Real-Time Immuno-PCR on the iCycler iQ™ System

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Introduction

A technique for antigen detection, called immuno-PCR, was developed by Sano et al. (1992). It combines the molecular recognition of antibodies with the high DNA amplification capability of PCR. The procedure is similar to conventional enzyme-linked immunosorbent assays (ELISA) but allows for more sensitive detection. Instead of an enzyme, a DNA molecule is linked to the detection antibody and serves as a template for PCR (Figure 1). The DNA molecule is amplified and the PCR product is measured by gel electrophoresis. An improvement of this method is to amplify the oligomer in a real-time PCR instrument, thereby eliminating post-PCR analysis (Sims et al. 2000). Further, real-time PCR is extremely accurate and sensitive, which should make it possible to quantitate very low amounts of DNA-coupled detection antibody with high accuracy. Here we present early results on the development of real-time immuno-PCR for prostate specific antigen (PSA) using the iCycler iQ system. PSA is a well-known tumor marker for prostate cancer and is widely used to detect, stage, and monitor the disease.

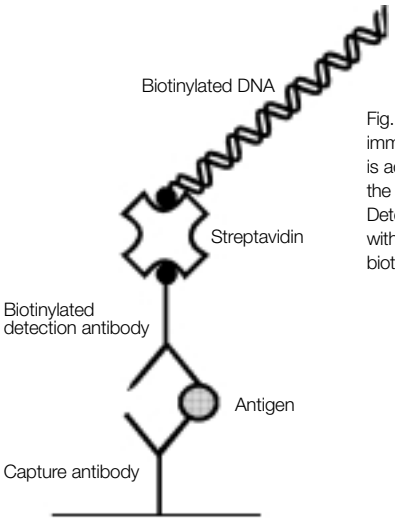

Fig. 1. Schematic of real-time immuno-PCR. Capture antibody is adsorbed to the surface of the wells in a microtiter plate. Detection antibody is tagged with a DNA marker through a biotin-streptavidin-biotin linkage.

Methods

Anti-PSA10 and anti-PSA66 from CanAg Diagnostics were used as capture and detection antibodies, respectively, in the sandwich immuno-PCR assay. Anti-PSA66 was biotinylated using biotinamido-caproate-N-hydroxysuccinimide ester. Biotinylated DNA was generated by amplifying a 1,098 bp fragment of the *gusA* gene (*E. coli* β-glucuronidase gene) with a 5'-biotinylated forward primer (biotin-AACTATGCCGG-AATCCATCG-3') and unmodified reverse primer (5'-ACATAT-CCAGCCATGCACAC-3'), and was purified with the QIAquick PCR purification kit (QIAGEN).

Real-time PCR was performed in the Bio-Rad iCycler iQ system. The primers used were 5'-GTTAGCCGGGCTGCA-CTC-3' and 5'-ACATATCCAGCCATGCACAC-3', which produced a 71 bp product. Reaction volumes were 25 µl, containing 1x PCR buffer (Sigma), 4 mM $MgCl_2$ (Sigma), 200 µM dNTPs (Sigma), 0.08 µg/µl BSA (Fermentas), 1 U JumpStart *Taq* polymerase (Sigma), 300 nM of each primer, and 0.5x SYBR Green I (Molecular Probes, Inc.) Cycling conditions were 95°C for 3 min, followed by 40 cycles of 95°C for 20 sec, 60°C for 20 sec, and 72°C for 25 sec. The fluorescence data used for quantitation were collected at the end of each 72°C step.

Results and Discussion

The sandwich real-time immuno-PCR system was assembled in a standard PCR plate from Bio-Rad as shown in Figure 1. To each well, 50 µl anti-PSA10 (10 µg/ml in 0.2 M phosphate buffer) was added and allowed to adsorb to the surface of the PCR plate overnight. The wells were then washed three times with wash buffer 1 (0.154 M NaCl, 5 mM Tris, pH 7.75, 0.005% Tween 20, 0.1% Germall II), and the surface was then blocked from further adsorption by incubating at room temperature overnight with blocking buffer (50 mM Tris, pH 7.0, 6% D-sorbitiol, 0.1% BSA, 0.05 % NaN_3). The wells were washed with wash buffer 1, followed by incubation at room temperature with 5 µl of PSA standards (containing 2.4×10^6 to 2.8×10^9 molecules) and 20 µl Tris-HCl buffered salt solution containing BSA for 1 hr (CanAg PSA EIA instructions). The samples were then washed three times with wash buffer 1. Biotinylated anti-PSA66 was diluted to 0.92 µg/L with phosphate buffer containing BSA and immunoglobulins to reduce nonspecific adsorption (CanAg PSA EIA instructions).

To each well, 25 µl of the diluted biotinylated anti-PSA66 was added and the samples were incubated for 1 hr at room temperature. After washing six times with wash buffer 1, the wells were incubated for 30 min at room temperature with 25 µl streptavidin solution (0.3 µg/ml streptavidin in 10 mM Tris-HCl, pH 7.5, 50 mM KCl, 2.5 mM $MgCl_2$). Thereafter, the samples were washed six times with wash buffer 1 and incubated with 25 µl of biotinylated DNA marker (15 pM). The wells were then washed six times with wash buffer 1, followed by four times with wash buffer 2 (0.154 M NaCl, 5 mM Tris, pH 7.75), and then left to stand in wash buffer 2 for 1 hr before removing the buffer. Finally, the PCR mix was added to the wells and real-time PCR was performed in the iCycler iQ system (Figure 2). Approximately 15–20 cycles were needed to reach threshold, set at 10,000 relative fluorescence units (RFU), which was substantially above the background but still in the exponential growth phase of the PCR. The C_T vs. log (concentration) plot was linear with a large correlation coefficient (r = 0.986), showing that the assay correctly and accurately reflects the amount of PSA in the studied range. However, PCR products that appear in negative control samples limit sensitivity. Analysis revealed two sources of these products. One is formation of primer-dimers, which form independently of the presence of template. There are several ways to suppress their formation through assay optimization techniques. The second source of error is nonspecific adsorption of the detection antibody or DNA. This is common to standard ELISA and can be reduced by proper blocking of the surface or by binding the capture antibody more tightly to allow for more extensive washing. These approaches are being developed.

The results presented here and also those reported previously (Sims et al. 2000) are promising indications that applications of real-time PCR will expand to protein detection. A challenging future task will then be to quantitate the levels of both mRNA and the corresponding protein in only a few cells, or perhaps even a single cell, to study the correlation between gene and protein expression.

Acknowledgements

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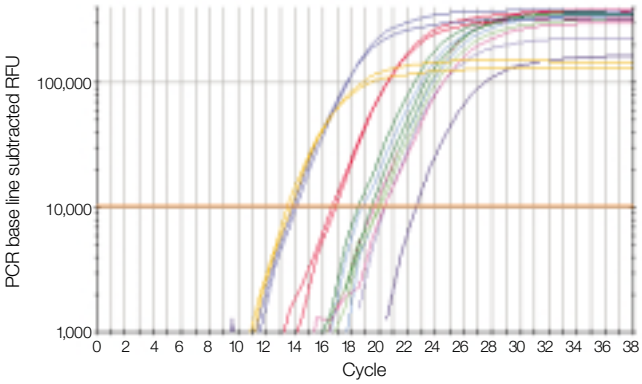

Fig. 2. Amplification plot of real-time immuno-PCR of PSA using anti-PSA10 and anti-PSA66 as capture and detection antibodies, respectively. Threshold was set to 10,000 RFU. Samples contained 2.8 x 10^9, 9.4 x 10^8, 9.4 x 10^7, 9.4 x 10^6, and 2.4 x 10^6 molecules of PSA; negative controls were without PSA.

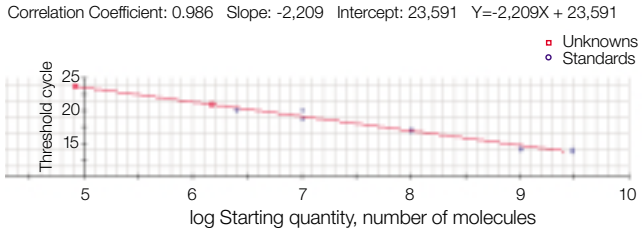

Fig. 3. C_T vs. log (concentration) plot. Samples shown in blue contain 2.4 x 10^6 to 2.8 x 10^9 molecules of PSA, while samples shown in red are negative controls without PSA.

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