Gibson Cloning Protocol

**Objective**

* Ligation of oligos via Gibson assembly, and cloning of plasmid constructs via heat shock method

**Protocol**

* DNA Assembly
  + In pcr strip
    - 2x NEBuilder HiFi DNA Assembly MM: 6uL
    - Linearized vector: 1-4 uL (aim for 150 ng digested vector per reaction)
    - Insert: 2 uL (usually single stranded oligos, liquefied at 100uM)
    - Dw: 1-4 uL (fill to 12 uL final volume per reaction)
  + Set up above reaction, incubate in 50C water bath for 1hr
    - If I’m cloning a new set of pooled oligos I would usually liquefy the pool in 500uL dw, and make a serial dilution (1x-10000x) of these oligos to see what concentration of oligos yields the greatest number of clones
* PSDNase treatment (optional)
  + This step is used to degrade any linearized vector that still remains in the Gibson reaction, in an effort to decrease our background noise from the negative control
  + In separate pcr strip:
    - Gibson reaction product: 12uL
    - ATP solution: 2uL
    - 10x Plasmid-Safe buffer: 5uL
    - Dw: 30uL
    - Plasmid-Safe ATP dependent DNase: 1uL
  + Incubate above reaction for 30 minutes in 37C water bath
* Bead purification
  + If no PSDNase treatment, after incubation is complete, add 38uL dw and 50uL AMPure SPRI bead to each 12uL Gibson reaction
    - Incubate RT 2 minutes at least
  + If yes PSDNase treatment, 50uL AMPure SPRI bead to each 50uL PSDNase reaction
    - Incubate at RT for 2 minutes (can pause here)
  + When the 2 minutes has passed, insert pcr tubes to magnetic rack
  + After about 1 minute, you should see a bead pellet stuck to the back of the tube
  + Decant the remaining liquid without disturbing the pellet
  + Wash 2x with 180uL of 80% ethanol
  + Dry the pellet for 3 minutes
  + When dry, remove tubes from magnetic rack and elute in 10uL dw
    - Here we can target the bead directly
  + Do not put the tubes back on the magnetic rack, you will add the entire 10uL elution (including the bead) to the competent cells for the transformation step
* Heat Shock transformation
  + Thaw competent cells on ice for about 15 minutes
  + In each tube with the 10uL eluted Gibson reaction, add 60uL competent cells
    - Pipette up and down very gently
  + Incubate on ice for 30 minutes
    - Here you can aliquot 300uL of SOC media for each reaction, and store the SOC in the warm room for the time being
  + After 30 minutes on ice, heat shock the cells in 42C water bath for 45 seconds
  + Incubate on ice for another 2 minutes
  + Transfer the now transformed bacteria to the previously aliquoted SOC
  + Incubate in warm room on shaker for 2 hours
    - Pre-warm LB + Cb agar plates for 30 minutes prior to plating bacteria
  + Spread cells onto LB + Cb agar plates, incubate in warm room overnight
* Colony PCR
  + In Applied Biosystems 96-well plate, add 50 uL of LB+CB to each well
  + Pick colonies from overnight growth plates with P20 tips, and swirl bacteria into each well
    - Typically pick 10 colonies from experimental plate with greatest number of colonies, and 2 colonies from negative control plate
  + Let the 96 well plate with the picked colonies sit in the warm room for at least an hour (at most 3 hours) for regeneration
  + In 384 well plate (PCR)
    - 2x PCR MM: 12.5uL
    - Dw: 11uL
    - Primer mix (10uL F + 10uL R + 80uL dw): 1uL
      * Most of my experiments I use oV114 (beginning of U6 promotor) and oV268 (after scaffold)
      * Could be different depending on the experiment so be careful which primers you use
    - DNA (bacteria from 96 well plate): 0.5uL
  + Cover the 384 well plate with adhesive film, vortex and spin down
  + Perform TD65 pcr protocol in pcr machine
  + Run 8uL of each sample on gel to check presence of pcr product and band size
  + Send necessary samples for Sanger sequencing
    - Forward primer dilution: 5 uL of 100uM forward primer + 255 uL dw
    - 13 uL forward primer (after above dilution) + 2 uL pcr product
    - Genewiz (Azenta) instructions
      * Order
      * PCR product purified
      * Service type premix
      * Purification type enzymatic

**Common Pitfalls**

* If Gibson assembly does not work, try decreasing Gibson incubation time in 50C to 30 minutes or even less
* For bead purification
  + Make sure 80% ethanol is fresh, it shouldn’t be more than a week old
    - Each time you open the tube, the concentration of ethanol decreases
  + When drying the bead pellet, don’t wait too long, usually 5 minutes is enough to dry, but 10 minutes at most
* Heat shock transformation
  + Thaw competent cells on ice, not room temp or in water, this will kill the cells
  + Don’t forget the heat shock step! (42C, 45 seconds)
* Colony pcr
  + Be careful with the amount of template DNA you use
    - We don’t want to inhibit the reaction with too much DNA, or have the reaction fail with too little DNA
    - Typically 0.5 uL of DNA is good
      * If the colonies are huge, or if the warm room incubation was for more than 2 hours, consider using less DNA