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Protocol

SIRT Combines Homologous Recombination, Site-Specific Integration, and Bacterial Recombineering for Targeted Mutagenesis in *Drosophila*

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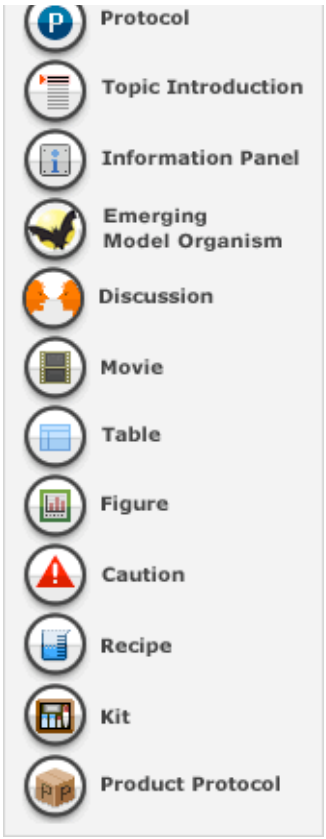
INTRODUCTION

Systematic mutational analysis is required for the comprehensive deciphering of gene function. However, repeated targeting of a single locus is labor intensive and has not been a routine approach for studies using multicellular organisms. We have developed the "site-specific integrase mediated repeated targeting" (SIRT) method to facilitate targeted mutagenesis in *Drosophila melanogaster*. In SIRT, homologous recombination is used to place a landing site for the phage phiC31 integrase in the vicinity of the target locus. All subsequent genetic modifications to the same gene are introduced by integrase-mediated precise insertion of plasmids directly injected into embryos. For SIRT mutagenesis, one must generate a series of plasmid vectors that contain various DNA elements placed at different positions in the target-homologous clone. Unlike traditional cloning methods, SIRT is not limited by the availability of convenient restriction cut sites. This protocol presents the details of SIRT plasmid construction, relying heavily on the method of bacterial recombineering and using a number of streamlined DNA elements.

RELATED INFORMATION

For more information about SIRT targeting, see Gao et al. (2008). The procedure described here for transforming SW102 cells closely follows "Recombineering Protocol 1" by S Warming (http://recombineering.ncifcrf.gov/Protocol.asp). Protocols are available for

Agarose Gel Electrophoresis (Sambrook and Russell 2006a), and **Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation** (Sambrook and Russell 2006b).



MATERIALS

Reagents

Bacterial strains:

- Standard bacterial cloning strains (e.g., DH10B or DH5α)
- SW102 recombineering cells (available from NCI, http://recombineering.ncifcrf.gov)

SW102 cells carry a heat-inducible lambda red system. It is crucial to grow them at or below 32°C at all times (except during induction). Other recombineering-ready strains are also available.

DNA ligase and 10X ligase reaction buffer

DNA minipreparation kit (commercially available) (optional; see Steps 12 and 18)

DNA polymerase with proofreading activities and 10X polymerase chain reaction (PCR) buffer

Drosophila stocks:

- *70FLP+70I-SceI* (transgenic line with heat-inducible *FLP* and *I-SceI* genes; Bloomington *Drosophila* Stock Center, #6934, #6935)
- *70FLP10* (transgenic line with a constitutively active *FLP* gene; Bloomington *Drosophila* Stock Center, #6938)
- *70I-CreI* (transgenic line with a heat-inducible *I-CreI* gene; Bloomington *Drosophila* Stock Center, #6937)
- *vasa-phiC31* (transgenic lines with *vasa-phiC31* on X or IV; www.frontiers-in-genetics.org/flyc31)
- White-eyed (*w*)

FseI restriction endonuclease and 10X reaction buffer

Genomic DNA template from *Drosophila*

Glycerol solution, sterile, ice-cold (10% v/v in H_2O) (~50 mL per batch of cells)

LB (Luria-Bertani) liquid medium and solid plates containing the appropriate antibiotic, at the following concentrations:

- Ampicillin (amp, 100 µg/mL)
- Chloramphenicol (cm, 50 µg/mL)
- Tetracycline (tet, 12.5 µg/mL)

LR Clonase II enzyme mix (Gateway; Invitrogen)

pCR8/GW/TOPO TA Cloning Kit (Invitrogen)

Plasmid templates containing the following cloning cassettes: Cm-attP, Cm-attB, and Cm-ISceI (available from authors upon request) (see Step 13)

PmeI restriction endonuclease and 10X reaction buffer

Primers for PCR amplification of Cm-attP, Cm-ISceI, and Cm-attB cloning cassettes (see Steps 13, 22, and 24)

Primers for PCR amplification of donor fragment (see "General Rules for Designing Plasmids for SIRT" and Step 1)

pTV2gw ends-in targeting vector (available from authors upon request)

Restriction enzymes for plasmid verification (see Steps 12 and 18)

SOC medium

Equipment

Capillary tubing for microinjection (FHC, #30-30-0) (optional; see Steps 25 and 32)

Centrifuge preset to 0ºC, with rotor for 15-mL tubes (Tomy)

Culture tubes, prechilled on ice (15-mL, Nalgene)

DNA sequencing equipment

Electroporator (MicroPulser, BioRad) and cuvettes (1-mm)

Equipment for *Drosophila* culture, including *Drosophila* vials

Equipment for Southern blot analysis

Flasks, Erlenmeyer (50-mL or 125-mL)

Ice slurry (H_2O in a bucket of ice)

Incubator preset to 30ºC

Incubator, shaking, preset to 30ºC

Microinjector (Eppendorf transjector p-5246) (optional; see Steps 25 and 32)

Micromanipulator for microinjection (Leica) (optional; see Steps 25 and 32)

Micropipette puller (Sutter P-97, Flaming/Brown micropipette puller) (optional; see Steps 25 and 32)

Microscope, dissecting, equipped for microinjection (optional; see Steps 25 and 32)

Pipettes, sterile, 10-mL

Spectrophotometer and prechilled cuvettes

Thermocycler

Towel, paper

Water bath, shaking, preset to 42ºC

Water baths, circulating, preset to 36ºC and 38ºC (Lauda)

METHOD

Vector Constructions

General Rules for Designing Plasmids for SIRT

- The genomic fragment used for targeting (the "donor" fragment) should contain enough homology to support homologous recombination. We routinely use fragments of 5-6 kb. For *attP* placement, the donor contains an *attP* site and an I-SceI cut site. For phiC31-mediated integration, it contains an *attB* site, as well as various

modifications such as mutations and epitope tags. For possible donor configurations, see Figure 1A .

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Figure 1. DNA elements for vector construction. (*A*) Configurations of the donor fragment for various purposes (listed to the *left* of the diagrams). To make a deletion, the *attB* and *attP* are placed at opposite sides of the desired deletion. (*B*) Cm cassettes for recombineering. The arrows indicate primer positions. For recombineering, attach 50 bp of homology arms 5' to these primers. (*C*) A map of the pTV2gw targeting vector. w^+: eye marker gene. FRT: FLP recombinase target. 3'P and 5'P: inverted repeats of P element.

- The *attP*/*attB* (*att*) site, which is ~250 bp long, needs to be placed such that it does not disrupt expression of the target gene. Thus, we recommend placing it in an intergenic region or within a large intron.

- The placement for the 18-bp I-SceI cut site is under fewer constraints, because it is usually precisely eliminated during the recombination process in the targeting step (Rong and Golic 2000; Rong et al. 2002). However, if possible, place the cut site in a "nonessential" region, such as the one used for the *att* site, and roughly in the center of the donor fragment (see Fig. 1A).

- The relative position between *attP* and the I-SceI cut sites can affect the efficiency at which *attP* is recovered in the reduction event (Rong et al. 2002). As a rule of thumb, place *attP* 0.5-1 kb from the cut site.

- In summary, to design a donor plasmid for SIRT, start by choosing a position for the *att* site. Next, place the I-SceI cut site 0.5-1 kb on either side of the *att* site, and then position the donor fragment so that the cut site is near the center.

Generating pTV[donor], the Master Plasmid Clone for SIRT

A flowchart depicting the use of recombineering in SIRT is shown in Figure 2 .

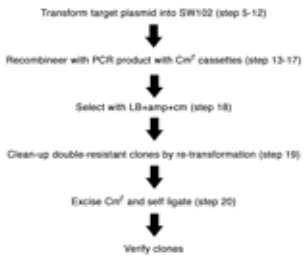

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Figure 2. The use of bacterial recombineering for vector construction in SIRT. For an overview of bacterial recombineering, see Sawitzke et al. (2007).

1. Amplify the donor fragment (5-6 kb) from genomic templates in a PCR using a polymerase with proofreading activities. Use agarose gel electrophoresis to check the specificity and efficiency of the PCR (see **Agarose Gel Electrophoresis** [Sambrook and Russell 2006a]).
See Troubleshooting.

2. Clone the donor fragment into the pCR8/GW/TOPO vector by TOPO TA cloning, according to Invitrogen's instructions.
See Troubleshooting.

3. Sequence several clones and choose one that does not contain mutations introduced by PCR.
Natural polymorphisms can be differentiated from mutations by looking for sequence variations that are present in multiple independent clones.

4. Subclone the donor fragment into the pTV2gw vector using the LR clonase enzyme mix according to Invitrogen's instructions.
This clone, pTV[donor], is the master clone used in subsequent recombineering steps for the placement of various DNA elements, such as att sites, an I-SceI cut site, and mutations.

Preparing Competent SW102

5. Inoculate a 5-mL culture of LB+tet with SW102 recombineering cells. Incubate the culture overnight at 30°C.

6. Dilute the overnight culture 50-fold by adding 600 µL to a flask with 30 mL of LB+tet. Incubate with shaking at 250 rpm at 30°C until the culture reaches an OD_{600} of 0.5-0.6 (~3 h).

7. Chill the culture by placing the flask in ice slurry and mixing vigorously. Transfer the cells to prechilled 15-mL culture tubes. Centrifuge the cells at 5000 rpm for 5 min at 0ºC.
From this step on, try to keep the cells at 0°C at all times.

8. Pour off as much supernatant as possible without letting the cells warm. Resuspend each cell pellet in 1 mL of sterile, ice-cold, 10% glycerol solution by jostling the tubes on ice. Fill the tubes to 10 mL with sterile, ice-cold, 10% glycerol solution, and mix by inverting several times. Centrifuge the cells at 5000 rpm for 5 min at 0ºC.
The initial pellets are relatively tight, so resuspension may take a few minutes.

9. Repeat Step 8 two more times.

10. Carefully pour off the supernatant (the pellets at this stage are very loose), and invert the tubes on a paper towel for a few seconds before returning them to ice. Resuspend the cells in the residual liquid, and keep the tubes on ice until electroporation in Step 11.

Transforming pTV[donor] into Competent SW102

11. Mix 1-10 ng of pTV[donor] from Step 4 with 25 µL of competent SW102 cells from Step 10. Transfer the mixture to prechilled cuvettes and electroporate according to the manufacturer's instructions. Add 500 µL of SOC medium, incubate for 1 h at 30°C, and plate the entire mixture on LB+amp. Incubate overnight at 30ºC.
Keep any unused competent SW102 cells in 25-µL aliquots at –80°C for future use.

12. Prepare miniprep cultures of several colonies in 5 mL of LB+amp. Incubate with shaking overnight at 30ºC. Isolate DNA from the cultures by alkaline lysis (see **Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation** [Sambrook and Russell 2006b]) or with commercially available miniprep kits. Perform restriction digests of the DNA to confirm transformation of the cells.

Generating pTV[donor-attP-ISceI], the Targeting Vector for attP Placement

Bacterial recombineering requires 50 bp of homology at each side of the position at which a DNA cassette is to be inserted. The right and left arms of homology are introduced as long PCR primers.

13. Amplify the Cm-attP cloning cassette from a diluted plasmid template (see Fig. 1B). Use the following pair of PCR primers:

5'-left homology arm (50 bp)-**GGCCGGCC**CTGTGGAACACC-3' (carries **FseI**; homologous to *Cm*^{r})

5'-right homology arm (50 bp)-TCGCGCTCGCGCGACTGACG-3' (homologous to *attP*)

14. Prepare SW102 cells carrying pTV2[donor] for induction:

i. Inoculate a 5-mL culture of LB+amp with SW102 recombineering cells carrying pTV2[donor] from Step 11. Incubate the culture overnight at 30°C.

ii. Dilute the overnight culture 50-fold by adding 600 µL to a flask with 30 mL of LB+amp. Incubate with shaking at 250 rpm at 30°C until the culture reaches an OD_{600} of 0.5-0.6 (~3 h).

15. Transfer 15 mL of the culture to a new flask. Induce this aliquot by incubating in a water bath with shaking at 250 rpm for exactly 15 min at 42ºC. Return the remaining culture to 30°C as the uninduced control.

16. Immediately after induction, wash the cells:

i. Chill the cultures by placing the flasks in an ice slurry and mixing vigorously. Transfer the cells to prechilled 15-mL culture tubes. Centrifuge the cells at 5000 rpm for 5 min at 0ºC.
From this step on, try to keep the cells at 0°C at all times. Treat the induced and uninduced cultures identically.

ii. Pour off as much supernatant as possible without letting the cells warm. Resuspend each cell pellet in 1 mL of ice-cold H_2O by jostling the tubes on ice. Fill the tubes to 10 mL with ice-cold H_2O and mix by inverting several times. Centrifuge the cells at 5000 rpm for 5 min at 0ºC.
The initial pellets are relatively tight, so resuspension may take a few minutes. H_2O is used instead of glycerol, because the induced cells cannot be saved for future use.

iii. Repeat Step 16.ii two more times.

iv. Carefully pour off the supernatant (the pellets at this stage are very loose), and invert the tubes on a paper towel for a few seconds before returning them to ice. Resuspend the cells in the residual liquid and keep the tubes on ice until electroporation in Step 17.

17. Mix 100-300 ng of the Cm-attP PCR product from Step 13 with 25 µL of induced SW102 cells from Step 16.iv. Transfer the mixture to prechilled cuvettes and electroporate according to the manufacturer's instructions. Add 500 µL of SOC medium, incubate for 1 h at 30°C, and plate the entire mixture on LB+amp+cm. Incubate at 30ºC.
Colonies may take 2 d to appear.
See Troubleshooting.

18. Prepare miniprep cultures of double-resistant colonies in 5 mL of LB+amp+cm. Incubate with shaking overnight at 30ºC. Isolate DNA from the cultures by alkaline lysis (see **Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation** [Sambrook and Russell 2006b]) or with commercially available miniprep kits. Perform restriction digests of the DNA based on the EcoRI and SmaI sites introduced with the cassette.
The uninduced control should produce very few colonies. Normally, fewer than 50 colonies are expected from

the induced culture, and most of them carry a mixture of both the correct plasmid and the original pTV[donor] plasmid.

19. To "clean up" the plasmid mixture, retransform the miniprep DNA from Step 18 into standard bacterial cloning cells (e.g., DH10B or DH5α) and plate on LB+amp+cm. Pick several colonies and repeat the restriction digests from Step 18. Most of the clones should contain only one plasmid; the correct plasmid is designated pTV[donor-Cm-attP]. Sequence several clones to ensure the integrity of *attP*.
See Troubleshooting.

20. Digest a few nanograms of pTV[donor-Cm-attP] with FseI. Self-ligate the digested DNA, transform the ligation reactions into standard bacterial cloning cells (e.g., DH10B or DH5α), and plate the transformed cells on LB+amp. Pick several colonies, prepare miniprep DNA, and sequence to verify the loss of *Cm*^{r}. The plasmid is now designated pTV[donor-attP].
See Troubleshooting.

21. Repeat Steps 5-12 to introduce pTV[donor-attP] into SW102 recombineering cells. Alternatively, use previously frozen, competent SW102 cells and repeat Steps 11 and 12.

22. Similar to Step 13, amplify the Cm-ISceI cassette (see Fig. 1B) with the following pair of primers:

5'-left homology arm (50 bp)-CTATATTACCCTGTTATCCCTA-3' (homologous to I-SceI cut site)

5'-right homology arm (50 bp)-**GTTTAAAC**AGCCAGTATACACTCCGCTA-3' (carries **PmeI**; homologous to *Cm*^{r}).

23. Repeat Steps 14-20, but use the pTV[donor-attP] in SW102 cells from Step 21 as the starting plasmid, and use the PCR products from Step 22 for electroporation. To excise Cm in Step 20, use PmeI instead of FseI. After sequencing, the resultant pTV[donor-attP-ISceI] plasmid can be used for germline transformation in Step 25.
See Troubleshooting.

Generating pTV[donor-attB] Vectors for Site-Specific Integration
These vectors are different from pTV[donor-attP-ISceI] in Step 23 in several ways:

- attB *replaces* attP *in the identical position, unless the vector is being used to make a deletion, in which case* attB *is placed in another position (see* Fig. 1*).*
- *There is no I-SceI cut site.*
- *pTV[donor-*attB*] vectors carry the designed genetic modification to the target gene.*

24. Start with SW102 cells that carry the pTV[donor] vector, from Step 12. Repeat Steps 13-21, but use Cm-attB PCR product instead of Cm-attP PCR product, and use the following primers:

5'-left homology arm (50 bp)-**GGCCGGCC**CTGTGGAACACC-3' (carries **FseI**; homologous to *Cm*^{r})

5'-right homology arm (50 bp)-CGACATGCCCGCCGTGACCG-3' (homologous to *attB*)
These steps lead to the creation of the pTV[donor-attB] plasmid, which can be further modified using recombineering and customized PCR products that contain the modifications attached to an excisable Cm cassette. The final series of modified pTV[donor-attB] plasmids will be used for microinjection in Step 32.
See Troubleshooting.

Genetic Crosses for SIRT

Placement of attP *by Ends-In Targeting*

For a detailed presentation on genetic crosses for gene targeting, see Maggert et al. (2008). For background on

Drosophila *ends-in gene targeting, see Rong and Golic (2000) and Rong et al. (2002).*

25. Use pTV[donor-attP-ISceI] from Step 23 to transform white-eyed (*w*) flies by microinjection. Alternatively, injection can be performed by a fee-for-service company. Recover red-eyed (w^+) transformants to establish donor lines for ends-in targeting.
For general information on microinjecting Drosophila *embryos, see Bachmann and Knust (2008).*

26. Mass-mate flies from two or three donor lines to flies from the *70FLP+70I-SceI* line in groups of four or five pairs of flies. Transfer the parents every 3 d, and heat-shock the progeny by incubating the vials of larvae in a circulating water bath for 1 h at 38°C.
Repeated heat shocks of the same vials are not necessary.

27. Recover female progeny with both the donor P element and the *70FLP*, *70I-SceI* genes. These flies should be mostly white-eyed, but some will show eye mosaicism. Mass-mate these females to males from the constitutive *70FLP10* line in groups of three or four pairs. Transfer the crosses once after 5 d.
As a rule of thumb, aim at setting up 1000 crosses spread over a few weeks.

28. Establish lines from progeny with solid eye pigmentation from Step 27. Map the w^+ marker in relationship to the target chromosome. Perform Southern blot and PCR analyses to confirm targeted events and the presence of *attP*.

29. Mass-mate targeted lines to flies with a *Sb*-marked *70I-CreI* gene on chromosome 3 in groups of four or five pairs. Transfer the parents every 3 d, and heat-shock the progeny by incubating the vials of larvae in a circulating water bath for 1 h at 36°C. Recover mosaic males carrying *Sb 70I-CreI* individually to females carrying chromosome 3 balancers. Recover a single fertile, white-eyed, and Sb^+ (without *70I-CreI*) male from every mosaic father. Establish 50 such stocks for further characterization. For a target locus on a chromosome other than the third chromosome, additional dominant markers are needed.

30. Perform Southern blot and PCR analyses to identify lines with a single, intact target locus carrying the targeted *attP* site.
These are the master stocks for future phiC31-mediated integration experiments.

Site-Specific Integration by phiC31 Integrase
The phiC31 integrase can be supplied as in vitro synthesized mRNA or from an endogenous phiC31 *transgene. We recommend the second method with a vasa-driven phiC31 inserted on chromosome X or 4 (Bischof et al. 2007).*
For background on phiC31-mediated site-specific integration in Drosophila*, see Groth et al. (2004), Bateman et al. (2006), and Bischof et al. (2007).*

31. Generate a stock with *vasa-phiC31* and the master *attP* line from Step 30. Expand this stock for microinjection experiments.

32. Inject pTV[donor-attB] plasmids (0.5 µg/µL in H_2O) from Step 24 into embryos. Alternatively, injection can be performed by a fee-for-service company. Cross survivors to *w* flies, and recover pigmented progeny as integrants. Verify the genomic structure of integrants by Southern blot, PCR, and sequencing analyses.
*In our hands, 100% of the integrants tested had the correct structure (*n*>30).*

33. Repeat Steps 29 and 30.
This repetition reduces the target copy. The above integrants carry a target duplication, only one of which has the desired genetic modification.
See Troubleshooting.

34. Confirm final reduction events with the desired modifications by molecular analysis.

TROUBLESHOOTING

Problem: Long PCR is not productive.

[Step 1]

Solution: To increase the yield of amplification products, try the following:

1. Test several pairs of primers from the same region.

2. Treat the template genomic DNA with RNaseA and phenol.

Problem: There are very few colonies after the TA reaction.

[Step 2]

Solution: TA cloning depends on the presence of a 3' A-overhang on the PCR products. This overhang is often removed by proofreading polymerases. To add 3' A-overhangs, add 0.2 µL of regular *Taq* polymerase directly to the completed PCR. Incubate the reaction for 10 min at 72ºC, and use in the TA cloning reaction.

Problem: The electroporator arcs.

[Step 17]

Solution: Arcing is caused by excess salt in the cell-DNA mixture.

1. To reduce the salt in the cell suspension, repeat the cell-washing in Step 16 an additional two times.

2. To reduce the salt in the DNA component of the mixture, do one or both of the following steps:

    i. Reduce the amount of PCR product added to the cells.

    ii. Ethanol-precipitate the PCR product, and wash the precipitate with warm 70% ethanol.

Problem: Cm-resistant clones are carrying the original plasmid template used for PCR amplification of the cloning cassette in Step 13.

[Step 19]

Solution: To minimize the amount of plasmid template in the DNA used for electroporation, digest the template with DpnI after cassette amplification in Step 13. Alternatively, perform a first round of PCR with the cassette primers only (without homology arms), dilute the PCR product 100- to 1000-fold, and use that as the template for the final PCR amplification (using primers with homology arms). The PCR reaction can be directly used for electroporation (0.5-4 µL of DNA per 25 µL of cells).

Problem: The plasmids are rearranged.

[Step 19]

Solution: Large constructs can be unstable in standard bacterial cloning strains. Using Stbl2 cells (Invitrogen) cultured at 30°C may help.

Problem: All clones remain double resistant (amp^r and cm^r).

[Steps 20, 23, 24]

Solution: Consider the following:

1. Perform a restriction digest to confirm that Cm is excisable; it is possible that the FseI or PmeI site was damaged during PCR.

2. The FseI enzyme may be inactive. Store FseI at −70ºC, because it is unstable at −20ºC.

Problem: Reduction frequency is very low and/or most white-eyed events do not have a single target locus.

[Step 33]

Solution: We experienced an increase in reduction frequency and fidelity when we implemented an additional step of FLP-mediated excision of the plasmid backbone before introducing *70I-CreI* (for more details, see Gao et al. 2008):

1. Cross the integrants from Step 32 to the constitutive *70FLP10* line.
2. Heat-shock the progeny for 1 h at 37°C during the first 3 d of their development.
3. Cross pigmented progeny to *70I-CreI* flies for reduction.

DISCUSSION

Targeted mutagenesis through homologous recombination has allowed researchers to mutate a particular locus in *Drosophila* in any desired way. SIRT expands this approach by targeting the *attP* landing site to the locus of interest. Through phiC31-mediated integration, several genetic variants can then be generated at the same location without having to perform multiple gene targeting experiments. The method has been used successfully in our laboratory to create an array of six mutants of the *nbs* locus, including a precise gene deletion (Gao et al. 2008). phiC31 can integrate DNA fragments that are larger than 100 kb into the *Drosophila* genome (Venken et al. 2006). In principle, SIRT would allow targeted mutagenesis to such a large region adjacent to an existing *attP* site (see Fig. 1A).

The greatest obstacle that one faces when using the SIRT method is the construction of the appropriate vectors that contain complex arrangements of several DNA elements. We simplify the process by taking advantage of the versatile bacterial recombineering methodology. This method increases flexibility and bypasses the shortcomings of traditional cloning, because it does not rely on the availability of restriction cut sites at the site of modification.

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Related Protocols

Agarose Gel Electrophoresis
Joseph Sambrook and David W. Russell
CSH Protocols 2006: 4020. [Extract] [Full Text]

Preparation of Plasmid DNA by Alkaline Lysis with SDS: Minipreparation
Joseph Sambrook and David W. Russell
CSH Protocols 2006: 4084. [Extract] [Full Text]