Ultrasmall volume molecular isothermal amplification in microfluidic chip with advanced surface processing

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Abstract. In this paper, we developed a metal micro-fluidic chip with advanced surface processing for ultra-small volume molecular isothermal amplification. This method takes advantages of the nucleic acid amplification with good stability and consistency, high sensitivity about 31 genomic DNA copies and bacteria specific gene identification. Based on the advanced surface processing, the bioreaction assays of nucleic acid amplification was dropped about 392nl in volume. A high numerical aperture confocal optical detection system was advanced to sensitively monitor the DNA amplification with low noise and high power collecting fluorescence near to the optical diffraction limit. A speedy nucleic acid isothermal amplification was performed in the ultra-small volume microfluidic chip, where the time at the inflexions of second derivative to DNA exponential amplified curves was brought forward and the sensitivity was improved about 65 folds to that of in current 25μl Ep-tube amplified reaction, which indicates a promising clinic molecular diagnostics in the droplet amplification.

1. Introduction

Nucleic acid amplification[1-7] is one of most important tools in life science fields and clinic molecular diagnostics, where specific nucleic acid sequence can be amplified with more than 10^6-10^9 copies within an hour and detected with high sensitivity and specificity. Based on nucleic acid amplification, the single molecular diagnostics has always been a dream of human for clinic application, because of the surface adsorption of bioreactor to DNA molecules, the amplified double stranded DNA to be unfastened under high amplified temperature, the photo-bleaching [8] from the excited light and the background of material of bioreactor and surroundings, etc. The current nucleic acid amplification in Ep-tube, 96 or 384 well microplate usually uses a 25μl bioreaction assays with more than 10^3 copies of genomic DNA to make enough more amplified double stranded DNA to ignore the above negative effects, and obtain a relative steady exponential mode amplified signal and good consistency to repeated detection.

Polymerase chain reaction (PCR) is most early current nucleic acid amplification process described by Saiki RK (1985)[1], but PCR needs a precise temperature cycling control to keep good specificity of amplification, there is a low efficiency in amplification cycling where only the extend phase can produce new strands and the valid time for real DNA amplification is about 50%. Loop-mediated
isothermal amplification (LAMP) is a novel high effective nucleic acid amplification method introduced by Notomi (2000) [2], where nucleic acid amplification is performed at a constant temperature, and there is a high efficiency of amplification about 100%. Compared with PCR, LAMP has some obviously advantages:

Firstly, the whole amplification reaction can be performed at an isothermal condition, and the temperature control is relatively easy to design and realize with low cost. Secondly, the amplification efficiency is significantly improved, reaches to 100%, because no extra time is needed to wait for thermal changes in LAMP reaction. Thirdly, the reaction is highly specific to the amplification target where six specific primers with complete match are required to startup amplification.

The polycarbonate (PC), polymethylmethacrylate (PMMA), polydimethylsiloxane (PDMS), Glass and silicon are current materials used for nucleic acid amplification reactors [3-7], because of adsorption on the coarse surfaces of bioreactor and the limits of detection methods, which general requires at least 20μl reaction assays, otherwise it is difficult to perform a steady exponential mode amplification and good consistency for many repeated detections. Ideal materials could be used for nucleic acid amplification in micro-nanoliter assay systems is absent.

In this paper, we developed a metal micro-fluidic chip with advanced surface processing for ultra-small volume molecular isothermal amplification. A high numerical aperture confocal optical detection system was advanced to sensitively monitor the DNA amplification with low noise and high power collecting fluorescence near to the optical diffraction limit. Nucleic acid amplification in microenvironment was studied for good stability and consistency, high sensitivity. A promising clinic molecular diagnostics in the droplet amplification was performed.

2. Materials and methods

2.1. Materials
Oligonucleotide primers were synthesized by Invitrogen Corporation (Shanghai, China). Bst DNA Polymerase, Large Fragment and ThermoPol Reaction Buffer were from New England Biolabs (Beijing) Ltd (Beijing, China). MgSO4 was from Beijing Chemical Reagents Company (Beijing, China). dNTPs were from TaKaRa Biotechnology Co., Ltd (Dalian, China). dUTP and Uracil-DNA Glycosylase were from Fermentas Inc (Burlington, Canada). Betaine and BSA were from Fluka Sigma-Aldich Inc (Missouri, USA). EvaGreen was from Biotium Inc (California, USA). Other reagents were analytical grade.

2.2. Design of primers
According to the sequence of the specific mip gene from GenBank (Accession No. DQ896988), oligonucleotide primers used for the isothermal amplified assay of Legionella pneumophila were designed, which is a bacterial species of pneumonia. Six primers, composing of two outer (F3 and B3), two inner (FIP and BIP) primers and two loop (LF and LB) primers were designed. FIP consists of a complementary sequence of F1 and a sense sequence of F2. Similarly, BIP consists of a complementary sequence of B1 and a sense sequence of B2. The schematic representation and sequence of primers are shown in Figure 1 and Table 1.
Figure 1. Schematic representation of primers used in this study.

Table 1. The sequences of the six specific oligonucleotide primers of *L. pneumophila*.

| Primers | Sequences               |
|---------|-------------------------|
| F3      | GCAAGACGCTATGAGTGG      |
| B3      | TGATTACTTTGTATTGCAAACCA |
| FIP     | GCCATCAAATCCTTCTGAACCTTGT-CTCAATTGGCTTTAACCAGAC  |
| BIP     | GCGGATGAAAATAAAGTAAAAGGGG-CTTGGGCAATACACAAACGC  |
| LF      | TAAGAACGCTTTCTATGCT     |
| LB      | CTGAAAACAAAAAACCAAGCCAG |

2.3. Bacterial DNA preparation
Peking University People's Hospital (Beijing, China) kindly provided *L. pneumophila* strain (LP-1). Genomic DNA of LP-1 was extracted and purified by using the QIAamp DNA Mini Kit (Qiagen Inc., CA, USA). DNA concentration was measured by using NanoDrop Spectrophotometer (ND-1000, NanoDrop Technologies, Inc., DE, USA). The number of genomic copies was calculated assuming a molecular size of 3.4 Mbp.

2.4. Microfluidic chip fabrication
At first, the microfluid chips with different structure and microenvironment 50nL $\rightarrow$ 7µL are designed by using the machine design software *Solidworks* 2006, and fabricated with metal aluminium materials in thickness 1.5mm by using the Computer Numerical Control Machining Center (JT-M960L, Jiatai Numerical Control Co., Quanzhou, China). An example of masking diagram of microfluid chip was shown in Figure 2(a), where the chip is 60mm in diameter. The center hole is used to fix the chip to detection instrument. The diameter of inlet hole and outlet hole is both of 1.2mm, which fits for Eppendorf tips to inject samples and reactants into the chip. The bioreactor cells were fabricated in different sizes (0.5-3.0mm in diameter and 0.2-1.0mm in depth) to obtain microliter to nanoliter volume ranges. The channel was fabricated with different sizes (0.1-0.5mm in both of width and depth) to limit reagents and sample consumption. All buffer cells are 3.0mm in diameter and 0.5mm in depth for collecting air bubble displaced during sample injection. The curved areas of tunnel near to
the center hole was used to block the connection of bioreactor cells by heated press molding, and limit diffusion of reactants from one cell to others during the isothermal amplification.

Secondly, the microfluidic chip was further processed as the processes in Figure 2(b) to make surface smooth and inert to biological molecules, where an advanced inert surface processing was listed as following steps:

a). Clean oil and dirt, in the acidic solution of 50g/L H2SO4, 8g/L Emulsifier OP-10 for five minutes at 25°C.

b). Erode the surface, in the alkaline solution of 50g/L NaOH and 10g/L C6H11O7Na for ten minutes at 60°C.

c). Polish the surface, in solution of 280g/L H3PO4, 100g/L HNO3 and 40g/L H2SO4 for five minutes at 25°C.

d). Anodic oxidation, in electrolytic solution of 300g/L H2SO4, 2g/L HOOC-COOH, 15g/L HOCH2COOH and 15g/L C3H4O4 for thirty minutes at 25°C. The electronic current density is 30mA/cm².

e). Electrolytic coloring, in a black coloring solution of 30% SnSO4, 30% NiSO4 and 15% CuSO4 for twenty minutes at 25°C. The AC voltage is about 6-10V.

f). Seal the surface, in a pH 6 solution of 4.5g/L NiSO4, 0.8g/L CoSO4 and 1.2g/L NaF for forty minutes at 25°C.

g). Clean the chip, in a three aqueous washes to remove un-reacted chemicals.

After the advanced inert surface processing, the chips is encapsulated tightly by a thin PC film from ABI Corporation for use.

![Figure 2. The fabrication of microfluidic chips](image)

2.5. Microfluidic chip isothermal amplification detection system

To sensitively detect in real time the fluorescent signal of DNA amplification, a confocal optical system was developed, which can detect the bioreactor cell in a limited spherical area with high excited power, while effectively limiting the background. This system used a high numerical aperture of objective NA = 0.72 to collect fluorescence on a focal plane near to the diffraction limit as shown in Figure 3, where there is a high efficiency of 92% at the focal plane. When the target is at the off focus position of 30μm, the efficiency of fluorescence signal collected is very low, about 0.96%. In the microfluidic chip detection, both the diameter and depth of bioreactor cell is 100μm or more, the collected fluorescence is positioned within the range of 50-60μm off the bottom of bioreactor, the
background from the material of bioreactor and surrounding is approximated to 0 and can be ignored. Therefore, our developed confocal optical system can effectively restrain the background signals from the materials of bioreactor and surroundings.

![Diffractive Limit](image.png)

**Figure 3.** The analysis of fluorescent collecting efficiency in developed confocal optical detection system

2.6. Amplification processes

The process of DNA amplification in micro-nanoliter fluidic chips (50nL $\rightarrow$ 7µL) is as Table 2.

| Temperature(℃) | Time(min) | Remark                      |
|----------------|-----------|-----------------------------|
| 37             | 5         | for ready                   |
| 65             | 40-45     | for real isothermal amplification |
| 80             | 5         | for limit life activity     |

When the microfluidic chip is used, the inlet and outlet holes are opened by using the Eppendorf tip with light pressure, the mixtures of the circular probes, reagents and DNA samples were injected into the bioreactor cells of microfluidic chip from the inlet hole by the Eppendorf. And then, the inlet and outlet holes were sealed by thin PC film, the channel between any two adjacent bioreactor cells was blocked by heating press to make all bioreactor cells free from others. Finally, the microfluidic chip was setup into our developed confocal optical detection system for DNA isothermal amplification and specific gene identification.

3. Results and discussion

The fluorescent detection of nucleic acid amplification usually is affected by many factors, including the surface adsorption of bioreactor, the amplified double stranded DNA, the photo-bleaching, the unfastened double stranded DNA under amplified temperature and the background, etc. Except the amplified double stranded DNA make a positive detection response, while the unfastened double stranded DNA, the surface adsorption and the photo-bleaching all cause a negative detection response and low measuring sensitivity or make the detection response of amplified signal late. The difference
from the unfastened double stranded DNA, the surface adsorption and the photo-bleaching usually causes the detection response of nucleic acid amplification unstable. And the background of surroundings and bioreactor material causes a high original value of dynamical amplified signal and low amplified efficiency. Therefore, current nucleic acid amplification in Eppendorf tube, 96 or 384 well microplate usually use a 25 μl reaction assays or more, to make enough amplified double stranded DNA to ignore all other negative factors, and get a relative stable exponential amplified curves and good repeated detection.

When the reaction assay is less than 10 μl, above negative factors are significant to the nucleic acid amplification as Figure 4. In figure 4, a PMMA microfluidic chip was used, where there are 16 positive amplified cells and 2 negative control cells, DNA template concentrations is 1.3 fM (10^{-15}M) and reaction mixtures is 7 μl in every bioreactor cells. The maximum fluorescent signal of nucleic acid amplification in 16 bioreactor cells is about 40000, and the minimum fluorescent signal of nucleic acid amplification in 16 bioreactor cells is about 8500, the percent deviation of fluorescent signal was about 129%. The times at the second derivative inflexions of DNA exponential amplified curves for 16 bioreactor cells is from 12’th minute to 23’th minute, the maximum of time difference at these inflexions was about 11 minutes.

The contrast experiment was performed in our advanced inert processing metal microfluidic chip with same DNA template concentration of 1.3 fM and reaction volume of 7 μl in every bioreactor cells, as Figure 5. In Figure 5, the maximum fluorescent signal of nucleic acid amplification in 16 bioreactor cells is about 46000, and the minimum fluorescent signal of nucleic acid amplification in 16 bioreactor cells is about 38000, the percent deviation of fluorescent signal was about 19%. The times at the second derivative inflexions of DNA exponential amplified curves for 16 bioreactor cells is from 11.42’th minute to 12.11’th minute, the maximum of time difference at these inflexions was about 0.7 minutes.

From Figure 4 and Figure 5, it is obvious that our developed inert processing metal microfluidic chips can outstanding improve the detection response of nucleic acid amplification in less than 10μl reaction assays.

In order to reduce further the reagents and sample consumption, isothermal nucleic acid amplification experiments were undertaken in different micro-nanoliter reaction volumes of 7.07 μl, 3.14 μl, 785 nl and 392 nl, each with the same concentrations of template DNA of 1.3 fM. The real time curves of DNA isothermal nucleic acid amplifications were obtained in Figure 6. It is obvious that the time at the inflexions of second derivative to the DNA exponential amplified curves is nearly the same in the different micro-nanoliter bioreactor cells, and the CVs of time at these inflexions is about 1.96%.

After the denatured double stranded DNA, the surface adsorption of bioreactor, the photo-bleaching and the background from the bioreactor material and surroundings are reduced, a fast response to the amplified double stranded DNA can be detected. With the same reaction mixtures and DNA template concentration, and differences only in reaction volume, nucleic acid amplification was performed respectively in PCR tube with reaction assay of 25μl and in our advanced metal microfluidic chip with a reaction assay of 7.07μl.
Figure 4. The DNA isothermal amplification in primary metal microfluidic chip with 18 bioreactor cells of 7μl in volume.

Figure 5. The DNA isothermal amplification in advanced inert processing metal microfluidic chip with same DNA template concentrations and reaction volume.
Figure 6. The microfluidic chip isothermal amplification in different micro-nanoliter reaction volumes

Figure 7 is the curves of isothermal nucleic acid amplification in the PCR tubes with a reaction volume of 25μl by using the ABI Fast Real-Time PCR System, where the DNA template copies were 2×10^6 copies, 2×10^5 copies, 2×10^4 copies, 2×10^3 copies and 2×10^2 copies, and the time at the second derivative inflexions of DNA exponential amplified curves for five different DNA template concentrations is 13'th minute, 15'th minute, 17'th minute, 20'th minute and without amplification to 45'th minute. When all amplifications were repeated 4 times, the CVs of the time at these inflexions were about 1%, 1%, 2% and 10% respectively.

Figure 7. The isothermal amplification in 25μl PCR tubes
Figure 8 corresponds the real curves of nucleic acid amplification in the advanced metal microfluidic chip by using our developed confocal optical detection system, where the DNA template copies of $5.7 \times 10^4$ copies, $5.7 \times 10^3$ copies, $5.7 \times 10^2$ copies, $5.7 \times 10^1$ copies and $3.1 \times 10^1$ copies were used, and the time at the second derivative inflexions of DNA exponential amplified curves of five different DNA template copies is 12' th minute, 14'th minute, 16'th minute, 19'th minute and 22'th minute. When all amplifications were repeated 5 times, the CVs of inflexion times are respectively 0.2%, 0.3%, 0.8%, 1.1% and 0.9%. From Figure 7 and Figure 8, it is clear that the time at the second derivative inflexions of DNA exponential amplified curves in the advanced metal microfluidic chip have been advanced significantly, the less the DNA template copies is, and the more the time at the second derivative inflexions was advanced. The detection sensitivity in advanced metal microfluidic chip was raised about 65 folds compared to that of in 25μl PCR-tube.

![Figure 8. The isothermal amplification in advanced metal microfluidic chip with a reaction assay of 7.07μl](image)

From Figure 5, Figure 6 and Figure 8, when using the advanced metal microfluidic chip and confocal optical detection system, a droplet nucleic acid isothermal amplification could be realized for promising clinic molecular diagnostics.

4. Conclusion
We have developed a metal micro-fluidic chip with advanced surface processing for micro-nanoliter volume DNA isothermal amplification. This method takes advantages of the nucleic acid amplification with good stability and consistency, high sensitivity about 31 genomic copies and bacteria specific gene identification. A high numerical aperture confocal optical detection system was developed to sensitively monitor the DNA amplification with low noise and high power collecting fluorescence near to the optical diffraction limit. Based on the advanced processing microfluidic chip and confocal optical detection system, the reaction assays of nucleic acid amplification could be reduced to nanoliter in volume. An accelerated nucleic acid isothermal amplification was detected in the ultrasmall volume microfluidic chip, where the time at the second derivative inflexions of DNA exponential amplified curves was advanced and the sensitivity was improved about 65 folds to that of in 25μl Ep-tube amplified reaction. The method and system in this paper indicates a promising clinic molecular diagnostics in the droplet amplification.
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