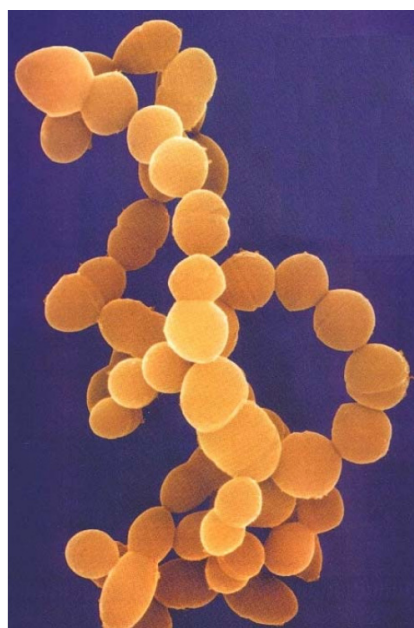
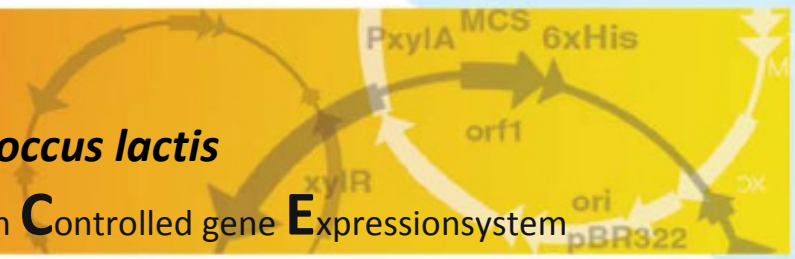


# NICE<sup>®</sup>

## Expression System for *Lactococcus lactis*

The effective & easy-to-operate **N**isin **C**ontrolled gene **E**xpression system



**Mo Bi Tec**  
MOLECULAR BIOTECHNOLOGY



## Content

<b>1 Introduction</b> .....	<b>3</b>
1.1 Nisin and the regulation of nisin biosynthesis.....	3
1.2 The nisin controlled gene expression system.....	4
1.3 <i>Lactococcus lactis</i> .....	4
1.4 Transfer of the NICE <sup>®</sup> system to other bacteria.....	4
1.5 Codon usage .....	5
<b>2 Products</b> .....	<b>6</b>
2.1 Host strains .....	6
Strains available at MoBiTec .....	6
2.2 Plasmids.....	6
pNZ8008, pNZ8148, pNZ8149 and pNZ8150.....	6
pNZ9530 .....	7
Plasmids available at MoBiTec .....	7
<b>3 Protocols</b> .....	<b>9</b>
3.1. Growth and nisin induction for the NICE <sup>®</sup> system in <i>Lactococcus lactis</i> .....	9
3.2. Media and growth conditions for <i>Lactococcus lactis</i> .....	9
3.3. Preparation of stocks for <i>Lactococcus lactis</i> .....	10
3.4. Plasmid DNA isolation from <i>Lactococcus lactis</i> , small scale.....	11
3.5. Transformation of <i>Lactococcus lactis</i> .....	12
3.6. Nisin induction of gene expression in <i>Lactococcus lactis</i> .....	13
3.7. Food grade selection of recombinant <i>Lactococcus lactis</i> .....	14
<b>4 Overview of applications of the NICE<sup>®</sup> system</b> .....	<b>15</b>
4.1 Over-expression of homologous and heterologous genes.....	15
4.2 The expression of genes of other organisms depends on the codon usage and the distribution of rarely used codons .....	15
4.3 Metabolic engineering.....	15
4.4 Expression of integral membrane proteins.....	16
4.5 Protein secretion and surface exposure of proteins .....	16
4.6 Expression and analysis of toxic or essential gene products.....	17
4.7 Large-scale applications .....	17
<b>5 Bottlenecks for gene expression in <i>L. lactis</i></b> .....	<b>18</b>
<b>6 References</b> .....	<b>19</b>
<b>7 Vector maps</b> .....	<b>20</b>
7.1. Vector map pNZ8148.....	20
7.2. Vector map pNZ8149.....	21
7.3. Vector map pNZ8150.....	22
7.4. Vector map pNZ8008.....	23
7.5. Vector map pNZ9530.....	24
<b>8 Order Information, Shipping and Storage</b> .....	<b>25</b>
<b>9 Contact and Support</b> .....	<b>25</b>



## 1 Introduction

The strictly **Nisin** controlled gene expression system (NICE<sup>®</sup>), developed at NIZO Food Research, NL, is easy-to-operate and has advantages for the following applications:

- (1) over-expression 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, R. et al. 2005; Ravn, P. et al. 2003; van Asseldonk et al. 1990; Vos, P. et al. 1989) and anchoring in the cell envelope,
- (5) expression of genes with toxic products and analysis of essential genes and
- (6) large scale applications.

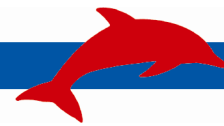
The major advantages of the NICE<sup>®</sup> system over other expression systems are:

- (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 inclusion bodies
- (VI) No spores
- (VII) Tightly controlled gene expression allows production of toxic proteins
- (VIII) Simple fermentation, scale-up and down stream processing

### 1.1 Nisin and the regulation of nisin biosynthesis

Nisin is a 34 amino acids anti-microbial peptide (lantibiotic). It first binds to lipidII 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 the 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*<sup>\*</sup> induces transcription from two of the three promoters in the nisin gene cluster: *P<sub>nisA</sub>* and *P<sub>nisF</sub>*. 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 autoinduction 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), creating the strain NZ9000. When a gene of interest is subsequently placed behind 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, the protein is expressed into the cytoplasm, into the membrane or secreted into the medium.

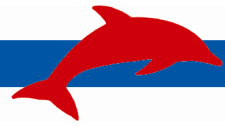
Studies with increasing amounts of nisin, using the  $\beta$ -glucuronidase gene as the reporter, show a linear dose–response curve. This shows that the NICE<sup>®</sup> system can be used not only for on/off gene expression studies but also to dose the target protein.

## 1.3 *Lactococcus lactis*

*Lactococcus lactis* is a homofermentative bacterium. Its primary function is rapid lactic acid production from lactose. 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 largely publicly available. This wealth of knowledge and experience has led to the use of lactococci in several fields of biotechnology, e.g. the expression of bacterial and viral antigens for safe vaccination via mucosal immunization, the production of human cytokines and other therapeutic agents for in situ treatments, the 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<sup>®</sup>) has been crucial for the development of many of these applications.

## 1.4 Transfer of the NICE<sup>®</sup> system to other bacteria

Because of its simplicity and its powerful induction characteristics, the NICE<sup>®</sup> 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  $\beta$ -glucuronidase or  $\beta$ -galactosidase as reporter gene, e.g. pNZ8008), the NICE<sup>®</sup> 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<sup>®</sup> 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 the 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 fits the codon usage pattern 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. The two genes are transcribed from their own constitutive promoter. For NZ9100 the genes were inserted into a neutral locus.

Strain NZ3900 was developed for food grade applications of the NICE<sup>®</sup> system. It is derived from 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 as described for NZ9000.

#### Strains available at MoBiTec

*Lactococcus lactis* NZ9000 – *pepN::nisRnisK*;

Standard host strain for nisin regulated gene expression (NICE<sup>®</sup>). 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 – *nisRnis*;

Standard host strain for nisin regulated gene expression (NICE<sup>®</sup>). The strain contains the regulatory genes *nisR* and *nisK* integrated in a neutral locus.

*Lactococcus lactis* NZ3900 – *lacF*, *pepN::nisRnisK*;

Standard strain for food grade selection based upon 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).

### 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). The two plasmids are compatible when cloned 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 replicon has a broad host range. 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*<sup>+</sup> strain like our MC1061 (order # VS-ELS10610-01). A *recA*<sup>-</sup> 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 like *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 pAM $\beta$ 1. This plasmid can only replicate in Gram-positive host strains, like *Lactococcus lactis*, *Lactobacillus plantarum* etc. (Simon and Chopin, 1988; Kleerebezem, 1997)

#### Plasmids available at MoBiTec

pNZ8148 – broad host range vector; Cm<sup>R</sup>; nisA promoter followed by an NcoI site for translational fusions at the ATG. Contains a terminator after the MCS. Sequence adaptation for cloning in NcoI can result in a change in the second amino acid of a protein (Mierau and Kleerebezem, 2005).

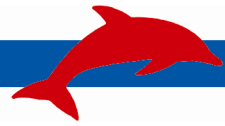
pNZ8149 – broad host range vector; *lacF* for food grade selection for growth on lactose; nisA promoter followed by an NcoI site for translational fusions at the ATG. Contains a terminator after the MCS. Sequence adaptation for cloning in NcoI can result in a change in the second amino acid of a protein (no reference, example: (Mierau *et al.*, 2005)).

pNZ8150 - broad host range vector; Cm<sup>R</sup>; nisA promoter followed by Scal 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 Scal site (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 (PnisA) (de Ruyter *et al.*, 1996).

pNZ9530 – Low copy plasmid with pAM $\beta$ 1 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 two plasmid system is used: e.g. pNZ9530 (*nisRnisK*) + pNZ8150 (+insert) (Kleerebezem *et al.*, 1997).

Strains	Plasmids	Medium
<i>Lactococcus lactis</i> NZ9000/NZ9100	pNZ8008	M17+0.5% glucose+10 µg/ml chloramphenicol
<i>Lactococcus lactis</i> NZ9000/NZ9100	pNZ8148	M17+0.5% glucose+10 µg/ml chloramphenicol
<i>Lactococcus lactis</i> NZ9000/NZ9100	pNZ8150	M17+0.5% glucose+10 µg/ml chloramphenicol
<i>Lactococcus lactis</i> NZ9000/NZ9100	pNZ9530	M17+0.5% glucose+10 µg/ml erythromycin
<i>Lactococcus lactis</i> NZ3900	pNZ8149	see page 9



All plasmids are lyophilized from water. Please use TE-buffer for reconstitution.

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 transformation.





## 3 Protocols

### 3.1. Growth and nisin induction for the NICE<sup>®</sup> 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  $OD_{600} = 0.2–0.5$  and induced with nisin (0.1–5 ng/ml). After that, the culture is continued for 0.5–3 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 densities because of lactic acid production and the consequent pH drop. Alternatively, the culture can be grown with pH control to higher cell densities. In this case, induction can be carried out at cell densities from  $OD_{600} = 1$  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 with *lacF* as food grade selection marker is selected for the ability to grow on lactose. The host strain *Lactococcus lactis* NZ3900 has all genes involved in the lactose fermentation on the genome, with a deletion of the *lacF* gene (Enzyme III of the Lac-PTS). The strain can grow on glucose, but in the presence of the *lacF* 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 only carbon source the lactose fermenting cells give yellow colonies.

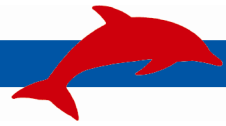
#### Elliker-medium

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 adjusting necessary

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, the yeast extract concentrations can be reduced to one half.

### 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 3000 x g or more (up to 6000 x g)
- Resuspend pellet in 250 µl THMS-buffer + 2 mg/ml lysozyme in Eppendorf cup
- 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 Eppendorf centrifuge
- Take out supernatant and add to new Eppendorf cup
- Fill the cup with 2-propanol
- Incubate 5-10 min at room temperature
- Spin 10 min in Eppendorf centrifuge
- Wash pellet carefully with 70% ethanol
- Dry the pellet (vacuum)
- Dissolve the pellet in 50 µl TE or sterile water

#### Materials

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

THMS buffer: 30 mM Tris-HCl pH 8  
3 mM MgCl<sub>2</sub>  
in 25% sucrose  
add lysozyme before use

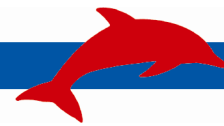
0.2 N Sodium hydroxide, 1% SDS (not older than 3 months)

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 G/L-SGM17B from a -80 °C stock, grow at 30 °C

Day 2:

Dilute the 5 ml culture in 50 ml G/L-SGM17B, grow at 30 °C

Day 3:

- Dilute the 50 ml culture in 400 ml G/L-SGM17B
- Grow until OD<sub>600</sub> is 0.2-0.3 (ca. 3 h.)
- Spin down cells for 20 min 6000 x g, at 4 °C
- Wash cells with 400 ml 0.5 M sucrose, 10% glycerol (4 °C) and spin down (6000 x g) (centrifugation speed may need to increase during successive washing steps)
- Resuspend the cells in 200 ml 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 0.5 M sucrose, 10% glycerol (4 °C) and spin down (6000 x g)
- Resuspend the cells in 4 ml 0.5 M sucrose, 10% glycerol (4 °C):
  - Use 40 µl per electroporation (keep on ice)
  - Or keep the cells in small portions in -80 °C, let them defreeze **on ice** before use

#### Electroporation:

- Place 40 µl cells in a pre-chilled electroporation cuvette with 1 µl DNA (reconstituted in TE-buffer) and keep the cuvette on ice
- Use the Biorad GenePulser with following adjustments:
  - 2000 V
  - 25 µF
  - 200 Ω
- Pulse (normal reading is 4.5-5 msec)
- Add 1 ml G/L-M17B + 20 mM MgCl<sub>2</sub> + 2 mM CaCl<sub>2</sub>
- 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-Bouillon 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

*Lactococcus lactis* grows very slowly in G/L-SGM17B. Leaving out the sucrose is possible (Wells et al. 1993) but can lower the transformation efficiency. The medium for cell recovery must contain MgCl<sub>2</sub> and CaCl<sub>2</sub>.



### 3.6. Nisin induction of gene expression in *Lactococcus lactis*

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

#### General protocol

Grow 5 ml culture overnight, 30 °C

Dilute 1/25 in 2 x 10 ml fresh medium (30 °C)

Grow until the OD<sub>600</sub> ≈ 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<sub>600</sub> 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 the 1 mg/ml nisin solution at -20 °C. Frozen aliquots are stable for at least 1 year.

**Note:** Prepare nisin dilution from aliquoted and frozen 1 mg/ml nisin stock solution in sterile water just before use and dispose this 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 *Lactococcus 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<sup>+</sup> transformants, see protocol 'Food grade selection of recombinant *Lactococcus lactis*'.

This 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 (waterfree)  
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

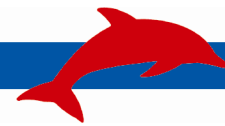
After sterilization:

add 0.5% lactose or 0.5% glucose (stock: 20% solution)

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

#### **Growth conditions**

*Lactococcus lactis* grows at 30 °C, without aeration.



## 4 Overview of applications of the NICE<sup>®</sup> system

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

### 4.1 Over-expression of homologous and heterologous genes

The NICE<sup>®</sup> 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  $\beta$ -glucuronidase gene of the Gram-negative *E. coli* has been expressed up to 20% of the total cellular protein.

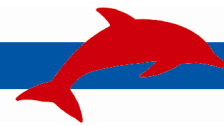
Furthermore, the NICE<sup>®</sup> system has been used to study the genetics and biology of pathogenic bacteria and to study genetic entities such as chromosomes and bacteriophage genomes. Genes of these various backgrounds can be expressed, however, with case-dependant yields (probably due to codon usage). Genes of closely related Gram-positive organisms (e.g. *Streptococcus*, *Enterococcus*, *Staphylococcus* and low-GC *Lactobacillus*) are almost always expressed effectively and hardly present any problems.

### 4.2 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 lysins, various peptidases and esterases to influence, for instance, flavour formation in dairy fermentations. Another important feature of the NICE<sup>®</sup> 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<sup>+</sup>-ATPase or the *rfb* operon of *L. lactis*.

### 4.3 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<sup>®</sup> 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<sup>®</sup> 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.



#### 4.4 Expression of integral membrane proteins

*Lactococcus lactis* is an excellent tool 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<sup>®</sup> 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.

#### 4.5 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 proteinases known:

- (1) the cell-wall-anchored proteinase PrtP (200 kD) and
- (2) the housekeeping membrane-bound proteinase 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 litre, 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 about 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 proteinase 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 on 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.

#### 4.6 Expression and analysis of toxic or essential gene products

One of the great strengths of the NICE<sup>®</sup> system is that it is tightly regulated, and genes that would otherwise have a detrimental effect on the cell can be cloned, analysed and expressed.

Toxic gene products: 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.

Essential genes: The tight control of gene expression displayed by the NICE<sup>®</sup> system also allows the functional study of essential genes in any background in which the NICE<sup>®</sup> system can be implemented. By integration of the *nisA* promoter upstream of the gene under study, expression of essential genes can be controlled by the addition of nisin to the medium, thereby allowing growth. Subsequent removal of nisin from the growth medium leads to increasing depletion of the essential gene product, allowing the investigation of the mutant phenotype. This application of the NICE<sup>®</sup> system raises possibilities for high-throughput essential gene screening methods.

#### 4.7 Large-scale applications

Nisin-induced gene expression can be scaled up from the 1- to 300-l scale and 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). The main reason for this limitation is the accumulation of lactic acid that eventually will stop the growth. 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 in the 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<sup>®</sup>-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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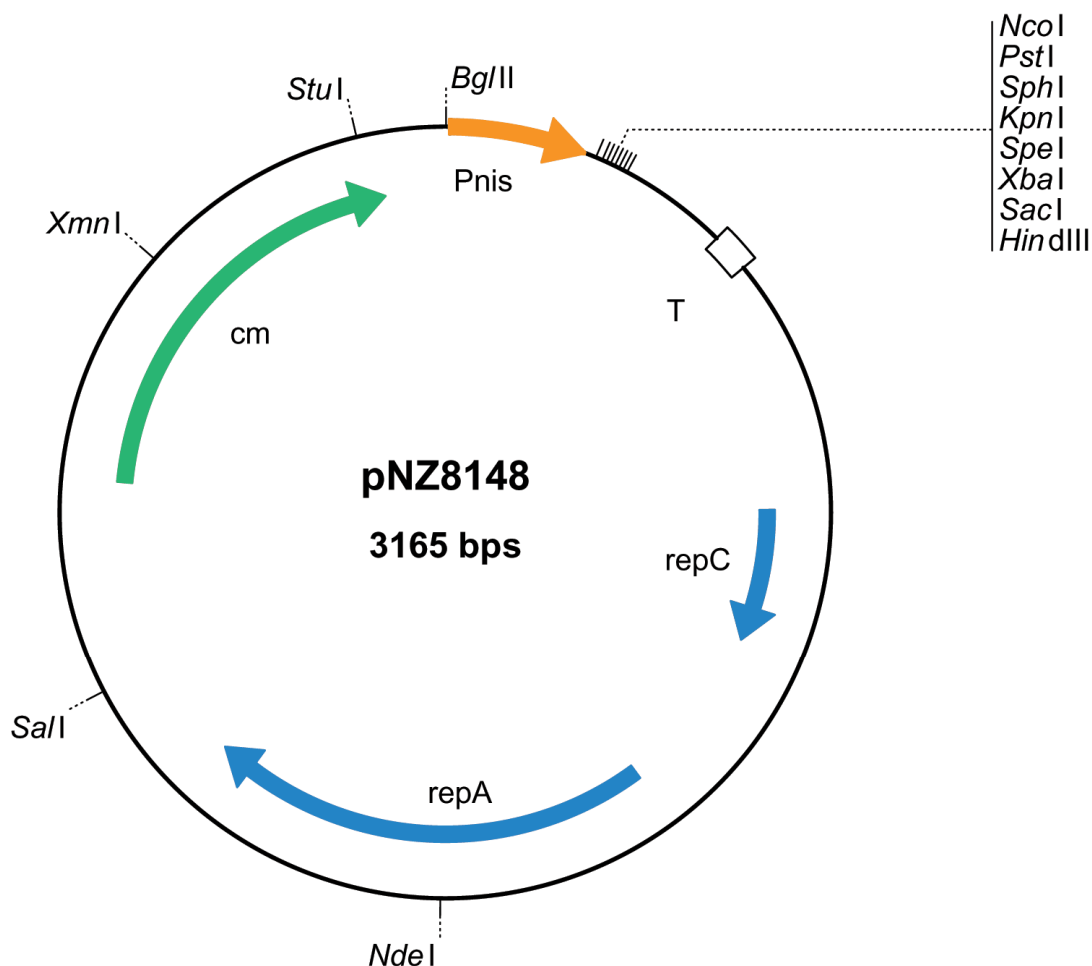


## 7 Vector maps

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

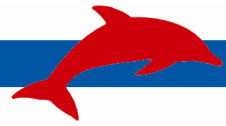
### 7.1. Vector map pNZ8148

3165 bps DNA Circular



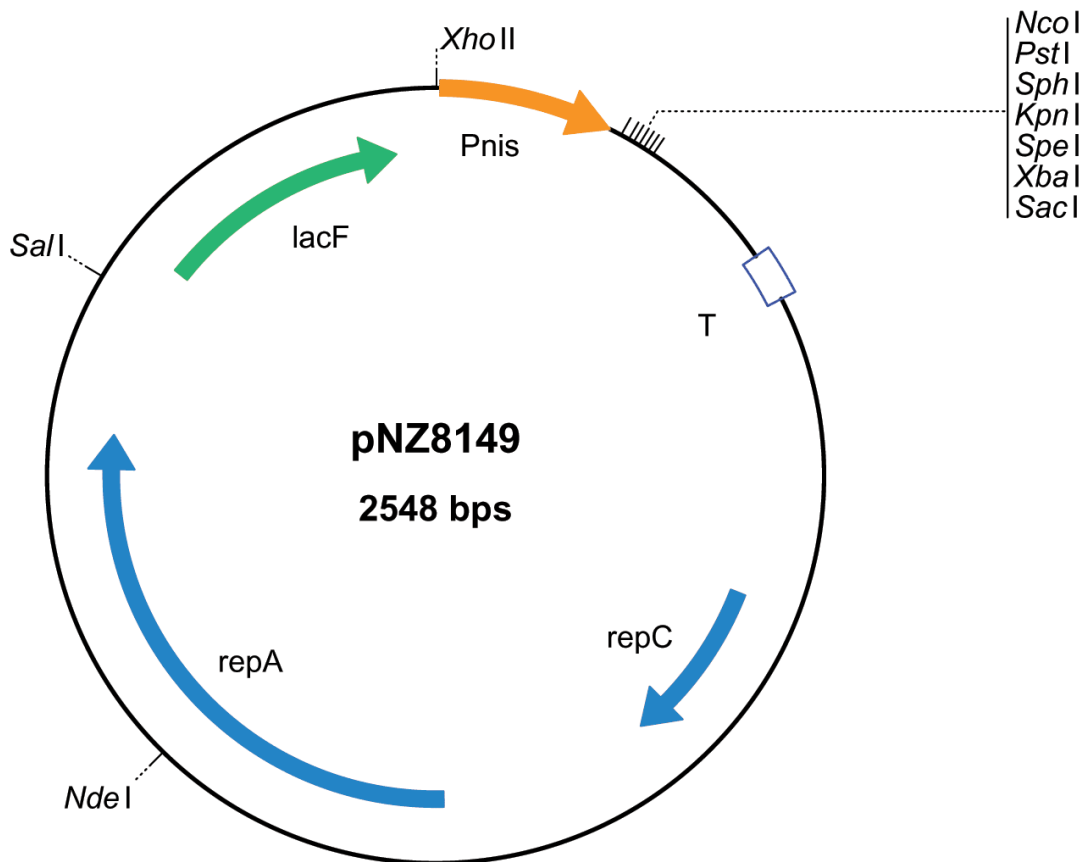
Molecule Features:

Type	Start	End	Name	Description
Region	5	188	Pnis	Nisin A promoter-region
Region	393	445	T	Terminator
Gene	787	996	repC	Replication gene C
Gene	1264	1962	repA	Replication gene A
Gene	2421	3071	cm	Chloramphenicol acetyltransferase gene



## 7.2. Vector map pNZ8149

2548 bps DNA Circular



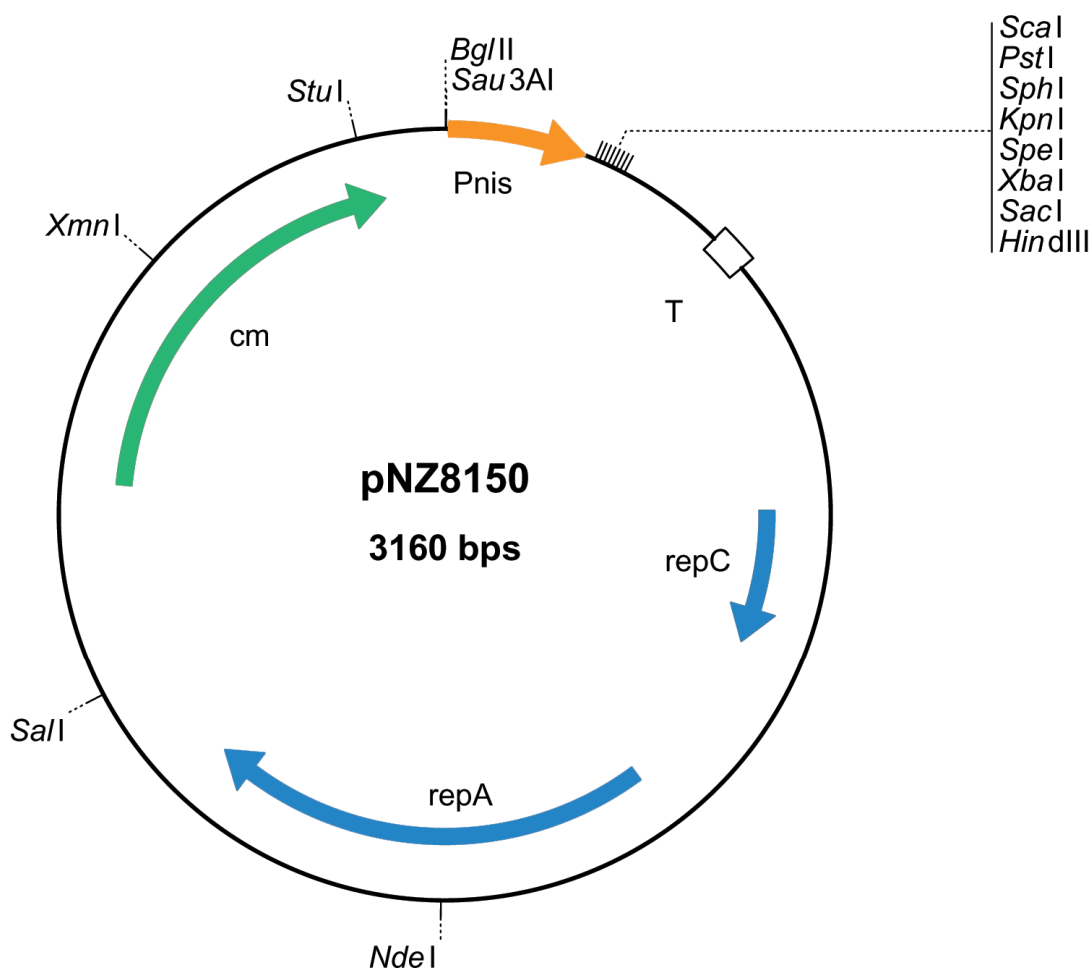
Molecule Features:

Type	Start	End	Name	Description
Region	5	188	Pnis	Nisin A promoter-region
Region	393	445	T	Terminator
Gene	787	996	repC	Replication gene C
Gene	1264	1962	repA	Replication gene A
Gene	2182	2499	lacF	Food grade selection marker, <i>L. lactis lacF</i> gene



### 7.3. Vector map pNZ8150

3160 bps DNA Circular



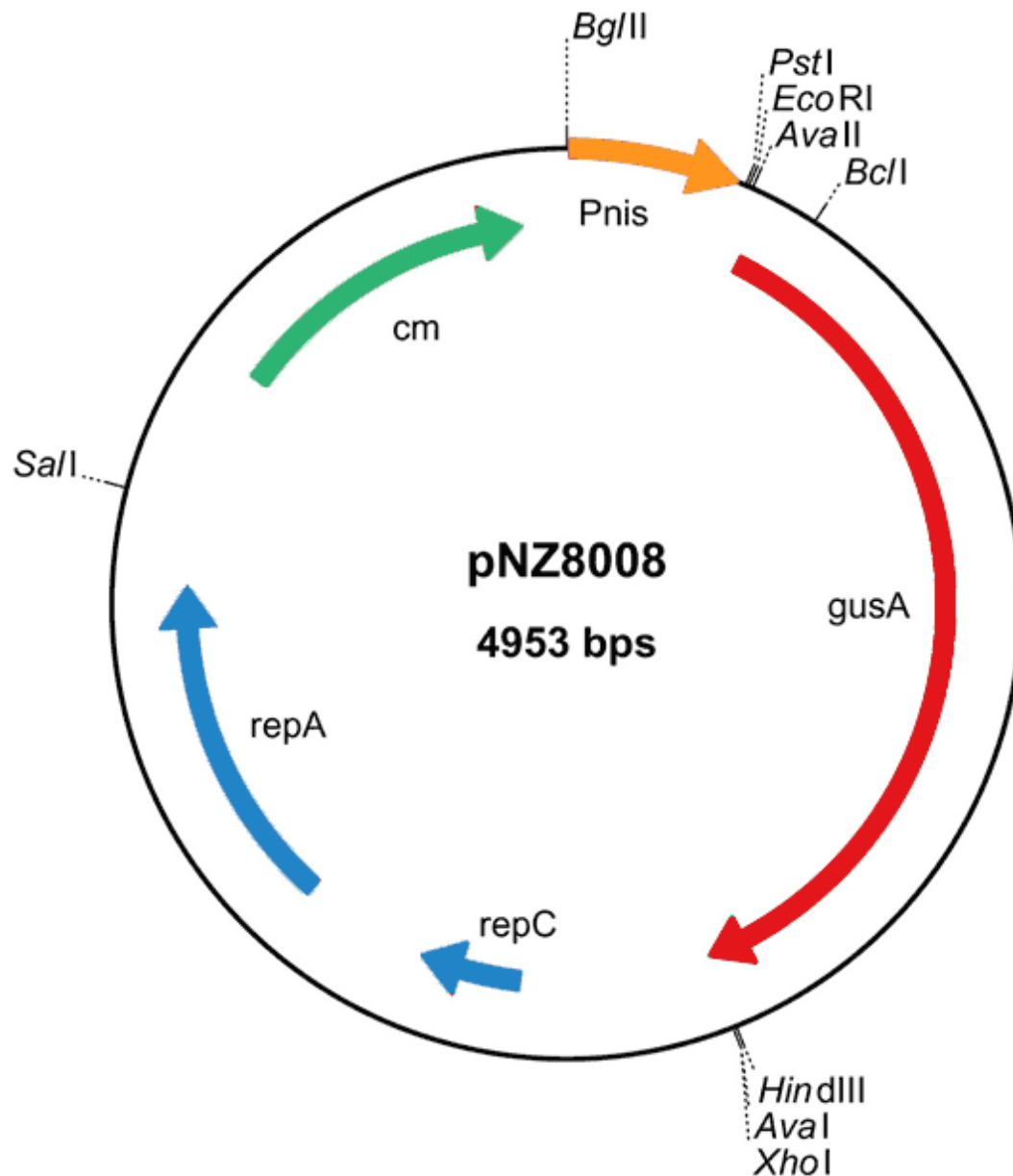
Molecule Features:

Type	Start	End	Name	Description
Region	5	188	Pnis	Nisin A promoter-region
Region	393	441	T	Terminator
Gene	783	992	repC	Replication gene C
Gene	1260	1958	repA	Replication gene A
Gene	2417	3067	cm	Chloramphenicol acetyltransferase gene



## 7.4. Vector map pNZ8008

4953 bps DNA Circular



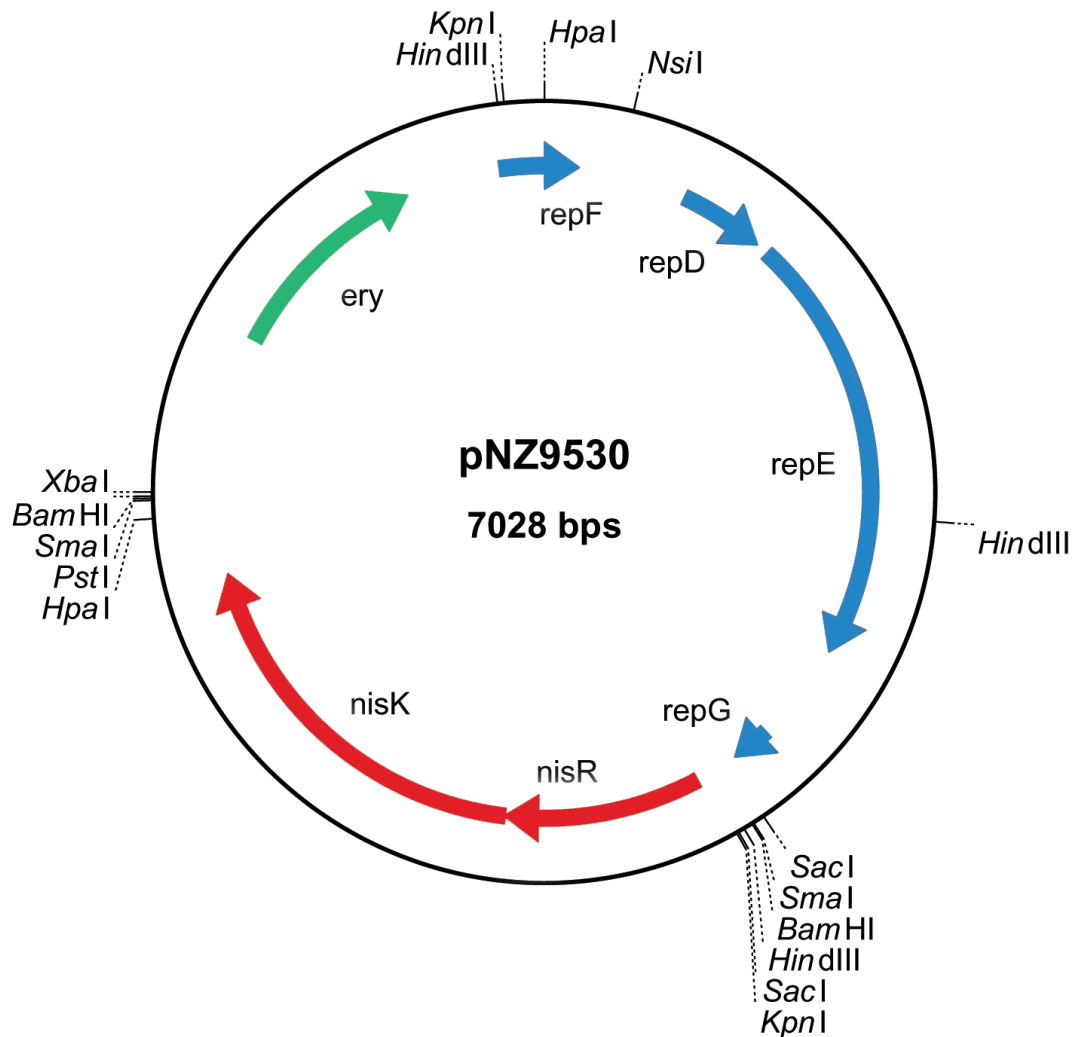
Molecule Features:

Type	Start	End	Name	Description
Region	5	305	Pnis	Nisin A promoter-region
Gene	366	2174	gusA	β-glucuronidase gene
Gene	2575	2784	repC	Replication gene C
Gene	3052	3750	repA	Replication gene A
Gene	4209	4859	cm	Chloramphenicol acetyltransferase gene



## 7.5. Vector map pNZ9530

7028 bps DNA Circular



Molecule Features:

Type	Start	End	Name	Description
Gene	497	796	repD	Replication gene D
Gene	839	2329	repE	Replication gene E
Gene	2679	2816	repG	Replication gene G
Gene	2966	3652	nisR	Response regulator
Gene	3648	4988	nisK	Histidine protein kinase
Gene	5810	6547	Ery	Erythromycin resistance gene
Gene	6874	120	repF	Replication gene F





## 8 Order Information, Shipping and Storage

Order#	Product	Quantity
VS-ELV00100-01	NICE <sup>®</sup> pNZ8008 Reference plasmid with gusA gene	10 µg
VS-ELV00200-01	NICE <sup>®</sup> pNZ8148 <i>Lactococcus lactis</i> expression vector, NcoI site	10 µg
VS-ELV00250-01	NICE <sup>®</sup> pNZ8150 <i>Lactococcus lactis</i> expression vector, Scal site	10 µg
VS-ELV00300-01	NICE <sup>®</sup> pNZ8149 <i>Lactococcus lactis</i> expression vector, food grade, NcoI site	10 µg
lyophilized from water, shipped at RT; store at -20 °C		
VS-ELV00500-01	NICE <sup>®</sup> pNZ9530 <i>Lactococcus lactis</i> nisRnisK vector, in Strain NZ9000	1 ml
VS-ELS09000-01	NICE <sup>®</sup> <i>Lactococcus lactis</i> expression Strain NZ9000	1 ml
VS-ELS09100	NICE <sup>®</sup> <i>Lactococcus lactis</i> expression Strain NZ9100	1 ml
VS-ELS03900-01	NICE <sup>®</sup> <i>Lactococcus lactis</i> expression Strain NZ3900, food grade	1 ml
VS-ELS10610-01	NICE <sup>®</sup> <i>E. coli</i> replication Strain MC1061	1 ml
shipped on dry ice; store at -80 °C		
VS-ELK01000-02	NICE <sup>®</sup> 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<sup>®</sup> system was developed by NIZO food research BV. NICE<sup>®</sup> is a trademark of NIZO food research BV. Patent EP0712935, EP0355036, EP0228726.

## 9 Contact and Support

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