

PLAN 1.0

Wednesday 18th

- Induce competence in 390 cells ✓
- Transform 390 cells with 379 plasmid ✓
- Make more 10% glycerol
- Autoclave 2 more 500ml flasks
- Plating the transformed cells (Kan, Chior, Spec plates) ✓

17

Thursday 19th

- Plate 21 cells if not already plated

Friday 20th

- Remove 21 plates from incubator and place in cold storage
↳ ask Sophie if this is ok, if not do not plate 21 cells Thursday.
- Collect DNA delivery (carbapenemase + gRNA)

Monday 23rd

- Make 21 + liquid culture (afternoon)
- PCR the 390 plasmid
- Gibson assembly the carbapenemase gene into the 390 plasmid

24

Tuesday 24th

- Induce competence in 21 cells
- Transform 21 cells with assembled plasmids
- Plate ~~21~~ ^{WD40} transformed 21 cells (WD40 cells!)

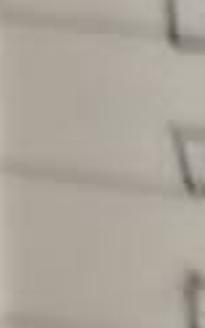
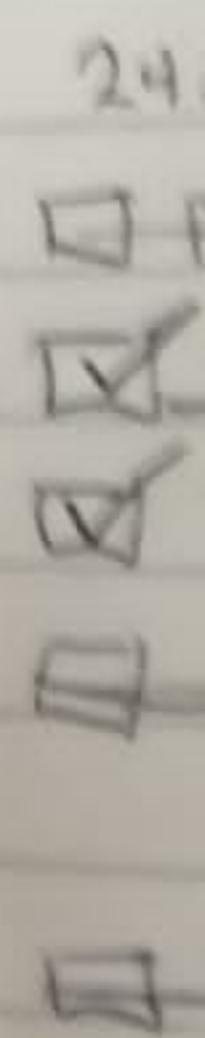
Wednesday 25th

- 25
- PCR the 379 plasmid
 - Goldengate assembly of gRNA into 379 plasmid
 - ~~Transform WD40 cells assembled p~~ - Prepare WD40 liquid culture

Thursday 26th

- Induce competence in WD40 cells
- Transform WD40 cells with assembled plasmid from Goldengate
- Plate resulting cells

Friday
Fluo



Friday 24/8

fluorescence experiment of new transformed LWD40 cells

STORES:

MORE LOOPS - green ✓

Wednesday 25/8

- Plate competent cells on C/S plates → need to make C/S plates x2 ✓
- Transform new aliquot with PAJ - 390
- Plate transformed cells
- Incubate both plates overnight
- Prepare ~~2~~ liquid culture for safety (SAS 351 from plate) for safety

→ Autoclave 2 flasks (500ml)

STORES:

- L-shaped spreader things
- More plates

24/8

- Prepare electrocompetent cells
- Autoclave 2 150ml & 500ml flasks } and autoclave aliquots eppendorfs after all day ✓
- Prep 10% glycerol
- Transform cells
- OR Prep liquid culture
- Prepare liquid culture (afternoon only) 25/8
- Prepare electrocompetent cells
- Transform cells
- Plate cells

200ml LBA

35 µg/ml of Chl → 7000 µg of Chl → 7mg

10 µg/ml of Spec → 2000 µg Spec → 2mg

50 µg/ml of Kan →

~~50 35 mg/ml = 285000 mg/ml~~

50mg/ml

1ml = 50mg

x ml = 2mg

$$x = \frac{2}{50} = \frac{1}{25} = 0.04 \text{ ml}$$

40 µl

$$10 \times 250 = 250 \text{ mg}$$

0.25mg

200 µl

$$x = 0.25 \text{ mg}$$

↓

$$x = \frac{0.25}{50} = 0.005 \text{ ml} \quad 20 \mu\text{l per plate}$$

↓ if 20 µl → 25 µl = 5 µl
4 µl per plate

5µl

25 µl of Chl
5 µl of Spec
25 µl of Kan

per 25ml

× 2 as we are prepping 2 plates
at once!

~~300 µl~~

15ml LBA

50 µg/ml of Kan → 750 µg Kan = 0.75 mg

1ml ... 50 mg Kan

x ml ... 0.75 mg

$$x = \frac{0.75}{500} = \frac{1,5}{1000} = 0.0015 \text{ ml} = 1.5 \mu\text{l}$$

100ml of Chl
20ml of Spec

per flask

for inducing competence

Preparing 2 plates with antibiotic (100 ml plates)

1. Wipe down materials and fume hood with ethanol before use
2. Add 50 ml LBA (25 ml per plate) into Falcon tube 10
3. Add ~~2~~ ~~3~~ ~~4~~ ~~5~~ ~~6~~ ~~7~~ ~~8~~ ~~9~~ ~~10~~ ~~11~~ ~~12~~ ~~13~~ ~~14~~ ~~15~~ ~~16~~ ~~17~~ ~~18~~ ~~19~~ ~~20~~ ~~21~~ ~~22~~ ~~23~~ ~~24~~ ~~25~~ ~~26~~ ~~27~~ ~~28~~ ~~29~~ ~~30~~ ~~31~~ ~~32~~ ~~33~~ ~~34~~ ~~35~~ ~~36~~ ~~37~~ ~~38~~ ~~39~~ ~~40~~ ~~41~~ ~~42~~ ~~43~~ ~~44~~ ~~45~~ ~~46~~ ~~47~~ ~~48~~ ~~49~~ ~~50~~ ~~51~~ ~~52~~ ~~53~~ ~~54~~ ~~55~~ ~~56~~ ~~57~~ ~~58~~ ~~59~~ ~~60~~ ~~61~~ ~~62~~ ~~63~~ ~~64~~ ~~65~~ ~~66~~ ~~67~~ ~~68~~ ~~69~~ ~~70~~ ~~71~~ ~~72~~ ~~73~~ ~~74~~ ~~75~~ ~~76~~ ~~77~~ ~~78~~ ~~79~~ ~~80~~ ~~81~~ ~~82~~ ~~83~~ ~~84~~ ~~85~~ ~~86~~ ~~87~~ ~~88~~ ~~89~~ ~~90~~ ~~91~~ ~~92~~ ~~93~~ ~~94~~ ~~95~~ ~~96~~ ~~97~~ ~~98~~ ~~99~~ ~~100~~ ~~101~~ ~~102~~ ~~103~~ ~~104~~ ~~105~~ ~~106~~ ~~107~~ ~~108~~ ~~109~~ ~~110~~ ~~111~~ ~~112~~ ~~113~~ ~~114~~ ~~115~~ ~~116~~ ~~117~~ ~~118~~ ~~119~~ ~~120~~ ~~121~~ ~~122~~ ~~123~~ ~~124~~ ~~125~~ ~~126~~ ~~127~~ ~~128~~ ~~129~~ ~~130~~ ~~131~~ ~~132~~ ~~133~~ ~~134~~ ~~135~~ ~~136~~ ~~137~~ ~~138~~ 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Preference and without money concern (see also above)

1. Break the ice to follow line
 2. Make a small circle going along this line and (Wagon Circle, 3rd 10) then
in this line a circle, with your stick a long
 3. Make a line
 4. Place with the diamond

W. 2000 to 2000 m.s.n.m. (Year) 1000 years old tree with basal diameter 1.2 m. (Year)

Right now, here's the instructions for the while:

If you haven't got your pure Z1 cells yet, then ask Satya.

^you need Pure MG1655 Z1 cells [just spec resistant]

if you have got your pure Z1 cells on Monday and are waiting for them to grow, then you can skip to step 3, and once your DNA is ready, you can freeze at -20C until your cells are ready for transformation.

Prepare your version of the 390 Plasmid, and make your WD40 cells:

1. Get your **pure Z1** cells (they grow on just Spec plates)
2. Make the Z1 cells competent (you shouldn't need many)
3. PCR The 390 plasmid with the forward and reverse primers.
4. Take the (now) linearized 390 plasmid and Gibson Assembly the 390-insert part [containing your carbapenemase] into the 390 plasmid
5. Transform the pure Z1 competent cells with your carbapenemase assembled 390 plasmid
6. Grow up for an hour, then plate [chlor and spec]
7. These are your WD40 cells. Make as many competent cells out of this (in one batch) as you can, the more the merrier - but don't stress yourself, we can always make more in future.

^label these WD40

At this stage you have your competent WD40 cells, ready to accept gRNAs for testing

Now PCR your 379 plasmid (the GFP one), perform GoldenGate assembly with BSAI type IIIs restriction enzyme [the one basically everyone uses for GG] and transform them into your WD40 cells -> plate them

After that you can conduct the Fluorescence experiment detailed previously with the PAJ cell line [to help debug] and your new cells. Do them in the same plate so we can plot them on the same graph.

There should be protocols on that 2018 document, and I'm sure the PhD's helping in the lab are all very familiar with all of the protocols above.

Here's a link to **Gibson [390, Carbapenemase, dCAS9, Chlor]**:

<https://international.neb.com/protocols/2012/09/25/gibson-assembly-master-mix-assembly>

Here's a link to **Goldengate [379, gRNA, GFP, Kan]**

<https://international.neb.com/protocols/2018/10/02/golden-gate-assembly-protocol-for-using-neb-golden-gate-assembly-mix-e1601>

If the kits in Chris' lab have instructions with them then they supersede my instructions

*Remember: the Goldengate enzyme **MUST be BsaI** [to cut GGTCTC]*

When making glycerol, sterilise after making the 10% solution

Always use sterile eppendorfs when working with live cells (e.g. when inducing competence). Ideally use sterile ones when transforming but this is not strictly necessary. Sterile eppendorfs are not needed when working with DNA only (e.g. minipreps)

Time	1mL ABS	500μL ABS	1=blank, 2=1mL, 3=500μL
2hrs	0.158	0.177	
3hrs	0.537	0.501	
2hrs	0.091	0.056	
3hrs	0.36	0.328	
3.5hrs	0.525	0.440	

26/8

Miniprep 379 plasmid

Transform SAJ-351 competent cells with 379 plasmid

Plate

Prep plates ($\frac{1}{2} \times$ Chlor/spec, $\frac{1}{2} \times$ Chlor/spec/kan)

Transform SAJ-351 with a different plasmid as a control
↓

If this doesn't work the issue is the cells

Transform SAJ-351 with a different plasmid

Transform different cells with the 379 plasmid

green - cutsmart

red - 3.1

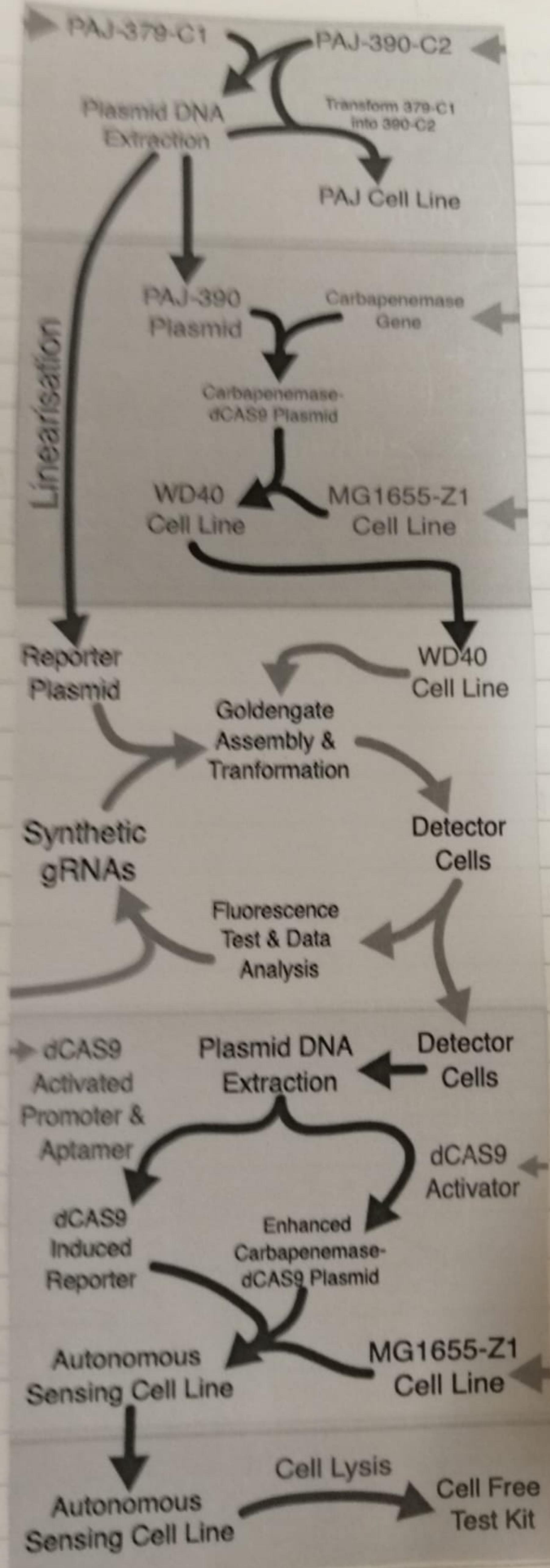
yellow - 1.1

add purple ↗

0x concentration

AUGUST 26th
2021

Version 1



reaction volume

- a 50μL reaction volume is recommended for digestion of 1μg of substrate

25μL rxn 5 units of RE 0.5μg of DNA, ^{25μL} 10X NEBuffer

To stop the reaction

- terminate with stop solution, 5μL per 25μL rxn

Protocol:

1. add DNA ~ 0.5 μg
2. add 10X NEBuffer ~ 2.5 μL (1x concentration)
3. ~~add~~ add Restriction Enzyme, 5 units, 0.5 μL
4. mix components by pipetting reaction up & down or by "flicking"
5. quick spin-down in a microcentrifuge (do not vortex)
6. incubate for an hour at appropriate temperature

↳ NEB at 37°C, check this

↳ NEB website
says 37°C

- done in epitubes? PCR tubes
- what is a quick-spin-down?
- 5 units x 0.5 μL? No

5μL on gel

1μL of dye

↓
Yes,
must be
10X

- NEBuffer, actually relates to buffer in the color?

- you said to add the purple dye, when would this be?

500ng of DNA

↳ 11.8 μL of DNA

so 12 μL of DNA add water up to 24 μL

↳ 18.65 μL of DNA 1% gel ~ 19 of Agarose up to 200 μL

so 19 μL of DNA

42.4 ng/μL - 371

26.8 ng/μL - 390

12 μL of DNA 2.5 μL of Buffer 0.5 μL of RE → 10 μL of H₂O

19 μL of DNA 2.5 μL of Buffer 0.5 μL of RE → 4 μL of H₂O

10 μL + 2 μL Dye

1 μL of Ladder

01/09/21

- liquid culture ~ if transformation works B transformation
- miniprep ~ if transformation doesn't work only one place
- chromatography

02/09/21

- fluorescence test ~ if old transformation works
- run digest miniprep new DNA, transform with old DNA

Se miniprep 26.5

$\Sigma V_d = 40.7$

$\Sigma A_d = 242.4$

$$\frac{C_1 V_1}{V} = \frac{C_2 V_2}{V}$$

↓ ↓
0.01 Mg/ml

06/09/21

- Replate transformed cells

07/09/21

- Make liquid culture

08/09/21 → 10/09/21

- Run fluorescence experiment

1M TRIS → $\frac{10}{1000} \text{ mM}$

6

/100 ml

0.5M EDTA → 0.1mM EDTA

/500

$$CV = CV$$

$$1M \cdot V = 10\text{mM} \cdot 5\text{mL}$$

$$1000\text{mM} \cdot V = 50$$

$$V = \frac{50}{1000} = 0.05\text{mL}$$
$$= 50\mu\text{L}$$

$$0.5M \cdot V = 0.1\text{mM} \cdot 5\text{mL}$$

$$500\text{mM} \cdot V = 0.5$$

$$V = \frac{0.5}{500} = \frac{1}{1000} = 0.001\text{mL}$$

$$= 1\mu\text{L EDTA}$$

Sept. 10th : Fluorescence test

Reanna		Chlorophenicol		ON TEST PAJ + ATC		Blank = LB IPTG [] = 100 μM ATC [] = 10 ng/mL
	+ control	- control	OFF TEST PAJ			
BLANK	SAJ-351 + IPTG	SAJ-351	PAJ			
Blank IPTG	+ control	- control	OFF TEST PAJ	ON TEST		990 μL
	SAJ-351 + IPTG	SAJ-351	PAT		PAJ + ATC	10 μL
990 μL 10 μL ↓ final [] of 10 ng/mL	Blank ATC	+ control SAJ-351 + IPTG	- control SAJ-351	OFF TEST PAJ	ON TEST PAJ + ATC	

Dilution []

$$3 \text{ mg of ATC} + 1 \text{ mL ethanol} = 3 \text{ mg/mL}$$

1 mg/mL of ATC

$$\frac{3 \text{ mg/mL}}{(1 \mu\text{g/mL})} \cdot 0.5 \text{ mL} = 1 \text{ mg/mL} \cdot x$$

$$1 \text{ mg/mL} \cdot 0.001 \text{ mL} = 0.001 \text{ mg/mL} \cdot x$$

$$x = 1 \text{ mL}$$

0.999 mL added of ethanol

(4000 g/mL)

$$\frac{3}{2} = 1 \cdot x$$

x = ~~1.5 mL~~ of ethanol
total volume

So add 1 mL of ethanol

31/08/21

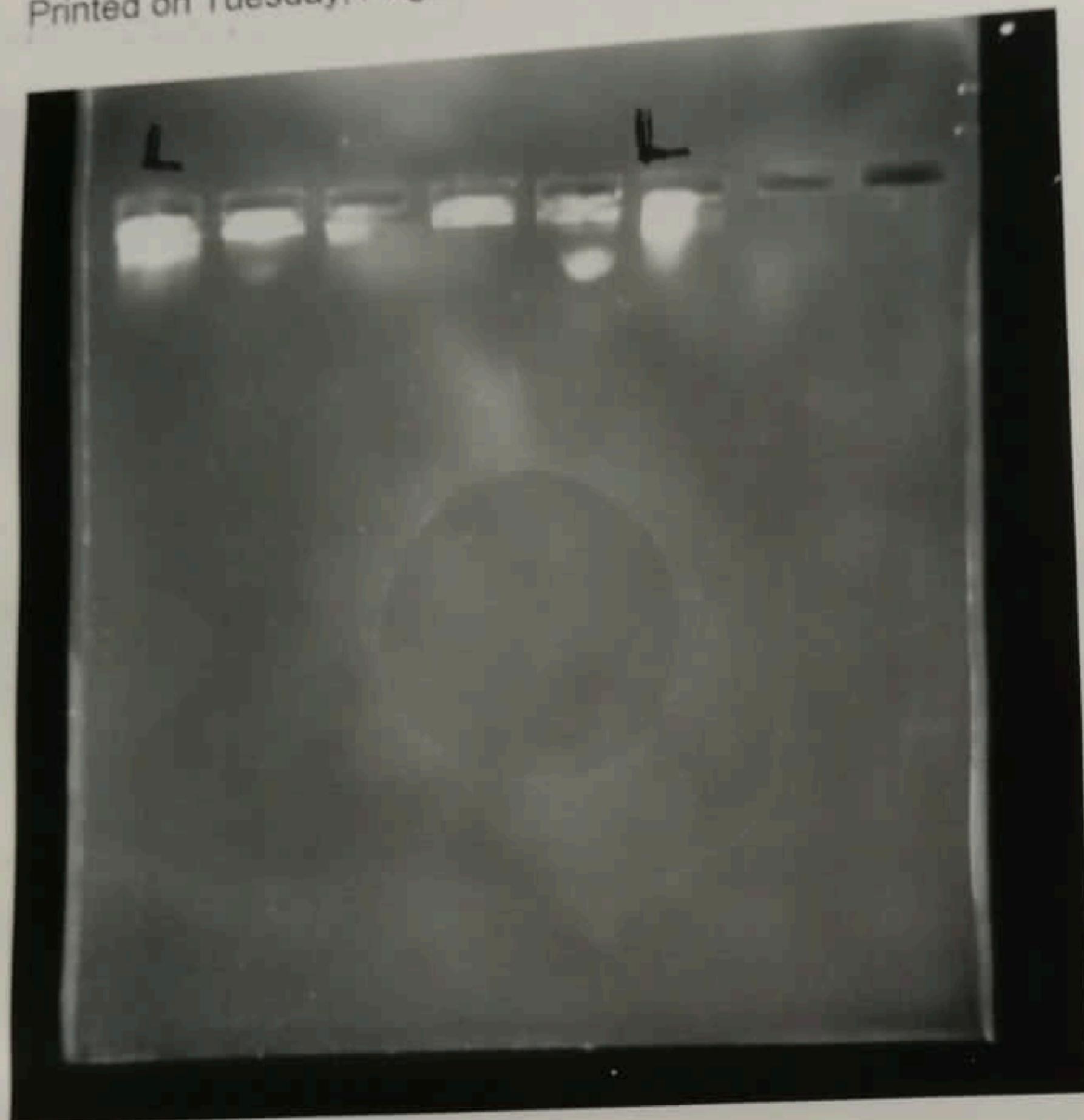
☐ transform

X run digest

☐ plate

100V for 40 mins

Printed on Tuesday, August 31, 2021 15:40:17 (PM)



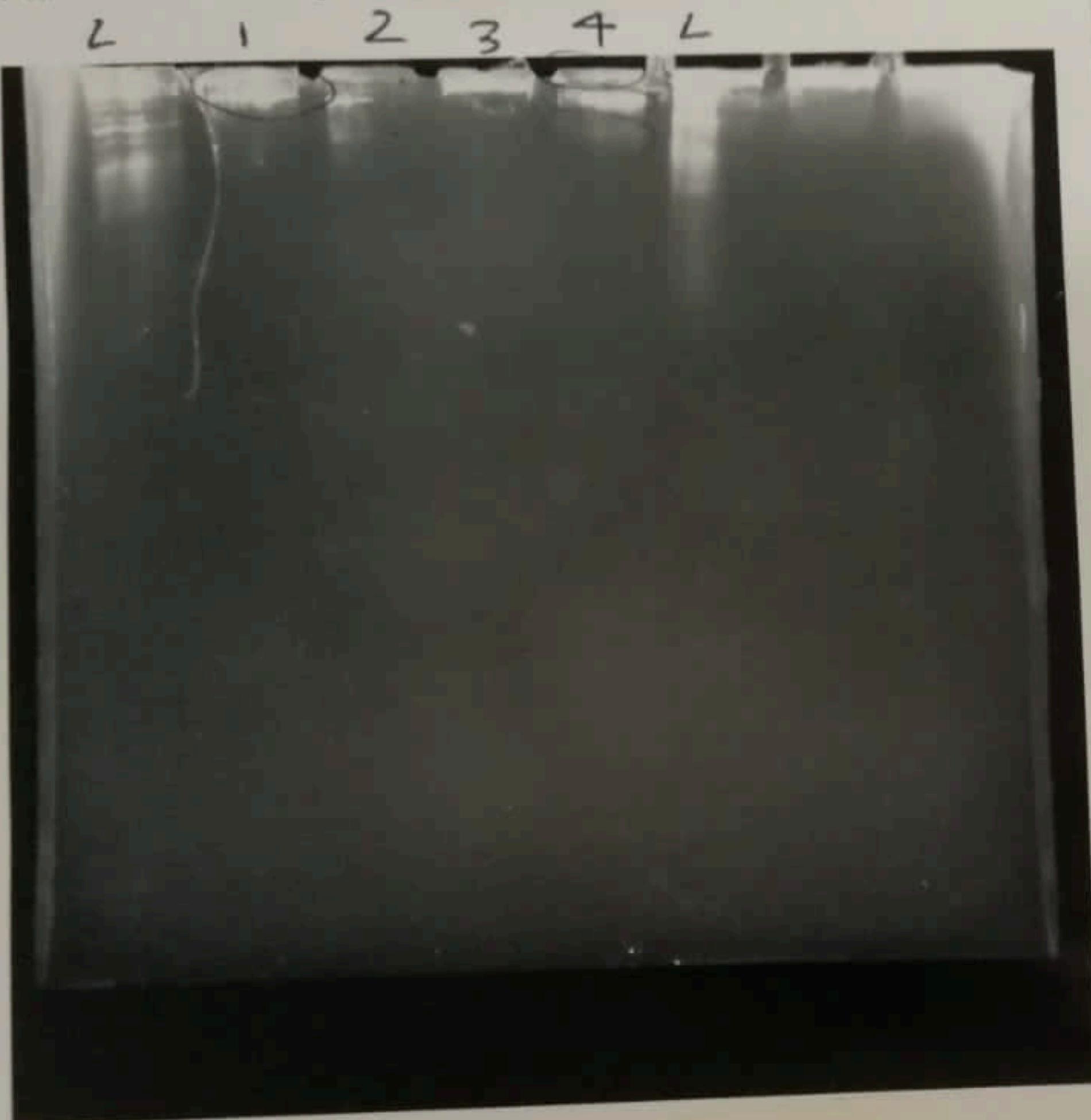
We reran the digest,

* note to self make sure to inspect for cracks, the wells must be covered with TAE in the entire time

after another 40 mins, still no ladder, we put it on for another 20 mins

100V for 100mins

Printed on Tuesday, August 31, 2021 17:01:50 (PM)



1. nL
looks like it could have worked, but not totally convinced!

→ make liquid culture to miniprep tmrw (01/09)
& will transform using this DNA just to see

the integrity of the gel was weak & it broke easily

PURIFICATION PROTOCOLS

Note

- Read IMPORTANT NOTES on p. 3 before starting.
- All purification steps should be carried out at room temperature.
- All centrifugations should be carried out in a table-top microcentrifuge at $>12000 \times g$ (10 000-14 000 rpm, depending on the rotor type).

Protocol A. DNA extraction from the gel using centrifuge

Step	Procedure
1	<p>Excise gel slice containing the DNA fragment using a clean scalpel or razor blade. Cut as close to the DNA as possible to minimize the gel volume. Place the gel slice into a pre-weighed 1.5 mL tube and weigh. Record the weight of the gel slice.</p> <p>Note. If the purified fragment will be used for cloning reactions, avoid damaging the DNA through UV light exposure. Minimize UV exposure to a few seconds or keep the gel slice on a glass or plastic plate during UV illumination.</p>
2	<p>Add 1:1 volume of Binding Buffer to the gel slice (volume: weight) (e.g., add 100 μL of Binding Buffer for every 100 mg of agarose gel).</p> <p>Note. For gels with an agarose content greater than 2%, add 2:1 volumes of Binding Buffer to the gel slice.</p>
3	<p>Incubate the gel mixture at 50-60 °C for 10 min or until the gel slice is completely dissolved. Mix the tube by inversion every 3 minutes to facilitate the melting process. Ensure that the gel is completely dissolved. Vortex the gel mixture briefly before loading on the column.</p> <p>Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 μL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.</p>
4 for ≤500 bp and >10 kb DNA fragments	<p>Optional: use this step only when DNA fragment is ≤500 bp or >10 kb long.</p> <ul style="list-style-type: none"> • If the DNA fragment is ≤500 bp, add 1 gel volume of 100% isopropanol to the solubilized gel solution (e.g. 100 μL of isopropanol should be added to 100 mg gel slice solubilized in 100 μL of Binding Buffer). Mix thoroughly. • If the DNA fragment is >10 kb, add 1 gel volume of water to the solubilized gel solution (e.g. 100 μL of water should be added to 100 mg gel slice solubilized in 100 μL of Binding Buffer). Mix thoroughly.
5	<p>Transfer up to 800 μL of the solubilized gel solution (from step 3 or 4) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.</p> <p>Note.</p> <ul style="list-style-type: none"> • If the total volume exceeds 800 μL, the solution can be added to the column in stages. After each application, centrifuge the column for 30-60 s and discard the flow-through after each spin. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1 g of total agarose gel per column. • Close the bag with GeneJET Purification Columns tightly after each use!

Step	Procedure
6	<p><i>Optional:</i> use this additional binding step only if the purified DNA will be used for sequencing.</p> <p>Add 100 μL of Binding Buffer to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.</p>
7	Add 700 μL of Wash Buffer (diluted with ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 1 min. Discard the flow-through and place the column back into the same collection tube.
8	<p>Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove residual wash buffer.</p> <p>Note. This step is essential to avoid residual ethanol in the purified DNA solution. The presence of ethanol in the DNA sample may inhibit downstream enzymatic reactions.</p>
9	<p>Transfer the GeneJET purification column into a clean 1.5 mL microcentrifuge tube (not included). Add 50 μL of Elution Buffer to the center of the purification column membrane. Centrifuge for 1 min.</p> <p>Note.</p> <ul style="list-style-type: none"> For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 μL does not significantly reduce the DNA yield. However, elution volumes less than 10 μL are not recommended. If DNA fragment is >10 kb, prewarm Elution Buffer to 65 °C before applying to column. If the elution volume is 10 μL and DNA amount is ≤ 5 μg, incubate column for 1 min at room temperature before centrifugation.
10	Discard the GeneJET purification column and store the purified DNA at -20 °C.

Protocol B. DNA extraction from the gel using vacuum manifolds

Step	Procedure
1	Excise a gel slice containing DNA fragment and completely dissolve it according to steps 1 - 4 in Protocol A on page 4. Do not exceed 1 g of total agarose gel per column.
2	Prepare the vacuum manifold according to the supplier's instructions. Place the GeneJET purification column(s) onto the manifold.
3	<p>Transfer up to 800 μL of the solubilized gel solution (from step 3 or 4 as in Protocol A) to the GeneJET purification column.</p> <p>Note. Close the bag with GeneJET Spin Columns tightly after each use!</p> <p>Note. If the total volume of solubilized gel solution exceeds 800 μL, the solution can be added to the column in stages. After each application, apply the vacuum and discard the flow-through. Repeat until the entire volume has been applied to the column membrane. Do not exceed 1 g of total agarose gel per column.</p>

100 μ L 50 μ L Fluorometer plate

	LB
① Control	100 μ L
② 1/10 SAJ	900 μ L LB + 100 μ L control
1+ATC	$1 + ATC = 495 \mu L$ control + 5 μ L ATC
2+ATC	$2 + ATC = 495 \mu L$ $\frac{1}{10}$ control (2) + 5 μ L ATC
SAJ + ATC	
$\frac{1}{10}$ SAJ + ATC	
PAJ	
$\frac{1}{10}$ PAJ	
PAJ + ATC	
$\frac{1}{10}$ PAJ + ATC	

	LB
③ SAJ + ATC	100 μL LB + 100 μL plasmid
④ 1/10 plasmid	$900 \mu L$ water + 100 μ L plasmid
3+ATC	$3 + ATC = 495 \mu L$ plasmid + 5 μ L ATC
4+ATC	$4 + ATC = 500 \mu L$ $\rightarrow 495 \mu L$ $\frac{1}{10}$ plasmid + 5 μ L ATC

\rightarrow 500 μ L

IN TRIP

MO BIO
Laboratories, Inc.

SAVING YOU TIME FOR LIFE

LB 100 μ L

LB 50 μ L

① NEAT CONTROL 100 μ L \rightarrow 50 μ L

② 1/10 CONTROL 100 μ L \rightarrow 50 μ L

① + ATC

100 μ L 50 μ L

② + ATC

100 μ L 50 μ L

③ PLASMID

100 μ L 50 μ L

④ 1/10 PLASMID

100 μ L 50 μ L

⑤ ATC

" "

⑥ + ATC

" "

1st time around, no fluorescence

as maybe we are using too many antibiotics & selection pressure on E. coli is too much for effective GFP production

Temporary:

- Grow an overnight culture with antibiotics
- Tomorrow morning, transfer culture to new tube without antibiotics and incubate with ATC for a while

RUN
 ① M
 ② M
 ③ P
 ④ P
 ⑤ A
 ⑥ S
 ⑦ R
 ⑧ V

epp. wt = 0.94 g

use the large tube with 'molecular grade'
on the side

RUNNING A GEL

TAE

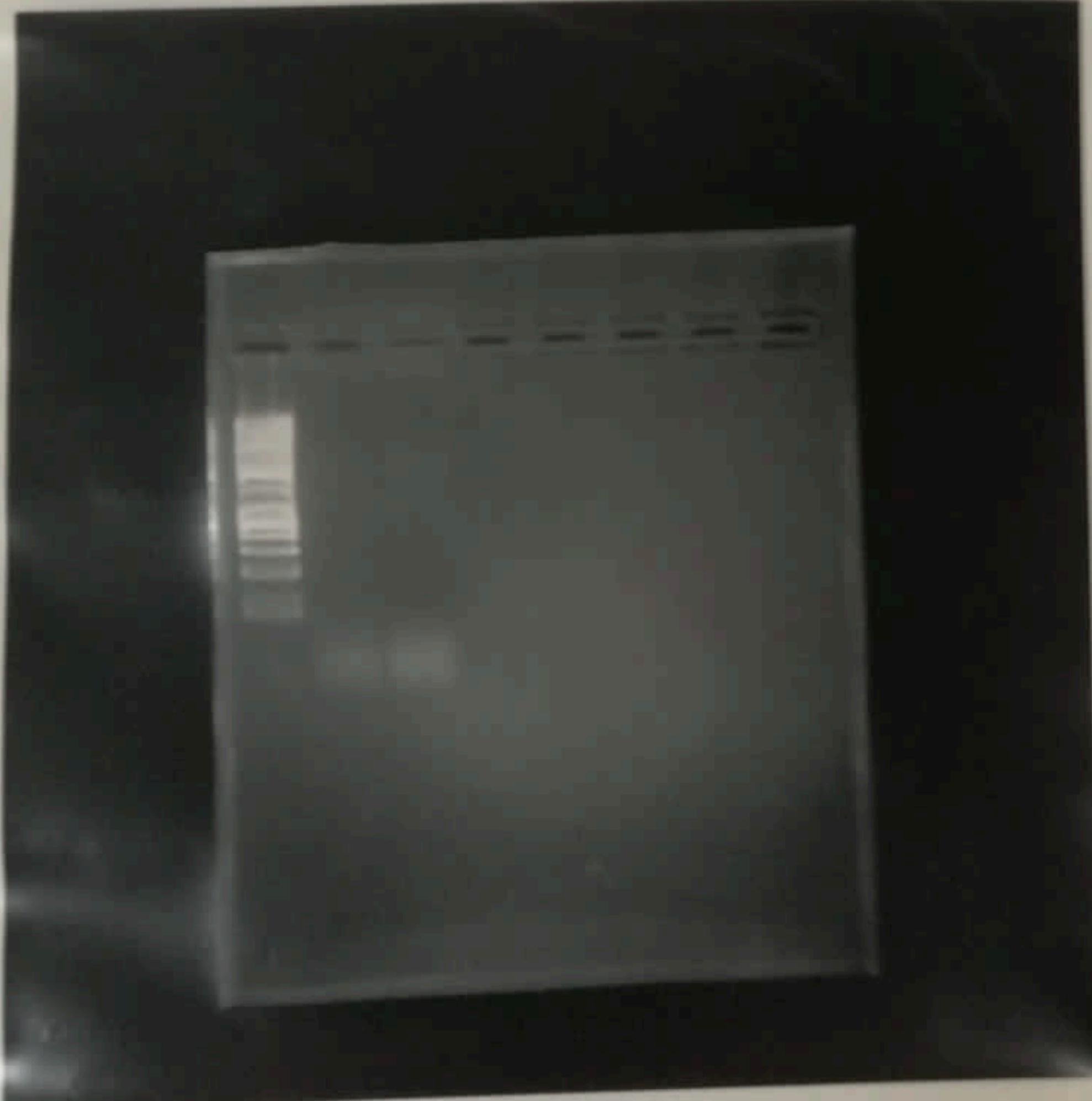
0.25 to 25ml

- ① Mix 1% agarose powder to distilled water (e.g. 1g per 100ml or ~~0.94g per 100ml~~)
(30s) → until clear/not cloudy *
- ② Microwave until and pour it into the mould. The gel will set around the comb, creating wells
- ③ Add DNA and move to the gel machine of an appropriate size
- ④ Add TAE until the wells are covered
* *
- ⑤ Add ladder and DNA then connect the electrodes (they are cross-connected)
- ⑥ Set voltage and time appropriately (asic if needed)
↓
15-20min for 25ml gels,
100V also
- ⑦ Run the gel
- ⑧ View under UV light

After microwaving,

* Then add 5μl loading dye per
100ml of gel
- aka 1.25μl for a 25ml gel

Printed on Tuesday, September 14, 2021 15:33:56 (PM)



Agarose gel showing the results of the PCR (unsuccessful, only a very faint band so it likely didn't work)

xx

Preparing a sample

- Pipette the desired amount of DNA and mix with the desired amount of loading dye
 - e.g. for PCR, 1 μl DNA and 1 μl loading dye
 - Pipette up and down to mix (or flick)

* use ice pack
eppendorf holder

13/09

1x [each PCR tube]

Q5 12.5 μl

H₂O 10.5 μl

primer 1 0.5 μl

2 0.5 μl

22.7 nmol
30.3 nmol
resuspend by flicking
not needed to
ensure DNA is not
damaged

- ① Resuspend primers to 100 μM (centrifuge first)
- ② Dilute primers to 10 μM (100 μl in an eppendorf) **
- ③ Make master mix then centrifuge quickly (3 seconds?)
- ④ 24 μl of master mix into each eppendorf (x4)
- ⑤ 1 μl of plasmid into each eppendorf
- ⑥ Centrifuge
- ⑦ Plug primer sequences into NEB Tm calculator
to get the PCR temperature
- ⑧ Thermocycler: * edit protocol, change step 3 to
specific primer temp, start.

PCR

[5x] master mix

Q5 62.5 μl

16

H₂O 52.5 μl

18

primer + 2.5 μl

2 2.5 μl

61°C

22.7 nmol

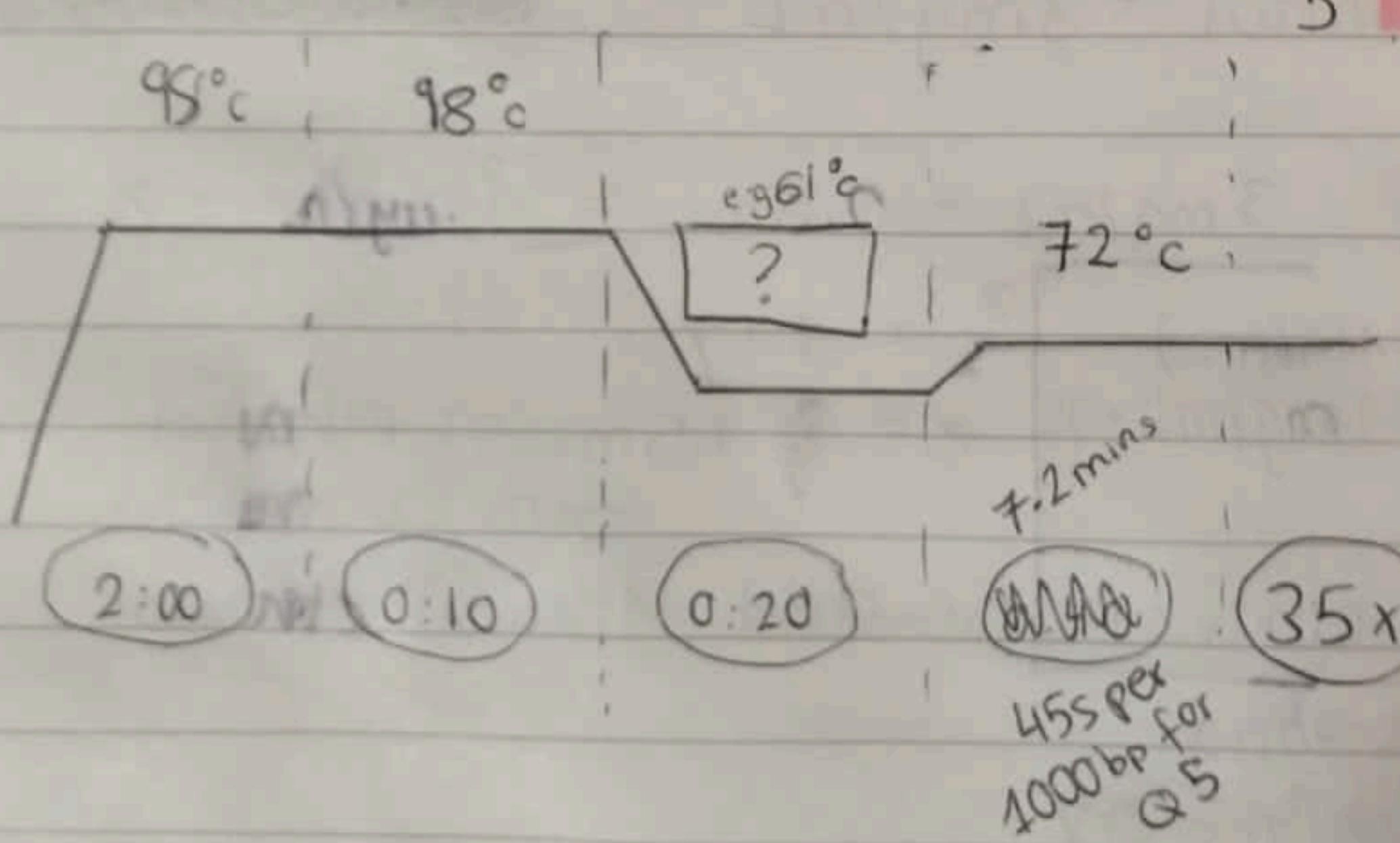
$$\frac{0.227 \text{ nmol}}{X} = \frac{100 \text{ nmol}}{L}$$

$$2.27 \times 10^{-4} L$$

$$\frac{0.227 \text{ nmol}}{227 \mu\text{l water}}$$

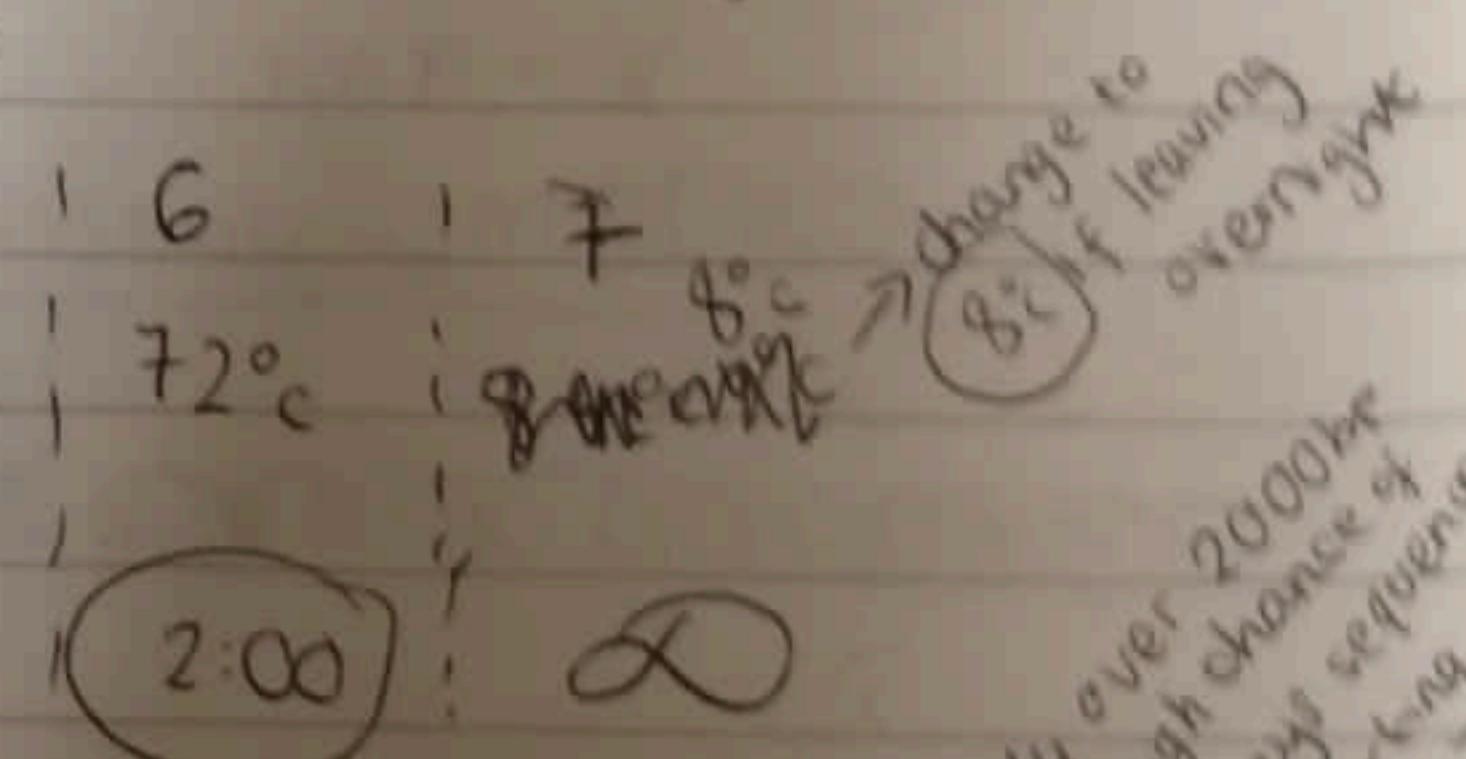
10μM = 10 μl primer, 90 μl water

* Saved protocols → Jingfan → 154155



After PCR, always run a gel to see whether or not it has worked.
Use 2 μl of DNA

** Make a larger stock if using these primers a lot
500 μl



generally over 2000x
gives a high chance of
error so always sequence
before inserting

14/09

Patrick

- run fluorescence test → fluorometer plates? 15.00 til morning
- Gibson Assembly → email PhDs (Jingfein, Sopida, Jack, Sophie)

GIBSON ASSEMBLY

① Nanodrop DNA to obtain its concentration (ng/ml)

② Calculate how much enzyme we need to add → NEB clonase (digestion → $DpnI$)

$$DNA = 1\text{ }\mu\text{g} \longrightarrow 20\text{ }\mu\text{l}$$

$$10x rQuesSmart Buffer = 5\text{ }\mu\text{l} \longrightarrow 5\text{ }\mu\text{l}$$

$$DpnI = 1\text{-}\mu\text{l (20 units)} \longrightarrow 20\text{ }\mu\text{l} 0.5\text{ }\mu\text{l}$$

$$\text{Nuclease free water to } 50\text{ }\mu\text{l} \longrightarrow 24.5\text{ }\mu\text{l}$$

Incubate

$$0.2 = 1\text{ ng/ml}$$

1.78

$\frac{2\text{ }\mu\text{g DNA}}{0.2\text{ }\mu\text{g insert}}$ of each
4

$$10 \times x = 0.2\text{ }\mu\text{g} \times 20\text{ }\mu\text{l}$$

~~= 2000 200~~
~~= 0.0002 ng~~
~~\times 200~~

~~204~~

③ Incubate at 37°C for 45-60 minutes → use the plate incubator

↳ This is the master mix

④ Dilute to 10 mM TRIS and ~~500~~ mM EDTA to make ~~TE~~ 5ml TE

⑤ Resuspend ~~DNAs~~ DNA fragments using TE buffer to 10ng/ml

⑥ Put DNA in a ~~320 bp~~

mini dry bath ~~0.3 pmol~~

for 20 mins ~~0.059 μg of DNA~~

⑦ Add correct amount of DNA and Gibson inserts to a ~~20 μl~~ total
([↳] backbone)

6

M

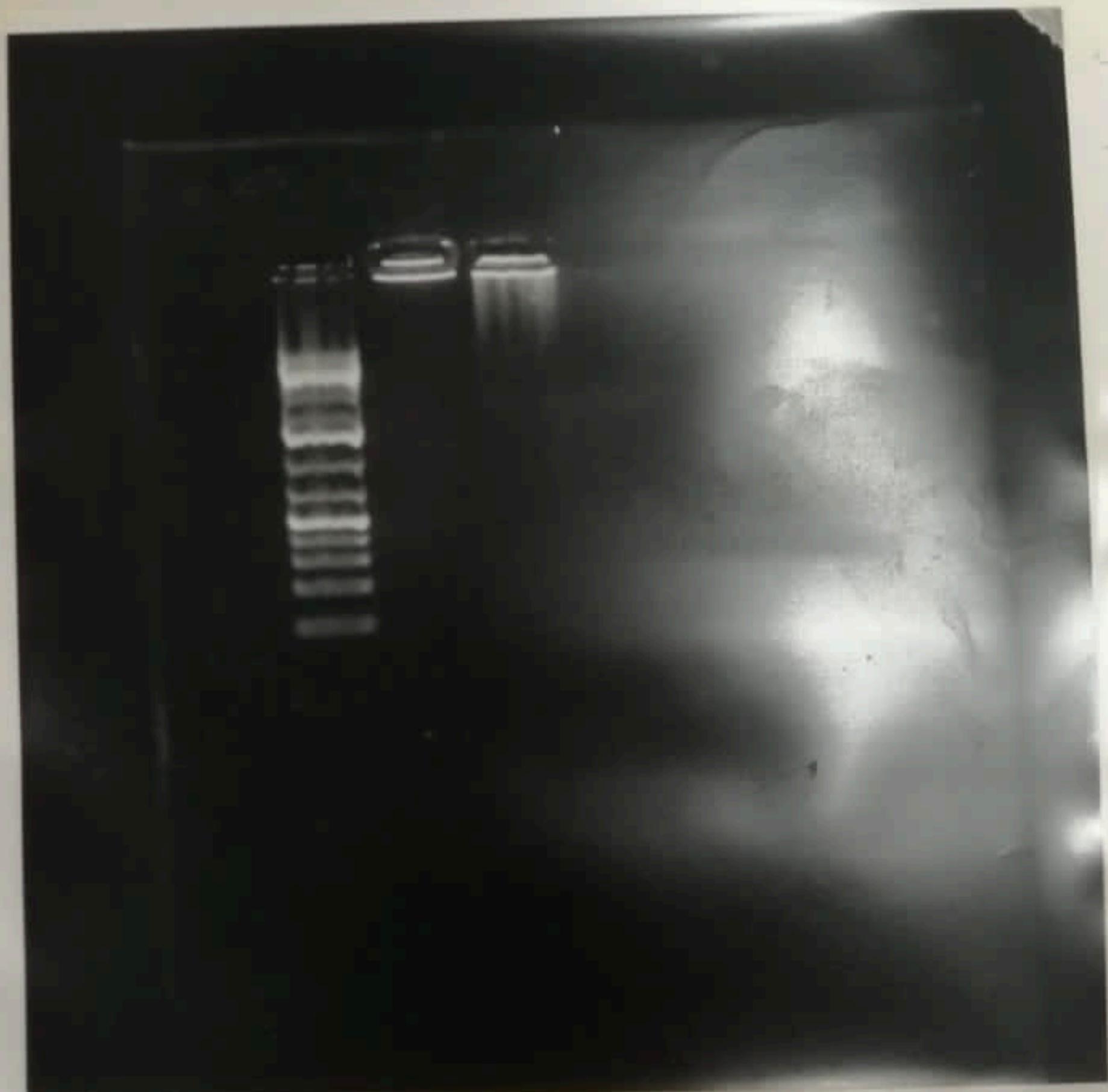
Printed on Wednesday, September 15, 2021 15:05:09 (PM)

021 15:05:09 (PM)



Second attempt at running the agarose gel - the ladder appeared successfully but the bands of DNA did not migrate properly

Printed on Wednesday, September 15, 2021 16:15:58 (PM)



Third attempt at running the agarose gel - the DNA did not migrate properly, meaning the PCR was unsuccessful.

Tomorrow: nanodrop 890
mini prep - blank with elution buffer

marker 100 0.1 Ochre, Hha I 999 1000 • rebiting 999
 SAJ-351 + Kan/Chlor/spec + ATC
 SAJ-351 + ATC
 PAJ + Kan/Chlor/spec + ATC
 PAJ + ATC

Assembly cloning Kit
 -80°C →
 -20°C assembly master mix, positive control, pUC19 control DNA
 44°C → SOC outgrowth

NEB® Golden Gate Assembly Kit (BsaI-HF v2)

S E1601S

20 reactions

Store at -20°C

First time users are strongly encouraged to read the manual before performing this short protocol.

To download full manual, visit www.neb.com/E1601

The NEB Golden Gate Assembly Kit contains the following components:

- Optimized mix of BsaI-HFv2 and T4 DNA Ligase
- pGGA destination plasmid (user provided plasmid can be substituted)
- T4 DNA Ligase Buffer (shown to support efficient and high fidelity assemblies)

Together these components can support assemblies of 2-20+ inserts or modules, or be used for single insert cloning or library creation from diverse populations.

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Protocol for a Routine PCR

Individual components prior to use,
assemble all reaction components on ice.

COMPONENTS	25 µl RXN	50 µl RXN	FINAL CONC.
5 pmol Forward Primer	1.25 µl	2.5 µl	0.5 µM
5 pmol Reverse Primer	1.25 µl	2.5 µl	0.5 µM
Template DNA	variable	variable	<1,000 ng
High-Fidelity 2X Master Mix	12.5 µl	25 µl	1X
Water-Free Water	to 25 µl	to 50 µl	

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NEBuilder® HiFi DNA Assembly Cloning Kit

S E5520S

10 rxns

To download full manual, visit www.neb.com/E5520

Important Note: Store the kit components at indicated temperatures: NEBuilder HiFi DNA Assembly Master Mix and positive controls at -20°C, competent cells at -80°C and SOC Outgrowth Medium at room temperature. Before use, thaw and vortex master mix thoroughly and keep on ice.

NEB recommends a total of 0.03–0.2 pmol of DNA fragments when 1 or 2 fragments are being assembled into a vector, and 0.2–0.5 pmol of DNA fragments when 4–6 fragments are being assembled. Efficiency of assembly decreases as the number or length of fragments increases. To calculate the number of pmol of each fragment for optimal assembly, based on fragment length and weight, we recommend the following formula:

$$\text{pmol} = (\text{weight in ng}) \times 1,000 / (\text{base pairs} \times 650 \text{ daltons})$$

50 ng of 5000 bp dsDNA is about 0.015 pmol

50 ng of 500 bp dsDNA is about 0.15 pmol

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PCR purification: after centrifugation (step 5)
air dry for 2 min.

Step 6: 20 μ l EB, 2 min. Make sure EB
is heated to 60°C, while washing.

$$\begin{aligned} \text{Total ladder vol} &= 2 \mu\text{l} \\ \text{Conc.} &= 0.5 \mu\text{g}/\mu\text{l} \\ &\quad \left. \begin{array}{l} \text{vol} = 2 \mu\text{l} \\ \text{conc.} = 0.5 \mu\text{g} \\ \text{mass} = 1 \mu\text{g} \end{array} \right\} \\ &\quad - 1000 \text{ ng} \end{aligned}$$

$$0.15 \cdot 500 = 15 \cdot 5 = 75 \text{ ng}/\mu\text{l}$$

$$A \text{ and } D \approx 250 \text{ ng}/\mu\text{l}$$

$$\textcircled{B} = 0.05 \cdot 500 = 25 \text{ ng}/\mu\text{l}$$

↓
lower that to 200,
to be safe

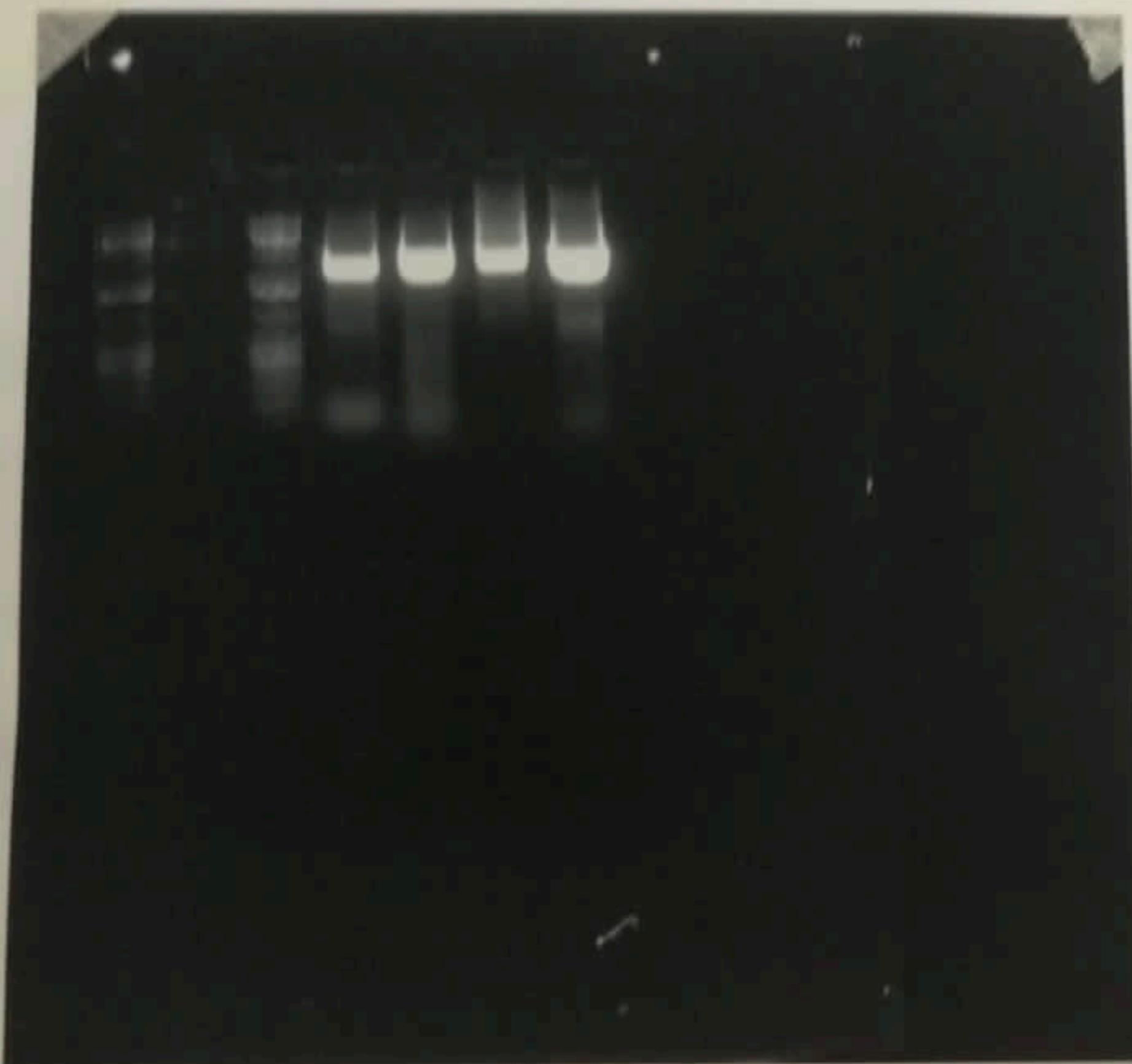
B = slightly less than A and D,
600 ng/ μ l

We repeat

A and C

been suc-

Printed on Monday, September 20, 2021 17:19:49 (PM)



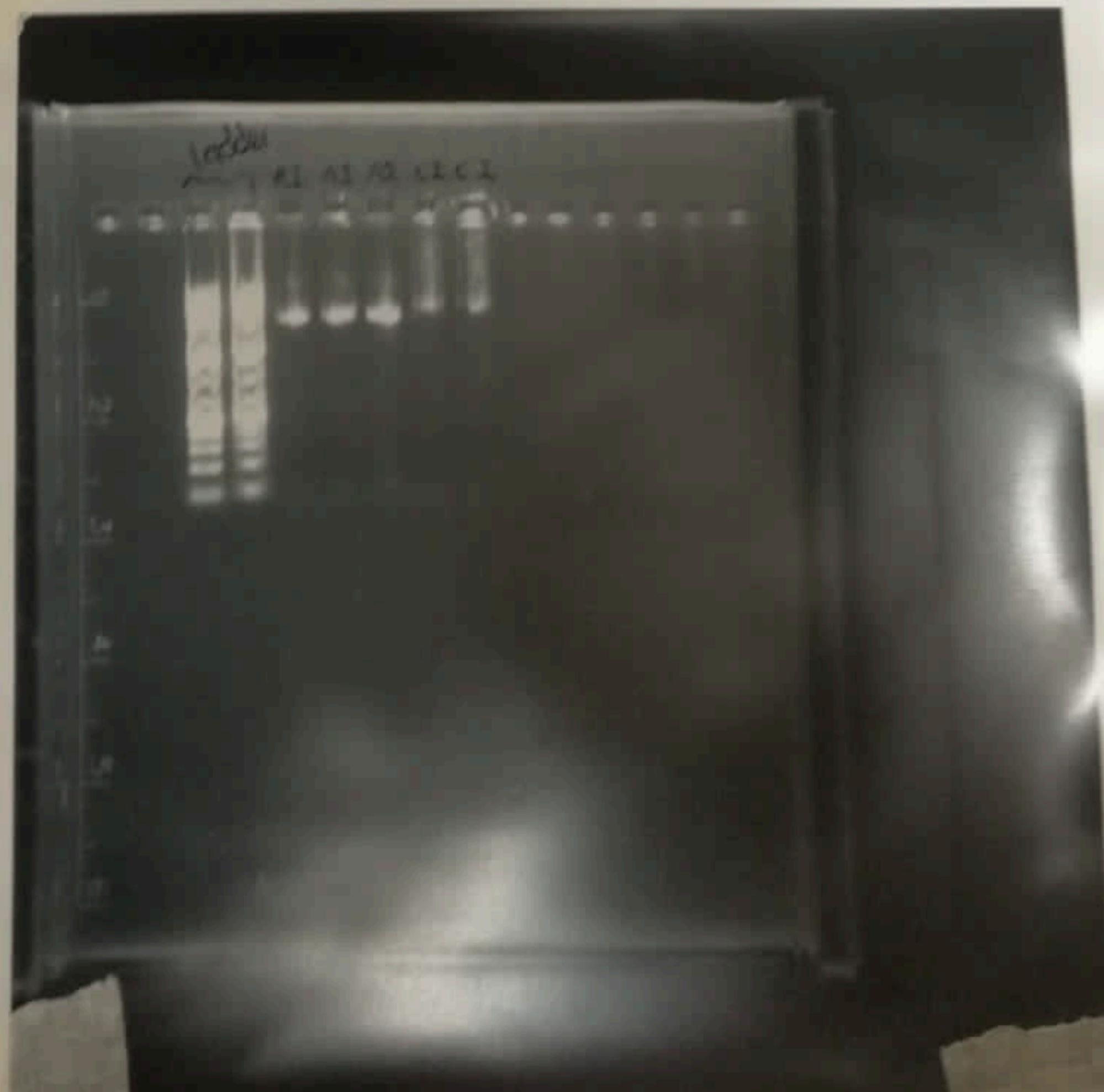
A successful PCR!

We then attempted to purify the PCR product but for some reason we were unsuccessful.

We had to redo the PCR as a result - the next two PCR attempts were unsuccessful.

Printed on Monday, September 27, 2021 16:59:47 (PM)

We repeated the PCR for backbones A and C and they appear to have been successful



WD40 plasmid recipe

6 Progenets:

- BBA = 100 fold to 115 ng/ μ l
- BBB = 10 fold to 12 ng/ μ l
- BBC = 10 fold to 14, 2 ng/ μ l

Goldengate

Backbone : 112 ng / reaction
Total : 1344 ng (60 fmol)

$$\begin{array}{r} 8 \mu\text{l} \times 143 \text{ ng} / \mu\text{l} \\ + \\ 2 \mu\text{l} \times 128 \text{ ng} / \mu\text{l} \\ \hline \end{array}$$

10 μl solution

Inserts : 120 fmol each (2×60
 $2:1$ ratio)
= 22,25 ng each

Conc = 10 ng/ μl \Rightarrow 2,25 μl of
each insert / reaction \Rightarrow

$30 \mu\text{l}$ of each for the big one (12 rxn)
Per reaction

$30 \times 8 = 240 \mu\text{l}$ of insert mix + $10 \mu\text{l}$ backbone

$250 \mu\text{l}$ of mastermix

$250/12 = 20,8 \mu\text{l}$ of mastermix/stage

Avg gRNA length = 130 bp

Need 120 fmol = $10 \mu\text{g}$ / reaction

$10 \mu\text{g}/\mu\text{l} \rightarrow 1 \mu\text{l}/\text{rxn}$

Components: $20,8 + 1 = 21,8 \mu\text{l}/\text{rxn}$

$3 \mu\text{l}$ of buffer (10x)
 $1,5 \mu\text{l}$ of assembly mix

26,3 components + 3,7 H₂O

30 μl total