YPD or SD with several colonies, 2–3 mm in diameter
2. Vortex vigorously for 5 min to disperse any clumps
3. Transfer this into a flask containing 50 ml of YPD or the appropriate SD medium
4. Incubate at 30°C for 16–18 hr with shaking at 250 rpm to stationary phase (OD600>1.5)
5. Transfer 30 ml of overnight culture to a flask containing 300 ml of YPD. Check the OD600 of the diluted culture and, if necessary, add more of the overnight culture to bring the OD600 up to 0.2–0.3
6. Incubate at 30°C for 3 hr with shaking (230 rpm). At this point, the OD600 should be 0.4–0.6. (We measure an OD600 of 0.58)

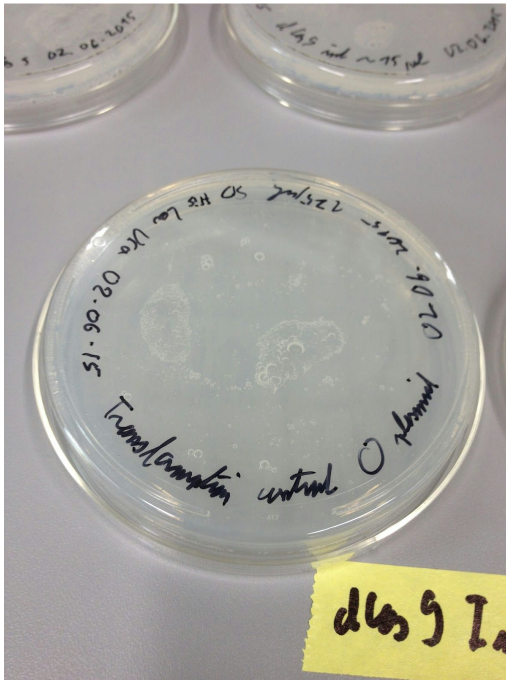
7. Place cells in 50-ml tubes and centrifuge at 1,000 x g for 5 min at room temperature (20–21°C)
8. Discard the supernatants and thoroughly resuspend the cell pellets in sterile TE. Pool the cells into one tube (final volume 25–50 ml)
9. Centrifuge at 1,000 x g for 5 min at room temperature.
10. Decant the supernatant
11. Resuspend the cell pellet in 1.5 ml of freshly prepared, sterile 1X TE/1X LiAc
12. Add at least 1 µg of linear DNA and 0.1 mg of salmon sperm DNA to a fresh 1.5-ml tube and mix
13. Add 0.1 ml of yeast competent cells to each tube and mix well by vortexing
14. Add 0.6 ml of sterile PEG/LiAc solution to each tube and vortex at high speed for 10 sec to mix
15. Incubate at 30°C for 30 min with shaking at 200 rpm
16. Add 70 µl of DMSO. Mix well by gentle inversion. Do not vortex !
17. Heat shock for 15 min in a 42°C water bath
18. Chill cells on ice for 1–2 min
19. Centrifuge cells for 5 sec at 14,000 rpm at room temperature. Remove the supernatant
20. Resuspend cells in 0.5 ml of sterile 1X TE buffer
21. Plate 100 µl on each SD agar plate that will select for the desired transformants We plated 3 plates of integration with 100µl and one with 200µl. One negative control and one positive medium control were plated with the tube without DNA. One positive control was plated on the Histidine- plate provided by Julie from Bart's lab.

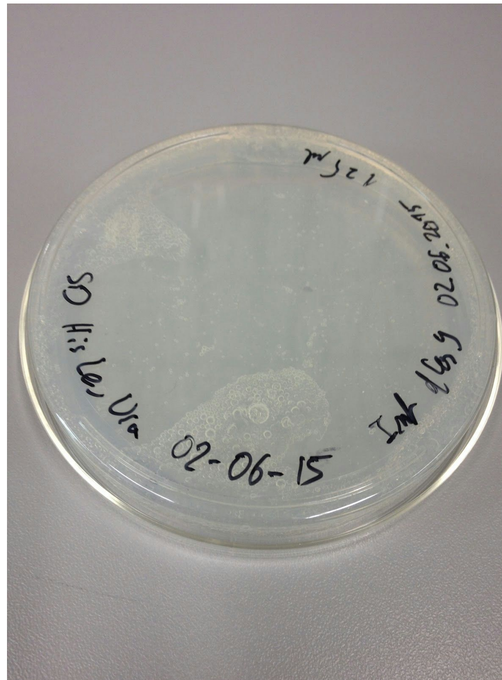
Results:

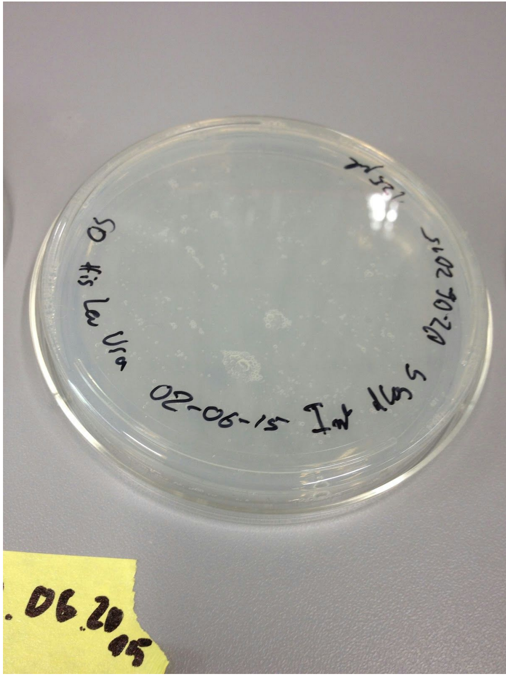
- The negative and positive control worked fine, but the transformation control didn't work. After investigation we found out that we didn't have an origin of replication in our plasmid thus not enabling our yeasts to replicate the plasmid and to grow.


SD His Leu Ura 02-06-15
control 02.06.2015

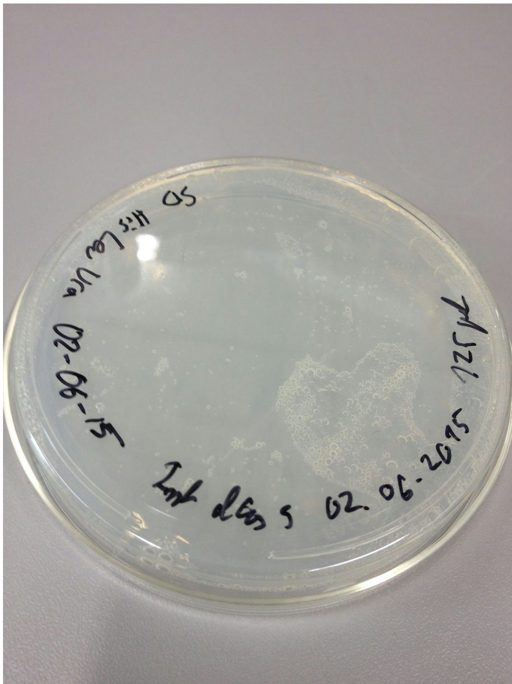

1.25 µl
control
IGEM 02.06.2015


control


SD His Leu Ura
02-06-15


SD His Leu Ura
02-06-15
02.06.2015

SD His Leu Ura
02-06-15
02.06.2015


SD His Leu Ura
02-06-15
02.06.2015