Pdr-3 Transformation Protocol

* Start with Pdr1 knockout strain. Culture overnight.
* Dilute overnight culture 1:50 (1ml + 49ml media) and shake for 3h at 30˚C. After 3h should be in midlog phase.
* Measure OD. Around 0.4-0.6.
* Spin down (4000rpm, 5min) 50ml cells and ruspend in 9.5ml TE/LiOAc solution.
  + Make TE/LiOAc solution by mixing 1ml x10 TE + 1ml x10 LiOAc + 8ml sterile water.
* Spin down and resuspend in 500µl TE/LiOAc solution.
* Heat up salmon sperm DNA in 90˚C heatblock to denature to ssDNA. Mix 150µg (15µl) salmon sperm DNA with 200-500ng transforming DNA (1-2µl of pdr3-HYG cassette stock). Remove salmon sperm DNA from heatblock 1 min before use.
* Add 200µl of TE/LiOAc/cell solution to DNA mixture, mix, and then add 700µl TE/LiOAc/40%PEG solution. Mix again with pipette.
  + Make TE/LiOAc/40%PEG solution by mixing 100µl x10 TE + 100µl x10 LiOAc + 800µl 50% PEG.
* Incubate at 30˚C for 30 minutes. No need to shake.
* Heat shock at 42˚C (heatblock) for 15 mins.
* Spin down and resuspend in 250µl YPD media. Plate on YPD agar plate and incubate at 30˚C overnight.
* Replica plate YPD plate onto YPD+HYG plate (velvet method).
* Incubate at 30˚C for two days.
* If colonies have formed on YPD + HYG plate streak out 4 colonies onto a new YPD + HYG plate. Divide the plate into quarters and streak out each colony in its own quarter.
* Store at 4˚C.
* To check correct incorporation of HYG cassette, perform three PCRs. Use three primers (positive control, negative control and test). Positive control primer is upstream or downstream of Pdr3 but not within the ORF. This tests whether PCR has worked. Negative control primer is complementary to Pdr3 gene. Should have been knocked out and come back negative. Test primer is complementary to HYG cassette. Perform PCRs and run on gel to see if PCR has elongated primers.

PCR Protocol

* 100µl total
  + 50µl Q5 mix 2x.
  + 2.5µl Forward primer 10µM.
  + 2.5µl Reverse primer 10µM.
  + 1µl template (HYG cassette).
  + 44µl nuclease free water.
* PCR cycle
* x7
  + 98 – denature
  + 64 – anneal
  + 72 – replicate
  + 98 – denature
* x20
  + 98 – denature
  + 72 – replicate
* Check length of 1.6kb product through electrophoresis.
  + 100 V, 30 mins
  + 1µl dye + 3µl buffer + 2µl PCR product. Mix on parafilm and load in well.
  + 6µl ladder product in well next to it.
  + Dye in gel in gel red. Visualise under UV light. Should be a band next to the 1.6kb band.
* PCR purification
  + 1:1 binding buffer + PCR product and mix.
  + Add to purification column. Centrifuge for 30-60s. DNA binds to matrix.
  + Add 700µl wash buffer. Pushes proteins etc through. Centrifuge and discard flowthrough.
  + Centrifuge empty purification column for 1min again to remove any residual wash buffer.
  + Transfer column to clean eppendorf.
  + Add 30µl of elution buffer to columns. The DNA enters this liquid phase and leaves the matrix membrane.
  + Test concentration with nanodrop. Use elution buffer to blank twice.