05-03-2023 paul's cloning protocol.

- **Workflow:**
  - **Generating Competent Bacteria**
  - **Digestion & Ligation (place desired insert into plasmid vector)**
  - **Bacterial Transformation**
  - **Plate Growth: Select for bacteria that uptake the vector**
  - **Colony PCR (low-resolution genotyping)**
  - **Sanger Sequencing (high-resolution genotyping)**
  - **Overnight culture (of the inoculated, genotyped, and correct clones)**
  - **Or Bacterial Freezer Stock Generation**
  - **Plasmid Purification**
  - **Repeat**

pCCo1 (Puro). { AmpR for bacteria
PuroR for cell.
- gRNA
- trRNA
- Cas9

wall
take up DNA

- **Generating Competent Bacteria by CaCl2 method**
  - Strain: Invitrogen E.coli Stbl3
  - 100-200x dilution from frozen stock
  - Grow 2-3 hrs in exponential phase
  - Incubate on ice (to synchronize all cells, not mitosis phase)
  - Wash with pre-chilled 100 mM CaCl2, while on ice
  - Store competent bacteria at -80 C
  - Expected Yield: 5e6 to 2e7 transformed colonies/ ug of plasmid DNA
  - Stage 1: Preparation of Cells
    - Pick a single colony from a plate that has been incubated overnight at 37 C.
    - Inoculate that colony into 100-mL LB broth in a 1-L flask
    - Incubate at 37 C for 3 hours
    - Begin harvesting when OD 600 reaches 0.35
    - Transfer bacteria to 50-mL conical tubes on ice for 10 min
    - Centrifuge at 2700 g for 10 min at 4 C.
    - Decant supernatant. Invert tubes on a paper towel to dry out all supernatant
    - Resuspend with 30 mL of ice-cold MgCl2+CaCl2 solution (80 mM MgCl2, 20 mM CaCl2). Vortex.
    - Centrifuge at 2700 g for 10 min at 4 C.
    - Decant supernatant. Invert tubes on a paper towel to dry out all supernatant
    - Resuspend with 4 mL of ice-cold 0.1 M CaCl2 (2 mL per 50 mL of original culture). Vortex.
    - Dispense the competent bacteria into aliquots and store at -80C

pRDA122 - perturb-seq

- **Generation of Insert** (Oligo can be encoding for gRNA to be inserted into H3 or pXPR_003 plasmid vectors)
  - Go to UCSC Genome Browser
    - Type in Gene Name that you want to Knock-Out
    - Scroll down to "Genes and Gene Prediction" section

- Display "CRISPR Targets" in "full"
- The green sequences are better NGG

- Order oligo synthesis from IDT
  - Find 20210621_gRNA_design.xlsx to use as template
  - Add "g" to 5' end of TOP strand
  - Complete TOP strand: cacc+g+seq
  - Reverse Complement of TOP to get BOTTOM
  - Add "aaac" to 5' end of BOTTOM strand
  - Name the ssDNA oligo to be purchased

5' → 3'
{ Caccg + gRNA
{ aaac + (RC) gRNA + c

start
→ - Liquidify oligos
  - 100 uM per oligo: 10 nmol mixed in 100 uL dw 2.38 x 100 = 238uL = 119 x 2
  - Formula for oligos mixture in PCR tube strip:
    - 5 uL TOP strand
    - 5 uL BOTTOM strand > 10 uL

dd H2O NF, not dw
use 10 x 23.8 = 238 uL
For both { gRNAs / primers

- Phosphorylation of 5' end of oligo (synthetic oligos do not have 5' phosphate)
  - Formula in PCR tube strip: T4 Polynucleotide kinase
    - 1 uL T4 DNA ligase 10X Buffer
    - 0.5 uL PNK Enzyme (polynucleotide kinase)
    - 6.5 uL dw
    - (2) uL oligos mixture
  - Put in PCR machine:
    - 37 C for 30 min
    - 95 C for 5 min
    - 28 Cycles cooldown
      - -2.5 C decrement per cycle
      - 30 sec per cycle

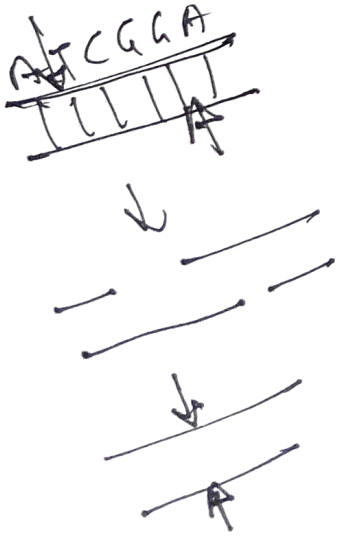
35+14 = 49

100x: 99 uL + 1 uL → Golden Gate

  - Before ligation, dilute the phosphorylated oligos 10X: ~~90 uL dw + 10 uL~~ → normal ligation oligos mixture

- **Digestion & Ligation**: Place desired insert into vector
  - **Digestion of vector (linearization)**
  - **Ligation**

Golden Gate Assembly



  - Formula in PCR tube strip:
    - 1 uL T4 DNA ligase
    - 1 uL T4 DNA ligase 10X Buffer
    - 1-2 uL linearized vector H3 (enough to get 50 ng of vector per rxn)
    - 2 uL phosphorylated oligos mixture
    - Top off with dw to get final volume of 10 uL per rxn
  - Mix by pipetting up and down or tap tap
  - Incubation RT 30
    - Sticky ends: room temperature for 10 min (or 16 C overnight)
    - Blunt ends: room temperature for 2 hours (or 16 C overnight)
  - Heat inactivate at 65 C for 10 min

① purity plasmid DNA.
2. 20 ml DW + 20 ml PCR ⇒ 0.6:1
3. 30 ml Bead

- Chill on ice

- **Bacterial Transformation by Heat-shock Method**
  - Get aliquots of competent E coli Stbl3 bacteria from -80 C. Thaw on ice for 15 min.
  - Distribute in Each PCR tube: 50 uL bacteria + 5 uL ligation product (plasmid DNA with insert) (10% ratio of DNA:bacteria) 10:1
  - Vortex to mix. Slight spin down with bench-top microfuge.
  - Incubate mixture on ice for 30 min.
  - Heat shock @ 42 C for 45 sec.
  - Incubate on ice for 2 min.
  - In 2-mL Eppendorf tubes, add 400 uL of pre-warmed SOC media per tube.
  - Transfer transformed bacteria into these SOC-filled Eppen tubes.
  - Keep in warm room on shaker for 2 hours for regeneration
  - Using a P1000 tip, suck up the bacteria and spread on to Cb+Lb petri dishes.
  - Leave dishes in warm room overnight.
  - Notes:
    - SOC media bought from Boston Bioproducts
    - Recipe

50×20=1000ul 2mL管

② 2× 80% EtOH wash

300ul

original bacteria as positive control ✓
negative controls !

Don't over grow-! move to 4°C

12 DW ↓ 6 ml or 5 ml with bead.

- **Colony PCR (low-resolution genotyping)**
  - Add 50-75 uL Lb+Cb broth per well to 96-well PCR plate (leave out the edges)
  - Pick 3 colonies per agar+Cb dish (triplicates) with sterile P20 tips
    - These colonies have picked up the plasmid
  - Incubate 1 hour in warm room 37 C
  - Formula in 384-well plate:
    - 12.5 uL ThermoScientific PCR 2X MM
    - ~~11~~ uL dw
    - 1 uL of vector-specific Primer Mix (10 uM each from 80dw+10F+10R mixture)
      *OV267-F,OV268-R for pLentiCRISPRV2
    - DNA template (heat lysis of bacteria)
    - Total Volume: 25 uL
  - Keep the 96-well PCR plate, if PCR genotype is fine → use it as stock
  - Touchdown PCR Phire 1kb TD65 check machine
    - 1st 10 cycles: 98-65-72
    - 2nd 10 cycles: 98-60-72
      - Non-permissive 65 C annealing temperature → permissive 60 C annealing temperature
      - Initially, only very specific hybridizations are allowed to occur
    - Temperature increment: -0.5 C per cycle
    - Primer design:
      - Spanning U6 promoter to after the gRNA scaffold (370 bp region)

aliquots

(6) BCA protein or 8-strip

Single colone ! pure

Phire Tissue green

0.5 ul

3 share 1:
OV 267
OV 268

1ul

dip just for PCR

1ul is too much.

(Time: 37 min)

colony PCR

(200 - 500 bp)

50×3×20

colony PCR
1. 2min - 98°C
2. 0.05min - 98°C ×3
3. 0.05min - 65°C ×3
4. 0:20min - 72°C ×3.
5. ...

3

SYBR safe DNA Gel Stain 5uL → 50mL
Thermo scientific 0 GeneRuler express DNA ladder steps

- **Gel Electrophoresis (to verify we have product)**
  - See agarose gel making protocol
- **Sanger Sequencing (high-resolution genotyping)**
  - Send pre-mixed template + primer
  - Diluted primer Formula
    - 5 uL FORWARD primer
    - 255 uL dw
    - Total Volume 260 uL
  - Formula of sample to submit to Genewiz
    - 13 uL diluted F primer
    - 2 uL PCR product
  - GeneWiz Instructions
    - PCR Product (Purified)
    - Service Type: Premix (Primer + Template)
    - "Purification": "Enzymatic"
    - Put in the bag a note: the barcode, # of samples submitted, Sanger Sequencing

10uL PCR.
5 uL ladder
dye. 5uL/50mL gel
120 V x 30 min

OVNIGHT Sanger
Just need 1 side

SYBR Safe
~300 bp band.

not bacteria

- **Overnight culture (of the inoculated, genotyped, and correct clones)**
  - Prepare 50 mL Lb + Cb in 1 50-mL conical tube for every correct clone.
  - Add ~75 uL (all) of the regenerated clone from the 96-well PCR plate to each conical tube
  - Vortex.
  - Split into 2 50-mL conical tubes (duplicates)
  - Overnight 37 C shaker for 14-16 hours (~~no more or less~~)

0.5mL
500 uL
1:1000

25mL Day

35uL each

make hole at top

16+16 = 32

- **Stock Generation by Freezing**
  - From overnight culture of the **inoculated, genotyped, and Sanger-correct clones**
  - Add 1m L overnight growth to 2-mL cryovial
    - 1.5-mL Eppendorf tubes can snap open at -80
  - Top off with 1 mL 50% glycerol (dilute 1:1 beforehand with dw)
  - Store at -80 C

long term.

0.5/0.5 or

Print label.

- **Plasmid Purification by QIAgen PlasmidPrep Midi Plus Kit; high-yield protocol**

Table 3. Maximum recommended culture volumes

Copy number	Standard protocol	High-yield protocol
High-copy plasmid*	20–25 ml	25–35 ml
Low-copy plasmid*†	50 ml	–

* For high-copy plasmids, expected yields are ▲100–200 µg for the QIAGEN Plasmid *Plus* Midi Kit using the standard protocol and ●150–250 µg for the QIAGEN Plasmid *Plus* Midi using the high-yield protocol. For low-copy plasmids, expected yields are ▲30–100 µg for the QIAGEN Plasmid *Plus* Midi Kit using these culture values with the standard protocol.

  - Collect bacteria from overnight culture, suspension total volume 50 mL

max.

dirty machine. 3000g → 15 min

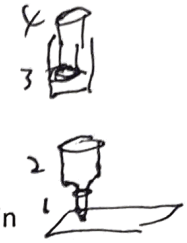
- Centrifuge 50-mL Falcon tubes for 10min at 3000g, discard supernatant 上清液
- Vortex Falcon tubes to break pellets. 沉淀

break wall Add 4 mL P1 Lysis Buffer (TEG, hypo-osmotic lysis) 20mL + 20ul RNase A. optional

- Shake, vortex or pipet until no cell clump. Wait 3-4 min

denature Add 4 mL P2 Buffer (blue, SDS detergent, basic pH) to denature bacterial & plasmid DNA

- Carefully flip tubes 10x to precipitate bacterial gDNA, protein, lipid
- Wait 3-4 min
- viscous lysate, mix until homogenize; the solution should be blue



neutral

- Add 4 mL S3 binding buffer (acetic acid) to quench
  - Carefully flip tubes 10x to mix till the blue color disappear
  - white precipitate = gDNA, proteins, cell debris, SDS
  - Do not wait go 3上
- Centrifuge for 5 min at 4500g, Prepare the vacuum manifold and QIAGEN spin columns
- Insert the plunger into the QIA filter cartridge and filter the cell lysate into a new tube
  *filter into the extension tube directly above the spin column

binding.

- Add 2 mL BB buffer to the cleared lysate, invert 4-6 times 1 mL not uL
  *or add BB to the extension tube Please mix !!!
- Transfer lysate to the spin column
- Switch on the vacuum source ~300 mbar

wash

- Wash DNA with 0.7 mL ETR buffer, switch on vacuum then switch off
- Wash DNA with 0.7 mL PE buffer, switch on vacuum then switch off
- Put the column inside a 1.5 mL container, centrifuge 10,000 g x 1 min to remove residual wash buffer

elution.

- Place a column into a new 1.5 mL tube; elute DNA with 200 uL EB buffer or water to the center of a spin column, wait 1 mins and centrifuge 10,000g x 1 min
  *Elute with 100 uL if high concentration needed
  *DNA stored in water may degrade over time
- Store DNA at -20*c

cap 帽盖

100ul x 2 times better

ds DNA

Suppose to be ~ 2000 one

QIAprep. 50 for PCR product bigger hole

25 for Plasmid small hole

Cautions:

1. Centrifuge can only be done in dirty machine!
2. Vacuum manifold must connect to storage!

Aug-1-2023

8 ul raw plasmid

↓

50 ul digested plasmid

Step 1: Digest plasmid/vector with bdsl enzyme in 1.5 ml tube (~1 day) (time: 1 day)

- 20 ug cellecta plasmid (conc is 3031 ug/ml, so add 6.5 ul pRSITEP-U6Tet)
- Esp3I 5ul bdsl enzyme (can be find in green enzyme box)
- 10 ul Rcutsmart buff (can be find in green enzyme box)
- Add up to 100ul (100-~~20~~-5-10=65 ul dw) 100-5-5-10=80
- 37 C° water bath overnight

20 / 4560 = 4.39 ul

~~20 / 251 = 79.7 ul~~

20 / 4400 = 4.55 ul

mix of everything

Step 2: Purify plasmid/vector using bead

- Magnetic bead (XP in 4 C°) 1:1 ratio (volume) relative affinity
- Vortex bead, mix with digested vector wait 1 min
- Put onto magnetic base
- Remove liquid
- Wash with 80% EtOH
- Wash with 80% EtOH
- Open lip, let bead dry
- Elution with 50ul dw water / bead compete for DNA

measure conc (should dilute 6x) ✓

Step 3: Phosphorylation of 5' end of oligo (time: 1 hours)

- bulk 8ul { 1 ul T4 DNA ligase 10X buff
- 0.5 ul PNK Enzyme
- 6.5 ul dw] bulk × 4 ×20

4, 2, 26 = 32. 8ul; 8, 4, 52 = 64.; 8, 3, 39 = 48

- 2 ul oligos mixture
- PCR machine (Phosphorylation profile) 37-95-28
- Dilute phosphorylated oligos 100x (~~1 ul product + 99 ul dw~~)

×20 = 20 = 10 = 160 = 130

key ※ **Step 4: Ligation (connect digested plasmid with oligos)** (time: 2-3 hours)

- 1 ul T4 DNA ligase
- 1 ul T4 DNA ligase 10X buffer
- ~~2ul + 198 dw dilute~~ 50 ng digested vector ~~5 ul~~ 88.2 ng/ul
- 2 ul phosphorylated oligos mixture ~~(diluted 100 times)~~
- Add up to 10 ul per Rxn

50ng / 88.2 ng/ul = 0.567 ul

10 - 2 - 2 - 5.6

Empty: 1 ul plasmid + 50 ul bacteria

Gibson Assembly

step 3: dilute dry oligos: 10nmol mixed in 100 ul dw

PCR tube 5 ul Top

5 ul Bottom

50ng / 3031 =

50ng / 3031 ng/ul = 0.016 ul