Gateway schedule :
from PCR product to an expression clone

- Design and order attBs primers (Operon).

For details (strategy, sequences, frame respect for fusion proteins) please refer to http://crfb.univ-mrs.fr/ciona/lemaire/ and download the « chordate Gateway vector manual ».

DAY 1 :

1. **PCR amplification for Entry clones.**

Amplify the DNA of interest by PCR using high fidely taq polymerase (eg : «AccuPrime Pfx DNA polymerase» (Invitrogen ; ref : 12344-024 ; for plasmid DNA), or « AccuPrime DNA polymerase » (ref : 12339-016 ; Invitrogen, for genomic DNA)).

Choose between classical PCR (full lengh primers) or « adaptor PCR » (2 rounds of PCR with shorter oligos).

2. **Gel verification : purification of attB PCR product.**

Load on gel the 1/10 of PCR product to check if the reaction worked.
If yes, then purify the PCR product, either loading all the PCR on gel and purify with a gel extraction kit (« Strataprep » from Stratagene or QIAEX II from Qiagen) or if the PCR is very clean (no unspecific amplification) use a PCR purifying kit (to remove oligos, like « qiaquick PCR purification kit » from Qiagen).
Load 1µl on agarose gel to check the concentration.

3. **BP Reaction :**

Use 1µl of BP clonase with 50 fentomole of each DNA (pDonor and purified PCR product), in 10µl of reaction mix.
Incubate over-night at room temperature.

Formula to convert ng in femtomoles (fmol):

ng of DNA = (x fmol) X (N) X ((660fmol X 1ng)/(1fmol X 10^6 fg))

x = number of femtomoles desired

N = size of DNA in bp

DAY 2 :

4. **Transformation of BP reaction.**

First apply PK treatment to kill the BP clonase (10µl of BP reaction + 1µl of PK (at 2µg/µl from the kit) at 37°C for 10min). (« Gateway BP Clonase enzyme mix », ref 11789-021 , Invitrogen).
Then use 1-2µl of BP reaction mix and incubate with DH5α electrocompetent bacteria.
Apply electric field then add SOC media (or 2YT) and incubate for 45min at 37°C.
Plate half (or less) on LB-Kanamycin plates.
Incubate O/N at 37°C.

DAY 3 :

5. PCR screen of entry clones (BP colonies) .

Pick one colony with a yellow tip, « dip » it in PCR mix (containing buffer, dNTPs, primers, polymerase) and transfer the yellow tip in 1,5ml tube containing 100µl of LB and keep it at room temperature.
Perform the PCR using « Go Taq » (from Promega) or « Quick load Taq 2X Master mix » (from New England Biolabs). (The PCR buffer contains in both cases the electrophoresis loading buffer).
The PCR can be assessed in 20µl, either using specific primers (the one used to amplify the PCR product : eg : attB3/attB5 ; attB1/attB2 etc…) or M13Fw/Rev primers located on pDonor-221 backbone and external to the attL cassette, or « attB adaptor-Fw/attB-adaptors-rev ».

- Load 2.5 µl of PCR on agarose gel to check the positive clones and correct insert size.
- Select the positive clone and start the culture in 4 ml of LB-Kanamycin of the selected one.

Incubate O/N at 37°C.

DAY 4 :

6. Analysis of Entry clone

- **Glycerol stock of the entry clone :**

700µl of bacteria culture + 300µl of 50% glycerol. Mix and store at -80°C

- **Miniprep of the entry clone :**

Prepare miniprep of the entry clone (eg kit « Wizard », Promega).

- **Restriction digest analysis**

Choose restriction enzymes according to your insert and the att-feet of Entry clone.

- **DNA concentration of the Entry clone**

Take the OD to know precisely the concentration of the plasmid.

- **Sequencing**

Eventually send few ng of the selected entry clone to be sequenced.

7. LR reaction

Use 10 femtomole of each plasmid in 10µl of reaction mix.
- when destination vector contains “R1-R2” and/or “R3-R5” gateway cassettes with the corresponding entry clones, use the classical LR clonase. (« Gateway LR Clonase enzyme mix », ref 11791-019, Invitrogen).

- For a LR reaction involving multisite (pEntry-L3-enhancer-L4 + pEntry-R4-basal promoter-L5 + pDest R3-R5-B1-reporter-B2), use high performance **« LR+ clonase »** (« Gateway LR Clonase Plus enzyme mix », ref 12538-013, Invitrogen,).

Incubate O/N at RT.

DAY 5 :

8. **Transformation of the LR reaction**

- Apply PK treatment for 10min at 37°C (same than the one applied in BP reaction, see above).
- For "classical LR", transform 1µl of the LR reaction in DH5α and plate half or all transformation on ampicillin plate.

- For multisite LR, transform 1-2µl of the reaction in high competent efficiency DH5α bacteria, **plate all** on ampicillin plate .

Incubate O/N at 37°C

DAY 6 :

9. **PCR screen of expression clone (LR colonies)**

Use the same process than the one described above to check Entry clones.
The PCR can be assessed in 20µl, using attB3 and attB5 adaptors primers. Because of the large sequence homology between B4R and B5, the PCR will give rise to 3 amplified bands (attB3-attB4 ; attB4-attB5 ; attB3-attB5) allowing to check the whole cis-regulatory cloning.

Mix for PCR :

1 colony/50ng of plasmid miniprep
5µl Go taq PCR buffer 5X ready to load (green)
2,5µl MgCl2
0,5µl dNTPs 20 µM
0,5µl attB3 fw adaptor 20µM
0,5µl attB4 fw adaptor 20µM
0,5µl attB5 rev adaptor 20µM
0.2µl Go Taq (Promega)
complete to 25µl with H2O.

Programm :

3 min 94°C
30 sec 94°C
30 sec 50°C 25 cycles
X sec 72°C
3 min 72°C

To check the ORF cloning, perform a PCR using attB1-attB2 adaptor primers.
Load 5µl of the PCR product on agarose gel to verify the lengh of amplified DNA

Select a positive clone and grow directly a maxiprep culture (100ml of LB-amp + 100µl LB containing the selected yellow tip).
Incubate O/N at 37°C

DAY 7 :

10. **Expression clone Maxiprep and analysis**

- **Glycerol stock**

As described in step 6.

- **Maxiprep**

Follow instruction of the provider (Qiagen, Macherey-Nagel…)

- **Restriction digest analysis**

Check the pattern with restriction digest, cutting Xba1/Xho1 to remove the cis regulatory regions (this Xho1 and Xba1 are the cloning sites of attR3-R5 cassette), and BsrG1 to remove the ORF (BsrG1 sites are within attB1 and attB2 sequences).