pX330 Protocols

Annealing and Digestion

sgRNA + pX330 cloning

Anneal oligos without phosphorylating

Forward primer (100uM):	1ul
Reverse primer (100uM):	1ul
T4 ligation Buffer	1ul
H2O	7ul

Program:
37C 30min
95C 5min
Ramp down 2C/sec
85C 1min
Ramp down 0.1C/sec
25C 5min
4C

Digest pX330 with BbsI-HF

pX330 mCherry (1ug)	1ul
Cut Smart Buffer	2ul
BbsI-HF	1ul
H2O	16ul
Total	20ul

37C for 1hr

Add ligation components directly to digest

10x T4 Ligase Buffer	2.5ul
Annealed oligo (1:25 diluted)	1ul
T4 DNA ligase	1.5ul
Total	25ul

37C for 1hr

Transform 2ul directly from above reaction to stbl3

Transformation

1. Perform transformation
    a. Thaw competent STBL3 cells on ice for 5-10 minutes (make sure completely thawed)
    b. Spin down briefly to make sure all of bacteria is at the bottom of the tube.
    c. Use 50 ul of competent cells in a 1.5 ml microcentrifuge tube (these tubes are lab stock and are pre-aliquoted)
    d. Add 3-5 ul (for us we found that we needed 4 uL to successfully generate colonies) of the assembled product from step 5 to the competent cells
    e. Mix gently by flicking the tube 4-5 times. Do not vortex. Place mixture on ice for 30 minutes.
    f. Heat shock at 42°C for exactly 45 seconds in water bath. Do not mix.
    g. Transfer tubes immediately onto ice for 2 minutes
    h. Add 1 mL of room temperature 1X LB media to the tubes containing the transformed bacteria (do not pipette to mix)
    i. Place the tubes at 30°C for 60 minutes. Shake vigorously at 220 rpm
    j. Warm appropriate selection plates (we used ampicillin – pay attention to what resistance your plasmid uses) to 30°C at the same time as the reaction is mixing.
    k. Once the tubes are done, centrifuge them at 13000 rpm for 1 minute
    l. Aspirate out the supernatant to leave a pellet, re-suspend each pellet with 100 ul of 1X LB media
    m. Create tools for spreading samples
        i. Pick out 1 glass pipette per sample
        ii. Start an open flame on a Bunsen burner, take a pipette and run it through the flame once or twice to sterilize it
        iii. Hold the pipette over the flame about 1 inch up from the tip to bend the tip to a 90° angle
        iv. Repeat for each pipette
    n. Spread 100 ul of the cells on to the plates with appropriate antibiotics. Our backbones called for Ampicillin plates
        i. Label plates before spreading.
        ii. Take up entire sample volume in a pipette and place in one drop in the middle of a plate
        iii. Take your spreading tool (bent glass pipette) and spread the volume to make a line spanning the plate's diameter
        iv. Rotate the plate with your left hand and make sweeping arcs with your tool in your right hand to spread the volume evenly across the plate's surface area. We spread until the sample appears to dry out. Avoid breathing/talking directly on to the plate while spreading.
        v. Repeat for each sample/plate
    o. Incubate plates overnight at 30°C with the agar side on top (this helps to prevent contamination).

2. Check plates for colonies the following morning (for one attempt we found no colonies on the first morning but left the plates at 30*C for a second night and found colonies the following day – this could be due to the 16*C overnight ligation step – perhaps room temp ligation for less time would improve colony formation over a single night).
3. Move the plates into 4*C storage until you are ready to pick colonies to prevent overgrowth.
4. If colonies are present, pick several per plate. Colony picking should ideally be performed in the afternoon as the colonies should only incubate at 30*C for about 12-16 hours.
    a. Be wary of plates that have an abundance of colonies (this could indicate that the digestion failed and likely the colonies are negative)
    b. Also be wary of your self ligation control plate (these should be free from colonies) if colonies are present could indicate some other issues with the procedure.
    c. Set up and label glass tubes (1 per colony) for picking with ~2ml of 1X LB+Ampicillin (Our Amp stock is 1000x so adjust accordingly to set up the 1X LB+Amp mixture; for example, if you have 8 colonies to pick, you will need 16 mL of LB + Amp mix. So you would take 20 ul of Ampicillin and add it to 20 mL of 1x LB (20 mL * 1000 = 20,000 / 1000X = 20 ul. Then aliquot 2mL of that mixture into each tube for each colony.)
    d. The number of colonies to pick is up to you (we picked 17 for our sgRNAs and all 17 were positive)
    e. Pick colonies by using the 10uL pipette tips attached to a pipette. Hold the open plate up to the light and gently touch the end of the tip to one of the colonies. Once you are sure it touched the colony detach the tip into the correctly labeled glass tube with LB+Amp. Close the plate in between colonies.
    f. Repeat this procedure for each colony to be picked.
    g. Place all tubes in 30*C and shake at 200rpm overnight.
    h. Collect the tubes in the morning and move them into 4*C if mini prep cannot be performed immediately. (Tubes are okay at 4*C for 1-2 weeks but it is good to perform mini and maxi preps as soon as possible)
    i. LB medium in the tube should be cloudy the next day to indicate that the bacteria expanded overnight. (clear LB means you probably didn't touch the colony or something else went wrong).
5. Perform Mini prep with ~1.5mL of the expanded bacteria (We use Qiagen DNA Plasmid Mini Kit I Cat#6942-02). Follow instructions and elute in about 30uL of recommended buffer and send for sequencing to confirm correct oligo insertion.
6. Perform Maxi prep (We used E.Z.N.A kit (VWR D6922-02)) on positive colonies after sequencing results are confirmed – kit recommends to elute in 1.5-3mL however we found that ~500uL is much better to make a concentrated stock.