Automated cloning using Takara Bio In-Fusion® Snap Assembly on the OT-2

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ABSTRACT

Automating the cloning workflow can increase throughput and minimize variability, but few cloning reagents are compatible with automation platforms. The automation of a rapid and simple cloning workflow on the Opentrons' OT-2 platform using the In-Fusion Snap Assembly kit was assessed.

Overview

- In-Fusion Snap Assembly reaction volumes of 5 µL and 10 µL were tested on a 96-well plate format.
- High cloning efficiency and accuracy were observed in samples prepared with the OT-2, demonstrating the ability of the OT-2 to accurately pipette low-range volumes.
- Automation of the In-Fusion Snap Assembly protocol on the OT-2 platform was successful, with the results comparative to those obtained using a manual pipetting setup.

INTRODUCTION

The development of high throughput cloning methods has enabled the rapid assembly of hundreds of genes in parallel for use in genomics and synthetic biology research. PCR-based, restriction enzyme-free cloning has replaced time-consuming methods like restriction enzyme-based ligation or TA cloning that are constrained by the sequences of vector and insert fragments. PCR-based, restriction enzyme-free cloning enables the insertion of any DNA fragment into any vector, at any locus, and within any sequence, and has become the preferred method for cloning.

Automation of the cloning workflow could increase throughput and minimize experimental variability, but few cloning reagents have been proven to be compatible with automation platforms. We have conducted a study to evaluate the automation of a rapid and simple cloning workflow on the Opentrons OT-2 platform using the In-Fusion Snap Assembly kit. In-Fusion cloning enables restriction enzyme-free, directional, and highly efficient (>95%) cloning with extremely low background with a simple protocol. We successfully demonstrate automation of the In-Fusion Snap Assembly protocol on the OT-2 platform.

METHODS

Overview of the automated cloning workflow

Figure 1 shows the experimental workflow for the In-Fusion cloning reaction in a 96-well plate using the OT-2 platform. While the entire cloning workflow demonstrated in the figure can be automated, we applied the automated function only to the set-up of the In-Fusion Snap Assembly reactions to verify the ability of the OT-2 liquid handler to accurately pipette the In-Fusion reagent.

Vector and insert preparation

The pUC19 vector (2.7 kb) was linearized with the Hind III restriction enzyme. The insert was a DNA fragment (3.7 kb) prepared by PCR amplification with a primer set including 15 bp sequences homologous to the ends of the linearized pUC19 vector.

Cloning reaction set-up

Using the OT-2 platform, thirty-five cloning reactions were assembled along with 'no insert' and 'water-only' negative controls. All reagents were pipetted into a 96-well plate (Bio-Rad 96 well plate # hsp9601) by the 8-channel pipette of the OT-2 (**Figure 2**). A pre-mixed solution containing 5X In-Fusion Snap Assembly Master Mix, linearized vector, and water was placed into the source plate in location 5 of the robot deck (**Table 1**).

In addition to the recommended In-Fusion reaction volume of 10 µL, a lower reaction volume of 5 µL was tested to assess the ability to scale-down the reaction volume (**Table 1**). The 9 µL premix solution (or 4 µL for the 5 µL reaction setup) was transferred into the wells of a 96-well destination plate (location 4 on the robot deck) by the 8-channel pipette. The 1 µL insert DNA fragment or water control was pipetted into each well of the destination plate by the 8-channel pipette and mixed by pipetting up and down 1 time at a speed of 2 µL/s.

Following completion of the protocol, the destination plate was removed from the OT-2 and sealed with a PCR film. The plate was incubated at 50°C for 15 minutes on a heat block, then stored at -20°C until further analysis.

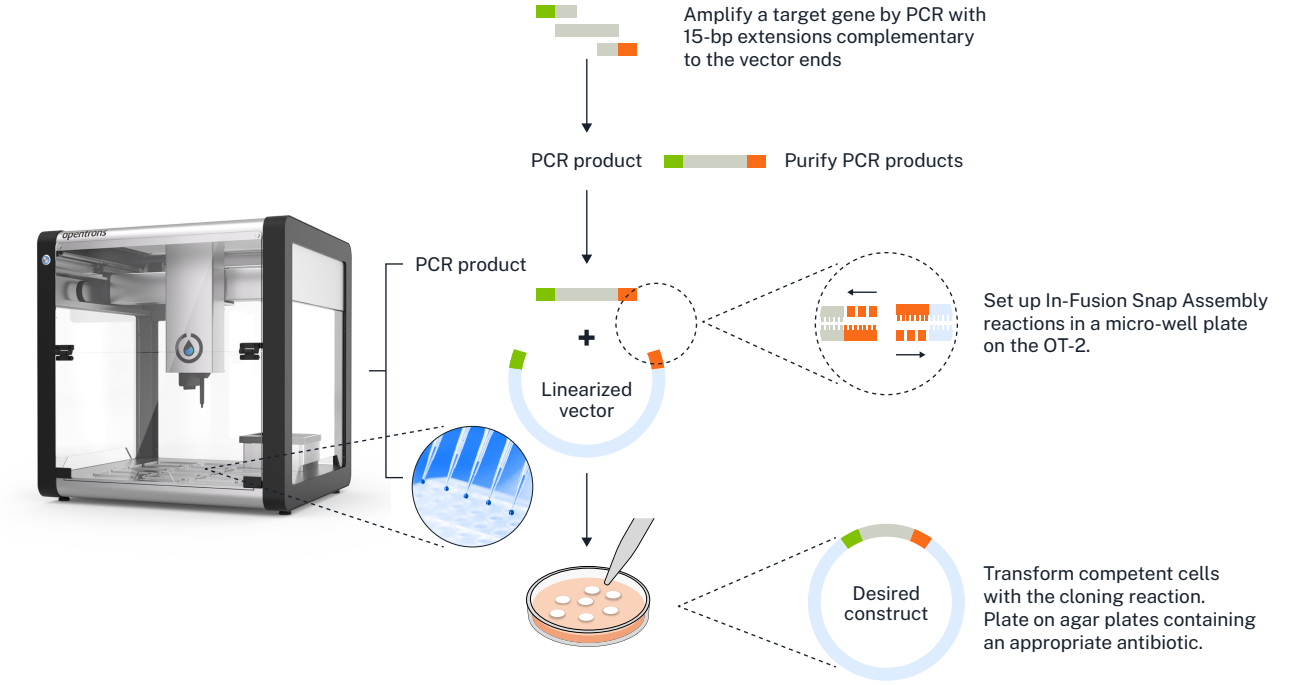

Figure 1. Automated In-Fusion Snap Assembly reaction setup using the OT-2 platform. A target sequence was PCR-amplified using forward and reverse primers with 5' ends homologous to the respective 5' and 3' ends of the linearized vector. After purification, the PCR product, a linearized vector, and In-Fusion Snap Assembly Master Mix were pipetted into a 96-well plate using an OT-2 liquid handler. The cloning reaction was transformed into competent cells and plated on selective plates for further analysis.

Reagent	10 µL reaction volume	5 µL reaction volume
5X In-Fusion Snap Assembly Master Mix	2 µL	1 µL
Vector	1 µL (53 ng)	1 µL (26.5 ng)
Insert	1 µL (147 ng)	1 µL (73.5 ng)
Water	6 µL	2 µL

Table 1. Required volume of reagents for 10 µL and 5 µL reaction volumes.

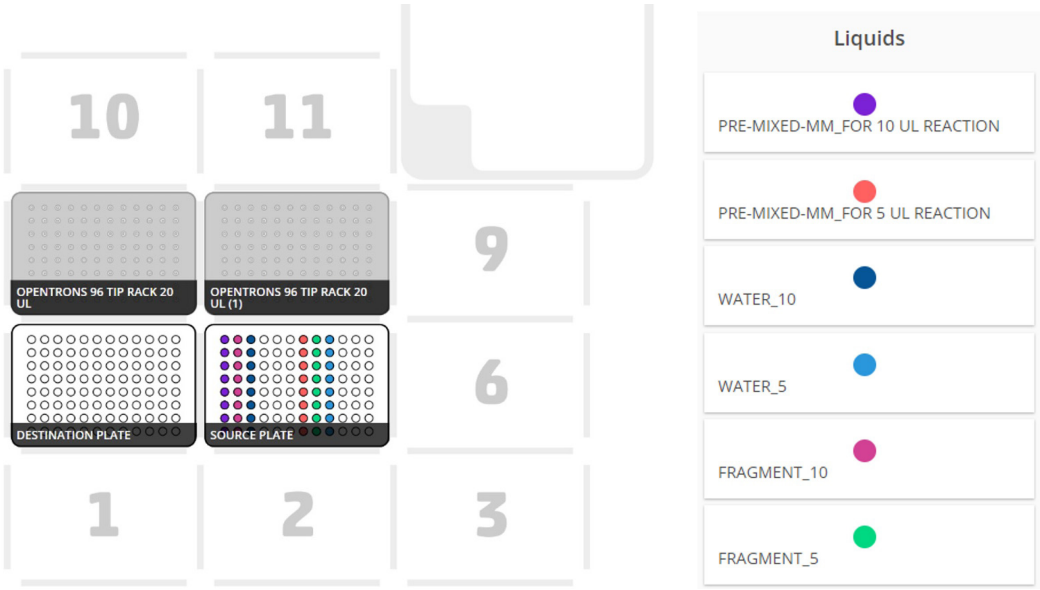

Figure 2. OT-2 work deck layout for In-Fusion Snap cloning reaction set-up. Purple: pre-mixed master mix for a 10 µL reaction volume. Red: pre-mixed master mix for a 5 µL reaction volume. Dark blue: water for a 10 µL reaction volume. Light blue: water for a 5 µL reaction volume. Magenta: insert fragment for a 10 µL reaction volume. Green: insert fragment for a 5 µL reaction volume.

Cloning product verification

Five samples from each reaction volume and a 'no insert' negative control group were randomly chosen from the 96-well plate and transformed into bacteria, respectively. 'Water-only' negative controls were also assessed for well-to-well cross contamination (data not shown). Ten random colonies were chosen from the array of plates corresponding to each reaction volume and analyzed by Sanger sequencing to determine the sequence accuracy. Sequences were required to be one hundred percent identical to the reference sequence to be counted as accurate.

RESULTS

Consistent CFUs

Both 10 µL and 5 µL reactions resulted in high numbers of CFUs (counts equivalent to observed CFUs resulting from samples prepared via manual pipetting, which suggested successful automation of the cloning workflow) (**Figure 3**). The small variability of the data suggests that automated cloning is consistent across replicate cloning reactions at each reaction volume. Minimal CFUs were present for the negative controls that had no DNA fragment insert, thus illustrating that there was minimal background from non-linearized vectors. No well-to-well cross-contamination was observed in 'Water-only' negative controls.

Sequence accuracy

Sanger sequencing was used to evaluate the assembled fragment sequences. The accuracy of the experiment was determined by the number of sequenced colonies that had a 100% sequence match to the reference sequence. All clones in the 5 µL reaction (10/10 or 100%) were proven accurate. One clone in the 10 µL reaction volume failed due to the presence of non-linearized vectors (9/10 or 90%).

CONCLUSION

The automation of a restriction enzyme-free and ligation-free cloning workflow using the In-Fusion Snap Assembly on the OT-2 platform was successfully demonstrated. The resulting number of CFUs was consistent with expected counts, while consistency was demonstrated among replicate reactions for two different reaction volumes. The accuracy of the cloning workflow was observed at levels above 90%. Our results suggest that the OT-2 liquid handler can successfully automate the cloning workflow using In-Fusion Cloning reagent.

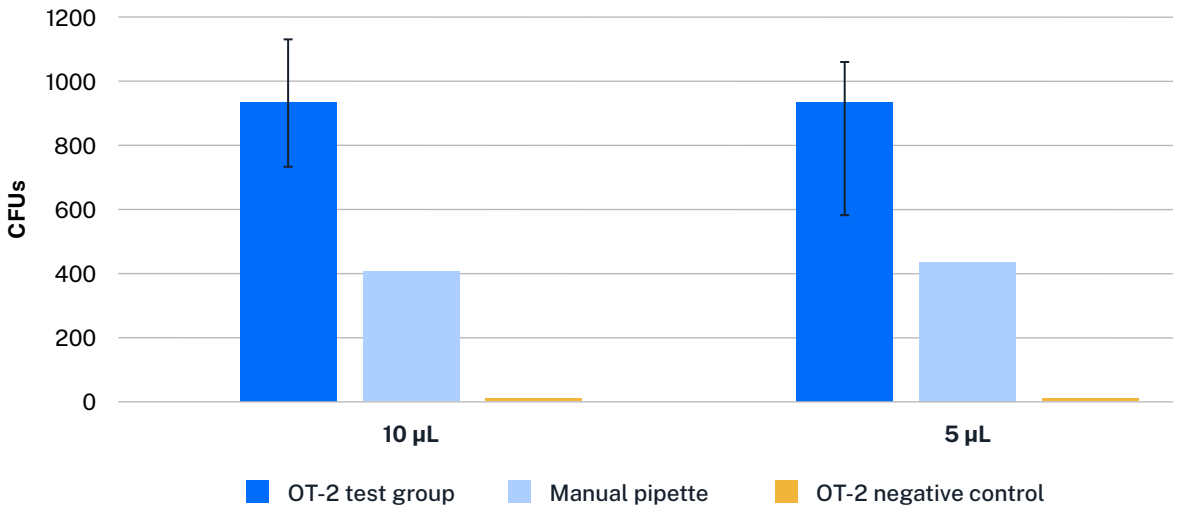

Figure 3. Enumeration of bacterial colony forming units (CFUs) after transformation with a reaction volume of 10 µL or 5 µL. Graphed values using the OT-2 platform are the average number of CFUs from three independent cloning reactions that either contained (OT-2 test group – Blue) or were missing (OT-2 negative control - Yellow) the DNA fragment insert. Error bars show ± standard deviations. A single cloning reaction using a manual pipette (Manual pipette - Light Blue) is shown as a reference.