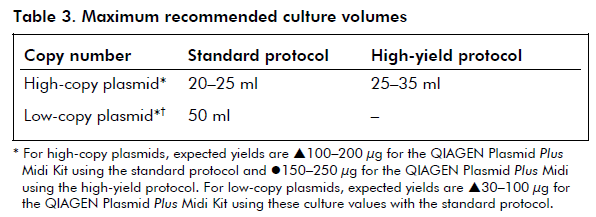
* **Workflow:**
  + **Generating Competent Bacteria**
  + **Digestion & Ligation (place desired insert into plasmid vector)**
  + **Bacterial Transformation**
  + **Plate Growth: Select for bacteria that uptake the vector**
  + **Colony PCR (low-resolution genotyping)**
  + **Sanger Sequencing (high-resolution genotyping)**
  + **Overnight culture (of the inoculated, genotyped, and correct clones)**
  + **Or Bacterial Freezer Stock Generation**
  + **Plasmid Purification**
  + **Repeat**
* **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
* **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
  + Liquidify oligos
    - 100 uM per oligo: 10 nmol mixed in 100 uL dw
    - Formula for oligos mixture in PCR tube strip:
      * 5 uL TOP strand
      * 5 uL BOTTOM strand
  + Phosphorylation of 5’ end of oligo (synthetic oligos do not have 5’ phosphate)
    - Formula in PCR tube strip:
      * 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
    - Before ligation, dilute the phosphorylated oligos 10X: 90 uL dw + 10 uL oligos mixture
* **Digestion & Ligation**: Place desired insert into vector
  + **Digestion of vector (linearization)**
  + **Ligation**
    - 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
      * 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
    - 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)
  + 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](http://cshprotocols.cshlp.org/content/2018/3/pdb.rec098863.full?rss=1)
* **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)
* **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
* **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)
* **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
* **Plasmid Purification by QIAgen PlasmidPrep Midi Plus Kit; high-yield protocol**



* + Collect bacteria from overnight culture, suspension total volume 50 mL
  + Centrifuge 50-mL Falcon tubes for 10min at 3000g, discard supernatant
  + Vortex Falcon tubes to break pellets.
  + Add 4 mL P1 Lysis Buffer (TEG, hypo-osmotic lysis)
    - Shake, vortex or pipet until no cell clump. Wait 3-4 min
  + 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
  + 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
  + 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

* Add 2 mL BB buffer to the cleared lysate, invert 4-6 times

\*or add BB to the extension tube

* Transfer lysate to the spin column
* Switch on the vacuum source ~300 mbar
* 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
* 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