METHODS

Cloning and transformation protocol – for iGEM Biobrick

The primers (synthesized by IDT)
Item Name: igem_F
Product: 25 nmole DNA Oligo
Purification: Standard Desalting
Sequence: GAA TTC GCG GCC GCT TCT AGA TGC GCA GAA CGA CAG GCA G
Quantity: 1
Item Name: igem_R
Product: 25 nmole DNA Oligo
Purification: Standard Desalting
Sequence: CGA CGC GGA CGG CCT GGT GAT ACT AGT AGC GGC CGC TGC AG

The PCR protocol
1. Gently vortex and briefly centrifuge Maxima Hot Start Green PCR Master Mix (2X) after thawing.
2. Add the following components for each 50 µl reaction at room temperature:

Maxima Hot Start Green PCR Master Mix (2X)	25 µl
Forward primer	0.1-1.0 µM
Reverse primer	0.1-1.0 µM
Template DNA	10 pg - 1 µg
Water, nuclease-free (#R0581)	to 50 µl
Total volume	50 µl

3. Gently vortex the samples and spin down.
4. When using a thermal cycler that does not contain a heated lid, overlay the reaction mixture with 25 µl of mineral oil.
5. Perform PCR using the recommended thermal cycling conditions outlined below:

Step	Temperature, °C	Time	Number of cycles
Initial denaturation / enzyme activation	95	4 min	1
Denaturation	95	30 s	25-40
Annealing	Tm-5	30 s	
Extension	72	1 min/kb	
Final Extension	72	5-15 min	1

6. Load 5-15 µl of PCR mixture directly on a gel.

DNA purification

Prior to the initial use of the kit, we diluted the Wash Buffer (concentrated) with ethanol (96-100%):

	50 preps #K0701	250 preps #K0702
Wash Buffer (concentrated)	9 mL	45 mL
Ethanol	45 mL	225 mL
Total Volume	54 mL	270 mL

All purification steps were carried out at room temperature. All centrifugations were carried out in a table-top microcentrifuge at >12000 × g

Step	Procedure
1	Add a **1:1 volume** of **Binding Buffer** to completed PCR mixture (e.g. for every 100 µL of reaction mixture, add 100 µL of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µL of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2 for DNA ≤500 bp	*Optional:* if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µL of isopropanol should be added to 100 µL of PCR mixture combined with 100 µL of Binding Buffer). Mix thoroughly. **Note.** If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
3	Transfer up to 800 µL of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through. **Notes.** 1. If the total volume exceeds 800 µL, the solution can be added to the column in stages. After the addition of 800 µL of solution, centrifuge the column for 30-60 s and discard flow-through. Repeat until the entire solution has been added to the column membrane. 2. Close the bag with GeneJET Purification Columns tightly after each use!
4	Add **700 µL** of **Wash Buffer** (diluted with the ethanol as described on p. 3) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
5	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. **Note.** This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
6	Transfer the GeneJET purification column to a clean 1.5 mL microcentrifuge tube (not included). Add **50 µL** of **Elution Buffer** to the center of the GeneJET purification column membrane and centrifuge for 1 min. **Note** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µL does not significantly reduce the DNA yield. However, elution volumes less than 10 µL are not recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65°C before applying to column. • If the elution volume is 10 µL and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
7	Discard the GeneJET purification column and store the purified DNA at -20°C.

Digestion:

We have used Thermo Scientific Fermentas restriction endonucleases (PstI and Eco RI) with the appropriate buffers of Thermo Scientific.

Ligation Protocol:

Double stranded oligonucleotide linkers are often used to generate compatible overhangs not found in the insert. Linkers normally contain restriction enzyme recognition sequences and are digested after ligation to generate overhangs compatible with cloning vectors. Alternatively, linkers may have overhangs which are ready for ligation with a cloning vector and do not require further manipulation following ligation.

1. Prepare the following reaction mixture:

Linear DNA	100-500 ng
Phosphorylated linkers	1-2 µg
10X T4 DNA Ligase buffer	2 µl
50% PEG 4000 solution	2 µl
T4 DNA Ligase	2 u
Water, nuclease-free	to 20 µl
Total volume	**to 20 µl**

2. Mix thoroughly, spin briefly and incubate for 1 hour at 22°C.
3. Heat inactivate at 65°C for 10 min or at 70°C for 5 min.

Note: T4 DNA Ligase is active in PCR and restriction digestion buffers (see table below). Therefore, linker ligation reactions can be performed in the restriction enzyme buffer optimal for the subsequent digestion. In this case, the ligation reaction should be supplemented with ATP to a final concentration of 0.5 mM. After inactivation of the T4 DNA Ligase, add the restriction enzyme directly to the reaction mixture and incubate according to the digestion protocol.

Activity in PCR and restriction digestion buffers		
Buffers		**Activity*, %**
PCR and RT buffers		75
Restriction buffers for restriction enzymes	FastDigest®, 1X / 2X Tango™, B, G, O, R, KpnI, BamHI, EcoRI	75-100
	EcI136II, SacI	50

** activity of T4 DNA Ligase in various buffers supplemented with 0.5 mM ATP.*

Transformation protocol (Heat shock protocol):

1. Take competent cells out of -80°C and thaw on ice (approximately 20-30min).
2. Take agar plates (containing the appropriate antibiotic) out of 4°C to warm up to room temperature or place in 37°C incubator.
3. Mix 1 to 5µl of DNA (usually 10pg to 100ng) into 20-50µL of competent cells in a microcentrifuge or falcon tube. GENTLY mix by flicking the bottom of the tube with your finger a few times.

Note: Transformation efficiencies will be approximately 10-fold lower for ligation of inserts to vectors than for an intact control plasmid.

4. Place the competent cell/DNA mixture on ice for 20-30min.
5. Heat shock each transformation tube by placing the bottom 1/2 to 2/3 of the tube into a 42°C water bath for 30-60 seconds (45sec is usually ideal, but this varies depending on the competent cells you are using).
6. Put the tubes back on ice for 2 min.
7. Add 250-500µl LB or SOC media (without antibiotic) and grow in 37°C shaking incubator for 45min.

Note: This outgrowth step allows the bacteria time to generate the antibiotic resistance proteins encoded on the plasmid backbone so that they will be able to grow once plated on the antibiotic containing agar plate. This step is not critical for Ampicillin resistance but is much more important for other antibiotic resistances.

8. Plate some or all of the transformation onto a 10cm LB agar platecontaining the appropriate antibiotic.

Note: We recommend that you plate 50µL on one plate and the rest on a second plate. This gives the best chance of getting single colonies, while allowing you to recover all transformants.

9. Incubate plates at 37°C overnight.

Cloning and transformation protocol – for pZA31 → E.coli Nissle

Primers:

CV2935, from the *Chromobacterium violaceum* was performed using the following primers (synthesized by IDT):

Item Name: ORF_F
Product: 25 nmole DNA Oligo
Purification: Standard Desalting
Sequence: /5Phos/AT GCG CAG AAC GAC AGG CA
Quantity: 1
Item Name: ORF_R
Product: 25 nmole DNA Oligo
Purification: Standard Desalting
Sequence: /5Phos/TC ACC AGG CCG TCC GCG TCG
Quantity: 1

A gradient PCR was performed using an Eppendorf Mastercycler Gradient thermocycler according to the following protocol:

1. 94°C for 3 minutes
2. 94°C for 40 seconds
3. 62°C ± 8°C for 40 seconds
4. 72°C for 2 minutes

Steps 2-4 were repeated 30 times

5. 72°C for 5 minutes

An optimal annealing temperature of 58°C was found, the above protocol was repeated with the annealing temperature set to 58°C. From this reaction, the PCR fragment was first treated with Fast Digest DpnI enzyme (Thermo Scientific) for 30 minutes at 37°C, then heat-inactivated at 80°C for 5 minutes. The PCR fragment was then cleaned using the Zymo Research DNA Clean & Concentrator kit according to the manufacturer's protocol.

The pZA31 plasmid (Lutz R. and Bujard H. *Nucl. Acids Res.* (1997) 25 (6): 1203-1210) was chosen as an expression vector, into which the chitinase gene was cloned. The plasmid backbone was amplified and linearized using Phusion High-Fidelity polymerase (Thermo Scientific) to avoid 3' overhangs. The plasmid-specific primers used for this amplification were pZA_F (5'-CACATATCGA GGTGAACATC and pZA_R (5'-GATGTTCACCTCGATATGTG). The following reaction conditions were used in the Eppendorf Mastercycler Gradient thermocycler:

1. 98°C for 30 seconds
2. 98°C for 10 seconds
3. 56°C for 30 seconds
4. 72°C for 90 seconds

Steps 2-4 were repeated 32 times

5. 72°C for 7 minutes

Electrophoresis of the plasmid backbone

The amplified and linearized plasmid backbone was then run on a 1.5 % agarose gel (SeaKem LE agarose (Lonza)) and the fragment was subsequently isolated from the gel using the Promega Wizard SV Gel and PCR Clean-Up System according to the manufacturer's protocol.

Ligation of chitinase gene

Blunt-end ligation of the cleaned chitinase gene insert and the linearized plasmid backbone was performed at a 3:1 molar ratio using T4 DNA-ligase (Thermo Scientific) for 1 hour at 22°C.

Preparation of electrocompetent cells

Starters of *Escherichia coli* Nissle strain 1917 (Schultz M. *Inflammatory Bowel Diseases* (2008) 14 (7): 1012-1018) were grown overnight at 37°C in Luria-Bertani-Lennox (LB^L) media (10 g tryptone, 5 g yeast extract, 5 g sodium-chloride per 1 l water). The cultures were then diluted 100-fold into 50 ml fresh media and grown at 37°C until reaching an $O.D._{600}$ value of 0.6. The cultures were chilled on ice for 20 minutes, then centrifuged at 4250 rpm for 10 minutes. The supernatant was discarded and the cells suspended in 50 ml ice cold ddH_2O. This was repeated twice more, with the second suspension in 4 ml 20% glycerol. Cells were centrifuged once more at 4250 rpm for 10 minutes, then finally suspended in 200 µl 20% gycerol and partitioned into 40 µl aliquots.

Electroporation of cells

Cells were subjected to electroporation in a pre-chilled 1 mm gap VWR Signature Electroporation cuvette (VWR, Cat no. 89047-206) using a BioRad MicroPulser electroporator applying the following parameters: 1800 V, 25 µF, 200 Ω. 5 ml prewarmed (37 °C) LB^L medium was immediately added to the electroporated cells, which were then transferred to culture flasks. Cells were allowed to recover for 60 minutes at 37 °C after which cells were harvested and plated onto solid LB^L media containing chloramphenicol (25 µg/ml) and grown overnight at 37°C.

GEL ELECTROPHORESIS

DNA is negatively charged due to the phosphate ions (PO_4^{3-}) backbone. When an electric field is applied across an agarose matrix containing DNA, the nucleic acid fragments move towards the positive cathode. This migration of DNA is dependent upon the size of the matrix pores and the length of the DNA in question. For a fixed pore size and potential difference, a particular DNA fragment migrates a distance proportional to the molecular weight of the molecule. This allows DNA fragments to be separated by size. The sizes are calculated by comparison with a 'ladder' of standard DNA fragments of known sizes. We used the Cleaver Scientific Multi Sub Mini + 7x10 cm geltray.

Procedure:

1. For 1% agarose gel , add 2g of agarose powder to 200 ml of 1x TAE buffer (obtained by diluting 10x TAE stock buffer with water).
2. Heat the mixture in the microwave until the powder has completely dissolved stirring the contents every so often.
3. Ensure electrophoresis chamber is clean and dry. Slot in the comb.
4. Add the gel to the electrophoresis chamber, and wait to set. The comb can then be removed from the chamber.
5. Fill the electrophoresis apparatus half-full with 1x TAE buffer solution (for good electrical contact) and place the set gel in the buffer. Ensure that there are no air bubbles (particularly in the wells created by the comb).
6. Add the ladder solution to the first well, and the DNA samples to subsequent wells. A loading dye (DNA Electrophoresis Sample Loading Dye Bio-Rad #166-0401EDU) should be added to the mixtures to aid visualization when loading into wells.
7. Connect the electrodes to the apparatus (the right way!). Set DC voltage at 80V (with current at approximately 3 mA) and run for 30-60 minutes.
8. After the run, take the gel out and put it into a dyeing dish. Pure Fast Blast DNA Stain (Fast Blast DNA Stain #166-0420EDU) onto the top of the gel. Wait for 3-5 minutes.
9. Pour the dye off the gel, the separated DNA should be visible now.

SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis is a method of resolving proteins of different molecular weights by mixing samples with SDS, loading samples into usually an acrylamide gel and passing an electric current through it.

SDS is an anionic detergent which denatures secondary and non–disulfide–linked tertiary structures, and applies a negative charge to each protein in proportion to its mass, allowing fractionation of proteins via electrophoresis similar to a DNA gel with longer proteins experiencing more difficulty moving through the gel than shorter proteins. Samples are often heated in boiling water prior to loading to shake up the molecules and allow improved binding with SDS. A dye, bromophenol blue is used to indicate the stopping point.

After electrophoresis the gel is rinsed in D.I water and stained with a dye, Coomassie Brilliant Blue for improving fainter bands for visualisation of the separated proteins. After staining the gel is rinsed again and left to de-stain to your desired amount either in D.I water or in a de-staining solution.

We ran our gels using Bio-Rad electrophoresis tanks.

Ingredients:
-100 µg protein per lane
-43.8 kDa (mass of the protein)
-15% slab gels
-samples:
  -0.0625 M Tris-HCl (pH 6.8)
  -2% (w/v) SDS
  -5% (v/v) ß-mercaptoethanol
  -10% (v/v) glycerol
  -0.001% (w/v) bromophenol blue
-stained: 0.2% (w/v) Coomassie Brilliant Blue R250 in 50% methanol/10% acetic acid for 16 h (or Simply Blue Safe Stain (Invitrogen))
-destaining: 12.5% isopropanol/10% acetic acid

SCREENING CHITINASE-ACTIVITY ON COLLOIDAL CHITIN AGAR

We found this method in the article of N. Murthy, B. Bleakley: Simplified Method of Preparing Colloidal Chitin Used For Screening of Chitinase- Producing Microorganisms.
We would like to use this method as a chitinase assay. In order to check that the chitinase gene was infiltrated and expressed in the genetically modified bacteria, we inoculate the bacteria onto colloidal chitin agar. ,,The colloidal chitin agar (pH 7.0+/-0.2) prepared had the following ingredients (in g/L): (K_2HPO_4, 0.7; KH_2PO_4, 0.3; $MgSO_4$ X $5H_2O$, 0.5; $FeSO_4$ X $7H_20$, 0.01; $ZnSO_4$, 0.001; $MnCl_2$, 0.001), amended with 2.0 % moist colloidal chitin. Bacto Agar (Difco) was added at 2.0% as a solidifying agent. The medium was sterilized by autoclaving at STP (standard temperature and pressure: 15 psi, 20 minutes, 121 ° C) and poured into sterile Petri plates (approximately 20 ml per plate)."
If the chitinase gene expresses, we'll see clearing zones around the colonies on the colloidal chitin agar, indicating the production of chitinase. From the ratio of the clearing zone to colony diameter, we can conclude the efficiency of the gene expression.

CHITINASE ACTIVITY MEASUREMENTS

We used a microplate reader and microtiter plates to measure the activity of chitinase enzyme. The 96-Well Microplate from Greiner Bio-One is a flat plate with multiple "wells" used as small test tubes. The Benchmark Microplate reader from Bio-Rad is a 16-channel vertical pathlength photometer that measures the absorbance of the contents of the wells of 96-well microtitration plates. It performs single or dual wavelength measurements between 340 and 750 nm, and reports absorbance values. The reader calculates the absorbance value of each well from Beer's Law. The absorbance is equal to the log10 of the ratio of the baseline measurement (Io) to the sample measurement (I): Absorbance = Log10*(Io/I)
We have ordered a Chitinase Assay Kit from Sigma-Aldrich to measure the activity of our used bacteria.
According to the table below, we have filled the microplate's appropriate wells:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
A	Blank	Blank	Blank	PC	PC	PC	S	S	S
B	1	2	3	4	5	6	7	8	
C	1	2	3	4	5	6	7	8	
D	9	10	11	12	13	14	15	16	
E	17	18	19	20	21	22	23	24	
F	25	26							
G	25	26							

Blank: 100 microliter of Substrate Solution
PC- Positive Control: 5 microliter of Chitinase Control Enzyme
S- Standard: 50 microliter of Standard Solution
1-8: 5 microliter of Culture Medium 1-8 + 2,5 microliter of PBS
9-16: 5 microliter of Culture Medium 1-8 + 2,5 microliter of healthy rat intestine
17-24: 5 microliter of Culture Medium 1-8 + 2,5 microliter of inflamed rat intestine
25: 5 microliter of PBS + 2,5 microliter of healthy rat intestine
26: 5 microliter of PBS + 2,5 microliter of inflamed rat intestine

To check the effects of several cultivating methods of Bacillus thuringiensis on the chitinase activity we have arranged 8 types of cultures according to the table below:

Culture Medium	**Strain**	**Medium CHITIN**	**CHITIN in liquid culture**	**Shaken/No**
1	01262	+	+	-
2	01262	+	-	-
3	01292	+	+	-
4	01292	+	-	-
5	01262	-	-	+
6	01262	+	-	+
7	01292	-	-	+
8	01292	+	-	+

We made another measurement:

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
A	Blank	Blank	Blank	PC	PC	PC	S	S	S
B	L1.S1	L2.S1	Tr1.S1	Tr1.S1	Tr2.S1	Tr2.S1	Soil1.S1	Soil2.S1	
C	L1.S2	L2.S2	Tr1.S2	Tr1.S2	Tr2.S2	Tr2.S2	Soil1.S2	Soil2.S2	
D	5.S1	5.S2	6.S1	6.S2					

L1: liquid culture of assumed transformed bacteria Nissle
L2: liquid culture of assumed transformed bacteria Nissle
Tr1: transformed Bacillus thuringiensis 01262 sample
Tr2: transformed Bacillus thuringiensis 01292 sample
Soil1: bacteria sample from the bank of river Tisza shown chitinase activity on colloidal medium
Soil2: bacteria sample from flower bed shown chitinase activity on colloidal medium
S1: substrate for measuring exochitinase activity
S2: substrate for measuring endochitinase activity

Procedure:

The chitinase hydrolysis is performed in an acidic environment (pH ~4.8) at 37 °C.6 The enzymatic hydrolysis liberates p-nitrophenol. 8 Addition of the basic Stop Solution causes ionization of the p-nitrophenol to form the yellow p-nitrophenylate ion. The absorbance of the p-nitrophenylate ion is measured at 405 nm.3 In order to quantitate the total chitinolytic activity; separate reactions should be run with the three substrates supplied in the kit. Profiling of the chitinolytic enzymes can be determined after separation of the chitinolytic enzymes by SDS-PAGE, using an agarose overlay containing fluorescent substrates.3,9 Note that in crude preparations there may be additive/synergist activity of different chitinases. It is recommended to perform the assays in duplicates. For each substrate, perform a separate activity assay according to the following instructions.

1. Equilibrate the Substrate Solution(s) and the Standard Solution to 37 °C by incubating for several minutes in a 37 °C water bath.
2. Set the plate reader at 405 nm.
3. Add the reaction components to the 96 well plate according to Table 1 and mix using a horizontal shaker or by pipetting. The substrate should be added first and the enzyme should be added last.

Table 1.
Reaction Scheme for 96 Well Plate Assays

	Substrate Solution	Sample	Standard Solution
Blank*	100 µl	–	–
Standard**	–	–	300 µl
Positive control***	90–99 µl	1–10 µl of Chitinase Control Enzyme	–
Test	90–99 µl	1–10 µl of sample	–

* A blank reaction (Substrate Solution without enzyme) should be run, since a portion of the substrate may hydrolyze spontaneously during the incubation time.
** A standard should be run when activity calculations are required.
*** The volume of the enzyme can range between 1–10 ml, depending on the reaction duration (i.e., for a shorter time a higher volume of the enzyme is required).The positive control enzyme is the 20-fold diluted enzyme. If required, the concentrated, non-diluted enzyme may be used.

4. Incubate the plate for 30 minutes at 37 °C. If required, the incubation time for highly active samples can be reduced to as low as 5 minutes.
5. Stop the reactions by adding 200 ml of Stop Solution to each well, except for the wells containing the Standard Solution. After the addition of the Stop Solution the reaction mixture will develop a yellow tint.
6. Measure the absorption at 405 nm no later than 30 minutes after ending the reaction.
Unit definition: One unit will release 1.0 mmole of p-nitrophenol from the appropriate substrate per minute at pH 4.8 at 37 °C.
Units/ml = (A405sample – A405blank)*0.05*0.3*DF / A405standard*time*Venz
A405sample – absorbance of the sample at 405 nm
A405blank – absorbance of the blank at 405 nm
0.05 – mmole/ml of p-nitrophenol in the Standard Solution
0.3 – final volume of the 96 well plate reaction after addition of the Stop Solution (ml)
DF - Dilution Factor - fold dilution of the original chitinase enzyme or biological solution to prepare sample for the test
A405standard – absorbance of the Standard Solution at 405 nm
time – minutes
Venz – volume of the sample (ml)