



***K. lactis* Protein Expression Kit**

Recombinant Protein Expression in Yeast

I n s t r u c t i o n M a n u a l

Catalog #E1000S
Store at -20°C



Version 1.1
8/05

Table of Contents:

| | |
|---|----|
| Supplied Kit Components | 2 |
| Method Overview | 3 |
| Strategies for Protein Expression Using pKLAC1 | 9 |
| Cloning Strategy I: Secretion of a protein with a native N-terminus | 12 |
| Cloning Strategy II: Secretion of a protein with a non-native N-terminus | 13 |
| Cloning Strategy III: Secretion of a protein with a C-terminal hemagglutinin epitope tag | 14 |
| Cloning a PCR Fragment into pKLAC1 | 15 |
| Example Protocol | 17 |
| Optional Method: Identification of Properly Integrated Cells | 25 |
| Optional Method: Identification of Multi-copy Integrants | 26 |
| Media and Solutions | 28 |
| References | 30 |
| Kit Components Sold Separately/Companion Products | 31 |

Notice to Buyer/User: The *K. lactis* Protein Expression Kit was developed from basic research at New England Biolabs, Inc. and DSM Biologics Company B.V. The buyer and user has a non-exclusive sublicense to use this system or any component thereof, including the *K. lactis* GG799 Competent Cells, for **RESEARCH PURPOSES ONLY**. A license to use this system for manufacture of clinical grade material or commercial purposes is available from New England Biolabs, Inc., or DSM Biologics Company B.V.

Supplied Kit Components:

The *K. lactis* Protein Expression Kit contains all reagents necessary to express a recombinant protein in the yeast *Kluyveromyces lactis*.

Vectors:

| | |
|---------------------|-------|
| pKLAC1 | 20 µg |
| pKLAC1- <i>malE</i> | 20 µg |

Restriction Enzyme:

| | |
|------------------|-------------|
| Sac II | 2,000 units |
| NEBuffer 4 (10X) | |

Integration Identification Primers:

| | |
|--------------------------------|----------|
| Integration Primer 1 (10X) | |
| 5' d(TACCGACGTATATCAAGCCCA) 3' | 500 µl |
| Integration Primer 2 (10X) | |
| 5' d(ATCATCCTTGTCAGCGAAAGC) 3' | 1,000 µl |
| Integration Primer 3 (10X) | |
| 5' d(CAGTGATTACATGCATATTGT) 3' | 500 µl |

Yeast Transformation Reagents:

| | |
|--|-------------|
| <i>K. lactis</i> GG799 Competent Cells | 5 reactions |
| NEB Yeast Transformation Reagent | 5 ml |

Yeast Media:

| | |
|---------------------------------------|----------|
| Yeast Carbon Base (YCB) Medium Powder | 12 grams |
| Acetamide Solution (100X) | 10 ml |

Method Overview:

The *K. lactis* Protein Expression Kit provides a method for cloning and expressing a gene of interest in the yeast *Kluyveromyces lactis* (Figures 1 and 2). Proteins may be expressed intracellularly or be secreted from the cell using the supplied expression vector pKLAC1 (Figure 3).

Secretion of proteins is the most common approach to yeast protein expression. To achieve protein secretion, the gene of interest is cloned into pKLAC1 downstream of the *K. lactis* α -mating factor domain (α -MF; Figure 4), resulting in expression of an α -MF fusion protein. The α -MF domain directs the fusion protein to be efficiently transported through the yeast secretory pathway. An α -MF fusion protein undergoes sequential processing by signal peptidase in the endoplasmic reticulum (ER) and the Kex protease in the Golgi, resulting in the secretion of a native form of the protein of interest into the growth medium (Figure 1).

Expression in yeast is driven by a mutant form of the strong *K. lactis* *LAC4* promoter ($P_{LAC4-PBI}$) that has been engineered to lack background *E. coli* transcriptional activity (1). Therefore, genes encoding products toxic to *E. coli* can be cloned into pKLAC1 in *E. coli* prior to their introduction into yeast cells. To achieve expression in yeast, pKLAC1 containing a cloned gene of interest is linearized by either Sac II or BstX I to produce an expression cassette that can integrate into the *K. lactis* genome at the *LAC4* locus by homologous recombination. A fungal acetamidase gene (*amdS*) in pKLAC1 provides for selection of yeast containing an integrated expression cassette by allowing their growth on nitrogen-free minimal medium containing acetamide. Only cells expressing *amdS* can break down acetamide to ammonia for use as a nitrogen source. An advantage of this selection method is that it enriches transformant populations for cells that have integrated multiple tandem copies of the expression cassette and therefore produce more recombinant protein. Finally, the supplied *K. lactis* GG799 strain is an industrial isolate that has no auxotrophies, rapidly grows to high cell density, and efficiently secretes heterologous proteins.

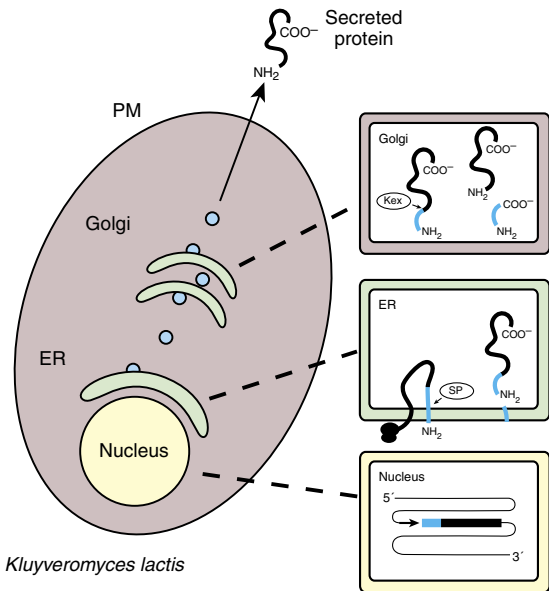


Figure 1: Secreted protein processing. In the nucleus, DNA encoding a protein fusion of the α -MF domain (blue) and a desired recombinant protein (black) is integrated into the yeast genome. Expression is driven by the $P_{LAC4-PBI}$ promoter (arrow). Upon expression of the fusion protein, a signal peptide in the α -MF domain directs translocation of the fusion protein into the lumen of the endoplasmic reticulum (ER) and is removed by signal peptidase (SP). Secretory vesicles (circles) transport the fusion protein to the Golgi where the Kex endoprotease cleaves the α -MF pro-domain, releasing a mature form of the desired protein. The protein of interest is then transported via vesicles to the plasma membrane (PM) where it is secreted from the cell.

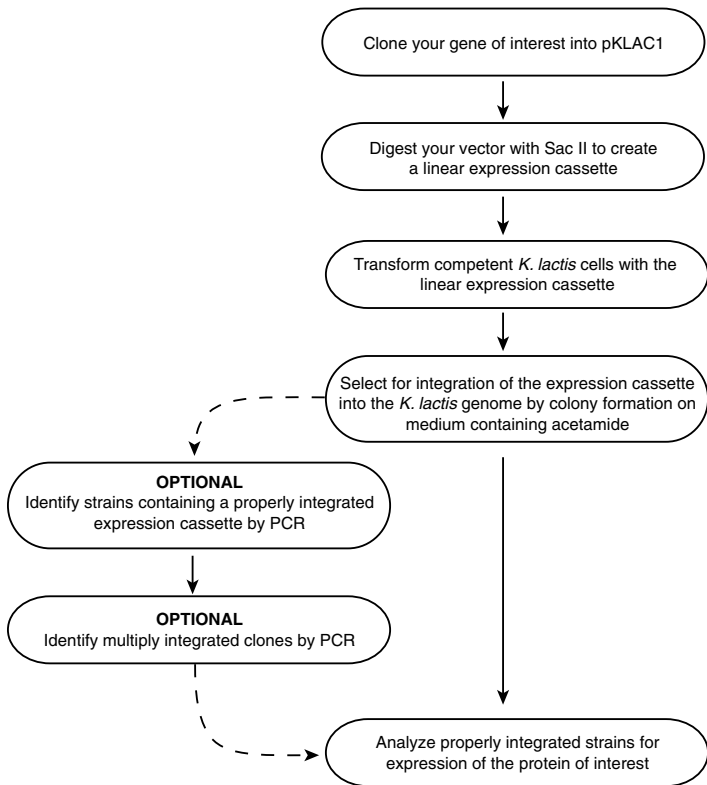


Figure 2: Method Overview of the *K. lactis* Protein Expression Kit.

Secretion of both eukaryotic (1-6) and prokaryotic proteins (7,8) from *K. lactis* has been achieved. Typically, proteins that are normally secreted from cells (e.g., cytokines, serum albumins, antibody fragments and glycosidases) produce the highest yields. In these cases, 10–50 mg of recombinant protein per liter can often be achieved in shake flasks, and yields can be further enhanced by high density cell fermentation. Secretion of other types of proteins is also possible. Secreted recombinant proteins are routinely detected in the growth medium of saturated cultures via SDS-PAGE and protein staining, Western analysis or enzyme assay. Secreted proteins may also bear post-translational modifications (e.g., asparagine-linked glycosylation) that can be removed by treatment with Endo H (NEB #P0702) or PNGase F (NEB #P0704).

Advantages:

- High level expression of recombinant proteins
- Rapid high cell density growth
- No background gene expression during *E. coli* cloning steps
- Easy and fast cell transformation procedure
- No expensive antibiotics required
- Attractive commercial sublicensing

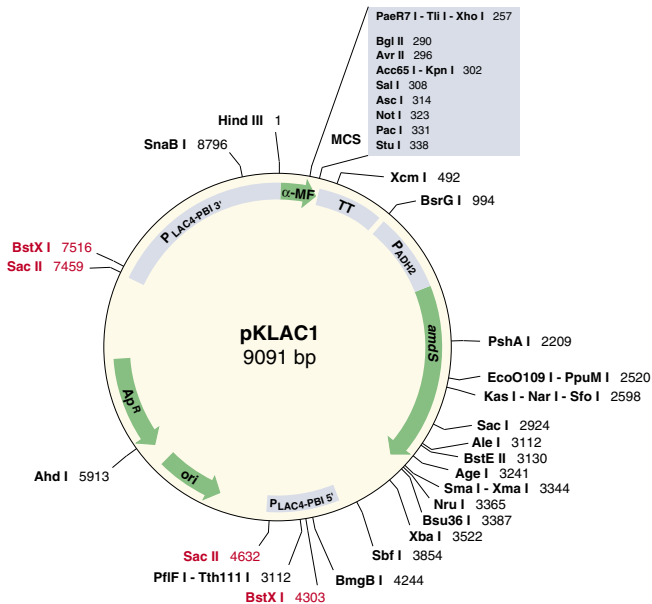


Figure 3: The pKLAC1 expression vector. pKLAC1 (9091 bp) contains the 5' and 3' ends of the LAC4 promoter ($P_{LAC4-PBI}$) separated by DNA encoding β -lactamase (Ap^R) and the pMB1 origin (ori) to allow for its propagation in *E. coli*. The *K. lactis* α -mating factor secretion leader sequence (α -MF), multiple cloning site (MCS), and the LAC4 transcription terminator (TT) lie immediately downstream of 3' $P_{LAC4-PBI}$. The yeast ADH2 promoter (P_{ADH2}) drives expression of an acetamidase selectable marker gene ($amdS$). The vector can be linearized by digestion with Sac II or BstX I to create a linear DNA fragment capable of inserting into the native LAC4 promoter region of the *K. lactis* genome.



Figure 4: pKLAC1 multiple cloning site. pKLAC1 (9091 bp) contains the *K. lactis* α -mating factor secretion leader sequence (blue background) and a polylinker immediately downstream of the $P_{LAC4-PBI}$ promoter. Unique polylinker restriction sites are indicated. Half-arrows show the positions of pKLAC1-specific sequencing primers available from New England Biolabs.

Strategies for Protein Expression Using pKLAC1:

pKLAC1 can be used for either intracellular or secreted expression of proteins of interest in *K. lactis*. The following sections describe various factors that influence how genes should be cloned into pKLAC1 to achieve the desired method of expression.

Secreted protein expression using pKLAC1

Secretion of a protein of interest from *K. lactis* cells is the most common approach to protein expression. Secretion results in production of proteins that are significantly pure, that do not require difficult lysis of yeast cells to isolate, and that may have desired post-translational modifications (e.g. glycosylation) that cytosolic proteins do not. Protein secretion using pKLAC1 is achieved by generating a fusion between the protein of interest and the α -MF secretion domain present in the vector. To do this, a gene or open reading frame of interest must be inserted into pKLAC1 so that it is in the same translational reading frame as the α -MF domain. It is also required to have a Kex protease processing site (KR↓) at the junction between the α -MF domain and the N-terminus of the protein of interest.

Because fusion proteins will be processed by the Kex protease in the Golgi, there are two strategies for making fusions to the α -MF domain. The first strategy generates a secreted recombinant protein with a native N-terminus and the second produces a protein with additional vector-encoded amino acids at its N-terminus. The first strategy makes use of the unique Xho I site that lies immediately upstream of DNA encoding the Kex protease cleavage site. A gene cloned into Xho I must contain DNA at its 5' end that reconstitutes the Kex protease cleavage site to allow for processing of the pro-protein (see Cloning Strategy I). In cases where the gene of interest contains an Xho I site or a native N-terminus is not required on the protein of interest, in-frame fusion with the α -MF domain may be achieved by cloning a gene into any of the other restriction sites that reside in the polylinker (see Cloning Strategy II). Fusion proteins made this way will contain additional amino acids encoded by vector DNA that resides between the Kex site and the polylinker restriction sites.

Native signal sequences

Proteins that are naturally secreted contain native secretion leader sequences. While various signal sequences have been used to direct secretion of certain proteins from *K. lactis* (2–8), it is recommended to replace the protein's native leader sequence with the *K. lactis* α -MF domain sequence present in pKLAC1. Predictive algorithms such as SIGNAL P (<http://www.cbs.dtu.dk/services/SignalP/>) can be used to determine if a protein contains a native secretion leader sequence.

Intracellular protein expression using pKLAC1

Because yeast cells are difficult to lyse, secretion is the most common approach to protein expression in *K. lactis*. However, it is possible to use pKLAC1 to express a protein intracellularly. In this case, it is not necessary to create a fusion to the α -MF domain. Therefore, a gene of interest should be cloned into pKLAC1 using the unique Hind III site that lies immediately upstream of the α -MF domain and any of the polylinker sites. This places the gene of interest immediately downstream of the strong P_{LAC4-PBI} promoter. In this scenario, it is important to ensure that the desired gene begins with a methionine codon to initiate translation. If the desired gene does not begin with a methionine codon, one should be added by PCR or site-directed mutagenesis.

It is important to note that when expressing a gene in this manner, identification of properly integrated strains or multiply integrated strains by PCR (see Optional Methods on pages 25 and 26) is not possible using Integration Primer 2. Integration Primer 2 anneals to a region of pKLAC1 DNA that lies between the Hind III site and the polylinker, and is removed during cloning for intracellular expression. Instead, a custom reverse primer can be designed that anneals to the 5' end of the gene of interest (for more details see *K. lactis* Protein Expression Kit FAQ #2.5 at www.neb.com) and can be used in place of Integration Primer 2 in the Optional Methods. Additionally, the α -Mating Factor Sequencing Primer (NEB #S1275, Figure 4) cannot be used to sequence the 5' end of genes cloned in this manner. The LAC4mt Promoter Sequencing Primer (NEB #S1274, Figure 4) should be used instead.

Epitope tagging

To allow for detection of secreted recombinant protein in the culture medium by western blotting, it may be desirable to create a fusion to a peptide epitope tag. Cloning Strategy III illustrates the use of PCR to incorporate DNA encoding a C-terminal hemagglutinin (HA) peptide epitope and a stop codon into the gene of interest.

Incorporation of stop codons

It is assumed that the gene of interest will include a stop codon (TAG, TAA or TGA) at its 3' end. If it does not, one should be engineered into the cloning strategy. Stop codons may be incorporated into reverse PCR primers as shown in Cloning Strategies I, II and III.

Cloned Genes must be free of Sac II or BstX I restriction sites

Vector pKLAC1 containing the gene of interest must be linearized with either Sac II or BstX I to create an expression cassette that can be stably integrated into the *K. lactis* genome at the *LAC4* locus upon its introduction into *K. lactis*. Therefore, the gene of interest must be free of either Sac II or BstX I sites. If the gene contains both restriction sites, then either all Sac II sites or all BstX I sites must be removed using site-direct mutagenesis prior to transforming *K. lactis* cells.

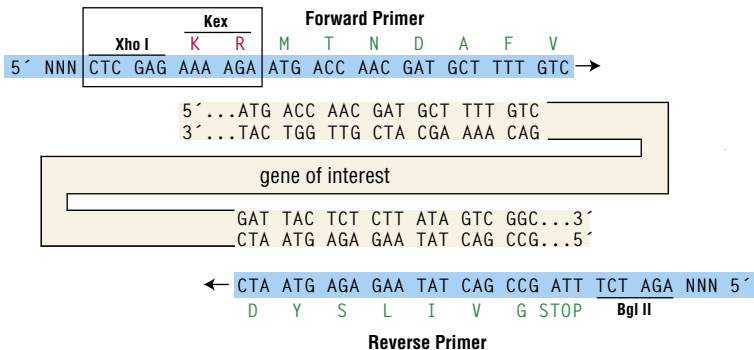
Sequencing

Genes cloned into vector pKLAC1 should be sequenced prior to transformation of *K. lactis*. The positions of pKLAC1-specific sequencing primers available from New England Biolabs are shown in Figure 1. The sequence of vector pKLAC1 (Genbank #AY968582) is available at www.neb.com or by email request to info@neb.com.

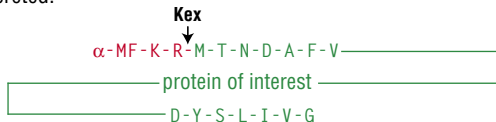
Cloning Strategy I:

Secretion of a protein with native N-terminus

Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) for cloning into pKLAC1. The Forward Primer must contain an Xho I site and a Kex protease cleavage site (boxed) immediately followed by the first codon of the gene's open reading frame. The Reverse Primer must include a restriction site for cloning into any of the polylinker sites of pKLAC1 (Bgl II shown).



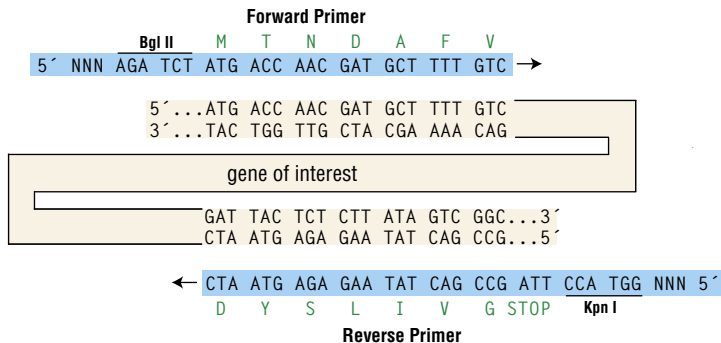
After Kex processing, the protein of interest (green) containing a native N-terminus is secreted.



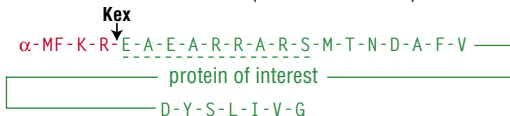
Cloning Strategy II:

Secretion of a protein with non-native N-terminus

In cases where the gene of interest contains an Xho I site, one may clone the gene in-frame with the α -MF domain using other sites in the pKLAC1 polylinker. This strategy results in vector-encoded amino acids being added to the protein's N-terminus. Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) for cloning into the Bgl II and Kpn I polylinker sites of pKLAC1.



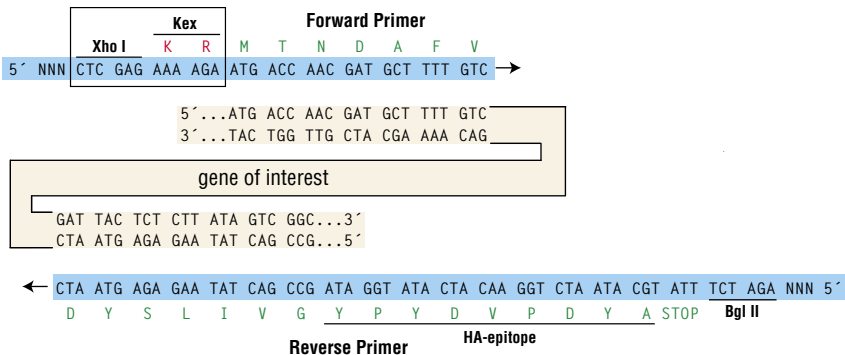
After Kex processing, the secreted form of the protein of interest (green) contains extra vector-encoded amino acids at its N-terminus (dashed underline).



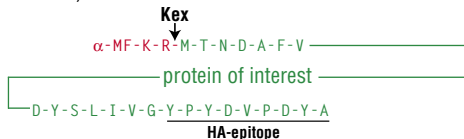
Cloning Strategy III:

Secretion of a protein with a C-terminal hemagglutinin epitope tag

Shown are PCR primers (blue background) designed for amplification of an example gene of interest (yellow background) having a C-terminal antibody epitope tag (hemagglutinin epitope [HA] shown). The Forward Primer should be designed as shown in Cloning Strategies I or II (strategy I shown). The Reverse Primer should contain DNA encoding an HA epitope tag, a stop codon, and a restriction site for cloning into the polylinker of pKLAC1 (Bgl II shown).



After Kex processing, the protein of interest (green) containing a C-terminal HA-epitope (underlined) is secreted.



Cloning a PCR Fragment into pKLAC1:

The procedure below is for cloning a fragment produced by PCR into pKLAC1. This example assumes that the PCR fragment contains a 5' Xho I site, and has a stop codon followed by a Bgl II site incorporated into its 3' end (see Cloning Strategy I).

1. Prepare a PCR fragment of the gene of interest as outlined in Cloning Strategy I on page 11.
2. Digest 0.5 μg of pKLAC1 DNA with 10 units of Xho I and 10 units of Bgl II in 20 μl of 1X NEBuffer 3 (supplied as a 10X stock) supplemented with 100 $\mu\text{g}/\text{ml}$ BSA (supplied as a 100 mg/ml stock) at 37°C for 2 hours.
3. Digest 0.5 μg of the PCR fragment with 10 units of Xho I and 10 units of Bgl II in 20 μl of 1X NEBuffer 3 supplemented with 100 $\mu\text{g}/\text{ml}$ BSA at 37°C for 2 hours.
4. Add an equal volume of phenol:chloroform (1:1, v/v) to the restriction digests, mix and remove the aqueous (top) phase to a fresh tube. Repeat using only chloroform.

Alternatively, the DNA fragments can be isolated using one of the many commercially available fragment purification kits. If a kit is used, skip to Step 8.

5. Add 10 μg glycogen or tRNA as carrier to both digests, then add a 1/10th volume 3M sodium acetate, mix and add an equal volume of 100% isopropanol. Incubate at room temperature for 10 minutes.
6. Microcentrifuge at 12,000 $\times g$ for 15 minutes. Pour off the supernatant and gently rinse the pellet with 70% ethanol. Allow the pellet to air-dry (~10 minutes).
7. Resuspend each sample in 25 μl of 10 mM Tris-HCl, 1 mM EDTA (pH 8.0).

8. Mix:
 - 2 μ l pKLAC1 digest (~100 ng)
 - 1 μ l PCR fragment (insert digest) (~300 ng)
 - 2 μ l 10X T4 DNA Ligase Buffer
 - 14 μ l deionized water
 - 1 μ l (~400 units) T4 DNA Ligase (NEB #M0202S)
9. Incubate at 16°C for 2 hours to overnight.
10. Mix the whole ligation reaction with 25–50 μ l competent *E. coli* cells and incubate on ice for 5–30 minutes. Heat to 42°C for 0.5–2 minutes.

Any competent E. coli strain can be used. However, blue-white screening is not possible with pKLAC1.
11. Add 0.1 ml LB and incubate at 37°C for 20 minutes. Spread on an LB plate containing 100 μ g/ml ampicillin. Incubate overnight at 37°C.
12. Prepare miniprep DNA (9) from several transformants. Digest each with an appropriate restriction endonuclease to determine the presence of a cloned insert.

Expression vectors may be stored frozen at –20°C indefinitely.

Example Protocol:

An example experiment is described to illustrate creation of a *K. lactis* strain that is capable of secreting a protein of interest. This is accomplished by linearizing pKLAC1 containing a cloned gene of interest, inserting the linearized expression vector into the *K. lactis* genome by integrative transformation and growing cells to achieve secretion of the desired protein into the growth medium. A control pKLAC1 vector containing the *E. coli* *malE* gene (pKLAC1-*malE*) that encodes the maltose binding protein (MBP) is supplied with the kit and can be processed in parallel using this protocol. Integration of pKLAC1-*malE* into the *K. lactis* genome routinely yields cells that secrete > 25 mg MBP per liter of culture.

Linearization of pKLAC1 for integrative transformation of *K. lactis*

pKLAC1 containing any desired gene must be linearized to allow it to insert into the *K. lactis* genome at the *LAC4* locus (Figure 5). This is accomplished by digesting the construct with either Sac II (supplied with kit) or BstX I to generate an “expression cassette” consisting of > 6.2 kb of DNA containing P_{LAC4-PBI}, the cloned gene and the *amdS* selection cassette, and a 2.8 kb fragment containing the remaining pKLAC1 vector DNA. The cloned gene must be free of Sac II sites (or BstX I sites if digesting with BstX I) to allow for generation of the proper expression fragment. It is not necessary to purify the expression fragment from the remaining vector DNA following digestion as only the expression fragment will integrate into the *K. lactis* genome upon transformation.

1. Digest 2 µg of pKLAC1 DNA containing the gene of interest with 20 units of Sac II in 50 µl of 1X NEBuffer 4 (supplied as a 10X stock) at 37°C for 2 hours.

The pKLAC1-malE control vector can be linearized only with Sac II due to the presence of a BstX I site in the malE gene.

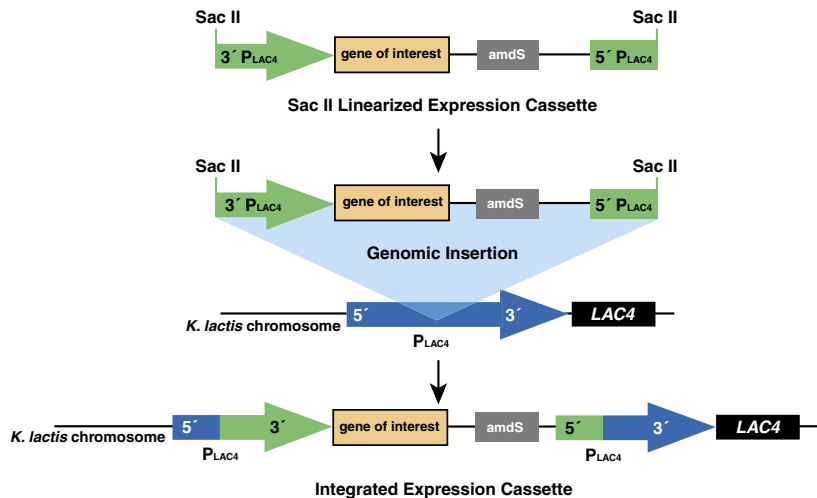


Figure 5: Genomic integration of a linear expression cassette. Vector pKLAC1 containing the gene of interest is digested with either *Sac II* or *BstX I* (*Sac II* shown) and introduced into *K. lactis* cells. The 5' P_{LAC4} and 3' P_{LAC4} sequences direct insertion of the cassette into the promoter region of the *LAC4* locus in the *K. lactis* genome.

2. Desalt digested DNA using a commercially available DNA fragment purification kit (e.g., Qiagen's QIAquick™ PCR Purification Kit).

A total of 1 µg of linearized DNA in a volume less than 15 µl will be needed to transform K. lactis cells. DNA may be stored frozen at -20°C for up to one month prior to transforming K. lactis cells.

Transformation of *K. lactis* GG799 cells

Introduction of the linearized expression cassette into *K. lactis* cells is achieved by chemical transformation using the *K. lactis* GG799 Competent Cells and NEB Yeast Transformation Reagent supplied with the kit. This procedure yields approximately 1×10^4 transformants per microgram of DNA. Transformants are selected by growth on Yeast Carbon Base (YCB) Agar Medium containing 5 mM acetamide (see Media & Solutions). YCB medium contains glucose and all nutrients needed to sustain growth of *K. lactis* GG799 cells except a simple nitrogen source. Cells can utilize acetamide as a source of nitrogen only after it is broken down to ammonia by acetamidase, the product of the *amdS* gene present in pKLAC1.

The following steps should be conducted using aseptic technique. Care should be taken to ensure that pipet tips, tubes, solutions and deionized water are sterilized prior to use.

1. Thaw a tube of *K. lactis* GG799 Competent Cells on ice. Add 620 µl NEB Yeast Transformation Reagent to the cells. Briefly shake or invert the tube until the solution is homogeneous.

Do not vortex.

2. Add 1 µg of linearized pKLAC1 DNA containing the gene of interest to the cell mixture. Briefly shake or invert the tube to mix.

Do not vortex. The total volume of transforming DNA should not exceed 15 µl.

3. Incubate the mixture at 30°C for 30 minutes.
4. Heat shock the cell mixture by incubation at 37°C for 1 hour.
5. Pellet cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant.
6. Resuspend the cell pellet in 1 ml sterile deionized water.
7. Pellet cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant.
8. Resuspend the cell pellet in 1 ml YPGlu medium (see Media & Solutions) and transfer the cell mixture to a sterile culture tube. Incubate with shaking (250–300 r.p.m.) at 30°C for 30 minutes.
9. Transfer the cell mixture to a sterile 1.5 ml microcentrifuge tube. Pellet the cells by microcentrifugation at ~7000 r.p.m for 2 minutes and discard the supernatant. Resuspend the cell pellet in 1 ml sterile deionized water.
10. Remove 1, 10 and 50 μ l of the cell suspension to separate fresh sterile 1.5 ml microcentrifuge tubes each containing 75 μ l of sterile deionized water. Mix briefly and spread the entire cell mixture from each tube onto separate YCB Agar Medium plates containing 5 mM acetamide (see Media & Solutions). Incubate plates inverted at 30°C for 3–4 days until colonies form.

Due to the high transformation efficiency of K. lactis GG799 Competent Cells, plating multiple dilutions of the cell mixture is necessary to ensure formation of plates with distinct single colonies. Growth time should not exceed 5 days as small colonies that lack an integrated expression fragment may form. Plates containing colonies can be stored at 4°C for up to two weeks.

11. Streak or patch 10–20 individual colonies onto fresh YCB Agar Medium plates containing 5 mM acetamide. Incubate at 30°C for 1–2 days.

Patches of approximately 1.0 cm² are recommended. Plates containing patched cells may be stored at 4°C for up to 3 days prior to performing whole-cell PCR (optional steps 12, 13).

12. [OPTIONAL] Transformants can be tested to verify that they have correctly integrated the expression fragment (see Identification of Properly Integrated Cells, page 25).
13. [OPTIONAL] Correctly integrated transformants can be further screened to identify cells that have integrated multiple tandem copies of the expression fragment (see Identification of Multicopy Integrants, page 26).

Growth of strains for detection of secreted protein

1. From the patch of each strain that contains an integrated expression fragment, harvest cells from an area approximately 2 mm² by scraping with a sterile pipette tip and resuspend the cells in 2 ml of YPGal medium (see Media & Solutions) in a sterile culture tube. Incubate the cultures with shaking (~250 r.p.m.) at 30°C.

The duration of growth will vary depending on the protein being secreted. As a general rule, allow a minimum of 2 days growth at 30°C to obtain a saturated culture (a culture density of > 30 OD₆₀₀ units/ml). Analysis of culture supernatant may be performed each day thereafter to determine the optimum growth time to achieve maximum secretion of the protein of interest. Culture sizes will ultimately depend on the desired application. For example, to determine the efficiency of secretion on previously untested cells, 2 ml cultures allow for simultaneous analysis of many strains. Larger cultures (e.g., ≥1 L) for protein purification should be inoculated 1:100 with a starter culture grown overnight at 30°C.

2. Microcentrifuge 1 ml of each culture for 30 seconds to pellet cells. Remove the culture supernatant to a fresh microcentrifuge tube and store on ice.

3. Since expression levels of recombinant proteins secreted from *K. lactis* vary from protein to protein, culture supernatant samples must be analyzed to determine if the protein of interest is being secreted. Polyacrylamide gel electrophoresis followed by Coomassie or silver staining of unconcentrated culture supernatant (15 μ l per lane) allows for visual detection of proteins that are highly secreted (e.g. > 10 mg/l) (Figure 6A). Alternatively, Western blotting can detect lesser quantities of secreted protein (Figure 6B). If an antibody to your protein of interest is unavailable, an antibody epitope tag (e.g. hemagglutinin (HA) peptide epitope) can be engineered as a C-terminal fusion to the protein of interest (see Cloning a PCR Fragment, Strategy III). Finally, if the protein of interest is an enzyme, culture supernatant may be analyzed for the presence of the protein directly by activity assay (Figure 7). In such cases, it is important to note that the absence of an enzyme activity in culture supernatant does not always indicate a lack of secretion. For example, the enzyme may be secreted in an inactive form or the nutrient rich growth medium may inhibit the activity assay. In such cases, absence of secretion of the protein of interest should also be confirmed by SDS-PAGE or Western analysis.

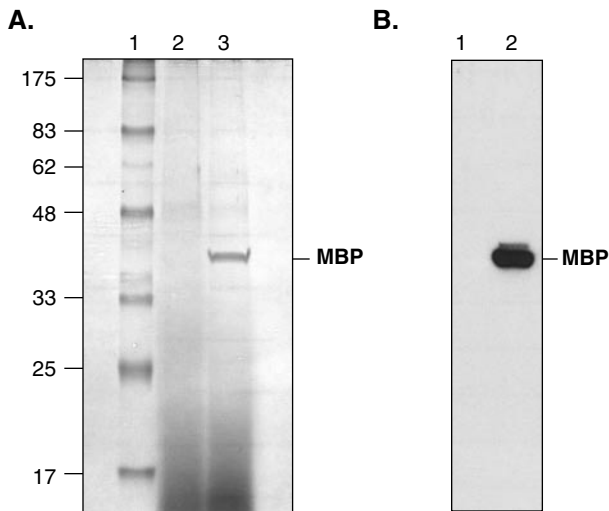


Figure 6: SDS-polyacrylamide gel electrophoresis of secreted recombinant maltose binding protein (MBP). (A) Detection of secreted MBP by Coomassie staining. Lane 1: Protein Molecular Weight Markers. Lane 2: spent culture medium (15 μ l) from wild-type *K. lactis* cells. Lane 3: spent culture medium (15 μ l) from *K. lactis* cells harboring an integrated expression cassette containing the *E. coli malE* gene. (B) Detection of secreted MBP by Western analysis using a polyclonal anti-MBP antibody (NEB #E8031S; 1:2000 dilution) and an anti-rabbit IgG-HRP secondary antibody (1:2000 dilution). Lane 1: culture medium from *K. lactis* cells (15 μ l). Lane 2: culture medium (15 μ l) from *K. lactis* cells containing the *E. coli malE* gene.

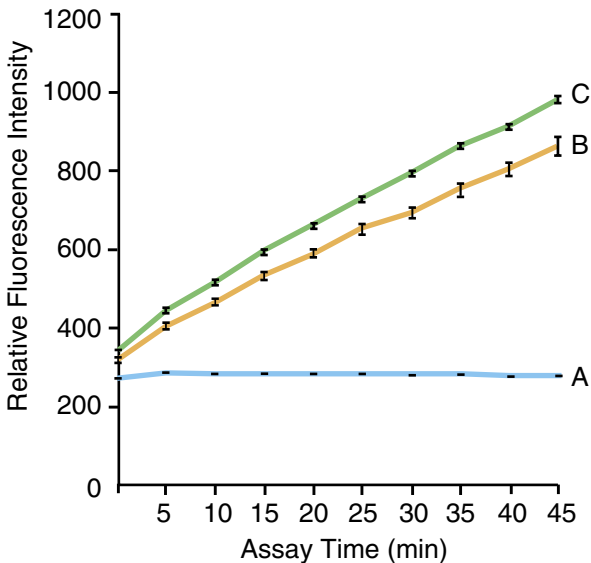


Figure 7: Activity of secreted bovine enterokinase. Enterokinase proteolytic activity measured in the spent culture medium of wild-type *K. lactis* cells as a control (A). *K. lactis* cells harboring a single integrated expression cassette containing a bovine enterokinase cDNA (B) and *K. lactis* cells harboring multiple integrations of the expression cassette (C). Activity was assayed by measuring an increase in fluorescence over time produced in reactions containing 25 μ l spent culture medium and a fluorogenic peptide.

Optional Method: Identification of Properly Integrated Cells

Transformants in which the expression cassette has correctly integrated into the *K. lactis* genome can be identified by PCR using supplied Integration Primers 1 and 2 to amplify a 1.9 kb product (Figure 8A). To facilitate simultaneous screening of many transformants, PCR using freshly grown cells as a source of template chromosomal DNA is recommended.

1. For each transformant patched and grown on YCB Agar Medium plates containing 5 mM acetamide (see Example Experiment step 1), harvest cells from an area approximately 2 mm² by scraping with a pipette tip and resuspend the cells in 25 µl of 1 M sorbitol containing 10 mg/ml Lyticase (Sigma #L-2524). Mix by vortexing. Incubate at 37°C for 1 hour.
2. Use 15 µl of Lyticase-treated cells as template in a PCR reaction containing Integration Primer 1 and Integration Primer 2 (each supplied as a 10X stock). A total reaction volume of 100 µl is recommended. Mix reaction mixtures containing cells by vortexing. Incubate reactions at 95°C for 10 minutes, then for 2 minutes at 80°C during which time Taq DNA Polymerase (NEB #M0267 or NEB #M0273) should be added. Thermocycling should consist of 30 rounds of successive incubations at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 2 min, then a final incubation at 72°C for 10 minutes.
3. Analyze 10 µl of each amplification reaction on a 1% agarose gel.

Integration of the expression fragment at the LAC4 locus in the K. lactis genome will result in amplification of a 1.9 kb product (Figure 8A).

4. Test strains harboring a properly integrated expression fragment for secretion of the protein of interest (see Growth of strains for detection of secreted protein, page 21).

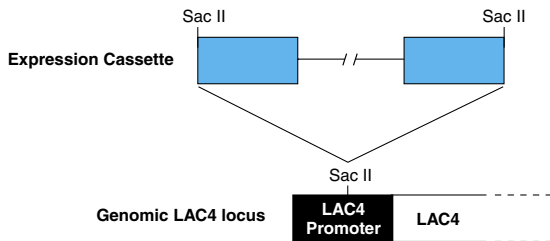
Optional Method: Identification of Multi-copy Integrants

It is possible for multiple copies (up to 10) of the expression cassette to randomly insert into the genome during transformation of *K. lactis* cells. Correspondingly, strains harboring multiple integrations often produce more secreted protein. An advantage of selection for *K. lactis* transformants on YCB Agar Medium containing acetamide is that it enriches for cells harboring multiple tandem integrations (Figure 8B). Multiply integrated cells can be identified using whole-cell PCR with Integration Primers 2 and 3.

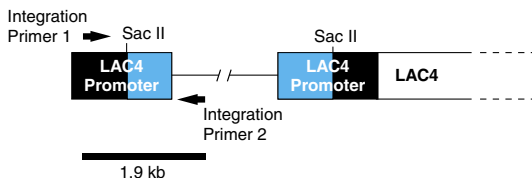
1. For each transformant patched and grown on YCB Agar Medium plates containing 5 mM acetamide (see Example Experiment step 1), harvest the cells from an area approximately 2 mm² by scraping with a pipette tip and resuspend them in 25 μ l of 1 M sorbitol containing 10 mg/ml Lyticase (Sigma #L-2524). Mix by vortexing. Incubate at 37°C for 1 hour.
2. Use 15 μ l of Lyticase-treated cells as template in a PCR reaction containing Integration Primer 2 and Integration Primer 3 (each supplied as a 10X stock). A total reaction volume of 100 μ l is recommended. Mix reaction mixtures containing cells by vortexing. Incubate reactions at 95°C for 10 minutes, then for 2 minutes at 80°C during which time Taq DNA Polymerase (NEB #M0267 or NEB #M0273) should be added. Thermocycling should consist of 30 rounds of successive incubations at 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 3 min, then a final incubation at 72°C for 10 minutes.
3. Analyze 10 μ l of each amplification reaction on a 1% agarose gel.

Cells harboring multiple tandem integrations of the expression fragment at the LAC4 locus in the K. lactis genome will result in amplification of a 2.3 kb product (Figure 8B).

4. Test strains harboring multiple copies of the expression fragment for secretion of the protein of interest (see Growth of strains for detection of secreted protein, page 21).



A. Single Copy Integration



B. Multi-Copy Integration

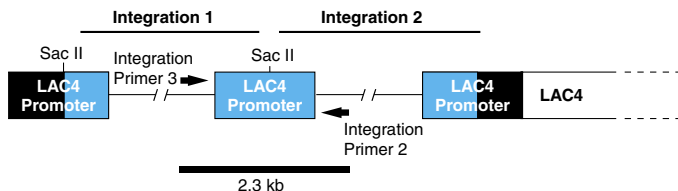


Figure 8: Integration of the expression cassette. Upon transformation of *K. lactis* cells with the linearized expression cassette, the fragment inserts into the promoter of the *LAC4* locus. Single-copy integration can be detected by PCR using Integration Primers 1 and 2 to amplify a 1.9 kb diagnostic fragment (A), whereas multi-copy integration can be detected using Integration Primers 2 and 3 to amplify a 2.3 kb fragment (B).

Media & Solutions

40% Glucose or 40% Galactose Stock Solutions (1 liter)

Add 400 g glucose or galactose to 500 ml deionized water (dH₂O)

Dissolve by stirring, warm to 50°C to aid dissolution if needed.

Adjust volume to 1 liter with dH₂O; dispense into 100 ml aliquots

Filter sterilize or autoclave for 20 minutes at 121°C.

Solutions may caramelize if autoclaved longer or at temperatures higher than recommended.

YPGlu & YPGal Media (1 liter)

Dissolve 10 g Yeast Extract and 20 g Bacto™ Peptone (Becton Dickinson #211820) in 950 ml dH₂O

Autoclave for 20 min at 121°C; let cool to room temperature

Aseptically add 50 ml of sterile 40% glucose (for YPGlu) or 40% galactose (for YPGal)

Solid YPGlu and YPGal media can be made by adding 20 grams of Bacto™ agar (Becton Dickinson #214050) to the recipe prior to autoclaving.

1 M Sodium Phosphate Buffer Stock Solution pH 7.0 (1 liter)

Solution A: Dissolve 138.0 g NaH₂PO₄•H₂O in 1 liter dH₂O

Solution B: Dissolve 142.0 g Na₂HPO₄ in 1 liter dH₂O

Mix 423 ml of Solution A with 577 ml of Solution B. Autoclave. Store at room temperature.

YCB Agar Medium with 5 mM acetamide (500 ml)

Mix in an autoclavable bottle:

15 ml 1 M Sodium Phosphate Buffer Stock Solution

5.85 g YCB Medium powder (supplied with kit)

10 g Bacto agar (Becton Dickinson #214050)

Bring volume up to 495 ml with dH₂O

Autoclave 20 minutes at 121°C. Let cool to ~60°C.

Aseptically add:

5 ml 100X Acetamide Stock Solution (supplied with kit)

Dispense into sterile disposable Petri dishes; close plates and let sit at room temperature until solid, then invert and let sit for 12–18 hours to dry prior to use.

*Yeast carbon base (YCB) medium contains glucose and all nutrients needed to sustain growth of *K. lactis* GG799 Competent Cells except a simple nitrogen source. Cells can utilize acetamide as a source of nitrogen only after it is broken down to ammonia by acetamidase (the product of the *amdS* gene present in *pKLAC1*). **Acetamide should not be autoclaved.***

References:

1. Colussi, P.A. and Taron, C.H. unpublished observations.
2. Fleer, R. et al. (1991) *Gene* 107, 125–295.
3. Fleer, R. et al. (1991) *Bio/Technology* 9, 968–975.
4. Rocha, T.L. et al. (1996) *Biochem. J.* 313, 927–932.
5. Tokunaga M. et al. (1997) *Yeast* 13, 699–707.
6. van den Berg et al. (1990) *Bio/Technology* 8, 135–139.
7. Walsh, D.J. and Bergquist, P.L. (1997) *Appl. Environ. Microbiol.* 63, 3297–3300.
8. Walsh D.J. et al. (1998) *Extremophiles* 2, 9–14.
9. Sambrook, J. et al. (1989). *Molecular Cloning: A Laboratory Manual*, (2nd ed.), (pp. 1.25–1.28). Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Kit Components Sold Separately:

| | |
|---|--------------|
| pKLAC1 #N3740S | 20 µg |
| pKLAC1 - <i>malE</i> #N3741S | 20 µg |
| Sac II #R0157S | 2,000 units |
| #R0157L | 10,000 units |
| Integration Primer 1 #S1277S | 500 µl |
| Integration Primer 2 #S1278S | 1,000 µl |
| Integration Primer 3 #S1279S | 500 µl |
| <i>K. lactis</i> GG799 Competent Cells #C1001S | 5 reactions |
| Yeast Carbon Base (YCB) Medium Powder #B9017S | 12 g |

Companion Products:

| | |
|---|----------------------------|
| BstX I #R0113S | 1,000 units |
| #R0113L | 5,000 units |
| LAC4mt Promoter Sequencing Primer #S1274S | 0.5 A ₂₆₀ units |
| α -Mating Factor Sequencing Primer #S1275S | 0.5 A ₂₆₀ units |
| pKLAC1 Reverse Sequencing Primer #S1276S | 0.5 A ₂₆₀ units |
| <i>Taq</i> DNA Polymerase with ThermoPol Buffer #M0267S | 400 units |
| #M0267L | 2,000 units |
| <i>Taq</i> DNA Polymerase with Standard Buffer #M0273S | 400 units |
| #M0273L | 2,000 units |
| Deoxynucleotide Solution Mix #N0447S | 8 µmol of each |
| #N0447L | 40 µmol of each |



New England Biolabs, Inc.

240 County Road
Ipswich, MA 01938
USA

Telephone (978) 927-5054
Toll Free (USA Orders) 1-800-632-5227
Toll Free (USA Tech) 1-800-632-7799
Fax (978) 921-1350
e-mail: info@neb.com
www.neb.com

New England Biolabs, Ltd.

1815 Ironstone Manor, Unit 6
Pickering, Ontario, L1W 3W9
Canada

Telephone (905) 837-2234
Toll Free 1-800-387-1095
Fax (905) 837-2994
Fax Toll Free 1-800-563-3789
e-mail: info@ca.neb.com

New England Biolabs (Beijing), Ltd.

Wangzhuang Lu No. 1
Tsinghua Tongfang High-Tech Plaza
Building B, 6th Floor B
Haidian District, Beijing 100083
China, People's Republic
Telephone 010-82378266
Fax 010-82378262
e-mail: beijing@neb-china.com

New England Biolabs GmbH

Brüningstrasse 50, Geb.G 810
65926 Frankfurt am Main
Germany
Telephone +49/(0)69/305 23140
Free Call 0800/246 5227 (Germany)
Fax +49/(0)69/305 23149
Free Fax 0800/246 5229 (Germany)
e-mail: info@de.neb.com

New England Biolabs (UK) Ltd.

73 Knowl Piece, Wilbury Way
Hitchin, Hertfordshire SG4 0TY
England, UK
Telephone (01462) 420616
Call Free 0800 318486
Fax (01462) 421057
Fax Free 0800 435682
e-mail: info@uk.neb.com



NEW ENGLAND
BioLabs^{Inc.}
the leader in enzyme technology