SELECTING CORES AND ANTIMICROBIAL PEPTIDES (AMPs)

Choosing the cores: Homomultimeric, self-assembling, protein cages with free N or C terminal for attaching AMPs and a reference of expression in E. coli

We have 14 cores : link

CORE CODE	NAME	PDB	N outside?	C outside?	B pairs	F-primer	R-primer	Ul-mer /g-block
DB.C01	Ferritin	4XGS	YES	NO	524	DB.P003	DB.P004	g-block
DB.C02	MS2	7msf	YES	YES	418	DB.P005	DB.P006	g-block
DB.C03	Qbeta	5vlz	NO	YES	1286	DB.P091	DB.P092	g-block
DB.C04	SMALL HEAT SHOCK PROTEIN	1SHS	NO	YES	467	DB.P009	DB.P010	g-block
DB.C05	ConcavalinA 2G4I	2g4i	YES	YES	742	DB.P093	DB.P094	g-block
DB.C06	PYRUVATE DEHYDROGENASE	1EAA	YES	NO	758	DB.P013	DB.P014	g-block
DB.C07	designed protein lyase cage	4QCC	YES	NO	899	DB.P095	DB.P096	g-block
DB.C08	Isoaspartyl Dipeptidase	5LP3	YES	NO	1196	DB.P097	DB.P098	g-block
DB.C09	GTP CYCLOHYDROLASE	1GTP	YES	NO	692	DB.P019	DB.P020	g-block
DB.C10	bacterial FucU isomerase	2WCV	NO	YES	446	DB.P099	DB.P100	g-block
DB.C11	Pizza3	3WW8	YES	YES	421	DB.P101	DB.P102	g-block
DB.C12	holo- synthase	5xu7	YES	YES	406	DB.P025	DB.P026	g-block
DB.C13	Pizza2	3WW7	YES	YES	295	DB.P103	DB.P104	g-block
DB.C14	RADA	x	YES	YES	79	DB.P029	DB.P030	ultramer

Choosing the AMPs: Variety of AMPs that target specifically the membrane. Generally cationic. Varied length (13-40 a. acids), from various origin (fish, toad, plant, synthetic). We also chose AMPs that have narrow spectrum, broad spectrum and toxic profile

We have 15 AMPs : link

REFERENCE ID	AMP CODE + PRIMERS						Peptide Name	Ultramer/g-block	B pairs
	N-ter	F-primer	R-primer	C-ter	F-primer	R-primer			
DRAMP03471	DB.A1	DB.P031	DB.P032	DB.A2	DB.P033	DB.P034	Cg-Def	g-block	158
DRAMP01082	DB.A3	DB.P035	DB.P036	DB.A4	DB.P037	DB.P038	Alyteserin-2a	ultramer	77
DRAMP03809	DB.A5	DB.P039	DB.P040	DB.A6	DB.P041	DB.P042	Pardaxin P-1	g-block	126
DRAMP00943	DB.A7	DB.P043	DB.P044	DB.A8	DB.P045	DB.P046	α-1-purothionin	g-block	162
DRAMP03173	DB.A9	DB.P047	DB.P048	DB.A10	DB.P049	DB.P050	Arenicin-1	ultramer	92
DRAMP03826	DB.A11	DB.P051	DB.P052	DB.A12	DB.P053	DB.P054	Ovispirin-1	ultramer	83
DRAMP04069	DB.A13	DB.P055	DB.P056	DB.A14	DB.P057	DB.P058	V6 peptide	ultramer	84
SNAPP_AMP	DB.A15	DB.P059	DB.P060	DB.A16	DB.P061	DB.P062	PolyVK-11	ultramer	117
SNAPP_AMP	DB.A17	DB.P063	DB.P064	DB.A18	DB.P065	DB.P066	PolyVK-12	ultramer	117
SNAPP_AMP	DB.A19	DB.P067	DB.P068	DB.A20	DB.P069	DB.P070	PolyVK-21	ultramer	117
BAC221	DB.A21	DB.P071	DB.P072	DB.A22	DB.P073	DB.P074	Bactofencin A	ultramer	93
BAC141	DB.A23	DB.P075	DB.P076	DB.A24	DB.P077	DB.P078	Aureocin A53	g-block	180
DRAMP00089	DB.A25	DB.P079	DB.P080	DB.A26	DB.P081	DB.P082	Bacteriocin E50-52	g-block	144
BAC088	DB.A27	DB.P083	DB.P084	DB.A28	DB.P085	DB.P086	Enterocin A	g-block	168
BAC083	DB.A29	DB.P087	DB.P088	DB.A30	DB.P089	DB.P090	Pediocin PA-1	g-block	159

DESIGNING THE *IN SILICO* GOLDEN-GATE ASSEMBLY USING GENEIOUS: link

Plasmid backbone: pDUET-17 (Chlorampehnicol resistance)
Nomenclature for primers + cores + AMPs + final plasmids = link

1. **Cores-** DB.C1-C14 (#1 to #14)
The cores are already prepared with golden-gates.

2. **AMPs-** DB.A1-A2 (#1 to #30)
N- terminal AMPs (for cores that can be tagged on N-terminal as it faces outside)
C- terminal AMPs (for cores that can be tagged on C-terminal as it faces outside)
The caps are tagged with their respective golden gates.

3. **Plasmid-** pDB.000 (duet plasmid with Chloramphenicol resistance)
This plasmid was cut with Avr2 and Nco1 sites to linearize for cloning
The plasmid is prepared/tagged with GG1 and GG4 golden-gate.

4. **Golden-gate overhangs**
Golden_gate sites (GGTCTC-N1-N5) are : undirected and Serine is the linker
Overhangs designed for golden-gate (phyto-bricks for reference - iGEM)
GG1: CATG
GG2: ATCA
GG3: TTCG
GG4: TGAC

5. Final designed plasmids: link
6. Final amino acid sequence: link
7. More details:
    DB.C01 to DB.C14: Cores: link
    DB.A01 to DB.A30 : AMPs: link
    pDB.000 to pDB.210: Plasmids: link
    DB.P01 to DB.P90: Primers: link

PREPARING (ULTRAMERS + G-BLOCKS + PRIMERS + VECTOR) FOR GOLDEN-GATE

Preparing aliquots of primers:

We received primers from IDT: DB.P01 to DB.P90 : use nuclease free H2O

1. Make freezer stock: resuspend primers @ 100 uM concentration
    a. Example: 30 nM primer + 300 ul dH2O
2. Make working stock @ 10 uM concentration
    a. Example: 1:10 dilution of freezer stock (20 ul F.stock + 180 ul dH2O)

Preparing the aliquots of g-blocks, received from IDT:

We received g-blocks @ 250 ng concentration

1. Resuspended in dH2O to make 2 ng/ul concentration
    a. Example: 250 ng G-block + 125 ul dH2O

Amplification of g-blocks:

1. PCR of g-blocks (13 CORES + 14 AMPs) Q-5 master mix protocol
2. Run a gel for verification (2% agarose gel for g-blocks shorter than 500 bp)
3. Do a PCR-clean-up for each of these g-blocks

Preparing the aliquots of ultramers, received from IDT:

We received ultramers @ 4nM concentration.

1. Resuspend in dH2O to make 100 pM/ul concentration.
    a. Example: 4 nM ultramer + 40 ul dH2O
2. Anneal the ultramers:
    a. Following reaction is made for each ultramer pair (forward + reverse):

Reagents	**Volume**
FastDigest buffer	10 ul
dH2O	70 ul
Each ultramer (forward+reverse) @ 100 pM/ul concentration	10 ul
Total volume per reaction	**100 ul**
Final concentration of ultramers	**10 pM/ul**

    b. Perform the annealing reaction in following steps:
        i. 99* C - 5 mins
        ii. 55* C - 5 mins
        iii. Bring to room temperature slowly
3. Dilute each annealed ultramer from 10 pM/ul to 0.1 pM/ul
    a. Example: 890 ul dH2O + 100 ul buffer + 10 ul annealed ultramer

Preparing plasmid backbone: p17

1. Make 30 ug/ml Chloramphenicol + LB agar plates (LB+Cm)
2. Streak the plasmid (received from Jake) on a LB+Cm plate - incubate 24 hrs - 37* C
    a. pacYcDUET-1 - p17 plasmid
    b. In NEB turbo strain
3. Pick a colony and transfer to 50 ml LB+Cm broth - incubate 24 hrs - 37* C + shaking
4. Do a plasmid mini-prep from the cell-suspension (protocol: 202-250 AA_Biotech)
5. We got 6 aliquots of plasmid after the mini-prep

1	24.4 ng/ul in 60 ul total volume	From 1.5 ml culture
2	47.7 ng/ul in 60 ul total volume	From 3.0 ml culture
3	84.4 ng/ul in 60 ul total volume	From 10 ml culture
4	90 ng/ul in 60 ul total volume	From 10 ml culture
5	100.2 ng/ul in 60 ul total volume	From 10 ml culture
6	118.6 ng/ul in 60 ul total volume	From 10 ml culture

6. We took aliquot no. 6 for further experiments
7. Digest pDB.000 with NotI to linearize
    a. 1 ul of FastDigest NotI can digest 1 ug of plasmid in 30 minutes
    b. So we used 5 ul of NotI to digest 2 ug plasmid for 90 minutes
    c. Details:

Reagents	**Volume**
Purified plasmid (~100 ng/ul)	20 ul
H2O	153 ul
10X FastDigest Buffer	20 ul
FastDigest NotI	5 ul
Alkaline phosphatase	2 ul
Total volume	**200 ul**

8. After digestion do a Clean-up with PCR purification column: JENA Bio kit
    a. Got 2 tubes, each has final volume 40 ul with DNA @ 1.4 ul concentration

9. Continue for a PCR using Q5 master-mix (NEB)
    a. Use primers DB.P01 (forward) and DB.P02 (reverse)
    b. Details: Make 10X of reaction mix to have 10 aliquots at the end of PCR

Reagents	**Volume per aliquot**
Template (plasmid)	1 ul
Primers (forward + reverse)	5 ul each (5+5 = 10 ul)
Q5 Master mix	50 ul
Nuclease free dH2O	39 ul
Total volume per aliquot	**100 ul**

10. Setup PCR machine : details = Q5 master-mix (NEB)

Steps	**Temperature *C**	**Time (plasmids)**	**Time (g-blocks)**
Initial denaturation	95	30 seconds	30 seconds
34 cycles	98	*5-10 seconds*	*5-10 seconds*
34 cycles	55	*10-30 seconds*	*10-30 seconds*
34 cycles	72	1.5 mins	15 seconds
Final extension	72	2 minutes	2 minutes
Hold	4-10	Infinite hold	Infinite hold

11. After PCR - run a gel to verify linearization of plasmid: do for each aliquot
    a. 8 ul of DNA + 2 ul loading dye or 2 ul of DNA + 2 ul loading dye + 6 ul dH2O
    b. 8 ul ladder DNA + 2 ul loading dye
    c. 100 Volts - 90 minutes
12. Continue with PCR clean-up for all of the 10 aliquots: JENA Bio kit
13. All plasmid aliquots (10 aliquots) were pooled into 3 aliquots of 50 ul volume each
    a. We have following DNA concentration in each aliquot

1	365 ng/ul in 50 ul total volume (verify on nanodrop)
2	438 ng/ul in 50 ul total volume (verified)
3	345 ng/ul in 50 ul total volume (verify on nanodrop)

14. We continue with aliquot no. 2 of pDB.000 for further experiments
    a. pDB.000 = linearized + has Golden-gate 1 and golden-gate 2

Golden-gate assembly: NEB golden gate

1. Requirements:
    a. SOC broth + golden-gate mix + DNA
    b. DNA requirements:
        i. 75–100 ng each plasmid (2:1 molar ratio = insert:vector)
        ii. We have plasmid @ 0.038 pM and ultramer inserts @ 0.1 pM
        iii. Our plasmid DNA conc= 438 ng/ul. Therefore we will use 2 ul of plasmid pDB.000 (aliquot no.2).
2. Reaction details:

Reagents	**Volume**
Plasmid pDB.000	2 ul
Insert DNA (each insert)	1 ul
Golden-gate Buffer	2 ul
G-G mix	1 ul
dH2O	14 ul (changes if you have more inserts)
Total volume per reaction	**20 ul**

3. After mixing well, let it react
    a. 37*C for 1 hour
    b. 55*C for 5 mins (imperatively for 5 mins only)
    c. This is the final G.G reaction: pDB.197 (DB.A03 + DB.C14 + DB.A04)
4. Transfect: 20 ul competent cells (NEB aplha/beta cells) with 2 ul G.G reaction
    a. Incubate on ice - 30 mins
    b. Heat shock for 30 seconds @ 42*C
    c. Bring back to ice for 5 mins
    d. Add 180 ul of SOC broth
    e. Plate at 2 concentrations: to have a hint on transformation efficiency
        i. 20 ul/plate - to expect low number of transformed colonies
        ii. 180 ul/plate - to expect high number of transformed colonies
        iii. **NOTE**: We used Jake's DH5a cells and NEB-a competent cells
        iv. **NOTE**: We will continue from the plate that gives the highest number of transformed CFU or the one that looks promising

Colony PCR and verification of Golden-gate assembly

1. Resuspend the selected colonies in 100 ul dH2O
    a. Take 3 colonies in total to check
2. From this:
    a. Take 50 ul and resuspend in 10 ml LB+Cm broth to preserve the clone.
    b. Take the rest 50 ul and heat @ 90*C for 2 minutes for cell lysis
        i. Vortex before and after the heating
3. Do a PCR: Dreamtaq GREEN
    a. Details:Choose the appropriate primers: We take the forward primer of the N-ter insert and reverse primer of the C-ter insert. This allows us to amplify the sequence if and only if it is assembled in proper orientation and order.
        i. Example: For plasmid no. pDB.197= DB.A03 - DB.C14 - DB.A04
            1. We use the Forward primer of DB.A03 (i.e DB.P35) and the reverse primer of DB.A04 (DB.P38).

Reagents	**Volume**
Lysed bacterial suspension (each colony)	2 ul
Primers each (Forward and reverse)	5 ul (5+5 = 10 ul)
DreamTaq Master Mix	50 ul
dH2O	38 ul
Total volume	**100 ul per reaction**

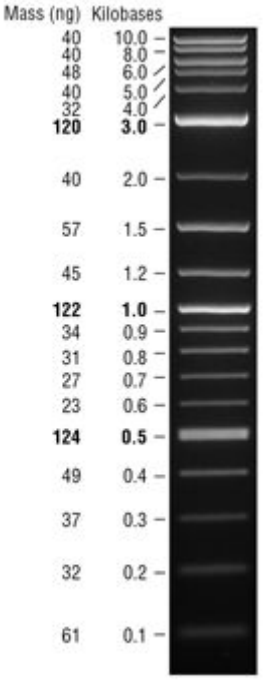
    b. Setup the PCR machine according to the instructions: Dreamtaq GREEN
        i. We have 3 colonies = 3 PCR reactions
        ii. Most of our primers have Tm ranging from 55-62, so our annealing temperature will be 55*C

Step	**Temperature *C**	**Time**	**Cycles**
Initial denaturation	95	1-3 min	1
Denaturation	95	30 s	34
Annealing	55	30 s	34
Extension	72	1 min	34
Final extension	72	5 min	1

    c. After PCR:
        i. Measure the DNA concentration by nanodrop
        ii. Run a gel for verification of inserts and assembly

DNA Gel-electrophoresis protocol

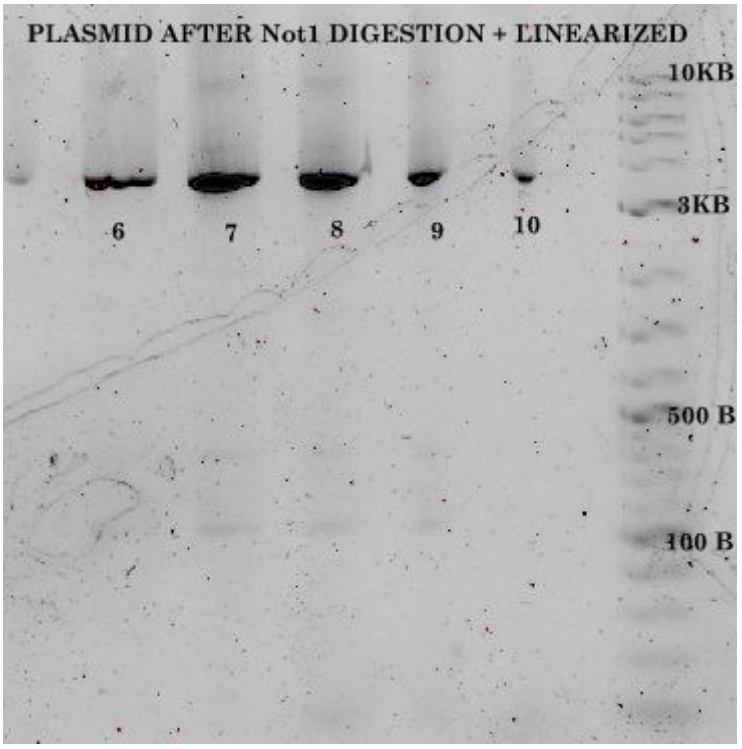
1. Protocol: protocol reference
    a. Loading: 2 ul DNA + 2 ul loading dye + 6 ul dH2O = (10 ul total)
    b. Ladder: 2 ul ladder + 2 ul loading dye + 6 ul dH2O = (10 ul total)
        i. Ladder reference = N0550S 2 log Ladder



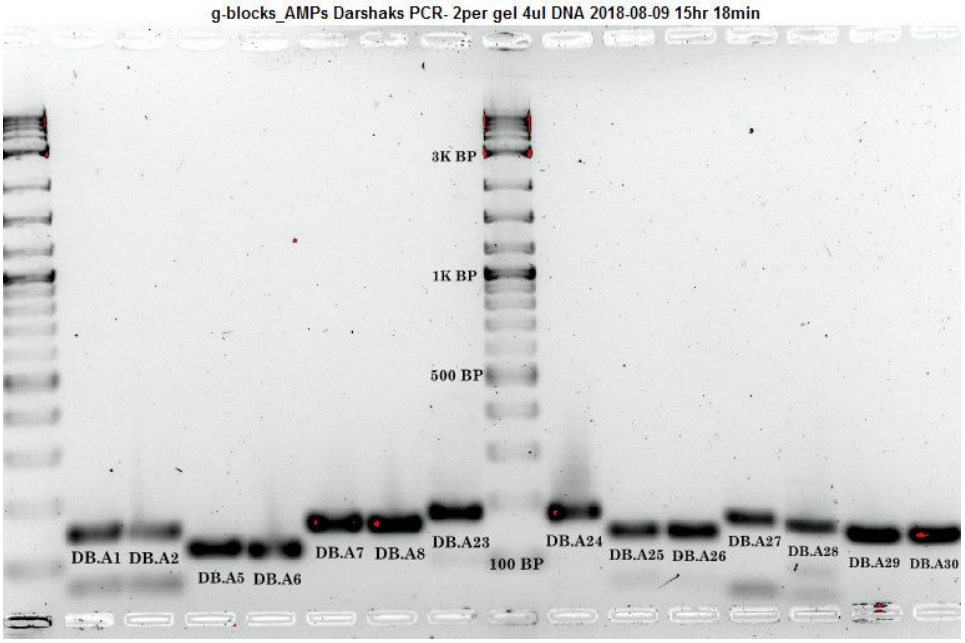
2. NOTES:
    a. If DNA is shorter than 200 base pairs, then use 2 % agarose gel
    b. If DNA is less concentrated then use 4 ul DNA / 10 ul total

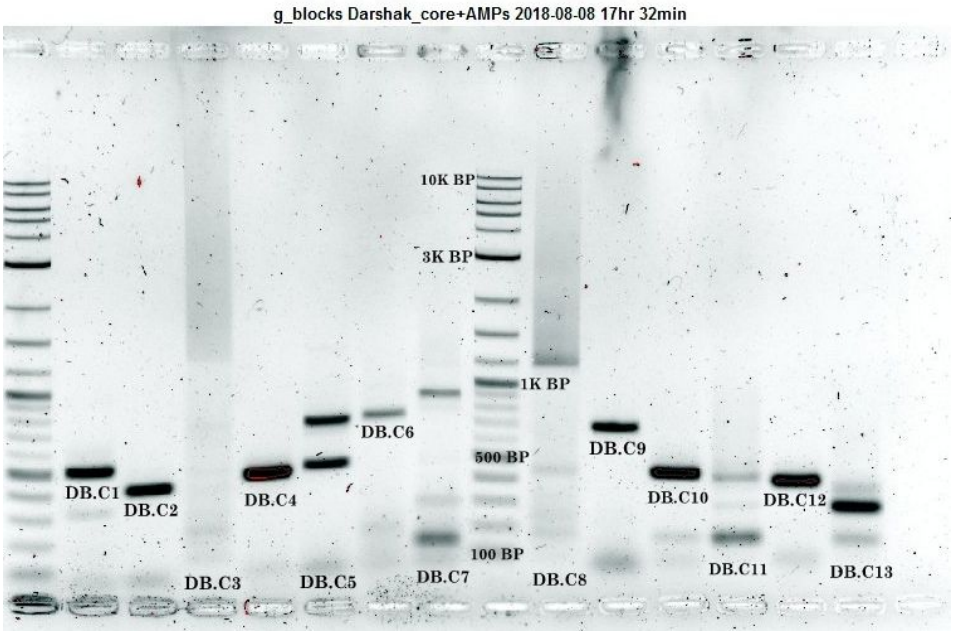
RESULTS

1. **Gel 1 :**
    a. Linearized plasmid-p17 + PCR with primers (DB.P01+DB.P02) to obtain pDB.000 (3 kb size)
    b. 1% gel + 2 ul DNA / 10 ul total

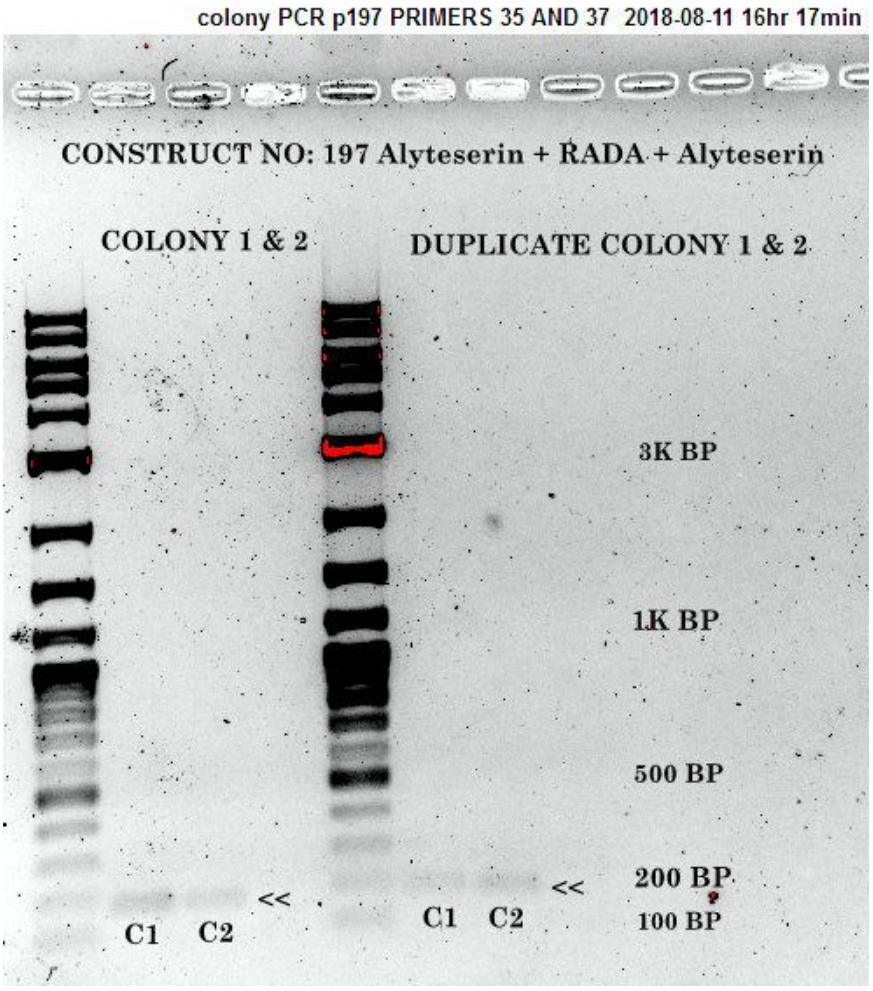


2. **Gel 2 :**
    a. G-blocks (cores and AMPs) amplified with respective primers via G5 m-mix
    b. 2% gel + 4 ul DNA / 10 ul total

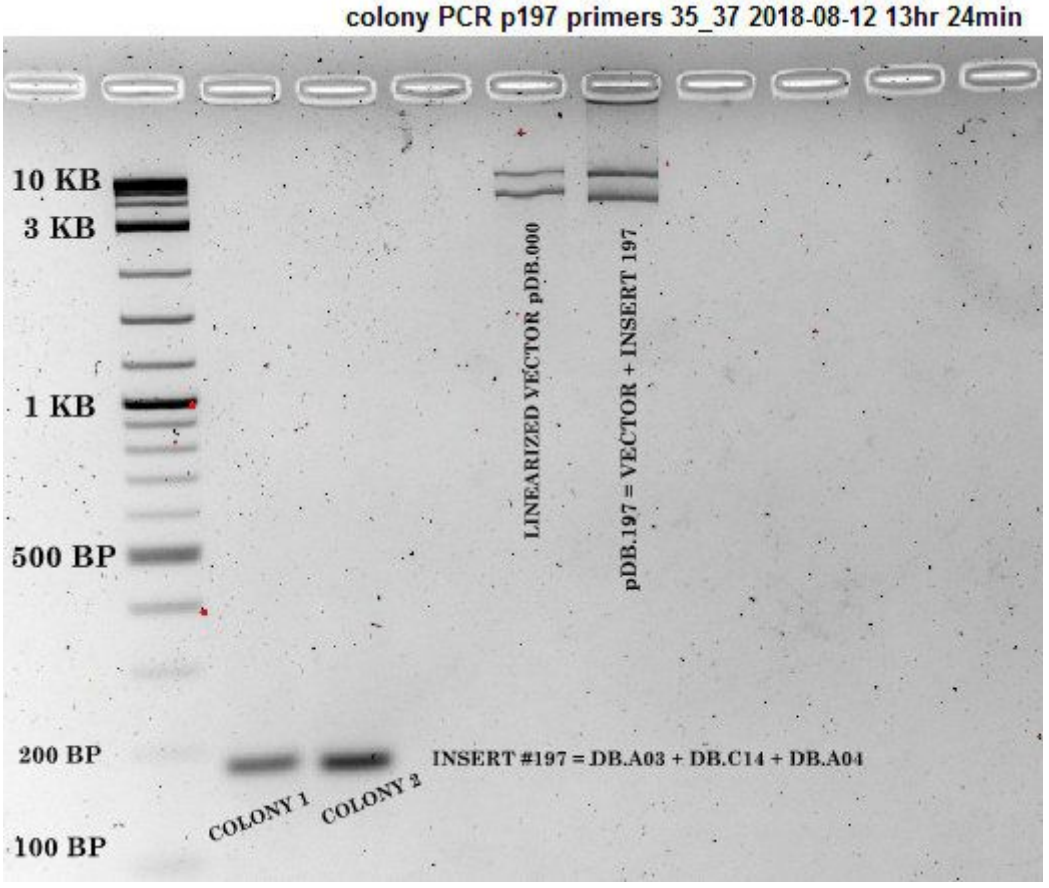




3. **Gel 3 :**
    a. Colony PCR for pDB.197 (with primers DB.P35+DB.P38)
    b. 1 % gel + 4 ul DNA / 10 ul total



4. **Gel 4 :**
    a. Colony PCR for pDB.197 (with primers DB.P35+DB.P38)
    b. Linearized plasmid pDB.000 (Not1 digested) and whole plasmid pDB.197
    c. 2 % gel + 6 ul DNA for amplified insert and 3 ul DNA for plasmids / 10 ul total



5. **New data**
6. **New data**