A simple and automated solution for miniaturising reaction volumes for Nextera NGS sample preparation

introduction

DNA sequencing plays a key role in academic research, personalised medicine and drug discovery. In the past decade, improvements in DNA sequencing technologies have made these techniques more reliable and faster than before. Alongside advances in automation, data handling and bioinformatics, the scope of applications and services has increased significantly, demanding high throughput gene sequencing applications to be routinely carried out. In a high throughput environment, the ability to reduce the volume of costly reagents, such as enzymes employed in cloning or next-generation sequencing (NGS) kits, can significantly reduce the cost of performing these reactions.

The introduction of miniaturised sample preparation provides an excellent opportunity to obtain the current level of data at a significantly reduced cost, or to generate more data. However, challenges in maintaining accuracy, precision and throughput must be overcome. The introduction of automated nanolitre volume liquid handling instrumentation revolutionises cloning and NGS workflows, provided that the necessary accuracy and precision are maintained across the wide range of different liquid types used in these reactions. The miniaturisation of plate set up and reagent addition steps would facilitate high throughput applications, which, in turn, would offer excellent cost saving advantages over traditional large volume workflows.

the key to successful assay miniaturisation

Although high throughput sequencing labs regularly employ automated liquid handlers, the need to accurately dispense low volumes (nanolitres to microlitres) can be a bottleneck in miniaturising these reactions. In addition, many enzymes are stored and dispensed in highly viscous solutions, such as 30-40% glycerol, to ensure enzyme activity and stability. Accurately dispensing liquids with high viscosity is a challenge for most low volume liquid handlers, other than TTP Labtech's mosquito®, which uses true positive-displacement pipetting technology.

TTP Labtech's mosquito liquid handling robot is extremely accurate within the 25 nL-1.2 µL and 0.5-5 µL volume ranges, across a wide range of liquid viscosities. The disposable micropipettes ensure zero cross-contamination between samples. These features address three key requirements for assay miniaturisation: accuracy, precision and throughput, thus conserving valuable samples and costly reagents.

This application note demonstrates that mosquito is well-suited to reduce the reaction volumes of NGS reactions for high throughput applications.

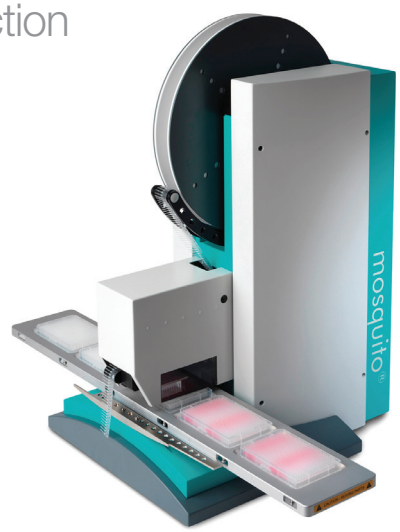

Fig 1. TTP Labtech's mosquito uses unique positive displacement, disposable micropipettes to ensure accuracy and no cross-contamination.

accurate dispensing of highly viscous solutions

A validation study compared the accuracy of mosquito's dispensing of TE buffer in the presence and absence of 50% glycerol across a volume range of 50 nL to 5 µL.

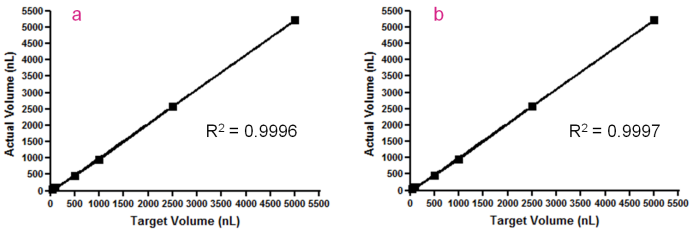

Fig 2. shows excellent accuracy across the lower end of mosquito's pipetting range, with average CVs of 3.0% for TE buffer alone (a), and less than 3.1% for 50% glycerol in TE buffer (b).

miniaturising Nextera NGS sample preparation

The Nextera NGS sample preparation protocol involves four major steps:

- tagmentation - fragmentation and adding the barcodes and adaptors
- clean up
- PCR amplification
- PCR clean up.

The tagmentation step is the most costly part of the Nextera sample preparation workflow, due to the use of tagment DNA enzyme, TDE1. Current protocols recommend using 5 µL of TDE1 in a final assay volume of 50 µL. This is due to the limitations of most available liquid handlers in transferring accurate and precise volumes of reagents, particularly the ones with high viscosity, such as enzymes.

Here, the effect of reducing the total reaction volume from 50 µL (original protocol) down to 1 µL was studied. At the lowest reaction volume (1 µL), 100 nL of TDE1 and 1 ng (400 nL) of plasmid DNA were added using mosquito (table below). mosquito easily handled the reagents and samples that were used in these reactions, down to 25 nL, with high accuracy and precision of 7 and 5%, respectively.

Volume (µL)	50	25	10	5	1
DNA (µL, ng)	20, 50	10, 25	4, 10	2, 5	0.4, 1
TD Buffer (µL)	25	12.5	5	2.5	0.5
TDE1 (µL)	5	2.5	1	0.5	0.1

In this study, samples were sequenced at 2 x 50 paired end reads. Following sequence analysis, reactions employing final volumes of 5 µL – and even 1 µL – showed 100% coverage for all 96 plasmids, with every nucleotide having at least 64 reads of coverage. The data clearly shows that miniaturisation did not affect the data quality in any way.

the effect of automated *vs.* manual mixing of enzymes

In a further study, the effect of manual versus automated mixing of an enzyme with cDNA was assessed. In this study, mosquito was used to transfer 80, 240, 320 and 400 nL of a restriction digest enzyme from a TTP Labtech 96-well microplate to 4 µL of cDNA and buffer solution in a Bio-Rad 384-well PCR plate. mosquito was alo used to predispense cDNA and buffer solutions from a Greiner V-bottom plate. The reactions were mixed 10 times using mosquito. As a positive control some of the wells were additionally mixed by a hand pipette. Also, 80, 240 and 400 nL of the restriction digest enzyme were added to some of the wells containing cDNA and buffer solutions without any further mixing, as a negative control. The reactions were incubated for an hour at 37ºC and then loaded and analyzed on a bioanalyzer gel.

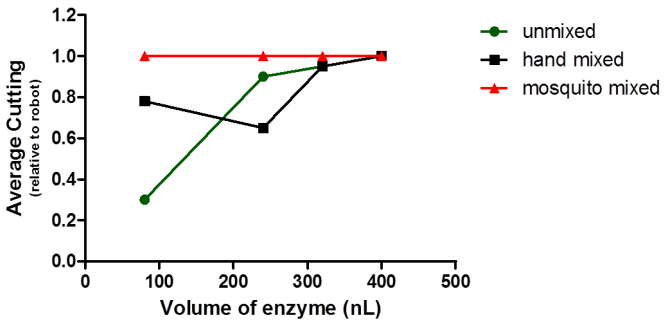

Fig 3. Comparison of average restriction enzyme digestion of a cDNA resulting from no sample mixing, double mixing using TTP Labtech's mosquito followed by manual mixing, or mixing using mosquito

Figure 3 and the bar graph in Figure 4 demonstrate that the mosquito-mixed samples were digested more completely and reproducibly than either the unmixed or the double (robot then manually) mixed samples. We believe that manual mixing is more vigorous, causing frothing and therefore denaturing of the enzyme, hence causing a lower degree of digestion efficiency by the enzyme (shown as double in the graphs).

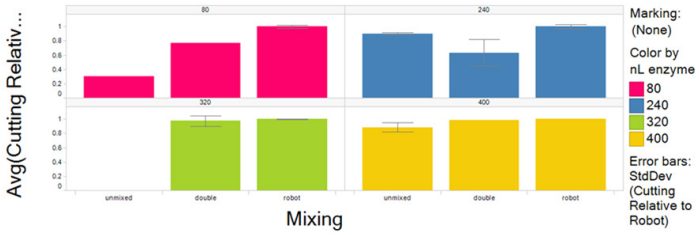

Fig 4. Superior efficiency of mixing of enzyme with cDNA for enzymatic digestion reactions using TTP Labtech's mosquito.

discussion

The high demand for low-cost sequencing has driven the development of high throughput technologies, such as NGS, that speed up and facilitate the sequencing process, producing thousands or millions of sequences simultaneously.

The introduction of nanolitre volume liquid handling instrumentation has revolutionised molecular biology workflows, allowing miniaturisation of plate set up and reagent addition steps for a wide range of high throughput applications. TTP Labtech's mosquito liquid handling robot offers an accurate and easy to use solution, which can be applied to miniaturising NGS reaction volumes within an automated, high throughput setting.

Automated dispensing of miniaturised assays provide a number of cost and time saving benefits for the research and drug discovery industries, not only reducing the amount of valuable compounds and reagents required per reaction but also eliminating the tedium of manual pipetting and its associated errors. The ability to use much lower amounts of reagents, DNA and enzymes while maintaining the same level of accuracy and precision as before, would allow more analyses to be run on the same amount of sample, and therefore obtains more data. Taking advantage of accurate, robust and easy to use automated nanolitre to microlitre liquid handling robots, such as mosquito, provides a unique opportunity to obtain the current level of data at a significantly lower cost, and/or to carry out larger scale studies at current costs.

conclusion

TTP Labtech's mosquito offers significant benefits for high throughput gene amplification and sequencing applications:

- reduced cost through miniaturisation of sample preparation
- lower consumption of genomic DNA
- gentle pipetting resulting in less shearing of DNA or frothing / denaturing of the enzyme
- ability to pipette liquids with high viscosities (such as enzymes in 50% glycerol) accurately and precisely.

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