Week 1 Project B

Tasks completed:

- Swapping resistance gene for plasmid pME6010 from tetracycline to kanamycin.

Notebook:

- First, we designed forward and reverse primers for KanR (with native RSB and promoter) which we isolated from plasmid pCM66. The 5' ends were complementary to the insert region of pME6010 (reaction 1).
- We then designed forward and reverse primers to amplify the pME6010 backbone (reaction 2).
- Further to this we redesigned each set of primers to incorporate an ampicillin promoter and optimised RBS (https://salis.psu.edu/software/) (B reactions) instead of the native promoter region (A reactions).
- The designed plasmids were as follows:

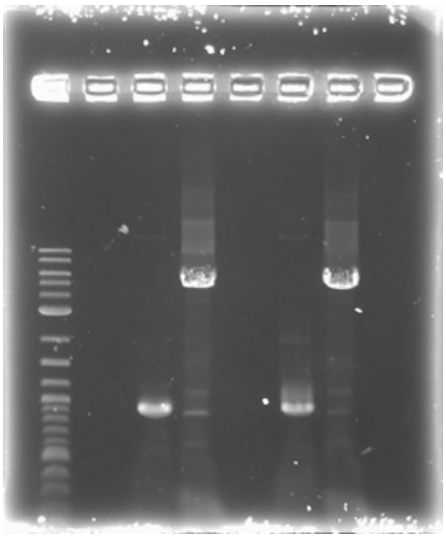
PCR reaction	Primer name	Primer sequence	Fragment length / bp
1A	KanRGib3	ggagcctatggaaaaacggcagataaaaatatatcatcatgaacaataaaactgtctgcttaca	920
	KanRGib4	ctatcgtttccacgagcaaattagaaaaactcatcgagcatcaaatgaaactgcaat	
1B	PampRBSKanRGib1	ggagcctatggaaaaacggcttcaaatatgtatccgctcatgagacaatTTATCTTCCGAGTCCTAGGAGGTATTAATTAatgagccatattcaacggg	920
	KanRGib4	ctatcgtttccacgagcaaattagaaaaactcatcgagcatcaaatgaaactgcaat	
2A	pME6010Gib3	atgatgatatatttttatctgccgtttttccataggctccgcc	4868
	pME6010Gib4	tgctcgatgagtttttctaatttgctcgtggaaacgatagggacgtcatatgg	
2B	pME6010Gib1	tgagcggatacatatttgaagccgtttttccataggctccgc	4848
	pME6010Gib4	tgctcgatgagtttttctaatttgctcgtggaaacgatagggacgtcatatgg	

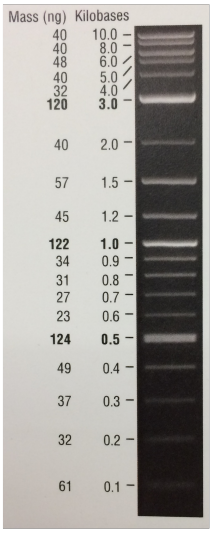
- We ran these 4 PCR reactions as follows using the NEB Q5 Protocol:

PCR reaction	Annealing temperature / ºC	Extension time / seconds
1A	70	30
1B	60	30
2A	70	150
2B	70	150

- A 0.8% agarose gel was used for the extraction as this offered good separation around 1kb and 5kb. This gel was run and the bands extracted according to our Gel Electrophoresis and Extraction protocol using NEB purple loading dye and 2-log purple ladder and QIAquick extraction kit.
- Gel obtained:

lane 1: 2 log purple ladder
lane 2: -
lane 3: 1A
lane 4: 2A
lane 5: -
lane 6: 1B
lane 7: 2B
lane 8: -





PCR product	Gel cut-out volume / μl	Concentration of first elution with 20μl EB / ng/μl	Concentration of second elution with 20μl EB / ng/μl
1A	200	53.7	22.8
1B	170	22.4	9.3
2A	190	41.8	26.1
2B	180	17.1	8.1

- As we later found out these NanoDrop readings are false since the QG buffer in the QIAGEN gel extraction kit interferes with the UV/Vis readings.
- Following extraction of our PCR products from the gel we used the NEB Gibson Assembly protocol to run an 8hr reaction over night. We ran an 'A' reaction which will insert the KanR gene into the pME6010 plasmid with the native promoter and a 'B' reaction that will insert the KanR gene with a pamp promoter and optimised RBS.

	Reaction A	Reaction B
PCR fragment 1 (fragment) / μl	1.04 (0.093 pmol)	2.53 (0.0942 pmol)
PCR fragment 2 (vector) / μl	2.4 (0.031 pmol)	5.85 (0.0314 pmol)
Gibson Assembly MM 2X / μl	5	10
dH2O / μl	1.56	1.62
TOTAL VOLUME / μl	10	20

- The volumes were chosen to satisfy 100ng vector with a 3-fold increase in the amount of insert. The insert amount must lie between 0.02 and 0.5 pmol and the total volume of total fragments cannot exceed 10μl.
- Following an overnight 8hr Gibson Assembly the reaction volumes were treated with Dpn1 restriction enzyme that cuts bacterial (methylated) DNA.
- We transformed the Gibson products into chemically competent DH5-alpha cells as well as into NEB alpha-5 cells. Unfortunately no colonies grew on a KanR plate!
- We know that the PCR products are correct so we think an issue may have arisen during the Gibson Assembly stage so we will re-do this part next week.