mosquito® LV
application note

successful miniaturisation of molecular biology reaction volumes: using mosquito® LV for Gateway® cloning, PCR amplification and Nextera library generation

introduction

The ability to rapidly generate and validate novel clones for protein expression and subsequent characterisation is central to both academic and industrial research. Over the past two decades, advances in molecular biology have enabled the development of high-throughput cloning pipelines. However, to prevent costs escalating, this increase in throughput necessitates a concomitant reduction in sample and reagent input at a number of steps.

This application note describes the benefits of using SPT Labtech's mosquito® LV liquid handler for miniaturising Gateway® cloning reactions, bacterial colony PCR reactions (to initially check cloning success) and NGS library preparation (to sequence and verify cloning). Miniaturising these reaction volumes reduces both reagent cost and amount of DNA required.

miniaturisation of Gateway cloning

The Gateway® recombination cloning technology (Invitrogen by Life Technologies Ltd, UK) has revolutionised molecular cloning; enabling DNA to be transferred between expression vectors easily, rapidly and in high-throughput. It is based on the bacteriophage site-specific recombination system which facilitates the integration of lambda into the *E.coli* chromosome and the switch between the lytic (catalysed by LR clonase™ enzyme mix) and lysogenic (catalysed by BP clonase™ enzyme mix) pathways. This system enables rapid and highly efficient transfer of DNA into multiple vector systems. Once a gene is cloned into an entry vector using BP clonase™, the DNA fragment can then be moved into one or more destination vectors using LR clonase™ (Fig 1). This method boasts a cloning efficiency of 99% and reduced cloning time from several days to 1 day, without the use of restriction enzymes or ligation to maintain orientation.

This application note validates the miniaturisation of reaction volumes for Gateway® cloning, PCR and NGS library preparation. In reducing these volumes, the cost and amount of DNA required is also reduced.

methods

BP and LR clonase reactions were tested at decreasing volumes using mosquito HTS. Volumes for these reactions are provided in Table 1. For each, the lowest total reaction volume was 1 µL, which included the addition of proteinase K to stop the reaction.

> Relative to the manufacturer's suggested volume of 22 µL, this represents a 22-fold decrease for each of the BP and LR clonase reactions and a minimum 10-fold decrease relative to the low-volume adaption of the Gateway cloning protocol at this site
>
> Dr. John Reece-Hoyes, Novartis Institutes for BioMedical Research (NIBR), Cambridge, USA

key benefits

automating and miniaturising liquid handling for Gateway cloning, PCR amplification and NGS sequencing:

- **reduces assay volume and cost**
- **enables fast and high-throughput studies**
- **reduces input DNA/RNA to pg values**

Gateway reactions were incubated at 25°C for 2 hours then transformed into 50 µL competent *E.coli* (10 µL of the recommended 22 µL reaction, or the entire 6 µL and 1µL reactions) and the entire transformation reaction plated on appropriate antibiotic-containing media.

The resulting resistant colonies were subjected to bacterial colony PCR (30 cycles) in reactions of decreasing volumes (Table 2). Miniprep DNA extracted from cultures of these colonies were subjected to full-length plasmid NGS sequencing using NGS Nextera library preparation kits (Illumina, USA) in reactions one-tenth of the recommended volume

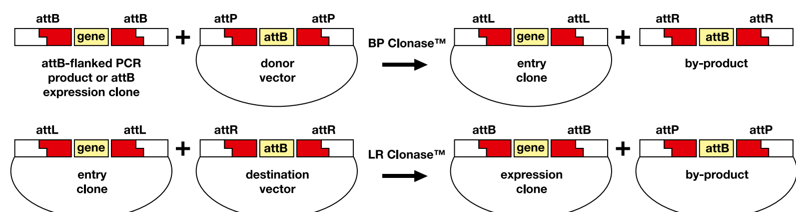

Fig 1. The Gateway cloning process involves cloning an attB-PCR product into a donor vector (attP-substrate) to form an attL-entry clone (BP recombination reaction). The entry clone is then recombined with a selected attR-destination vector to produce an attB-expression clone of choice (LR recombination reaction). (Image is modified from invitrogen, ThermoFisher Scientific, USA https://tools.thermofisher.com/content/sfs/manuals/gatewayman.pdf)

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(Table 3). The use of mosquito liquid handlers for miniaturising NGS library preparation is described in additional app notes, please see website for details.

results

Antibiotic-resistant colonies were obtained for all transformations regardless of volumes used for the BP and LR clonase reactions. While reduction of the reaction volume was accompanied by a reduction in the number of resulting colonies, even the 1 µL reactions yielded more than 100 colonies (Fig 2).

For each reaction, a series of resulting colonies were screened via bacterial colony PCR to ensure an insert of the correct length was obtained. All colonies screened in this manner indicated that the BP and LR clonase reactions were successful (Fig 3).

Final expression constructs were fully validated via NGS sequencing from miniaturised Nextera NGS library preparation reactions. Full coverage of plasmid sequences was achieved at the miniaturised reaction volumes.

conclusions

Miniaturisation represents an effective and simple approach to increasing throughput, reducing costs and sample input in molecular biology workflows. Here, we have reported significant reductions in reaction volumes and costs for Gateway cloning reactions, bacterial colony PCR reactions, and Nextera NGS library generation reactions. Total reaction volumes were reduced from a typical 25 µL volume to as little as 1 µL using the mosquito LV liquid handler – representing a tremendous opportunity for significant cost savings.

Table 1. Miniaturising BP and LR reaction volumes compared to the recommended volumes (µL)

BP or LR reaction	22 µL (recommended vol)	6 µL	1 µL
pDONR or pDEST vector 50 ng/µL	[2]	[1]	[0.2]
att-PCR product or entry clone 50ng/µL	2	1	0.2
BP/LR clonase II	[4]	[1]	[0.15]
TE buffer	[12]	[2]	[0.15] (0.15)
Proteinase K	(2)	(1)	(0.15)

Table 2. Miniaturising colony PCR reaction volumes compared to the recommended volumes (µL)

Colony PCR reaction	22 µL (recommended vol)	5 µL	1 µL
Single bacterial colony suspended in water	2	1	0.2
10x Taq reaction buffer with dNTPs	[2.5]	[0.5]	[0.1]
10 µM forward and reverse primers	[0.5]	[0.1]	[0.02]
Taq polymerase (25 U/mL)	[0.125]	[0.025]	[0.005]
Water	[20]	[3.4]	[0.675]

Table 3. Miniaturising Nextera NGS library preparation reaction volumes compared to the recommended volumes (µL)

Nextera reaction	50 µL (recommended vol)	5 µL
Plasmid DNA (5 ng/µL)	10	2.4
TD buffer	[25]	[2.5]
TDE1	[5]	[0.1]
Water	[10]	[0]

(square brackets "[,]" indicate master mix, round brackets '(,)' indicate addition after initial incubation step).

SPT Labtech's true positive-displacement liquid handlers provide:

- fast, accurate and reliable low volume pipetting (25 nL – 5 µL)
- reduced cost
- decreased DNA/RNA input
- low dead volume
- simplicity of use
- a small footprint, low-cost instrument
- easy to integrate into automated workflows

acknowledgements

We would like to thank Dr. John Reece-Hoyes and Aye Chen, Novartis Institutes for BioMedical Research (NIBR), Cambridge, MA, USA for providing the data presented in this application note.

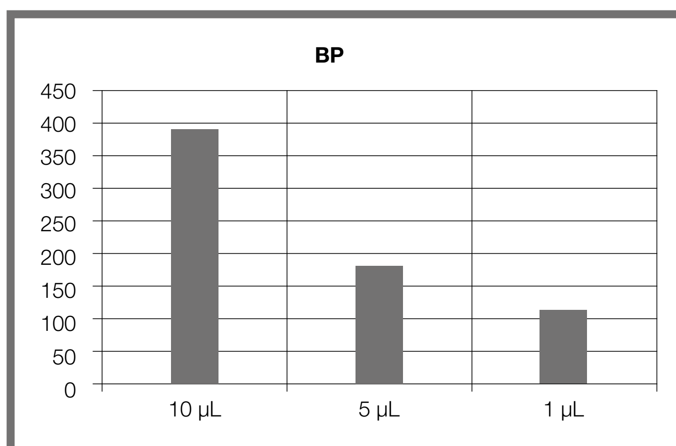

Fig 2. Colony counts from one series of BP clonase reactions comparing the transformation volumes of 1 µL and 5 µL to the recommended 10 µL (LR clonase reactions provided similar results).

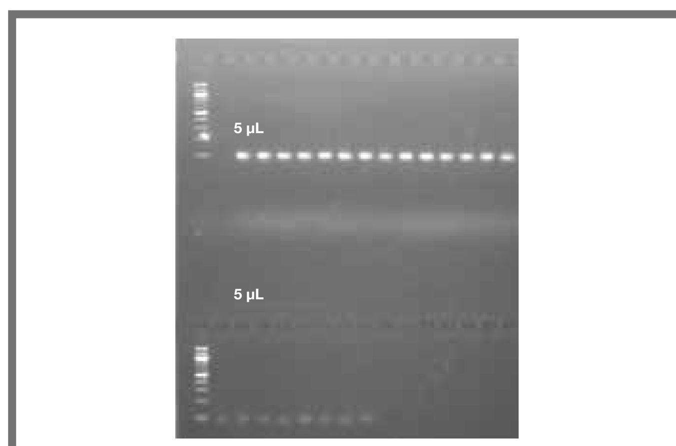

Fig 3. Comparison of 1 µL and 5 µL total reaction volumes for PCR-based bacterial colony screening following the BP clonase reaction (LR reactions provided similar results).

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