fM to aM nucleic acid amplification for molecular diagnostics in a non-stick-coated metal microfluidic bioreactor

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A sensitive DNA isothermal amplification method for the detection of DNA at fM to aM concentrations for pathogen identification was developed using a non-stick-coated metal microfluidic bioreactor. A portable confocal optical detector was utilized to monitor the DNA amplification in micro- to nanoliter reaction assays in real-time, with fluorescence collection near the optical diffraction limit. The non-stick-coated metal microfluidic bioreactor, with a surface contact angle of 103°, was largely inert to biomolecules, and DNA amplification could be performed in a minimum reaction volume of 40 nL. The isothermal nucleic acid amplification for Mycoplasma pneumoniae identification in the non-stick-coated microfluidic bioreactor could be performed at a minimum DNA template concentration of 1.3 aM, and a detection limit of three copies of genomic DNA was obtained. This microfluidic bioreactor offers a promising clinically relevant pathogen molecular diagnostic method via the amplification of targets from only a few copies of genomic DNA from a single bacterium.

Molecular diagnostics is one of the most important tools currently in use for clinical pathogen detection due to its high sensitivity and specificity. Novel methods have been reported to improve nucleic acid amplification by optimizing the conditions and materials used. Saiki et al. developed the polymerase chain reaction (PCR) in 1985, which requires precise temperature cycling to achieve DNA amplification specificity and is still the most popular method for nucleic acid amplification. For instance, Guatelli et al. performed self-sustained sequence replication (3SR) reactions at a relatively low temperature with compromised specificity in 1990. Compton advanced nucleic acid sequence-based amplification (NASBA) in 1991, which is an efficient method specific for target RNA detection at 41°C that uses three enzymes (T7 RNA polymerase, RNase H, and avian myeloblastosis virus (AMV) reverse transcriptase) and two specific primers. Walker et al. developed strand displacement amplification (SDA) with four primers and iso-thermal conditions for amplification. Liu et al. reported rolling circle amplification (RCA) for isothermal DNA polymerization in 1996, which is based on circular single-stranded DNA probes with sense primers as everlasting templates. Tsugunori et al. invented loop-mediated isothermal amplification (LAMP) in 2000, requiring a DNA polymerase and a set of four special primers to recognize a total of six distinct sequences on target DNA; 106 copies of target can be obtained within 1 h with this method.

In studies of materials, polycarbonate (PC) is the main component of reactors for nucleic acid amplification, in the form of Eppendorf tubes (Ep-tube) and 96- or 384-well micro-plates, where a ≥ 25-µL reaction volume is typically required to obtain stable amplification curves and ignore the material adsorption and fabrication roughness of reactors, detection method limits, etc. Glass and silicon are used for nucleic acid amplification in capillaries with ion-beam milling but with compromised stability. Polymethylmethacrylate (PMMA) and polydimethylsiloxane (PDMS) have also been applied for some initial nucleic acid amplification experimentations with the drawbacks of low sensitivity and stability.

Other studies have been performed to enhance detection sensitivity, reduce reagent cost, and construct miniaturized high-throughput systems. Primer generation–rolling circle amplification was developed with a high sensitivity of 50.7 molecules of synthetic sample DNA and 60 molecules of genomic DNA. Li et al.
reported a DNA-encapsulating liposome for protein detection with a limit of detection of 0.08 fg/mL. Jishan et al. used gold nanoparticle aggregates for highly sensitive identification of single-nucleotide polymorphisms in a mutated target at a concentration as low as 70 fM. Daniel et al. introduced ultra-small volume analysis, ranging from micro- to nanoliters. Xueen et al. demonstrated microloop-mediated isothermal amplification (μLAMP) with a sample volume of 0.4 μL under isothermal conditions (63°C) in 2010. Matthias et al. performed quantitative PCR from the micro- to picogram level, and Brunetto et al. reported a novel digital droplet PCR (ddPCR) system for high-throughput single DNA molecule isothermal amplification and analysis. Further, an ultrafast miniaturized real-time 40-cycle PCR that can be completed in 6 min has been reported, and a portable chip-based RT–PCR system for specific nucleic acid amplification to detect RNA-based viruses has been described.

When it comes to molecular reaction detectors, Nathaniel et al. reported an integrated capture, concentration, PCR, and capillary electrophoretic analysis of pathogens on a chip. Yongki et al. demonstrated a field-effect enzymatic amplifying detector with a picomolar detection limit. Zhu et al. developed a highly sensitive electrochemical sensor for mercury(II) ions using a mercury-specific oligonucleotide probe and gold nanoparticle-based amplification. Lee et al. reported the diffractometric detection of proteins using microbead-based rolling circle amplification. Wu et al. reported an electrochemical aptameric recognition system for a sensitive protein assay based on specific target binding-induced rolling circle amplification. Tong et al. reported a portable fluorescence detector for end-point analysis using cycling probe technology. Emory et al. developed a simple and portable compact fluorescence single-molecule detector for single-molecule detection (SMD).

Some superior coatings have been used to improve surface uniformity and make surfaces inert to bio-molecules, including anodic oxidation surface treatment and nanostructured Teflon-like superhydrophobic coatings. The anodic oxidation surface treatment made the surface of an aluminum bioreactor uniform and inert to biological reagents with a 93.8° liquid droplet contact angle on the surface of the processed bioreactor, which remarkably improved the amplification of nucleic acids in the micro-nanofluidic chip. However, the fabrication process is complicated and involves serious environmental pollution due to the use of NaOH, H3PO4, HNO3, and H2SO4. The nanostructured Teflon-like superhydrophobic coatings display super hydrophobic behavior with a water contact angle of 165° and are widely used on an industrial scale in stainless steel shell and non-stick cookware as an important cooking utensil. Unfortunately, there are few reports concerning these coatings for micro-nanofluidic chips.

When nucleic acid amplification is performed with reaction assays in micro- to nanoliters or picoliters, DNA molecule adsorption to the surface of the reactor, denatured double-stranded DNA (dsDNA) due to high amplification temperatures, photobleaching, and difference in the background from the material of the reactor and surroundings are also significant in comparison to the amplified molecules. In other words, their effects are negative relative to the stability of nucleic acid amplification and detection sensitivity. Therefore, to lower these negative effects, improve amplification efficiency, reduce reagent costs, and shorten assay times, it is important to advance nucleic acid amplification for Micro Total Analysis Systems.

In this paper, we describe isothermal DNA amplification from template DNA at fM to aM concentrations via isothermal amplification in a non-stick-coated metal microfluidic bioreactor with a minimum reagent consumption of 40 nL and detection limit of three genomic copies.
bioreactors. When the negative effects from the surface adsorption of the bioreactor, photo-bleaching of the excited light, residual denatured DNA under high amplification temperature, and the background from the bioreactor material and surroundings were limited, fast isothermal nucleic acid amplification could be performed. Further, the time at the inflexion of the second derivative of the exponential DNA amplification curve was significantly advanced, and the minimum DNA template concentration for sequence-specific molecular identification was 1.3 aM. Thus, the detection limit was improved to as little as three genomic copies. This novel micro-nanoliter fluidic detection system and method could be used to develop a Micro Total Analysis System for promising clinical pathogen molecular diagnostics via droplet amplification with several copies of genomic DNA from a single bacterium.

Results
Non-stick-coated metal micro-fluidic chip for stable DNA amplification. The surface adsorption of DNA molecules to a bioreactor has a significant effect on the dynamic detection of the nucleic acid amplification signal in micro-nanoliter assays and usually delays the detection response and renders the fluorescent signal of DNA amplification unsteady. The surface adsorption is mainly due to surface roughness and the surface not being inert. The surface structure of a bioreactor on metal micro-nanoliter fluidic chips is usually rough from machine fabrication. Thus, a non-stick silicone coating was used to coat the metal micro-nanoliter fluidic chips to make the surfaces of the bioreactor smooth and inert, yielding a high and steady DNA amplification signal. The surface structure of uncoated and non-stick-coated bioreactors was imaged using a scanning electron microscope (S-3000N, Hitachi Corporation, Japan) as shown in Figure 2. In Figure 2, (a) and (b) are the surface structure of the original bioreactor at 40× and 2000× magnification, respectively, displaying a rough microscopic structure from machine fabrication. (c) corresponds to the contact angle of a liquid droplet on the surface of the original bioreactor (71.3°) after the surface was washed with anhydrous ethanol (tested by the OCA-15plus contact angle system, DataPhysics Instruments GmbH, Germany). Figure 2 (d) and (e) are the surface structure of the non-stick-coated bioreactor at 40× and 2000× magnification, respectively, where the surfaces of the bioreactor were covered with an even silicone protective film of 10–20 μm in thickness. (f) corresponds to the contact angle of a liquid droplet on the surface of the non-stick-coated bioreactor (103.1°). The silicone protective film is inert to biological samples and general reagents, can withstand a carving stress of >2000 kg/mm², and has a melting point 280°C.

Comparison of isothermal DNA amplification in uncoated and non-stick-coated metal micro-nanoliter fluidic chips. Using the same DNA template concentration of 1.3 fM (10⁻¹⁵ M) and 7-μL reaction mixtures in every bioreactor cell, isothermal DNA amplification in uncoated and non-stick-coated metal micro-nanoliter fluidic chips was performed using our portable confocal detector. The contrast between DNA isothermal amplified curves is shown in Figure 3. Figure 3 (a) corresponds to isothermal DNA amplification in the uncoated metal micro-nanoliter fluidic chip, where the times at the second derivative inflexions of the exponential DNA amplification curves for the five bioreactor cells were 24.85, 27.29, 27.80, 28.18, and 29.53 min. The maximum of the time difference at these inflexions was ~4.68 min. At the top of the amplification curves, the fluorescence intensities of the five bioreactor cells ranged from 3600–6900; the percent deviation of the fluorescence intensity was ~91.7%. Figure 3 (b) is isothermal DNA amplification in the non-stick-coated metal micro-nanoliter fluidic chip, where the times at the second derivative inflexions of the exponential DNA amplification curves for the five bioreactor cells corresponded to 19.42, 19.45, 19.59, 19.60, and 19.92 min. The maximum of the time differences at these inflexions was 0.5 min. At the top of the amplification curves, the fluorescence intensities of the five bioreactor cells ranged from 4000–4500; the percent deviation of the fluorescence intensity was ~11%. Figure 3 (c) shows the isothermal DNA amplification curves in Figure 3 (b) after normalization processing, which is generally used in commercial RT-PCR setups.

Sensitive isothermal DNA amplification and fast detection response in the non-stick-coated metal micro-nanoliter bioreactor. Using the same DNA template concentration and reaction mixtures but different assay volumes, nucleic acid amplification was performed in PC Eppendorf tubes (25 μL) and in the non-stick-coated metal micro-nanoliter fluidic bioreactors (7 μL). Our portable real-time fluorescent confocal detector showed a sensitive and fast detection response of isothermal DNA amplification in the non-stick-coated metal micro-nanoliter fluidic bioreactor (Fig. 4). Figure 4 (a) shows the real time curve of isothermal DNA amplification in the Eppendorf tube using an ABI 7700 Fast Real-Time PCR System, where the DNA template

Figure 2 | Contrast of the surface characteristics of the bioreactor with and without the silicone coating. (a) and (b) are the surface structure of the uncoated bioreactor at 40× and 2000× magnification, respectively; (c) is the contact angle of the liquid droplet on the surface of the uncoated bioreactor, ~71.3°; (d) and (e) are the surface structure of the non-stick-coated bioreactor at 40× and 2000× magnification, respectively; (f) corresponds to the contact angle of the liquid droplet on the surface of the non-stick-coated bioreactor, ~103.1°.
Figure 3 | Comparison of DNA amplification in uncoated and non-stick-coated metal micro-nanoliter fluidic chips. (a) Isothermal DNA amplification in the uncoated metal micro-nanoliter fluidic chip, which displays obvious differences among the five parallel bioreactors. (b) Isothermal DNA amplification in the non-stick-coated metal micro-nanoliter fluidic chip, where the amplification in the five parallel bioreactors displays good consistency and the time difference at the second derivative inflexions of the exponential DNA amplification curves for the five bioreactors are within 0.5 min. (c) Normalization processing of the isothermal DNA amplification curves in (b).
concentrations were $1.3 \times 10^2$ fM, $1.3 \times 10^1$ fM, $1.3$ fM, $1.3 \times 10^{-1}$ fM (130 aM), $1.3 \times 10^{-2}$ fM (13 aM), and $1.3 \times 10^{-3}$ fM (1.3 aM), and the times at the second derivative inflexions of the exponential DNA amplification curves for the six different DNA template concentrations were 16.3, 18.4, 20.8, 23.7, and 28.9 min and 45 min without amplification. The CVs (Coefficient of Variation) of time at these inflexions of correct amplification were 1.2, 1.1, 1.6, 6.1, and 22.7%, respectively, when all amplifications were repeated five times. Figure 4(b) shows the real-time curves of nucleic acid amplification in the non-stick-coated metal micro-nanoliter fluidic bioreactors measured using our portable real-time fluorescent confocal detector, where the DNA template concentrations were the same as above, and the times at the second derivative inflexions of the exponential DNA amplification curves for the six different DNA template concentrations corresponded to 14.8, 17.4, 19.6, 21.5, 24.5, and 28.7 min. The CVs of time at these inflexions were 0.4, 2.2, 0.6, 2.2, 3.6, and 7.4%, respectively, when all amplifications were repeated five times. Comparing Figure 4(a) to Figure 4(b), the times at the second derivative inflexions of the exponential DNA amplification curves in the non-stick-coated fluidic bioreactors measured using our portable real-time fluorescent confocal detector, where the DNA template concentrations were the same as above, and the times at the second derivative inflexions of the exponential DNA amplification curves for the six different DNA template concentrations corresponded to 14.8, 17.4, 19.6, 21.5, 24.5, and 28.7 min. The CVs of time at these inflexions were 0.4, 2.2, 0.6, 2.2, 3.6, and 7.4%, respectively, when all amplifications were repeated five times. Comparing Figure 4(a) to Figure 4(b), the times at the second derivative inflexions of the exponential DNA amplification curves in the non-stick-coated fluidic bioreactors measured using our portable real-time fluorescent confocal detector, where the DNA template concentrations were the same as above, and the times at the second derivative inflexions of the exponential DNA amplification curves for the six different DNA template concentrations corresponded to 14.8, 17.4, 19.6, 21.5, 24.5, and 28.7 min. The CVs of time at these inflexions were 0.4, 2.2, 0.6, 2.2, 3.6, and 7.4%, respectively, when all amplifications were repeated five times.

Figure 4 | Comparison of the isothermal nucleic acid amplification reactions in an Eppendorf tube vs. the non-stick-coated metal micro-nanoliter fluidic bioreactor. (a) Real-time curves of common 25-μL isothermal nucleic acid amplification reactions in the Eppendorf tube using an ABI 7700 Fast Real-Time PCR System, where the DNA template concentrations were $1.3 \times 10^2$, $1.3 \times 10^1$, $1.3 \times 10^0$, $1.3 \times 10^{-1}$, $1.3 \times 10^{-2}$ fM and the times at the second derivative inflexions of the exponential DNA amplification curves were 16.3, 18.4, 20.8, 23.7, and 28.9 min and 45.0 min without amplification. (b) Real-time curves of 7-μL nucleic acid amplification reactions in the non-stick-coated metal micro-nanoliter fluidic bioreactor measured using our portable real-time fluorescent confocal detector, where the DNA template concentrations were $1.3 \times 10^2$, $1.3 \times 10^1$, $1.3 \times 10^0$, $1.3 \times 10^{-1}$, $1.3 \times 10^{-2}$, and $1.3 \times 10^{-3}$ fM and the times at the second derivative inflexions of the exponential DNA amplification curves were 14.8, 17.4, 19.6, 21.5, 24.5, and 28.7 min.
metal micro-nanoliter fluidic bioreactor were obviously advanced for the same DNA template concentration (as Table 1). Indeed, the lower the DNA template concentration was, and the grater the detection response was advanced. For instance, the time at the second derivative inflexion of the exponential DNA amplification curve for the template concentration of $1.3 \times 10^{-3}$ fM was advanced by 4.4 min. Thus, the sensitivity in the non-stick-coated metal micro-nanoliter fluidic bioreactor was improved by $\sim$10-fold relative to that of the 25-μL reaction in the Eppendorf tube.

**Table 1 | Comparison of the times at the inflexions of the second derivative of the exponential DNA amplification curves between our advanced metal micro-nanoliter fluidic bioreactors and in the general 25-μL Eppendorf tubes**

| DNA template (fM) | $1.3 \times 10^{-1}$ | $1.3 \times 10^{-2}$ | $1.3 \times 10^{-3}$ |
|------------------|---------------------|---------------------|---------------------|
| In 7-μL non-stick-coated metal micro-nanoliter fluidic bioreactors measured using our Portable Confocal Detection System | 14.8 | 17.4 | 19.6 |
| In 25-μL Eppendorf tubes measured using an ABI 7700 Fast Real-Time PCR System | 16.3 | 18.4 | 20.8 |

Stable DNA amplification in different reaction volumes (μL to nL). With the same concentration of bio-reaction mixtures but different (micro- to nanoliter) reaction volumes, isothermal nucleic acid amplification for gene-specific identification was performed in the non-stick-coated metal micro-nanoliter fluidic bioreactor (Fig. 5). In Figure 5, the DNA template concentration was $1.3 \times 10^{-3}$ fM, and the isothermal amplifications in six non-stick-coated metal micro-nanoliter fluidic bioreactors with assay volumes of 7 μL, 3 μL, 785 nL, 392 nL, 98 nL, and 40 nL are shown. The same time at the second derivative inflexion of the exponential DNA amplification curves were determined for the various reaction volumes when the DNA template concentration was the same, indicating the potential for isothermal amplification for gene-specific identification using microliter to nanoliter consumption of samples and reagents.

The detection limit of DNA amplification for gene-specific identification in the non-stick-coated metal micro-nanoliter fluidic bioreactors. Sensitivity analysis of the isothermal DNA amplification for different copy numbers of DNA template was performed in the non-stick-coated metal micro-nanoliter fluidic bioreactors. Six different DNA template samples ($5.6 \times 10^6$, $5.6 \times 10^5$, $5.6 \times 10^4$, $5.6 \times 10^3$, 6, and 3 copies) were used, and the amplification curves are displayed in Figure 6. The isothermal amplification for every DNA template concentration was repeated five times. The times at the second derivative inflexions of the exponential DNA amplification curves for the six different DNA template concentrations were 17.32, 19.42, 21.34, 23.26, 28.71, and 30.62 min. The time CVs of five repeated measurements for every group of inflexions were 2.2, 0.6, 2.2, 3.6, 7.4, and 7.8%, respectively. As shown in Figure 6, the minimum DNA template copy number for isothermal amplification was three copies of genomic DNA.

**Discussion**

The fluorescent detection response of nucleic acid amplification is related to many factors, including the number of amplified dsDNA molecules, the number of denatured dsDNAs at the reaction temperature, the surface adsorption of the bioreactor, photo-bleaching, and background noise. General nucleic acid amplification in Eppendorf tubes and 96- or 384-well microplates is usually performed in a $\geq 25$-μL reactions to increase the signal of the amplified dsDNA far above the lost signal from the above-mentioned negative factors, as well as to obtain a relatively steady exponential mode of amplified signal and good consistency for repeated detection. When the reaction assay is reduced to <10 μL, all of the above factors are significant detriments to the nucleic acid amplification. Here, we report that a non-stick-coated metal micro-nanoliter fluidic chip and portable confocal detector obviates these negative effects, making the nucleic acid amplification stable, the detection sensitive, and the response rapid in micro-nanoliter reaction assays. This was theoretically analyzed in the Supporting Information and proved by our experimental results in Supplementary Figures S1 and S2 and Figures 3–6.

Figure 3 illustrates fast and stable isothermal DNA amplification in the non-stick-coated metal micro-nanoliter fluidic chip. Supplementary Figure S2 demonstrates that our portable confocal detector can collect fluorescence near the optical diffraction limit and effectively limit the background noise. Figure 4 shows that the time at the second derivative inflexion of the exponential DNA amplification curve in the non-stick coating fluidic bioreactor was significantly advanced by 4.4 min at a DNA template concentration of $1.3 \times 10^{-3}$ fM. Figure 5 proves that stable detection of nucleic acid amplification can be obtained in the non-stick-coated metal micro-nanoliter fluidic bioreactor with reaction volumes from 7 μL to 40 nL. Figure 6 indicates that the sensitivity of the non-stick-coated metal micro-nanoliter fluidic bioreactor is three genomic copies, which is an $\sim$62-fold improvement compared to that of a normal 25-μL reaction in an Eppendorf tube (limit of detection $= 1.3 \times 10^{-3}$ fM, $\sim$196 copies; Fig. 4(a)). Therefore, fast and sensitive detection of the amplified nucleic acid for gene-specific pathogen diagnostics in a micro-nanoliter reaction assay can be obtained using our method and system.

The technology and methods in this paper are of value to develop Micro Total Analysis Systems for general lab research and clinical molecular diagnostics, which indicates that there is promise for single-molecule amplification in 1-nL droplet assays when all negative effects to nucleic acid amplification are further reduced.

**Methods**

**Materials and reagents.** EvaGreen was purchased from Biotium Inc (California, USA). Bst DNA Polymerase, Large Fragment, and ThermoPol Reaction Buffer were from New England Biolabs Ltd (Beijing, China). MgSO₄ was purchased from Beijing Chemical Reagents Company (Beijing, China). dNTPs were obtained from TaKaRa Biotechnology Co., Ltd (Dalian, China). BIP consists of the complementary sequence of B1 and the sense sequence of F2. The primer sequences are listed in Supplementary Figure S4. BIP was designed to recognize eight distinct regions on the target sequence (Supplementary Figure S3). BIP consists of the complementary sequence of 81 and the sense sequence of B2. The primer sequences are listed in Supplementary Figure S4.

**Design of primers.** Oligonucleotide primers were designed for the isothermal amplification assay according to the sequence of the *Mycoplasma pneumoniae* P1 gene from GenBank (Accession No. M18639). Six primers, including two loop primers (LF and LB), two outer primers (F3 and B3), and two inner primers (FIP and RIP), were designed to recognize eight distinct regions on the target sequence (Supplementary Figure S3). BIP consists of the complementary sequence of 81 and the sense sequence of B2. The primer sequences are listed in Supplementary Figure S4.

**Factors for successful gene-specific detection in the non-stick-coated metal micro-nanoliter fluidic bioreactor.** Supposedly, one of the best conditions for high copy number of DNA template in the non-stick-coated metal micro-nanoliter fluidic bioreactor is the non-stick effect, which obviates the negative effects due to adsorption, photo-bleaching, and background noise. General nucleic acid amplification in Eppendorf tubes and 96- or 384-well microplates is usually performed in a $\geq 25$-μL reactions to increase the signal of the amplified dsDNA far above the lost signal from the above-mentioned negative factors, as well as to obtain a relatively steady exponential mode of amplified signal and good consistency for repeated detection. When the reaction assay is reduced to <10 μL, all of the above factors are significant detriments to the nucleic acid amplification. Here, we report that a non-stick-coated metal micro-nanoliter fluidic chip and portable confocal detector obviates these negative effects, making the nucleic acid amplification stable, the detection sensitive, and the response rapid in micro-nanoliter reaction assays. This was theoretically analyzed in the Supporting Information and proved by our experimental results in Supplementary Figures S1 and S2 and Figures 3–6.

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DNA preparation from bacteria. *M. pneumoniae* strain FH was supplied by the People’s Hospital of Peking University (Beijing, China). Genomic DNA from FH was extracted and purified using a QIAamp DNA Mini Kit (Qiagen Inc., CA, USA). The DNA concentration was measured using an ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., DE, USA). The number of genomic copies was calculated according to the molecular size of 0.8 Mbp.

Optimizing the isothermal amplification assay of nucleic acids for pathogen molecular diagnostics. A 25-μL isothermal nucleic acid amplification assay for pathogen molecular diagnostics consisted of 0.2 μM each of F3 and B3, 1.6 μM each of FIP and BIP, 0.4 μM each of LF and LB, 8 U of the Bst DNA Polymerase, Large Fragment, 0.1 mM dUTP, 0.4 mM dNTPs, 0.5 mg/ml BSA, 0.6× EvaGreen, 0.8 M betaine, 6 mM MgSO₄, 0.1 U/ml Uracil-DNA Glycosylase, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.8 at 25°C), 10 mM KCl, 0.1% Triton X-100, and template DNA (2 μL). As LAMP, LF, and LB were used to advance the amplified reaction, BSA was used to decrease nonspecific adsorption by the bioreactor, Uracil-DNA Glycosylase and dUTP were used to eliminate contamination, EvaGreen was added to the reaction mixture as a real-time fluorescent reporter of amplified dsDNA, and 6 mM MgSO₄ was used to increase the efficiency of amplification.
DNA amplification in micro-nanoliter reaction assays, where a non-stick coating methods are available. Here, we explored a non-stick coating surface processing to Machining Center. In contrast to normal PC, PMMA, PDMS, glass, and silicon, the Al were fabricated from aluminum (Al) using a Computer Numerical Control Machining Center JT-M960L (Jiatai Numerical Control Co., Ltd, Quanzhou, China), Figure 7(b). First, the bioreactor cell, channel, buffer cell, inlet hole, outlet hole, and chip. Micro-nanoliter fluidic chips were fabricated by the process shown in Figure 7(a), where the chip is 60 mm in diameter and 2.5–3.0 mm in thickness. The fixed hole in the center of the chip was used to fasten the chip to the rotation stage of the detection system. Both the inlet and outlet holes are 1.2 mm in diameter to load reagents and sample by the Eppendorf head. The bioreactor cell was produced with different micro-nanoliter volumes of 40 nL to 7 μL, and the channel was carved with different sizes (0.5–0.1 mm) in both width and depth to decrease reagent consumption. The buffer cell was 3.0 mm in diameter and 0.5 mm in depth, which is used to collect air from the injected sample to eliminate bubbles from the bioreactor cells. The inlet hole was connected to the buffer cell by the channel on the back of the chip. Micro-nanoliter fluidic chips were fabricated by the process shown in Figure 7(a), First, the bioreactor cell, channel, buffer cell, inlet hole, outlet hole, and fixed hole were produced on the Al plate with a Computer Numerical Control Machining Center JT-M960L (Jiatai Numerical Control Co., Ltd, Quanzhou, China), and the primary micro-nanoliter fluidic chip was obtained. Second, the surface of the micro-nanoliter fluidic chip was covered with the non-stick coating layer to approximately 10–20 μm thickness to make the surface of the chip smooth and inert to DNA molecules. Then, the chip was washed using anhydrous ethanol and dried by approximately 10–20 min at 80–120 °C. CoriLon 9800 is a Teflon-like coating40–42 with the chemical structure of (CF₂–CF₃)ₐ and was obtained from Shenzhen Sovilor Science and Technology Develop Co. Ltd (Shenzhen, China). It is a high-stability material with both organic groups and inorganic structure. Sintering solidification. After spray-painting, the chip was sintered at 280 °C for 30 min, and the non-stick coating on the surface of the chip solidified into a thin protective layer with a thickness of 10–20 μm.

Non-stick-coated metal microfluidic chip fabrication. The metal microfluidic chips were fabricated from aluminum (Al) using a Computer Numerical Control Machining Center. In contrast to normal PC, PMMA, PDMS, glass, and silicon, the Al has excellent thermal properties, is readily machinable, and rich surface processing methods are available. Here, we explored a non-stick coating surface processing to make the surface of the Al microfluidic chip inert for sensitive and stable isothermal DNA amplification in micro-nanoliter reaction assays, where a non-stick coating layer was formed with a polymeric silicon dioxide layer (SiO₂–SiO₂)ₙ, binding a polytetrafluoroethylene (PTFE) (CF₂–CF₃)ₙ, The structure of the micro-nanoliter fluidic chips was designed as shown in Figure 7(a), where the chip is 60 mm in diameter and 2.5–3.0 mm in thickness. The fixed hole in the center of the chip was used to fasten the chip to the rotation stage of the detection system. Both the inlet and outlet holes are 1.2 mm in diameter to load reagents and sample by the Eppendorf head. The bioreactor cell was produced with different micro-nanoliter volumes of 40 nL to 7 μL, and the channel was carved with different sizes (0.5–0.1 mm) in both width and depth to decrease reagent consumption. The buffer cell was 3.0 mm in diameter and 0.5 mm in depth, which is used to collect air from the injected sample to eliminate bubbles from the bioreactor cells. The inlet hole was connected to the buffer cell by the channel on the back of the chip. Micro-nanoliter fluidic chips were fabricated by the process shown in Figure 7(a), First, the bioreactor cell, channel, buffer cell, inlet hole, outlet hole, and fixed hole were produced on the Al plate with a Computer Numerical Control Machining Center JT-M960L (Jiatai Numerical Control Co., Ltd, Quanzhou, China), and the primary micro-nanoliter fluidic chip was obtained. Second, the surface of the micro-nanoliter fluidic chip was covered with the non-stick coating layer to approximately 10–20 μm thickness to make the surface of the chip smooth and inert to DNA molecules. Then, the chip was washed using anhydrous ethanol and dried by nitrogen. Finally, a thin polycarbonate film (~0.1 mm) from ABI Corporation was tightly affixed to the surface of the micro-nanoliter fluidic chip for encapsulation.

The metal micro-nanoliter fluidic chip was coated with the non-stick coating layer using the following fabrication of the non-stick coating surface processing:

1. Surface preparation of the chip. Oil and dirt on the surface of chip were removed with 75% ethanol or ~5 min, and then the chip was heated at a temperature of 360 °C for 30 min.
2. Surface roughening and hardening. The surface of the chip was roughened using 50–150 μm Corundum Sand under a pressure of 0.4–0.6 mPa for 12–14 s and then hardened by depositing a polymeric silicon dioxide layer (SiO₂–SiO₂)ₙ on the roughening surface using initiated chemical vapor deposition (iCVD)40.
3. Spray-painting the surface. CoriLon 9800 was spray-painted for ~12 s using a sprayer at a pressure of 0.2–0.3 mPa. The distance from the nozzle of the sprayer to the chip was ~30 cm. Then, the chip was dried for approximately 15 min at 80–120 °C. CoriLon 9800 is a Teflon-like coating40–42 with the chemical structure of (CF₂–CF₃)ₐ and was obtained from Shenzhen Sovilor Science and Technology Develop Co. Ltd (Shenzhen, China). It is a high-stability material with both organic groups and inorganic structure.
4. Sintering solidification. After spray-painting, the chip was sintered at 280 °C for 30 min, and the non-stick coating on the surface of the chip solidified into a thin protective layer with a thickness of 10–20 μm.

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**Acknowledgments**

This work was supported by the National Natural Science Foundation of China (81327005, 61361160418), the National Supporting Plan of China (2012BAI23B01), the National Foundation of High Technology of China (2012AA020102, 2013AA041201), the Projects 973 of China (2011CB707701), the National Key Foundation for Exploring Scientific Instruments (2013YQ190467), the Beijing Municipal Natural Science Foundation (4142025), the Beijing Lab Foundation, and Tsinghua Lab Innovate Foundation (110020003).

**Author contributions**

G.L.H. supervised and directed the overall project and wrote the main manuscript text. Q.H. conceived the methods and wrote part of the manuscript text. L.M., X.B.L., Z.X.Z. and B.P. built the setup and performed the experiments. R.L.W., J.Q.Z., Q.L., R.X.F. and J.C.Y. designed the bioreactors and performed the experiments. All authors contributed to the review of the manuscript.

**Additional information**

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

How to cite this article: Huang, G. *et al.* From micrograms to picograms: quantitative PCR reduces the material demands of high-throughput sequencing. *Nucleic Acids Res.* **34**, e77 (2006).

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