Applications of bacteriophage Mu *in vitro* transposition reaction and genome manipulation via electroporation of DNA transposition complexes

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Running title: Mu *in vitro* transposition applications

i. Summary/Abstract

The capacity of transposable elements to insert into the genomes has been harnessed during the past decades to various *in vitro* and *in vivo* applications. This article describes in detail the general protocols and principles applicable for Mu *in vitro* transposition reaction as well as the assembly of DNA transposition complexes that can be electroporated into bacterial cells to accomplish efficient gene delivery. These techniques with their modifications potentiate various gene and genome modification applications, which are discussed briefly here and the reader is referred to the original publications.

ii. Key Words transposition technology, phage Mu, transpososome

1. Introduction

Bacteriophage Mu transposition is one of the best characterized DNA transposition systems, and it is the first, for which an *in vitro* reaction was established [1]. Thereafter, a substantially simpler version of the original reaction has been developed, and it requires only a simple buffer and three purified macromolecular components: transposon DNA, MuA transposase, and target DNA [2, 3]. This minimal *in vitro* reaction has further been modified to yield a variety of elaborate applications, e.g. for DNA sequencing [4], protein engineering [5-10], SNP discovery [11, 12], and constructing of gene targeting vectors [13-16]. The reaction has found its utility also in functional analyses of proteins, genes, and entire genomes [17-23]. With an additional *in vivo* step, the minimal system can be used for efficient gene delivery not only in a variety of bacteria but also in yeast and mammalian cells [24-28]. The gene delivery technology is characteristically species non-specific, and it can be used to generate exhaustive insertion mutant libraries for many types of micro-organisms, with the latest development widening the scope of the technology also to archaeal species [17]. All of the abovementioned methodologies are critically dependent on the high efficiency and low target site selectivity of the Mu *in vitro* transposition reaction [2, 29-31], which makes the system ideal for a wide variety of applications.

In this paper we describe general protocols and principles for Mu *in vitro* transposition reaction as well as the assembly of DNA transposition complexes (Mu transpososomes) and their subsequent electroporation into bacterial cells as an example of the gene delivery methodology.

2. Materials

MuA transposase protein:

MuA can be purified by using the published protocol [32]. The protein preparation is recommended to be flash-frozen and stored at -80 °C. Alternatively, you may obtain MuA from Thermo Fisher Scientific (Waltham, USA). As this product is not frozen, the manufacturer recommends its storage at -20 °C.
(http://www.thermoscientificbio.com/mutagenesis/transposon-products/).

MuA storage buffer:

25 mM Hepes pH7.6, 0.1 mM EDTA, 1 mM DTT, 20% (w/v) glycerol, 500 mM KCl. This buffer is recommended for long term storage of MuA preparations made in house.

MuA dilution buffer:

0.3M NaCl, 25 mM Hepes pH 7.6, 0.1 mM EDTA, 1 mM DTT, 10% (w/v) glycerol.
Use this buffer to dilute MuA from the stock solution directly prior to use. Store at -20 °C.

5x MuA stop:

0.1% bromophenol blue, 2.5% SDS, 50 mM EDTA, 25% ficoll 4000. Store at -20 °C.

Triton X-100:

Prepare 1.25% solution from 10% stock solution by diluting with H_2O directly prior to use.

2 x MIX:

50 mM Tris 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.

1M Hepes pH 7.6:

Dissolve 13,015 g Hepes (sodium salt) and 11,915 g Hepes (free acid) in 100 ml H_2O. Filter sterilize. Do not try to adjust additionally the pH of the solution!

5 x complex buffer:

750 mM Tris pH 6, 0.125 % Triton TX-100, 750 mM NaCl, 0.5 mM EDTA. Filter sterilize. Store at -20 °C (e.g. in 1 ml aliquots). This buffer is used for transpososome assembly.

Transposon DNA:

Mu transposon DNA can be prepared in house from carrier plasmids by using the published protocol [2] (*see* **Note 1**). Several ready-to-use transposons (called Entransposons) are available from Thermo Fisher Scientific (Waltham, USA). (http://www.thermoscientificbio.com/mutagenesis/transposon-products/).

TAE buffer:

To prepare a concentrated (50x) stock solution of TAE dissolve 242 g Tris base in approximately 750 ml deionized water. Add 57.1 ml of glacial acetic acid and 100 ml of 0.5 M EDTA (pH 8.0). Adjust the final volume to 1 liter with deionized water.

SOB:

2 % Bacto tryptone, 0.5 % Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl. Autoclave. This SOB solution is made without the addition of $MgCl_2$.

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.

***E. coli* cells:**

Any standard *E. coli* strain that is suitable for cloning can be used for the methods described.

3. Methods

3.1. General protocol for Mu *in vitro* transposition reactions

A standard reaction protocol is described here, but it can be modified in many ways (*see* **Notes 2 and 3**).

Reagent	Volume
2 x MIX	12.5 µl
Target DNA (typically 50 – 500 ng)	typically 1-2 µl
Transposon DNA (0.5 pmol/µl)	1 µl
2.5 M NaCl	1 µl
1.25% Triton X-100 (freshly diluted)	1 µl
0.25M $MgCl_2$	1 µl
H_2O	up to 24 µl
MuA (220 ng/µl)	1 µl
	∑ 25 µl

1. Dilute MuA into a final concentration of 220 ng/µl with cold MuA dilution buffer. Keep MuA preparation on ice.

2. Assemble the reactions on ice without MuA.

3. Add MuA to start the reaction and incubate at 30°C, typically for 1 hour (*see* **Note 4**).

4. Stop the reaction by the addition of 25 µl of 1% SDS and incubate at room temperature for 30 min. Add 50 µl of water to reduce the salt concentration. Electrotransformation can be done with this preparation using 1 µl aliquots (*see* **Note 5**).

5. Alternatively, for gel analysis, stop the reaction by the addition of 5 x MuA stop (e.g. 5 µl sample + 1.5 µl 5x MuA stop). Electrophorese the products using an agarose gel in TAE buffer and, following the run, stain the gel with ethidium bromide to visualize the reaction products.

3.2. General protocol for the assembly of Mu transpososomes and their subsequent electroporation into *E. coli*

3.2.1 *In vitro* assembly of Mu transpososomes

A standard 20 µl reaction protocol is described here, but the reaction can be scaled up to include the reaction volume of 80 µl.

Reagent	Volume
5 x complex buffer	4 µl
Glycerol	10 µl
H_2O	4 µl
Transposon DNA (1.1 pmol/µl)	1 µl
MuA (400 ng/µl)	1 µl
	∑ 20 µl

1. Adjust the transposon concentration to 1.1 pmol/μl.

2. Dilute MuA into a final concentration of 400 ng/μl with cold MuA dilution buffer. Keep MuA preparation on ice.

3. Assemble the reaction with 5 x complex buffer, glycerol, and H_2O at room temperature, transfer the tube on ice.

4. Add transposon to the reaction.

5. Add MuA to start the reaction and incubate at 30°C, typically for 2 h (*see* **Note 6**).

6. Transpososome assebly can be monitored using native agarose gel electrophoresis. Prepare 2% agarose gel (NuSieve 3:1, Lonza) containing 87 μg/ml BSA (Sigma) and 87 μg/ml heparin (Sigma) in TAE buffer. Run the gel using buffer circulation. Electrophorese at 5.3 V/cm for 2h at 4 ºC. Prior to loading, add 0.2 volume of 25% Ficoll 400 to the samples. Stain the gel after the run to visualize stable protein-DNA complexes, i.e. transpososomes.

3.2.2 Electroporation of Mu transpososomes into *E. coli*

1. Dilute the Mu transpososome preparation 1:5 or 1:10 with H_2O to reduce the salt concentration onto a level suitable for electroporation.

2. Thaw competent *E. coli* cells on ice (*see* **Note 7**).

3. Add 1 μl of diluted transpososome preparation into 25 μl of electrocompetent cells in a cold tube. Mix gently.

4. Rapidly transfer the mixture into an ice-cold electroporation cuvette (0.1 cm electrode spacing, Bio-Rad).

5. Electroporate immediately using the following pulse settings: voltage 1.8 kV, resistance 200 ohms, and capacitance 25 μF (*see* **Notes 8 and 9**).

6. Add 1 ml SOC (room temperature solution), transfer to a microcentrifuge tube.

7. Incubate at 37 °C by shaking (220 rpm) for 40 min (*see* **Note 10**).

8. Spread the cells onto appropriate selection plates.

4. Notes

Note 1. 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. Also the R-end DNA can be modified, at least to some extent, as restriction sites and translation stop signals have been engineered successfully into these ends to allow downstream processing possibilities. These R-end modifications enable efficient protein engineering applications, by which short insertions [2, 5], deletions [6, 7], single amino acid substitutions [8], or domain additions [9] can be produced.

Note 2. The standard reaction described generates reaction products in amounts sufficient for a majority of applications. However, if a more efficient reaction is needed for the product generation, the concentration of MuA and donor DNA (=transposon) can be increased. The Mu transposition reaction proceeds within the context of the Mu transpososome that contains four molecules of MuA synapsing two transposon ends. Therefore, the stoichiometry between MuA and transposon ends should be kept relatively constant. With our standard transposons (1-2 kb in length) we have used the stoichiometry of 1 pmol transposon ends (equals to 0.5 pmol of mini-Mu transposon DNA) and 2.7 pmol (220 ng) MuA. MuA is used in a moderate excess, as it binds not only to its binding sites in the R-ends but also sequence non-specifically along the entire transposon DNA, albeit with a lower affinity. Thus, if longer transposons or more target DNA need to be used, also the MuA concentration in the reaction should be increased. MuA binds to plastic surfaces, e.g. pipet tips and tube walls. Therefore, it is worth minimizing such contacts when working with the enzyme. The volume of the standard reaction is 25 µl, but it can be increased at least up to 100 µl.

Note 3. Make sure that your target DNA does not contain the same selectable marker gene that is present in the transposon DNA that you are using. The use of different selectable markers in the target and donor DNA allows the easy selection of proper integration products.

Note 4. Depending on the transposon DNA, different incubation times may be needed to allow the reaction to proceed into completion. In particular, extended incubation times are needed with long transposons, or if the transposon used contains modified R-ends.

Note 5. The SDS treatment disassembles transpososomes. The protocol described allows a quantitative analysis of reaction products by the use of biological selection. This is, reaction products are transformed or electroporated into *E. coli* and scored as colonies on

appropriate selection plates. Transpososomes may also be disassembled using phenol extraction. This is recommended particularly if further downstream processing is included in the application protocol. In short, reaction products from several reactions may be pooled, extracted with phenol and subsequently with chloroform, ethanol precipitated, and resuspended in a buffer appropriate for further processing.

Note 6. MuA transposase and transposon DNA assemble transpososomes in the absence of divalent metal ions. Under these conditions transpososomes are inactive but can be activated by the addition of Mg^{2+}. Extended complex assembly time may be needed with long transposons, or if the transposon used contains modified R-ends. Transpososomes are stable under the conditions used for the assembly. The transpososome preparation can be flash-frozen under liquid nitrogen and stored at -80 ^{o}C for later use.

Note 7. It is important that the recipient cells used are kept in a solution devoid of Mg^{2+} ions to prevent the activation of transposition chemistry prior to electroporation.

Note 8. Mu transpososomes will encounter Mg^{2+} ions inside the recipient cell and become activated for transposition. Subsequently, transpososomes are able to integrate the delivered transposon DNA into the host chromosome.

Note 9. The protocol has been developed for Genepulser II electroporation apparatus (Bio-Rad). If other brand is used, optimal pulse parameters may differ.

Note 10. During the incubation, the cells will recover from the stress inflicted by freezing and electrical pulse. To avoid cell duplication prior to plating, the incubation time may need to be adjusted depending on the bacterial strain used.

Note 11. The electroporation protocol described has been optimized for gram negative bacteria [24]. Its further optimization for gram positive bacteria has been published [25].

Note 12. Electroporation of transpososomes into yeast, mouse ES cells, human HeLa, and human ES cells is also feasible [26].

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