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pPIC3.5K/pAO815

Pichia vectors for multicopy integration and intracellular expression

Catalog no. V173-20, V180-20

Rev. Date: 14 July 2010
Manual part no. 25-0156

MAN0000038

User Manual

Contents

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Kit Contents and Storage

Shipping and Storage

pPIC3.5K and pAO815 vectors are shipped on wet ice. Upon receipt, store vectors at –20°C.

Kit Contents

All vectors are supplied as detailed below. **Store the vectors at –20°C.**

Catalog no.	Vector	Composition	Amount
V173-20	pPIC3.5K	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg
V180-20	pAO815	40 µL of 0.5 µg/µL vector in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0	20 µg

Intended Use

For research use only. Not intended for any animal or human therapeutic or diagnostic use.

Kit Contents and Storage, Continued

Materials Supplied by the User

For the procedures described in this manual, you will need:

- Manual from the Pichia Expression System
- Microbiological equipment
- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation (page 44). You need 3–4 tubes of competent cells per experiment. For protocols to prepare competent *E. coli* and transformation protocols, see Current Protocols (Ausubel, et al., 1990) or Molecular Biology: A Laboratory Manual (Sambrook, et al., 1989)
- *Eco*R I, *Bam*H I and *Bgl* II restriction enzymes and appropriate buffers
- Agarose and low-melt agarose
- S.N.A.P.™ Gel Purification Kit (see page 44) or glass milk
- Sterile water
- CIP (calf intestinal phosphatase, 1 unit/µL)
- 10X CIP Buffer
- Phenol/chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/µL)
- 10X Ligation Buffer (with ATP)
- LB medium
- LB-ampicillin plates (50–100 µg/mL ampicillin)
- 16°C, 37°C, and 65°C water baths or temperature blocks
- Geneticin® antibiotic (see page 44)
- YPD-Geneticin® plates (see Recipes, page 36)
- 50 mL conical centrifuge tubes
- Hemacytometer
- 30°C and 37°C incubator
- Microtiter plates (optional)

Important

Registered *Pichia* users should already have the *Pichia* Expression System and the current manual. Procedures for transformation into *E. coli* and *Pichia*, analysis of recombinants, and expression are described in the *Pichia* manual.

Introduction

Product Overview

Description of the System

Multiple copy integration of recombinant genes in *Pichia* has been demonstrated to increase expression of the desired gene in some cases (Brierley, *et al.*, 1994; Clare, *et al.*, 1991a; Cregg, *et al.*, 1993; Romanos, *et al.*, 1991; Scorer, *et al.*, 1993; Scorer, *et al.*, 1994; Thill, *et al.*, 1990; Vedvick, *et al.*, 1991). The two vectors included in this kit allow isolation and generation of multicopy inserts by *in vivo* or *in vitro* methods to test whether increasing the copy number of your recombinant gene will lead to a subsequent increase in protein expression. The *in vivo* method utilizes hyper-resistance to Geneticin® (G418 sulfate) to screen for possible multicopy inserts, while the *in vitro* method produces tandem inserts of your gene by ligation.

Frequency of Multicopy Inserts

Multiple plasmid integration events occur spontaneously in *Pichia* at a frequency between 1 and 10% of all His$^+$ transformants. The *in vivo* method allows you to screen for the His$^+$ transformants that may have multiple inserts of your gene. The *in vitro* method allows you to construct multimers by ligation. When His$^+$ transformants are selected, they have a high probability of containing the multimers that you constructed *in vitro*.

Generating Multicopy Inserts *in vivo*

pPIC3.5K contains the bacterial kanamycin gene (*kan* from Tn*903*) that confers resistance to Geneticin® in *Pichia*. Note that *kan* does not confer resistance to kanamycin in *Pichia*. The level of Geneticin® resistance roughly depends on the number of kanamycin genes integrated. A single copy of pPIC3.5K integrated into the *Pichia* genome confers resistance to Geneticin® to a level of ~0.25 mg/mL. Multiple integrated copies of pPIC3.5K can increase the Geneticin® resistance level from 0.5 mg/mL (1–2 copies) up to 4 mg/mL (7–12 copies). Because of the genetic linkage between the kanamycin gene and the "expression cassette" (P$_{AOX1}$ and your gene of interest), one can infer that Geneticin® resistant clones contain multiple copies of your gene. Protein expression may increase because of a gene dosage effect. Thus, the presence of the *kan* gene on pPIC3.5K can be used as a tool to detect pPIC3.5K transformants that harbor multiple copies of your gene. The graphic on the following page shows multiple insertion and linkage of the *kan* gene to your expression cassette.

Continued on next page

1

Product Overview, Continued

2nd Insertion Event

3rd Insertion Event, etc.
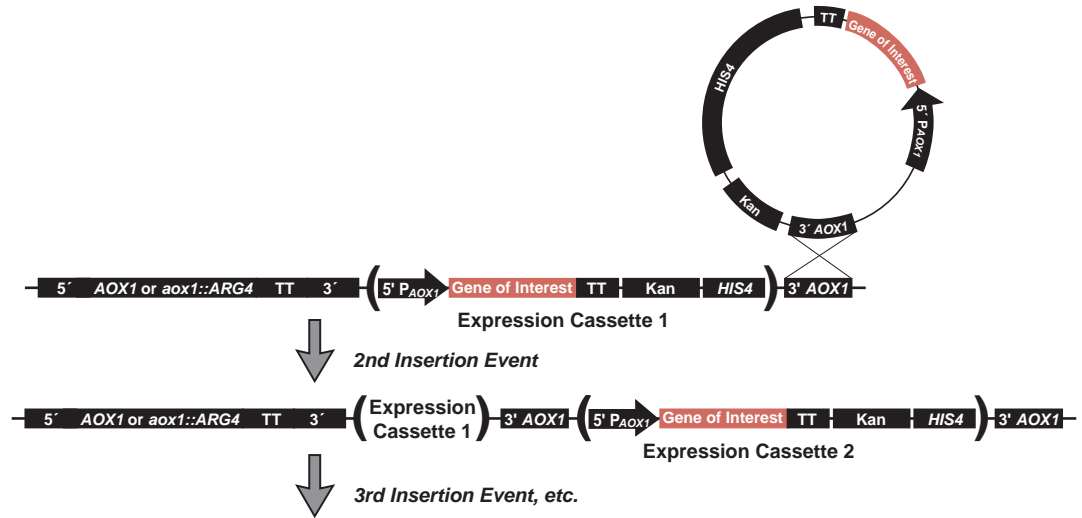
Screening on Geneticin®

Direct selection of Geneticin® resistance in yeast does not work well because newly transformed cells need time to express sufficient amounts of the resistance factor. Since yeast grow much more slowly than bacteria, significant numbers of recombinant yeast are killed before they accumulate enough of the resistance factor to survive direct plating on antibiotic. **Do not use Geneticin® resistance as a selectable marker.** The procedure to generate Geneticin® resistant clones requires an initial selection of His+ transformants followed by a screen for varying levels of Geneticin® resistance. Resistance to Geneticin® conferred by the kanamycin gene present on pPIC3.5K is used as a SCREEN, not as a SELECTION for multicopy integrants.

Product Overview, Continued

The graphic below shows how pAO815 is used to generate multiple expression cassette copies in a single vector prior to transformation into *Pichia*. The gene of interest is inserted into the vector at a unique *Eco*R I site. The resulting expression cassette (the P$_{AOX1}$ plus your gene) is flanked on the upstream side by a unique *Bgl* II site and on the downstream site by a unique *Bam*H I site (see A below).

The vector containing the gene of interest is digested with *Bgl* II and *Bam*H I to excise the expression cassette. The cassette is then reinserted at the *Bam*H I site to create a tandem repeat of the cassette. The reinsertion process can be repeated to generate a series of vectors that contain an increasing number of cassettes linked to a single *HIS4* gene (see B below).

Transformation of *Pichia* with these *in vitro*-formed multimers increases the frequency of multicopy expression cassette recombinants. *Pichia* recombinants may be custom-designed to contain a defined number of multicopy inserts. For more information, see page 12.
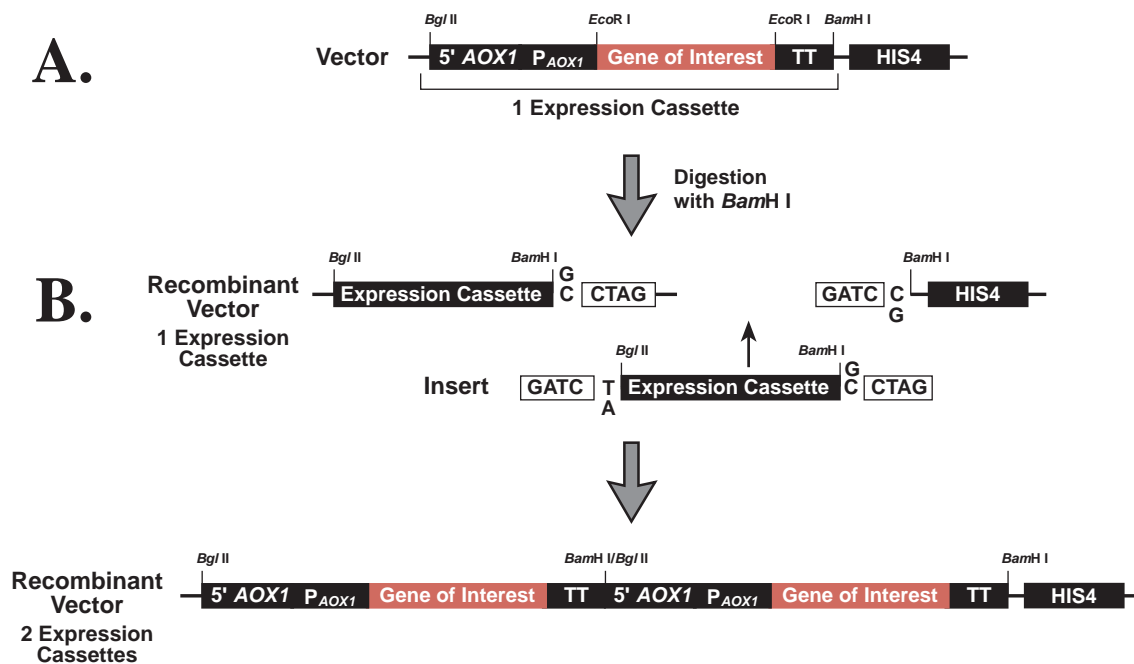
Continued on next page

Product Overview, Continued

We recommend trying both methods to generate or isolate multicopy inserts of your gene. A summary of the advantages and disadvantages of each method is presented in the lists below. The "best" method is the one that works for your protein; unfortunately, there is no way to predict beforehand which method will work for your protein.

In vivo Method (pPIC3.5K)

Advantages	Disadvantages
• Easier to initiate experiment because only one copy of your gene is cloned into pPIC3.5K before transforming into *Pichia.*	• Qualitative screen – Geneticin® resistance may not necessarily correlate with the number of copies of your gene.
• Identifies the 1–10% of spontaneous His$^+$ transformants that have multiple inserts.	• Screening His$^+$ transformants may involve more work because you need thousands of His$^+$ transformants to generate enough Geneticin® resistant colonies to test.
• Average size of vector is similar to other *Pichia* expression vectors.	• The number of multiple inserts is unknown (although this can be determined through Southern or dot blot analysis).
• Multiple inserts are located at a single locus.	• Screening on Geneticin® is sensitive to the density of the cells and may result in the isolation of false positives.

In vitro Method (pAO815)

Advantages	Disadvantages
• Quantitative – construction of a defined number of multimers.	• More work up front to clone defined number of multimers.
• Most of the His$^+$ transformants will contain the proper, defined number of inserts.	• Size of the vector may become quite large depending on the size of your gene and the number of copies you create.
• Isolation of recombinants with multiple inserts is easier because most of the His$^+$ transformants contain multiple copies of your gene.	• Rearrangements in *E. coli* may occur.
• *In vitro* construction allows step-wise analysis of copy number effects on protein expression.	
• Multiple inserts are located at a single locus.	
• No need for a second drug resistance marker in the vector.	

Product Overview, Continued

pPIC3.5K

pPIC3.5K is a plasmid designed to allow you to identify *in vivo* multiple integrations of your gene in the *Pichia* genome.

- 9004 bp vector
- Five unique restriction sites in the multiple cloning site: *Bam*H I, *Sna*B I, *Eco*R I, *Avr* II, *Not* I
- Intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990)
- *HIS4* selection in *Pichia*
- For insertion at *AOX1* in GS115 or KM71, linearize with *Sac* I (generates His$^+$ Mut$^+$ in GS115 and His$^+$ Mut^S in KM71)
- For insertion at *HIS4*, linearize with *Sal* I (generates His$^+$ Mut$^+$ in GS115 and His$^+$ Mut^S in KM71)
- For a gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates His$^+$ Mut^S)

See page 24 for alternate restriction sites if your insert DNA has a *Bgl* II, *Sac* I, or *Sal* I site.

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pAO815

pAO815 is a plasmid designed for *in vitro* generation of multimers of your gene for integration into the *Pichia* genome.

- 7709 bp vector
- One unique restriction site: *Eco*R I
- Intracellular expression of your gene
- Requires an initiating ATG codon in a Kozak consensus sequence for proper translation initiation of your gene (Cavener and Stuart, 1991; Kozak, 1987; Kozak, 1990)
- *HIS4* selection in *Pichia*
- For insertion at *HIS4*, linearize with *Sal* I or *Stu* I (generates His$^+$ Mut$^+$ in GS115 and His$^+$ Mut^S in KM71)
- For a gene replacement at *AOX1* in GS115, linearize with *Bgl* II (generates His$^+$ Mut^S)

See page 24 for alternate restriction sites if your insert DNA has a *Bgl* II, *Stu* I, or *Sal* I site.

Methods

Cloning into pPIC3.5K and pAO815

General Molecular Biology Techniques

For help with DNA ligations, *E. coli* transformations, restriction enzyme analysis, purification of single-stranded DNA, DNA sequencing, and DNA biochemistry, refer to *Molecular Cloning: A Laboratory Manual* (Sambrook *et al.*, 1989) or *Current Protocols in Molecular Biology* (Ausubel *et al.*, 1994) (See References, page 47).

- We recommend that you ligate your insert into both pPIC3.5K and pAO815 so that you can try both methods to isolate multiple integrants. Below are some guidelines to consider when developing a cloning strategy for these vectors. The multiple cloning sites for each vector are presented on the pages 9–10 for your convenience.

- We recommend that you transform the two supercoiled *Pichia* expression vectors into *E. coli*, so that you have a permanent stock and a way to make more plasmid. Transform competent *E. coli* with 1–2 µL of the supplied vector stock solution and select on LB with 50–100 µg/mL ampicillin (LB-Amp).

General Considerations

The following general considerations are applicable to both vectors.

- The codon usage in *Pichia* is believed to be the same as *Saccharomyces cerevisiae.*

- Many *Saccharomyces* genes have proven to be cross-functional in *Pichia.*

- Maintain plasmid constructions in a *rec*A, *end*A mutant *E. coli* strain such as TOP10. Electrocompetent TOP10 cells are available from Invitrogen (see page 44).

- The native 5´ end of the *AOX1* mRNA is noted in each multiple cloning site. This is needed to calculate the size of the expressed mRNA of the gene of interest if you need to analyze mRNA for any reason.

- Translation termination is determined by either stop codons in the gene of interest or in the 3´ *AOX1* sequence. The stop codons in the 3´ *AOX1* sequence are noted in each figure on pages 9–10.

- The premature termination of transcripts because of "AT rich regions" has been observed in *Pichia* and other eukaryotic systems (Henikoff and Cohen, 1984; Irniger, *et al.*, 1991; Scorer, *et al.*, 1993; Zaret and Sherman, 1984). If you have problems expressing your gene, check for premature termination and AT rich regions. It may be necessary to change the sequence to express your gene (Scorer, *et al.*, 1993).

Cloning into pPIC3.5K and pAO815, Continued

General Cloning Strategies

Strategies generally fall into three different categories:

1. Ligation of a compatible restriction fragment:

    a. Forced (directional) insertion involving the use of two different sites in the multiple cloning site (pPIC3.5K).

    b. Ligation of the fragment with the same restriction end on both ends into a single, compatible site (e.g. *Eco*R I cloning in pAO815). Note that you will need to dephosphorylate pAO815 to ligate into the *Eco*R I site.

2. PCR amplification of the fragment containing the gene of interest in such a way that compatible restriction ends are generated for ligation into the appropriate vector.

3. Direct cloning of an amplified fragment containing the gene of interest via the TA Cloning® Kit (see page 44) followed by subcloning of a compatible fragment into the appropriate *Pichia* expression vector.

Important

If your insert has an *Eco*R I site and you are trying to clone into the *Eco*R I site of pAO815, we recommend the following:

1. An enzyme like *Bsa* I has the following restriction recognition site:

    ```
    5´-GGTCTCN˘
    3´-CCAGAGNNNNN^
    ```

2. An *Eco*R I site may be engineered into the recognition site for *Bsa* I.

    ```
    5´-GGTCTCG˘AATTC.....
    3´-CCAGAGCTTAA^G.....
    ```

3. This sequence may be added to your DNA fragment by integrating it into your PCR primer or created *in vitro* as an adaptor to another restriction site.

4. Digest your PCR or adapted ligation product with *Bsa* I. This will generate *Eco*R I overhangs on both ends of your fragment without digesting with *Eco*R I.

    ```
    5´- AATTC......
        3´-G......
    ```

5. Ligate into dephosphorylated pAO815. Other enzymes that may be used are *Bsm*A I or *Bsm*B I.

Cloning into pPIC3.5K and pAO815, Continued

Cloning Procedures

Refer to (Ausubel, *et al.*, 1990), pages 3.16.1–3.17.3. or (Sambrook, *et al.*, 1989), pages 5.10–5.13. for help with cloning.

Bacterial Transformation

Once you have decided on a cloning strategy, you can use electrocompetent or chemically competent *E. coli* for transformation.

Cloning into pPIC3.5K and pAO815, Continued

P_{AOX1} and Multiple Cloning Site of pPIC3.5K

The sequence below shows the details of the multiple cloning site and surrounding sequences.

```
AOX1 mRNA 5´ end (824)                                    5´ AOX 1 Primer Site (855-875)
    ↓                                                      ┌─────────────────
TTATCATCAT TATTAGCTTA CTTTCATAAT TGCGACTGGT TCCAATTGAC


─────┐
AAGCTTTTGA TTTTAACGAC TTTTAACGAC AACTTGAGAA GATCAAAAAA


                   BamH I      SnaB I    EcoR I   Avr II        Not I
                        ┌          ┌        ┌       ┌             ┌
CAACTAATTA TTCGAAGGAT CCTACGTAGA ATTCCCTAGG GCGGCCGCGA


ATTAATTCGC CTTAGACATG ACTGTTCCTC AGTTCAAGTT GGGCACTTAC


                                     3´ AOX 1 Primer Site (1055-1075)
                                      ┌─────────────────
GAGAAGACCG GTCTTGCTAG ATTCTAATCA AGAGGATGTC AGAATGCCAT


─────┐
TTGCCTGAGA GATGCAGGCT TCATTTTTGA TACTTTTTTA TTTGTAACCT
            AOX1 mRNA 3´ end (1146)
                           │
ATATAGTATA GGATTTTTTT TGTCATTTTG TTTCTTC
```

Special Considerations

- For pPIC3.5K, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC <u>ATG</u> G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener and Stuart, 1991; Kozak, 1987; and Kozak, 1990). **Note:** There is an ATG upstream of the *Sna*B I site.

- Be sure to analyze the 5´ untranslated region of the mRNA for secondary structure formation. Secondary structure in the mRNA may have a negative effect on expression of the recombinant protein.

- If you are digesting with *Bam*H I and *Sna*B I or *Sna*B I and *Eco*R I, digest with *Sna*B I first. If you digest with *Bam*H I or *Eco*R I first, the *Sna*B I site will be too close to the end of the DNA and will not be digested properly.

Cloning into pPIC3.5K and pAO815, Continued

P_{AOX1} and Multiple Cloning Site of pAO815

The sequence below shows the details of the multiple cloning site and surrounding sequences.

AOX1 mRNA 5´ end (824)   5´ AOX 1 primer site (855-875)

```
TTATCATCAT TATTAGCTTA CTTTCATAAT TGCGACTGGT TCCAATTGAC


AAGCTTTTGA TTTTAACGAC TTTTAACGAC AACTTGAGAA GATCAAAAAA

                       EcoR I
CAACTAATTA TTCGAAACGA GGAATTCGCC TTAGACATGA CTGTTCCTCA


GTTCAAGTTG GGCACTTACG AGAAGACCGG TCTTGCTAGA TTCTAATCAA
```

3´ AOX 1 primer site (1024-1044)

```
GAGGATGTCA GAATGCCATT TGCCTGAGAG ATGCAGGCTT CATTTTTGAT
```
                                  AOX1 mRNA 3´ end (1115)
```
ACTTTTTTAT TTGTAACCTA TATAGTATAG GATTTTTTTT GTCATTTTGT
```

Special Considerations

- For *in vitro* multimerization, you need to analyze your insert for *Bam*H I and *Bgl* II restriction sites. If your insert has a *Bam*H I or *Bgl* II site, we recommend that you use the *in vivo* method (pPIC3.5K) to isolate multiple inserts of your gene.

- For pAO815, the fragment containing the gene of interest should have a Kozak consensus sequence for proper translation initiation, although this requirement is not as stringent in yeast. For example, **ACC ATG G** is a Kozak consensus sequence, where the ATG corresponds to the initiating ATG for your gene of interest (Cavener and Stuart, 1991; Kozak, 1987; and Kozak, 1990).

- Be sure to analyze the 5´ untranslated region of the mRNA for secondary structure formation. Secondary structure in the mRNA has a negative effect on expression of the recombinant protein.

Transformation

At this point you should have ligation reactions that you will transform by chemical means or electroporation into competent *E. coli* cells such as TOP10 (see page 44 for ordering) using your method of choice.

Analyzing *E. coli* Transformants

Analyzing Transformants

1. After transformation, plate 10 μL and 100 μL of the transformation mix onto LB plates with 50–100 μg/mL ampicillin and select ampicillin resistant colonies.

2. Pick 10 ampicillin resistant transformants and inoculate into 2 mL LB medium with 50–100 μg/mL ampicillin. Grow overnight at 37°C with shaking.

3. Isolate plasmid DNA by miniprep for restriction analysis and sequencing (see below). To sequence the *Pichia* expression vectors (pPIC3.5K and pAO815 **with only one insert**), use the 5′ *AOX1* and the 3′ *AOX1* sequencing primers.

4. Make a glycerol stock of your desired clone by combining 0.85 mL of a overnight bacterial culture with 0.15 mL of sterile glycerol. Mix by vortexing and transfer to a labeled storage tube. Freeze the tube in liquid nitrogen or a dry ice/ethanol bath and store at –80°C.

Sequencing Recombinant Clones

We strongly recommend that you sequence your construct in pPIC3.5K and pAO815 (with only one insert) before proceeding further to confirm that the ATG is in the proper context for eukaryotic translation initiation. Use the 5′ and 3′ *AOX1* sequencing primers to sequence your constructs.

Sequencing primers are available from Invitrogen (see page 44).

After Sequencing

Once you have cloned and sequenced your insert, proceed as directed below:

- If you cloned your insert into pAO815, proceed to *In Vitro* **Multimerization**, next page.

- If you cloned your insert into pPIC3.5K, generate enough plasmid DNA to transform *Pichia* (5–10 μg of each plasmid per each transformation). Proceed to **Transformation into *Pichia***, page 23.

pAO815–*In Vitro* Multimerization Protocol

Introduction
At this point you should have your gene cloned into the *Eco*R I site of pAO815 (recombinant pAO815). You will use this vector for two purposes. First you will use it to generate a *Bgl* II-*Bam*H I expression cassette consisting of the *AOX1* promoter and your gene. Second, you will linearize the vector using *Bam*H I to allow cloning of multiple copies of the *Bgl* II-*Bam*H I expression cassette. Note that the linearized vector already contains one copy of your expression cassette. To generate multiple copies of your expression cassette:
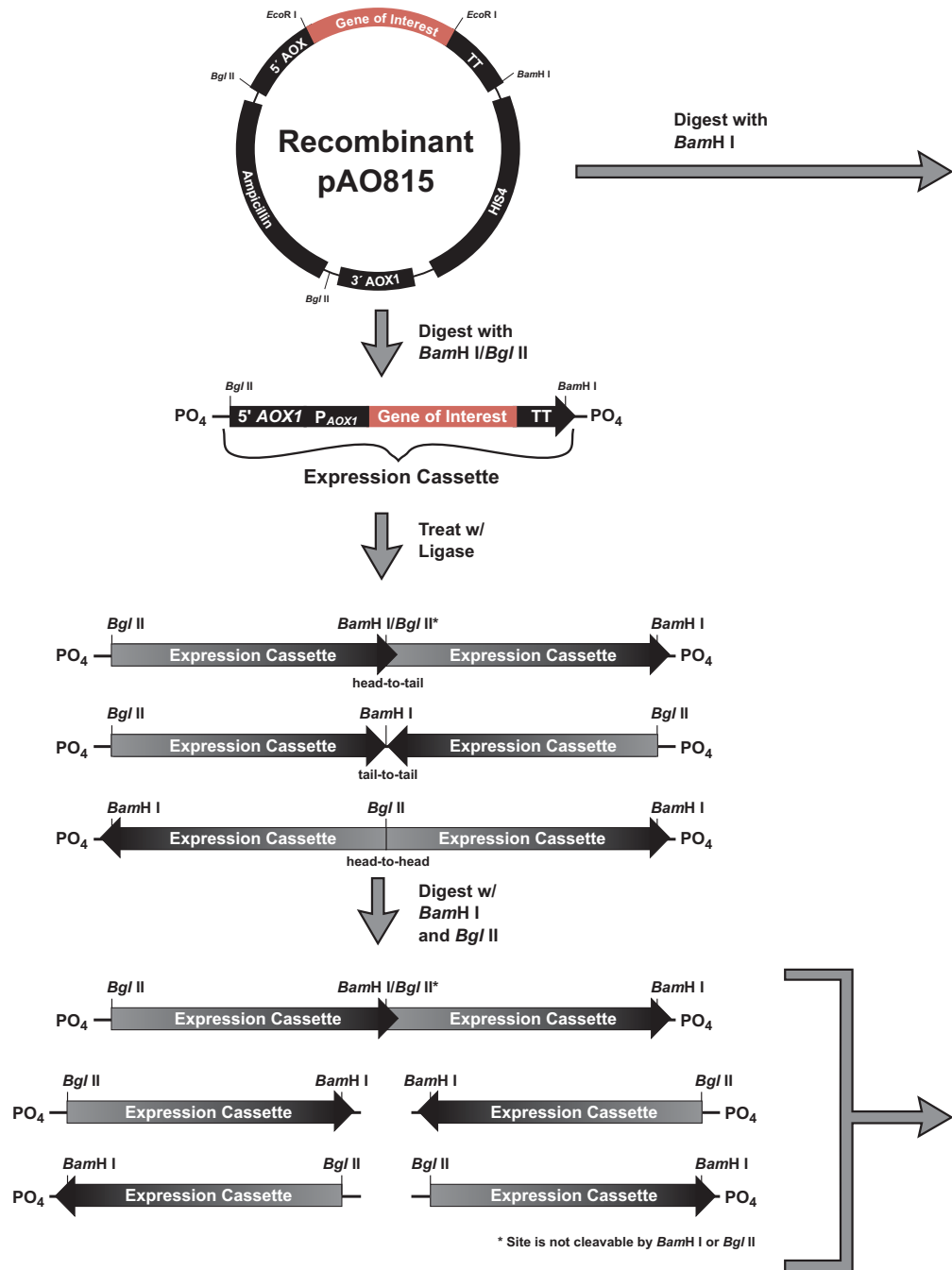
Step	Description
1	Treat your *Bgl* II-*Bam*H I expression cassette with ligase *in vitro*. Note that *Bgl* II and *Bam*H I share 4 bases in common between their recognition sites.
2	Generate head-to-tail, head-to-head, and tail-to-tail multimers (Head-to-tail ligation, which is the correct orientation for expression, will destroy both the *Bam*H I and *Bgl* II sites).
3	Treat the ligation mix with *Bam*H I and *Bgl* II to eliminate head-to-head and tail-to-tail multimers.
4	Ligate into *Bam*H I-linearized recombinant pAO815.
5	Transform into *E. coli* and analyze recombinant plasmids for copy number by digesting with *Bgl* II and *Bam*H I.

pAO815–*In Vitro* Multimerization Protocol, Continued

Flow Chart of Multimerization Process

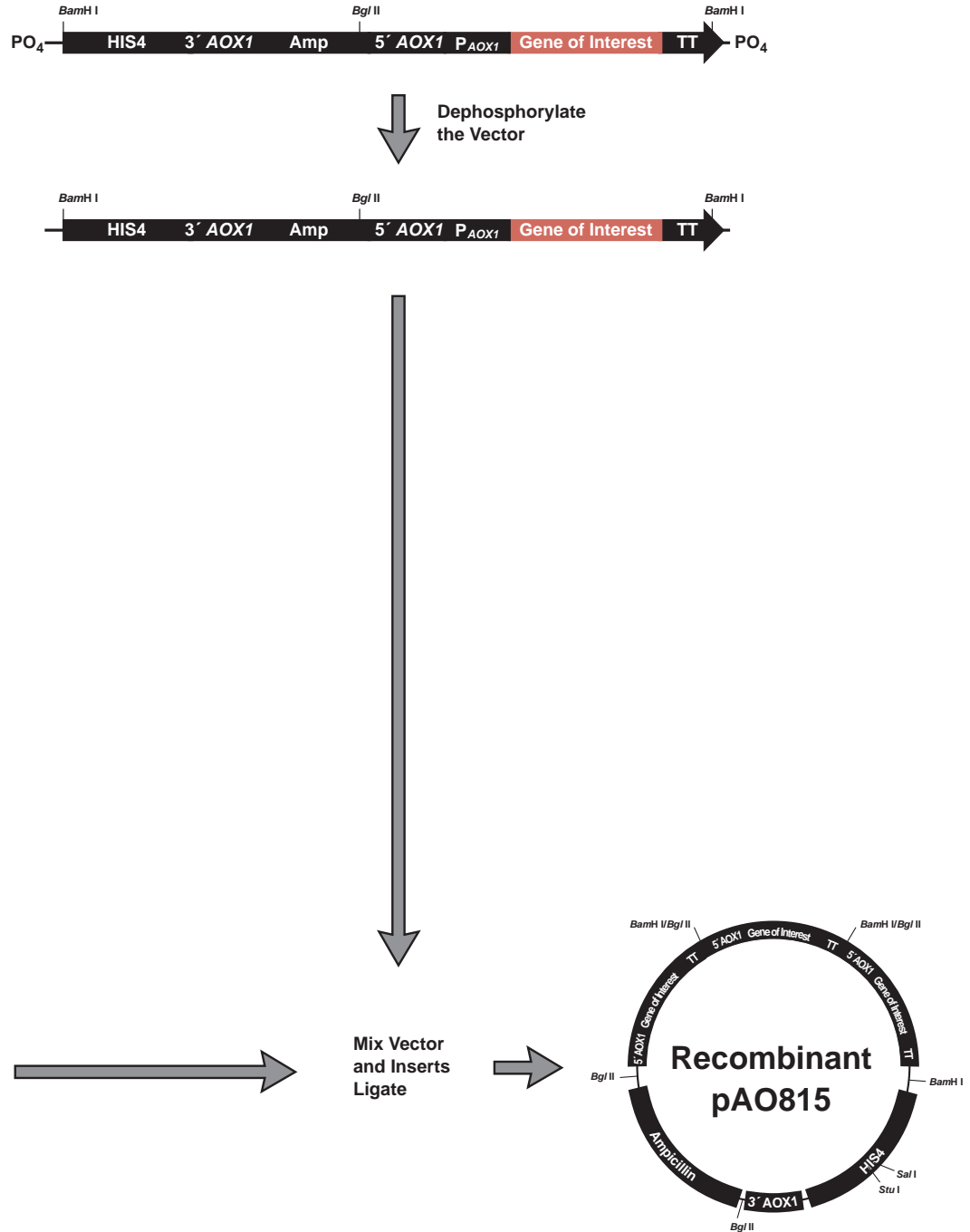The figure below and on the following page outlines the multimerization process.

Flow Chart of Multimerization Process, Continued

pAO815–*In Vitro* **Multimerization Protocol,** Continued

Alternative Procedure

You may wish to build each desired multimer in increments by ligating each additional expression cassette one (or two) at a time into pAO815. For example:

Step	Description
1	Digest pAO815 with one copy of your gene using *Bam*H I.
2	Ligate a single copy of the *Bgl* II-*Bam*H I expression cassette into the vector.
3	Transform *E. coli* and analyze the transformants for the vector with 2 copies of your insert.
4	Isolate and digest this vector (with 2 copies of your gene) with *Bam*H I and *Bgl* II to isolate a cassette with 2 copies of your gene (optional).
5	Digest the vector with 2 copies of your gene with *Bam*H I and ligate 1 or 2 copies (see Step 4) of the expression cassette into the vector.
6	Transform *E. coli* and analyze the transformants for the vector with 3 or 4 copies of your insert.
7	Repeat until the desired multimer is reached.

Materials Needed

- Electrocompetent or chemically competent *E. coli* (must be *rec*A, *end*A) for transformation. You will need 3–4 tubes of competent cells per experiment. See page 44 for ordering information.
- *Eco*R I, *Bam*H I and *Bgl* II restriction enzymes and appropriate buffers
- Low-melt agarose
- S.N.A.P.™ Gel Purification Kit (see page 44) or glass milk
- Sterile water
- CIP (calf intestinal alkaline phosphatase, 1 unit/μL)
- 10X CIP Buffer
- Phenol/chloroform
- 3 M sodium acetate
- 100% ethanol
- 80% ethanol
- T4 Ligase (2.5 units/μL)
- 10X Ligation Buffer (with ATP)
- LB-Amp plates (50–100 μg/mL ampicillin)
- 16°C, 37°C, and 65°C water baths or temperature blocks

Continued on next page

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pAO815–*In Vitro* Multimerization Protocol, Continued

Controls

To evaluate your transformants and expression data later on, we recommend transforming *Pichia* with pAO815 (the parent vector) and pAO815 containing one copy of your expression gene. This will allow you to compare expression levels to see if multiple copies significantly increase the amount of protein produced. Also, if you elect to determine how many copies of your gene are in a recombinant by dot or Southern blot, the strain with the parent vector will control for background hybridization and the strain with the single copy gene will provide a signal to normalize your data.

Digesting Recombinant pAO815

Set up two separate digestions of recombinant pAO815 containing one copy of your gene:

1. Double digest 1–2 µg recombinant pAO815 with 10 units each of *Bgl* II and *Bam*H I. Use a 20 µL reaction volume and digest for 1–2 hours at 37°C to release your expression cassette. Proceed to **Producing Expression Cassettes for Multimerization**, Step 1.

2. Digest 2 µg recombinant pAO815 with 10 units of *Bam*H I only. Use a 20 µL reaction volume and digest for 1–2 hours at 37°C to linearize recombinant pAO815. Proceed to **Dephosphorylating the Vector**, Step 1.

Producing Expression Cassettes for Multimerization

The S.N.A.P.™ Gel Purification Kit available from Invitrogen (see page 44 for ordering) allows you to rapidly purify DNA fragments from regular agarose gels. Alternatively, you may use glass milk. To use the S.N.A.P.™ Gel Purification Kit, follow the steps below:

1. Electrophorese your digest from Step 1, above, on a 1 to 5% regular TAE agarose gel. **Note**: Do not use TBE to prepare agarose gels. Borate interferes with the sodium iodide step, below.

2. Cut out the gel slice containing the PCR product and melt it at 65°C in 2 volumes of the 6 M sodium iodide solution.

3. Add 1.5 volumes Binding Buffer.

4. Load solution (no more than 1 mL at a time) from Step 3 onto a S.N.A.P.™ column. Centrifuge 1 minute at 3,000 × *g* in a microcentrifuge and discard the supernatant.

5. If you have solution remaining from Step 3, repeat Step 4.

6. Add 900 µL of the Final Wash Buffer.

7. Centrifuge 1 minute at full speed in a microcentrifuge and discard the flow-through.

8. Repeat Step 7.

9. Elute the purified DNA in 15 µL of sterile water. Store on ice if proceeding immediately to **Ligating the Expression Cassette**, page 18. Store at –20ºC for long-term storage.

Continued on next page

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pAO815–*In Vitro* Multimerization Protocol, Continued

Dephosphorylating the Vector

Dephosphorylation is necessary to prevent self-ligation of the vector.

1. Take your digest from **Digesting Recombinant pAO815**, Step 2 and phenol extract, then ethanol precipitate the DNA. Resuspend in 17 µL of sterile water.

2. Set up the dephosphorylation reaction in a microcentrifuge tube as follows:

*Bam*H I digested recombinant pAO815 (page 16, Step 2)	17 µL
10X CIP Buffer	2 µL
CIP (1 Unit/µL)	1 µL
Total Volume	20 µL

3. Incubate at 37°C for 15 minutes.

4. Add 30 µL sterile water to the reaction to make a final volume of 50 µL.

5. Add 50 µL phenol/chloroform and extract your DNA solution. Transfer the aqueous solution to a new tube.

6. Precipitate the DNA by adding 5 µL 3 M sodium acetate and 110 µL 100% ethanol. Incubate on ice for 30 minutes.

7. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.

8. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol.

9. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.

10. Resuspend the pellet in 8 µL sterile water. Save on ice if you plan to ligate your insert immediately (see **Ligating and Digesting the Expression Cassette**, next page) or store at –20°C.

pAO815–*In Vitro* **Multimerization Protocol,** Continued

Ligating and Digesting the Expression Cassette

Ligation of the expression cassette will generate head-to-tail, head-to-head, and tail-to-tail multimers. Creation of head-to-tail multimers will be in the correct orientation for expression and will destroy both the *Bam*H I and *Bgl* II sites between the expression cassettes. Digestion of the multimers with *Bam*H I and *Bgl* II will eliminate those multimers with tail-to-tail and head-to-head orientation. After digestion with these two restriction enzymes, you will have a mixture of multimers containing 1, 2, 3, etc. copies of your gene that can be ligated into *Bam*H I-linearized, recombinant pAO815.

1. Set up a 20 μL ligation reactions as follows:

Bgl II-*Bam*H I digested expression cassette	15 μL
Sterile water	2 μL
10X Ligation Buffer (with ATP)	2 μL
T4 DNA Ligase (2.5 units/μL)	1 μL
Total Volume	20 μL

2. Incubate at 16°C for 2.5 hours.

3. Heat inactivate the ligase by incubating at 65°C for 20 minutes.

4. Add the following reagents for restriction enzyme digestion (cut-back). Note that *Bam*H I and *Bgl* II may be used with the same restriction buffer:

Sterile water	23 μL
10X restriction enzyme buffer	5 μL
Bgl II (10 units/mL)	1 μL
*Bam*H I (10 units/mL)	1 μL
Total Volume	30 μL

5. Incubate the reaction at 37°C for 2 hours.

6. Add 50 μL phenol/chloroform and extract the restriction enzyme digestion to remove the enzymes. Transfer the aqueous solution to a new microcentrifuge tube.

7. To ethanol precipitate the DNA, add 5 μL 3 M sodium acetate and 110 μL 100% ethanol.

8. Centrifuge at maximum speed in a microcentrifuge for 10 minutes at 4°C. Carefully decant the supernatant.

9. Wash the nucleic acid pellet with 80% ethanol, centrifuge 2 minutes, and remove the ethanol. Centrifuge again for 1 minute, remove residual ethanol, and air dry the pellet.

10. Resuspend pellet in 4 μL sterile water. Save on ice if you plan to ligate your insert immediately or you can store at –20°C. Proceed to **Ligating Multimers into Linearized Vector**, next page.

pAO815–*In Vitro* Multimerization Protocol, Continued

Note	You may wish to combine the ligation reaction with the restriction enzyme digestion. T4 ligase will retain most of its activity in all of the four New England BioLabs buffers. Remember to add 1 mM ATP to the reaction to ensure ligase activity.

Ligating Multimers into Linearized Vector

You are now ready to ligate the mixture of multimers generated in Step 10, above, into dephosphorylated, linearized vector.

1. Set up the following ligation reactions:

Dephosphorylated vector (page 17, Step 10)	4 µL
Expression cassette multimers (Step 10, above)	4 µL
10X Ligation Buffer	1 µL
T4 DNA Ligase (2.5 units/µL)	1 µL
Total volume	10 µL

    For the vector only control:

Dephosphorylated vector	4 µL
Sterile water	4 µL
10X Ligation Buffer	1 µL
T4 DNA Ligase (2.5 units/µL)	1 µL
Total volume	10 µL

2. Incubate overnight at 16°C.

3. You may store the ligation reactions at –20°C until ready to use, or transform 1–10 µL of each ligation mix into competent *E. coli*. Note that the amount of the ligation mixture you transform depends on whether you use electrocompetent or chemically competent cells. You may have to decrease the amount you to transform into electrocompetent cells to prevent arcing.

Transforming *E. coli*

Remember to include the "vector only" and "cells only" controls to evaluate your experiment. The "vector only" will indicate whether your vector was dephosphorylated. Since the CIP reaction is not 100% and because you often get degradation of the ends, there might be a few colonies on this plate. The "cells only" plate should have no colonies at all.

1. Transform competent *E. coli* by your method of choice.

2. After adding medium to the transformed cells and allowing them to recover, plate 10 µL and 100 µL of each transformation mix onto LB plates with 50–100 µg/mL ampicillin. Save the remainder of your transformation mix at 4°C.

3. Incubate overnight at 37°C. If you do not get transformants or very few transformants, plate out the remainder of the transformation mix onto LB-ampicillin plates.

pAO815–*In Vitro* Multimerization Protocol, Continued

**Analyzing
Transformants**

1. Pick 20 transformants and inoculate 2 mL LB containing 50–100 µg/mL ampicillin. Grow overnight at 37°C.

2. Isolate plasmid DNA and digest with *Bgl* II and *Bam*H I to release any multimers from pAO815.

   (Be sure to include *Bgl* II-*Bam*H I digested pAO815 as a control. It is possible to get vector rearrangements and deletions with large vectors in *E. coli*. Including *Bgl* II-*Bam*H I digested pAO815 will allow you to detect these rearrangements-deletions in the vector backbone.)

3. Analyze your digests on a 1% agarose gel. You should see bands corresponding to 1 copy, 2 copies, 3 copies, etc. of your expression cassette along with the vector backbone.

   (The number of copies you obtain may depend on how well a large vector is tolerated by the host strain.)

4. Once you have identified plasmids with multiple copies of your expression cassette, be sure to purify by streaking for single colonies and confirming your construct.

5. Prepare frozen glycerol stocks of *E. coli* containing each of your multimeric constructs.

6. Prepare 5–10 µg of each plasmid for transformation into *Pichia*. Proceed to **Transformation into *Pichia***, page 23.

pAO815–*In Vitro* Multimerization Protocol, Continued

Troubleshooting   The table below will help you optimize formation and isolation of multimers in *Pichia*.

Problem	Possible Reason	Solution
No multimers or low number of multimers in your vector after transformation into *E. coli*	CIP defective	Use fresh CIP.
		Add more CIP. Add 1 unit of CIP and incubate 15 more minutes at 37°C. This is somewhat risky as CIP can degrade the ends of your DNA.
	Not enough insert DNA to ligate	Digest more pAO815 containing 1 copy of your expression cassette.
	Construct is unstable in *E. coli*	Use the *in vivo* method to isolate multimers (see page 27).
	Multimers are too long to ligate efficiently	Try ligating each expression cassette separately.
Recombinant vector rearranges and deletions are detected	Construct is unstable in *E. coli*	Use the *in vivo* method to isolate multimers (see page 27).
Pichia His[+] transformants do not have multimers	Vector was linearized with the wrong enzyme. Restriction enzymes in the 5´ AOX1 region are duplicated when multimers are created.	Linearize your construct with *Sal* I or *Stu* I to insert the construct into his4.
		Analyze your construct for other unique restriction sites in the vector backbone that are near the 5´ *AOX*1 region or the 3´ *AOX*1 region. These sites will preserve your multimers and allow recombination with *AOX*1.

pAO815–*In Vitro* Multimerization Protocol, Continued

For More Information

There are a number references in the literature you can consult to optimize synthesis of *in vitro* multimers. A partial list is provided below:

Cohen, B. and Carmichael, G. G. (1986) A Method for Constructing Multiple Tandem Repeats of Specific DNA Fragments. *DNA* **5**: 339-343.

Eisenberg, S., Francesconi, S. C., Civalier, C. and Walker, S. S. (1990) Purification of DNA-Binding Proteins by Site-specific DNA Affinity Chromatography. *Methods Enzymol.* **182**: 521-529.

Graham, G. J. and Maio, J. J. (1992) A Rapid and Reliable Method to Create Tandem Arrays of Short DNA Sequences. *BioTechniques* **13**: 780-789.

Rudert, W. A. and Trucco, M. (1990) DNA Polymers of Protein Binding Sequences Generated by Polymerase Chain Reaction. *Nucleic Acids Res.* **18**: 6460.

Simpson, R. T., Thoma, F. and Brubaker, J. M. (1985) Chromatin Reconstituted from Tandemly-repeated Cloned DNA Fragments and Core Histones: A Model System for the Study of Higher-order Structure. *Cell* **42**: 799-808.

Takeshita, S., Tezuka, K.- i., Takahashi, M., Honkawa, H., Matsuo, A., Matsuishi, T. and Hashimoto-Gotoh, T. (1988) Tandem Gene Amplification *in vitro* for Rapid and Efficient Expression in Animal Cells. *Gene* **71**: 9-18.

Taylor, W. H. and Hagerman, P. J. (1987) A General Method for Cloning DNA Fragments in Multiple Copies. *Gene* **53**: 139-144.

Transformation into *Pichia*

Introduction

At this point you should have your gene cloned as multimers in pAO815 and singly in pPIC3.5K. You should also have about 5–10 µg of each construct for each transformation into *Pichia*. For methods to transform *Pichia* and select His⁺ transformants, refer to the *Pichia* Expression System manual. To linearize your construct prior to transformation, see below.

Linearizing Plasmid DNA

To linearize your construct in pPIC3.5K or pAO815, read the following:

1. If you cloned your insert into pPIC3.5K, you will need to linearize your insert prior to transformation using:

    *   *Bgl* II for replacement at *AOX1* (GS115)

    *   *Sac* I for insertion at *AOX1* (GS115 or KM71)

    *   *Sal* I for insertion at *HIS4* (GS115 or KM71)

2. If you cloned your insert into pAO815, you will need to linearize your insert prior to transformation using:

    *   *Bgl* II for replacement at *AOX1* (GS115)

    *   *Sal* I or *Stu* I for insertion at *HIS4* (GS115 or KM71)

    Note that multiple *Sac* I sites are formed if there are 2 or more multimers in pAO815.

If your insert contains any of these restriction sites, see the table on the next page for alternate sites.

We recommend that you linearize your vector in such a manner to generate both Mut⁺ and Mut^S recombinants. It is possible that one phenotype will express your multicopy integrant better than the other.

If you want only Mut⁺ recombinants:

*   Linearize pPIC3.5K with *Sac* I or *Sal* I for insertion at *AOX1* or *his4*, respectively, and transform GS115.

*   Linearize pAO815 with *Sal* I or *Stu* I for insertion at *his4* and transform GS115.

If you wish to have only Mut^S recombinants:

*   Use strain KM71 which is already Mut^S and linearize for insertion at *AOX1* or *his4*.

*   Linearize pPIC3.5K with *Bgl* II for gene replacement at *AOX1* and transform GS115.

*   Linearize pAO815 with *Bgl* II for gene replacement at *AOX1* and transform GS115.

Transformation into *Pichia*, Continued

Alternate Restriction Sites

The table below describes alternate restriction sites for linearizing your construct before transformation into *Pichia*.

pPIC3.5K. Note that an additional *Stu* I site was added with the inclusion of the *kan* gene, so that the *Stu* I site in *HIS4* is no longer unique.

Restriction Enzyme	5´ *AOX1*	3´ *AOX1*	Vector backbone	*HIS4* gene
Sac I	209	--	--	--
Pme I	414	--	--	--
Bpu 1102 I	589	--	--	--
Xcm I	699	--	--	--
Aat II*	(8843)	--	--	--
Tth III I*	--	(6775)	--	--
Bgl II†	2	6616	--	--
Dra I†	414	6454	6596, 7787, 7806	--
Sal I	--	--	--	2919
*Bsp*E I	--	--	--	3580

pAO815. Note that if more than one expression cassette is created in pAO815, most of the unique sites in the 5´ *AOX1* region are now duplicated and no longer unique.

Restriction Enzyme	5´ *AOX1*	3´ *AOX1*	Vector backbone	*HIS4* gene
Aat II*	(7535)	--	--	--
Tth III I*	--	(5467)	--	--
Bgl II†	2	5307	--	--
Sal I	--	--	--	2863
Stu I	--	--	--	2948
*Bsp*E I	--	--	--	3580

*Restriction sites are outside the *AOX1* sequences in the vector backbone, but they are close enough for efficient recombination to occur.

†Restriction sites are used to generate gene replacements at *AOX1* in GS115 only.

Transformation into *Pichia*, Continued

Controls
We recommend that you include the following controls when transforming *Pichia*.

* The parent vector linearized in the same manner as your construct. This is used as a control to confirm integration via PCR (see the *Pichia* Expression Manual for a protocol) and as control for background for the expression analysis and the quantitative dot blots or Southern analysis.

* pAO815 or pPIC3.5K containing one copy of your expression cassette. Be sure to linearize pAO815 in the same manner as your multimer. Most of the His$^+$ transformants created by transforming with recombinant pPIC3.5K will only have one copy. Make sure that the transformant you pick is **only** resistant to 0.25 mg/mL Geneticin®. The single copy controls created using pPIC3.5K and pAO815 should have the same Mut phenotype as the putative multimeric recombinants you are testing. These recombinants will be used as a control to compare expression levels with multiple copies of your expression cassette and as a single copy control for quantitative dot blot or Southern analysis. This is a very important control as increasing the copy number of the desired gene does not always lead to increased expression of recombinant protein.

Transforming *Pichia*
Refer to the *Pichia* Expression Manual for procedures to prepare *Pichia* for transformation, transformation procedures, and selection of His$^+$ recombinants. Invitrogen also offers the EasyComp™ Kit for preparation and transformation of competent *Pichia* cells (see page 44).

Analysis of His$^+$ Transformants
Once you have generated His$^+$ transformants using recombinant pPIC3.5K, proceed to *In Vivo* **Selection of Multiple Inserts**, page 27.

For His$^+$ transformants generated using recombinant pAO815, you will need to analyze recombinants for the presence of your insert. Refer to **PCR Analysis of *Pichia* Integrants** in the *Pichia* Expression System manual. Analyze for the presence of your insert by PCR (see the *Pichia* Expression System manual for a protocol). **Note**: The size of the PCR product for pAO815 is 189 bp.

Important
PCR will only indicate if your gene is present but will not indicate how many copies of your gene are integrated or at which locus it is integrated. PCR can reasonably be done on 12–20 transformants. Remember to include vector only and original construct controls to analyze your PCR experiment.

Since there is no guarantee that multiple copies will actually increase the amount of protein expressed, most people elect to proceed directly to expression to see if any of these colonies overexpress their protein. Be sure to include a single copy insert as a control. Test all your multimeric His$^+$ transformants for their Mut phenotype so that you induce expression properly. Refer to the *Pichia* Expression System manual for methods to express your protein.

Transformation into *Pichia*, Continued

Determining Copy Number

If you find that your His⁺ recombinants significantly overexpress your protein, you may wish to quantify the copy number of your gene. Copy number may be analyzed by Southern or quantitative dot (slot) blots (see page 41). It is very important to include genomic DNA isolated from the host strain, *Pichia* recombinants transformed with the parent vector, and *Pichia* recombinants transformed with pPIC3.5K or pAO815 containing a single copy of your gene as controls to evaluate your experiment.

Be sure to purify your clones by streaking for single colonies and making frozen, glycerol stocks of all your Geneticin® resistant colonies. Always initiate expression studies from frozen stocks, not old plates.

pPIC3.5K–*In Vivo* Screening of Multiple Inserts

Introduction

You will need as many His⁺ transformants as you can conveniently generate. Note that statistically 1–10% of the His⁺ transformants will have more than one insert. This means that if the frequency of multicopy inserts is 1%, you will have to screen 1000 His⁺ transformants to get 10 Geneticin® resistant colonies to test. This may require 1–5 plates containing His⁺ transformants. It is not unusual to screen thousands of colonies. Once you have Geneticin® resistant colonies, you can then characterize them for their Mut phenotype.

Methods to Screen for Geneticin® Resistant Transformants

There are two methods used to screen His⁺ transformants for Geneticin® **resistance**:

- **Method 1** is technically easier and screens a greater number of clones, but is less reliable. After initial selection of His⁺ transformants, they are pooled and plated on YPD-Geneticin® plates containing increasing concentrations of Geneticin®. Method 1 is applicable to spheroplast or electroporation transformation methods.

- **Method 2** is technically more difficult and screens fewer numbers of clones, but it is more reliable. It involves growing clones in microtiter plates until all clones are at the same density. The cultures are then spotted on the YPD-Geneticin® plates and scored for Geneticin® resistance.

Important

There is a tendency to isolate false positives when screening with Geneticin®. It is very important to purify your putative Geneticin® resistant clones by streaking for single colonies on YPD and then confirming Geneticin® resistance on YPD-Geneticin® plates. For this reason, we do not recommend replica-plating as a method to screen for Geneticin® resistance. If you do elect to replica-plate, be sure to confirm Geneticin® resistance.

Before Starting

Prepare 4 YPD plates containing the following concentrations of Geneticin®: 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL (see **Recipes**, page 36).

pPIC3.5K–*In Vivo* Screening of Multiple Inserts, Continued

**Method 1,
(Spheroplasts)**

Use this procedure if you transformed *Pichia* spheroplasts. Start with plates containing His$^+$ transformants.

1. Using a sterile spreader, remove the top layer of the soft agar containing the His$^+$ transformants and place into a sterile, 50 mL conical centrifuge tube.

2. Add 10 to 20 mL of sterile water. There should be a 2X volume of water above the settled agar. Vortex vigorously for 1 to 2 minutes.

3. Set centrifuge tube upright on bench and let agar pieces settle (about 1 minute).

4. Determine the cell density of the supernatant by using a hemacytometer. You need at least 5×10^5 cells/mL so you can plate ~10^5 cells in 200 µL or less.

   (If the cells are too dilute, transfer the liquid to a fresh tube and centrifuge the cells. Resuspend the cell pellet in sterile water in a volume sufficient to give 5×10^5 cells/mL.).

5. Plate 10^5 cells on YPD-Geneticin® plates containing Geneticin® at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL. Use four plates for each concentration.

   (You may want to confirm the titer of the cells on the YPD plates without Geneticin® to calculate the percent of Geneticin® resistant colonies you obtain for each Geneticin® concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100 to 200 µL per plate.).

6. Incubate plates at 30°C and check daily. Geneticin®-resistant colonies will take 2 to 5 days to appear while cells plated on YPD without Geneticin® will take 2–3 days. Proceed to **Analyzing the Results**, page 31.

pPIC3.5K–*In Vivo* Screening of Multiple Inserts, Continued

Method 1, (Electroporation)

Use this procedure if electroporation was used to transform *Pichia*. Transformants will not be plated in top agar. Start with plates containing His⁺ transformants.

1. Pipette 1 to 2 mL sterile water **over** the His⁺ transformants on each plate. Use all the plates that have His⁺ transformants.

2. Resuspend the His⁺ transformants into the water by using a sterile spreader and running it across the top of the agar. Be careful not to tear the agar.

3. Transfer and pool the cell suspension into a sterile, 50 mL conical centrifuge tube and vortex briefly (5 to 10 seconds).

4. Determine cell density using a spectrophotometer ($1\ OD_{600} = 5 \times 10^7$ cells/mL).

    Note: Any agar present will interfere with a spectrophotometer reading.

5. Plate 10^5 cells on YPD plates containing Geneticin® at a final concentration of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL.

    (You may want to confirm the titer of the cells on the YPD plates without Geneticin® to calculate the percent of Geneticin® resistant colonies you obtain for each Geneticin® concentration and determine whether you are getting multimers at 1–10% of the transformants plated. Prepare 10^{-5}, 10^{-6}, and 10^{-7} dilutions of the pooled transformants using sterile water. Plate 100 to 200 µL per plate.)

6. Incubate plates at 30°C and check daily. Geneticin®-resistant colonies will take 2 to 5 days to appear while cells plated on YPD will take 2–3 days. Proceed to **Analyzing the Results**, page 31.

Note

If you do not plate all of the cell suspension from either of the methods above, add sterile glycerol to 15% and freeze in convenient aliquots at –80°C. You may thaw the aliquots and analyze for Geneticin® resistant colonies at a later date.

pPIC3.5K–*In Vivo* Screening of Multiple Inserts, Continued

Method 2   You will need three sets of two microtiter plates (6 total) to screen ~180 His$^+$ recombinants. It is important to grow your clones to approximately the same cell density by successive inoculations to ensure that equivalent numbers of cells are spotted on Geneticin® plates. If you plated your transformants in top agar, it may be necessary to extract them from the agarose and re-plate them on minus histidine plates (see *Pichia* Expression System manual) to pick colonies. Remember to include controls for strain background and one copy of your gene. For every 180 colonies, you can expect to isolate 1–10 Geneticin® resistant colonies.

1. Using sterile technique, add 200 µL YPD to each microtiter well.

2. Inoculate each well of the first set of plates with a single His$^+$ transformant using a sterile toothpick and stirring to resuspend cells.

3. Cover the microtiter plate and incubate at 30°C for 2 days (shaking not required).

4. After 2 days, take new microtiter plates and add 190 µL of YPD to each well.

5. Inoculate the second set of microtiter plates with 10 µL from the first set of microtiter plates by using a multichannel pipette. Make sure the second set of plates is marked and oriented in such a way that you can keep track of wells.

6. Cover and incubate the second set of plates overnight at 30°C.

7. The next day, repeat Steps 5 and 6, creating a third set of microtiter plates. **Note:** Successive growth and passage of clones will bring them all to the same cell density.

8. After incubation, take the third set of plates and resuspend the cells in each well by pipetting up and down with a multichannel pipette set on 100 µL volume.

9. Spot 10 µL from each well on YPD plates containing Geneticin® at a final concentration of 0, 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL. Spot in a regular pattern using the multi-channel pipette or a grid underneath the plate.

10. Let the liquid soak in, then incubate plates at 30°C, and check after 2, 3, 4, or 5 days for Geneticin® resistant clones. Proceed to **Analyzing the Results**, next page.

Continued on next page

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Analyzing the Results

There may be only a few Geneticin® resistant colonies, and they may be of different sizes, but the colony morphology should be the same. Pick all Geneticin® resistant colonies and purify by streaking for single colonies. Be sure to confirm the observed level of Geneticin® resistance.

You may not find colonies resistant to 2.0, 3.0, or 4.0 mg/mL Geneticin®. "Jackpot" clones resistant to these high levels of Geneticin® are very rare. You may have to screen thousands of His[+] transformants to isolate colonies resistant to 2–4 mg/mL Geneticin®.

Analyze for the presence of your insert by PCR (see the *Pichia* Expression System manual for a protocol). PCR will only tell you if your gene is present. It will not tell you how many copies of your gene are integrated or at which locus the integration occurred. PCR can reasonably be done on 12–20 transformants. Remember to include the vector only and original (one copy) construct controls to analyze your PCR experiment.

Since there is no guarantee that multiple copies will actually increase the amount of protein expressed, you may elect to proceed directly to expression to see if any of these colonies overexpress their protein. Be sure to include a single copy insert as a control. Test all your Geneticin® resistant colonies for their Mut phenotype so that you induce expression properly. Refer to the *Pichia* Expression System manual for methods to express your protein.

Be sure to purify your clones by streaking for single colonies and making frozen, glycerol stocks of all your Geneticin® resistant colonies. Always initiate expression studies from frozen stocks, not old plates.

Determining Copy Number

If you find that your Geneticin®-resistant His[+] recombinants significantly overexpress your protein, you may wish to quantify the copy number of your gene. Copy number may be analyzed by Southern or quantitative dot (slot) blots (see page 41). It is very important to include genomic DNA isolated from *Pichia* recombinants transformed with pPIC3.5K alone and pPIC3.5K with a single copy of your gene as controls to evaluate your experiment.

Continued on next page

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pPIC3.5K–*In Vivo* Screening of Multiple Inserts, Continued

Troubleshooting

Since there is a tendency to isolate false positives (colonies which appear to be Geneticin® resistant, but are not), it is very important to purify your putative Geneticin® resistant colonies and confirm the observed level of Geneticin® resistance before proceeding further.

The other most common problem with the *in vivo* method is that very few Geneticin® resistant colonies are isolated requiring screening of more His+ transformants. Remember that you are isolating spontaneous, multiple integration events. These occur at a frequency of 1–10% which may mean that you need to screen thousands of His+ transformants as opposed to hundreds. In addition, to isolate recombinants with the most copies of your gene inserted, you must screen more His+ transformants. Successive multiple insertions are simply more rare.

If you find that your transformation efficiency is low, try electroporation instead of spheroplasting. This may increase the transformation efficiency and help you isolate more His+ transformants.

Appendix

Vectors

Introduction

The vectors pPIC3.5K and pAO815 share many of the same features (see below). Both are functional in *Pichia* strains GS115 and KM71. However, pPIC3.5K has a more extensive multiple cloning site and contains the kanamycin gene for *in vivo* screening of multiple copy inserts. It is identical to pPIC3.5 except for the presence of the kanamycin gene. pAO815 is similar to pHIL-D2 except that it does not contain an f1 origin.

Features

The table below describes the general features of the pPIC3.5K and pAO815 *Pichia* expression vectors.

Feature	Description	Benefit
5´ *AOX1*	An ~1,000 bp fragment containing the *AOX1* promoter	Allows methanol-inducible high level expression in *Pichia.* Targets plasmid integration to the *AOX1* locus.
MCS	Multiple Cloning Site	Allows insertion of your gene into the expression vector.
TT	Native transcription termination and polyadenylation signal from *AOX1* gene (~260 bp)	Permits efficient transcription termination and polyadenylation of the mRNA.
HIS4	*Pichia* wild-type gene coding for histidinol dehydrogenase (~2.4 kb) and used to complement *Pichia his4* strains	Provides a selectable marker to isolate *Pichia* recombinant strains.
3´ *AOX1*	Sequences from the *AOX1* gene that are further 3´ to the TT sequences (~650 bp)	Targets plasmid integration at the *AOX1* gene.
Amp pBR322 origin	Ampicillin resistance gene *E. coli* origin of replication	Allows selection, replication, and maintenance in *E. coli.*
*Bam*H I *Bgl* II *Not* I *Sac* I *Sal* I *Stu* I	Unique restriction sites (**Note:** *Stu* I is not unique to pPIC3.5K)	Permits linearization of vector for efficient integration into the *Pichia* genome and generation of either Mut$^+$ or Mut^S recombinants.
kan	Kanamycin resistance gene from Tn*903* which confers resistance to Geneticin® in *Pichia* and kanamycin resistance in *E. coli* (for pPIC3.5K only)	Allows *in vivo* screening for multicopy inserts by increased resistance to Geneticin®. Also allows selection for kanamycin resistance in *E. coli.*

Note

There is no yeast origin of replication in any of the *Pichia* expression vectors available from Invitrogen. His$^+$ transformants can only be isolated if recombination occurs between the plasmid and the *Pichia* genome (i. e. integration of the plasmid).
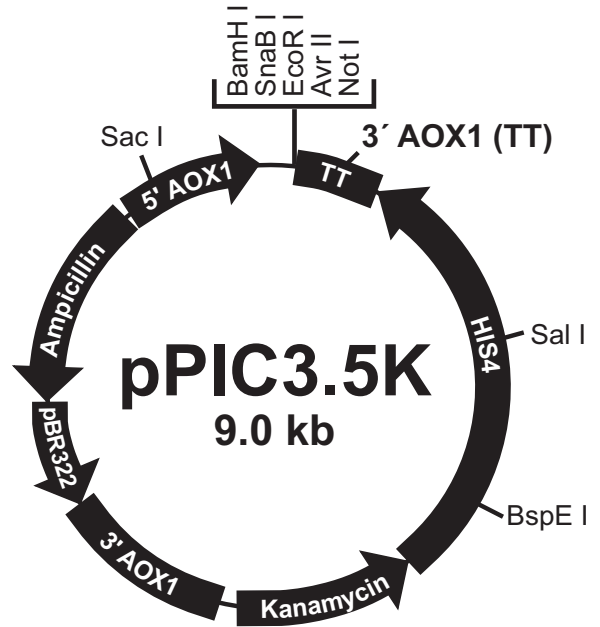
pPIC3.5K

The figure below shows the map of pPIC3.5K. Details of the multiple cloning site are shown on page 9.

Comments for pPIC3K:
   9004 nucleotides

5´ *AOX1* promoter fragment: bases 1-937
5´ *AOX1* primer site: bases 855-875
Multiple Cloning Site: bases 938-968
3´ *AOX1* primer site: bases 1055-1075
3´ *AOX1* transcription
        termination (TT): bases 981-1314
HIS4 ORF: bases 4242-1708
Kanamycin resistance gene: bases 5458-4656
3´ *AOX1* fragment: bases 5850-6607
pBR322 origin: bases 7689-7016
Ampicillin resistance gene: bases 8694-7834

pAO815

Map of pAO815   The figure below shows the map of pAO815. Details of the multiple cloning site are shown on page 10.

Comments for pAO815:
    7709 nucleotides

5´ *AOX1* promoter fragment: bases 1-940
5´ *AOX1* primer site: bases 855-875
*Eco*R I Site: bases 943-948
3´ *AOX1* primer site: bases 1024-1044
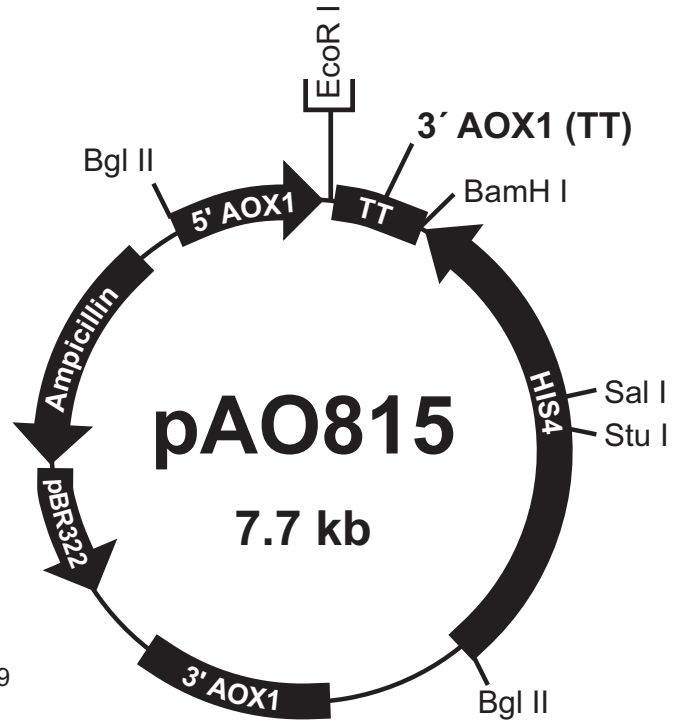3´ *AOX1* transcription
        termination (TT): bases 950-1277
HIS4 ORF: bases 4199-1665
3´ *AOX1* fragment: bases 4554-5310
pBR322 origin: bases 6394-5740
Ampicillin resistance gene: bases 7399-6539

Recipes

**YPD-Geneticin®
plates**

<u>Y</u>east <u>E</u>xtract <u>P</u>eptone <u>D</u>extrose Medium
1% yeast extract
2% peptone
2% dextrose (glucose)
1.5% agar
Variable amounts of Geneticin®

10X D (20% Dextrose)
Dissolve 200 g of D-glucose in 1,000 mL of water. Autoclave for 15 minutes or filter sterilize. The shelf life of this solution is approximately one year.

100 mg/mL Geneticin®
Geneticin® is available from Invitrogen (see page 44)

Prepare 30 mL of 100 mg/mL Geneticin® stock solution in sterile water. Filter sterilize and store frozen at –20°C. You will use this solution to make YPD plates containing Geneticin® at final concentrations of 0.25, 0.5, 0.75, 1.0, 1.5, 1.75, 2.0, 3.0, and 4.0 mg/mL.

For 250 mL (8 to 10 plates of a single Geneticin® concentration):

1. Combine 2.5 g yeast extract, 5 g peptone, and 5 g agar in 225 mL deionized water.

2. Autoclave for 20 minutes on liquid cycle.

3. Add 25 mL of 10X D and mix well.

4. Cool YPD to approximately 55–60°C and add appropriate volume of Geneticin® stock (see chart below). Remember to also make several YPD plates **without** Geneticin®.

5. Mix well by swirling, but be careful to minimize bubble formation.

6. Pour agar solution into 10 cm petri plates. Let plates harden, invert, and store bagged at 4°C. Plates are stable for at least 6 months.

Final [Geneticin®] (mg/mL)	mL Geneticin® stock/250 mL YPD
0.25	0.625
0.50	1.25
0.75	1.875
1.00	2.5
1.50	3.75
1.75	4.375
2.00	5.0
3.00	7.5
4.00	10.0

Pichia Genomic DNA Isolation

Introduction	The protocol below allows you to isolate DNA from the desired His[+] recombinant and untransformed GS115 or KM71. The DNA isolated is suitable for Southern blot analysis, dot/slot blot analysis or genomic PCR. See *Current Protocols in Molecular Biology*, pages 13.11.1 to 13.11.4 (Ausubel, *et al.*, 1990), *Guide to Yeast Genetics and Molecular Biology*, pages 322–323 (Strathern and Higgins, 1991), or Holm, *et al.*, 1986 for other methods to isolate DNA from *Pichia*.
	In addition to the protocol listed below, we use our Easy-DNA™ Kit (see page 44) to isolate DNA from *Pichia* for PCR and quantitative dot (slot) blots. See page 41 for this protocol.
	Lastly, there is a fast DNA isolation protocol for multiple samples (24) which has been reported (Wach, *et al.*, 1994).

Solutions	You need to prepare the following solutions.

- Minimal Medium (MD, MGY)

- Sterile water

- **Fresh**, SCED (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT)

- Zymolyase, 3 mg/mL stock solution in water (Seikagaku America, Inc.)

- 1% SDS in water

- 5 M potassium acetate, pH 8.9

- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)

- 7.5 M ammonium acetate, pH 7.5

- Phenol:chloroform (1:1 v/v)

Preparation	1. Grow the recombinant strain and the parent strain at 30°C to an OD_{600} of 5–10 in 10 mL of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115).
	2. Collect the cells by centrifugation at 1500 × g for 5–10 minutes at room temperature.
	3. Wash the cells with 10 mL sterile water by centrifugation as in Step 2.

Spheroplasting and Lysis	1. Resuspend the cells in 2 mL of SCED buffer, pH 7.5. Make this solution fresh.
	2. Add 0.1–0.3 mg of Zymolyase (mix well before adding to the cells). Incubate at 37°C for 50 minutes to achieve ~80% spheroplasting.
	3. Add 2 mL of 1% SDS, mix **gently**, and set on ice for 5 minutes.
	4. Add 1.5 mL of 5 M potassium acetate, pH 8.9, and mix **gently**.
	5. Centrifuge at 10,000 × *g* for 5–10 minutes at 4°C and save the supernatant.

Pichia Genomic DNA Isolation, Continued

Precipitating DNA

1. Transfer the supernatant from Step 5, previous page, and add 2 volumes of ethanol to the supernatant. Incubate at room temperature for 15 minutes.

2. Centrifuge at 10,000 × g for 20 minutes at 4°C.

3. Resuspend the pellet **gently** in 0.7 mL TE buffer, pH 7.4 and transfer to a microcentrifuge tube.

4. **Gently** extract with an equal volume of phenol:chloroform (1:1 v/v) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Split the aqueous layer into two microcentrifuge tubes.

5. Add 1/2 volume of 7.5 M ammonium acetate, pH 7.5, and 2 volumes of ethanol to each tube. Place on dry ice for 10 minutes or at –20°C for 60 minutes.

6. Centrifuge at 10,000 × g for 20 minutes at 4°C and wash the pellets once with 1 mL of 70% ethanol. Briefly air dry the pellets and resuspend each one in 50 μL of TE buffer, pH 7.5. Determine the concentration of the DNA sample. The two samples can be stored separately or combined, and stored at –20°C until ready for use.

Easy-DNA™ Protocol for Isolation of DNA from *Pichia*

Solutions

You will need to prepare the following solutions.

- Minimal Medium (MD, MGY)

- TE buffer, pH 7.4 (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8.0)

- 1 M Sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol (make fresh)

- Zymolyase, 3 mg/mL stock solution in water (Seikagaku America, Inc.)

- SCED (1 M sorbitol, 10 mM sodium citrate, pH 7.5, 10 mM EDTA, 10 mM DTT, make fresh)

- Easy-DNA™ Kit (to purchase, see page 44)

- Chloroform

- Isopropanol

- 70% or 80% ethanol

- RNase A

Preparing Cells

1. Grow the recombinant strain and the parent strain at 30°C to an OD_{600} of 5-10 in 2–5 mL of minimal media such as MD or MGY (recombinant) or MDH or MGYH (GS115 or KM71).

2. Harvest 1.5 mL of the culture by centrifuging at maximum speed in a microcentrifuge for 1–2 minutes at room temperature.

3. Resuspend cells in 1.5 mL TE and centrifuge as in Step 2.

4. Resuspend cells in 1 mL fresh 1 M Sorbitol, 100 mM EDTA, 14 mM β-mercaptoethanol. Vortex to resuspend.

5. Add 1.5 µL of 3 mg/mL Zymolyase to each tube of cells and incubate at 30°C for 1 hour.

6. Centrifuge at 1,700 × *g* in a microcentrifuge for 8 minutes at room temperature. It is important to centrifuge with less force as the cells are fragile because of digestion with Zymolyase.

7. **Gently** resuspend the cells in 200 µL **fresh** SCED and incubate at 37°C for 1 hour.

Easy-DNA™ Protocol for Isolation of DNA from *Pichia*, Continued

Isolating DNA

1. Add 350 µL Easy-DNA™ Solution A to the cell suspension from Step 7, above, vortex, and incubate at 65°C for 10 minutes.

2. Add 150 µL of Easy-DNA™ Solution B and vortex.

3. Add 600 µL chloroform and vortex.

4. Centrifuge at maximum speed for 20 minutes at room temperature.

5. Transfer the aqueous layer to a fresh tube, add 600 µL isopropanol, and mix by inversion. Incubate at room temperature for 10 minutes.

6. Centrifuge sample at maximum speed for 20 minutes at 4°C.

7. Wash pellet with cold 70 or 80% ethanol, centrifuge at maximum speed for 2 minutes at 4°C, remove ethanol, and air-dry.

8. Resuspend the pellet in 50 µL TE containing 50 µg/mL RNase A and incubate overnight at room temperature. Quantify the amount of DNA. We generally use 5 µL of this DNA solution in a 50 µL PCR reaction.

Determining Copy Number of Multiple Integrants

General Guidelines
- Use standard procedures and solutions for Southern blotting as outlined in *Molecular Cloning: A Laboratory Manual* (Sambrook, *et al.*, 1989), pages 9.31–9.58.

- Isolate genomic DNA and quantify using fluorometry. Be sure to eliminate RNA. It is very important to load the same amount of DNA into each lane to accurately determine copy number.

- Probe your Southern blot with probes to both *HIS4* and your gene. Note that the point mutation in the *his4* gene in the host strain will not interfere with hybridization if you make the probe complementary to the wild-type gene.

- If you used pPIC3.5K to generate multimers, use *Bgl* II to digest your DNA (Clare, *et al.*, 1991a). Note that if you used pPIC3.5K that all multimers are NOT necessarily in a head-to-tail configuration. Some multimers may be head-to-head and others tail-to-tail. We recommend that you think about what products may be produced. An expression cassette in the opposite orientation may produce a different band. The number of multiple copies will cause one or two bands (depending on orientation) in the Southern blot to increase in intensity once you are >2 copies.

- If you used pAO815 to generate multimers, use *Bgl* II and *Bam*H I to digest the genomic DNA and release the multimer. The molecular weight of the band should allow you to determine the number of multimers. If this multimer is too large, you may wish to digest with an enzyme like *Sac* I. This will collapse the multimer into single fragments containing your gene. These will produce a band that will be quite intense. The relative intensity of this band versus a band containing a single copy of your gene will allow you to determine the copy number.

- *Bgl* II digested DNA from GS115 and GS115 transformed with pPIC3.5K or pAO815 will produce a bands of 2.8 kb (the genomic copy of *HIS4)*, and ~6.7 kb (the vector derived copy of *HIS4)*, respectively, when probed with a complementary fragment to *HIS4.*

Determining Copy Number of Multiple Integrants, Continued

Introduction   You may wish to determine the actual number of gene copies in your *Pichia* recombinant. You may use quantitative dot blots or Southern hybridization to analyze gene copy number (Brierley, *et al.*, 1994; Clare, *et al.*, 1991a; Romanos, *et al.*, 1991; Scorer, *et al.*, 1993; Scorer, *et al.*, 1994). This requires isolation of genomic DNA from *Pichia* recombinants transformed with the parent vector (0 copies of your gene), pAO815 or pPIC3.5K containing 1 copy of your gene (single copy control), and the *Pichia* recombinants containing multiple copies of your gene. Use the protocols detailed on the pages 37 and 39 to isolate genomic DNA.

Quantitative Dot Blot Solutions   You will need the following solutions, 10–15 mL of each for each dot blot.

- 50 mM EDTA, 2.5% β-mercaptoethanol pH 9.0

- 1 mg/mL Zymolyase 100T in water (Seikagaku America, Inc.)

- 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate

- 2X SSC (1X = 0.15 M NaCl, 0.015 M sodium citrate)

- 3MM paper

Quantitative Dot Blot Procedure   The following protocol is a summary of a rapid DNA dot blot technique to detect multiple integrants (Romanos, *et al.*, 1991). It is very important to spot equivalent numbers of cells onto filters to quantify copy number.

1. Grow Mut$^+$ or Mut^s transformants in individual wells of a 96-well microtiter plate in 200 µL of YPD broth at 30°C until all wells have approximately the same density. This may necessitate several passages; see page 27 for more details. Alternatively, individual transformants may be grown in culture tubes and the absorbance at 600 nm normalized with the addition of medium.

2. Filter 50 µL of each sample onto a nitrocellulose or nylon filter placed into a dot (slot) blot apparatus using multichannel pipettor. Air dry filters.

3. To lyse the cells on the filter, treat the filter with four solutions as follows: place two sheets of 3MM paper in a tray and soak with 10–15 mL of 50 mM EDTA, 2.5% β-mercaptoethanol pH 9.0. Make sure that the paper is uniformly soaked and that there are no puddles. Place the nitrocellulose filter face down on the treated 3MM paper. Incubate for 15 minutes at room temperature.

4. Remove the nitrocellulose filter from the 3MM paper and replace the 3MM paper with two new sheets. Soak the paper with 10–15 mL of 1 mg/mL Zymolyase 100T as described in Step 3. Place the nitrocellulose filter face down on the 3MM paper and incubate for 4 hours at 37°C.

5. Remove the nitrocellulose filter from the paper and replace the paper with two new sheets. Soak the paper with 10–15 mL of 0.1 N NaOH, 1.5 M NaCl, 0.015 M sodium citrate. Place the nitrocellulose filter face down on the paper and incubate for 5 minutes at room temperature.

Determining Copy Number of Multiple Integrants, Continued

Quantitative Dot Blot Procedure, Continued

6. Remove the nitrocellulose filter and replace with two new 3MM sheets. Soak with 10–15 mL 2X SSC. Place the nitrocellulose filter face down on the 3MM paper and incubate for 5 minutes at room temperature. Repeat.

7. Bake nitrocellulose filters at 80°C or UV-crosslink DNA to nylon. The filters may be probed with a nonradioactive-labeled or random-primed, ^{32}P-labeled probe complementary to your gene.

Multi-copy integrants can be identified by a strong hybridization signal relative to the single copy control. Dot blots can then be quantified for copy number by densitometry of the film or blot, or by using a β-scanner (if radiolabeled).

Southern Blot Analysis

For a detailed description of this technique as applied to *Pichia pastoris*, see (Clare, *et al.*, 1991a). It is very important to digest your DNA with the right restriction enzyme(s) to generate a blot of digested and gel-separated genomic DNA. It is also important to understand that your strategy will be different if you use pPIC3.5K versus pAO815 to generate your multiple copies. Digestion of DNA from *Pichia* recombinants containing multiple copies will produce a band that will vary in intensity depending on the number of copies of your gene. It is very important to include a control to show the intensity of a single copy gene. The band intensities can be relatively quantified using densitometry to estimate gene dosage.

Controls

It is very important to include DNA from the host strain alone (GS115 or KM71), the host strain transformed with the parent vector (pPIC3.5K or pAO815), and the host strain transformed with a vector containing one copy of your gene. It is also a very good idea to make a probe to the *HIS4* gene as an internal control for single copy in addition to a probe to your gene. Note that if your gene inserts into *his4*, two copies of the *HIS4* gene are created, one mutant and the other wild-type (see **Recombination and Integration in *Pichia***, *Pichia* Expression Kit manual).

Accessory Products

Introduction

The following products may be used with the pPIC3.5K and pAO815 vectors. For details, visit www.invitrogen.com or contact **Technical Support** (see page 45).

Item	Amount	Catalog no.
Electrocomp™ TOP10F´	5 × 80 µL	C665-55
One Shot® TOP10F´ (Chemically Competent cells)	20 × 50 µL	C3030-03
S.N.A.P.™ Gel Purification Kit	25 preps	K1999-25
Geneticin®	1 gram	11811-023
	5 grams	11811-031
	25 grams	11811-098

Primers

For your convenience, Invitrogen offers a custom primer synthesis service. Visit www.invitrogen.com for more details.

Other *Pichia* Products

Other *Pichia* products available from Invitrogen are described below:

Item	Purpose	Amount	Catalog no.
Pichia Expression Kit	Complete Kit for Gene Expression in *Pichia pastoris.*	1 kit	K1710-01
Spheroplast Kit	Preparation of *Pichia* spheroplasts	1 kit	K1720-01
Pichia EasyComp™ Transformation Kit	Rapid preparation and transformation of competent *P. pastoris* cells.	1 kit	K1730-01
Easy-DNA™ Kit	Isolation of DNA from *Pichia* for PCR.	1 kit	K1800-01
pPICZ A, B, & C	For simple selection on Zeocin™ and intracellular expression of recombinant proteins containing a C-terminal histidine tag.	20 µg each	V190-20
pPICZα A, B, &C	For simple selection on Zeocin™ and secreted expression of recombinant proteins containing a C-terminal histidine tag.	20 µg each	V195-20
pPIC9K	For *in vivo* isolation of multiple copy inserts for secreted expression.	20 µg	V175-20

Technical Support

Web Resources

Visit the Invitrogen website at **www.invitrogen.com** for:

- Technical resources, including manuals, vector maps and sequences, application notes, SDSs, FAQs, formulations, citations, handbooks, etc.

- Complete technical support contact information

- Access to the Invitrogen Online Catalog

- Additional product information and special offers

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Tel (Toll Free): 1 800 955 6288	Tel: 81 3 5730 6509	Tel: +44 (0) 141 814 6100
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E-mail: tech_support@invitrogen.com	E-mail: jpinfo@invitrogen.com	E-mail: eurotech@invitrogen.com

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You may contact Research Corporation Technologies at the following address: Bennett Cohen, Ph.D., Research Corporation Technologies, 101 North Wilmot Road, Suite 600, Tucson, Arizona 85711-3335. Tel: 520-748-4443, Fax: 520-748-0025.

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