

Visual detection of glial cell line-derived neurotrophic factor based on a molecular translator and isothermal strand-displacement polymerization reaction

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Background: Glial cell line-derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many types of neurons. Detection of GDNF is vital to monitoring the survival of sympathetic and sensory neurons. However, the specific method for GDNF detection is also un-discovered. The purpose of this study is to explore the method for protein detection of GDNF.

Methods: A novel visual detection method based on a molecular translator and isothermal strand-displacement polymerization reaction (ISDPR) has been proposed for the detection of GDNF. In this study, a molecular translator was employed to convert the input protein to output deoxyribonucleic acid signal, which was further amplified by ISDPR. The product of ISDPR was detected by a lateral flow biosensor within 30 minutes.

Results: This novel visual detection method based on a molecular translator and ISDPR has very high sensitivity and selectivity, with a dynamic response ranging from 1 pg/mL to 10 ng/mL, and the detection limit was 1 pg/mL of GDNF.

Conclusion: This novel visual detection method exhibits high sensitivity and selectivity, which is very simple and universal for GDNF detection to help disease therapy in clinical practice.

Keywords: lateral flow biosensor, molecular translator, isothermal strand-displacement polymerization reaction

Introduction

Glial cell line-derived neurotrophic factor (GDNF) is a small protein that potently promotes the survival of many types of neurons and is able to prevent apoptosis of motor neurons induced by axotomy. The detection of GDNF is vital to monitoring the survival and maintenance of sympathetic and sensory neurons.¹⁻⁶ Several approaches have so far been devised for the detection of GDNF. Conventional antibody-based methods, including Western blotting, dot blotting, immunohistochemistry, and enzyme-linked immunosorbent assay, are widely used analytical approaches in both clinical diagnosis and biological research.⁷ However, these methods always suffer from the limitations of long assay time, complex experimental procedures, high cost, and low sensitivity. Therefore, continuing efforts have been made to seek ideal methods for rapid, sensitive, low-cost, and user-friendly detection of GDNF.

A molecular translator can be employed as a signal transducer by highly converting the input target molecules into unique output deoxyribonucleic acid (DNA) for signal amplification. For example, Picuri et al⁸ have developed a novel nucleic acid detection method through converting any target nucleic acids to a predesigned output DNA.

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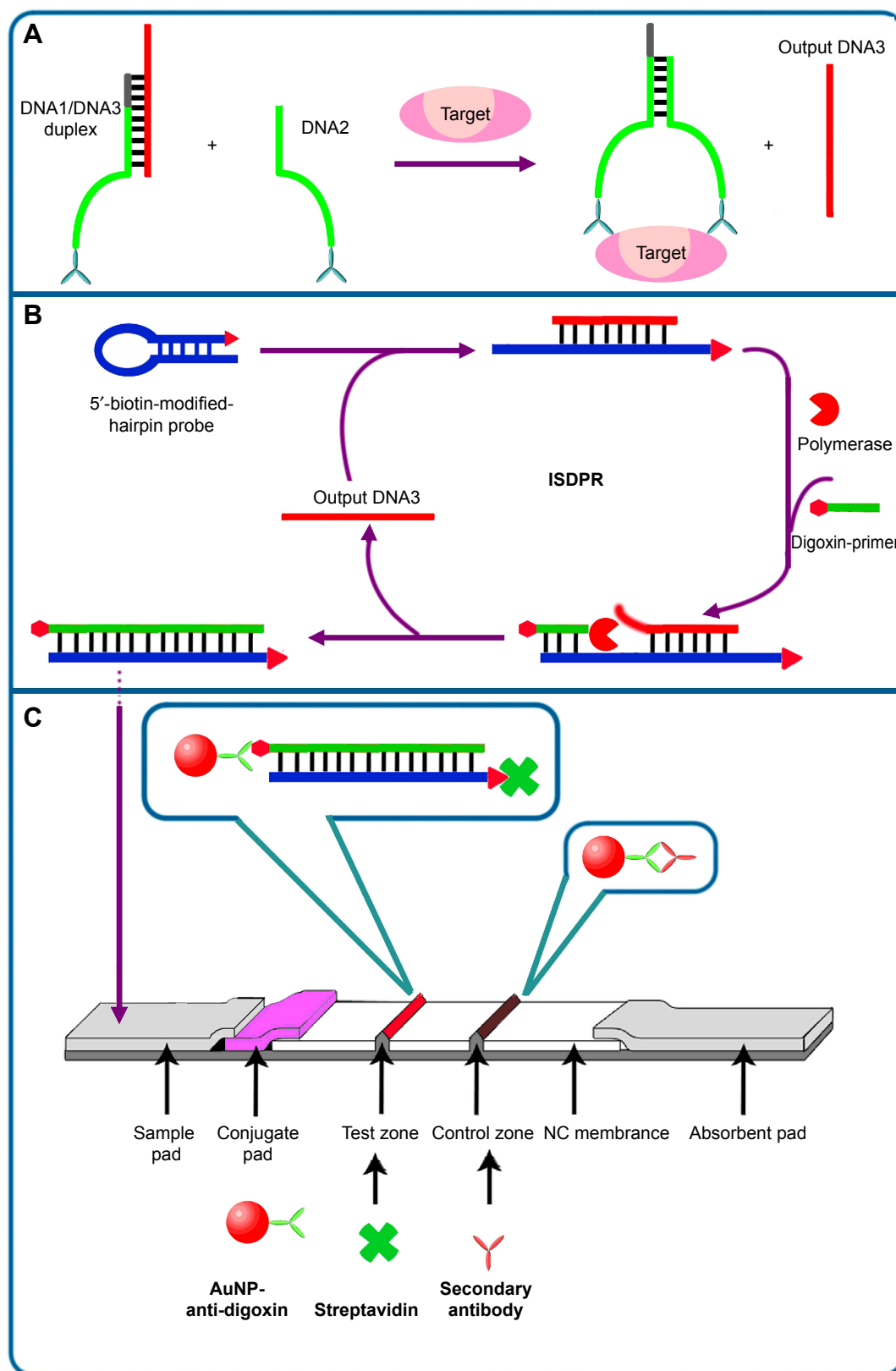


Figure 1 Process of molecular translator and ISDPR.

Notes: (A) Principle of molecular translator, the input GDNF is converted into output DNA3 by the binding-induced strand-displacement activity of the molecular translator. (B) The output DNA3 triggers ISDPR for signal amplification by producing a large amount of duplex DNA. (C) Schematic illustration of the strip biosensor for visual detection of duplex DNA. AuNP-anti-digoxin antibody is dispensed on the conjugate pad. Streptavidin and secondary antibody are dispensed on the test zone and control zone, respectively. **Abbreviations:** ISDPR, isothermal strand-displacement polymerization reaction; GDNF, glial cell line-derived neurotrophic factor; DNA, deoxyribonucleic acid; AuNP, gold nanoparticle; NC, nitrocellulose.

of AuNP–antidigoxin conjugates onto the glass fiber using a dispenser. The conjugate pad was dried at room temperature for 12 hours and stored at 4°C. Streptavidin (1 mg/mL) and rabbit antimouse immunoglobulin G (IgG) antibody (30 µL, 1 mg/mL) were dispensed onto the nitrocellulose membrane to form a test zone and a control zone, respectively, with a lateral flow dispenser. The distance between the test zone and the control zone was approximately 5 mm. The membrane was dried at room temperature for 12 hours and stored at 4°C. Finally, the four components of the lateral flow DNA biosensor were assembled on plastic adhesive backing (6×30 cm). Each part overlapped by 2 mm to ensure solution migration through the strip during the assay. Strips were cut into 3 mm width with a paper cutter.

Detection of GDNF by LFB

A DNA probe for the molecular translator was prepared at a final concentration of 5 µM by mixing 20 µL 50 µM mouse anti-GDNF antibody conjugated DNA1 with 13.3 µL 50 µM output DNA3 in 166.7 µM Tris–ethylenediaminetetraacetic acid (TE) buffer (containing 10 mM MgCl₂ and 0.05% Tween 20), heating to 90°C for 5 minutes, and allowing solution to cool down to 25°C slowly in a period of 3 hours. Strand displacement of molecular translator was performed in 20 µL TE buffer, 10 nM DNA1/DNA3 complex, 10 nM DNA2. The reaction mixture was incubated at 25°C for 45 minutes.

ISDPR was performed in a 25 µL 50 nM Tris-HCl (pH 8.0) buffer containing 50 nM short primer, 3U polymerase Klenow fragment exo-, 50 µM dNTPs, 6% dimethyl sulfoxide, 0.1% BSA, 1 mM DTT, 5 mM MgCl₂, and 2 µL of product of strand displacement of molecular translator. This mixture was incubated at 42°C for 30 minutes. Finally, the ISDPR product was loaded onto the sample pad together with 20 µL PBS. The red bands were observed within 5 minutes, and the biosensor was scanned with a portable LFB reader. The optical intensities of the test zone and the control zone were recorded simultaneously by the reader, which automatically located the red bands in a fixed reaction area and then measured strip parameters such as peak height and area integral.

Process of molecular translator and ISDPR

For the process of molecular translator and strand-displacement amplification, DNA1 conjugated with the GDNF antibody is initially hybridized to DNA3 to form a stable DNA1/DNA3 duplex. DNA2 sequences are designed in such a way that the complementary sequences between DNA1 and DNA2

are 4-nt shorter than the complementary sequences between DNA1 and DNA3. In the absence of GDNF, the strand displacement activity is poor between DNA2 and DNA3 at 25°C, and the displacement of output DNA3 by competing DNA2 is extremely minimal. However, in the process of GDNF, the binding of the same target protein to its antibodies that are coupled with DNA1 and DNA2 brings DNA1 and DNA2 to a close proximity, resulting in an increasing in their local effective concentration. This process triggers the strand-displacement reaction between DNA2 and DNA3. As a result, the output DNA3 is released for the next signal amplification (Figure 1A).

The releasing output DNA3 recognizes and hybridizes with the loop region of the hairpin probe, which is composed of an eleven base pair stem, a 25-nt loop, and a biotin at the 5' end, causing the hairpin probe to undergo a conformational change and leading to stem separation. The digoxin-coupled primer (8-nt) complementary to the stem region of the hairpin probe at the 3' end thus anneals with the open stem and triggers a polymerization reaction in the presence of dNTPs and DNA polymerase. In the process of primer extension, the output DNA3 is released by DNA polymerase with strand-displacement activity, after which complementary DNA is synthesized, leading to the formation of a hairpin–DNA complex. To start the next cycle, the releasing output DNA3 hybridizes with another hairpin probe, triggering another polymerization reaction. Through this cyclic reaction comprising release of output DNA3 and hybridization of output DNA3 with remaining hairpin probe, a great amount of duplex DNA products with a biotin and digoxin tag at the 5' end are synthesized. The hairpin probe retains its original stem-loop structure in the absence of GDNF, the primer is unable to anneal to the hairpin to initiate a polymerization, and no duplex DNA is produced (Figure 1B).

An LFB consists of four components: sample pad, conjugate pad, nitrocellulose membrane, and absorption pad. The antidigoxin antibodies conjugated with AuNPs are dispensed on the conjugate pad. Streptavidin and goat antimouse IgG antibody (secondary body) are dispensed on the nitrocellulose membrane, respectively, to form the test zone and the control zone. The sample solution containing the ISDPR product and running buffer is applied on the sample pad of the LFBs. The solution migrates along the LFB by capillary action to the conjugate pad, where antidigoxin–AuNP conjugates have been deposited. Antidigoxin antibody couples with digoxin of the ISDPR duplex DNA product to form a biotin–duplex DNA–digoxin–antidigoxin–AuNP complex. The complexes are captured at the test zone by the specific

reaction between preimmobilized streptavidin and biotin. The accumulation of AuNPs on the test zone is then visualized as a characteristic red band. Excess antidigoxin–AuNP conjugates continue to migrate and are captured at the control zone by immunoreactions between antidigoxin antibody and the preimmobilized secondary antibody on the AuNP surface, thus forming a second red band. In the absence of GDNF, no ISDPR duplex DNA product is produced; therefore, no red band is observed at the test zone. In this case, the red band at the control zone indicates that the LFB is working properly. Visual detection is performed by observing the color change caused by the accumulation of AuNPs on the test zone, and quantitative detection can be realized by recording the color intensity of the red band on the test zone with a portable LFB reader (Figure 1C).

Statistical analysis

The data in this study were analyzed with SPSS statistics software version 19.0 (IBM Corporation, Armonk, NY, USA). The comparison between groups was analyzed by independent *t*-tests. A *P*-value of less than 0.05 was considered as statistical significance.

Results

Principle of visual detection of GDNF

The molecular translator is composed of target recognition elements (DNA1 and DNA2) and a signal output element (DNA3), which is illustrated in Figure 1A. The output DNA3 triggers ISDPR for signal amplification by producing a large amount of duplex DNA (Figure 1B). Figure 1C illustrates the schematic illustration of the strip biosensor for visual detection of duplex DNA.

Verification of the signal amplification of ISDPR

We investigated the optical response of 10 nM DNA3 on the LFB with and without ISDPR amplification. In the case without ISDPR, there was no polymerase in the reaction buffer. As shown in Figure 2, the response of the sample with ISDPR was 35 times higher compared with that without ISDPR or the blank group ($P < 0.01$).

Optimization of ISDPR

The experimental parameters, such as ISDPR temperature and reaction time that could affect the analytical performance, were optimized systematically. We compared the Signal to Noise (S/N) ratio of 10 ng mL⁻¹ and 1 ng mL⁻¹ GDNF with four different ISDPR temperatures (25°C, 37°C, 42°C, and 50°C). As

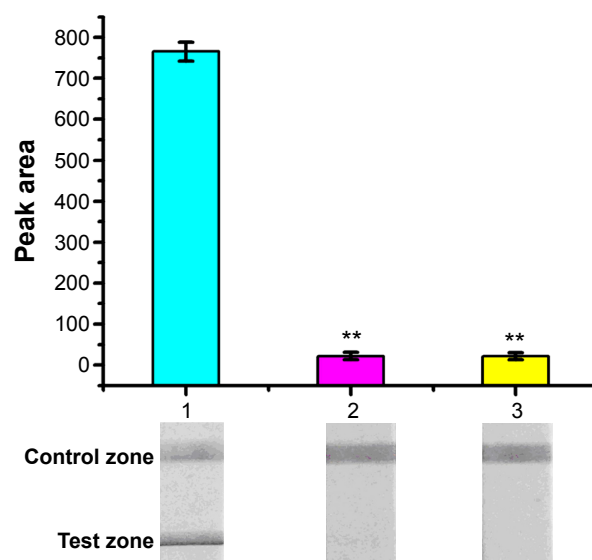


Figure 2 Verification of the signal amplification of isothermal strand-displacement polymerization reaction (ISDPR): 1: with ISDPR; 2: without ISDPR; 3: blank.

Note: ** $P < 0.05$ represents the peak area in sample 2 and sample 3 compared with sample 1.

shown in Figure 3A, the S/N ratios increased with increasing reaction temperature in the range from 25°C to 42°C. The S/N ratio decreased when the temperature was over 42°C.

As shown in Figure 3B, the optical response elevated gradually with the augmenting of reaction time from 10 minutes to 30 minutes. The peak area of the 10 ng/mL GDNF and 1 ng/mL GDNF was significantly higher compared with the blank group (Figure 3B, $P < 0.05$). Further increasing the reaction time led to the increasing background signal. A longer reaction time at 42°C may cause stem separation of the hairpin probe, even in the absence of output DNA3, resulting in a high background signal. The results indicated that 30 minutes is the optimal reaction time (Figure 3B).

Analytical performances

We have examined the sensitivity and dynamic range of the LFB using different concentrations of GDNF under optimal conditions. As shown in Figure 4A, the color intensities of the red bands on the test zone increased with the increasing of GDNF concentrations. No distinct red band was observed on the test zone in the absence of GDNF. For quantitative detections, the optical responses were further confirmed by recording the color intensities of the red bands by a portable strip reader. The peak areas versus the incremental concentrations of GDNF have been plotted in Figure 4B, which followed a sigmoid increase. The resulting calibration curve shows that the peak areas are proportional to the logarithm of GDNF concentrations in the range from 1 pg mL⁻¹ to 10 ng mL⁻¹ (Figure 4C).

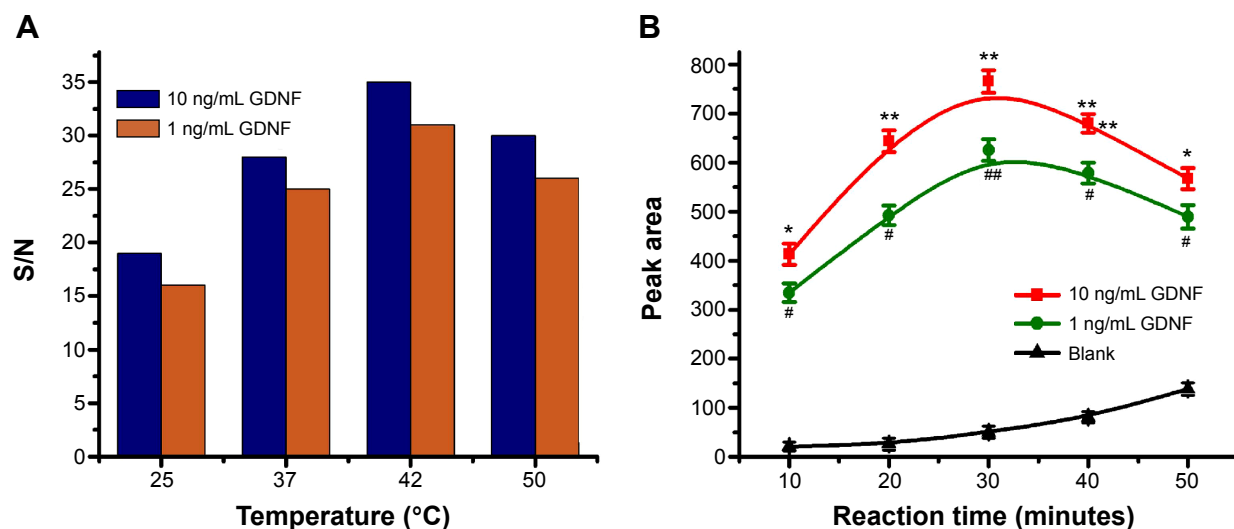


Figure 3 Effects of the isothermal strand-displacement polymerization reaction (ISDPR) temperature and ISDPR reaction time. **(A)** Effect of ISDPR temperature on the S/N ratio of 10 ng/mL and 1 ng/mL glial cell line-derived neurotrophic factor (GDNF) assay. **(B)** Effect of the ISDPR reaction time on the peak area of 10 ng/mL and 1 ng/mL GDNF assay.

Notes: * $P < 0.05$ and ** $P < 0.01$ represent the peak area in 10 ng/mL GDNF group compared with the blank group. # $P < 0.05$ and ## $P < 0.01$ represent the peak area in 1 ng/mL GDNF group compared with the blank group.

Abbreviation: S/N, Signal to Noise.

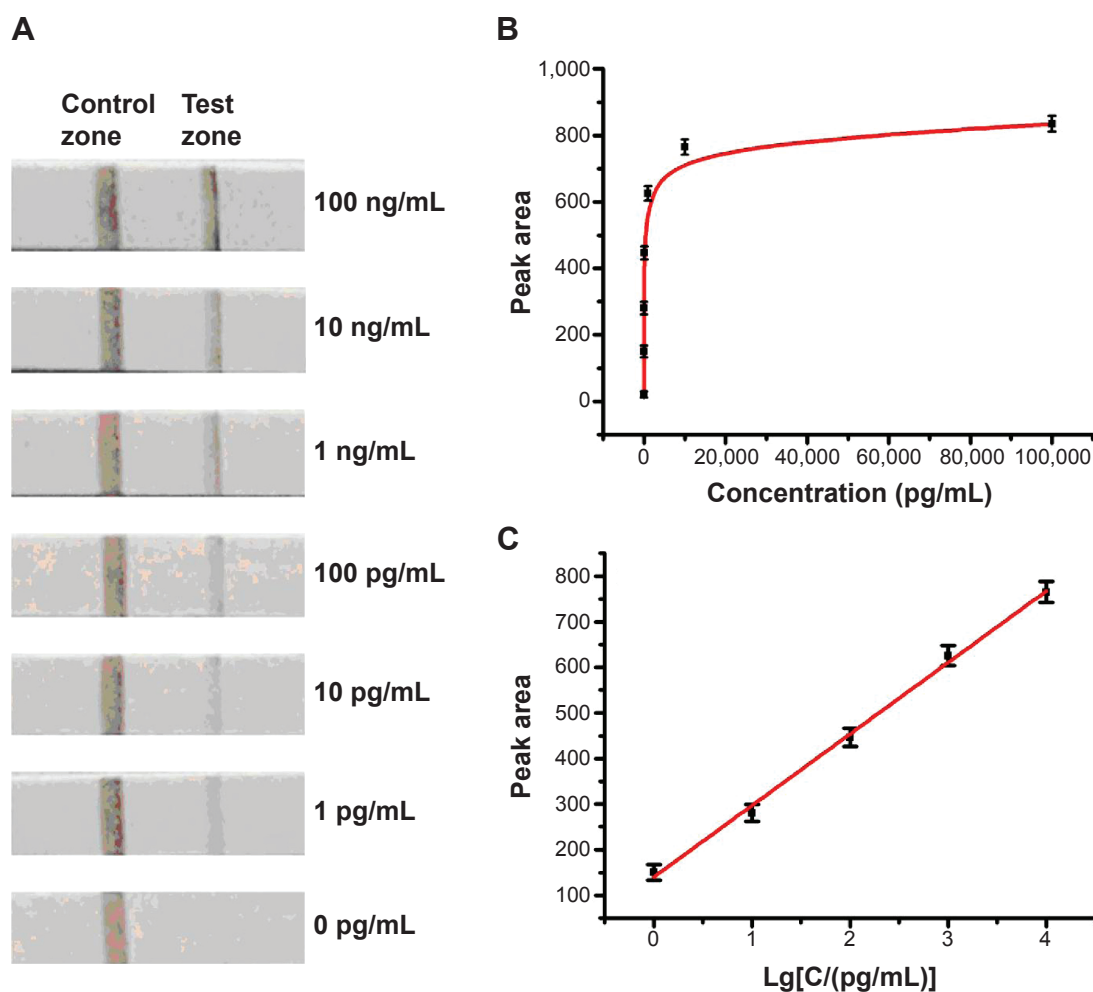


Figure 4 Examination for glial cell line-derived neurotrophic factor (GDNF) with different concentrations.

Notes: **(A)** Photo images of detection results of the lateral flow biosensor with different concentrations of GDNF. The images were recorded with a digital camera. **(B)** The calibration curve plotted with the peak areas versus GDNF concentrations. **(C)** The linear relationship between the peak areas and the logarithm of GDNF concentrations. Error bars represent standard deviation, $n=3$.

The specificity of the LFB for GDNF was studied by testing the responses of the assay to other kinds of growth factors, including PDGF, TGF- α , TGF- β , EGF, IGF-I, and α FGF, which are similar to GDNF. As shown in Figure 5A, 1 ng/mL GDNF produced a visible bright red band on the test zone of LFB, whereas all other growth factors at a concentration of 100 ng/mL did not yield a red band on the test zone of LFB.

We studied the stability and performance of LFBs after different periods of storage. The prepared LFBs were sealed and stored at 37°C for the following time periods: 12 hours, 24 hours, 14 days, and 1 month. As shown in Figure 5B, there were no significant performance differences among different storage times, indicating that the stability of the assay is acceptable.

Discussion

A molecular translator can be employed as a signal transducer by highly converting the input target molecules into a unique output DNA for signal amplification.^{17,18} ISDPR is a commonly used approach for the DNA signal amplification.^{16–18} Therefore, we combined the molecular translator and ISDPR method to detect the GDNF in cells.

In this work, ISDPR was used to produce a large amount of digoxin- and biotin-coupled duplex DNA for visual

detection of GDNF. Compared with the solutions without ISDPR, the response of the sample with ISDPR was 35 times higher. These results showed that the ISDPR did occur as expected for signal amplification. These results were consistent with Liu et al's¹⁹ reports.

Figure 3A shows that the S/N ratios increased with increasing reaction temperatures in the range from 25°C to 42°C, however S/N ratios decreased with temperatures over 42. These results could be explained by conformational change of the hairpin probe at high temperature, which leads to the stem separation of the hairpin probe. The primer can anneal with the open hairpin probe and trigger a polymerization reaction, even in the absence of output DNA3, resulting in a high background signal. Therefore, an ISDPR temperature of 42°C was selected in the following experiments.

The process of signal amplification by ISDPR is strongly affected by the reaction time. Usually, the ISDPR can be enhanced with increasing the reaction time. In this study, 30 minutes was selected as the reaction time throughout the experiments to obtain the highest signal.

As shown in Figure 4, the red band on the test zone was observed with as little as 1 pg mL⁻¹ of GDNF, which can be considered the limit of detection. Based on the molecular weight of GDNF (13.5 kDa), the limit of detection was calculated to be 74 fM. The detection limit of our

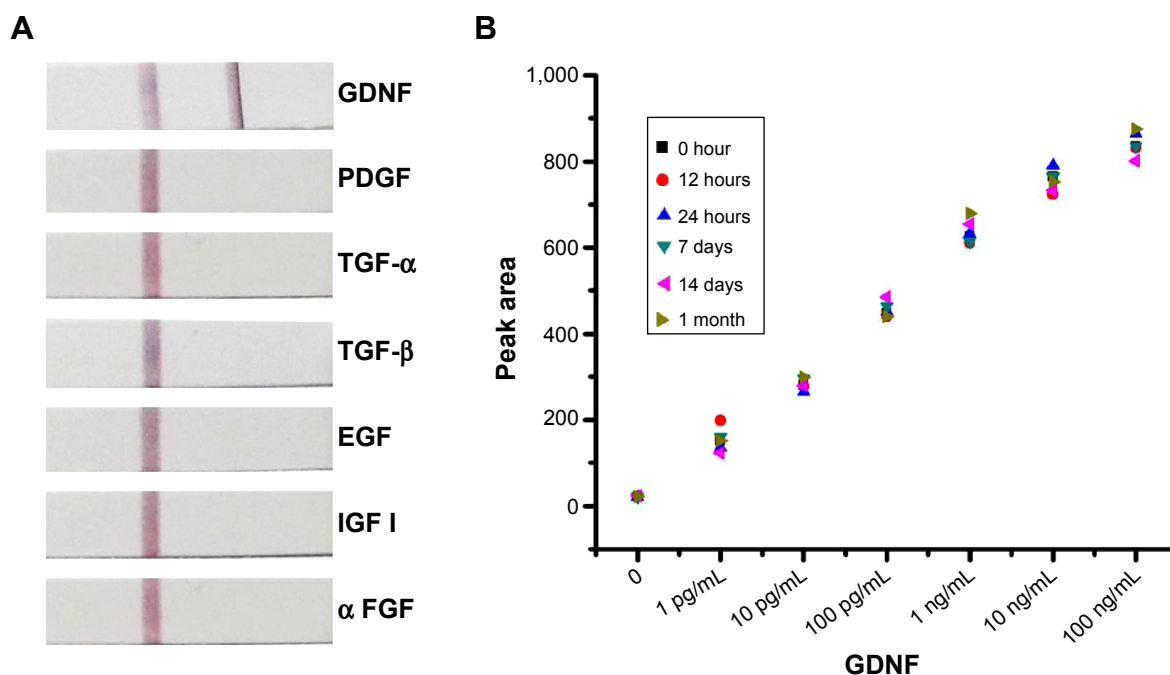


Figure 5 Specificity analysis for the GDNF.

Notes: (A) Photo image of the lateral flow biosensors for specificity analysis with GDNF and other kinds of growth factors that are similar to GDNF. (B) The optical response of the lateral flow biosensors at different periods of storage with various concentrations of GDNF.

Abbreviations: GDNF, glial cell line-derived neurotrophic factor; PDGF, platelet-derived growth factor; TGF, transforming growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; FGF, fibroblast growth factor.

proposed approach is two orders of magnitude better than the antibody-based immunoassay.⁷ Generally, human basal serum GDNF concentrations are between 20 pg/mL and 30 pg/mL. This LFB holds great promise in monitoring GDNF by naked eyes with its superior detection sensitivity and large dynamic range.

From the results of Figure 5A, 1 ng/mL GDNF produced a visible bright red band, while the other growth factors (such as TGF- α , TGF- β , EGF, IGF-I, and α FGF) did not yield a red band even at a concentration of 100 ng/mL. These results indicated that our constructed LFB exhibited excellent selective response to GDNF. Our results also showed that this method is very stable after different periods of storage.

Conclusion

In summary, we have successfully fabricated an LFB for amplified detection of GDNF using a molecular translator for converting the input protein to output DNA signal and ISDPR for signal amplification. Compared with other reported antibody-based immunoassays, the sensitivity of the LFB developed here is higher. By using this assay, GDNF as low as 1 pg mL⁻¹ could be detected by naked eyes within 30 minutes. The response of the optimized assay is highly linear over a range of 1 pg/mL to 10 ng/mL. This new GDNF detection method is expected to be a great potential platform for the detection of protein, which would be valuable for clinical therapy for some diseases related to GDNF.

Disclosure

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