Characterizing neomycin resistance in E. coli (BBa_K1323013)

Project: Laboratory Notes
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TUESDAY, 6/25/2019

Aim: To characterize the part responsible for kanamycin resistance (**BBa_K1323013)** in E. coli using OD measurements at 600 nm in the presence or absence of a plasmid containing the resistance sequence.

Cloning kanamycin resistance intro HFP365_pUC19 plasmid

Kanamycin resitance sequence aquired from the 2018 Distribution Kit (Plate 5 well 10F)
The aim is to overlap-PCR up a kanamycin resistance fragment with 200 basepair homologous overlap overhangs with sequences in the pUC19 plasmid in order to insert the resistance sequence into this plasmid. The plasmid would be cut with a restriction enzyme and ligated based on homology then grown on kanamycin plates.

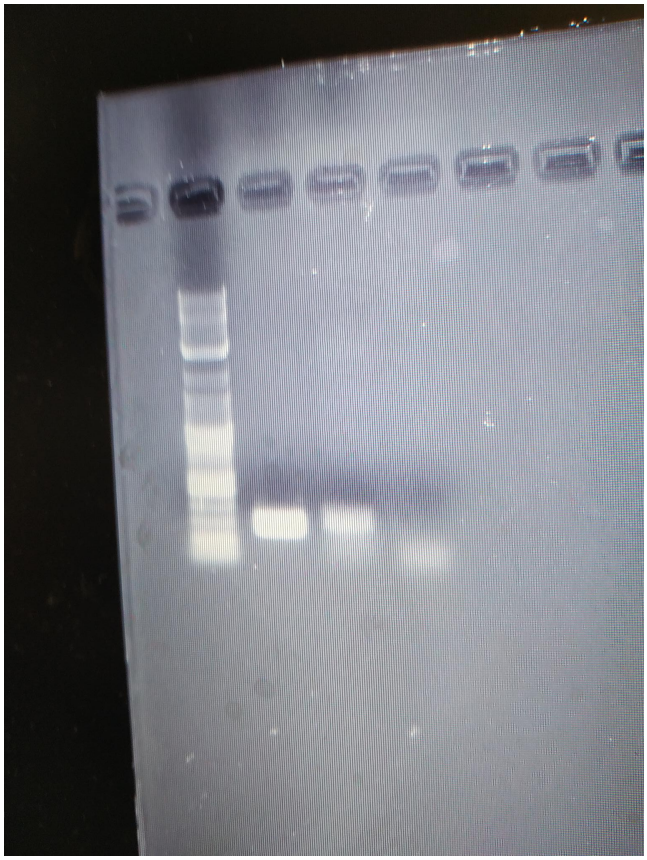
PCRing up DNA fragments for overlap PCR

- Primers KRF (kanamycin resistance forward), KRR (kanamycin resistance reverse), HR1F (homology region 1 forward), HR1R (homology region 1 reverse), HR2F (homology region 2 forward), HR2R (homology region 2 reverse) were diluted to 100 µM. 2 µL of each primer solution was added to 18uL of dH_2O to make 10uM solutions, later used in PCR. Kept on ice.
- Kanamycin resistance DNA was diluted with 10 µL water in the iGEM distribution kit (Plate 5 well 10F)
- 3 PCR reaction where prepared - 50 µL reaction volume was prepared with 19.5 µL dH_2O, 2.5 µL of the appropriate forward and reverse primers and 25 µL master mix
    - +0.5 µL of the kanamycin template solution
    - +2 seperate 0.5 µL mixtures of the pUC19 plasmid to PCR up the homology regions
- Run a PCR - NEB Q5-HF Kit protocol with an annealing temperature of *52°C* and an extension time of 20 s

Gelelectrophoresis

- A 1% agarose gel was run with the fragments to confirm the succes of the PCR reaction at 90V fro 30 minutes

PCR of homology region 1, homology region 2 and kan amycin resistance in this order



Succesful PCR of homology regions
Unsuccesful kanamycin PCR. Later identified that incorrect primers were used so new primers were ordered from IDT.

MONDAY, 7/1/2019

Aim: Reattempting the kanamycin resistance fragment PCR, this time with the correct primers.

PCR of kanamycin with correct primers:

- PCR reaction where prepared - 50 µL reaction volume was prepared with 19.5 µL dH_2O, 2.5 µL of the forward and reverse primers and 25 µL master mix + 0.5 µL of the kanamycin template solution
- Anneling temperature 47°C

The agarose gel showed no PCRd fragment - reason unknown

TUESDAY, 7/2/2019

Reattempting the kanamycin resistance PCR 2.

PCR reaction where prepared - 50 µL reaction volume was prepared with 19 µL dH_2O, 2.5 µL of the forward and reverse primers and 25 µL master mix + 1 µL of the kanamycin template solution

Anneling temperature 50°C

1% agarose gel was run to confirm the PCR

No band on the gel - unsuccesful PCR - reason unknown

WEDNESDAY, 7/3/2019

Aim: Redirecting focus on characterising neomycin resistance (**BBa_K1313004)**. The neomycin resistance fragment confers resistance against geneticin, kanamycin and neomycin. Kanamycin and neomycin are readily available in the lab so we'll focus on firstly kanamycin resistance characterization.

Acquired plasmid from the David Evans research group (PLDE1) that contains neomycin resistance as a selection marker. The DNA sequence is identical to **BBa_K1313004.**

Transformed DH5Alpha cells with the plasmid containing neomycin resistance (PLDE1) using standard transformation protocol with 90 minutes incubation time and 300 uL LB media added.
Since the plasmid used also contains ampicillin resistance the cells were plated on 10 uL on ampicillin and kanamycin plates and incubated overnight at 37°C in an inverted position

Realized that PLDE1 only expresses in mammalian cell lines so the initial idea was abandoned and a new plasmid was acquired from the Bee Research Group (PLBDE2) from the Evans Laboratory.

Transformed this plasmid into DH5Alpha' s (standard transformation porocol with 90 minutes incubation, 300 uL LB added)
Plated 10 uL of the cells on kanamycin plates and incubated overnight at 37°C inverted.

THURSDAY, 7/4/2019

The cells containing the PLDE1 plasmid did not grow on kanamycin plates but grew on ampicilin plates. Transformation was successful but no kanamycin resistance acquired. Since the plasmid is not subject for further work this line of work was abandoned.

PLBDE2 grew on kanamycin plates, although the cultures are extremely small so it was left to grow for an extra 5 hours.

A 10 mL LB vial with the working concentration of kanamycin (10 ul stock) was inoculated from a PLBDE2 culture and left for overnight at 37°V in the shaking incubator.

FRIDAY, 7/5/2019

The 10 mL LB vial was empty and no cells survived. The colony picked might have been unviable so regrowing cells next week with different colonies.

TUESDAY, 7/9/2019

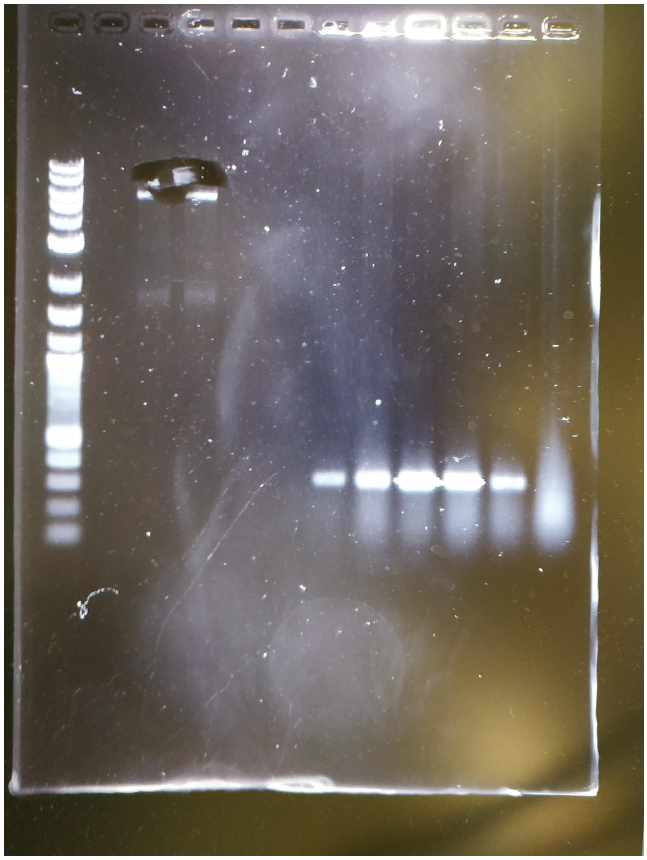
Aim: The plasmid used in very large (~12 kb) so the cells might strugle to grow. Digesting the plasmid with a restriction enzyme that has to sites on the plasmid then ligating yields a smaller plasmid an more viable cells.

Digestion reaction

15 uL of plasmid, 3 uL 10x Buffer, 9 uL water, 3 uL NheI enzyme
Incubate for 30 minutes at 37°C

Run an agarose gel of the digestion and gel extracted the larger band using standard gel extraction protocol, 50°C incubation for 7 minutes to dissolve the gel fragment.

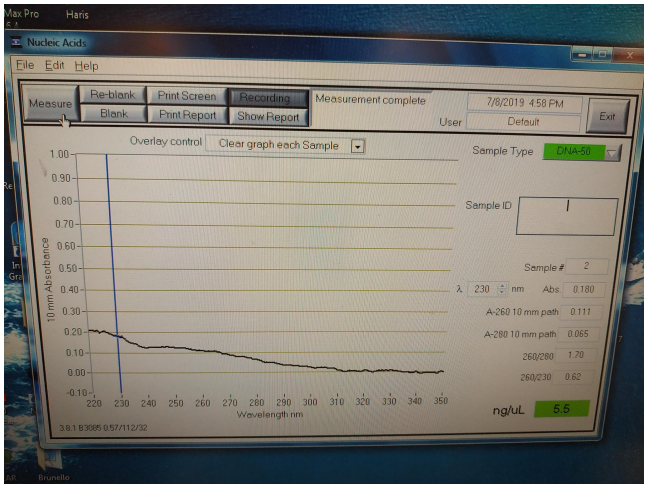
Agarose gel run for the digestion reaction in two wells d ue to the large volume of digestion reaction



Succesful digestion reaction as shown on the left of the gel, a smaller fragment and a larger one containing the resistance sequence.

Nanodropped the gel extracted plasmid

Nanodrop of the cut and gel extracted PLBDE2



Very low concentration but enough for a ligation.

Set up a ligation

10 uL of extracted DNA, 1 uL T4 ligase, 2 uL buffer, 7 uL of water.

Left for 1 hour to incubate.

Standard, "hard" transformation protocol - 2uL of ligation reaction into 100 uL of competent DH5Alpha cell, followed by 10 minutes on ice, 30 s at 42°C, 2 minutes on ice, 300 uL of LB and 60 minutes in 37°C shaking incubator.
Plated on a kanamycin plate, left in the incubator at 37°C overnight.

WEDNESDAY, 7/10/2019

Succesful tranformation and the colonies grew on the plate are larger.
The plate is kept at 4°C until further use.

A 10 mL kanamycin vial was **inoculated** with a larger colony from this plate.

THURSDAY, 7/11/2019

- The DH5 alpha cells with the digested and ligated PLBDE2 (PLDBE3) were transferred to a 15 mL falcon tube and spun in a centrifuge for 4 min. A relatively small pellet formed.
- The supernatant was discarded and the pellet was resuspended in fresh LB media.
- 8 vials with different kanamycin concentrations were prepared in LB media.

Table 1. The amount of kanamycin in each vile, needed to reach the specific concentration

	0 ug/uL	10 ug/uL	20 ug/uL	30 ug/uL	40 ug/uL	50 ug/uL	100 ug/uL	200 ug/uL
1	0 uL	2 uL	4 uL	6 uL	8 uL	10 uL	20 uL	40 uL

- 100 ul of the resuspended DH5 alpha bacteria with PLBDE3 were added to each vial.
- Cells were incubated at 37 degrees for 30 minutes and an OD measurment at 600 nm was taken.
- OD was then taken every 30 min for 1 hour.

Table 2. Absorbance of cultures at 30 minutes and 1 hours past inoculation

	0 ug/uL	10 ug/uL	20 ug/uL	30 ug/uL	40 ug/uL	50 ug/uL	100 ug/uL	200 ug/uL
1	0.0	-	-	-	-	-	-	0.01
2	0.0	0.01	0.0	0.0	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.01	0.01	0.0	0.0	-

The cells seem to not grow again => cultures were left in the incubator for 2 h and 30 min to see if something grows at all.

- *No cell further growth was observed, so the experiment failed.*

The cultures from today were left in the shaking incubator overnight at 37°C to see if they would grow if left for longer.

FRIDAY, 7/12/2019

The cells grew overnight in the kanamycin containing vials - most likely a very few cells are actually viable.

The experiment was repeated but the cells were taken from these double overnight cultures, assuming that these would be the most viable cells.
100 ul of cells from the vial with 50 ug/uL kanamycin concentration was put into 8 new vial with fresh LB media with different kanamycin concentrations as follows:

Table 3. The amount of kanamycin needed to reach the specific concentrations

	0 ug/uL	10 ug/uL	20 ug/uL	30 ug/uL	40 ug/uL	50 ug/uL	100 ug/uL	200 ug/uL
1	0 uL	2 uL	4 uL	6 uL	8 uL	10 uL	20 uL	40 uL

Cells were put in the shaking ocubator at 37 degrees and measurments of the absorbence were taken every 30 min.

Table 4. The change in OD in a certain time with different kanamycin concentrations

	Time of Abs taken	Abs of 0 ug/uL	Abs of 10 ug/uL	Abs of 20 ug/uL	Abs of 30 ug/uL	Abs of 40 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL
1	**0 min (10:04)**	-	-	-	-	-	-	-	0.05
2	30 min (10:34)	0.03	0.04	0.03	0.04	0.02	0.03	0.03	0.03
3	60 min (11:14)	0.07	0.09	0.08	0.07	0.09	**0.08**	0.10	0.03
4	90 min (11:57)	0.21	0.28	0.27	0.25	0.25	0.28	0.27	0.25
5	120 min (12:40)	0.46	0.55	0.55	0.55	0.54	0.61	0.62	0.53
6	150 min (13:29)	0.97	0.99	0.99	1.00	0.98	1.19	1.03	0.94
7	180 min (14:39)	1.28	1.36	1.36	1.41	1.34	1.96	1.48	1.39
8	210 min (15:09)	1.39	**1.45**	1.48	1.52	1.35	2.00	1.38; 1.46; 1.48	1.41
9	240 min (15:58)	1.49	1.55	1.61	1.60	1.52	2.00	1.56	1.48

A spillage happened after the measurment was taken. The volume of the vial had decreased => may affect further readings.
When 1 ml of the vial was taken in order to measure OD, it was thrown out instead of returning back to vial => decreased volume => may affect further readings.

Cells grew up over time with an expected growth speed. These cells are the most viable. Further reading are going to be taken on a further date to acquire data over a larger timescale.

MONDAY, 7/15/2019

A colony taken from the same plate was used to inoculate a new 10 mL kanamycin vial overnight at 37°C and was used to repeat the experiment performed on 12/07.

TUESDAY, 7/16/2019

A new blank was prepared with 1mL of Lb media + 1 uL of kanamycin
8 vials were prepared with new, different kanamycin concentrations as 100 uL of the DH5 alphas with neomycin resistance were put into each vial.
OD measurements were taken every 30 minutes.

Table 5.: Kanamycin concentraions

	0 ug/uL	20 ug/uL	50 ug/uL	100 ug/uL	200 ug/uL	500 ug/uL	1000 ug/uL	1500 ug/uL
1	0 uL	4 uL	10 uL	20 uL	40 uL	100 uL	200 uL	300 uL

Table 5. Kanamycin stock solutions used to reach a specific concentration in 10 mL of LB media.

Table 6.

	Time	Abs of 0 ug/uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL	Abs of 1500 ug/uL
1	0 min, 10:52	-0.02	-	-	-	-	-	-	-
2	30min, 11:22	0.02	0.02	0.01	0.00	0.01	0.01	0.01	0.00
3	60min, 12:14	0.02	0.01	0.01	0.02	0.02	0.01	0.02	0.02
4	90 min, 13:01	0.02	0.01	0.01	0.01	0.01	0.01	0.00	0.01
5	120 min, 13:53	0.01	0.01	0.01	0.00	0.00	0.01	0.00	0.00
6	150 min, 14:40	0.03	0.01	0.00	0.02	0.01	0.00	0.01	0.01

Table 6. Growth of neomycin resistant bacteria in the presence of different kanamycin concentrations.

For cells B2 B3 B4 blank with 1 uL of kanamycin was used.
After measurements had been taken from cell B4 when returning the cuvette the blank was poured into the 50 ug/uL sample by mistake.
A new blank only with 1 mL LB media was prepared.
8 other vials were prepared with new, different kanamycin concentrations and 100 uL of the DH5 alphas without neomycin resistance were put into each vial.

Table 7.

	Time	Abs of 0 ug/uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL	Abs of 1500 ug/uL
1	0 min, 10:52	0.00	-	-	-	-	-	-	-
2	30 min, 11:22	0.05	0.02	0.04	0.02	0.02	0.03	0.02	0.02
3	60 min, 12:14	0.12	0.03	0.04	0.00	0.02	0.02	0.02	0.02
4	90 min, 13:01	0.25	0.03	0.03	0.01	0.02	0.02	0.02	0.02
5	120 min, 13:53	0.42	0.02	0.02	0.01	0.00	0.01	0.01	0.01
6	150 min, 14:40	0.60	0.02	0.02	0.01	0.01	0.02	0.02	0.01

Table 7. Growth on non-resistant bacteria with changing kanamycin concentrations

DH5 alpha bacteria without neomycin resistance grow when in Lb media without kanamycin present and do not grow when kanamycin is present.
The cells containing the resistance did not grow even without any resistance. Plasmid influences cell fitness and viability quite substentially or it is possible that overnight cultures do not survive every time for an unknown reason.

5 different colonies were picked up from the same plate, from which bacteria were inoculated for the prevous experiments, and were inoculated overnight.

WEDNESDAY, 7/17/2019

Colonies III and IV were chosen for the experiment from the 5 inoculated colonies since the vials from these colonies had the highest OD. **OD of colony III. - 0.75, OD of colony IV. - 0.80**
To increase the chances of the growth of the colonies, the LB media was preheated prior use.
A new blank was prepared.

14 vials with different kanamycin concentration were prepared, one for each concentration for both colony III. and IV.
OD measurements were taken every 30 minutes.

Table 8.: Kanamycin concentrations

	0 ug/uL	20 ug/uL	50 ug/uL	100 ug/uL	200 ug/uL	500 ug/uL	1000 ug/uL	1500 ug/uL
1	0 uL	4 uL	10 uL	20 uL	40 uL	100 uL	200 uL	300 uL

Table 8. Volume of kanamycin stock soltuion required to reach a specific concentration in 10 mL of LB media.

For the first set of 7 vials with bacteria from colony III, only 100 uL of DH5 alpha bacteria were used per vial.

Table 9.: Colony III

	Time	Abs of 0 ug/ uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL
1	0 min, 10:52	-0.02	-	-	-	-	-	-0.02
2	30 min, 11:22	-0.03	-0.05	-0.05	-0.03	-0.04	-0.03	-0.02
3	60 min, 12:04	-0.03	-0.04	-0.03	-0.04	-0.04	-0.04	-0.02
4	90 min, 13:47	0.02	-0.04	-0.03	-0.05	-0.05	-0.05	-0.03
5	120 min, 13:33	0.00	-0.05	-0.05	-0.06	-0.06	-0.06	-0.05
6	150 min, 14:15	-0.08	-0.09	-0.05	-0.09	-0.09	-0.09	-0.08

Table 9. Growth of DH5Alpha cells under different kanamycin concentrations (colony III.)

For the second set of 7 vials with bacteria from colony IV, 200 uL of DH5 alphas were used per vial.

Table 10: Colony IV

	Time	Abs of 0 ug/ uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL
1	0 min, 12:17	-0.05	-	-	-	-	-	-0.04
2	30 min, 12:47	-0.04	-0.04	-0.04	-0.04	-0.04	-0.04	-0.04
3	60 min, 13:33	-0.06	-0.07	-0.06	-0.06	-0.07	-0.07	-0.07
4	90 min, 14:15	-0.08	-0.08	-0.08	-0.06	-0.07	-0.08	-0.06

Table 10. Growth of DH5Alpha cells under different kanamycin concentrations (colony IV.)

Expermiment was unsuccessfull since no bacteria grew at all even at 0 ug/uL kanamycin concentration, Negative values may have been due to contaminated cuvette

The vials were left in the shaking incubator at 37°C overnight to see if double overnight work or not.

THURSDAY, 7/18/2019

All of the vials showed significant cell growth, including the ones with high kanamycin concentrations.

The overnight containing 50 ng/uL initial kanamycin conentrations were used to **inoculate further colonies**. The double overnights seem to produce viable, resistant cells.

Both colony III. and IV: cultures were used again for inoculation.
ODs of colonies have reached significantly higher values: **OD (III)- 1.78; OD (IV)- 1.27**
14 vials were prepared with different kanamycin concentrations; 2 of each concentration; the same experiment was carried out with 2 different colonies so 7 vials per colony.
OD was measured every 30 min.

For the first set of 7 vials with bacteria from colony III, only 200 uL of DH5 alpha bacteria were used per vial.

Table 11: Colony III

	Time	Abs of 0 ug/ uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL
1	0 min, 10:27	0.05	-	-	-	-	-	0.06
2	30 min, 10:57	0.07	0.07	0.07	0.06	0.12	0.06	0.07
3	60 min, 11:36	0.10	0.09	0.10	0.10	0.11	0.11	0.07
4	90 min, 12:16	0.15	0.16	0.21	0.17	0.15	0.14	0.13
5	120 min, 12:54	0.29	0.26	0.31	0.30	0.29	0.22	0.18
6	150 min, 13:32	0.48	0.47	0.48	0.44	0.46	0.41	0.36
7	180 min, 14:42	0.65	0.63	0.65	0.62	0.60	0.57	0.43
8	210 min, 15:10	0.80	0.78	0.75	0.80	0.77	0.63	0.56
9	245 min, 16:00	1.06	1.04	1.00	1.00	0.96	0.74	0.60
10	275 min, 16:50	1.28	1.28	1.19	1.10	1.06	0.87	0.60

Table 11. Growth of DH5alpha cells (from colony III.) with the changing kanamycin concentrations based on OD 600 measurments

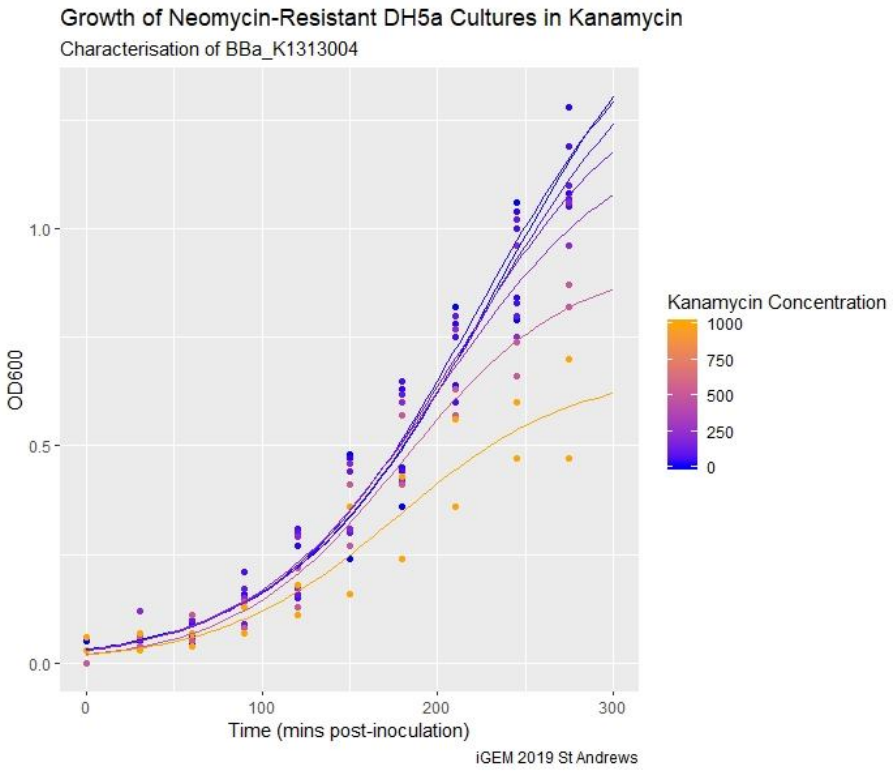
Table 12: Colony IV

	Time	Abs of 0 ug/ uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL
1	0 min, 10:27	0.03	-	-	-	-	-	0.03
2	30 min, 10:57	0.04	0.04	0.05	0.04	0.03	0.04	0.03
3	60 min, 11:36	0.05/ 0.04	0.05	0.06	0.05	0.05	0.05	0.04
4	90 min, 12:16	0.09	0.09	0.09	0.09	0.08	0.08	0.07
5	120 min, 12:54	0.13	0.17	0.15	0.16	0.17	0.13	0.11
6	150 min, 13:32	0.24	0.27	0.30	0.30	0.31	0.27	0.16
7	180 min, 14:42	0.36	0.45	0.44	0.42	0.42	0.41	0.24
8	210 min, 15:10	0.56	0.64	0.60	0.57	0.57	0.57	0.36
9	245 min, 16:00	0.79	0.84	0.83	0.80	0.75	0.66	0.47
10	275 min, 16:50	1.05	1.08	1.05	1.07	0.96	0.82	0.47

Table 12. Growth of DH5alpha cells (from colony IV.) with the changing kanamycin concentrations based on OD 600 measurments

Cell grew overtime, and the vials containing high kanamycin concentrations produced slower cell growth as depicted on the graph produced:

Growth of Neomycin-Resistant DH5alpha cultures in the presence of kanamycin



MONDAY, 7/22/2019

A 10 mL LB vial was inocluated with non-resistant DH5Alpha bacteria from glycerol stocks.

TUESDAY, 7/23/2019

Non-resistant bacteria were used to measure the cell growth in different kanamycin concentration.
200 uL of the overnight culture was used to inoculate new vials containing the appropriate kanamycin concentrations.

Cells only grew in the non-kanamycin vials. Two measurements were missed hence an hour and a half difference between measurement 5 and 6.

Table 13: Growth of non-resistant bacteria

	Time	Abs of 0 ug/uL	Abs of 20 ug/uL	Abs of 50 ug/uL	Abs of 100 ug/uL	Abs of 200 ug/uL	Abs of 500 ug/uL	Abs of 1000 ug/uL
1	0 min, 10:29	0.03	-	-	-	-	-	0.03
2	30 min, 10:59	0.11	0.07	0.05	0.04	0.04	0.04	0.03
3	60 min, 11:36	0.21	0.08	0.05	0.04	0.04	0.04	0.05
4	90 min, 12:14	0.42	0.10	0.05	0.03	0.03	0.04	0.03
5	120 min, 12:52	0.59	0.06	0.04	0.03	0.03	0.03	0.02
6	150 min, 14:32	1.19	0.10	0.04	0.04	0.03	0.03	0.04
7	240 min, 15:05	1.36	0.07	0.04	0.04	0.03	0.03	0.05
8	275 min, 15:40	1.38	0.08	0.05	0.04	0.04	0.04	0.06

Table 13. Growth on non-resitant bacteria based on OD 600 measurments

Cells only grow in the vial containing no kanamycin, since these cells are non-resistant.