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ERRATUM

Erratum to: An integrated disposable device for DNA extraction and helicase dependent amplification

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Abstract In the original manuscript, we reported the demonstration of an integrated microfluidic chip that performed helicase dependent amplification (HDA) on samples containing live bacteria. Bacterial lysis, nucleic acid extraction, and DNA amplification with a fluorescent reporter were incorporated into a disposable polymer cartridge format. We reported that the device was able to detect as few as 10 colony-forming units (CFU) of *E. coli* in growth medium. While the main conclusions of the original paper remain sound, the data presented in support of those conclusions contained errors that we detail, discuss and correct here. In short, we misidentified a non-specific product as a specific product of our HDA reaction. We incorrectly called reactions containing the non-specific product (length 70 bp) positive. Further investigation demonstrated that our primer set was faulty and not capable of amplifying the specific product. Here we redesigned primers, sequenced all of the products and reran all of the experiments reported previously to generate a new, verified dataset.

Keywords DNA · Bacteria · Helicase dependent amplification · Isothermal amplification · Microfluidic chip · Plastic · Solid phase extraction

1 Introduction

Soon after the original paper was published (Mahalanabis et al. 2010), it was brought to our attention by Mr. Gerd Grosshauser, Application Specialist, Qiagen GmbH, Hilden, Germany, that our 70 base pair product was likely a non-specific product. We immediately reran our experiments with the primer sets that were used in the original paper, sequenced the products, and confirmed that indeed our 70 base pair product was non-specific. We then notified the editors of *Biomedical Microdevices* and took steps to rectify our error.

First, we attempted to design new primers for the same gene that would only amplify the specific product we had originally sought, the *dxs* gene in *E. coli*. After consulting with experts in helicase dependent amplification (Vincent et al. 2004), and after designing and trying several new primer pairs, we were unable to perform quantitative studies with the *dxs* gene. Our failure was due to the fact that the commercial helicase enzyme mix preparations contain detectable background *E. coli* genomic DNA. In order to validate our assay, we needed to target a gene that was not present in this background DNA but was present in our test strain. As a result, we stopped using *E. coli* DH5α as our test

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strain and designed an assay for a gene specific to a pathogenic *E. coli* (EHEC 0157:H7; ATCC, Cat #700728) as suggested by Mr. Grosshauser and his colleagues at Qiagen to avoid non-specific background amplification.

The methods, results and discussion presented here describe our efforts to generate correct data that support the conclusions of the original paper. We believe we have now been successful, and we regret the errors in our previous report and any inconvenience they may have caused other investigators.

2 Methods

2.1 Chip design and chip fabrication

The chip design and the chip fabrication methods are the same as in the original paper (Mahalanabis et al. 2010).

2.2 Primer redesign and assay development

The new primer sequences used here were given to us by Mr. Grosshauser at Qiagen and target the *cesT* gene as determined from whole genome sequence annotations of strains enterohemorrhagic *E. coli* (EHEC) 0157:H7, EDL933 and TW14359 (Elliott et al. 1999). The target region is in the *cesT* (chaperone for E. coli-secreted protein Tir) coding sequence, mapping to position 4668461-4668532 of EDL933 (accession NC_002655.2), noted as Orf U hypothetical protein, and position 4658467-4658937 of TW14359 (accession NC_013008).

This region is within a pathogenicity island encoding genes essential for attaching and effacing functions that cause EHEC disease including the *tir, cesT*, and *eae* (Elliott et al. 1999; Kulasekara et al. 2009).

2.3 Bacterial culture

The target gene had to be changed to *cesT*, a gene present in pathogenic *E. coli,* EHEC 0157:H7 (ATCC, Cat#700728), but not in the HDA enzyme mix preparations or in the original strain used, DH5α. The bacteria samples were prepared as described in the original paper with the following changes. The bacteria were grown in nutrient broth and nutrient agar media instead of LB broth and LB agar. Also, EHEC 0157:H7 was pelleted by centrifugation at 12,000 g for 15 min to prepare the dilutions.

2.4 PCR and HDA amplification and detection

The PCR and HDA amplification reagents and cycling conditions are the same as those used in the original paper except for the following. The *cesT* primers used for PCR and HDA amplification are forward primer 5′ CCCA TATCTGTGCTATGAGGCTGGAG 3′ and reverse primer 5′ GGTAGCATCATCGAGAGGGAAACGTAA 3.′ The amplicons generated were 73 bp in length. Additionally, after publication of the original paper, a newer generation of the HDA enzyme kit became available (BioHelix, Beverly, MA, IsoAmp® III Enzyme Mix, Cat# H1020S). We used the new kit to generate the data in this report.

2.5 Sequencing protocols

In light of our difficulty separating non-specific from specific products using both gel and capillary electrophoresis, in this report we have sequenced all relevant products. Some of the sequencing data is presented here and some of it is presented as supplementary data. Sequencing the HDA products required cloning the products into the pGEM-Teasy vector (Promega, Madison, WI). The cloning strategy required the addition of restriction enzyme sites with 4 bp overhangs at the 5′ ends of both the forward and reverse primers used for the HDA reactions. The oligos used to generate HDA amplicons for cloning into identical restriction sites in the vector are forward oligo 5′ CTATACTAGT CCCATATCTG TGCTATGAGG CTGGAG 3′ and reverse oligo 5′ GAATGACGTC GGTAGCATCA TCGA GAGGGA AACGTAA 3′. The forward oligo contains an Spe I site (ACT AGT) preceded with the 4 bp overhang CTAT. The reverse oligo contains an Aat II site (GAC GTC) preceded with the overhang GAAT. Amplicons were purified with phenol chloroform extraction, digested overnight at 37°C (SpeI and Aat II from New England Biolabs (NEB), Beverly, MA), gel purified, ligated to the vector for 1 h at room temperature (T4 ligase, NEB) and transformed into TOP10F' cells (Invitrogen, Carlsbad, CA). One to two successful transformants were sequenced bi-directionally with M13 forward and reverse primers (Invitrogen) and compared to *E. coli* O157:H7 reference strains in GenBank.

3 Results

In this section, we report results to replace the incorrect results in the original manuscript. Specifically, the data in Table 1 and Table 2 replace the original Fig. 2 and the data in Fig. 1 replaces the original Fig. 3. Supplementary data showing PCR validation (Fig. S1), HDA validation (Fig. S1) and amplicon sequence alignments (Fig. S2) are included.

3.1 Off-chip amplification from on-chip bacterial DNA extraction

The qPCR and HDA amplification assays were compared in tube using chip-extracted samples (Tables 1 and 2). The

Table 1 HDA and PCR threshold cycles for increasing dilutions from 10^1–10^5 CFU/ml of *E. coli* in growth medium. Although not a strict linear relationship, the threshold values show an increasing trend as the input bacteria concentration decreases for the HDA reactions. The trend is much more linear in the case of PCR

CFU/ml	Avg Ct ± SD		*p*–value*
	HDA	PCR	
10^1	20.4±4.9	34.1±1.4	<0.0001
10^2	18.4±0.9	33.4±1.2	<0.0001
10^3	26.4±2.8	31.2±1.2	0.0014
10^4	19.6±1.2	29.8±0.4	<0.0001
10^5	17.8±0.4	26.8±0.5	<0.0001

*two-tailed unpaired *T*-test, 99% CI

average C_T value and time to detection of the HDA amplicon was significantly lower and faster, respectively, than the PCR amplicon. The endpoint product yield as measured using capillary electrophoresis was similar for both on-chip and themocycler methods at all dilutions (Fig. 1(a)).

E. coli cells were resuspended into dilutions of 10^5 to 10^1 CFU/ml. DNA was extracted from each dilution using microSPE columns. The DNA extracted from each channel (N=5) was amplified using the qPCR assay *in tube* as described above. 5 extractions were amplified via qPCR for all of the dilutions. 5/5 channels had amplifiable DNA using off chip PCR (Fig. 1(b)).

DNA from N=3 channels was amplified off chip using the redesigned HDA assay. At the higher concentrations (10^5 and 10^4 CFU/ml), 3/3 extractions amplified in the tube (Fig. 1(c)). For the lower concentrations (10^3–10^1), only 1/3 channels yielded amplifiable DNA by HDA in the tube reactions. The endpoint DNA amplicon yield following PCR and HDA in tube were comparable for 10^3 CFU and greater (Fig. S1). It is notable that in the case of 10^2, the

Table 2 Time to detection of amplicons for both amplification methods. The HDA method is faster to result in most cases, but the differences between PCR and HDA are much smaller than in the original report

CFU/ml	Avg time (min) to Ct ± SD		*p*-value*
	HDA	PCR	
10^1	40.7±9.8	54.7±1.8	0.0023
10^2	36.8±1.8	53.8±1.4	<0.0001
10^3	52.7±5.5	51.0±1.5	0.3735
10^4	39.2±2.3	49.3±0.6	<0.0001
10^5	35.6±0.9	45.5±0.6	<0.0001

*two-tailed unpaired *T*-test, 99% CI

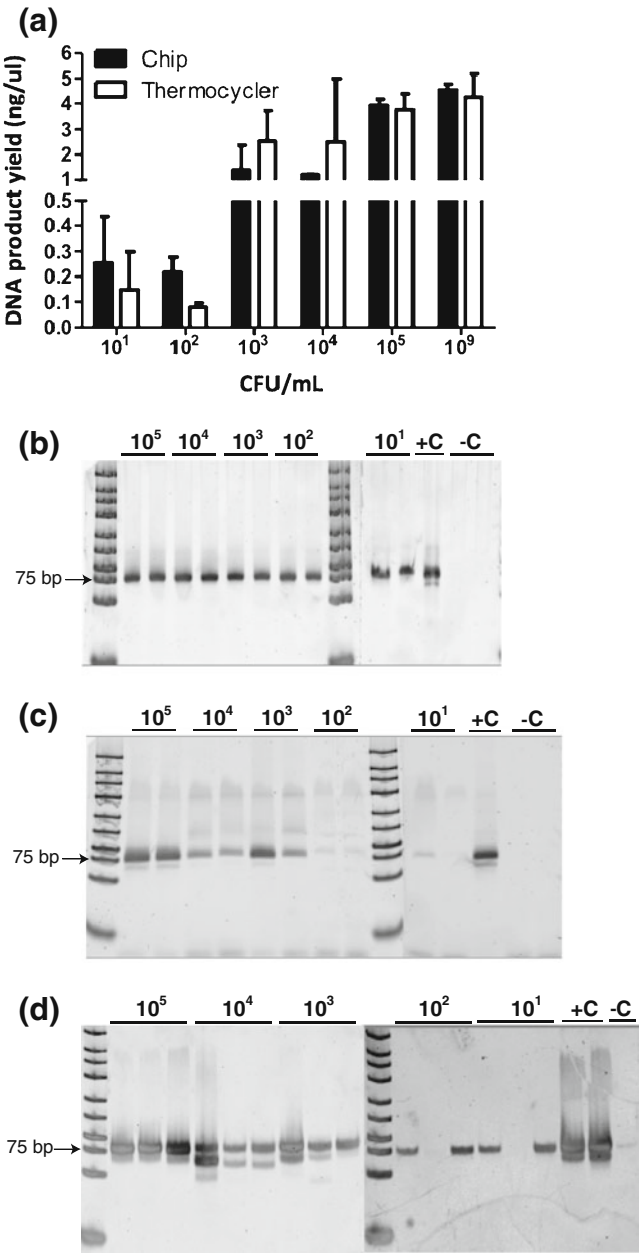

Fig. 1 (**a**) Sensitivity of *E. coli* O157:H7 HDA on chip and tube. (**b**–**d**) Amplicon size verification of PCR and HDA in tube thermocycler products and HDA on-chip products on 12% PAGE. "-C" is the template negative control, and "+C" is PCR/HDA reaction with *E. coli* genomic DNA. (**b**) Representative PCR thermocycler products from one out of five extractions is shown. Duplicates seen on the gel are duplicate PCR reactions performed using DNA extracted at multiple CFU dilutions, (**c**) Representative HDA thermocycler products from one out of three extractions is shown. Duplicates seen on the gel are duplicate HDA reactions performed using DNA extracted at multiple CFU dilutions, (**d**) Representative on-chip HDA products from one out of three extractions is shown. Triplicates seen on the gel are triplicate HDA reactions performed for DNA extracted at multiple CFU dilutions

bands are very faint, and in the 10^1 case, only one lane shows a product, both indicating that the threshold of the limit of detection for on-chip extraction followed by in tube HDA amplification is around 10^2 CFU/ml.

Both 12% PAGE gels and sequencing reactions confirmed that the amplicons were real products (Figs. 1(b–d) and S2).

3.2 Detection of bacteria on the integrated μSPE–HDA chip

The same five dilutions (10^5 to 10^1 CFU/ml) were made, extracted with microSPE channels and subsequently amplified using HDA *on-chip*. The 12% PAGE gel in Fig. 1(d) shows the correct amplicon size. Again, in the case of 10^2 and 10^1 CFU/ml case, only one lane of three shows a product, both indicating that the limit of detection for on-chip extraction followed by in tube HDA amplification is above 10^2 CFU/ml.

Tables 1 and 2 list the average C_T and time to detection for both sets of reactions. The PCR C_T values decrease roughly linearly as the number of CFU increases, as expected. A similar trend is expected with the HDA reactions. While it is true that the C_T values decrease with increased concentration, the trend is not linear. While we see a linear relationship in this assay with spiked genomic DNA, we do not see a clean linear effect with whole bacteria even with repeated trials.

Sequencing of on-chip and in tube HDA amplicons was performed by amplifying *E. coli* DNA from a 10^9 CFU/ml sample and cloning the products. All clones showed identical sequences to the reference *E.coli* 0157:h7 strains as expected in the correct target *cesT* gene (Fig. S2).

4 Discussion

We are confident at this point that the data presented is accurate, and that this new data supports our original conclusions with the following modifications. First, the speed of the HDA assay is not appreciably faster than our quantitative PCR at lower bacteria concentrations. Second, designing suitable primers for HDA is significantly more difficult than designing comparable primers for PCR, so this tempers our enthusiasm a bit.

We include here a short discussion about the origins of our errors in the original paper. First, we misidentified a nonspecific product as a specific product in both capillary electrophoresis and in slab gel electrophoresis. The products of HDA reactions are small by design, and can be confused with long nonspecific products. Thus, we highly recommend sequencing all products until a reasonable confidence in any new assay is achieved. Second, we should have been alerted to our error when the threshold cycle values in our spiked dilution experiments did not increase with decreasing amounts of genomic DNA in the HDA reactions. We saw the appropriate threshold value increases in our quantitative PCR, but not in our HDA. We accepted these results as real based on literature reports that stated that HDA is not always linear in this respect (Chow et al. 2008; Goldmeyer et al. 2007, 2008; Tong et al. 2008). These explanations were not appropriate for the observed discrepancies in our data.

5 Conclusion

We present these corrected experiments in good faith, and regret any inconvenience our errors may have caused other researchers.

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