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1 **Chapter 5. Implementation of transposon mutagenesis in *Bifidobacterium***

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9 Running Head: Transposon mutagenes in bifidobacteria

10

1 **Abstract**

2 Random transposon mutagenesis allows relatively rapid, genome-wide surveys to
3 detect genes involved in functional traits, by performing screens of mutant libraries.
4 This approach has been widely applied to identify genes responsible for activities of
5 interest in multiple eukaryote and prokaryote organisms, although most studies on
6 microorganisms have focused on pathogenic and clinically relevant bacteria. In this
7 chapter we describe the implementation of an *in vitro* Tn5-based transposome
8 strategy to generate a large collection of random mutants in the gut commensal
9 *Bifidobacterium breve* UCC2003, and discuss considerations when applying this
10 mutagenesis system to other *Bifidobacterium* species/strains of interest.

11 **Keywords:** Tn5, transposon, *Bifidobacterium*

12

1 **1. Introduction**

2 Bifidobacteria are Gram-positive commensal microorganisms whose presence in the
3 human gastrointestinal tract has been associated with beneficial effects on host health
4 (1). Consequently, significant scientific and commercial efforts have been made to
5 discern the mechanisms responsible of their purported beneficial attributes and cross-
6 talk with the human host and with other members of the intestinal microbiota (2). In
7 this context, gene inactivation may be considered to represent the gold standard
8 methodology to unequivocally prove the function of specific genes although most
9 *Bifidobacterium* strains remain recalcitrant to genetic manipulation with only a few
10 species and strains having been successfully mutated to date (3–6). An increasing
11 number of genome-based studies has allowed insights into the reasons why genetic
12 manipulation of this group of commensal microorganisms is so difficult, and has
13 provided new opportunities to design tailor-made tools to achieve gene inactivation in
14 various *Bifidobacterium* species/strains (4, 5, 7–10).

15 Transposon-based random mutagenesis approaches generally involve the *in vivo*
16 delivery of a conditional vector carrying a transposon piece of DNA containing a
17 selective marker (usually an antibiotic resistance cassette) and the transposase
18 encoding gene. Tight control of transposase expression allows for the transposon to
19 jump into (random positions in) the targeted genome, thereby inactivating any gene in
20 which it had been inserted. Subsequent plasmid curing, which removes the
21 transposase-encoding gene from the cells, fixes the transposon in the original insertion
22 position of the host genome (11). This approach allows the generation of an unlimited

1 number of transposon insertion mutants from a single transformant carrying the
2 delivery vector. However, substantial limitations exist to apply such an approach in
3 bifidobacteria as conditional replicative vectors are currently not available for most
4 species/strains. Besides, most heterologous expression systems in bifidobacteria have
5 made use of strong constitutive promoters, which are not appropriate to control the
6 expression of a transposase as high expression of a transposase in the presence of the
7 corresponding transposon, may destabilize the transposon position and ultimately
8 affect cell viability. An alternative to this *in vivo* transposon delivery approach was
9 proposed early in 2002 when certain mobile genetic elements, i.e. Tn5 and Mu
10 transposons, were shown to form functional transposon-transposase complexes by
11 means of *in vitro* assembly. These complexes were reported to be stable enough to be
12 directly electroporated into the targeted host, where they successfully transposed into
13 the host DNA (12, 13). The practical implementation of this system to new strains
14 requires achieving: (i) high transformation efficiencies in the selected host, and (ii) the
15 use of an appropriate antibiotic selection marker within the transposon as it needs to
16 be efficiently expressed to confer antibiotic resistance so as to allow selection for
17 (single-copy) transposon insertion events. The development of these *in vitro*
18 transposon delivery approaches represented a huge advancement as they offered new
19 opportunities to tackle transposon-based random mutagenesis in microorganisms,
20 such as bifidobacteria.

21 **2. Materials**

1 **2.1. Materials and equipment for molecular biology techniques used for transposon**
2 **construction, purification and assembly; and for identifying transposon insertion**
3 **points in the mutants obtained**

4 1. Standard molecular biology reagents (see **Note 1**): PCR master mix; appropriate
5 restriction enzymes for transposon end pruning (PshAI or PvuII if using pMOD2-TetW),
6 and, if relevant, for cloning a new antibiotic resistance cassette in between the Tn5
7 mosaic ends to create a new transposon (see **Note 2**); plasmid mini-preparation kits to
8 conduct plasmid extraction from bacterial cultures ; PCR purification kits; agarose, 1X
9 TAE buffer (40 mM Tris-HCl pH 7.6, 20 mM acetic acid, 1 mM EDTA), ethidium
10 bromide.

11 2. Replicative vector containing an antibiotic resistant Tn5 transposon, e.g. pMOD2-
12 TetW from Ruiz et al. (14) or, alternatively, another customized transposon (see **Note**
13 **2**). This vector will be used as a source of the transposon piece of DNA and can be
14 stably maintained into appropriate host cells as it does not contain the transposase-
15 encoding gene (and therefore will not destabilize the transposon piece of DNA). If
16 using pMOD2-TetW, most *E. coli* cloning hosts are capable to support its replication,
17 although *endA*⁻ strains are recommended in order to achieve high plasmid yields (see
18 **Note 3**).

19 3. Oligonucleotides to amplify the Tn5 transposon from the replicative vector into
20 which it is being maintained (e.g. pMOD2-TetW). If using pMOD2-TetW,
21 pMOD<MCS>Fw (5'-ATTCAGGCTGCGCAACTGT-3') and pMOD<MCS>Rev (5'-

1 *GTCAGTGAGCGAGGAAGCGGAAG-3'*) can be used as previously described (Ruiz et al.,
2 2013).

3 3. Purified EZ-Tn5 transposase (commercially available by Lucigen Corporation,
4 Middleton, WI, US - www.lucigen.com -), it is a mutated version of the original Tn5
5 transposase that displays a transposition frequency which is 1,000 fold higher than the
6 one exhibited by the original Tn5 transposase.

7 4. Molecular grade glycerol.

8 **2.2. Microbiology reagents for culturing bacteria**

9 1. Luria Bertani (LB) culture media: 10 g L⁻¹ tryptone (Merck, Darmstadt, Alemania), 5 g
10 L⁻¹ yeast extract (Merck), 10 g L⁻¹ sodium chloride (Merck), sterilized by autoclaving.

11 This medium will be used for routinely growth of *E. coli* cells or, supplemented with 10
12 µg ml⁻¹ tetracycline in order to select for *E. coli* strains harbouring pMOD2-TetW.

13 2. de-Man-Rogosa-Sharpe (MRS) adjusted to pH 6.8: Proteose Peptone (10 g L⁻¹) (Difco,
14 Franklin Lakes, NJ, US), beef extract (10 g L⁻¹) (Difco), yeast extract (5 g L⁻¹) (Difco),
15 polysorbate (tween 80) (1 ml L⁻¹) (Sigma Aldrich; St Louis, MO, US), tri-ammonium
16 citrate (2 g L⁻¹) (Sigma Aldrich), magnesium sulfate heptahydrated (0.525 g L⁻¹) (Merck),
17 manganese sulfate tetrahydrated (0.12 g L⁻¹) (Merck), di-potassium phosphate (3 g L⁻¹)
18 (Merck), potassium phosphate (3 g L⁻¹) (Merck), pyruvic acid (0.2 g L⁻¹) (Sigma Aldrich),
19 cysteine-HCl (0.3 g L⁻¹) (Sigma Aldrich), ferric sulphate heptahydrate (0.034 g L⁻¹)
20 (Sigma Aldrich), sterilized by autoclaving. This medium will be used to grow the

- 1 appropriate *Bifidobacterium* strain for competent cell preparation prior to
- 2 electroporation.

- 3 3. 10 % D-Lactose solution prepared in distilled water and sterilized by filtration (0.2
- 4 μm diameter pore) (see **Note 4**).

- 5 4. 10 % L-cysteine-HCl solution prepared in distilled water and sterilized by filtration
- 6 (0.2 μm diameter pore).

- 7 5. Washing buffer for preparation of electrocompetent cells of *Bifidobacterium*: 1 mM
- 8 citrate, 0.5 M sucrose buffer, pH 5.8, sterilized by autoclaving.

- 9 6. Reinforced Clostridial Medium (RCM) (Oxoid) for routine growth of *Bifidobacterium*
- 10 cells, resuscitation and recovery of electrotransformed cells.

- 11 7. Reinforced Clostridial Agar (RCA) (Oxoid) plates supplemented with appropriate
- 12 antibiotic concentrations for transposon insertion selection. In the particular case of
- 13 using the Tn5-TetW transposon described for *B. breve* UCC2003 or *B. breve* NCFB2258
- 14 strains (14), use a final concentration of 10 $\mu\text{g ml}^{-1}$ of tetracycline (see **Note 5**).

- 15 8. 10 mg ml^{-1} tetracycline stock solution dissolved in 50 % ethanol and sterilized by
- 16 filtration (0.2 μm diameter pore). The filtered sterilized stock solution can be stored at
- 17 -20°C (see **Note 6**).

- 18 9. Plasmid capable of replicating into the selected *Bifidobacterium* host, preferably
- 19 harbouring the same antibiotic resistance cassette than the transposon, to be used as
- 20 a positive control to test transformation efficiency of the prepared competent cells. If

1 using pMOD2-TetW, the *E.coli-Bifidobacterium* shuttle vector pAM5 (15) can be used
2 as a control plasmid.

3 **2.3. Other equipment required**

4 1. Thermocycler.

5 2. Microcentrifuge.

6 3. Electroporator and electroporation cuvettes.

7 4. Device for spectrophotometric DNA quantification, e.g. Nanodrop or Qubit systems

8 5. Electrophoresis unit and transilluminator for DNA visualization.

9 6. Rotatory shaker incubator

10 7. Anaerobic work station (10 % H₂, 10 % CO₂, 80 % N₂).

11 8. Refrigerated centrifuge with rotor for bottles of at least 50 ml volume.

12 **3. Methods**

13 A schematic overview of the main steps which are detailed in the following sections is
14 represented in Figure 1.

15 ***3.1. Transposon construction and preparation***

16 1. A customized transposon can be constructed by cloning the desired antibiotic
17 resistance cassette, or any alternative selective marker to identify transposon insertion
18 events, in between the Tn5 mosaic ends recognized by the Tn5 transposase (Johnson

1 and Reznikoff, 1983) in an *E. coli* replicative vector (e.g. pMOD2 vector from Lucigen)
2 (Figure 1). The *tetW* resistance cassette used in pMOD2-TetW was originally isolated
3 from a *Bifidobacterium* species (17) and has been shown to be efficiently expressed to
4 provide sufficient tetracycline resistance in order to achieve selection of clones
5 harbouring a single chromosomal copy of the corresponding gene in multiple
6 *Bifidobacterium* strains (4, 9). For the above reasons, this is a good marker to be
7 included in a transposon to be used in tetracycline-sensitive *Bifidobacterium* strains
8 (14).

9 2. Plasmid mini-preparations from the transposon containing construct (e.g. pMOD2-
10 TetW or alternative customized constructs as indicated in Note 2) will be used to
11 generate large quantities of a TetW-Tn5 transposon ready for assemblage with the
12 purified EZ-Tn5 transposase. Plasmid miniprep extractions need to be performed on *E.*
13 *coli* cells harbouring the transposon-containing plasmid (e.g. pMOD2-TetW). An
14 appropriate volume of growing cells must be used according to guidelines of the
15 plasmid mini-preparation kit provider. Extracted plasmid DNA must be verified by
16 restriction profiling followed by agarose gel electrophoresis. For instance, to verify the
17 pMOD2-TetW construct, digest 10 µl of a plasmid mini-preparation in a final reaction
18 volume of 20 µl by using SphI and XbaI according to indications from the restriction
19 enzymes provider. Following incubation for at least 1 hour at 37°C, mix 10 µl of
20 restricted plasmid DNA with 2 µl of commercial loading buffer dye and load on a 1 %
21 agarose gel. Apply a voltage of 1.5 V/cm until the dye reaches about two thirds of the
22 gel length; and stain the gel for 30 minutes with ethidium bromide (0.5 µg ml⁻¹).
23 Visualize DNA bands in a UV transilluminator. SphI and XbaI digested pMOD2-TetW

1 should be visible as bright bands of 2.5 Kb (pMOD2 backbone) and 2.8 Kb (tetracycline
2 resistance Tn5 transposon).

3 3. PCR-amplify the transposon piece of DNA using as a template the plasmid mini-
4 preparation of the construct harbouring it, e.g. pMOD2-TetW, isolated from *E. coli* cells
5 in the previous step. Oligonucleotides annealing immediately upstream of the inverted
6 repeats flanking the Tn5 transposon in the plasmid harbouring the transposon must be
7 used. If using a transposon constructed within a pMOD2 vector, as the one described
8 above for bifidobacteria, oligonucleotides pMOD<MCS>Fw and pMOD<MCS>Rev
9 (Lucigen) can be used. A suggested PCR cycling scheme is as follows: (i) denature the
10 template at 94°C for 2 minutes; (ii) perform 30 cycles denaturing at 94°C for 30
11 seconds; annealing at 60°C for 45 seconds and extending at 72°C for 1 minute for every
12 kb of expected product. Since a good amount of transposon DNA needs to be
13 generated, it is recommended to perform at least 5-10 PCR reactions in a final volume
14 of 50 µl each to guarantee a sufficiently large quantity of transposon DNA (as a general
15 guideline, it would be recommended to generate at least 50-60 µg of PCR product).

16 3. To verify that PCR reactions have amplified a fragment of the desired size, load 5 µl
17 of the PCR reactions in a 1 % agarose gel as previously described in step 2. The TetW-
18 Tn5 transposon from Ruiz et al. (14) should produce a band of about 2.8 kb. Each PCR
19 product must be purified on a silica column (see **Note 7**).

20 4. The efficiency of the transposition process is maximized in the presence of
21 phosphorylated transposon ends. In order to get phosphorylated ends, the TetW-Tn5
22 transposon amplified from pMOD2-TetW as described in the previous step, can be

1 pruned by restriction with PshAI or PvuII, as these restriction sites are located
2 immediately upstream of the transposon inverted repeats. The restriction reactions
3 must be set up according to guidelines issued by the restriction enzyme provider (see
4 **Note 8**). Following restriction, the transposon is cleaned up using a column PCR
5 purification system and each DNA preparation is concentrated by eluting it in a small
6 volume in the final step of the cleaning procedure (20 μl per purification column).

7 5. Pool all transposon preparations and measure DNA concentration for instance using
8 spectrophotometric methods (e.g. nanodrop or Qubit based nucleic acid
9 quantification). The concentration of transposon ends need to be adjusted to desired
10 values taking into consideration the transposon length, by following instructions from
11 the Tn5 transposase provider. The TetW-Tn5 transposon previously used for
12 bifidobacteria was adjusted to a final concentration of 400 $\text{ng } \mu\text{l}^{-1}$.

13 6. The transposon-transposase complexes are assembled according to instructions
14 from the transposase provider. As a standard guideline, mix 2 μl of transposon DNA
15 preparation (dissolved in TE Buffer (10 mM Tris-HCl (pH 7.5), 1 mM EDTA)); 4 μl of EZ-
16 Tn5 transposase (1 U/ μl) and 2 μl of 100 % glycerol, vortex and incubate at room
17 temperature for 30 minutes. This preparation can be directly electroporated into
18 freshly prepared *Bifidobacterium* competent cells, or stored at $-20\text{ }^{\circ}\text{C}$ until use.

19 7. Assembled transposome complexes can then be electroporated into freshly
20 prepared *Bifidobacterium* electrocompetent cells by following the instructions
21 provided in the following section.

1 **3.2. Preparation of electrocompetent cells of *Bifidobacterium***

2 In order to achieve maximum transformation efficiencies, electroporation needs to be
3 conducted employing freshly prepared *Bifidobacterium* competent cells. Growth
4 media, wash buffer and antibiotic containing plates are recommended to be prepared
5 freshly the day before the experiment starts.

6 DAY 1

7 1. An isolated colony of the strain to be mutated is inoculated into 10 ml of RCM broth
8 and grown overnight (~16 hours) at 37°C in standing tubes into an anaerobic chamber
9 (10 % CO₂, 10 % H₂, 80 % N₂ atmosphere).

10 2. 50 ml tubes containing de Man-Rogosa-Sharpe broth supplemented with a final
11 concentration of 0.05 % L-cysteine (freshly added from a 10 % filter sterilized stock
12 prepared in distilled water); and 1 % of an appropriate carbon source (for *B. breve*
13 UCC2003 or *B. breve* NCFB2258, use D-lactose freshly added from a 10 % filter
14 sterilized stock solution prepared in distilled water) can be pre-reduced and stored
15 overnight in the same anaerobic chamber.

16 DAY 2

17 2. The pre-reduced and pre-warmed de Man-Rogosa-Sharpe broth supplemented with
18 the carbon source and L-cysteine, is inoculated (2 % v/v) with the culture grown
19 overnight. Incubate the standing tubes at 37°C in the anaerobic chamber until the
20 cultures reach an OD_{600nm} of about 0.5-0.7.

1 3. While the culture(s) for competent cell preparation are growing, the citrate-sucrose
2 buffer must be placed in an ice box and an appropriate centrifuge, 1.5 ml eppendorf
3 tubes, pipettes and electroporation cuvettes must be refrigerated by keeping them in
4 an ice box.

5 4. Incubate cultures for competent cells preparation until they reach an optical density
6 at 600 nm between 0.5-0.7 (5-7 hours). At this point, take the cultures out of the
7 anaerobic incubator and spin down the cells at 4 000 g in a cold rotor (4°C) for 10
8 minutes. From this point onwards the cells need to be kept ice-cold.

9 5. Wash the cells twice by decanting the supernatant and gently swirling the cell pellet
10 into the same volume (50 ml) of ice-cold 1 mM citrate-0.5 M sucrose buffer.

11 6. Resuspend the washed cell pellet obtained from 50 ml of culture into 500 µl of ice-
12 cold citrate-sucrose buffer.

13 7. In 1.5 ml eppendorf tubes dispense adequate amounts of Tn5 transposase-
14 transposon complexes assembled as previously described in section 1. In parallel, in
15 one of the tubes add 5 µl of a plasmid capable of replicating into the *Bifidobacterium*
16 strain used, e.g. pAM5 (15), in order to be used as a positive control to verify the
17 quality of the prepared competent cells and *Bifidobacterium* electrotransformation
18 procedure.

19 8. Add to each tube containing the transposase-transposon complexes or positive
20 control plasmid DNA, 45 µl of freshly prepared electrocompetent cells. Mix by gently

1 pipetting up and down and transfer the whole volume to an ice cold electroporation
2 cuvette (see **Note 9**).

3 9. Apply a pulse of 2.0 KV, 200 ohms, 25 μ F in an electroporator (e.g. Gene Pulser II
4 Porator Electroporation System from Bio Rad). Immediately following application of
5 the electric pulse, aseptically add 950 μ l of RCM and gently suspend the cells by
6 pipetting up and down a few times.

7 10. Incubate the electroporation cuvettes with the cell suspensions for 30 minutes at
8 37°C in the anaerobic incubator (see **Note 10**).

9 11. Plate 100 μ l aliquots of electroporated cells onto freshly prepared plates of RCA
10 supplemented with 10 μ g ml⁻¹ of tetracycline added from a filter-sterilized 1000X stock
11 solution as previously described (section 2.2).

12 12. Incubate the plates anaerobically at 37°C for 2-3 days.

13 **2.3. Recovery of tetracycline-resistant colonies and identification of transposon** 14 **insertion sites**

15 If no antibiotic resistant colonies are grown following electroporation of transposome
16 complexes, verify the presence of antibiotic resistant colonies in the reaction
17 performed with the positive control plasmid DNA. If transformation efficiency of this
18 control plasmid is under 10⁴ cfu μ g⁻¹, repeat the experiment or consider optimizing the
19 competent cells preparation or transformation procedure (see **Note 11**).

1 If (sufficient) antibiotic resistant colonies are grown following electroporation of
2 transposome complexes:

3 1. Using a sterile pipette tip, inoculate them into RCM broth supplemented with
4 tetracycline ($10 \mu\text{g ml}^{-1}$) and cultivate them overnight anaerobically at 37°C .

5 2. The following day, add 30 % sterile glycerol and stock the clones at -80°C for further
6 analysis.

7 3. The transposon presence in obtained clones can be verified through a) Southern blot
8 hybridization; b) PCR using oligonucleotides targeting the transposon mosaic ends or
9 targeting internal sequence fragments within the transposon.

10 4. Identification of transposon insertion sites in specific clones can be performed
11 through inverse-PCR coupled to Sanger sequencing as previously described (14) (see
12 **Note 12**). As a general guideline, the inverse-PCR procedure to identify the genome
13 positions where a transposon is inserted in a specific clone includes the following
14 steps: 1) extract *Bifidobacterium* DNA (see **Note 13**); 2) digest $1 \mu\text{g}$ of DNA in a final
15 volume of $50 \mu\text{l}$ of reaction, using a restriction enzyme which does not cut within the
16 transposon; 3) purify digested DNA; a suggested protocol is as follows: add $50 \mu\text{l}$ of
17 distilled water and $100 \mu\text{l}$ of phenol/chloroform pH 8.0, mix and spin down ($\geq 12\ 000\ \text{g}$,
18 10 minutes), transfer upper phase to a fresh tube, add 1/10 volumes of sodium acetate
19 $3\ \text{M}$ pH 5.8 and 1 volume of cold ethanol; spin down 30 minutes, $\geq 12\ 000\ \text{g}$, 4°C ;
20 remove supernatant; wash with 70 % ethanol; air dry and resuspend in $10 \mu\text{l}$ of
21 distilled water; 4) quantify DNA spectrophotometrically; 5) set up self-ligation

1 reactions, using 0.2 or 0.4 µg of restricted DNA in a final volume of 50 µl; 6) incubate
2 ligations overnight at room temperature; 7) purify ligated DNA using the same
3 procedure described in step 3; 8) set up PCR reactions using each ligation reaction as
4 template DNA and oligonucleotides annealing with the transposon ends, outwards
5 facing (if using the TetW-Tn5 previously used for bifidobacteria, the oligonucleotides i-
6 PCR-Fw 5'-GCATACCGTACTGATCTG-3' and i-PCR-Rev 5'-CAATCATACCGGCTTCC-3' can
7 be used) (see **Note 14**); 9) verify the PCR amplification by loading 5 µl in a 1 % agarose
8 gel as previously described (section 3.1. step 2); 10) sequence the PCR products using
9 nested oligonucleotides, located within the transposon ends, upstream of the position
10 where the inverse-PCR oligonucleotides annealed e.g. if using the TetW-Tn5
11 transposon previously described for bifidobacteria, sequencing can be performed using
12 oligonucleotides pMOD-fw-seq 5'-GCCAACGACTACGCACTAGCC-3' and pMOD-rev-seq
13 5'-GAGCCAATATGCGAGAACACC-3' (14).

14 **Notes**

15 1. Molecular biology grade reagents need to be used for transposon construction and
16 transposase-transposon assembly. Standard caution to prevent nuclease
17 contamination of transposon DNA preparation needs to be taken (use molecular
18 biology grade reagents, gloves and filter tips).

19 2. Virtually any piece of DNA can be included into a transposon, in between the mosaic
20 ends recognized by the corresponding transposase. For further instructions and
21 background information please check Lucigen guidelines.

1 3. If using pMOD2-TetW grow the *E. coli* host cells harbouring the vector in LB
2 supplemented with 10 µg ml⁻¹ of tetracycline. Grow *E. coli* cultures into sterile tubes,
3 ensuring to leave a sufficient empty space in the tubes to allow for appropriate
4 aeration during incubation. Grow the cultures by incubating them overnight at 37°C in
5 a rotary shaker (200 r.p.m.).

6 4. D-Lactose might need to be replaced by an alternative carbon source depending on
7 the particular needs of the strain used (14). In order facilitate preparation of a 10 %
8 stock solution of D-lactose, the suspension might need to be warmed up at 37°C, e.g.
9 incubate it in a water bath at 37°C for 5 minutes and then vortex until the
10 carbohydrate is fully dissolved in the suspension. Then filter sterilize (0.2 µm pore
11 filters).

12 5. After autoclaving agar containing media, leave the bottles to cool down to
13 approximately 50°C before adding the antibiotic solution as it might be heat labile.
14 Gently mix the medium bottles and pour the plates.

15 6. Discard the tetracycline solution stored at -20°C if precipitation is observed.

16 7. Alternative methods to purify the PCR-amplified transposon so as to eliminate salts,
17 and excess of nucleotides and primers, can be used (for example polyethylene glycol
18 precipitation or standard ethanol precipitation).

19 8. When designing a new customized transposon construct, ensure the absence of
20 PshAI or PvuII internal restriction sites in the constructed transposon. Alternatively,
21 whenever these two sites have target sequences within the transposon sequence, it is

1 possible to PCR-amplify the transposon piece of DNA by employing a single
2 phosphorylated oligonucleotide, annealing with the Tn5 mosaic end (5'-
3 *CTGTCTCTTATACACATCT*-3'). If the transposon is amplified in this way from the pMOD-
4 derived constructs, there is no need to trim the transposon ends with PshAI or PvuII
5 restriction.

6 9. Avoid the presence of bubbles in the sample dispensed in the electroporation
7 cuvette.

8 10. Time of incubation following electroporation might need to be adjusted depending
9 on the strains used and transformation efficiencies. Longer times increase the number
10 of antibiotic resistant colonies obtained, but this may be the result of growth rather
11 than increased transposition efficiency.

12 11. When optimizing transposon mutagenesis strategies in new strains, it is critical to
13 achieve high transformation efficiencies and to guarantee that the antibiotic selective
14 marker included within the transposon is efficiently expressed in the selected host and
15 allows accurate and reliable selection for transposition events. Type I and Type II
16 endogenous restriction-modification systems encoded in the host genome have been
17 demonstrated as key bottlenecks limiting transformation efficiencies in bifidobacteria
18 (4, 9). Therefore, if the (recognition sites of the) restriction-modification systems of the
19 host genome are known, it is advisable to avoid the presence of the corresponding
20 restriction sites in the transposon sequence.

1 12. For alternative procedures to simultaneously identify transposon insertion points
2 in large collections of transposon insertion mutants, see (18).

3 13. Multiple procedures have been available in order to extract DNA from
4 *Bifidobacterium* cells. As a general guideline, cell pellets obtained from 6 ml of
5 *Bifidobacterium* cells grown overnight need to be lysed enzymatically by suspending
6 the cell pellets into TE buffer supplemented with lysozyme (30 mg ml⁻¹) and
7 mutanolysin (5 U ml⁻¹) and incubated for at least 30 minutes at 37°C. Then proceed
8 with DNA purification procedures either using column-based kits or standard
9 phenol/chloroform purification coupled to ethanol precipitation (19).

10 14. In the inverse-PCR programme, include a long elongation time in each cycle (at
11 least 8 minutes).

12 **References**

- 13 1. Tojo R, Suárez A, Clemente MG et al (2014) Intestinal microbiota in health and
14 disease: role of bifidobacteria in gut homeostasis. *World J Gastroenterol*
15 20:15163–15176. doi: 10.3748/wjg.v20.i41.15163
- 16 2. Bottacini F, van Sinderen D, Ventura M (2017) Omics of bifidobacteria: research
17 and insights into their health-promoting activities. *Biochem J* 474:4137–4152.
18 doi: 10.1042/BCJ20160756
- 19 3. Brancaccio VF, Zhurina DS, Riedel CU (2013) Tough nuts to crack: site-directed
20 mutagenesis of bifidobacteria remains a challenge. *Bioengineered* 4:197–202.
21 doi: 10.4161/bioe.23381

- 1 4. O'Callaghan A, Bottacini F, O'Connell Motherway M, van Sinderen D (2015)
2 Pangenome analysis of *Bifidobacterium longum* and site-directed mutagenesis
3 through by-pass of restriction-modification systems. BMC Genomics 16:832. doi:
4 10.1186/s12864-015-1968-4
- 5 5. Hidalgo-Cantabrana C, Sánchez B, Álvarez-Martín P et al (2015) A single
6 mutation in the gene responsible for the mucoid phenotype of *Bifidobacterium*
7 *animalis* subsp. *lactis* confers surface and functional characteristics. Appl
8 Environ Microbiol 81:7960–7968. doi: 10.1128/AEM.02095-15
- 9 6. Sakaguchi K, He J, Tani S et al (2012) A targeted gene knockout method using a
10 newly constructed temperature-sensitive plasmid mediated homologous
11 recombination in *Bifidobacterium longum*. Appl Microbiol Biotechnol 95:499–
12 509. doi: 10.1007/s00253-012-4090-4
- 13 7. O'Connell Motherway M, Watson D, Bottacini F et al (2014) Identification of
14 restriction-modification systems of *Bifidobacterium animalis* subsp. *lactis* CNCM
15 I-2494 by SMRT sequencing and associated methylome analysis. PLoS ONE
16 9:e94875, 1-10. doi: 10.1371/journal.pone.0094875
- 17 8. Hirayama Y, Sakanaka M, Fukuma H et al (2012) Development of a double-
18 crossover markerless gene deletion system in *Bifidobacterium longum*:
19 functional analysis of the α -galactosidase gene for raffinose assimilation. Appl
20 Environ Microbiol 78(14):4984-4994. doi: 10.1128/AEM.00588-12
- 21 9. O'Connell Motherway M, O'Driscoll J, Fitzgerald GF, Van Sinderen D (2009)

- 1 Overcoming the restriction barrier to plasmid transformation and targeted
2 mutagenesis in *Bifidobacterium breve* UCC2003. *Microb Biotechnol* 2:321–32.
3 doi: 10.1111/j.1751-7915.2008.00071.x
- 4 10. Bottacini F, Morrissey R, Roberts RJ, et al (2018) Comparative genome and
5 methylome analysis reveals restriction/modification system diversity in the gut
6 commensal *Bifidobacterium breve*. *Nucleic Acids Res* 46:1860–1877. doi:
7 10.1093/nar/gkx1289
- 8 11. Picardeau M (2010) Transposition of fly mariner elements into bacteria as a
9 genetic tool for mutagenesis. *Genetica* 138:551–558 . doi: 10.1007/s10709-009-
10 9408-5
- 11 12. Reznikoff WS, Goryshin IY, Jendrisak JJ (2004) Tn5 as a molecular genetics tool:
12 *in vitro* transposition and the coupling of *in vitro* technologies with *in vivo*
13 transposition. *Methods Molec Biol* 260:83-96. doi: 10.1385/1-59259-755-6:083
- 14 13. Lamberg A, Nieminen S, Qiao M, Savilahti H (2002) Efficient insertion
15 mutagenesis strategy for bacterial genomes involving electroporation of *in vitro*-
16 assembled DNA transposition complexes of bacteriophage mu. *Appl Environ*
17 *Microbiol* 68:705–712. doi: 10.1128/AEM.68.2.705-712.2002
- 18 14. Ruiz L, Motherway MO, Lanigan N, van Sinderen D (2013) Transposon
19 mutagenesis in *Bifidobacterium breve*: construction and characterization of a
20 Tn5 transposon mutant library for *Bifidobacterium breve* UCC2003. *PLoS One*
21 8:e64699. doi: 10.1371/journal.pone.0064699

- 1 15. Alvarez-Martin P, Belen Florez A, Margolles A et al (2008) Improved cloning
2 vectors for bifidobacteria, based on the *Bifidobacterium catenulatum* pBC1
3 replicon. Appl Environ Microbiol 74:4656–4665. doi: 10.1128/AEM.00074-08
- 4 16. Johnson RC, Reznikoff WS (1983) DNA sequences at the ends of transposon Tn5
5 required for transposition. Nature 304:280–282. doi: 10.1038/304280a0
- 6 17. Flórez AB, Ammor MS, Alvarez-Martín P et al (2006) Molecular analysis of tet(W)
7 gene-mediated tetracycline resistance in dominant intestinal *Bifidobacterium*
8 species from healthy humans. Appl Environ Microbiol 72:7377–73799. doi:
9 10.1128/AEM.00486-06
- 10 18. Barquist L, Boinett CJ, Cain AK (2013) Approaches to querying bacterial genomes
11 with transposon-insertion sequencing. RNA Biol 10:1161–1169. doi:
12 10.4161/rna.24765
- 13 19. Sambrook J, Russell DW (2006) Purification of nucleic acids by extraction with
14 phenol:chloroform. CSH Protoc 2006(1). doi: 10.1101/pdb.prot4455

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16 **Figure Legends**

17 Figure 1. Schematic overview of the main steps required to create transposon insertion
18 mutants in bifidobacteria, by using EZ-tn5 transposome complexes and a customized
19 tetracycline resistant Tn5- transposon encompassing a TetW cassette.

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