MEANS FOR GENERATING ADENOVIRAL VECTORS FOR CLONING LARGE NUCLEIC ACIDS

Field of the invention

The present invention is related to a first nucleic acid molecule, a second nucleic acid molecule, a third nucleic acid molecule, a combination of the first and the second nucleic acid molecule, a combination of the second and the third nucleic acid molecule, a fourth nucleic acid molecule, a fifth nucleic acid molecule, methods for the generation of nucleic acid molecules coding for a virus, methods for the generation of a library of nucleic sequences, a plurality of the fourth nucleic acid molecule, a plurality of the fifth nucleic acid molecule, a plurality of individual adenoviruses and kits containing at least one of these nucleic acid molecules.

Background of the Invention

The development of recombinant viruses for gene expression since the '80s led to their widely application as gene expression vectors in vitro as well as in vivo. Cloning and expression of numerous genes, including non-coding nucleic acids such as small interfering RNAs using viral or non-viral expression libraries, is recognized as a most powerful tool in functional genomics and already led to the discovery and validation of new drug target genes. Generating virus-based expression libraries requires a cloning procedure yielding a large number of accurate clones, preferably with no need for screening positive recombinants, and ensuring stability of the viral genomes in the DNA-based constructs during amplification.

Particularly, preferred viral vectors are adenoviral vectors. The construction of adenoviral vectors can be effected by various means. The first protocols provided in the literature involved co-transfection of permissive cells, usually gene complementing cell lines such as 293 or 911 cells, with a shuttle plasmid containing the left end of the viral genome, where the E1 region typically was replaced with foreign DNA, and isolated viral DNA cut near the left end of the genome by an appropriate restriction enzyme. Homologous recombination occurs in vivo between overlapping sequences of the shuttle plasmid and the adenoviral DNA yielding a recombined virus genome that can replicate. The applicability of this

technology for vector construction is limited by the inefficient transfection of large isolated viral DNA fragments and moreover vector preparations can be contaminated by wild type adenoviruses due to only partial digestion of the adenovirus DNA.

One variation of this system comprises the use of two plasmids each providing a part of the adenovirus genome individually unable to replicate which are co-transfected into the complementing production cell line to produce replicable viral DNA through homologous recombination. This method has been described in detail (Bett et al. J. Virol. 67:5921-5921, 1993). The disadvantage of wild type virus contamination, also referred to as wt-Virus contamination, has been overcome by this variation. The use of this method to generate large numbers of recombinant adenovirus vectors is limited by the low recombination efficiency and transfection efficiency of large vector DNAs in producer cells such as 293, however. In general, adenovirus vector construction through homologous recombination between two DNA entities in eukaryotic cells supporting replication of E1-deleted adenoviruses is time consuming, and requires screening and purification of individual virus clones by plaque purification.

Site-specific recombinases as involved in the recombination processes of the viral DNA fragments, are proteins that have both endonuclease and ligase properties and exist in multiple organisms. These recombinases recognize specific sequences of bases in DNA and exchange the DNA segments flanking those segments. Thus the resulting recombination product either consists as an insertion of the first nucleic acid into the second nucleic acid. In such case the plasmids are circular plasmids containing one recombinase recognition sequence on each nucleic acid. Alternatively, there is an excision of the nucleic acid fragment in between two recombinase recognition sequences on the same nucleic acid, or an exchange of parts of nucleic acids between two nucleic acids having each of the exchanged nucleic acid in between two recognition sites present on each of the nucleic acids. Two plasmids having each one *lox*P, or other recombinase binding sites, such as, e.g., a Frt recombinase recognition site, will form a mixture of monomer, dimer, trimer, ect. product. Numerous recombination systems from various organisms have been described. (Landy A., Curr Opin Genet Dev. 3:699-707, 1993; Hoess RH., et al. Proc. Natl. Acad. Sci. USA 79:3398-3402,1982; Abremski et al., J Biol Chem 261:391-396, 1986; Esposito D, Scocca

JJ, Nucl Acids Res 25:3605-3614, 1997). The best-studied members of the integrase family of recombinases are the Integrase/att system from bacteriophage lamda, (Landy A., Current Opinions in Genetics and Devel. 3:699-707, 1993$_4$, the Cre/loxP system from bacteriophage P1 (Hoess RH, Abremski K. "The Cre-lox Recombination System," (1990) In Nucleic Acids and Molecular Biology, vol. 4. Eds.: Eckstein and Lilley, Berlin-Heidelberg: Springer-Verlag; pp. 90-109, and the Flp/FRT system from the Saccharomyces cerevisiae 2.mu. circle plasmid (Broach JR., et al., Cell 29:227-234, 1982). A system was developed for construction of adenovirus vectors by site-specific recombination mediated by Cre from bacteriophage P1 Hardy et al., J. Virol. 71:1842-1849, 1997). This method provides a means to generate E1-substituted adenoviruses with insertion of foreign DNA in this region upon recombination between a shuttle plasmid containing the gene transduction unit and one loxP site, and a helper adenovirus vector deleted for its packaging signal through intramolecular recombination between two loxP sites in Cre-expressing cells. An application of this method for construction of recombinant adenoviruses through Cre-lox mediated site-specific recombination between two plasmids in 293Cre cells was disclosed in U.S. patent 6,379,943, herein incorporated by reference.

In a different approach Farmer and Quinn (US patent application US2003/0054555) describe a method for the generation of recombinant adenoviral vectors using Cre-lox mediated site-specific recombination between a donor vector and an acceptor vector encoding a gene-deleted adenovirus genome. The use of high copy plasmids as vectors, as described in this method of the prior art does not allow for the generation of certain types of non-adenovirus-type 5 'serotype' recombinant adenovirus expression vectors. Genome instability was observed when the genome of the adenovirus type 19a was cloned into plasmids when propagated in E.coli. A solution was provided by cloning the adenovirus genome into a BAC, allowing amplification and genome modification in bacteria without the plasmid-associated genome instability (Ruzsics Z et al. J. Virol. 80:8100-8113, 2006). Farmer and Quinn also describe the use of an acceptor vector encoding a gene-deleted adenovirus genome (deleted for the E region) including both ITRs, and being able to be complemented and propagated in a complementing cell line such as 293 cells. Upon site-specific recombination between the donor and acceptor vector the resulting recombination product contains the insertion nucleic acid of the donor vector. In this method of the prior art

provided for construction of recombinant adenoviral genomes, a donor plasmid containing two sequence-specific recombination target sites that are arranged in a way allowing recombination between these two sequences, is used. The acceptor plasmid contains one sequence-specific recombination site. The donor and acceptor constructs are reacted in vitro or in a host cell with site-specific recombinase, and the resulting recombination product, which is a recombinant adenovirus genome construct, contains the desired donor fragment. In a further embodiment a selectable marker (i.e. sacB) is split between the donor and acceptor vector, and the first part of the marker is present on the acceptor vector, and the second part of the selectable marker present on the donor vector. Upon site-specific recombination mediated by expression of a site-specific recombinase (Cre recombinase) both parts form a functional selectable marker in the resulting recombination construct. The generation of a selectable marker enables the selection of reaction products. The Cre-mediated recombination reaction catalyzes both reactions at the same time, excision and insertion, ultimately leading to an equilibrium containing a mixture of reaction products. Applying this method of the prior art using an acceptor and donor vector yielded 80% desired recombination products with a total of 10 clones analyzed in the example provided being recombinant adenoviral vectors. This method of the prior art described herein is prone to the generation of multiple reaction products, especially since no mechanism applies that limits the number of site-specific recombinations between the acceptor and the donor vector to exactly one. This, however, is an unsolved technical problem and a prerequisite for the generation of a pure and complex adenoviral vector expression library without the need for sequencing and characterization of individual clones.

Another method for construction of helper-dependent gutless adenovirus vectors was described (Parks et al., Proc. Natl. Acad. Sci. U.S.A. 93:13565-13570, 1996), and disclosed as a method for "High-efficiency Cre/loxP based system for construction of adenovirus vectors" in U.S. patent 6,379,943. Genetic elements incorporated into the adenoviral (AdV) AdV genome that are flanked by LoxP sites are subject to spontaneous excision during propagation of adenovirus vectors, however (Anton M. and Graham F.L., " J.Virol. 69:4600-4606, 1995). In another application Cre-mediated recombination was used to generate adenovirus vectors after two sequential recombination events and negative selection against

an adenovirus deleted for its packaging signal after recombination (Hardy S et al., J. Virol. 71:1842-1849, 1997).

Methods using Cre-mediated recombination between two nucleic acids for generation of infectious adenovirus genomes in E.coli or in eukaryotic cells fail to generate stable, unbiased libraries. The ability of Cre-recombinase to catalyze the reaction in both directions, results in adenovirus preparations that still can be contaminated by the non-recombined parental adenovirus. Moreover, two mechanisms limit the use of Cre/loxP site specific recombination for construction of genomic libraries. Due to the small size of the recognized sequence by Cre recombinase, cryptic loxP sites in genomes are present, inducing either recombination between compatible sites, or introducing single- or double-strand breaks, affecting the ability to grow and modify BACs, PACs, Cosmids or Fosmids containing loxP sites in E.coli strains expressing Cre, even if an inducible system is used for Cre expression (Semprini S et al. Nucleic Acids Res. 35:1402-1410, 1997). This process also occurs in mammalian cells and organisms; here recombination events between cryptic (pseudo) loxP sites within the genomes of mice and humans leads to genome instability inducing illegitimate chromosome rearrangements (Schmidt EE et al., Proc. Natl. Acad. Sci. U. S. A. 97:13702-13707, 2000; Sauer B. J. Mol. Biol. 223:911-928, 1992). A Library of adenovirus vector genomes constructed by site-specific Cre-mediated homologous recombination thus can be subject to a significant degree of contamination, requiring intensive cell culture work and virologic methods to get single clones.

A method using Cre-lox mediated recombination to construct adenoviruses was further refined and described in Graham et al., U.S. patent 7,132,290, incorporated herein by reference23. The use of DNA-TP complexes was embodied in said patent to overcome limitations related to the low infectivity of adenovirus encoding nucleic acids when transfected in producer cells such as 293. It is known by those skilled in art that infectivity of adenovirus DNA is augmented 100-fold if DNA-TP complexes are used instead of plasmid derived DNA. The viral DNA is purified such that the terminal protein, which is attached to the 5' end of each strand of the duplex adenovirus, is left intact. Co-transfection of DNA-TP complexes harboring a loxP site together with a second plasmid yielding replication competent adenoviral DNA upon site-specific recombination in the presence of

Cre recombinase can increase the number of viral plaques generated per μg viral DNA transfected significantly (Sharp PA et al., Virology 75:442-456, 1976; Chinnadurai G et al., J. Virol. 26:195-199, 1978). The use of DNA-TP complexes (DNA-TPC) is at risk to be contaminated with parental infectious adenovirus DNA form which the DNA-TP complexes are derived from by restriction digestion.

The construction of recombinant adenovirus genomes through homologous recombination of two fragments in 293 cells using DNA-TPC was further used in combination with a positive selection with library efficiency (Elahi SM et al., Gene Ther. 9:1238-1246, 2002); a patent application for this method has been filed and the reader is referred for technical details to U.S. Pat. Appl. No. 2006210965. Here co-transfection of a plasmid harboring the left end ITR and the adenovirus protease expression cassette along with viral DNA-TPC deleted for the adenovirus protease gene yielded high amounts of recombinant viral vectors. A Library of adenovirus vector genomes constructed by site-specific or homologous recombination in 293 cells, however, can be subject to a significant degree of bias due to selection of virus mutants which have a variable growth properties (e.g in the case of cDNA expression libraries where the expression of the cDNA confers a growth advantage or disadvantage), and thus are over- or underrepresented in the library population. Propagation of such a library is critical, and moreover requires intensive cell culture work and virologic methods to get single clones.

Several methods of the prior art allow the construction of recombinant adenoviruses without any background of parental adenovirus genomes. Methods using direct ligation of DNA fragments to the adenovirus genome for construction of recombinant adenoviruses have been developed early on (Ballay A et al., EMBO J. 30: 3861-5, 1985). However, ligation of large fragments is little efficient and scarcity of unique restriction sites limit the use of this method for construction of viral genome libraries.

Recombination between genetic elements in bacteria rather than in eukaryotic cells can be used to construct adenovirus vectors without the need for plaque purification. In an application commercialized as AdEasy® system (He T-C et al., Proc. Natl. Acad. Sci. U.S. 95:2509-2514, 1998) recombination between a co-transfected supercoiled adenovirus

genome and a shuttle plasmid occurs in BJ5183 bacteria. This bacterial strain has favorable properties for the maintenance of genetic stability of adenovirus genomes. According to the information given by the manual of the producer more than 20% of the colonies are correct recombination products. In another method described by Chartier et al. (Chartier C et al., J. Virol. 70 :4805-4810, 1996) the increased length of the two homology arms increases the recombination efficiency. An improvement of this method was described by Crouzet et al.. Proc. Natl. Acad. Sci USA, 94 :1414- 1419, 1997. Here the number of background colonies was reduced by introducing a negative selection marker. However, the efficiency and genetic stability of the system is not sufficient for large library generation, since DNA sequences cloned in plasmid vectors harboring direct repeats or repetitive DNA sequences suffers from genetic instability. This is especially true for plasmid vectors which replicate with high copy numbers in E.*coli*. In another successful attempt to use this method for construction of recombinant adenoviruses, homologous recombination in yeast was established. This method, however, relies on linearization of DNA to induce the recombination between identical sequences. The scarcity of unique restriction sites in adenoviral genomes in addition to low YAC DNA yields obtained from large (typically 500ml) yeast spheroblast cultures limit this application.

The Gateway™ system as commercialized by Invitrogen Corp. uses site specific recombination for recombination in vitro between nucleic acids generating a third nucleic acid being selectable in host cells. For technical details it is referred to US Patent No. 7,282,326 (Invitrogen Corp.), and U.S. Pat No. 5,888,732 (Life Technologies), both incorporated herein by reference. This system yields the recombinant plasmid with high efficiency and accuracy with no background from the non-recombined plasmid vectors, and circumvents the unpredictable recombination events occurring during recombination in E.coli hosts. Recombinant adenovirus genomes, (commercialized as ViralPower™), can be generated with high efficacy, typically >90% correct recombined viral genomes (own observation and according to the manual) using this method.

However, the efficiency of the in vitro recombination decreases with the size of the DNA fragments (Katzen, F.Gateway® recombinational cloning: a biological operating system. Expert Opinion Drug Discovery 2:571-589, 2007), and in case of adenovirus genomes the

resulting number of colonies obtained after transformation of appropriate E.*coli* host cells are decreased several fold, if compared to in vitro recombination between small DNA molecules, thus limiting the use of the Gateway system for construction of sized large-DNA libraries. Moreover, the efficiency of bacterial transformation, which here is the limiting factor for library construction, decreases with the size of the transformed DNA in a bacterial strain dependent way (Sheng Y et al., , Nucleic Acids Res. 23:1990-1996, 1995).

Using BACs instead of plasmid vectors circumvents the instability of genomic sequences cloned into plasmid vectors in E.*coli*. Examples apply to large viral genomes cloned in plasmid vectors (Bzymek M and Lovett ST, Proc Natl Acad Sci U S A. 98:8319-8325, 2001; and adenovirus vector genomes from other subgroups (Ruzsics Z et al., J. Virol. 80:8100-8113, 2006). The viral vectors are instable if propagated on plasmid vector in E.coli hosts and require propagation as stable genomes on bacterial artificial chromosome (BAC) plasmids. Although genomes can be maintained and manipulated in BACs, the selection procedure involves multiple steps and no method is available yet for construction of large libraries of such genomes.

The occurrence of genomic instability of genomic BACs due to the presence of cryptic Frt sites and Flp has not been observed to date. This system therefore is used here in this invention for genomic library construction. Targeted exchange of parts of nucleic acids between two nucleic acids can be achieved by use of Flp-mediated site-specific recombination if two non identical Frt sites are used. A method for targeted modification of a genome of a eukaryotic cell has been claimed in the PCT patent application WO 1999/025854, incorporated herein by reference. Adaptation of this system for construction of genome libraries would require the exchange of a selectable marker and the gene transduction unit. However, similar to the gateway system, the efficiency of this reaction decreases with the size of the nucleic acid fragment to be exchanged and is not 100% reliable, thus making an extensive characterization of the obtained library necessary. The construction of a library of adenovirus vector genomes using Cre-mediated site-specific homologous recombination was only achieved in eukaryotic cells and therefore subject to a significant degree of contamination, requiring intensive cell culture work and virologic methods to get single clones. Usage of Cre-mediated site-specific recombination in E.*coli* is

associated with genomic instability and cannot be used with state of the art high copy plasmid systems. Especially if the virus library is constructed in eukaryotic cells, a significant degree of library bias occurs due to selection of virus mutants which have variable growth properties, leading to a library with over- or underrepresented viruses. Stable propagation of such a library is critical, and moreover requires intensive cell culture work and virologic methods to get single clones. Moreover, the use of DNA-TPC fragments to enhance the infectivity of the viral DNA is at risk to be contaminated with parental infectious adenovirus DNA from which the DNA-TP complexes are derived from by restriction digestion. The use of methods involving site-specific recombination mediated double-reciprocal exchange of nucleic acid sequences between two non-identical recombination sites for genomic library construction are limited by the efficiency and fidelity of the reaction, making an extensive screening and characterization of the resulting library necessary. Alternative systems using in vitro site-specific recombination are limited by the efficiency of the recombination reaction especially if large plasmids are used, and moreover suffer from decreased transformation efficiency of the resulting large plasmids into E.*coli*.

The problem underlying the present invention is to overcome the shortcomings of the methods of the prior art in the generation of adenovirus genomes, and to provide improved respective methods and means for performing such methods.

A still further problem underlying the present invention is to provide methods, and means for performing such methods, which allow the cloning of large nucleic acid sequences such as genomic nucleic acid sequences with high efficiency.

Summary of the invention

These and other problems underlying the instant invention are solved by the subject matter of the independent claims. Preferred embodiments may be taken from the dependent claims.

The problem underlying the present invention is solved in a first aspect, which is also the first embodiment of the first aspect, by a first nucleic acid molecule comprising

(1) a nucleic acid molecule comprising, the following elements:

(a) a site-specific recombination site, preferably exactly one site-specific recombination site;

(b) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker;

(c) a first part of a genome of a virus;

(d) a transcription unit; and

(e) optionally a first restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO:1 and/or SEQ ID No:15; or

(3) a nucleic acid molecule being similar or identical to the nucleic acid molecule contained in the organism deposited with the DSMZ according to the Budapest treaty under accession number DSM 23753, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

wherein the first nucleic acid molecule is either a circular or a linear molecule.

In a second embodiment of the first aspect which is also an embodiment of the first embodiment of the first aspect, the first nucleic acid molecule comprises a packaging signal.

In a third embodiment of the first aspect which is als an embodiment of the first and the second embodiment of the first aspect, the first part of a genome of a virus comprises a terminal sequence of a genome of a or the virus, preferably of a genome of the virus.

In a fourth embodiment of the first aspect which is also an embodiment of the third embodiment of the first aspect, the terminal sequence of a genome of a or the virus comprises a terminal repeat of the genome of a or the virus, preferably a viral inverted terminal repeat.

In a fifth embodiment of the first aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the first aspect, the first restriction site is absent in the first part of a or the viral genome and the transcription unit.

In a sixth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the first aspect, the first restriction site is selected from the group comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a seventh embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth and the sixth embodiment of the first aspect, the virus is an adenovirus.

In an eighth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the first aspect, the virus is a human adenovirus type 5 or the virus is a human adenovirus type 19a.

In a ninth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the first aspect, the elements (a) to (e) are arranged in a 5'-> 3' direction.

In a tenth embodiment of the first aspect which is also an embodiment of the ninth embodiment of the first aspect, the terminal repeat is a viral terminal repeat, preferably a left viral terminal repeat.

In an eleventh embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the first aspect, the first nucleic acid molecule is a linear molecule, wherein elements (a) to (d), preferably upon cleavage of the circular molecule of the first nucleic acid molecule with the first restriction enzyme which recognizes and cleaves at the first restriction site, are arranged in a 5'-> 3' direction in the following sequence:

1. the first part of a genome of a virus;
2. the transcription unit;
3. the site-specific recombination site;
4. the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker.
5. optionally a restriction site which is referred to as first restriction site.

In a twelfth embodiment of the first aspect which is also an embodiment of the eleventh embodiment of the first aspect, the first part of a or the genome of a virus comprises a terminal sequence of a genome of a virus, preferably a terminal repeat sequence of a genome of a virus, and more preferably an inverted terminal repeat sequence of a genome of a virus.

In a thirteenth embodiment of the first aspect which is also an embodiment of the twelfth embodiment of the first aspect, the terminal sequence of a genome of a virus comprises a terminal repeat of a or the genome of a virus, preferably a first left terminal repeat of a or the genome of a or the virus and more preferably the terminal sequence is a first left inverted terminal repeat of a or the genome of a virus.

In a fourteenth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth and the thirteenth embodiment of the first aspect, the inverted terminal repeat is the inverted terminal sequence of adenovirus and preferably has any length from about 18 to 103 base pairs.

In a fifteenth embodiment of the first aspect which is also an embodiment of the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth and fourteenth embodiment of the first aspect, preferably to the extent they directly or indirectly refer to the ninth embodiment of the first aspect, the packing signal is a packing signal of an adenovirus.

In a sixteenth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the first aspect, the transcription unit comprises a promoter, optionally a nucleic acid sequence to be expressed, and a termination signal, whereby preferably, the promoter and the nucleic acid to be expressed are operably linked to each other, more preferably to promoter, the nucleic acid and the termination signal are operably linked to each other.

In a seventeenth embodiment of the first aspect which is also an embodiment of the sixteenth embodiment of the first aspect, the promoter is selected from the group comprising eukaryotic promoters, viral promoters, promoters recognized by RNA Polymerase II and promoters recognized by RNA Polymerase III , wherein, preferably, the promoters recognized by RNA polymerase II are selected from the group comprising the PGK promoter and the CMV promoter, and wherein, preferably, the promoters recognized by RNA polymerase are selected from the group comprising the U6 promoter, the H1 promoter, the tRNA promoter and the adenovirus VA promoter.

In an eighteenth embodiment of the first aspect which is also an embodiment of the sixteenth and the seventeenth embodiment of the first aspect the sequence to be expressed is a sequence selected from the group comprising a nucleic acid coding for a peptide, a nucleic acid coding for a polypeptide, a nucleic acid coding for a protein, a non-coding RNA, an siRNA, a microRNA.

In a nineteenth embodiment of the first aspect which is also an embodiment of the sixteenth, the seventeenth and the eighteenth embodiment of the first aspect, the termination signal is selected from the group comprising eukaryotic, viral termination signals and termination signals for RNA Polymerase III-dependent promoters, preferably the termination signal is a polyA signal.

In a twentieth embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the

seventeenth, the eighteenth and the nineteenth embodiment of the first aspect, the site-specific recombination site is selected from the group comprising the recombination site for Flp recombinase.

In a twenty-first embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the first aspect, the bacterial sequences for conditional replication comprise a replication origin.

In a twenty-second embodiment of the first aspect which is also an embodiment of the twenty-first embodiment of the first aspect, the bacterial nucleotide sequences for conditional replication comprise an origin of replication, whereby preferably the origin of replication is the minimal origin of phage gR6K.

In a twenty-third embodiment of the first aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first and the twenty-second embodiment of the first aspect, the sequence providing for a first selection marker is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding for an enzyme.

In a twenty-fourth embodiment of the first aspect which is also an embodiment of the twenty-third embodiment of the first aspect, the resistance is resistance against an agent, preferably against an antibiotic, wherein more preferably such agent comprising gentamycin, kanamycin, zeocin, chloramphenicol, ampicillin, tetracycline.

In a twenty-fifth embodiment of the first aspect which is also an embodiment of the twenty-fourth embodiment of the first aspect, the resistance conferring gene is selected from the group comprising *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, *cml*A, ble, *aad*A, *aad*B, *sac*B and *tet*A.

The problem underlying the present invention is solved in a second aspect, which is also the first embodiment of the second aspect, by a second nucleic acid molecule comprising

(1) a nucleic acid molecule comprising the following elements:

(a) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;

(b) a site-specific recombination site, preferably exactly one site-specific recombination site;

(c) a second part of a genome of a virus; and

(d) optionally a restriction site which is referred to as second restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID No:2 and/or SEQ ID NO:13 and/or SEQ ID NO:14; or

(3) a nucleic acid molecule being similar or identical to the nucleic acid molecule contained in the organisms deposited with the DSMZ according to the Budapest treaty under the accession numbers DSM 24298 and/or DSM 24299, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

wherein the second nucleic acid molecule is either a circular or a linear molecule.

In a second embodiment of the second aspect which is also an embodiment of the first embodiment of the second aspect, the second part of a genome of a or the virus results in a complete genome, which is replication competent if combined with one or several other parts of a or the genome of a or the virus.

In a third embodiment of the second aspect which is also an embodiment of the first and the second embodiment of the second aspect, the site-specific recombination site is selected from the group comprising the recombination site for Flp recombinase.

In a fourth embodiment of the second aspect which is also an embodiment of the first, the second and the third embodiment of the second aspect, the virus is an adenovirus.

In a fifth embodiment of the second aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the second aspect, the virus genome is a human adenoviral type 5 genome or a human adenoviral type 19a genome.

In a sixth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the second aspect, the second part of the genome of a or the virus comprises a terminal sequence of a genome of a virus, preferably of a genome of the virus.

In a seventh embodiment of the second aspect which is also an embodiment of the sixth embodiment of the second aspect, the sequence of a genome of a or the virus comprises a terminal repeat, preferably a viral terminal sequence and more preferably a terminal repeat of a or the virus and further more preferably an inverted terminal repeat of a or the virus, and even further more preferably a right inverted terminal repeat of a or the virus.

In an eighth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the second aspect, the second restriction site is absent in the second part of the genome.

In a ninth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the second aspect, the second restriction site is selected from the group comprising the restriction sites for restriction enzymes AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI, whereby the virus is preferably human adenovirus type 5.

In a tenth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth and the ninth embodiment of the second aspect, the bacterial nucleotide sequences for replication, preferably single copy replication, comprise a replication origin for single copy maintenance in prokaryotic host cells.

In an eleventh embodiment of the second aspect which is also an embodiment of the tenth embodiment of the second aspect, the replication origin is a single copy origin derived from the f-episomal factor or a P1 origin of replication.

In a twelfth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth and the eleventh embodiment of the second aspect, the second nucleic acid molecule is a linear molecule, wherein elements (a) to (d), preferably upon cleavage of the circular molecule of the second nucleic acid molecule with the second restriction enzyme which recognizes and cleaves at the second restriction site, are arranged in a 5'-> 3' direction in the following sequence:

1. a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;
2. a site-specific recombination site, preferably exactly one site-specific recombination site;
3. a second part of a genome of a virus; and
4. optionally a restriction site which is referred to as second restriction site.

In a thirteenth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh and the twelfth embodiment of the second aspect, the nucleotide sequence providing for a second selection marker is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding to an enzyme.

In a fourteenth embodiment of the second aspect which is also an embodiment of the thirteenth embodiment of the second aspect, the resistance is resistance against an agent selected from the group of positive selection markers comprising gentamycin, kanamycin, zeocin, chloramphenicol, ampicillin and streptomycin.

In a fifteenth embodiment of the second aspect which is also an embodiment of the thirteenth and the fourteenth embodiment of the second aspect, the gene conferring resistance is selected from the group comprising *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, cmlA, ble, *aadA, and aadB.*

In a sixteenth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the second aspect, the second nucleic acid molecule is a linear molecule, wherein elements (a) to (c), preferably upon cleavage of the circular molecule of the second nucleic acid molecule with the second restriction enzyme which recognizes and cleaves at the second restriction site, are arranged in a 5'-> 3' direction

(1) the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker
(2) the site-specific recombination site; and
(3) the second part of a genome of a virus.

In a seventeenth embodiment of the second aspect which is also an embodiment of the sixteenth embodiment of the second aspect, the second part of a or the genome of a or the virus comprises a terminal sequence of a genome of a or the virus, preferably of a genome of the virus.

In an eighteenth embodiment of the second aspect which is also an embodiment of the seventeenth embodiment of the second aspect, the terminal sequence of a or the virus comprises a terminal repeat of a or the virus, preferably a right terminal repeat of a or the virus.

In a nineteenth embodiment of the second aspect which is also an embodiment of the sixteenth, the seventeenth and the eighteenth embodiment of the second aspect, the second part of a or the genome of a or the virus comprises a packaging signal.

In a twentieth embodiment of the second aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the second aspect, the second nucleic acid molecule is a BAC.

The problem underlying the present invention is solved in a third aspect, which is also the first embodiment of the third aspect, by a combination of a first nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth ,the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth and the twenty-fifth embodiment of the first aspect and a second nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect.

In a second embodiment of the third aspect which is also an embodiment of the first embodiment of the third aspect, both the first nucleic acid molecule and the second nucleic acid molecule are present as circular closed nucleic acid molecules.

In a third embodiment of the third aspect which is also an embodiment of the first and the second embodiment of the third aspect, the first nucleic acid molecule and the second nucleic acid molecule are present as separate molecules.

In a fourth embodiment of the third aspect, which is also an embodiment of the first, the second and the third embodiment of the third aspect, the virus is an adenovirus.

In a fifth embodiment of the third aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the third aspect, the first restriction site and

second restriction site are the same on both the first nucleic acid molecule and the second nucleic acid molecule.

In a sixth embodiment of the third aspect which is also an embodiment of the fifth embodiment of the third aspect, the first and second restriction site is selected from the group comprising the restriction sited for AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a seventh embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth and the sixth embodiment of the third aspect, the first part of a or the genome of a or the virus and the second part of a or the genome of a or the virus form, if taken together, the complete virus genome.

In an eighth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the third aspect, the first part of a or the genome of a or the virus and the second part of a or the genome of a or the virus form, if taken together, a or the virus genome which is replication competent in permissive cells.

In a ninth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the third aspect, the first selection marker and the second selection marker is an enzyme which is conferring resistance against an antibiotic, wherein such antibiotic is selected from the group comprising kanamycin, streptomycin, neomycin, puromycin, ampicillin, zeocin, gentamycin and chloramphenicol wherein the first selection marker is different from the second selection marker.

In a tenth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the third aspect, the first selection marker is an enzyme which is conferring resistance for kanamycin, and the second selection marker is an enzyme which is conferring a resistance for chloramphenicol.

In an eleventh embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the third aspect, the packing signal is provided by either the first or the second nucleic acid molecule.

In a twelfth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth and the eleventh embodiment of the third aspect, one terminal repeat is provided by the first nucleic acid molecule and one terminal repeat is provided by the second nucleic acid molecule.

In a thirteenth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh and the twelfth embodiment of the third aspect, the first nucleic acid provides both the first part of a or the genome of a or the virus and preferably the terminal sequence of a or the genome of a or the virus, and the second part of a or the genome of a or the virus and preferably the terminal sequence of a or the genome of a or the virus, and the second nucleic acid molecule does provide neither the first part of a or the genome of a or the virus and preferably the terminal sequence of a or the genome of a or the virus, nor the second part of a or the genome of a or the virus and preferably the terminal sequence of a or the genome of a or the terminal repeat of a or the virus.

In a fourteenth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth and the thirteenth embodiment of the third aspect, the first nucleic acid provides the two inverted terminal repeats and the second nucleic acid molecule does not provide any inverted terminal repeat or the second nucleic acid molecule provides at least one inverted terminal repeat.

In a fifteenth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth and the fourteenth embodiment of the third aspect,

the first restriction site and the second restriction site are absent in the transcription unit, in the first and in the second part of a or the genome.

In a sixteenth embodiment of the third aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the third aspect, the virus is an adenovirus, more preferably human adenovirus serotype 5.

The problem underlying the present invention is solved in a fourth aspect, which is also the first embodiment of the fourth aspect, by a method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) providing a first nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth and the twenty-fifth embodiment of the first aspect;

b) providing a second nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect; or

c) providing a combination according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth and the sixteenth embodiment of the third aspect;

d) allowing the first nucleic acid molecule and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably a single copy of a or the genome of a or the virus, whereby the genome is a

complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

e) optionally selecting the recombination product; and

f) optionally cleaving the recombination product with the first restriction enzyme or the second restriction enzyme, or cleaving with both, the first and second restriction enzyme.

In a second embodiment of the fourth aspect which is also an embodiment of the first embodiment of the fourth aspect, the first nucleic acid molecule and the second nucleic acid molecule are reacted in a prokaryotic host cell and the recombination product is selected, and wherein preferably the prokaryotic host cell is *E. coli*, more preferably the host cell is the organism or an organism similar to such organism which has been deposited with the DSMZ according to the Budapest treaty under accession number DSM 23743 and/or DSM 23742,

In a third embodiment of the fourth aspect which is also an embodiment of the first and the second embodiment of the fourth aspect, the host cell is selected from a group of *E.coli* strains lacking the F-factor and being sensitive to the first and the second selection marker.

In a fourth embodiment of the fourth aspect which is also an embodiment of the first, the second and the third embodiment of the fourth aspect, the host cell is the *E. coli* K12-derived type, preferably DH10B.

In a fifth embodiment of the fourth aspect which is also an embodiment of the first embodiment of the fourth aspect, the first nucleic acid molecule and the second nucleic acid molecule are reacted in a eukaryotic host cell, more preferably the host cell is the organism or an organism similar to such organism which has been deposited with the DSMZ according to the Budapest treaty under accession number DSM ACC3077m or DSM ACC3077, in the presence of the site-specific recombinase and the step of selecting the recombination product is absent, wherein preferably the eukaryotic host cell is a permissive host cell.

In a sixth embodiment of the fourth aspect which is also an embodiment of the fifth embodiment of the fourth aspect,the site-specific recombinase is provided, and wherein the permissive host cell is a cell which is selected from the group comprising 293 cells, 911 cells, PER.C6 cells and CAP cells.

In a seventh embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth and the sixth embodiment of the fourth aspect, the first selection marker is kanamycin and the second selection marker chloramphenicol.

In an eighth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the fourth aspect, the first nucleic acid molecule and the second nucleic acid molecule are reacted in the presence of the recombinase.

In a ninth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the fourth aspect, the recombinase is interacting with the site-specific recombination site provided by the first nucleic acid molecule and the site-specific recombination site provided by the second nucleic acid molecule.

In a tenth embodiment of the fourth aspect which is also an embodiment of the eighth and the ninth embodiment of the fourth aspect, the recombinase is Flp recombinase.

In an eleventh embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the fourth aspect, the recombinase is encoded by either the first nucleic acid molecule or the second nucleic acid molecule.

In a twelfth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the fourth aspect, the recombinase is provided or produced by the prokaryotic host cell or the eukaryotic host cell.

In a thirteenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh and the twelfth embodiment of the fourth aspect, after the recombination, preferably the site-specific recombination, the recombinase is inactivated.

In a fourteenth embodiment of the fourth aspect which is also an embodiment of the first embodiment of the fourth aspect, the recombinase and preferably the Flp recombinase is controlled by an inducible promoter or a temperature-sensitive repressor.

In a fifteenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth and the fourteenth embodiment of the fourth aspect, the restriction enzymes are selected from the group comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a sixteenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the fourth aspect, the method comprises as a further step transfecting the complemented genome of a or the virus which is preferably a replicable virus genome into a permissive host cell.

In a seventeenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth and the sixteenth embodiment of the fourth aspect, the expression of the recombinase is controlled by a temperature-sensitive origin of replication.

In an eighteenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth and

the seventeenth embodiment of the fourth aspect, the method is used in the construction of vectors for gene transfer, vaccine or therapeutic applications.

In a nineteenth embodiment of the fourth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth and the eighteenth embodiment of the fourth aspect, the method is used in the construction of a library of virus genomes.

The problem underlying the present invention is solved in a fifth aspect, which is also the first embodiment of the fifth aspect, by a third nucleic acid molecule comprising

(1) a nucleic acid molecule comprising the following elements:
   (a) optionally, a first part of a genome of a virus;
   (b) a nucleotide sequence, preferably a genomic nucleotide sequence, or atranscription unit;
   (c) a regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;
   (d) a site-specific recombination site, preferably exactly one site-specific recombination site;
   (e) a nucleotide sequence providing for a negative selection marker;
   (f) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker;
   (g) optionally a first restriction site;

wherein the bacterial nucleotide sequence with regulatory activity in a prokaryote, the site-specific recombination site, and the nucleotide sequence providing for a negative selection marker are arranged in a 5' to 3' direction.

or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO:6; or

(3) a nucleic acid moelcule being similar or identical to the nucleic acid molecule contained in the organism deposited with the DSMZ according to the Budapest treaty under accession number DSM 23754, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

wherein the third nucleic acid molecule is either a linear or a circular molecule.

In a second embodiment of the fifth aspect which is also an embodiment of the first embodiment of the fifth aspect, the third nucleic acid molecule is a linear molecule, wherein elements (a) to (f), preferably upon cleavage of the circular molecule of the third nucleic acid molecule with the first restriction enzyme which recognized and cleaves at the first restriction site, are arranged in a 5' -> 3' direction in the following sequence as follows:

1. optionally the first part of a genome of a virus;
2. the nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit;
3. the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;
4. the site-specific recombination site;
5. the nucleotide sequence providing for a negative selection marker; and
6. the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker.

In a third embodiment of the fifth aspect which is also an embodiment of the first and the second embodiment of the fifth aspect, the third nucleic acid molecule further comprises the first part of a genome of a virus and, wherein, preferably the first part comprises one or more terminal sequences of a or the genome of a or the virus.

In a fourth embodiment of the fifth aspect which is also an embodiment of the third embodiment of the fifth aspect, the first part of a genome of a virus mediates or is needed for the replication and/or packaging of a or the virus in a permissive cell.

In a fifth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the fifth aspect, the transcription unit is a transcription unit for a nucleic acid sequence, whereby such nucleic acid sequence is a heterologous nucleic acid sequence.

In a sixth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the fifth aspect, the third nucleic acid molecule is a bacterial plasmid or a bacterial artificial chromosome.

In a seventh embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth and the sixth embodiment of the fifth aspect, the third nucleic acid molecule further comprises a first restriction site.

In an eighth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the fifth aspect, the third nucleic acid molecule further comprises a first part of a genome of a virus.

In a ninth embodiment of the fifth aspect which is also an embodiment of the eighth embodiment of the fifth aspect, the first part of a genome of a virus comprises a packaging signal.

In a tenth embodiment of the fifth aspect which is also an embodiment of the ninth embodiment of the fifth aspect, the packaging signal is derived from an adenovirus genome, preferably the packaging signal is the packaging signal Ψ5 from human adenovirus type 5.

In an eleventh embodiment of the fifth aspect which is also an embodiment of the eighth, the ninth and the tenth embodiment of the fifth aspect, the first part of a or the genome of a virus comprises the terminal sequence of a or the genome of a or the virus or one or several parts of the terminal sequence.

In a twelfth embodiment of the fifth aspect which is also an embodiment of the eleventh embodiment of the fifth aspect, the terminal sequence of a or the virus comprises one or several terminal repeats of a or the genome of a or the virus.

In a thirteenth embodiment of the fifth aspect which is also an embodiment of the eighth, the ninth, the tenth, the eleventh and the twelfth embodiment of the fifth aspect, the first part of a or the genome of a or the virus is a first part of the genome of an adenovirus, preferably a human adenovirus and more preferably the adenovirus is human adenovirus type 5, and most preferably the entire left end of adenovirus type 5 upstream of the TATA box of the E1 transcription unit, or one or several parts thereof.

In a fourteenth embodiment of the fifth aspect which is also an embodiment of the eleventh, the twelfth and the thirteenth embodiment of the fifth aspect, the terminal sequence of a or the genome of a or the virus or one or several parts of the terminal sequence comprises an inverted terminal repeat, wherein preferably the inverted terminal repeat is the inverted terminal repeat of an adenovirus and more preferably the inverted terminal repeat is from the left end of the human adenovirus type 5.

In a fifteenth embodiment of the fifth aspect which is also an embodiment of the fourteenth embodiment of the fifth aspect, the inverted terminal repeat comprises any length from about 18 to 103 base pairs.

In a sixteenth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the fifth aspect, the first restriction site is absent from both the first part of a or the genome of a or the virus and the transcription unit.

In a seventeenth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth and the sixteenth embodiment of the fifth aspect, the restriction site is absent form an adenoviral genome.

In an eighteenth embodiment of the fifth aspect which is also an embodiment of the sixteenth and the seventeenth embodiment of the fifth aspect, the virus is an adenovirus, preferably a human adenovirus, and more preferably human adenovirus type 5.

In a nineteenth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth and the eighteenth embodiment of the fifth aspect, the restriction site is a restriction site for a restriction enzyme, whereby the restriction enzyme is selected from the group comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a twentieth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the fifth aspect, the third nucleic acid molecule comprises, preferably in a 5' -> 3' orientation,

1. the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;
2. the site-specific recombination site;
3. the nucleotide sequence providing for a negative selection marker;
4. the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker;
5. the first restriction site;
6. the first part of a or the genome of a or the virus; and
7. the transcription unit.

In a twenty-first embodiment of the fifth aspect which is also an embodiment of the twentieth embodiment of the fifth aspect, the first part of a or the genome of a or the virus comprises a terminal repeat, whereby preferably said terminal repeat is a left terminal repeat

and more preferably the left terminal repeat is the rigth inverted terminal repeat of an adenovirus genome.

In a twenty-second embodiment of the fifth aspect which is also an embodiment of the twentieth embodiment of the fifth aspect, the first part of a or the genome of a or the virus comprises a terminal repeat, preferably an inverted terminal repeat, more preferably a right terminal repeat of a virus genome, and even more preferably the right terminal repeat of an adenovirus.

In a twenty-third embodiment of the fifth aspect which is also an embodiment of the twenty-second embodiment of the fifth aspect, the right terminal repeat of an adenovirus comprises at least 103 nucleotides of the genome of a human adenovirus type 5 virus genome.

In a twenty-fourth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the fifth aspect, the first part of a or the genome of a or the virus comprises a packaging signal, whereby preferably the packaging signal is an adenoviral packaging signal, and more preferably the adenoviral packaging signal is the Ψ5 of the left end of the human adenovirus type 5.

In a twenty-fifth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third and the twenty-fourth embodiment of the fifth aspect, preferably of the twentieth, the twenty-first, the twenty-second, the twenty-third and the twenty-fourth embodiment of the fifth aspect, the transcription unit comprises a promoter, a nucleic acid to be expressed, and a termination signal.

In a twenty-sixth embodiment of the fifth aspect which is also an embodiment of the twenty-fifth embodiment of the fifth aspect, the promoter is active in eukaryotic cells, preferably the promoter is selected from the group comprising eukaryotic or prokaryotic promoters.

In a twenty-seventh embodiment of the fifth aspect which is also an embodiment of the twenty-fifth and twenty-sixth embodiment of the fifth aspect, the promoter is one which is recognized by RNA polymerase II, whereby such promoter is preferably selected from the group comprising the PGK promoter and CMV promoter.

In a twenty-eighth embodiment of the fifth aspect which is also an embodiment of the twenty-fifth and twenty-sixth embodiment of the fifth aspect, the promoter is one which is recognized by RNA polymerase III, whereby such promoter is preferably selected from the group comprising the U6 promoter, the H1 promoter, the tRNA promoter, the adenovirus VA promoter.

In a twenty-ninth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, twenty-sixth, the twenty-seventh and the twenty-eighth embodiment of the fifth aspect, prefereably of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, twenty-sixth, the twenty-seventh and the twenty-eighth embodiment of the fifth aspect, the nucleic acid to be expressed is selected from the group comprising coding nucleic acids and non-coding nucleic acid sequences.

In a thirtieth embodiment of the fifth aspect which is also an embodiment of the twenty-ninth embodiment of the fifth aspect, the nucleic acid to be expressed is a coding nucleic acid, whereby the coding nucleic acid codes for a protein, a polypeptide or a peptide.

In a thirty-first embodiment of the fifth aspect which is also an embodiment of the twenty-ninth embodiment of the fifth aspect, the nucleic acid to be expressed is a non-coding RNA,

preferably the nucleic acid to be expressed is a microRNA, a small interfering RNA (siRNA) or a shRNA.

In a thirty-second embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest and the thirty-first embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest and the thirty-first embodiment of the fifth aspect, the termination is signal is a termination signal of a eukaryotic or a viral gene, preferably the termination signal is selected from the group comprising a polyA signal and termination signals for RNA Pol III-transcribed genes.

In a thirty-third embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first and the thirty-second embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first and the thirty-second embodiment of the fifth aspect the site-specific recombination site is the site for the Flp recombinase.

In a thirty-fourth embodiment of the fifth aspect which is also an embodiment of the thirty-third embodiment of the fifth aspect, the site for the Flp recombinase is the wild type site or a derivative thereof, whereby the derivative thereof is suitable to bind to the Flp recombinase.

In a thirty-fifth embodiment of the fifth aspect which is also an embodiment of the thirty-fourth embodiment of the fifth aspect, the derivative of the site for the Flp recombinase is a minimal recombination site having a length of 34 nucleotides and comprising the R2, the U and the R3 element of the wild type FRT site.

In a thirty-sixth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth and the thirty-fifth embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth and the thirty-fifth embodiment of the fifth aspect, the bacterial nucleotide sequences for conditional replication comprise an origin of replication, whereby preferably the origin of replication is the minimal origin of phage gR6K.

In a thirty-seventh embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth and the thirty-sixth embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth and the thirty-sixth embodiment of the fifth aspect, the positive selection marker is mediating resistance against a selecting agent.

In a thirty-eighth embodiment of the fifth aspect which is also an embodiment of the thirty-seventh embodiment of the fifth aspect, the selecting agent is selected from the group comprising ampicillin, zeocin, gentamycin, chloramphenicol, kanamycin, neomycin and puromycin.

In a thirty-ninth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh and the thirty-eighth embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh and the thirty-eighth embodiment of the fifth aspect, the nucleotide sequence providing for a positive selection marker is gene selected from the group of genes comprising *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, *ble*, and *cmlA*.

In a fortieth embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth and the thirty-ninth embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth,

the thirty-sixth, the thirty-seventh, the thirty-eighth and the thirty-ninth embodiment of the fifth aspect, the regulatory sequence which has a regulatory activity in a prokaryote is a sequence which directs expression of a nucleotide sequence in a prokaryote, preferably in a prokaryotic host cell.

In a forty-first embodiment of the fifth aspect which is also an embodiment of the fortieth embodiment of the fifth aspect, the regulatory sequence is a promoter, preferably according to the fortieth embodiment of the fifth aspect a prokaryotic promoter, and even more preferably according to the fortieth embodiment of the fifth aspect the E.*coli* galaktokinase promoter.

In a forty-second embodiment of the fifth aspect which is also an embodiment of the forty-first embodiment of the fifth aspect, the promoter is an inducible promoter, preferably an inducible prokaryotic promoter.

In a forty-third embodiment of the fifth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, the thirty-ninth, the fortieth, the forty-first and the forty-second embodiment of the fifth aspect, preferably of any of the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, thirty-ninth, the fortieth, the forty-first and the forty-second embodiment of the fifth aspect, the negative selection marker or the expression of the nucleotide sequence providing for a negative selection marker mediates or confers sensitivity to a selecting agent and/or a selecting condition.

In a forty-fourth embodiment of the fifth aspect which is also an embodiment of the forty-third embodiment of the fifth aspect, the nucleotide sequence providing for a negative selection marker is a gene selected from the group comprising the *galK*, *tetAR*, *pheS*, *thyA*, *lacy*, *ccdB* and *rpsL* gene.

In a forty-fifth embodiment of the fifth aspect which is also an embodiment of the forty-third and forty-fourth embodiment of the fifth aspect, the selecting agent is selected form the group comprising lipophilic compounds, sucrose, *p*-chlorophenylalanine, trimethoprim, *t-o*-nitrophenyl-beta-D-galactopyranoside and streptomycin.

The problem underlying the present invention is solved in a sixth aspect, which is also the first embodiment of the sixth aspect, by a combination of a second nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect and a third nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, the thirty-ninth, the fortieth, the forty-first, the forty-second, the forty-third, the forty-fourth and the forty-fifth embodiment of the fifth aspect.

In a second embodiment of the sixth aspect which is also an embodiment of the first embodiment of the sixth aspect, both the third nucleic acid and the second nucleic acid are present as circular closed nucleic acid molecules.

In a third embodiment of the sixth aspect which is also an embodiment of the first and the second embodiment of the sixth aspect, the second and the third nucleic acid molecule are each present as a separate molecule.

In a fourth embodiment of the sixth aspect which is also an embodiment of the first, the second and the third embodiment of the sixth aspect, the third nucleic acid molecule is a plasmid, and the second nucleic acid molecule is a BAC.

In a fifth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the sixth aspect, the virus is an adenovirus.

In a sixth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the sixth aspect, the first part of a or the genome of a or the virus and the second part of a or the genome of a or the virus form, if taken together, the complete genome of a virus.

In a seventh embodiment of the sixth aspect which is also an embodiment of the sixth embodiment of the sixth aspect, the virus is adenovirus, preferably human adenovirus, more preferably human adenovirus type 5.

In an eighth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth and the seventh embodiment of the sixth aspect, the complete genome contains one, preferably exactly one transduction unit.

In a ninth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh and the eighth embodiment of the sixth aspect, the first restriction site and the second restriction site are absent from both the first part of a or the genome of a or the virus and the second part of a or the genome of a or the virus, whereby preferably the virus is an adenovirus and more preferably a human adenovirus and even more preferably a human adenovirus type 5.

In a tenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth and the ninth embodiment of the sixth aspect, the first restriction enzyme and the second restriction enzyme is selected from the group comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In an eleventh embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the sixth aspect, the complete genome can be released by digestion with the first and the second restriction enzyme, whereby preferably the first restriction enzyme and the second restriction enzyme are the same, more preferably the first and the second restriction enzyme is PacI.

In a twelfth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth and the eleventh embodiment of the sixth aspect, the virus comprising the complete genome is viable and replication competent in a permissive host cell, preferably a permissive cell line.

In a thirteenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh and the twelfth embodiment of the sixth aspect, the complete genome comprises a deletion of parts of the genome of a or the virus, preferably a deletion of one or several genes or coding regions.

In a fourteenth embodiment of the sixth aspect which is also an embodiment of the thirteenth embodiment of the sixth aspect, the virus is an adenovirus and the deletion is a deletion of a region of the adenoviral genome, whereby the region is selected from the group comprising the E1 region, E2 region, E3 region, E4 region and combinations thereof.

In a fifteenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth and the fourteenth embodiment of the sixth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against kanamycin, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to streptomycin, and the second selection marker of the second nucleic acid molecule is conferring resistance for chloramphenicol.

In a sixteenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the sixth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against kanamycin, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to streptomycin, and the second selection marker of the second nucleic acid molecule is conferring resistance against chloramphenicol but not to kanamycin and/or streptomycin.

In a seventeenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the sixth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against a first selecting agent, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to a second selecting agent, and the second selection marker of the second nucleic acid molecule is conferring resistance against a third selecting agent, wherein the first selecting agent, the second selecting agent and the third selecting agent are different form each other.

In an eighteenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth and the seventeenth embodiment of the sixth aspect, the nucleotide sequence providing for a negative selection marker is under the control of a promoter, preferably under the control of a prokaryotic promoter.

In a nineteenth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth and the eighteenth embodiment of the sixth aspect, the second nucleic acid molecule comprises a bacterial nucleotide sequence for single copy replication.

In a twentieth embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the sixth aspect, the combination, upon having been introduced into a host cell, allows only the replication of the second nucleic acid molecule.

In a twenty-first embodiment of the sixth aspect which is also an embodiment of the twentieth embodiment of the sixth aspect, the bacterial nucleotide sequences for conditional replication comprises the minimal origin of phage gR6K, and the sequences for single copy replication comprises a nucleotide sequence which codes for factors which are parts of or the F-factor origin of replication.

In a twenty-second embodiment of the sixth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth and the twenty-first embodiment of the sixth aspect, the third nucleic acid molecule provides one terminal repeat and the second nucleic acid molecule provides one terminal repeat.

The problem underlying the present invention is solved in a seventh aspect, which is also the first embodiment of the seventh aspect, by a fourth nucleic acid molecule comprising:

the following elements of the second nucleic acid molecule as preferably defined in the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect, namely

(a) the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;

(b) the site-specific recombination site;
(c) the second part of a genome of a virus; and
(d) the restriction site which is referred to as second restriction site;

and the following elements of the first nucleic acid molecule as preferably defined in the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-forth and the twenty-fifth embodiment of the first aspect, namely

(a) the site-specific recombination site;
(b) the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker;
(c) the first part of a genome of a virus;
(d) the transcription unit; and
(e) the first restriction site

wherein the fourth nucleic acid molecule is preferably a circular molecule, whereby, preferably, the fourth nucleic acid molecule is obtainable by a method according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the fourth aspect.

The problem underlying the present invention is solved in an eighth aspect, which is also the first embodiment of the eighth aspect, by a method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) providing a third nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the

ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, the thirty-ninth, the fortieth, the forty-first, the forty-second, the forty-third, the forty-fourth and the forty-fifth embodiment of the fifth aspect;

b) providing a second nucleic acid molecule according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect; or

c) a combination according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first and the twenty-second embodiment of the sixth aspect;

d) allowing the third and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably single copy of the genome of a or the virus, whereby the genome is a complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

e) optionally selecting the recombination product; and

f) optionally cleaving the recombination product with the first and second restriction enzyme.

In a second embodiment of the eighth aspect which is also an embodiment of the first embodiment of the eighth aspect, the third and the second nucleic acid molecule are reacted

in a prokaryotic host cell preferably *E. coli*, more preferably the host cell is the organism or an organism similar to such organism which has been deposited with the DSMZ according to the Budapest treaty under accession number DSM 23743.

In a third embodiment of the eighth aspect which is also an embodiment of the first and the second embodiment of the eighth aspect, the virus is an adenovirus, preferably a human adenovirus, and more preferably human adenovirus type 5,

In a fourth embodiment of the eighth aspect which is also an embodiment of the first, the second and the third embodiment of the eighth aspect, the host cell is selected from a group of *E.coli* strains lacking the F-factor origin of replication and sensitive to the selection markers provided by both the third and the second nucleic acid molecule.

In a fifth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third and the fourth embodiment of the eighth aspect, the host cell is an *E. coli* strain which is deficient in the expression of pi protein.

In a sixth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the eighth aspect, the host cell is selected from a group comprising *E. coli* K12-derived cells, preferably DH10B.

In a seventh embodiment of the eighth aspect which is also an embodiment of the first embodiment of the eighth aspect, the third nucleic acid molecule and the second nucleic acid molecule are reacted in a eukaryotic host cell and the step of selecting the recombination product is absent, wherein preferably the eukaryotic host cell is a permissive host cell.

In an eighth embodiment of the eighth aspect which is also an embodiment of the seventh embodiment of the eighth aspect, the permissive host cell is a cell which is selected from the group comprising 293 cells, 911 cells, PER.C6 cells and CAP cells.

In a ninth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth and the fifth embodiment of the eighth aspect, the number of recombination events is limited to one recombination event.

In a tenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth and the ninth embodiment of the eighth aspect, the selection for the recombination product is performed by selecting the host cell(s) which harbour a recombination product providing the positive selection marker of the third nucleic acid molecule and the second selection marker of the second nucleic acid molecule, and which are not sensitive to the negative selection marker.

In an eleventh embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the eighth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against kanamycin, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to streptomycin, and the second selection marker of the second nucleic acid molecule is conferring resistance against chloramphenicol, and wherein the selection for the recombination product is obtained by subjecting the prokaryotic host cell to kanamycin, streptomycin and chloramphenicol.

In an twelfth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the tenth embodiment of the eighth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against kanamycin, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to streptomycin, and the second selection marker of the second nucleic acid molecule is conferring resistance against chloramphenicol but not to kanamycin and/or streptomycin, and wherein the selection for the recombination product is obtained by subjecting the prokaryotic host cell to kanamycin, streptomycin and chloramphenicol.

In an thirteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth and the

tenth embodiment of the eighth aspect, the positive selection marker of the third nucleic acid molecule is conferring resistance against a first selecting agent, the negative selection marker of the third nucleic acid molecule is conferring sensitivity to a second selecting agent, and the second selection marker of the second nucleic acid molecule is conferring resistance against a third selecting agent, wherein the first selecting agent, the second selecting agent and the third selecting agent are different form each other, and wherein the selection for the recombination product is obtained by subjecting the prokaryotic host cell to the first, the second and the third selecting agent.

In an fourteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth and the thirteenth embodiment of the eighth aspect, the third and the second nucleic acid molecule are reacted in the presence of a recombinase.

In an fifteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth and the fourteenth embodiment of the eighth aspect, the recombinase is interacting with the site-specific recombination site provided by the third nucleic acid molecule and the site-specific recombination site provided by the second nucleic acid molecule.

In an sixteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth and the fifteenth embodiment of the eighth aspect, the recombinase is Flp recombinase.

In an seventeenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth and the sixteenth embodiment of the eighth aspect, the recombinase is encoded by either the first nucleic acid molecule or the second nucleic acid molecule.

In an eighteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth and the sixteenth embodiment of the eighth aspect, the recombinase is provided by the prokaryotic host cell or the eukaryotic hose cell.

In a nineteenth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth and the eighteenth embodiment of the eighth aspect, after the recombination the recombinase is inactivated.

In a twentieth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the eighth aspect, the recombinase and preferably the Flp recombinase is controlled by a conditional or inducible promoter.

In a twenty-first embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the eighth aspect, the expression of the recombinase is controlled by a temperature-sensitive origin of replication.

In a twenty-second embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth and the twenty-first embodiment of the eighth aspect, the restriction enzyme is selected from the group comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a twenty- third embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first and the twenty-second embodiment of the eighth aspect, the third and the second nucleic acid molecule are separately introduced into the host cell.

In a twenty-fourth embodiment of the eighth aspect which is also an embodiment of the twenty-third embodiment of the eighth aspect, the second nucleic acid molecule is introduced into the host cell prior to the third nucleic acid molecule.

In a twenty- fifth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third and the twenty-fourth embodiment of the eighth aspect, the cleaving of the recombination product provides a complemented complete virus genome which is replication competent in permissive cells.

In a twenty-sixth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth and the twenty-fifth embodiment of the eighth aspect, the method comprises as a further step transfecting the complemented complete virus genome into a complementing host cell.

In a twenty-seventh embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth and twenty-sixth embodiment of

the eighth aspect, the method is used in the construction of vectors for gene transfer, vaccines or therapeutic applications.

In a twenty-eighth embodiment of the eighth aspect which is also an embodiment of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, twenty-sixth and the twenty-seventh embodiment of the eighth aspect, the method is used in the construction of a library of virus genome.

The problem underlying the present invention is solved in a ninth aspect, which is also the first embodiment of the ninth aspect, by a fifth nucleic acid molecule comprising:

the following elements of the second nucleic acid molecule as preferably defined in the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect, namely

(a) the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;
(b) the site-specific recombination site;
(c) the second part of a genome of a virus; and
(d) the restriction site which is referred to as second restriction site;

and the following elements of the third nucleic acid molecule as preferably defined in the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-

seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, the thirty-ninth, the fortieth, the forty-first, the forty-second, the forty-third, the forty-fourth and the forty-fifth embodiment of the fifth aspect, namely

(a) optionally the first part of a genome of a virus;
(b) the nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit;
(c) the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;
(d) the site-specific recombination site;
(e) the nucleotide sequence providing for a negative selection marker;
(f) the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker; and
(g) the first restriction site,

wherein the fifth nucleic acid molecule is preferably a circular molecule, whereby, preferably, the fifth nucleic acid molecule is obtainable by a method according to any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh and the twenty-eighth embodiment of the eighth aspect.

The problem underlying the present invention is solved in a tenth aspect, which is also the first embodiment of the tenth aspect, by a method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes and each viral genome contains a single one of the individual nucleotide sequences, comprising the steps of the method as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the

fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh and the twenty-eighth embodiment of the eighth aspect, wherein the individual nucleotide sequence is part of the transcription unit of the third nucleic acid molecule.

The problem underlying the present invention is solved in an eleventh aspect, which is also the first embodiment of the eleventh aspect, by a method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes, comprising the steps of the method as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth and the nineteenth embodiment of the fourth aspect, wherein the individual nucleotide sequence is part of the transcription unit of the third nucleic acid molecule.

In a second embodiment of the tenth and the eleventh aspect which is also an embodiment of the first embodiment of the tenth aspect and of the first embodiment of the eleventh aspect, the individual nucleotide sequence is present in the complete virus genome as a single copy.

In a third embodiment of the tenth and the eleventh aspect which is also an embodiment of the first and the second embodiment of the tenth aspect and of the first and the second embodiment of the eleventh aspect, the individual nucleotide sequence is the nucleic acid to be expressed.

The problem underlying the present invention is solved in a twelfth aspect, which is also the first embodiment of the twelfth aspect, by a plurality of a fourth nucleic acid molecule according to the seventh aspect and preferably of the first and any other embodiment of the seventh aspect, wherein the plurality of the nucleic acid molecule consists of a number such individual the nucleic acid molecules, wherein the individual nucleic acid molecules differ from each other in the nucleic acid to be expressed which is part of the transcription unit.

The problem underlying the present invention is solved in a thirteenth aspect, which is also the first embodiment of the thirteenth aspect, by a plurality of a fifth nucleic acid molecule according to the ninth aspect and preferably of the first and any other embodiment of the ninth aspect, wherein the plurality of the nucleic acid molecule consists of a number such individual the nucleic acid molecules, wherein the individual nucleic acid molecules differ from each other in element (b) of the third nucleic acid molecule, preferably in the nucleotide sequence of element (b) or in the nucleic acid to be expressed which is part of the transcription unit of element (b).

In a second embodiment of the tweflth and the thirteenth aspect which is also an embodiment of the first embodiment of the tweflth aspect and of the first embodiment of the thirteenth aspect, the virus is an adenovirus.

The problem underlying the present invention is solved in a fourteenth aspect, which is also the first embodiment of the fourteenth aspect, by a plurality of individual adenoviruses, wherein the individual adenoviruses contain an individual nucleic acid as defined in any of the first and the second embodiment of the twelfth aspect and of the first and the second embodiment of the thirteenth aspect.

The problem underlying the present invention is solved in a fifteenth aspect, which is also the first embodiment of the fifteenth aspect, by a kit comprising optionally a package insert, and, in (a) suitable container(s), at least a first nucleic acid molecule as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth and the twenty-fifth embodiment of the first aspect, a second nucleic acid molecule as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth and the twentieth embodiment of the second aspect, optionally a permissive cell line providing the site-specific recombinase as defined in the twelfth embodiment of the fourth aspect, a combination of the first nucleic acid molecule and the

second nucleic acid molecule as defined in any of the the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth embodiment of the third aspect, a third nucleic acid molecule as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh, the twenty-eighth, the twenty-ninth, the thirtiest, the thirty-first, the thirty-second, the thirty-third, the thirty-fourth, the thirty-fifth, the thirty-sixth, the thirty-seventh, the thirty-eighth, the thirty-ninth, the fortieth, the forty-first, the forty-second, the forty-third, the forty-fourth and the forty-fifth embodiment of the fifth aspect, a combination of the third nucleic acid molecule and the second nucleic acid molecule as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first and the twenty-second embodiment of the sixth aspect, a fourth nucleic acid molecule as defined in the first embodiment of the seventh aspect, a fifth nucleic acid molecule as defined in the first embodiment of the ninth aspect, a plurality of a fourth nucleic acid molecule as defined in the first and the second embodiment of the twelfth aspect, a plurality of a fifth nucleic acid molecule as defined in the first and the second embodiment of the thirteenth aspect, or a plurality of individual adenoviruses as defined in the first embodiment of the fourteenth aspect.

In a second embodiment of the fifteenth aspect which is also an embodiment of the first embodiment of the fifteenth aspect, the nucleic acid molecule(s) is/are contained in a ready-to-use form.

In a third embodiment of the fifteenth aspect which is also an embodiment of the first and the second embodiment of the fifteenth aspect, the kit is for use in a method for the generation of nucleic acid molecules coding for a virus as defined in any of the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the

seventeenth, the eighteenth and the nineteenth embodiment of the fourth aspect and the first, the second, the third, the fourth, the fifth, the sixth, the seventh, the eighth, the ninth, the tenth, the eleventh, the twelfth, the thirteenth, the fourteenth, the fifteenth, the sixteenth, the seventeenth, the eighteenth, the nineteenth, the twentieth, the twenty-first, the twenty-second, the twenty-third, the twenty-fourth, the twenty-fifth, the twenty-sixth, the twenty-seventh and the twenty-eighth embodiment of the eighth aspect.

In a fourth embodiment of the fifteenth aspect which is also an embodiment of the first, the second and the third embodiment of the fifteenth aspect, the kit is for use in a method for generating a library of nucleotide sequences as defined in any of the first, the second and the third embodiment of the eleventh aspect.

Further preferred embodiments of the various aspects of the instant invention are as follows.

Embodiment 1. A nucleic acid molecule, which is also referred to as third nucleic acid molecule, wherein the third nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) optionally, a first part of a genome of a virus;

(b) a nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit;

(c) a regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;

(d) a site-specific recombination site;

(e) a nucleotide sequence providing for a negative selection marker;

(f) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker; and

(g) optionally a first restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 6; or

(3) a nucleic acid molecule identical or similar to the nucleic acid molecule contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 23754, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

wherein the third nucleic acid molecule is either a linear or a circular molecule.

Embodiment 2: The third nucleic acid molecule according to Embodiment 1, wherein in the nucleic acid molecule of (1) the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote, the site-specific recombination site and the nucleotide sequence providing for a negative selection marker are arranged in a 5' to 3' direction.

Embodiment 3: The third nucleic acid molecule according to any one of Embodiments 1 to 2, wherein the third nucleic acid molecule contains exactly one site-specific recombination site.

Embodiment 4: The third nucleic acid molecule according to any one of Embodiments 1 to 3, wherein the third nucleic acid molecule is a linear molecule, wherein elements (a) to (f), preferably upon cleavage of the circular molecule of the third nucleic acid molecule with the first restriction enzyme which recognized and cleaves at the first restriction site, are arranged in a 5' -> 3' direction in the following sequence as follows:

1. optionally the first part of a genome of a virus;

2. the nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit;

3. the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;

4. the site-specific recombination site;

5. the nucleotide sequence providing for a negative selection marker; and

6. the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker.

Embodiment 5: The third nucleic acid molecule according to any one of Embodiments 1 to 4, wherein the third nucleic acid molecule further comprises a first part of a genome of a virus.

Embodiment 6: The third nucleic acid molecule according to Embodiment 5, wherein the first part of a or the genome of a virus comprises the terminal sequence of a or the genome of a or the virus or one or several parts of the terminal sequence.

Embodiment 7: The third nucleic acid molecule according to any one of Embodiments 5 to 6, wherein the first part of a or the genome of a or the virus is a first part of the genome of an adenovirus, preferably a human adenovirus and more preferably the adenovirus is human adenovirus type 5, and most preferably the entire left end of adenovirus type 5 upstream of the TATA box of the E1 transcription unit, or one or several parts thereof.

Embodiment 8: The third nucleic acid molecule according to any one of Embodiments 1 to 7, preferably Embodiment 7, wherein the bacterial nucleotide sequences for conditional

replication comprise an origin of replication, whereby preferably the origin of replication is the minimal origin of phage gR6K.

Embodiment 9: The third nucleic acid molecule according to any one of Embodiments 1 to 8, preferably any one of Embodiments 7 to 8, wherein the regulatory sequence which has a regulatory activity in a prokaryote is a sequence which directs expression of a nucleotide sequence in a prokaryote, preferably in a prokaryotic host cell.

Embodiment 10: The third nucleic acid molecule according to any one of Embodiments 1 to 9, preferably any of Embodiments 8 to 9, wherein the negative selection marker or the expression of the nucleotide sequence providing for a negative selection marker mediates or confers sensitivity to a selecting agent and/or a selecting condition.

Embodiment 11: The third nucleic acid molecule according to Embodiment 10, wherein the nucleotide sequence providing for a negative selection marker is a gene selected from the group comprising the *galK*, *tetAR*, *pheS*, *thyA*, *lacy*, *ccdB* and *rpsL* gene.

Embodiment 12: A combination of a third nucleic acid molecule as defined in any of Embodiment 1 to 11 and a nucleic acid molecule which is also referred to as second nucleic acid molecule,

wherein the second nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;
(b) a site-specific recombination site;
(c) a second part of a genome of a virus; and
(d) optionally a restriction site which is referred to as second restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 2 and/or SEQ ID NO: 13 and/or SEQ ID NO: 14; or

(3) a nucleic acid molecule identical or similar to the nucleic acid molecule contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 24298 and/or DSM 24299, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

wherein the second nucleic acid molecule and the third nucleic acid molecule each and independently is either a linear molecule or a circular molecule, preferably the second nucleic acid molecule is a circular molecule and the third nucleic acid molecule is a circular molecule.

Embodiment 13: A combination of a nucleic acid molecule which is also referred to as first nucleic acid molecule, and a nucleic acid molecule which is also referred to as second nucleic acid molecule,

wherein the first nucleic acid molecule comprises

(1) a nucleic acid molecule comprising, the following elements:
   (a) a site-specific recombination site;
   (b) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker;
   (c) a first part of a genome of a virus;
   (d) a transcription unit; and
   (e) optionally a first restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO:1 and/or SEQ ID No:15; or

(3) a nucleic acid molecule being similar or identical to the nucleic acid molecule contained in the organism deposited with the DSMZ according to the Budapest treaty under accession number DSM 23753, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

and wherein the second nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker;
(b) a site-specific recombination site;
(c) a second part of a genome of a virus; and
(d) optionally a restriction site which is referred to as second restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 2 and/or SEQ ID NO: 13 and/or SEQ ID NO: 14; or

(3) a nucleic acid molecule identical or similar to the nucleic acid molecule contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 24298 and/or DSM 24299, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

and wherein the first nucleic acid molecule and the second nucleic acid molecule each and independently is either a linear molecule or a circular molecule, preferably the first nucleic acid molecule is a circular molecule and the second nucleic acid molecule a circular molecule.

Embodiment 14: The combination according to Embodiment 13, wherein the first nucleic acid molecule contains exactly one site-specific recombination site.

Embodiment 15: The combination according to any one of Embodiments 13 and 14, wherein the genome of a virus of the first nucleic acid molecule is a human adenovirus genome, preferably a human adenovirus genome which is different from human adenovirus type 5 genome, more preferably the genome of a virus of the first nucleic acid molecule is a human adenoviral type 19a genome.

Embodiment 16: The combination according to any one of Embodiments 13 to 15, wherein the bacterial nucleotide sequences for conditional replication of the first nucleic acid molecule comprise an origin of replication.

Embodiment 17: The combination according to any one of Embodiments 13 to 16, wherein the sequence providing for a first selection marker of the first nucleic acid molecule is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding for an enzyme.

Embodiment 18: The combination according to any one of Embodiments 13 to 17, wherein the first part of a genome of a virus of the first nucleic acid molecule is a viral terminal repeat, preferably an adenoviral terminal repeat.

Embodiment 19: The combination according to any one of Embodiment 13 to 18, wherein the first part of a genome of a virus of the first nucleic acid molecule contains the adenoviral promoter pIX, more preferably the adenoviral promoter pIX is a pIX promoter from human adenovirus 19a.

Embodiment 20: The combination according to any one of Embodiments 12 to 19, wherein the second nucleic acid molecule contains exactly one site-specific recombination site.

Embodiment 21: The combination according to any one of Embodiments 12 to 20, wherein the virus genome of the second nucleic acid molecule is a human adenovirus genome, whereby in case of the combination according to Embodiment 12 the virus genome

of the second nucleic acid molecule is preferably a human adenovirus type 5 genome or a human adenoviral type 19a genome and in case of the combination according to Embodiment 13 the virus genome of the second nucleic acid molecule is preferably a human adenovirus genome which is different from human adenovirus type 5 genome, more preferably the virus genome of the second nucleic acid molecule is a human adenoviral type 19a genome .

Embodiment 22: The combination according to any one of Embodiments 12 to 21, wherein the bacterial nucleotide sequence for single copy replication of the second nucleic acid molecule comprises a replication origin for single copy maintenance in prokaryotic host cells.

Embodiment 23: The combination according to any one of Embodiments 12 to 22, wherein the nucleotide sequence providing for a second selection marker of the second nucleic acid molecule marker is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding to an enzyme.

Embodiment 24: The combination according to any one of Embodiments 12 to 23, wherein the second part of a genome of a virus of the second nucleic acid molecule comprises an inverted terminal repeat of a virus, preferably an adenoviral inverted terminal repeat and more preferably an adenoviral right inverted terminal repeat.

Embodiment 25: A method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) providing a third nucleic acid molecule as defined in any one of Embodiments 1 to 11;

b) providing a second nucleic acid molecule as defined in Embodiment 12; or

c) a combination of a third nucleic acid molecule and a second nucleic acid molecule according to any one of Embodiments 12 to 24;

d) allowing the third and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably single copy of the genome of a or the virus, whereby the genome is a complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

e) optionally selecting the recombination product; and

f) optionally cleaving the recombination product with the first and second restriction enzyme.

Embodiment 26: A method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) a combination of a first nucleic acid molecule and a second nucleic acid molecule according to any one of Embodiments 13 to 24;

b) allowing the first and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably single copy of the genome of a or the virus, whereby the genome is a complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

c) optionally selecting the recombination product; and

d) optionally cleaving the recombination product with the first and second restriction enzyme.

Embodiment 27: The method according to Embodiment 25, wherein the third and the second nucleic acid molecule are reacted in a prokaryotic host cell preferably *E. coli*, being

similar or identical to the deposited organisms at the DSMZ with the accession numbers according to the Budapest treaty DSM 23743.

Embodiment 28: The method according to Embodiment 26, wherein the first and the second nucleic acid molecule are reacted in a prokaryotic host cell preferably *E. coli*, being similar or identical to the deposited organisms at the DSMZ with the accession numbers according to the Budapest treaty DSM 23743.

Embodiment 29: A method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes and each viral genome contains a single one of the individual nucleotide sequences, comprising the steps of the method as defined in any of Embodiments 25 and 27, wherein the individual nucleotide sequence is part of the transcription unit of the third nucleic acid molecule.

Embodiment 30: A method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes and each viral genome contains a single one of the individual nucleotide sequences, comprising the steps of the method as defined in any of Embodiments 26 and 28, wherein the individual nucleotide sequence is part of the transcription unit of the first nucleic acid molecule.

Embodiment 31: A kit comprising optionally a package insert, and, in (a) suitable container(s), at least a third nucleic acid molecule as defined in any one of Embodiments 1 to 11 and/or a combination of the third nucleic acid molecule and the second nucleic acid molecule according to any one of Embodiments 12 to 24.

Embodiment 32: A kit comprising optionally a package insert, and, in (a) suitable container(s), at least a first nucleic acid molecule as defined in any one of Embodiments 13 to 19 and/or a combination of the first nucleic acid molecule and the second nucleic acid molecule according to any one of Embodiments 13 to 24.

Embodiment 33: The kit according to any one of Embodiments 31 and 32, wherein the nucleic acid molecule(s) is/are contained in a ready- to-use form and/or wherein the kit contains instructions for use.

It will be understood by a person skilled in the art that the term "to provide" or "providing" as used herein in connection with the various methods preferably also means that the nucleic acid molecule which is provided, is available for the performance of any step subject to such methods and that there is no need to synthesize such nucleic acid molecule before or immediately before such step. Rather such nucleic acid molecule may be taken from any stock of such nucleic acid molecule.

The methods of the present invention allow the construction of infectious viral vector genomes irrespectively of the number of recombination events when using site-specific recombination in bacteria, and limit the number of recombination events to one when using site-specific recombination as described for the second nucleic acid system, whereby a first such system consists of a combination of the first nucleic acid molecule according to the present invention and the second nucleic acid molecule according to the present invention, and a second such system consists of a combination of the third nucleic acid molecule according to the present invention and the second nucleic acid molecule according to the present invention. It will be acknowledged by the persons skilled in the art that in accordance with the present invention, a certain percentage of the recombination events results in multiple recombinations. Preferably, such percentage is less than 5 %, preferably less than 3 % and more preferably less than 2.5 %. The occurrence of multiple recombination events is a contamination making it necessary to screen and characterize the recombinants. Such screening is performed in connection with the methods of the invention for the generation of nucleic acid molecules coding for a virus and is referred to as selecting the recombinant product.

Both systems disclosed can be used to generate recombinant virus vector genomes. The resulting recombination products in both systems contain exactly one copy of a virus genome irrespectively of the number of integration events. This invention provided a solution to eliminate multiple recombinations and avoid screening of a library containing a plurality of viral vector genomes. Such virus genome or plurality of virus genomes is a

complemented and complete genome of a virus, preferably of an adenovirus. The genome of such virus is released by restriction digest with a unique restriction enzyme thereby removing all bacterial sequences connected to the virus genome. Therefore, the virus genomes generated by these methods are essentially free of any unwanted bacterial sequences.

The arrangement of the genetic elements in the first, the second, and the third nucleic acid molecules imply that the resulting recombination products contain exactly one copy of a complemented complete virus genome irrespectively of the number of integration events. A method is therefore disclosed in this invention in which a linearized form of the first or the third nucleic acid is reacted with a linear form of the second nucleic acid in a eukaryotic host cell permissive for the or an adenovirus in the presence of the site-specific recombinase. This method does not require the step of selecting for the recombination product.

The methods of the present invention substantially overcome current limitations of technologies making use of site-specific recombination as, e.g., subject to the Gateway ™ system, for the construction of adenovirus genomes or a plurality of adenovirus genomes. More specifically, recombination between one Frt site present on either the first or the third nucleic acid molecule according to the present invention, and on the second nucleic acid molecule circumvents the disadvantages associated with recombination between two non-identical recombination sites in vitro, and allows the generation of a plurality of viral genomes with high efficiency and fidelity.

The methods of the present invention also solve the problem of chimerism in yeast artificial chromosomes (YACs) and genomic DNA instability in multicopy cosmid or plasmid vectors. In connection the such methods this effect is mediated by the use of bacterial artificial chromosome (BAC) which replicate using the single-copy F-factor replicon (Kim UJ et al., Nucleic Acids Res., 20:1083-1085, 1992; Shizuya H and Kouros-Mehr H., Keio J. Med. 50:26-30,2001) Moreover, the methods of the present invention overcome the limitation of site-specific recombination for construction of pure libraries, through limiting the number of recombination events to one for recombination reactions occurring in E.coli subject to the above considerations on the percentage of multiple recombination events. The

library can be stably maintained in single copy BACs, and thus overcomes the limitation of current viral expression libraries. In the case of adenovirus, libraries are maintained as life viruses and thus subject to a bias due to selection of virus mutants which have a growth advantage, which is due in the case of cDNA expression libraries where the expression of the cDNA confers a growth advantage or disadvantage, and thus are over- or underrepresented in the library population. Furthermore, by use of a selection or screening according to the methods of the present invention, those recombination products having multiple insertions are altogether eliminated. As preferably used herein, selection, in case of the method for the generation of a nucleic acid molecule coding for a virus using the third nucleic acid molecule and the second nucleic acid molecule, means that the reaction product can be selected by means of use of a combination of positive and negative selection markers provided by both nucleic acid molecules, whereby the positive selection marker provided by the third nucleic acid molecule confers resistance against a selecting agent, the negative selection marker provided by the third nucleic acid confers sensitivity to a selecting agent, and the second selection marker provided by the second nucleic acid, provides resistance against a second selecting agent.

Finally, the instant invention is related to recombination system allowing the construction of large DNA libraries and a solution to apply the Frt/Flp system for construction of recombinant vector genomes *in vitro* and *in vivo*.

The instant inventor has surprisingly found that a system comprising site-specific recombination between two nucleic acids, each with one recognition site for the recombinase Flp, in an E.*coli* host harboring a plasmid which allows conditional recombinase expression was able to overcome the above mentioned limitations and allowed the construction of an adenovirus genome, and a plurality of adenovirus genomes with high efficiency, accuracy, preserving the genetic stability of the adenovirus genomes in the second nucleic acid and in the resulting recombination products, and limiting the number of recombinations between the two nucleic acids to one. Consequently the problem of the present invention is also solved by a two nucleic acid system for site specific recombination mediated by Flp recombinase, providing a high efficient reliable and simple method for construction of viral vectors based on site-specific recombination in E.*coli*.

It is a further objective of the invention to use the Flp-Frt-mediated site-specific recombination together with the disclosed two vector DNA systems, i.e. the system comprising the first nucleic acid molecules and the second nucleic acid molecule, and the system comprising the second nucleic acid molecule and the third nucleic acid molecule. This provides a high efficient, reliable, and simple method for construction of a plurality of recombined nucleic acids containing a nucleotide sequence. Preferably such nucleotide sequence is a genomic nucleotide sequence of a virus, preferably adenovirus, or (a) part(s) thereof. It is possible to use this aspect of the invention for stably maintaining genomes in excess to 300kb, in order to preserve a good transformation efficiency, however, the size of the first and the third nucleic acids should be less than 100kb and, if a library of nucleic acids shall be constructed, preferably less than 40kb, and even more preferably less than 10kb.

The invention also relates to nucleic acid molecules, vectors and methods using site-specific recombination to recombine said nucleic acid molecules in *E.coli*. Methods are disclosed for the generation of nucleic acid libraries, whereby in a preferred embodiment of the method of the present invention the method is for the generation of virus genomes and pluralities of virus genomes thereof. The nucleic acid molecules individually or as a combination, and methods disclosed in the present invention are more reliable and expand the use of site-specific recombination systems, allowing, among others, the construction of complemented and complete, respectively, viral genomes, and the generation of nonbiased stable, and libraries of viral genomes.

The invention also relates to methods for generation of nucleic acid molecules, generation of virus genomes, or a plurality of virus genomes thereof, using site-specific recombination to recombine said nucleic acid molecules in permissive eukaryotic host cells in the presence of the site-specific recombinase. Methods are disclosed for the generation of virus genomes or a plurality of virus genomes, whereby in a preferred embodiment of the method of the present invention the method is for the generation of adenovirus vectors. The methods disclosed in the present invention are considerably faster than established methods for adenovirus generation, and expand the use of site-specific recombination systems, allowing,

the generation of virus genomes or a plurality of virus genomes, which are replication competent in a complementing cell line.

The present invention provides a first nucleic acid system in E.coli with high efficiency for site-specific recombination mediated by Flp recombinase, to join two DNAs that separately are non-replicating, and form a complemented complete genome of a virus, preferably an adenovirus, after recombination. One application of nucleic acid molecules of the present invention, and more specifically the first nucleic acid molecule, the second nucleic acid molecule and the third nucleic acid molecule, is the construction of adenovirus genomes deleted for E1 and optionally E3 genes, containing foreign DNA instead of the E1 gene (first-generation adenovirus vectors).
In a further embodiment of this invention a first nucleic acid system is provided in E.coli, with high efficiency for site-specific recombination mediated by Flp recombinase, to join two DNAs that separately are non-replicating, and form a complemented complete genome of human non-type 5 adenovirus, preferably a human type 19a adenovirus after recombination. One application of the nucleic acid molecules of the present invention, and more specifically the first nucleic acid molecule, and the second nucleic acid molecule, is the construction of human non-type 5 adenovirus genomes deleted for E1 and optionally E3 genes, containing foreign DNA instead of the E1 gene (first-generation serotype adenovirus vectors).

In accordance with the present invention a second nucleic acid molecule which is preferably a BAC, is disclosed, containing one wild type Frt site, identical or similar to the vector pBACSir2 (SEQ ID No.2), or pBACSir Ad19a (SEQ ID No. 14) comprising a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequence for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker, a site-specific recombination site, preferentially a wild type Frt site (SEQ. ID 8), a second part of a genome of a virus, preferentially a second part of a genome of an adenovirus, and even more preferentially a second part of an human adenovirus type 5 or human adenovirus type 19a, and optionally a second restriction site. Furthermore a plasmid identical or similar to pDonorSir1(SEQ. ID No.1) or to the plasmid pDonorSir19a (SEQ ID No. 15), corresponding to the first nucleic acid molecule comprising a first part of a genome of a virus,

preferentially a first part of a genome of an adenovirus, and even more preferentially a second part of an adenovirus type 5 or human adenovirus type 19a, a transcription unit, a site specific recombination site, preferentially a minimal Frt site (SEQ. ID No.7), a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker, is provided.

Another embodiment of the second nucleic acid system disclosed is a plasmid similar or identical to pDonorSir2 (SEQ ID No.6) corresponding to the third nucleic acid molecule of the present invention, containing optionally a first part of a genome of a virus, preferentially a first part of a genome of an adenovirus, and even more preferentially a second part of an adenovirus type 5 a nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit, a regulatory nucleic acid sequence which has regulatory activity in a prokaryote, preferentially a bacterial promoter, a site-specific Frt site, preferentially a minimal Frt (SEQ. ID No.7), a nucleotide sequence providing for a negative selection marker, a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker, and optionally a first restriction site.

One application of this method using the two nucleic acid systems is the construction of virus genomes, or a plurality of virus genomes thereof, preferentially adenovirus genomes, or a plurality of adenovirus genomes, and more preferentially adenovirus type 5 genomes or a plurality of adenovirus type 5 genomes. One further application of the disclosed methods is the generation of viral expression libraries. The nucleic acid systems according to the present invention and the methods for the generation of a nucleic acid molecule coding for a virus genome are applicable by analogy to the manipulation and construction of equivalent non-adenovirus type 5 genomes as well. In a further embodiment such nucleic acid molecules and methods, respectively, may be applied in the mirror setting, and nucleotide sequences rescued into the right end of the adenovirus genome (e.g. E3), or applied to delete E4 genes, or to generate adenovirus genomes, or a plurality of adenovirus genomes carrying such nucleotide sequences. One application of the use of the two nucleic acid systems in the "mirror setting" is the construction of an adenovirus genome or a plurality of adenovirus genomes with mutated viral proteins.

The present invention discloses the use of a combination comprising the first nucleic acid molecule and the second nucleic acid molecule, and the use of a combination comprising the third nucleic acid molecule and the second nucleic acid molecule, respectively. The first with the second or the third with the second nucleic acid molecule is reacted in E.*coli* with high efficiency by means of site-specific recombination mediated by Flp recombinase. In the present invention the second nucleic acid molecule is a BAC and contains one, preferably a wild type Frt site, whereby the second nucleic acid molecule is identical or similar to the vector pBACSir1, pBACSir2, or BacSir19a. Furthermore, a third nucleic acid identical or similar to pDonorSir2 is disclosed. One application of this system is the construction of a fourth, a fifth nucleic acids or a plurality of fourth or fifth nucleic acid molecules, each containing one copy of a complemented complete virus genome.

Brief description of the examples

The reaction product, corresponding to a fourth nucleic acid molecule according to the method provided in this invention, results from combination followed by site-specific recombination in an E.*coli* host cell between the nucleic acid molecules pDonorSir1 or pDonorSir19a and a nucleotide acid molecule identical or similar to pBACSir1 or pBACSir19a, respectively. The reaction products resulting from site-specific recombination between the two nucleic acid molecules pDonorSir1 and pBACSir1 were characterized by restriction analysis using XhoI (Figure 2A). Restriction analysis of the once recombined reaction product pRAB1x (SEQ. ID 4) and the twice recombined reaction product pRAB2x (SEQ. ID5) is shown in Fig. 2A. The obtained reaction products pRAB1x and pRAB2x contained exactly one copy of a complemented complete human adenovirus type 5 genome, which were released from the reaction products by restriction digest with PacI. The PacI-digested DNA was transfected into 293 cells, and a recombinant adenovirus obtained. DNA from the adenoviruses isolated after productive infection of 293 cells, was analyzed by restriction digest with XhoI (Fig. 2A). A schematic representation of the Flp-mediated recombination reaction and the resulting once and twice recombined reaction products is given in figure 1.

A plurality of reaction products, corresponding each to a fifth nucleic acid molecule according to the method provided in this invention, resulting from a combination and site-specific recombination in an E.*coli* host cell of pDonorSir2 and pBACSir2 were characterized by restriction analysis using XhoI (Figure 2B). Restriction analysis with *Xho*I of the once recombined reaction product pRAB_RPSL_1x (SEQ. ID 11) and the twice recombined reaction product pRAB_RPSL_2x (SEQ. ID 12) is shown in figure 2B. The majority (83/88) of the obtained reaction products resulted from single recombination between pDonorSir2 and pBACSir2 (Fig.2B). The restriction digest pattern of (2/88) recombination products corresponded to the double recombined product pRAB_RPSL_2x. A schematic representation of the reaction scheme and the reaction products is given in figure 3. In the single recombined product pRAB_RPSL_1x the regulatory nucleic acid sequence which has regulatory activity in a prokaryote, here the E.*coli* galaktokinase promoter, and the negative selection marker, here the RPSL gene, are functionally separated from each other, whereas, surprisingly, for the double recombined product pRAB_RPSL_2x the negative selection marker in combination with the two positive selection markers was highly functional, leading to a minimal background of double-recombined reaction products. Based on the results obtained in this example (Fig.3) a matrix describing possible combinations of positive and negative selection markers for high efficient counter-selection of double recombination products pRAB_RPSL_2x is given in Fig. 6.

In another example the site-specific recombination mediated by Flp recombinase between the first nucleic acid molecule and the second nucleic acid molecule was done in a eukaryotic host cell providing the site-specific recombinase. The nucleic acid molecules pDonorSir1-EGFP (Seq. ID No.9) and pBACSir2 were digested with an enzyme recognizing the first and second restriction site, respectively, and the linearized nucleic acid molecules transfected into 293 cells stably expressing the Flp recombinase. The resulting recombination products contained one copy of a complemented complete human adenovirus type 5 genome expressing the EGFP gene being replication competent in 293 cells (Fig. 4).

We were able to transfer the here described FRT/Flp based technique to another adenovirus serotype, and generated recombinant adenovirus type Ad19a vectors expressing GFP. The adenovirus type 19a genome was cloned into a BAC (pBACSir19a) deleted for the left end of the Ad19a genome including the left ITR, the packaging site and E1 gene region including its poly-adenylation site, with a FRT site introduced at this site to allow Flp mediated insertion of a donor plasmid, which carried a copy of the deleted elements. Moreover, the pIX promoter which is necessary for expression of the pIX gene coding for a minor capsid protein was preserved in the nucleic acid pBACSir19a. In addition pBACSir19a also had the E3 region deleted. The sequence of pBACSir19a vector is provided in Seq. ID No.14). The pIX promoter and more specifically the adenovirual pIX promoter is known in the art and its sequence can be retrieved from publicly available data banks. In connection therewith it will be acknowledged by a person skilled in the art that the pIX promoter as preferably used herein, is a promoter operably linked to a pIX coding sequence in an adenovirus, whereby such adenovirus is preferably an adenovirus type 5 or an adenovirus type 19a. In an embodiment the pIX promoter is a minimal promoter, wherein said minimal promoter is a 70 nucleotide DNA element derived from the promoter region upstream of the adenovirus pIX gene. In a further embodiment the minimal pIX promoter comprises a TATA box and a Sp1 box, and, in Ad5, corresponds to nucleotides 3511 to 3580 of the adenoviral genome. Many other (sero)types of adenovires contain the pIX gene and its upstream promoter as well, and the minimal promoters derived from these pIX promoters are encompassed by this invention as well.

The donor nucleic acid pDonorSir19a carries a PacI site, Ad19a ITR and packaging signal and an EGFP transcription unit, and is comparable to the donor vector pDonorSir1 but all viral cis-elements are replaced by sequences from human adenovirus type 19a (Seq.ID No.15). The reaction products obtained according to the method provided in this invention contained an entire replication competent recombinant adenovirus type 19a genome resulting from combination followed by site-specific recombination in an E.coli host cell between the nucleic acid molecules pDonorSir19a and a nucleotide acid molecule identical or similar to pBACSir19a, respectively. The reaction products obtained were characterized by restriction analysis using KpnI (Fig.7). Two independent clones (lane 1 and lane 4) of the resulting nucleic acid were purified and digested with PacI and 293 cells transfected,

resulting in viable recombinant Ad19a vectors.

A plurality of reaction products, corresponding each to a fifth nucleic acid molecule according to the method provided in this invention, results from a combination and site-specific recombination in an E.*coli* host cell of pDonorSir2_Ad19a. The plasmid pDonorSir2_Ad19a is an embodiment of the third nucleic acid molecule of the present invention and differs from pDonorSir2 regarding all Ad5-derived sequences, which have been replaced by the corresponding Ad19a sequences. The recombination between pDonorSir2_ad19a and pBACSir19a results, according to the present invention in a single recombination product. In this single recombined product the regulatory nucleic acid sequence which has regulatory activity in a prokaryote, here the E.*coli* galactokinase promoter, and the negative selection marker, here the RPSL gene, are functionally separated from each other, whereas, surprisingly, for the double recombined product the negative selection marker in combination with the two positive selection markers is highly functional, leading to a minimal background of double-recombined reaction products. Based on the results obtained in this example a matrix describing possible synergistic combinations of positive and negative selection markers for high efficient counter-selection of double recombination products is provided in Fig. 6.

Description of the preferred embodiments

The present invention discloses a first nucleic acid with a first part of a genome of a virus which is combined with a second nucleic acid molecule comprising a second part of a genome of a virus, whereby the first and the second nucleic acid molecule are combined and reacted by site-specific recombination in *E.coli* host cells providing a site-specific recombinase. The resulting nucleic acid molecule contains exactly one copy of a complemented complete genome of a or the virus, whereby the virus genome is replication competent in permissive cells. A schematic illustration of this invention is shown in Fig.1. In a further embodiment of this invention a third nucleic acid molecule is described. The third nucleic acid is combined and reacted by site-specific recombination with a second nucleic acid molecule in *E.coli* host cells providing a site-specific recombinase. The organization of

the genetic elements in the third nucleic acid molecule is inventive, and according to the method provided in this invention restricts the number of recombination events to one in >97.5% of cases. This efficiency is sufficient for the construction of a plurality or library of fifth nucleic acid molecules and solves the problem of the need for screening such plurality of nucleic acid or library for single recombined products. A diagrammatic representation of this method is shown in Fig. 3.

The first nucleic acid molecule comprises a first part of a genome of a virus, preferentially a first part of a genome of an adenovirus, and even more preferentially a first part of the human adenovirus type 5 or a human adenovirus type 19a. Moreover, the first nucleic acid molecule comprises a transcription unit, a site specific recombination site, preferentially a minimal Frt site (SEQ. ID No.7), a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker. In a preferred embodiment the first nucleic acid is a bacterial plasmid containing a first part of a or the genome of a virus, whereby the first part of a genome of a or the virus is a terminal repeat, preferably an inverted terminal repeat. In a more preferred embodiment the virus is an adenovirus, and the terminal repeat is an inverted terminal repeat of an adenovirus. In a most preferred embodiment, the virus is the human adenovirus type 5 and the first part of the genome of the human adenovirus type 5 is the left inverted terminal repeat.

In one embodiment of the present invention the first part of a genome of a virus comprises a packaging signal. In a preferred embodiment the packaging signal is part of the terminal sequence, whereby in a more preferred embodiment the packaging signal is the packaging signal (Ψ5) from human adenovirus type 5 extending from nt194 to nt385 of the AV5 genome Packaging of adenoviral vectors depend on a series of 7 'A' repeats that are used in a hierarchical order with some being more important than others. Therefore it is possible to define synthetic or minimal packaging sequences by combining parts of sequences derived from this region. The location of these cis-acting packaging elements to the left part of the adenovirus genome has been experimentally confirmed for many other types of adenoviruses. Moreover, the identification of trans-acting factors for the packaging process has identified several adenovirus proteins acting in a subtype specific way, allowing only

packaging of viral DNA if the encapsidation signal Ψ and the trans acting factors are derived from the same subtype or are compatible.

In a further embodiment of the invention the first part of a genome of a virus comprising the entire or parts of the left end of AV5 genome upstream of the TATA box of the E1 transcription unit from nt1 to nt 342 (SEQ ID 10).

In a further embodiment of this invention the first part of a genome of a virus contains an inverted terminal repeat (ITR), whereby in a preferred embodiment the inverted repeat is derived from the left end of the human adenovirus type 5 (AV5) and comprises the left inverted terminal repeat. The length of the left inverted terminal repeat sequence (left ITR) extends from nucleotide 1 to nucleotide 103 of the AV5 sequence. The size of the ITRs vary among the serologically distinct types of adenoviruses, and minimal terminal ITR sequences as short as 18bp (nt1 to nt18) supporting human Adenovirus type 4 virus replication in vivo can be defined. Although the terminal 18-bp of the ITR supports basal level of DNA replication, the auxiliary region is needed for maximum efficiency in subgroup C adenoviruses, AV2 and AV5, respectively. Other virus types (e.g. adeno-associated viruses AVVs) do also rely on the presence of an ITR for virus replication. For human AAV type 2 the length of the ITR is 145 nucleotides and is an essential terminal sequence required for virus replication. The principle also applies to other types of viruses that contain terminal sequences other than ITRs (e.g. SV40, baculovirus, gamma herpesviruses) needed for replication and encapsidation. As an example, the alpha sequence of the cytomegalovirus genome functions as a cleavage/packaging signal for herpes simplex virus defective genomes.

In one embodiment the invention the first nucleic acid comprises a first restriction site, whereby this sequence is absent in the first part of the genome of a virus and in the transcription unit present in the first nucleic acid. In a preferred embodiment of this invention the restriction site is chosen from a group of restriction sites absent in the genome of an adenovirus. In a more preferred embodiment, the restriction site is selected from a group of sites absent in human adenovirus type 5 (AV5) comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

The first nucleic acid molecule comprises the following elements: a site-specific recombination site, a bacterial nucleotide sequence comprising (i) bacterial nucleotide sequences for conditional replication, and (ii) a nucleotide sequence providing for a first selection marker, a first restriction site, a first part of a genome of a virus, and a transcription unit, whereby in a preferred embodiment the virus is an adenovirus, and in more preferred embodiment, the virus is a human adenovirus type 5 or human adenovirus type 19a.

In a further embodiment the preset invention provides a first nucleic acid molecule comprising the following elements in a 5`to 3`orientation obtained after linearization of the first nucleic acid molecule optionally with the first restriction enzyme: the first part of a genome of a virus, a transcription unit, a site-specific recombination site, the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker, optionally a first restriction site, By an inventive matter this is the preferred orientation of the genetic elements of the first nucleic acid molecule.

In one embodiment of this invention the first nucleic acid contains the genetic elements in a "mirror conformation", comprising in a in 5'-> 3'orientation: a site specific recombination site, a transcription unit, a first part of a genome of a virus, optionally a first restriction site, and bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication, and (ii) a sequence providing for a first selection marker. By an inventive matter this is a further orientation of the genetic elements of the first nucleic acid molecule.

In a further embodiment the first part of a genome of a virus in a "mirror conformation" comprises a terminal repeat. In a preferred embodiment the first part of a genome of a virus comprises an inverted terminal repeat. In a more preferred embodiment it comprises a right terminal repeat of a virus genome, and even more preferably the right terminal repeat of an adenovirus, being the last 103 nucleotides of the genome of a human adenovirus type 5 virus genome.

A further embodiment of the invention relates to a transcription unit containing a promoter, optionally a nucleic acid sequence to be expressed, and a termination signal.

A further embodiment of the invention the first nucleic acid contains a transcription unit comprising a nucleic acid sequence to be expressed operable linked to a a promoter, and a termination signal, whereby the promoter shall be selected from the group of eukaryotic or viral promoters recognized by eukaryotic RNA Pol II such as PGK, and CMV, or from the group of eukaryotic or viral promoters recognized by RNA Pol III such as U6, H1, tRNA, and Adenovirus VA promoter.

A further embodiment of the invention relates to a transcription unit containing a promoter, a nucleic acid sequence to be expressed, and a termination signal, whereby the nucleic acid to be expressed is chosen from the group of nucleic acids encoding a protein, a peptide, a nucleic acid encoding non-coding RNA, including microRNAs, and small interfering RNAs (siRNAs), and shRNAs.

In another aspect of the invention the transcription unit contains a promoter, a sequence to be expressed, and a transcriptional termination signal, whereby the termination signal is derived from eukaryotic or viral genes such as a poly A signal, termination signals for RNA PolIII-transcribed genes, such as a stretch T nucleotides.

In a further embodiment of this invention the first nucleic acid comprises a site-specific recombination site for Flp recombinase. The Frt site used in the first nucleic acid molecule is based on the wild type Frt site from μ plasmid of *S. cerevisiae*. In one embodiment of the invention the Frt site used is not restricted to forms derived from the wild type 48 Frt site (SEQ ID. 8). It may be chosen from a group of other Frt sited including mutated Frt sites known in the art. (Schlake T. and Bode J. Biochemistry 33:12746-12751, 1994[54]; WO/1999/025854). In a preferred embodiment of this invention the Frt site used in the first nucleic acid molecule is a minimal recombination site of 34nt length (SEQ. ID 7) containing the R2, the U and the R3 element of the wt FRT site (Cherepanov PP and Wackernagel W. Gene 158:9-14, 1995).

In one embodiment of the invention the first nucleic acid molecule with a bacterial sequence unit comprises (i) bacterial sequences for conditional replication and (ii) a sequence providing for a first selection marker, whereby the bacterial sequences for replication contain an origin of replication (ori).

In another embodiment of the invention the first nucleic acid molecule with a bacterial sequence unit comprises (i) bacterial sequences for conditional replication and (ii) a sequence providing for a first selection marker, whereby the bacterial sequences for replication contain an origin of replication for conditional replication in special *E. coli* strains or in normal *E. coli* strains under specific conditions where the bacterial cell provides all functions necessary. In a preferred embodiment, bacterial sequences in the first nucleic acid molecule contain the minimal ori of phage gR6K as conditional replicon which can be maintained only in the presence of pi protein expression (Shafferman A et al., J. Mol. Biol. 161:57-76, 1982).

One embodiment of this invention provides a first nucleic acid molecule with a sequence providing for a first selection marker, whereby the selection marker is a nucleic acid that confers resistance to a cell harboring such nucleic acid against a selecting agent. In a preferred embodiment of the invention, the first selection marker encodes a gene, and in a more preferred embodiment the first selection marker preferably mediates resistance against an antibiotic including ampicillin, zeocin, gentamycin, chloramphenicol, tetracycline, and kanamycin among others known in the art. In a most preferred embodiment of this invention the first selection marker mediates resistance against kanamycin.

The first selection marker can be selected from a group of genes mediating resistance to antibiotics, including *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, *ble*, and *cml*A, *aad*A, *aad*B, *sac*B, *and tet*A genes among other genes known in the art. In a preferred embodiment a gene encoding a protein mediating resistance to kanamycin is the first selection marker.

One embodiment of the invention is a second nucleic acid molecule comprising the following elements: a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequence for single copy replication, and (ii) a nucleotide sequence providing for a second

selection marker, a site-specific recombination site, a second part of a genome of a virus, and optionally a restriction site which is referred to as second restriction site.

One embodiment of the invention is a second nucleic acid molecule containing a second part of a genome of a virus, whereby the second part of a genome of a virus combined with the first part of a genome of a virus form a complemented complete virus genome able to replicate in a complementing cell line.

In a preferred embodiment, the second nucleic acid contains the second part of an adenovirus genome, and in a more preferred embodiment the second nucleic acid contains the second part of a human adenovirus type 5 (AV5) genome. In a more preferred embodiment the second part of a virus genome is the AV5 genome deleted for the left ITR, the E1 region and the E3 region of AV5, and optionally for the encapsidation signal Ψ5, whereby the first nucleic acid complements this virus genome for the left ITR and optionally the encapsidation signal. Moreover, the deletions of the second part of the AV5 genome are not limited to E1 and E3, since additional sequences from the E2 or the E4 region may be deleted as well, provided, that a permissive cell line can complement for the deleted sequences in cis or trans.

In another preferred embodiment the second nucleic acid contains the second part of an adenovirus genome, and in a more preferred embodiment the second nucleic acid contains the second part of a human adenovirus type 19a (AV19a) genome. In a more preferred embodiment the second part of a virus genome is the AV19a genome deleted for the left ITR, the E1 region and the E3 region of AV19a, and optionally for the encapsidation signal Ψ5, whereby the first nucleic acid complements this virus genome for the left ITR and optionally the encapsidation signal. Moreover, the deletions of the second part of the AV19a genome are not limited to E1 and E3, since additional sequences from the E2 and/or the E4 region may be deleted as well, provided, that a permissive cell line can complement for the deleted sequences in cis or trans. Moreover, the pIX promoter which is necessary for expression of the pIX gene encoding for a minor capsid protein was preserved in the nucleic acid pBACSir19a.

One embodiment of the invention is a second nucleic acid containing a site specific recombination site. The site-specific recombination site is selected from the group comprising the recombination site for Flp recombinase. In a preferred embodiment of the invention the Frt site used is the wild type Frt48 site from μ plasmid of *S. cerevisiae* without being restricted to it. Other Frt sites can be used, including mutated Frt sites known in the art.

In a further embodiment of this invention, the second part of the genome of a virus comprises a terminal repeat, preferably a viral terminal repeat, and more preferably an inverted terminal repeat. Preferably, the inverted terminal repeat is the right inverted terminal repeat from an adenovirus genome, and in a most preferred embodiment the adenovirus genome is derived from human adenovirus type 5 or human adenovirus type 19a, and the inverted terminal repeat is the right inverted terminal repeat of human adenovirus type 5 or human adenovirus type 19a.

One embodiment of the invention provides a second nucleic acid molecule comprising a second restriction site, whereby the restriction site is absent in the second part of the genome of a virus. The restriction site is used for linearization of the nucleic acids contained according to the methods disclosed in this patent. Moreover, the second restriction site is absent in the first part of the genome of a virus provided by the first nucleic acid molecule, and in the sequence part of the first nucleic acid ranging from the first restriction site to the recombination site and encompassing the first part of the virus genome. In a more preferred embodiment of this invention the restriction site is chosen from a group of restriction sites absent in the genome of an adenovirus. In an even more preferred embodiment, the restriction site is selected from a group of sites absent in human adenovirus type 5 (AV5) comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI, among other restriction sites known by persons skilled in the art, including other types of sites recognized by homing endonucleases and synthetic binding sites for zinc finger nucleases.

One embodiment of the invention provides a second nucleic acid containing a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication and (ii) a nucleotide sequence providing for a second selection marker. In a

preferred embodiment the bacterial nucleotide sequences for replication (ori) contain all elements necessary low copy, preferably singly copy maintenance in *E. coli*. In a more preferred embodiment the ori in the second nucleic acid is based on the f-episomal factor (F-factor), and contains all elements which are necessary for replication and maintenance in E.*coli*.

One further embodiment of this invention is a second nucleic acid molecule comprising the following elements in a 5'-> 3' direction upon linearization of the second nucleic acid molecule with a restriction enzyme, preferably a restriction enzyme recognizing and cutting at the second restriction site: a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequence for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker, a site-specific recombination site, a second part of a genome of a virus, and optionally a second restriction site.

In a further embodiment of this invention the second nucleic acid molecule contains a sequence providing for a second selection marker coding for a resistance mediating gene, and more preferably for an resistance mediating gene encoding for an enzyme. The selection marker used in the second nucleic acid is different from the selection marker present in the first nucleic acid molecule. In a preferred embodiment the second selection marker confers resistance against antibiotics, including ampicillin, zeocin, gentamycin, chloramphenicol, and kanamycin among others known in the art. In a most preferred embodiment the second selection marker mediates resistance against chloramphenicol.

The second selection marker in the second nucleic acid molecule can be selected from a group of genes mediating resistance to antibiotics, including *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, *ble*, *aad*A, *aad*B, and *cmlA* genes among other genes known in the art. In a more preferred embodiment the second selection marker is a gene encoding a protein mediating resistance to chloramphenicol.

In a further embodiment of the invention, referred to as "the mirror conformation" the second nucleic acid molecules provides the following elements in a 5'-> 3' direction: optionally a second restriction site, a second part of a or the genome of a virus, a

recombination site, and a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication and (ii) a nucleotide sequence providing for a second selection marker, whereby the second part of the genome of the virus provides a left terminal repeat, and in a preferred embodiment the left inverted terminal repeat (ITR) of a virus genome. In a more preferred embodiment, the virus is an adenovirus, and in an even more preferred embodiment, the adenovirus is the human adenovirus type 5 or human adenovirus type 19a.

In a further embodiment of the invention the second nucleic acid molecules in a"mirror conformation" provides the second part of a or the genome of the virus providing a packaging signal, whereby in a preferred embodiment the virus is an adenovirus, and in an more preferred embodiment, the adenovirus is the human adenovirus type 5 or human adenovirus type 19a.

In one embodiment of the present invention the second nucleic acid replicates as a single copy vector in *E. coli*, whereby the ori used is based on F-factor or a P1 replicon. In a preferred embodiment the second nucleic acid is a bacterial artificial chromosome (BAC) without being limited to a BAC. However, the system requires low copy, preferably single copy maintenance of the second nucleic acid in E.*coli* in order to retain full functionality. In a more preferred embodiment, the BAC vector identical or similar to pBACSir1, pBACSir2, or pBAC Sir19a encodes a first generation E1 and E3 deleted Ad vector genome deleted for the left ITR and the encapsidation signal, and contains the *parS* the *parA, parB and parC* genes as elements of the origin of replication which are necessary for single copy maintenance.

One embodiment of the invention is the combination of a circular closed form of the first nucleic acid molecule with a circular closed form of the second nucleic acid molecule, whereby in a preferred embodiment the first nucleic acid used is a plasmid and the second nucleic acid is a BAC vector.

A further embodiment of the invention is a combination of the first and the second nucleic acid molecule, whereby both nucleic acid molecules are present as separate molecules. The

term "separate molecules" means that each molecule is dissociable in physical distinct compartments. In a preferred embodiment of this invention the first nucleic acid molecule is a plasmid and the second nucleic acid molecule is a BAC.

One embodiment of the invention is the combination of a first nucleic acid molecule with the second nucleic acid molecule, whereby the first part of a genome of a virus provided by the first nucleic acid, and the second part of a genome of a virus provided by the second nucleic acid, if taken together form a complete virus genome. The term "complete virus genome" describes a nucleic acid encoding a viral genomic sequence which upon transfection into a eukaryotic cell lines gives rise to viable and replication competent virus. Such a cell line is termed a permissive cell line. In a preferred embodiment of the invention the virus genome is an adenovirus genome, and in a more preferred embodiment of the invention the virus genome is the human adenovirus type 5 genome or human adenovirus type 19a genome.

One embodiment of the invention is the combination of a first nucleic acid molecule with the second nucleic acid molecule, whereby the first restriction site provided by the first nucleic acid molecule and the second restriction site provided by the second nucleic acid molecule are chosen from a group comprising restriction sites that are absent in the first part and the second part of the genome of a virus, and the transcription unit. In a preferred embodiment the restriction sites are selected from the group that does not cut in the human adenovirus type 5 genome: AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI. In a further preferred embodiment the first and the second restriction site are identical, an in an even more preferred embodiment the first and second restriction site is PacI.

In one embodiment of the invention the complete virus genome is an adenovirus genome, whereby in a more preferred embodiment the adenovirus is the human adenovirus type 5 or human adenovirus type 19a. In an even more preferred embodiment, the complete virus genome is a first generation – E1 and E3-deleted human adenovirus type 5 or human adenovirus type 19a without being limited to this type of adenovirus genome, since additional sequences from the E2 region may be additionally deleted or multiple regions

changed, or even the complete virus genome except for the left and right ITR and the packaging signal deleted in gutless adenovirus vectors.

One embodiment of the invention is the combination of a first nucleic acid molecule with the second nucleic acid molecule, whereby the first selection marker provided by the first nucleic acid molecule and the second selection marker provided by the second nucleic acid molecule, is a gene preferably encoding for an enzyme conferring resistance agianst an antibiotic. The gene may be chosen from a group conferring resistance against kanamycin, neomycin, puromycin, ampicillin, zeocin, gentamycin, and chloramphenociol among others known in the art. In a preferred embodiment the first selection marker is a gene confers resistance against kanamycin, and the selection marker is a gene conferring resistance against chloramphenicol but not kanamycin. It is know in the art, that several genes mediate resistance against more than one selection agent, especially if the selection agent is an antibiotic (Tenorio C et al. J. Clin. Microbiol. 39:824-825, 2001), limiting the possible combinations of selection markers for the first and second nucleic acid.

One embodiment of the invention is the combination of a first nucleic acid molecule with the second nucleic acid molecule, whereby the bacterial sequences for replication provided by the first nucleic acid molecule allow for conditional replication in special *E. coli* strains or in normal *E. coli* strains under specific conditions where the bacterial cell provides all functions necessary. Moreover, the combination of the sequences for replication of the first and the second nucleic acid allow only for replication of the second nucleic acid in a host cell. It is known in the art, that the combination of sequences for bacterial replication is restricted to the presence of factors provided by the host cell or the nucleic acid itself (Scott JR. Regulation of plasmid replication. Microbiol. Rev. 48:1-23, 1984). In a preferred embodiment, bacterial sequences in the first nucleic acid molecule contain the minimal ori of phage gR6K as conditional replicon which can be maintained only in the presence of pi protein expression, and the sequences for replication of the second nucleic acid are based on the F-factor and allow for single copy maintenance in E.*coli* cells.

One embodiment of the invention is the combination of a first nucleic acid molecule providing the first part of a or the genome of a virus and a second nucleic acid molecule

providing the second part of a or the genome of a virus, whereby the packaging signal may be provided by either the first or the second part of the genome of a virus. In a preferred embodiment the virus is an adenovirus, and in a more preferred embodiment the virus is a human adenovirus and in an even more preferred embodiment the virus is human adenovirus type 5 (AV5) or human adenovirus type 19a, and the packaging signal is derived from AV5 or the human adenovirus type 19a (Ψ19a) and provided by the first nucleic acid molecule.

One embodiment of the invention is the combination of a first nucleic acid molecule with the second nucleic acid molecule, whereby a first terminal repeat sequence is part of the first part of the genome of a or the virus provided by the first nucleic acid molecule, and a second terminal repeat sequence is part of the second part of a genome of a virus provided by the second nucleic acid molecule.

In a further embodiment of this invention either the first or the second nucleic acid molecule can provide all terminal repeat sequences. In a preferred embodiment the terminal repeat sequences are the inverted terminal repeat sequences derived from an adenovirus, and in an even more preferred embodiment the inverted terminal repeats are derived from AV5 or human adenovirus type 19a.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus, comprising a combination of a first nucleic acid molecule with a second nucleic acid molecule, whereby both nucleic acids are reacted through their site-specific recombination sites forming a recombination product, whereby the recombination product is selected and contains only one copy of a complete virus genome, and whereby the recombination product is cleaved with the first and second restriction enzyme.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus where the first and the second nucleic acid molecules are combined and reacted through their site-specific recombination sites in a prokaryotic host cell. The host cell is preferably a bacteria cell and can accept nucleic acids by either being electroporated or made chemically competent according to standard methods. In a preferred embodiment the bacterial host cell harbors the second nucleic acid molecule and accepts the

first nucleic acid molecule by means of electroporation. In a most preferred embodiment the bacteria is E.*coli*.

In a further embodiment of this invention the bacterial host cell is selected from a group of E.*coli* cells lacking the F-factor and being sensitive to the first and second selecting agent. In a preferred embodiment the E.*coli* strain is K12-derived and does not provide or express the pi protein. The pi protein sustains the replication of the first nucleic acid molecule, but not of the second nucleic acid molecule. In a more preferred embodiment the E.*coli* strain is sensitive to kanamycin and chloramphenicol, and selected from a group comprising DH5alpha, DH10B, among others known in the art.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus, whereby a first nucleic acid molecule with a first selection marker and a second nucleic acid molecule with a second selection marker are combined and reacted through their recombination sites in the presence of a site-specific recombinase, forming a recombination product in a prokaryotic host cell. The method is such, that the reaction product is selected in the host cell by conferring resistance against both selection markers. The use of a conditional origin of replication in the first nucleic acid ensures that the method selects exclusively for reacted products. In a preferred embodiment, the first selection marker is kanamycin, and the second selection marker is chloramphenicol.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus where a first nucleic acid molecule and a second nucleic acid molecule are combined and reacted in the presence of a site-specific recombinase which catalyses without the need of a source of energy like ATP the recombination between the first site-specific recombination sites provided by the first nucleic acid molecule and the second site-specific recombination site provided by second nucleic acid molecule. In a preferred embodiment the site-specific recombinase is Flp, whereby it mediates the recombination between the Frt site-specific recombination site present on the first nucleic acid and the Frt site-specific recombination site present on the second nucleic acid. Flp catalyzes the site-specific recombination between Frt sites, whereby the recognized site-specific recombination sites are large enough to be statistically absent in the human and

bacterial genome. According to the invention, a minimal wild type Frt34 site is used in the first nucleic acid and reacted with a wild type Frt48 site present in the second nucleic acid. However, other site-specific recombinases known in the art may be used, provided they function with equally high selectivity and efficiency.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus whereby a first nucleic acid molecule and a second nucleic acid molecule are combined and reacted in the presence of a site-specific recombinase, and whereby the recombinase is inactivated. It is generally acknowledged that a prolonged presence of a site-specific recombinase in *E.coli* interferes with genome stability. In the case of Cre, cryptic loxP sites are recognized in the mammalian genome causing genetic instability, and limiting the use of Cre-containing E.coli for receiving BAC and PAC vectors (Semprini S et al. Cryptic loxP sites in mammalian genomes: genome-wide distribution and relevance for the efficiency of BAC/PAC recombineering techniques. Nucleic Acids Res. 35:1402-1410, 1997). Preferably, transient expression of the site-specific recombination is desired when nucleic acid molecules need to be recombined and further propagated in E.*coli*, and even more preferably, expression of the site specific recombinase is fully eliminated after the recombination has occurred and during the growth of the bacteria.

In a preferred embodiment of the method for the generation of nucleic acid molecules coding for a virus the Flp expression is controlled by a temperature sensitive repressor from lambda phage. The Flp expression is induced by shifting the culture temperature to 43$_o$C. This procedure allows elimination (curing) of the plasmid at the same time. Other systems for conditional and/or inducible expression of a site-specific recombinase may be used instead, for example, without being limited to it, use of an arabinose-inducible *AraC-P$_{BAD}$* promoter to induce expression (Lee EC., et *al.* Genomics 73:56-65, 2001).

In a further embodiment of the method for the generation of nucleic acid molecules coding for a virus conditional expression for a site-specific recombinase in bacterial cells is used, whereby the replication of a plasmid harboring an expression unit for the Flp site-specific recombinase is controlled by a temperature-sensitive origin of replication. In a preferred embodiment E.*coli* host cell harboring the second nucleic acid molecule and a bacterial

plasmid (pCP20) providing a Flp expression unit, can be maintained and propagated at 30oC in the presence of ampicillin. The Flp expression is induced by shifting the culture temperature to 43oC. This procedure allows elimination (curing) the pCP20 in the same time (Cherepanov PP and Wackernagel W, Gene 158:9-14, 1995).

In the method disclosed for the generation of nucleic acid molecules coding for a virus, the nucleic acid coding for the complete virus can be released by restriction digest with the first and second restriction enzyme. In a preferred embodiment of this invention the restriction site is chosen from a group of restriction sites absent in the genome of an adenovirus, and in an even more preferred embodiment, the restriction site is selected from a group of sites absent in human adenovirus type 5 (AV5) comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

One embodiment of the invention discloses a method for the generation of nucleic acid molecules coding for a virus, comprising a combination of a linear form of a first nucleic acid molecule with a linear form of the second nucleic acid molecule, whereby both nucleic acids are reacted through their site-specific recombination sites forming a recombination product in a permissive cell, whereby the recombination product is not selected, and whereby it contains only one copy of a complete virus genome, and whereby the site-specific recombinase is provided by the permissive cell, and whereby the site-specific recombinase is either expressed in a constitutive, a conditional or in an induced way. Conditional or induced expression of the site-specific recombinase can be achieved with the tetracyclin-regulated expression system among other systems known in the art. In a preferred embodiment of the invention the permissive cell expresses the site-specific recombinase stably, whereby the permissive cell is selected from a group comprising 293, 911, Per.C6 and CAP cells. In an even more preferred embodiment the permissive cell is 293, and the site-specific recombinase Flp is constitutively expressed.

One embodiment of the invention provides a method for the generation of nucleic acid molecules coding for a virus where a first nucleic acid molecule and a second nucleic acid molecule are combined and reacted in the presence of a site-specific recombinase, whereby according to the invention the resulting nucleic acid molecule contains one copy of a

complete virus genome, which can be released by restriction digest with the first and second restriction enzyme, and generates a viable replication-competent virus when transfected into a permissive cell line, whereby the permissive cell is selected from a group comprising 293, 911, Per.C6 and CAP cells. In an even more preferred embodiment the permissive cell is 293.

One embodiment of the invention provides a method for the generation of nucleic acid molecules coding for a virus whereby the virus can be used as gene transfer vector, as vaccine or used for therapeutic applications.

One embodiment of the invention provides a method for the generation of nucleic acid molecules coding for a virus whereby the method can be used to generate large numbers of viruses or a library of viruses expressing nucleic acids.

The present invention discloses a third nucleic acid, whereby the third nucleic acid molecule comprises the following elements: optionally a first part of a or the genome of a or the virus, a nucleotide sequence, preferably a genomic nucleotide sequence or a transcription unit, a regulatory nucleic acid sequence which has regulatory activity in a prokaryote, a site-specific recombination site, a nucleotide sequence providing for a negative selection marker, a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker, and optionally a first restriction site.

In a further embodiment of the invention the third nucleic acid molecule comprises the following elements in a 5`to 3`orientation preferably upon cleavage with the first restriction enzyme: optionally the first part of a or the genome of a or the virus, the nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit, the regulatory nucleic acid sequence which has regulatory activity in a prokaryote, a site-specific recombination site, a nucleotide sequence providing for a negative selection marker, a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker

In one embodiment of the invention the first part of a or the genome of a or the virus provided by the third nucleic acid contains parts of the terminal sequence. In a preferred embodiment of this invention the terminal sequences comprise a terminal repeat. Moreover, the first part of a or the genome of a or the virus must be present in order to be a complete virus genome able to replicable in a permissive cell line. In a more preferred embodiment of the invention, the first part of a or the genome of a or the virus is derived from an adenovirus genome, and in an even more preferred embodiment, the first part of the human adenovirus genome is derived from a human adenovirus type 5 (AV5) genome, comprising the entire or parts of the left end of AV5 genome upstream of the TATA box of the E1 transcription unit (nt1 to nt 342) (SEQ.ID. No.10). In a further embodiment of this invention the third nucleic acid molecule provides a first part of a or the genome of a or the virus comprising a packaging signal as part of the viral genome. In a more preferred embodiment the packaging signal is derived from an adenovirus genome, and in an even more preferred embodiment, the first part of the adenovirus genome contains the packaging signal Ψ5 derived from the left end of the human adenovirus type 5 (AV5).

In one embodiment of this invention the first part of a or the genome of a or the virus provided by the third nucleic acid molecule comprises the terminal sequence of a or the genome of a or the virus. In a more preferred embodiment the terminal sequence is an inverted terminal repeat (ITR), and even more preferred the inverted repeat is derived from an adenovirus genome, and in a most more preferred embodiment, the first part of the adenovirus genome contains an inverted terminal repeat derived from the left end of the human adenovirus type 5 (AV5).

In one embodiment the invention the third nucleic acid comprises a first restriction site, whereby the first restriction site is absent in the first part of a or the genome of a or the virus and in the transcription unit present in the third nucleic acid. In a preferred embodiment of this invention the restriction site is chosen from a group of restriction sites absent in the genome of an adenovirus, and in a more preferred embodiment, the restriction site is selected from a group of sites absent in human adenovirus type 5 (AV5) comprising AbsI, BstBI, PacI, PsrI, SgrDI, and SwaI.

In a further embodiment the third nucleic acid molecule provides in a mirror confirmation, preferably upon cleavage with the first restriction enzyme in 5'-> 3'orientation: a regulatory nucleic acid sequence which has activity in a prokaryote, a site specific recombination site, a nucleotide sequence providing for a negative selection marker, a bacterial nucleotide sequence comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker, a first restriction site, a first part of a or the genome of a or the virus, and a transcription unit, whereby the first part of a or the genome of a or the virus comprises a terminal repeat. In a preferred embodiment the first part of a or the genome of a or the virus provided by the third nucleic acid in the mirror confirmation comprises an inverted terminal repeat. In a more preferred embodiment the inverted terminal repeat is a right terminal repeat of a virus genome, and even more preferably the right terminal repeat of an adenovirus, whereby in a most preferred embodiment the right terminal repeat of an adenovirus is the right ITR from human adenovirus type 5 encompassing the last 18-103 nucleotides of AV5

In one embodiment of the invention the third nucleic acid molecule comprises a gene transcription unit, whereby the transcription unit contains a promoter, a nucleic acid sequence to be expressed, and a termination signal. The promoter is selected from the group of eukaryotic or viral promoters recognized by eukaryotic RNA Pol II such as PGK, and CMV, or from the group of eukaryotic or viral promoters recognized by RNA Pol III such as U6, H1, tRNA, and Adenovirus VA promoter. The nucleic acid to be expressed is chosen from the group of nucleic acids encoding a protein, a peptide, a nucleic acid encoding non-coding RNA, including microRNAs, and small interfering RNAs (siRNAs), and shRNAs. The termination is signal is derived from eukaryotic or viral genes such as a poly A signal, termination signals for PolIII-transcribed genes, such as a stretch T nucleotides.

In a further embodiment of this invention the third nucleic acid comprises a site-specific recombination site recognized by the Flp recombinase. The Frt site used in the third nucleic acid molecule is based on the wild type Frt site from μ plasmid of *S. cerevisiae*. In a further embodiment of the invention the Frt site used is not restricted to forms derived from the wild type 48 Frt site. It may be chosen from a group of other Frt sited including mutated Frt sites

known in the art. In a preferred embodiment of this invention the Frt site used in the third nucleic acid molecule is a minimal recombination site of 34nt length (Frt34 site, SEQ.ID. 7) containing the R2, the U and the R3 element of the wt FRT48 site (SEQ.ID.NO. 8).

In one embodiment of the invention the third nucleic acid molecule with a bacterial sequence unit comprises (i) bacterial sequences for conditional replication and (ii) a sequence providing for a positive selection marker, whereby the bacterial sequences for conditional replication contain an origin of replication (ori) for replication in special *E. coli* strains or in normal *E. coli* strains under specific conditions where the bacterial cell provides all functions necessary. Replication of plasmid vectors in gram negative bacteria is controlled by host enzymes and determinants that are provided by the plasmid. Replication of plasmids only occur if all the factors necessary for replication are present in cis or in trans in the bacterial host (Kües U and Stahl U. Microbial reviews 53:491-516). In a preferred embodiment, bacterial sequences in the third nucleic acid molecule contain the minimal ori of phage gR6K as conditional replicon which can be maintained only in the presence of pi protein expression.

One embodiment of this invention provides a third nucleic acid molecule with a sequence providing for a positive selection marker, whereby the selection marker is a nucleic acid coding for an enzyme, and the enzyme mediates resistance against a selecting agent, whereby the positive selection marker can be selected from a group of genes mediating resistance against antibiotics, including *bla*, *ant(3'')-Ia, aph(3')-II*, *aph(3')-II*, *ble*, and *cmlA,* genes among other genes known in the art. In a preferred embodiment a gene encoding a protein mediating resistance to kanamycin is used as the positive selection marker.

The third nucleic acid molecule disclosed in this invention provides a negative selection marker, whereby the selection marker is a nucleic acid coding for an enzyme mediating sensitivity to a selecting agent and conditions, whereby the expression of the negative selection marker in a prokaryotic host cell is controlled by a nucleotide sequence which has regulatory activity in a prokaryote. In a preferred embodiment the regulatory nucleotide sequence is a promoter, whereby the promoter is preferentially selected from the group of

prokaryotic promoters. In an even more preferred embodiment the promoter is the E.*coli* galactokinase promoter.

In a further embodiment of the invention, the regulatory nucleotide sequence can be chosen from the group of inducible prokaryotic promoters, whereby the activity of the promoter can be regulated by various means including derepression of operons, induction of genes by ions and molecules, regulation of promoter activity by temperature, among other methods and systems known in the art.

In a further embodiment of the invention the negative selection marker provided by the third nucleic acid molecule is chosen from a class of genes coding for an enzyme, whereby the enzyme confers sensitivity to a selecting agent or condition including: sensitivity to streptomycin, lipophilic compounds (fusaric and quinaric acid), sucrose, *p*-chlorophenylalanine, trimethoprim, *t-o*-nitrophenyl-β-D-galactopyranoside among others known in the art. In a preferred embodiment of this invention the enzyme coded by the negative selection marker mediates sensitivity to streptomycin. Accordingly, the nucleic acid encoding the negative selection marker can be selected from a group of genes including *galK*, *tetAR*, *pheS*, *thyA*, *lacy*, *ccdB, and rpsL* among other genes known in the art. In a preferred embodiment the *rpsL* gene encoding a protein dominantly mediating sensitivity to streptomycin is used as the negative selection marker (Reyrat JM et al., Gene 15:99-102, 1981).

A further embodiment of the invention is a combination of the third and the second nucleic acid molecule, whereby both nucleic acids are present as circular closed molecules. In a preferred embodiment the third nucleic acid molecule used is a plasmid and the second nucleic acid is a BAC vector.

A further embodiment of the invention is a combination of the third and the second nucleic acid molecule, whereby both nucleic acid molecules are present as separate molecules. The term "separate molecules" means that each molecule is dissociable in physical distinct compartments. In a preferred embodiment of this invention the third nucleic acid molecule is a plasmid and the second nucleic acid molecule is a BAC.

In a preferred embodiment the nucleic acid provided by third nucleic acid provides a first part of the genome of a virus, and a second nucleic acid molecule provides a second part of the genome of a virus. The resulting nucleic acid after combination of the third with the second nucleic acid molecule contains one copy of a complete virus genome. In a preferred embodiment of the invention the virus genome is an adenovirus genome, and in a more preferred embodiment of the invention the virus genome is the human adenovirus type 5 genome.

A further embodiment of the invention is a combination of a third nucleic acid with a second nucleic acid, whereby the nucleic acid provided by third nucleic acid is the first part of a or the genome of a or the virus and contains a gene transduction unit, and is combined with a second nucleic acid molecule providing a second part of a or the genome of the virus. The resulting nucleic acid molecule contains one copy of the complete virus genome containing exactly one gene transduction unit. In a preferred embodiment of the invention the virus genome is an adenovirus genome, and in a more preferred embodiment of the invention the virus genome is the human adenovirus type 5 genome.

According to the invention the resulting complete virus genome can be released by restriction digest with the first and second restriction enzyme. In a preferred embodiment of the invention the first and the second restriction sites are identical on both nucleic acid molecules, and in a more preferred embodiment of the invention the restriction site recognized by *Pac*I enzyme is used.

In one embodiment of the invention a third nucleic acid molecule providing the first part of a or the genome of a virus with a first restriction site and a transcription unit is combined with a second nucleic acid molecule providing the second part of a or the genome of a virus with a second restriction site, whereby the restriction sites are chosen from a group comprising restriction sites that are absent in the first part and the second part of a or the genome of a or the virus, and the transcription unit. In a preferred embodiment the restriction sites are selected from the group that does not cut in the human adenovirus type

5genome: *Abs*I, *BstB*I, *Pac*I, *Psr*I, *SgrD*I, and *Swa*I. In an even more preferred embodiment of the invention, the first and second restriction site is *Pac*I

One embodiment of the invention is the combination of a third nucleic acid molecule providing the first part of a or the genome of a virus with a second nucleic acid molecule providing a second part of a or the genome of a e virus, whereby both nucleic acids can be recombined through their Frt sites to form a molecule which contains one copy of a complete complemented virus genome. The resulting complete complemented virus genome can be released by restriction digest with the first and second restriction enzyme, accordingly, and is viable and replication competent if transfected into a permissive cell line. In a preferred embodiment of the invention the virus is an adenovirus, and in a more preferred embodiment the adenovirus is the human adenovirus type 5 (AV5). In an even more preferred embodiment, the resulting complete virus genome is a first generation – E1 and E3 deleted AV5, whereby the composition of the adenovirus genome is not limited to E1 and E3 deleted genomes, since additional sequences from the E2 an E4 region may be additionally deleted or multiple regions changed, or even the complete virus genome except for the left and right ITR and the packaging signal deleted in gutless adenovirus vectors. The cell line used for reconstitution and propagation of the virus, also termed a "permissive cell line", is able to complement for all the deleted or changed regions in cis or trans. In case of the first generation AV5 virus genome, a cell line complementing for E1 may be used, such as 293, 911, Per.C6, N52.E6 among others known in the art. Other cell lines providing additional components of the viral genome in trans may be used as well if required Moreover, transient or conditional expression of said deleted components may also be used to allow virus reconstitution and replication.

In an embodiment of the invention the third nucleic acid molecule provides a positive selection marker and a negative selection marker. The second nucleic acid molecule provides a second selection marker. Upon combination of the third with the second nucleic acid molecule the resulting nucleic acid molecule comprises the positive and the negative selection marker from the third nucleic acid and the second selection marker form the second nucleic acid molecule. In a preferred embodiment of the invention, the positive selection marker of the third nucleic acid confers resistance against kanamycin and the

negative selection marker confers sensitivity to streptomycin, and the selection marker provided by the second nucleic acid molecule confers resistance against chloramphenicol.

A third nucleic acid molecule is combined with a second nucleic acid molecule, whereby the second selection marker provided by the second nucleic acid molecule is a resistance mediating gene coding for an enzyme conferring resistance against a selecting agent distinct from the positive selecting agent and negative selection agent provided by the third nucleic acid molecule. In a preferred embodiment of the invention, the positive selection marker is a gene conferring resistance against kanamycin, and the negative selection marker is a gene conferring sensitivity to streptomycin, and the second resistance marker provided by the second nucleic acid confers resistance against chloramphenicol but not kanamycin or streptomycin. It is know in the art, that several genes mediate resistance to more than one selection agent, especially if the selection agent is an antibiotic (Tenorio C et al. J. Clin. Microbiol. 39:824-825, 2001), limiting the possible combinations of selection markers in the third and second nucleic acid.

A further embodiment of this invention is the combination of a third nucleic with a second nucleic acid, and the third nucleic acid molecule provides a positive selection marker and a negative selection marker, whereby the activity of the negative selection marker is controlled by a nucleic acid sequence provided by the third nucleic acid, which has regulatory activity in a prokaryote. In a preferred embodiment the nucleic acid sequence controlling the activity of the negative selection marker is a promoter, and in a more preferred embodiment a prokaryotic promoter. In an even more preferred embodiment the promoter is the *E.coli* galactokinase promoter.

One embodiment of the invention is the combination of a third nucleic acid molecule comprising a bacterial nucleotide sequences for conditional replication, with a second nucleic acid molecule comprising a further nucleotide bacterial sequences for single copy replication. Thereby the bacterial sequences for replication of the third nucleic acid molecule allow for conditional replication in special *E. coli* strains or in normal *E. coli* strains under specific conditions, whereby the bacterial cell provides all functions necessary. In a preferred embodiment, the bacterial nucleotide sequence unit in the third nucleic acid

molecule contains the minimal ori of phage gR6K as conditional replicon, which can be maintained only in the presence of pi protein expression, and the sequences for replication of the second nucleic acid are based on the F-factor and allow for single copy maintenance in *E.coli* cells (Scott JR. Regulation of plasmid replication. Microbiol. Rev. 48:1-23, 1984).

One embodiment of the invention is the combination of a third nucleic acid molecule providing the first part of a or the genome of a virus and a second nucleic acid molecule providing the second part of a or the genome of a virus, whereby the packaging signal may be provided by either the first or the second part of a or the genome of a virus. In a preferred embodiment the virus is an adenovirus, and in a more preferred embodiment the virus is AV5, and the packaging signal is the packaging signal of AV5 and provided by the third nucleic acid molecule.

One embodiment of the invention is the combination of a third nucleic acid molecule providing the first part of a or the genome of a virus and a second nucleic acid molecule providing the second part of a or the genome of a virus, whereby at least one terminal repeat sequence is provided by the third and one terminal repeat sequence is provided by the second nucleic acid. In a further embodiment of this invention one nucleic acid can provide all terminal sequences, however in this case the resulting nucleic acid will then contain a complete viral genome containing the bacterial nucleotide sequence unit of one of the nucleic acid molecules. In a preferred embodiment of this invention the terminal repeat sequence is the inverted terminal sequence (ITR) of a or the genome of a virus. In an even more preferred embodiment the ITR is from derived from an adenovirus, whereby the third nucleic acid molecule provides the left ITR, and the second nucleic acid molecule provides the right ITR. In a most preferred embodiment the ITR is the ITR from the human adenovirus type 5.

According to the invention the third and second nucleic acids can be combined and reacted in a host cell through their Frt recombination sites by action of a site-specific recombinase. The resulting nucleic acid molecule contains exactly one copy of a complemented complete virus genome, which can be released by restriction digest with the first and second restriction enzyme, whereby the restriction site are being absent in the transcription unit

provided by the third nucleic acid, the first part of a or the genome of a virus, and the second part of a or the genome of a virus. In a more preferred embodiment of this invention the restriction site is chosen from a group of restriction sites absent in the genome of an adenovirus, and in an even more preferred embodiment the restriction site is selected from a group of sites absent in human adenovirus type 5 (AV5) comprising *Abs*I, *BstB*I, *Pac*I, *Psr*I, *SgrD*I, and *Swa*I.

A preferred embodiment of the invention is the combination of a third nucleic acid molecule with a second nucleic acid, whereby the virus is an adenovirus, and in a more preferred embodiment the virus is the human adenovirus type 5.

According to the method provided in this invention the recombination product of a first with a second nucleic acid molecule is a fourth, preferable circular, nucleic acid molecule comprising preferably the following elements: a bacterial nucleotide sequence unit comprising (i) bacterial sequences for single copy replication, and (ii) a nucleotide sequence providing for a second selection marker, the site-specific recombination site, the second part of a genome of a virus, optionally a second restriction site, a further site-specific recombination site, a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication, and (ii) a nucleotide sequence providing for first selection marker, the first part of a genome of a virus, preferably a transcription unit, and the first restriction site.

In this invention a method for the generation of a fourth nucleic acid molecule coding for a virus genome is disclosed, whereby a first nucleic acid molecule and a second nucleic acid molecule are provided and combined and allowed to react so that site-specific recombination occurs and a site-specific recombination product forms. Preferably the site-specific recombination occurs in a host cell, whereby more preferably the host cell is E.*coli*. The recombination product may be optionally selected, and contains a copy, preferably a single copy of a or the genome of a or the virus, whereby the genome of a or the virus is a complemented and complete virus genome, and upon optional cleavage of with the first and second restriction enzyme the resulting nucleic acid can be transfected into a permissive cell line and a or the virus generated and propagated in this permissive cell line. In a most

preferred embodiment the virus is an adenovirus, and in even more preferred embodiment the virus is the human adenovirus type 5.

In one embodiment of the invention a method for the generation of a nucleic acid molecule coding for a virus is provided, whereby a first nucleic acid molecule and a second nucleic are combined and allowed to react in a host cell so that site-specific recombination occurs and a site-specific recombination product forms. The host cell allows selection of the reaction product, whereby the host cell genome is deficient for parts of or the F-factor which allows single copy replication of the second nucleic acid, and whereby the host cell is deficient for expression of factors that allow conditional replication of the first nucleic acid. This allows selection against any non reacted first nucleic acid molecule in the host cell. In a preferred embodiment the host cell is a prokaryotic host cell, and more preferably E.*coli*. In an even more preferred embodiment the host cell is selected from a group comprising K12-derived E.*coli* host cells including DH10B among others known in the art.

In a further embodiment of the invention a method for the generation of a nucleic acid molecule coding for a virus is provided, whereby a first nucleic acid molecule and a second nucleic acid molecule are provided. The first and second nucleic acid molecules are combined and allowed to react in a host cell so that site-specific recombination occurs and a site-specific recombination product forms, whereby the reaction product does not need to be selected and contains one complete complemented genome of a or the virus. According to the method provided the host cell is an eukaryotic host cell, and the first and second nucleic acid molecules preferably are linear nucleic acid molecules, preferably upon cleavage with the first and second restriction enzymes, and whereby the eukaryotic host cell preferably is a permissive host cell, and even more preferably the host cell is selected from a group allowing replication of the human adenovirus type 5 comprising 293, 911, Per.C6, CAP cells among others known in the art. If the adenovirus is other than the human adenovirus type 5, a permissive host cell is defined as such, that it will allow replication of this virus. It is know in the art that linear nucleic acid molecules which contain one complete adenovirus genome are replicable in a permissive host cell. The efficiency of virus replication is optimal if the ends of the adenovirus genome are exactly ending with the ITRs of the adenovirus, and even more efficient if the terminal protein is attached to the left end of the adenovirus genome,

however, this is not a prerequisite for adenovirus replication in a permissive cell, since nucleic acid molecules containing a complete adenovirus genome with sequences extending the ITRs will also be replicated.

In this invention a method for the generation of a fifth nucleic acid molecule coding for a virus is provided, whereby a third nucleic acid molecule and a second nucleic acid molecule are combined and allowed to react so that site-specific recombination occurs and a site-specific recombination product forms, and whereby a reaction product is generated wherein the number of recombination events is limited to one. Preferably the site-specific recombination occurs in a host cell, whereby more preferably the host cell is E.*coli*, and whereby the selection of the recombination product is performed by selecting the host cell(s) which harbor the recombination product providing the positive selection marker of the third nucleic acid molecule, the negative selection marker of the third nucleic acid molecule, and the second selection marker of the second nucleic acid molecule, and whereby the host cell is not sensitive to the negative selection marker. According to this method, a host cell is used that is not sensitive to the negative selecting agent, whereby preferably the negative selecting agent is streptomycin. In a preferred embodiment the host cell is E.*coli*, expresses a mutant form of the *rps*L gene conferring resistance to streptomycin. The host cells can thus be selected from a group of E.*coli* cells expressing the mutant form of the *rps*L gene, and preferably the host cells are selected from group comprising DH10B among others know in the art. In a preferred embodiment, the selecting agents used to select host cells harboring the reaction product are kanamycin for the positive selection marker, chloramphenicol for the second selection agent, and streptomycin as the negative selecting agent. The negative selection marker encodes the wild type form of *rps*L, whereby the resistance to streptomycin conferred by the host cells expressing the mutant *rps*L is recessive if both the wild-type and mutant alleles of *rps*L are expressed in the same host cell strain, resulting in sensitivity to streptomycin (Reyrat JM et al. Infect. Immun. 1998, 66:4011-4017; Lederberg J. Streptomycin resistance: a genetically recessive mutation. J. Bacteriol. 1951, 61:549–550).

According to this method the third nucleic acid molecule providing a site-specific recombination site is combined with a second nucleic acid molecule providing a site-specific recombination site, and both nucleic acid molecules are allowed to react by site-specific

recombination in the host cell, whereby the site-specific recombinase is provided by the prokaryotic host cell. Thereby the site-specific recombinase is provided preferably by the host cell, either as part of the genome a host cell, or as extrachromosomal element. In a preferred embodiment the host cell provides the site-specific recombinase as an extrachromosomal plasmid, whereby in a more preferred embodiment the site-specific recombinase is Flp and the plasmid is pCP20. According to this method the expression of a site-specific recombinase is controlled during the reaction, whereby the control of the expression can be achieved by various ways including the use of a inducible expression system such as the arabinose-inducible *AraC-P*$_{BAD}$ promoter to induce expression (Lee EC., *et al.* Genomics 73:56-65, 2001) without being limited to this. In a preferred embodiment the expression of the site-specific recombinase and the replication of this plasmid is controlled by temperature, whereby in an even more preferred embodiment expression Flp is controlled by a temperature sensitive repressor from lambda phage, and replication of the plasmid controlled by a temperature-sensitive origin of replication, and whereby the temperature-sensitive FLP expression plasmid pCP20 is used (Cherepanov PP and Wackernagel W.Gene 158:9-14, 1995; Bubeck A, et al., J. Virol. 78:8026-8035, 2004).

According to this method the selected reaction product resulting from a combination of a third nucleic acid molecule and a second nucleic acid molecule and the subsequent site-specific recombination reaction in a host cell comprises a complete virus genome, whereby the selected nucleic acid molecule harbors a first restriction site, and a second restriction site, preferably being absent in an adenovirus genome, and more preferably being selected from a group of restriction sites comprising *Abs*I, *BstB*I, *Pac*I, *Psr*I, *SgrD*I, and *Swa*I, being absent in the genome of a human adenovirus type 5. According to this method the third and the second nucleic acid molecules can be introduced separately into the prokaryotic host cell, whereby in a preferred embodiment the host cell harbors the second nucleic acid and is made competent for transformation with a third nucleic acid molecule using state-of-the- art techniques. According to the method provided, the selected recombination product comprises a complete complemented virus genome, which can be released from the reaction product upon restriction digest, preferably upon restriction digest with one or more restriction enzymes binding and cleaving the nucleic acid at the first and second restriction site, respectively. This method comprises a further transfection step, whereby the released

virus genome is introduced into a permissive eukaryotic host cell using standard methods, preferably using the transfection reagent polyethylenenimine (PEI) or the calcium phosphate transfection method, among other methods known in the art. Transfection of the complete complemented virus genome into the eukaryotic permissive host cell yields a replication competent adenovirus vector, whereby the vector is used for gene transfer, vaccine or any therapeutic applications.

In this invention a method is provided for the generation of a library of nucleic acid molecule coding for a virus genome, whereby a plurality of third nucleic acid molecules and a second nucleic acid molecule is provided, whereby the plurality of third nucleic acid molecules and a plurality of second nucleic acid molecules are combined and allowed to react so that site-specific recombination occurs and a plurality of nucleic acid molecules is formed, whereby the plurality in its totality forms a library, and whereby the library consists of a plurality of fifth nucleic acid molecules, comprising the following elements of the second nucleic acid molecule: the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication, and (ii) a nucleotide sequence unit providing for a second selection marker, the site-specific recombination site, the second part of a genome of a virus, and the restriction site which is referred to as second restriction site, and the following elements from a third nucleic acid molecule comprising optionally the first part of a genome of a virus the nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit, the regulatory nucleic acid sequence which has regulatory activity in a prokaryote, the site-specific recombination site, the nucleotide sequence providing for a negative selection marker, the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker, and the first restriction site.

One embodiment of the invention discloses a method for the generation of a plurality of or a library of fifth nucleic acid molecules each coding for a complete complemented virus genome, whereby a plurality of third nucleic acid molecules and the second nucleic acid molecule are combined and reacted according to the method provided in a prokaryotic host cell, and whereby the host cell is preferably a bacteria cell and can accept nucleic acids by either being electroporated or made chemically competent according to standard methods. In

a preferred embodiment the bacterial host cell harbors the second nucleic acid molecule and accepts a third nucleic acid molecule by electroporation. In a more preferred embodiment the bacteria is *E.coli*.

According to the method provided the library of nucleic acid molecules does not need to be screened for multiple recombined products. The method is such, that host cell harboring the recombination product confers resistance to the second selection marker and the positive selection marker, and is not sensitive to the negative selection agent without expression of the negative selection marker. Moreover, according to the method provided, conditional replication of the third nucleic acid molecule ensures that the host cells will only replicate the reaction product avoiding any unwanted contaminating nucleic acid molecule. In a preferred embodiment, the positive selection marker confers resistance to kanamycin, the negative selection maker confers sensitivity to streptomycin, and the second selection marker provided by the second nucleic acid confers resistance to chloramphenicol

According to the method provided in this invention, a method for the generation of a library of nucleotide sequences comprising a plurality of individual nucleotide sequences is provided, whereby the library is represented by a plurality of virus genomes, each containing a single one of the individual nucleotide sequences, whereby the nucleotide sequence is part of a transcription unit. In a preferred embodiment of the invention the nucleic sequence is a nucleic acid to be expressed, and the nucleotide sequence is present in the complete virus genome as a single copy. In a more preferred embodiment the virus is an adenovirus and the method provides a mean to construct a plurality of individual adenoviruses. In an even more preferred embodiment the adenovirus is the human adenovirus type 5. The resulting adenovirus virus library can be used for identification of gene functions, in screening applications, for the construction of an expression or genomic library, and for gene transfer.

According to the method provided, a plurality of complete complemented adenovirus genomes each containing a nucleotide sequence, whereby each complete complemented adenovirus genome can be released by restriction digest with the first and second restriction enzyme, generating a viable replication-competent adenovirus upon transfection into a permissive host cell. The permissive cell line used for reconstitution and propagation of the

adenovirus is able to complement for all the deleted regions in cis or trans. In case of the first generation AV5 virus genome the permissive cell line complementing for the E1 function may be used, whereby the cell line may be chosen from a group comprising 293, 911, Per.C6, CAP, among others known in the art. Other cell lines providing additional components of the viral genome in trans may be used as well if required. Moreover, transient expression of said deleted components may also be used to allow virus reconstitution and replication.

In one embodiment of the invention a kit is provided, comprising optionally a package insert, and, in (a) suitable container(s), at least a first nucleic acid molecule, a second nucleic acid molecule, optionally a permissive cell line providing the site-specific recombinase, a combination of the first nucleic acid molecule and the second nucleic acid, a third nucleic acid molecule, a combination of the third nucleic acid molecule and the second nucleic acid molecule, a fourth nucleic acid molecule, a fifth nucleic acid, a plurality of a fourth nucleic acid molecule, a plurality of a fifth nucleic acid molecule, or a plurality of individual adenoviruses.
In a further embodiment of the invention, the kit comprises a first nucleic acid molecule, parts of or a second nucleic acid, preferably a linear form of the second nucleic acid, and a permissive cell line providing the site-specific recombinase. In a preferred embodiment the part of the second nucleic acid comprises at least the site-specific recombination site and the second part of a genome of a virus. According to the method provided in this invention a nucleic acid or library of said nucleic acid can be constructed comprising a nucleotide sequence or library of nucleotide sequences, each in a complete complemented virus genome, whereby the nucleic acid molecules are ready to be used and can be directly introduced into said permissive cell line in order to generate an adenovirus, or plurality of individual adenoviruses, whereby in a preferred embodiment the cell line is 293 and the recombinase is the wild type Flp recombinase, and the adenovirus the human adenovirus type 5.

In connection with the present invention it is preferred that if one part of a genome of a virus is subject to recombination or is to be subject to recombination with a or a different part of a genome of a virus as in case of recombination between the first nucleic acid molecule of the

present invention with the second nucleic acid molecule of the present invention or between the second nucleic acid molecule of the present invention with the third nucleic acid molecule of the present invention, the viruses are of the same species and preferably of the same serotype. More specifically, if the first nucleic acid molecule of the present invention conatins a part of a genome of an adenovius type 19a and is subject to recombination or is to be subject to recombination with a second nucleic acid molecule of the present invention, said second nucleic acid molecule of the present invention also contains a part of an adenovirus type 19a. Also, if the first nucleic acid molecule of the present invention conatins a part of a genome of an adenovius type 5 and is subject to recombination or is to be subject to recombination with a second nucleic acid molecule of the present invention, said second nucleic acid molecule of the present invention also contains a part of an adenovirus type 5. Likewise, if the third nucleic acid molecule of the present invention conatins a part of a genome of an adenovius type 19a and is subject to recombination or is to be subject to recombination with a second nucleic acid molecule of the present invention, said second nucleic acid molecule of the present invention also contains a part of an adenovirus type 19a. Also, if the third nucleic acid molecule of the present invention conatins a part of a genome of an adenovius type 5 and is subject to recombination or is to be subject to recombination with a second nucleic acid molecule of the present invention, said second nucleic acid molecule of the present invention also contains a part of an adenovirus type 5.

As used herein the term nucleic acid and nucleic acid are preferably used in a synonymous manner.

The invention will now be described by reference to the following figures and examples which are merely illustrative and are not to be considered as a limitation of the scope of the invention.

FIG. 1 is a diagrammatic representation showing a method for constructing a first generation adenovirus genome, whereby a first nucleic acid identical or similar to pDonorSir1, and a second nucleic acid molecule identical or similar to pBACSir1 are combined and reacted through their recombination sites forming a fourth nucleic acid (pRAB) as recombination product which can be selected and contains

exactly one copy of a complete complemented virus genome. Bacteria harboring the fourth nucleic acid containing the first and second selection marker and can be selected with the first and second selecting agent. The composition of a fourth nucleic acid molecule resulting from a single recombination event (pRAB1x) is given in figure 1A, the composition of a fourth nucleic acid molecule resulting from a double recombination event (pRAB2x) is given in figure 1B.

FIG. 2A illustrates the composition of DNA from recombinant adenovirus BACs analyzed by restriction digest with a restriction enzyme, and the composition of the DNA from two reconstituted complemented first generation adenovirus viruses generated from these BACs using the disclosed method of example 1 und example 2

FIG. 2B illustrates the composition of DNA from recombinant adenovirus BACs analyzed by restriction digest with a restriction enzyme obtained after site-specific recombination in E.*coli* using the disclosed method of example 3

FIG. 3 is a diagrammatic representation of the method disclosed in example 3 for constructing a plurality or library of fifth nucleic acid molecules. A third nucleic acid identical or similar to pDonorSir2, and a second nucleic identical or similar to pBACSir2 acid are combined and reacted through their recombination sites forming a fifth nucleic acid as recombination product which can be selected and contains exactly one copy of a complete complemented virus genome (Fig. 3A). Bacteria harboring the fifth nucleic acid containing the positive, the negative selection marker from the third nucleic acid, and the second selection marker, can be selected. The schematic composition of a fifth nucleic acid molecule resulting from a single recombination event (pRAB_RPSL1x) is given in figure 3A, the schematic composition of a fifth nucleic acid molecule resulting from a double recombination event ((pRAB_RPSL2x)) is given in figure 3B.

FIG. 4 shows GFP expressing adenovirus vectors obtained after direct transfection of linearized forms of the first and the second nucleic acid in 293 cells expressing the site-specific recombinase Flp using the disclosed method of example 5.

FIG. 5 illustrates the selective inhibition of growth of bacteria in medium containing the negative selecting agent at different concentrations harboring a double recombined BAC (pRAB_RPSL2x) according to the disclosed method of example 3.

Fig. 6 shows the combinations of positive and negative selection marker useful for generation of a plurality of fifth nucleic acid molecules according to the method provided by this invention.

Fig. 7 illustrates the composition of DNA from recombinant human adenovirus type 19a BACs analyzed by restriction digest with a restriction enzyme using the disclosed method of example 6

Brief Description of the Figures

FIG. 1 Diagrammatic representation showing a method for constructing a first generation adenovirus genome, whereby two nucleic acids are combined and reacted through their recombination sites forming a recombination product corresponding to a fourth nucleic acid molecule according to a disclosed method of example 1 and 2. The fourth nucleic acid generated can be selected and contains exactly one copy of a complete complemented virus genome. The reaction product can be cleaved optionally with a first and a second restriction enzyme in order to release a complete virus genome that can be replicated in a permissive cell. According to example 1 a first nucleic acid vector identical or similar to pDonorSir1 (Seq. ID. No. 1) contains a minimal Frt34 recombination site (SEQ.ID. No. 7) derived from the wild type Frt site, a bacterial nucleotide sequence comprising (i) bacterial nucleotide sequences for conditional replication (OriR6K) and a nucleotide sequence providing for a first selection marker conferring a host cell resistance against kanamycin (KnR), a first restriction site (RS1), a first part of a genome of a virus containing the left ITR of an adenovirus genome (ITRleft) and the packaging signal ES, and a transcription unit (TU). The second nucleic acid is a BAC vector identical or similar to pBACSir1 (Seq. ID NO. 13) or pBACSir2 (Seq. ID No.2), comprising a wild type Frt48 recombination (SEQ.ID. No. 8) site, a second part of the genome of an adenovirus (Ad) comprising the right ITR of an

adenovirus genome (ITRrigth), a second restriction site (RS2), and a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication (F'ori), and (ii) a nucleotide sequence providing for a second selection marker conferring a host cell resistance against chloramphenicol (CmR). Both nucleic acid molecules are reacted through their recombination sites in a bacterial host cell in the presence of Flp recombinase. The resulting fourth nucleic acid molecules are recombinant adenovirus BACs (pRABs) either consisting of single recombined products, or multiple recombined products. In figure 1A the schematic composition of a single recombination product (pRAB1x) (Seq.ID. No. 4) is given. In figure 1B the schematic composition of a double (pRAB2x) recombination product (Seq.ID. No. 5) is given. Upon digestion of the DNA of a fourth nucleic acid with the first and the second restriction enzyme, a complete adenovirus genome is released from the pRABs containing the left and the right ITR, the packaging signal, and the transcription unit (Fig. 1C). Viable first generation adenovirus vectors are obtained in 97% of the cases if the DNA of a fourth nucleic acid obtained according to the disclosed method in example 1 and digested with the first and second restriction enzyme according to the disclosed method in example 2 is transfected into permissive 293 cells.

FIG. 2A illustrates the composition of two reconstituted first generation adenovirus viruses obtained using the method disclosed in example 1. The DNA of two types of reaction products are pRAB1x and pRAB2x, resulting from single and double insertion of pDonorSir1 into pBACSir1, whereby pDonorSir1 is identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 23753, and whereby pBAcSir1 is identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 24298. The respective reaction products were isolated from a growing culture of DH10B bacteria according to standard protocols, and characterized by restriction digest with *Xho*I (FIG.2A, lanes 3-4). In lane 1 a nucleotide length marker was loaded, providing reference fragments with defined length between 1 and 10 kb. In lane 2 the restriction pattern of the recombinant adenovirus BAC vector pRABref (Seq. ID. No. 3) is given as a reference. The in silico generated *Xho*I restriction pattern of pRABref is as follows: 14.5kb, 10.274kb, 7.403kb, 2.466kb, 1.445kb, and 0.595kb. Analysis of the single recombination product RAB1x (lane 3) yields a characteristic additional pair of bands of 6.266kb and 4.187kb length, respectively. The *in silico* generated pattern for

digestion with *Xho*I of the single recombined reaction product RAB1x (Seq. ID. No. 4) is as follows: 14.5kb, 10.274kb, 6.266kb, 4.187kb, 2.466kb, 1.445kb, and 0.595kb. In case of the double recombined product, a third additional band of 3.05kb appears in the (lane4). The in silico generated pattern for digestion with *Xho*I of the double recombined reaction product RAB2x (Seq. ID. No. 5) is as follows: 14.5kb, 10.274kb, 6.266kb, 4.187kb, 3.05kb, 2.466kb, 1.445kb, and 0.595kb. In a further experiment the RAB1x and RAB2x DNA was isolated and cut with *Pac*I restriction enzymes (corresponding to RS1 and RS2 in figure 1, respectively), and transfected into permissive 293 cells. Figure 2A shows the restriction pattern of virus DNA isolated from 293 cells transfected with *Pac*I-restricted RAB1x (lane 6) and RAB2x (lane7). The *in silico* generated pattern for digestion with *Xho*I of the viral DNA obtained from both reconstituted recombinant adenoviruses, RAB1x and RAB2x respectively, is as follows: 14.5kb, 8.499kb, 3.365kb, 2.466kb, 1.445kb, and 0.595kb. The restriction fragment pattern is identical for both viruses since the identical complete complemented adenovirus genome is liberated from the pRABs upon digestion with PacI. The restriction pattern of RAB1x or RAB2x was compared to an empty adenovirus, being essentially the same as RAB1x and RAB2x, but lacking the transcription unit (Fig.2, lane 5). The *in silico* restriction fragment pattern upon digestion with *Xho*I of the adenovirus DNA isolated form RAB1x or RAB2x, respectively, is as follows: 14.5kb, 8.499kb, 3.365kb, 2.466kb, 1.445kb, and 0.595kb.

FIG. 2B shows the restriction fragment analysis of the recombination products between pDonorSir2 and pBACSir2 obtained according the method disclosed in example 3, whereby pDonorSir2 is identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 23754, and whereby pBACSir2 is identical to the deposited organism at the DSMZ with the accession number according to the Budapest treaty DSM 24299. Selection of recombination products took place onto agar plates which contained kanamycin (25µg/ml) chloramphenicol (25µg/ml), and streptomycin sulphate (50µg/ml) as selecting agents. Under these conditions E.*coli* contained recombined *recombinant adenovirus BACs* (pRABs), and contained pRAB_RPSL_1x (Seq. ID. No. 11) as reaction product in 83 out of 88 analyzed reaction products. In only 2 out of 88 cases a double recombined reaction product pRAB_RPSL_2x (Seq. ID. No. 12) was obtained. Single colonies were picked from the selection plate, subcultured in liquid media containing

chloramphenicol (25µg/ml) and BAC DNA from subsequent subcultures of the colonies containing pRABs was isolated and the integrity of the reaction products analyzed by restriction fragment analysis upon digestion with *Xho*I (figure 2B). All the 6 analysed recombinants analyzed, contained pRAB_RPSL_1x. To test the reliability of the method, the experiment was repeated and a further 82 clones were picked from the selection plates and characterized as above. We could find only 2 clones which contained multiple insertion products corresponding to pRAB_RPSL_2x marked with D in Fig. 2B; clones number #47 and #53, respectively. Further 7 BAC DNA preparations were contaminated by the parental vector pBACSir2 (marked with V, clone number #9,#17,#22,#39,#41,#62,#67), and 3 were recombination products resulted from rearrangements (marked with r, clone number #25,#46,#68. In a further experiment a total of 44BACs corresponding to a fifth nucleic acid were analyzed, whereby only 1 recombination product corresponded to pRAB_RPSL_2x and 43 of the 44 clones corresponded to pRAB_RPSL_1x. Altogether 126/132 BACs corresponded to the single recombination product pRAB_RPSL_1x (95,5% of the recombination products analyzed) and multiple recombination was observed in 3/132 corresponding to 2,3% of the clones.

FIG. 3 Diagrammatic representation showing the method disclosed in example 3 for constructing complemented complete adenovirus vector genomes, or a plurality or library of those. The recombination between a third nucleic acid molecule and a second nucleic acid molecule are combined and reacted through their Frt recombination sites forming a recombination product which can be selected, and whereby the number of recombination events is limited to one. According to example 3 a third nucleic acid molecule identical or similar to pDonorSir2 containing a prokaryotic promoter (PK promoter), a minimal Frt34 recombination site derived from the wild type Frt site, a negative selection marker (Rpsl), a bacterial nucleotide sequence comprising (i) bacterial nucleotide sequences for conditional replication (OriR6K) and a nucleotide sequence providing for a positive selection marker conferring a host cell resistance to kanamycin (KnR), a first restriction site (RS1), a first part of a genome of a virus containing the left ITR of an adenovirus genome (ITRleft) and the packaging signal ES, a transcription unit or gene of interest (GOI). The second nucleic acid, which is identical or similar to pBACSir2 (SEQ. ID. No. 2) comprises a wild type Frt48 recombination site (SEQ.ID. No. 8), a second part of the genome of an adenovirus

comprising the right ITR of an adenovirus genome (ITRrigth), a second restriction site (RS2), and a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for single copy replication (F'ori), and (ii) a nucleotide sequence providing for a second selection marker conferring a host cell resistance to chloramphenicol (CmR). Both nucleic acid molecules are reacted through their recombination sites in a bacterial host cell in the presence of Flp recombinase. Using the method disclosed, the resulting recombinant BACs predominantly (>95%) consist of single recombined products. In figure 3A the schematic composition of a single recombination product pRAB_RPSL_1x is given. In figure 3B the double recombination is depicted schematically. In the recombination product pRAB_RPSL_2x the prokaryotic promoter (PK Promoter) is in proximity to the open reading frame of the negative selection marker (Rpsl). This product is observed in less than 2,5% of the recombination products according to the disclosed method in example 3.

FIG. 4 shows the reconstitution of complemented infectious adenovirus viruses in 293 Flp cells expressing Flp recombinase according to a disclosed method in example 5, whereby 293 Flp cells are identical to the deposited organism at the DSMZ with the accession number according to the Budapest treaty DSM ACC3077. 293Flp cells were transfected with a first nucleic acid molecule corresponding to pSirDonor1-EGFP (SEQ. ID No.9), and a second nucleic acid molecule corresponding to pBACSir2, whereby both nucleic acids were treated with *Pac*I prior to transfection. After 3 days cultivation at 37°C under standard cell culture conditions comet-shaped fluorescent conglomerates of cells showing cytopathic effect (CPE) characteristic for productive adenovirus production in 293 cells were microscopically detected.

FIG. 5 shows the selective inhibition of the growth by streptomycin of E.*coli* DH10B cells carrying pRAB_RPSL_2x obtained from double recombination of pDonorSir2 with pBACSir2. Growth curves were generated from E.coli DH10B cells transformed with the empty vector pBACSir2, the single recombined pRAB_RPSL_1x_#1, or two double recombined adenovirus BAC vectors pRAB_RPSL_2x_#47, and pRAB_RPSL_2x_#53, respectively. Growth of bacterial cultures was done at different concentrations of streptomycin starting from a diluted saturated overnight culture as starting material, and monitoring of the OD600 over time. After the average of the OD600 of the replicate culture

containing the control BAC vector pBACSir2 reached 0.8, usually 8 hours post inoculation, the OD600 values were measured and the optical density calculated and referenced to the average OD600 of the control culture which was set to 100%. The results of 5 independent experiments were plotted for each growth conditions and standard deviations of the relative optical densities within the plotted 5 experiments included as error bars. The E. coli clones carrying the control BAC vector pBACSir2 or the single recombined BAC vector pRAB_RPSL_1x grew well even in the presence of very high concentration (200μg/ml) of streptomycin. In contrast the growth of the clones carrying a double insertion of pDonorSir2 (pRAB_RPSL_2x #47) and pRAB_RPSL_2x_#53) was blocked by 50μg/ml streptomycin, some inhibition was detectable already in the presence of 25μg/ml streptomycin.

FIG. 6 shows a synergy matrix of selection markers. Based on the mode of action of antibiotics, positive and negative selction markers have the potential to work synergistically in the presence of kanamycin. Combinations of positive and negative selction markers that are expected to work synergistically for the counter-selection of multiple-recombined products according to the method of the present invention described herein for the generation of recombinant adenovirus virus vectors are marked "+" in the table, and combinations that are not expected to work synergistically are marked with "0".

FIG. 7 illustrates the composition of two recombinant first generation adenovirus type 19a vectors obtained using the method disclosed in example 6. The reaction products were isolated from a growing culture of DH10B bacteria according to standard protocols, and characterized by restriction digest with *Kpn*I. In the first lane marked with M a nucleotide length marker was loaded, providing reference DNA fragments with defined length between 1 and 10 kb. Restriction analysis with *Kpn*I of the single recombination product pRAB19a1x (lane 1,2,3) and double recombination products (lanes 4) are shown. The *in silico* generated pattern for digestion with *Kpn*I of the single recombined reaction product pRAB19a1x (Seq. ID. No. 16) is as follows: 11.361kb, 6.254kb, 5.447kb, 4.443kb, 3.271kb, 2.016kb, 1.886kb, 1.868kb, 1.585kb, and 28bp. In case of the double recombined product, an additional band of 3.364kb appears (lane 4). The in silico generated pattern for digestion with *Kpn*I of the double recombined reaction product pRAB19a2x (Seq. ID. No. 17) is as follows: 11.361kb,

6.254kb, 5.447kb, 4.443kb, 3.364kb, 3.271kb, 2.016kb, 1.886kb, 1.868kb, 1.585kb, and 28bp.

Examples

Example 1: Construction of recombinant Adenovirus BACs using site-specific recombination in E.coli expressing Flp recombinase.

For construction of a recombinant adenovirus genome, a first nucleic acid pDonorSir1 and a second nucleic acid molecule pBACSir1were combined and reacted in DH10B *E.coli* cells harbouring pBACSir1and the plasmid pCP20 for conditional expression of FLP recombinase, whereby pDonorSir1 is identical to the deposited organism at the DSMZ with the accession number according to the Budapest treaty DSM 23753, and whereby pBACSir1 is identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM24298, and whereby E.*coli* cells harbouring pBACSir1 and pCP20 are identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 23742. The plasmid pDonorSir1 was introduced into the DH10B *E.coli* cells by means of electroporation using a standard protocol. The nucleic acid molecule pBACSir1 is a derivative of the pKSO BAC vector (Messerle et al. Proc. Natl. Acad. Sci. U. S. A. 94:14759-14763, 1997) and contain the right part of the human adenovirus type 5 (AV5) genome deleted for the E1 region and the E3 region. The nucleic acid molecule pBACSir1 was maintained in *E.coli* DH10B (or equivalent *E.coli* K12-derived strains lacking the F- factor) harbouring a conditional expression system for expression of Flp. Here, in example 1, the DH10B cells harboured the adenovirus BAC pBACSir1, and the Flp recombinase was provided by the plasmid pCP20, which replication is controlled by a temperature-sensitive origin of replication (Bubeck A. et al., J. Virol. 78:8026-8035, 2004). DH10B cells harbouring pBACSir1 and the pCP20 were maintained at 30°C in the presence of ampicillin (50µg/ml) and chloramphenicol (25µg/ml). Next, these DH10B cells were electro-transformed with pDonorSir1 and cultured for 60 minutes at 42°C in the absence of any antibiotics. The expressed Flp induced site-specific recombination

between FRT sites present on pDonorSir1 and pBACSir1, respectively. At the same time the elimination of Flp expression also started, since pCP20 cannot replicate in *E. coli* at elevated temperature. The transformed culture was plated onto agar plates which contained kanamycin (25µg/ml) and chloramphenicol (25µg/ml) as selecting agents. Under these conditions *E.coli* containing recombined *recombinant adenovirus BACs* (pRABs) were selected in which at least one pDonorSir1 plasmid had recombined with pBACSir1. DNA from growing cultures of DH10B cells containing pRABs was isolated and the integrity of the reaction products analyzed by restriction digestion with *Xho*I (Fig. 2A (lanes2-4). All the recombination products analyzed contained pRABs, either being single (pRAB1x) or multiply recombined products (pRAB2x).

Example 2: Reconstitution of recombinant adenoviruses generated by site-specific recombination in E.coli expressing Flp recombinase

The two predominant types of BAC vectors obtained from site-specific recombination according to the disclosed method in example 1 were pRAB1x and pRAB2x, respectively. The pRABs generated by the Flp-recombination in DH10B cells contained one, and only one continuous sequences of a complete complemented adenovirus genome, which was replication competent in 293 cells. The DNA of pRABs was purified from saturated E.coli over night cultures (100ml) in LB medium using a kit for plasmid preparation. Here, the Nucleobond PC-100 kit from Macherey and Nagel, Germany was used according to the manufacturer's recommendations. The identity of the pRBAs obtained was verified by means of restriction analysis of the pRAB DNAs (Fig. 2A, lanes 2-4). For virus reconstitution purified pRAB DNA was treated with 10U *Pac*I per µg DNA for 2h according to the manufacture's recommendations. Subsequently the *Pac*I-digested pRAB1x and pRAB2x DNAs were purified using phenol-chloroform according to standard protocols prior to transfection into 293 cells. In brief, 10µg pRAB DNA was digested in a volume of 100µl for 1,5 h at 37°C in a water bath. Subsequently 50 µl phenol/chloroform (1:1mixture) was added to the reaction tube (Eppendorff cup size 1,5ml, Eppendorf AG, Hamburg, Germany) and vortexed for 20 sec. here, the Vortexer MS-3 basic was used (IKAIKA® Werke GmbH & Co. KG, Staufen, Germany). The tube was centrifuged in a table top

centrifuge at maximum speed (20000 x g) for 5 min at room temperature and 80 µl of the aqueous upper phase was transfered into a fresh tube and 10 µl 3 M NaAc (pH 4,5) and 200 µl EtOH was added. All reagents and chemicals were purchased from Sigma-Aldrich, St Louis, USA. The tube was mixed with the finger tips until the precipitated DNA became visible. Moreover, the tube was incubated for 5 min at room temperature and the DNA was pelleted in a table top centrifuge at maximum speed for 15 min at room temperature. The supernatant was quantitatively removed and the pellet immediately dissolved in 20 µl sterile deionized water. Transfection of 293 cells was done using lipofection. Here, the Superfect transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendation. The resulting adenoviruses were replication competent in 293 cells and could be propagated according to standard protocols (Green M and Loewenstein P, Human Adenoviruses: Propagation, Purification, Quantification, and Storage in Current Protocols in Microbiology79). The identity of the recombinant adenovirus vectors obtained according to the disclosed method in this example was verified by restriction digest of adenovirus vector DNA with *Xho*I and analysis of DNA fragments using agarose gel electrophoresis (Fig. 2A). For preparation of genomic adenovirus vector DNA, 293 cells ($2{,}5x10_7$ cells) were infected with a MOI of 3 with the recombinant adenovirus vectors obtained after transfection of the *Pac*I-digested pRABs into the 293 cells. After the cytopathic effect (CPE) was complete the infected cells were washed once in PBS, scraped from the plates and resuspent in PBS. Cells (~$4x10_6$ cells/ml) were lysed by adding an equal volume of TST buffer (2% TritonX-100, 400mM NaCl, 20mM Tris-HCl pH8.0) to the cell suspension followed by incubation on ice for 30 minutes. Cell debris were removed by centrifugation at 20,000g for 10 minutes at 4_oC and the supernatant was treated with 50µg/ml proteinase K (Roche) in the presence of 0.5% SDS for 60 minutes at 56_oC. After extraction of the nucleic acids by phenol/chloroform and ethanol precipitation the extract was treated with RNase A (Sigma). RNA-free viral DNA was again phenol/chloroform extracted and precipitated with ethanol. The *Xho*I restriction pattern of reconstituted virus derived from pRAB1x and pRAB2x corresponded to the *in silico* generated pattern, confirming the integrity of the adenovirus genome in the recombinant adenovirus viruses obtained (Fig.2A lanes 5-7).

Example 3: Generation of recombinant RABs with controlled recombination through negative selection

To avoid multiple insertions and improve the construction of an adenovirus expression library, we constructed pDonorSir2 which is an embodiment of the third nucleic acid molecule of the present invention, whereby pDonorSir2 is identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 23754. pDonorSir2 differs from pDonorSir1 at its FRT locus, next to this pDonorSir2 contains a strong E.*coli* galaktokinase promoter (Warming, S., N. Costantino, Court DL, N. A. Jenkins, and N. G. Copeland. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res 2005, **33**:e36) upstream to the FRT site and downstream of the FRT site a *rps*L open reading frame, which mediated Streptomycin sensitivity if expressed (Reyrat JM, Pelicic V, Gicquel B, Rappuoli R. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. 1998, **66**:4011-4017). The use of pDonorSir2 is exemplified as follows: DH10B cells harbouring pBACSir2 and pCP20 were maintained at 30°C in the presence of ampicillin and chloramphenicol, whereby pBACSir2, which is an embodiment of the second nucleic acid molecule of the present invention, is identical to the deposited organism at the DSMZ with the accession number according to the Budapest treaty DSM 24299, and whereby E.*coli* cells harbouring pBACSir2 and pCP20 are identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM 23743. Next, the DH10B cells were electro-transformed with pDonorSir2 and cultured for 150 minutes at 42°C in the absence of any antibiotics. The expressed Flp induced site-specific recombination between FRT sites present on pDonorSir2 and pBACSir2, respectively. At the same time the elimination of Flp expression also started, since pCP20 cannot replicate in *E. coli* at elevated temperature. The transformed culture was plated onto agar plates which contained kanamycin (25µg/ml) chloramphenicol (25µg/ml) and streptomycin sulphate (50µg/ml) as selecting agents. Under these conditions E.*coli* containing recombined *recombinant adenovirus BACs* (pRAB_RPSL) were selected, in which the pDonorSir2 plasmid had recombined with pBACSir2. Single colonies were picked from the selection plate, and cultured in 10ml liquid LB media containing chloramphenicol (25µg/ml) over night at 37°C in a shaking incubator. All chemicals and media used were purchased from Sigma-Aldrich, St Louis, USA.

pRAB_RPSL DNA from these cultures was subsequently isolated according to the manufacture's recommendations using a DNA-plasmid isolation kit, and the integrity of the reaction products analyzed by restriction digestion with *Xho*I (fig. 2B). Here, the Nucleobond PC-100 kit from Macherey and Nagel, Germany was used for isolation of pRAB_RPSL-DNA according to the manufacturer's recommendations. The *Xho*I restriction pattern of all 6 pRABs analysed corresponded to single recombined products (pRAB_RPSL_1x). To test the reliability of the applied counter selection we picked further 82 clones from the selection plates ant tested as above. Only 2 clones contained multiple insertion products (marked by 'D' in figure 2B), further 7 clones were contaminated by pBACSir2 (marked by 'V' in figure 2B), and 3 contained other unidentified rearrangements (marked by 'r' in figure 2B). Altogether the great majority (83/88) of the colonies contained only pRAB_RPSL_1x (Fig. 2B). In a further experiment a total of 44clones were analyzed were analyzed, whereby only 1 recombination product corresponded to pRAB_RPSL_2x, and 43 of the 44 clones corresponded to pRAB_RPSL_1x. Altogether 126/132 BACs corresponded to the single recombination product pRAB_RPSL_1x (95.45% of the recombination products analyzed) and multiple recombination was observed in 3/132 corresponding to 2.3% of the clones.

Example 4: Determination of the average library efficiency for generation of recombinant adenovirus BAC libraries

To test the efficiency of our E. coli recombination system and avoid the contamination of pRAB_RPSL DNA preparations according to example 3 with non-recombined pBACSir2 vector, the experiment described in Example 3 was repeated two more times with the following modifications:

i) To test the primary cloning efficiency we took 50µl of a 10ml post-transformation culture and serial 10-fold dilutions were plated on a triple selection agarose plate containing kanamycin (25µg/ml), chloramphenicol (25µg/ml), and streptomycin sulphate (50µg/ml) as selecting agents (Experiment 2). All chemicals and media used were purchased from Sigma-Aldrich, St Louis, USA. After 60 minutes the rest of the culture was incubated for another

90 minutes giving finally 150 minutes total post-transformation culture time as above (Experiment 3). Two plates were inoculated by 200µl out of 1ml final volume of each dilution of the 50µl post-transfection culture ($1:10^1$, $1:10^2$, and $1:10^3$) from each experiment.

ii) After the colonies containing the pRABs appeared on selection plates we made replica plates on a second round of triple selection plates containing kanamycin (25µg/ml), chloramphenicol (25µg/ml), and streptomycin sulphate (50µg/ml) as selecting agents. This procedure minimized the contamination by both the vector and the multiple insertion products, whereby the generation of replica plates is applied regularly in maintaining E. coli libraries.

The colony counts on replica plates were 52 and 89 in experiment 2, and 204 and 129 in experiment 3 with the dilution $1:10^2$. Taking in account that 50ng DNA of pDonorSir2 was used, and the volume of the post-transformation culture was 50µl, and one fifth of each dilution was plated, the average cloning efficiency was $1{,}85x10^6$ colony for 1µg input.

Example 5: Generation of replication competent adenovirus in 293 cells expressing FLP recombinase

For construction of HEK 293 Flp cells expressing Flp recombinase $2{,}5 \times 10^5$ HEK 293 cells were transfected using lipofection with 10 µg of the plasmid pFlp-Puro linearized with *Pvu*I, whereby 293 Flp cells are identical to the deposited organism at the DSMZ according to the Budapest treaty with the accession number DSM ACC3077. Here, the Superfect transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendation.. The transfected cells were incubated for 48 h at 37°C under standard cell culture conditions (95% humidity, 5% CO2). The cell culture medium used was DMEM containing 10% FCS, 2 mM Glutamin, and 1% penicillin/streptavidin (P/S)). For selection puromycin was added to a final concentration of 1 µg/µl to the medium, and cells were cultivated under selective conditions for 12 days to obtain 293 cells stably expressing FLP recombinase. All chemicals and media used were purchased from Sigma-Aldrich, St Louis, USA. The stable cell pool was expanded and a master cell bank established. For

reconstitution of recombinant adenovirus 2 x 10^5 293 FlpP cells per well were plated onto a 6 well plate and 5 hours after plating cells were co-transfected with 0,8 µg pDonorSir2-EGFP (SEQ. ID No. 9) and 2,5 µg pBACSir2, both linearized with *Pac*I, using Lipofection. Here, the Superfect transfection reagent (Qiagen, Hilden, Germany) was used according to the manufacturer's recommendation. Following a 3 days cultivation at 37°C under standard cell culture conditions, cells were harvested by scraping and collected by subsequent centrifugation for 5 min at 200 x g. Cell pellets were resuspent in 400 µl cell culture medium (DMEM, 10% FCS, 2 mM Glutamin, 1% P/S) and subjected to three successive freeze/thaw cycles. Cell debris was separated from soluble material by centrifugation at 4.400 x g for 15 min. In order to demonstrate a successful rescue of adenovirus vectors expressing the EGFP gene, 2 x 10^5 HEK-293 cells/well were plated onto a 6 well plate and infected 12 h later with 200 µl of the freeze/thaw lysate followed by 3 days incubation at 37°C under standard cell culture conditions. At this time point comet-shaped fluorescent conglomerates of cells showing cytopathic effect (CPE) characteristic for productive adenovirus replication were microscopically detectable (see Fig. 4). The method thus allowed for the generation of first generation recombinant replication competent adenovirus vectors by co-transfection of a third nucleic acid molecule with a second nucleic acid molecule into 293 Flp cells.

Example 6: Construction of recombinant Adenovirus type 19a BACs using site-specific recombination in E.coli expressing Flp recombinase.

For construction of a human non-type 5 recombinant adenovirus genome, a first Ad19a nucleic acid pDonorSir19a, which is an embodiment of the first nucleic acid molecule of the present invention, and a second Ad19a nucleic acid molecule pBACSir19a, which is an embodiment of the second nucleic acid molecule of the present invention, were combined and reacted in DH10B *E.coli* cells harbouring pBACSir19a and the plasmid pCP20 for conditional expression of FLP recombinase. The plasmid pDonorSir19a was introduced into the DH10B *E.coli* cells by means of electroporation using a standard protocol. The nucleic acid Ad19a molecule pBACSir19a was maintained in *E.coli* DH10B (or equivalent *E.coli* K12-derived strains lacking the F- factor) harbouring a conditional expression system for Flp. Here, in example 6, the DH10B cells harboured the adenovirus type 19a BAC

pBACSir19a, and the Flp recombinase was provided by the plasmid pCP20, which replication is controlled by a temperature-sensitive origin of replication. DH10B cells harbouring pBACSir19a and the pCP20 were maintained at 30°C in the presence of ampicillin (50µg/ml) and chloramphenicol (25µg/ml). Next, these DH10B cells were electro-transformed with pDonorSir19a and cultured for 60 minutes at 42°C in the absence of any antibiotics. The expressed Flp induced site-specific recombination between FRT sites present on pDonorSir19a and pBACSir19a, respectively. At the same time the elimination of Flp expression also started, since pCP20 cannot replicate in *E. coli* at elevated temperature. The transformed culture was plated onto agar plates which contained kanamycin (25µg/ml) and chloramphenicol (25µg/ml) as selecting agents. Under these conditions *E.coli* containing recombined recombinant adenovirus type 19a BACs (pRAB19a) were selected in which at least one pDonorSir19a plasmid had recombined with pBACSir19a. DNA from growing cultures of DH10B cells containing pRAB19a's was isolated and the integrity of the reaction products analyzed by restriction digestion with *Kpn*I (Fig. 7). All the recombination products analyzed contained pRAB19a's, either being single (pRAB19a1x Seq ID. No.16) or multiple recombined products (pRAB19a2x, Seq ID No.17).

Example 7: Generation of human non-adenovirus type 5 recombinant RABs with controlled recombination through negative selection

For construction of a plurality or library of human non-type 5 recombinant adenovirus genomes, a third Ad19a nucleic acid pDonorSir2_19a, which is an embodiment of the third nucleic acid molecule of the present invention, and a second Ad19a nucleic acid molecule pBACSir19a, which is an embodiment of the second nucleic acid molecule of the present invention, are combined and reacted in DH10B *E.coli* cells harbouring pBACSir19a and the plasmid pCP20 for conditional expression of FLP recombinase. The plasmid pDonorSir2_Ad19a differs from pDonorSir2 at its FRT locus, next to this pDonorSir2_Ad19a contains a strong E.*coli* galaktokinase promoter (Warming SN et *al.* Nucleic Acids Res 2005, **33**:e36) upstream to the FRT site and downstream of the FRT site a *rps*L open reading frame, which mediated Streptomycin sensitivity if expressed (Reyrat JM

et *al.* Infect. Immun. 1998, **66**:4011-4017). The donor nucleic acid pDonorSir2_19a carries a PacI site, Ad19a ITR and packaging signal.

The use of pDonorSir2_Ad19a is exemplified as follows: DH10B cells harbouring pBACSir19a and pCP20 are maintained at 30°C in the presence of ampicillin and chloramphenicol. Next, the DH10B cells are electro-transformed with pDonorSir2_Ad19a and cultured for 150 minutes at 42°C in the absence of any antibiotics. The expressed Flp induces site-specific recombination between FRT sites present on pDonorSir2_Ad19a and pBACSir19a, respectively. At the same time the elimination of Flp expression starts, since pCP20 cannot replicate in *E. coli* at elevated temperature. The transformed culture is plated onto agar plates which contain kanamycin (25µg/ml) chloramphenicol (25µg/ml) and streptomycin sulphate (50µg/ml) as selecting agents. Under these conditions E.*coli* containing recombined recombinant adenovirus BACs are selected, in which the pDonorSir2_Ad19a plasmid has recombined with pBACSir19a. Single colonies are picked from the selection plate, and cultured in 10ml liquid LB media containing chloramphenicol (25µg/ml) over night at 37°C in a shaking incubator. All chemicals and media used are purchased from Sigma-Aldrich, St Louis, USA. DNA from recombination products from these cultures is subsequently isolated according to the manufacture's recommendations using a DNA-plasmid isolation kit, and the integrity of the reaction products analyzed by restriction digestion with *Kpn*I. Here, the Nucleobond PC-100 kit from Macherey and Nagel, Germany is used. The *Kpn*I restriction pattern corresponds to single recombined products.

Biological material

The invention uses and/or relates to biological material deposited under the Budapest Treaty. More specifically, the following depositions have been made with "Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ)", also referred to herein as DSMZ:
DSM 23753; DSM 24298; DSM 24299; DSM 23743; DSM 23742; DSM ACC3077m; DSM ACC3077; and DSM 23754.

The features of the present invention disclosed in the specification, the claims and/or the drawings may both separately and in any combination thereof be material for realizing the invention in various forms thereof.

Claims

1. A combination of a nucleic acid molecule, which is also referred to as third nucleic acid molecule, and a nucleic acid molecule, which is also referred to as second nucleic acid molecule,

wherein the third nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) a first part of a genome of an adenovirus, wherein the first part of a genome of an adenovirus comprises exactly one inverted terminal repeat of an adenovirus;

(b) a nucleotide sequence, preferably a genomic nucleotide sequence, or a transcription unit;

(c) a regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;

(d) exactly one site-specific recombination site recombining in the presence of a corresponding recombinase;

(e) a nucleotide sequence providing for a negative selection marker;

(f) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker; and

(g) a first restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 6; or

(3) a heterologous nucleic acid molecule comprising a third nucleic acid molecule as defined in (1) contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 23754;

and

wherein the second nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) a bacterial nucleotide sequence unit comprising
(i) bacterial nucleotide sequences for single copy replication, and
(ii) a nucleotide sequence providing for a selection marker;

(b) exactly one site-specific recombination site recombining in the presence of a corresponding recombinase;

(c) a second part of a genome of an adenovirus, wherein the second part of a genome of an adenovirus comprises exactly one inverted terminal repeat of an adenovirus; and

(d) a restriction site which is referred to as second restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 2 and/or SEQ ID NO: 13 and/or SEQ ID NO: 14; or

(3) a heterologous nucleic acid molecule comprising a second nucleic acid molecule as defined in (1) contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 24298 and/or DSM 24299;

wherein the second nucleic acid molecule and the third nucleic acid molecule each and independently is either a linear molecule or a circular molecule, preferably the second nucleic acid molecule is a circular molecule and the third nucleic acid molecule is a circular molecule;

wherein the second nucleic acid molecule and the third nucleic acid molecule complement each other to form a complete viral genome; and

wherein the recombinase recombining the recombination site of the third nucleic acid molecule is the same as the recombinase recombining the recombination site of the second nucleic acid molecule.

2. The combination according to claim 1, wherein in the nucleic acid molecule of (1) the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote, the site-specific recombination site and the nucleotide sequence providing for a negative selection marker are arranged in a 5’ -> 3’ direction.

3. The combination according to any one of claims 1 to 2, wherein the third nucleic acid molecule is a linear molecule, wherein elements (a) to (f), preferably upon cleavage of the circular molecule of the third nucleic acid molecule with the first restriction enzyme which recognizes and cleaves at the first restriction site, are arranged in a 5’ -> 3’ direction in the following sequence as follows:

1. the first part of a genome of an adenovirus;

2. the nucleotide sequence, preferably the genomic nucleotide sequence, or the transcription unit;

3. the regulatory nucleic acid sequence which has a regulatory activity in a prokaryote;

4. the site-specific recombination site recombining in the presence of a corresponding recombinase;

5. the nucleotide sequence providing for a negative selection marker; and

6. the bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a positive selection marker.

4. The combination according to any one of claims 1 to 3, wherein the adenovirus is a human adenovirus and preferably the adenovirus is human adenovirus type 5, and more preferably the entire left end of adenovirus type 5 upstream of the TATA box of the E1 transcription unit, or one or several parts thereof.

5. The combination according to any one of claims 1 to 4, preferably claim 4, wherein the bacterial nucleotide sequences for conditional replication comprise an origin of replication, whereby preferably the origin of replication is the minimal origin of phage gR6K.

6. The combination according to any one of claims 1 to 5, preferably any one of claims 4 to 5, wherein the regulatory sequence which has a regulatory activity in a prokaryote is a sequence which directs expression of a nucleotide sequence in a prokaryote, preferably in a prokaryotic host cell.

7. The combination according to any one of claims 1 to 6, preferably any of claims 5 to 6, wherein the negative selection marker or the expression of the nucleotide sequence providing for a negative selection marker mediates or confers sensitivity to a selecting agent and/or a selecting condition.

8. The combination according to claim 7, wherein the nucleotide sequence providing for a negative selection marker is a gene selected from the group comprising the *galK*, *tetAR*, *pheS*, *thyA*, *lacy*, *ccdB* and *rpsL* gene.

9. A combination of a nucleic acid molecule which is also referred to as first nucleic acid molecule, and a nucleic acid molecule which is also referred to as second nucleic acid molecule,

wherein the first nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) exactly one site-specific recombination site recombining in the presence of a corresponding recombinase;

(b) a bacterial nucleotide sequence unit comprising (i) bacterial nucleotide sequences for conditional replication and (ii) a nucleotide sequence providing for a first selection marker;

(c) a first part of a genome of an adenovirus, wherein the first part of a genome of an adenovirus comprises exactly one inverted terminal repeat of an adenovirus;

(d) a transcription unit; and

(e) a first restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO:1 and/or SEQ ID No:15; or

(3) a heterologous nucleic acid molecule comprising a first nucleic acid molecule as defined in (1) contained in the organism deposited with the DSMZ according to the Budapest treaty under accession number DSM 23753;

and wherein the second nucleic acid molecule comprises

(1) a nucleic acid molecule comprising the following elements:

(a) a bacterial nucleotide sequence unit comprising
(i) bacterial nucleotide sequences for single copy replication, and
(ii) a nucleotide sequence providing for a selection marker;

(b) exactly one site-specific recombination site recombining in the presence of a corresponding recombinase;

(c) a second part of a genome of an adenovirus, wherein the second part of a genome of an adenovirus comprises exactly one inverted terminal repeat of an adenovirus; and

(d) a restriction site which is referred to as second restriction site; or

(2) a nucleic acid molecule comprising a nucleotide sequence according to SEQ ID NO: 2 and/or SEQ ID NO: 13 and/or SEQ ID NO: 14; or

(3) a heterologous nucleic acid molecule comprising a second nucleic acid molecule as defined in (1) contained in the organism deposited with the DSMZ under the Budapest treaty under accession number DSM 24298 and/or DSM 24299, wherein preferably the nucleic acid molecule contained in the organism is a heterologous nucleic acid molecule;

and wherein the first nucleic acid molecule and the second nucleic acid molecule each and independently is either a linear molecule or a circular molecule, preferably the first nucleic acid molecule is a circular molecule and the second nucleic acid molecule is a circular molecule;

wherein the first nucleic acid molecule and the second nucleic acid molecule complement each other to form a complete viral genome; and

wherein the recombinase recombining the recombination site of the first nucleic acid molecule is the same as the recombinase recombining the recombination site of the second nucleic acid molecule.

10. The combination according to claim 9, wherein the genome of an adenovirus of the first nucleic acid molecule is a human adenovirus genome, preferably a human adenovirus genome which is different from human adenovirus type 5 genome, more preferably the genome of an adenovirus of the first nucleic acid molecule is a human adenoviral type 19a genome.

11. The combination according to any one of claims 9 to 10, wherein the bacterial nucleotide sequences for conditional replication of the first nucleic acid molecule comprise an origin of replication.

12. The combination according to any one of claims 9 to 11, wherein the sequence providing for a first selection marker of the first nucleic acid molecule is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding for an enzyme.

13. The combination according to any one of claims 9 to 12, wherein the first part of a genome of an adenovirus of the first nucleic acid molecule is an adenoviral terminal repeat.

14. The combination according to any one of claims 9 to 13, wherein the first part of a genome of an adenovirus of the first nucleic acid molecule contains the adenoviral promoter pIX, preferably the adenoviral promoter pIX is a pIX promoter from human adenovirus 19a.

15. The combination according to any one of claims 1 to 14, wherein the adenovirus genome of the second nucleic acid molecule is a human adenovirus genome, whereby in case of the combination according to claim 1 the adenovirus genome of the second nucleic acid molecule is preferably a human adenovirus type 5 genome or a human adenoviral type 19a genome and in case of the combination according to claim 9 the adenovirus genome of the second nucleic acid molecule is preferably a human adenovirus genome which is different from human adenovirus type 5 genome, more preferably the virus genome of the second nucleic acid molecule is a human adenoviral type 19a genome.

16. The combination according to any one of claims 1 to 15, wherein the bacterial nucleotide sequence for single copy replication of the second nucleic acid molecule comprises a replication origin for single copy maintenance in prokaryotic host cells.

17. The combination according to any one of claims 1 to 16, wherein the nucleotide sequence providing for a selection marker of the second nucleic acid molecule is a nucleic acid sequence coding for an enzyme which is conferring resistance to a host cell harbouring such nucleic acid sequence coding to an enzyme.

18. The combination according to any one of claims 1 to 17, wherein the second part of a genome of an adenovirus of the second nucleic acid molecule comprises an adenoviral right inverted terminal repeat and the first part of a genome of an adenovirus of the first nucleic acid molecule and the third nucleic acid molecule comprises an adenoviral left inverted terminal repeat.

19. A method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) providing a third nucleic acid molecule as defined in any one of claims 1 to 8;

b) providing a second nucleic acid molecule as defined in any one of claims 1 to 8; or

c) providing a combination of a third nucleic acid molecule and a second nucleic acid molecule according to any one of claims 1 to 8 and 15 to 18;

d) allowing the third and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably single copy of the genome of a or the virus, whereby the genome is a complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

e) selecting the recombination product; and

f) optionally cleaving the recombination product with the first and second restriction enzyme.

20. A method for the generation of a nucleic acid molecule coding for a virus comprising the following steps

a) providing a combination of a first nucleic acid molecule and a second nucleic acid molecule according to any one of claims 9 to 18;

b) allowing the first and the second nucleic acid molecule to react so that a site-specific recombination occurs, wherein the site-specific recombination is mediated by a site-specific recombinase and the site-specific recombination forms a recombination product comprising a copy, preferably single copy of the genome of a or the virus, whereby the genome is a complemented complete genome and the complemented complete genome is complemented by the site-specific recombination;

c) selecting the recombination product; and

d) optionally cleaving the recombination product with the first and second restriction enzyme.

21. The method according to claim 19, wherein the third and the second nucleic acid molecule are reacted in a prokaryotic host cell, preferably in *E. coli*.

22. The method according to claim 20, wherein the first and the second nucleic acid molecule are reacted in a prokaryotic host cell, preferably in *E. coli*.

23. A method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes and each viral genome contains a single one of the individual nucleotide sequences, comprising the steps of the method as defined in any of claims 19 and 21, wherein the individual nucleotide sequence is part of the transcription unit of the third nucleic acid molecule.

24. A method for generating a library of nucleotide sequences, wherein said library comprises a plurality of individual nucleotide sequences, wherein said library is represented by a plurality of viral genomes and each viral genome contains a single one of the individual nucleotide sequences, comprising the steps of the method as defined in any of claims 20 and 22, wherein the individual nucleotide sequence is part of the transcription unit of the first nucleic acid molecule.

25. A kit comprising optionally a package insert, and, in (a) suitable container(s), at least a combination of the third nucleic acid molecule and the second nucleic acid molecule according to any one of claims 1 to 8 and 15 to 18.

26. A kit comprising optionally a package insert, and, in (a) suitable container(s), at least a combination of the first nucleic acid molecule and the second nucleic acid molecule according to any one of claims 9 to 18.

27. The kit according to any one of claims 25 and 26, wherein the nucleic acid molecule(s) is/are contained in a ready- to-use form and/or wherein the kit contains a permissive cell line providing a recombinase and instructions for use.