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(54) **LINEAR VECTORS, HOST CELLS AND CLONING METHODS**

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C12N 15/73	(2006.01)
C12N 9/90	(2006.01)
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(52) **U.S. Cl.**

CPC ***C12N 15/73*** (2013.01); ***C12N 9/90*** (2013.01); ***C12N 15/70*** (2013.01); ***C12P 19/34*** (2013.01)

(58) **Field of Classification Search**

None

See application file for complete search history.

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(57) **ABSTRACT**

Linear vectors derived from bacteriophage of *E. coli* and host cells suitable for cloning are provided. The linear vectors include a left arm comprising a left telomere and a first selectable marker, a right arm comprising a right telomere and a second selectable marker and a cloning region located between the left arm and the right arm. Optional further components of the vector include transcriptional termination sequences, multiple cloning sites and reporter stuffer regions.

32 Claims, 10 Drawing Sheets

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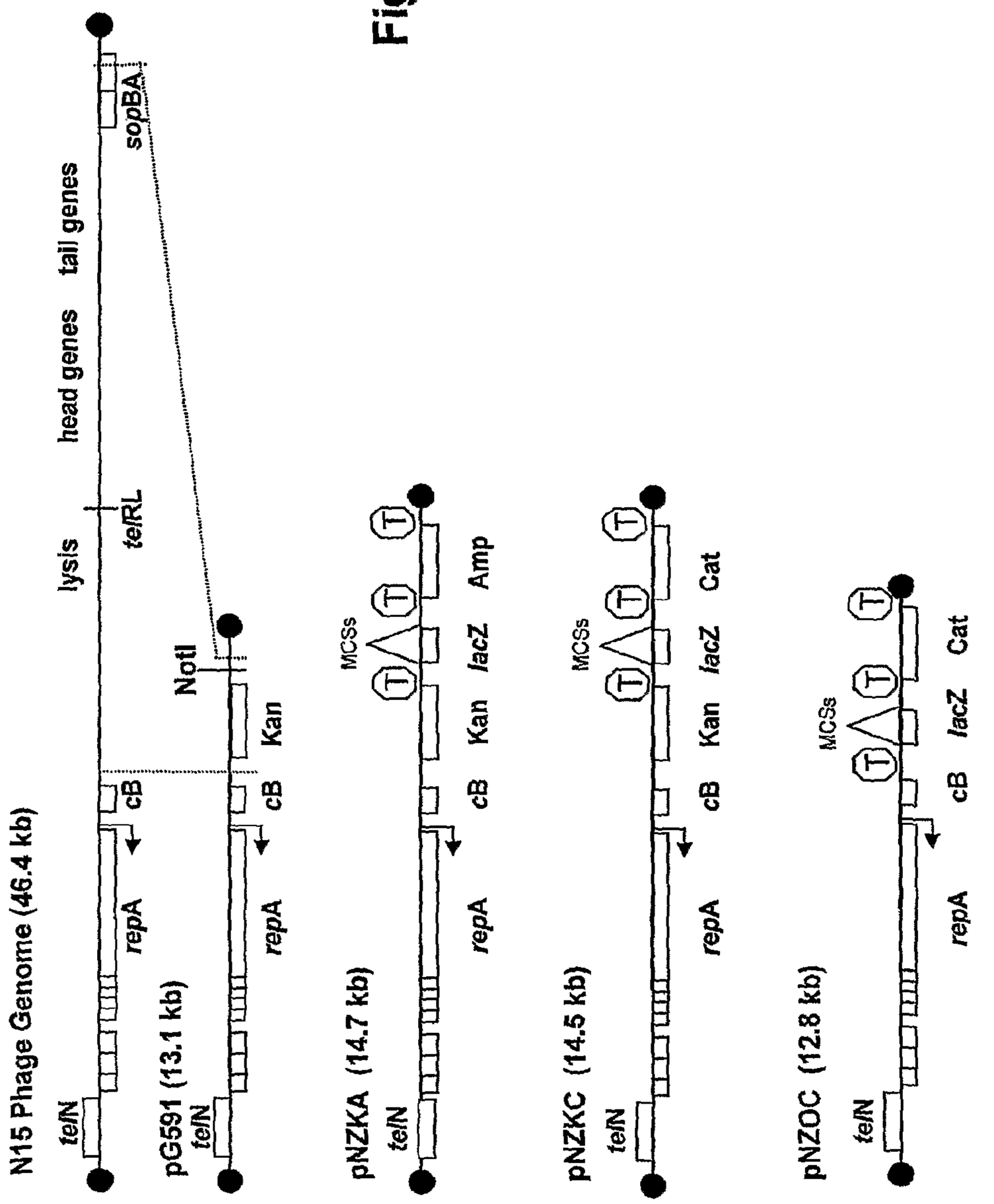
Fig. 1


N15 Phage Genome (46.4 kb)
telN
repA
cB
pG591 (13.1 kb)
telN
repA
cB
NotI
Kan
pNZKA (14.7 kb)
telN
repA
cB
MCSs
Kan
lacZ
Amp
pNZKC (14.5 kb)
telN
repA
cB
MCSs
Kan
lacZ
Cat
pNZOC (12.8 kb)
telN
repA
cB
MCSs
lacZ
Cat
lysis
telRL
head genes
tail genes
sopBA

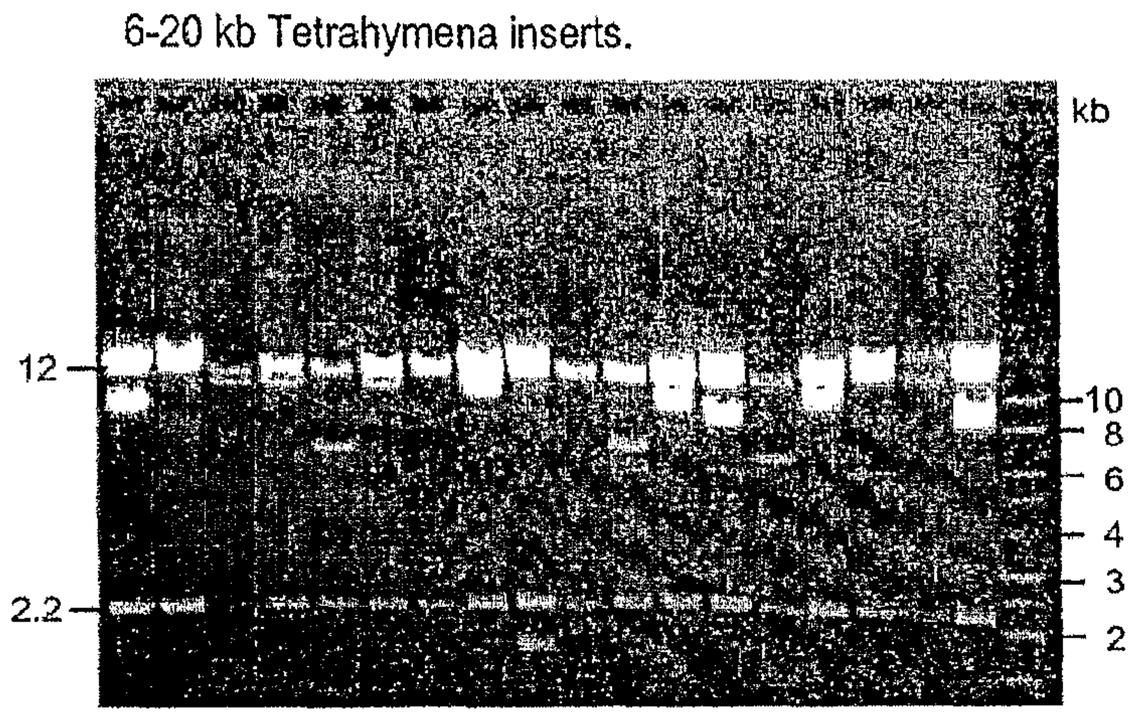


Fig. 2

Fig. 3

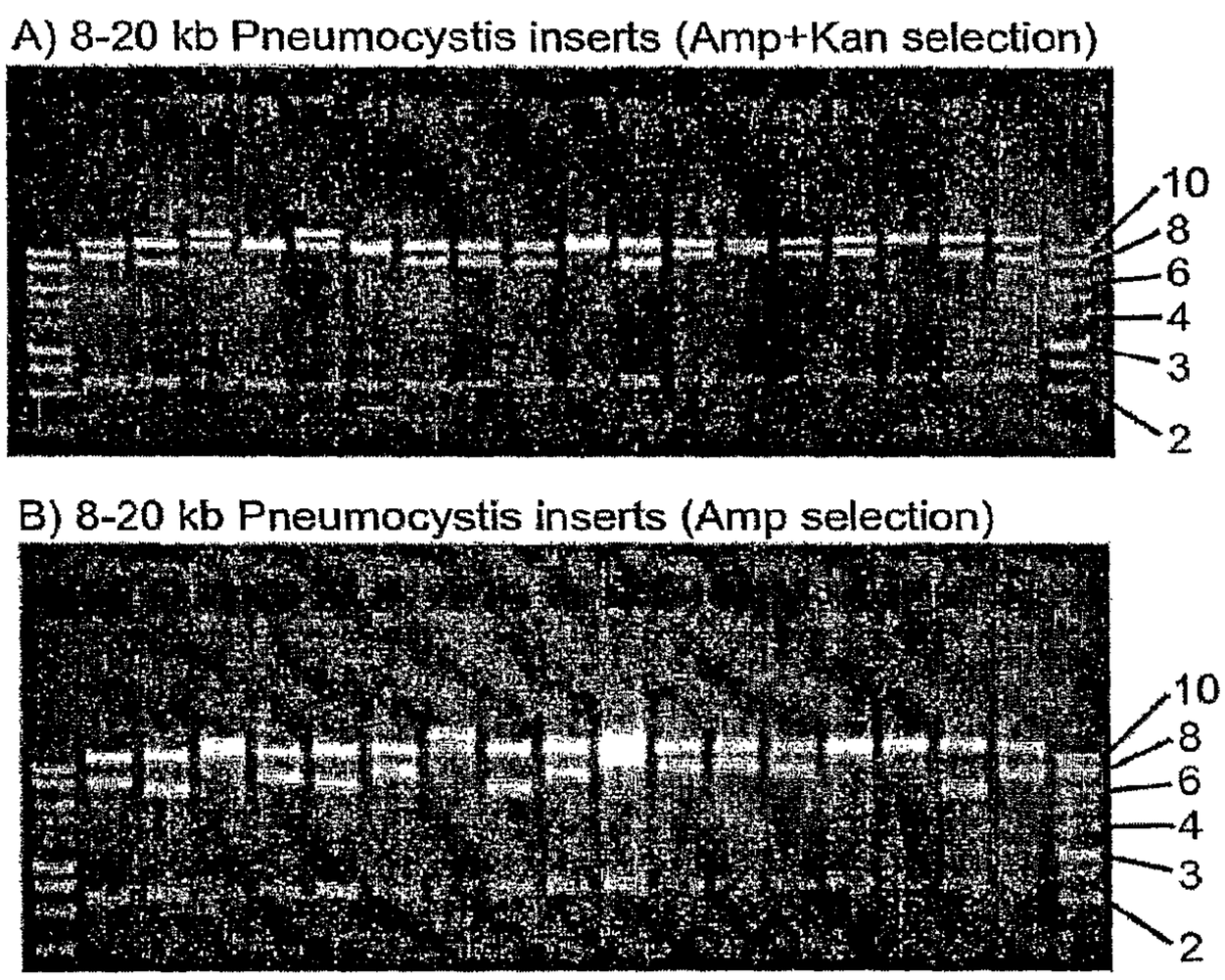

A) 8-20 kb Pneumocystis inserts (Amp+Kan selection)
10
8
6
4
3
2
B) 8-20 kb Pneumocystis inserts (Amp selection)
10
8
6
4
3
2

Fig. 4

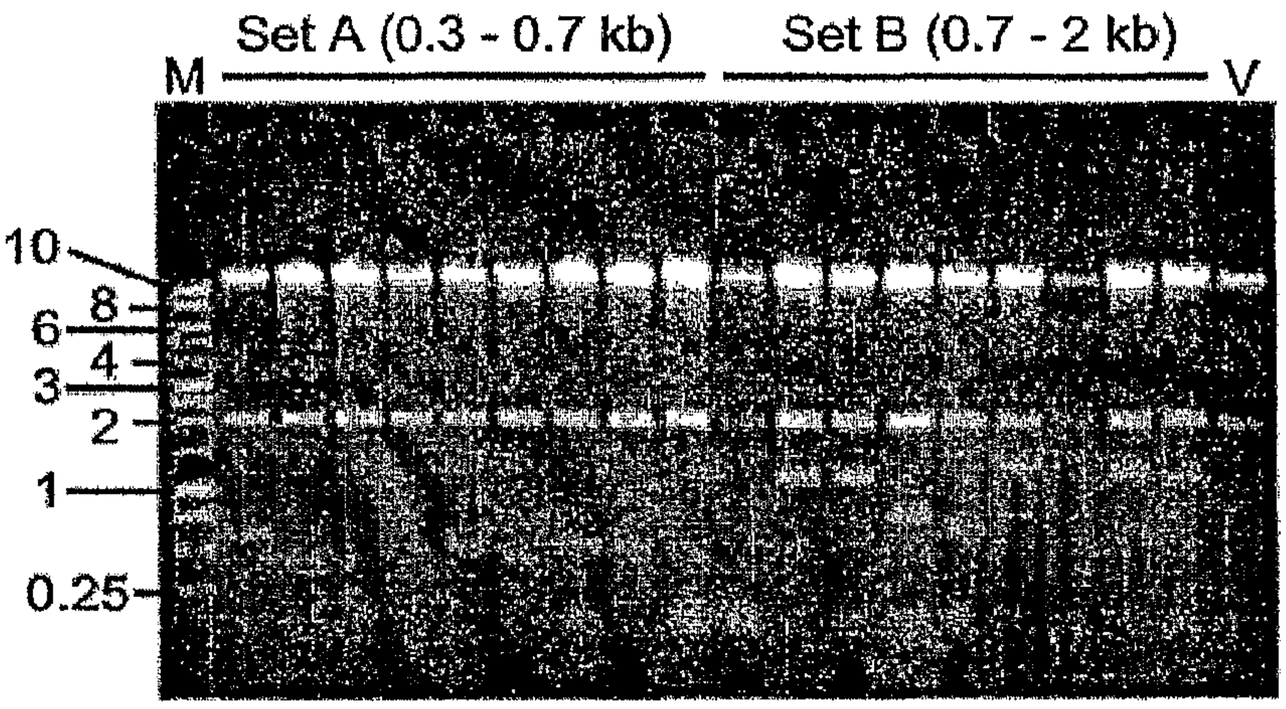


Fig. 5

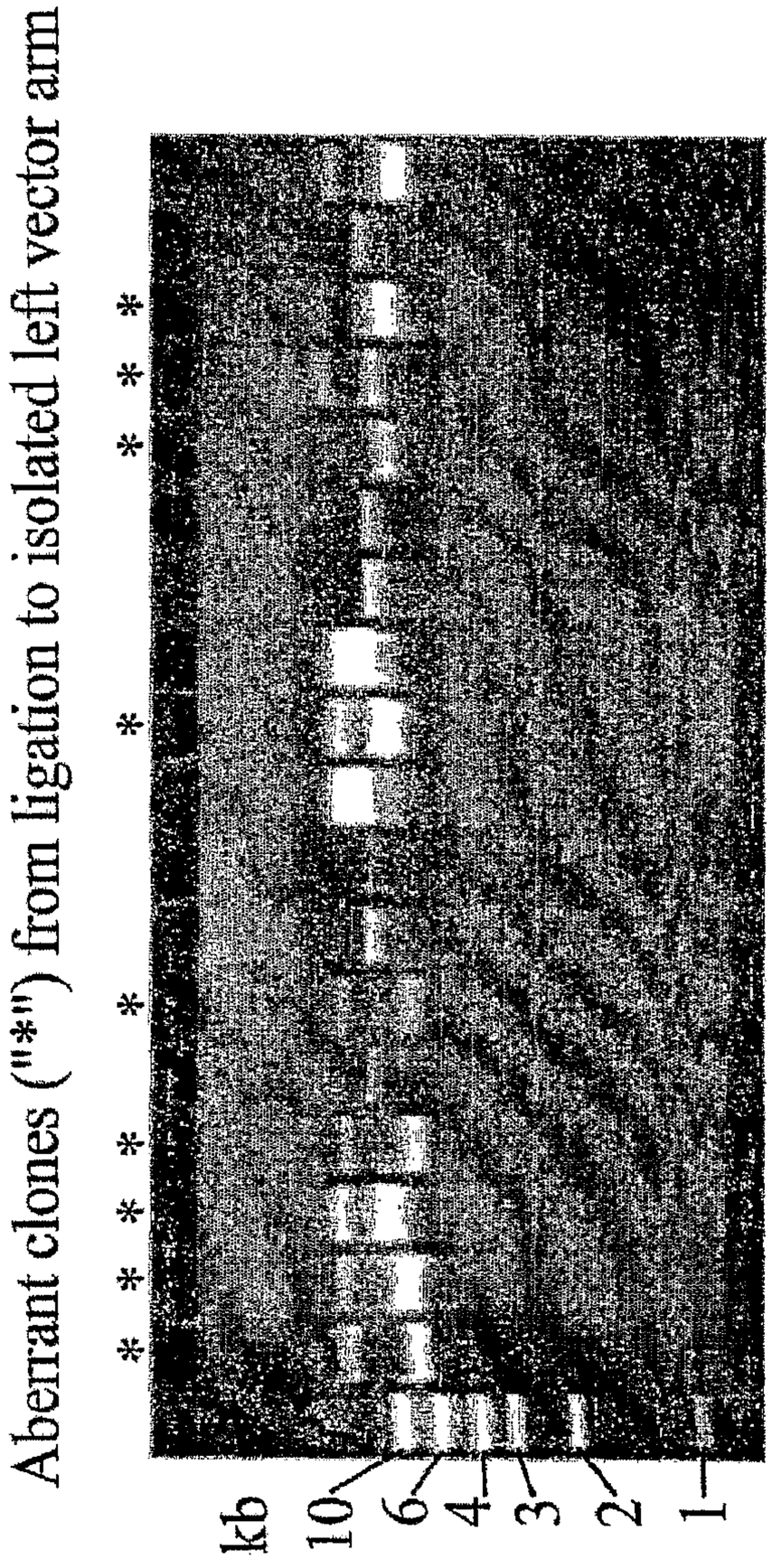

Aberrant clones ("*") from ligation to isolated left vector arm
* * * * * * * *
kb
10
6
4
3
2
1

Fig. 6

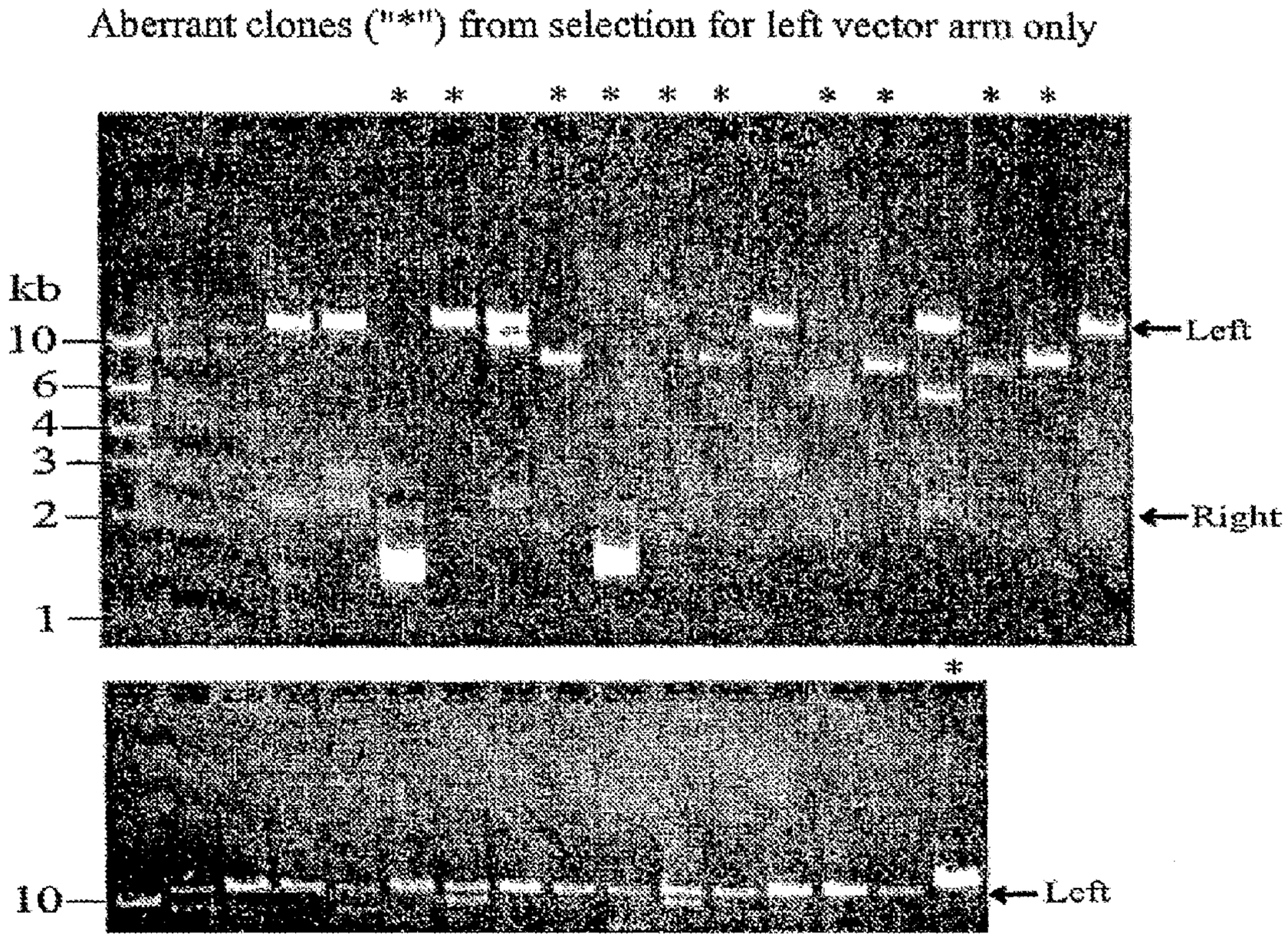

Aberrant clones ("*") from selection for left vector arm only
kb
10
6
4
3
2
1
Left
Right
10
Left

Fig. 7

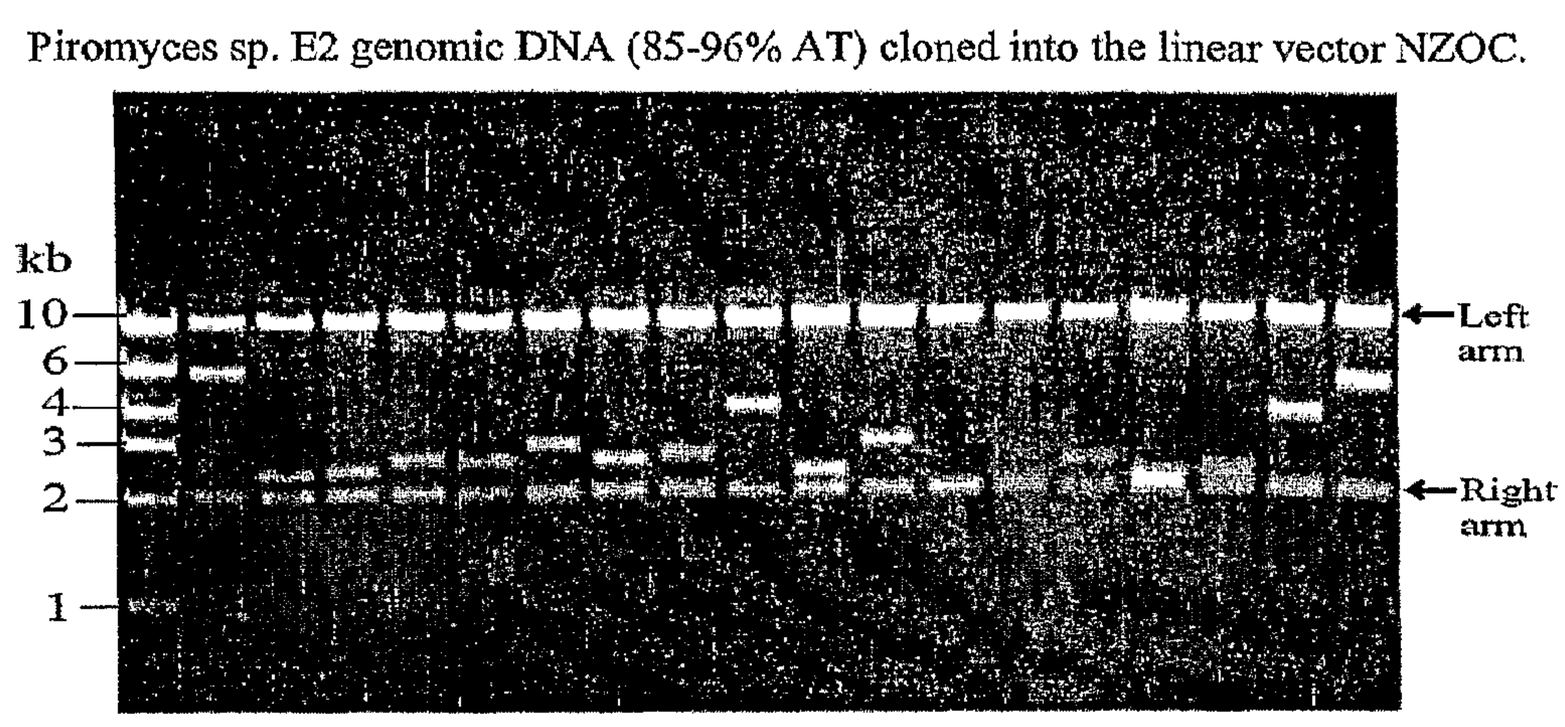

Piromyces sp. E2 genomic DNA (85-96% AT) cloned into the linear vector NZOC.
kb
10
6
4
3
2
1
Left arm
Right arm

Fig. 8

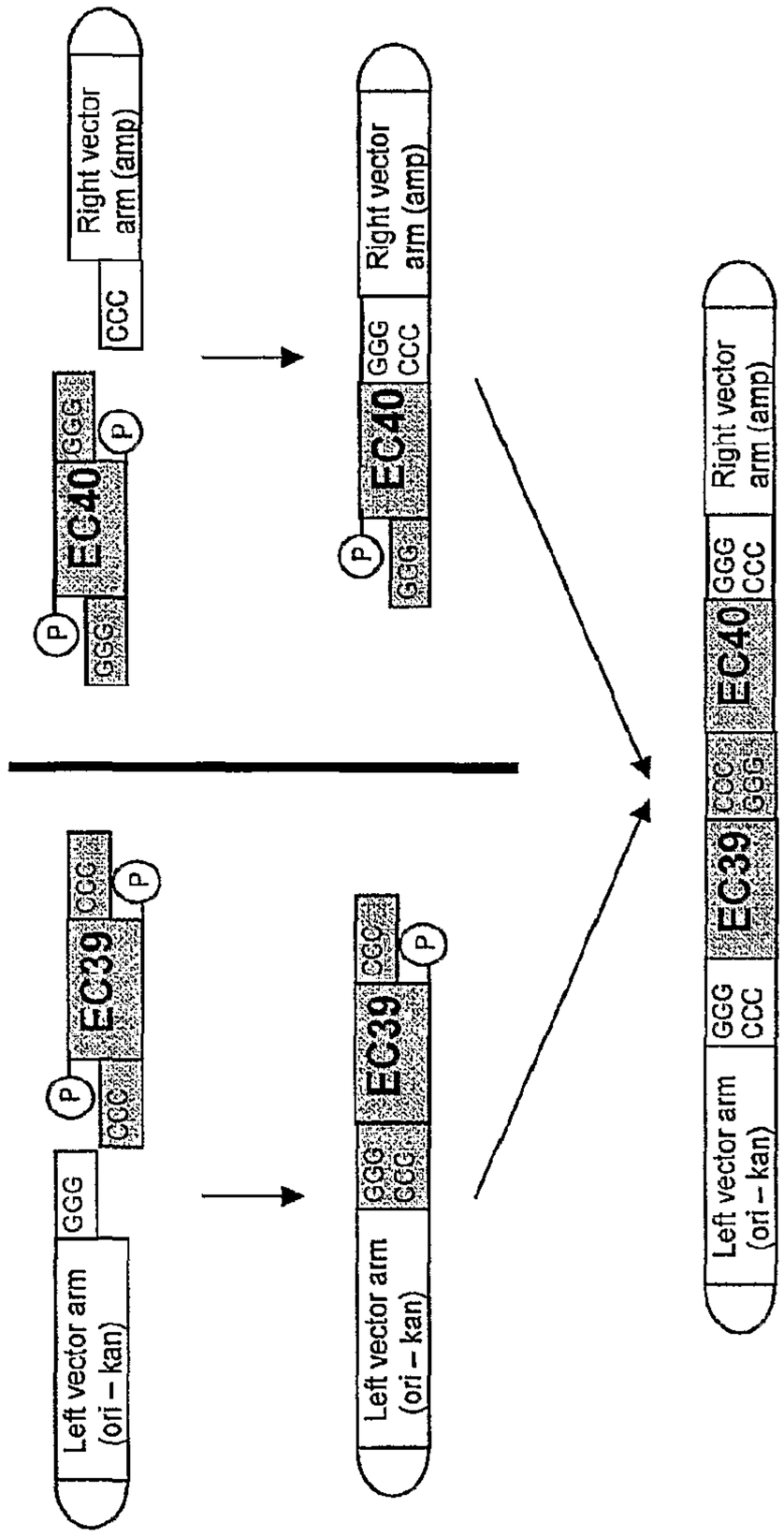
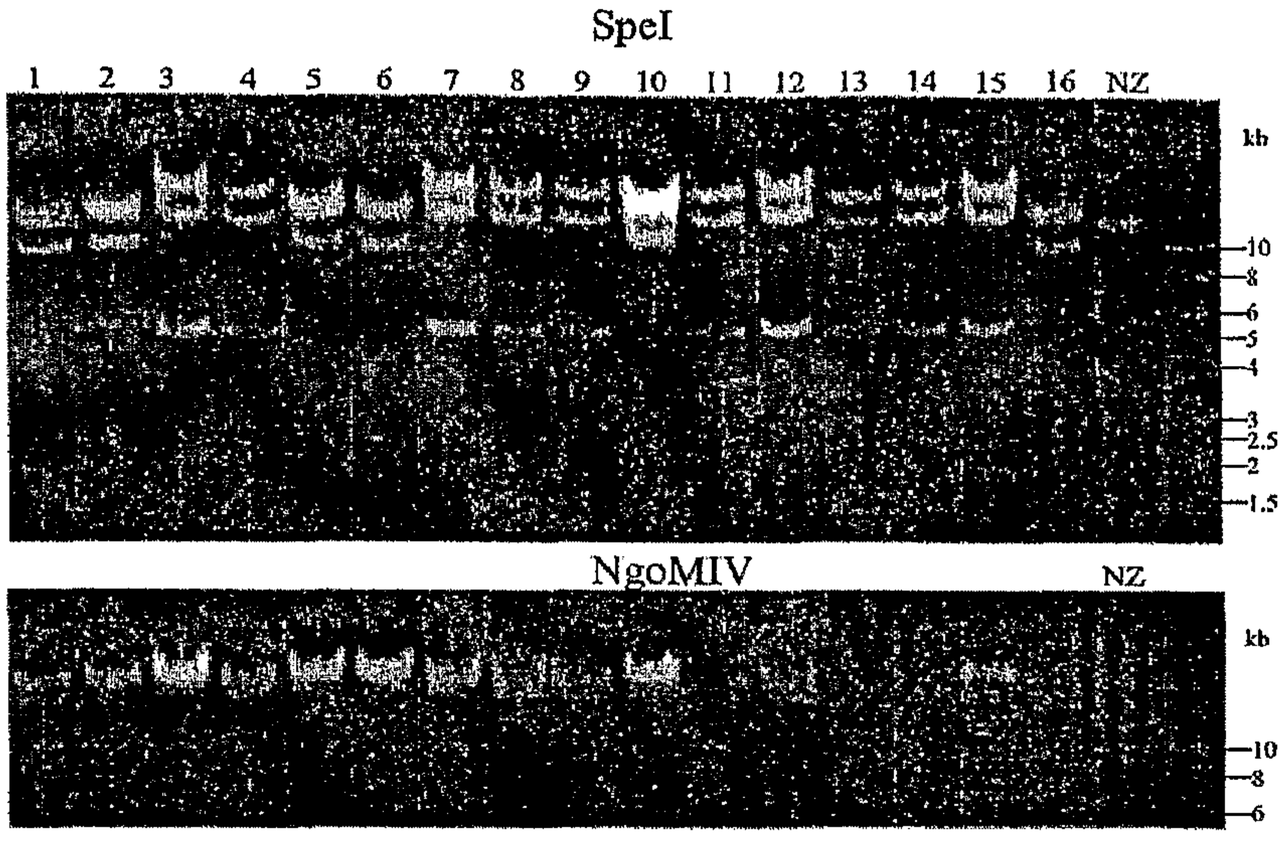
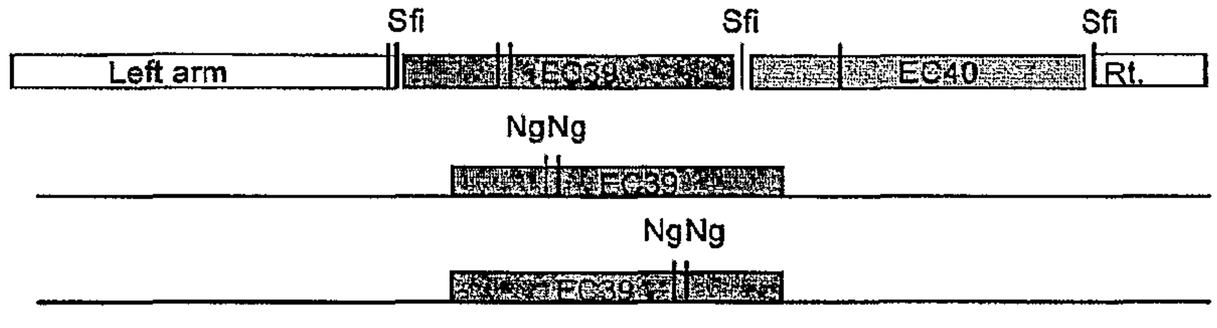

Left vector arm (ori – kan)
GGG
CCC
EC39
P
Right vector arm (amp)
GGG
CCC
EC40
P

Fig. 9

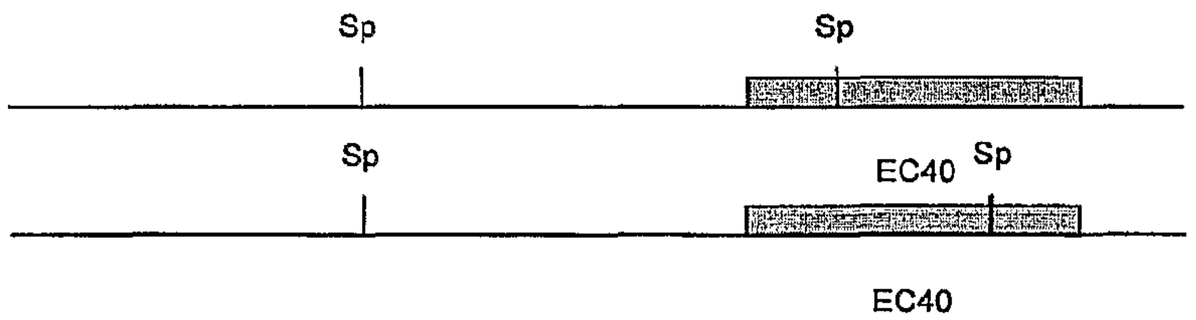
A.



B.



NgoMIV Digest: (15 kb, 0.4 kb, 19 kb) or (19 kb, 0.4 kb, 15 kb)



SpeI Digest: (12 kb, 13 kb, 9 kb) or (12 kb, 17 kb, 5 kb)

Fig. 10

LINEAR VECTORS, HOST CELLS AND CLONING METHODS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation of U.S. patent application Ser. No. 12/159,956, filed on May 15, 2009, which is a national stage filing under 35 U.S.C. 371 of International Application No. PCT/US2007/060500, filed on Jan. 12, 2007, which claims the benefit of U.S. Provisional Patent Application Ser. Nos. 60/758,479, filed Jan. 12, 2006 and 60/747,733, filed May 19, 2006, which are incorporated by reference herein.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

The invention was made with United States government support awarded by the National Institutes of Health (Grant No. 1R43HG002627-01). The United States has certain rights in the invention.

INTRODUCTION

Dramatic advances in high-throughput sequencing technology have resulted in the nearly complete deciphering of the human genome and genomes of several other species. In stark contrast to these technical achievements, there has been little improvement in the vectors or host cells used to generate the recombinant clone libraries necessary for genomic sequence analysis. Numerous genetic elements are unstable or unclonable in currently available vector/host systems due to toxicity, secondary structure, replication errors, and other poorly understood characteristics. Conventional vectors replicate to high copy number, actively induce transcription and translation of inserted fragments, and allow cloned promoters to interfere with plasmid maintenance. Moreover, conventional methods of cloning have primarily utilized supercoiled plasmid DNA, which causes instability due to torsional stress and enzymatic processing of secondary structures. Instability caused by these factors leads to sequence stacking, clone gaps, sequence gaps, and other difficulties.

The standard vectors for construction of libraries, pUC18 and its derivatives, contain many features useful for general cloning, including blue/white screening capability, large multiple cloning sites, and high copy number, as well as the ability to generate RNA transcripts from bacteriophage promoters and single-stranded DNA from the M13 origin of replication. However, many of these attributes are incompatible with stable maintenance of certain inserts, leading to clone gaps and seemingly "unclonable" DNA fragments. Such problematic sequences are typically characterized by high AT-content, strong secondary structure, deleterious open reading frames, or cis-acting functions (e.g., transcriptional promoters or replication origins).

Linear vectors provide an alternative to the use of circular supercoiled plasmids for cloning. Linear vectors are not subject to the supercoiling found in circular plasmids and therefore may stably maintain inserts that have primary or secondary structures that are unstable when supercoiled. This additional stability may result in improved sequencing data and reductions in the number of sequence gaps and cloning gaps in genomic assemblies. Linear vectors also exhibit the ability to clone larger inserts using standard methods. Linear vector cloning systems may stably clone

DNA in the mid-size range (10-50 kb), without the use of packaging systems required with cosmid or fosmid cloning. Linear vector cloning systems may also be used to clone fragments in the large size range (>100 kb), without the extensive vector purification needed for BAC cloning.

One linear cloning vector that has been investigated is derived from the *E. coli* double-stranded DNA ("dsDNA") phage N15. In contrast to typical temperate bacteriophages that physically integrate their prophage DNA into the host's chromosome during the establishment of lysogeny, N15 replicates in the lysogen as a low-copy-number, extrachromosomal linear plasmid that has covalently closed hairpin loop telomeres. Nearly half the genome of N15, including the head and tail genes, has extensive homology with that of bacteriophage lambda (λ). The elements that control transcription and determine prophage immunity have homologues in the repressor, operator, and anti-terminator of λ and P22. The lytic development of N15 resembles that of λ, resulting in virions with λ-like morphology; and it lysogenizes at similar frequencies. The portions of phage N15 required for replication and maintenance of the linear prophage have no known equivalents in phage lambda. Conversely, the head and tail genes of phage KO2 of *Klebsiella oxytoca* are completely distinct from those of N15, but the genes for replication and maintenance of the linear prophages of N15 and KO2 are highly homologous. (Sherwood R C et al., J. Bacteriol., 186(6): 1818-32 (March 2004), the disclosure of which is incorporated herein by reference).

The replication of linear N15 vectors requires three components: an origin of replication (Ori), the replication initiation protein RepA, and the protelomerase TelN for resolution of the replicated telomeres. To form the prophage, the cohesive ends of the injected linear DNA are joined to create a circular intermediate. The protelomerase recognizes a unique palindromic site (tel RL), located near the center of the previously linear molecule. It processes the linear or circularized DNA to produce a linear molecule with closed ends telL and telR, both in vitro and in vivo. The only N15 gene required for replication of the circular form of the plasmid is repA, which contains helicase, primase, and origin binding activities.

The origin itself is within the repA gene, and replication proceeds bidirectionally using the host *E. coli* DNA polymerase. N15 replication is independent of the host genes polA, dnaA, dnaJ, dnaK, grpE, and recA. The N15 genome also contains a partition system (sopBA), having homology to F' plasmid genes, but with a dispersed set of centromere sites.

The N15 virus has previously been modified into a 13.8 kb cloning vector, pG591 (SEQ ID NO:1). The pG591 vector, which is schematically shown in FIG. **1**, retains the genes essential for replication and copy number regulation, including telN (protelomerase), repA (replicase or replication factor), and cB (prophage repressor or copy number regulator), but the phage structural genes have been removed. It also lacks the partition genes sopBA necessary for stable maintenance of the vector. Instead, the sop functions may be supplied in trans using *E. coli* strain DH10B31sop, which has a chromosomally integrated N15 sop operon and anti-repressor gene, the latter under control of an arabinose-inducible promoter.

BRIEF SUMMARY OF THE INVENTION

Although it is functional as a vector, molecular cloning results using pG591 have revealed several major drawbacks. First, the left arm containing the 12 kb NotI vector fragment

(telN-repA-cB-KanR) is capable of transforming cells without the addition of an insert or the right telomere fragment, which lacks a selectable marker. Even if self-ligation of the vector is prevented via dephosphorylation, many aberrant clones and some non-recombinant clones are generated. Aberrant recombinant clones include dimers of the 12 kb fragment or circular permutations of the vector with or without various deletions between telN and repA. Thus, because pG591 generates a high frequency of empty vector background and alternate structures, it is not acceptable for molecular cloning purposes. In addition, pG591 has only a single restriction site (NotI) available for cloning, so it is not convenient for library construction or restriction analysis of clones. Moreover, a strong promoter is directed from the right telomeric region toward the NotI site, which is likely to reduce the stability of cloned inserts by transcribing them.

The present invention relates to improved linear cloning vectors and host cells suitable for propagating the improved linear cloning vectors, kits that include both the linear cloning vectors and a strain of host cells, and methods of cloning polynucleotide sequences using the linear cloning vectors. The invention permits cloning of large or "difficult" polynucleotide sequences which may otherwise not be cloned using conventional circular plasmid vectors. For example, the linear vector of the invention can maintain fragments that are unstable in the supercoiled plasmid form. The linear mode of replication imparts high fidelity replication of repeats, large palindromes, and AT-rich DNA. In addition, the invention allows for simplified molecular analysis of cloned sequences.

In one aspect, the invention provides a linear cloning vector derived from a bacteriophage capable of being maintained *E. coli*. The linear cloning vector of the invention includes a left arm comprising a left telomere and a first selectable marker; a right arm comprising a right telomere and a second selectable marker; and a cloning region located between the left arm and the right arm.

The invention also provides host cells suitable for use with the linear vector. In some embodiments, the invention provides a recombinant host cell having a polynucleotide sequence encoding a protelomerase integrated into the host cell genome.

In a further aspect, the invention provides a kit comprising a linear cloning vector of the invention and a suitable host cell.

In yet another aspect, the invention provides a method of cloning a polynucleotide sequence. The method includes steps of processing a linear cloning vector of the invention to separate the right arm from the left arm; ligating the first end of the polynucleotide sequence to the right arm and the second end of the polynucleotide sequence to the left arm to provide a ligation product; transforming a host cell with the ligation product; and growing the transformed host cell on medium, such that selection is provided for the first and second selectable markers of the linear cloning vector.

In an additional aspect, the invention provides a method of cloning at least two distinct polynucleotides. The method includes steps of processing each of the polynucleotides to provide a linking sequence on both termini of the polynucleotides; processing a linear cloning vector of the invention to separate the right arm from the left arm and to provide a linking sequence on the terminus opposite the telomere of each arm; forming a ligation product comprising the polynucleotides and the right and left arms, wherein the arms are noncontiguous with each other and are separated by both of the polynucleotides to be cloned; transforming a host cell with the ligation product; and growing the transformed host cell on medium, such that selection is provided for the first and second selectable markers of the linear cloning vector, wherein multiplication of the host cell results in cloning of the polynucleotides.

The objects and advantages of the invention will appear more fully from the following detailed description of the preferred embodiment of the invention made in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic diagram of N15 phage, plasmid pG591, and three linear cloning vectors of the invention: pNZKA, pNZKC, and pNZOC. As shown, pNZKA, pNZKC, and pNZOC carry the genes telN, repA, and cB, which are essential for replication and regulation of copy number. Promoters are indicated by arrows and transcriptional terminators by "T". Dark circles represent the telomeres. The lacZ "stuffer" fragment, situated between a pair of multiple cloning sites (MCSs), is removed before ligation to inserts.

FIG. **2** is a photograph of an electrophoretic gel used to resolve NotI digests of clones of *Tetrahymena* genomic DNA clones produced in pNZKA, a linear vector of the invention. Migration of the left and right arms of the vector at 12 and 2.2 kb, respectively, is indicated.

FIG. **3** is a photograph of an electrophoretic gel used to resolve NotI digests of *Oxytricha* genomic clones produced in pNZKA, a linear vector of the invention.

FIG. **4** is a photograph of an electrophoretic gel used to resolve NotI digests of *Pneumocystis* genomic clones produced in pNZKA, a linear vector of the invention. FIG. **4**A shows the results of selection on kanamycin plus ampicillin and FIG. **4**B shows the results of selection on ampicillin only.

FIG. **5** is a photograph of an electrophoretic gel used to resolve NotI digests of a cone snail cDNA library contained in pNZKC vectors. Set A shows 0.3-0.7 kb inserts; set B shows 0.7-2 kb inserts. The lane labeled "M" designates a size marker; "V" designates empty vector control.

FIG. **6** is a photograph of an electrophoretic gel used to resolve uncut *Thauera selenatis* genomic DNA clones ligated in the presence of purified left arms of pNZKC. Aberrant clones migrating as circular molecules are indicated.

FIG. **7** is a photograph of an electrophoretic gel used to resolve NotI digests of *Tetrahymena* genomic DNA clones produced in pNZKC and selected on kanamycin plates (upper panel) or chloramphenicol plates (lower panel). Aberrant clones migrating as circular or deleted linear molecules are indicated. Migration of left and right arms of the linear vector are indicated.

FIG. **8** is a photograph of an electrophoretic gel used to resolve NotI digests of *Piromyces* sp. E2 genomic DNA clones produced in pNZOC and selected on chloramphenicol plates.

FIG. **9** is a schematic diagram showing the process of dual insert cloning using a linear vector of the invention (pNZ-Sfi). Vector and insert fragments are not drawn to scale.

FIG. **10** shows (A) a photograph of an electrophoretic gel used to resolve restriction digests of NZSfi dual-insert recombinants and (B) a schematic diagram showing expected restriction fragments.

DETAILED DESCRIPTION OF SEVERAL EMBODIMENTS

Before any embodiments of the invention are explained in detail, it is to be understood that the invention is not limited

in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the following figures and examples. The invention is capable of other embodiments and of being practiced or of being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting. The terms "including," "comprising," or "having" and variations thereof are meant to encompass the items listed thereafter and equivalents thereof as well as additional items.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a vector" includes a mixture of two or more vectors. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

All publications, patents and patent applications referenced in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

It also is specifically understood that any numerical value recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification. The values listed above are only examples of what is specifically intended.

Linear Vectors

In one embodiment, the invention provides a linear cloning vector derived from a bacteriophage (also referred to herein as "phage") that is capable of replicating in *E. coli* as an extrachromosomal linear plasmid. Suitably, the bacteriophage used to derive the cloning vector is a temperate phage, i.e., it has a characteristic lysogenic life cycle. As used herein, a cloning vector is said to be "derived from" a bacteriophage when a genomic structure is isolated from the bacteriophage (or its host cell) and subjected to further molecular manipulation to produce a linear cloning vector. Such molecular manipulation may include deletion of structural genes or regulatory sequences, and/or introduction of one or more sequences such as promoter, operator or enhancer sequences, restriction sites, telomeres, sequences encoding selectable markers and/or transcriptional terminator sequences. As will be appreciated, a cloning vector will be considered to be "derived from" a bacteriophage whether it is directly produced (e.g., purified from a colony) or indirectly produced (e.g., through multiple rounds of cloning and subcloning, PCR amplification or synthetic methods). Most suitably, the phage from which the linear vector is derived is suitably selected from lambda, N15, KO2, PRD1 or PY54.

The linear plasmid pKO2 present in *K. oxytoca* CCUC 15788 is a prophage that is related to *E. coli* phage N15 and *Yersinia* phage PY54 and that apparently has a lifestyle that is very similar to theirs. The N15, PY54, and ϕKO2 genomes are mosaically related. Some regions are sufficiently similar that the N15 and ϕKO2 plasmid partitioning proteins, replicase, protelomerases (hairpin end generation), and prophage repressors are thought to have the same or extremely similar target site specificities in both phages (Sherwood R. Casjens et al. (2004) *J. Bacteriol.* 186(6):1818-32).

The ϕKO2 early region, which is similar to but mosaically related to those of phages N15 and PY54, consists of 43 predicted genes in two large divergent operons and several smaller transcription units. As in other lambdoid phages, the putative ϕKO2 prophage repressor gene (gene 36 in Sherwood R. Casjens et al. (2004) *J. Bacteriol.* 186(6):1818-32) lies between the divergent early left and early right operons. It and the putative Cro repressor (encoded by gene 37 as shown in Sherwood R. Casjens et al. (2004) *J. Bacteriol.,* 186(6):1818-32) are 88 and 87% identical to the N15 cB repressor and Cro, respectively. This high level of similarity and the fact that their putative three OR and two OL operator binding sites all match the N15 5'-TTATAN$_6$TATAA early operator consensus (Ravin, V., N. Ravin, S. Casjens, M. E. Ford, G. F. Hatfull, and R. W. Hendrix. (2000) *J. Mol. Biol.* 299:53-73) suggest that N15 and ϕKO2 have the same repressor target specificity.

The linear cloning vectors of the invention include a left arm having a left telomere and a first selectable marker, a right arm having a right telomere and second selectable marker, and a cloning region located between the left arm and the right arm. As used herein, a "telomere" refers to a polynucleotide or polypeptide structure on the end (or ends) of a linear DNA molecule that protects the termini of the DNA from recombination and/or exonucleolytic degradation. Suitable telomeres include covalently closed ends, sequences capable of binding terminal proteins (e.g., as in PRD1), and tracts of polynucleotide repeats (e.g., poly A, C, G, T or U tracts). Examples of telomeres useful in constructing the linear vectors of the present invention include those derived from bacteriophages lambda, N15, KO2, PRD1 and PY54, as well as from some linear chromosomes, e.g., those from *Borrelia* spp. and *Agrobacterium tumefaciens.*

As used herein, a "selectable marker" refers to a phenotypic trait conferred on transformed cells that protects them from a selective agent in their environment, i.e., the growth media. Examples of selectable markers include, but are not limited to, antibiotic resistance markers (e.g., genes encoding resistance to kanamycin, ampicillin, chloramphenicol, gentamycin, or trimethoprim) and metabolic markers (e.g., amino acid synthesis genes or transfer RNA genes). As is appreciated in the art, the origin of replication can also be used as a selectable marker. In some cases, the first and second selectable markers will be antibiotic resistance markers, and will be different from each other. In other cases, the first or second selectable marker may be an origin of replication (Ori). Incorporating different selectable markers on each arm of the linear vector allows for simultaneous selection of both arms among recombinant clones. Selection for both arms ensures that the structure of the recombinants is correct, having exactly one left arm and one right arm.

The cloning region of the linear vector may include a restriction site, or may be a multiple cloning site (MCS) including more than one restriction site. One or more of the restriction sites are suitably unique restriction sites, i.e., they do not occur in the vector arms. Suitably, the cloning region may include a reporter stuffer region, e.g., the lacZα gene or a lethal gene. The reporter stuffer region may be flanked by restriction sites, or more suitably, MCSs, so that the entire reporter stuffer region may be replaced by one or more polynucleotides to be cloned. This configuration advanta-

geously permits cloning of coding sequences which may be toxic to the cells, because strong promoters in or adjacent to the reporter region are eliminated, thus preventing transcription (and subsequent translation) of the toxic insert. In some embodiments, the total cloning capacity of the vector is approximately 50 kb.

Optionally, the linear cloning vector includes two or more transcriptional terminator regions. As used herein and in the art, a "transcriptional terminator region," is a regulatory sequence which induces dissociation of a transcription complex in prokaryotic cells. In some embodiments, the linear cloning vector includes a pair of transcriptional terminator regions flanking the cloning region. The use of transcriptional terminator regions in this configuration reduces or eliminates transcription from the cloning region into the vector, thereby preventing interference with the function of the selectable markers, such as an antibiotic resistance coding sequence or origin of replication. Optionally, the linear cloning vector includes a third transcriptional terminator region after the selectable marker of the right arm to prevent transcription into the telomere region. Suitable transcriptional terminators are palindromic sequences which can form hairpin loop structures. The transcriptional terminator regions may be the same or different, but use of different transcriptional terminator regions may result in a more stable vector construct due to a reduced likelihood of deletions caused by recombination between identical terminator sequences. Transcriptional terminators may be unidirectional or bidirectional. Bidirectional terminators advantageously block transcription into the insert from vector promoters and into the vector from promoters within the insert. Suitably, the transcriptional terminator following the selectable marker on the right arm is a bidirectional transcriptional terminator. Most suitably, the transcriptional terminators are functional in the absence of host factors (i.e., are rho independent). Suitable transcriptional terminator sequences include the trpA terminator, T3 terminator, T7 terminator, rrnB T1 terminator, and others as described by Reynolds, et. al, J. Mol. Biol. (1992) 224:31-51, the disclosure of which is incorporated herein by reference in its entirety.

Exemplary suitable configurations for linear cloning vectors in accordance with the present invention are designated "pNZKA," (or "pJAZZ-KA," or "NZAN," SEQ ID NO:3) "pNZKC" (SEQ ID NO:45), and "pNZOC" (or "NZTC3," or "pJAZZ-OC," SEQ ID NO: 2). These linear vector constructs are shown schematically in FIG. **1**. The vectors may be provided in undigested form, or may be provided as pre-digested and dephosphorylated linear vector arms.

The linear cloning vectors described herein have at least four advantages over lambda and circular plasmid vectors. First, efficient ligation of insert to vector can be driven to completion by a molar excess of vector arms. In contrast, plasmid vectors may require numerous titrations to optimize the vector:insert ratio, as excess vector will result in independent vector molecules ligating to each end of the insert, creating a non-viable recombinant molecule. Second, in vitro lambda DNA packaging extracts limit the insert sizes to a narrow size range of approximately 35-45 kilobases (kb), whereas linear vector insertions have no minimum size, and the maximum size may be about 30-50 kb. Third, the linear vector maintains inserts as large as those of the bacteriophage lambda vector while simplifying use and production of vectors and recombinants. Fourth, the linear vector system can be used with a simple, conventional protocol for ligation, transformation and DNA isolation, and additional components are not required (e.g., lambda packaging extracts that are required for cosmid/fosmid cloning).

Conditions for high efficiency ligation favor the linear vector over circular plasmids. Formation of a circular recombinant plasmid occurs in a two step reaction: an intermolecular reaction between the plasmid and insert, followed by an intramolecular reaction between the ends of the hybrid molecule to form a circle. Ligation of circular plasmid vector and large insert DNAs are typically performed in dilute reactions of about 100-150 microliters to facilitate intermolecular joining of one insert molecule to one much smaller vector molecule. The requirement for subsequent recircularization favors smaller inserts over larger ones, requiring stringent size selection and vector dephosphorylation to achieve acceptable results. In contrast, ligation reactions occur most efficiently at high DNA concentrations or under macromolecular crowding conditions. Unfortunately, these conditions favor intermolecular joining, which is optimal for forming concatamers, but not useful for creating circular plasmids. A linear vector preparation contains a left and right vector arm, each with only one end capable of ligation, so high vector-to-insert ratios can be used to drive the joining reaction. Thus, linear vectors provide an improved method for generating large insert libraries by lowering the bias against large inserts.

Host Cells

In further embodiments, the invention provides host cells suitable for propagating the linear cloning vectors. A "host cell" is any cell that may be transformed with heterologous DNA, i.e., any cell that is a competent cell. Suitably, the host cell is an *E. coli* cell. Suitable strains of *E. coli* are known, e.g., DH10B cells or E. CLONI 10G cells (Lucigen, Middleton, Wis.). In some embodiments, host cells may be engineered to enhance transformation efficiency and/or maintenance of the linear vector.

Host cells may contain a coding sequence for a prokaryotic telomerase, which is referred to herein and in the art as "protelomerase" (or, alternatively, "telomere resolvase"), either on a conventional plasmid, or stably integrated into the host cell genome. A suitable protelomerase is the N15 protelomerase, referred to herein and in the art as "TelN." Optionally, host cells may express protelomerase prior to transformation with the linear cloning vector. Suitably, the transformation efficiency of linear vectors in host cells expressing a protelomerase such as TelN is 10-100 fold higher than in host cells not containing a protelomerase coding sequence.

In addition to a coding sequence for a protelomerase, host cells may further contain a coding sequence for partitioning proteins. The partitioning proteins suitably provide segregation stability to ensure accurate, non-random distribution of replicated linear plasmid molecules between the daughter cells, such that each daughter cell will receive the linear plasmid. The coding sequence for the partitioning proteins may be maintained in the host cells on a conventional plasmid, or stably integrated into the host cell genome. Suitably, the partitioning proteins are the sopA and sopB genes encoded by the sopBA region of the N15 genome.

In addition to a protelomerase coding sequence and/or a coding sequence for partitioning proteins, host cells may further contain a coding sequence for an antirepressor. One suitable antirepressor coding sequence is the N15 antirepressor gene (antA), which is known to counteract cB repression that, in turn, is believed to control the expression of RepA protein. Thus, induction of antA leads to higher expression of RepA, thereby stimulating N15 replication and increasing prophage copy number. The N15 antA gene

is suitably placed under the control of an inducible promoter and may be contained on a plasmid or stably integrated into the genome of the host cell.

In some embodiments, the host cell contains a coding sequence for a suitable polymerase for replication of the linear vector, either contained on a plasmid or stably integrated into the genome of the host cell. As an example, the coding sequence for the PRD1 polymerase (Bamford et al., Virology 183(2):658-676 (1991), the disclosure of which is incorporated herein by reference) may suitably be introduced into host cells designed to replicate linear vectors derived from PRD1.

Kits

In further embodiments, the invention provides kits containing a linear cloning vector of the invention and host cells, as described herein. Suitably, the host cells are electrocompetent or chemically competent cells modified to enhance transformation efficiency or maintenance of the linear cloning vector included in the kit. Linear cloning vectors provided in kits may be optionally pre-digested and dephosphorylated. Other optional components of the kits may include ligation buffer, ligase, control insert DNA for ligation, sequencing primers, restriction endonucleases, a phosphatase, a polymerase and/or a kinase. The kit may also suitably provide instructions for using the kit in accordance with the methods described herein.

Cloning Methods

In some embodiments, the invention provides methods of cloning a polynucleotide sequence. The polynucleotide sequence to be cloned is suitably linear, i.e., having a first end and a second end. The steps of the method include at least processing the linear cloning vector to separate the right arm from the left arm, ligating the first end of the polynucleotide sequence to the right arm and the second end of the polynucleotide sequence to the left arm to provide a ligation product, transforming a suitable host cell with the ligation product, and growing the transformed host cell on medium that selects for the first and second selectable markers of the linear cloning vector.

In some embodiments, linear cloning vectors of the invention are suitably used to clone at least two distinct polynucleotides, or insert sequences. These embodiments may be suitably employed, for example, in the cloning and expression of multi-subunit polypeptides, (e.g., the heavy and light chains of an antibody). Such vectors are also suitably used to analyze an interaction between two or more known polypeptides (e.g., a receptor and its ligand(s)), an interaction between a known polypeptide and unknown polypeptides produced from, e.g., a library; or an interaction between two or more unknown polypeptides produced from, e.g., one or more libraries. As will be appreciated by those of skill in the art, simultaneously cloning two or more inserts also provides a means of sequencing multiple sequences via one sequencing reaction, i.e., "multiplex sequencing."

The linear cloning vectors of the invention suitably provide capacity for simultaneously cloning at least two insert sequences. In some embodiments, three inserts may be cloned. In some embodiments, four inserts may be cloned. In some embodiments, five inserts may be cloned. In some embodiments six inserts may be cloned. In some embodiments, seven inserts may be cloned. In some embodiments, eight inserts may be cloned. In some embodiments, nine inserts may be cloned. In some embodiments, ten or more inserts may be cloned. It will be appreciated that the upper limit of the number of inserts that may be cloned using the linear cloning vectors of the invention depends on their collective size. In other words, the upper limit depends on the total capacity of the vector, e.g., 50 kb in some embodiments.

In some embodiments, at least one of the polynucleotides to be cloned is of unknown sequence. In particular embodiments, each of the polynucleotides is of unknown sequence.

In some embodiments, the sequence of at least one polynucleotide is known. In other embodiments, at least a portion of one of the sequence of at least one of the polynucleotides is known (e.g., 5, 10, 15, 20, 25 bases are known). In some embodiments, the polynucleotides to be cloned are derived from a "library," which herein refers to a collection of insert sequences derived from a source of DNA such as, e.g., an environmental source or a genome, or cDNA derived from a particular tissue or organism.

Methods of cloning at least two distinct polynucleotides, or insert sequences, include a step of processing each of the insert sequences to provide a linker sequence on both termini. A "linker sequence," as used herein, is a sequence of nucleotides that is compatible with another linker sequence in a ligation reaction. Each of the polynucleotides to be cloned are suitably processed to provide either: a) a linker sequence on one terminus that is compatible with a linker sequence on one of the vector arms and a terminus of one other insert sequence, or b) a linker sequence on each terminus that is compatible with a linker sequence on a terminus of two other insert sequences, or c) a linker sequence on each terminus that is compatible with a linker sequence on a terminus of one other insert sequence and one vector arm. A linker sequence may be provided, e.g., by restriction, PCR amplification and/or ligation of an oligonucleotide to the termini of the insert. The linker sequence is suitably less than 12 nucleotides in length. In some embodiments, the linker sequence is homopolymeric. Non-limiting examples of suitable linker sequences include AAA, TTT, CCC and GGG. Other examples include GTG, CAC, GTGT, and CACA. In some embodiments, one linker sequence is a blunt end. In some embodiments, the termini of each insert sequence are not compatible with each other, i.e., the insert sequences cannot self-ligate. Self-ligation may suitably be prevented by providing incompatible linker sequences on the termini or removing free 5' phosphate groups.

In a further step, the linear cloning vector of the invention is processed to separate the right and left arms. In an optional further step, the right arms may be purified away from the left arms. In a further step, the arms are treated to provide a linker sequence on the terminus opposite the telomere on each arm. Optionally, separated vector arms may be processed to prevent re-ligation, e.g., by treating the 5' ends with a phosphatase. In some embodiments, the vector arms are processed to provide "fixed orientation" multiple insert cloning, wherein the insert sequences can assemble only in a fixed orientation relative to each other and to the vector arms upon ligation.

In a further step, a ligation product is formed. The ligation product includes the insert sequences and the right and left vector arms, wherein the arms are noncontiguous with each other and are separated by the insert sequences. Most suitably, each of the insert sequences is present and present only once in the ligation product. In some embodiments, one ligation reaction provides a mixture of the desired ligation product (containing each insert and a right and left vector arm), as well as undesired ligation products lacking one or more of the inserts. However, one of skill in the art may readily determine which ligation product in the mixture is the desired product using standard techniques, for example,

sequencing or restriction analysis. The single-ligation embodiment of the invention is suitable for cloning fewer polynucleotides, e.g., two or three inserts; it is also suitable for cloning a larger number of inserts, e.g., concatamers of insert sequences.

Alternatively, the ligation product may be the ultimate product of multiple ligation reactions which may be employed in a suitable scheme based on the number of insert sequences. One suitable scheme for cloning two inserts is demonstrated in Example 12. Suitable schemes for cloning three or more inserts are also envisioned. Some schemes may employ linkers designed such that a specific end of each insert can be ligated only to a specific end of another insert. The resulting recombinants suitably have all inserts in a fixed orientation relative, to each other and to the vector. Ligation of a vector arm to an insert sequence suitably results in a ligation product that has only one end available for further ligation, the other end being the "inert" telomere. Thus, in some embodiments, iterative ligations are performed, wherein an additional insert sequence is iteratively added to the product of the previous ligation. A viable recombinant clone is produced only upon addition of a fragment containing the opposing vector arm. Suitable schemes for cloning additional inserts may be determined by those of ordinary skill in view of the present disclosure.

Further steps in the method of cloning at least two polynucleotides include transforming a host cell with the ligation product and growing the transformed host cell on medium, such that selection is provided for the first and second selectable markers of the linear cloning vector. It is appreciated that multiplication of the host cell results in cloning of each of the polynucleotides. Verification of the identity and orientation of the cloned polynucleotides may be accomplished by standard methods, such as, e.g., restriction analysis or sequencing.

The following examples are provided to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be further illustrative of the invention and are not limiting upon the reasonable scope of the appended claims.

EXAMPLES

Example 1

Construction of Host Strains for Linear Vectors

A standard, commercially available strain of competent *E. coli* (E. CLONI 10G, Lucigen, Middleton, Wis.) was used to prepare host cells for efficient transformation with linear vectors of the invention. To create one host strain (referred to herein as E. CLONI® 10G-iTel), the telN gene was PCR-amplified from phage N15 DNA (Ravin et al., J. Mol. Biol. 299(1):53-73 (May 2000), the disclosure of which is incorporated herein by reference) using the following primers:

telN-F:
(SEQ ID NO: 42)
GCGGATCCCGATATCCAGAGACTTAGAA
(BamHI site underlined)

telN-R:
(SEQ ID NO: 43)
CGAAGCTTCTTTTAGCTGTAGTACGTTTC
(HindIII site underlined)

The resulting PCR product was cloned into the BamHI/HindIII sites of pGZ119EH, which allows cloning of the target gene under control of IPTG-inducible Ptac promoter (Lessl et al., 1992, J. Bacteriol., 174: 2493-2500, incorporated herein by reference). The recombinant vector, designated pGZ-telN, expresses telN protein and encodes resistance to chloramphenicol. pGZ-telN DNA was transformed into E. CLONI 10G cells by electroporation to create the chloramphenicol resistant strain E. CLONI 10G-ptel.

To integrate the telN gene into the attB site in the chromosome of 10G cells, the fragment containing Ptac-telN was excised from pGZ-telN and cloned into the chromosome-integration vector pJW22 (Wild J, Hradecna Z, and Szybalski W (2002), Genome 12:1434-44, incorporated herein by reference), which encodes resistance to ampicillin. The resulting integration vector, pJW-telN, was digested with NotI to excise the fragment containing Ptac-telN, which was purified by gel-electrophoresis and circularized by self-ligation. The circularized fragment was transformed into E. CLONI 10G cells (Lucigen, Middleton, Wis.) carrying the integrase-producing plasmid pJW289t. Colonies that contained an integrated telN gene and which had lost the pJW289t plasmid were selected as described by Wild J, Hradecna Z, and Szybalski W (2002), Genome 12:1434-44, incorporated herein by reference. The resulting ampicillin resistant strain was designated E. CLONI 10G-iTel.

The influence of telN expression was tested by comparing the efficiency of transformation of E. CLONI 10G-iTel and parental E. CLONI 10G with linear pG591 DNA. The efficiency of transformation was 10- to 100-fold higher in E. CLONI 10G-iTel than in E. CLONI 10G. (Data not shown).

Next, the sopBA region of N15 along with a chloramphenicol resistance marker was integrated into the chromosome of E. CLONI 10G cells to create chloramphenicol resistant strain DH10B31sop. This strain also contains the anti-repressor AntA under control of the araBAD promoter. To add the telomerase gene to this strain, the ampicillin resistance gene of the plasmid pGZ-TelN was replaced with a gene encoding gentamycin resistance. This plasmid was transformed into DH10Bsop31 to generate strain GTS-8 (chloramphenicol and gentamycin resistant). GTS-8 allows highly efficient transformation with pNZKA, and copy number can be induced by addition of arabinose.

A cassette containing the telN gene, the sopBA operon, and the antA gene was integrated onto the chromosome of E. CLONI 10G cells as follows: a DNA fragment comprising phage N15 sopBA operon (under control of its own promoter) and the antA antirepressor gene (under control of arabinose-inducible $araP_{BAD}$ promoter) was excised from plasmid pCD31sop (Mardanov A. V., and Ravin N. V., Abstracts of the conference "Lomonosov-2004", v. 1, p. 21, Moscow, Russia (2004), the disclosure of which is incorporated herein by reference) as an XhoI-MroNI fragment and cloned into the HindIII site of plasmid pJWtelN, described above, which contains the telN gene inserted into the vector pJW22. The resulting vector, pJW-telN31sop, was partially digested with NotI to excise the fragment containing telN-sopBA-antA, which was purified by gel-electrophoresis and circularized by self-ligation. The circularized fragment was transformed into E. CLONI 10G cells carrying the lambda integrase-producing plasmid pJW289t. Colonies that contained an integrated fragment comprising telN gene, sopBA operon and antA antirepressor, and which had lost the pJW289t plasmid were selected as described by Wild J, Hradecna Z, and Szybalski W, Genome 12:1434-44 (2002), the disclosure of which is incorporated herein by reference. The resulting ampicillin resistant strain, designated E.

CLONI 10G-telN31S (or, alternatively, BIGEASY TSA Cells), allow efficient transformation with the linear vector and permit induction of copy number.

Example 2

Construction of Linear Vectors

a) Construction of NZCK3

A linear vector suitable for general cloning was derived from pG591 (SEQ ID NO:1). (Ravin et al., Nucleic Acids Res. 31(22):6552-60 (2003), the disclosure of which is incorporated herein by reference) pG591 was digested with NotI and treated with a mixture of DNA repair enzymes that generates blunt, phosphorylated ends (DNATERMINTOR® Kit, Lucigen, Middleton, Wis.). The 12 kb fragment containing the left telomere, telN, repA, and kanamycin resistance was gel isolated. pG591 was also digested with BglII, and the 1.3 kb fragment containing the right telomere was gel isolated.

A fragment containing the lacZalpha and ampicillin genes was constructed as follows: The lacZalpha gene of the vector pEZ BAC (SEQ ID NO: 15, nucleotides 155-598) was PCR amplified using the two overlapping forward primers T7RC-NotF (SEQ ID NO:16) and NSAS-LacZ-F (SEQ ID NO:17) plus the reverse primer NNASA-LacZ-R (SEQ ID NO:18) to create a fragment called TerZ. The ampicillin resistance gene was amplified from pSMART-HCAmp (SEQ ID NO:44, nucleotides 97-1063) by PCR with the two overlapping forward primers rrn-Fd (SEQ ID NO:19) and rrn-pCmF2 (SEQ ID NO:20) plus the reverse primer TonAmpR (SEQ ID NO:21). The resulting fragment was re-amplified with rrn-Fd and TonB-R (SEQ ID NO:22) to generate the fragment TAmpT. The TerZ and TAmpT fragments were each digested with NcoI and ligated. A band corresponding to the size of the ligation product of TerZ plus TAmpT was gel isolated and re-amplified with the primers T7del (SEQ ID NO:23) and TonBR2 (SEQ ID NO:24). This PCR product was ligated into the HincII site of pSMART HCKan, and excised from the vector by digestion with EcoRV and BglII. The EcoRV-BglII fragment was ligated to the blunt 11 kb fragment and the BglII fragment of pG591, generating the linear vector NZAN (SEQ ID NO:3, also referred to herein as "pJAZZ™.-KA").

The lacZ fragment of the vector NZAN was amplified by PCR with the primers LacANN-For (SEQ ID NO:25) and LacANN-Rev (SEQ ID NO:26). The resulting PCR product was re-amplified with the primers LacApSA-For (SEQ ID NO:27) and LacAsSA-Rev (SEQ ID NO:28). The product was digested with ApaI and AscI, ligated to the 12 kb ApaI fragment and the 2 kb AscI fragment of the vector NZAN, transformed into E. CLONI 10G-pTel cells, and selected on plates containing ampicillin plus kanamycin. The resulting linear vector was designated NZASA (SEQ ID NO:4).

To add additional cloning sites and binding sites for sequencing primers, the lacZ fragment was amplified from NZASA using primers LacE-SL1-F (SEQ ID NO:29) and LacA SR2-Rev (SEQ ID NO:30). The resulting PCR product was digested with AflIII, ligated to the end-repaired 10-kb NotI fragment and the 3-kb NcoI fragment of the vector NZASA, transformed into E. CLONI 10G-pTel cells, and selected on plates containing ampicillin plus kanamycin. The resulting linear vector was designated NZAhd (SEQ ID NO:5).

To create a version of the linear vector for use with BIGEASY TSA cells, the ampicillin resistance gene of NZAhd was replaced with a chloramphenicol resistance gene. The AhdI restriction site in the vector backbone was also destroyed to allow cloning into AhdI sites in the multiple cloning site. The resulting vector, designated NZCK3 (SEQ ID NO:6), was created by ligation of four fragments. The first (left-most) fragment was the 7.8 kb AhdI fragment of NZAhd encompassing the left telomere, telN gene, and part of the repA gene. The second fragment was a region of approximately 4.5 kb amplified from NZAhd by PCR with the primers 7847-F2 (SEQ ID NO:31), which introduces a mutation that destroys the AhdI site, and LacA-SR2-Rev (SEQ ID NO:30). This fragment was treated with Tfl DNA polymerase in the presence of dGTP to add a single G tail to the 3' termini. It was further digested with SpeI to remove the lacZ region from the right side of the fragment. The third fragment was a region of ~1.3 kb containing the lacZ region flanked by multicloning sites (MCSs), followed by the chloramphenicol resistance gene. This fragment was amplified from NZAhd by PCR with the primers LacE-SL1-F (SEQ ID NO:29) and CamTonB-Rev (SEQ ID NO:32); it was subsequently digested with SpeI and BglII. The fourth fragment was the 1.3 kb BglII fragment of NZAN that contains the right telomere. A ligation reaction containing these four fragments was transformed into E. CLONI 10G-pTel cells, and recombinants containing NZCK3 were selected on plates containing chloramphenicol plus kanamycin.

b) Construction of NZOC

A linear vector employing the origin of replication as selectable marker on the left arm and the chloramphenicol resistance gene as a selectable marker on the right arm was constructed. This vector, designated NZTC2 (SEQ ID NO:7), was created by ligation of three fragments. The first (left-most fragment) was a 10 kb XbaI fragment from NZASA (SEQ ID NO:4), containing the left telomere, telN gene, and repA gene. The XbaI restriction site was made blunt by treatment with T4 DNA polymerase in the presence of dNTPs. The second fragment, containing the lacZ gene and flanking DNA, was amplified from NZCK3 by PCR with the primers T7-RC-Del (SEQ ID NO:8) and pCmOR (SEQ ID NO:9) and digested with AscII. The third fragment was a ~2.2 kb AscI fragment from NZCK3 containing the chloramphenicol resistance gene and the right telomere. The ligation reaction of these fragments was transformed into E. CLONI 10G iTel cells (as prepared in Example 1) and plated on agarose containing chloramphenicol. The correct NZTC2 clone was confirmed by sequencing.

NZTC2 contained an AhdI site in the repA gene. A derivative lacking this site was created from three fragments. The first (left-most) fragment was the 7.8 kb AhdI fragment of NZAhd (SEQ ID NO:5) encompassing the left telomere, telN gene, and part of the repA gene. The second fragment was a region of approximately 4.5 kb amplified from NZTC2 by PCR, using as forward primers a mixture of NZg7847a-F2 (SEQ ID NO:10) and NZg7847a-F3 (SEQ ID NO:11), which introduce a mutation that destroys the AhdI site, and the reverse primer NZ-RevB (SEQ ID NO:12). This fragment was re-amplified with NZg7847a-F2 as the forward primer and a mixture of NZ-RevA (SEQ ID NO:13) and NZ-RevC (SEQ ID NO:14) as reverse primers. This fragment was treated with Tfl DNA polymerase in the presence of dGTP to add single 3' G overhangs to the ends, and further digested with SwaI to generate a blunt site on the right side of the fragment. The third fragment was the approximately 2.2 kb SwaI fragment of NZTC2 that contains the right telomere. The ligation reaction of these three fragments was transformed into E. CLONI BIGEASY TSA cells, and recombinants containing NZOC (also referred to

as "NZTC3" or "pJAZZ-OC") (SEQ ID NO:2) were selected on plates containing chloramphenicol. The correct clone was confirmed by sequencing.

Example 3

Construction of a *Tetrahymena thermophila* 6-20 kb Genomic Library

T. thermophila is a free-living, widely distributed, ciliated protozoan. The cellular, structural, and functional complexity of this organism is comparable to that of human and other metazoan cells. The macronuclear (somatic) genome consists of 160 Mb processed in vivo into ~300 sub-chromosomal fragments. Constructing libraries with inserts of >6 kb is extremely problematic for this genome, presumably because the AT content ranges from 75-85%.

The linear pNZKA vector was used to successfully clone libraries of large AT-rich fragments. A library of 5-10 kb fragments of the *Tetrahymena* genome was created by ligation of sheared, end-repaired macronuclear DNA to a blunt digest of pNZKA. Of 54 clones analyzed, 51 had inserts of the expected size (data not shown).

A library was also successfully created with clones in the range of 6-20 kb, which to our knowledge, represents a library of the largest *Tetrahymena* fragments ever cloned. Genomic DNA was sheared to 6-20 kb, end-repaired, gel-purified, and ligated to a SmaI digest of pNZKA. Ligations were electroporated into DH10B31sop cells and selected on plates containing kanamycin plus ampicillin. The clones produced large colonies on selective plates, grew vigorously in culture, and yielded relatively high amounts of linear plasmid DNA from standard alkaline lysis minipreps. One-fifth of the DNA from each miniprep was incubated with NotI to excise the insert and subjected to gel electrophoresis. The results are shown in FIG. **2**. Vector bands are 12 kb and 2.2 kb. Inserts are in the range of 6-20 kb. Furthermore, sequencing reactions required only 150 ng of DNA from clones made with the linear vector.

Example 4

Oxytricha trifallax 8-20 kb Genomic Library

Another genome that has been very problematic to clone is that of the ciliated protozoan *Oxytricha trifallax*. The DNA in the somatic macronucleus of *Oxytricha* is processed in vivo into "nanochromosomes" of ~2-40 kb (75% AT), each fragment typically containing a single gene. Using circular vectors, previous attempts have been made to create a library of this DNA has been created for genomic sequencing, with the largest cloned insert being less than ~6 kb.

To make a large-insert genomic library of the *Oxytricha* macronuclear genome, the DNA was end-repaired to generate blunt ends, size selected to 8-20 kb, and ligated to pNZKA. The ligation was transformed into *E. coli* DH10B31sop cells, which contain the SopBA region, and into E. CLONI 10G-pTel, prepared as in Example 1. The transformed cells were plated on media containing kanamycin and ampicillin, to select for both arms of the vector. (Both cell lines are resistant to chloramphenicol.) E. CLONI 10G-pTel yielded approximately 12-fold more colonies than the DH10B31sop cells. For each library, 18 clones were analyzed. As shown in FIG. **3**, 15-17 clones had inserts in the range of 8-20 kb, and vector bands are 12 kb and 2.2 kb. A library with *Oxytricha* inserts of this size has not been created previously.

Example 5

Construction of a *Pneumocystis carinii* 8-20 kb Genomic Library

The pNZKA vector was used to clone the genomic DNA from *Pneumocystis carinii*, the causative agent of a severe pneumonia in immuno-compromised patients. The epidemiology of *P. carinii* infection is poorly understood and its life cycle remains obscure. Large-scale sequencing of the *P. carinii* genome will help elucidate the molecular basis of the pathogenicity and speed development of drug and vaccine targets. Cloning the DNA of *P. carinii* into circular vectors has previously proven problematic, even for small fragments.

Genomic *P. carinii* DNA was sheared to 8-20 kb, end-repaired to generate blunt ends, size selected to 8-20 kb, and ligated to SmaI-digested pNZKA. The ligation was transformed into *E. coli* DH10B31sop and into E. CLONI 10G-pTel. To show that the origin of replication can be used successfully as a selectable marker, the library was plated on media containing kanamycin plus ampicillin (FIG. **4**A) and on media containing only ampicillin (FIG. **4**B). Colonies were randomly picked for analysis. Each lane in FIG. **4** contains $\frac{1}{5}^{th}$ of the DNA from a 1.5 ml miniprep, cut with NotI to excise the insert. Vector bands are 12 kb and 2-2 kb. Inserts were in the range of 8-20 kb. As shown in FIG. **4**, both the number of colonies and the fraction of recombinants clones with the correct structure remained unchanged, regardless of the presence of kanamycin. This result indicates that the origin of replication can serve as a selectable marker, as it is essential for viability of the clones. Therefore, drug selection for the left arm of the vector, which contains the origin of replication, is redundant.

Example 6

Introduction of a Selectable Marker on the Right Arm Results in Fewer Non-Recombinants

To investigate the effects of a selectable marker on the right arm of the vector, a control insert of 2 kb, containing the lacZα gene fragment, was ligated to the vector pNZKC. Both the vector and the insert were digested with NotI, and the vector was further treated by dephosphorylation. The vector preparation was also self-ligated or unligated (i.e., incubated without ligase or insert DNA). The ligations were transformed into E. CLONI 10G-iTel cells, which are ampicillin resistant, and plated on chloramphenicol to select for the right arm of the vector. They were also transformed into E. CLONI 10G-pTel cells, which are chloramphenicol resistant, eliminating any selection for the right arm of the vector. Transformation into cells that allow selection for the right arm of the vector resulted in fewer than 0.1% non-recombinants, whereas lack of right arm selection led to nearly 20% non-recombinant, white colonies, as shown in Table 1:

TABLE 1

Cloning into the linear vector pNZKC with or without selection for the right vector arm.

E. cloni 10G-iTel(AmpR)			E. cloni 10G-iTel(CamR)
Pos. Control	Self-ligated	Unligated	Pos. Control
1360 Blue 1 White	0 Blue 0 White	1 Blue 0 White	300 Blue 70 White

Example 7

Construction of a Cone Snail cDNA Library

An example of a particularly difficult insert to clone is the cDNA derived from the poison duct of the cone snail (*Conus* sp.). cDNA was generated from cone snail poison duct RNA, end-repaired, and fractionated into size ranges of 0.3-0.7 kb ("set A") and 0.7-2 kb ("set B"). Linkers were ligated to the cDNA, and it was amplified by PCR using primers complementary to the linkers. The PCR products were ligated into pNZKC, transformed into E. CLONI iTel cells, and plated on kanamycin plus chloramphenicol media. Plasmid DNA was isolated from randomly chosen colonies, digested with NotI, and size fractionated using gel electrophoresis.

As shown in FIG. **5**, the linear vector produced only clones in the expected size range of 0.3 to 2 kb. Ligation and transformation of the cone snail cDNAs into conventional circular plasmids resulted in predominantly empty vectors or inserts of <100 bp (data not shown).

Example 8

Linear Vectors Containing Only the Left Arm Convert to a Circular Plasmid

pNZKC was digested with SmaI, and the 12 kb left arm was gel purified away from the lacZ stuffer region and the right arm. 1-2 kb fragments of DNA isolated from the genome of *Thauera selenatis* were prepared by shearing (using a HYDROSHEAR device, Gene Machines, San Carlos, Calif.). The fragments were end-repaired, gel-purified, and ligated to a SmaI digest of the purified left arm of pNZKC. Ligations were electroporated into E. CLONI 10G-iTel cells and selected on plates containing kanamycin. Eighteen kanamycin resistant colonies were randomly picked for analysis and $\frac{1}{5}^{th}$ of the DNA from a 1.5 ml miniprep was resolved using agarose gel electrophoresis.

The results, shown in FIG. **6**, show that at least 10 out of the 18 clones were converted to a circular plasmid, showing supercoiled and relaxed circular forms, while the remaining 8 clones appeared to be linear. In all 18 cases, the clones were not able to survive on plates containing chloramphenicol, indicating they lacked a right arm. Aberrant clones are indicated by "*" in FIG. **6**.

Example 9

Selection for Both Left and Right Arms Favors the Linear Vector Form

Tetrahymena genomic DNA was sheared to 4-10 kb, end-repaired, gel-purified, and ligated to the left and right arm of a SmaI digest of pNZKC. Ligations were electroporated into E. CLONI 10G-iTel cells and selected on plates containing kanamycin only (which selects only for the left arm of the vector) or kanamycin plus chloramphenicol (which selects for both arms of the vector). Colonies were randomly picked for analysis, and ⅕ of the DNA from a 1.5 ml miniprep was cut with NotI to excise the insert and resolved using agarose gel electrophoresis.

As shown in FIG. **7**, when the right arm was not selected by chloramphenicol, 8 out of 18 clones appeared to be linear molecules missing the expected 2.2 kb NotI fragment, which represents the right arm of the vector (FIG. **7**, upper panel), and 2 of the clones (lanes 6 and 10) appeared to be circular plasmids instead of linear vector. In contrast, when the right arm was under selection by chloramphenicol, 17 out of 18 clones contained the expected 2.2 kb NotI right arm fragment (FIG. **7**, lower panel). Since the origin of replication is essential for viability of the vector, the left arm is under selection regardless of the antibiotic used.

Example 10

Improved Transformation Efficiency with Strains Containing the telN Gene

Genomic DNA from *Oxytricha trifallax* and from *Pneumocystis carinii* was sheared to 8-20 kb, end-repaired, gel fractionated, and purified. The linear vector pNZKA was digested with SmaI and dephosphorylated. Approximately 300 ng of each prepared genomic DNA was ligated in separate reactions to 50 nanograms of the prepared linear vector. The ligation reactions were heat-inactivated and transformed into host strains that had been rendered electrocompetent. The host strains included E. CLONI 10G-pTel, which contains a telN protelomerase coding sequence, and DH10B31sop cells, which does not contain a telN coding sequence. One-tenth of the transformed cells were plated onto media containing kanamycin, ampicillin, XGAL, and IPTG. After overnight growth, each ligation reaction yielded ~12-fold more colonies in the telN strain (Table 2).

TABLE 2

Improved transformation efficiency of the linear vector in a host strain containing telN.

	pNZKA plus *Pneumocystis* DNA	pNZKA plus *Oxytricha* DNA
E. CLONI 10G-ptelN	800	2400
DH10B31sop	62	200

In separate experiments, the two host strains were shown to have similar transformation efficiency when electroporated with pUC19 DNA, indicating the ability to take up DNA was similar for the two strains. The linear vector was maintained in both strains of cells after several rounds of freezing, dilution, and re-growth, indicating that the linear plasmid was stably maintained in both strains.

Example 11

Construction and Use of a Single-Antibiotic-Resistant Linear Vector

Piromyces sp. E2 is a fungus of the phylum Chytridiomycota. The genomic DNA from this microbe is approximately 85% AT, and cloning fragments even as small as 2 kb is very difficult in standard circular vectors. In contrast, fragments of this genome as large as 2-6 kb could be successfully cloned in the NZOC vector.

Ten micrograms of *Piromyces* DNA was physically sheared to 2-6 kb using a HydroShear Device (Gene Machines), and the ends were repaired using the DNATERMINATOR® kit (Lucigen, Middleton, Wis.). The DNA was quantitated against a DNA mass standard using gel densitometry software (Alpha Innotech, San Leandro, Calif.), and ligated to a blunt digest of the pJAZZ® OC vector. The ligation reaction contained approximately 100 ng of insert DNA, 50 ng of digested pJAZZ® OC, ligase buffer, and 2 U T4 DNA ligase. The reaction was incubated at room

temperature for 2 hours, heat treated for 15 minutes at 70 .degree. C., and used to transform electrocompetent E. CLONI® BIGEASY™ TSA cells. Cells were spread on to an agar plate containing 12.5 ug/ml chloramphenicol, XGAL, and IPTG. Linear plasmid DNA was isolated using standard alkaline lysis purification with binding to diatomaceous earth. The DNA was digested with Not I and assayed by agarose gel electrophoresis. As shown in FIG. **8**, nearly all of the recombinant clones contained inserts of 2-6 kb. Twelve samples were sequenced to confirm that they contained genomic DNA from *Piromyces*. The AT content of some of these clones approached 96%; obtaining clones with this level of AT content has not been reported previously.

Example 12

Dual-Insert Cloning in a Linear Vector

The present Example describes construction of a dual-insert library in a linear vector. The insert DNAs were defined fragments of 10 kb amplified from *E. coli* genomic DNA by PCR using the Phusion polymerase (New England Biolabs) according to the manufacturer's recommendations. The primers used for PCR amplification were:

```
Primer 1:
                                        (SEQ ID NO: 33)
TTCTTATGGCCAGGGAGGCCGCTCTGGGTATAAGCGTAAGG

Primer 2:
                                         SEQ ID NO: 34)
AACTAGTGGCCAGGGAGGCCATCAGCCAGGCGACGAATCAG

Primer 3:
                                        (SEQ ID NO: 35)
GGACTTGGGCCACCCAGGCCTTGTAAATGCAGTATGGATTG

Primer 4:
                                        (SEQ ID NO: 36)
ATCCTAGGGCCACCCAGGCCAGATATTGGAGAGTTGGACCAG
```

One PCR product, termed "EC39" was amplified using Primers 1 and 2; a second product, "EC40," was amplified using Primers 3 and 4. The primers also contain the recognition site for the restriction enzyme SfiI (underlined above), which after digestion leaves a 3 base pair overhang on the 3' strand of the double-stranded DNA amplification product.

EC39 was digested with SfiI to produce a 3' extension of -CCC; digestion of EC40 by SfiI created a 3' extension of -GGG. The digested products of the EC39 insert are therefore not able to ligate to themselves to form concatamers. Similarly, the digested EC40 products cannot self-ligate. Consistent with the scarcity of SfiI sites in most genomes, the regions chosen for amplification do not have internal SfiI sites. The 10 kb SfiI digestion products were purified and quantitated.

Vector pNZ-Sfi (SEQ ID NO:37) was derived from pNZKA by replacing the multiple cloning sites and the lacZ stuffer of pNZKA with a DNA fragment containing the lacZ stuffer flanked by different multiple cloning sites, including sites for the restriction enzyme SfiI. The new lacZ stuffer was generated by PCR amplification of the lacZ region of the vector NZAhd using the primers lacFSfi (SEQ ID NO:38) and lacRSfi (SEQ ID NO:39). The primers were phosphorylated with T4 polynucleotide kinase prior to performing the PCR. The resulting PCR product was purified and ligated to a SmaI digest of the vector NZAhd.

The ligation reaction was transformed into E. cloni GTS-8 cells, and transformants were selected on agarose plates containing ampicillin, kanamycin, XGAL, and IPTG. The correct pNZ-Sfi clone was confirmed by sequencing.

The SfiI sites of the vector were designed such that digestion with SfiI creates a 10-kb left arm with a 3' extension of -GGG, a 2-kb right arm with a 3' extension of -CCC, and a 0.5 kb lacZ stuffer fragment. The -GGG extension on the left arm is compatible with the -CCC extension created by SfiI digestion of EC39; similarly, the right arm is compatible with the SfiI digest of EC40. The 5' phosphates were removed from the vector SfiI fragments by treatment with Calf Intestinal Phosphatase to prevent religation of the vector arms. The digested vector fragments were fractionated on an agarose gel, and the bands were individually excised, purified, and quantitated.

Dual insert cloning was performed as diagrammed in FIG. **9**. The SfiI-digested left arm was ligated to an equimolar amount of SfiI-digested EC39. In a separate ligation reaction, the SfiI-digested right arm was ligated to an equimolar amount of SfiI-digested EC40. After allowing the ligation reactions to proceed to at least 50% completion, aliquots of the two ligation reactions were combined with each other. Further incubation was carried out to facilitate ligation of the left arm/EC39 molecules to the EC40/right arm molecules. The final ligation reaction was heat-inactivated, and the products were transformed into GTS 8 cells (Lucigen, Middleton, Wis.). An additional ligation reaction was performed with only the Sfi-I-digested left and right vector arms to measure the frequency of self-ligation.

One-tenth of the transformants were plated on media containing kanamycin and ampicillin to select for both arms of the linear vector. The plates also contained XGAL plus IPTG to screen against uncut vector or recombinants containing the lacZ stuffer fragment. The dual-insert ligation/transformation reaction produced ~2200 white colonies and 26 blue colonies. The self-ligation/transformation yielded 208 white colonies and 23 blue colonies.

Thirty white colonies were randomly selected from the dual-insert plate, and grown overnight in TB media containing kanamycin plus arabinose Plasmid DNA was isolated by standard alkaline lysis methods, and restriction-digested with SpeI, NgoMIV, or NotI. SpeI has a single recognition site near the cloning site of the left arm and one site within the EC40 fragment. NgoMIV has a single site within the EC39 fragment, but no sites in the vector or in EC40. NotI has a site near each of the cloning sites, and no sites within EC39 or EC40; it therefore excises the entire dual insert. In all thirty clones analyzed, restriction analysis with these enzymes confirmed the presence of exactly one copy of each fragment and each vector arm in the expected relative positions of Left arm-EC39-EC40-Right arm, as shown in FIG. **10**.

Example 13

Derivation of a Linear Vector from Phage PRD1

Genomic DNA from phage PRD1 is digested with BsrBI to remove the left telomere and its associated terminal protein from the genomic DNA. The 3-kb BsrBI fragment is isolated by agarose gel electrophoresis. Another aliquot of phage genomic DNA is digested with Xmni to remove the right telomere and its associated protein. The 1-kb XmnI fragment is isolated by agarose gel electrophoresis. PCR is used to amplify a DNA fragment containing a selectable marker and, optionally, a visual screening marker. Creation of such a fragment, containing the TAmpT and TerZ segments, is described in Example 2.

The DNA polymerase of phage PRD1 (GenBank ACCESSION NC 001421) is amplified by PCR with the primers PRD1 POL-F (SEQ ID NO:40) and PRD POL R (SEQ ID NO:41). The 1.7 kb PCR product is purified, digested with SphI, and cloned into a bacterial expression vector (e.g., pET24, Novagen).

The PRD POL expression vector is transformed into E. CLONI cells, and a clone expressing the PRD1 polymerase gene is confirmed by sequence analysis. Expression of the PRD1 polymerase is verified by presence of an additional band at approximately 65 kD on an acrylamide gel.

Alternatively, the PRD1 polymerase gene is appended to a promoter sequence and integrated into the genome of E. CLONI cells, using e.g. methods described for integration of the telN gene in Example 1.

The PRD1 expression clone is made competent by standard techniques. A ligation reaction containing the 1-kb BsrBI fragment, the TAmpT-TerZ fragment, and the 3-kb XmnI fragment is transformed into the competent PRD1 expression cells. Colonies that express blue color and are ampicillin resistant are selected for further growth. The presence of the PRD-AmpLacZ vector is confirmed by restriction analysis and sequencing of plasmid DNA.

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ttcaagtcta atgg 74

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<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer NZ-RevC

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<210> SEQ ID NO 17
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer NSAS-LacZ-F

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cgggcagtg 69

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cgccattcag 70

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<223> OTHER INFORMATION: Primer rrn-pCmF2

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<220> FEATURE:
<223> OTHER INFORMATION: Primer TonB-R

<400> SEQUENCE: 22

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<210> SEQ ID NO 23
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer T7del

<400> SEQUENCE: 23

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<210> SEQ ID NO 24
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<212> TYPE: DNA
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ttaccaatgc ttaatcagtg aggcacc 87

<210> SEQ ID NO 25
<211> LENGTH: 48
<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Primer LacANN-For

<400> SEQUENCE: 25

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<210> SEQ ID NO 26
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<210> SEQ ID NO 27
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<223> OTHER INFORMATION: Primer LacApSA-For

<400> SEQUENCE: 27

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<210> SEQ ID NO 28
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<210> SEQ ID NO 29
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<210> SEQ ID NO 30
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<212> TYPE: DNA
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<223> OTHER INFORMATION: Primer LacA SR2-Rev

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<212> TYPE: DNA
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<220> FEATURE:
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<210> SEQ ID NO 32
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<220> FEATURE:
<223> OTHER INFORMATION: Primer CamTonB-Rev

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<210> SEQ ID NO 33
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<220> FEATURE:
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<400> SEQUENCE: 33

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<210> SEQ ID NO 34
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
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<400> SEQUENCE: 34

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<210> SEQ ID NO 35
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<212> TYPE: DNA
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<220> FEATURE:
<223> OTHER INFORMATION: Primer 3

<400> SEQUENCE: 35

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<210> SEQ ID NO 36
<211> LENGTH: 42
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: Primer 4

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<210> SEQ ID NO 37
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<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Vector pNZ-Sfi

<400> SEQUENCE: 37

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The invention claimed is:

1. A linear cloning vector comprising:

a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site;

a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site;

a cloning region located between the left arm and the right arm; and

an origin of replication.

2. The vector of claim **1**, further comprising a pair of transcriptional terminator regions flanking the cloning region, wherein the transcriptional terminator regions are the same or different and are unidirectional or bidirectional.

3. The vector of claim **2**, further comprising a transcriptional terminator region after the second selectable marker, wherein one of the pair of transcriptional terminator regions flanking the cloning region and the transcriptional terminator region after the second selectable marker together flank the second selectable marker.

4. The vector of claim **1**, wherein the cloning region comprises one or more restriction sites, wherein each of the one or more restriction sites is unique.

5. The vector of claim **1**, wherein the cloning region comprises a multiple cloning region, wherein the multiple cloning region comprises a plurality of restriction sites, wherein each of the plurality of restriction sites in the multiple cloning region is unique.

6. The vector of claim **1**, wherein the cloning region comprises a stuffer region.

7. The vector of claim **6**, wherein the stuffer region comprises a reporter gene flanked by a pair of restriction sites which are the same or different.

8. The vector of claim **1**, wherein the first and second selectable markers are antibiotic resistance markers or wherein the first or second selectable marker is the origin of replication.

9. The vector of claim **1**, further comprising one or more genes outside the cloning region, the one or more genes being selected from the group consisting of a replication initiation protein gene and a prophage repressor gene.

10. The vector of claim **1**, further comprising genes outside the cloning region, the genes comprising a replication initiation protein gene and a prophage repressor gene.

11. The vector of claim **10**, wherein the cloning region comprises one or more restriction sites, wherein each of the one or more restriction sites is unique.

12. The vector of claim **1**, wherein the first and second selectable markers are antibiotic resistance markers.

13. A host cell comprising:

a linear cloning vector comprising:

a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication;

a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and

a cloning region located between the left arm and the right arm; and

a polynucleotide sequence encoding a protelomerase, wherein the polynucleotide sequence encoding the protelomerase is integrated into the host cell genome.

14. A kit comprising:

a linear cloning vector comprising:

a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication;

a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and

a cloning region located between the left arm and the right arm; and

a recombinant host cell comprising a polynucleotide sequence encoding a protelomerase, wherein the polynucleotide sequence is integrated into the host cell genome.

15. A method of cloning a polynucleotide sequence having a first end and a second end using a linear cloning vector, the linear cloning vector comprising: a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication; a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and a cloning region located between the left arm and the right arm, the method comprising:

a) processing the linear cloning vector in the cloning region to separate the right arm from the left arm;

b) joining the first end of the polynucleotide sequence to the right arm and the second end of the polynucleotide sequence to the left arm to provide a joined product;

c) transforming a host cell with the joined product; and

d) growing the transformed host cell on medium, such that selection is provided for the first and second selectable markers of the linear cloning vector.

16. The method of claim **15**, wherein the host cell used in step c) is a recombinant host cell comprising a polynucleotide sequence encoding a protelomerase, wherein the polynucleotide sequence is integrated into the host cell genome.

17. The method of claim **15**, wherein the cloning region comprises one or more restriction sites, wherein each of the one or more restriction sites is unique.

18. A linear cloning vector comprising:

a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication;

a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and

a cloning region located between the left arm and the right arm, wherein the cloning region comprises one or more restriction sites, wherein each of the one or more restriction sites is unique.

19. A host cell comprising the vector of claim **18**.

20. The host cell of claim **19**, further comprising a polynucleotide sequence encoding a protelomerase integrated into the host cell genome.

21. The host cell of claim **19**, further comprising a polynucleotide sequence encoding a partitioning protein, a polynucleotide sequence encoding an antirepressor, or combinations thereof.

22. The vector of claim **18**, further comprising a pair of transcriptional terminator regions flanking the cloning region, wherein the transcriptional terminator regions are the same or different and are unidirectional or bidirectional.

23. The vector of claim **22**, further comprising a transcriptional terminator region on a side of the second selectable marker distal from the cloning region, wherein one of the pair of transcriptional terminator regions flanking the cloning region and the transcriptional terminator region on the side of the second selectable marker distal from the cloning region together flank the second selectable marker.

24. The vector of claim **18**, wherein the cloning region comprises a multiple cloning region, wherein the multiple cloning region comprises a plurality of restriction sites, wherein each of the plurality of restriction sites in the multiple cloning region is unique.

25. The vector of claim **18**, wherein the cloning region comprises a stuffer region.

26. The vector of claim **25**, wherein the stuffer region comprises a reporter gene flanked by a pair of restriction sites which are the same or different.

27. The vector of claim **18**, wherein the second selectable marker is an antibiotic resistance marker.

28. The vector of claim **18**, further comprising one or more genes outside the cloning region, the one or more genes being selected from the group consisting of a replication initiation protein gene and a prophage repressor gene.

29. The vector of claim **18**, further comprising genes outside the cloning region, the genes comprising a replication initiation protein gene and a prophage repressor gene.

30. A method of cloning at least two distinct polynucleotides using a linear cloning vector, the linear cloning vector comprising: a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication; a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and a cloning region located between the left arm and the right arm, the method comprising:

a) processing each of the polynucleotides to provide a linking sequence on both termini of the polynucleotides;

b) processing the linear cloning vector in the cloning region to provide a linking sequence on a terminus opposite the telomere of each arm;

c) forming a joined product comprising the polynucleotides and the right and left arms, wherein the arms are noncontiguous with each other and are separated by the polynucleotides;

d) transforming a host cell with the joined product; and

e) growing the transformed host cell on medium, such that selection is provided for the first and second selectable

markers of the linear cloning vector, wherein multiplication of the host cell results in cloning of the polynucleotides.

31. A method of cloning a polynucleotide sequence using a linear cloning vector, the linear cloning vector comprising: a left arm comprising a left telomere and a first selectable marker, wherein the left telomere comprises a covalently closed end comprising a protelomerase target site and wherein the first selectable marker is an origin of replication; a right arm comprising a right telomere and a second selectable marker, wherein the right telomere comprises a covalently closed end comprising a protelomerase target site; and a cloning region located between the left arm and the right arm, the method comprising:

a) inserting the polynucleotide sequence between the right arm and the left arm of the linear cloning vector to provide a joined product; and

b) growing a host cell comprising the joined product, such that selection is provided for the first and second selectable markers of the linear cloning vector.

32. The method of claim **31**, wherein the cloning region comprises one or more restriction sites, wherein each of the one or more restriction sites is unique.

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