Transposon insertion mutagenesis for archaeal gene discovery

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Running title:

Summary

Archaea constitute the third domain of life, but studies on their physiology and other features have lagged behind bacteria and eukarya, largely due to the challenging biology of archaea and concomitant difficulties in methods development. The use of genome-wide *en masse* insertion mutagenesis is one of the most efficient means to discover genes behind biological functions, and such a methodology is described here for a model archaeon *Haloferax volcanii*. The strategy successfully employs efficient *in vitro* transposition in combination with gene targeting *in vivo* via homologous recombination. The methodology is general and should be transferable to other archaeal species.

Key words: Insertion mutant library, gene discovery, *Haloferax volcanii*, halophilic archaea, MuA transposase

1. Introduction

Archaea constitute the third domain of life, are ubiquitous in different types of environments, and often live in habitats with extremely harsh conditions. Thus, these organisms are biologically intriguing and potentially constitute an enormous resource for biotechnology applications. Yet, largely due their challenging biology and concomitant difficulties in methods development, biochemical pathways and genetic basis behind many unique archaeal features remain poorly characterized or entirely uncharacterized. Advanced methodologies are thus warranted for these organisms.

Insertion mutant libraries, that contain randomly distributed genomic alterations in each gene, provide a valuable resource for studies aimed at delineating molecular mechanisms behind biological functions. Such libraries have proven their immense usefulness for gene discovery, particularly in microbiological research. Until recently, archaea were underrepresented in such studies, as adequate insertion libraries had only been made for two archaeal species, methanogens *Methanosarcina acetivorans* and *Methanococcus maripaludis* ***(1, 2)***.

Now a recent study has widened the scope and applicability of insertion mutant libraries to halophilic archaea. The paper by Kiljunen et al. (2014) ***(3)*** describes a transposition-based method to generate a comprehensive insertion mutant library for the easily cultivable model archaeon, *Haloferax volcanii*, and moreover, the use of the library for gene discovery. The methodology used exploited MuA transposase catalyzed *in vitro* transposition reaction and combined it with *in vivo* gene targeting by homologous recombination. As a result, a robust and widely applicable strategy was devised. The strategy entails the following steps: (i) Isolation of genomic DNA, its fragmentation, and tagging with a transposon. (ii) Cloning of the tagged genomic fragments in *E.*

coli to generate a plasmid library that covers the entire chromosome with overlapping fragments. (iii) Amplification of the library, isolation of tagged chromosomal fragments, and their size selection. (iv) Transformation of the fragments into *H. volcanii* and *en masse* gene targeting via homologous recombination. The end product is a *H. volcanii* insertion mutant library, in which each clone harbors a single transposon insertion in its genome. The library is an ideal resource for efficient gene discovery, and it facilitates the identification of non-essential genes behind any specific biochemical pathway. The strategy used for the construction of the library should readily be transferable to other archaeal species.

2. Materials

H. volcanii is sensitive to trace contaminants of detergents as well as to impurities in culture media. Therefore, it is advisable to rinse the glassware thoroughly with water and use for media preparation only those commercial products specified in this protocol. (*See* **Note 1**)

2.1. Materials needed for preparation of transposon-tagged plasmid library

2.1.1. Materials needed for *H. volcanii* chromosomal DNA isolation and fragmentation

1. 18% Modified Growth Medium (MGM) agar dishes and liquid medium: Prepare first 30% salt water (SW): Dissolve 240 g NaCl, 30 g $MgCl_2 \bullet 6H_2O$, 35 g $MgSO_4 \bullet 7H_2O$, and 7 g KCl into ~800 ml water. Add slowly 5 ml 1 M $CaCl_2$. Adjust the pH to 7.5 with 1M Tris-Cl, pH 7.5 and add water to the final volume of 1000 ml. For liquid 18% MGM, dissolve 5 g peptone (Oxoid) and 1 g yeast extract (Difco) into 600 ml SW and 367 ml water. Adjust the pH to 7.5 with 1 M Tris base and fill the volume to 1000 ml with water. For solid medium, add Bacto-agar (Difco) 15 g/l. ***(3)*** (*See* **Note 1**.)

2. Incubator and shaker for cell culture
3. Tabletop centrifuge, microcentrifuge
4. ST buffer: 1 M NaCl, 20 mM Tris-Cl, pH 7.5
5. Lysis solution: 100 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 0.2% SDS
6. Phenol: Saturated, pH 7.9 (Amresco ,Solon, OH, USA)
7. Heat block / water incubator
8. Chloroform
9. Ethanol; absolute and 70 %.
10. TE buffer: 10 mM Tris-Cl, pH 7.5, 1 mM EDTA
11. RNase A stock solution (10 mg/ml)
12. 3M Sodium acetate, pH 7.0
13. AciI, HpaI, TaqI restriction enzymes (several manufacturers)

2.1.2 Materials needed for preparation of transposon DNA

1. Transposon carrier plasmid pMPH20 ***(4)***
2. BglII restriction enzyme (several manufacturers)
3. Equipment for ion exchange chromatography or preparative agarose gel electrophoresis
4. TE buffer: 10 mM Tris-Cl, pH 7.5, 1 mM EDTA

2.1.3 Materials needed for Mu *in vitro* transposition reaction and size selection

1. 2 x MIX: 50 mM Tris-Cl pH 8.0, 200 μg/ml BSA (bovine serum albumin), 30% (w/v) glycerol. Use high quality molecular biology grade BSA. Store at -70 °C.

2. Triton X-100: Prepare 1.25% solution from 10% stock solution by diluting with H_2O directly prior to use.

3. 2.5 M NaCl, 0.25 M $MgCl_2$

4. MuA transposase protein (Thermo Fisher Scientific) 220 ng/µl in MuA dilution buffer: 0.3M NaCl, 25 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol.

2.1.4 Materials needed for preparation of vector DNA

1. pBlueScriptSK+ plasmid (several manufacturers)

2. Plasmid DNA isolation kit (several manufacturers)

3. ClaI restriction enzyme (several manufacturers)

4. Alkaline phosphatase (several alternatives)

2.1.5 Materials needed for generation of plasmid library

1. T4 DNA ligase (several manufacturers)

2. *E. coli* electrocompetent DH10B cells. Store at -70 °C.

3. Electroporation apparatus and cuvettes (several manufacturers)

4. SOB: 2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl. Autoclave.

5. SOC: Add to 100 ml of SOB solution one milliliter of both 2 M $MgCl_2$ and 2 M glucose from stock solutions sterilized by filtration through a 0.22 micron filter.

6. LB: 1 % Bacto tryptone, 0.5 % Bacto yeast extract, 1 %NaCl. For solid medium, add 1.5% Bacto-agar. Supplement LB with ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) when needed.

2.1.6 Materials needed for amplification and CsCl purification of plasmid library

1. *E. coli* DH5α electrocompetent cells. Store at -70 °C.

2. See ***(7)*** for the reagents and equipments of CsCl purification.

2.2. Materials needed for the preparation of *H. volcanii* insertion mutant library

2.2.1. Materials needed for isolation and purification of transposon-tagged fragments of chromosomal DNA

1. Restriction endonucleases (several manufacturers).
2. Equipment for preparative gel electrophoresis

2.2.2. Materials needed for transformation of *H. volcanii* cells

1. Tabletop centrifuge that accommodates 50 ml centrifuge tubes
2. Buffered spheroplasting solution: 1M NaCl, 27 mM KCl, 50 mM Tris-Cl, pH 8.5, 15% (w/v) sucrose. ***(3)***
3. Buffered spheroplasting solution with 15% glycerol: 1M NaCl, 27 mM KCl, 50 mM Tris-Cl, pH 8.5, 15% (w/v) sucrose, 15% (v/v) glycerol. ***(3)***
4. 0.5 M EDTA, pH 8.0
5. Unbuffered spheroplasting solution: 1M NaCl, 27 mM KCl, 15% (w/v) sucrose. Adjust pH to 7.5 with 1 M NaOH (~10 μl / 100 ml). ***(3)***

6. 60% PEG 600 solution: 1500 µl PEG 600 (Merck) + 1000 µl unbuffered spheroplasting solution. ***(3)***
7. Spheroplast dilution solution: Dissolve 15 g sucrose in 76 ml 30% SW, and add water up to 100 ml. After autoclaving, add 0.75 ml of 0.5 M $CaCl_2$. ***(3)***
8. Regeneration solution: Prepare first 10 x YPC: dissolve 1.25 g Yeast Extract (Difco), 0.25 g Peptone (Oxoid), and 0.25 g Casamino acids (Difco) in ~19 ml water. Adjust pH to 7.5 with 1 M KOH. Adjust volume to 25 ml with water. For regeneration solution, dissolve 37.5 g sucrose in 150 ml 30% SW and 25 ml 10 x YPC. Adjust the volume to 250 ml with water. After autoclaving, add 1.5 ml of 0.5 M $CaCl_2$. ***(3)***
9. Transformant dilution solution: Dissolve 37.5 g sucrose in 150 ml 30% SW and adjust the volume to 250 ml with water. After autoclaving, add 1.5 ml of 0.5 M $CaCl_2$. ***(3)***
10. 80% glycerol-6% SW: Mix 80 ml glycerol and 20 ml 30% SW. After autoclaving, add 200 µl of 0.5 M $CaCl_2$. ***(3)***
11. Hv-Ca: Prepare first 10 x Ca: Dissolve 1.7 g Casamino acids (Difco) in ~25 ml water. Add 800 µl 1M KOH and adjust volume to 33 ml with water. To prepare Hv-Ca agar, measure into a 500 ml bottle: 5 g Agar (Difco), 100 ml of water and 200 ml of 30% SW. Boil to dissolve and add 10 x Ca (33 ml). Autoclave. Cool to ~60^{o}C and add slowly 2 ml of 0.5 M $CaCl_2$ and 300 µl of a mixture of Thiamine (0.89 mg/ml) and Biotin (0.11 mg/ml). For *H. volcanii* H295 strain also add 340 µl of uracil (50 mg/ml in DMSO). ***(3)***

3. Methods

3.1 Cloning of transposon-tagged genomic DNA fragments to generate a plasmid library

3.1.1. *H. volcanii* chromosomal DNA isolation and fragmentation

Carry out all procedures at room temperature (RT) unless otherwise indicated.

1. Culture *H. volcanii* cells on 18% MGM agar dishes at 45ºC for 3-5 days. Inoculate 1-4 colonies into 5 ml of 18% MGM liquid medium and culture with shaking at 45ºC for ~32 h, until a late exponential culture phase is reached.

2. Collect cells from 3 ml of the cell suspension by centrifuging at 6000 rpm for 8 min. Add 200 µl of ST buffer and suspend the cell pellet. Transfer the suspension into a 1.5 ml microtube. To lyse the cells, add 200 µl of lysis solution and mix carefully by gently rotating the tube (to avoid extra DNA shearing, do not use vortex or pipette for mixing).

3. Add 400 µl of phenol. Incubate with a table-top rotator for 30 min. Transfer the tube into a heat block (or water bath) and incubate at 60ºC for 1h. To separate phases, use microcentrifuge at maximum g for 5 min, and transfer the viscous supernatant into another microtube. Repeat the phenol extraction procedure but without the 60ºC incubation step. Extract the supernatant twice with 600 µl of chloroform.

4. Add 1 ml of ethanol and mix gently for a few minutes to precipitate DNA. Transfer the thread-like DNA precipitate (e.g. with a pipette tip) into a tube containing 1 ml of 70% ethanol. Centrifuge at maximum g for 10 min, remove the supernatant, and air-dry the DNA pellet. Dissolve DNA in 300-500 µl of TE buffer. A complete dissolution may require up to 3 days. (*See* **Note 3**)

5. Remove RNA by incubating with RNase A (at 0.2 mg/ml) for 30 min at 37 ºC. Remove the enzyme by extracting with phenol and twice with chloroform. Ethanol precipitate DNA with 1/10 vol of 3M sodium acetate (pH 7.0). Dissolve DNA in TE buffer as above.

6. Digest genomic DNA partially with three different enzymes (AciI, HpaII and TaqI) in separate reactions (*See* **Note 4**). Aim at DNA fragment size distribution, in which a large proportion of fragments falls within the size range of 2-4 kb. Remove the enzyme by phenol and chloroform extractions as above. Ethanol precipitate and dissolve DNA in water. (*See* **Note 5**).

3.1.2 Preparation of transposon DNA (*See* **Note 6**)

1. TrpA-cat-Mu transposon DNA (*See* **Note 7**) is released from its carrier plasmid pMPH20 ***(4)*** by BglII digestion. The 2,212 bp linear transposon can be purified in large quantities by the use of anion exchange chromatography ***(6)***. Preparative agarose gel electrophoresis is a convenient means to purify several micrograms of transposon DNA for a few experiments. Store the purified transposon DNA in TE buffer.

3.1.3 Mu *in vitro* transposition reaction and size selection

1. Use DNA digested with each enzyme (AciI, HpaII and TaqI) as a target in separate transposition reactions. With each of them, assemble several (*See* **Note 8**) standard *in vitro* DNA transposition reactions on ice as specified in Table 1. Add MuA transposase as the last component just prior to incubation.

Reagent	**Standard reaction**
2 x MIX	25 µl
Digested genomic DNA as target (800 – 1000 ng)	typically 2-6 µl
TrpA-cat-Mu transposon as donor DNA (0.5 pmol/µl)	2 µl (1 pmol)
2.5 M NaCl	2 µl
1.25% Triton X-100 (freshly diluted)	2 µl
0.25M $MgCl_2$	2 µl
H_2O	up to 48 µl
MuA (220 ng/µl)	2 µl (440 ng, 5.4 pmol)
	∑ 50 µl

2. Incubate the reactions at 30ºC for 60 min and pool all reaction products in one tube.

3. Phenol and chloroform extract and ethanol-precipitate as above. Dissolve DNA in TE buffer.

4. Isolate from 4 to 6 kb fragments by the use of anion exchange chromatography as described ***(6)***. Alternatively, use preparative agarose gel electrophoresis for size selection.

3.1.4 Preparation of vector DNA for cloning

1. Isolate pBlueScript SK+ plasmid DNA (*See* **Note 9**) using any standard commercial plasmid isolation kit.

2. Digest 10 μg of pBlueScript SK+ plasmid DNA with ClaI restriction enzyme (4 U/μg) at 37ºC overnight.

3. Treat the vector DNA with calf intestinal phosphatase as recommended by the supplier.

4. Isolate the dephosphorylated linear vector DNA using preparative agarose gel electrophoresis.

3.1.5 Generation of plasmid library

1. Ligate the ClaI-digested vector with size-selected *in vitro* transposition reaction products using T4 DNA ligase and reaction conditions recommended by the supplier. Typically 50 ng of digested plasmid DNA is ligated with three molar excess of DNA inserts in a reaction volume of 15 μl.

2. Thaw competent *E. coli* DH10B cells on ice (*see* **Note 10**).

3. Add 1 μl of ligation mixture into 25 μl of electrocompetent cells in a cold tube. Mix gently. Transfer the mixture into an ice-cold electroporation cuvette (0.1 cm electrode spacing, Bio-Rad). (*see* **Note 11**).

4. Electroporate using the following pulse settings: voltage 1.8 kV, resistance 200 ohms, and capacitance 25 μF (*see* **Note 12**).

5. Add 1 ml SOC (room temperature solution), transfer into a microcentrifuge tube and incubate at 37 °C by shaking (220 rpm) for 30 min.

8. Spread the cells on culture plates containing ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) and incubate at 37 °C overnight.

9. Collect a suitable number of colonies (*see* **Note 13**) by adding per plate 1 ml LB medium supplemented with ampicillin (100 μg/ml) and chloramphenicol (10 μg/ml) (LB-amp-cam), and

scraping the colonies into a single pool. (*see* **Note 14**). Add a suitable volume of fresh LB-amp-cam medium and grow at 37 °C by shaking (220 rpm) for 2.5 hours. (*see* **Note 15**).

10. Isolate plasmid DNA using a plasmid isolation kit of suitable capacity. This plasmid pool represents the primary plasmid library with the diversity defined by the number of the collected colonies used for its preparation. (*see* **Note 16**).

3.1.6 Amplification and CsCl gradient purification of plasmid library (*see* **Note 17**).

1. Electrotransform aliquots from the primary plasmid library into *E. coli* DH5α, selecting for ampicillin and chloramphenicol as above. (*see* **Note 18**).

2. Collect a suitable number of colonies (*see* **Note 19**) and isolate plasmid DNA as above.

3. Purify supercoiled plasmid forms from the plasmid library by CsCl gradient ultracentrifugation. (*See* **Note 20**). The published procedure is recommended ***(7)***.

3.2. Preparation of *H. volcanii* insertion mutant library

3.2.1. Isolation and purification of transposon-tagged fragments of chromosomal DNA

1. Use two different pairs of restriction endonucleases to release transposon-tagged *H. volcanii* DNA fragments from the plasmid library (*see* **Note 21**).

2. Isolate suitable-sized (4-6 kb) fragments using preparative agarose gel electrophoresis. Prepare enough purified DNA (several micrograms) to be used in the subsequent transformation step.

3.2.2. Transformation of *H. volcanii* cells

Perform all steps at room temperature unless otherwise indicated.

1. Prepare *H. volcanii* competent cells (*See* **Notes 22 and 23**): Inoculate with ~4 *H. volcanii* colonies into 5 ml of 18% MGM and culture with shaking (200 rpm) at 45ºC for 24 h. Transfer 0.5

ml of the cell culture into 50 ml of fresh 18% MGM and culture as above for ~20 h until the absorbance at 600 nm is 0.8-1.0.

2. Divide the cell culture into two 25 ml aliquots in 50 ml centrifuge tubes and centrifuge in a table-top centrifuge (4,500 g) for 10 min. Remove the supernatant and resuspend each pellet in 10 ml of buffered spheroplasting solution. Centrifuge as above and resuspend each pellet in 2.5 ml of buffered spheroplasting solution with 15% glycerol. Pool the suspensions and divide into suitable (e.g. 600 μl) aliquots. Flash-freeze the cells in liquid nitrogen and store at -80ºC. *H. volcanii* competent cells can be stored for several months. However, a decrease in the transformation efficiency is expected during a prolonged storage. (*See* **Note 24**)

3. Use 200 μl of competent cells per transformation. Thaw cells at room temperature and transfer them into a 2 ml centrifuge tube. Add 20 μl of 0.5 M EDTA, pH 8.0. Mix gently by inverting the tube and incubate for exactly 10 min to form spheroplasts.

4. Mix 10 μl (1 μg) of DNA to be transformed (*See* **Note 25**), 15 μl unbuffered spheroplasting solution, and 5 μl 0.5 M EDTA, pH 8.0.

5. Following the spheroplasting incubation (see step 3), add the DNA mixture onto the tube wall and by gently rotating mix the solution with spheroplasts. Incubate for 5 min. Add 250 μl of freshly prepared 60% PEG 600 solution by slowly pipetting along the tube wall. Mix gently as above and incubate for 30 min.

6. Add 1.5 ml of spheroplast dilution solution and incubate for 2 min. Centrifuge in microsentrifuge at 6,000 rpm for 10 min. Remove the supernatant. To regenerate, add gently 1 ml of regeneration solution onto spheroplasts and incubate undisturbed at 45ºC for 1.5-2h. Resuspend and incubate with a slow rotation (~100 rpm) at 45ºC for 4 h.

7. Centrifuge at 6,000 rpm for 8 min. Remove the supernatant and resuspend into 1 ml of transformant dilution solution.

8. You can plate the transformed cells directly onto appropriate selection plates, or you can freeze the suspension for later use. (*See* **Note 26**)

9. To store the cell suspension, add 335 µl of 80% glycerol-6% SW, divide into suitable (e.g. 200 µl) aliquots, flash-freeze in liquid nitrogen, and transfer for storage at -80°C.

10. Spread the cells onto appropriate selective plates (*See* **Note 27**) as follows. Thaw the frozen cells at room temperature and spread (with a suitable dilution) onto a selection plate. Use e.g. 100 µl per standard petri dish and culture at 45°C for one week. Note that some insertion mutants have a reduced growth rate, resulting in variable colony sizes among the member clones of the library.

3.3. Library validation

3.3.1. It is advisable to validate the library with regard the insertion copy number (*See* **Note 28**).

3.3.2. Insertion site in the genome of a library clone can be determined by DNA sequencing (*See* **Note 29**).

4. Notes

1. It has been noticed that the quality of media reagents may differ substantially among manufacturers, and certain impurities inhibit the growth of haloarchaeal cells. More details about the purity requirements can be found in Halohandbook ***(3)***.

2. Also other rich media can be used. More alternatives can be found in Halohandbook ***(3)***.

3. Alternatively, a genomic DNA isolation kit may be used. Most commercial kits should be suitable for *H. volcanii* DNA isolation.

4. We recommend partial digestions for DNA fragmentation and the usage of several enzymes. This guarantees an extensive coverage of the genome with overlapping fragments and in practice eliminates any potential bias caused by restriction site distribution. The enzymes implicated in this protocol (AciI, HpaII, TaqI) each recognize a 4-bp sequence and generate a protruding 5-GC overhang that is compatible with ClaI site in cloning. The conditions for partial digestions need to be adjusted for each restriction enzyme separately. It is advisable to use otherwise constant reaction conditions but different amounts of enzyme. The size distribution of DNA fragments can be analyzed by the use of standard agarose gel electrophoresis.

5. It is advisable to use 5-10 μg of genomic DNA for digestions. It is important to dissolve the digested DNA fragments in water, as extra salt is inhibitory in the subsequent Mu *in vitro* DNA transposition reaction. The concentration of the fragmented DNA should preferably be ≥ 200 ng/ μl.

6. Mini-Mu transposons utilized in *in vitro* reactions are linear DNA molecules that contain in each of their ends, in an inverted relative orientation, a 50 bp segment from the right end of phage Mu genome. This so-called R-end segment contains a pair of MuA transposase binding sites. The DNA between the R-ends can be of any origin and modified with regard the needs of each particular application.

7. The TrpA-cat-Mu transposon ***(4)*** contains two selectable markers: *cat* for *E. coli* and *trpA* for *H. volcanii*. It is maintained within its carrier plasmid pMPH20 that can be obtained by request from H. Savilahti.

8. Material from from 3-6 reactions per enzyme digestion should yield enough DNA for the subsequent cloning step.

9. Other standard *E. coli* cloning vectors may be used. However, a suitable vector needs to harbor a unique ClaI site, which is flanked by at least a two pairs of unique restriction sites not present in the TrpA-cat-Mu transposon (the transposon sequence is available upon request).

10. Although several *E. coli* strains may be used for cloning, DH10B is recommended as it yields good quality plasmid DNA and can be electroporated efficiently. High efficiency electrocompetent cells can be prepared using the protocol described ***(5)***, or they may be obtained from commercial vendors.

11. Perform several parallel electroporations to yield a desired number of transformants for the generation of a plasmid library. The number defines the maximum diversity of potential transposon insertion sites within this library.

12. The protocol has been developed for Genepulser II electroporation apparatus (Bio-Rad). If other brand is used, optimal pulse parameters may differ.

13. The desired colony number (see note 11) varies according to the gene number of the target organism. A ten-fold excess over the gene number is recommended, as it guarantees a comprehensive library, in which each gene will be tagged with a very high likelihood. For the calculation of the probability see Kiljunen *et al*. 2014 ***(4)***.

14. Standard (diameter 9 cm) plates can accommodate up to 1,000 separate colonies, although this number may vary among different strains. If larger plates are used, the volume of the medium added should be increased proportionally. For example, 10 ml is suitable for large (25 x 25 cm) square plates.

15. The volume recommended depends on the number of collected colonies. For example, 200 ml of medium may be used for 40,000 colonies.

16. It is advisable to minimize the time used for liquid cultures, as the growth rate of the clones may differ. Accordingly, longer culture times may bias the original plasmid diversity.

17. Amplification of the plasmid library guarantees a sufficient amount of plasmid DNA for the purification by CsCl gradient ultracentrifugation.

18. Standard chemical transformation may also be used here given the efficiency is high enough to obtain the desired number of transformants. Although several *E. coli* strains may be used, DH5α is recommended. This strain yields very high quality plasmid DNA with the majority of the molecules being supercoiled.

19. To retain the original plasmid diversity with a very high probability, collect at least 20 times more colonies than what was collected for the primary plasmid library. Notice that you will need a substantial amount of DNA for the next step. We recommend isolating 1-2 mg plasmid DNA at this stage.

20. Alkaline plasmid preparation methods produce a fraction of collapsed supercoiled plasmid forms, which enter the cells effectively and cannot be digested with restriction endonucleases. It is important to remove them, as they would generate false positive colonies upon transformation into *H. volcanii*. See ***(4)*** for more details. CsCl gradient ultracentrifugation is a recommended means to remove collapsed supercoiled plasmid forms.

21. In the study of Kiljunen *et al*. (2014) ***(4)***, pBlueScript SK+ plasmid was used as the cloning vector. The restriction enzyme pairs used for the successful fragment release in that study were XhoI/HindIII and KpnI/EcoRV. It is advisable to digest a substantial amount of DNA at this stage (e.g. 100-200 μg). Use conditions recommended by the enzyme supplier.

22. In principle, any *H. volcanii* strain could be used. We used the strain H295, which is devoid of Mre11 and Rad50. Due to these deficiencies, its homologous recombination activity is increased 100-fold ***(8)***.

23. More information about *Haloferax* transformation procedures can be found in Halohandbook ***(3)***.

24. Transformation efficiency of competent cells can be tested with any *H. volcanii* plasmid and appropriate selection plate.

25. DNA may be in any commonly used buffer (such as TE) or in water.

26. The expected transformation efficiency is 10^4-10^5 c.f.u. per microgram of transformed DNA.

27. With the tryptophan auxotrophy marker gene (*TrpA*), the selection plate used is Hv-Ca. See Halohandbook for more information about selection plates and required additives ***(3)***.

28. A genome-wide insertion mutant library used as a gene discovery tool should contain mutants with single-copy insertions. Southern hybridization or quantitative PCR can be used to evaluate this. In the protocol described, the stoichiometry in the transformation step favors single-copy genomic integrations, i.e. one out of 2,000 target cells become transformed ***(4)***. Thus, the vast majority of the clones in the library is expected to contain a single genomic insertion.

29. The transposon insertion site in the genome of a mutant can be determined by initially cloning the transposon with its genomic flanks from the chromosomal DNA using a restriction enzyme that does not cut within the transposon DNA. The sequences bordering the transposon DNA are then determined using standard Sanger sequencing ***(5)***. However, with the fast development of Next Generation Sequencing (NGS) techniques, whole-genome sequencing by NGS may soon be the fastest and most cost-effective way to determine the insertion site in a microbial genome.

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