MicroCAP: Microfluidic Centrifuge Assisted Precipitation for DNA Quantification on Lab-on-DVD

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ABSTRACT

We report for the first time the MicroCAP technique, for rapid DNA detection and quantification, that does not require any purification or fluorescent labelling of DNA. The invention is based on DNA interacting with a detection dye (Gelred) to form a complex, that forms a visible precipitate within seconds of centrifugation. MicroCAP can be used for DNA quantification, when combined with the Lab-on-DVD with inbuilt centrifugation and sub- micron imaging resolution. We quantify PCR and LAMP assay products using MicroCAP on the integrated Lab-on- DVD platform, and demonstrate a detection limit of 10 ng/$\boldsymbol{\mu l}$.

KEYWORDS: MicroCAP, DNA detection, Centrifuge,Precipitate, LAMP, PCR.

INTRODUCTION

Detection of amplified DNA is often based on measurement of turbidity, fluorescence (after staining with a detection dye) or absorbance. Commercially available instruments for DNA quantitation can be broadly divided into two categories: UV instruments based on absorbance (such as spectrophotometers, e.g. Nanodrop or Nanophotometer) and instruments based on measurement of a fluorescent dye (such as plate readers). One bottleneck in quantifying amplified DNA in a nucleic acid amplification test (NAAT) reaction, based on absorbance measurement technique, is the bias introduced due to the presence of the isothermal amplification buffer, dNTPs and other reagents. Each reagent or buffer may have an absorbance density at around 260 nm, elevating the apparent concentration measured by the device compared to the actual value. Hence, for most quantitation based NAATs, it is important to include an extra DNA purification step, which may result in non-negligible loss of the amplified product and increases the cost of the purification kit. Measurements based on fluorescence mostly use fluorescent dyes that are potentially hazardous for handling. In addition, fluorescence based quantitation methods require time consuming labelling and washing steps.

In this report, we describe a new method, termed microfluidic centrifugation assisted precipitation (microCAP), involving quantification and detection of DNA based on precipitation of nucleic acids. The basis of the method is formation of a visible precipitate when GelRed, a nucleic acid intercalacting dye commonly used in gel electrophoresis, is mixed with DNA and centrifuged. A visible precipitate is formed after just a few seconds of centrifugation and enables rapid detection of the presence of DNA in a sample. To the best of our knowledge, the visible precipitate formed as a product of centrifuging GelRed mixed with DNA has not been reported before. We showed that the DNA GelRed complex is dense enough compared to water to precipitate upon centrifugation. Further, we extended the μCAP method to the Lab-on-DVD platform[1] to quantify the DNA concentration from images generated using the optical DVD reader instrument. The modified DVD player was able to image the precipitate formed up to a detection limit of 10 ng/μl of DNA. For calibration of the images, known quantities of a purified PCR product were used to identify the relationship between the amounts of DNA and precipitate formed. We applied the method to quantify an unknown quantity of LAMP amplicons from a LAMP assay for a HIV-1B type genome containing plasmid on the Lab-on-DVD platform. A sensitivity limit of 10 ng/μl of DNA was achieved, comparable with that of a Nanophotometer.[18] The results demonstrated that the method is able to quantitatively detect the presence of DNA in a sample in a few seconds without any purification step.

EXPERIMENTAL

The Lab-on-DVD system was employed for spinning and imaging the precipitate product using a modified DVD drive, as mentioned in our previous report.[1] We began by dispensing the sample in the design chamber, adding GelRed dye (at a concentration of 4000X in water) and centrifuging the mixture at 1200 rpm. Figure 1a and 1b

show schematics of the DNA sample precipitation process conducted in test tubes and the DVD platform, respectively. We used known amounts of a PCR product to calibrate the quantity of precipitate to the DNA concentration. We used a HIV genome amplified from 50 ng of plasmid pNL4.3 using the primers 0776F and 6231R.[2] To evaluate the sensitivity of DNA detection of our system, we used the amplified products from a LAMP assay. The sensitivity of LAMP primers was tested on DNA from pNL4.3 (a HIV-1B genome containing plasmid). A 25X LAMP primer mix was prepared according to Curtis et al.,[3] using the same template DNA sequence, set of primers and DNA polymerase. Eight concentrations (each being 5 µl volume) of the HIV-1B genome containing plasmid (pNL4.3) were tested, starting from 1 **ng/μl** serially diluted to 1 fg/μl. Two negative controls were also prepared, one without DNA and primers and one without primers. The total reaction volume was increased to 30 µl (instead of 25 µl used in Curtis et al.[3]) by multiplying every component volume in the reaction by a factor of 1.2. Fabrication of the multilayer microfluidic Disc followed the same procedure as described in our previous report.[1] The Lab-on-DVD system was used to generate images of the precipitation zone. To quantify the amount of precipitate, an image processing script was written in MATLAB software (Mathworks, USA).

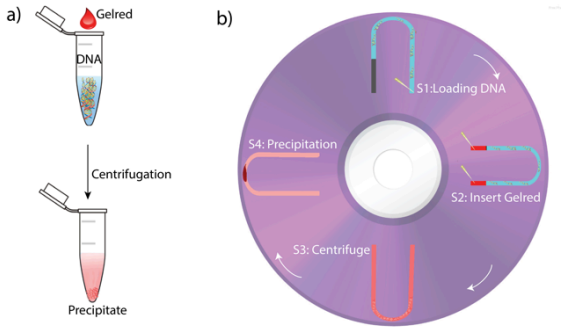

Fig.1 Schematics of the precipitation process. a) Sample of DNA in contact with GelRed in a test tube, forms a high-density complex which precipitates upon centrifugation. b) Precipitation steps on the DVD. Step 1: loading DNA into a U-shaped channel, Step 2: inserting GelRed, Step 3: centrifuging, Step 4: formation of precipitate.

RESULTS AND DISCUSSION

MicroCAP was found to be suitable for determining the presence of DNA in a sample, We carried out the LAMP assay in Eppendorf tubes in an oven set at 65°C. After 45 minutes, 3 µl of 10,000X GelRed in water was added to two tubes of 30 µl volume each, one having an unknown concentration of LAMP amplified DNA and the other one with no DNA template as a control. After centrifugation for approximately 5 seconds, a visible precipitate was formed in the tube containing amplified DNA, whereas no precipitate was formed in the control tube (Fig. 2a). 10 µl volume of DNA was inserted into a U shaped channel of the DVD alongwith 1 µl of 10,000X GelRed in water, which was the same ratio of DNA sample to Gelred as used in the test tube. An imageable precipitate was observed in the Lab on DVD custom imaging software (fig.2b).

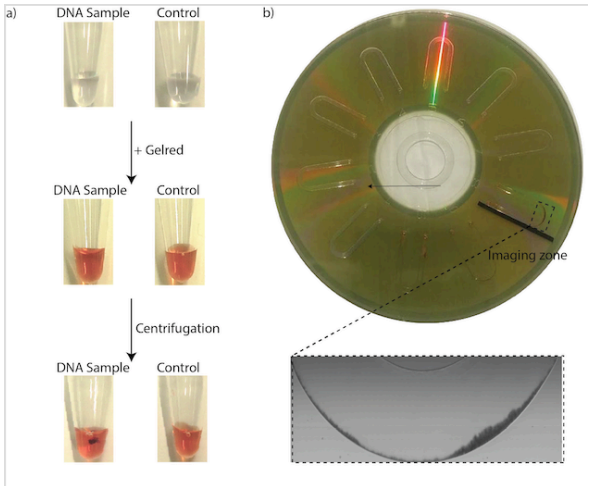

Fig.2 a) Qualitative detection of DNA in Eppendorf tubes upon addition of GelRed followed by centrifugation. b) Generated image of the precipitate formed on the DVD.

A Matlab script was used for image analysis in which an original image(fig.3a) was transformed into a binary image (fig.3b) by defining a threshold pixel value, exploiting the difference in intensity of the precipitate from its background. The entire area to the left of the threshold line in the histogram (Fig. 3c), i.e. from value 0 to the threshold value (normally 90), was summed to estimate the total area of the precipitate.

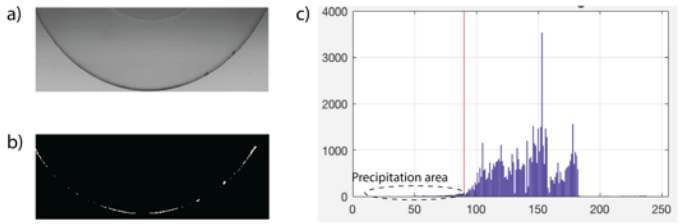

Fig.3 Image analysis code using MATLAB to convert a) original image to b) binary image, based on selection of a threshold value, c) in the histogram the entire area to the left of the threshold value was summed to evaluate the precipitation area.

For DNA quantification, known concentrations of a PCR product was used for calibration. The initial concentration of purified PCR product was 129 ng/µl, measured with a Nanophotometer (in triplicates) after purification with a GeneJet PCR purification kit. The purified PCR product was subsequently diluted serially several times and each diluted concentration was measured again with the Nanophotometer (in triplicate). The measurements were then repeated with the Lab-on-DVD method. Fig. 4a shows four images recorded at four known concentrations together with their binary threshold images. Fig. 4b shows the precipitation area calculated from the images plotted against the known DNA concentrations, showing a linear relationship. 10 ng/µl was the lowest concentration detectable in the DVD images.

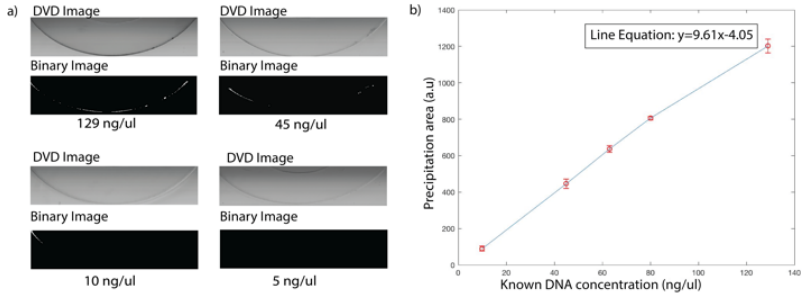

Fig.4 a) DVD generated images of a known PCR product. b) Precipitation area vs. known DNA concentration for the generated images.

For quantification of unknown quantities of nucleic acids, we carried out the LAMP assay on HIV-1B genome containing plasmid DNA using serial dilutions (10-fold dilutions from 1 ng/µl to 0.1 fg/µl) to evaluate the limit of detection (Fig.5). Two negative controls were also prepared, one comprising primers and no DNA template and second, no DNA template and no primers.

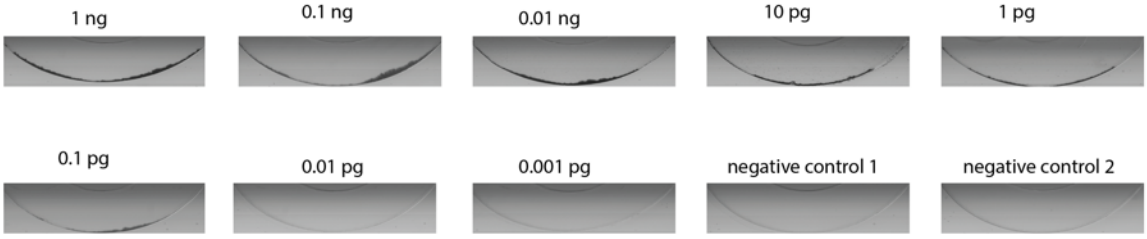

Fig.5 Images generated from DVD with eight serials 10 fold dilutions of plasmid DNA starting with a template concentration of 1 ng in 10 µl and two negative controls. Images generated for each of these sample concentrations of DNA shown. Negative control 1 denotes the LAMP assay without primers but DNA polymerase while negative control 2 depicts the absence of both primers and DNA polymerase.

Fig. 6 shows the precipitation area plotted against the starting concentration of DNA template. It shows that the amplification in the LAMP assay is not linear for all the starting concentrations of DNA template. The error bars in the figure show the

standard deviation for a particular concentration. For a LAMP assay, which fluctuates somewhat in its yield of amplified products, we believe that this error range is acceptable.

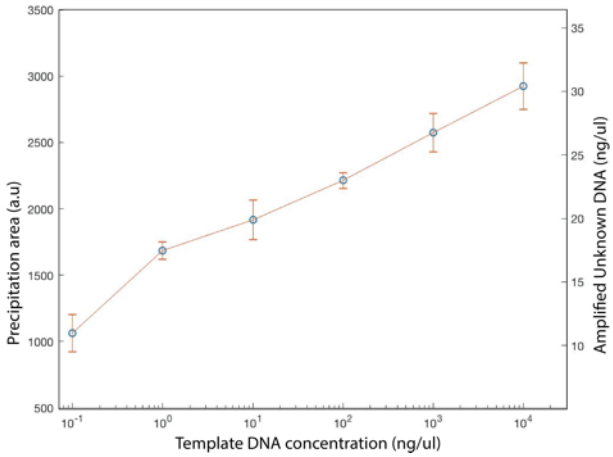

Fig.6 Starting DNA template concentration vs. precipitation area calculated from triplicate images using the MATLAB based image processing code (left-hand y-axis). Starting DNA template concentration vs. actual yield of amplified DNA produced due to the LAMP reaction (right-hand y-axis).

The precipitation area was converted to an actual yield of DNA products for each of the concentrations. This conversion was based on the linear empirical equation generated from the calibration curve presented earlier in Fig. 4b, given by:

$$y = 9.61x - 4.05 \quad (1)$$

Here, y denotes the precipitation area in arbitrary units while x denotes the DNA concentration.

CONCLUSION

We demonstrated an extremely fast visual DNA quantification method (μCAP) that can be made quantifiable on a Lab-on-DVD platform. The approach was based on DNA forming a precipitate upon centrifugation when in contact with the GelRed dye. Results using HIV-1B genome containing plasmid DNA revealed a detection limit of 0.01 pg/μl or total amount of 0.1 pg of starting DNA template, which is an acceptable standard for resource limited settings. The limit of detection of DNA with the Lab-on-DVD platform was found to be 10 ng/μl, which is almost comparable to the detection limits reported by commercially available instruments, such as the Nanophotometer. However, the μCAP method offers a distinct advantage over other state-of-the-art techniques as it does not require additional purification of the DNA. We believe the μCAP technique combined with the Lab-on-DVD platform provides a simple and low cost technology that can fulfil the need for a point-of-care device for DNA quantification.

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