An assay to monitor the activity of DNA transposition complexes yields a general quality control measure for transpositional recombination reactions

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Transposon-based technologies have many applications in molecular biology and can be used for gene delivery into prokaryotic and eukaryotic cells. Common transpositional activity measurement assays suitable for many types of transposons would be beneficial, as diverse transposon systems could be compared for their performance attributes. Therefore, we developed a general-purpose assay to enable and standardize the activity measurement for DNA transposition complexes (transpososomes), using phage Mu transposition as a test platform. This assay quantifies transpositional recombination efficiency and is based on an *in vitro* transposition reaction with a target plasmid carrying a lethal *ccdB* gene. If transposition targets *ccdB*, this gene becomes inactivated, enabling plasmid-receiving *Escherichia coli* cells to survive and to be scored as colonies on selection plates. The assay was validated with 3 mini-Mu transposons varying in size and differing in their marker gene constitution. Tests with different amounts of transposon DNA provided a linear response and yielded a 10-fold operational range for the assay. The colony formation capacity was linearly correlated with the competence status of the *E.coli* cells, enabling normalization of experimental data obtained with different batches of recipient cells. The developed assay can now be used to directly compare transpososome activities with all types of mini-Mu transposons, regardless of their aimed use. Furthermore, the assay should be directly applicable to other transposition-based systems with a functional *in vitro* reaction, and it provides a dependable quality control measure that previously has been lacking but is highly important for the evaluation of current and emerging transposon-based applications.

Introduction

Transposable genetic elements are discrete segments of DNA capable of moving between different chromosomal locations in their host's genome or between different genomes. DNA transposons form a class of elements that use a special form of DNA recombination for their movement, called transpositional recombination or transposition, and this process is not dependent on sequence homology between the transposon DNA and a target site.[1] As transposons move from one locus to another, they supply new genetic material and provoke genome instability, unavoidably influencing the evolution of organisms.[2] Transposable elements (TEs) have been found to exist in almost every organism studied so far, although the fraction of the genome that they cover varies among species. For instance, 45% of the human genome[3] and 85% of the maize genome[4] are related to TEs. In contrast to eukaryotes, prokaryotic genomes commonly carry moderate numbers of transposons, although the number of TEs can vary greatly even between closely related strains of a species.[5]

The inherent capability of transposons to break and rejoin DNA have enabled the construction of a diverse set of efficient molecular tools, which can be utilized for both single gene and whole genome studies. Applications based on transposable elements include insertional mutagenesis,[6] genome manipulation and transgenesis,[7-9] functional genomics studies,[10,11] gene therapy,[12] and generation of induced pluripotent stem cells.[13,14]

Transposon-based methodologies are currently under active development, and novel applications are expected to emerge in the near future.

Traditionally transposon-based applications were based on *in vivo* transposition reactions, in which the reaction needs either mobilization of an endogenous transposon or introduction of a transposon-containing plasmid. Increased understanding on transposition mechanisms has enabled the establishment of *in vitro* systems for many types of transposons. Typical prokaryotic examples include the *in vitro* reactions of Tn3,[15] Tn5,[16,17] Tn7,[18] Tn10,[19] Tn552,[20] IS911,[21] ISY100,[22] IS608,[23] and bacteriophage Mu,[24,25]. *In vitro* transposition systems developed for eukaryotic transposons include e.g. the *in vitro* reactions of yeast Ty1;[26] mariner/Tc1 family transposons Tc1,[27] Himar1,[28] and Mos1;[29] piggyBac;[30] and hAT superfamily transposon Hermes.[31]

Bacteriophage Mu was the first transposition system, for which an *in vitro* transposition reaction was established.[25] This phage uses two modes of DNA transposition during its life cycle: (i) upon infection it integrates without replication into its host genome, and (ii) during lytic growth it uses replicative transposition to produce copies of itself.[32] Mu DNA transposition proceeds through transposition complexes or transpososomes, that are formed when MuA transposase proteins initially bind to sequence-specific binding sites in the transposon ends, after which a transpositionally active protein-DNA complex, Mu transpososome, is formed.[33] While Mu transposition in a natural context is a complex process, a substantially simplified version of the reaction can be reproduced *in vitro*. In the minimal reaction, only MuA transposase protein, transposon DNA, and target DNA are required.[24,34,35] This minimal *in vitro* reaction has proven to be highly efficient, and it has a low target site selectivity.[24,36,37] To date, Mu transposition reaction has been utilized in a variety of molecular biology,[38-47] protein engineering,[48-52] and genomics applications,[10,53-55] and also for efficient gene delivery in bacteria, yeast, and mammalian cells.[56-59]

Although *in vitro* transposition reactions have been widely used in various applications, a universal activity measurement assay to quantify transpositional activity is lacking. A single assay utilizing a common target, and applicable to all types of different transposons with a functional *in vitro* reaction, would allow a direct comparison of transpositional activities within or between particular experimental systems. We therefore developed an assay for the standardization of the measurements on transpositional activity. It is well-suited for all types of transposons and particularly useful with transposons that do not contain selectable marker genes or contain markers functional only in eukaryotic cells.

Results

Assay design

All transposon-based technologies would greatly benefit from a single activity measurement assay to quantify transposition. To meet this demand for quality control, we used phage Mu DNA transposition as a test platform and set up an *in vitro* transposition assay that utilizes plasmid pZErO-2 as the target for transposition
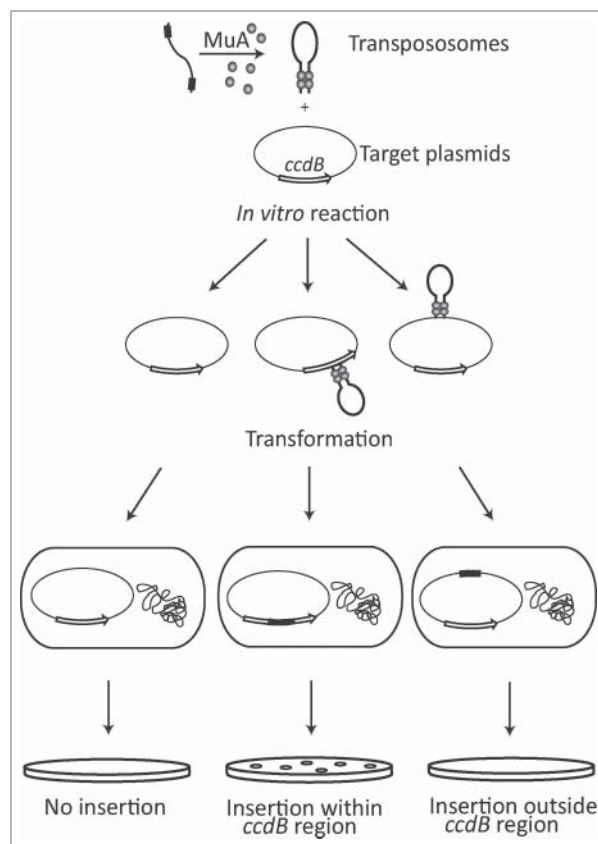

Figure 1. Assay design. *In vitro* transposition reaction with preassembled transposition complexes and pZErO-2 as a target plasmid. Target plasmid contains kanamycin resistance gene and lethal *ccdB* gene. *In vitro* transposition reaction products are introduced into *E.coli* cells by transformation and selected against kanamycin resistance on antibiotic selection plates. Inactivation of lethal *ccdB* gene by transposon insertion results into plasmid propagation and antibiotic resistance colonies.

(**Fig. 1**.). This plasmid contains a kanamycin resistance cassette for selection in *E. coli* and a lethal *ccdB* gene for direct selection of insertions.[60-62] Expression of CcdB protein in wild type *E. coli* cells causes cell death by inhibiting DNA gyrase, an essential enzyme that generates negative supercoils in DNA.[63] A transposon insertion into *ccdB* inactivates the gene, allowing the propagation of the plasmid. In this study, preassembled transpososomes are mixed with pZErO-2 target plasmid and incubated to generate transposition reaction products. The products are then introduced into *E. coli* cells by transformation, and the cells are selected for kanamycin resistance. The colonies scored represent events where *ccdB* in the target plasmid has been inactivated.

Validating assay procedure

To evaluate general properties of the assay, we performed *in vitro* transposition reactions with MuA transposase, Cat-Mu transposon DNA (encoding chloramphenicol resistance), and pZErO-2 target plasmid (encoding kanamycin resistance); transformed the transposition reaction products into DH10B and DB3.1 *E. coli* cells; and compared the results between the two strains (**Table 1.**). DB3.1 allows pZErO-2 propagation, as it

Table 1 Number of colonies detected following transformation of *in vitro* transposition reactions into bacterial strains

Reaction	DNA transformed (ng)		Incubation with MuA	No. of antibiotic resistant colonies (CFU/µg target DNA)					
				DH10B			DB3.1[a]		
	Donor	Target		Km	Cm	Km+Cm	Km	Cm	Km+Cm
Standard	Cat-Mu[b] (11.4)	pZerO-2[c] (500)	Yes	4.6×10^3	5.1×10^3	5.7×10^3	5.5×10^5	1.2×10^4	$1. \times 10^4$
	Cat-Mu (11.4)	pZerO-2 (500)	No	2.0×10^1	—[e]	—	7.73×10^5	—	—
				Ap	Cm	Ap+Cm	Ap	Cm	Ap+Cm
Control	Cat-Mu (11.4)	pUC19[d] (500)	Yes	7.9×10^6	2.1×10^4	1.9×10^4	1.1×10^6	1.1×10^4	8.6×10^3
	Cat-Mu (11.4)	pUC19 (500)	No	9.9×10^6	—	—	9.7×10^5	—	—

[a]DB3.1 contains a specific mutation in *gyrA* gene and can be used for propagating pZerO.
[b]Contains chloramphenicol acetyltransferase gene (Chloramphenicol resistance, Cm).
[c]Contains neomycin phosphotransferase gene (Kanamycin resistance, Km), and lethal *ccdB* gene.
[d]Contains β-lactamase gene (Ampicillin resistance, Ap).
[e]No colonies detected.

encodes the antidote for the lethal *ccdB* gene. Reactions incubated without MuA transposase did not produce colonies, whereas reactions with MuA yielded chloramphenicol and kanamycin double resistant colonies ($6 \times 10^3 - 1 \times 10^4$ CFU/µg target DNA) with pZErO-2 target plasmid and chloramphenicol and ampicillin double resistant colonies ($2 \times 10 - 9 \times 10^3$ CFU/µg target DNA) with pUC19 control plasmid (not harboring a lethal gene). As only the samples that had been incubated with MuA yielded colonies on double selection plates, the result indicates that the scored colonies resulted from transposon integration into the target plasmids. The number of colonies obtained from the pZErO-2 target plasmid reactions with DH10B recipient cells varied only slightly on different selection plates (kanamycin, chloramphenicol, double selection), verifying that all of these colonies originated from Cat-Mu integrations into pZErO-2. The reactions without MuA transposase incubation with pZErO-2 target plasmid produced a large number (8×10^5 CFU/µg DNA) of kanamycin resistant colonies with the antidote-encoding DB3.1 strain, while the standard laboratory strain DH10B produced only few colonies (2×10^1 CFU/µg DNA). Thus, the frequency of spontaneous *ccdB*-inactivating mutations (2.6×10^{-5}) is negligible, and therefore it has no significance for the utility of the assay.

Validating applicability with mini-Mu transposons

To test the applicability of the assay, we set up a time course experiment using 3 different transposons (**Fig. 1.**). Mu transpososomes were assembled by incubating the Cat-Mu (encoding chloramphenicol resistance gene), Kan/Neo-Mu (encoding kanamycin resistance gene), and Puro-eGFP-Mu (encoding no antibiotic resistance gene for bacteria) transposons with MuA protein for different periods of time (0, 10, 60, 120, 240 minutes). An aliquot of each assembly reaction from different time points was then incubated with pZErO-2 target plasmid
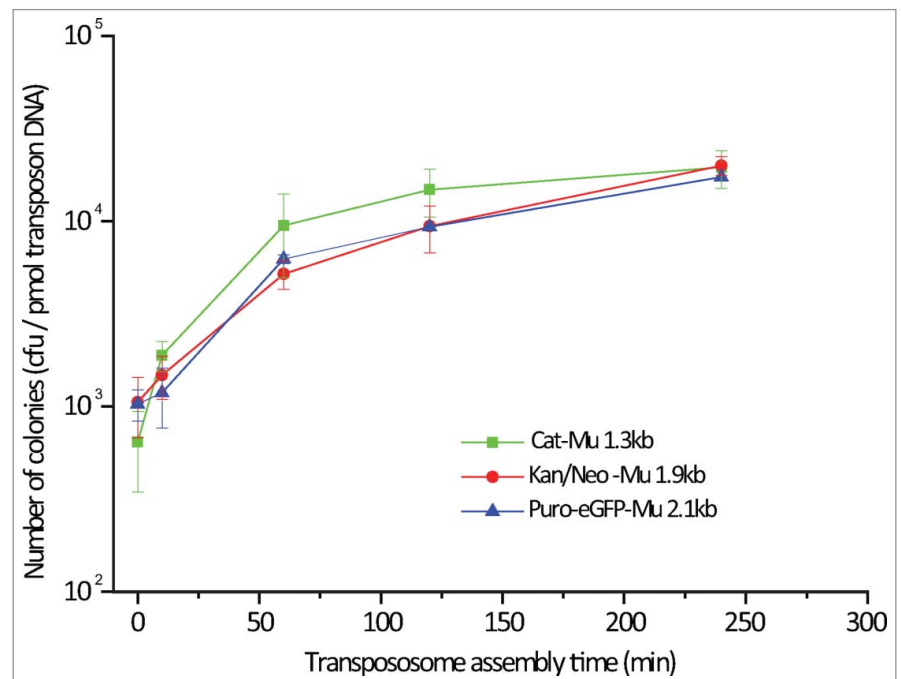

Figure 2. Validating applicability with mini-Mu transposons. Transposons used in the study: Cat-Mu (size 1.3 kb, contains chloramphenicol resistance marker), Kan/Neo-Mu (size 1.9 kb, contains kanamycin resistance marker) and Puro-eGFP-Mu (size 2.1 kb, no antibiotic resistance marker for bacteria). Transposon DNA was incubated with MuA transposase for different periods of time (0, 10, 60, 120, 240 minutes). *In vitro* transposition reactions with pZErO-2 and Cat-Mu transpososomes from different time points (0–240 minutes of incubation) were transformed into *E. coli* strain DH10B and selected against kanamycin resistance. Results with the mean and standard deviation are shown for 3 replicates. Cat-Mu is shown in green, Kan/Neo-Mu in red, and Puro-eGFP-Mu in blue.

for 5 minutes, $MgCl_2$ was then added to initiate strand transfer, and the reactions were let to proceed for 2 minutes, after which the *in vitro* transposition reaction products were transformed into *E. coli* DH10B cells (**Fig. 2.**). Bacterial clones were selected for kanamycin resistance, and the colonies produced were enumerated to reveal the relative amount of transposition reaction products for each time point. The experiment was thus designed to quantify assembled functional transpososomes. With all the three transposons, the number of colonies increased rapidly following the initiation of incubation, and the number reached a matching level following 4 hours of incubation. The assembly was somewhat slower with the 2 longer transposons, but otherwise all the three transposons used yielded very similar results, illustrating the assay's suitability for transposons differing in their marker composition.

Defining operational range

The general applicability of the assay requires functionality over a wide DNA range. To assess this, we used different amounts of transpososomes in the assay. The critical aspects evaluated were: (i) is the number of colonies sufficient for a reliable activity measurement even with a small amount of transpososomes, and (ii) does the amount of transpososomes correlate linearly with the number of colonies produced. Initially, we assembled Cat-Mu transpososomes for 4 hours, after which they were concentrated ∼10-fold, yielding a final concentration of 520 ng/μl for transposon DNA. Different amounts of this preparation were then used in the assay (**Fig. 3**). The highest amount

of transpososomes (520 ng transposon DNA) yielded almost 1.8×10^4 kanamycin resistant colonies, and the reaction with the most diluted sample (40 ng transposon DNA) produced 1.4×10^3 colonies. The number of colonies increased linearly with the amounts of transpososomes tested, indicating at least a 10-fold dynamic range for the activity measurement assay.

Influence of competence status

The competence status of recipient cells is one factor influencing the number of colonies produced following transformation of *in vitro* transposition reaction products into competent *E. coli* cells. To define whether there is a correlation between colony formation and competence status of *E. coli* cells, we transformed transposition products from Cat-Mu *in vitro* reaction into different batches of competent DH10B *E. coli* cells (**Fig. 4.**). The efficiency of these competent cells varied from ∼1×10^7 to 7×10^7 (CFU/μg pUC19 DNA). The number of colony-forming cells with transposon-tagged target plasmids increased linearly with the competence status of the *E. coli* cells used. Thus, as expected, the capacity for colony formation correlated with the competence status of the recipient cells, enabling comparison of results obtained with different cell batches.

Discussion

A variety of *in vitro* DNA transposition reactions have provided a general methodology arsenal for functional genetic analyses and molecular biology applications. The integration events into target plasmids can be scored by introducing products from *in vitro* reactions into *E. coli* cells by transformation. Transposon insertion events are usually scored by the use of antibiotic selection, selecting simultaneously for transposon and target plasmid resistance markers. Although these types of assays have performed well in the context, in which they have been used, they are limited to transposons with suitable bacterial markers. This excludes the activity measurement of transposons without selectable markers or those constructed solely for eukaryotic use. Furthermore, the selection is dependent on the marker each particular transposon carries, which is not an ideal situation when comparing the activities of different transposons. The assay developed in this study facilitates and standardizes the product analyses of various *in vitro* transposition reactions, as a common target plasmid (pZErO-2) and selection (for kanamycin resistance) is used regardless of the transposons employed.

The selection vector pZErO-2 has originally been designed to be used as a cloning vector,[62] but it can also be used as a target
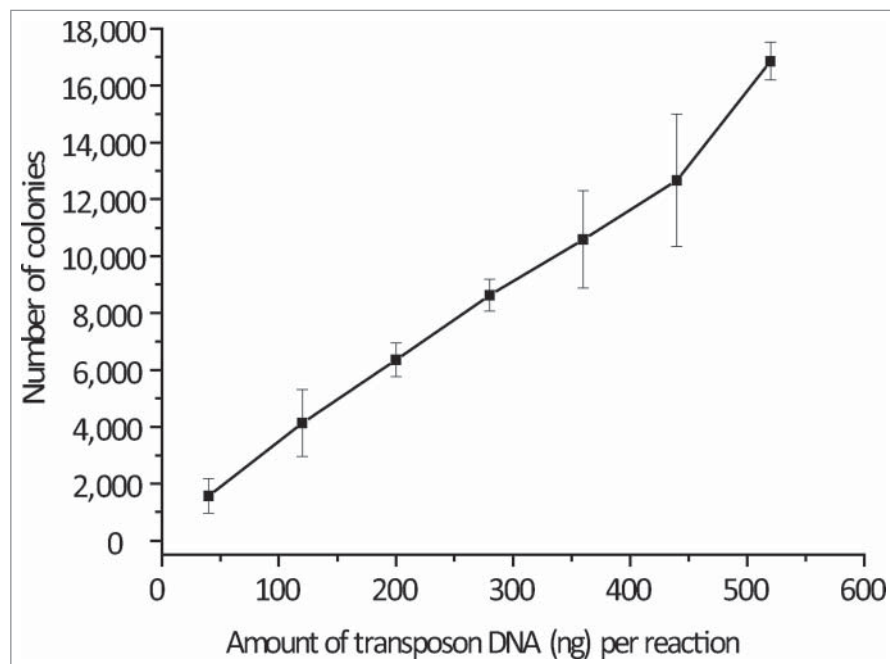

Figure 3. Defining operational range. *In vitro* transposition reactions with pZErO-2 and variable amounts of preassembled and concentrated Cat-Mu transpososomes (40 ng to 520 ng transposon DNA) were transformed into *E. coli* strain DH10B. Results with the mean and standard deviation are shown for 3 replicates.
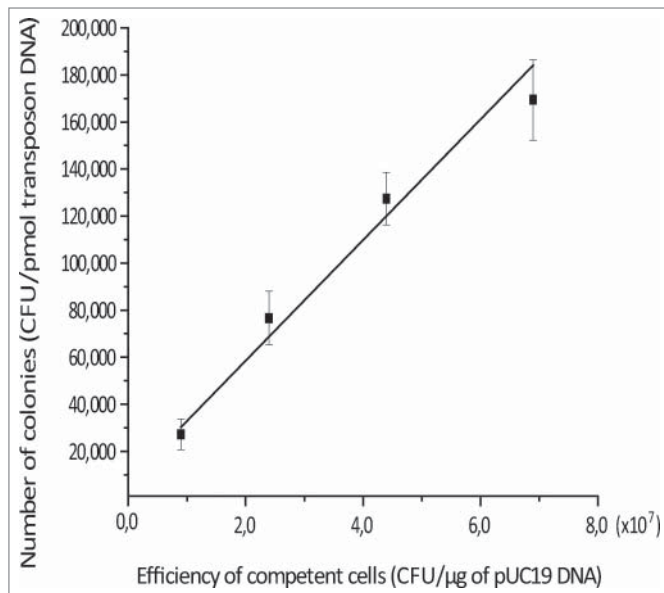
Figure 4. Influence of competence status. *In vitro* transposition reactions with pZErO-2 and preassembled and concentrated Cat-Mu transpososomes were transformed into different batches of DH10B *E. coli* cells. A linear fit mean analysis is shown as a line. Results with the mean and standard deviation are shown for 3 replicates. The equation of straight line is y = a+b*x, where slope is 0.00256 and intercept 7246. Adjusted R-square is 0.97.

plasmid selecting for DNA transposition events. Previously it has been used in demonstrating the relation between transposition and V(D)J recombination,[64] and for investigating the process of non-homologous end-joining.[65] It contains a lethal gene *ccdB*, and following selection toward the antibiotic resistance encoded by the vector, only those cells which contain an interrupted *ccdB* gene will survive.[60-62] The lethal *ccdB* gene region constitutes approximately one fourth of pZErO-2, and therefore, in our setup, on the average, one fourth of transposon insertions into it will become visible as colonies following transformation.

The frequency of spontaneous *ccdB* mutations resulting in the host survival and thus false positive colonies is 10^{-5}, as shown by the transformation of selective host cells with pZErO-2 and analogous pKIL plasmids,[61,62,66] and our results conform with these observations. Single-ended transposon integration events into *ccdB* may potentially produce false positive colonies if erroneous DNA repair processes generate a lesion at the original transposon end integration site. Although such mutagenic repair probably is very rare in *E. coli*, the issue needs to be kept under consideration if the assay is used with a transposition system producing a large fraction of single-ended integrations. With Mu transposition this is of no concern, as the frequency of one-ended integrations is negligible under standard *in vitro* reaction conditions with wild type MuA.[24]

The applicability of the assay was demonstrated with three different transposons in a time course experiment, in which transposition reaction products yielded colonies. Comparisons between different transposons showed that with Cat-Mu (1.3 kb), the amount of reaction products increased faster than with Puro-eGFP-Mu (2.1 kb) and Kan/Neo-Mu (1.9 kb). However, with

longer incubation times the differences evened out. The observed differences between different transposons most probably originate from size differences, and one explanation is that with longer transposons the assembly of transposition complexes occurs slower than with shorter transposons. It has been shown that as the transposon length increases, transposition efficiency decreases. This phenomenon is known as length-dependence, and it has been studied with several transposons, including Mu.[67-71] There are several possible explanations for length-dependence that have not been well studied. The transposition complex formation may be more difficult with longer DNA fragments, or the transformation efficiency may be less powerful for larger complexes. It has been also proposed that the length-dependence could be due to suicidal autointegration (intramolecular transposition), suggesting that the transposon may be more prone to insert into itself as the length increases.[72] Our results (**Fig. 2**.) show that the assembly of transposition complexes with the longer transposons Puro-eGFP-Mu (2.1 kb) and Kan/Neo-Mu (1.9 kb) is somewhat slower than that with the shorter transposon Cat-Mu (1.3 kb). However, since the transposons that we used in this study were all relatively short, to be able to draw any further conclusions on the effect of transposon length, further studies are warranted with appropriate sets of longer transposons. Comparable colony numbers were obtained, regardless whether transposition events were selected toward kanamycin marker encoded by the transposon (Kan/Neo-Mu) in addition to the *ccdB* gene, or solely by the *ccdB* gene (Puro-eGFP-Mu). These results prove that the assay is suitable for various transposons with different selection markers, and therefore it is especially suitable for transposons containing solely eukaryotic marker genes or entirely lacking markers. Our activity measurement assay gave very similar results as compared to a previous study where Cat-Mu transposon was analyzed,[56] showing that the data from the activity measurement assay is consistent with the earlier data.

The operational range of the assay was defined by using different quantities of Cat-Mu transposition complexes in *in vitro* transposition reactions (**Fig. 3**.). The amount of colonies increased linearly with the amount of transposon DNA (from 40 to 520 ng of transposon DNA per *in vitro* transposition reaction). The results showed that the adjustability of the assay spans a wide range of DNA concentrations, generating at least a 10-fold dynamic range.

Several batches of DH10B *E. coli* strain with different competence status were used to determine the effect of the competence status variation when transforming *in vitro* transposition reaction products into standard competent cells. The capacity for colony formation correlated linearly with the competence status, enabling normalization and therefore comparisons between the results with different batches of competent cells. The reaction products could as well be electrotransformed into electrocompetent cells, although then the variation between transformations presumably would be greater, somewhat reducing the reliability of the data.

We have established a general activity measurement assay that has been shown to be functional to quantify Mu transposition complexes. The assay measures transposition end products and provides a flexible and simple tool for measuring and comparing

the activity of different transpososome preparations regardless of the selection marker the transposon carries. Thus, it will ease particularly the evaluation of transposons containing solely eukaryotic selection markers. In addition to measuring the activity of preassembled transposition complexes, it can as well be used in the context of *in vitro* transposition reactions without prior complex assembly. Even though the developed assay has been shown to be functional with Mu transposons, it should be applicable to quantify *in vitro* reaction products of any other transposon with an established *in vitro* system. We envision that the developed assay could be regarded as a dependable quality control measure for various *in vitro* DNA transposition technology applications, including those aimed for mammalian genetics research and future gene therapy.

Methods

Bacteria, plasmids, and transposons

The target plasmid in activity measurements was pZErO-2 (Invitrogen by Life Technologies), and the control plasmid was pUC19 (New England Biolabs). *E. coli* strains DH10B[73] and DB3.1 (Invitrogen by Life Technologies) were used for transformations. Transposons have been described earlier: Cat-Mu, 1.3 kb,[24] Kan/Neo-Mu, 1.9 kb,[57] and Puro-eGFP-Mu, 2.1 kb.[57] Each transposon was released from its corresponding vector plasmid by BglII digestion that leaves 4 nucleotide 5′-overhangs, generating a precut end configuration. Following digestion transposons were purified using anion exchange chromatography as described.[24] All three transposons contain a 50-bp Mu right end segment (including R1 and R2 MuA binding sites) at their termini. As a selectable marker, Cat-Mu contains the gene for chloramphenicol resistance, Kan/Neo-Mu for kanamycin/neomycin resistance, and Puro-eGFP-Mu for puromycin resistance. Cat-Mu is selectable in *E. coli* and Puro-eGFP-Mu in eukaryotic cells. Kan/Neo-Mu is selectable both in *E. coli* and eukaryotic cells.

Competent cells

Competent *E. coli* cells were prepared essentially as described.[74] Briefly: *E. coli* cells were grown over night in Luria-Bertani[74] (LB) medium. The culture was diluted 1/100 in a total volume of 250 ml with SOC medium[67] and grown to an optical density at 600 nm of 0.4. Cells were harvested by centrifugation at 3000 rpm in a Sorvall GSA rotor at 4°C for 10 minutes. The cell pellet was resuspended in 100 ml of ice-cold buffer (100 mM RbCl, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, 15% glycerol). Following centrifugation at 3500 rpm for 15 minutes, the cell pellet was resuspended in 4 ml of ice-cold buffer (75 mM $CaCl_2$, 10 mM RbCl, 10 mM MOPS, 15% glycerol). The cells were incubated on ice for 1 h, frozen as aliquots using liquid nitrogen, and stored at −80°C.

Reagents and enzymes

MuA transposase was from Finnzymes. PEG6000 was from J. T.Baker, Triton X-100 from Fluka, Glycerol from BDH, DTT from Sigma, and EDTA from Gibco. Culture media for bacteria were from Scharlau.

Transpososome assembly and concentration of transpososomes

The *in vitro* transpososome assembly was conducted as previously described.[50] The assembly reaction (80 μl) contained 4.4 pmol transposon DNA, 19.6 pmol MuA, 150 mM Tris-HCl pH 6, 50 % (v/v) glycerol, 0.025 % (w/v) Triton X-100, 150 mM NaCl and 0.1 mM EDTA. The reaction was carried out at 30°C for 4 hours, unless otherwise stated in the text. A successful assembly of transpososomes was verified using agarose/BSA/heparin gels as previously described.[56] Transpososomes were concentrated using polyethylene glycol (PEG 6000) precipitation as described in.[58,75] Storage buffer for concentrated complexes contained 10 mM Tris-HCl pH 6, 0.5% glycerol, and 0.1 mM DTT.

In vitro transposition reactions with preassembled transposition complexes and transformation of complexes into *E. coli* cells

The *in vitro* transposition reaction was done as previously described.[39] The reaction (19 μl) contained variable amount of transpososomes as specified in each experiment, 2 μg of target DNA pZErO-2, 25 mM Tris pH 8.0, 110 mM NaCl, 0.05 % (w/v) Triton X-100, and 10 % (v/v) glycerol. The reaction was first incubated for 5 minutes at 30°C, and following the addition of one microliter of 200 mM $MgCl_2$, incubation was continued for 2 minutes at 30°C. The reaction was terminated by incubation at 75°C for 10 minutes. The transposition reaction products (5 μl) were transformed into *E. coli* cells (100 μl). Following transformation, 900 μl of LB medium was added, and bacteria were grown for 50 minutes at 37°C on 220 rpm shaking and plated on chloramphenicol (10 μg/ml), kanamycin (25 μg/ml), or double selection plates.

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