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APPLICATION NOTE No. 458

Automating Takara In-Fusion® Snap Assembly Cloning on the Eppendorf ep*Motion*®

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Abstract

PCR-based seamless cloning approach is essential to obtain reliable results from scaled-up experiments. On the Eppendorf ep*Motion* 5075t workstation, high accuracy brings a higher value to automation. This application note demonstrates the capability of the ep*Motion* to automate restriction enzyme- and ligation-free cloning using low reaction volumes. This reduces the per-reaction cost without compromising the high accuracy offered by In-Fusion Snap Assembly cloning.

Platform: Eppendorf ep*Motion*® 5075t

Kit: Takara In-Fusion® Snap Assembly

Application: Automated cloning reaction using 96-well and 384-well plates

Processing time and tip usage: 96-well plate format: processing 40 samples (10 µL volume) takes 8 min 20 sec (excluding 15 min incubation time) and uses 108 x 10 µL tips. 384-well plate format: processing 40 samples (5 µL volume) takes 14 min 30 sec (excluding 15 min incubation time) and uses 128 x 10 µL tips.

Introduction

Modern scientific trends lean towards greater convenience and lower reaction volumes while increasing throughput and reproducibility. Many molecular engineering techniques have been adapted to high-throughput platforms, and molecular cloning is no exception. High-throughput cloning methodologies have been developed in the past decade to rapidly clone hundreds to thousands of genes in parallel, especially in the fields requiring high-throughput capabilities such as antibody therapy, proteomics, and synthetic biology.

Assay setups in the 384-well format, for example, can be very time-consuming and tedious if done manually, especially when handling very small volumes. Imprecision and pipetting errors caused by the operator can lead to massive variations in the results.

The Eppendorf ep*Motion* family of automated pipetting systems is an essential tool for many laboratories looking to achieve consistent results. The ep*Motion* 5075t and other models in the Eppendorf family pipette volumes ranging from 0.2 µL to 1 mL with extreme efficiency and accuracy.

PCR-based restriction enzyme-free cloning is a mainstream approach for high-throughput platforms, allowing for inserting any DNA fragment(s) into any vector at any locus without any sequence constraints. In-Fusion seamless cloning allows for restriction enzyme-free, directional, and highly efficient cloning (95%) with extremely low background following a simple protocol. The simplicity of the In-Fusion cloning system makes it ideal for high-throughput workflows without compromising the robustness of cloning technology.

Adapting the manual procedure of setting up cloning reactions into liquid handling instrumentations, however, is not always straightforward. In most cases, the manufacturer's protocol from reagent suppliers is developed based on manual pipetting of a limited number of cloning reactions and does not fully support an automation platform.

Here, we established a high throughput cloning workflow using In-Fusion Snap Assembly on the Eppendorf ep*Motion* 5075t to allow scientists easily to adapt a manual procedure to 96-well and 384-well plate formats for an automated platform.

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Material and Methods

Automation

10 µL In-Fusion Snap Assembly cloning reaction setup
> ep*Motion* 5075t
> epT.I.P.S.® Motion 10 µL PCR-clean, with filter, sterile
> TM10 multi-channel dispensing tool
> Eppendorf PCR Sealing Film
> MixMate®

10 µL In-Fusion Snap Assembly cloning reaction setup
> Thermoadapter for skirted PCR plates, 96- and 384-well
> Eppendorf twin.tec® PCR Plate 96 LoBind®, skirted, PCR clean
> Eppendorf twin.tec® PCR Plate 384 LoBind®, skirted, PCR clean
> 5X In-Fusion Snap Assembly Master Mix
> Linearized vector (2.7kb, 53 ng/µL) epT.I.P.S.® Motion 10 µL
PCR-clean, with filter, sterile
> Purified PCR fragment (3.7 kb, 147 ng/µL)
> DNAase/RNAase-free distilled water

Methods

In this experiment, the control vector pUC19 (2.7 kb) was linearized with Hind III restriction enzyme and the insert DNA fragment (3.7 kb) was PCR-amplified with a primer set including 15 bp-sequences homologous to the ends of the linearized pUC19 vector. The primers were designed using the In-Fusion Cloning Primer Design Tool at Takara.com.
Two reaction volumes, 10 µL and 5 µL, were tested on a 96-well and a 384-well plate, respectively. Within each reaction volume, two automated pipetting approaches were tested for setting up In-Fusion Snap Assembly cloning reactions. The first approach was a separate sequential pipetting, in which each reaction component, water, In-Fusion Master Mix, linearized vector, and insert fragments were individually added ("Separate" in Table 1). In the second approach, a reaction master mix of water, In-Fusion Master Mix, and linearized vector ("PreMix" in Table 1) was dispensed into the reaction wells first, followed by the transfer of the insert fragment ("PreMix" in Table 1).
The experiment was performed on the ep*Motion* 5075t as shown in the worktable setup in Figure 1. Before initiating the procedure, all reagents were dispensed into a skirted 96-well or 384-well PCR plate. The ep*Motion* 5075 was programmed to dispense a series of target volumes (1, 2, 4, and 8 µL). The first reagent was delivered with free-jet dispensing, and the following solutions were delivered with a mixing step to ensure no unmixed reagents remain in the tips.

Table 1: Set up the In-Fusion Snap Assembly cloning reaction in applications

		ddH_2O	Master Mix (MM)	Linearized vector (LV)	Premixed H_2O, MM, LV	DNA fragment or Ctrl ddH_2O
10 µL	**Separate**	4 µL	2 µL	2 µL		2 µL
	PreMix				8 µL	2 µL
5 µL	**Separate**	2 µL	1 µL	1 µL		1 µL
	PreMix				4 µL	1 µL

Material and Methods

Methods (cont.)
Forty PCR cloning reactions were performed along with 8 water reactions for negative controls for each condition setup (10 µL-separate, 10 µL-premix, 5 µL-separate, 5 µL-premix). After all the reagents for In-Fusion Snap Assembly cloning were dispensed to the plate, the PCR plate was removed from the ep*Motion*, sealed with Eppendorf PCR film, and mixed at 1200 rpm for 10 seconds on an MixMate®. The PCR plate was then placed back onto the ep*Motion*, and incubated at 50 °C for 15 min, then stored at -20 °C until further analysis.

Five wells from the 10 µL reactions and ten wells from the 5 µL reactions were randomly picked from the plates for the transformation procedure (Figure 2). One negative control (no insert) well from each condition was selected (Figure 2). Ten random colonies were chosen from the array of plates corresponding to each reaction and analyzed by Sanger sequencing to determine the cloning accuracy. Sequences were required to be one hundred percent identical to the reference sequence to be counted as accurate.

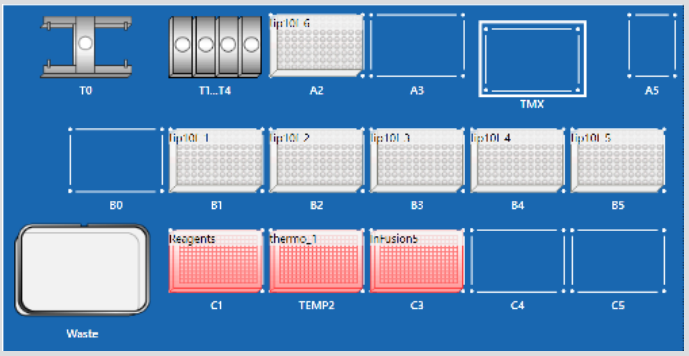
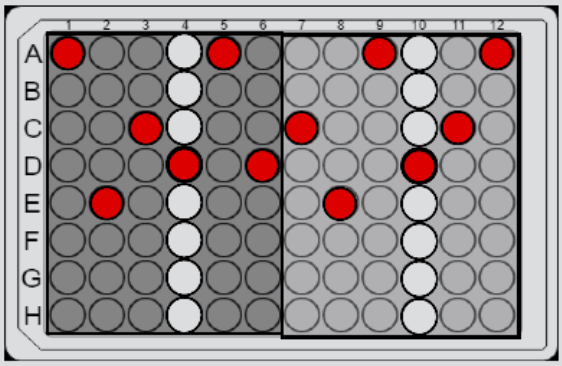


Figure 1: ep*Motion* 5075t worktable for In-Fusion Snap cloning setup

A. 96-well plate (10 µL)



B. 384-well plate (5 µL)

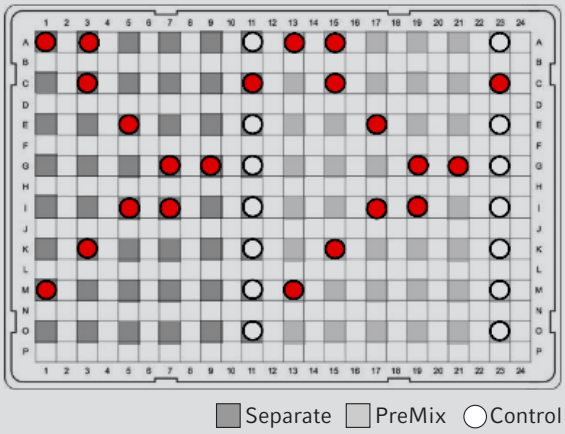


Figure 2: Experimental plates, a 96-well (A) and a 384-well (B), setup and sample positions in red for testing cloning efficiency

Results and Discussion

The schematic image (Figure 3) shows an experimental workflow of In-Fusion cloning reaction in a multi-well plate format using ep*Motion* platform. While the entire workflow in the figure can be automated, we applied the automated function only to the In-Fusion enzymatic reaction to focus on this specific reaction step. Information for automating the upstream PCR purification steps using various liquid handling systems is available in separate application notes.

We previously confirmed that premixed cloning reaction solution (5X In-Fusion Snap Assembly Master Mix with linearized vector) was stable at room temperature (RT) or 4°C for 2 hours (data not shown). In a high-throughput workflow, linearized vector is often premixed with a cloning reaction master mix and pipetted into each well of multi-well plates ahead of adding unique DNA insert fragments to each well. In this study, we took advantage of the stability of In-Fusion premix solution to test two pipetting approaches.
As shown in Table 2, each positive sample yielded 400-1200 colonies after transformation, where >100 colony results represent a successful cloning procedure.

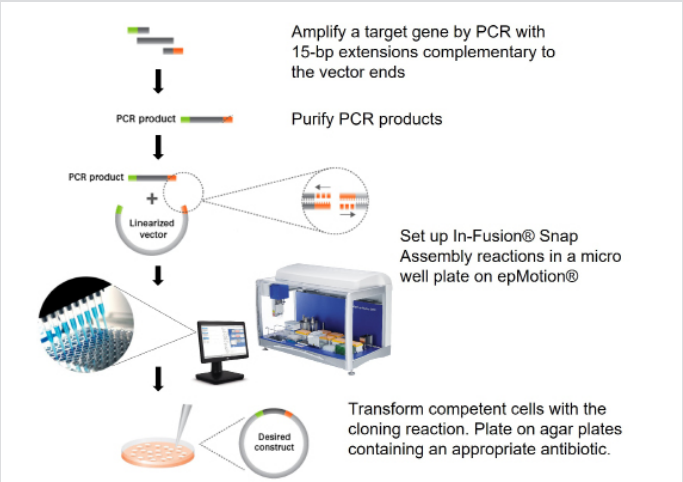


Figure 3: At-a-glance image of automated cloning workflow. A target sequence was PCR amplified using forward and reverse primer with 5' ends homologous to the respective 5' and 3' ends of the linearized vector. After purification, the In-Fusion cloning reaction was set up on a 96- or 384-well plate using ep*Motion* 5075t. The construct was transformed into competent cells and plated on selective plates for further analyses.

Table 2: Colony count for different pipetting approaches and reation volumes

Program	Well	Colony Count	Program	Well	Colony Count	Program	Well	Colony Count
10 µL Separate	A1	448	5 µL Separate	A1	1080	5 µL PreMix	A13	416
	E2	560		A3	816		A15	832
	C3	672		C3	680		C15	864
	A5	432		E5	768		E17	640
	D6	496		G7	832		G19	688
	D4 (Ctrl)	2		G9	704		G21	464
10 µL PreMix	C7	464		I5	432		I17	480
	E8	704		I7	896		I19	848
	A9	576		K3	1232		K15	616
	C11	496		M1	768		M13	736
	A12	544		C11 (Ctrl)	2		C23 (Ctrl)	3
	D10 (Ctrl)	0						

All four automated liquid handling conditions showed consistently high efficiency (i.e., colony numbers) and accuracy (i.e., Sanger sequencing; Figure 4). These results indicate that ep*Motion* can handle both
5 µL and 10 µL of In-Fusion Snap Assembly cloning setup and is successful on both separated and premixed pipetting approaches.

The sequencing result from a total of 40 positive samples shows >95% cloning accuracy, which indicates utilizing ep*Motion* for automated liquid handling can successfully perform In-Fusion Snap Assembly cloning setup without compromising the high accuracy of the technology. Few colonies in the no insert control are within the normal level of background from un-linearized vectors.

Results and Discussion (cont.)

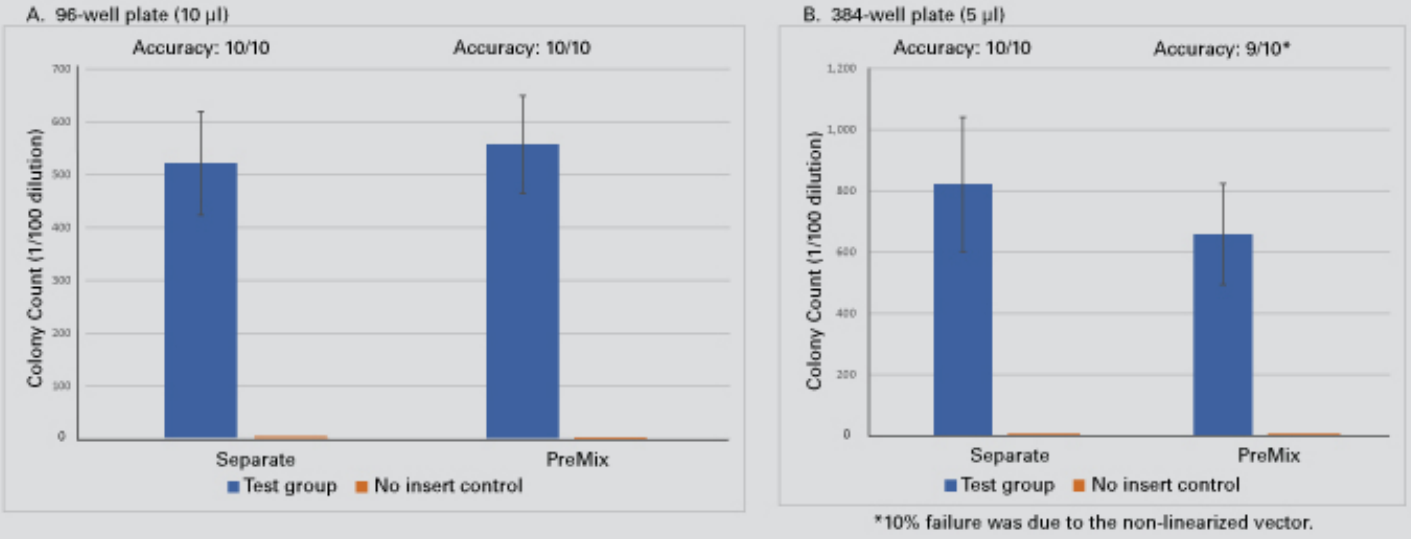


Figure 4: In-Fusion cloning reaction set-up using the ep*Motion*. Two pipetting approaches, separate (labeled as 'Separate') and pre-mixed (labeled as 'Premix') pipetting, were taken to set up the In-Fusion reaction with the ep*Motion*. Graphed values are the mean colony counts of independent cloning reactions. Error bars show ± standard deviations. Accuracy was determined by Sanger sequencing.

Conclusion

In this application note, we demonstrate that the highly efficient restriction enzyme- and ligation-free In-Fusion cloning technology can be implemented on Eppendorf ep*Motion* systems. Furthermore, the ep*Motion* was able to pipette low range volumes with extreme efficiency and accuracy in each tested pipetting approach, thereby ensuring reproducibility and also reducing per-reaction costs.

Product Use Limitations and Safety Information

Please read the In-Fusion Snap Assembly manual before performing the method for the first time.

Takara Ordering Information		
Product	**Size**	**Order no.**
In-Fusion® Snap Assembly Master Mix	10 Rxns	638947
In-Fusion® Snap Assembly Master Mix	50 Rxns	638948
In-Fusion® Snap Assembly Master Mix	250 Rxns	638949
In-Fusion® Snap Assembly Master Mix	500 Rxns	638943
In-Fusion® Snap Assembly Master Mix	1,000 Rxns	638944
In-Fusion® Snap Assembly EcoDry™ Master Mix	8 Rxns	638954
In-Fusion® Snap Assembly EcoDry™ Master Mix	32 Rxns	638955
In-Fusion® Snap Assembly EcoDry™ Master Mix	96 Rxns	638956

Eppendorf Ordering Information		
Product	**Order no. (INT)**	**Order no. (ENA)**
ep*Motion*® 5075t	5075000302	5075006022
Thermoadapter for skirted PCR plates, 96-well	5075787008	960002199
Thermoadapter for skirted PCR plates, 384-well	5075788004	960002091
Eppendorf twin.tec® PCR Plate 96 LoBind®, skirted, PCR clean	0030129512	0030129512
Eppendorf twin.tec® PCR Plate 384 LoBind®, skirted, PCR clean	0030129547	0030129547
epT.I.P.S.® Motion 10 µL PCR-clean, with filter, sterile	0030014391	0030014391
TM 10-8 eight-channel dispensing tool	5280000304	5280000304
Eppendorf PCR Sealing Film	0030127781	0030127781
MixMate®	5353000510	5353000529

For more information about Takara, please visit: https://www.takarabio.com/

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