

Expression of Recombinant *Aspergillus fumigatus* Antigens Using OverExpress™ C43(DE3) Cells

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Introduction

A large number of saprophytic fungi present in our environment can cause disease in humans. Among the known allergenic fungi, *Aspergillus fumigatus* is the most common species that causes invasive mycoses in immunocompromised patients. Patients at highest risk for acquiring the disease are those with a significant reduction in the number or function of granulocytes, such as leukemia patients undergoing chemotherapy or patients on long-term treatment with immunosuppressive drugs, especially cortisone (e.g., organ transplant patients). Despite antimycotic treatment, the fatality rate of invasive aspergillosis for both patient populations is approximately 58% (1). To prevent or reduce the incidence of invasive aspergillosis, we are developing a unique approach to immunization against *A. fumigatus*. Our studies are focused on developing a vaccine based on candidate *A. fumigatus* antigens that are believed to be relevant targets for providing protective immune responses against aspergillosis. Recently, we used OverExpress C43(DE3) cells to express four difficult-to-express, recombinant *A. fumigatus* antigens fused with a hydrophobic domain: AspF3-HD, AspF9-HD, Asp-hemolysin-HD, and Asp-rodA-HD. The fusion proteins will be formulated in a liposomal vaccine delivery system (2), and then tested in relevant murine models for their ability to protect against both systemic and pulmonary *A. fumigatus* challenge.

Materials and Methods

Recombinant DNA for each of the five *A. fumigatus* antigen genes was synthesized, ligated into the pET28a vector, and transformed into OverExpress C43 (DE3) *E. coli* cells. Cells were plated onto LB-agar* plates containing 30 µg/ml kanamycin. Positive transformants were identified by restriction enzyme analysis and DNA sequencing. One clone for each *A. fumigatus* antigen was selected for protein expression and purification.

Each positive clone was grown in 0.5 L of TB medium with 30 µg/ml kanamycin. Protein expression was induced with 0.75 mM IPTG, when the culture reached an OD₆₀₀ of 0.5, and then incubated for an additional 6 hours. The cells were pelleted at 6,000 x g for 30 min, resuspended in an 8 M urea buffer, and lysed by microfluidization at 15,000 psi. Following centrifugation at 30,000 x g for 1 h, clarified lysate was loaded onto an 8-ml Ni-NTA agarose column, washed with 5 volumes of 8 M urea buffer, 5 volumes of 0.3% sodium deoxycholate buffer, and 5 volumes of 50 mM sodium phosphate buffer. The 6xHis-tagged proteins were eluted with 5 volumes of 250 mM imidazole elution buffer. Column fractions were analyzed by SDS-PAGE. Protein concentrations were estimated using both UV absorbance at 280 nm and the bicinchoninic acid assay. Endotoxin levels for each protein preparation were estimated using the Limulus amoebocyte lysate assay.

Results and Discussion

Four recombinant *A. fumigatus* antigens were successfully purified after expression in OverExpress C43 (DE3) cells. Each protein eluted from the Ni-NTA agarose column with an elution buffer containing 250 mM imidazole. SDS-PAGE (Figure 1) showed that each protein was expressed and purified. After purification, the following protein concentrations were estimated (per liter of fermentation): 0.9037 mg/L for AspF3-HD, 3.6437 mg/L for AspF9-HD, 1.3006 mg/L for Asp-hemolysin-HD, and 1.8105 mg/L for Asp-rodA-HD.

Conclusions

Using OverExpress C43(DE3) *E. coli* cells, we successfully expressed four recombinant *A. fumigatus* antigens: AspF3-HD, AspF9-HD, Asp-hemolysin-HD, and Asp-rodA-HD. Because of the high efficiency of expression for these hydrophobic fusion proteins, which have proved difficult to express in other cells, we will now be able to assess the protective immune response elicited by each of the five *A. fumigatus* antigens.

References

1. Lin, S. J. et al. Aspergillosis case-fatality rate: systematic review of the literature. Clin. Infect. Dis. 32, 358-366 (2001).
2. Ernst, W. A. et al. Protection against H1, H5, H6 and H9 influenza A infection with liposomal matrix 2 epitope vaccines. Vaccine. 24, 5158-5168 (2006).

*Lucigen now recommends plating on YT agar plates for even better results.

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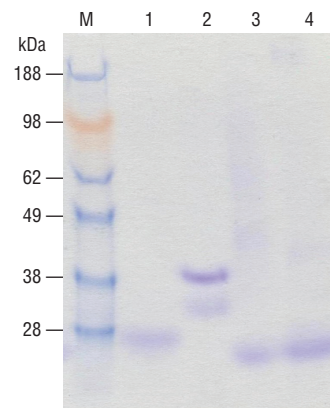


Figure 1. High protein yield. SDS-PAGE on a 4-20% Tris-HEPES gel shows high protein yield for each of the four *A. fumigatus* antigens. Lane M, molecular weight marker; Lanes 1-4, purified protein samples: (1)AspF3-HD; (2)AspF9-HD; (3)Asp-hemolysin-HD; (4)Asp-rodA-HD. 20 µg of sample was loaded per well.

NEW BigEasy® Long PCR Cloning Kit

- Easy cloning of amplicons up to 30 kb
- Highest stability cloning system known
- Get every gene and sequence
- No insert size bias
- Inducible copy number for high DNA yields

The BigEasy Long PCR Cloning Kits contain everything needed to efficiently clone PCR products up to 30 kb into an unbiased, high-fidelity cloning vector (Figure 1). The two different versions of the kit are compatible with either proofreading or non-proofreading PCR polymerases. They can also be used to clone any blunt or G-tailed DNA up to 30 kb. These vectors incorporate Lucigen's exclusive CloneSmart® transcription-free cloning technology to reduce bias and maximize cloning efficiency.

Proofreading PCR polymerases (Pfu, Vent®, etc.)

Proofreading DNA polymerases (and mixtures of proofreading and non-proofreading polymerases) produce primarily blunt-ended PCR products. The BigEasy Long PCR Kit with the ultra high-stability, linear pJAZZ®-OK Blunt vector is for cloning long PCR products from these enzymes (Figure 2).

Non-proofreading PCR polymerases (Taq, Tfl, Tth, etc.)

The BigEasy Long PCR Kit with the ultra high stability, linear pJAZZ-OK GC vector utilizes a newly discovered attribute of non-proofreading DNA polymerases: these enzymes can add a single 3'-G residue to the ends of DNA molecules. The 3'-G tailing occurs during PCR with a non-proofreading polymerase. The pJAZZ-OK GC vector (for non-proofreaders) contains a single 3'-C overhang, which is compatible with the 3'-G overhang generated by the non-proofreading polymerase (Figure 2). The unique combination of a G-tailed insert and C-tailed vector is the basis for GC Cloning (patents pending).

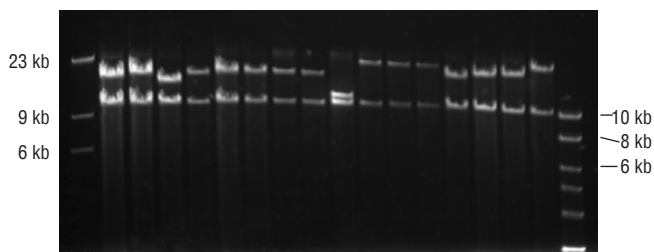


Figure 1. Large PCR products (15-25 kb) cloned into BigEasy Long PCR Cloning Kit. Bands from left vector arms are at 10 kb. The bands from the right vector arm ran off the gel.

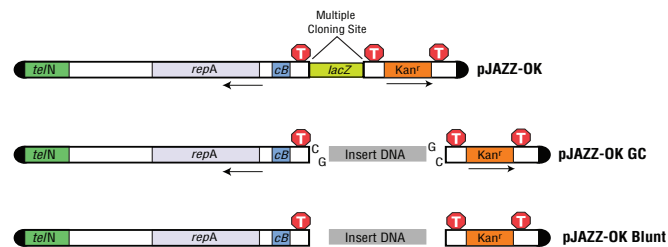


Figure 2. Processing and ligation of the pJAZZ-OK GC and Blunt vectors. Top panel – Before processing, the pJAZZ-OK vector (12.9 kb) contains a *lacZ* stuffer fragment. Middle panel – After processing, the vector has 3'-C tails and dephosphorylated ends. The left arm is 10 kb and the right arm is 2.2 kb. G-tailed insert DNA is ligated to vector arms. Or Bottom panel – after processing, the vector has blunt dephosphorylated ends and blunt insert DNA is ligated to vector arms. *telN*, protelomerase gene; *repA*, replication factor and origin of replication; *cBI*, regulator of copy number; *Kanr*, kanamycin resistance gene. Approximate positions of transcription terminators (T) are indicated.

Note: Lucigen's BigEasy-TSA™ Electrocompetent Cells must be used for high efficiency transformation and copy number induction with pJAZZ vectors. These cells yield $\geq 4 \times 10^{10}$ cfu/ μ g of supercoiled control plasmid.

BigEasy-TSA Genotype: F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80 *dlacZ* Δ M15 Δ *lacX74* *endA1* *recA1* *araD139* Δ (*ara, leu*)7697 *galJ* *gaK* *rpsL* *nupG* λ - *tonA* *Amp*^r *sopAB* *teN* *antA*

ORDER INFORMATION

Each BigEasy Long PCR Cloning Kit contains: pre-cut pJAZZ-OK vector (Blunt or C-tailed overhang), CloneSmart DNA Ligase, CloneDirect™ 10X Ligation Buffer, PCR Control Template and Primers, Sequencing Primers, T4 Polynucleotide Kinase, 10X Primer Kinase Buffer, BigEasy-TSA Electrocompetent Cells, Positive Control Plasmid, Recovery Medium, Arabinose Induction Solution, and complete protocols.

Product	Size	Cat. No.	Price
BigEasy Long PCR Cloning Kit (pJAZZ-OK Blunt) For proofreaders	5 rxns	43054-1	\$291
	10 rxns	43054-2	\$495
	20 rxns	43054-3	\$887
BigEasy Long PCR Cloning Kit (pJAZZ-OK GC) For non-proofreaders	5 rxns	43066-1	\$291
	10 rxns	43066-2	\$495
	20 rxns	43066-3	\$887

CloneSmart Technology is covered by U.S. Patent 6,709,861, and other patents issued or pending in the U.S. and other countries and assigned to Lucigen. Inducible Copy Amplification is covered by U.S. Patent 5,874,259, and other patents issued or pending in the U.S. and other countries and licensed to Lucigen. Vent is a trademark of New England Biolabs, Inc.

PyroPhage® 3173 DNA Polymerase

Tom Schoenfeld, Vinay Dhodda, David Mead

Lucigen Corporation

Introduction

DNA polymerases are critical components in some of the most widely used methods in life sciences. Virtually all conventional and newly emerging methods of DNA amplification, sequencing, and SNP detection use DNA polymerases in at least one stage. Even DNA polymerases considered to be “low fidelity” are extraordinarily precise in inserting the correct nucleotide. However, most methods of amplification and analysis are also limited by the available DNA polymerases.

Bacteriophage polymerases are much more diverse than microbial enzymes and have remarkable biochemical characteristics that have enabled several moderate-temperature applications. T7 DNA polymerase (Sequenase®) represented a huge leap forward in reliability, quality, and sequencing read length. This improvement was attributed to its extraordinary processivity and efficient incorporation of chain-terminating nucleotide analogs. No enzyme has improved on T7 DNA polymerase for isothermal DNA sequencing. The unparalleled processivity of phi29 DNA polymerase has enabled remarkable isothermal amplification of plasmid templates and whole genomes. T4 DNA polymerase has one of the highest fidelities of any DNA polymerase. Retroviral reverse transcriptases are indispensable in research into transcription, RNA viruses, and other areas in which RNA may be the template.

Many of the most important amplification and thermocycled sequencing applications rely on thermostable DNA polymerases. The compelling attributes of phage DNA polymerases would improve some of these applications, but unfortunately no thermostable phage DNA polymerases have been available. Despite decades of effort and development of hundreds of enzymes, all of the commercially available thermostable DNA polymerase fall into one of two groups of relatively high similarity (bacterial and archaeal). While these enzymes have been extraordinarily useful, significant improvements will require a new source of thermostable enzymes.

Traditional cultivation-based methods have not yielded thermophilic phage DNA polymerases. We used a non-traditional approach of screening metagenomic libraries constructed from phage isolated from hot springs in Yellowstone National Park (Figure 1). In collaboration with DOE's Joint Genome Institute, we sequenced nearly 30,000 clones, resulting in the identification of hundreds of apparent DNA polymerase homologs, 59 of which appear full length. Ten of these have been expressed to produce thermostable DNA polymerases. Of these, clone 3173 has been developed as the first PyroPhage DNA polymerase.

PyroPhage 3173 DNA Polymerase (patent pending) is the first thermostable enzyme to offer the superior DNA replication performance of a phage replicase. As expected, this enzyme has important advantages in template utilization, fidelity, and overall performance compared to previously available microbial enzymes.

Its thermostability allows thermocycling methods, including PCR. Its potent strand-displacing activity allows for isothermal DNA synthesis through double-stranded DNA. The enzyme initiates efficiently at nicks, allowing amplification to be initiated either with primers or at enzymatically-introduced nicks. PyroPhage 3173 DNA Polymerase also has an efficient reverse transcription activity. The enzyme can perform single-tube, single-enzyme reverse-transcription PCR of RNA transcripts. The potent 3'-5' exonuclease (proofreading) activity of the Wild Type enzyme results in the highest fidelity available in a PCR enzyme (see Table 1, next page).



Figure 1. “Fortress” Hot Spring in Yellowstone National Park. Lucigen is using its novel cloning technologies to identify and isolate new enzymes from rare, previously inaccessible hyperthermophilic microorganisms that inhabit the water column of such hot springs.

PyroPhage® 3173 Properties

Table 1. Established characteristics

PyroPhage 3173 DNA Polymerase	Wild Type	Exo Minus
3'-5' exonuclease	strong	none
5'-3' exonuclease	none	none
Strand displacement	strong	strong
Extension from nicks	strong	strong
Thermostability (T _{1/2} @95° in PCR buffer)	10 min.	10 min.
K _m dNTPs	40 μM	40 μM
K _m DNA	5.3 nM	5.3 nM
Processivity	not determined	47 nt
Fidelity	8 X 10 ⁴	1.5 X 10 ⁴
3' ends of amplicons	blunt	single base A and G overhangs
Template	DNA or RNA	DNA or RNA

PyroPhage 3173 in PCR amplification

PyroPhage 3173 DNA Polymerase is much more effective than current PCR enzymes in amplifying certain difficult templates (Figure 2). PyroPhage 3173 DNA Polymerase effectively amplifies most templates of up to 4 kb.

Fidelity

PyroPhage 3173 DNA Polymerase (Wild Type) demonstrates strong proofreading activity, resulting in extremely high fidelity (Figure 3). Because of this very active nuclease activity, use of phosphorothioate primers is recommended when using the Wild Type enzyme for DNA amplification. When fidelity is not critical, the Exonuclease Minus enzyme is generally easier to use.

Amplification efficiency

With certain primer/template sets, PCR with PyroPhage 3173 DNA Polymerase results in higher product yields in fewer cycles compared to available enzymes (Figure 4, next page).

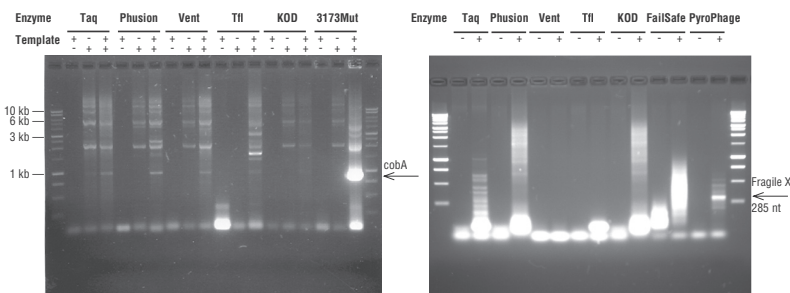


Figure 2. PCR amplification of difficult templates. PCR amplification of the *Bacillus cobA* gene (left) and the human Fragile X gene (right) using PyroPhage 3173 (Exo Minus) or the indicated DNA polymerase.

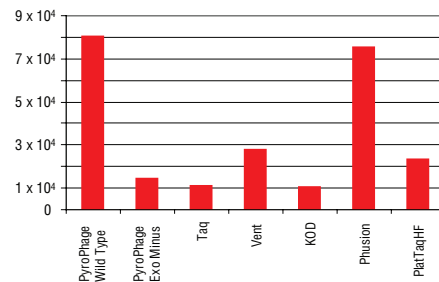
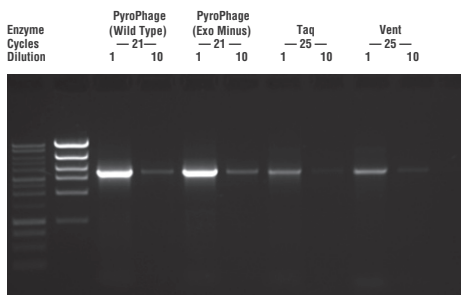


Figure 3. Fidelity measurements, shown as the ratio of correct to incorrect nucleotide incorporations, are based on the *LacI*⁺ forward mutation assay. PyroPhage 3173 DNA Polymerase (Wild Type & Exo Minus) was compared to various commercially available enzymes.



PyroPhage 3173	Cycles	Yield	Efficiency
Wild Type	21	18 µg	43%
Exo Minus	21	18 µg	43%
Taq	25	3 µg	26%
Vent	25	3 µg	26%

Figure 4. PCR amplification efficiency of PyroPhage 3173 DNA Polymerases was compared to that of Taq and Vent DNA polymerases using the fidelity assay template. Reaction products formed after the indicated number of cycles were quantified by densitometry of the gel bands. Cycle numbers for the PCRs are shown. Efficiency was calculated by published methods.

Isothermal amplification

PyroPhage® 3173 DNA Polymerase (Wild Type & Exo Minus) is effective in isothermal replication of linear, circular, or supercoiled DNA, either single- or double-stranded, with or without primers. Using PyroPhage 3173 DNA Polymerase on enzymatically nicked genomic or plasmid DNA yields a 10,000-fold isothermal amplification in 2 hours (Figure 5).

Reverse Transcribed (RT)-PCR

The Exo Minus enzyme can also be used for single-tube, single-enzyme, single-buffer reverse transcription PCR (RT-PCR) (Figure 6).

Convenient and easy to use

PyroPhage 3173 DNA Polymerase includes PyroPhage 3173 2X PCR Buffer containing magnesium, PyroPhage 3173 10X Isothermal Buffer, PCR Control + Primers, and complete protocols. For PCR, the PyroPhage 3173 DNA Polymerase can be used in essentially the same way as Taq DNA polymerase.

Usage Information

Certain primer/template sets are more effectively amplified by PyroPhage 3173 DNA Polymerase. Amplification of other apparently similar primer/template sets can be problematic. The basis of this inconsistency is not understood. Because of this variability, no predictions can be made on the effectiveness of PyroPhage 3173 DNA Polymerase in amplifying a given primer/template set. Variability may be significantly reduced by use of alternative primer sets or common PCR additives, such as betaine or ectoine.

References

1. Arezi, B., Xing, W., Sorge, J. A., Hogrefe, H. H. (2003). Amplification efficiency of thermostable DNA polymerases. *Anal Biochem.* **321**(2), 226.
2. Hogrefe HH, Cline J, Lovejoy AE, Nielson KB. (2001). DNA polymerases from hyperthermophiles. *Methods Enzymol.* **334**, 91.

ORDER INFORMATION

PyroPhage 3173 DNA Polymerase (Wild Type and Exo Minus) is provided with PyroPhage 3173 2X PCR Buffer, PyroPhage 3173 10X Isothermal Buffer, and PCR Control + Primers. 2X PCR Buffer and 10X Isothermal Buffer are also available separately. The 2,500 U and 5,000 U package sizes of PyroPhage 3173 DNA Polymerase are provided as multiples of the 500 U package size.

Product	Size	Cat. No.	Price
PyroPhage 3173 DNA Polymerase			
Wild Type	500 U	30051-1	\$97
	2,500 U	30051-2	\$375
	5,000 U	30051-3	\$598
Exo Minus	500 U	30053-1	\$97
	2,500 U	30053-2	\$375
	5,000 U	30053-3	\$598
PyroPhage 3173 10X Isothermal Buffer, 1,000 rxns	5 ml	30071-1	\$55
PyroPhage 3173 2X PCR Buffer, 500 rxns	12.5 ml	30072-1	\$89

The PyroPhage DNA Polymerases and methods of using the PyroPhage DNA Polymerases are covered by pending patents assigned to Lucigen Corporation. Lucigen does not encourage or support the unauthorized or unlicensed use of the PCR process, reverse transcription PCR process, or isothermal DNA synthesis. It is the sole responsibility of the buyer to ensure that use of the product does not infringe the patent rights of third parties.

Vent is a trademark of New England Biolabs, Inc. Sequenase is a trademark of GE Healthcare, Inc. Phusion is a trademark of Finnzymes Oy. FailSafe is a trademark of Epicentre Biotechnologies.

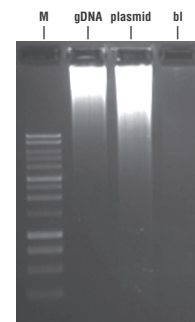


Figure 5. Primer-free isothermal amplification. PyroPhage 3173 Exo Minus DNA Polymerase was used to amplify *E. coli* gDNA and a supercoiled plasmid. Reactions were incubated two hours at 55°C. In the absence of added template (bl), no product is seen.

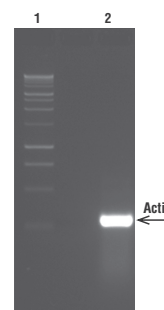


Figure 6. Single-tube, single-enzyme RT-PCR with PyroPhage 3173 DNA Polymerase (Exo Minus). Mouse liver RNA was used as a template with actin-specific primers. A single band was produced at the expected size of 283 bp.

NEW Competent Cells Strains & Custom Competent Cells

Table 1. Characteristics of cloning and expression strains.

Cloning Strains

Strain Attribute	10G (DH10B)	5-alpha (DH5 α)
T1 Phage Resistant	✓	
Blue/White Screen	✓	✓
<i>lacIq</i> Repressor		
Endonuclease I Deficient	✓	✓
Methylated DNA Cloning	✓	
Express M13 Phage (F')		
RecA Deficient	✓	✓
Expression Strain		
Strain Attribute	TG1	
Blue/White Screen	✓	
<i>lacIq</i> Repressor	✓	
Protease Deficient		
Methylated DNA Cloning	✓	
Express M13 Phage (F')	✓	

Table 2. Examples of Custom competent cell transformation efficiencies

Starting Sample	Resulting Transformation Efficiency (cfu/ μ g)
Strain "A"	3.0×10^{10}
Strain "B"	2.4×10^{10}
Strain "C"	1.8×10^{10}
Strain "D"	2.5×10^9

***E. coli*[®] 5-alpha Chemically Competent Cells** are versatile and useful in a wide variety of applications, including routine cloning, subcloning, and plasmid isolation with or without blue/white screening. The efficiency of transformation is $\geq 1 \times 10^8$ cfu/ μ g pUC19 DNA. These economical cells are equivalent to DH5 α and are packaged as DUOs (two transformations per tube) or in 96-well plates (4 \times 24 well sections). See Table 1 for Attributes and Applications.

TG1 Electrocompetent Cells deliver $\geq 2 \times 10^{10}$ cfu/ μ g of DNA, at least twice the transformation efficiency of TG1 cells from other vendors. TG1 cells are the cells of choice for phage display protein expression. They are also suitable for M13 phage work, general cloning, blue/white screening, and protein expression. TG1 cells are packaged as DUOs.

In addition to these strains, ***E. coli* 10G Chemically Competent Cells are now available in 96-well plate format** with 4 X 24-well sections. *E. coli* 10G cells are equivalent to DH10B, except they have been optimized for high efficiency transformation ($\geq 1 \times 10^8$ cfu/ μ g pUC19 DNA). The chemically competent cells in microplates are perfect for high throughput applications. They give high yield and high quality plasmid DNA due to the *recA* and *endA1* mutations. *E. coli* 10G cells contain the *mcr* and *mrr* mutations, allowing methylated genomic DNA that has been isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. See Table 1 for Attributes and Applications. Electrocompetent *E. coli* 10G cells are also available with transformation efficiencies up to $\geq 4 \times 10^{10}$ cfu/ μ g.

Genotypes:

***E. coli* 5-alpha (DH5 α)**

fhuA2 Δ (*argF-lacZ*)U169 *phoA glnV44* Φ 80 Δ (*lacZ*)M15 *gyrA96 recA1 relA1 endA1 thi-1 hsdR17*

TG1

supE thi-1 Δ (*lac-proAB*) Δ (*mcrB-hsdSM*)5(rK- mK-) [F' *traD36 proAB lacIq* Δ M15]

***E. coli* 10G (DH10B)**

F - *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1 recA1* Φ 80d*lacZ* Δ M15 Δ *lacX74 araD139* Δ (*ara,leu*)7697 *galJ galK rpsL nupG* λ - *tonA*

Transformance™ Custom Competent Cell Service

Need to make your cells highly competent for transformation? Take advantage of Lucigen's unrivalled expertise and proprietary methods of competent cell manufacturing. Lucigen's capabilities are proven by our success in developing cells with the highest transformation efficiencies available: *E. coli* ELITE ($\geq 2 \times 10^{10}$ cfu/ μ g DNA) and SUPREME ($\geq 4 \times 10^{10}$ cfu/ μ g DNA) Competent Cells.

Lucigen's Transformance Custom Competent Cell Service offers

- Construction of chemically competent or electrocompetent cells
- High transformation efficiencies
- Fast turnaround
- Quality control performance testing
- Large or small quantities

Excellent results are obtained with a wide variety of *E. coli* strains (see Table 2 for examples). Lucigen has also increased transformation efficiencies of low competency strains by 10-100 fold in just a few days. Contact Lucigen for a free quote.

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Competent Cells include Control DNA and Recovery Medium.

Product	Size	Cat. No.	Price
<i>E. coli</i> 5-alpha Chemically Competent Cells (DUOs)	12 rxns	60602-1	\$91
	24 rxns	60602-2	\$158
<i>E. coli</i> 5-alpha Chemically Competent Cells (96-well)	1 plate	60696-1	\$335
	4 plates	60696-4	\$1179
TG1 Electrocompetent Cells (DUOs)	12 rxns	60502-1	\$129
	24 rxns	60502-2	\$223
<i>E. coli</i> 10G Chemically Competent Cells (96-well)*	1 plate	60096-1	\$335
	4 plates	60096-4	\$1179

*PLEASE NOTE: *E. coli* 10G Electrocompetent Cells are available in BTX 25-well electroporation strips and 96-well electroporation plates. Contact Lucigen for availability and pricing.

DH10B and DH5 α are trademarks of Invitrogen Corporation.

NEW CopyRight® v2.0 BAC Cloning Kits

- High efficiency BAC library construction
- General cloning of any DNA

Lucigen's new CopyRight v2.0 vectors and BAC-Optimized Replicator™ v2.0 Electrocompetent Cells combine the revolutionary, patented CloneSmart® transcription-free technology, inducible copy amplification, and a *lacZ-sacB* stuffer fragment that eliminates uncut vector from recombinants. The result is unprecedented accuracy, reliability, and efficiency in BAC cloning and library construction. Unlike other BAC cloning systems, Lucigen's CloneSmart technology assures that even "unclonable" inserts are stably maintained. Sequences are not lost or rearranged in the cloning process. A simple "on command" amplification system built into the CopyRight v2.0 vectors and BAC-Optimized Replicator v2.0 Electrocompetent Cells gives a 20-50 fold increase in copy number for high yields and easy purification (Wild, J., *et al.* (2002). *Genome Res.* 12,1434).

CopyRight pSMART® BAC vectors

The CopyRight pSMART BAC vector (Figure 1) incorporates transcription-free cloning for the highest stability BAC cloning. The vector is supplied pre-cut at a choice of BamHI, EcoRI, or HindIII sites, with dephosphorylated ends. Insert DNAs with 5'-phosphorylated ends are ligated to the CopyRight vector and transformed into Lucigen's BAC-Optimized Replicator v2.0 Electrocompetent Cells.

The new pSMART BAC vectors have a *lacZ-sacB* stuffer region, allowing uncut vector to be detected by blue/white screening and selected against by plating on sucrose. This feature completely eliminates uncut vector background, improving the efficiency of colony picking. However, unlike other cloning vectors, the promoter and the coding sequence of the stuffer fragment are completely removed during processing. This design prevents active expression of the insert DNA by the *lacZ* or *sacB* promoter, contributing greatly to plasmid stability, especially for inserts containing toxic coding sequences, strong secondary structure, or other deleterious features. It also prevents selection against clones that contain promoters, because the coding regions of the *lacZ* and *sacB* genes have been removed.

Advantages

Greater cloning fidelity. Transcription-free, single-copy cloning minimizes cloning bias and sequencing gaps. Clone even "unclonable" DNAs such as centromeric regions, toxic genes, strong promoters, repeats, hairpins, and AT-rich sequences with the pSMART BAC transcription-free vector (see Figure 1).

Maximum insert stability. CloneSmart transcription terminators in the pSMART BAC vector minimize deletions and rearrangements.

Higher throughput. Zero background eliminates wasted time in colony picking and analysis.

Inducible Copy Number. Increase DNA yields 20-50 fold with simple chemical induction.

Fast and easy cloning protocol. Simply add your DNA, incubate, and transform.

Higher cloning and sequencing efficiencies. Ultra-high efficiency BAC-Optimized Replicator v2.0 Electrocompetent Cells ($\geq 4 \times 10^{10}$ cfu/ μ g pUC 19) maximize recombinant yields.

Better Value. CopyRight Kits offer superior performance for BAC cloning (Figure 2) at a much better price than other available kits.

CopyRight Kits are also available with pEZ BAC vector for blue/white screening. Please see the Lucigen website (www.lucigen.com) for more information.

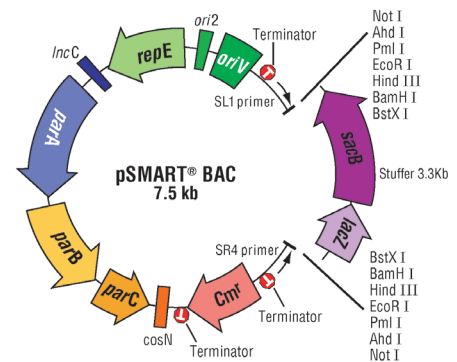


Figure 1. Diagram of CopyRight vector pSMART BAC. *ori2*, *repE*, *IncC* - origin of replication (single copy); *oriV* - inducible origin of replication; *parA,B,C* - partition genes; *Cm^r* - chloramphenicol-resistance gene; *cosN* - lambda packaging signal; T - CloneSmart transcription terminators; *sacB*, sucrose gene; *lacZ*, alpha peptide portion of the β -galactosidase gene. Approximate positions of sequencing primers and transcription terminators are indicated.

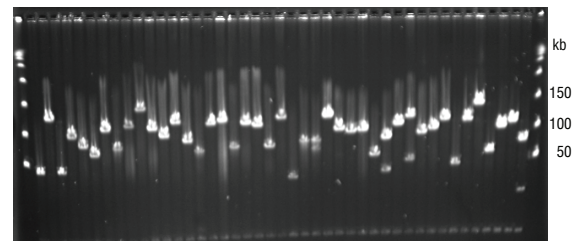


Figure 2. *Arabidopsis* Random Shear BAC Library in the pSMART BAC vector. 7,680 clones were obtained with an average insert size of 105 kb and ~5X genome coverage.

CopyRight® v2.0 BAC Cloning Kits *continued*

ORDER INFORMATION

Each CopyRight v2.0 BAC Cloning Kit contains: pre-cut vector (pSMART® BAC with a choice of BamHI or EcoRI or HindIII sites), CopyRight DNA Ligase, CopyRight 5X Ligation Buffer, Positive Control Insert DNA, Sequencing Primers, BAC-Optimized Replicator™ v2.0 Electrocompetent Cells in DUO packaging (2 transformations per tube), Positive Control Insert DNA, Recovery Medium, Arabinose Induction Solution, and complete protocols.

Product	Size	Cat. No.	Price
CopyRight v2.0 BAC Cloning Kit, pSMART BAC BamHI w/BAC-Optimized Replicator v2.0 Electrocompetent Cells	10 rxns	42030-1	\$435
	20 rxns	42030-2	\$776
CopyRight v2.0 BAC Cloning Kit, pSMART BAC EcoRI w/BAC-Optimized Replicator v2.0 Electrocompetent Cells	10 rxns	42031-1	\$435
	20 rxns	42031-2	\$776
CopyRight v2.0 BAC Cloning Kit, pSMART BAC HindIII w/BAC-Optimized Replicator v2.0 Electrocompetent Cells	10 rxns	42032-1	\$435
	20 rxns	42032-2	\$776

CloneSmart Technology is covered by U.S. Patent 6,709,861, and other patents issued or pending in the U.S. and other countries and assigned to Lucigen. Inducible Copy Amplification is covered by U.S. Patent 5,874,259, and other patents issued or pending in the U.S. and other countries and licensed to Lucigen.

\$500 Reward!

If you develop a novel application or would like to share technical insights related to our products, we would like to hear from you. Please email us (lucigen@lucigen.com) with comments or ideas for articles. We offer a \$500 cash honorarium for articles published in **eLucidations**.

NEW ChimeraFree™ Cloning Kits

The only way to ensure one, and only one, insert per vector

The CloneSmart® ChimeraFree Cloning Kits (patent pending) are designed for cloning one, and only one, insert per vector, saving time and work in downstream analyses. These Kits incorporate Lucigen's exclusive GC Cloning technology and unique DNA end-blocking chemistry. A single 3'-G overhang is added to blunt-ended DNA using the included PyroPhage® DNA Polymerase. The G-tailed DNA is ligated to a single 3'-C overhang on the appropriate cloning vector (Figure 1). The presence of the blocked G tail prevents ligation of multiple inserts, dramatically reducing chimeras (Figure 2). These vectors also incorporate Lucigen's exclusive CloneSmart® transcription-free cloning technology to reduce bias and maximize cloning efficiency.

All ChimeraFree vectors are supplied pre-cut with dephosphorylated 5' ends and single 3'-C overhangs. The copy number of the high copy pSMART® GC HK and pOT GC vectors is similar to that of pUC plasmids (~300 copies/cell); the copy number of the low copy pSMARTGC LK vector is similar to that of pBR322 (~20/cell).

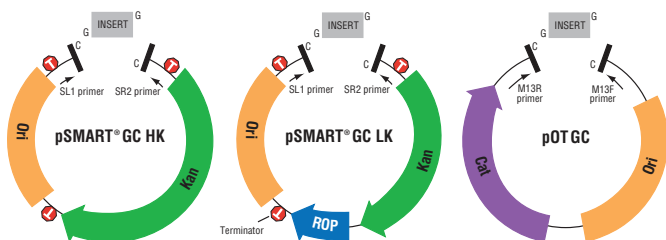


Figure 1. ChimeraFree vectors. Ori, origin of replication; Kan, Kanamycin-resistance gene; Cat, chloramphenicol-resistance gene; ROP, Repressor of primer (lowers plasmid copy number). *TerM*, protelomerase gene; *repA*, replication factor and origin of replication; *cB*, regulator of copy number; *Camr*, chloramphenicol resistance gene; *Kanr*, kanamycin resistance gene. Approximate positions of sequencing primers and CloneSmart transcription terminators (T) are indicated.

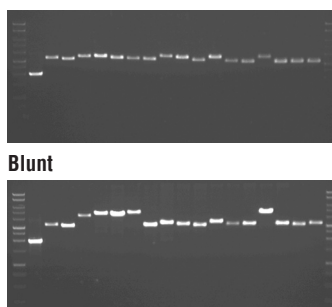
ORDER INFORMATION

CloneSmart ChimeraFree Cloning Kits contain: Vector Premix, CloneSmart DNA Ligase, Positive Control Insert DNA, Sequencing Primers, PyroPhage DNA Polymerase Tailing Enzyme and Buffer, DNATerminator® End Repair Enzyme and Buffer, a choice of *E. coli* ELITE 10G or 10GF' ($\geq 2 \times 10^{10}$ cfu/ μ g) or 10G SUPREME ($\geq 4 \times 10^{10}$ cfu/ μ g) Electrocompetent Cells, or 10G Chemically Competent Cells ($\geq 1 \times 10^9$ cfu/ μ g), as indicated, packaged in one (SOLD) or two (DUO) transformations per tube, with Recovery Medium and Positive Transformation Control, and complete protocols. Kits can also be used with any *E. coli* chemically competent or electro-competent cells.

CloneSmart Technology is covered by U.S. Patent 6,709,861, and other patents issued or pending in the U.S. and other countries and assigned to Lucigen. Inducible Copy Amplification is covered by U.S. Patent 5,874,259, and other patents issued or pending in the U.S. and other countries and licensed to Lucigen.

This product or portions thereof is manufactured and sold under license from GE Healthcare under U.S. Patent 5,614,365 and other pending and foreign patent applications.

ChimeraFree



ChimeraFree Cloning

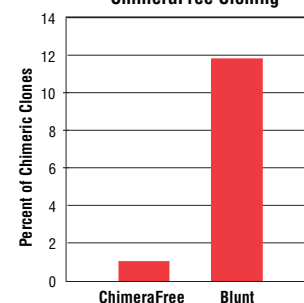


Figure 2. Cloning single copy inserts in ChimeraFree vectors (upper panel) vs. blunt vectors (lower panel). ChimeraFree vectors greatly reduce the presence of cloned chimeric inserts (bar graph). A total of 102 clones were analyzed.

Product	Size	Cat. No.	Price
CloneSmart ChimeraFree Cloning Kit (pSMART GC HK) w/10G ELITE Electrocompetent Cells	10 rxns	41140-1	\$316
	20 rxns	41140-2	\$538
CloneSmart ChimeraFree Cloning Kit (pSMART GC HK) w/10G SUPREME Electrocompetent Cells	10 rxns	41141-1	\$388
	20 rxns	41141-2	\$683
CloneSmart ChimeraFree Cloning Kit (pSMART GC HK) w/10G Chemically Competent Cells	10 rxns	41142-1	\$274
	20 rxns	41142-2	\$465
CloneSmart ChimeraFree Cloning Kit (pSMART GC LK) w/10G ELITE Electrocompetent Cells	10 rxns	41150-1	\$316
	20 rxns	41150-2	\$538
CloneSmart ChimeraFree Cloning Kit (pSMART GC LK) w/10G SUPREME Electrocompetent Cells	10 rxns	41151-1	\$388
	20 rxns	41151-2	\$683
CloneSmart ChimeraFree Cloning Kit (pSMART GC LK) w/10G Chemically Competent Cells	10 rxns	41152-1	\$274
	20 rxns	41152-2	\$465
CloneSmart ChimeraFree Cloning Kit (pOT GC) w/10G ELITE Electrocompetent Cells	10 rxns	41160-1	\$316
	20 rxns	41160-2	\$538
CloneSmart ChimeraFree Cloning Kit (pOT GC) w/10G SUPREME Electrocompetent Cells	10 rxns	41161-1	\$274
	20 rxns	41161-2	\$683
CloneSmart ChimeraFree Cloning Kit (pOT GC) w/10G Chemically Competent Cells	10 rxns	41162-1	\$274
	20 rxns	41162-2	\$465

New Custom Libraries & Sequencing Services

Lucigen specializes in creating libraries from difficult-to-clone genomes and nanogram amounts of DNA. We are the only provider of truly unbiased plasmid, fosmid, large insert, or BAC libraries with high complexity, low background, and dramatically reduced gaps. Lucigen also provides custom competent cells using our proprietary *E. coli*[®] technology for high transformation efficiencies (see p. 6 of this *eLucidations* issue).

Lucigen now introduces high throughput colony picking and sequencing, to complete our “one-stop shop” for custom libraries.

Custom Library Sequencing

We offer high throughput (96-well) library sequencing as an economical, fast-turnaround custom service. Library sequencing is done using ABI 3730xl sequencers. This fully integrated library construction and sequencing service offers unique benefits.

- **Perfect for shotgun BAC, metagenomic, or small genomic libraries.**
Simply provide us with your BAC, fosmid, genomic, or phage DNA. We construct the library, sequence it, and provide you with the sequence and the clones.
- **Chimera-free libraries!**
Lucigen’s exclusive ChimeraFree™ technology, based on a novel DNA end-blocking chemistry (patent pending), ensures one, and only one, insert per vector, simplifying sequencing and downstream applications. ChimeraFree cloning is described on p. 9 of this *eLucidations* issue.
- **Dramatically reduced finishing costs.**
Transcription-free, high stability vectors with Lucigen’s patented CloneSmart® technology prevent lost genes and sequence deletions or rearrangements, even with AT-rich inserts, toxic genes, nucleotide repeats, and other difficult-to-clone DNAs.
- **Unequaled expertise in library construction and sequencing.**
Technologies pioneered by Lucigen – including Random Shear BAC library construction, NanoClone® library construction from trace amounts of DNA, and the linear pJAZZ® vector – have greatly improved the quality and usefulness of genomic libraries. We have constructed hundreds of shotgun, fosmid, and BAC libraries for satisfied customers worldwide. Our high throughput sequencing group has many years of automated sequencing experience, including whole genome shotgun libraries with large and small inserts and transposon sequencing. The head of our sequencing group previously managed the University of Wisconsin-Madison, Genetics Sequencing and Genomics Services Center (GSGSC).

Automated Colony Picking, Re-arraying, & Duplicating

Lucigen offers custom colony picking, re-arraying, and duplicating, either as a stand-alone service or as part of our library construction services (Random Shear or conventional BAC, fosmid, GapFree™ shotgun, and NanoClone® libraries). Colony picking is an automated method for transferring colonies from a solid to liquid medium in 96- or

384-well plates. From directly transformed clones or frozen glycerol stocks, colonies are first grown on an agar tray and then robotically picked into plates for further growth and storage. Lucigen’s Genetix QPix2 bench-top colony picker (Figure 1) uses a camera to assess colony roundedness, size, and color before picking and transferring to the 384-well plate. BAC libraries are delivered as clones frozen in 384-well plates. The average BAC insert size is >100 kb for random-sheared DNA or >150 kb for conventional BAC clones.



Figure 1. Genetix QPix2

Gridding & High Density Colony Filters

To facilitate screening large numbers of DNA clones, Lucigen can also robotically print viable bacterial cells from 96 or 384 well plates onto high-density, 22.2 × 22.2 cm nylon filters (Table 1 and Figure 2). Over 36,000 individual colonies can be spotted in duplicate onto each filter. After overnight growth of the clones, the DNA from each clone is permanently fixed to a known position on the filter, allowing rapid screening using standard DNA hybridization procedures. Positive clones can be identified and retrieved from the original plates. Each nylon filter can be screened 3-5 times. Lucigen provides 22.2 cm × 22.2 cm or 8 cm × 12 cm filters printed at a choice of two densities. All clones are printed in duplicate. We can also provide customized filters to meet your needs.

Table 1. Types of High Density Colony Filters

Filter Type	Filter Size	# of Fields	Pattern	Spotting	# of Clones	# of Spots
1 *	22.2cm×22.2cm	6	4×4	Double	18,432	36,864
2	22.2cm×22.2cm	6	3×3	Double	9,216	18,432
3	8cm×12cm	1	4×4	Double	3,072	6,144
4	8cm×12cm	1	3×3	Double	1,536	3,072

*Lucigen can meet special needs: for example, different spotting patterns, single spotting, etc.

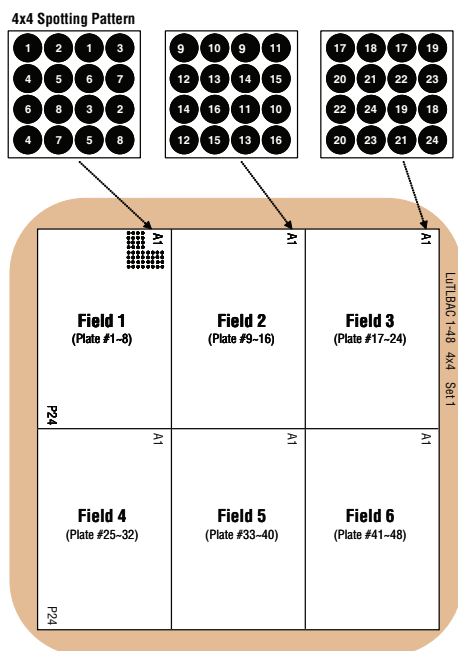


Figure 2. Lucigen gridded filter (4x4). The membrane (filter) is divided into 6 fields. Each field contains 384 squares. The 384 squares represent the row and column identification of the BAC clone. Within each square there are 16 positions where 8 clones are spotted in duplicate. The pattern of the spotted clones will generate the plate address of the BAC. The numbers in the spotting pattern indicate the plate numbers. Each grid corresponds to each well of the original 384-well plates, starting from A1 from the left-top corner. Each Lucigen filter includes the identification code of every clone within the grid.

Recent Citations of Lucigen Products

CloneSmart® Cloning Kits

Umphress, S.M. (2008). *Lumbriculus variegatus* mitochondria DNA (mtDNA) analysis. *FASEB J.* **22**, 193.

Schoenfeld, T., Patterson, M., Richardson, P.M., Wommack, E., Young, M., and Mead, D.A. (2008). Assembly of viral metagenomes from Yellowstone hot springs. *Appl. Envir. Microbiol.* **74**, 4164.

Osborne, R.J., and Thornton, C.A. (2008). Cell-free cloning of highly expanded CTG repeats by amplification of dimerized expanded repeats. *Nucleic Acids Res.* **36**, e24.

Riemann, L., Leitert, C., Pommier, T., Simu, K., Holmfeldt, K., Larsson, U., and Hagström, Å. (2008). The native bacterioplankton community in the central Baltic Sea is influenced by freshwater bacterial species. *Appl. Envir. Microbiol.* **74**, 503.

E. cloni® Competent Cells for Cloning

Bidlingmaier, S., and Liu, B. (2007). Interrogating yeast surface-displayed human proteome to identify small molecule-binding proteins. *Mol. Cell. Proteomics.* **6**, 2012.

Pradhan, A., Lambert, Q.T., and Reuther, G.W. (2007). Transformation of hematopoietic cells and activation of JAK2-V617F by IL-27R, a component of a heterodimeric type I cytokine receptor. *PNAS.* **104**, 18502.

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E. cloni® BL21(DE3) Competent Cells for Protein Expression

Opperman, D.J., Piater, L.A., and van Heerden, E. (2008). A novel chromate reductase from *Thermus scotoductus* SA-01 related to old yellow enzyme. *J. Bacteriol.* **190**, 3076.

EconoTaq® DNA Polymerase

Kan, R., Sun, X., Kolas, N.K., Avdievich, E., Kneitz, B., Edelmann, W., and Cohen, P.E. (2008). Comparative analysis of meiotic progression in female mice bearing mutations in genes of the DNA mismatch repair pathway. *Biol. Reprod.* **78**, 462.

DNATerminator® End Repair Kit

Snyder, M., Huang, X., and Zhang, J. (2008). Identification of novel direct *Stat3* target genes for control of growth and differentiation. *J. Biol. Chem.* **283**, 3791.



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