Automated capture and on-column detection of biotinylated DNA on a disposable solid support

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Received 13th April 2007, Accepted 14th June 2007
First published as an Advance Article on the web 2nd July 2007
DOI: 10.1039/b705617f

This work comprises the development of a technique for the capture of single-stranded DNA on a solid support combined with *in situ* quantification. The capture is based on the strong and selective interaction between biotinylated DNA and streptavidin-coated agarose beads. Sequential Injection in the lab-on-valve format allows for automated manipulation of all components including the building and disposal of bead columns. Detection was accomplished using the OliGreen fluorescent dye and optimization of the assay achieved a limit of detection of 111 pg ssDNA, with a total assay time of roughly 2.5 min per sample.

Introduction

The purpose of this work is to develop a highly sensitive method for the capture and quantification of biotinylated DNA (b-DNA) on a solid phase. Sequential Injection in the lab-on-valve format (μSI-LOV) lends itself very well to this goal as evidenced by several solution-phase techniques for quantifying DNA having been already translated to Sequential Injection.[1,2] The use of Bead Injection can further enhance the method by allowing for the pre-concentration of DNA samples onto a solid phase with subsequent elution and solution-phase detection.[3,4] This work employs Bead Injection to pre-concentrate the sample; however, instead of eluting DNA for post-column detection the signal is collected by on-column detection, as has previously been described for protein determination.[5,6] A benefit of on-column detection is the ability to detect strongly or irreversibly bound molecules, as in the interaction between biotinylated DNA fragments and streptavidin-derivatized surfaces.[7]

Many methods exist for quantifying DNA, including the use of fluorescent dyes,[8,9] absorbance spectrometry (both native[10] and indicator based[11,12]), chemiluminescence,[13,14] and light scattering.[15,16] The choice of detection technique is primarily dependent on the particular application and the sensitivity that the application requires. While UV absorbance is by far the easiest method of quantification available, fluorescent probes, such as OliGreen, allow for much more sensitive and selective detection.[17]

In this work, OliGreen and biotinylated, single-stranded DNA (b-ssDNA) are used in a Bead Injection technique that provides high sensitivity and selectivity for the detection of DNA samples in an environment where bead columns can easily be built and discarded. This work is part of a larger effort to develop an automated method for detecting the activity of the telomerase enzyme. The ability to pre-concentrate the DNA on a bead column by using biotinylated telomerase substrate may be critical to the development of a non-PCR alternative to the telomere repeat amplification protocol (TRAP), which is becoming of greater interest in the field of telomere biology.[18] The approach presented here represents significant progress towards the development of an assay for telomerase activity using Sequential Injection technology.

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Experimental

Instrumentation

The experiments were carried out using a FIALab 3000 Sequential Injection instrument (FIALab Instruments, www.flowinjection.com). The system (Fig. 1) was configured with a 500 μL syringe pump, a 500 μL holding coil and a lab-on-valve manifold (LOV) mounted on a six-port multi-position valve. The flow-cell of the LOV was configured with 600 μm ID fiber optic probes oriented at a 90° angle to each other and the remaining channel plugged, forming a 1.6 mm diameter cylindrical flow cell for the entrapment of beads as described previously.[19] The horizontally oriented probe was connected to the light source, which was fabricated in house using a 470 nm Cree Xlamp (Superbright LEDs, www.superbrightleds.com) to provide light for fluorescence excitation. The output of the light source was filtered with a 500 nm shortpass filter. The vertically oriented probe was connected to a PMT for the detection of fluorescence emission. A 520 nm ± 15 nm bandbass filter was placed between the collection fiber and the PMT to reject scattered excitation light.

Reagents

TE buffer was prepared from 20X TE buffer (T11493, Molecular Probes, probes.invitrogen.com) by diluting the concentrated buffer 1 : 20 with DNAse free water (W4502, Sigma, www.sigma-aldrich.com) containing 0.1% Tween 20. This gave a final buffer of 10 mM Tris-HCl, 1 mM EDTA, pH 7.5 and 0.1% Tween 20. This buffer was used for all further reagent preparation and as the carrier solution.

OliGreen reagent was prepared from Quant-iT OliGreen ssDNA Reagent in DMSO (O7582, Molecular Probes,

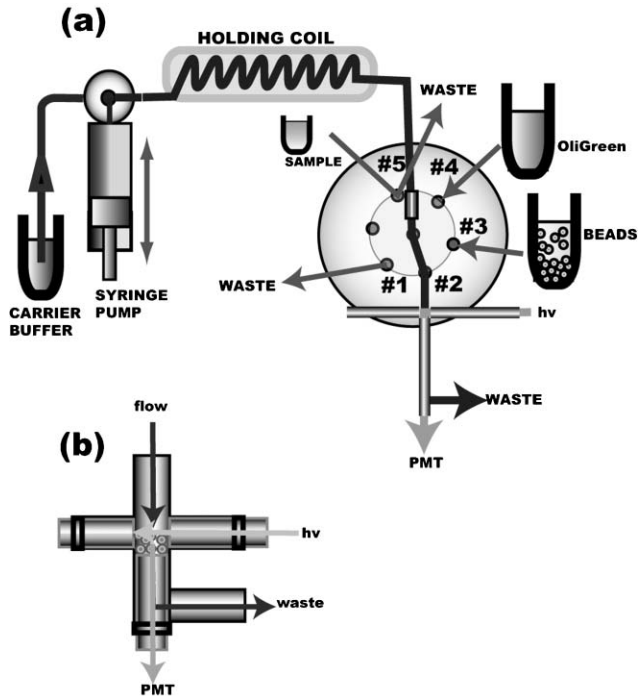

Fig. 1 Schematic of the Sequential Injection system. (a) The system consists of a 500 μL syringe pump, two-way valve, 500 μL holding coil and a lab-on-valve (LOV) manifold with the following port assignments: 1 – waste, 2 – flow cell, 3 – bead suspension, 4 – OliGreen reagent and 5 – sample with the sixth port unused and blocked. (b) Enlarged view of the flow cell port of the LOV. For solution-phase measurements, the flow cell is filled with solution. For Bead Injection experiments, the beads are retained between the fiber optic probes, as shown.

probes.invitrogen.com) by diluting the concentrated solution 1 : 200 with TE buffer immediately prior to the experiments.

Biotinylated, single-stranded DNA (b-ssDNA) solutions were prepared from 26- and 50-base, GT repeating, biotinylated oligomers (Operon, www.operon.com). The solid b-ssDNA was dissolved in TE buffer to 99.3 μg mL^{-1} DNA stock solution. This solution was further diluted in TE buffer to working conditions immediately prior to the experiments.

Beads were prepared by washing streptavidin-coated Sepharose 4B beads (43-4341, Zymed, www.zymed.com) with TE buffer several times. The washed beads were then suspended (1 : 8) in TE buffer.

Development of methods

Bead injection with fluorescence detection. The capture of b-ssDNA on beads and subsequent labeling with OliGreen was controlled with a software protocol (Table 1). Beads were drawn from the suspension and sent to the flow cell. The resulting bead column filled the flow cell to just slightly above the incident light beam. Monitoring began after the formation of the column and continued as a 10 μL bolus of b-ssDNA was perfused through the column at a slow flowrate (1 μL s^{-1}), followed by 30 μL of carrier serving as a wash. A 10 μL bolus of OliGreen dye was then perfused through the beads, followed by 30 μL of carrier, at 1 μL s^{-1}. Finally, the monitoring ceased and the bead column was removed by flow reversal and sent to waste. This protocol was repeated three times for each sample and typical data generated are shown in Fig. 2.

Table 1 Bead Injection software protocol

Start loop	
Loop Start (#) 3	
Pre-load syringe	
SyringePump Valve In	
SyringePump Flowrate	100 μL s^{-1}
SyringePump Aspirate	350 μL
SyringePump Delay Until Done	
SyringePump Valve Out	
Build bead column	
Valve beads	
SyringePump Flowrate	5 μL s^{-1}
SyringePump Aspirate	60 μL
SyringePump Delay Until Done	
Valve flowcell	
SyringePump Flowrate	10 μL s^{-1}
SyringePump Dispense	50 μL
SyringePump Delay Until Done	
Valve beads	
SyringePump Dispense	50 μL
SyringePump Delay Until Done	
Start PMT	
Analyte New Sample	
PMT Start Scans	
Aspirate b-DNA	
Valve sample	
SyringePump Flowrate	10 μL s^{-1}
SyringePump Aspirate	10 μL
SyringePump Delay Until Done	
Deliver b-DNA to beads	
Valve flowcell	
SyringePump Flowrate	1 μL s^{-1}
SyringePump Dispense	40 μL
SyringePump Delay Until Done	
Aspirate dye	
Valve dye	
SyringePump Flowrate	10 μL s^{-1}
SyringePump Aspirate	10 μL
SyringePump Delay Until Done	
Label captured b-DNA	
Valve flowcell	
SyringePump Flowrate	1 μL s^{-1}
SyringePump Dispense	40 μL
SyringePump Delay Until Done	
PMT Stop Scans	
Discard column	
SyringePump Flowrate	20 μL s^{-1}
SyringePump Dispense	150 μL
SyringePump Delay Until Done	
SyringePump Flowrate	20 μL s^{-1}
SyringePump Aspirate	100 μL
SyringePump Delay Until Done	
Valve waste	
SyringePump Flowrate	100 μL s^{-1}
SyringePump Empty	
SyringePump Delay Until Done	
Loop End	

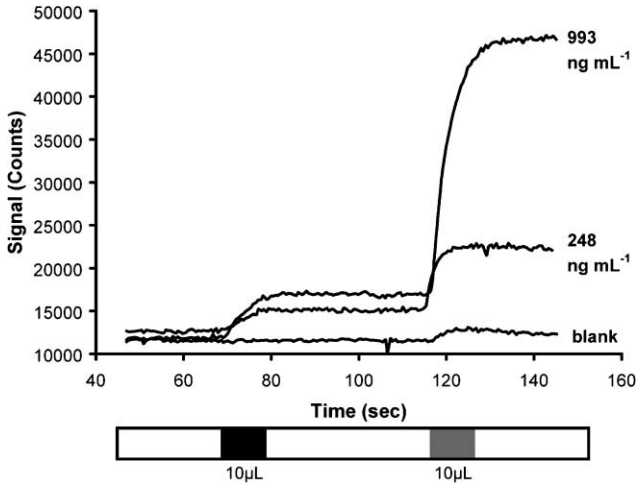

Fig. 2 Biotinylated-ssDNA (b-ssDNA) captured on beads. The black bar represents the delivery of b-ssDNA, the gray bar represents the delivery of OliGreen and the white bars represent carrier solution. Flowrate: 1 μL s^{-1}.

Experiments were performed to determine the optimum volume of beads to aspirate from the 1 : 8 bead suspension. The volumes aspirated ranged from 52 to 100 μL and a 99.3 ng mL^{-1} b-ssDNA solution was used for all measurements. Signal magnitudes were determined from the collected data and plotted *versus* the volume of bead suspension aspirated (Fig. 3). Volumes less than 60 μL resulted in a bead column not being large enough to fill any noticeable portion of the flow cell directly in the path of the incident light. At volumes of 68 μL or more the bead column was sufficiently large such that the beads occupied the entire path of the incident light. A 68 μL aspirated bead volume was chosen for all subsequent experiments in order to maximize the intensity of signals and minimize the effects of variation in the bead column size. This column occupies approximately 2.2 μL.

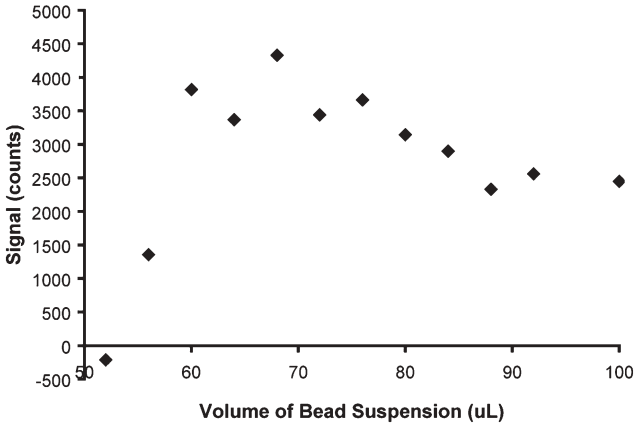

Fig. 3 Optimization of bead-sampling volume. Bead columns were built using aspirated volumes of bead suspension ranging from 52 to 100 μL and were evaluated using the signals obtained for the capture of 99.3 ng mL^{-1} b-ssDNA and its subsequent labeling. 68 μL was determined to be the optimal volume of bead suspension to aspirate.

Stopped-flow, solution-phase measurements. Solution-phase measurements were performed using a protocol (Table 2) comprised of the following steps: 20 μL of OliGreen were aspirated into the holding coil, followed by 10 μL of b-ssDNA and another 10 μL of OliGreen, each aspirated at 5 μL s^{-1}. 15 μL of the stacked zones were then delivered to the flow cell at a flowrate of 1 μL s^{-1} and the flow was stopped for 60 s. Subsequently, all solutions were washed out of the flow cell with 450 μL carrier at a flowrate of 50 μL s^{-1}. Monitoring of the reaction began just prior to the delivery of solution to the flow cell and was stopped just prior to washing out the flow cell. This protocol was repeated three times for each sample and typical results are shown in Fig. 4.

Signal processing. All signals were calculated as the difference between the average of the last 10 data points and the average of the first 10 data points for each run.

Table 2 Solution-phase software protocol

Start loop	
Loop Start (#) 3	
Start PMT	
Analyte New Sample	
PMT Start Scans	
Pre-load syringe	
SyringePump Valve In	
SyringePump Flowrate	100 μL s^{-1}
SyringePump Aspirate	450 μL
SyringePump Delay Until Done	
SyringePump Valve Out	
Aspirate dye segment 1	
Valve dye	
SyringePump Flowrate	5 μL s^{-1}
SyringePump Aspirate	20 μL
SyringePump Delay Until Done	
Aspirate DNA	
Valve sample	
SyringePump Aspirate	10 μL
SyringePump Delay Until Done	
Aspirate dye segment 2	
Valve dye	
SyringePump Aspirate	10 μL
SyringePump Delay Until Done	
Deliver to flow cell	
Valve flowcell	
SyringePump Flowrate	1 μL s^{-1}
SyringePump Dispense	15 μL
SyringePump Delay Until Done	
Stopped flow	
Delay	60 s
PMT Stop Scans	
Wash flow cell	
SyringePump Flowrate	50 μL s^{-1}
SyringePump Empty	
SyringePump Delay Until Done	
Loop End	

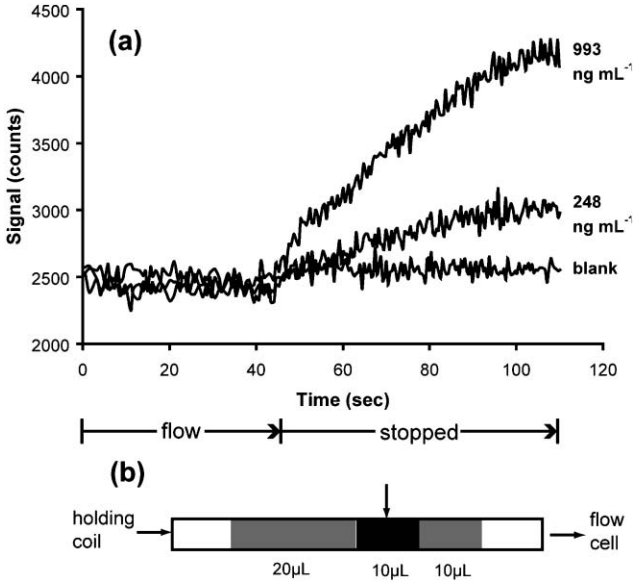

Fig. 4 Solution-phase measurement of b-ssDNA. Part (a): b-ssDNA reacting with OliGreen in the absence of beads and under stopped flow. Arrows indicate the flow and stopped-flow regions of the signals. Part (b): the order in which the zones of DNA, OliGreen and carrier solution were stacked in the holding coil and the point at which flow was stopped during delivery to the flow cell. The black bar represents DNA, the gray bars OliGreen, and the white bars carrier solution. Flowrate: 1 μL s^{-1}.

Results and discussion

The Bead Injection system was calibrated using solutions of a biotinylated, 50-base oligomer ranging in concentration from 0 ng mL^{-1} to 993 ng mL^{-1}. Data are shown (Fig. 2) for the capture of b-ssDNA on beads and subsequent labeling with OliGreen dye. The first increase in signal observed corresponds to the delivery of a 10 μL bolus of b-ssDNA solution. The second, much larger increase in signal corresponds to the delivery of a 10 μL bolus of OliGreen dye and its reaction with the captured DNA.

Calibration of the solution-phase system was performed using the same set of b-ssDNA and reagents as for the Bead Injection method. Following delivery of 15 μL of the stacked sample and reagent zones to the flow cell, the flow was stopped for 60 s to allow the ssDNA to react with the OliGreen. Data from the stopped-flow reactions are shown in Fig. 4a. The position of the stacked zones (Fig. 4b) for the stopped-flow period was chosen in order to maximize the concentration of DNA in the flow cell while ensuring that sufficient dye was available to label the DNA in front of the detector. After the stopped-flow period, flow was resumed to wash out the flow cell and bring the signal back to the baseline. No data were collected during this time.

Calibration curves for solution-phase and Bead Injection measurements demonstrate good linearity with calibration equations of $y = 1.89x + 41.5$ ($r^2 = 0.992$) and $y = 35.4x + 1.21 \times 10^3$ ($r^2 = 0.998$), respectively from triplicate runs. Comparison of these curves reveals that the signals observed are significantly higher for Bead Injection measurements than

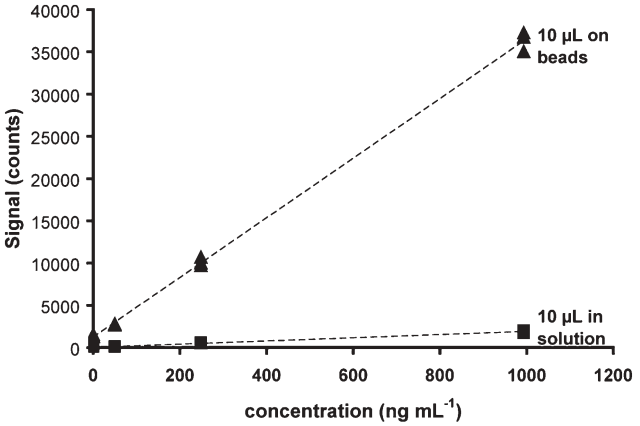

Fig. 5 Comparison of solution-phase and Bead Injection methods for detection of labeled DNA. Each sample is shown in triplicate. The lines demonstrate the difference in sensitivity provided by the two approaches.

for solution-phase measurements (Fig. 5). This can primarily be attributed to the capture of DNA on the bead column concentrating the sample in the detection flow cell whereas solution-phase measurements can only monitor a fraction of the sample at any given time.

Optimization of the delivery flowrate for Bead Injection experiments was accomplished by delivering several samples of b-ssDNA, with identical concentrations (99.3 ng mL^{-1}) and constant volumes (10 μL), to the column at varying flowrates from 0.5 μL s^{-1} to 4 μL s^{-1} (Fig. 6). DNA capture was followed by the delivery of 10 μL OliGreen at a constant flowrate of 1 μL s^{-1} and the signal was calculated as previously described. The signals observed show a distinct trend towards increasing capture of DNA with decreasing flowrates, however, the time required for the assay also increases significantly with decreasing flowrates. A flowrate of 1 μL s^{-1} was chosen for delivery of DNA to the bead column in order to balance signal strength with assay time.

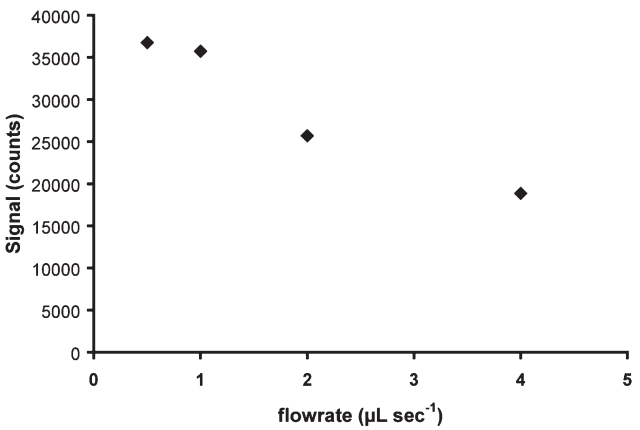

Fig. 6 Summary of flowrate optimization for the capture of b-ssDNA on beads. 10 μL of 99.3 ng mL^{-1} b-ssDNA solution were delivered to the bead column at flowrates ranging from 0.5 μL s^{-1} to 4 μL s^{-1}. 1 μL s^{-1} was chosen as the optimal delivery rate for all subsequent experiments.

As verification that this assay could handle varying lengths of ssDNA, calibration curves were generated for biotinylated 50-base and 26-base oligomers. The injected mass ranged from 0 to 9.93 ng ssDNA. As expected, the same mass of ssDNA delivered to the column as either a 26- or 50-base oligomer yielded very similar calibration curves of $y = 33.3x + 665$ ($r^2 = 0.998$) and $y = 35.4x + 1.21 \times 10^3$ ($r^2 = 0.998$), respectively.

Conclusion

The ultimate goal of our research is the development of a practical assay for the determination of telomerase activity. The selectivity of the desired telomerase assay is based on the elongation of a biotinylated primer and subsequent selective capture on beads *via* the biotin–streptavidin interaction. Due to the strength of this bond, derivatization with a fluorescent probe on-column and measurement *in situ* is the most practical way to assay the amount of elongated DNA present. The work presented here is an essential step toward the realization of this goal.

We believe that this novel approach for the selective assay of biotinylated DNA will become useful for applications other than telomerase that we cannot yet foresee.

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