Making Competent Cells

Need: 1-50ml Falcon tube of $CaCl_2$
5-1.5 ml minicentrifuge tubes
2-15 ml Falcon tube

Night Before

Prepare Stock of DH5 α cells

1. Add 5ml of LB broth into 15ml Falcon tube
2. Add loop of cells from plate
3. Incubate in shaking incubator at 37°C O/N

Next Day

Add 100 µl DH5 α mixture + 9.9ml LB broth in 15 ml Falcon tube

↓ 3-4 hours in shaking incubator

[Add 500 µl of vial into 1.7 ml minicentrifuge tubes] x 5

↓ centrifuge at 10 000 RPM for 5 min
- if no pellet at base raise RPM or add more culture

Remove Supernatant x 5

↓

[Add 500 µl $CaCl_2$ - resospend solution] x 5 → 15min on ice

↓ centrifuge at 10 000 RPM for 5 min

Remove supernatant x 5

↓

[Add 100 µl $CaCl_2$ - resuspend solution] x 5

↓

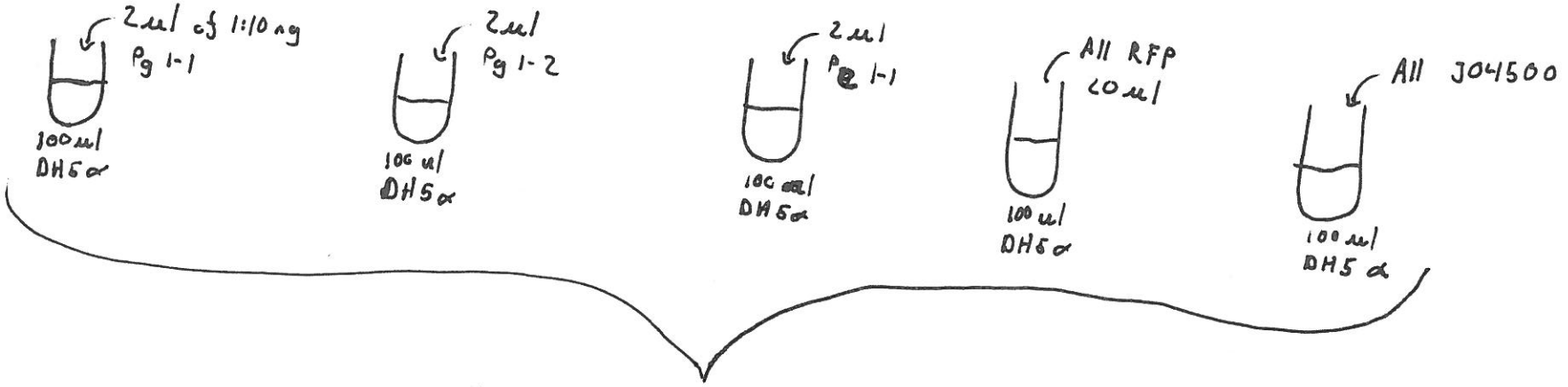
30 min on ice → add DNA within this time

Result 5 minicentrifuge tubes containing 100 µl competent DH5 α cells

Transformation

① Made 100ng/mL original stock
② Diluted 1:10

Need: 5 - 1.7 mL vials competant DH5α cells (100 µL) ON ICE + 3 vials chitinase enzymes made according to instructions ON ICE + RFP (J04460) ON ICE + J04500 ON ICE



Heat Shock in floater at 42°C for 60 seconds
↓
Ice in floater 5min
↓
Add 500 µl per tube LB broth
↓
Incubate at 37°C for 60 min
↓
Spin down for 30 secands at 10 000 RPM
↓
Remove supernatant
↓
Resuspend in 200 µl LB broth
↓
Plate 200 µl of each vial in appropriate antibiotic plate ⇒ Incubate at 37°C O/N

J04500/J04450 - pSB1C3
- chlor resistant

Chi enzymes - pUC57
- amp resistant

Mini Prep

1-2 days before Mini Prep

Select single colony from freshly streaked transformed plate using inoculating loop

↓

add to 10-15 mL LB media
+
5 µL Amp in 50 mL Falcon tube

↓

Incubate 12-16 hours at 37°C in Shaking incubator

↓

Prepare Reagents

↓

1 mL of culture to 1.7 mL minicentrifuge tube

↓

Spin at 8000 rpm for 1 min

↓

Decant

↓

Add 1 more mL of culture to 1.7 mL centrifuge tube

↓

Spin at 8000 rpm for 10 min

↓

Remove supernatant in beaker with bleach

thoroughly resuspend cells in 500 µL RNase A

↑

Add 400 µL solution II (lysis buffer)

↑

invert + rotate tube several times (get clear lysate)

↑

Add 600 µL solution III (neutralization buffer) invert until white precipitate forms

↓

Spin at 14000 rpm for 10 min. Want to see pellet debris.

↓

Insert HiBind DNA mini Column into 2 mL Collection tube (label all tubes)

↓

Add 700 µL clear lysate from spun tube aspirating (swirling around edge of mini column) into HiBind DNA mini column. Leave pellet undisturbed

↓

Spin at 14000 rpm for 1 min

↓

Pull out mini column tubes, discard liquid, put column tubes back into collection tubes.

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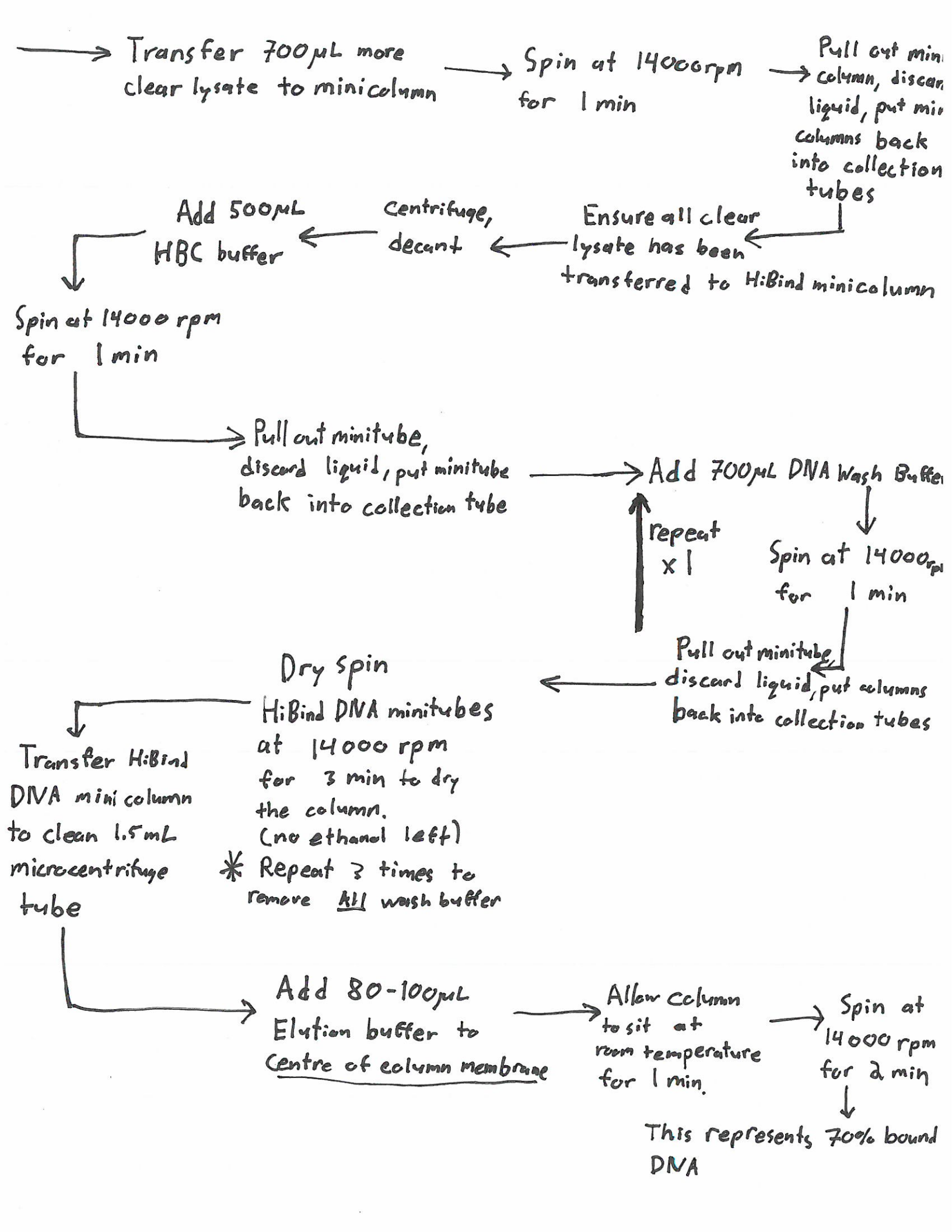
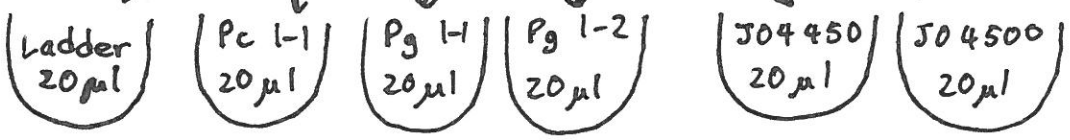

Transfer 700µL more clear lysate to minicolumn
Spin at 14000rpm for 1 min
Pull out min column, discar liquid, put mi columns back into collection tubes
Ensure all clear lysate has been transferred to HiBind minicolumn
Centrifuge, decant
Add 500µL HBC buffer
Spin at 14000 rpm for 1 min
Pull out minitube, discard liquid, put minitube back into collection tube
Add 700µL DNA Wash Buffe
repeat x 1
Spin at 14000 rpm for 1 min
Pull out minitube, discard liquid, put columns back into collection tubes
Dry Spin
HiBind DNA minitubes at 14000 rpm for 3 min to dry the column. (no ethanol left)
* Repeat 3 times to remove All wash buffer
Transfer HiBind DNA mini column to clean 1.5mL microcentrifuge tube
Add 80-100µL Elution buffer to Centre of column membrane
Allow column to sit at room temperature for 1 min.
Spin at 14000 rpm for 2 min
This represents 70% bound DNA

Gel Electrophoresis

Make gel agarose
- 1g agarose in 100 mL TAE
- Microwave 30s × 2 + 3rd time for 10s
  ↳ saran wrap top with a hole in the top
- Wait until cool enough to touch for 3s
- Add 5µl SYBR green
- Swirl until colour is fully dissolved
- Pour into mould (covered by masking tape)
- Insert comb at end slot

- After gel is solidified (~30 mins), remove comb & tape
- Place into gel electrophoresis machine
- Fill with TAE until top of gel is covered

4µl loading dye into tubes



- Pipette all into each well (spin down first if necessary
- 125 V for 40 min, checking every 10 min
- Ladder → compared to ~3700 bp for chitinases + PUC 57
  ~3100 bp for J04500 + pSB1C3
  ~2200 bp for J04450 + pSB1C3

Restriction Digest

In 1.7mL centrifuge tube, add:

30.5µl dd H_2O
5µl buffer cutsmart
12.5µl chitinase inserts
1µl Spe I
1µl Pst I

↙ ↓ ↘

Pc 1-1 Pg 1-1 Pg 1-2

In 1.7mL centrifuge tube, add:

30.5µl dd H_2O
50µl buffer cutsmart
12.5µl J04... vectors

J04450	J04500
1µl Xbal	1µl Spe I
1µl Pst I	1µl Pst I

↙ ↘

J04460 J04500

↘ ↙

Pipette gently 5x

↓

Centrifuge @ max for 10s

↓

Incubate @ 37°C for 1 hr

↙ ↓

remainder freeze @ -20°C for ligation

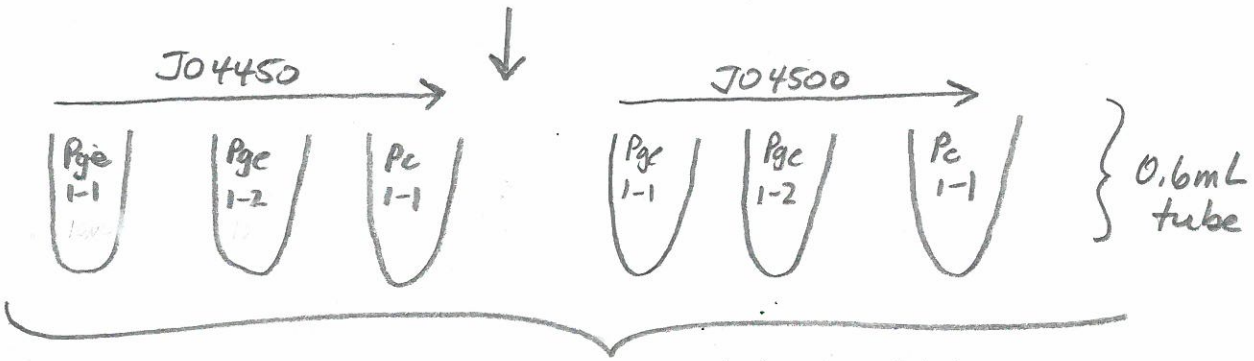
Gel electrophoresis to check if digest worked (want 2 bands - one of enzyme + one of backbone

Ligation

(F)

80°C for 20min
water bath to
deactivate restriction
enzymes

J04450 | J04500 | Pge1-1 | Pe1-1 | Pge1-2



in each 0.6 mL tube add:

2 µL vector (J04....)
6.5 µL insert (chitinases)
1 µL T4 DNA buffer
0.5 µL T4 DNA ligase
10 µL

pipette gently 3x

spin @ max for 5s

RT 10 min

4 µL for Transformation

rest stored @ -20°C

Transform

4 µL → 100 µL DH5α comp

4 µL → 100 µL DH5α comp

4 µL → 100 µL DH5α comp

4 µL → 100 µL DH5α comp

4 µL → 100 µL DH5α comp

4 µL → 100 µL DH5α competent cells

from ligation

leave to grow for 24 hours.