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## Transformation Techniques for the Anaerobic Hyperthermophile *Thermococcus kodakarensis*

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### Abstract

Genetic manipulation is an essential tool to investigate complex microbiological phenomena. In this chapter we describe the techniques required to transform the model hyperthermophilic, anaerobic archaeon *Thermococcus kodakarensis*. *T. kodakarensis* can support two modes of genetic manipulation, dependent either on homologous recombination into the genome or through retention of autonomously replicating plasmids. The robust genetic system developed in *T. kodakarensis* offers a variety of selectable and counterselectable markers for complex, accurate and iterative genetic manipulations offering greater flexibility to probe gene function *in vivo*.

### Keywords

Transformation; Archaea; Hyperthermophile; Genetics; Anaerobic; *Thermococcus*

### 1 Introduction

Many Archaea can survive and often thrive in the environmental extremes, ranging from saturating salinities to high temperatures within hot springs and near marine vents [1–7]. Archaeal systems are increasingly employed for production of specialized and commodity bio-products, bioremediation efforts, and archaeal enzymes that function at the extremes of pH, temperature, and salinity are often used in molecular biology and biotechnological applications [5, 8–15]. Efforts to understand and take advantage of the unique characteristics of archaeal organisms demand a reliable means of genetic manipulation for rational and iterative strain construction.

*Thermococcus kodakarensis* is a naturally competent archaeal organism that thrives in an anaerobic, hyperthermophilic (85 °C optimal growth temperature) environment [16–19]. The genetic system for *T. kodakarensis* has been continuously refined through the combined efforts of multiple laboratories and these foundational and improved techniques are now regularly employed for many euryarchaeal species [20–26]. Initial efforts focused on homologous-recombination dependent manipulation of genomic sequences, with systems

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maturing to allow repetitive and markerless alterations. The absence of naturally occurring autonomously replicating plasmids leads to the development of novel shuttle vectors that provide a complementary method to alter the genotypes and phenotype of *T. kodakarensis*.

Although multiple strains of *T. kodakarensis* have been used as parental strains, the genetic system described here is optimized for, and reliant on strain TS559 ( $\Delta$ TK2276;  $\Delta$ TK0254::TK2276;  $\Delta$ TK0149;  $\Delta$ TK0664) [23], which is an agmatine and tryptophan auxotroph strain of *T. kodakarensis*. We describe two techniques to manipulate TS559. The first is based on integration and subsequent excision of nonreplicative plasmids into the genome of TS559 to restore the agmatine biosynthesis pathway via reintroduction of TK0149, encoding a pyruvoyl-dependent arginine decarboxylase, and counterselective pressures afforded by TK0664, a hypoxanthine guanine phosphoribosyltransferase [23]. The second transformation procedure uses a shuttle vector system that restores the tryptophan biosynthesis pathway via reintroduction of TK0254, which encodes for the large subunit of anthranilate synthase, and expresses PF1848 (a hydroxymethylglutaryl coenzyme A reductase gene from *Pyrococcus furiosus*) providing resistance to statin-based antibiotics.

## 2 Materials

## 2.1 Microbial Culture

All solutions are sterilized by autoclaving unless noted otherwise. Solutions are made with water purified to 18 MΩ resistance. All solutions are adjusted to their final volume after the addition of dry ingredients.

### 2.1.1 *Escherichia coli* Media

1. Luria–Bertani (Ec-LB-Amp) liquid media (1 L): 10 g tryptone, 10 g NaCl, 5 g yeast extract, 100 µg/mL ampicillin.
2. LB solid media (Ec-LB-Amp plates): 10 g tryptone, 10 g NaCl, 5 g yeast extract, 20 g agar, 100 µg/mL ampicillin.
3. 100 mg/mL ampicillin.

## 2.1.2 *Thermococcus kodakarensis* Media

1. KOD Vitamins (*see Notes 1 and 6*) (1000×) (1 L): 0.2 g niacin, 0.08 g biotin, 0.2 g pantothenate, 0.2 g lipoic acid, 0.08 g folic acid, 0.2 g thiamine, 0.2 g riboflavin, 0.2 g pyridoxine, 0.2 g cobalamin.
2. 1 M Agmatine sulfate (1000×).
3. Wolfe's Trace Minerals (200×) (1 L): 0.5 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 1 g  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.1 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 g  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 0.01 g  $\text{AlK}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$ , 0.01 g  $\text{H}_3\text{BO}_3$ , 0.01 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ .
4. Artificial Sea Water (ASW)-YT (1 L): 5 g tryptone (*see Note 2*), 5 g yeast extract (*see Note 3*), 1× Wolfe's trace mineral solution, 1× KOD vitamins, 20 g NaCl, 3 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 6 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1 g  $(\text{NH}_4)_2\text{SO}_4$ , 0.2 g  $\text{NaHCO}_3$ , 0.3 g

CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.5 g KCl, 0.420 g KH<sub>2</sub>PO<sub>4</sub>, 0.050 g NaBr, 0.020 g SrCl<sub>2</sub>·6H<sub>2</sub>O, 0.010 g Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O.

- 5. Elemental Sulfur (flowers of sulfur; powdered).
- 6. 2× ASW (see Note 4) (1 L): 40 g NaCl, 6 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 12 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 2 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.4 g NaHCO<sub>3</sub>, 0.6 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 1 g KCl, 0.840 g KH<sub>2</sub>PO<sub>4</sub>, 0.100 g NaBr, 0.040 g SrCl<sub>2</sub>·6H<sub>2</sub>O, 0.020 mg Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O.
- 7. Gelzan<sup>TM</sup> CM.
- 8. Polysulfides solution (500×) (see Note 5) (15 mL): 10 g Na<sub>2</sub>S·9H<sub>2</sub>O, 3 g sulfur.
- 9. 0.8× ASW (1 L): 16 g NaCl, 2.4 g MgCl<sub>2</sub>·6H<sub>2</sub>O, 4.8 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.800 × g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.160 g NaHCO<sub>3</sub>, 0.240 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.400 g KCl, 0.336 g KH<sub>2</sub>PO<sub>4</sub>, 0.040 g NaBr, 0.016 g SrCl<sub>2</sub>·6H<sub>2</sub>O, 0.008 mg Fe (NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>·6H<sub>2</sub>O.
- 10. 20 amino acid solution (20×) (see Note 6) (200 mL): 1 g cysteine, 1 g glutamic acid, 1 g glycine, 0.500 g arginine, 0.500 g proline, 0.400 g asparagine, 0.400 g histidine, 0.400 g isoleucine, 0.400 g leucine, 0.400 g lysine, 0.400 g threonine, 0.400 g tyrosine, 0.300 g alanine, 0.300 g methionine, 0.300 g phenylalanine, 0.300 g serine, 0.300 g tryptophan, 0.200 g aspartic acid, 0.200 g glutamine, 0.200 g valine.
- 11. 19 amino acid solution lacking tryptophan (20×) (see Note 6) (200 mL): 1 g cysteine, 1 g glutamic acid, 0.500 g arginine, 0.500 g proline, 0.400 g asparagine, 0.400 g histidine, 0.400 g isoleucine, 0.400 g leucine, 0.400 g lysine, 0.400 g threonine, 0.400 g tyrosine, 0.300 g alanine, 0.300 g methionine, 0.300 g phenylalanine, 0.300 g serine, 0.200 g aspartic acid, 0.200 g glutamine, 0.200 g valine.
- 12. 100 μM 6-methylpurine
- 13. 25 mM mevinolin
- 14. *T. kodakarensis* solid complete media (Tk-ASW-YT): 0.5 g yeast extract, 0.5 g tryptone, 1.0 g Gelzan<sup>TM</sup>, 500 μL Wolfe's Trace minerals (200×), 200 μL polysulfide solution (500×), 5.0 mL 20 amino acid mixture (20×), 100 μL KOD vitamins (1000×), 50 mL H<sub>2</sub>O, 50 mL 2× ASW.
- 15. *T. kodakarensis* solid minimal media (Tk-ASW-min): 500 μL Wolfe's trace minerals (200×), 1.0 g Gelzan<sup>TM</sup>, 50 mL H<sub>2</sub>O, 50 mL 2× ASW, 200 μL polysulfide solution (500×), 100 μL KOD vitamins (1000×), 5.0 mL 20 amino acid mixture (20×).
- 16. *T. kodakarensis* solid minimal 19 amino acid media (TK-ASW--min-(–Trp)): 500 μL Wolfe's trace minerals (200×), 1.0 g Gelzan<sup>TM</sup>, 50 mL H<sub>2</sub>O, 50 mL 2× ASW, 200 μL polysulfide solution (500×), 100 μL KOD vitamins (1000×), 5.0 mL 19 amino acid mixture (20×).
- 17. *T. kodakarensis* solid minimal mevinolin media (TK-ASW-min-mev): 500 μL Wolfe's trace minerals (200×), 1.0 Gelzan<sup>TM</sup>, 50 mL H<sub>2</sub>O, 50 mL 2× ASW,

200  $\mu$ L polysulfide solution (500 $\times$ ), 100  $\mu$ L KOD vitamins (1000 $\times$ ), 12.5  $\mu$ M mevinolin, 5.0 mL 20 amino acid mixture (20 $\times$ ).

### 2.1.3 Equipment

1. 1 mL syringe.
2. Microcentrifuge.
3. 125 mL serum bottles.
4. Large aluminum pot with loose-fitting lid.
5. Autoclave.
6. Anaerobic chamber (Coy Labs).
7. 10% hydrogen, 90% nitrogen (v/v) compressed gas mix.
8. 100% nitrogen compressed gas.
9. Glass Petri plates (*see* Note 7).
10. Plastic Petri plates (*see* Note 8).
11. Floor model centrifuge and compatible rotor(s).
12. Thermal cycler.
13. GasPak EZ Anaerobe Container System.
14. Pipettes.
15. Enzyme Cooler, Isotherm System.
16. Forced air incubator (37 °C and 85 °C).
17. Dry block heater.
18. Anaerobic canister.
19. 1.7 mL microcentrifuge tubes.
20. 0.2 mL PCR tubes.
21. Two-leg lyophilization serum bottle stoppers.
22. 20 mm aluminum seals.
23. 20 mm Crimper, Standard Seal.
24. 20 mm Decapper.
25. Polycarbonate centrifuge tubes.
26. Cell spreader.
27. 10 mL serum bottles
28. Face shields, lab coats, nitrile gloves, and autoclave gloves.
29. Paper towels.

30. *T. kodakarensis* strain TS559 [23] (see Note 9).
31. Nonreplicative plasmid pTS700 (see Note 9).
32. Autonomously replicating plasmid pLC70 (see Note 9).
33. ZR Plasmid Miniprep™ Classic Kit.
34. Qubit™ ds DNA BR Assay Kit.
35. NucleoSpin Gel and PCR Clean-up, Mini kit.
36. In-Fusion® Snap Assembly.
37. Agilent QuikChange-II Kit.

### 3 Methods

#### 3.1 Microbial Methods

*T. kodakarensis* transformation techniques are reliant on plasmid DNAs isolated from *Escherichia coli*. Standard growth and DNA preparations from *E. coli* yield plasmid DNA of sufficient quality for successful transformations of *T. kodakarensis*. Details are provided to generate (1) nonreplicative plasmids, based on pTS700, for genomic modifications and (2) autonomously replicating plasmids, based on pLC70, for ectopic expression, in Subheadings 3.2.1 and 3.2.3, respectively. The choice of *E. coli* strain has minimal impacts on successful plasmid isolations.

##### 3.1.1 *E. coli* Media and Cultivation—*E. coli* LB medium containing ampicillin: Ec-LB-Amp

1. Prepare Ec-LB-Amp and dispense 5 mL aliquots to sterile culture tubes.
2. Inoculate cultures with plasmid containing *E. coli* strains, incubate at 37 °C for 12–16 h with agitation (~200 rpm).

*E. coli* LB plate medium with ampicillin: Ec-LB-Amp plate

1. Prepare Ec-LB-Amp plate media, autoclave, cool to ~50–55 °C (see Note 10), and aliquot ~25 mL per plastic Petri plate (see Note 11) and allow to solidify at room temperature. Plates should be inverted and left to partially dry overnight before storing in a sealed bag at 4 °C.

##### 3.1.2 *Thermococcus kodakarensis* Media and Cultivation—*T. kodakarensis* strains are typically grown in 100 mL aliquots in nutrient-rich media (ASW-YT) (see Note 12) to provide large numbers of cells for transformations. Strain TS559 must be supplemented with agmatine even when grown in nutrient-rich media. Smaller (~3–5 mL) cultures of *T. kodakarensis*, prepared with the same media formulations, suffice to yield sufficient DNA from transformants for use in diagnostic PCR to confirm the genotypes of transformed cells.

1. Prepare *T. kodakarensis* complete liquid medium by combining ASW-YT and Wolfe's Trace Minerals inside an anaerobic chamber (see Note 13). Aliquot 100

mL of mineral-supplemented ASW-YT media into 125 mL serum bottles, then seal with stoppers and aluminum seals, autoclave (see Note 14), and store sterile media at room temperature.

2. Immediately prior to inoculation with *T. kodakarensis* cultures, and inside an anaerobic chamber, add 0.2 g of elemental sulfur and 100  $\mu$ L KOD vitamins (1000 $\times$ ). When appropriate, add 100  $\mu$ L 1 M agmatine sulfate solution (1000 $\times$ ).
3. Using a syringe, withdraw 1 mL of an active culture of TS559 (or other *T. kodakarensis* strain), inject the inoculum into the now completely supplemented media, seal the culture with a stopper and aluminum seal (see Note 15), and incubate at 85 °C without agitation for ~13 h (cultures entering stationary phase yield the highest percentage of transformants).

*Thermococcus kodakarensis* complete solid medium: Tk-ASW-YT plate.

Clonal populations of transformants from the transformations must be selected on solid media. Special protocols are necessary to generate solid media that remains solid at 85 °C; solid media is prepared from two independently autoclaved solutions that are mixed and rapidly distributed (~20–30 s) into glass Petri plates before setting at room temperature. Depending on the transformation procedure and selective pressures applied, transformed cells are typically plated on nutrient-rich solid media lacking agmatine supplementation (for genomic modifications) or minimal, 19 amino acid-based solid media containing agmatine supplementation (for ectopic modifications).

Nutrient-rich *T. kodakarensis* solid media: Tk-ASW-YT plate

1. Inside an anaerobic chamber, combine 50 mL 2 $\times$  ASW, 0.5 mL Wolfe's Trace Minerals (200 $\times$ ), 0.5 g tryptone, and 0.5 g yeast extract in a 125 mL serum bottle. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.
2. Prepare a second 125 mL serum bottle by mixing 50 mL water and 1 g Gelzan™ CM. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.
3. Immediately after autoclaving, bring both halves of the media formulations inside the anaerobic chamber. Remove stoppers and aluminum seals from both bottles. To the bottle containing 2 $\times$  ASW add 100  $\mu$ L KOD vitamins (1000 $\times$ ) and 200  $\mu$ L polysulfides (500 $\times$ ) (see Note 16), then immediately combine with the bottle containing the dissolved Gelzan (see Note 17). Swirl to mix then aliquot the contents into 4 glass Petri plates (~25 mL/plate). Plates will solidify within seconds and can be used immediately or stored inverted, within the anaerobic chamber at room temperature for 1–2 days (see Note 18).

*Thermococcus kodakarensis* minimal 20 amino acid solid medium: Tk-ASW-min plate

1. Inside an anaerobic chamber, combine 50 mL 2 $\times$  ASW and 0.5 mL Wolfe's Trace Minerals (200 $\times$ ) in 125 mL serum bottle. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.

2. Prepare a second 125 mL serum bottle by mixing 50 mL water and 1 g Gelzan™ CM. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.
3. Immediately after autoclaving, bring both halves of the media formulations inside the anaerobic chamber. Remove stoppers and aluminum seals from both bottles. To the bottle containing 2× ASW add 100 µL KOD vitamins (1000×), 100 µL 1 M agmatine sulfate solution (1000×), 5 mL 20 amino acid solution (20×), 100 µL 100 µM 6-Methylpurine or 50 µL 25 mM mevinolin and 200 µL polysulfides (500×), then immediately combine with the bottle containing the dissolved Gelzan. Swirl to mix, then aliquot the contents into 4 glass Petri plates (~25 mL/plate). Plates will solidify within seconds and can be used immediately or stored inverted, within the anaerobic chamber at room temperature for 1–2 days (see Note 18).

*Thermococcus kodakarensis* minimal 19 amino acid solid medium lacking tryptophan: Tk-ASW-min-(–Trp) plate

1. Inside an anaerobic chamber, combine 50 mL 2× ASW and 0.5 mL Wolfe's Trace Minerals (200×) in a 125 mL serum bottle. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.
2. Prepare a second 125 mL serum bottle by mixing 50 mL water and 1 g Gelzan™ CM. Seal the bottle with a stopper and aluminum seal, then sterilize via autoclaving.
3. Immediately after autoclaving, bring both halves of the media formulations inside the anaerobic chamber. Remove stoppers and aluminum seals from both bottles. To the bottle containing 2× ASW add 100 µL KOD vitamins (1000×), 100 µL 1 Magmatine sulfate solution (1000×), 5 mL 19 amino acid solution (20×), 100 µL 100 µM 6-Methylpurine and 200 µL polysulfides (500×), then immediately combine with the bottle containing the dissolved Gelzan. Swirl to mix then aliquot the contents into 4 glass Petri plates (~25 mL/plate). Plates will solidify within seconds and can be used immediately or stored inverted, within the anaerobic chamber at room temperature for 1–2 days (see Note 18).

### 3.2 Design and Construction of Plasmid DNAs Used to Transform *Thermococcus kodakarensis*

We first detail the steps to generate pTS700-based plasmids that permit desired modifications to the genome of TS559 (see Figs. 1, 2, and 4). pTS700 contains a unique *Swa*I cutsite that permits linearization and insertion of amplicons (see Fig. 1a). Given that many genomic targets are ultimately targeted both for deletion and modification, it is practical to generate a common plasmid (termed an "A"-plasmid; see Fig. 1) from which two unique plasmids can be generated, one for deletion of the target locus (termed a "B"-plasmid; see Fig. 2b) and another for modification of the target locus (termed either a "C"- or "D"-plasmid; see Fig. 2c, d). Standard plasmid preparations of "B"-, "C"-, and "D"-plasmids from most *E. coli* strains yield high-quality constructs that can be transformed

into TS559 (see Fig. 4) to first generate transformants with intermediate genomes that can be resolved to generate the desired final genomic modifications (see Fig. 4b–d).

Following the use of pTS700-based vectors for genomic modifications, we then detail the use of autonomously replicating plasmids, based on the pLC70 vector, to generate transformants wherein selectable phenotypes are based on retention of and expression from ectopic vectors (see Fig. 3).

### 3.2.1 Generating Linearized pTS700 to Accept Amplicons for Genomic Modifications. Construction of “A”-Plasmids

1. Linearize ~2 µg of pTS700 plasmid using SwaI endonuclease, resolve the reactions via agarose gel electrophoresis, and purify the linear products using the NucleoSpin Gel extraction protocols (see Fig. 1a). Recovered products are quantified using the Qubit™ dsDNA BR Assay Kit. SwaI-linearized pTS700 can be prepared in bulk, purified and stored at –20 °C for use in construction of “A”-plasmids for multiple genomic targets (see Note 19).
2. Generate a PCR-amplicon, using *T. kodakarensis* genomic DNA as a template, containing the target gene with ~700 bp of upstream and downstream genomic sequences (see Fig. 1a). The primer complementary to sequences upstream of the target locus should begin with the sequence, 5' GGAGGTGAATT (see Note 20) followed by ~25 nucleotides of complementarity to the genome. The primer complementary to sequences downstream of the target locus should begin with the sequence, 5' GGTGAAGGATT (see Note 20) followed by ~25 nucleotides of complementarity to the genome. Resolve the reactions via agarose gel electrophoresis, and purify the linear products using the NucleoSpin Gel extraction protocols. Recovered products are quantified using the Qubit™ dsDNA BR Assay Kit.
3. Use In-Fusion® Snap Assembly to insert the target gene fragment into pTS700-SwaI in a 2:1 ratio (gene fragment: linearized vector) (see Fig. 1b). Transform 2.5 µL of the In-Fusion® Snap Assembly reaction into Stellar (see Note 21) competent cells and plate the cells on Ec-LB-Amp plate. Incubate at 37 °C overnight (12–14 h). Successful insertion of the target gene into pTS700 is typically confirmed by colony PCR using primers specific to pTS700 that flank the SwaI cutsite (see Note 22). Once colonies containing the presumptive “A”-plasmid are identified, they are grown in 5 mL Ec-LB-Amp overnight at 37 °C with shaking. 5 mL Ec-LB-Amp grown cultures are sufficient to yield sufficient plasmid DNAs first for sequencing and ultimately for transformation into *T. kodakarensis*.
4. Purify the target gene A-plasmid from the *E. coli* cells using ZR Plasmid Miniprep™—Classic and quantify the concentration of the plasmid using Qubit™ dsDNA BR Assay Kit. Store at –20 °C in a freezer. The full sequence of the amplicon should be confirmed via Sanger sequencing (or alternative sequencing techniques) prior to transformation into *T. kodakarensis*.

### 3.2.2 Generating “B”-and “C”-Plasmids from “A”-Plasmids—Mutagenic PCR for deletion of the target gene: generation of the B-plasmid (see Fig. 2a, b)

1. Design a primer pair that permit the deletion of the target gene sequence for use in QuikChange-II reactions. Primers typically are ~60 nt in length, with 30 nt of complementarity to sequences immediately upstream and downstream of the target locus. Use the primer pairs in a QuikChange-II reaction using the A-plasmid as the template DNA following manufacturer’s instructions, inclusive of the transformation into *E. coli* and plating of Ec-LB-Amp plates. Allow colonies to form during overnight incubation at 37 °C. As an alternative, the target gene sequence can be deleted via inverse PCR and ligation of the linear product.
2. The success of removing the target gene sequence can be evaluated via colony PCR as in Subheading 3.2.1, **step 3**. Once *E. coli* clones harboring presumptive B-plasmids are identified, growth of small cultures, plasmid recovery and sequencing should be completed as in Subheading 3.2.1, **step 4**. Plasmids can be stored at –20 °C for extended periods prior to transformation into *T. kodakarensis*.

Mutagenic PCR for tagging of the target gene: generation of C-plasmid and D-plasmids (see Fig. 2c–f).

C-plasmids are generated by addition of sequences that encode HA- and 6xHis-tags immediately prior to the stop codon of the target gene (see Fig. 2c, e). D-plasmids are generated by addition of sequences that encode HA- and 6xHis-tags immediately downstream of the start codon of the target gene (see Fig. 2d, f).

1. Design a primer pair for use with Quikchange-II reactions that permit the addition of the tag-encoding sequences to either the start or end of the target genes. Primers are typically ~95 nt in length, with 25 nt of complementarity immediately upstream and downstream of the insertion site (50 nt total) and an additional 45 nt encoding the 9 amino acid HA-tag and 6xHis- tags. Use the primer pairs in Quikchange-II reactions using the A-plasmid as the template DNA following manufacturer’s instructions, inclusive of transformation into *E. coli* and plating of Ec-LB-Amp plates. Allow colonies to form during overnight incubation at 37 °C.
2. The success of adding sequences to extend the target gene at the 5' or 3' end can be evaluated via colony PCR as in Subheading 3.2.1, **step 3** using primer pairs that flank the site of the sequence insertion. Amplicons from desirable C- or D-plasmids harbored in *E. coli* transformants are ~45 bp longer than amplicons generated from A-plasmid that lack the tag-encoding sequences. Once *E. coli* clones harboring presumptive C- and D-plasmids are identified, growth of small cultures, plasmid recovery and sequencing should be completed as in Subheading 3.2.1, **step 4**. Plasmids can be stored at –20 °C for extended periods prior to transformation into *T. kodakarensis*. As an alternative method to identify successful addition of tag-encoding sequences, amplicons generated

with primers that flank the site of insertion will generate a product that has a new BspEI endonuclease recognition site that will permit identification of C- and D-plasmids from A-plasmids following digestion of the amplicons with BspEI.

**3.2.3 Generating pLC70-Based Expression Plasmids**—pLC70 contains selectable markers to phenotypically select desired transformants (see Fig. 3a). Typically, pLC70 vectors are modified to express genes of interest by insertion of an expression cassette—a *T. kodakarensis* promoter and associated open reading frame—into pLC70 prior to transformation into *T. kodakarensis* strains (see Fig. 3b–d). The expression cassette (see Fig. 3c) can be constructed through various procedures but should ultimately be cloned into pLC70 taking advantage of unique SalI and NotI sites. Cloning procedures similar those detailed for amplicon insertion to pTS700 yield consistent results.

**3.2.4 *Thermococcus kodakarensis* Strain Construction**—Initial transformation to confirmation of final strains typically takes 2–3 weeks.

1. Grow strain TS559 in ASW-YT-Agm as directed in Subheading 3.1.2. Incubate at 85 °C for 12–13 h.
2. Harvest TS559 biomass by anaerobically transferring the culture into high-speed centrifuge tubes followed by centrifugation at ~18,500  $\times g$  for 10 min at 4 °C (see Note 23) Inside the anaerobic chamber, carefully decant the supernatant and keep the undisturbed pellet.
3. Directly in the anaerobic chamber, gently resuspend the TS559 cell pellet in 3 mL of ice-cold 0.8× ASW. ~200  $\mu$ L of resuspended cells are typically used per transformation, and by preparing several aliquots, multiple transformations can be performed simultaneously. Aliquot 200  $\mu$ L of the resuspended cells into 1.7 mL microcentrifuge tubes and incubate anaerobically on ice for 30 min (see Note 24).
4. Add ~2–4  $\mu$ g of the transformation plasmid (either a pTS700- or pLC70-based plasmid) to the 200  $\mu$ L of incubated cells and extend incubation anaerobically on ice for an additional 60 min.
5. Heat shock cells at 85 °C for 45 s in a dry heat block. Immediately chill on ice for 10 min following heat shock (see Note 25).
6. Carefully spread the now transformed cells to Tk-ASW-YT plates (for pTS700 based plasmids) or Tk-ASW-min-(–Trp) plates (for pLC70 based plasmids). Gently spread cells with a sterile cell spreader inside the anaerobic chambers. Once the transformation media has been absorbed into the plates, invert the plates and transfer to an air-tight anaerobic metal canister, packed with paper towels (see Note 26) and a Gas Pak EZ anaerobic container system packet. Seal the canister inside of the anaerobic chamber, then remove and incubate the sealed vessel in an 85 °C incubator for 48–96 h.
7. Return the sealed anaerobic vessel to the anaerobic chamber and remove the incubated plates, being careful to remove condensation. Identify colonies formed

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from successful transformations; *T. kodakarensis* colonies are nearly transparent and may be difficult to identify at first (see Note 27). Transformants from the transformation with pLC70-based plasmids are likely to contain the desired final genotype and phenotype but are typically replated on Tk-ASW-min-mev plates to ensure the transformants are resistant to mevinolin before being transferred to liquid media for experimental use (see Fig. 3). In contrast, colonies resultant from transformation with pTS700-based plasmids only contain an intermediate genome (see Fig. 4c) and require additional manipulations to generate the desired modified final genome.

8. Initial transformants from pTS700-based transformations that contain the intermediate genome wherein the entire pTS700-plasmid has integrated via homologous recombination into the TS559 genome must be picked from the plates and used to inoculate 3 mL Tk-ASW-YT liquid cultures containing agmatine in 5 mL serum bottles. Seal the serum bottles anaerobically using stoppers and aluminum seals, followed with an overnight (12–13 h) incubation at 85 °C. Growth in the presence of agmatine permits the spontaneous excision of the integrated vector (see Fig. 4d). Regardless of the nature of the excision event, the resulting genomes will lack both TK0149 and TK0664, rendering cells agmatine auxotroph that are resistant to 6-methylpurine. If the excision event captures the target gene, the resulting cells will also lack the target gene (for B-plasmid based transformations) or encode a modified target gene sequences (for C- and D-plasmid based transformations) (see Fig. 4d).
9. 1 mL of overnight ASW-YT + Agm grown intermediate cultures should be anaerobically harvested, concentrated via centrifugation five-fold, then plated on Tk-ASW-min plates containing 6-methylpurine. Gently spread cells with a sterile cell spreader inside the anaerobic chambers. Invert plates with transformants, transfer to an air-tight anaerobic metal canister, packed with paper towels (see Note 26) and a Gas Pak EZ anaerobic container system packet. Seal the canister inside the anaerobic chamber, then remove and incubate the sealed vessel in an 85 °C incubator for 48–96 h.
10. Return the sealed anaerobic vessel to the anaerobic chamber and remove the incubated plates, being careful to remove condensation. Identify colonies formed from successful excision events from the genome. Colonies must be picked from the plates and used to inoculate 3 mL Tk-ASW-YT liquid cultures containing agmatine in 5 mL serum bottles. Seal the serum bottles anaerobically using stoppers and aluminum seals, followed with an overnight (12–13 h) incubation at 85 °C.
11. 1 mL of overnight cultures is anaerobically removed to prepare genomic DNA for diagnostic PCRs and sequencing to identify strains wherein the excision event resulted in generation of the desired genotype. Sequencing of PCR amplicons provides nucleotide level confidence of the desired modifications. We also recommend whole genome sequencing of all strains to ensure that

spontaneous modifications were not accidentally introduced elsewhere in the genome.

## 4 Notes

1. This solution is light-sensitive and should be protected by either wrapping a transparent tube in aluminum foil, or by storing in an opaque, amber conical tube.
2. *T. kodakarensis* requires casein peptone that is enzymatically digested using pancreatic enzymes. Other sources of tryptone are suitable for *E. coli* media.
3. AMRESCO, catalog number: J850; For *E. coli* media, any yeast extract is suitable, however *T. kodakarensis* requires this source of yeast extract.
4. 2× ASW can be stored outside of the anaerobic chamber.
5. Dissolve solution using heat, it will be a deep red color when both sulfur and sodium sulfide are completely dissolved.
6. Autoclaving will degrade this solution.
7. Plastic Petri plates may melt at *T. kodakarensis* growth temperatures (85 °C), requiring the use of glass Petri plates.
8. Plastic Petri plates will not melt at *E. coli* growth temperature (37 °C).
9. Please contact corresponding author to obtain plasmid. Details of the plasmid sequences can be found at references [16, 18, 26].
10. Ampicillin is unstable at temperatures above 50 °C, do not add before autoclaving.
11. There must be enough media to cover the bottom of the plate.
12. Water used in the preparation of ASW-YT should be boiled before it is combined with dry ingredients. Boiling releases dissolved oxygen.
13. ASW-YT must be prepared anaerobically.
14. Autoclave liquid cycle, 30 min sterilization time.
15. *T. kodakarensis* released H<sub>2</sub>S and H<sub>2</sub> gasses as part of its metabolism. Using a stopper alone to seal the bottle is inadequate, as the gasses produced will push the stopper out of the bottle.
16. Polysulfides replace elemental sulfur in solid *T. kodakarensis* media.
17. Most selective agents for solid media should be added to the ASW solution after autoclaving. Supplemented ASW-based solutions should then be poured into the autoclaved Gelzan solution before pouring plates. This order of combination ensures homogeneous solid media.
18. For pouring multiple sets of plates at once, keep autoclaved solutions on a hot plate to prevent premature solidification.

19. *SwaI* endonuclease creates a blunt cutsite, reducing the likelihood of the linearized vector reannealing to itself, allowing for stable long-term storage.
20. This sequence is homologous to regions near the *SwaI* cutsite.
21. This strain is the recommended cell line by Takara; Stellar cells do not contain a plasmid prior to transformation, increasing confidence in isolated products after transformation.
22. 700 Forward and 700 Reverse are the primers typically used in this reaction. Further details can be found at [16, 18, 26].
23. Centrifuge bottles cannot be opened outside of the chamber. Ensure centrifuge bottles have a rubber seal in their cap to maintain anaerobicity. When transferring centrifuge bottles between the centrifuge and the anaerobic chamber, maintain the bottles in an inverted configuration to prevent the supernatant from washing over the pellet and reducing overall yield.
24. Excess cells cannot be stored for future transformations.
25. This step increases transformation efficiency.
26. Packing the anaerobic canister with paper towels reduces shifting of the canister's contents when being moved, reducing the risk of breaking a glass Petri plate and compromising cell cultures. Additionally, condensation will build up in the canister during incubation, and paper towels serve to absorb some moisture in the canister.
27. *T. kodakarensis* colonies are transparent puncta on the surface of media; most colonies are no larger than 1 mm in diameter and appear similar to bubbles on the surface. Occasionally, salt precipitates from media will look like *T. kodakarensis* colonies on solid media.

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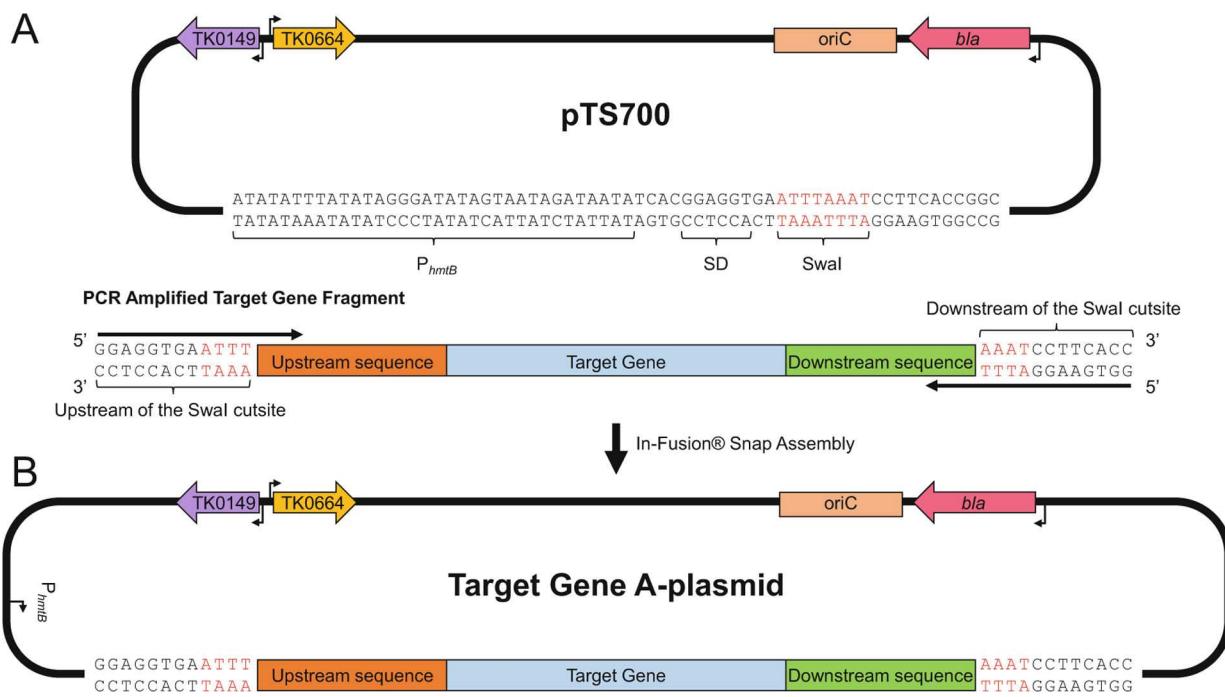
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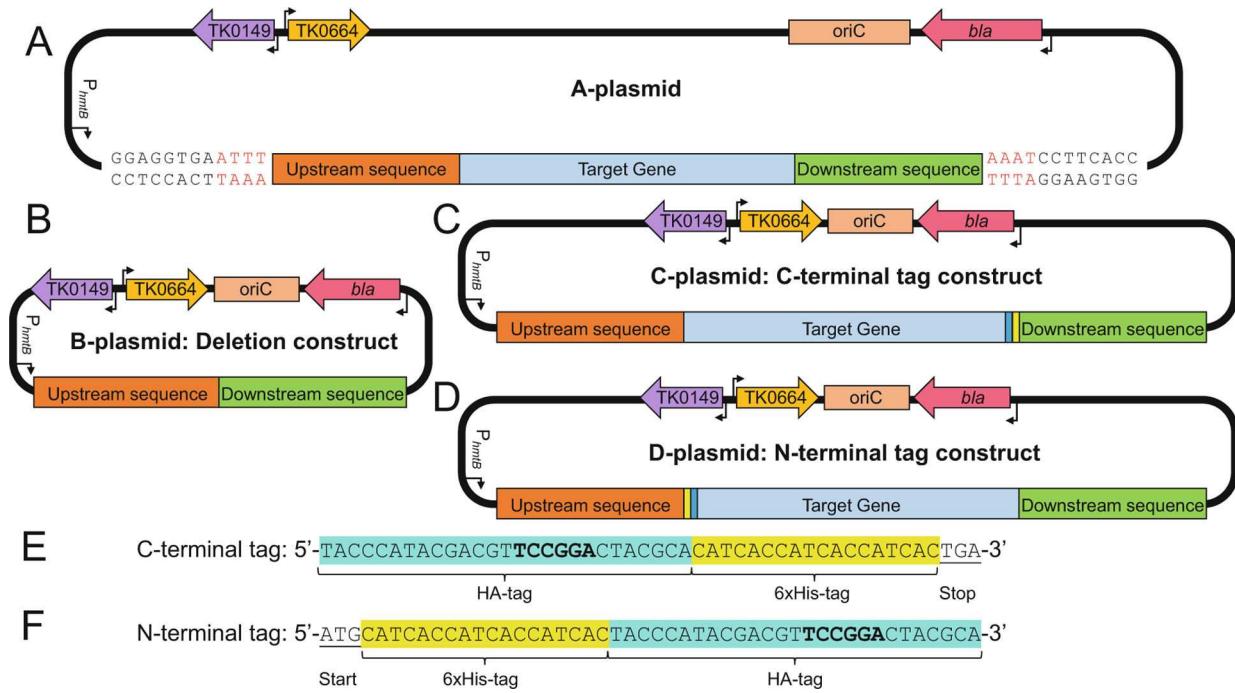
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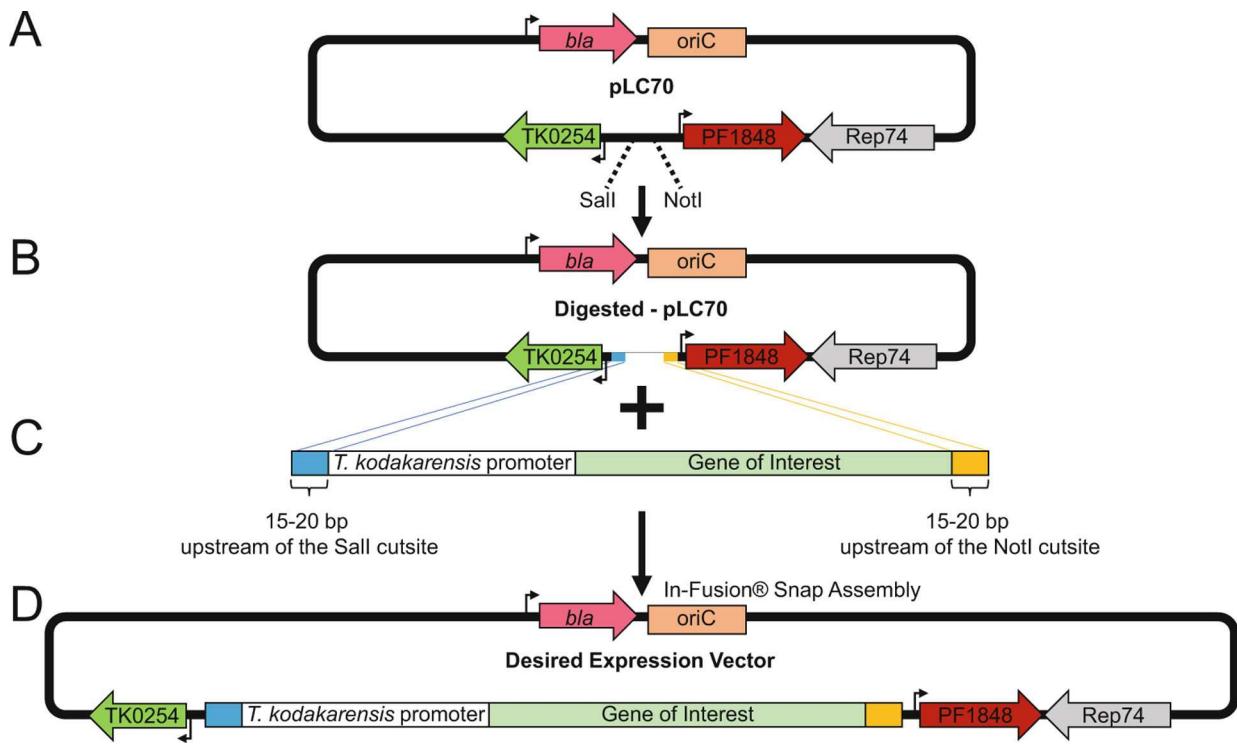
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**Fig. 1.**

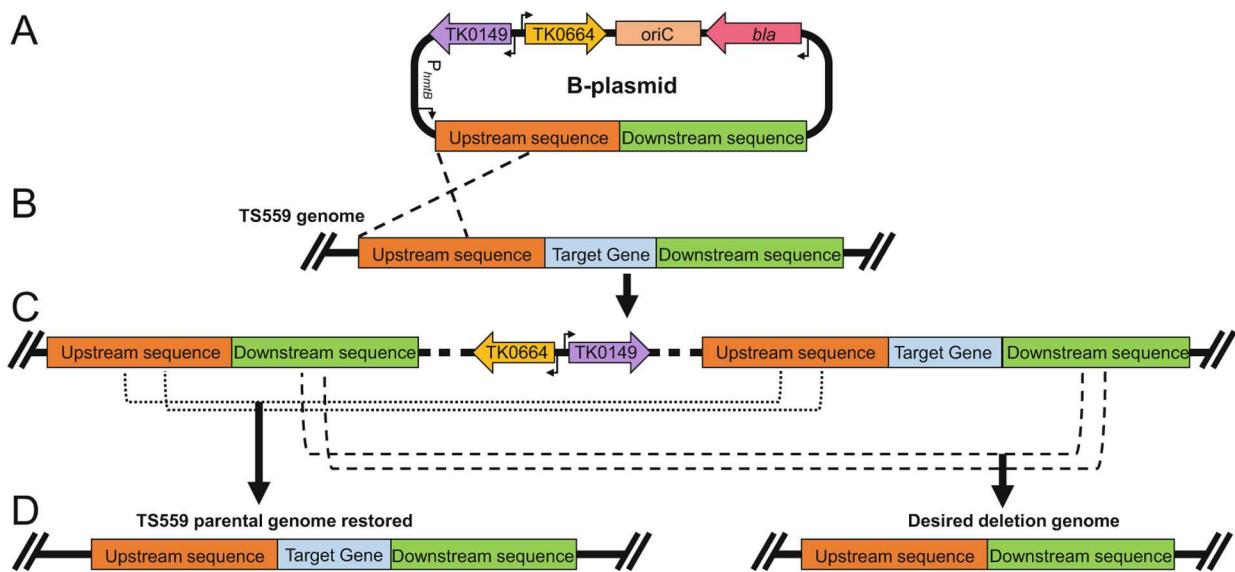
Generating an A-plasmid for the targeted *T. kodakarensis* genomic locus. (a, top) The pTS700 plasmid encoding TK0149, an agmatine prototrophic selectable marker (purple left arrow), TK0664, a hypoxanthineguanine-phosphoribosyltransferase, that serves as a 6-methylpurine sensitive counter selectable marker (orange right arrow), oriC, bacterial plasmid origin of replication (salmon box), and  $\beta$ -lactamase (*bla*), ampicillin resistance gene (pink left arrow). The strong constitutive archaeal promoter ( $P_{hmtB}$ ) and the Shine-Dalgarno sequence (SD) are included to promote expression in instances where recombination of the plasmid into the genome of *T. kodakarensis* displaces genes from their native promoters. (a, bottom) The PCR amplified target gene fragment contains 12 bp sequences complementary to both the upstream and downstream regions of the Swal cutsite in pTS700. The amplicon contains ~700 bp of upstream sequences (red box), ~700 bp of downstream sequences (green box), and the target gene sequence (blue box). (b) In-Fusion cloning results in production of a complete A-plasmid for the target gene

**Fig. 2.**

Design of integrative vectors to delete or modify genomic targets in *Thermococcus kodakarensis*. (a) The parental A-plasmid containing ~700 bp of upstream sequence (red box), the target gene (blue box), and ~700 bp of downstream sequence (green box). (b) B-plasmids are used to generate deletion strains of *T. kodakarensis*. The B-plasmid is generated from the A-plasmid by deleting the sequences encoding the target gene while retaining the upstream- and downstream-sequences. (c) & (d) The C- and D-plasmids are used to generate strains of *T. kodakarensis* wherein the genomic target locus is extended to encode for HA- and 6xHis-tags. The C-plasmid is generated from the A-plasmid by the inclusion of sequences encoding the HA-tag (cyan box) followed by the 6xHis-tag (yellow box) before the stop codon. The D-plasmid is generated from the A-plasmid by the inclusion of sequences encoding the 6xHis-tag (yellow box) followed by the HA-tag (cyan box) after the start codon. (e) & (f) The nucleotide sequences and positions of the C- and N-terminal tags highlighting the introduced BspEI sites (bold)

**Fig. 3.**

The autonomously replicating pLC70 vector permits ectopic gene expression in *Thermococcus kodakarensis*. (a) The pLC70 vector contains the  $\beta$ -lactamase (*bla*) ampicillin resistance gene (pink left arrow), the bacterial origin of replication, oriC (salmon box), the archaeal DNA replication protein Rep74 (gray left arrow), the *Pyrococcus furiosus* PF1848 gene conferring resistance to statin-based antibiotics (red right arrow), and the TK0254 gene which provides a tryptophan prototrophic selectable marker (green left arrow). (b) The pLC70 vector is linearized using SalI and NotI endonucleases to accept expression cassettes. (c) An expression cassette composed of the sequence for a *T. kodakarensis* promoter (white box), sequences for the target gene of interest (sage box), and terminal flanking sequences homologous to regions near the SalI cutsite (blue box) and the NotI cutsite (orange box) for insertion into the linearized pLC70 vector. (d) The complete vector with inserted expression cassette can be transformed into *T. kodakarensis* and selected based on restoration of tryptophan prototrophy and resistance to statin-based antibiotics

**Fig. 4.**

Example of target gene locus modification in *Thermococcus kodakarensis* using a B-plasmid to generate a deletion strain. (a) The sequence-confirmed B-plasmid of the target gene is transformed into the parental strain (TS559) and integrates into the chromosome via homologous recombination. (b) Homologous recombination (black dashed lines) of the B-plasmid into the parental (TS559) genome integrates the entire B-plasmid into the genome, resulting in restoration of agmatine prototrophy and introduction of 6-methylpurine sensitivity. Recombination through upstream sequences (red boxes) is shown, however it is equally probable that the B-plasmid will recombine with the genome via homologous recombination through downstream sequences (green boxes). (c) The genotype of the resulting intermediate strain is shown, highlighting the duplication of upstream- and downstream-sequences that ultimately permit the spontaneous excision of the integrated plasmid sequences. Transformants containing the intermediate genome are selected based on agmatine prototrophy. Intermediate strains whose genotype is confirmed by diagnostic PCR are grown with agmatine supplementation to permit for spontaneous excision of the B-plasmid sequence, then plated on media containing 6-methylpurine to select for transformants that have lost TK0664. (d) Excision of the B-plasmid from the genome can result in two potential homologous recombination events: upstream recombination (black dotted line) resulting in the restoration of the parental (TS559) genome, or downstream recombination (black dashed line) resulting in the desired deletion of the target locus in the *T. kodakarensis* genome. 6-methylpurine resistant strains are picked to liquid medium (ASW-YT + Agm) and their genotypes are confirmed via diagnostic PCR or whole genome sequencing