

CLONEPLEX[®] -AK

Library Construction Kit

IMPORTANT!
-86°C and -20°C Storage Required
Immediately Upon Receipt

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Technical Support

Lucigen is dedicated to the success and satisfaction of our customers. Our products are tested to assure they perform as specified when used according to our recommendations. It is imperative that the reagents supplied by the user, especially the DNA targets to be cloned, are of the highest quality. Please follow the manual carefully and contact our technical service representatives if additional information is necessary. We encourage you to contact us with your comments regarding the performance of our products in your applications. Thank you.

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Components & Storage Conditions

The ClonePlex-AK Library Construction Kit is shipped on dry ice in two containers. The ligation components of the ClonePlex-AK Kits and the DNA Terminator components are shipped in container 1. These store at **-20°C**. The *E. coli*[®] Electrocompetent Cells are shipped in Container 2, which must be stored at **-86°C**. Additional DNA Terminator Kits (cat. #40035-1; 40035-2) and *E. coli* Supreme Electrocompetent Cells may be purchased separately.

Container 1: ClonePlex-AK Ligation Components.

Store at **-20°C**

ClonePlex-AK Library Construction Kit	Cat #40043-1 5 Reactions	Cat #40043-2 10 Reactions	Storage
4X ClonePlex-AK Vector Premix (ligation-ready pLEXX [™] -AK, ATP, buffer)	12.5 µl	2 x 12.5 µl	-20°C
Positive Control Insert DNA (500 ng/µl lambda <i>Hind</i> III)	5 µl	5 µl	-20°C
CloneSmart [™] DNA Ligase (2 U/µl)	10 µl	10 µl	-20°C
Positive Control Transformation Plasmid (pAKL)	10 µl	10 µl	-20°C
SL1 Primer (3.2 pmol/µl)	200 µl	200 µl	-20°C
SR2 Primer (3.2 pmol/µl)	200 µl	200 µl	-20°C
KanL Primer (3.2 pmol/µl)	200 µl	200 µl	-20°C
KanR Primer (3.2 pmol/µl)	200 µl	200 µl	-20°C
DNATerminator 5X End Repair Buffer	100 µl	100 µl	-20°C
DNATerminator End Repair Enzyme	25 µl	25 µl	-20°C

Container 2: *E. coli*[®] 10G Supreme Electrocompetent Cells.

Store at **-86°C**

	Cat #60080-1 12 rxns	Storage
<i>E. coli</i> [™] 10G Supreme Electrocompetent Cells	6 x 50 µl	-86°C
Positive Control Transformation Plasmid (1 ng/µl pUC19); 10 µl	---	-20° or -86°C
Recovery Medium; 12 mls	---	-20° or -86°C

Description

The ClonePlex-AK Library Construction Kit is a new system for high throughput cloning and sequencing. For the first time, two DNA fragments can be cloned simultaneously and independently into two sites of a single vector, allowing four sequencing reactions to be performed from each template. There is no need to screen for recombinant clones, as a selection scheme ensures insertion of exogenous DNA fragments into more than 99.9% of the cloning sites. By reducing the number of template purifications, the ClonePlex Kit improves cloning throughput two-fold relative to standard plasmid vectors and four-fold relative to phage M13mp18, saving time, labor, and reagent costs. Additional savings are realized by eliminating the costs and uncertainties of vector preparation and XGAL/IPTG screening.

Conventional plasmids, which contain a single cloning site, are limited to just one independent DNA insertion event per vector. In contrast, the pLEXX-AK[™] cloning vector supplied with the ClonePlex-AK Kit contains two dispersed blunt cloning sites, each flanked by two different antibiotic resistance genes (Figure 1). This arrangement of dispersed cloning sites and selectable markers allows for two independent DNA insertion events per plasmid. Unique sequences suitable for primer annealing also flank each insertion site. As a

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result, four independent sequencing reactions can be performed from each template, yielding up to 2,800 bases of data per clone. Thus, a single 96-well plate of pLEXX-AK recombinant clones provides over 250 kb of sequence, sufficient to sequence a cosmid with 5-fold redundancy or a 100 kb BAC vector with 2-fold redundancy. A ClonePlex ligation reaction using 500 ng of sheared DNA typically yields 30,000-70,000 duplex clones.

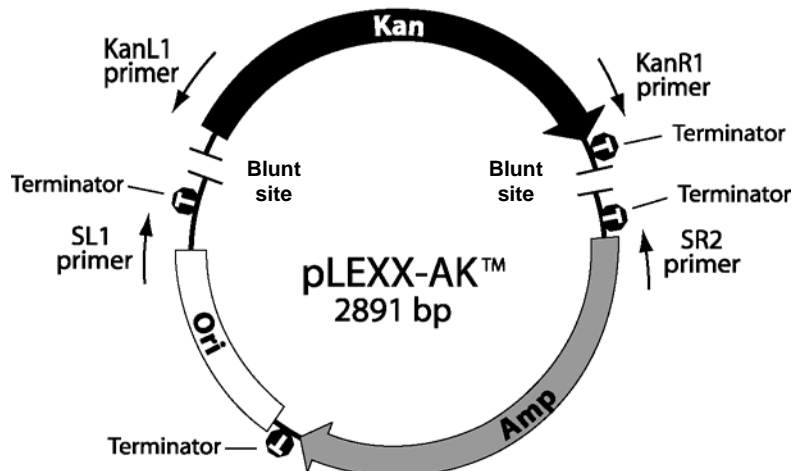


Figure 1. Diagram of the pLEXX-AK[™] duplex cloning vector.

The ultra-low background of empty vector in the ClonePlex system is unparalleled. When transformed into the supplied *E. coli*[®] electro-competent cells, over 99.9% of clones have inserts at both sites. Therefore, all colonies from a ClonePlex library can be grown for template purification, eliminating the need for any screening protocol. In contrast, conventional vectors utilizing the blue/white screen can generate a dense background of blue colonies. These vectors also produce ambiguous “light blue” colonies, which may contain inserts but are often discarded. The DNA contained in such clones is consequently thought to be “unclonable”, leading to gaps in sequence contigs.

The vector pLEXX-AK[™] also has been designed to reduce the cloning bias commonly found with standard plasmids by eliminating transcription both into and out of the insert DNA (Figure 1). In conventional plasmids, strong promoters are used to transcribe an indicator gene such as *lacZ* α or a negative selection gene such as *ccdB*. DNA cloned into these vectors can be lost due to plasmid instability caused by transcription of toxic coding sequences, strong secondary structure, or other deleterious features. The pLEXX-AK vector does not use a promoter or an indicator gene, so transcription across the insert is eliminated. Conventional plasmids can also be lost due to transcription initiating from inserts containing *E. coli*-like promoters, which can cause instability by inappropriately transcribing into essential regions of the vector. Strong transcription terminators flanking the cloning sites in pLEXX-AK block this transcription (Figure 1), eliminating another source of cloning bias and sequencing gaps.

The ClonePlex vector has been designed to reduce the growth of “satellite” or “feeder” colonies, which often grow near colonies harboring conventional ampicillin resistant plasmids. Combined with the dual selection used in the ClonePlex system, contamination of recombinant clones with non-transformed bacteria is eliminated.

Constructing a random library with the ClonePlex system is very convenient and easy. The kit supplies all the components needed for library construction, including enzymes for highly efficient ligation of fragmented DNA; pre-digested, dephosphorylated duplex cloning vector; competent cells; sequencing primers, and control insert and plasmid DNAs.

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pLEXX-AK[™] Vector

The pLEXX-AK vector is supplied predigested (with blunt ends) and dephosphorylated. Blunt-ended, 5'-phosphorylated insert DNA is ligated to pLEXX-AK, transformed into the supplied high efficiency *E. cloni* electroporation competent cells, and spread on plates containing kanamycin and ampicillin. A ClonePlex-AK library does not require additional screening against clones containing empty vector, as they typically are not present at detectable levels. **Please note:** After ligation to insert DNA, the Kan^R portion of the vector, and the associated KanL and KanR primers, may be in either orientation relative to the AmpR portion of the vector (see Vector Map on p. 14).

E. cloni[®] 10G Electrocompetent Cells

E. cloni 10G Supreme Electrocompetent Cells are provided at transformation efficiencies of $\geq 4 \times 10^{10}$ cfu/ μ g supercoiled pUC19 DNA. This strain is required for high-efficiency cloning and plasmid propagation of ClonePlex derived libraries. To achieve the stated efficiencies of the ClonePlex Kit, **do not** substitute another cell strain for the 10G Supreme cells.

E. cloni 10G SupremeGenotype:

F⁻ *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1 recA1* ϕ 80*dlacZ* Δ M15 Δ *lacX74* *araD139* Δ (*ara,leu*)7697 *galU galK rpsL nupG* λ . *tonA*

E. cloni 10G cells are ideal for cloning and propagation of BAC, cosmid, or plasmid clones. They produce high yield and high quality plasmid DNA due to the *endA1* mutation. This strain contains the inactive *mcr* and *mrr* mutations, allowing methylated genomic DNA isolated directly from mammalian or plant cells to be cloned without deletions or rearrangements. *E. cloni* 10G cells can be used to generate unbiased and complete plasmid or Bacterial Artificial Chromosome (BAC) libraries. This strain lacks the F plasmid.

The plasmid pAKL is provided as a control for transformation of *E. cloni* cells with a ClonePlex-AK library. It contains phage lambda DNA fragments of 1.2 kb and 2.2 kb inserted into the cloning sites of pLEXX-AK. The molar concentration of pAKL is equivalent to 10 pg/ μ l pUC19. It is resistant to ampicillin and kanamycin.

End Repair of Sheared DNA

Shotgun cloning entails a fragmentation step to randomly shear the DNA, an end repair step to generate blunt ends, and a fractionation step to size-select the material. Physical methods of DNA fragmentation (e.g., nebulization, sonication, hydrodynamic shearing) are often preferred over enzymatic methods, as they are random and reduce the bias of sequencing projects (1). However, physical fragmentation results in a heterogeneous mix of blunt and 3'- and 5'-overhanging ends. Successful library construction requires an efficient method to convert these sheared ends to blunt-ended molecules.

Lucigen has developed the DNATerminator[®] End Repair Kit (Cat. # 40035-1 & 40035-2) to provide an efficient and convenient method for repairing DNA fragments. The DNATerminator End Repair components are included with the ClonePlex Kit to clone sheared or restriction-digested DNA.

For random shotgun library construction, Lucigen recommends using the HydroShear[®] instrument by GeneMachines[®] to fragment high molecular weight DNA. Fragments generated by the HydroShear device are repaired more efficiently than those produced through sonication or nebulization, resulting in larger libraries from a given amount of DNA. HydroShear fragmentation has the added advantage of generating a tight distribution of fragments in any desired size range, increasing the proportion of DNA available for

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cloning (2). Finally, the results obtained with HydroShear fragmentation are very predictable and reproducible.

Purification and Size Fractionation of DNA

DNA must be purified from restriction or repair enzymes before ligation to pLEXX-AK™. Agarose gel electrophoresis, which is commonly used to size fractionate DNA fragments, is sufficient for purification. If the DNA is not fractionated by electrophoresis after repair or digestion, it must be purified by phenol/chloroform extraction or binding to a purification column.

Sensitivity of DNA to Short Wavelength UV Light

Following gel fractionation, DNA is typically stained and visualized under ultraviolet light. However, exposure to short wavelength ultraviolet light (e.g., 254, 302, or 312 nm) can reduce cloning efficiencies by several orders of magnitude (Fig.3). Note that the wavelength of UV transilluminators specifically sold for DNA visualization is commonly 302 nm or 312 nm.

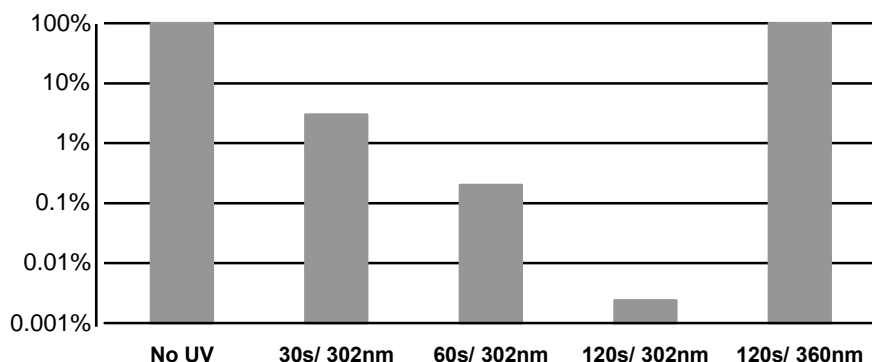


Figure 3. Relative cloning efficiency of pUC19 after exposure to short or long wavelength UV light. Intact pUC19 DNA was transformed after no UV exposure ("No UV") or exposure to 302 nm UV light for 30, 60, or 90 seconds ("30s/302nm, 60s/302nm, 120s/302nm") or to 360 nm UV light for 120 seconds ("120s/360nm"). Cloning efficiencies were calculated relative to un-irradiated pUC19 DNA.

IMPORTANT: Avoid exposure to genotoxic short wavelength UV light (e.g., 254, 302, or 312 nm) when preparing samples for cloning. Use a long UV wavelength (e.g., 360 nm) low intensity lamp and short exposure times.

Materials and Equipment Needed

The ClonePlex-AK Library Construction Kit supplies many of the items needed to efficiently repair the ends of sheared DNA and construct a duplex shotgun clone library. Successful construction of a ClonePlex library requires proper planning for each step. It is important to read the entire manual and to prepare the necessary equipment and materials before starting. It is assumed that common molecular biology equipment, supplies, and reagents are readily available. The following items are required for this protocol:

- Microcentrifuge and tubes.
- Electroporation apparatus and 1 mm gap-width cuvettes. Successful results are obtained with cuvettes from BTX (Model 610) or BioRad (Cat. #165-2089). Users have reported difficulties using *E. coli* cells with 1 mm Invitrogen cuvettes (Cat. # 65-0030).
- Sterile 17 x 100 mm culture tubes.
- Terrific Broth.

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- 2X TY agar plates containing ampicillin and kanamycin (see Appendix).
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Detailed Protocol

Preparation and Purification of Insert DNA

Generation of Blunt-Ended Fragments

DNA fragments created by digestion with blunt-cutting restriction enzymes (e.g., *EcoRV* or *HincII*) can be used with the ClonePlex-AK Kit without further processing. However, an end-repair reaction is required for cloning fragments generated by physical shearing (e.g. sonication or hydrodynamic shearing) or by restriction enzymes that leave 3' or 5' overhangs. PCR amplification products produced by polymerases with terminal transferase activity (e.g. Taq or Tfl) have a single-base 3' extension that also must be removed. The end-repair reaction must generate blunt, 5' phosphorylated ends.

Lucigen's DNATerminator[®] End Repair components are included with the ClonePlex Kit for cloning physically sheared DNA or DNA fragments created by restriction digestion. For cloning PCR products that have single-base 3' extensions, we recommend using T4 DNA Polymerase followed by T4 Polynucleotide Kinase.

Purification of Repaired Fragments

If repaired fragments are subsequently fractionated by gel electrophoresis, no further purification is necessary to remove the repair enzymes. Use of short-wavelength UV light (e.g., 254, 302, or 312 nm), common in UV transilluminators designed for nucleic acid visualization, **must** be avoided. After electrophoresis, DNA may be isolated using your method of choice.

If the repaired DNA is to be used *without* gel fractionation, it must be purified by phenol/chloroform extraction or by affinity chromatography (e.g., spin columns containing glass fibers or diatomaceous earth).

Ligation to the pLEXX-AK[™] Vector

In the ClonePlex-AK ligation reaction, the pre-processed pLEXX-AK vector is ligated with fragmented, repaired, fractionated insert in a total volume of 10 μ l. We recommend using 300-500 ng of insert DNA in the size range of 1000 to 4000 bp. Use of lower amounts of insert will result in significantly fewer transformants. The ligation is performed as follows:

1. Mix the Vector Premix before use by pipeting up and down several times. Combine the following components in a 1.5-ml tube, adding the ligase last:
 - x μ l Insert DNA (300-500 ng, blunt-ended, phosphorylated)
 - y μ l H₂O
 - 2.5 μ l 4X ClonePlex Vector Premix (pLEXX-AK, ATP, buffer)
 - 1.0 μ l CloneSmart[™] DNA Ligase (2 U/ μ l)
 - 10.0 μ l total reaction volume
2. Mix by gently pipeting the reaction mixture up and down. Incubate at room temperature (21-25°C) for 2 hours.

Optional Control Reactions include the following:

Vector Background	To determine the background of empty vector, use water instead of insert in the above reaction.
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Positive Control Insert DNA	To determine the ligation and transformation efficiency with a known insert, use 1 μ l (500 ng) of the supplied <i>HincII</i> digested lambda DNA.
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Preparation for Transformation

1. Heat denature the ligation reaction at 70°C for 15 minutes.
2. Cool at room temperature for 15 seconds and on ice for 15 seconds.
3. Spin 1 minute at 12000 rpm to collect condensation and pellet precipitated material.
4. The sample is ready for transformation; precipitating the DNA is not necessary.

Transformation

To ensure successful multiplex cloning results, Lucigen's *E. cloni*[®] 10G Supreme Electrocompetent Cells are provided with the ClonePlex Kit. These cells are provided in 50 μ l aliquots, sufficient for two transformation reactions using 25 μ l of cells each. Suggested settings for electroporation are listed below. Typical time constants are 3.5 to 4.5 msec.

Optimal Setting	Alternate Settings (~ 20-50% lower efficiencies)
1.0 mm cuvette 10 μ F 600 Ohms 1800 Volts	1.0 mm cuvette 25 μ F 200 Ohms 1400 – 1600 Volts
Bio-Rad Micro Pulser #165-2100 Bio-Rad E. coli Pulser #165-2102	Bio-Rad Gene Pulser II #165-2105 BTX ECM630 Electroporation System

1. Have room temperature Recovery Medium and 17 x 100 mm sterile culture tubes readily available (one tube for each transformation reaction). Transformation efficiency may decrease with SOC or other media.
2. Place electroporation cuvettes (1 mm gap, BTX or BioRad brand) and microcentrifuge tubes on ice (one cuvette and one tube for each transformation reaction).
3. Remove *E. cloni* cells from the -86°C freezer and thaw completely on wet ice (20-30 minutes).
4. Add 25 μ l of *E. cloni* cells to the chilled microcentrifuge tube.
5. Add 1 μ l of the ClonePlex ligation reaction to the 25 μ l of cells on ice. Stir briefly with pipet tip; **do not** pipet up and down to mix, which can introduce air bubbles and warm the cells.
6. Carefully pipet 25 μ l of the cell/ligation mixture into a chilled electroporation cuvette without introducing bubbles. Quickly flick the cuvette downward with your wrist to deposit the cells on the bottom of the well. Electroporate using the conditions described above.
7. Within 10 seconds of the pulse remove the cells from the electroporation cuvette by rinsing with the 975 μ l Recovery Medium. Transfer the cells and Recovery Medium to the culture tube.
8. Place the tubes in a shaking incubator at 250 rpm for 1 hour at 37°C.
9. Spread the following amounts of experimental and control reactions on the indicated antibiotic plates. Spread no more than 100 μ l of the experimental insert transformation per 100 mm petri plate.

Reaction Plate	Microliters/Plate	Antibiotic
Experimental Insert	100	Amp + Kan
Lambda <i>HincII</i> Positive Control Insert	100	Amp + Kan
No-Insert Control (Vector Background)	100	Amp + Kan
pAKL Supercoiled Plasmid Control (10 pg)	2	Amp + Kan

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10. Incubate the plates overnight at 37°C.

EXPECTED RESULTS

The results presented below are expected when cloning 500 ng of intact, purified DNA fragments, with blunt ends and 5' phosphate groups, into Lucigen's *E. coli* 10G Supreme Electrocompetent Cells, with BTX or BioRad cuvettes. The number of recombinant clones is typically 1000-fold greater than the background of self-ligated pLEXX[™]-AK vector. The background number of empty pLEXX[™]-AK vectors is constant (≤ 1 colony per 100 μ l of cells plated), unless kinase is introduced as a contaminant. However, use of too little insert DNA, or insert DNA that is improperly end-repaired, or modified DNA that is not repairable yields significantly lower recombinant cloning efficiencies. Cloning AT-rich DNA and other recalcitrant sequences may also lead to fewer colonies.

Reaction	CFU/Plate	Efficiency
Experimental Insert	> 300	> 99.9% inserts
Lambda <i>HincII</i> Insert (Positive Control)	> 300	> 99.9% inserts
No Insert (Negative Control)	≤ 1	< 0.1% background
pAKL Supercoiled Plasmid Control (10 μ g)	> 200	> 1×10^{10} cfu/ μ g plasmid

1. A 100- μ l aliquot of transformed cells from the experimental or positive control reaction can be expected to yield > 300 colonies with > 99.9% inserts.
2. A 100- μ l aliquot of the empty vector control reaction should produce not more than 1 colony, representing less than 0.1% background.
3. A 2- μ l aliquot of transformed cells from the supercoiled pAKL or pUC19 reaction (diluted into 90 μ l of TB) should yield > 200 colonies, or > 1×10^{10} colonies per μ g plasmid.

No Screening Required

No additional screening for recombinant colonies is required, as the design of the ClonePlex system ensures that more than 99.9% of the colonies obtained from a typical transformation contain recombinant plasmid. Because the background of empty vector transformants is nearly undetectable, colonies can be picked at random for growth and plasmid purification.

DNA Isolation & Sequencing

Grow transformants in TB medium plus 30 μ g/ml kanamycin. Addition of ampicillin to the growth medium is not necessary. Use your favorite method to isolate plasmid DNA suitable for sequencing. The pLEXX-AK plasmid contains the high copy number pUC origin of replication and produces DNA yields similar to other pUC-based plasmids. The *E. coli*[®] competent 10G cells are *recA endA* deficient and will provide high quality plasmid DNA. The four ClonePlex Sequencing Primers are provided with the Kit. The sequence of the primers and their orientation relative to the pLEXX-AK plasmid markers is shown in the Appendix.

References

1. Sambrook, J. and Russell, D.W. *Molecular Cloning: A Laboratory Manual* (Third Edition). 2001. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

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2. Thorstenson YR, Hunicke-Smith SP, Oefner PJ, Davis RW. 1998. An automated hydrodynamic process for controlled, unbiased DNA shearing. *Genome Res* 8: 848-55.

Appendix A: Media Recipes

YT+amp+low kan Agar Medium for Plating of Transformants

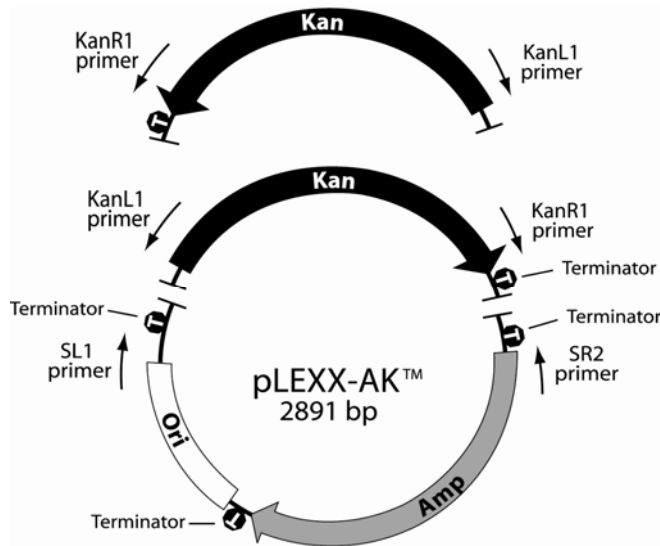
Per liter: 8 g Bacto-tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, 10 mg kanamycin, 50 mg ampicillin. Mix all components except antibiotics; autoclave and cool to 55°C. After cooling, add ampicillin to 50 µg/ml and kanamycin to 10 µg/ml. Pour into petri plates.

TB+kan Culture Medium

Per liter: 11.8 g Bacto-tryptone, 23.6 g yeast extract, 9.4 g dipotassium hydrogen phosphate (anhydrous), 2.2 g potassium dihydrogen phosphate (anhydrous), 0.4% glycerol. Mix all components except glycerol; autoclave and cool to 55°C. Add 8 ml filter-sterilized 50% glycerol prior to using. Add kanamycin to 30 mg/l.

Appendix B: Vector Map

The pLEXX-AK[™] vector is supplied pre-digested at the *HincII* sites. The total size is 2891 bp, consisting of the Amp^R+Ori fragment of 1833 bp and the Kan^R fragment of 1058 bp. Note that after ligation to insert DNA, the Kan^R portion of the vector may be in either orientation. The sequence of the Amp^R+Ori fragment can be obtained from GenBank (AF399742). It is identical to the sequence Lucigen's pSMART-HCAmp vector. The sequence of the Kan^R fragment can be obtained from Lucigen upon request. The primer sequences are shown in Appendix E.



The pSMART[®] vectors are supplied predigested, with blunt, dephosphorylated ends. Transcriptional terminators border the cloning site to prevent transcription from the insert into the vector. Another terminator at the 3' end of the ampicillin or kanamycin resistance gene prevents this transcript from reading into the insert DNA.

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Appendix C: Troubleshooting Guide

Problem	Probable Cause	Solution
Very few or no transformants	Inefficient end repair.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair if necessary.
	Contaminating enzymes in ligation reaction.	Heat-denature end repair reaction 10 minutes at 70°C. Purify DNA after end repair reaction or restriction digestion.
	No DNA or molar ratio of insert to vector is incorrect.	Check the insert DNA by gel electrophoresis. Determine concentration of insert and add the correct amount. Use the supplied control insert to test the system.
	Ligation reaction failed.	Check the insert DNA for self-ligation by gel electrophoresis. Repeat end repair if necessary. Be sure insert DNA is phosphorylated. Use the supplied control insert to test ligation.
	Inadequate heat denaturation after ligation reaction.	DO heat denature for 15 min at 70°C. Skipping this step may lower the number of transformants 2-3 orders of magnitude.
	Loss of DNA during precipitation.	DO NOT precipitate DNA after ligation reaction. It is not necessary with this protocol and these cells.
	Improper electroporation conditions.	Use BTX or BioRad electroporation cuvettes with a gap of 0.1 cm. Pre-chill cuvettes on ice. Add the 1 µl of DNA to 25 µl of pre-aliquotted cells on wet ice; DO NOT add the cells to the DNA.
	Incorrect recovery media.	DO NOT use SOC or other recovery media. DO use TB (Terrific Broth) for optimal results.
	Addition of XGAL/DMSO to competent cells.	DO NOT add additional compounds to competent cells, as they are fragile.
High background of transformants that do not contain inserts.	Incorrect amount of Ampicillin or Kanamycin in agar plates.	Add the correct amount to molten agar at 55°C before pouring plates. DO NOT spread Kan and Amp onto the surface of agar plates.
	Contaminating enzymes in ligation reaction.	Purify DNA after DNATerminator reaction. DO NOT add T4 DNA Kinase to the ligation reaction.
	Did not use 10G electrocompetent cells.	Repeat transformation with 10G electrocompetent cell strain.
	Incorrect amounts of one or both antibiotics in agar plates.	DO NOT spread Kan and Amp onto the surface of agar plates. Add the correct amount to molten agar at 55°C before pouring plates.

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Appendix D: Abbreviated Protocol (Please see Manual for detailed instructions.)

Insert DNA Preparation

1. Fragment target DNA
 2. If necessary, repair the DNA ends with the DNATerminator[®] End Repair Enzyme and 5X Buffer.
 3. Purify DNA by phenol/chloroform extraction, column chromatography, or gel electrophoresis. **Do NOT use 256, 302, or 312nm UV light to visualize the DNA.**
-

Ligation

1. Combine the following components in a 1.5-ml tube. Add Ligase last.
 - x μ l Insert DNA (300-500 ng, 1-4 kb, blunt-ended, 5'-phosphorylated)
 - y μ l H₂O
 - 2.5 μ l 4X ClonePlex Vector Premix (pLEXX-AK[™], ATP, buffer)
 - 1.0 μ l CloneSmart[™] DNA Ligase (2 U/ μ l)
 - 10.0 μ l total reaction volume
 2. Incubate 2 hours at room temperature.
 3. Heat denature the ligation reaction 15 minutes at 70°C.
 4. Cool 15 seconds at room temperature and 15 seconds on ice.
 5. Spin 1 minute at 12,000 rpm.
-

Transformation

1. Chill electroporation cuvettes (0.1 cm gap) and microcentrifuge tubes on ice (one cuvette and one microcentrifuge tube per transformation).
 2. Place 1 ml of room temperature Recovery Medium into 17 x 100 mm sterile culture tube (one tube per transformation).
 3. Thaw *E. coli* 10G cells on wet ice. Add 25 μ l of cells to the chilled microcentrifuge tube on ice.
 4. Add 1 μ l of heat-treated ligation reaction to the 25- μ l aliquot of cells.
 5. Carefully pipet the cell/DNA mixture to a chilled electroporation cuvette so as not to introduce bubbles.
 6. Electroporate and immediately transfer cells to 1 ml of Recovery Medium. Shake at 225 rpm for 1 hour at 37°C.
 7. Spread up to 100 μ l per plate on 2X TY+amp+low kan agar plates. Incubate overnight at 37°C.
-

Colony Growth

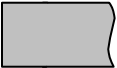
1. Pick colonies at random and grow in TB+kan medium.
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CLONEPLEX[®] Library Construction Kit



Appendix E: Primer Sequences

Kanamycin Gene Primers

KanL1 Primer → **DraI** **Insert DNA**



ATCTTGTGCAACGTGACATCAGAGATTTTGAGACACAACGTTTAAACGGACGACGACCGTATCGGCTGCAGGATCCAGATATGCGTC...
TAGAACACGTTGCACTGTAGTCTCTAAACTCTGTGTTGCAAATTTGCCTGCTGCTGGCATAGCCGACGTCCTAGGTCTATACGCAG... 

KanR1 Primer → **BstXI** **Insert DNA**


TTCGTTTTAATCTGGAAAACCACCCTGGCGCTGCAGGTTCCAGATTCCTGGTT... 
AAGCAAATTAGACCTTTTTGGTGGGACCGCGACGTCCAAGGTCTAAGGACCAA... 

Ampicillin Gene Primers

SL1 Primer → **EcoRV Hind III EcoRI** **Insert DNA**

CAGTCCAGTTACGCTGGAGTCTGAGGCTCGTCCTGAATGATATCAAGCTTGAATTCGTT... 
GTCAGGTCAATGCGACCTCAGACTCCGAGCAGGACTTACTATAGTTCGAACTTAAGCAA... 

SR2 Primer **SwaI** → **EcoRV XbaI EcoRI** **Insert DNA**

CTTTCTGCTAT**GGAGGTCAGGTATGATTTAAATG**GTCAGTATTGAGCGATATCTAGAGAATTCGTC... 
GAAAGACGATACCTCCAGTCCATACTAAATTTACCAGTCATAACTCGCTATAGATCTCTTAAGCAG... 