NICE®
Expression System for *Lactococcus lactis*
The effective & easy-to-operate
NIsin **C**ontrolled gene **E**xpression system

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Mo Bi Tec
MOLECULAR BIOTECHNOLOGY

Content

1 Introduction

The strictly **Ni**sin **c**ontrolled gene **e**xpression system (NICE®), developed at NIZO Food Research, NL, is easy-to-operate and has advantages for the following applications:

(1) Overexpression of homologous and heterologous genes for functional studies and to obtain large quantities of specific gene products
(2) Metabolic engineering
(3) Expression of prokaryotic and eukaryotic membrane proteins (Kunji *et al.* 2003; Kunji *et al.* 2005; Monné *et al.* 2005)
(4) Protein secretion (Novotny *et al.* 2005; Ravn *et al.* 2003; van Asseldonk *et al.* 1990; Vos *et al.* 1989) and anchoring in the cell envelope
(5) Expression of genes with toxic products and analysis of essential genes
(6) Large scale applications

The major advantages of the NICE® system over other expression systems are:

(I) Expression of membrane proteins
(II) Secretion of proteins into the medium
(III) Less endogenous and no exogenous proteases
(IV) Endotoxin-free food grade expression system
(V) No spores
(VI) Tightly controlled gene expression allows production of toxic proteins
(VII) Simple fermentation, scale-up and downstream processing

1.1 Nisin and regulation of nisin biosynthesis

Nisin is a 34 amino acids anti-microbial peptide (lantibiotic). It first binds to lipid II and then forms, together with this cell wall synthesis precursor, small pores in the cytoplasmic membrane that lead to leakage of small molecules, including ATP, and subsequently to cell death. Because of its broad host spectrum, it is widely used as a preservative in food. Initially, nisin is ribosomally synthesized as a precursor. Subsequent enzymatic modifications introduce the unusual chemical and structural features of the molecule. Finally, the modified molecule is translocated across the cytoplasmic membrane and processed into its mature form.
Biosynthesis of nisin is encoded by a cluster of 11 genes, of which the first gene, *nisA*, encodes the precursor of nisin. The other genes direct the synthesis of proteins that are involved in the modification, translocation and processing of nisin (*nisB*, *nisC*, *nisP*, and nisT), in the immunity against nisin (nisI, nisF, nisE, and nisG) and in the regulation of the expression of nisin genes (nisR and nisK). NisR and NisK belong to the family of bacterial two-component signal transduction systems. NisK is a histidine-protein kinase that resides in the cytoplasmic membrane and is proposed to act as a receptor for the mature nisin molecule. Upon binding of nisin to NisK, it autophosphorylates and transfers the phosphate group to NisR, which is a response regulator that becomes activated upon phosphorylation by NisK. Activated NisR* induces transcription of two out of three promoters in the nisin gene cluster: PnisA and PnisF. The promoter driving the expression of nisR and nisK is not affected. Because nisin induces its own expression the accumulation of small amounts of nisin in a growing culture leads to an auto-induction process.

1.2 The nisin controlled gene expression system

For exploitation of the auto-induction mechanism of nisin for gene expression, the genes for the signal transduction system nisK and nisR were isolated from the nisin gene cluster and inserted into the chromosome of *L. lactis* subsp. *cremoris* MG1363 (nisin-negative), resulting in the strain NZ9000. When a gene of interest is subsequently placed downstream of the inducible promoter PnisA on a plasmid [e.g., pNZ8148] or on the chromosome, expression of that gene can be induced by the addition of sub-inhibitory amounts of nisin (0.1-5 ng/ml) to the culture medium. Depending on the presence or absence of the corresponding targeting signals, protein is expressed into the cytoplasm, into the membrane, or secreted into the medium.
Studies with increasing amounts of nisin, using the β-glucuronidase gene as a reporter, show a linear dose-response curve. This shows that the NICE® system can be used not only for on/off gene expression studies, but also to dose the target protein.

1.3 *Lactococcus lactis*

Lactococcus lactis is a homofermentative bacterium. Its primary function is rapid lactic acid production from sugar. Functional characteristics that have extensively been studied in lactococci include the carbon metabolism, the extracellular and intracellular proteolytic system, the production of antibiotic substances, and their interaction with and resistance to bacteriophages. At present, the genome information of at least 5 strains of *L. lactis* is publicly available. This wealth of knowledge and experience has led to the use of lactococci in several fields of biotechnology, e.g., expression of bacterial and viral antigens for safe vaccination via mucosal immunization, production of human cytokines and other therapeutic agents for *in situ* treatments, use of lactococci as a cell factory for the production of specific compounds, and the pilot production of pharmaceutical products. The availability of an easy-to-operate and strictly controlled gene expression system (NICE®) has been crucial for the development of many of these applications.

1.4 Transfer of the NICE® system to other bacteria

Because of its simplicity and its powerful induction characteristics, the NICE® system has been transferred to other low-GC Gram-positive bacteria. Using the dual plasmid system pNZ9520/30 and one of the nisA promoter vectors (typically with β-glucuronidase or β-galactosidase as reporter gene, e.g., pNZ8008), the NICE® system was introduced into *Leuconostoc lactis*, *Lactobacillus brevis*, *Lactobacillus helveticus*, *Lactobacillus plantarum*, *Streptococcus pyogenes*, *Streptococcus agalactiae, Streptococcus pneumoniae, Streptococcus zooepidemicus, Enterococcus faecalis,* and *Bacillus subtilis*. In many cases regulated gene expression can be established, however, growth of several species is retarded by the introduction of the dual plasmid system. This is the case for *Leu. lactis, Lb. helveticus,* and *Lb. plantarum*, but not for *Streptococcus* species and *B. subtilis*. Other strategies have been employed for *E. faecalis, Lb. plantarum,* and *Lactobacillus gasseri*. For *E. faecalis*, a vector (pMSP3535) has been developed that carries both the nisRK genes and the nisA promoter on one plasmid, considerably simplifying the transfer procedure. In *Lb. plantarum* and *Lb. gasseri*, the nisRK genes were integrated as single copies into the chromosome. In general, the NICE® system can successfully be transferred to other Gram-positive bacteria, however each case is different because of variations in nisin sensitivity, in the primary amino acid sequence of RNA polymerase (possible interaction with NisR), and in other factors.

1.5 Codon usage

Until very recently codon usage was an important factor in the possibility and efficiency to express heterologous genes in *L. lactis* (GC content of the DNA of 35-37%). When a gene donor organism is closely related to *L. lactis*, or the DNA GC content is similar to that of *L. lactis*, the probability that a gene can successfully be expressed is high. With the availability of cheap and reliable custom DNA synthesis, there are no longer restrictions as to the origin of a specific target gene, since, from a known amino acid sequence, a gene can be designed that matches the optimal codon usage of the host organism. In addition to a general codon optimization, specific codon tables can be used, such as the codon table for the highly expressed ribosomal protein genes, to further increase product formation.

2 Products

2.1 Host strains

All supplied strains are derivatives of *L. lactis* subsp. *cremoris* MG1363, a plasmid-free progeny of the dairy starter strain NCDO712.
The most commonly used host strain is NZ9000. To construct this strain the genes for *nisK* and *nisR* were integrated into the *pepN* gene of MG1363. Two genes are transcribed from their own constitutive promoter. In the strain NZ9100 nisin genes were inserted into a neutral locus.
Strain NZ3900 was developed for food grade applications of the NICE® system. It is derived of strain NZ3000, which is a *lacF* deletion mutant of strain MG5267, a strain with a single chromosomal copy of the lactose operon of the dairy starter strain NCDO712. The lactose operon was transferred to strain MG1363 by transduction, creating strain MG5267. Due to the *lacF* deletion, strain NZ3000 is unable to grow on lactose. However, growth on lactose can be restored by providing *lacF* on a plasmid such as pNZ8149. Finally, NZ3900 was obtained by inserting *nisRK* into the *pepN* gene using a similar procedure as described for NZ9000.

Strains available at MoBiTec

Lactococcus lactis NZ9000 – *pepN*::*nisRK;*
Standard host strain for nisin regulated gene expression (NICE®). The strain contains the regulatory genes *nisR* and *nisK* integrated into the *pepN* gene (broad range amino peptidase) (Kuipers *et al.*, 1998; Mierau and Kleerebezem, 2005).

Lactococcus lactis NZ9100 – *nisRK*;
Standard host strain for nisin regulated gene expression (NICE®). The strain contains the regulatory genes *nisR* and *nisK* integrated in a neutral locus.

Lactococcus lactis NZ3900 – *lacF*$^-$, *pepN*::*nisRK;*
Standard strain for food grade selection based on the ability to grow on lactose. This strain is a progeny of NZ3000, a strain in which the lactose operon, that is generally present on plasmids, has been integrated into the chromosome and the *lacF* gene was deleted. Deletion of the *lacF* gene makes this strain unable to grow on lactose unless *lacF* is provided on a plasmid (de Ruyter *et al.*, 1996a).

Lactococcus lactis NZ3910 – *lacF*$^-$, *nisRK;*
Same as NZ3900 for food grade *lacF* selection, but with *nisRnisK* integrated in a neutral locus.

Lactococcus lactis NZ9130 – *alr*$^-$, *nisRK*;
This strain is the same as N9100 used for nisin regulated gene expression. It carries an *alr* deletion (*Δalr*) that encodes for alanine racemase. Deletion of the *alr* gene results in auxotrophy for D-alanine suggesting that the strain is unable to grow on media without alanine unless *alr* is provided on a plasmid. The *alr* gene serves here as food grade complementation marker (Bron *et al.*, 2002).

2.2 Plasmids

All plasmids are based on the pSH71 rolling circle replicon (de Vos, 1987) except for plasmid pNZ9530, which is based on the theta-type replicon of pAMβ1 (Simon and Chopin, 1988). Two plasmids can stably be maintained when introduced into one bacterial strain.

pNZ8008, pNZ8148, pNZ8149, and pNZ8150

The replicon of the vectors pNZ8008, pNZ8148, pNZ8149, and pNZ8150 are identical and came originally from the *Lactococcus lactis* plasmid pSH71. However, this is a broad-host-range replicon. Plasmids with this replicon can replicate in many Gram-positive bacteria such as *Lactobacillus plantarum* and *Streptococcus thermophilus*. They can also replicate in *E. coli*, but require a *recA*+ strain like MC1061 (order # VS-ELS10610-01). A *recA*- strain, like DH5α, cannot be used for these vectors. In case of problems with the *E. coli* host please see the next paragraph.
In some cases, cloning with these vectors in *E. coli* can cause instability and/or reorganization of the plasmid, because in *E. coli* the nisin promoter is not completely repressed, allowing expression of gene products that can be toxic or lethal. Therefore, in these cases it is preferred to transform a ligation mixture directly to a *Lactococcus lactis* strain.
The vector pNZ8149 has the *lacF* gene as food grade selection marker. To enable selection of transformants, this vector needs a host strain with the lactose operon without the *lacF* gene, such as *Lactococcus lactis* NZ3900. Due to the Gram-positive food grade selection principle this plasmid cannot be used in *E. coli* (de Vos, 1987; De Ruyter 1996).

pNZ9530

The replication genes from the plasmid pNZ9530 originally came from the *Enterococcus faecalis* plasmid pAMß1. This plasmid can only replicate in Gram-positive host strains, for instance, *Lactococcus lactis, Lactobacillus plantarum,* etc. (Simon and Chopin, 1988; Kleerebezem, 1997).

Plasmids for intracellular expression available at MoBiTec

pNZ8148: broad-host-range vector; Cm^R; nisin A promoter (*PnisA*) followed by an NcoI site for translational fusions at the ATG. Contains a terminator after the MCS. Sequence adaptation for cloning into NcoI site can result in a change in the second amino acid of a protein (Mierau and Kleerebezem, 2005).

pNZ8149: broad-host-range vector; *lacF* for food grade selection for growth on lactose; nisA promoter followed by an NcoI site for translational fusions at the ATG. Contains a terminator after the MCS. Sequence adaptation for cloning into NcoI can result in a change in the second amino acid of a protein (Mierau *et al.*, 2005).

pNZ8150: broad-host-range vector; Cm^R; nisA promoter followed by ScaI site for translational fusions precisely at the ATG. Blunt end fragments are generated by PCR and cannot be cut out again after ligation to the ScaI site (Mierau and Kleerebezem, 2005). Contains a terminator after the MCS.

pNZ8151: broad-host-range vector; *lacF* gene as food grade selection marker; nisA promoter followed by an ScaI site for translational fusions at the ATG start codon. Contains a terminator after the MCS (Mierau and Kleerebezem, 2005).

pNZ8152: broad-host-range vector; *alr* gene as food grade selection marker, nisA promoter followed by an ScaI site for translational fusion at the ATG. Contains a terminator after the MCS (Mierau and Kleerebezem, 2005).

pNZ8008: reference plasmid for testing the nisin induction in *Lactococcus* and other lactic acid bacteria genera. A *gusA* gene without promoter was fused to the nisin A promoter (de Ruyter *et al.*, 1996).

pNZ9530: low copy plasmid with pAMβ1 origin of replication, which carries the *nisR* and *nisK* genes. For cloning in *Lactococcus* strains and in strains of other lactic acid bacteria genera that do not have the regulatory genes integrated into the chromosome. In this case for nisin induced expression a dual plasmid system is used: e.g., pNZ9530 (*nisRnisK*) + pNZ8150 (+insert) (Kleerebezem *et al.*, 1997).

Secretion vectors available at MoBiTec

pNZ8120: broad-host-range vector; Cm^R; NICE vector for protein secretion with the signal sequence of the lactococcal cell wall proteinase PrtP (Vos *et al.*); cloning via a NaeI site; mature protein starts with its own first amino acid (unpublished data).

pNZ8121: broad-host-range vector; Cm^R; NICE vector for protein secretion with the signal sequence of the lactococcal cell wall proteinase PrtP (Vos *et al.*); cloning via an EcoRV site; mature protein starts with the first amino acid of PrtP (Asp) after the signal cleavage site. This setting can contribute to a greater efficiency in removing the signal peptide (unpublished data).

pNZ8122: broad-host-range vector; Cm^R; NICE vector for protein secretion with the signal sequence of the *Lactobacillus brevis* SlpA protein (Novotny *et al.*, 2005); cloning via an NruI site; mature protein starts with the first amino acid of SlpA (Ser) after the signal cleavage site. This setting can contribute to a greater efficiency in removing the signal peptide (results not published; for this construct there is no alternative without this additional amino acid).

pNZ8123: broad-host-range vector; Cm^R; NICE vector for protein secretion with the signal sequence of the lactococcal major secreted protein Usp45; cloning via a NaeI site; mature protein starts with its own first amino acid (unpublished data).

pNZ8124: broad-host-range vector; Cm^R; NICE vector for protein secretion with the signal sequence of the lactococcal major secreted protein Usp45; cloning via an EcoRV site; mature protein starts with the first amino acid (Asp) of Usp45 (van Asseldong *et al.*, 1993 & 1990) after the signal cleavage site. This setting can contribute to a greater efficiency in removing the signal peptide (unpublished).

Note: *There is a choice of three signal peptides: ss-usp45, ss-prtP, and ss-slpA (from Lactobacillus brevis). As efficient protein secretion depends on the combination of the signal peptide with the target gene, and as there is at this moment no way to predict the pairing, we advise to choose a particular vector, or to use a few to determine what works best.*

Strains	Plasmids	Medium
Lactococcus lactis NZ9000/NZ9100	pNZ8008	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8148	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8150	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ9530	M17+0.5% glucose+10 µg/ml erythromycin
Lactococcus lactis NZ3900	pNZ8149	see pages 14 and 15
Lactococcus lactis NZ3910	pNZ8149	see pages 14 and 15
Lactococcus lactis NZ9000/NZ9100	pNZ8120	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8121	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8122	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8123	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ9000/NZ9100	pNZ8124	M17+0.5% glucose+10 µg/ml chloramphenicol
Lactococcus lactis NZ3900/NZ3910	pNZ8151	see pages 14 and 15
Lactococcus lactis NZ9130	pNZ8152	see page 11

- All plasmids are lyophilized from water. Use TE buffer or water for reconstitution of plasmid DNA.

For propagation of NICE® vectors, see comments above, we recommend *E. coli* strain MC1061, VS-ELS-10610-01 (10 µg/ml chloramphenicol; growth of colonies may take 2 days):

Genotype: araD139, Δ(ara, leu)7697, ΔlacX74, galU-, galK-, hsr-, hsm+, strA

Never thaw frozen bacterial stock in glycerol. Use a sterile loop, sterile wooden stick, or sterile disposable pipette to scratch the surface of the stock. Streak appropriate agar plate (TY) for single colonies. Recap the frozen stock and return to storage at -80 °C. Incubate the plate overnight at 37 °C. Use single colonies for preparation of competent cells.

3 Protocols

3.1 Growth and nisin induction for the NICE® system in *Lactococcus lactis*

Various media are available for growth of lactococci. The most commonly used laboratory medium is M17 supplemented with glucose, lactose, or other sugars as carbon source, and a relevant antibiotic for plasmid selection. The basic ingredients for a large-scale medium are 1-3% peptone, 0.5-2% yeast extract, 1-10% carbon source, and small amounts of magnesium and manganese ions. Individual processes need specific optimization of the medium components and fermentation conditions.
For experiments in which specific metabolites are addressed or cell components need to be labelled, a chemically defined medium can be used. Lactococci are auxotrophic for a number of amino acids that can be added in a labelled form and are then integrated into newly formed proteins.
At a laboratory scale, an overnight culture is most commonly inoculated into fresh medium with a dilution of 1:100, grown to an optical density (OD_{600} of 0.2 to 0.5) and induced with nisin (0.1-5 ng/ml). After that, the culture is continued growing for 0.5 to 3 h and then harvested for further use or testing. In this set-up pH is not controlled, and the culture will stop growing at low cell density because of lactic acid production and the consequent pH drop. Alternatively, the culture can be grown with pH control to higher cell density. In this case, induction can be carried out at the OD_{600} of 1 to 5 or even higher, leading to a substantial yield increase.
For cloning purposes in *E. coli* with the vectors that have Cm^R a *rec* A^+ strain such as *E. coli* MC1061 should be used.

3.2 Media and growth conditions for *Lactococcus lactis*

Lactococcus lactis vector pNZ8149 harboring *lacF* as food grade selection marker is selected for the ability to grow on lactose. The host strain *L. lactis* NZ3900 has all genes involved in the lactose fermentation on the genome, with a deletion of the *lac*F gene (Enzyme III of the Lac-PTS). The strain can grow on glucose, but in the presence of the *lac*F gene on a plasmid, it can also grow on lactose.

A special medium that can be used for selection of Lac^+ colonies is Elliker medium. On this rich medium all cells can grow, Lac^+ or Lac^-, but when lactose is added as sole carbon source the lactose-fermenting cells give yellow colonies.

Elliker medium

20	g/l	Tryptone
5	g/l	Yeast extract*
4	g/l	Sodium chloride
1.5	g/l	Sodium acetate (water free)
0.5	g/l	L(+) Ascorbic acid

for agar: 15 g/l agar

The pH is about 6.8, no adjustment required

Sterilization: 15 min 121 °C

After sterilization:

+ 0.5% lactose (stock: 20% solution)
+ 0.004% Bromocresolpurple (0.4% stock solution, filter sterilized)

* For less background of lactose negative colonies, half the yeast extract concentration can be used.

Lactococcus lactis strain NZ9130 is auxotroph for alanine. It is able to grow on M17 broth supplemented with 0.5% of glucose and 200 µg/ml of alanine. The strain will not require alanine for growth after introducing a plasmid harboring *alr* gene. The *alr* gene serves here as a food grade selectable genetic marker.

3.3 Preparation of stocks for *Lactococcus lactis*

Lactococcus lactis can grow on M17 broth with 0.5% sugar. For a strain with plasmid 10 µg/ml chloramphenicol or erythromycin is added to maintain the plasmid.
On plate or slant, the strain will survive 2-3 weeks.

Stock preparation
- Inoculate 5 ml broth with cells from the slant
- Grow the cells overnight at 30 °C
- Add 3 ml fully grown culture to 1 ml 60% glycerol and store at -80 °C

Materials
Medium: M17 broth with: 0.5% lactose or glucose
10 µg/ml chloramphenicol or erythromycin

Sterile (15 min 121 °C) 60% glycerol in a -80 °C tube

3.4 Plasmid DNA isolation from *Lactococcus lactis*, small scale

Method
- Use 5 ml full-grown culture
- Spin down 10 min at 3000-6000 x g
- Resuspend pellet in 250 µl THMS-buffer + 2 mg/ml lysozyme in reaction tube
- Incubate 10 min at 37 °C
- Add 500 µl 0.2 N NaOH + 1% SDS, shake carefully (no vortex)
- Incubate 5 min on ice
- Add 375 µl ice-cold 3 M potassium acetate pH 5.5, shake carefully
- Incubate 5 min on ice
- Spin 5 min in benchtop centrifuge
- Take out supernatant and add to new reaction tube
- Fill the cup with 2-propanol
- Incubate 5-10 min at room temperature
- Spin 10 min in benchtop centrifuge
- Wash pellet carefully with 70% ethanol
- Vacuum dry the pellet
- Dissolve the pellet in 50 µl TE or sterile water

Materials

Medium: M17 broth containing: 0.5% lactose or glucose
10 µg/ml chloramphenicol or erythromycin

THMS buffer: 30 mM Tris-HCl pH 8
3 mM $MgCl_2$
in 25% sucrose
add lysozyme before use

0.2 N sodium hydroxide, 1% SDS (make sure that SDS is fully dissolved prior to use, depending on room temperature the expiration date should not exceed three months)

3 M potassium acetate pH 5.5

2-propanol

70% ethanol

TE: 10 mM Tris-HCl pH 8
1 mM EDTA pH 8

3.5 Transformation of *Lactococcus lactis*

Preparation of the cells:

Day 1:
Inoculate 5 ml of G/L-SGM17B medium with *L. lactis* glycerol stock from -80 °C and grow at 30 °C, without aeration, overnight

Day 2:
Inoculate 50 ml of G/L-SGM17B with pre-culture in a dilution of 1:100 and grow at 30 °C, without aeration, overnight

Day 3:
- Add 50 ml full-grown culture to 400 ml of G/L-SGM17B medium
- Grow the culture until OD_{600} is 0.2-0.3 (ca. 3 h)
- Spin down cells for 20 min at 6000 x g, 4 °C
- Wash cells with 400 ml of 0.5 M sucrose, 10% glycerol (4 °C) and spin down at 6000 x g (centrifugation speed may need to be increased during successive washing steps)
- Resuspend the cells in 200 ml of 0.5 M sucrose, 10% glycerol, 50 mM EDTA (4 °C), keep the suspension on ice for 15 min and spin down
- Wash cells with 100 ml of 0.5 M sucrose, 10% glycerol (4 °C) and spin down (6000 x g)
- Resuspend the cells in 4 ml of 0.5 M sucrose, 10% glycerol (4 °C):
  - Use 40 µl per electroporation (keep on ice)
  - Or store the cells in small portions at -80 °C, let them defreeze **on ice** before use

Electroporation:

- Place 40 µl cells in a pre-chilled electroporation cuvette with 1 µl DNA (100-500 ng vector DNA reconstituted in TE-, Tris-buffer, or distilled water; for transforming cells with ligation product use 500-1000 ng DNA) and keep the cuvette on ice

- Use Biorad Genepulser with following adjustments:
  2000 V
  25 µF
  200 Ω
- Pulse (normal reading is 4.5-5 msec)
- Add 1 ml of G/L-M17B + 20 mM $MgCl_2$ + 2 mM $CaCl_2$
- Keep the cuvette for 5 min on ice and incubate 1-1.5 h at 30 °C
- Plate 10 µl, 100 µl, 900 µl on M17agar with glucose or lactose and antibiotics (depends on plasmid)
- Incubate 1-2 days at 30 °C

Materials:
- G/L-SGM17B:
  M17-Broth with: 0.5 M sucrose
  2.5% glycine
  0.5% glucose or 0.5% lactose (strain dependent)
  Add the sucrose and glycine to the M17-B and sterilize 20 min 121 °C. Add sterile glucose or lactose after cooling down.

- 0.5 M sucrose/ 10% glycerol
- 0.5 M sucrose/ 10% glycerol/ 0.05 M EDTA

L. lactis grows very slowly on G/L-SGM17B. Leaving out the sucrose is possible (Wells *et al.*, 1993) but can decrease the transformation efficiency.
The medium for cell recovery must contain $MgCl_2$ and $CaCl_2$.

3.6 Nisin induction of gene expression in *Lactococcus lactis*

The growth and induction conditions are optimized for *L. lactis*. When other species are used, growth temperature and amount of nisin used for induction can be different.

General protocol
Grow 5 ml culture overnight at 30 °C

Dilute 1/25 in 2 x 10 ml fresh medium (30 °C)
Grow until the $OD_{600} \approx 0.4$

Induce one 10 ml culture with 0.5-5 ng/ml nisin (typically 1 ng/ml is used) and keep the other 10 ml culture as negative control.

Incubate 2-3 hours, measure the OD_{600} to monitor growth of the induced and non-induced cultures.

Collect cells by centrifugation; resuspend the pellet in a suitable buffer or sterile water. Make a cell free extract and test for protein production by SDS-PAGE, enzyme assay, etc.

Nisin stock solution
1 mg/ml nisin in 0.05 % acetic acid.

When using cat. # VS-ELK01000-02, prepare a 40 mg/ml solution in 0.05 % acetic acid (supplied 5 % acetic acid diluted with dist. water) for obtaining a final concentration of 1 mg/ml pure nisin. Let it dissolve for 5-10 min at RT and spin to pellet insoluble material. The nisin itself is dissolved completely in the clear supernatant.

Store aliquoted vials of 1 mg/ml nisin stock solution at -20 °C. Frozen aliquots are stable for at least 1 year.

Note: Prepare nisin dilution from aliquoted and frozen 1 mg/ml stock solution with sterile water just before use and dispose dilution after use. A diluted nisin solution is not stable.

3.7 Food grade selection of recombinant *Lactococcus lactis*

M17 medium
M17 medium is the commonly used growth medium for *L. lactis*. This medium is commercially available without carbon source.

Addition of carbon source for growth:

> 0.5% glucose or 0.5% lactose (all strains can grow on glucose; for growth on lactose a strain needs the lactose operon)

Elliker medium
Elliker medium is used for selection of Lac^+ transformants, see protocol "Food grade selection of recombinant *L. lactis"*.

Elliker medium is not commercially available without carbon source.

Ingredients:

20 g/l Tryptone
5 g/l Yeast extract*
4 g/l Sodium chloride
1.5 g/l Sodium acetate (water free)
0.5 g/l L(+) Ascorbic acid

For agar: 15 g/l agar

The pH is about 6.8, no adjusting necessary

Sterilization: 15 min 121 °C

After sterilization:

add 0.5% lactose or 0.5% glucose (20% stock solution)
+ 0.004% Bromocresolpurple (0.4% stock solution, filter sterilized)

***** For less background of lactose negative colonies, the yeast extract concentrations can be reduced to one half.

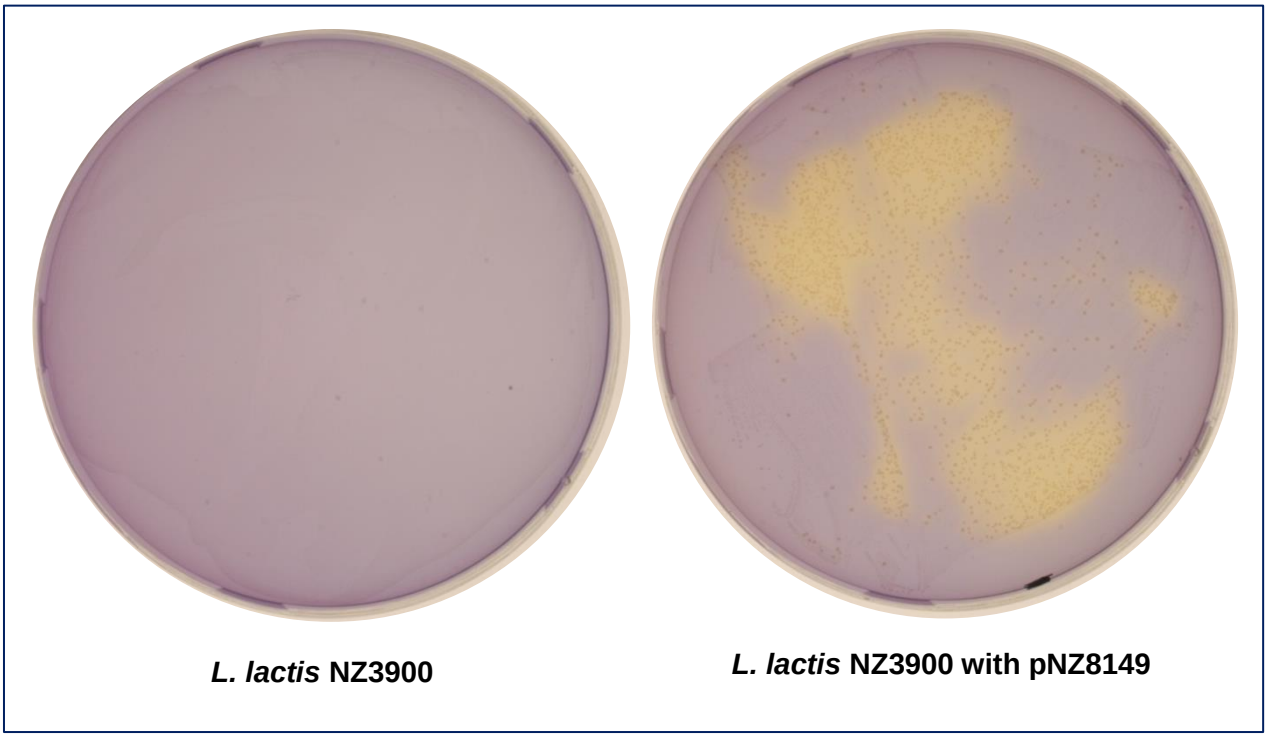

Figure 1. Food grade selection system based on the *lacF* gene: growth of *L. lactis* bacterial colonies without or with the vector pNZ8149 on Elliker agar containing 0.5% lactose at 30 °C. Lactose-positive strain can metabolize lactose that results in growing colonies in yellow color.

Both media M17 broth and Elliker supplemented with lactose can be used for selection of transformants harbouring a plasmid with *lacF* gene. The advantage of Elliker medium is that it allows color-based identification of lactose positive strains. This is convenient as there will be background of lactose-negative strains that grow on small quantities of sugars present in the other components of the medium.

Growth conditions

Lactococcus lactis grows at 30 °C, without aeration.

4 Overview of Applications of the NICE® System

In the following section we will give an overview of known applications of the NICE® system with some pertinent examples.

4.1 Overexpression of homologous and heterologous genes

The NICE® system has been used to express genes of various different backgrounds (Gram-positive, Gram-negative, and eukaryotic) to study metabolic and enzyme function and to produce larger amounts of an enzyme for food, medical, or technical applications. With the homologous *pepN* gene of *L. lactis* it has been shown that protein production up to 50% of the total cellular protein is possible without the formation of inclusion bodies. The β-glucuronidase gene of the Gram-negative *E. coli* has been expressed up to 20% of the total cellular protein.
Furthermore, the NICE® system has been used to study the genetics and biology of pathogenic bacteria and to study genetic entities such as chromosomes and bacteriophage genomes. Genes of these various backgrounds can be expressed, however, with case-dependent yields (probably due to codon usage). Genes of closely related Gram-positive organisms (e.g., *Streptococcus, Enterococcus, Staphylococcus,* and low-GC *Lactobacillus*) are almost always expressed effectively without any problems.
The expression of genes of other organisms depends on the codon usage and the distribution of rarely used codons:
Important examples are the expression of phage lysines, various peptidases, and esterases to influence, for instance, flavor formation in dairy fermentations. Another important feature of the NICE® system is that it is possible to control not only the expression of one gene but of a whole operon, as shown for the eight-gene (F1F0) H^+-ATPase or the *rfb* operon of *L. lactis*.

4.2 Metabolic engineering

Metabolic engineering is based on the application of genetic engineering methods to manipulate cellular processes and structures with the aim to study, improve, or redirect cellular functions. *L. lactis* has widely been used as a model system for metabolic engineering studies because it has a rather simple carbon and energy metabolism in which the carbon source is mainly transformed into lactic acid via the central metabolite pyruvate. The possibility to dose the expression of a gene of interest by varying the amount of nisin that is added for induction is unique and makes the NICE® system an ideal instrument to study gradual changes in a metabolic route. Therefore, nisin-dosed expression has been used extensively to study, engineer, and model sugar catabolism in *L. lactis* and the conversion of pyruvate into various alternative end products, like diacetyl and L-alanine. As mentioned above, the NICE® system cannot only be used to drive expression of single genes, but also of whole operons. This feature has also been used to study and manipulate complex metabolic pathways, like the production of exopolysaccharides and of the vitamins folate and riboflavin, leading to higher product yields.

4.3 Expression of integral membrane proteins

Lactococcus lactis is an excellent model species for the expression and study of integral membrane proteins of both prokaryotes and eukaryotes. In addition to straightforward cloning and cultivation procedures,

(1) many strains of *L. lactis* are auxotrophic, allowing the incorporation of various labels,
(2) the tightly regulated NICE® system allows the cloning and induction of membrane proteins that are often toxic for the cell,
(3) expressed membrane proteins are only targeted to the cytoplasmic membrane,
(4) the cells have a weak proteolytic activity (*htrA*) that can easily be eliminated,
(5) *L. lactis* has only one membrane, allowing direct functional studies with either intact bacteria or isolated membrane vesicles, and
(6) the membrane proteins can easily be solubilized with various detergents.

Examples:
L. lactis has been used for the expression and functional analysis of various classes of prokaryotic integral membrane proteins such as ATP-binding cassette (ABC) transporters, ABC efflux pumps, major facilitator superfamily proteins, peptide transporters, mechano-sensitive channel, ATP/adenosine diphosphate (ADP) transporters, etc. These transporters have been expressed to sometimes very high levels of up to 30% of all membrane proteins and, in general, to 1-10% of all membrane proteins. *L. lactis* is also suitable for the expression of eukaryotic membrane proteins, like the KDEL receptor and different mitochondrial and hydrogenosomal carriers. These proteins could not only be expressed at between 0.1 and 5% of all membrane proteins, but they were also functionally intact and showed the characteristics that they have in their natural environment.

4.4 Protein secretion and surface exposure of proteins

Lactococcus lactis is a Gram-positive bacterium and therefore has only one cellular membrane. This makes it an ideal host for protein secretion with subsequent membrane- or cell wall anchoring, or export into the fermentation medium. Another advantage is the low extracellular proteinase activity in lactococci. To date there are only two proteases known:
(1) the cell wall anchored protease PrtP (200 kD) and
(2) the housekeeping membrane-bound protease HtrA.

The first is plasmid-encoded and absent in the plasmid-free host strains. For the second a viable mutation can be constructed that helps to stabilize secreted proteins. In comparison to the aerobically growing *B. subtilis*, which can secrete several grams of protein per liter, protein secretion in *Lactococcus* is less effective. Nonetheless, *Lactococcus* is an interesting host for, e.g., surface display of various antigens and the development of live vaccines and other *in situ* applications. One of the latest areas of application has been the expression and secretion of a surface-layer protein with a yield of 100 mg/l for the exploration of nanobiotechnological applications.
Two signal peptides are mainly used to effect protein secretion:
(1) the signal peptide of the major lactococcal-secreted protein Usp45 and
(2) the signal peptide of the cell-wall-associated protease PrtP.
In general, the signal peptide of Usp45 gives better results and is more widely used than that of PrtP.

For the exposure of proteins in the cell wall two principal systems have been developed:

(1) the sortase system that uses the LPXTG motive at the N- or C-terminus of the protein and
(2) the cell-wall anchor of the major autolysin of *L. lactis* that also can be attached either N- or C-terminally.

4.5 Expression and analysis of toxic products

One of the great strengths of the NICE® system is that it is tightly regulated, and genes that would otherwise have a detrimental effect on the cell can be cloned, analyzed, and expressed.
In very rare cases the residual leakage of the system can lead to unsuccessful cloning attempts when genes are cloned that encode toxic gene products. This can be amended by placing also the nisA promoter in single copy on the chromosome.

4.6 Large-scale applications

Fermentations in which nisin-induced gene expression is performed have been demonstrated to be scalable up to the 3,000-l scale, with almost identical fermentation characteristics and product yields (100 mg/l and more). The downstream processing is straightforward. With four unit operations -microfiltration, homogenization, second microfiltration, and chromatography- a product with 90% purity could be obtained. A substantial increase in yield can be achieved with careful optimization of the complete process.

5 Bottlenecks for Gene Expression in *L. lactis*

Aerobic bacteria can be grown to cell densities far above 100 g/l dry biomass concentration. Because of the fermentative metabolism this is not possible with *L. lactis*. In a simple acidifying buffered culture in, for instance, M17 medium, the maximum cell density is about OD_{600} = 3 (1 g/l dry cell mass). Growth will stop when a pH of about 5.0 is reached. With neutralization using NaOH or NH_4OH the cell density can rise to OD_{600} = 15 (5 g/l dry cell mass). There have been attempts to develop high cell density cultivation methods for lactic acid bacteria, but so far none of these have been applied to increase gene expression. Efficient methods to extend logarithmic growth of *L. lactis* would allow further increase of product yield. Recently, it has been rediscovered that lactococci can grow under aerobic conditions when haem is added to the medium. Under these conditions the growth period and the long-term survival of the cells is greatly extended. This observation can be employed to considerably increase the cell density of lactococcal cultures and initiate nisin-controlled gene expression at higher cell densities, leading to increased product formation.
Alternatively, NICE®-like systems that display highly similar characteristics, but can be employed in respiring bacteria, such as SURE (SUbtilin Regulated gene Expression) in *B. subtilis*, can potentially overcome the fermentative biomass yield restrictions encountered with *L. lactis*.

6 References

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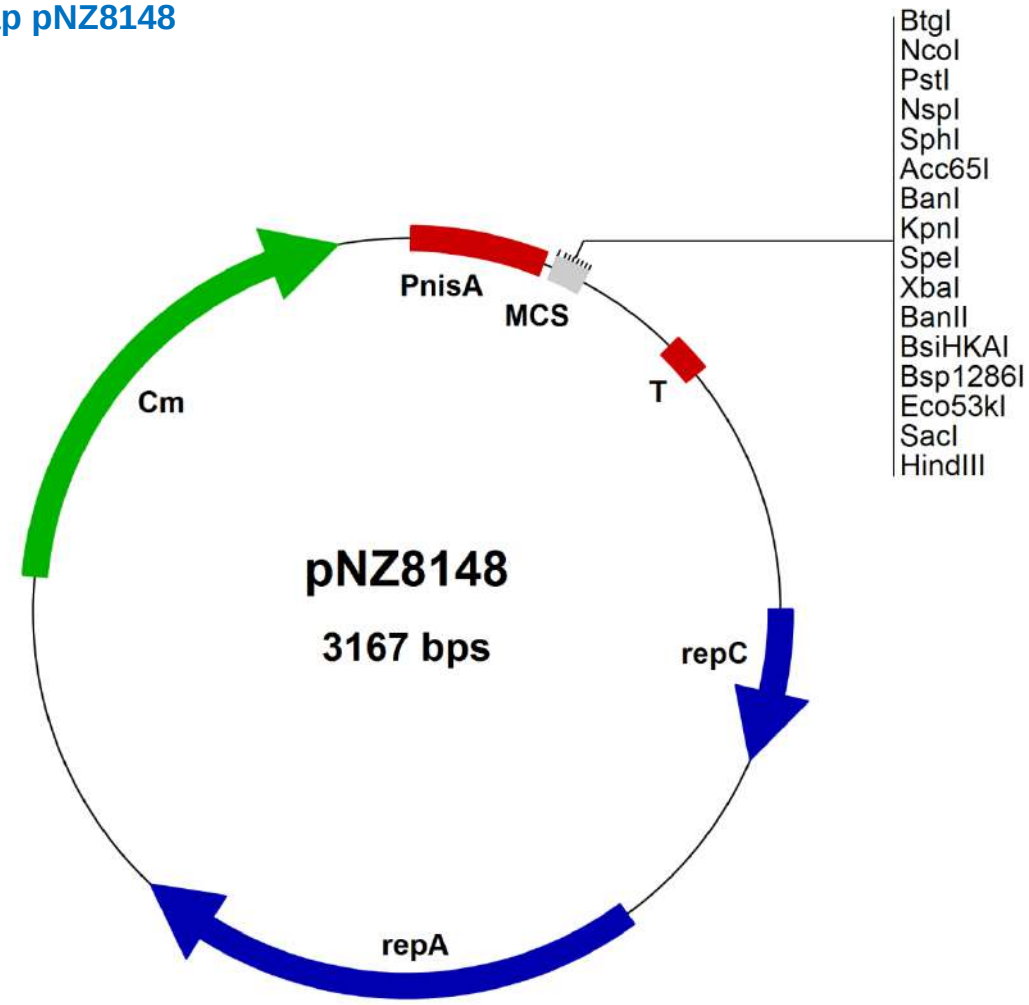
7 Vector Maps

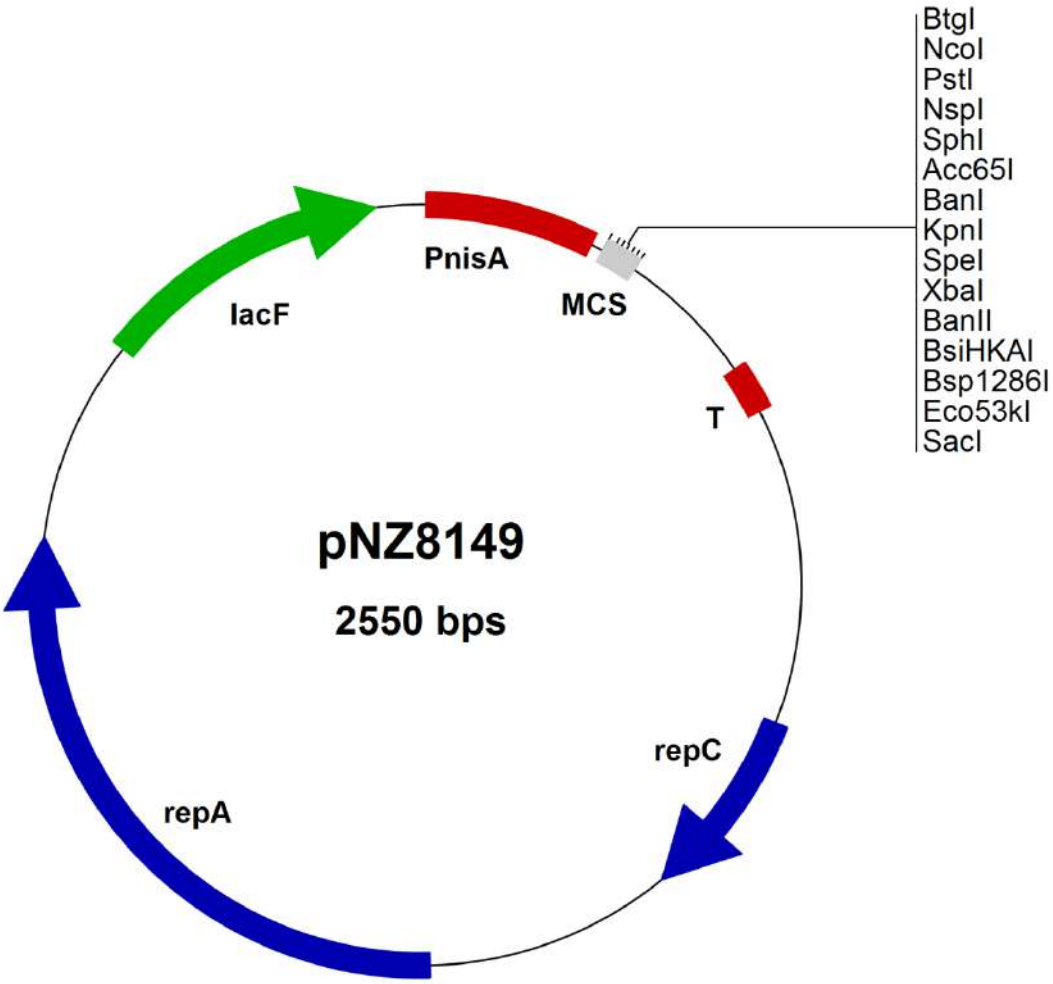
All vector maps and the complete DNA sequences are available for download on our web page http://www.mobitec.com.

7.1 Vector map pNZ8148



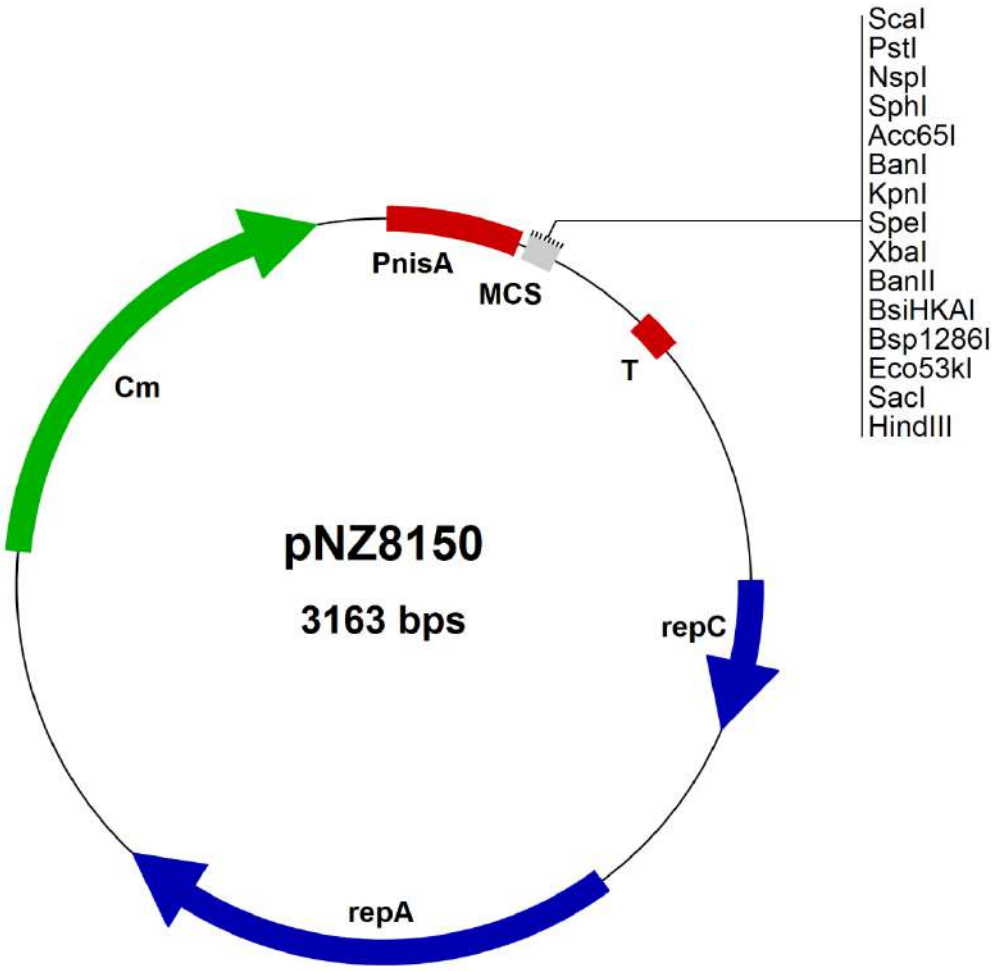
	Type	Start	End	Name	Description
	Promoter	5	188	PnisA	*nisin A* promoter region
	Region	202	247	MCS	Multiple Cloning Site
	Terminator	393	445	T	Termination sequence of *pepN* gene
	Gene	788	997	*repC*	Replication gene *C*
	Gene	1266	1964	*repA*	Replication gene *A*
	Selectable Marker	2424	3074	*Cm* (*cat*)	Chloramphenicol resistance

7.2 Vector map pNZ8149



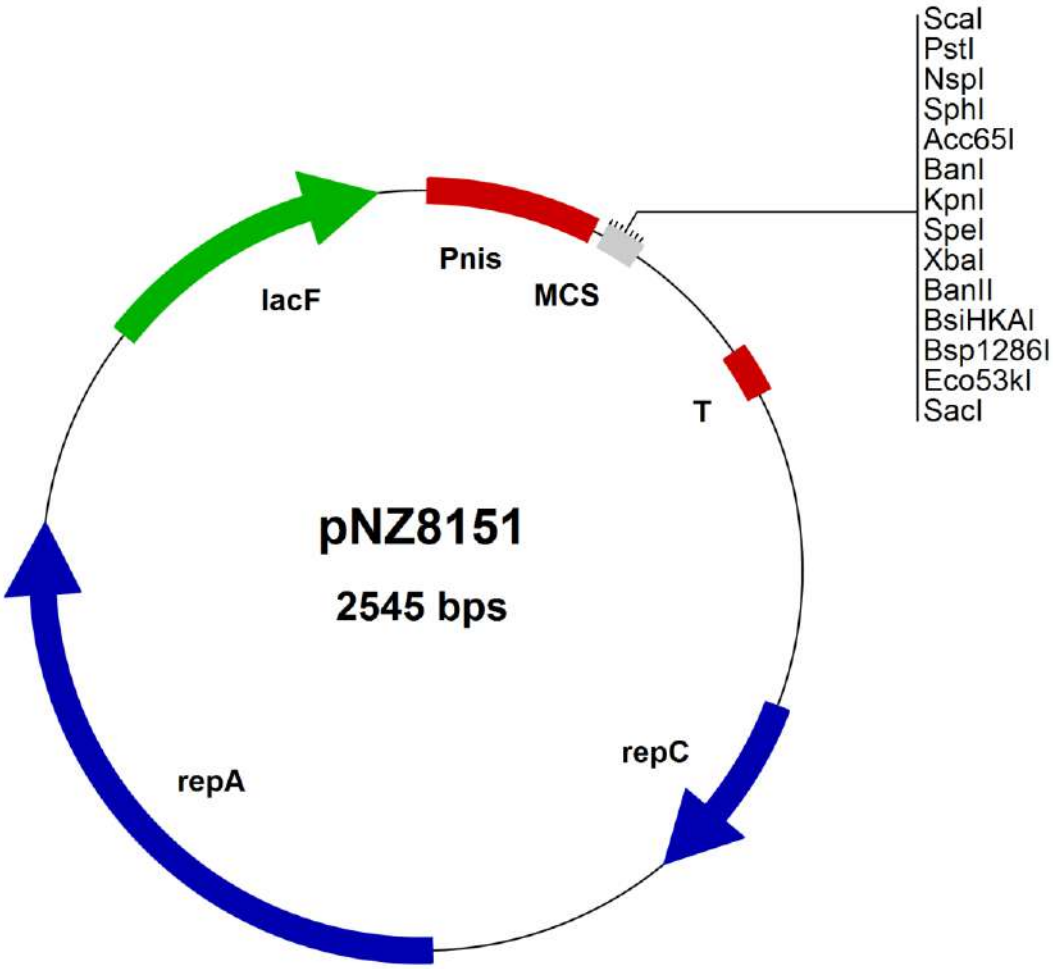
	Type	Start	End	Name	Description
	Promoter	5	188	PnisA	*nisin A* promoter region
	Region	202	241	MCS	Multiple Cloning Site
	Terminator	393	445	T	Termination sequence of *pepN* gene
	Gene	788	997	*repC*	Replication gene *C*
	Gene	1266	1964	*repA*	Replication gene *A*
	Selectable Marker	2184	2501	*lacF*	Food grade selection marker, *L. lactis lacF* gene

7.3 Vector map pNZ8150



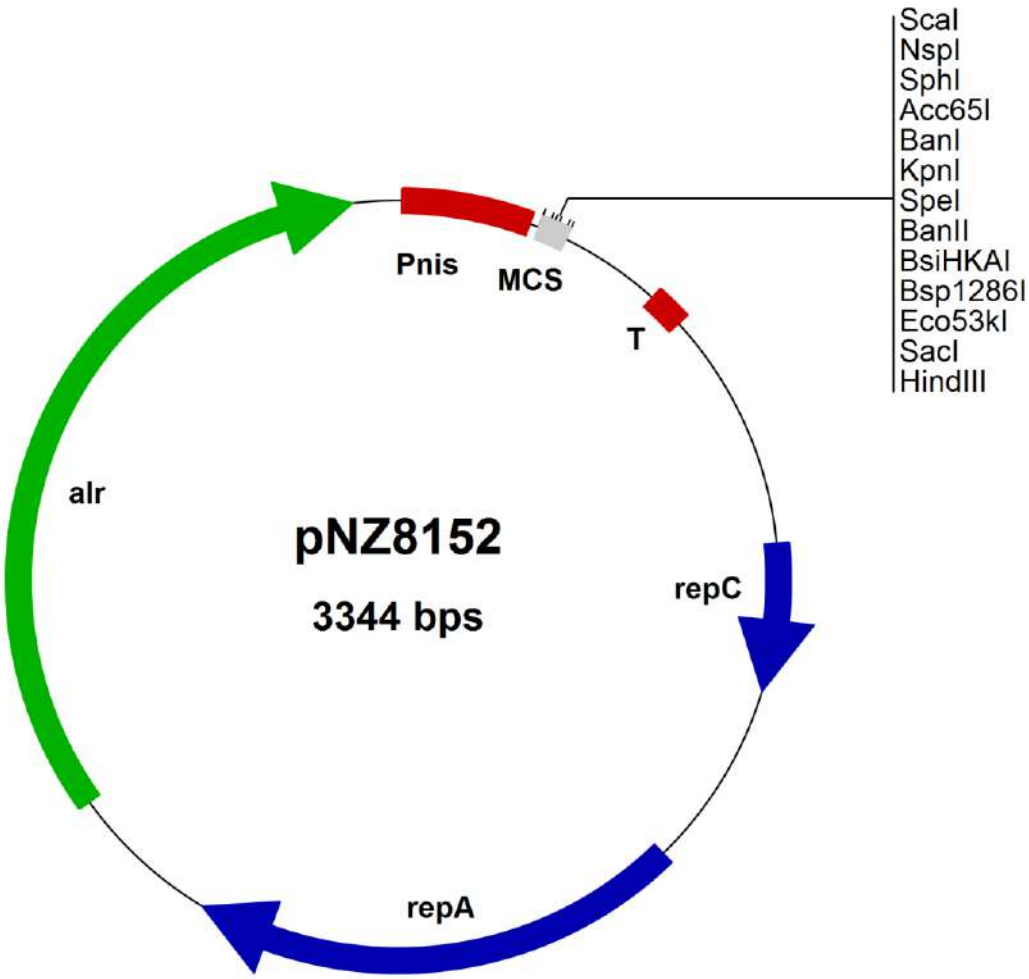
	Type	Start	End	Name	Description
	Promoter	5	188	PnisA	*nisin A* promoter region
	Region	201	243	MCS	Multiple Cloning Site
	Terminator	389	441	T	Termination sequence of *pepN* gene
	Gene	784	993	*repC*	Replication gene *C*
	Gene	1262	1960	*repA*	Replication gene *A*
	Selectable Marker	2420	3070	*Cm* (*cat*)	Chloramphenicol resistance gene

7.4 Vector map pNZ8151



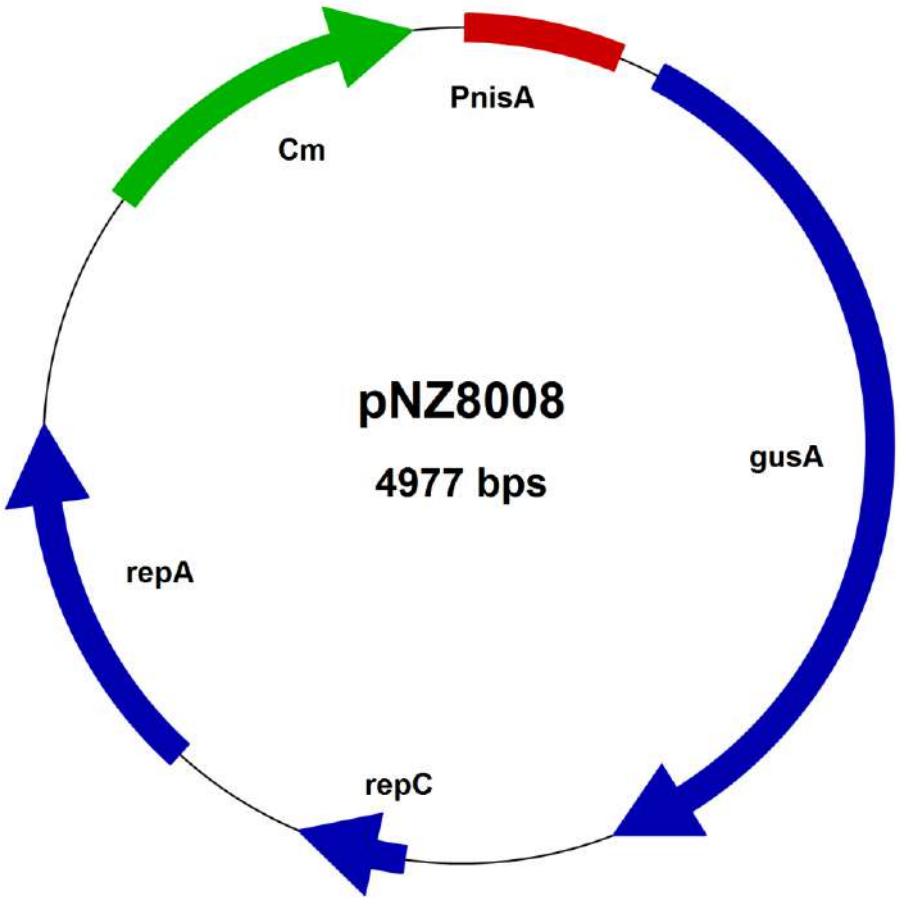
	Type	Start	End	Name	Description
	Promoter	5	188	PnisA	*nisin A* promoter region
	Region	201	241	MCS	Multiple Cloning Site
	Terminator	389	441	T	Termination sequence
	Gene	784	993	*repC*	Replication gene *C*
	Gene	1262	1960	*repA*	Replication gene *A*
	Selectable Marker	2180	2497	*lacF*	Food grade selection marker, *L. lactis lacF* gene

7.5 Vector map pNZ8152



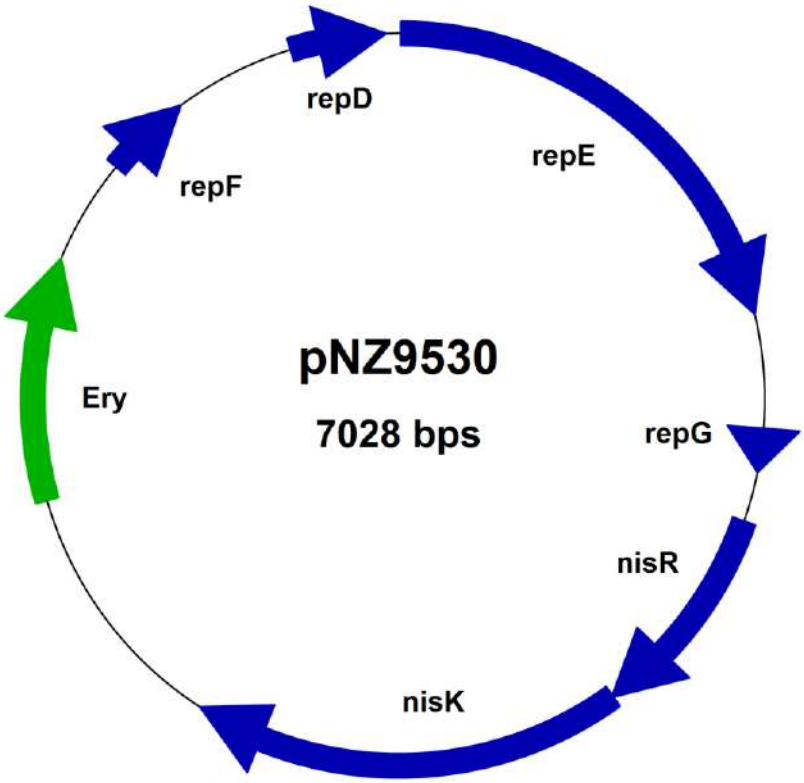
	Type	Start	End	Name	Description
	Promoter	5	188	PnisA	*nisin A* promoter region
	Region	201	243	MCS	Multiple Cloning Site
	Terminator	389	441	T	Termination sequence
	Gene	784	993	*repC*	Replication gene *C*
	Gene	1262	1960	*repA*	Replication gene *A*
	Selectable Marker	2177	3280	*alr*	Food grade selection marker, *L. lactis alr* gene

7.6 Vector map pNZ8008



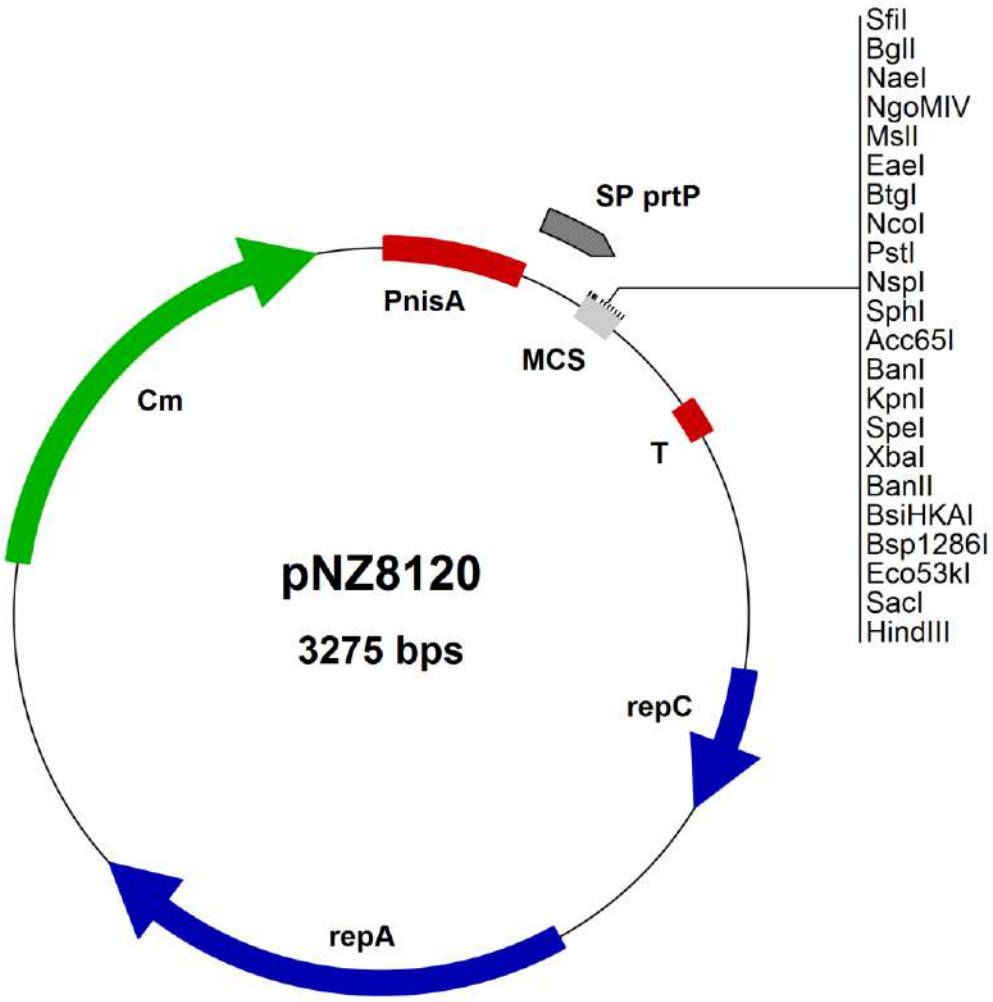
	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region including ribosomal binding site
	Gene	366	2174	*gusA*	ß-glucuronidase gene
	Gene	2576	2785	*repC*	Replication gene *C*
	Gene	3054	3752	*repA*	Replication gene *A*
	Selectable Marker	4212	4862	Cm (*cat*)	Chloramphenicol resistance

7.7 Vector map pNZ9530



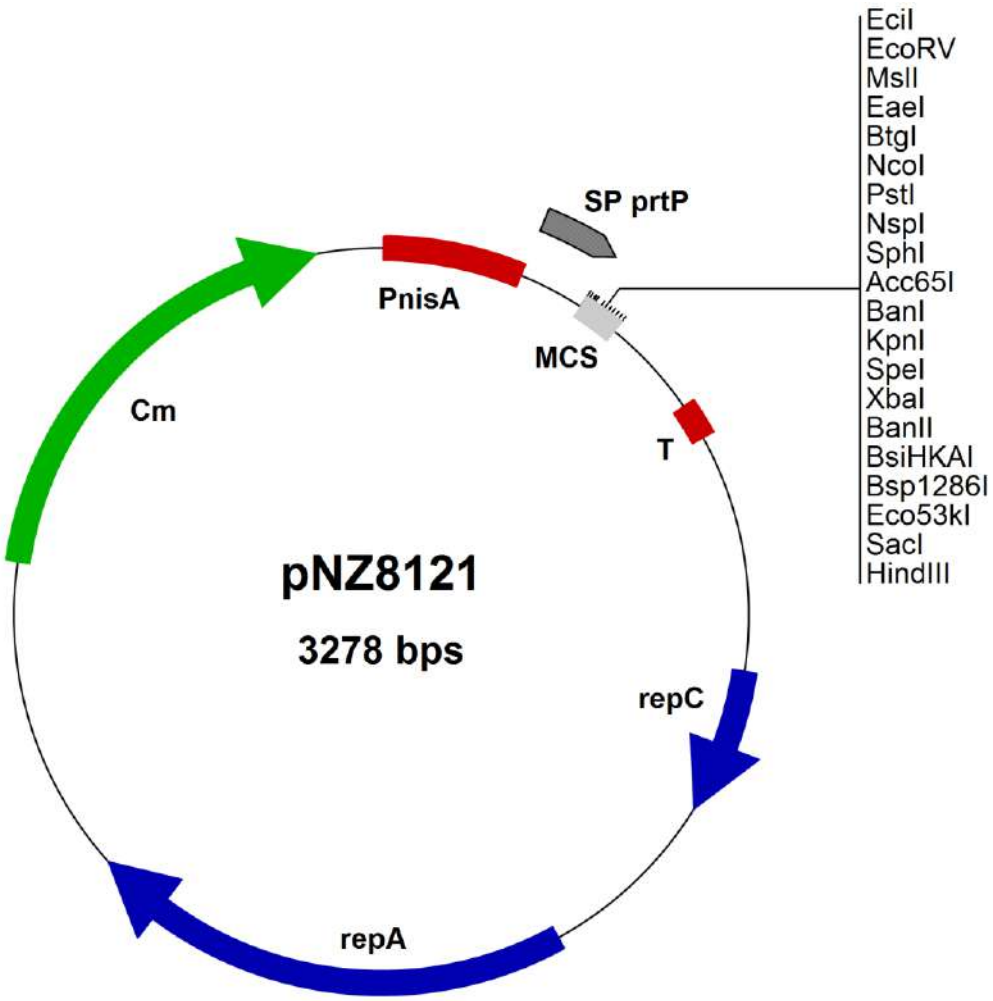
	Type	Start	End	Name	Description
	Gene	5	1495	*repE*	Replication gene *E*
	Gene	1845	1982	*repG*	Replication gene *G*
	Gene	2135	2821	*nisR*	Response regulator
	Gene	2814	4157	*nisK*	Histidine protein kinase
	Selectable Marker	4958	5715	Ery	Erythromycin resistance gene
	Gene	6073	6323	*repF*	Replication gene *F*
	Gene	6691	6990	*repD*	Replication gene *D*

7.8 Vector map pNZ8120



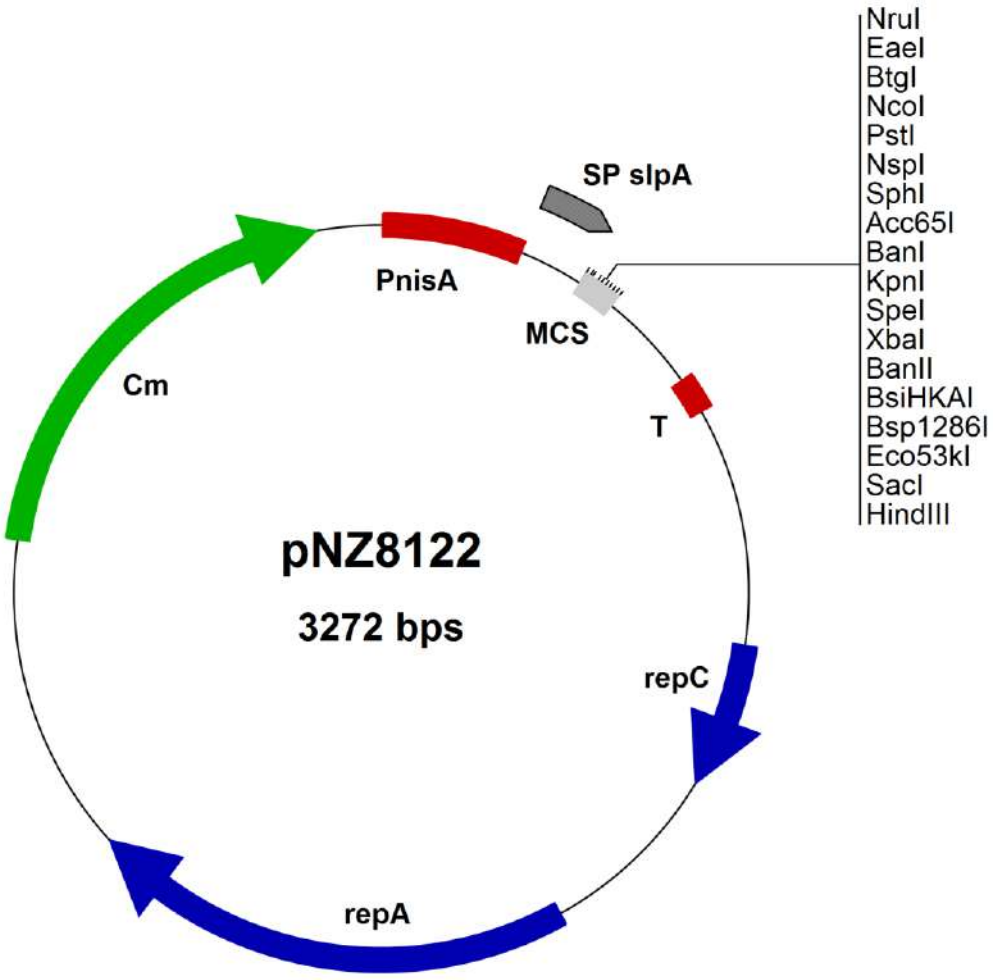
	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region
	Region	204	302	SP prtP	Signal sequence of *prtP* gene
	Region	299	355	MCS	Multiple Cloning Site
	Terminator	501	553	T	Termination sequence of *pepN* gene
	Gene	896	1105	*repC*	Replication gene *C*
	Gene	1374	2072	*repA*	Replication gene *A*
	Selectable Marker	2532	3182	Cm (*cat*)	Chloramphenicol resistance

7.9 Vector map pNZ8121



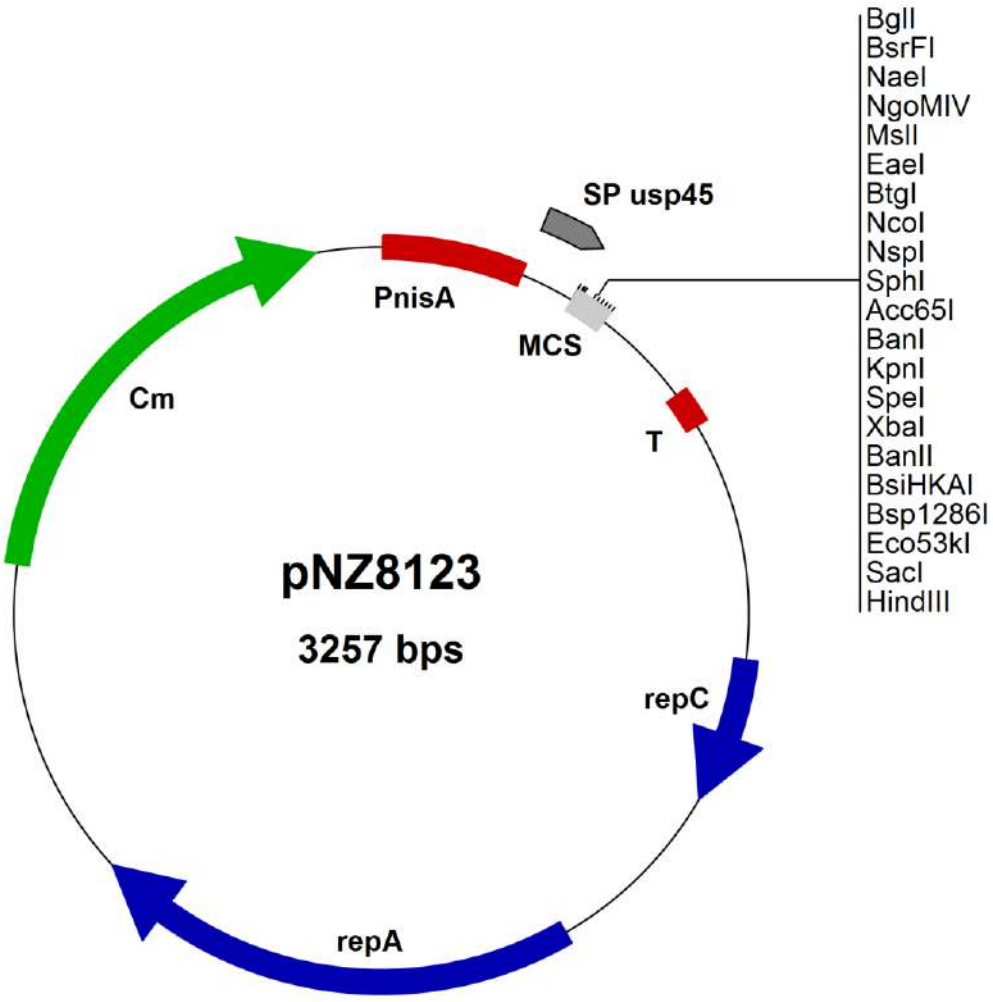
	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region
	Region	204	302	SP prtP	Signal sequence of *prtP* gene
	Region	299	358	MCS	Multiple Cloning Site
	Terminator	504	556	T	Termination sequence of *pepN* gene
	Gene	899	1108	*repC*	Replication gene *C*
	Gene	1377	2075	*repA*	Replication gene *A*
	Selectable Marker	2535	3185	Cm (*cat*)	Chloramphenicol resistance

7.10 Vector map pNZ8122



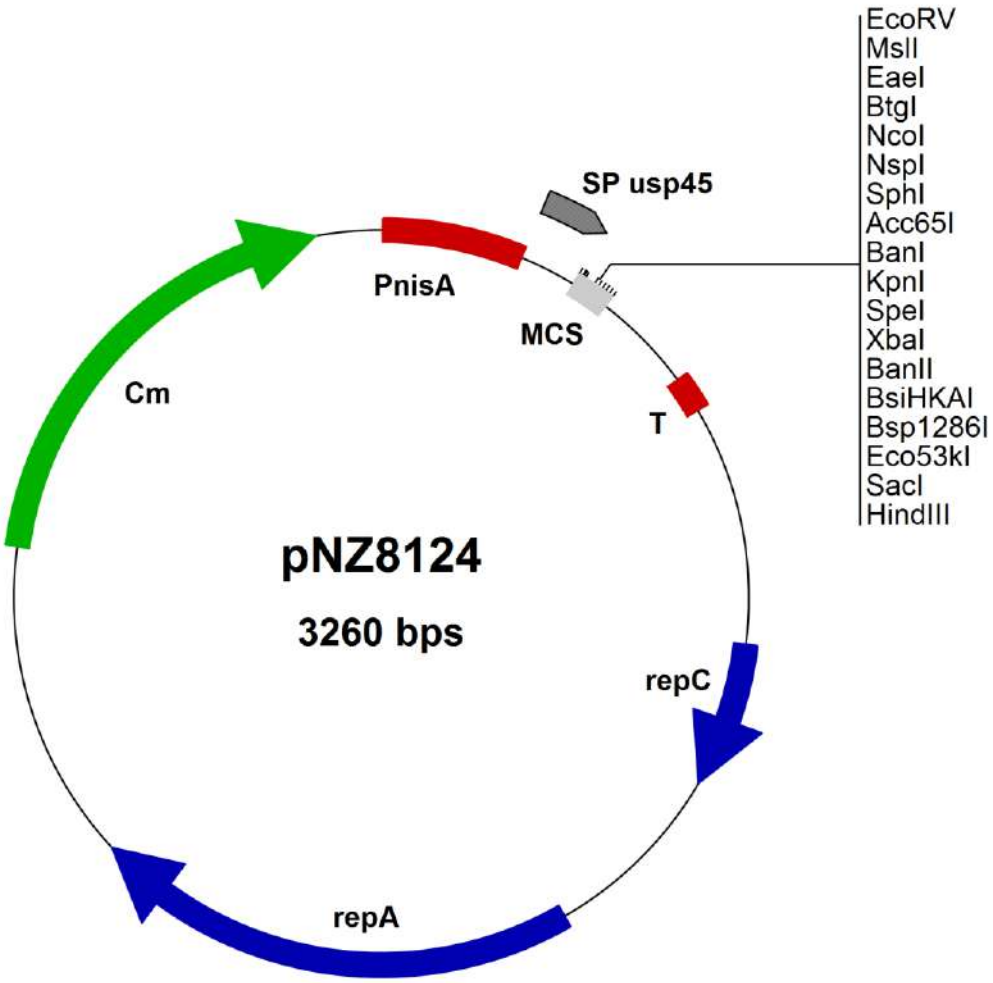
	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region
	Region	204	296	SP slpA	Signal sequence of *slpA* gene
	Region	297	352	MCS	Multiple Cloning Site
	Terminator	498	550	T	Termination sequence of *pepN* gene
	Gene	893	1102	*repC*	Replication gene *C*
	Gene	1371	2069	*repA*	Replication gene *A*
	Selectable Marker	2529	3179	Cm (*cat*)	Chloramphenicol resistance

7.11 Vector map pNZ8123



	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region
	Region	204	284	SP usp45	Signal sequence of *usp45* gene
	Region	282	337	MCS	Multiple Cloning Site
	Terminator	483	535	T	Termination sequence of *pepN* gene
	Gene	878	1087	*repC*	Replication gene *C*
	Gene	1356	2054	*repA*	Replication gene *A*
	Selectable Marker	2514	3164	Cm (*cat*)	Chloramphenicol resistance

7.12 Vector map pNZ8124



	Type	Start	End	Name	Description
	Promoter	3	203	PnisA	*nisin A* promoter region
	Region	204	284	SP usp45	Signal sequence of *usp45* gene
	Region	285	340	MCS	Multiple Cloning Site
	Terminator	486	538	T	Termination sequence of *pepN* gene
	Gene	881	1090	*repC*	Replication gene *C*
	Gene	1359	2057	*repA*	Replication gene *A*
	Selectable Marker	2517	3167	Cm (*cat*)	Chloramphenicol resistance

8 Order Information, Shipping, and Storage

Order#	Product	Quantity
VS-ELV00100-01	NICE® pNZ8008 Reference plasmid with gusA gene	10 µg
VS-ELV00200-01	NICE® pNZ8148 *Lactococcus lactis* expression vector, NcoI site	10 µg
VS-ELV00250-01	NICE® pNZ8150 *Lactococcus lactis* expression vector, ScaI site	10 µg
VS-ELV00300-01	NICE® pNZ8149 *Lactococcus lactis* expression vector, food grade, NcoI site	10 µg
VS-ELV00310-01	NICE® pNZ8151 *Lactococcus lactis* expression vector, food grade (lac F), ScaI site	10 µg
VS-ELV00400-01	NICE® pNZ8152 *Lactococcus lactis* expression vector, food grade (alr), ScaI site	10 µg
VS-ELV00600-01	NICE® pNZ8120 *Lactococcus lactis* secretion vector (SP PrtP/NaeI)	10 µg
VS-ELV00650-01	NICE® pNZ8121 *Lactococcus lactis* secretion vector (SP PrtP/EcoRV)	10 µg
VS-ELV00700-01	NICE® pNZ8122 *Lactococcus lactis* secretion vector (SP SlpA/NruI)	10 µg
VS-ELV00750-01	NICE® pNZ8123 *Lactococcus lactis* secretion vector (SP Usp45/NaeI)	10 µg
VS-ELV00800-01	NICE® pNZ8124 *Lactococcus lactis* secretion vector (SP Usp45/EcoRV)	10 µg
lyophilized from water, shipped at RT; store at -20 °C		
VS-ELV00500-01	NICE® pNZ9530 *Lactococcus lactis* nisRnisK vector, in Strain NZ9000	1 ml
VS-ELS09000-01	NICE® *Lactococcus lactis* expression Strain NZ9000	1 ml
VS-ELS09100	NICE® *Lactococcus lactis* expression Strain NZ9100	1 ml
VS-ELS03900-01	NICE® *Lactococcus lactis* expression Strain NZ3900, food grade, *ΔlacF*	1 ml
VS-ELS03910-01	NICE® *Lactococcus lactis* expression Strain NZ3910, food grade, *ΔlacF*	1 ml
VS-ELS09130-01	NICE® *Lactococcus lactis* expression Strain NZ9130, food grade, *Δalr*	1 ml
VS-ELS10610-01	NICE® *E. coli* replication strain MC1061	1 ml
VS-ELS10710-01	MC1061 Chemically Competent *E. coli*	5×100 µl
shipped on dry ice; store at -80 °C		
VS-ELK01000-02	NICE® Nisin kit, 1 g nisin (concentration 2.5% (balance sodium chloride and denatured milk solids)), 1 ml 5% acetic acid	Kit
shipped at RT; store at 4 °C		

The patented NICE® system was developed by NIZO food research BV. Patent # EP0712935, EP0355036, EP0228726 NICE® is a trademark of NIZO food research BV, The Netherlands.

9 Related Products

Order#	Product	Quantity
PBS023	*Bacillus subtilis* strain NZ8963	1 ml
PBS024	*Bacillus subtilis* strain NZ8900	1 ml
PBS025	*Bacillus subtilis* strain NZ8901	1 ml
PBS031	pNZ8901 vector, lyophilized plasmid DNA	10 µg
PBS032	pNZ8902 vector, lyophilized plasmid DNA	10 µg
PBS033	pNZ8910 vector, lyophilized DNA	10 µg
PBS034	pNZ8911 vector, lyophilized DNA	10 µg

10 Contact and Support

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