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FAQs

Table of Contents

GENERAL QUESTIONS

1. What type of proteins express well in *K. lactis*?

2. What promoter drives heterologous gene expression in *K. lactis*?

3. Is $P_{LAC4\text{-}PBI}$ an inducible/repressible promoter?

4. Does pKLAC2 replicate as an episomal plasmid in *K. lactis*?

CLONING STRATEGIES

1. What affinity tags can be used with pKLAC2?

2. Is it possible to secrete a protein with an amino-terminal antibody epitope tag?

3. What leader sequences can be used to direct protein secretion in *K. lactis*?

4. Can pKLAC2 be used for cytosolic protein expression?

STRAINS AND COMPETENT CELLS

1. What is the origin of strain GG799?

2. Will pKLAC2 work with *K. lactis* strain CBS2359?

3. Why does *K. lactis* strain GG799 have no auxotrophic markers?

4. Can I re-freeze *K. lactis* GG799 Competent Cells?

TRANSFORMATION AND SELECTION

1. How does acetamide selection work?

2. What are the advantages of acetamide selection?

3. Can I use yeast nitrogen base medium in place of yeast carbon base medium?

4. Why is whole-cell PCR recommended for identification of integrants?

5. How many copies of pKLAC2 can be tandemly integrated?

STRAIN GROWTH AND PROTEIN EXPRESSION

1. How stable are strains containing an integrated pKLAC2 expression construct?

2. Can I store spent culture medium containing a secreted protein?

3. When I analyze my secreted protein by SDS-PAGE and/or Western analysis there is a discrepancy between the predicted and the observed mass. What could cause this?

4. How do I store *K. lactis* strains for the long term?

FAQs for *K. lactis* Protein Expression Kit

GENERAL QUESTIONS

1. What type of proteins express well in *K. lactis*?

Many types of proteins can be secreted from yeast cells. As a general rule, those that tend to secrete best are proteins that are also secreted by their native host (e.g. glycosidases, serum albumins, cytokines, etc). However, there are numerous examples in the literature of normally non-secreted proteins that have been successfully secreted from various yeasts. Therefore, when in doubt, it is always best to try secreted expression. Additionally, intracellular protein expression in yeast is also possible for a wide range of proteins and is a great alternative to bacterial protein expression.

2. What promoter drives heterologous gene expression in *K. lactis*?

The *K. lactis* Protein Expression Kit vector (pKLAC2) contains a variant of the strong *K. lactis LAC4* promoter ($P_{LAC4\text{-}PBI}$) for expression of a desired gene in *K. lactis*. The major advantage of the $P_{LAC4\text{-}PBI}$ promoter is that it is transcriptionally silent while in *E. coli*. In contrast, the wild-type P_{LAC4} promoter shows background transcriptional activity in *E. coli* which can be detrimental to the process of assembling or amplifying expression constructs in *E. coli* prior to their introduction into yeast cells. This is especially problematic if the cloned gene of interest encodes a translated product that is toxic to *E. coli* cells. Therefore, pKLAC2 is well-suited for the cloning and yeast expression of genes encoding proteins that are toxic or otherwise detrimental to bacteria.

3. Is $P_{LAC4\text{-}PBI}$ an inducible/repressible promoter?

This depends upon the *K. lactis* strain background. Glucose repression of the *LAC4* locus in *K. lactis* is mediated by the transcriptional activator Lac9p (KlGal4p). Subtle differences in the expression levels of Lac9p from strain to strain dictate the level of glucose repression of P_{LAC4}. In some laboratory *K. lactis* strains, the wild-type P_{LAC4} promoter shows up to 100-fold greater expression in growth medium containing galactose or lactose than in medium containing glucose. However, expression is never completely repressed in glucose medium. In the GG799 strain background, glucose repression of P_{LAC4} is not as dramatic, thereby limiting its utility as an inducible promoter. However, to achieve maximum expression of a desired protein in strain GG799, it is recommended that the cells be grown in galactose-containing medium. Additionally, glucose is unable to repress galactose-induced expression in GG799 cells grown in medium containing both carbon sources.

4. Does pKLAC2 replicate as an episomal plasmid in *K. lactis*?

No. pKLAC2 is an integrative expression vector that inserts into the promoter region of the *LAC4* locus of the *K. lactis* genome upon its introduction into *K. lactis* cells. While *K. lactis* episomal plasmids do exist, they can present some problems for large-scale protein production. For example, plasmids are easily lost by cells in the absence of a selection. For large-scale fermentation, plasmid selection using antibiotics can be too costly, and selection using an auxotrophic marker can reduce yields (see FAQ #3 in

"Strains and Competent Cells"). Integrative expression vectors are attractive because they insert into the genome, thus becoming part of the host chromosome, and are therefore quite stable in the absence of selection.

CLONING STRATEGIES

1. What affinity tags can be used with pKLAC2?

In the *K. lactis* Protein Expression Kit manual, a PCR method for the addition of a carboxy-terminal hemagglutinin (HA) antibody epitope tag to a secreted protein is described (See Cloning Strategy III). This example can be adapted for the addition of other antibody epitope tags (e.g. FLAG or c-*myc*) or a hexahistidine (6-His) tag by directly substituting the desired tag's DNA sequence for the HA epitope DNA sequence in the reverse PCR primer. Additionally, a chitin-binding domain has been used as a tag for capture of secreted proteins onto inexpensive chitin beads directly in spent medium (1).

2. Is it possible to secrete a protein with an amino-terminal antibody epitope tag?

Yes. An adaptation of Cloning Strategy I in the *K. lactis* Protein Expression Kit manual can be used. In this strategy, a new forward PCR primer is used that contains (5' to 3'): an Xho I restriction site, codons encoding the Kex protease cleavage site (KR) immediately followed by DNA encoding the desired tag's sequence and DNA homologous to the 5' end of the desired gene or cDNA. The reverse primer is designed as described in Cloning Strategy I and the amplified product is cloned into pKLAC2. After Kex protease processing of the expressed protein in the Golgi, a protein bearing the desired tag at its amino-terminus is produced and secreted.

3. What leader sequences can be used to direct protein secretion in *K. lactis*?

Many different secretion leader sequences have been be used for protein secretion in *K. lactis*. Some examples include the *K. lactis* killer toxin leader (2,3,5,7), the human serum albumin leader (3), the b-lactoglobulin leader (4), and a synthetic leader (6). The vector pKLAC2 contains the native *K. lactis* a-mating factor secretion domain that includes a secretion leader sequence and a pro-domain that are sequentially removed by signal peptidase in the endoplasmic reticulum and the Kex protease in the Golgi, respectively.

4. Can pKLAC2 be used for cytosolic protein expression?

Yes. The gene of interest must be cloned into pKLAC2 in a manner that places it downstream of $P_{LAC4\text{-}PBI}$ without the a-mating factor secretion domain being present. This can be accomplished by cloning the gene's 5' end into the unique Hind III restriction site of pKLAC2 and the 3' end into any of the polylinker restriction sites. Genes expressed in this manner must include a methionine as their first codon, to initiate translation.

STRAINS AND COMPETENT CELLS

1. What is the origin of strain GG799?

K. lactis strain GG799 is a haploid (a) wild-type industrial isolate that has no genetic markers. It was originally chosen as a host strain in the food industry because of its ability to grow to very high cell density and efficiently secrete heterologous proteins.

GG799 is <u>not</u> the same strain as CBS2359 which is the *K. lactis* type strain for the genome sequencing project.

2. Will pKLAC2 work with *K. lactis* strain CBS2359?
Yes. pKLAC2 integrates into the *LAC4* locus and strongly promotes gene expression in the CBS2359 strain background. Additionally, strain CBS2359 can be transformed with pKLAC2 using acetamide selection (see FAQ #1 in "Transformation & Selection").

3. Why does *K. lactis* strain GG799 have no auxotrophic markers?
While auxotrophic markers have historically been used for genetic manipulation of yeasts, they are not always desirable to achieve maximum protein expression. In some cases, an auxotrophy (e.g. uracil) can cause a significant reduction in the strain's ability to produce a heterologous protein even if exogenous uracil or uridine is provided in the growth medium.

4. Can I re-freeze *K. lactis* GG799 Competent Cells?
No. The chemically competent cells that New England Biolabs provides lose significant transformation efficiency upon re-freezing. They are packaged for one transformation per tube.

TRANSFORMATION AND SELECTION

1. How does acetamide selection work?
This is a nitrogen source selection. A cell transformation mixture (containing a population of cells that are either transformed or untransformed by vector pKLAC2) is spread onto yeast carbon base (YCB) medium agar containing 5 mM acetamide. YCB medium contains all of the nutrients and carbon source required for K. lactis cells to grow, but lacks a nitrogen source. The acetamide provided in the medium can be utilized as source of nitrogen only if it is broken down to ammonia by the enzyme acetamidase (expressed from the amdS gene present on pKLAC2). Therefore, only transformed cells are able to grow into colonies.

2. What are the advantages of acetamide selection?
There are two main advantages, cost and selection of multiple integrants. Acetamide is significantly less expensive than antibiotics. Additionally, acetamide selection enriches transformant populations for cells that have integrated multiple tandem copies of the pKLAC2 expression vector. Multi-copy integrants are desirable because they often produce more recombinant protein than single integrants.

3. Can I use yeast nitrogen base medium in place of yeast carbon base medium?
No. Yeast nitrogen base (both with and without ammonium sulphate) contains nitrogen that can be used by cells instead of acetamide. To select for transformants, it is essential to plate the cells on YCB medium containing 5 mM acetamide (#B9017S).

4. Why is whole-cell PCR recommended for identification of integrants?
Whole-cell PCR protocols are recommended so that numerous transformants can be rapidly screened simultaneously for integrated (or multiply integrated) expression constructs without the need for purification of genomic DNA from each strain. This

dramatically reduces the amount of time needed to test numerous strains for the desired integration event.

5. How many copies of pKLAC2 can be tandemly integrated?

The majority of multiply integrated strains contain 2-5 integrated copies of pKLAC2. While higher copy number strains may be attainable, they will be significantly more rare within a given population of transformants due to the reduced probability of their formation and to mitotic instability.

STRAIN GROWTH AND PROTEIN EXPRESSION

1. How stable are strains containing an integrated pKLAC2 expression construct?

The stability of pKLAC2-integrated strains depends on the nature of the protein being expressed and secreted. For example, in a population of cells expressing a protein that is toxic to K. lactis a certain percentage of cells may revert to wild-type by losing the integrated expression vector. The extent of reversion depends largely on the competitive growth advantage the wild-type strain has over the strain expressing the toxic protein. In our experience, *K. lactis* strains expressing proteins that do not adversely affect the health of cells can be grown continuously for >30 generations in non-selective medium with less than 5% reversion, as determined by Southern analysis.

2. Can I store spent culture medium containing a secreted protein?

Yes. Spent culture medium containing a secreted protein that has been cleared of cells can be stored for up to 24 hours at 4°C or frozen at –20°C indefinitely.

3. When I analyze my secreted protein by SDS-PAGE and/or Western analysis there is a discrepancy between the predicted and the observed mass. What could cause this?

The most likely cause for a discrepancy between the predicted and observed mass of a secreted protein is the presence of a covalent modification like an N-linked oligosaccharide. Treatment of proteins with endo-glycosidases that remove N-linked sugars (Endo-H #P0702S or PNGase F #P0704S) can help diagnose if the secreted protein is glycosylated.

4. How do I store *K. lactis* strains for the long term?

K. lactis cells can be stored at –70°C suspended in a final concentration of 20% (v/v) sterile glycerol. For example, 500 ml of a culture of freshly grown cells can be diluted with 500 ml of a sterile 40% glycerol solution (to give a 20% final glycerol concentration). Alternatively, a scoop of cells from a freshly grown streak on agar medium can be scraped from the plate using a sterile loop or pipet tip and resuspended directly in 20% sterile glycerol. It is important to make sure that all solutions and tubes that contact the cells are sterilized prior to their use. To revive frozen *K. lactis* cells containing an integrated pKLAC2 construct, streak a small aliquot of frozen cells on YCB agar medium supplemented with 5 mM acetamide. After this initial growth on YCB agar medium, the cells can be grown without selection in rich medium for protein expression.

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