For the amplification of the promoter and the gene we transformed them into Escherichia coli JM-109 and Dh5-Alpha bacteria.

	Sample ID	Method	Competent cell type	Volume	Annotation
Promoter	P1	thermal shock	JM 109	150 µL	
	P2	thermal shock	JM 109	150 µL	
	P3	thermal shock	JM 109	150 µL	no colony
	P4	electroporation	DH5-Alpha	200 µL	
	P5	electroporation	DH5-Alpha	200 µL	
	P6	electroporation	DH5-Alpha	200 µL	no colony
Gene	G1	thermal shock	JM 109	150 µL	
	G2	thermal shock	JM 109	150 µL	
	G3	thermal shock	JM 109	150 µL	
	G4	electroporation	DH5-Alpha	200 µL	
	G5	electroporation	DH5-Alpha	200 µL	
	G6	electroporation	DH5-Alpha	200 µL	

We used both heat shock and electroporation in order to get safe results.
During thermal shock the reaction mixes were incubated at 37 °C for 10 min. After the incubation, we transformated E.coli JM109 competent cells with the samples of table (number) using thermal shock by the following protocol.

1. On ice, to 50 µL of E. coli JM109 competent cell solution 5 µL of the reaction mix was confused in an Eppendorf-tube, then the solution was left on ice for 30 minutes. During that, SOC-containing soil was placed on ice as well.
2. After 30 minutes, thermal shock was accomplished at 42 °C for 60 seconds.
3. When the thermal shock was ready, the Eppendorf-tubes were placed back on ice, and right after it, 200 µL of the SOC-containing soil was pipetted into each Eppendorf-tube.

The mixtures were incubated at 37 °C for 1 hour.

In the case of electroporation we used 0.1 cm tubes, and we applied 1.8 kV.

In 10 of 12 Petri-dishes containing LB and Ampicillin, the colonies have successfully developed and we were able to select individual ones and grow them in test tubes. We used a growth medium containing LB and Ampicillin as well, therefore only successfully transformated bacteria with were able to produce.

For the amplification of plasmids pMHE5, pMHE7 and pDSK5 we cultivated the bacteria containing them in mediums including antibiotic. In the case if bacteria containing pDSK5 they were grown in LB medium with Kanamycin and bacteria with pMHE5 and pMHE7 were cultivated in LB medium containing Streptomycin. With this method, only the certain plasmid-containing bacteria were able to grow, preventing being infected by other ones. From every Petri-dishes 16 colonies were separated and put in individual test tubes.

Petri-dishes	Test-tubes	Plasmid	Antibiotic
1.	pM5 01	pMHE5	Streptomycin
	pM5 02	pMHE5	Streptomycin
	pM5 03	pMHE5	Streptomycin
	pM5 04	pMHE5	Streptomycin
	pM5 05	pMHE5	Streptomycin
	pM5 06	pMHE5	Streptomycin
	pM5 07	pMHE5	Streptomycin
	pM5 08	pMHE5	Streptomycin
	pM5 09	pMHE5	Streptomycin
	pM5 10	pMHE5	Streptomycin
	pM5 11	pMHE5	Streptomycin
	pM5 12	pMHE5	Streptomycin
	pM5 13	pMHE5	Streptomycin
	pM5 14	pMHE5	Streptomycin
	pM5 15	pMHE5	Streptomycin
	pM5 16	pMHE5	Streptomycin
2.	pM7 01	pMHE7	Streptomycin
	pM7 02	pMHE7	Streptomycin
	pM7 03	pMHE7	Streptomycin
	pM7 04	pMHE7	Streptomycin
	pM7 05	pMHE7	Streptomycin
	pM7 06	pMHE7	Streptomycin
	pM7 07	pMHE7	Streptomycin
	pM7 08	pMHE7	Streptomycin
	pM7 09	pMHE7	Streptomycin
	pM7 10	pMHE7	Streptomycin
	pM7 11	pMHE7	Streptomycin
	pM7 12	pMHE7	Streptomycin
	pM7 13	pMHE7	Streptomycin
	pM7 14	pMHE7	Streptomycin
	pM7 15	pMHE7	Streptomycin
	pM7 16	pMHE7	Streptomycin
3.	pD5 01	pDSK5	Kanamycin
	pD5 02	pDSK5	Kanamycin
	pD5 03	pDSK5	Kanamycin
	pD5 04	pDSK5	Kanamycin
	pD5 05	pDSK5	Kanamycin
	pD5 06	pDSK5	Kanamycin
	pD5 07	pDSK5	Kanamycin
	pD5 08	pDSK5	Kanamycin
	pD5 09	pDSK5	Kanamycin
	pD5 10	pDSK5	Kanamycin
	pD5 11	pDSK5	Kanamycin
	pD5 12	pDSK5	Kanamycin
	pD5 13	pDSK5	Kanamycin
	pD5 14	pDSK5	Kanamycin
	pD5 15	pDSK5	Kanamycin
	pD5 16	pDSK5	Kanamycin

At this point we had 18 test tubes with the E coli containing the synthetized gene and 12 test tubes with the E coli containing the synthetized promoter. We vortexed the test tubes and then we united them in groups of three (Figure 1)into Eppendorf tubes and then centrifuged them thus one Eppendorf tube holds bacteria from 3 test tubes and then we discarded the supernatant.

For the miniprep we used the GeneJET Plasmid Miniprep Kit(Thermo Fisher Scientific, Catalog number: K0502) which contains 15 mL Resuspension Solution, 15 mL Lysis Solution, 20 mL Neutralization Solution, 20 mL Wash Solution, 0.15 mL RNase A and 4 mL Elution Buffer (10 mM Tris-HCl, pH 8.5) 50 pieces of GeneJET Spin Columns 50 pieces of Collection Tubes (2 mL). To prepare the buffer we had to add the RNase A solution to the Resuspension Solution and mix, add 35mL ethanol (96%) to the Wash Solution.

Before starting the protocol of the kit we had 6 Eppendorf tubes with the E.coli containing the synthetised gene and 4 with the E. coli containing the synthesised promoter.(Figure 1).

1. We resuspended the pelleted cells in 250 µL of the Resuspension Solution in 2mL Eppendorf tubes. We had the bacteria resuspended by vortexing until no cell clumps remained.
2. Then we added 250 µL of the Lysis Solution and mixed it by inverting the tube 4-6 times until the solution became viscous and slightly clear.
3. We added 350 µL of the Neutralization Solution and mixed it immediately by inverting the tube 4-6 times. The neutralized bacterial lysate became cloudy. We centrifuged them for 5 minutes to pellet cell debris and chromosomal DNA
4. We transfered the supernatant to the GeneJET spin columns by decanting. We centrifuged them for 1 minute, discard the flow-through and placed the column back into the same collection tube.
5. We added 500 µL of the Wash Solution to the GeneJET spin column, centrifuged them for 1minute and discarded the flow-through and placed the column back into the same collection tube.
6. We repeated the wash procedure using 500 µL of the Wash Solution, discard the flow-through and centrifuged for an additional 1 min to remove residual Wash Solution.
7. We transfered the GeneJET spin column into a fresh 1.5 mL Eppendorf tube then we added 50 µL of the Elution Buffer(which we rewarmed to 70°C)to the center of GeneJET spin column membrane to elute the plasmid DNA.
8. We incubated them for 2 minute at room temperature and centrifuged them for 2 minute. Finally we discard the column and stored the purified plasmid DNAs at -20°C.

test tubes	After protocol Eppendorf tubes	content
G11	G1	Plasmid cointaining LDH gene +50µL Elution Buffer
G12		
G13		
G21	G2	Plasmid cointaining LDH gene +50µL Elution Buffer
G22		
G23		
G31	G3	Plasmid cointaining LDH gene +50µL Elution Buffer
G32		
G33		
G41 X	G4 X	Plasmid cointaining LDH gene +50µL Elution Buffer
G42 X		
G43 X		
G51	G5	Plasmid cointaining LDH gene +50µL Elution Buffer
G52		
G53		
G61	G6	Plasmid cointaining LDH gene +50µL Elution Buffer
G62		
G63		
P11	P1	Plasmid cointaining promoter +50µL Elution Buffer
P12		
P13		
P21	P2	Plasmid cointaining promoter +50µL Elution Buffer
P22		
P23		
P41	P4	Plasmid cointaining promoter +50µL Elution Buffer
P42		
P43		
P51	P5	Plasmid cointaining promoter +50µL Elution Buffer
P52		
P53		

Miniprep

We obtained three plausible working plasmids: pMHE5, pMHE7 and pDSK5. We amplified them by letting the E. coli divide which had these plasmids inside them. We had 16 test tubes with bacterial solution for each plasmid. We vortexed the test tubes and then we united them in groups of four(Figure 1)into Eppendorf tubes and then centrifuged them thus one Eppendorf tube holds bacteria from 4 test tubes and then we discarded the supernatant.

For the miniprep we used the GeneJET Plasmid Miniprep Kit(Thermo Fisher Scientific, Catalog number: K0502) which contains 15 mL Resuspension Solution, 15 mL Lysis Solution, 20 mL Neutralization Solution, 20 mL Wash Solution, 0.15 mL RNase A and 4 mL Elution Buffer (10 mM Tris-HCl, pH 8.5) 50 pieces of GeneJET Spin Columns 50 pieces of Collection Tubes (2 mL). To prepare the buffer we had to add the RNase A solution to the Resuspension Solution and mix, add 35mL ethanol (96%) to the Wash Solution.

Before starting the protocol of the kit we had 4 Eppendorf tubes for each type of plasmid(a total of 12 Eppendorf tube).(Figure 1).

1. We resuspended the pelleted cells in 250 µL of the Resuspension Solution in 2mL Eppendorf tubes. We had the bacteria resuspended by vortexing until no cell clumps remained.
2. Then we added 250 µL of the Lysis Solution and mixed it by inverting the tube 4-6 times until the solution became viscous and slightly clear.
3. We added 350 µL of the Neutralization Solution and mixed it immediately by inverting the tube 4-6 times. The neutralized bacterial lysate became cloudy. We centrifuged them for 5 minutes to pellet cell debris and chromosomal DNA
4. We transfered the supernatant to the GeneJET spin columns by decanting. We centrifuged them for 1 minute, discard the flow-through and placed the column back into the same collection tube.
5. We added 500 µL of the Wash Solution to the GeneJET spin column, centrifuged them for 1minute and discarded the flow-through and placed the column back into the same collection tube.
6. We repeated the wash procedure using 500 µL of the Wash Solution, discard the flow-through and centrifuged for an additional 1 min to remove residual Wash Solution.
7. We transferred the GeneJET spin column into a fresh 1.5 mL Eppendorf tube then we added 50 µL of the Elution Buffer(which we rewarmed to 70°C)to the center of GeneJET spin column membrane to elute the plasmid DNA.
8. We incubated them for 2 minute at room temperature and centrifuged them for 2 minute. Finally we discard the column and stored the purified plasmid DNAs at -20°C

plasmids	test tubes(16pieces/plasmid)	before protocol Eppendorf tubes	after protocol Eppendorf tubes	content
pMHE5	pM5 01	pM5 1-4	pMHE5 1-8	pMHE5 plasmids from test tubes pM5 01-08 +100µL Elution Buffer
pMHE5	pM5 02			
pMHE5	pM5 03			
pMHE5	pM5 04			
pMHE5	pM5 05	pM5 5-8		
pMHE5	pM5 06			
pMHE5	pM5 07			
pMHE5	pM5 08			
pMHE5	pM5 09	pM5 9-12	pMHE5 9-16	pMHE5 plasmids from test tubes pM5 09-16 +100µL Elution Buffer
pMHE5	pM5 10			
pMHE5	pM5 11			
pMHE5	pM5 12			
pMHE5	pM5 13	pM5 13-16		
pMHE5	pM5 14			
pMHE5	pM5 15			
pMHE5	pM5 16			
pMHE7	pM7 01	pM7 1-4	pM7 1-8	pMHE7 plasmids from test tubes pM7 01-08 +100µL Elution Buffer
pMHE7	pM7 02			
pMHE7	pM7 03			
pMHE7	pM7 04			
pMHE7	pM7 05	pM7 5-8		
pMHE7	pM7 06			
pMHE7	pM7 07			
pMHE7	pM7 08			
pMHE7	pM7 09	pM7 9-12	pM7 9-16	pMHE7 plasmids from test tubes pM7 09-16 +100µL Elution Buffer
pMHE7	pM7 10			
pMHE7	pM7 11			
pMHE7	pM7 12			
pMHE7	pM7 13	pM7 13-16		
pMHE7	pM7 14			
pMHE7	pM7 15			
pMHE7	pM7 16			
pDSK5	pD5 01	pD5 1-4	pDSK 1-8	pDSK5 plasmids from test tubes pD5 01-08 +100µL Elution Buffer
pDSK5	pD5 02			
pDSK5	pD5 03			
pDSK5	pD5 04			
pDSK5	pD5 05	pD5 5-8		
pDSK5	pD5 06			
pDSK5	pD5 07			
pDSK5	pD5 08			
pDSK5	pD5 09	pD5 9-12	pD5 9-16	pDSK5 plasmids from test tubes pD5 09-16 +100µL Elution Buffer
pDSK5	pD5 10			
pDSK5	pD5 11			
pDSK5	pD5 12			
pDSK5	pD5 13	pD5 13-16		
pDSK5	pD5 14			
pDSK5	pD5 15			
pDSK5	pD5 16			

To separate our gene and promoter from the plasmid they were synthesised into first we had to digest them with the right type of restriction enzyme.(figure 1)

Eppendorf tube	G1E1	G1E2	G1E3	G1E4	G1E5	G1E6	G1E7
NcoI	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
Green Buffer	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
G1	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
Nuclease-free water	14 µL	14 µL	14 µL	14 µL	14 µL	14 µL	14 µL
Total	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

Eppendorf tube	P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P1E7
BglII	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
Green Buffer	2 µL	2 µl	2 µL	2 µL	2 µL	2 µL	2 µL
P1	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL	2µL
Nuclease-free water	14 µL	14 µL	14 µL	14 µL	14 µL	14 µL	14 µL
Total	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

Then we filled them into the pockets of two gels (figure 2) and run them with gel electrophoresis, we used the settings 110V with 400mA for 40 minutes. After the run we shined UV lights on the gels for just for 1 or 2 seconds in a dark room and quickly cut out the gel parts where the lines were furthest away from the pockets because those were our gene and promoter. (it is important to use UV light just for a short time not to damage the DNA)

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	G1E1	G1E2	G1E3	G1E4	G1E5	G1E6	G1E7
volume (µL)	5	10	10	10	10	10	10	10

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	PE1E	P1E2	P1E3	P1E4	P1E5	P1E6	P1E7
volume (µL)	5	10	10	10	10	10	10	10

Following this we used the GeneJET Gel Extraction Kit (Catalog number: K0692) on the sliced gel parts.

This kit contains:

- Binding Buffer (150 mL)
- Wash Buffer (concentrated) (45 mL)
- Elution Buffer (10 mM Tris-HCl, pH 8.5) (30 mL)
- GeneJET Purification Columns (preassembled with collection tubes) (250pieces)

Then we followed the protocol:

1. We placed the gel slices into a pre-weighed 1.5 mL tube and recorded the weight of the gel slices.
2. We added 1:1 volume of Binding Buffer to the gel slices (e.g. 100 µL of Binding Buffer for every 100 mg of agarose gel)
3. We incubated the gel mixture at 60 °C for 10 min or until the gel slice is completely dissolved. (Mixed the tube by inversion every few minutes to facilitate the melting process). We ensured that the gel is completely dissolved, vortexed the gel mixture briefly before loading on the columns.
4. This step was only necessary for the promoter because it was shorter than 500bp. We added 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)and mixed thoroughly.
5. We transferred up to 800 µL of the solubilized gel solution to the GeneJET purification columns. We centrifuged for 1 min, then discarded the flow-through and placed the column back into the same collection tube.
6. We added 700 µL of Wash Buffer to the GeneJET purification columns then centrifuged them for 1 min, discarded the flow-through and placed the column back into the same collection tube.
7. We centrifuged the empty GeneJET purification columns for an additional 1 min to completely remove residual wash buffer.
8. We transferred the GeneJET purification columns into clean 1.5 mL microcentrifuge tubes. We added 25 µL of Elution Buffer to the center of each purification column membrane and centrifuged them for 1 min.
9. Finally we discarded the GeneJET purification columns and stored the purified DNA at -20 °C.

Tube name	mass (g)	Binding Buffer (µL)	Wash Buffer (µL)	Elution Buffer (µL)	pH buffer	isopropanol (µL)	Eppendorf tube after protocol	Final volume (µL)
G1E1	0,88	880	700	25	-	-	G1E1T	25
G1E2	0,57	570	700	25	-	-	G1E2T	25
G1E3	0,66	660	700	25	-	-	G1E3T	25
G1E4	0,64	640	700	25	-	-	G1E4T	25
G1E5	0,6	600	700	25	-	-	G1E5T	25
G1E6	0,7	700	700	25	-	-	G1E6T	25
P1E1	0,4	400	700	20	-	400	P1E1T	20
P1E2	0,54	540	700	20	-	540	P1E2T	20
P1E3	0,44	440	700	20	-	440	P1E3T	20
P1E4	0,39	390	700	20	-	390	P1E4T	20
P1E5	0,38	380	700	20	-	380	P1E5T	20
P1E6	0,29	290	700	20	-	290	P1E6T	20
P1E7	0,38	380	700	20	-	380	P1E7T	20

To separate our gene and promoter from the plasmid they were synthesised into first we had to digest them with the right type of restriction enzyme, this time we cut out the gene with NcoI and EcoRV too.(figure 1)

Eppendorf tube	G1G1	G1G2
NcoI	1,5 µl	1,5 µl
EcoRV	1,5 µL	1,5 µL
Green Buffer	3 µl	3 µl
G1	5 µL	5 µL
Nuclease-free water	19µL	19 µL
Total	30 µL	30 µL

Eppendorf tube	P1G12	P1G34	P1G56
BglII	2 µL	2 µL	2µL
Green Buffer	2 µl	2 µl	2 µl
P1	8 µl	8 µl	8 µl
Nuclease-free water	8 µL	8 µL	8 µL
Total	20 µl	20 µl	20µl

Then we filled them into the pockets of two gels (figure 2) and run them with gel electrophoresis, we used the settings 110V with 400mA for 40 minutes. After the run we shined UV lights on the gels for just for 1 or 2 seconds in a dark room and quickly cut out the gel parts where the lines were furthest away from the pockets because those were our gene and promoter. (it is important to use UV light just for a short time not to damage the DNA)

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	G1G1	G1G1	G1G1	G1G2	G1G2	G1G2	-
volume (µL)	5	10	10	10	10	10	10	-

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	P1G12	P1G12	P1G34	P1G34	P1G56	P1G56	-
volume (µL)	5	10	10	10	10	10	10	-

Following this we used the GeneJET Gel Extraction Kit (Catalog number: K0692) on the sliced gel parts.

This kit contains:

- Binding Buffer (150 mL)
- Wash Buffer (concentrated) (45 mL)
- Elution Buffer (10 mM Tris-HCl, pH 8.5) (30 mL)
- GeneJET Purification Columns (preassembled with collection tubes) (250pieces)

Then we followed the protocol:

1. We placed the gel slices into a pre-weighed 1.5 mL tube and recorded the weight of the gel slices.
2. We added 1:1 volume of Binding Buffer to the gel slices (e.g. 100 µL of Binding Buffer for every 100 mg of agarose gel)
3. We incubated the gel mixture at 60 °C for 10 min or until the gel slice is completely dissolved. (Mixed the tube by inversion every few minutes to facilitate the melting process). We ensured that the gel is completely dissolved, vortexed the gel mixture briefly before loading on the columns.
4. This step was only necessary for the promoter because it was shorter than 500bp. We added 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)and mixed thoroughly.
5. We transferred up to 800 µL of the solubilized gel solution to the GeneJET purification columns. We centrifuged for 1 min, then discarded the flow-through and placed the column back into the same collection tube.
6. We added 700 µL of Wash Buffer to the GeneJET purification columns then centrifuged them for 1 min, discarded the flow-through and placed the column back into the same collection tube.
7. We centrifuged the empty GeneJET purification columns for an additional 1 min to completely remove residual wash buffer.
8. We transferred the GeneJET purification columns into clean 1.5 mL microcentrifuge tubes. We added 20 µL of Elution Buffer to the center of each purification column membrane and centrifuged them for 1 min.
9. Finally we discarded the GeneJET purification columns and stored the purified DNA at -20 °C.

Eppendorf tube	mass (g)	Binding Buffer (µL)	Wash Buffer (µL)	Elution Buffer (µL)	isopropanol	Eppendorf tube after protocol	final volume (µL)
G1G1	0,30	300	700	20	-	G1G1T	40
G1G2	0,26	260	700	20	-		
G1G3	0,24	240	700	20	-	G1G2T	40
G1G4	0,22	220	700	20	-		
G1G5	0,20	200	700	20	-	G1G3T	40
G1G6	0,32	320	700	20	-		
P1G1	0,93	930	700	40	930	P1G1T	40
P1G2	0,80	800	700	40	800	P1G2T	40
P1G3	0,92	920	700	40	920	P1G3T	40

To provide place for the promoter in the pMHE5 and pMHE7 plasmids first we digested them with BglII.(figure 1)

Eppendorf tube	M5RB1	M5RB2	M5RB3	M7RB1	M7RB2	M7RB3
sample	pMHE5	pMHE5	pMHE5	pMHE7	pMHE7	pMHE7
sample volume	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL
Green Buffer	2 µL	2 µL	2 µL	2 µL	2 µL	2 µL
BglII	2 µL	2µL	2 µL	2 µL	2 µL	2 µL
nuclease-free water	12 µL	12 µL	12 µL	12 µL	12 µL	12 µL
Total	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

Then we separated the DNA fragments from all the other parts of this solution with gel electrophoresis. We filled them into the gel pockets(figure 2) and run them on the settings 110V with 400mA for 40 minutes. After the run we shined UV lights on the gels for just for 1 or 2 seconds in a dark room and quickly cut out the gel parts where the lines were. (it is important to use UV light just for a short time not to damage the DNA)

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	M5RB1	M5RB1	M5RB2	M5RB2	M5RB3	M5RB3	-
volume (µL)	5	10	10	10	10	10	10	-

gel pocket	1.	2.	3.	4.	5.	6.	7.	8.
content	ladder	M7RB1	M7RB1	M7RB2	M7RB2	M7RB3	M7RB3	-
volume (µL)	5	10	10	10	10	10	10	-

Following this we used the GeneJET Gel Extraction Kit (Catalog number: K0692) on the sliced gel parts.

This kit contains:

- Binding Buffer (150 mL)
- Wash Buffer (concentrated) (45 mL)
- Elution Buffer (10 mM Tris-HCl, pH 8.5) (30 mL)
- GeneJET Purification Columns (preassembled with collection tubes) (250pieces)

Then we followed the protocol:

1. We placed the gel slices into a pre-weighed 1.5 mL tube and recorded the weight of the gel slices.
2. We added 1:1 volume of Binding Buffer to the gel slices (e.g. 100 µL of Binding Buffer for every 100 mg of agarose gel)
3. We incubated the gel mixture at 60 °C for 10 min or until the gel slice is completely dissolved. (Mixed the tube by inversion every few minutes to facilitate the melting process). We ensured that the gel is completely dissolved, vortexed the gel mixture briefly before loading on the columns.
4. We transferred up to 800 µL of the solubilized gel solution to the GeneJET purification columns. We centrifuged for 1 min, then discarded the flow-through and placed the column back into the same collection tube.
5. We added 700 µL of Wash Buffer to the GeneJET purification columns then centrifuged them for 1 min, discarded the flow-through and placed the column back into the same collection tube.
6. We centrifuged the empty GeneJET purification columns for an additional 1 min to completely remove residual wash buffer.
7. We transferred the GeneJET purification columns into clean 1.5 mL microcentrifuge tubes. We added 20 µL of Elution Buffer to the center of each purification column membrane and centrifuged them for 1 min.
8. Finally we discarded the GeneJET purification columns and stored the purified DNA at -20 °C.

Tube name	mass (g)	Binding Buffer (µL)	Wash Buffer (µL)	Elution Buffer (µL)	pH buffer	isopropanol (µL)	Eppendorf tube after protocol	Final volume (µL)
M5E1	0,49	490	700	20	-	-	M5E1T	40
M5E2	0,28	280	700	20	-	-		
M5E3	0,39	390	700	20	-	-	M5E2T	40
M5E4	0,45	450	700	20	-	-		
M5E5	0,38	380	700	20	-	-	M5E3T	40
M5E6	0,36	360	700	20	-	-		
M7E1	0,39	390	700	20	-	-	M7E1T	40
M7E2	0,38	380	700	20	-	-		
M7E3	0,43	430	700	20	-	-	M7E2T	40
M7E4	0,33	330	700	20	-	-		
M7E5	0,36	360	700	20	-	-	M7E3T	40
M7E6	0,38	380	700	20	-	-		

Protocol for ligation of the LDH gene and the plasmid

Plasmid-LDH ligation

The ligation of the lactate-dehydrogenase (LDH) gene and the pMHE5 / pMHE7 plasmid was carried out with the Thermo Scientific T4 DNA ligase. Plasmid DNA concentration in the samples are shown by the table.

Sample ID	DNA concentration (ng/μL)
G1G1T	11.65
G1G2T	6.54
G1G3T	11.06
P1G1T	5.76
P1G2T	3.37
P1G3T	3.92
M5E1T	4.49
M5E2T	3.55
M5E3T	2.57
M7E1T	2.24
M7E2T	2.03
M7E3T	2.46

Table

Since we knew the exact DNA content of the samples, we could carry out the ligation. The ligation mixture was composed as shown by table.

	I. reaction mixture	II. reaction mixture	III. reaction mixture	IV.reaction mixture
Plasmid sample	M5E1T	M5E2T	M7E1T	M7E3T
Plasmid sample volume (µL)	10	13	20	20
Gene sample	G1G3T	G1G2T	G1G1T	G1G3T
Gene sample volume (µL)	3	4	2	2
Buffer volume (µL)	4	4	4	4
Thermo Scientific T4 DNA ligase volume (µL)	1	1	1	1
Nuclease-free water volume (µL)	2	-	-	-
Total volume (µL)	20	22	27	27

Table

The reaction mixtures were incubated at 37 °C for 10 minutes.

After the incubation, the putative construct of the ligated plasmid and gene were transferred into Escherichia coli JM109 competent cells with thermal shock by the following protocol:

1. On ice, to 50 µL of E. coli JM109 competent cell solution 5 µL of the reaction mix was confused in an Eppendorf-tube, then the solution was left on ice for 30 minutes.
   During that, SOC-containing soil was placed on ice as well.
2. After 30 minutes, thermal shock was accomplished at 42 °C for 60 seconds.
3. When the thermal shock was ready, the Eppendorf-tubes were placed back on ice, and right after it, 200 µL of the SOC-containing soil was pipetted into each Eppendorf-tube.
4. The mixtures were incubated at 37 °C for 1 hour.

Scaling up plasmid DNA

Under Laminar Air-Flow 200 µL of each samples was placed on Petri-dishes containing LB and Streptomycin, and as a control, 50 µL of each samples was placed on Petri-dishes containing only LB. The samples were incubated at 37 °C for a day. At the end of the incubation time, the result was 5 colonies containing the putative pMHE5-LDH construct, and 11 colonies containing the putative pMHE7-LDH construct, both on Petri-dishes containing LB and Streptomycin. Each colony on the LB and Streptomycin containing Petri-dishes was transferred into test tubes containing 2 mL of LB and Streptomycin. These bacterial cultures were incubated at 37 °C for 2 days.

DNA purification

Plasmid DNAs were purified using the Thermo Scientific GeneJET Plasmid Miniprep Kit.

1. We resuspended the pelleted cells in 250 µL of the Resuspension Solution in 2 mL Eppendorf tubes. Bacteria were resuspended by vortexing until no cell clumps remained.
2. 250 µL of the Lysis Solution was added and mixed by inverting the tube 4-6 times until the solution became viscous and slightly clear.
3. 350 µL of the Neutralization Solution was added and mixed immediately by inverting the tube 4-6 times. The neutralized bacterial lysate became cloudy. They were centrifuged for 5 minutes to pellet cell debris and chromosomal DNA.
4. We transferred the supernatant to the GeneJET spin columns by decanting. We centrifuged them for 1 minute to discard the flow-through and placed the column back into the same collection tube.
5. We added 500 µL of the Wash Solution to the GeneJET spin column then centrifuged them for 60 seconds, discarded the flow-through and placed the column back into the same collection tube.
6. We repeated the wash procedure using 500 µL of the Wash Solution, discarded the flow-through and centrifuged for an additional 60 seconds to remove residual Wash Solution.
7. We transferred the GeneJET spin column into a fresh 1.5 mL Eppendorf tube then we added 50 µL of the Elution Buffer (which was rewarmed to 70°C) to the center of GeneJET spin column membrane to elute the plasmid DNA.
8. We incubated them for 2 minute at room temperature and centrifuged them for 2 minutes. Finally we discarded the column and stored the purified plasmid DNAs at -20°C.

Control of the putative pMHE5-LDH and pMHE7-LDH construct

We had to make sure that the ligation of the pMHE5 / pMHE7 and the LDH was successful. Therefore, we carried out a gel electrophoresis analysis. As figure shows, the (here the exact number) samples contained an approximately 1000 base pairs long DNA sequence, which must be due to the successfully inserted LDH sequence, so the ligation of the pMHE5 / pMHE7 and the LDH was proven successful.

Restriction of the purified plasmids with BglII

The purified pMHE5-LDH and pMHE7-LDH constructs were restricted with the Thermo Scientific FastDigest BglII restriction kit. The composition of the reaction mixes are shown by table.

BglII volume (µL)	3
Buffer volume (µL)	3
Volume of plasmid sample (µL)	8
Volume of nuclease-free water (µL)	16
Total volume (µL)	30

Table

Ligation of the pMHE5-LDH / pMHE7-LDH constructs and the putative promoter

The pMHE5-LDH (S5.1M-S5.5M) / pMHE7-LDH (S7.1M-S7.7M) constructs and the promoter were ligated using the Thermo Scientific T4 DNA Ligase kit. The composition of the reaction mixtures are shown by table.

	I. RM	II. RM	III. RM	IV. RM	V. RM	VI. RM	VII. RM	VIII. RM	IX. RM	X. RM
Plasmid sample	S5.1M	S5.1M	S5.2M	S5.3M	S5.4M	S5.5M	S7.1M	S7.2M	S7.6M	S7.7
Plasmid sample volume (µL)	10	10	10	10	10	10	10	10	10	10
Promoter sample	P1G1T	P1G1T	P1G1T	P1G1T	P1G1T	P1G1T	P1G3T	P1G3T	P1G3T	P1G3T
Promoter sample volume (µL)	5	5	5	5	5	5	7	7	7	7
Buffer volume (µL)	5	5	5	5	5	5	5	5	5	5
T4 DNA ligase volume (µL)	2	2	2	2	2	2	2	2	2	2
Total volume (µL)	22	22	22	22	22	22	24	24	24	24
Sample ID	LP1	LP2	LP3	LP4	LP5	LP6	LP7	LP8	LP9	LP10

RM: abbrev. of Reaction Mixture
Table

The reaction mixes were incubated at 37 °C for 10 min. After the incubation, we transformated E.coli JM109 competent cells with the samples of table (number) using thermal shock by the following protocol.

1. On ice, to 50 µL of E. coli JM109 competent cell solution 5 µL of the reaction mix was confused in an Eppendorf-tube, then the solution was left on ice for 30 minutes.
   During that, SOC-containing soil was placed on ice as well.
2. After 30 minutes, thermal shock was accomplished at 42 °C for 60 seconds.
3. When the thermal shock was ready, the Eppendorf-tubes were placed back on ice, and right after it, 200 µL of the SOC-containing soil was pipetted into each Eppendorf-tube.
4. The mixtures were incubated at 37 °C for 1 hour.

Under Laminar Air-Flow 200 µL of each samples was placed on Petri-dishes containing LB and Streptomycin, and as a control, 50 µL of each samples was placed on Petri-dishes containing only LB. The samples were incubated at 37 °C for a day. At the end of the incubation time, the result was 5 colonies containing the putative pMHE5-LDH-promoter construct, and 11 colonies containing the putative pMHE7-LDH-promoter construct, both on Petri-dishes containing LB and Streptomycin. Each colony on the LB and Streptomycin containing Petri-dishes was transferred into test tubes containing 2 mL of LB and Streptomycin. These bacterial cultures were incubated at 37 °C for 2 days.
After 2 days there were colonies on the Petri-dishes containing LB and Streptomycin:

Sample ID	LP1	LP2	LP3	LP4	LP5	LP6	LP7	LP8	LP9	LP10
Number of colonies	1	1?	2	7	2	-	-	-	1	4

Table

The colonies were transferred into test tubes containing LB and Streptomycin.

1. **Cultivation of Methylococcus capsulatus**

The Methylococcus capsulatus (Bath) bacteria were cultivated on solid LB soil containing nitrate mineral salts (NMS), under methane-air (1:1) mixture in glass flasks. The supply of methane was ensured by a balloon (figure).

1. We attach a balloon to a tube which supply the methane gas from a methane-containing bottle.
2. The balloon is blown up by the gas. The volume of the balloon is almost the same as the volume of bottle containing the Methylococcus bacteria.
3. We close the neck of the balloon with a clip.
4. The balloon is removed into the bacteria-containing tank.
5. Finally, we open the way between the balloon and the tank.

Methylococcus capsulatus bacteria are cultivated on NMS (Nitrate mineral salts) medium at 45°C.

The components of NMS medium are:

ATCC medium: 1306 Nitrate mineral salts medium (NMS)

$MgSO_4 \cdot 7H_2O$ .....1.0 g
$CaCl_2 \cdot 6H_2O$ .....0.20 g
Chelated Iron Solution (see below).....2.0 ml
KNO_3.....1.0 g
Trace Element Solution (see below).....0.5 ml
KH_2PO_4 .....0.272 g
$Na_2HPO_4 \cdot 12H_2O$.....0.717 g
Purified Agar (e.g., Oxoid L28).....12.5 g
Distilled deionized water.....1.0 L

Adjust pH to 6.8. Autoclave at 121C for 15 minutes.

Chelated Iron Solution:
Ferric (III) ammonium citrate*.....0.1 g
EDTA, sodium salt.....0.2 g
HCl (concentrated).....0.3 ml
Distilled deionized water.....100.0 ml

*0.05 g of Ferric (III) chloride may be substituted.

Use 2.0 ml of this chelated iron solution per liter of final medium.

Trace Element Solution:
EDTA.....500.0 mg
$FeSO_4 \cdot 7H_2O$ .....200.0 mg
$ZnSO_4 \cdot 7H_2O$ .....10.0 mg
$MnCl_2 \cdot 4H_2O$ .....3.0 mg
H_3BO_3 .....30.0 mg
$CoCl_2 \cdot 6H_2O$ .....20.0 mg
$CaCl_2 \cdot 2H_2O$ .....1.0 mg
$NiCl_2 \cdot 6H_2O$ .....2.0 mg
$Na_2MoO_4 \cdot 2H_2O$.....3.0 mg
Distilled water.....1.0 L

Autoclave at 121C for 15 minutes.

Lactate assay kit

Based on the homepage of the product; Catalog No. MAK064

Procedure

Lactate Standards for Colorimetric Detection Dilute

10 mL of the 100 nmole/mL Lactate standard with 990 mL of Lactate Assay Buffer to generate a 1 nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 ml of the 1 nmole/mL Lactate standard into a 96 well plate, generating 0 (blank), 2, 4, 6, 8, and 10 nmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL. Lactate Standards for Fluorometric Detection Prepare a 1 nmole/mL standard solution as for the colorimetric assay. Dilute 10 mL of the 1 nmole/mL standard solution with 990 mL of the Lactate Assay Buffer to make a 0.01 nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 mL of the prepared 0.01 nmole/mL standard solution into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL. Sample Preparation Both the colorimetric and fluorometric assays require 50 mL of sample for each reaction (well). Tissue or cells can be homogenized in 4 volumes of the Lactate Assay Buffer. Centrifuge the samples at 13,000 g for 10 minutes to remove insoluble material. Samples should be deproteinized with a 10 kDa MWCO spin filter to remove lactate dehydrogenase. The soluble fraction may be assayed directly. Serum samples (0.5–10 mL/assay) can be assayed directly by adding in duplicate to 96 well plate. If lactate dehydrogenase activity is present, samples should be deproteinized with a 10 kDa MWCO spin filter. Bring samples to final volume of 50 mL/well with Lactate Assay Buffer. For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range. Note: Lactate Dehydrogenase (LDH) will degrade lactate. There, samples containing LDH (such as culture medium or tissue lysate) should be kept –80 °C for storage, and filtered through a 10 kDa cut-off spin filter. Complete medium containing FBS should be deproteinized due to high LDH content.

Lactate Standards for Fluorometric Detection:

Prepare a 1 nmole/mL standard solution as for the colorimetric assay. Dilute 10 mL of the 1 nmole/mLstandard solution with 990 mL of the Lactate Assay Buffer to make a 0.01 nmole/mL standard solution. Add 0, 2, 4, 6, 8, and 10 mL of the prepared 0.01 nmole/mL standard solution into a 96 well plate, generating 0 (blank), 20, 40, 60, 80, and 100 pmole/well standards. Add Lactate Assay Buffer to each well to bring the volume to 50 mL.

Sample Preparation:

Both the colorimetric and fluorometric assays require 50 mL of sample for each reaction (well). Tissue or cells can be homogenized in 4 volumes of the Lactate Assay Buffer. Centrifuge the samples at 13,000 ´ g for 10 minutes to remove insoluble material. Samples should be deproteinized with a 10 kDa MWCO spin filter to remove lactate dehydrogenase. The soluble fraction may be assayed directly. Serum samples (0.5–10 mL/assay) can be assayed directly by adding in duplicate to 96 well plate. If lactate dehydrogenase activity is present, samples should be deproteinized with a 10 kDa MWCO spin filter. Bring samples to final volume of 50 mL/well with Lactate Assay Buffer. For unknown samples, it is suggested to test several sample volumes to make sure the readings are within the standard curve range.

Note: Lactate Dehydrogenase (LDH) will degrade lactate. There, samples containing LDH (such as culture medium or tissue lysate) should be kept –80 °C for storage, and filtered through a 10 kDa cut-off spin filter. Complete medium containing FBS should be deproteinized due to high LDH content.

Assay Reaction

1. Set up the Master Reaction Mix according to the scheme in Table 1. 50 mL of the Master Reaction Mix is required for each reaction (well).

Reagent	Master Reaction Mix
Lactate Assay Buffer	46 ≒L
Lactate Enzyme Mix	2 ≒L
Lactate Probe	2 ≒L

2. Master Reaction MixAdd 50 mL of the Master Reaction Mix to each of the wells. Mix well using a horizontal shaker or by pipetting, and incubate the reaction for 30 minutes at room temperature. Protect the plate from light during the incubation.
3. For colorometric assays, measure the absorbance at 570 nm (A570). For fluorometric assays, measure fluorescence intensity (lex = 535/lem = 587 nm).

Calculations

The background for either assay is the value obtained for the 0 (blank) lactate standard. Correct for the background by subtracting the blank value from all readings. Background values can be significant and must be subtracted from all readings. Use the values obtained from the appropriate lactate standards to plot a standard curve. The amount of lactate present in the samples may be determined from the standard curve. Note: A new standard curve must be set up each time the assay is run.