

High-Throughput *arcA* Genotyping of *E. coli* O26:H11 using the LightCycler® 1536 System

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Introduction

Escherichia coli O26:H11 are important enteric pathogens causing severe diarrhea and life threatening haemolytic and uremic syndrome (HUS). Among *E. coli* O26:H11, one can distinguish two groups of strains that differ in their pathogenicity: EPEC strains responsible for diarrhea, mainly in infants and children, and the more severe EHEC strains associated with hemorrhagic colitis and HUS.

These two groups cannot be easily distinguished phenotypically or based on their biochemical profiles. It has been shown that combining the molecular tools, *wzx*O26, *fliC*_{H11}, *eae*-beta, *stx*, *espK* and *arcA* genotyping is suitable for a rapid and specific identification of the most pathogenic strains of *E. coli* O26:H11 (Miko *et al.*, 2010; Bugarel *et al.*, 2011). The methods for detecting *wzx*O26, *fliC*_{H11}, *eae*-beta, *stx* and *espK* have been successfully adapted to high-throughput qPCR (Bugarel *et al.*, 2011).

Conventional real-time PCR genotyping of the *arcA* (aerobic respiratory control protein A) gene is based on a single-nucleotide difference (C/T) (SNP class I) between the published *arcA* sequences of an EHEC O26 and an EPEC O26. This single-nucleotide change remains to be established for high-throughput testing. In the present study, we assessed the feasibility of high-throughput *arcA* genotyping of *E. coli* O26:H11 using the LightCycler® 1536.

Samples

E. coli strains tested include 148 strains previously tested for their allelic type using direct nucleotide sequencing (Miko *et al.*, 2010; Neto M., personal communication), as well as 33 additional O26:H11 strains of unknown genotype. The concentrations of the samples were determined using a Nanodrop 2000 (Thermo), and ranged from 0.2 to 2.0 ng/ μ l.

Real-time PCR

For PCR setup of the LightCycler® 1536 Multiwell Plates, the Bravo Automated Liquid Dispenser platform (Agilent), equipped with a chiller and the PlateLoc thermal microplate sealer (Agilent) were used. The Bravo used a dispensing pattern of 1 mix versus 1536 samples with a final reaction volume of 1 μ l. This particular program uses the full 96-tips head of the robot. Using the same tip, the robot first aspirates 0.5 μ l of master mix, then aspirates an air bubble equivalent to 0.3 μ l, and then aspirates 0.5 μ l of sample, before dispensing everything in the LightCycler® 1536 Multiwell Plate.

This pipeting scheme is very fast, requiring only 15 minutes to load the full LightCycler® 1536 Multiwell Plate. This includes the time to change the source plate, and thorough mixing of the sample and master mix. The qPCR mix contained 0.5 μ l sample (corresponding to 0.1 to 1.0 ng DNA per reaction), and 0.5 μ l master mix containing 2x RealTime ready DNA

Probes master, 1 μ M each primer and 400 nM each probe, corresponding to 500 nM and 200 nM final, respectively.

During the dispensing process the destination plate was kept at a maximum temperature of +6°C to prevent evaporation. Before sealing, the plate was centrifuged for 1 min 30 at 1500 g. Sealing parameters of the PlateLoc were adjusted to +165°C and 2s.

The LightCycler® 1536 Real-Time PCR system was used with the following thermal profile: +95°C for 1 min, followed by 45 cycles of +95°C for 0 s (ramp: 4.8°C/s), and +60°C for 30 s (ramp: 2.5°C/s), and a final cooling step at +40°C for 30s. Software settings were Dual color hydrolysis probes /UPL probes and Master Control.

Resulting data were analyzed using the LightCycler® 1536 Endpoint Genotyping Analysis Tool.

Primers and MGB-conjugated probes were as described in (Bugarel *et al.*, 2011). MGB-conjugated probes were purchased from Applied Biosystems. The LNA-substituted probes arcA-P1b (5'-HEX-CAAATTCAGTCTC+G+T+GC-3') and arcA-P2b (5'-FAM-CAAATTCAGTCCC+G+TGC-3') were also used (+ indicates LNA bases). LNA-substituted probes were purchased from Tib Mol Biol.

Results and Discussion

The aim of our study was to assess the feasibility of high-throughput *arcA* genotyping of *E. coli* O26:H11 with the LightCycler® 1536 System using primers and probes previously developed for a conventional competitor system (96-well format). A secondary aim was to compare MGB-conjugated and LNA-substituted probes for detecting a class I SNP using the LightCycler® 1536 System.

For each type of probe (*i.e.*, probes with conjugated MGB and LNA-substituted probes), we analyzed in duplicate a set of 192 samples, including one no template control (NTC). The 192 samples tested included 148 samples that had previously been tested for their allelic type by nucleotide sequencing (Miko *et al.*, 2010; Neto M., personal communication).

The system proved to be robust and adaptable as the primers and probes developed for a competitor system (MGB-conjugated probes) were directly applied on the LightCycler® 1536 System without modification or optimization. Similar-

ly, the LNA-substituted probes were designed just so as to keep the same sequence and melting temperature of the probes and were used without protocol modification.

In total, 768 reactions were performed, and there were no PCR failures. The sensitivity of the LightCycler® 1536 was very satisfactory. The Cp-values obtained using 0.1 to 1 ng of template DNA ranged from 19.23 to 27.44 and from 18.91 to 26.71 using the MGB-conjugated FAM and VIC probes respectively (Figure 1). Cp-values ranged from 18.94 to 26.28 and from 17.46 to 28.02 using the LNA-substituted FAM and HEX probes, respectively (Figure 2). Cp-values were slightly higher than expected for sample concentrations spread over one log, however samples were not homogeneous due to the different extraction methods used for different samples. Thus, different DNA quality and presence of external components best explain this expanded range in Cp-values. One sample, although highly concentrated (2 ng/ μ l), consistently produced late Cp values (>30) in duplicates, using both types of probes. This was most likely

due to poor DNA quality or presence of inhibitory substances in the sample. This sample was not included in the analysis.

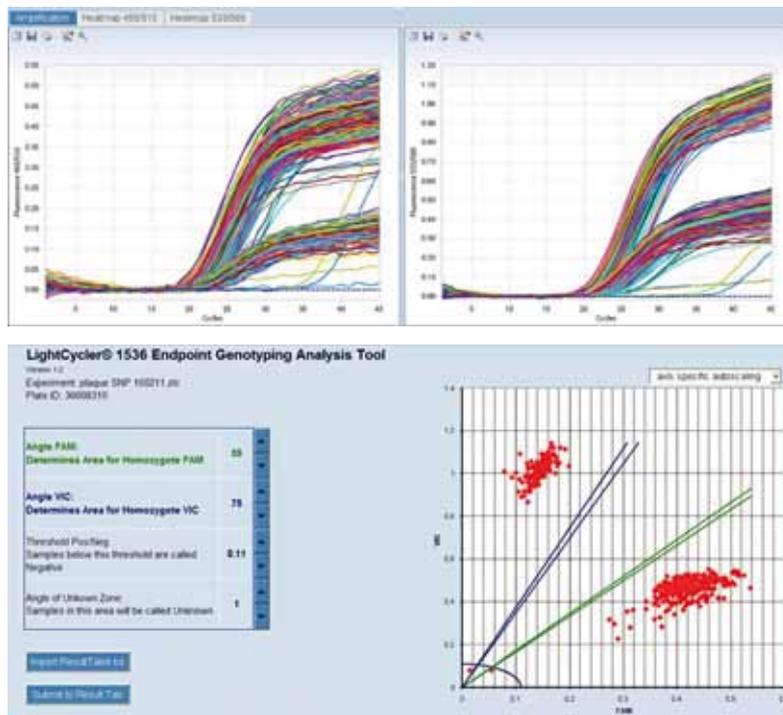
The analysis software was able to call each genotype. The results obtained showed excellent reproducibility, as all duplicates gave the same result. Similarly, the results obtained with the MGB-conjugated probes and with the LNA-substituted probes were identical, indicating that both types of probes can be used for genotyping class I SNP.

For the 148 samples previously characterized by sequencing, we obtained 100% concordance, demonstrating that high-

throughput *arcA* genotyping of *E. coli* O26:H11 can easily be implemented using the LightCycler® 1536 System.

As shown in Figure 2, panel A (compare with Figure 1, panel A), the FAM-labeled LNA-substituted probe showed a reduced nonspecific binding compared to the MGB-conjugated probe, as it did not bind to the mismatched sequence. This lower rate of mismatch detection was obtained without optimization in the design of the LNA-substituted probes. In all likelihood this binding to the mismatched sequence could be completely eliminated by carefully choosing the nucleotides substituted, for example by substituting the polymorphic nucleotide with an LNA base.

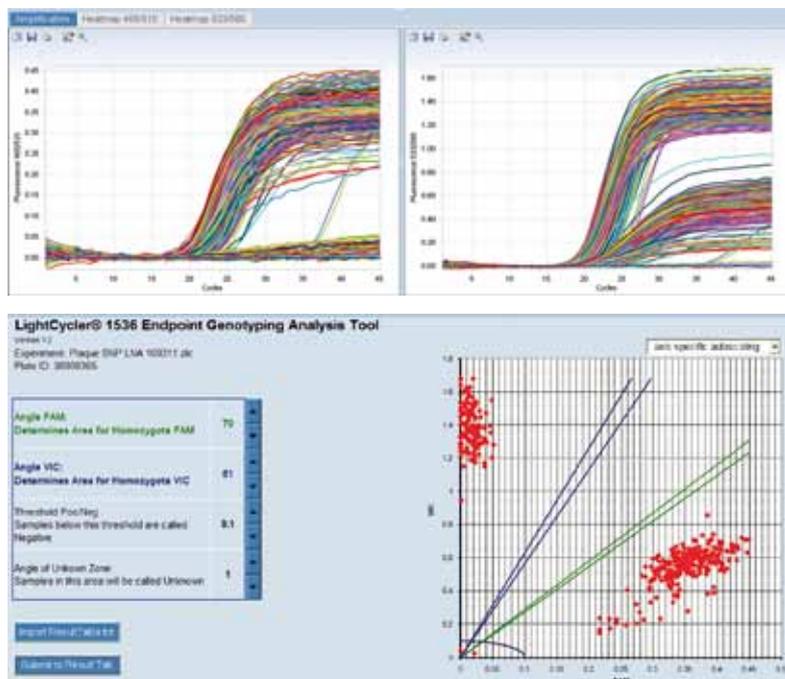
Figure 1: *arcA* genotyping of *E. coli* O26 using the MGB-conjugated probes



Panel A: Amplification curves using the MGB-conjugated probes. FAM channel is shown on the left and VIC/HEX channel is shown on the right.

Panel B: Automated genotype calling using the LightCycler® 1536 Endpoint Genotyping Analysis Tool.

Figure 2: *arcA* genotyping of *E. coli* O26 using the LNA-substituted probes



Panel A: Amplification curves using the LNA-substituted. FAM channel is shown on the left and VIC/HEX channel is shown on the right.

Panel B: Automated genotype calling using the LightCycler® 1536 Endpoint Genotyping Analysis Tool.

Conclusion

In this study, we successfully adapted high-throughput *arcA* genotyping of *E. coli* O26:H11 using the LightCycler® 1536 System. We demonstrate that the LightCycler® 1536 System can be used for high-throughput *arcA* genotyping, in concordance with the other developed tools (detection of *wzx*O26, *fliC_{H11}*, *eae*-beta, *stx* and *espK*), for rapid and specific identification of the most pathogenic strains of *E. coli* O26:H11. The system was robust and reliable. We obtained a call rate of

100% and 100% concordance with sequencing, as well as 100% concordance between duplicates (intra- and inter-plates). The LightCycler® 1536 used in combination with the Bravo liquid dispenser automat (Agilent) proved to be fast. We can set up the reactions, load the plates, run the reactions and analyze the results in less than 90 minutes. The small volume used by the LightCycler® 1536 System allowed us to conserve reagent and precious sample amounts.

References

1. Bugarel, M., Beutin, L., Scheutz, F., Loukiadis, E., Fach, P., 2011. Identification of genetic markers for differentiation of Shiga toxin-producing, enteropathogenic, and avirulent strains of *Escherichia coli* O26. *Appl Environ Microbiol* 77, 2275-2281.
2. Miko, A., Lindstedt, B.A., Brandal, L.T., Lobersli, I., Beutin, L., 2010. Evaluation of multiple-locus variable number of tandem-repeats analysis (MLVA) as a method for identification of clonal groups among enteropathogenic, enterohaemorrhagic and avirulent *Escherichia coli* O26 strains. *FEMS Microbiol. Lett.* 303, 137-146.

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