iGEM 2020 Lab Notebook

Team IISER - Pune - India

Training

Tue 22 Sep

Sample PCR 1.

- Total rxn of 100 µl.

1. 10 x Buffer - 10 µl
2. dNTPs - 2 µl
3. Template - 2 µl
4. Primers (usually 100 µm) - 2 µl each (forward & reverse)
5. MilliQ - 80 µl
6. Enzyme Polymerase - 2 µl {for homemade}
   (Pfu)

* Make primer stock to 100 µm
* Do not add DNA / enzyme before making buffer solution in water.

Vial Temp

1 : 45°
2 : 46.8°
3 : 52.8°
4: 61.0°
5 : 65.0°

} for Gradient PCR.

PCR Protocol :

Lid : 105°C Vol. : 20 ul.

Step 1: 95°C for 10:00 min.
2: 95°C for 0:30 min.
3: 45.0°C / 65.0°C for 1:00 min (Gradient)
4: 68° for 14:00 min.
5: Goto Step 2, 30 times.
6: 72°C for 10:00 min
7: 4°C forever
8: End

Wed 23 Sep

* For running gels:
The product should be around 6 kb (for abv. PCR).

* Conc. of gel depends on size of DNA.
- can vary from 0.5% to 2% w/v.

Smaller the DNA, higher the concentration.

→ 1% for this run.

TAE : Tris Acetic Acid EDTA Buffer

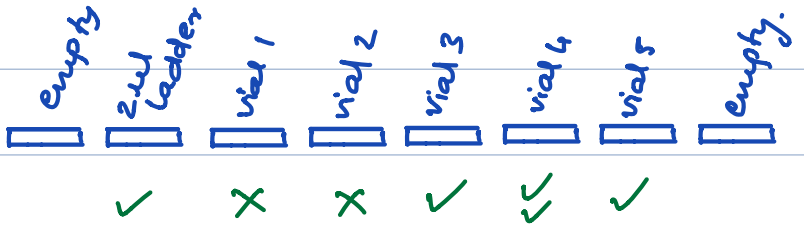
Things to Check:

- Check for leaks
- Check for levelness.
- Check for residual agarose in the ETBR flask.
- While melting agarose, check if all the granules have melted.

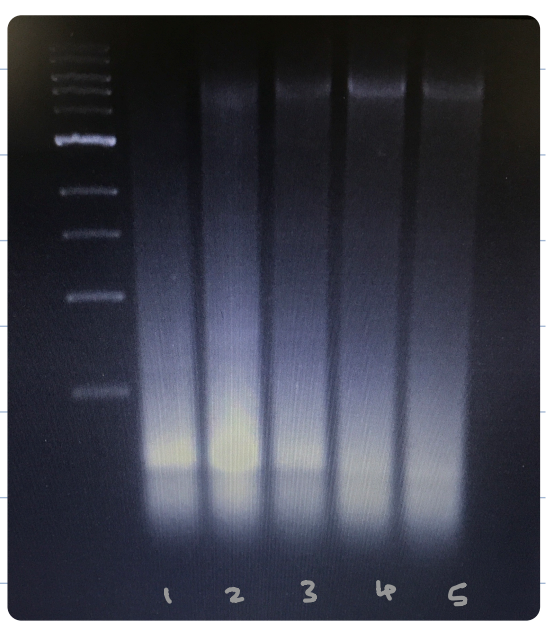
*Use loading dye. - *dilute from 5x conc to 1x

→ Added 5ul of green dye to each vial to make the total volume 25 ul.

→ Load entire volume (25ul) to the wells.



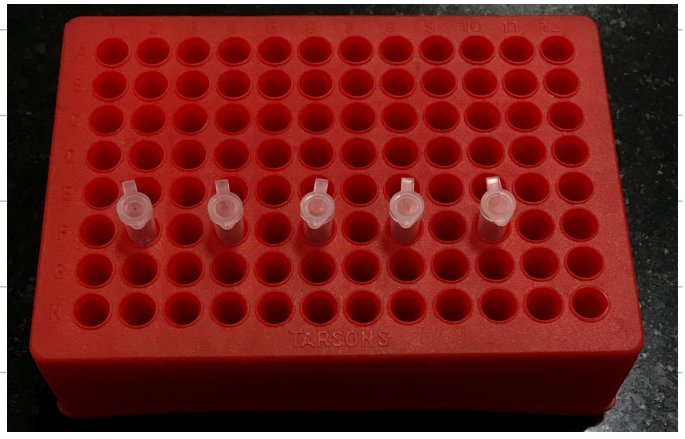
visible! ✓ × × ✓ ✓✓ ✓



PCR 2

→ for 100 µl

- ✓ 10 x Buffer – 10 µl
- ✓ dNTPs – 2 µl
- ✓ Template – 2 µl
- ✓ Primers (usually 100 µm) – 2 µl each (forward & reverse)
- ✓ MilliQ – 80 µl
- ✓ Enzyme Polymerase – 2 µl {for homemade}
  (Pfu)



PCR Protocol :

Lid : 105°C Vol. : 20 µl.

Step 1: 95°C for 10:00 min.
2: 95°C for 0:30 min.
3: 61°C for 1:00 min.
4: 68° for 14:00 min.
5: Goto Step 2, 30 times
6: 72°C for 10:00 min
7: 4°C forever
8: End

Fri 25 Sep

* Gel Extraction using PCR#2 product

Step 1 : Make Agarose Gel.

- Make Standard 1% w/v agarose gel (50 ml TAE)
- Fuse 3 wells of the comb using tape.
- Check the level of the mould using a spirit levellers
- Check water proofing of the mould.
- Make sure all the agarose beads have dissolved while heating in the microwave.
- Add 4 µl of EtBr for 50 ml TAE.
- Let sit for 20 - 30 min.

Step 2 : Run the product on the gel

- Add 25 µl of 5x GoTaq Flexi dye to 100 µl of the PCR product.
- Spin down and pipette into the fused well.
- Pippette out 2 µl of the ladder into one of the wells.
- Make sure to leave a space of 1 well b/w the ladder and the product (useful for gel extraction)

→ Weight of extracted gel piece = 280mg

→ Add 3x (weight) of gel piece of denaturation

buffer to the tube.

→ Let sit in a 55°C water bath for 10 min.

Mon 28 Sep.

PCR 3 : 2 PCR tubes, 1 with primers (Experiment)
1 without primers (Control)

- ✓ 10x Buffer – 2.5 µl
- ✓ dNTPs – 0.5 µl
- ✓ Template – 0.5 µl
- ✓ Primers (usually 100 µm) – 0.5 µl each (forward & reverse)
- ✓ MilliQ – 20 µl
- ✓ Enzyme Polymerase – 0.5 µl {for homemade}
  (Pfu)

$$\frac{x}{25+x} = \frac{1}{5} \Rightarrow 5x = 25 + x$$

$$4x = 25$$

$$x = \frac{25}{4} = 6.25 \text{ µl}$$

$$\frac{x}{5+x} = \frac{1}{5}$$

$$5x = 5 + x$$

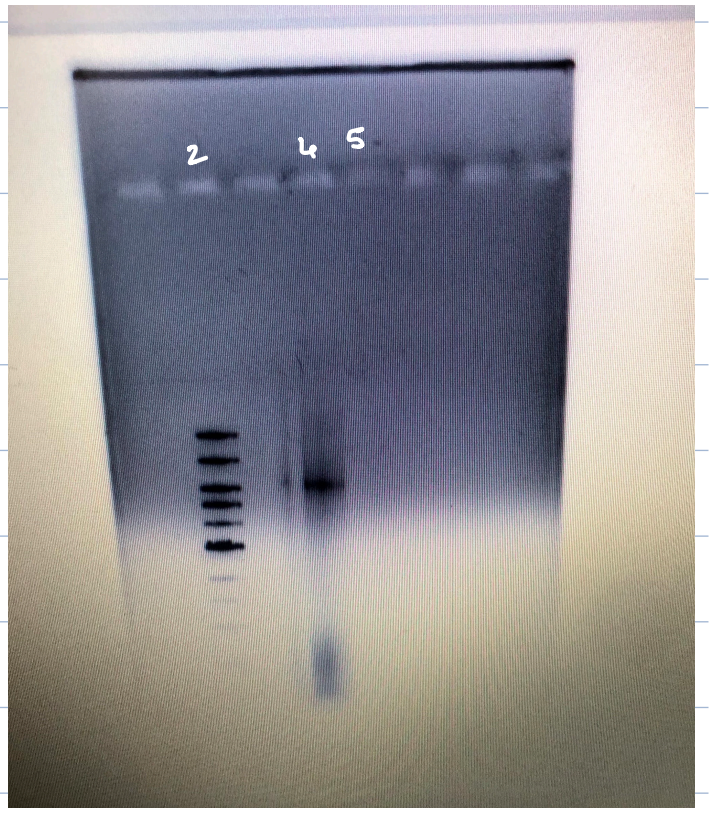
$$4x = 5$$

$$x = 1.25$$

→ Amount of 5x gel dye to be added.

Gel Synthesis + Electrophoresis

result →



2 : Ladder : 1kb NEB

4 : Experiment

5 : Control

* Dpn1 treatment

0.5 ul of Dpn1 (20,000 enzyme units/ml) in 20ul of PCR product.

Overnight treatment in sealed tube at 37 °C.

Tue 29 Sep

* Cell Transformation

Material : Competent Cells (E. coli DH5 α) - ___ ul
PCR product

Protocol :

Comp Cells (kept @ -80°C)

↓ Thaw on ice

On ice

↓ + Plasmid (PCR product)

Heat Shock

@ 42°C for 60 - 90 sec.

backup measure: hold the tube in your hand (37°C) for 3 min.

↓

on ice for 2 - 5 min

↓

Add LB ⟶ Incubate at 37°C for 60 min ⟶ Plate for 12 - 16 hrs

Fri 2 Oct

Colony PCR :

	Negative Control	Colony #1 (Experiment)	Positive Control
✓ Milliq	20 µl	20 µl	20 µl
✓ Buffer	2.5 µl	2.5 µl	2.5 µl
✓ dNTPs	0.5 µl	0.5 µl	0.5 µl
✓ Template	-	colony	0.5 µl
✓ New oligos FP	0.5 µl	0.5 µl	0.5 µl
✓ New oligos RP	0.5 µl	0.5 µl	0.5 µl
Pfu	0.5 µl	0.5 µl	0.5 µl

Primer dilution: 8 x dilution : 2 µl for 100 µl

↳ 12.5 in 100

0.25 in 2 µl

2.5 in 20 µl

PCR Protocol :

Lid : 105°C Vol. : 20 µl.

Step 1: 95°C for 10:00 min.

2: 95°C for 0:30 min.

3: 55°C for 1:00 min.

4: 68° for 0:40 min.

5: Goto Step 2, 35 times

6: 72°C for 10:00 min

7: 4°C forever

8: End

Sat 3 Oct

Gel Electrophoresis

Step 1: Make Agarose Gel.

- Make Standard 1% w/v agarose gel (50 ml TAE)
- Fuse 3 wells of the comb using tape.
- Check the level of the mould using a spirit leveller
- Check water proofing of the mould.
- Make sure all the agarose beads have dissolved while heating in the microwave.
- Add 4 µl of EtBr for 50 ml TAE.
- Let sit for 20 - 30 min.

Alkaline Lysis – Sat 3 Oct 1:15 pm

Alkaline lysis is the method of choice for isolating circular plasmid DNA, or even RNA, from bacterial cells. It is probably one of the most generally useful techniques because it is a fast, reliable and relatively clean way to obtain DNA from cells. If necessary, DNA from an alkaline lysis prep can be further purified.

Alkaline lysis depends on a unique property of plasmid DNA. It is able to rapidly anneal following denaturation. This is what allows the plasmid DNA to be separated from the bacterial chromosome.

Typically, you will grow E coli cells that contain the plasmid you want to isolate, then you will lyse the cells with alkali and extract the plasmid DNA. The cell debris is precipitated using SDS and potassium acetate. This is spun down, and the pellet is removed. Isopropanol is then used to precipitate the DNA from the supernatant, the supernatant is removed, and the DNA is resuspended in buffer (often TE). A mini prep usually yields 5-10 ug. This can be scaled up to a midi prep or a maxi prep, which will yield much larger amounts of DNA (or RNA).

Specific protocols for alkaline lysis differ widely from lab to lab, and even from scientist to scientist. The basic principles behind the procedure, however, are fairly uniform. Here they are:

1. Spin down your cells. Your DNA is still in the cells, so it is in the pellet at this stage.

2. Discard the supernatant. Pieces of cell wall are released from the bacteria and are floating around in the supernatant. These cell wall pieces can inhibit enzyme action on your final DNA, so it is important to get rid of all of the supernatant and to even invert the tube and wipe the lip with a Kim-wipe or Q-tip.

3. Resuspend the cells in buffer (often Tris) and EDTA. EDTA chelates divalent metals (primarily magnesium and calcium). Removal of these cations destabilizes the cell membrane. It also inhibits DNases. Glucose should also be added to maintain osmolarity and prevent the buffer from bursting the cells.

4. Lyse the cells with sodium hydroxide (NaOH) and SDS. This highly alkaline solution gave rise to the name of this technique. Mix this by gentle inversion and incubate on ice for five minutes (but no longer, or your DNA will be irreversibly denatured). Three things happen during this stage:

> a.SDS pops holes in the cell membranes. SDS (sodium dodecyl - lauryl sulfate) is a detergent found in many common items such as soap, shampoo and toothpaste.
>
> b.NaOH loosens the cell walls and releases the plasmid DNA and sheared cellular DNA.
>
> c.NaOH denaturesthe DNA. Cellular DNA becomes linearized and the strands are separated. Plasmid DNA is circular and remains topologically constrained.

5. Renature the plasmid DNA and get rid of the garbage. Add potassium acetate (KAc), which does three things:

> a.CircularDNA is allowed to renature. Sheared cellular DNA remains denatured as single stranded DNA (ssDNA).
>
> b.The ssDNAis precipitated, since large ssDNA molecules are insoluble in high salt.
>
> c.Adding potassium acetate to the SDS forms KDS, which is insoluble. This will allow for the easy removal of the SDS from your plasmid DNA.

Now that you've made it easy to separate many of the contaminants, centrifuge to remove cell debris, KDS and cellular ssDNA. Your plasmid DNA is in the supernatant, while all of the garbage is in the pellet.

6. Precipitate the plasmid DNA by alcohol precipitation (ethanolor isopropanol) and a salt (such as ammonium acetate, lithiumchloride, sodium chloride or sodium acetate) and spin this down. DNA is negatively charged, so adding a salt masks the charges and allows DNA toprecipitate. This will place your DNA in the pellet.

7. Rinse the pellet—your plasmid DNA—in ice-cold 70% EtOH and air-dry for about 10 minutes to allow the EtOH to evaporate.

8. Resuspend your now clean DNA pellet in buffer (often Tris) and EDTA plus RNases to cleave any remaining RNA. Your DNA is now back in solution.

DNA of this purity is good for a number of uses, such as in vitrotranscription or translation or cutting with some enzymes. If you are sequencing or transforming this DNA into mammalian cells, you'll want to use additional purification techniques such as phenol extraction, Qiagen column purification, or silica-based purification.

Ratio = I : II : III = 1 : 2 : 1.5

Pellet Size : 3ml Culture (Vinayak's lab)

Step 0. Add RNAse to Sol I vial

Step 1. 200 ul Solution I

2. 400 ul Solution II

3. 300 ul Solution III

4. Incubate on ice for 15 min and Precool centrifuge to 4°C

5. Centrifuge twice @ 4°C, 13,000 RPM for 12 min.

6. Add Room Temp isopropanol : 0.8 x total volume of sols I+II+III.
   for 7-10 min
   or at 4°C for 30 min. = 0.8 x 600 ul
   ≈ 500 ul of isoprop

* keep Sol II at Room Temp,

7. Leave at 4°C for 30 min.
8. Split the volume into 2 tubes and centrifuge at 4°C, 13000 rmp for 12 min.
9. Remove the supernatant (Plasmid DNA is in the pellet).
10. Add 500 µl of 70% Ethanol on ice.
11. Spin down for 5-7 min at 4°C, 13000 rpm
12. Remove supernatant, and let excess ethanol evaporate till the pellet becomes transparent.
13. Add autoclaved water depending on the size of the pellet. [20 µl in this case].
14. Put on shaker for ~5 min.
15. Add 0.5 µl of 10 mg/ml RNAse to one vial.
16. Let sit at 37°C (static, no shaker) for ~40 min.

Transformation (II)

* Cell Transformation

Material : Competent cells (E. coli DH5 α) - ~250 µl
PCR product

Protocol :

Comp cells (kept @ -80°C)
| Thaw on ice

On ice

↓ + Plasmid (PCR product)

Heat Shock

@ 42°C for 60 - 90 sec.

backup measure: hold the tube in your hand (37°C) for 3 min.

↓

on ice for 2 - 5 min

↓

Add LB → Incubate at 37°C for 60 min → Plate for 12 - 16 hrs

Diluting the vector :

2 ng / µl

2 ng / µl = 2000 ng / µl

need 100 ng / µl. 1 µl in 19 µl H_2O

Mon 5 Oct

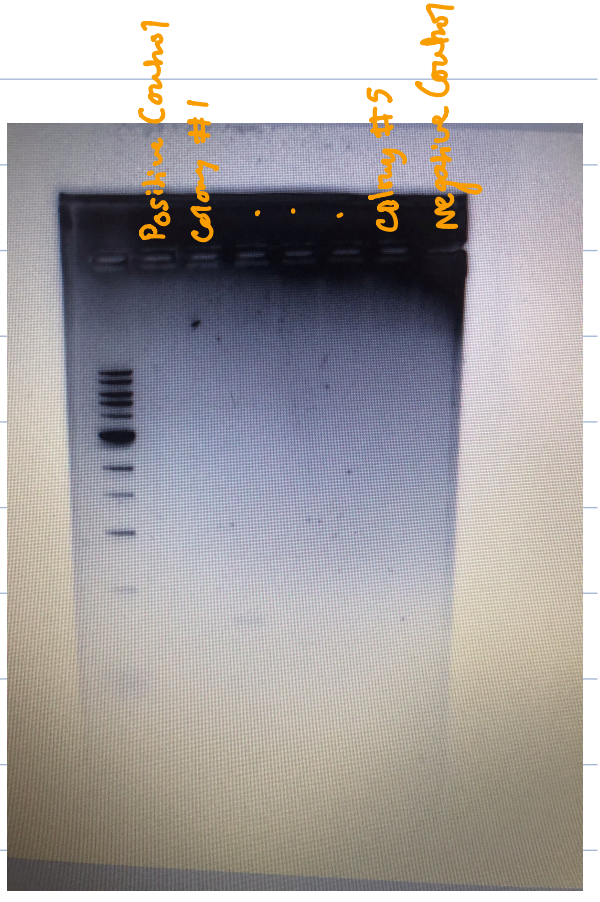
Colonies obtained :

* Colony PCR using 5 colonies.

	Negative Control	Colonies 1-5 (Experiment)	Positive Control
✓ MilliQ	20 µl	20 µl	20 µl
✓ Buffer	2.5 µl	2.5 µl	2.5 µl
✓ dNTPs	0.5 µl	0.5 µl	0.5 µl
✓ New oligos FP	0.5 µl	0.5 µl	0.5 µl
✓ New oligos RP	0.5 µl	0.5 µl	0.5 µl
Pfu	0.5 µl	0.5 µl	0.5 µl
Template	-	colony	✓ 0.5 µl

Tue 6 Oct

Gel Results for Colony PCR:
- verdict: Wrong Primers used.



But, colonies were successfully incubated. All 5 grew in 1.7 ml of LB broth

Plasmid Extraction : Colonies #4, #5

[Follow protocol from "Alkaline lysis".]

Ratio : I : II : III :: 1 : 2 : 1.5

from previous calculation for the 3rd culture:

Solution I : $\frac{1.7\ ml}{3\ ml} \times 200\ ml \approx 120\ ml$

* Add RNAse to Sol I before starting! See conc. below.

(Per vial)

∴ Sol I = 120 ml + 0.5 ml [of 10 mg/ml RNAse]

Sol II = 240 ml

Sol III = 180 ml

Mon 12 Oct

Protocol :

Comp Cells (kept @ −80°C) one vial

↓ Thaw on ice – 10 min

On ice

↓ + Plasmid – 100ng (PCR product)

backup measure:

Heat Shock
② 42°C for 90s
↓
on ice for 2 min
↓
Add LB → Incubate at → Plate
100 µl 37°C for 10 min for 12-16 hrs

} hold the tube in your hand (37°C) for 3 min.

Tuesday 13 Oct

① Colony PCR + Innocolation & Incubation (8 hrs)

② Make gel
③ Get ladder
④ Run gel

⑤ Check in fridge ← Aniruddh

→ Transformation failed.

Trouble shooting. What could have gone wrong?
↳ what changed?

① Comp Cells - Krish's lab vs Vinayak's lab

② Amount of LB added - 200 µl vs. 100 µl

③ On ice (after heatshock - 5 min vs. 2 min.

④ Incubation time - 60 min vs 10 min @ 37°C (after adding LB)

⑤ Volume plated - 100 µl from total vs. everything in the vial.

⑥ Plate size - small KAN plate vs. large KAN plate.

Repeating transformation (varying a few factors).
*KAN plates available: 4.

1. Control - Same as yesterday's

2. Change Plate to AMP plate.

3. Change incubation time to 60 min.

4. Change comp cells?

5. Change amount of LB added to 200µl.

* Colony PCR using 3 colonies.

	Negative Control	Colonies 1-3 (Experiment)	Positive Control
✓ Milliq	20 µl	20 µl	20 µl
✓ Buffer	2.5 µl	2.5 µl	2.5 µl
✓ dNTPs	0.5 µl	0.5 µl	0.5 µl
✓ New oligos FP	0.5 µl	0.5 µl	0.5 µl
New oligos RP	0.5 µl	0.5 µl	0.5 µl
Pfu	0.5 µl	0.5 µl	0.5 µl
Template	-	colony	0.5 µl

— 1 hr incubation

Mon 19 Oct

Plasmid Extraction

Tips · autoclave + Look for DNA

→ Estimate Plasmid conc.

50 µl H_2O added!

Nanodrop Conc. Estimation:

B: 180 ng/µl

A1: 107.6 ng/µl

A2: 55.6 ng/µl

* Plasmid Prep columns (kit)
* Polymerase (Pfu) +

PP HP Inhi

PCR ②

__°C

megaprimer

PCR

final construct

Digest

sequenced

* Digest pET28a+ (200 ng)

10 µl

Plasmid – ~ 200 ng

(10×) CutSmart – 1 µl

(Aniroddh) REast – 0.8 µl – 1 µl

water –

Expected size ?

} 37°C for 1–2 hrs

↓

1% Agaro gel

Qubit estimation of Plasmid conc.

→ 199 ul of Buffer

→ 1 ul of DNA specific dye.

→ 1 ul of Plasmid Sample

} Broadrange.

→ Mix well

↘

* for calibration: 190 µl of mix + 10 µl of standard

Plasmid Stalk : 1120 ng/µl

A1 : Out of range (too low)

A2 : 6.6 ng/µl

B : 7.2 ng/µl

Conclusion: Either scanty growth or DNA was lost somewhere in the process.

Transformation - with pET28(a)+ & comp cells from Anirudh's lab.

Protocol:

Comp cells (kept @ -80°C) one vial

↓ Thaw on ice - ~~10 min~~ 2 min only!

On ice

↓ + Plasmid - 100ng ✓
(PCR product)

Incubate on ice
(15 min) !

↓

Heat Shock
@ 42°C for 90s

backup measure:
hold the tube in your hand (37°C) for 3 min.

↓

On ice for ~~2 min~~ 5 min.

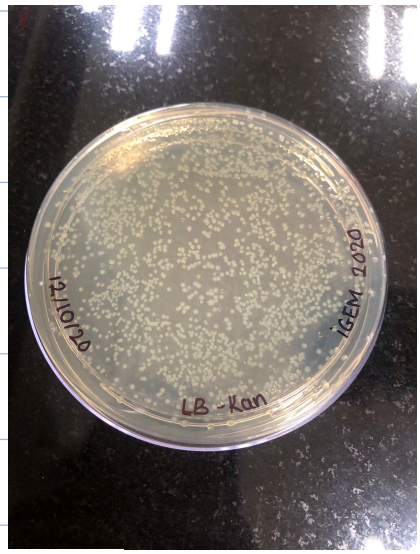
↓

Add LB → Incubate at → Plate
100 µl 37°C for 10 min for 12-16 hrs

Things we need:
→ 10x Thermopol buffer
→ Pfu

Tue 20 Oct

Transformation was a success!



To-do for today:

To-do list [20th Oct]

- [ ] Plasmid restriction digestion (in Main Building) - To confirm that we're working with the correct plasmid
- [ ] Make gel
- [ ] Enquire about DNA (Purchase section)
- [ ] Have lunch
- [ ] Run gel after lunch
- [ ] Meeting with mentors while gel is running
- [ ] Course work
- [ ] Remove cultures from incubator at around 8pm
- [ ] List item

1 ticked item

- [x] ~~Inoculate colonies~~

Restriction digestion

Water	To 10 µL	Water	5.5 µL
(10X) Buffer*	- 1 µL	Enzyme(s)	1 µL
(p)DNA	150 ng	⇒ pET-28a(+)	3 µL
Enzyme(s)	0.5 µL (each)	BamHI-HF	0.5 µL

Total	10 µL

* Must be selected from short.

Total	10 µL

Day 1: PCR for making mega primer. (x3)

- Run gel for verification (2 w)
- Column purification → 5-10 min
- conc. estimation → 5-10 min.

} 2-3 hours

↳ PCR using mega primer

- 4 min for extension
- 4-5 hours.

Parasite Protein :

5LGD Human Protein

Day 1 : All cloning procedures - PCR ① - 1.5 hrs

PCR ② - 4.5 hrs

DPNI - 2 hrs

Day 2 :

Transformation (14 hrs)

maybe 7am to 9pm

- 5ml cultures (5 cultures) - 16 hrs [done by 12 pm on Day 3]

(Plasmid Prep)

Day 3 - Plasmid Extraction - 0.5 hrs

- Gel for confirmation + Send for sequencing.

→ Cloning Complete

- Transformation into BL21 (14 hrs)

[

Day 4 - Grow secondary culture [6 hrs] (0.6 OD)

- Express protein - [3 hrs]

- Run SDS page for confirmation

→ Protein Expression Complete

The goal is to achieve this before 27th Oct.

To do tomorrow:

- Make LB
- Make LB Agar
- Make Kan plates.
- Autoclave water
- Get buffer and PBS (Aniruddh)

} autoclave both

wed 21 Oct

- ✓ LB + LB Agar
- ✓ Autoclave - Agar + Tips + water
- ✓ Take buckets (x2)
- ✓ Get enzymes + buffer
- Plasmid prep kit (+ mentor)

} ✓ find Housekeeping
✓ Fill water
clean fridge.

Recipe for LB + LBAgar

- 25 g of LB + 15 g Agar in 1 l of H_2O

∴ in 150 ml H_2O : 3.75 g of LB + 2.25 g of Agar

Fri 23 Oct - DNA Arrived from IDT!

Actual Work Begins

1. Mega primer PCR

→ for 100 µl

- ✓ 10 x Buffer - 10 µl →
- ✓ dNTPs - 2 µl
- ✓ Template - 2 µl
- ✓ Primers (usually 100 µm) - 2 µl each (forward & reverse)
- ✓ MilliQ - 80 µl
- ✓ Enzyme Polymerase - 2 µl {for homemade}
  (Pfu)

PCR Protocol:

Lid: 105°C Vol.: 20 µl.

Step 1: 95°C for 10:00 min.
2: 95°C for 0:30 min.
3: 55°C for 0:30 min.
4: 68° for 1:30 min.
5: Goto Step 2, 30 times
6: 72°C for 10:00 min
7: 4°C forever

8: End

Step 1 :

PCR for Mega Primer:

50 µl rxn:

Rxn no.	1 I: 5436p	2 PP: 5376p	3 HP: 14376p
10x buffer	5 µl	5 µl	5 µl
dNTPs	2 µl	2 µl	2 µl
Template	5 µl	5 µl	5 µl
Primer(F) [20 µM]	1 µl	1 µl	1 µl
Primer(R) [20 µM]	1 µl	1 µl	1 µl
Pfu	0.8 µl	0.8 µl	0.8 µl
Water	35.2 µl	35.2 µl	35.2 µl.

PCR Protocol :

Lid : 105°C Vol. : 50 µl

Step 1: 95°C for 10:00 min.

2: 95°C for 0:30 min.

3: 55°C for 0:30 min.

4: 68° for 1:30 min.

5: Goto Step 2, 30 times

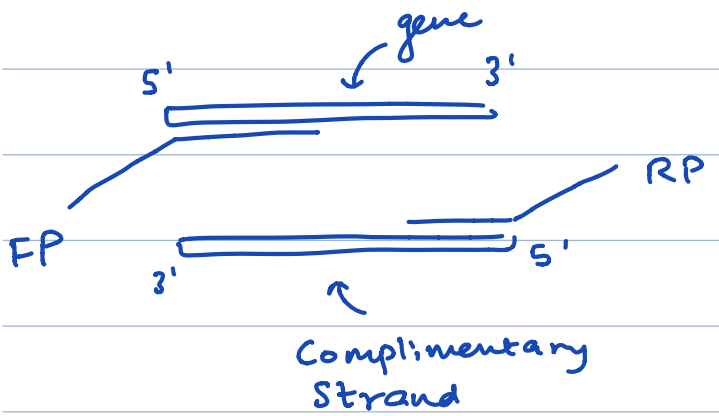
6: 72°C for 10:00 min

7: 4°C forever

8: End

Sat 4 Oct

Primer Schematic:



RP + FP both are binding

2 Gradient PCRs:

1. DMSO 3% + Pfu
2. Taq

PCR 1

~~10x~~ buffer	1 µl	× 17	17 µl
dNTPs	0.4 µl		6.8 µl
Template	0.5 µl		8.5 µl
Primer(F) [20 µM]	0.2 µl		3.4 µl
Primer(R) [20 µM]	0.2 µl		3.4 µl
Pfu	0.2 µl		3.4 µl
Water	7.5 µl		127.5 µl
DMSO – 3% (5.1 µl)	10 µl		170 µl

10× Paq buffer	1 µl	x9	9 µl
dNTPs	0.5 µl		4.5 µl
FP	0.5 µl		4.5 µl
RP	0.5 µl		4.5 µl
Template	0.1 µl		0.9 µl
Paq5000 (2kb/min)	0.1 µl		0.9 µl
H_2O	7.3 µl		65.7 µl
	10 µl		90 µl

Protocol AX : 30°–45° gradient
Block B
Pfu + DMSO.

Protocol A5 : 45°–65° gradient
Block A

Left : Pfu + DMSO
Right : Paq

Gel :

–	ladder	8 Pfu+DMSO (30-45)	– –	ladder	(4) Pfu+DMSO (45-60) –
–	ladder	(4) Pfu+DMSO 45-66	– –	ladder	(8) Paq5000 –

~~10×~~ Paq buffer	1 µl		40 µl
~~dNTPS~~	0.5µl		20 µl
~~FP~~	0.5µl		20 µl
~~RP~~	0.5µl	×40	20 µl
~~Template~~	0.1 µl		4 µl
Paq5000 (2kb/min)	0.1 µl		4 µl
H_2O	7.3 µl		292 µl
	10 µl		400 µl

Sun 25 Oct

① : 49.5°C

② : 52.8°C

③ : 57.5°C (Best one)

Mon 26 Oct

PCR 1: Megaprimer Amplification

10x Paq buffer	1 µl
dNTPs	0.5 µl
FP	0.5 µl
RP	0.5 µl
Template (megaprimer)	0.2 µl
PaqSOOO (2kb/min)	0.1 µl
H_2O	7.2 µl
	10 µl

replace with high fidelity

PCR Protocol : (1) (assuming Pfu)
subject to change with enzyme.

Lid : 105°C Vol. : 20

Step 1: 95°C for 10:00 min.
2: 95°C for 0:30 min.
3: 57°C for 0:30 min.
4: 68° for 1:30 min.
5: Goto Step 2, 30 times
6: 72°C for 10:00 min
7: 4°C forever
8: End

PCR 2 : RF PCR.

		For 100
10x Paq buffer	5	10
dNTPS	2.5 ul	5
FP	} 1000 ng ≈	} 20
RP	} = 10 ul	
Template (plasmid)	120ng = 0.107 ul // or dilute by 10x then add 1.5 ul (3)	
Paq5000 (2kb/min)	0.5	1.5
H_2O	30.5	69
	50 ul	100 ul.

PCR Protocol : (2)

Lid : 105°C Vol. : 20

Step 1: 95°C for 10:00 min.

2: 95°C for 0:30 min.

3: 42–58°C for 0:30 min.

4: 72°C for 6:30 min.

5: Goto Step 2, 30 times

6: 72°C for 10:00 min

7: 4°C forever

8: End

(3) PCR with GXL :

5X GXL buffer	6 µL.
dNTPS	1.5 µL
FP (megaprimer) / RP	4 µL
Template (plasmid)	1 µL
GXL	0.35 µL
H_2O	17.15 µL
	30 µL

PCR mix : 90 µl (9 rxns - gradient)

Plasmid : 270 ng → 10 µl → Plasmid = 30ng

Megaprimer : 1384.ng. → M. Prim = 153ng

↳ remaining = 50 µL

Add 1 µL dpnI

3-4 hrs at 37°C

then transform

as control : equivalent plasmid

+ 1.2 µL dpnI

50 µL (150 ng)

1.5 µL

of 100 ng/µL

5 µL buffer

+ 1.5 µL plasmid

+ 43.5 µL water.