Pdr-3 Transformation Protocol

At the same time as this run a control where the hyg linear construct isn’t added. No colonies should form on this plate (as it shouldn’t have hyg resistance).

When making the selection plates. Add hyg and g418 to agar that is <50 ˚C to prevent stability issues. 300µl of Hyg in 50ml media and 167µl of g418 in 50ml media.

* 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 for 5 mins to denature to ssDNA. Mix 150µg (15µl) salmon sperm DNA with 200-500ng transforming DNA (1-2µl of pdr3-HYG cassette stock, try 4µl). 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 (4000rpm, 3 mins) and resuspend in 1ml YPD media. Leave on side overnight at room temperature\*.
* Spin down, resuspend in 250µl YPD and plate onto YPD+HYG+KAN plate.
* Incubate at 30˚C for two days.
* If colonies have formed on YPD + HYG + KAN plate streak out 4 colonies onto a new YPD + HYG + KAN 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 2 PCRs. Do them both in the same tube to see if the pcr has worked and if the transformation has worked.
  + The test with a downstream reverse primer and mid cassette forward. If the cassette isn’t there this will fail.
  + A positive control with 2 primers that amplify a 300 bp section of the genome (mif2-ORF-F/mif2-DSS-R).

\* - If the transformation is left on the bench overnight this allows the transformed cells to start expressing Hyg resistance and decreases competition from non transformed cells, due to sub optimal growth conditions, thereby decreasing the chance of transformed cells being outcompeted.

**Fast yeast genomic DNA extraction without phenol**

* Pick one yeast colony from plate or spin down 100-200 μl of liquid yeast culture. Resuspend cells in 100 μl of 200mM LiOAc, 1%SDS solution.
* Incubate for 5 minutes at 70 °C.
* Add 300 μl of 96-100 % ethanol and vortex.
* Spin down DNA and cell debris at 13000 rpm for 3 minutes. Remove supernatant being carefully not to disturb the white pellet.
* Wash pellet with 500 μl of 70% ethanol and spin down at 13000 rpm for 1 minute
* Remove ethanol as much as you can without disturbing the white pellet. Dissolve pellet in 100 μl of H2O or TE and spin down at 13000 rpm for 30 seconds to pellet cell debris. If you want to use the DNA for *E.coli* transformation dissolve pellet in H2O.
* Use 1 μl of supernatant for PCR.

**Reference: Looke *et al.*, 2011. Extraction of genomic DNA from yeast for PCR-based applications. Biotecniques 50 (5), 325-328**

**DNA Concentration Check with Nanodrop**

* Use 1 μl of supernatant for Nanodrop.
* Blank with 1µl of elution buffer twice.

**PCR DreamTaq Green (remember fresh 10µM primer stocks)**

* 2.5µl F primer
* 2.5µl R primer
* 1µl of DNA extraction supernatant
* 25µl DreamTaq green
* 19µl nuclease free water (or milli-q)

|  |  |  |  |
| --- | --- | --- | --- |
|  | temp | time | no. cycles |
| Initial denaturation | 95 | 3 | 1 |
| Denaturation | 95 | 30s | 30 |
| Annealing | 55 | 30s | 30 |
| Extension | 72 | 1 (1 min extra every 1kb above 2kb) | 30 |
| Final Extension | 72 | 5-15 | 1 |

**Gel Electrophoresis**

* Make Gel
  + 50ml TAE x1 in gel only conical flask
  + Add 0.5g agarose (electrophoresis grade)
  + microwave until it boils (40s)
  + swirl then boil again
  + cool under tap
  + add gel red (5µl) wear gloves and be careful
  + pour into cassette and push bubbles to the side
  + Leave to set for 20 mins
* 30 mins, 100V.
* Make sure gel in the correct orientation so the DNA runs towards the anode (red).

PCR Protocol

Spin down primers before adding water to ensure solid isn’t in cap. Dilute to 100µM stock.

* 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.