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Feasible and reliable quantification of mRNA in *Arabidopsis thaliana* using optical thin-film biosensor chips

Sulan Bai [a, *], Fan Wang [a], Zhen Zhang [a], Shucheng Li [a], Jie Zhang [b], Yaochuan Zhang [b, *]

[a] *College of Life Sciences*, *Capital Normal University*, *Beijing 100048*, *China*
[b] *Beijing Vocational College of Agriculture*, *Beijing 102442*, *China*

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Abstract

mRNA quantification is very important in molecular biological researches. Traditional spectrophotometric method cannot distinguish DNA, rRNA and tRNA species from mRNA. Northern blot can be used for mRNA quantification but is known to be time consuming. To rapidly detect mRNA levels, we developed an optical thin-film biosensor chip based method, to quantify mRNA in samples. After total RNA was extracted, the mRNA with poly(A) tails was reverse transcribed with oligo$(dT)_{20}$ primers and dNTPs mixed with digoxigenin(DIG)-11-dUTP. The transcribed first strand cDNA was hybridized with oligo$(dA)_{20}$ nucleotide probes spotted on optical thin-film biosensor chips. Excess first strand cDNA, single-strand RNA, and mis-matched DNA/DNA hybrids were removed by washing. The perfect-matched DNA/DNA hybrid was detected with anti-DIG-AP (alkaline phosphatase) conjugate and then incubated with NBT/BCIP substrate for color development. The range of the color is from purplish red to blue, according to the cDNA mass deposited on chip surface. Detection of mRNA levels from *Arabidopsis* samples proved that this method is feasible for mRNA quantification, and has great potential for application in mRNA quantification in various organisms.

Keywords: mRNA quantification; optical thin-film biosensor chip; assay

Introduction

Quantification of mRNA in samples is necessary for a number of different molecular biology experiments, such as generating cDNA probes for microarray analysis, determining RNA concentrations prior to northern blot analysis, creating of cDNA library, as well as reverse transcriptase PCR (RT-PCR), and differential display PCR.

Traditionally, the concentration of mRNA can be determined by spectrophotometry accomplished by measuring light absorbance at 260 nm. Though easy to perform, this method has several disadvantages. Free nucleotides, proteins and common contaminants in RNA preparations can contribute significant amounts of interfering signal when present. DNA, rRNA and tRNA species cannot be distinguished from mRNA with ultraviolet (UV) light absorbance. In addition, absorbance is relatively insensitive and lack of precision (Anno et al., 1998).

The quantity and quality of isolated RNA may be checked by denaturing agarose gel electrophoresis (Sambrook and Russell, 2001), but this requires up to 1 mg of total RNA.

Northern blot is applied for semi-quantification of

Abbreviations: DIG, digoxigenin; NBT/BCIP, nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; AP, alkaline phosphatase; UV, ultraviolet.

* Corresponding author. Tel & Fax: +86-10-6898 1191.
E-mail address: yaochuanzhang@sina.com (Y. Zhang);
sulanb@sina.com (S. Bai)

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mRNA. RNA is separated by electrophoresis in denaturing agarose gel and transfer to nylon membrane. The membrane is hybridized with ^{32}P-labeled or biotinylated oligo (dT). Labeled oligo (dT) hybridize to poly(A) mRNA and the total strength of signal represents the amount of poly(A) tails on membrane (Anno et al., 1998; Dong et al., 2009). This method can avoid the interference of other RNA species and comtaminations, but it is time consuming and there is risk of exposure to isotope radiation.

For accurate and rapid detection of mRNA levels from samples, we developed an optical thin-film biosensor chips based method to quantify mRNA. This method has been proven to be sensitive, feasible, time saving and specific for poly(A) mRNA detection, and has great potential for application in mRNA quantification in various organisms.

Materials and methods

Plant growth condition and total RNA extraction

The *Arabidopsis* seedlings were grown on Petri dishes with Murashige and Skoog (MS) agar medium at 22°C under a 16-h light/8-h dark photoperiod regime for 5 days. Total RNA was isolated by using the Trizol reagent (Invitrogen, China) according to manufactural directions. Concentration of total RNA was measured by spectrophotometry NanoDrop Instrument (NanoDrop Technologies, USA).

The thin-film biosensor chip

Thin-film biosensors are capable of transducing specific molecular interactions into signals that can be visualized by unaided human eyes because mass deposited on the thin-film surface by enzymatic catalysis alters the wavelength of light reflected by the optical layer resulting in a perceptible color change on the surface. The biosensors used here were prepared by coating the surface with a 475 Å layer of silicon nitride (Si_3N_4), which served as the optical layer (Zhong et al., 2003; Bai et al., 2007). To facilitate covalent attachment of biomolecules, the chip surface was coated with poly (Phe-Lys) to provide hydrazine for future covalently linking the capture probes.

Oligonucleotide synthesis

Oligo $(dA)_{20}$ served as the capture probe was synthesized by Invitrogen. Its 5′ end was modified with an aldehyde group that could react with a hydrazine group on the thin-film biosensor chip surface and covalently immobilize capture probe to the chip (Fig. 1, A and B). The capture probe was prepared as 1.0 mol/L oligonucleotide stocks in 0.1 mol/L sodium phosphate buffer, pH 7.8 and 200 nL per spot was loaded on the chip.

Preparation of standard mRNA samples

The standard mRNA sample, 200 g/mL neo poly(A) "sense" RNA, was purchased from Roche Diagnostics (USA). Standard mRNA and plant total RNA used in this experiment were reverse transcribed using the RevertAid M-MULV Rrverse Transcriptase (Fermentas, Canada) and dNTPs mixed DIG labeled dUTP (1 mmol/L dATP, dCTP, dGTP (each), 0.65 mmol/L dTTP, 0.35 mmol/L DIG-11-dUTP, Roche, USA).

Hybridization and signal detection

Target first strand cDNA was incubated with chips at 45°C in hybridization buffer (5 × SSC and 5 mg/ mL acid-treated casein, ATC) for 10 min. Then the chips were washed with 1 × SSC to remove all mismatched molecules. The DIG labeled cDNA on chips is detected by incubation with anti-DIG-AP antibody (Roche) for 10 min in hybridization buffer. The chips were rinsed with 0.1 × SSC for three times, and then incubated in AP buffer (100 mmol/L Tris-HCl, 100 mmol/L NaCl , 50 mmol/L $MgCl_2$, pH 9.5) for 5 min at room temperature. Then 100 μL of NBT/BCIP (Roche; 1:50 dilution in AP buffer) was added to the chip surface and incubated for 5 min at room temperature in dark. The chips were rinsed in double distilled water (ddH_2O) and air dried. Finally, the results were scored readily by unaided human eyes or photographed under a simple dissection microscope.

Results

Strategy for mRNA quantification on thin-film biosensor chip

Because mRNAs are not stable and easy to be degraded, the quantification of sample mRNA was conducted by re-

verse transcribed target mRNA to first strand cDNAs and labeled with DIG. The cDNA was then hybridized with capture probes covalently arrayed on thin-film biosensor silicon chips (see Methods and Materials and Fig. 1). The amount of cDNA deposited on the chip can be detected with anti-DIG antibody and conjugated alkaline phosphatase (AP). The reaction of AP with NBT/BCIP substrate make a distinguishable color change from purple to blue on the chip surface depending on the quantity of mass deposited on chip surface and can be read either by unaided human eyes or simple digital-imaging systems. Based on the colors we can estimate the quantity of cDNA that correlated to the quantity of mRNA.

Defining sensitivity parameters of capture probe on biosensor chip

In order to establish the suitable concentration of capture probe for spotting on a biosensor chip, we diluted the probe to various concentrations. As shown in Fig. 2, 200 nL of solution containing the capture probes at the concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 mol/L each were manually spotted onto the chip surface. Identical chips were hybridized with initiative mRNA at the gross of 10 ng and 50 ng in a 100 μL total reaction volume. As expected, the signal intensity increased as the quantity of initiative mRNA increase, as little as 0.01 mol/L capture probe can be detected when 50 ng initiative mRNA used. Spotting of probes from stock solutions higher than 1 μmol/L did not increase detection sensitivity, leading us to adopt the 1 μmol/L probe concentration for subsequent spotting.

Color change correlated to mRNA quantity on biosensor chip

We then established the correlation between the color and the quantity of initiative mRNA. After reverse transcribing the standard mRNA to first strand cDNA, various quantity of cDNA was used for detection. As shown in Fig. 3A, 100 μL of reaction solution containing 0, 1, 5, 10, 50, 100, 200 and 500 ng of initiative mRNA were hybridized

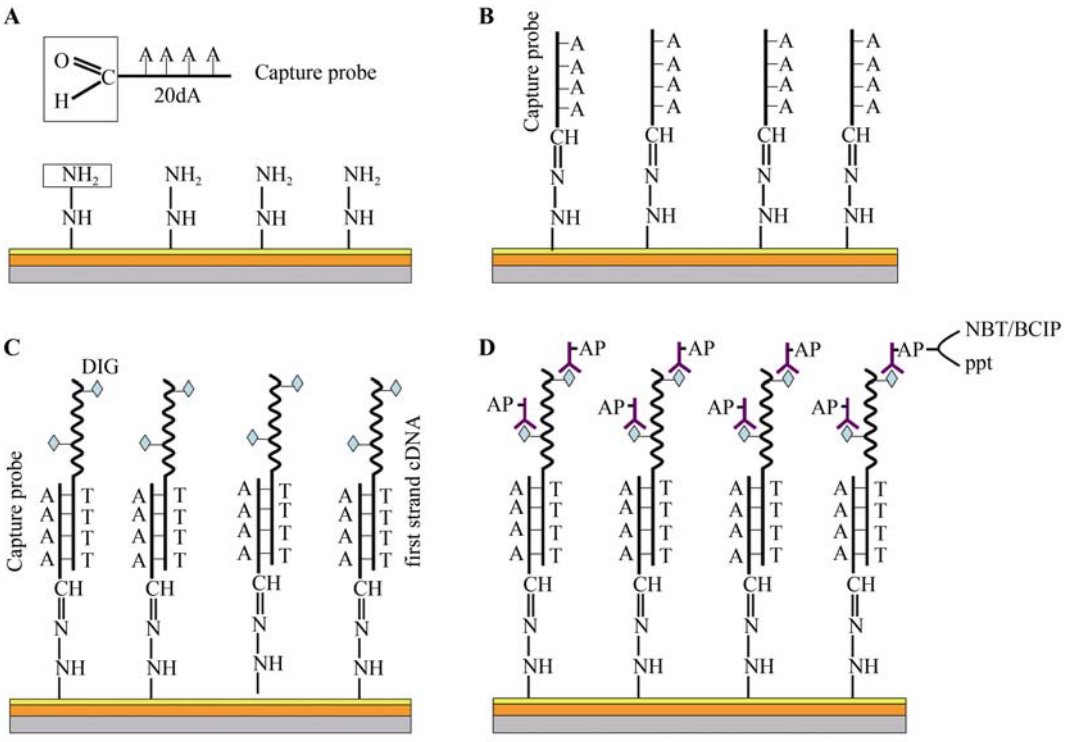

Fig. 1. Principles underlying thin-film biosensor chip assays for detecting first strand cDNA. **A** and **B:** immobilization of capture probes on thin-film biosensor chip surface by a chemical reaction between an aldehyde group at the 5′ terminus of capture probes and a hydrazine group at the chip surface. **C** and **D:** strategy for detection of cDNA on thin-film biosensor chips. DIG-labeled first strand cDNA with $(dT)_{20}$ complementary to the capture probe with $(dA)_{20}$ on biosensor chips can specifically hybridize (**C**), and the subsequent immunoreaction and precipitation require anti-DIG-AP antibody and NBT/BCIP substrate for color development as described in experimental procedures and (**D**). ppt, precipitations.

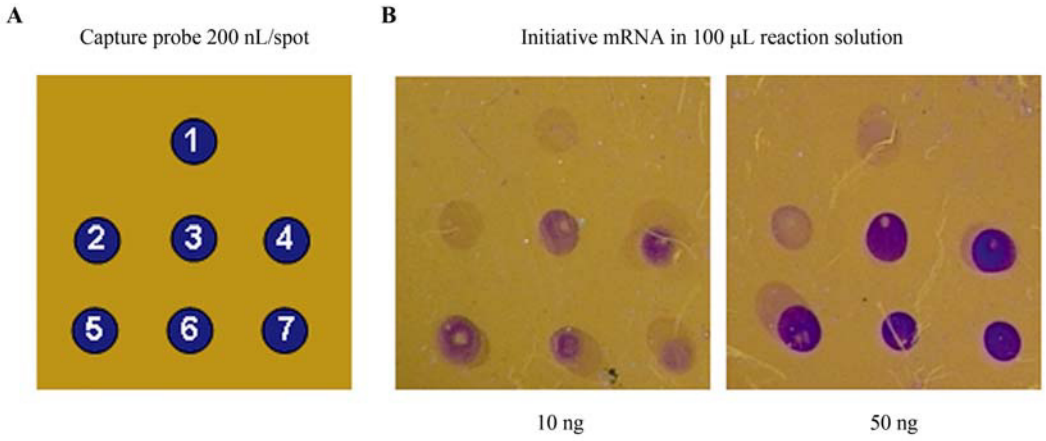

Fig. 2. Defining sensitivity parameters of capture probes on thin-film biosensor chips. Sensitivity of mRNA is detected on a chip with capture probes manually spotted at various concentrations. Capture probes were spotted at a volume of 200 nL and concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0 and 10.0 μmol/L shown in panel **A** at the position 1 to 7 respectively. The initiative mRNA at 10 and 50 ng, respectively, in 100 μL reaction solution were hybridized to two identical chips shown in panel **B**.

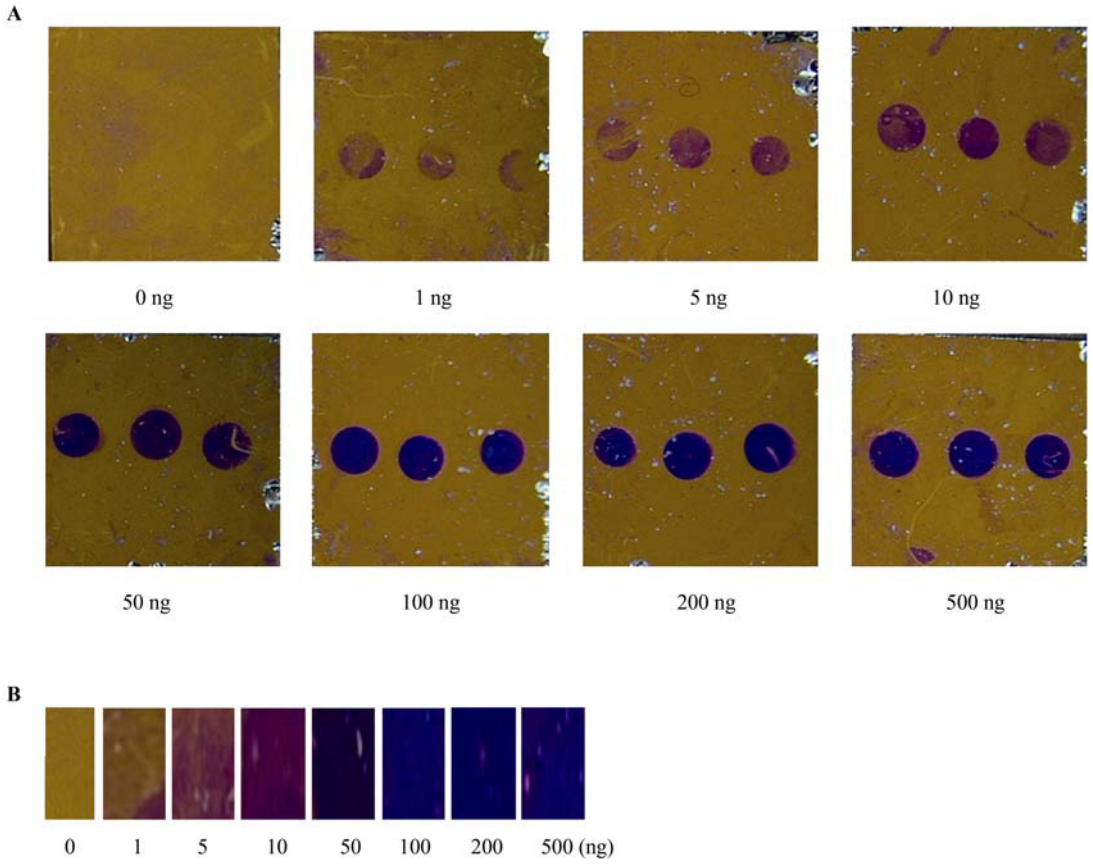

Fig. 3. Color change correlated to the quantity of mRNA. **A:** the capture probes spotted manually at 1 μmol/L for three repeats. The different colors of spots were developed when the quantity of initiative mRNA changed from 0 ng to 500 ng. **B:** standard color plate correlated to the quantity of initiative mRNA.

respectively with chips spotted with 1 μmol/L capture probe each spot and all probes had 3 replicates. As expected, the signal colors displayed from purple to blue correlated to the quantity of initiative mRNA. From the change of the signal colors, we could estimate the quantity of initiative mRNA in samples. Then the standard color plate correlated to the quantity of initiative mRNA was established (Fig. 3B).

Quantification of mRNA in Arabidopsis samples using optical thin-film biosensor chips

We have known that the signal colors on optical thin-film biosensor chip co-related to the quantity of initiative mRNA. To test the possible application of this method for mRNA quantification in real samples other than standard mRNA, we isolated total RNA from *Arabidopsis* wild-type seedlings in Columbia background. As shown in Fig. 4A, we first confirmed its integrality by agarose gel electrophoresis and measured its concentration by spectrophotometry. Then first strand cDNA was reverse transcribed, identical chips were hybridized with cDNAs corresponding to 8 and 40 ng initiative mRNA, respectively (Typically, 1%–5% of total RNA is mRNA; Sambrook and Russell, 2001). The results showed that the quantity of initiative mRNA was consistent with the corresponding colors and all of the quantification assay can be finished in about 30 min. So the method, optical thin-film biosensor chip technique, for mRNA quantification has been established successfully.

Discussion

We have demonstrated that using optical thin-film biosensor chips can detect and quantitate very small amount of mRNA from 1 ng to 200 ng in 100 μL of reaction solution. High concentrations of initiative mRNA (above 200 ng in 100 μL of reaction solution) did not result in further color change. One could provide more dilutions of the mRNA in order to increase the upper range.

The ability of this assay to detect such small amounts of RNA (approximately 1 ng per 100 μL of reaction solution) makes it particularly useful. Poly(A) mRNA, which is in considerably smaller amounts than ribosomal RNA, can be quantitated on optical thin-film biosensor chips without using a large amount of sample. As this method is based on cDNA/DNA hybrid, the first strand cDNA synthesis is most important and the high quality of RNA is required.

This method, optical thin-film biosensor chips, is sensitive, feasible and time saving for poly(A) mRNA detection. The optical thin-film biosensor has been developed to detect DNA, such as detection of single nucleotide polymorphisms (SNPs) and point mutations in human and *Arabidopsis*, SNP marker-assisted breeding in tomato, detection of genetically modified organisms/food (GMO) etc (Zhong et al., 2003; Bai et al., 2007). For its obvious advantage on the visible quantification data that can be read directly by unaided human eyes or photographed under a simple dissection microscope, it has great potential for application in mRNA quantification in many other organisms.

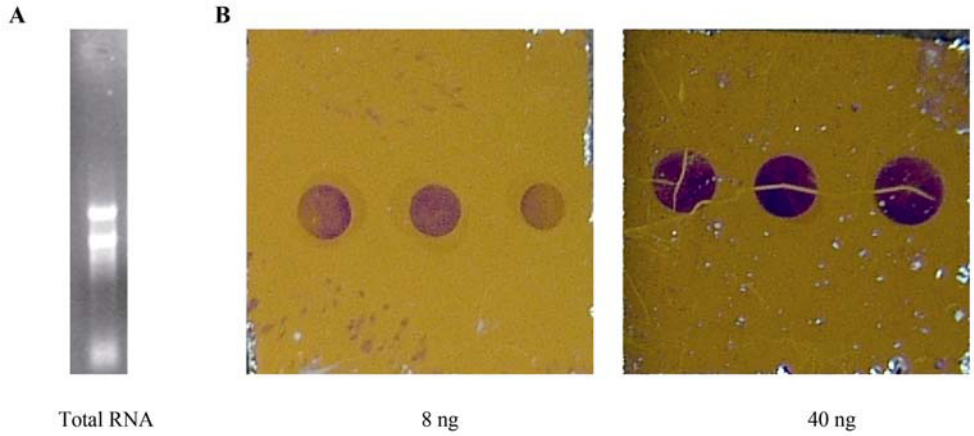

Fig. 4. Detection of initiative mRNA in *Arabidopsis* using optical thin-film biosensor chips. **A:** total RNA extracted from *Arabidopsis* was checked by agarose gel electrophoresis. **B:** the initiative mRNA at about 8 ng and 40 ng in 100 μL of reaction solution were detected and the spotting colors were consistent with the quantity of mRNA (comparing with the standard color plate, in Fig. 3B).

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