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A "turn on/off" scorpion biosensor targeting point mutation of *SMN* genes for diagnosis of spinal muscular atrophy†

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A "turn on/off" biosensor for diagnosis of exon 7 of the *SMN1* gene was developed by employing a "scorpion primer". This scorpion primer was based on the principle of fluorescence resonance energy transfer using a fluorophore, a blocker and a quencher. It was successfully applied to detect 10 volunteer samples, and not only to *in vitro* testing.

Genotyping is an important technique for prenatal diagnosis and personalized medicine. Polymerase chain reaction (PCR) is the most commonly used technique for DNA amplification and requires an appropriate amount of DNA or RNA for the reaction.[1] Fluorometric detection of PCR products is a popular detection mode which simplifies the readout procedure and can use probes for diagnosis.[2] Fluorescence resonance energy transfer (FRET) is a process in which the energy from an excited fluorophore is transferred to an acceptor moiety at distances of up to 7–10 nm.[3,4] At present, there are some probe-based reporter systems, such as TaqMan,[5,6] molecular beacons[7–9] and the scorpions.[10,11] Nazarenko *et al.* used scorpion-like primers to amplify the genes in PCR, and following this concept,[10] some research groups have used the scorpion primers as a diagnostic tool specifically for detection of PCR products in real-time PCR (RT-PCR).[11–13] The scorpion probe has some basic elements in all formats, including: (1) a PCR primer; (2) a PCR stopper to prevent PCR read-through of the probe element; (3) a specific probe sequence; and (4) a fluorescence detection system containing at least one fluorophore and a quencher.[11,14] This technology could be used for allelic discrimination and is effective in single nucleotide polymorphism (SNP) genotyping. Although the detection of SNP or other nucleotide variations by using scorpion primers often depends upon the RT-PCR instrument, it is not applied for traditional PCR. For RT-PCR at least two detection probes have to be designed which are labeled with different fluorophores to distinguish the point mutation, as it would produce some interferences when the probe is only labeled with a single fluorophore in the RT-PCR system and affects the determination of the results. Therefore, a scorpion primer system equipped with a simple technique, such as capillary gel electrophoresis (CGE), may be a good replacement for detection of SNPs.

Spinal muscular atrophy (SMA) is one of the common autosomal recessive diseases. The frequency of the carrier is one in 40 to 50 subjects, and the incidence of SMA is 1 in 10 000 live newborns.[15] In clinical analysis, SMA is subdivided into four types based on the age of onset, best motor function and life expectancy.[16] This severe neuromuscular disorder is controlled by the survival motor neuron gene (*SMN*), located on chromosome 5q13,.[17] Two almost identical copies of the *SMN* gene have been identified, telomeric *SMN* (*SMN1*) and centromeric *SMN* (*SMN2*). The *SMN1* gene is different from *SMN2* gene only by two nucleotide variants in exon 7 (c.840 C>T) and exon 8 (G>A). In approximately 94% of clinically typical SMA-affected patients, either deletion or conversion of *SMN1* has been reported.[18] So in SMA diagnosis, detection of *SMN1* is an important standard procedure for diagnosis of this disease. Some previous studies have used capillary electrophoresis for quantification of these two genes.[19–22]

In this study, a detection technique combining scorpion primer PCR with CGE-laser induced fluorescence (LIF) was employed to develop a "turn on/off" biosensor for specific analysis of the *SMN1* gene. This method is different from RT-PCR and it is the first scorpion-PCR study on the SMA diagnosis of the point mutation of exon 7 (c.840 C>T). We combined the CGE-LIF separation ability which could help us to determine the genotype of DNA samples. With the only additional requirement of an extra

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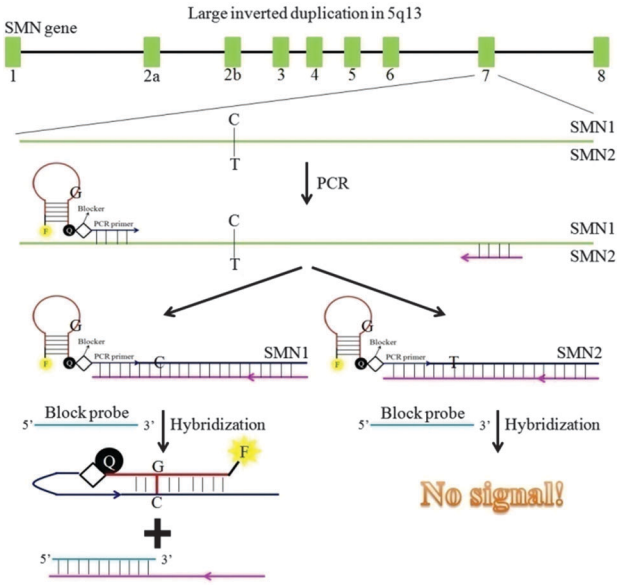

Scheme 1 The principle of the scorpion-PCR for *SMN1* and *SMN2* genes which led to the development of a "turn on/off" biosensor.

hybridization step after PCR, the *SMN1* gene can be resolved by the scorpion-PCR-CGE-LIF method.

Scheme 1 shows the mechanism of this design used for detection of the *SMN1* gene. A scorpion primer was labeled with 6-carboxyfluorescein (FAM) at the 5′-end, Black Hole Quencher 1 (BHQ1) serving as a quencher and hexaethylene glycol (HEG) serving as a primer blocker at the middle site of the scorpion primer. This probe had two parts, one was the hairpin structure, which was used to self-bind to the single amplified sequence, and the other section was a specific sequence used for PCR. First, the scorpion primer and the reverse primer were mixed with the matrices for PCR. After PCR, all the amplicons possessed the section of the hairpin structure. Second, the block probe was added into the solution to combine with the complementary strand of PCR products, and the other strand containing the scorpion section was dissociated. During the hybridization procedure, the hairpin structure of the scorpion section was denatured, to identify the self-strand. If the sequence coincided completely, the fluorescence of FAM could be observed. In contrast, if the scorpion section was not hybridized to the self-strand, it would re-anneal and the fluorescence would be quenched. In this study, the sequence of the scorpion primer was designed to recognize the sequence of exon 7 of the *SMN1* gene. By this method, the subjects could be quickly diagnosed with or without the *SMN1* gene.

The conditions of the scorpion PCR were optimized, including the PCR annealing temperature and cycles, volume of the block probe, hybridization temperature and hybridization time. During investigation of the PCR annealing temperature, YOPRO-1 was used as a fluorescent intercalating dye to confirm the PCR efficiency. We found that when the annealing temperature was too low, non-specific DNA fragments were amplified, resulting in the appearance of multiple bands. However, when the annealing temperature was too high, the yield and purity of the desired product was reduced due to poor annealing of primers.[23] Finally, when the temperature was set at 52 °C, the maximum signal could be obtained (Fig. S1, ESI†).

The other important factor was the control over PCR cycles. Although a larger number of PCR cycles could enhance the signal, it also induced some non-specific PCR products.[24] In this study, several noises resulted from the PCR products, when the number of PCR cycles was over 30. Finally, the number of PCR cycles was set at 30 (Fig. S2, ESI†).

The key points of the procedure of hybridization were focused on the volume of the block probe, hybridization temperature and time. Generally, the scorpion probe was used in RT-PCR research, and it could be denatured by heating. When an appropriate temperature is applied, a part of the hairpin structure opens and self-combines with the single amplified strand to hybridize the specific DNA sequence.[25] Therefore, the complementarity between the PCR amplicons had to be excluded to avoid the interference at the hybridization step. Hence, the block probe was added to combine with complementary DNA and for enhancing the specificity of this study. We tested different concentrations of the block probe,

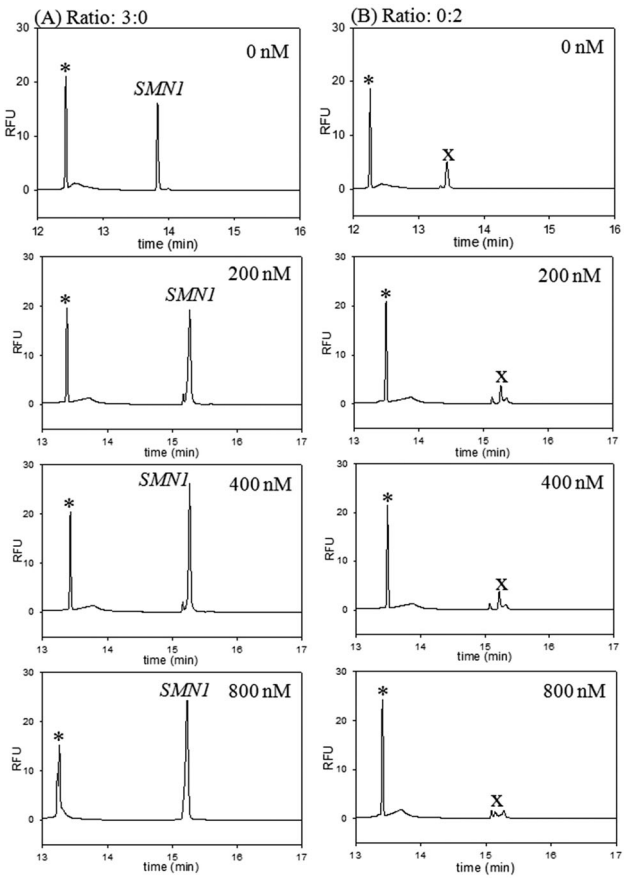

Fig. 1 The effects of the concentration of the block probe which demonstrate DNA samples for (A) *SMN1*/*SMN2* = 3 : 0; (B) *SMN1*/*SMN2* = 0 : 2. The "x" is not identified as a substantial signal for this study, and the (*) is the peak attributed to the primer residue. The CE conditions that followed were sample loading, −10 kV for 20 s; separation voltage, −6 kV; capillary temperature, 25 °C; and separation matrix, 1.2% PEO in 1X TBE buffer.

including 0 nM, 200 nM, 400 nM and 800 nM, and the results indicated that the 400 nM block probe was able to produce better peak signals. It could also be observed that the addition of the block probe improved the signal sensitivity (Fig. 1). The hybridization temperature and time also play an important role in the hybridization process. The temperature around 55–65 °C and 30–120 min duration for hybridization was tested (Fig. S3 and S4, ESI†). Finally, the optimized conditions of scorpion PCR were annealing temperature of 52 °C for 30 cycles of the reaction, hybridization at 60 °C for 30 min using the 400 nM block probe.

Stability of the self-annealing scorpion structure was measured at 0, 3, 6, 12, 24 and 48 hours after hybridization. The results demonstrated good stability for at least 48 hours (Fig. S5, ESI†). In some of the previously reported studies using fluorescence detection, 24 hours was the limitation for the storage time.[26] As shown in the data, this method provides good stability within 48 h and is thus suitable for screening large samples at the same time.

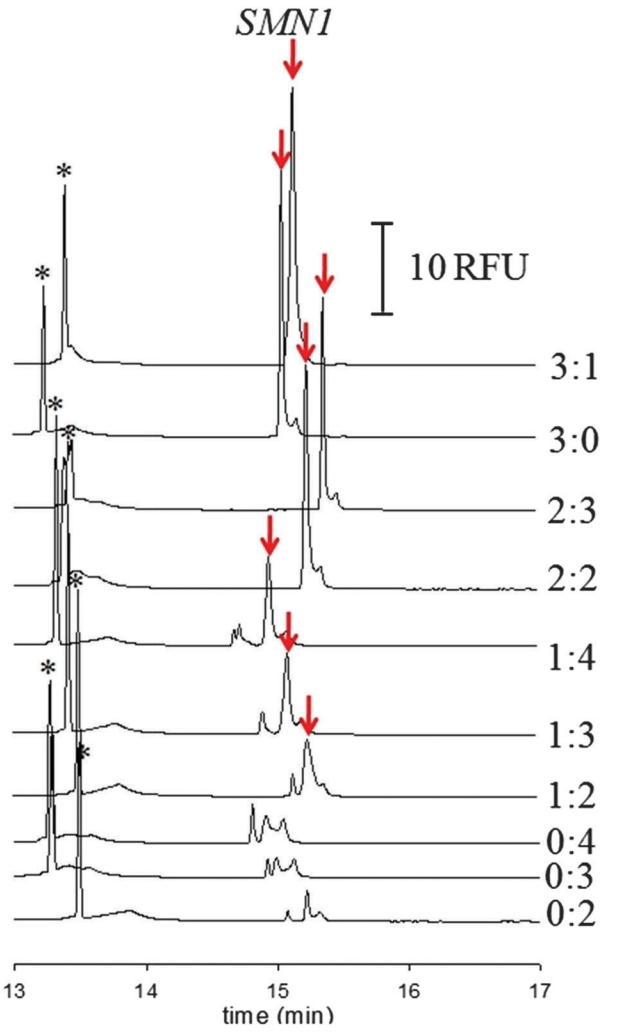

Fig. 2 The real 10 samples with different *SMN1*/*SMN2* gene ratios applied for this method. The peak attributed to *SMN1* is indicated by the red arrow, and the (*) is the peak attributed to the primer residue. Other conditions were the same as in Fig. 1.

We also optimized the CE conditions, the separation voltages were examined at −3, −6 and −9 kV. According to the results, we decided to use −6 kV, because of the best speed and resolution. Based on the above investigation, CE separation was carried out by using 1.2% PEO in 1X TBE buffer and the separation voltage was set at −6 kV (Fig. S6, ESI†).

This study was successfully applied for detection of 10 real DNA samples, including 3 from SMA patients and 7 from normal controls, to distinguish whether the *SMN1* gene is deleted or not. Anyone who has only 1 copy of the *SMN1* gene is not a patient. Therefore, in this study, we observed that subjects having at least one copy of the *SMN1* gene had a higher signal of 5 relative fluorescence unit (RFU) than those having no *SMN1* gene (Fig. 2). Compared with the previous study, we saved more time for polymer matrix production and the separation time.[19]

In conclusion, we developed the scorpion-PCR combined with the CGE method for detection of the nucleotide variant at exon 7 of *SMN* genes. This method did not require RT-PCR and only required the traditional PCR instrument and reagents. This method is the first study on the principle of a ''turn on/off'' system to detect the nucleotide variant and to distinguish *SMN1* genes. It was successfully applied to detection of real samples. This method is very specific for detection the existence of *SMN1*. Till now this is the first biosensor method to detect such genes in real samples.

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