Journal of Experimental Microbiology and Immunology (JEMI)

Characterization of Action and Efficiency of Tn5 Transposase Through Comparative qPCR

Derek Bogdanoff, Timothy Jou, Brian Lee
Department of Microbiology and Immunology, University of British Columbia

We describe initial functional profiling of an in-house cloned Tn5 transposase in the transposition based sequencing library preparation reaction termed tagmentation. We provide the basis for a quantitative procedure to compare efficiency of different Tn5 enzymes in the tagmentation reaction. Fragment size distributions after tagmentation by our transposase varied greatly from those previously described utilizing Illumina's Nextera derivative, suggesting differences in enzyme activity. A comparative qPCR assay of tagmentation efficiency was unable to resolve these differences present within our tagmentation libraries after reaction attenuation, however a simulation of differential fragment length, created by library size selection, was resolvable by this method. Together, our findings highlight the need of a quantitative transposase activity assay, their feasibility, and the difficulty of providing such assays in a cost-effective high-throughput manner.

Next generation sequencing technology has improved dramatically over the past decade, allowing higher sample throughput at a significantly cheaper cost (1). These advancements in turn have spurred the rapid development of novel sequencing applications and methods, as researchers have begun to tailor the methodologies of next generation sequencing platforms to their specific needs (1, 2). Tagmentation is a recently developed transposition-based method of sequencing library preparation; designed to be compatible with Illumina's sequencing technologies (2, 3). Utilizing a hyperactive Class 2 DNA transposase to concurrently fragment a genomic template and ligate the necessary sequencing adaptors, tagmentation has been shown to cut preparation time and genomic input down to fractions of the amounts needed in traditional, multi-step library approaches (1). Since its initial description in 2010, a number of daughter methods based on tagmentation have been utilized to probe the transcriptome (2), epigenome (3, 4) and metagenomic systems, demonstrating the method's popularity and adaptability. Presently, the widespread use and experimentation with tagmentation-based methods is impeded by the high cost of reagents, available commercially through Illumina's Nextera kits. In-house cloning and purification of the kit's key reagent, the hyperactive Tn5 transposase, has recently been shown to be a viable way of obtaining the reagent at low cost (5) opening the door for more unique and cost effective uses of the technology.

Although the utilization of in-house cloned Tn5 transposase is projected to greatly reduce the assay's cost and allow researchers to modify the reagents as they see fit, protein cloning parameters and methods will unavoidably add variance to its enzymatic efficiency. Regarding tagmentation based sequencing library preparation, efficiency of Tn5 must be of a certain level to produce viable libraries: if efficiency is low, the reaction may yield genome fragments that are too long and unsuitable for sequencing. To assay for fragment length prior to sequencing, libraries are often run on a gel or bioanalyzer instrument (2); however, readouts from these methods are not fully quantitative, and are feasible only for low numbers of libraries tested at a single time.

It is true that reagents and protocols commercially available from Illumina remove one's need to optimize, yet as researchers begin to push the envelope in sequencing advancements, the kind of 'black box' methods provided commercially will ultimately be succeeded by more flexible and economic workarounds. The aim of our work here is to determine whether a comparative quantitative PCR based method can quantify tagmentation efficiency, with the ultimate goal of developing a simple assay, with high throughput potential, capable of differentiating tagmentation efficiencies to a stringency level acceptable for sequencing applications (FIG. 1**)**. Efficiency of the tagmentation reaction can be extrapolated from fragment

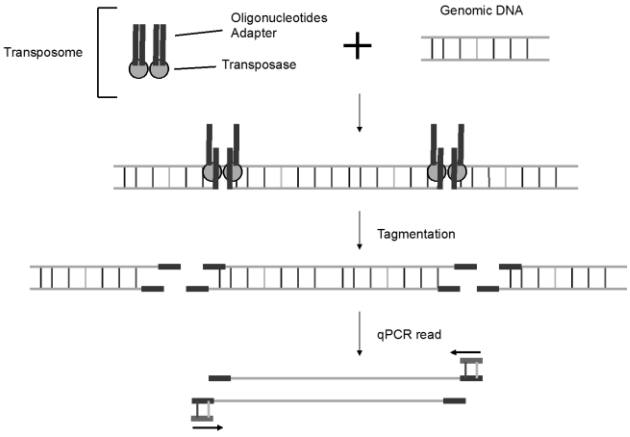

FIG 1 Schematic of tagmentation and experimental approach. Oligonucleotide adapters are complexed with Tn5 transposase to generate active transposome. Transposome units insert randomly into a genomic template resulting in concerted fragmentation and ligation of adapter oligonucleotide sequences. Generated fragment libraries can be amplified using primers complimentary to the ligated adapter regions and the library contents can be quantified by qPCR. Fragment quantifications can used to directly call the efficiency tagmentation.

size of the output library, and therefore, characterization of an enzyme's abilities could be assayed for by PCR. We hypothesize that such a qPCR system will be able to accurately quantify tagmentation efficiency based on mean library fragment length.

MATERIALS AND METHODS

Cell culturing. Single colonies of pUC19 and DH5α were inoculated into 5 ml of Luria Broth (10g tryptone, 5g yeast extract, 10g NaCl, H_20 to 1L). The cultures were incubated on the shaker overnight at 37°C.

Quantitative polymerase chain reaction. Quantitative PCR was performed by using SYBR Green Supermix from BioRad (172-5264) on a BioRad CFX connect Real-Time system. In a qPCR reaction, 2μL of both forward and reverse primers, 1 ng of template, 10 mL of the SYBR Green Supermix, and dH2O to a volume of 20 ml were used. The primers used for the extension time optimization and the primers for the tagmented sequence are listed below (Table 1 and 2). Primers were ordered from Integrated DNA Technologies (IDT), and diluted in ddH2O to a concentration of 100 μM. The transposase oligo primers were used to amplify the tagmented product, while primers 1F-5R were used to create libraries of known length fragments using pUC19 plasmid DNA as template in order to optimize Ct resolution and extension time. Primer 1F and primer 2R generates a product size of 158bp, Primer 1F and Primer 3R generates a product size of 228 bp, and primer 1F and Primer 5R generate a product size of 1250 bp. Two separate experiments were therefore undertaking using this methodology, the initial fragment-length resolution assays using pUC19 as template, and the assays of tagmentation fragment length, using DH5α genomic DNA as template.

Table 1 Primers used to amplify tagmented product

Primer Designation	Sequence
Transposase Oligo1 primer	5'AATGATACGGCGACCACCGAGATCTACACTAGATCGCTCGTCGGCAGCGTC 3'
Transposase Oligo2 primer	5'CAAGCAGAAGACGGCATACGAGATTAAGGCGAGTCTCGTGGGCTCGG 3'

Table 2 Primers used for extension time optimization control experiment

Primer Designation	Sequence
Primer 1F	5'CGGCATCAGAGCAGATTGTA 3'
Primer 2R	5'CTGGCGTAATAGCGAAGAGG 3'
Primer 3R	5'AACGTCGTGACTGGGAAAAC 3'
Primer 5R	5'CTACCAGCGGTGGTTTGTTT 3'

Isolation of plasmid DNA. Plasmid DNA was extracted from pUC19 cultures using the PureLink Quick Plasmid Miniprep Kit (Invitrogen K210010). Cells were harvested by centrifugation. The media was removed, and the pellet was resuspended in resuspension buffer with added RNase A. Cells were lysed by adding the kit lysis buffer and DNA was precipitated in the kit precipitation buffer. After centrifugation, the supernatant was loaded onto a spin column and washed with wash buffer and ethanol twice. The final isolated plasmid DNA was eluted by adding TE buffer and centrifugation. Plasmid DNA was checked for purity on a Nanodrop 2000c UV-Vis Spectrophotometer and stored at -20°C for long-term storage.

Isolation of genomic DNA. Genomic DNA was extracted from DH5α cultures by using the PureLink Genomic DNA mini kit (Invitrogen K1820-01). Cells were pelleted by centrifugation and resuspended in 180 μL PureLink Genomic digestion buffer with 20 μL of proteinase K to lyse the cells. Samples were incubated at 55^0C for 30 minutes. RNase A, Genomic lysis/binding buffer and ethanol were mixed with the lysate to yield a homogeneous solution. The lysate was transferred to a spin column and washed with kit buffers 1 and 2. Lastly, 200 μL of elution buffer was added to the column to elute the sample into a sterile microfuge tube. Genomic DNA was was checked for purity on a Nanodrop 2000c UV-Vis Spectrophotometer and stored at -20^0C for long-term storage.

Annealing of oligonucleotides adapter. Oligonucleotides for transposase loading were synthesized in single strand form and annealed to yield the double stranded functional units. Adapters were purchased from IDT (Table 3) and resuspended in TE buffer to 100μM, creating a 50 mM adapter stock when combined. The combined oligonucleotides were annealed on ice for 30 minutes, and stored at -20°C.

Table 3 Adapter sequences used for annealing of oligonucleotides

Oligonucleotides for Transposase Loading	Sequence
Transposase Oligo 1	5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG 3'
Transposase Oligo 2	5'CTGTCTCTTATACACATCTCCGAGCCCACGAGA 3'

Preparation of transposome for tagmentation reaction. Transposase functions as a dimerized unit bound to a double stranded transposon sequences. Modified for tagmentation, a functional transposome consists of the transposase dimer bound to adapter sequences to be ligated to fragment ends after transposase fragmentation. Cloned and purified transposase stock was graciously gifted by Dr. Van Loenhout (Carl Hansen lab, NCE, University of British Columbia) at a concentration of 8 mM in enzyme storage buffer (50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton X-100, and 1 mM dithiothreitol). Transposase stock was mixed with annealed adapters at a 1:1 ratio, and placed on ice for 5 minutes to allow binding. Constructed transposome was stored at -20°C in aliquots of 10 μL for future use.

Tagmentation reaction. Tagmentation reactions were carried out by mixing 5μL of prepared transposome stock with 40ng genomic in 95μL of tagmentation buffer (50mM Taps pH 8.5, 25mM $MgCl_2$, 50% DMF). Mixes were placed in a thermocycler (Applied Biosystems 9800 Fast Thermal Cycler) and heated to at 55°C for 5 minutes. The finished tagmentation reaction was then analyzed using qPCR with the corresponding primers and by Agilent Bioanalyzer analysis. To simulate differences in Tn5 efficiency, a series of attenuated tagmentation reactions were created by varying the volume of transposome stock used in the reaction from 5μL down to 1μL, TE buffer as an effective diluent of the enzyme stock in the reaction.

Bioanalyzer analysis using DNA gel on chip. Fragment length distributions were obtained by Agilent Bioanalyzer, following the manufacturer's protocol. Following amplification, libraries were quantified by Nanodrop 2000c UV-Vis Spectrophotometer, and diluted to within the range of 5-15ng/μL. Samples were loaded onto a prepared High Sensitivity DNA Chip (Agilent 5067-4626) and the chip run and scanned with an Agilent 2100 Bioanalyzer system to provide electropherogram readouts of the fragment length distribution within the library (fragment length in bp against a fluorescent unit of quantification).

Library size selection. 8μL of each Fragment library was mixed with 2μL of 5X loading buffer and ran on a 6% PAGE gel for 60 minutes at 120 V. After running the gel was stained with

SYBR Gold nucleic acid gel stain (Life S-11494) in a container for 2–3 minutes and viewed the gel on a UV transiluminator. Bands at the 200 bp-250 bp and 700bp -800 bp ranges were cut and removed from the gel, and placed into prepared 0.5mL tubes in which holes were pricked into the bottom with a 0.8 gauge needle. The 0.5mL were placed with 2mL tubes, and spun down at 12,000g for 5 minutes in order to sheer the gel. The collected gel was then soaked in 250 µL DNA Gel Elution Buffer (1X) and rotated at room temperature overnight. Gel elutions were transferred to Spin-X tube filters (Corning CLS8160) and spun at 14,000g for 2 minutes. Eluate was recovered and mixed with 1 µL Linear Acrylamide, 25 µl 3M sodium acetate pH 5.2 and 750 µl of 100% ethanol. Solutions were precipitated on dry ice for 30 minutes and spun down at 14,000g for 30 minutes at 4°C. Supernatant was removed and the pellet was washed 80% ethanol and spun down at 14,000g for 5 minutes at 4°C. Ethanol was removed and the pellet was air-dried for 10 minutes at room temperature. Dry pellets were resuspended in dH_20, quantified by Nanodrop 2000c UV-Vis Spectrophotometer, and immediately used in our qPCR assay.

RESULTS

Quantitative PCR based assay is capable of resolving fragment lengths with large size variances. To investigate the use of qPCR to resolve libraries of different mean fragment lengths, we sought an optimal PCR extension time, at which amplification of different sized libraries surpassed the background threshold at different program cycles, corresponding to the Ct value readout. Extension times tested were based upon the rate at which Taq polymerase functions, and our target fragment sizes. A clear Ct value difference corresponding to required amplification cycles was observed at all extension times between our large fragment library of 1250 bp and, and the smaller two at 158bp and 228bp (FIG. 2). However, the extension times tested were not able to resolve the 70 bp difference between the smaller libraries. A 4 second extension time was chosen for all subsequent experiments based on these results.

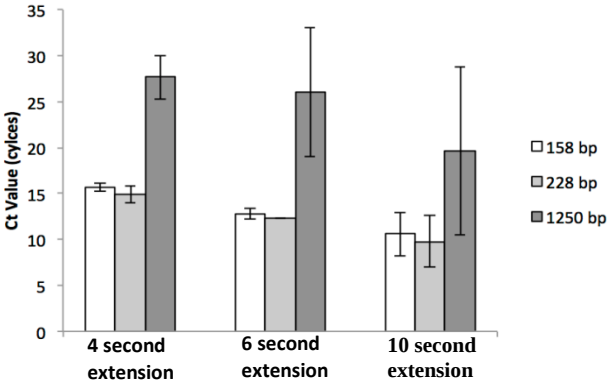

FIG 2 Fragment length resolution by qPCR. Data represents 3 replicates for all parameter combinations. Extension times of 4, 6 and 10 seconds were used in an attempt to resolve the Ct values of different library fragment lengths. Resolution was obtained between our high weight library at 1250 bp and our two lower weight libraries at 158 and 228 bp for all extension times tested. Lower weight libraries remained unresolved at all extension times tested. Error bars represent the standard deviation between the replicates.

Tagmentation with Tn5 does not show unimodal distribution. Initial tests of tagmentation activity of our constructed Tn5 transposase showed a non-unimodal fragment length, unlike those described previously (1) (FIG.3). Fragment length within a single library varied in a uniform distribution, spanning a range from approximately 200 bp to lengths outside the detectable size measured by the Agilent Bioanalyzer (>10,380bp). Above this baseline, fragment lengths peaked at 250 bp, a size considered to be acceptable for tagmentation libraries (1). Of note, a secondary fragment peak at 3400bp was present within all tagmentation libraries constructed, creating a bi-modal distribution above the uniform baseline.

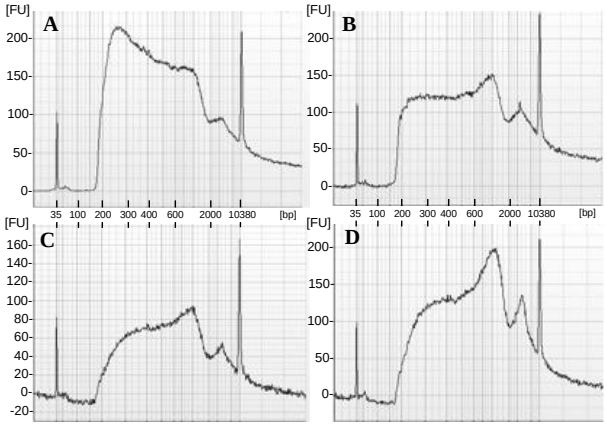

FIG 3 Fragment Length Distribution Changes With Tagmentation Attenuation. Electropherogram plots describing the fragment length distributions of tagmentation reactions under normal and attenuated reaction conditions. FU (fluorescent units) describes provides a quantification of material corresponding to the fragment length given in base pairs. Peak at 35bp and 10,380bp represent assay markers, and are not constituent of the fragment library. Concentration of Tn5 transposase varies with attenuation conditions: **a.** 400nM Tn5, **b.** 270nm Tn5, **c.** 80nm Tn5, **d.** 200nm Tn5.

Shifting of tagmentation efficiency observed under attenuated reaction conditions. As a means of simulating different transposase enzyme efficiency, attenuation of the tagmentation reaction was carried out by varying the concentration of Tn5 transposome from 400nm progressively down to 80nm. Our efficiency models showed a gradual shift in peak fragment lengths within our libraries, from 250 bp to 800 bp, correlating with changes in the amount of active transposase within the reaction (FIG. 3). While shifts in the peak fragment length were present in response to attenuation, much of the remaining fragment distribution remained static with shifts appearing above the baseline fragment length distribution only.

Direct comparison quantitative PCR is unable to differentiate shifts in fragment length distributions arising from tagmentation attenuation. Preliminary testing of our described qPCR based assay was unable to differentiate our tagmentation-based libraries. Comparing libraries constructed under normal, and highly attenuated tagmentation conditions showed no significant difference in Ct value (FIG. 4a) A Ct value of approximately 6.5

cycles was reached uniformly by the samples involved, a value at least 2 fold less than those observed in our initial optimizations for fragments lengths of a similar peak size.

Simulation of differential tagmentation efficiency by library size selection is resolvable by quantitative PCR. Amplified libraries were size selected at the 200 bp - 250 bp and 700bp - 800 bp ranges. These derived, sub-libraries were purified, quantified and immediately subject to amplification by qPCR. Ct values between the two sub-libraries were resolvable, with values approximately 2-fold higher for the 200bp library compared to the 800bp library (FIG. 4b). Ct values remained 2-3 folds lower than those measured in our initial optimizations for fragments lengths of a similar peak size.

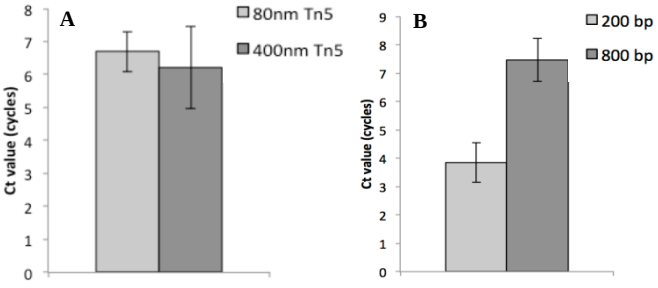

FIG 4 a. Fragment length of attenuated libraries is unresolvable by qPCR. Data represents 4 replicates. Highly attenuated tagmentation (80 nM Tn5) was compared against the unattenuated reaction (400nM Tn5). Error bars represent standard deviation between the replicates **b. Size selected tagmentation libraries show resolvable Ct values, correlating with mean fragment length of the library**. Data represents 4. Material from a single library was size selected to generate sub-libraries of approximately 50 bp in length distribution, with means at 200 bp and 800 bp. Error bars represent standard deviation between the replicates.

DISCUSSION

The potential of transposase based sequencing library construction as a paradigm shifting method is significant, as the protocol's low time, skill and equipment requirements makes next-generation sequencing feasible for many researchers outside the realm of large scale sequencing facilities. We hope our goal of profiling the tagmentation ability of a Tn5 transposase enzyme cloned in house, and develop a quick and potentially high-throughput method for future benchmarking of similarly derived enzymes, can help lead to the removal of the last and most significant factor barring this technology from widespread use, its cost.

Upon addition to genomic DNA lacking bound factors or chromatin constituents, active transposase has been shown to fragment genomic DNA in a statistically uniform manner with little sequence bias, resulting in a unimodal fragment distribution in the constructed library of 200-400 bp in length (2). Fragment size distributions acquired from our Tn5 tagmentation assays showed an alternative pattern, comprised of a baseline, uniform distribution spanning a very large range of fragment sizes (200-10,880bp), from which peak fragment lengths emerged. One explanation provided by Illumina in regards to its own Nextera tagmentation based library prep kits, claims that the observation of larger than expected sized fragments in gel or bioanalyzer type readouts can be accounted for by entwinement of tagged fragments at homologous adapter regions (8). Termed "bird-nesting", this artifact of tagmentation library construction may also explain a common second peak present among our libraries at 3400bp, as a fragment aggregate of mutual size. Although this phenomenon can explain the higher than expected fragment sizes observed, the baseline nature of our distributions extends to fragment sizes smaller than the average size peak. These small fragments outside of the peak fragment size are present within all libraries prepared by our methods, regardless of tagmentation attenuation, suggesting a potential sequence bias present in the function of our Tn5 leading to genomic transposase binding in a non-random manner (4). Shallow sequencing of a library constructed using our cloned Tn5 would provide details on whether such a sequence bias is present. Furthermore, direct comparison of the action of our Tn5 to that of Illumina's reagent from the Nextera line of sequencing prep kits would better help define the action of our cloned variant.

Although our initial attempts at calling tagmentation efficiency by direct-comparison qPCR failed, this method should still be able to call tagmentation efficiency of unimodal fragment size libraries, based on the results of our size-selected sub-libraries. Unimodal shaped distributions are to be expected from transposase binding and fragmenting a genomics template in a completely random manner (4), with fragment size range and mean position varying upon changes in reaction condition and efficiency of the transposase. Our results show that resolution between size-selected unimodal libraries is possible through our procedure, however the narrow 50 bp range of size selection does not reflect the Gaussian shaped distributions observed in most tagmentation libraries within the literature (2, 4, 5), which may possibly give unique Ct readouts upon qPCR assaying, due to the greater range in fragment sizes present.

Within our naturally tagmented libraries, a common Ct value between the attenuated and non-attenuated reactions was observed, and could perhaps be accounted for by the common baseline fragment size distribution present within all of these samples. Furthermore these Ct values were significantly lower than the Ct outputs called for similarly sized peaks (158bp and 228bp) in our optimization qPCR runs, suggesting that with a larger fragment distribution the threshold fluorescence value is overcome within fewer amplification cycles.

While larger fragments within the distribution require a greater extension time to amplify, they're size accumulates more fluorescent dye, perhaps pushing these libraries to lower Ct values (9). This notion however, does not provide an explanation for the 3-fold difference in Ct value between our size-selected 200-250bp-range library and our 225bp optimization, where it appears that the existence of a 50bp range alone accounts for the change. More work should be completed in this regard, to show a relation between the effect of multiple fragment sizes in a qPCR amplification, and fluorescence output. Traditionally, qPCR involves the amplification of a singe site to create a library of a single fragment length; our observation may therefore be the result of a mere technical artefact in the equipment used. Nonetheless, our assay was able to resolve Ct readout of between our size-selected libraries, which mirror the fragment size range of some tagmentation based libraries previously reported (1, 3).

Despite these findings, the degree to which this assay would be beneficial as a direct, quantifiable enzyme-function comparator has undoubtedly become diminished. Functional parameters of the transposase itself, such as potential sequence bias and enlarged range of output fragment sizes does not appear to be resolvable or even detectable by our method in its current state, and would have a dramatic effect on sequencing library construction and the downstream quality of sequencing data. At this time, it is likely that a thorough comparison of the abilities of transposase enzymes will require multiple assays. Due to the relatively new nature of the tagmentation method, patterns in the output libraries, such as those we observed in ours, have yet to be completely described and confidently attributed to one aspect of the library construction. As the method gains more use, it is likely that such library attributes as baseline and bimodal fragment length distributions will be annotated, and high-throughput testing of in-house derived transposase will be more feasible.

FUTURE DIRECTIONS

Primary to the aims of this project is the characterization of the tagmentation activity of the Tn5 transposase used here, as it is difficult to create an assay for functional activity, without complete knowledge of how your controls function. Sequencing on the fragment libraries produced by our transposase would define whether a sequence bias is present in transposase binding or activity, a hypothesis that potentially explains the non-standard fragment length distributions observed. Likewise, obtaining and comparing Illumina's Nextera transposome reagent, the current standard for tagmentation based library construction, would allow direct testing of our methods and reagents using transposase of known activity.

Future work to show comparative qPCR resolution of different sized fragment libraries would also be beneficial in the development of a comparative assay. Pinpointing an extension time length at which fragments differing in 100bp can be resolved could be readily undertaken following a similar methodology outlined here. Varying fragment length libraries can be created using a more extensive primer set to allow testing of a greater range of fragment lengths. Incremental variations in extension time within the 0-2 second range, using non-integer values, may prove promising as well, the effective of which having yet to be shown.

Concerning the assay itself, further steps using different fragment distribution sub-libraries acquired from gel size-selection could be used to demonstrate and test its efficacy. This approach, while artificial in nature, is practical when working with a transposase enzyme of unknown functionality. Based on the large fragment distributions from which these libraries would be derived, these sub-libraries can vary in size and distribution themselves. In this regard, the complex nature of the fragment distribution provided by our tagmentation tests could actually prove beneficial; certain distribution motifs can be picked and combined, such as uniform-baseline and bimodal distributions, and tested to see if they afford unique or characteristic qPCR Ct value outputs. Such work could potentially have value outside of a simple assay of enzyme efficiency, in fields looking to describe the physical characteristics of interactions at the sub-cellular level, in processes such as DNA amplification and transposition.

ACKNOWLEDGEMENTS

We would like to thank Dr. Marijn Van Loenhout of the Dr. Carl Hansen's lab, University of British Columbia and Dr. John Q. Zhang of the UC Davis Genome Center for their help and gracious donation of the Tn5 transposase used in our experiments. We would also like to thank Dr. David Oliver, and his teaching assistants Jia and Celine for their advice, guidance, and overall help throughout the course of this project.

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