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Strand displacement amplification for ultrasensitive detection of human pluripotent stem cells

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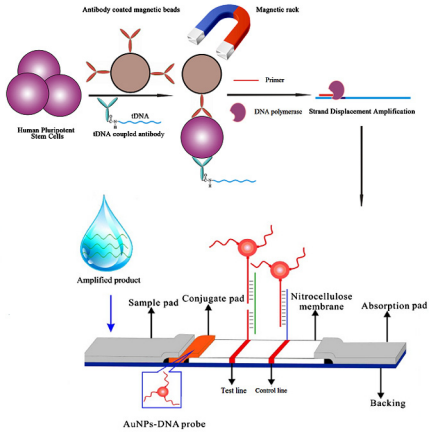
HIGHLIGHTS

- The limit of detection was 19 cells by strip reader and 100 cells by naked eye.
- This assay shows good specificity and sensitivity of whole stem cells detection.
- The gold-nanoparticle based lateral flow biosensor enables visual detection.

GRAPHICAL ABSTRACT



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ABSTRACT

Human pluripotent stem cells (hPSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), provide a powerful model system for studies of cellular identity and early mammalian development, which hold great promise for regenerative medicine. It is necessary to develop a convenient method to discriminate hPSCs from other cells in clinics and basic research. Herein, a simple and reliable biosensor for stem cell detection was established. In this biosensor system, stage-specific embryonic antigen-3 (SSEA-3) and stage-specific embryonic antigen-4 (SSEA-4) were used to mark human pluripotent stem cells (hPSCs). Antibody specific for SSEA-3 was coated onto magnetic beads for hPSCs enrichment, and antibody specific for SSEA-4 was conjugated with carboxyl-modified tDNA sequence which was used as template for strand displacement amplification (SDA). The amplified single strand DNA (ssDNA) was detected with a lateral flow biosensor (LFB). This biosensor is capable of detecting a minimum of 19 human embryonic stem cells by a strip reader and 100 human embryonic stem cells by the naked eye within 80 min. This approach has also shown excellent specificity to

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distinguish hPSCs from other types of cells, showing that it is promising for specific and handy detection of human pluripotent stem cells.

1. Introduction

Over the past decade, stem cell research has elicited continuous scientific, commercial, and public interest [1–3]. Human pluripotent stem cells (hPSCs), such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs), have a gene expression program that allows them to differentiate to all types of somatic cells in response to developmental cues [4]. Meanwhile, somatic cells can reprogram to iPSCs by ectopic expression of several transcription factors, originally Oct4, Sox2, Klf4 and Myc (OSKM) [5,6]. These two types of cell conversion hold great promise for regenerative medicine and provide a powerful model system for studies of cellular identity and development [7]. Therefore, an efficient and convenient approach for the discrimination of stem cells from somatic cells is highly required in both research and clinics.

Traditional cell lysate-based methods for stem cell identification, including reverse transcription-polymerase chain reaction (RT-PCR), western blot, and northern blot, are labor intensive, time consuming, and costly. They use specific proteins and messenger RNAs (mRNAs) in stem cells as markers. It usually takes several hours even days to obtain confirmed results. Besides, protein/DNA extraction also adds complexity to the detection [8–10]. Thus, rapid whole cell-based immunoassays, such as flow cytometry and immunofluorescence, for stem cell detection have been developed [11,12]. They use specific protein markers in stem cells as target. These methods have better performance in sensitivity and specificity, but expensive, specialized equipments and highly trained personnel are still required. To avoid these disadvantages, a lateral flow biosensor for the detection of hPSCs has been developed [13]. The surface proteins were used as markers, and a red band produced by gold nanoparticle (AuNP) accumulation in test zone was used as the positive signal. Although this biosensor is visual, cheap, rapid and convenient, the low sensitivity (10,000 cells) is still a major limitation. Therefore, signal amplification and magnetic bead based enrichment emerge may be effective for improving the sensitivity.

Nucleic acid is cheap, stable, and can be chemically synthesized in high purity and modified with relative ease. Moreover, the virtue of high amplification efficiency, simplicity, extensively validated standard operating procedure and good reproducibility gives nucleic acid more superiority to other signal molecules. Nucleic acid amplification, especially isothermal amplification, is a valuable molecular tool not only in basic research but also in application oriented fields [14–16]. With these advantages, various strategies for signal conversion from protein/small molecules to nucleic acid have been reported [17–19]. These strategies include two types, one involves specific DNA binding with target protein/small molecules, and the other uses modified DNA conjugated to protein/small molecules. The former only suits for some special protein/small molecules, such as transcription factors and proteins with known aptamers. The latter is a general DNA-based protein detection platform. For example, thiol-modified random DNA sequence can conjugate to protein A and the complementary DNA modified with fluorescent molecule is used as signal reporter for multiple detections of green fluorescent protein (GFP), *Renilla* luciferase (RL), and heat shock factor (HSF) [19]. Herein, we established a platform for stem cell detection by using two types of antibodies specifically binding to surface markers of stem cells. Briefly, magnetic beads coated with one type of antibodies were used to enrich target cells and carboxyl-modified template DNA (tDNA) conjugated to another type of antibodies was used as amplification template. Based on the pre-analytical sample enrichment by magnetic beads and isothermal strand displacement amplification (SDA) for signal amplification, the specificity and sensitivity of this biosensor was highly improved.

In this study, a simple and reliable biosensor for hPSC detection was constructed, in which the surface proteins, SSEA-3 and SSEA-4, were used to mark human pluripotent stem cells (hPSCs). Magnetic beads coated with antibodies for SSEA-3 were used for hPSC enrichment. Carboxyl-modified tDNA sequence conjugated to antibodies for SSEA-4 was used as template for SDA. The amplified single strand DNA (ssDNA) was detected with a lateral flow biosensor (LFB) (Fig. 1). The LFB has the following features: (i) high sensitivity (limit of detection of 100 cells), (ii) short detection time (within 1 h), (iii) no cell lysis needed for the detection, (iv) visual detection by the naked eye, and (v) no need for complex and expensive instrumentation.

2. Materials and methods

2.1. Reagents and chemicals

Human SSEA-3 and SSEA-4 antibodies were purchased from R&D Systems (Minneapolis, USA). Epoxy-modified magnetic beads and magnetic rack were purchased from INNOSEPBIO (Zhengzhou, China). Oligonucleotides were purchased from Shanghai Sangon Biological Engineering Technology (Shanghai, China). Klenow (exo$^-$) DNA polymerase, Nt.BbvCI nicking enzyme, deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs (New England, USA). Bovine serum albumin (BSA) and $HAuCl_4$ were purchased from Sigma–Aldrich (Steinheim, Germany). mTeSR™1 medium was purchased from STEMCELL Technologies Inc. (Vancouver, Canada). Matrigel™ hESC-qualified matrix was purchased from BD Biosciences (San Diego, USA). DMEM/F-12 medium was purchased from Hyclone (Logan, USA). Nitrocellulose (NC) membranes were purchased from Shantou Ealon (Shantou, China). All other reagents were purchased from standard commercial sources and were of analytical grade. The strip reader was purchased from Kinbio (Shanghai, China). All buffer solutions used in this study were prepared using ultrapure water (resistance of 18 MΩ).

2.2. Construction of lateral flow biosensor

Thirty microliters of 100 μM DNA probes (test line probe and control line probe) was dispensed onto the nitrocellulose membrane simultaneously with a lateral flow dispenser (Shanghai Kinbio, Shanghai, China). The membrane was then dried at room temperature for 12 h. Fiberglass was used as sample pads after being soaked in sample pad buffer (0.5% Triton, 1% BSA, 2% sucrose, 50 mM boric acid, pH 8.0). It was dried and stored in low-humidity at room temperature. Sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were attached along the long axis of an adhesive plate with an overlap of 1–2 mm, and cut into 4-mm-wide strips using a paper cutter (Fig. 1B).

2.3. Cell culture

For culture ware coat, diluted Matrigel™ was used, 1 mL per well of 6-well plate. The plate was swirled to spread the solution

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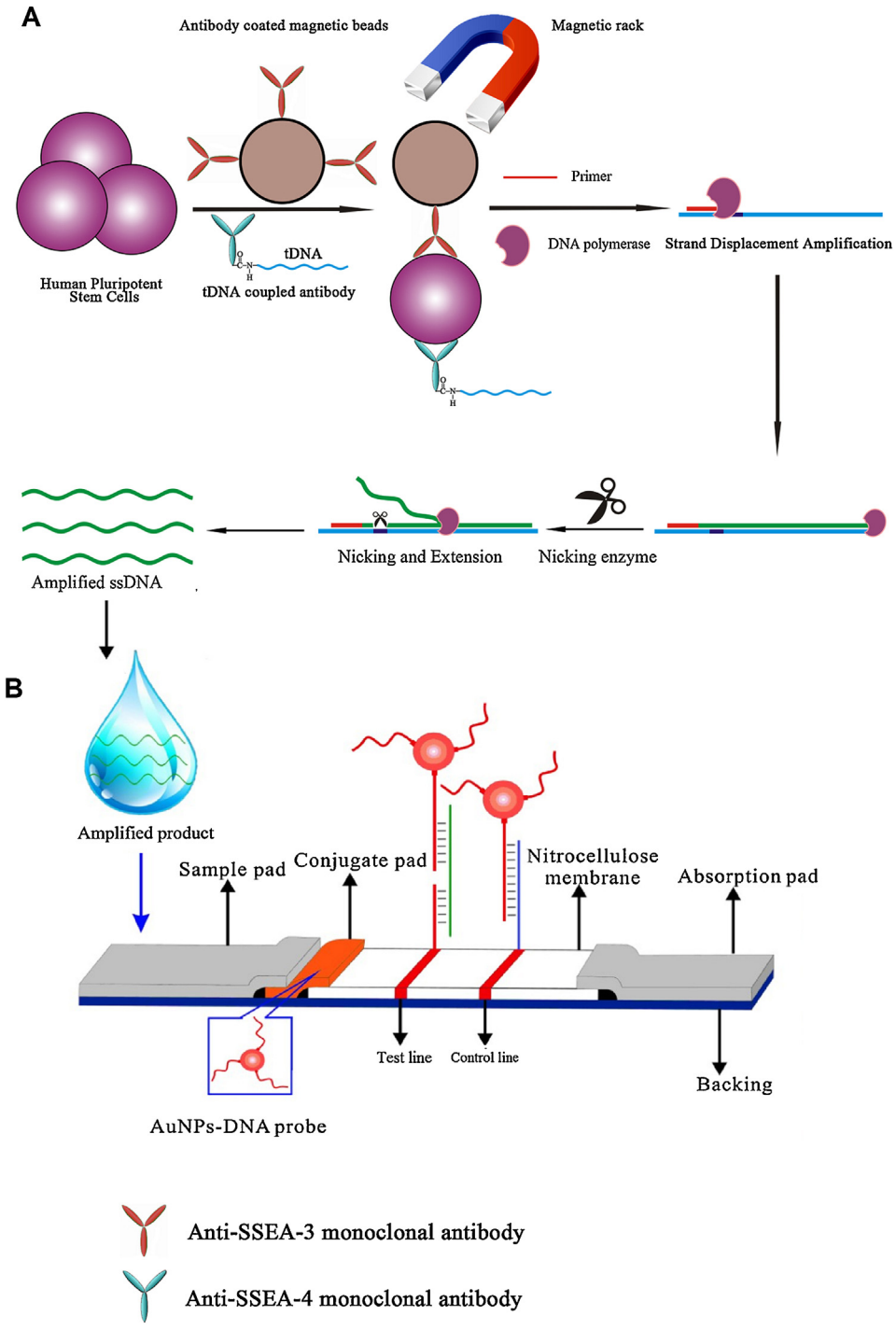

Fig. 1. Schematic illustration of the LFB for the detection of human pluripotent stem cells (hPSCs). (A) A schematic illustration of the magnetic enrichment of hPSCs and SDA amplification of tDNA. hPSCs are first mixed with excess amount of ssDNA coupled SSEA-4 antibody. After incubation, SSEA-3 antibody coated magnetic beads (MB) are added, and the MB–hPSCs–tDNA complexes are collected by a magnet rack. The collected MB–hPSCs–tDNA complexes are directly amplified by isothermal strand displacement amplification. (B) The amplified product is loaded onto the sample pad for visual detection.

evenly across the surface. Coated culture ware was kept at room temperature (15–25 °C) for at least 1 h before use. Matrigel™ solution in the plate was air-drought until they are ready for use. The hiPSCs were quickly thawed in a 37 °C water bath by gently shaking the cryovial continuously until only a small frozen pellet remains. A pipette of 2 mL was used to transfer the contents of the cryovial to a 15 mL conical tube. Warm mTeSR™ of 5–7 mL was added drop by drop to the tube, gently mixing as the medium is added [20].

Cells were centrifuged at 300 × *g* for 5 min at room temperature (15–25 °C). The cell pellet was kept intact after aspirating the medium. The cell pellet was gently resuspended in 1 mL of mTeSR™ by using a 2 mL pipette, taking care to maintain the cells as aggregates avoiding cell loss. The Matrigel™ was removed from a coated tissue culture plate by gently tilting the plate and allowing the excess Matrigel™ solution to collect in the corner. The solution was removed using a serological pipette or by aspiration. It should be noted that the tip of the pipette does not scratch the coated

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surface. The appropriate amount of medium containing the cell aggregates was transferred to a Matrigel™-coated 6-well plate. Ensure that clumps are evenly distributed between wells. The plates were put into a 37 °C incubator and moved side to side, forward to back motions to evenly distribute the clumps within the wells. Cells were cultured at 37 °C, with 5% CO_2 and 95% humidity. Culture medium was daily changed and the optimal passage time was determined.

2.4. Preparation of SSEA-4 antibody–tDNA conjugates

Dissolve the COOH-modified tDNA, SSEA-4 antibody and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) in PBS (0.1 M, pH 7.4). Add EDC to the COOH-modified tDNA to obtain at least a 1.5-fold molar excess of EDC over the amount of tDNA for carboxyl activation. Mix and react for 30 min at room temperature. Then, add SSEA-4 antibody to the above solution, in which tDNA is in a 30-fold molar excess of antibody. React for 2 h at room temperature. Purify the conjugate by dialysis using PBS overnight [21].

2.5. Preparation of SSEA-3 antibody coated magnetic beads

The epoxy-magnetic beads were washed for three times with PBS (0.1 M, pH 7.4) to remove the original supernatant buffer. Washed and resuspended beads of 1 mL were transferred to a tube. The tube was put in a magnet for 1 min and discarded the supernatant. The tube was removed from the magnet. PBS was used to resuspend the beads and 100 μg of SSEA-3 antibody was added during mixing to reach a total coupling volume of 1 mL for incubate for 15 min. BSA was added for block with a final concentration of 0.1% w/v, incubated for 16–24 h at 4 °C with gentle tilting and rotation. The supernatant was discarded by magnetic separation. PBS of 1 mL was added to wash the beads by gentle tilting and rotation. Remove the tube from the magnet and resuspend the beads in 1 mL of PBS [22].

2.6. Preparation of gold-nanoparticle (AuNP)–DNA conjugates

AuNPs with an average diameter of 15 nm were prepared according to our previously reported method [18,23]. Briefly, 4 mL of 1% trisodium citrate was added into 100 mL of a rapidly stirred and boiling $HAuCl_4$ solution (0.01%) in a 500-mL round bottom flask. After turning red, the solution was boiled for additional 10 min. It was cooled to room temperature with gentle stirring. The AuNPs were collected and concentrated four times by centrifugation (12×10^3 rpm, 20 min). The resulting AuNP solution was stored at 4 °C until use.

To prepare AuNP–DNA conjugate, 100 μL of ultra-pure water (18.2 MΩ cm^{-1}, Millipore, Billerica, MA, USA) containing 1 OD of thiolated DNA was added to 1 mL of the AuNP solution (four times concentrated) and shaken gently overnight at 4 °C. The DNA coated AuNPs (1.1 mL) were subjected to "aging" by adding 110 μL 100 mM phosphate buffer (pH 7.0) with 1% sodium dodecyl sulfate and 1.5 M NaCl. The solution was kept at 4 °C for 12 h. Particles were centrifuged (12×10^3 rpm, 20 min) and rinsed three times with rinsing buffer (20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20 and 10% sucrose) to remove any unbound DNA. The red pellet was resuspended in 150 μL of rinsing buffer and then stored in a refrigerator at 4 °C until use. The AuNP–DNA conjugate solution was dispensed onto fiberglass as conjugate pad [24].

2.7. Detection of stem cells

Stem cells were first mixed with excess amount of SSEA-4 antibody–tDNA conjugates in a 500 μL of PBS supplied with 0.1 mg mL^{-1} of salmon sperm DNA. The ultimate concentration of tDNA was 1 μM. The mixture was incubated at room temperature for 20 min. 5 μL of SSEA-3 coated MBs was then added to the mixture. The mixture was incubated at room temperature for 10 min with shaking. MB–cell–tDNA complexes were collected using a magnet rack. The precipitant was transferred to another tube and washed three times with PBST (PBS supplied with 0.05% Tween-20). The collected MB–cell–tDNA complexes were resuspended with 5 μL of ultrapure water. For the SDA based detection, the cell suspension was added to 15 μL of reaction solution (1 μM primer, 1 U Nt.BbvCI, 5 U polymerase Klenow fragment exo^-, 50 μM dNTPs, and 1 × polymerase Klenow fragment exo^- reaction buffer), and then incubated at 37 °C for 30 min. After amplification, 10 μL of the product was diluted in 50 μL of 4 × saline–sodium citrate (SSC) buffer and loaded onto the sample pad of the LFB. Test band and control band in LFB were visually determined in 5 min.

3. Results and discussion

3.1. Principle of the LFB for stem cell detection

For stem cell detection, antibodies specifically binding with surface markers of stem cells were used to recognize target cells. The stem cells used here were iPS-S cell line, which were induced from human foreskin fibroblast cells by transducing with a lentiviral cocktail of carrying 6 factors (OCT4, NANOG, SOX2, LIN28, C-MYC, and KLF4) [25]. In order to achieve high sensitivity, magnetic beads coated with antibodies were used for cell enrichment and pre-modified tDNA conjugated with antibodies was used as template for SDA to produce single-stranded DNA (ssDNA). At last, a lateral flow biosensor (LFB) was used to detect the amplified product ssDNA.

Firstly, antibodies for two surface markers (SSEA-3 and SSEA-4) of human pluripotent stem cells (hPSCs), were used in this assay [26]. The SSEA-4 antibody was pre-conjugated with tDNA. The SSEA-4 antibody was first incubated with hPSCs, and then SSEA-3 MBs were added. The SSEA-3 antibody was pre-coated onto epoxy-modified magnetic beads (MBs), by which hPSCs were captured for enrichment. After magnetic separation, the enriched tDNA that anchored on the cell surface was used as template for single direction strand displacement amplification (Fig. 1A).

Table 1
Oligonucleotides used in this work.

Oligonucleotide	Sequence (5′–3′)	Length (nt)
tDNA	CGCTGACATCGGTTCCACCTTCGGTCGCTGAGGCAATAGCTCCAG[a,b]	45
sPrimer	CTGGAGCTATTG	12
C-line DNA	GAACCGATGTCAGCG	15
T-line DNA	CACCTTCGGTCGCTG	15
AuNP probe	S-*n*-TTTTTCGCTGACATCGGTTC[c]	20

[a] Bold blue characters indicate the recognition sequence of Nt.BbvCI.
[b] Solid underlines indicate the additional primer binding sequence.
[c] S indicates thiol modification and *n* indicates a C6 linker.

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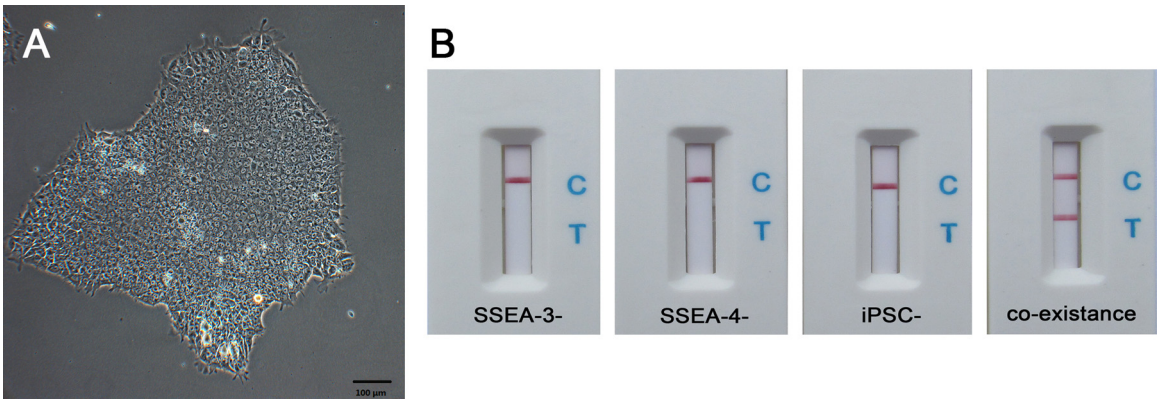

Fig. 2. Verification of the LFB for human pluripotent stem cell detection. (A) Morphology of undifferentiated iPSC cultured in the mTeSR system on Matrigel™ approximately 3 days after the cells were passaged. For iPSC culture, 10^4 cells/well were seeded in a 6-well plate and the culture medium was changed once a day. Before microscopy, culture medium was removed and cells in plates were washed three times with PBS. For microscopy, cells were suspended with 500 μL of PBS per well and observed on a microscope (magnification 100×). Scale bar: 100 μm. (B) Typical images of the biosensor for iPSC detection. From left to right are detection without SSEA-3 antibody, SSEA-4 antibody, iPSC and co-existence of both antibodies and iPSC. Cultured iPSCs were digested by 200 μL of 150 mM EDTA solution, washed with DMEM twice, washed with PBS twice, and then 100 μL of PBS was used to resuspend cells. After cell counting, 10^4 cells were used for each test. The amplification lasted for 30 min and 50 μL of loading buffer was used. At last, the image photos were captured by a camera.

Secondly, nicking enzyme assisted SDA was introduced for signal amplification. A Nt.BbvCI recognition site was designed to hybridize with the 3′-terminal of the tDNA for SDA amplification. An SDA reaction, comprising primer (sPrimer), DNA polymerase and nicking enzyme, is initiated by the enriched tDNA. The primer anneals with the tDNA and triggers a polymerization reaction in the presence of dNTPs and polymerase Klenow exo⁻. During primer extension, the synthesized complementary strand is cut by Nt.BbvCI nicking enzyme at its recognition site. The cut strand is then displaced by primer extension. Through this repeated process, a large number of amplified single stranded DNA (ssDNA) are produced and detected with a LFB (Fig. 1A).

Thirdly, in order to detect the amplified ssDNA, a nucleic acid LFB was constructed according to principle of DNA complementary pairing. A DNA probe (AuNP-probe) was modified with a thiol group at the 5′-terminal for attaching gold nanoparticle (AuNPs) by Au—S bond. The labeled AuNPs with AuNP-probe were immobilized onto the conjugate pad. The AuNP-probe was complementary to 3′-terminal of the ssDNA. When the ssDNA migrated through the conjugate pad, the ssDNA would be captured by AuNPs. Moreover, DNA immobilized on the test line (T-line DNA) was complementary to 5′-terminal of the ssDNA. When AuNPs captured with ssDNA arrived at the test line, a sandwich link between T-line DNA and AuNPs was formed and a red band can be observed in the test line (Table 1). As the AuNPs flowed along the nitrocellulose membrane, DNA immobilized on the control line (C-line DNA), which was complementary to the AuNP-probe, could capture the excess labeled AuNPs to form another red band in the control line (Fig. 1B). Therefore, a simple and rapid biosensor for hPSC detection is then constructed.

3.2. Validation of the LFB for human iPSC detection

In order to validate the LFB for hPSC detection, we selected human induced pluripotent stem cells (iPSCs) as the target, which were reprogrammed from human fibroblast. Fig. 2A shows the morphology of iPSCs used in our work. Undifferentiated iPSCs in the TeSR™ system grew as compact, multicellular colonies. When viewed under high magnification (i.e., 100× magnification), individual cells exhibited a high nuclear-to-cytoplasmic ratio and prominent nucleoli. Fig. 2B shows the typical photo images of the LFB. No test line was observed in the absence of iPSCs, SSEA-3 antibody or SSEA-4 antibody. The distinctive red test line could only be observed in coexistence of two antibodies and iPSCs. The results suggested that this LFB works well in iPSC detection. Moreover, in order to verify the specificity of sequences, mutant T-line DNA and mutant target ssDNA was synthesized to challenge our LFB (Supplemental information, Figs. S1 and S2). These results suggested that the signal was from the specific binding of perfect-match target-ssDNA with T-line DNA in the LFB.

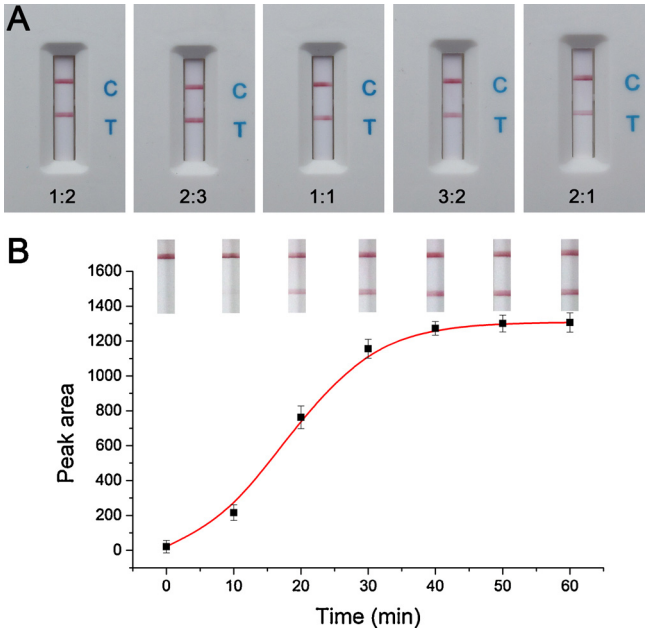

Fig. 3. Optimization of experimental conditions to improve sensitivity. (A) Typical images of biosensor for iPSC detection with various molar ratios of SSEA-3 antibody and SSEA-4 antibody. From left to right, the ratios of are 1:2, 2:3, 1:1, 3:2, 2:1. After cell counting, 10^4 cells were used for each test. The amplification lasted for 30 min and 50 μL of loading buffer was used. At last, the image photos were captured by a camera. (B) Effects of amplification time for detection of iPSCs. The measurements were performed after different time of amplification. After cell counting, 10^4 cells were used for each test. The top typical images of biosensor showed iPSC detection within various amplification times. The peak area values were measured by a strip reader. The illustrated error bars represent the standard deviation of three repetitive measurements.

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3.3. Optimization of experimental conditions to improve sensitivity

The SSEA-4 antibody–tDNA complex was used as template for signal amplification, and the SSEA-3 antibody–MB conjugation was used for template enrichment. As both SSEA-3 and SSEA-4 antibodies bind to the surface antigens of hPSCs, the molar ratio of the two antibodies determines the initial number of template and the efficiency of enrichment. We tested the two antibodies with various ratios for experimental optimization. As shown in Fig. 3A, the brightest test line was observed when the ratio of SSEA-3 antibody/SSEA-4 antibody reached 2:3. The two antibodies competitively bind to the surface of cells to form space steric hindrance between each other, which would affect the binding of SSEA-4 antibody–tDNA to cells, therefore the amount of ssDNA template. On the other hand, certain amount of SSEA-3 antibody is needed to provide enough magnetic force for pulling down the cells efficiently. Inefficient enrichment caused weaker signal on the test line. The optimum ratio of SSEA-3 antibody/SSEA-4 antibody at 2:3 was used in the following experiments.

The amplification time determines the total amount of SDA product ssDNA and the sensitivity of the assay. The SDA mixture was incubated at 37 °C for different periods of time and the performance of LFB for different amplification time are shown in Fig. 3B. The intensity of the test lines increased gradually in the first 30 min and remained stable for another 30 min, therefore, 30 min was chosen for the SDA reaction.

3.4. Sensitivity of the LFB for human iPSC detection

The sensitivity of the LFB was examined by using seven different concentrations of iPSCs suspended in 1 mL of PBS. For each test, 5 μL of magnetic beads with different amounts of iPSCs was added to 20 μL of SDA reaction solution. After incubation at 37 °C for 30 min, the SDA product ssDNA was loaded onto the LFB. Fig. 4A shows the typical images together with the corresponding optical responses of the biosensors when loaded with various amounts of iPSCs. No red band was observed at the test zone of the LFB in the absence of iPSC (negative control). A red band was observed by naked eyes at the test zone when as low as 100 cells of iPSCs were loaded. Well-defined peaks were observed, and the peak areas increased with the increase of the number of iPSCs. The

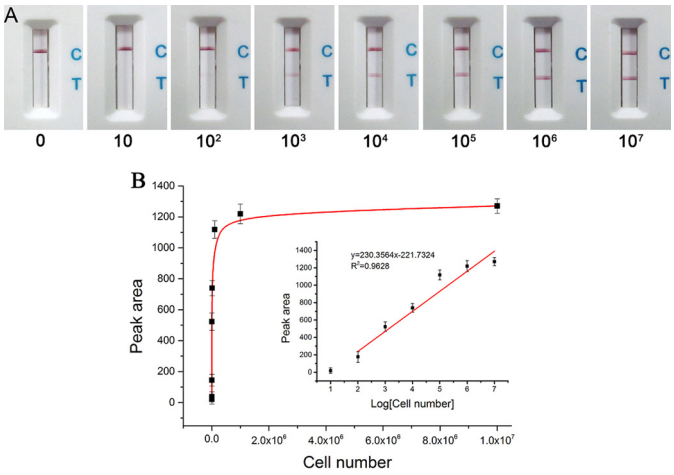

Fig. 4. Sensitivity of biosensor for iPSCs detection. (A) Typical images of biosensor for hPSC detection with different amounts of iPSCs, ranging from 0 to 10^7 cells. After cell counting, different amounts of cells were tested with this LFB. The amplification lasted for 30 min and 50 μL of loading buffer was used. The image photos were captured by a camera. (B) Calibration curve of the assay with different amounts of stem cells. The peak area values were measured by a strip reader. The peak areas were averages of three measurements.

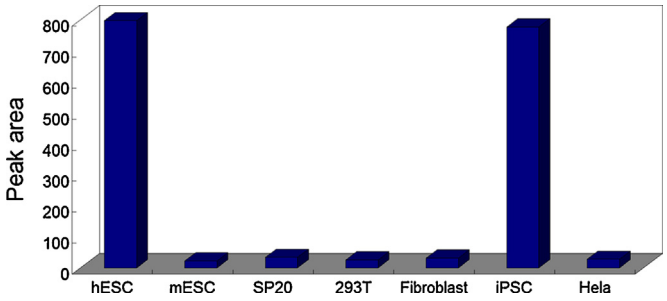

Fig. 5. Selectivity of biosensor for human pluripotent stem cell detection. Typical images of the biosensor for the detection of different types of cells. 10^4 cells of different types were collected to challenge this LFB. After incubation with antibodies, the enriched cells with MB were added into SDA reaction buffer for amplification for 30 min, and the amplified products were loaded onto the LFB. 5 min later, the peak area values were recorded by a strip reader.

resulting calibration plot of peak area versus target cell concentration was basically linear between 100 cells/mL and 10^6 cells/mL (Fig. 4B). The limit of detection (LOD) was calculated by 3-fold standard deviation of blank. The value of peak area in triplicate blanks was 42.15 ± 8.91. Therefore, the LOD of LFB was calculated to be 19 cells according to the linear equation ($y = 230.3564x - 221.7324$). This number is considered as the limit of detection (LOD). This LOD is 500 folds lower than that of previously published result (10,000 cells) [13].

3.5. Selectivity of the LFB for hPSC detection

For the specificity assay, cell lines, including mESC, SP20, 293T, fibroblast and Hela, without the expression of SSEA-3 and SSEA-4 were used as negative controls. Besides, human embryonic stem cells (hESCs) and iPSCs were also tested by the LFB. As shown in Fig. 5, high peak area was observed on the test line of the biosensor when the hESCs and iPSCs were loaded, but no test band was observed when mouse embryonic stem cells, SP20 cell line, 293T cell line, human fibroblast cells, or HeLa cells were tested. The peak area detected by other cells were a little bit lower than the LOD (value of 68.88), 30.67 for mESC, 51.24 for SP20, 49.32 for 293T, 50.18 for Fibroblast, and 40.28 for Hela cells, respectively. All of these cells could be considered as negative control. These results demonstrate that the biosensor is specific for human pluripotent stem cells, not for stem cells from other species or human somatic cells.

Stem cell detection is very important in two aspects: induction of pluripotent stem cells from somatic cells and direct differentiation of stem cells to somatic cells. Hence, we further challenged the LFB with a cell mixture of iPSCs and human fibroblast cells. Serially diluted iPSCs were added into 50,000 cells of human fibroblast cells, resulting 0%, 2%, 5%, 10%, 50% of iPSCs in the mixture. No test line was observed in the absent of iPSCs. The test line became brighter as the increase of spiked iPSCs, and the LFB can detect as low as 200 cells in spiked cell mixture. The corresponding peak area of the test line in cell mixture was compared with that of pure

Table 2
Recovery of this method for analytical spiked iPSC detection (10^5 human fibroblast cells). According to the equation of Log [cell number] *VS* Peak Area, the number of iPSC was calculated (calculated number of iPSC). The recovery = calculated number/spiked number × 100%. Standard deviations were calculated from three independent experiments.

Spiked number	Calculated number	Standard deviation	Recovery (%)
25,000	24,575	143.7	98.3
10,000	9320	120.3	93.2
5000	4585	112.8	91.7
2000	1784	87.9	89.2
200	193	17.4	96.5

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iPSC suspension. The recovery rates for the detection of cell mixture samples are listed in Table 2. The recovery ranged from 89.2% to 98.3%, and the good recovery rates can be explained by the efficient separation by SSEA3 antibody modified MBs, which can reduce the unspecifically bound cells and enrich tDNA for the SDA. These results suggest that the LFB has good selectivity for human pluripotent stem cell detection, and the proposed sensor can be used in cell mixture samples.

This LFB is essentially a combination of three different technologies: magnetic enrichment of specific cells, isothermal strand displacement amplification, and lateral flow biosensor based DNA detection, which render many advantages to this stem cell LFB. First, in the SDA-based amplification, tedious DNA extraction and purification is avoided, instead, the captured tDNA on cell surface is amplified. Consequently, some inherent problems in PCR based methods such as carry over contamination and false-positive results can be largely reduced. Secondly, with reduced handling and simpler equipment, this assay provides a simple and rapid alternative to conventional methods. Moreover, this method can be easily modified to detect other types of cells by changing the specific antibodies.

4. Conclusion

In conclusion, we have developed a LFB for simple and sensitive detection of hPSCs. The LFB is capable of detecting a minimum of 100 hPSCs within 80 min (20 min for antibody binding, 10 min for magnetic enrichment, 15 min for washing, 30 min for SDA, 5 min for detection) by the naked eye. The response of the optimized LFB is highly linear over a range of 100–10^6 of hPSCs. Meanwhile, the LFB has good selectivity, even in a cell mixture. The LFB can have significant applications in the field of regenerative medicine such as the determination of the efficiency of iPSC induction from somatic cells and the efficiency of direct differentiation from ES or iPS cells to somatic cells, including hematopoietic cells, cartilage cells, and adipose cells. This approach can also be extended to detect different types of stem cells. For example, antibodies to mouse stem cell surface markers, SSEA-1 and Thy-1, can be used in the LFB for the detection of mouse stem cells. Furthermore, it is possible to develop a stem cell biosensor for the simultaneous detection of two types of stem cells. Because such an LFB does not require any specialized equipment or skills, it is inexpensive, rapid and, thus, holds great potential for wide applications, especially in stem cell clinical therapy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.aca.2015.04.003.

References

[1] M. Politis, O. Lindvall, Clinical application of stem cell therapy in Parkinson's disease, BMC Med. 10 (2012) 1.
[2] S. Takashima, D. Tempel, H.J. Duckers, Current outlook of cardiac stem cell therapy towards a clinical application, Heart 99 (2013) 1772–1784.
[3] M.G. Engelmann, W.M. Franz, Stem cell therapy after myocardial infarction: ready for clinical application? Curr. Opin. Mol. Ther. 8 (2006) 396–414.
[4] J. Rossant, Stem cells and early lineage development, Cell 132 (2008) 527–531.
[5] K. Takahashi, S. Yamanaka, Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, Cell 126 (2006) 663–676.
[6] S. Yamanaka, K. Takahashi, Induction of pluripotent stem cells from mouse fibroblast cultures, Tanpakushitsu Kakusan Koso 51 (2006) 2346–2351.
[7] R.A. Young, Control of the embryonic stem cell state, Cell 144 (2011) 940–954.
[8] J. Su, X.H. Xu, Q. Huang, M.Q. Lu, D.J. Li, F. Xue, F. Yi, J.H. Ren, Y.P. Wu, Identification of cancer stem-like $CD44^+$ cells in human nasopharyngeal carcinoma cell line, Arch. Med. Res. 42 (2011) 15–21.
[9] C. Brandl, C. Florian, O. Driemel, B.H. Weber, C. Morsczeck, Identification of neural crest-derived stem cell-like cells from the corneal limbus of juvenile mice, Exp. Eye Res. 89 (2009) 209–217.
[10] H. Kubo, M. Shimizu, Y. Taya, T. Kawamoto, M. Michida, E. Kaneko, A. Igarashi, M. Nishimura, K. Segoshi, Y. Shimazu, K. Tsuji, T. Aoba, Y. Kato, Identification of mesenchymal stem cell (MSC)-transcription factors by microarray and knockdown analyses, and signature molecule-marked MSC in bone marrow by immunohistochemistry, Genes Cells 14 (2009) 407–424.
[11] S. Awasthi, D.L. Matthews, R.A. Li, N. Chiamvimonvat, D.K. Lieu, J.W. Chan, Label-free identification and characterization of human pluripotent stem cell-derived cardiomyocytes using second harmonic generation (SHG) microscopy, J. Biophotonics 5 (2012) 57–66.
[12] N. Tiptanavattana, C. Thongkittidilok, M. Techakumphu, T. Tharasanit, Characterization and in vitro culture of putative spermatogonial stem cells derived from feline testicular tissue, J. Reprod. Dev. 59 (2013) 189–195.
[13] W. Wu, L. Yu, Z. Fang, P. Lie, L. Zeng, A lateral flow biosensor for the detection of human pluripotent stem cells, Anal. Biochem. 436 (2013) 160–164.
[14] J.N. Southard, Protein analysis using real-time PCR instrumentation: incorporation in an integrated, inquiry-based project, Biochem. Mol. Biol. Educ. (2013) .
[15] M. Fakruddin, K.S. Mannan, A. Chowdhury, R.M. Mazumdar, M.N. Hossain, S. Islam, M.A. Chowdhury, Nucleic acid amplification: alternative methods of polymerase chain reaction, J. Pharm. Bioallied Sci. 5 (2013) 245–252.
[16] M. Yoshino, H. Watari, T. Kojima, M. Ikedo, J. Kurita, Rapid, sensitive and simple detection method for koi herpesvirus using loop-mediated isothermal amplification, Microbiol. Immunol. 53 (2009) 375–383.
[17] L. Kashefi-Kheyrabadi, M.A. Mehrgardi, Aptamer-based electrochemical biosensor for detection of adenosine triphosphate using a nanoporous gold platform, Bioelectrochemistry 94 (2013) 47–52.
[18] Z. Fang, C. Ge, W. Zhang, P. Lie, L. Zeng, A lateral flow biosensor for rapid detection of DNA-binding protein c-jun, Biosens. Bioelectron. 27 (2011) 192–196.
[19] T.N. Tran, J. Cui, M.R. Hartman, S. Peng, H. Funabashi, F. Duan, D. Yang, J.C. March, J.T. Lis, H. Cui, D. Luo, A universal DNA-based protein detection system, J. Am. Chem. Soc. 135 (2013) 14008–14011.
[20] S. Lin, P. Talbot, Methods for culturing mouse and human embryonic stem cells, Methods Mol. Biol. 690 (2011) 31–56.
[21] K. Seno, K. Matumura, K. Oshita, M. Oshima, S. Motomizu, Determination of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride by flow-injection analysis based on a specific condensation reaction between malonic acid and ethylenediamine, Anal. Sci. 25 (2009) 389–393.
[22] X. Yu, J. He, S. Mongkhoune, Y. Peng, Y. Xie, J. Su, S.F. Zhou, X.X. Xie, G.R. Luo, Y. Fang, X. Li, N. Zhou, Y.X. Zhao, X.L. Lu, Artificial antigen-presenting cells plus IL-15 and IL-21 efficiently induce melanoma-specific cytotoxic $CD8^+$ $CD28^+$ T lymphocyte responses, Asian Pac. J. Trop. Med. 6 (2013) 467–472.
[23] P. Lie, J. Liu, Z. Fang, B. Dun, L. Zeng, A lateral flow biosensor for detection of nucleic acids with high sensitivity and selectivity, Chem. Commun. (Camb.) 48 (2012) 236–238.
[24] Z. Fang, W. Wu, X. Lu, L. Zeng, Lateral flow biosensor for DNA extraction-free detection of salmonella based on aptamer mediated strand displacement amplification, Biosens. Bioelectron. 56 (2014) 192–197.
[25] D. Pei, The magic continues for the iPS strategy, Cell Res. 18 (2008) 221–223.
[26] T. Enver, M. Pera, C. Peterson, P.W. Andrews, Stem cell states, fates, and the rules of attraction, Cell Stem Cell 4 (2009) 387–397.

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