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Inserting site-specific DNA lesions into whole genomes

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Running head: DNA lesions into genomes

i: Summary / Abstract:

Here we describe a methodology that allows the insertion of site-specific DNA lesions into genomes in living cells. The technique involves the integration of a plasmid containing a site-specific lesion engineered *in vitro* into a precise location in the genome via the site-specific recombination reaction from phage lambda. The notion of DNA lesion is not restricted to chemically modified nucleotides but also refers to unusual DNA structures. This method will be instrumental to study qualitatively and quantitatively the genetic consequences of site-specific lesions *in vivo*; moreover, it does also allow to analyze the molecular structure of stalled replication forks at well-defined locations.

ii: Key Words: DNA adducts, unusual DNA structures, DNA damage tolerance, site-specific recombination, Translesion Synthesis, Damage Avoidance, Homology-Directed Recombination

1. Introduction

In cells, replication-blocking lesions are tolerated via two major pathways, mutagenic Translesion synthesis (TLS) and error-free Damage Avoidance (DA). Currently most molecular and genetic studies of TLS and mutagenesis are achieved by using plasmids probes carrying site-specific single adducts. However, it became clear that plasmids are inappropriate tools for the study of DA mechanisms (1, 2) Indeed, replication fork uncoupling (3, 4) that occurs locally at the site of a transiently blocked replication fork leads to complete unwinding of the parental strands in plasmids thus preventing DA pathways to be functional. To overcome this difficulty we developed a method to insert a construct containing a single lesion into a whole genome by harnessing the site-specific recombination machinery of phage lambda. The methodology, described in the present chapter for the insertion of a single lesion into the *E. coli* genome, can be transposed to yeast or mammalian cells. The methodology can be applied to the study of chemical lesions (UV induced, lesions, adducts formed by chemical carcinogens) or to sequences potentially forming unusual DNA structures (G4 quadruplex, H-DNA, cruciforms...) or to short ribonucleotides stretches embedded in DNA. This approach not only allows the genetic consequences induced by a site-specific lesion in a genome to be analyzed, it also allows the structure and the dynamics of a single well-defined stalled replication fork to be dissected at the molecular level. The genetic and molecular consequences as a function of the site of stalling (close to replication origins, to replication termini...) can be studied.

2. Material

- LB liquid media
- LB agar plates
- SOC (0.5% yeast extract, 2% tryptone, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose)
- IPTG (Isopropyl β-D-1-thiogalactopyranoside). Stock at 200mM in H₂O.
- X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Stock at 80mg/ml in N,N-Diméthylformamide
- Ampicillin. Stock at 100mg/ml in H₂O.
- Kanamycin. Stock at 50mg/ml in H₂O.
- Chloramphenicol. Stock at 30mg/ml in ethanol
- EtOH/Na-acetate solution
dissolve 21.8g of Sodium Acetate (MW= 136) into 20ml of water. Complete to 1 liter with 100% Ethanol.
- 5X GM buffer:
 - 0,5ml TrisHCl 1M pH7 (50mM final)
 - 0,1ml NaCl 5M (50mM final)
 - 0,1ml EDTA 0,5M (5mM final)
 - H₂O complete to 10ml
 - Filter sterilize and store at 4°C

- 10X Gap Duplex Ligation Buffer:

 - 500µl TrisHCl 1M pH7,5 (100mM final)

 - 500µl MgCl₂ 1M (100mM final)

 - 500µl DTT 1M (100mM final)

 - 3500µl H₂O

- Isopropyl-alcohol / TE / CsCl saturated

Saturate TE solution with CsCl. Then add Isopropyl-alcohol.

- Equipment:

Electroporation apparatus: BioRad Gene Pulser

Ultracentrifuge type Beckman Optima™ L-90K and rotor NVT 65.2

Ultracentrifuge type Beckman Optima™ Max-XP with rotor TLN120

Not required but useful:

Automatic serial diluter and plater EasySpiral Dilute (Interscience)

Scan 1200 automatic colony counter (Interscience)

3. Methods

3.1. Construction of the recipient cell

The recipient cell must contain a unique attR integration site in fusion with the 3' end of *lacZ* gene. We use MG1655 strain in which the original λ attB site was replaced by an artificial promoter-less operon carrying attR fused to the 3' end of *lacZ* upstream of the *aadA* gene (conferring Spectinomycin resistance) and between *ybhC* and *ybhB* (5). This locates the integration site around minute 17 of the chromosome (Figure 1A). Two parental strains were obtained, FBG151 where the integration of the lesion occurs on the lagging strand, and FBG152 where *lacZ* is inverted in order to locate the integrated lesion on the leading strand (Figure 1C).

pVP135 plasmid expresses the xis/int operon from phage lambda under the control of a *trc* promoter that has been weakened by mutation in the -35 and the -10 regions (6) in order to obtain optimal expression level of integrase. This plasmid is transformed into this recipient strain and maintained by growing the cells with 50µg/ml of kanamycin.

3.2. Control plasmid to measure transformation efficiency

In order to normalize integration efficiency between the damaged vector and the non-damaged control, we use an internal standard (pVP146) in the electroporation experiment. pVP146 is derived from pACYC184 plasmid where the chloramphenicol resistance gene has been deleted by BsaAI digestion and re-ligation. This vector, that carries only the tetracycline resistance gene, serves as an internal control for transformation efficiency.

3.3. Construction of the damaged integrating vector by the "gapped duplex" method (7)

3.3.1. Description of the parental plasmids

Two plasmids are required for the construction of the damaged integrating vector by the gapped duplex method. One plasmid named "parental" plasmid contains the sequence where the lesion-containing oligonucleotide will hybridize. The other plasmid named "helper" is lacking the sequence of the oligonucleotide allowing to generate a precise gap into which the oligonucleotide will be incorporated.

In the following example, we will use the combination of plasmid pVP141 (helper) and pVP142 (parental) that allows to probe non-frameshift TLS events at the *NarI* locus modified by the G-AAF lesion (1, 2)

Plasmids pVP141 and pVP142 plasmids contain the following characteristics:

-the *bla* gene that confers resistance to ampicillin thus allowing for the selection of the integration events.

-the R6K replication origin that prevents autonomous replication of the plasmid in the recipient strain. This plasmid can be propagated in a strain that carries the *pir* gene (8).

-the 5' end of *lacZ* gene in fusion with the attL site of phage lambda. The P3' site of attL has been mutated (AATGATTAT to AATTATTAT) to avoid excision of the plasmid once integrated (9).

3.3.2. Gap duplex assembly of the damaged vector

3.3.2.1. Production of the vectors

Plasmids are produced in strain EC100D pir-116 (from Epicentre Biotechnologies - cat# EC6P0950H) (*see Note 1*)

Plasmids are purified using the MaxiPrep kit from Qiagen.

In order to obtain plasmid of higher purity, we further purify the DNA by cesium chloride / Ethidium bromide equilibrium centrifugation on Beckman Beckman Optima™ L-90K ultracentrifuge with rotor NVT 65.2 (10).

3.3.2.2. Digestion of the vectors (Figure 2A)

Helper plasmid (pVP141) is digested with EcoRV, whereas parental plasmid (pVP142) is digested with ScaI.

pVP141

50µl pVP141 at 1µg/µl (=50µg)

40µl NEB buffer cutsmart

4µl EcoRV-HF (20U/µl)

306µl H₂O (final 400µl)

pVP142

50µl pVP142 at 1µg/µl (50µg)

40µl NEB buffer cutsmart

4µl ScaI-HF (20U/µl)

306µl H₂O (final 400µl)

- Incubate 3 hours at 37°C
- Take a sample to check that the digestion is complete on agarose gel electrophoresis
- Ethanol precipitate by adding 1.2ml of EtOH/Na-acetate solution
- Spin and wash the pellet with 1ml 70% Ethanol
- Spin and dry the pellet under vacuum

- Resuspend in 50µl of water

3.3.2.3. Gapped-duplex formation (Figure 2B)

All the following steps are executed in duplicate, one being used for the non-damaged control, the other for the lesion-containing plasmid.

- Mix in a 30ml glass tube, type Corex 30 (*see Note 2*):

pVP141 digested EcoRV (1µg/µl)	25µl
pVP142 digested ScaI (1µg/µl)	25µl
GM buffer 5x	1ml
H ₂ O	3.9ml

→ freeze a 20µl aliquot for electrophoresis checking (STEP 0)

- Incubate 3.5min at 99°C with constant and vigorous shaking

- Immediately after, cool off in a mix of water + crushed ice while shaking for 2 min.

Leave 3 additional minutes without shaking

→ freeze a 20µl aliquot for electrophoresis checking (STEP 1)

- Add 40µl of 5M NaCl, vortex

- Incubate at 55°C in a water bath

- After 2h incubation, shut off the water bath and allow to slowly cool down to room temperature overnight.

→ freeze a 20µl aliquot for electrophoresis checking (STEP 2)

- Control the efficiency of denaturation and gapped-duplex formation by migration of the aliquots of Steps 0, 1 and 2 on a 1.4% agarose gel electrophoresis without ethidium bromide (in order to better resolve the gapped duplex from the linear forms).

After migration, stain the gel in TAE buffer + 0.5µg/ml ethidium bromide (Figure 3A).

3.3.2.4. Ligation of the oligonucleotides (Figure 2C)

The 15mer oligonucleotide containing the single lesion (G-AAF in our example) and the non-damaged control oligonucleotide are phosphorylated and purified on a polyacrylamide gel electrophoresis.

- To the 5ml gapped-duplex reaction (still in the glass tube), add a 1.5 to 3-fold excess of oligonucleotide (*see Note 3*).

- Add 0.5ml of 10x Gap Duplex ligation buffer

- Incubate 2 min at 65°C

- Incubate 15 min at 16°C

- Add 50µl of 0.1M ATP and 25µl of 10mg/ml BSA

→ take a 20µl aliquot for electrophoresis checking (STEP 3)

- Add 2µl of T4 DNA ligase (400U/µl)

- Vortex briefly and incubate 3 minutes at 16°C (*see Note 4*).

- Stop the reaction on ice

→ take a 20µl aliquot for electrophoresis checking (STEP 4)

- Control the ligation on a 0.8% agarose gel electrophoresis with ethidium bromide (Figure 3B).

- Inactivate the ligase by incubating 10 min at 65°C

- Transfer the reaction to a 50ml falcon tube

- Precipitate the DNA by adding 15ml of Ethanol/NaAcetate solution and incubate Over Night at -20°C

3.3.2.5. Purification of the ligated gapped-duplex on CsCl gradient

- Recover the precipitated DNA by centrifugation 30 min at 12000g.
- Resuspend in 1.2ml TE/CsCL/Ethidium Bromide mix
- Transfer to an ultracentrifugation tube 8x35mm (ref Beckman 361082) and seal the tube
- Centrifuge 5 hours at 120.000rpm at 20°C on Beckman Optima™ Max-XP ultracentrifuge in rotor TLN120.
- After centrifugation, recover the lower band under UV light (312nm) using a syringe and a needle. The recovered volume is usually around 100µl

3.3.2.6. Removal of the ethidium bromide

- add 1 volume of isopropyl-alcohol / TE / CsCl saturated
- Vortex
- eliminate the upper phase where the ethidium bromide is dissolved
- Repeat 2 additional times
- Add 2 volumes of water to the aqueous phase
- Precipitate by adding 2 volumes (of the total volume) of 100% EtOH and incubate 1h at -20°C
- Recover the precipitated DNA by centrifugation
- Wash the pellet with 150µl of 100% EtOH
- Dry and resuspend the pellet in TE
- Quantify and adjust the concentration of the damaged and non-damaged plasmid by quantification on agarose gel electrophoresis (Figure 3C – **Note 5**).

3.4. Integration protocol

3.4.1. Competent cells

- Inoculate 3ml of LB + 50µg/ml kanamycin with the recipient strain and grow O/N at 37°C with shaking
- Inoculate 100ml of LB + 50µg/ml kanamycin + 200µM IPTG with 1ml of the O/N starter culture
- Grow at 37°C under shaking until OD⁶⁰⁰=0.4
- Spin and wash the cells with 100ml of ice cold water
- Spin and wash the cells with 50ml of ice cold water
- Spin and wash the cells with 2ml of ice cold 10% glycerol
- Spin and resuspend the cells in 200µl of ice cold 10% glycerol
- Divide the competent cells in 40µl aliquot and freeze at -80°C

3.4.2. Electroporation

- Add 1µl of plasmid mix (gap-duplex + transformation control plasmid pVP146) at 1ng/µl each to the 40µl of thawed competent cell
- Transfer to an electroporation cuvette (2mm gap) and Pulse 2.5kV, 25µF, 200Ω (time constant should be ~5ms)

- Immediately add 1ml of SOC + 200 μ M IPTG (pre-warmed at 37°C). Mix by pipetting and transfer 500 μ l to 2ml of LB + 200 μ M IPTG (pre-warmed at 37°C) (*see Note 6*).

- Incubate at 37°C for 40 min with shaking

- Dilute and plate the cells.

Integrations events: on LB-agar + 50 μ g/ml Ampicillin + 80 μ g/ml X-gal

Transformation control: on LB-agar + 30 μ g/ml Chloramphenicol

- Incubate O/N at 37°C

3.4.3. Analysis of the plates (Figure 4)

Blue and white colonies are counted

Blue colonies represent TLS events. The relative integration efficiencies of damaged vectors compared to their non-damaged homologs, and normalized by the transformation efficiency of pVP146 plasmid in the same electroporation experiment, allow the overall rate of tolerance of the lesion to be measured.

4. Modification of the system

As discussed above different DNA lesions can be inserted into the genome of living cells by using the gapped duplex methodology. In addition to the lesion of choice, genetic markers (i.e. mismatches) can be introduced upstream and/or downstream from the lesion in order to study strand-switching / recombination reactions. Care should be taken to identify proper mismatches that will be resistant to mismatch repair correction *in vivo*. For that purpose mismatch repair defective strains should be tested. We recently implemented a modified version of the above-described system that allow to monitor homologous recombination events at the lesion site. This was achieved by the introduction of an additional mismatch upstream the lesion site, allowing to distinguish the damaged from the non-damaged strand, and to evidence any exchange of genetic information between the two strands (11).

5. Notes

1. Vectors containing the R6K origin can only be replicated in a strain that express the *pir* gene. In order to increase the yield of our plasmid prep, we use the strain EC100D pir-116 (from Epicentre Biotechnologies - cat# EC6P0950H), in which the pir-116 allele supports higher copy number of R6K origin plasmids.
2. It is important to perform all the following steps in glass tubes, as plastic containers do not allow sufficient thermal exchange to insure proper plasmid denaturation efficiency.
3. Expected yield: the heat denaturation / renaturation cycle will produce both the two reconstituted linear plasmid forms (50%) and gapped-duplexes (~50%). Two molecular forms of gapped-duplexes will be generated with gaps of complementary sequences (denoted as “good” and “wrong” in Figure 2B). Thus, the maximal yield of gapped duplex carrying the proper gap will be $\approx 25\%$. From an initial amount of 50 μ g of parental plasmids (25 μ g each), the expected yield of proper gapped-duplex will

corresponds to ~7pmol of DNA (parental plasmid size 2.5kb). Therefore, 10-21pmol of oligonucleotide is added.

4. Since only 3 nicks have to be sealed, a 3 min ligation at 16°C is enough. Further incubation will lead to re-circularization of the linear forms that we want to avoid.
5. There is a slight difference in migration between the construct and the control plasmid (fig 3C). This difference is due to differences in the supercoiling status, the covalently closed circular (ccc) construct having essentially no supercoils.
6. Recovery of the cells after electroporation in SOC only or LB only greatly reduce the integration efficiency. For this reason, cells are resuspended in SOC after electroporation and diluted in LB for the incubation.

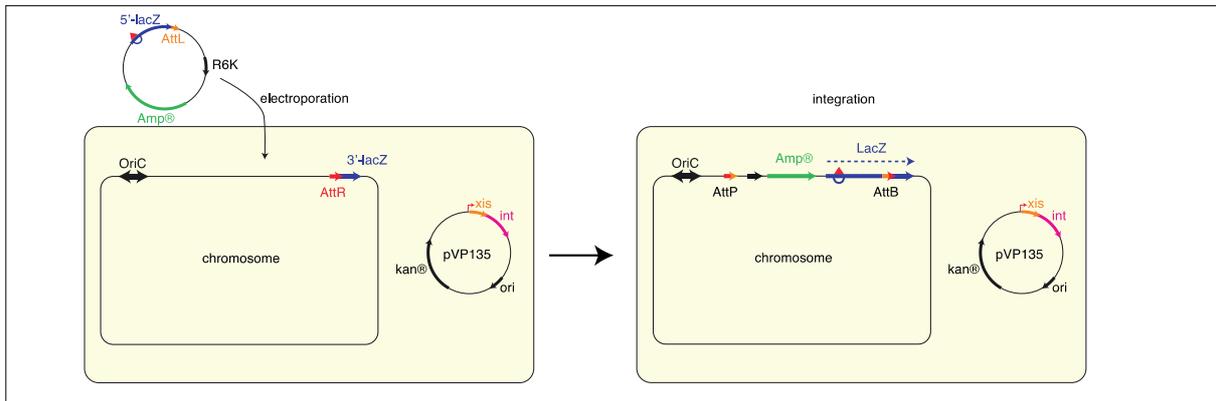
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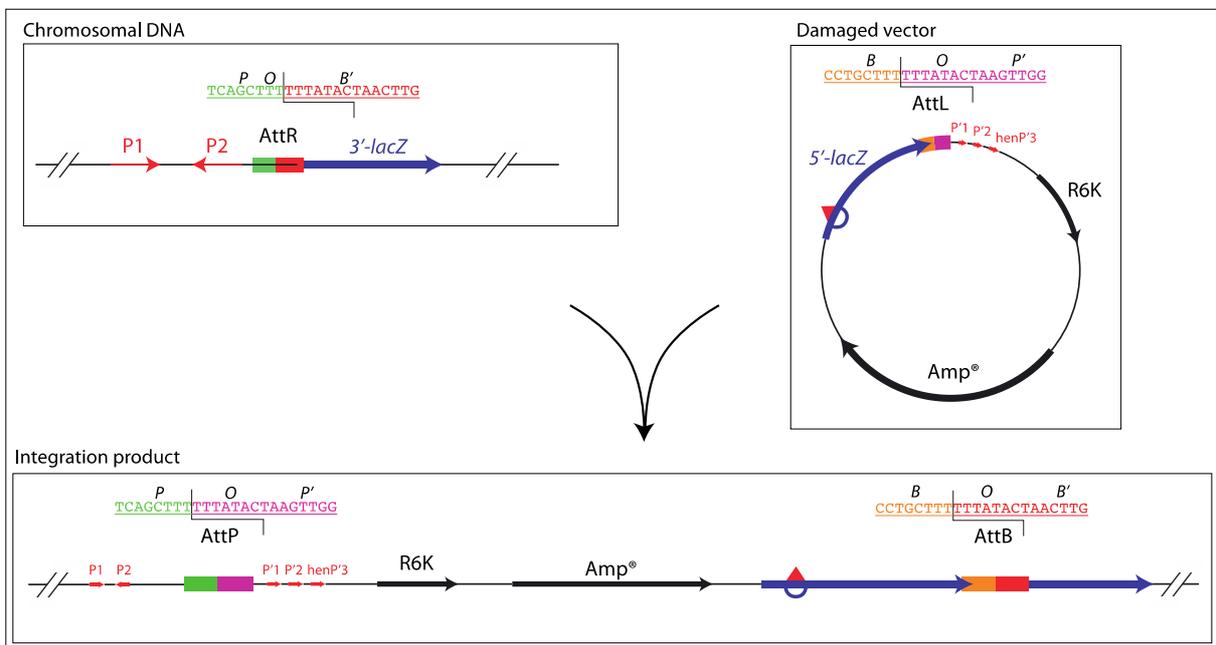
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A. Outline of the integration system



B. Detail of the phage lambda integration system



C. Map of the strains

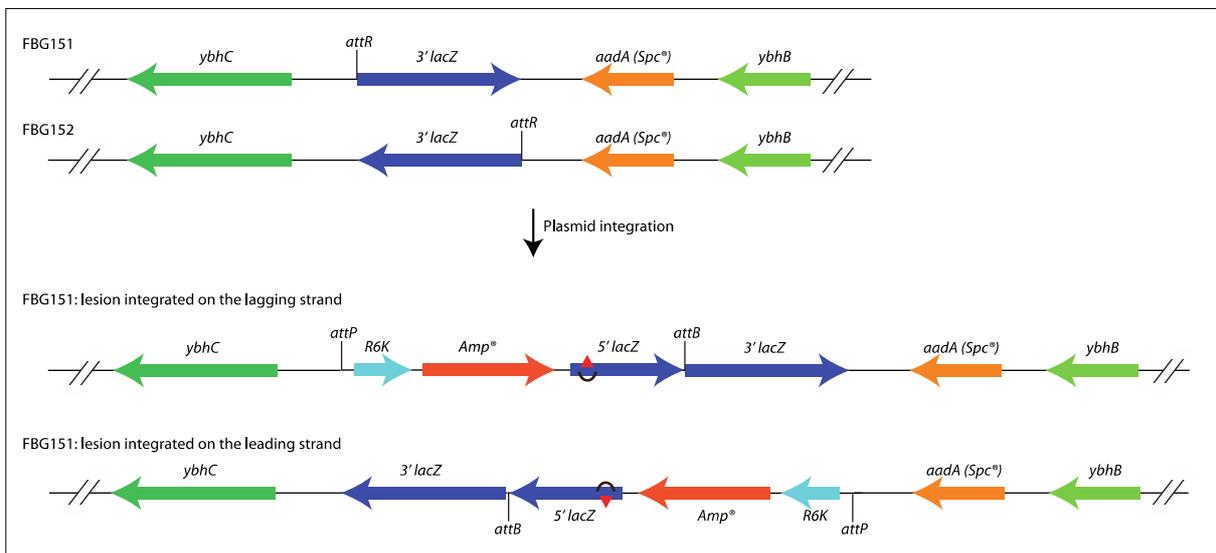


Figure 1.

A. Outline of the integration system:

The recipient strain contains a single attR integration site in fusion with the 3' end of lacZ gene at min 17 in the *E. coli* chromosome. Following ectopic expression of phage lambda int_{xis}, the lesion-carrying construct is introduced by electroporation. Its attL site will recombine with the chromosomal attR, leading to integration of the entire lesion-containing construct. Integration events are selected on the basis of their resistance to ampicillin. Integration at nucleotide level resolution restores a functional *lacZ* gene allowing these events to be monitored on X-gal indicator plates.

B. Detail of the phage lambda integration system:

The figure show the detail of the core sequences of the site-specific recombination sites of phage lambda: attR, attL, attB and attP.

C: Map of the strains before and after integration. In strain FBG151, the lesion is integrated in the lagging strand. In strain FBG151, attR-lacZ has been inverted in order to integrate the lesion in the leading strand.

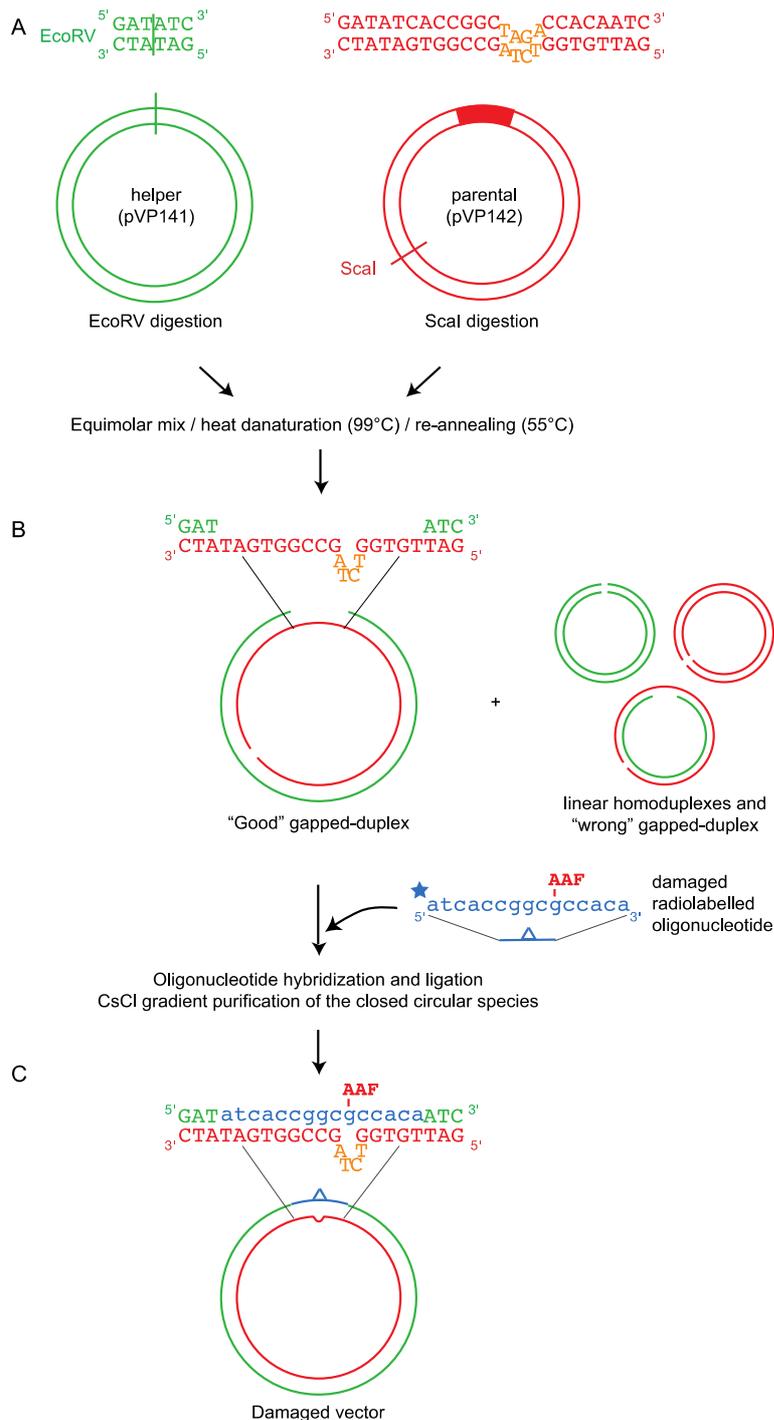


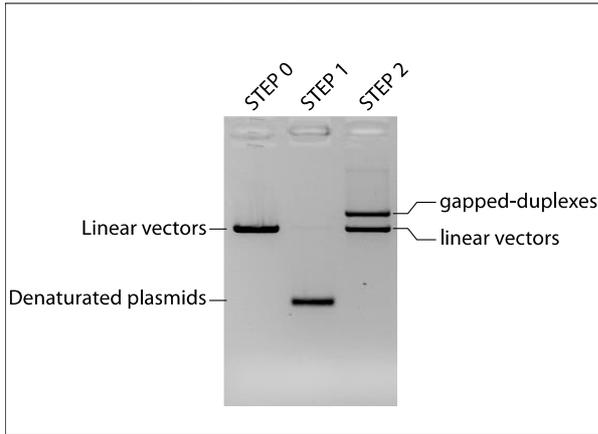
Figure 2. Gapped duplex method to produce vectors with a single lesion

A. Helper and parental plasmid are linearized by a restriction endonuclease generating blunt ends.

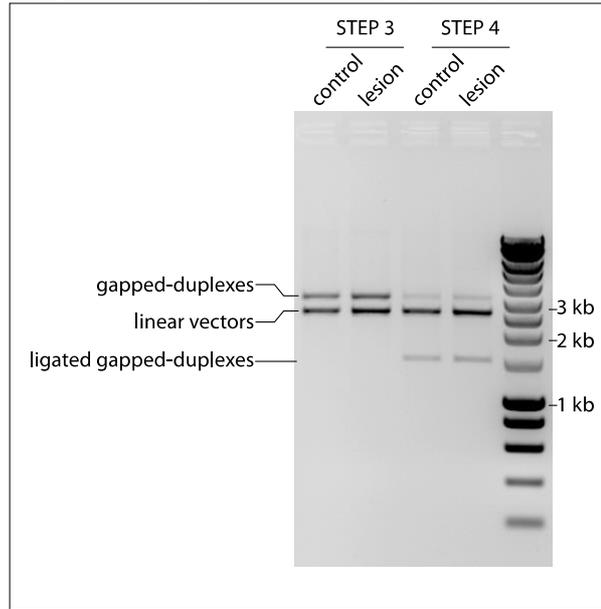
B. Equal amounts of the two plasmids are mixed, heat-denatured and allowed to re-anneal in order to form to gapped-duplex molecules. Two molecular forms of gapped-duplexes will be generated, one with a gap that is complementary to the damaged oligonucleotide (denoted "good" gapped duplex) and one that has the same sequence (denoted "wrong").

C. Ligation of the control or damaged oligonucleotide in the gapped-duplex and isolation of the closed circular species by CsCl gradient purification allows to recover high purity damaged vector.

A. Formation of the gapped duplexes



B. Ligation of the oligonucleotides in the gapped-duplexes



C. Gel quantification of the constructions

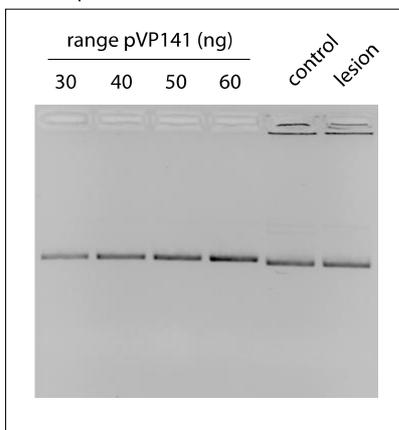


Figure 3. Controlling the steps of vector production on agarose gel electrophoresis

A. Visualization of the three steps leading to the formation of the gapped duplexes. Step 0: linear vectors after digestion. Step 1: denatured plasmids. Step 2: after re-annealing, formation of ~50% of linear homoduplexes and ~50% of gapped heteroduplexes.

B. Control of the ligation of the oligonucleotides (control and damaged) into the gapped-duplex molecule. Successful ligation events generate circular, covalently closed constructs that migrate faster during agarose gel electrophoresis in the presence of ethidium bromide.

C. Quality control and quantification of the construction: a range of the helper plasmid (pVP141) allows to estimate the concentration of the construction after CsCl gradient purification. (note that the electrophoretic migration of the circularized duplex is slightly faster than the pVP141 plasmid. Indeed, pVP141 was produced *in vivo* and therefore contains negative supercoiling, whereas the constructed duplex was ligated *in vitro* and contains thus no supercoils).

Plates after integration

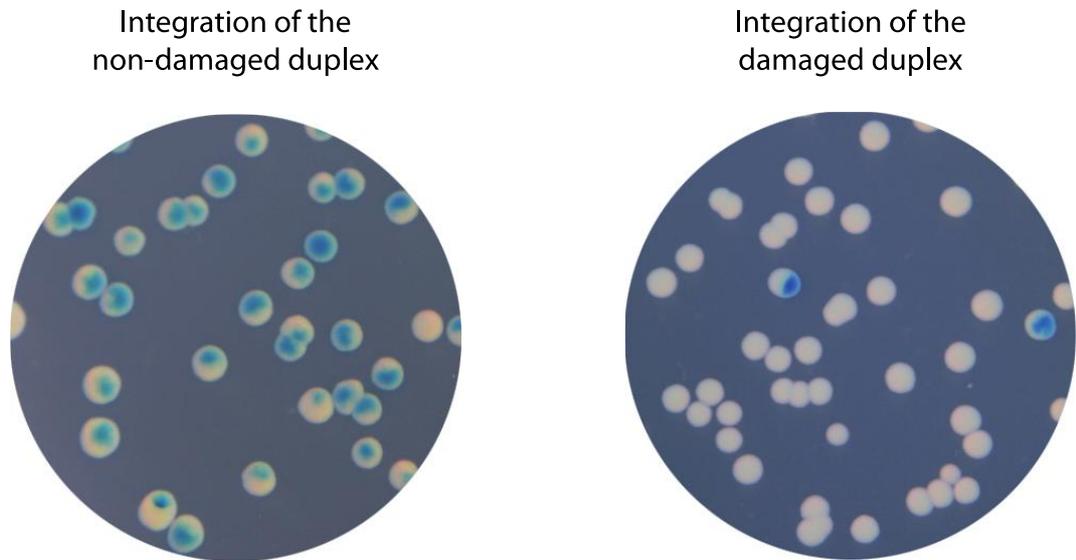


Figure 4. Colonies following integration.

After integration of the non-damaged construct, all colonies exhibit blue/white sectors; the white and blue sectors represent the bacterial progeny that stems from replication of the *lacZ*⁻ and *lacZ*⁺ strand of the initial construct, respectively. After integration of the single lesion-containing vector, only cells in which a TLS event occurred give rise to a sectorial blue/white colony. Phenotypic monitoring of DA events requires a specific construct (11).