Automating Sensitive Polymerase Chain Reaction Protocols Using the Opentrons Thermocycler on the Opentrons OT-2 Platform

ABSTRACT

There is an increase in demand for sensitive polymerase chain reaction (PCR) techniques to be automated. In this application note, we demonstrate that the Opentrons Thermocycler and the OT-2 generate the expected base-pair length for PCR amplicons when compared to a popular off-deck third-party thermocycler. The results show the expected base-pair length of PCR amplicons for both nested and multiplex PCR, which require precise and accurate primer concentrations for efficient amplification.

INTRODUCTION

There are challenges with the efficiency and accuracy of complex protocols, such as nested PCR and multiplexed PCR, due to the possibility of contamination, technical errors, and time constraints during sample preparation.

Multiplex PCR experiments are utilized for, GMO (genetically modified organisms) detection, forensics, food analysis, mutation and polymorphism detection, gene deletion analysis, template quantitation, linkage analysis, and many more applications.[1] These multiplex reactions are highly efficient and result in the product of two or more amplicons in a well.

Nested PCR, another highly specific technique, can be employed as a useful diagnostic tool for identifying pathogens in biological samples such as periodontal pathogens in atherothrombotic plaques, metastatic breast cancer cells, and viral pathogens such as *Mycobacterium tuberculosis* and *Penicillium marneffei.*[2,3,4,5] Unlike standard PCR that only requires one amplification step, nested PCR has a two-step amplification process. The PCR amplicons generated during the first PCR amplification step are used as the template for the second amplification step.

These compound PCR assays can be tedious and long depending on your final product. The Opentrons OT-2 can perform these PCR assays with very minimal manual preparation and variability across reactions.

MATERIALS AND METHODS

Multiplex PCR Protocol

Multiplex PCR was performed with genomic DNA isolated from *P. aeruginosa* and *S. aureus.* Forward and reverse primers specific for multiple loci regions were designed for *P. aeruginosa* (*lasI*, *lasR*, *gyrB*) and *S. aureus* (16s rRNA, *clfA*, *mecA*, Eubacterial 16S rRNAb) based on previous studies.[6] The expected products per well are 600, 700 and 222 bp. Twenty microliter multiplex reactions were prepared in a 0.1 ml 96-well Full-Skirt NEST plate on the Opentrons OT-2 on-deck thermocycler. The products were analyzed on a 2% agarose gel. The same experiment was prepared manually and run on a third-party manual thermocycler for comparison.

Nested PCR Protocol

Nested PCR is used to identify pathogens in biological samples isolated from lambda phage.[7] Forward and reverse primers specific for multiple loci regions were designed for *P. aeruginosa* (*lasI*, *lasR*, *gyrB*) and *S. aureus* (16s rRNA, *clfA*, *mecA*, Eubacterial 16S rRNAb) based on previous studies.[6] The OT-2 platform was supplied with PCR components that were transferred to the 0.1 ml 96-well NEST plate. The DNA template is added last, before mixing and automated sealing on the Opentrons Thermocycler Module. For the second amplification step, this process was repeated with a 0.1 ml non-skirted NEST plate, sealed, and placed on the third-party thermocycler. The amplicon from the first amplification was used as the

positive control. PCR products were analyzed on a 2% agarose. All experiments were quantified with Qubit BR and the coefficient of variation was calculated to evaluate amplification uniformity.

RESULTS

Automating the multiplex PCR protocol using the Opentrons Thermocycler on the OT-2 shows comparable amplification to the third-party thermocycler.

The experiment layout of the multiplexed PCR protocol was adopted from Kim *et al.* 2018, which shows that the sample wells were labeled green, and the negative control was marked blue **(Figure 1)**.

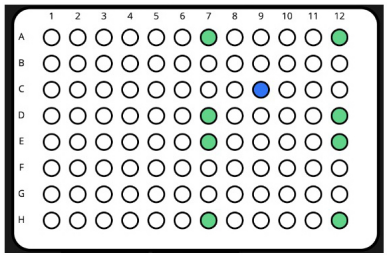

Figure 1: Wells tested for automated multiplex PCR experiments. Green wells indicate samples, blue wells indicate negative controls.

To evaluate the high sensitivity and specificity of automating the multiplex PCR protocol, the PCR products were visualized on a gel. The PCR amplicons generated on the OT-2 thermocycler not only showed clearer and evident bands but was the expected base-pair length (*lasI*, 600bp; *lasR*, 700bp; and *gyrB*, 222bp, respectively) **(Figure 2A)** compared to PCR amplicons generated by the third-party thermocycler **(Figure 2B)**.

To further demonstrate efficiency of the OT-2, an automated multiplex PCR protocol was performed using primers specific for S. aureus (16s rRNA, *clfA*, *mecA*, Eubacterial 16s rRNA) with expected base pair (bp) lengths of (791bp, 638bp, 499bp, 371bp, respectively) were visualized on an agarose gel **(Figure 2C)**. In parallel, manual multiplex PCR protocol utilizing a third-party thermocycler was performed using the same PCR components **(Figure 2)**. The multiplexed PCRs were automated on the OT-2 and manually performed using a third-party thermal cycler showed a similar amplification of P. aeruginosa **(Figure 2A, 2B)**.

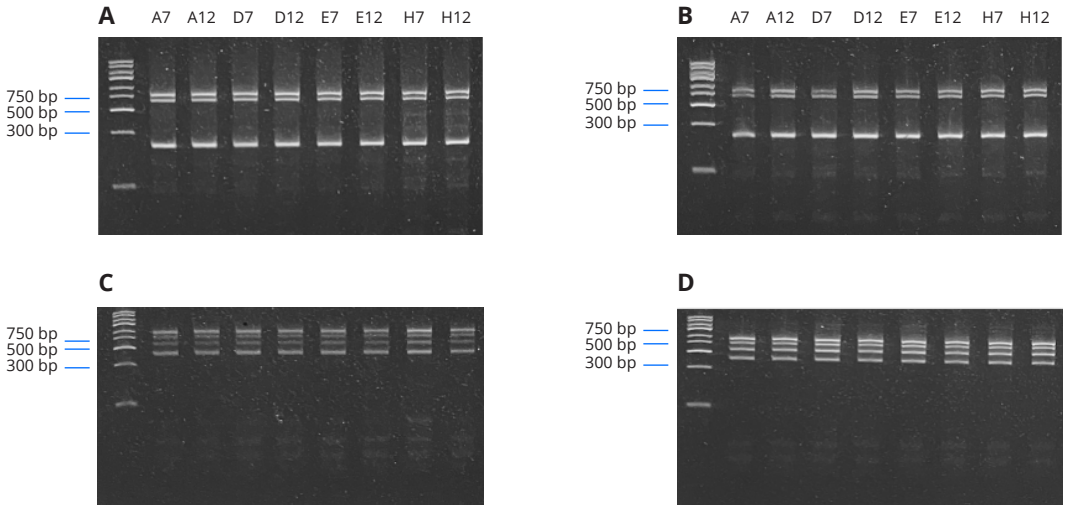

Figure 2: Uniform and efficient automated multiplex PCR of *P. aeruginosa* and *S. aureus* targets. The expected targets in *lasI*, *lasR* and *gyrB* are 600, 700 and 222 bp were amplified from *P. aeruginosa* genomic DNA in each well tested. A) Lanes 1 - 8 show products after automated pipetting and thermocycling on the OT-2. B) Lanes 1 - 8 show products after manual pipetting on a third-party thermocycler. The expected targets in 16s rRNA, *clfA*, *mecA*, Eubacterial 16S rRNAb are 791, 638, 499 and 371 bp were amplified from *S. aureus* genomic DNA in each well tested. C) Lanes 1 - 8 show products after automated pipetting and thermocycling on the OT-2. D) Lanes 1 - 8 show products after manual pipetting on a third-party thermocycler.

To validate the precision of the PCR products generated on the OT-2 versus third-party thermal cycler, the coefficient of variation (CV%) was analyzed and showed a lower or comparable amplification uniformity for multiplex and nested PCR experiments CV% on the OT-2 than the third-party thermocycler **(Table 1)**.

Table 1: Highly uniform amplification demonstrated across diverse template types and PCR assays. CV, coefficient of variation, was calculated after quantitation by Qubit is shown for each experiment amplified on the OT-2 and third-party thermocyclers.

PCR type	Organism	Thermocycler	CV (%)
Multiplex	*S. aureus*	OT-2	8.4
Multiplex	*S. aureus*	Third party	13.4
Multiplex	*P. aeruginosa*	OT-2	2.8
Multiplex	*P. aeruginosa*	Third party	9.1
Nested	Lambda (Outer)	OT-2	7.3
Nested	Lambda (Outer)	Third party	9.1
Nested	Lambda (Inner)	OT-2	9.9
Nested	Lambda (Inner)	Third party	8.8

Automating a sensitive nested PCR protocol on the OT-2 yielded PCR amplicons that were similar to the products generated by the third-party thermocycler

The layout for the nested PCRs show sample wells labeled in green and the negative control wells labeled in blue **(Figure 3)**.

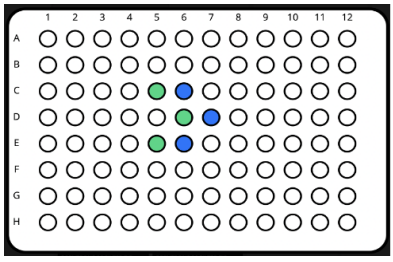

Figure 3: Wells tested for nested PCR experiments. Green wells indicate samples, blue wells indicate negative controls.

Two sets of primers were designed to detect 500-bp and 247-bp regions of the lambda phage template DNA. The 500-bp region was detected in the first PCR run, while the 247-bp region in the second PCR run. Samples were repeated in triplicate for nested PCRs, and then after the PCR run, the amplicons were analyzed.

The PCR amplicons generated on the OT-2 versus the third-party thermocycler were analyzed on an agarose gel **(Figure 4)**. The expected size of the PCR amplicon for the outer amplicon is 500 bp and the inner amplicon is 247 bp. The PCR amplicons showed similar yields and CV% **(Table 1)** highlighting the comparable performance to manual and third party i.e. no pipetting loss.

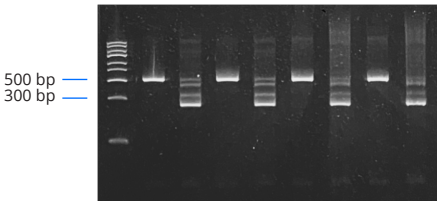

Figure 4: The Opentrons on-deck thermocycler is capable of reliably performing nested PCR. The outer amplicon is 500 bp and the inner amplicon is 247 bp. Lanes 1-4 show amplicons produced on the OT-2 in wells C5, D6 and E5 and lanes 5-8 were from a third-party thermocycler in wells C5, D6 and E5.

CONCLUSION

The comparative analysis between the Opentrons automated Thermocycler and the third-party manual thermocycler demonstrated precise amplification with expected base-pair length for PCR amplicons and a better amplification for PCR products on the OT-2. Additionally, Opentrons OT2 demonstrated accurate PCR of multiple targets in one well and the ability to amplify iteratively for long nested PCR assays.

REFERENCES

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