

Short Communication

Universal insertion/deletion-enrich PCR

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Accepted 3 October 2011

Abstract

Objective: Insertions and deletions (indels) are sometimes critical genetic events and can lead to various diseases. Identifying indel types by DNA sequencing is difficult and labor intensive. The sensitivity of DNA sequencing techniques such as Sanger's direct sequencing, which broadly screens all types of microindels in predefined areas, is somewhat limited. Other techniques such as indel variant-specific primer sets are sensitive and specific, but are expensive, labor intensive, less efficient and only cover known indel variants.

Materials and Methods: We report on the universal insertion/deletion-enrich PCR (Unindel-PCR) which has ultra-high sensitivity and can be used to screen for known and unknown indels in a predefined area. In brief, the Unindel-PCR consists of one forward, one reverse and one blocking primer. At very condition, blocking primer is superior to forward primer in recognizing the wild-type template and prevent nucleotide extension and wild-type amplification during PCR cycles. Conversely, forward primer is superior to blocking primer in recognizing the indel template and promote indel-type amplification during PCR cycles.

Results: Unindel-PCR performs well in detecting any indels in a wild-type background. The sensitivity is 1% in Unindel-PCR and 0.01% in nested Unindel-PCR. The wild-type inhibition capacity of Unindel-PCR is 10^7 . One thousand copies of deletion type plasmids are sufficient for detection by Unindel-PCR, whereas nested Unindel-PCR requires only 10 copies.

Conclusion: Unindel-PCR will remold the capability of PCR-based genetic testing, especially in the field of cancer molecular diagnosis, infectious disease and identification of minor populations of alternative splicing variants of RNA transcribed.

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Keywords: EGFR; High sensitive; Indel; Unindel-PCR; Universal insertion/deletion-enrich PCR

Introduction

Universal indel detection is a dream come true now. Not only is amplification indel-specific, but also the indel involving sequences can be detected at the same time. Universal insertion/deletion-enrich PCR (Unindel-PCR) is a simple one-step PCR method. Unindel-PCR is able to perform simultaneous wild-type inhibition with type-independent mutant enrichment. It is a cheap, easy, quick and ultra-high sensitive screening method for all known and unknown types of indel within a predefined area. To determine the efficiency of Unindel-PCR, we examined the EGFR exon 19 indel.

Materials and methods

In brief, the Unindel-PCR consists of one forward, one reverse and one blocking primer. The whole experiment can be completed using regular PCR reagents on a conventional PCR machine. The blocking primer (P^B) is designed to target the EGFR exon 19 indels hot spot but also the upstream region. The 3' end of P^B is modified in order to prevent nucleotide extension during PCR. The recognition site of P^B at the 5' end is partially overlapped with the 3' end of the forward primer (P^F), i.e., P^F or P^B can recognize the upstream of exon 19 indels hot spot region at very condition. The T^m of P^B is higher than that of P^F (Fig. 1A).

The P^B is superior to P^F in recognizing the wild-type template under P^B specific T^m (Fig. 1B). The wild-type template is "blocked" and cannot be extended further by P^F or amplified during PCR cycles. With regard to the insertion/

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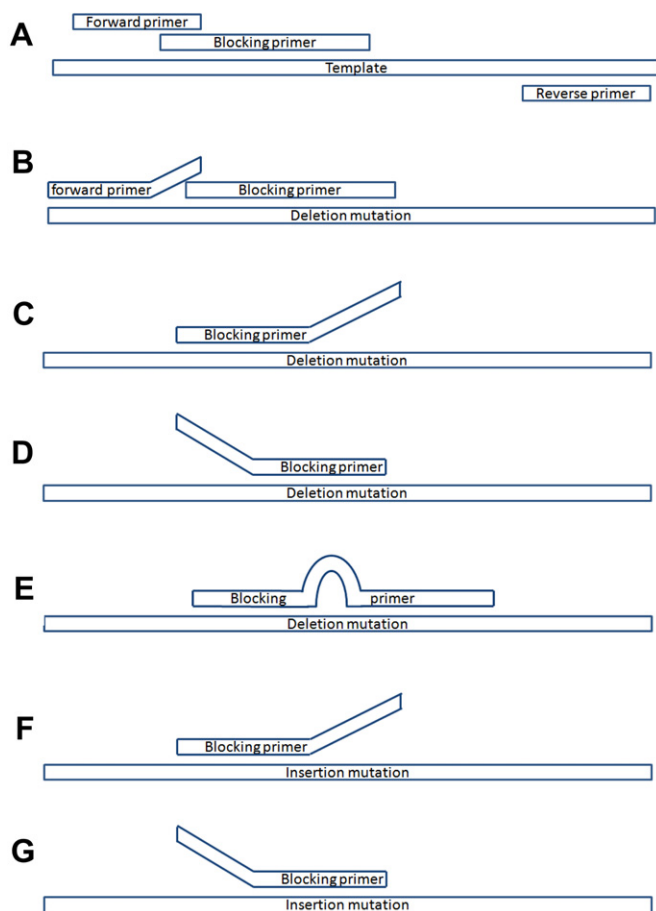


Fig. 1. Primer designed of Unidel-PCR. (A) Three kinds of primer were designed for Unidel-PCR. The relative recognition sites on nucleotide sequence are shown. The forward primer (P^F) is partially overlapping with blocking primer (P^B). (B) In the presence of the wild-type nucleotide sequence, the P^B not only occupies its binding site, but also prevent P^F from extension. When the deletion mutation occurs at the 3' (C), 5' (D) or internal (E) sequence of the P^B recognition site, there will be conformation change of P^B , and a decrease in its binding stability. The same theory can also apply to the condition of insertion (F) and (G).

deletion mutation templates under P^F specific T^m , the P^B changes its conformation into a free end or loop (Fig. 1C–G). At this point, the T^m of P^B becomes lower than that of P^F . The deletion mutation template “kicks out” P^B , is extended by P^F and is amplified during the PCR cycles. The PCR products of Unidel-PCR reflect the exact sequence of the insertion/deletion mutation template, and the insertion/deletion type can be confirmed by the sequencing or genotyping method. During the whole PCR cycle, the inhibition effect on the wild-type template is faithful and persistent. None or only a few wild-type PCR products are produced. On the contrary, all types of indels, known or unknown, are exclusively amplified.

Results

Improvement of deletion detection and wild-type inhibition via Unidel-PCR

EGFR exon 19 was used to determine the sequence in order to confirm the preponderance of Unidel-PCR. Indels

preferentially occur at exon 19 of EGFR in lung adenocarcinoma. Two kinds of plasmid containing the EGFR exon 19 sequence, wild type and E746-A750del, were selected to confirm the efficiency of Unidel-PCR. Both of the two plasmid types were serially diluted from 10^7 copies, and mixed in different percentages (Fig. 2). Once analysis by the Unidel-PCR and nested Unidel-PCR was complete, the PCR products were observed by gel electrophoresis.

The sensitivity in Unidel-PCR is 1% (Fig. 2A) and 0.01% in nested Unidel-PCR (Fig. 2B). The amplicons from the nested Unidel-PCR were clearly seen even if only 10 copies of the deletion type plasmid were present in 10^5 copies of the wild-type plasmid. There was no wild-type amplification observed in the presence of 10^5 wild-type copies and under the nested Unidel-PCR. Regardless of the concentration of wild-type plasmid, only 1000 copies of deletion-type plasmids were sufficient for detection by Unidel-PCR (Fig. 2C).

Improvement of sequencing technologies via Unidel-PCR

Traditional PCR and Unidel-PCR were performed to amplify the same tissue sample from primary lung adenocarcinoma. Sanger's direct sequencing technique was used on the PCR products. Amplicons from the traditional PCR revealed both wild-type and deletion-type sequences on an electropherogram (Fig. 3A), whereas those from the Unidel-PCR disclosed only the deletion type (Fig. 3B) with the wild type totally inhibited. This deletion type is L747-P749, A750P, a complex in-frame deletion with point mutation, indicating

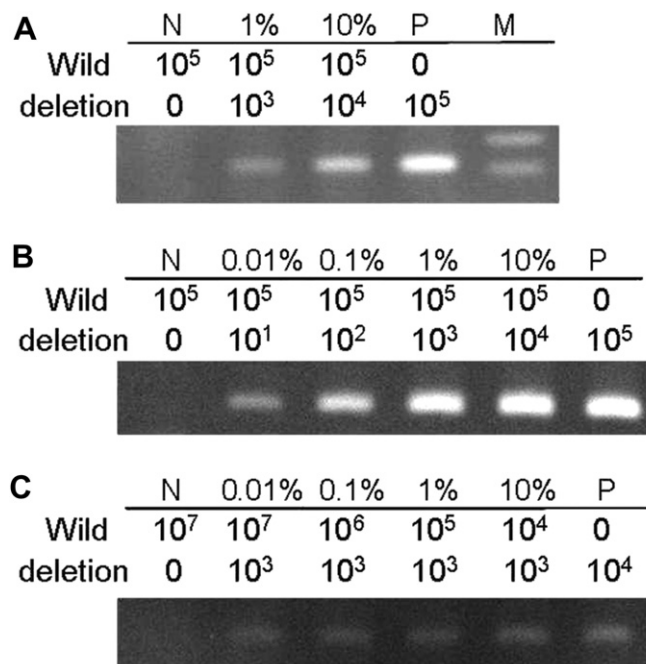


Fig. 2. The detection sensitivity and specificity of Unidel-PCR. (A) Result of testing 10–100 folds in the background of the wild-type plasmid by Unidel-PCR. (B) In nested Unidel-PCR, the sensitivity and specificity of Unidel-PCR was at 0.01% of the rate of mutant type to wild type. (C) Result of Unidel-PCR in the same copy numbers of mutant-type plasmid.

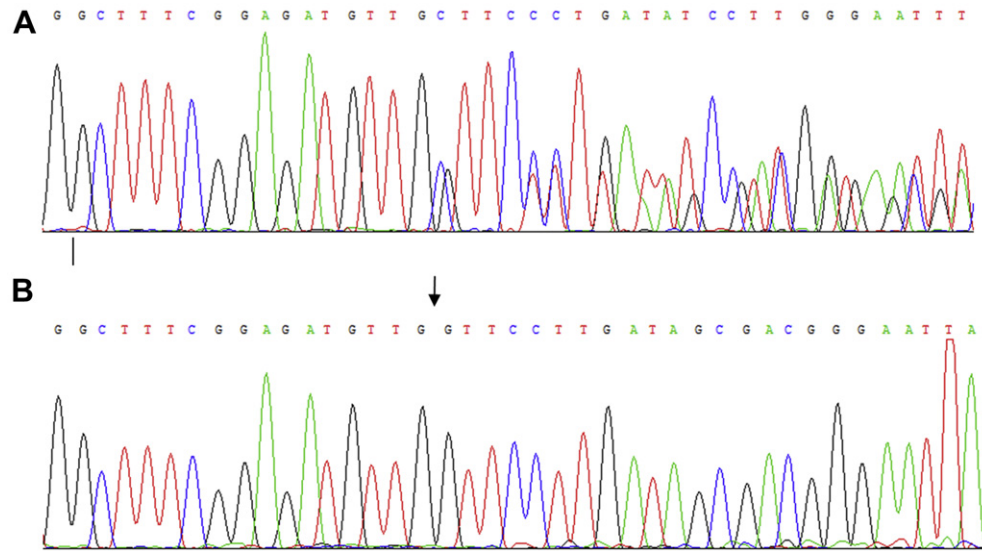


Fig. 3. The sequencing result of traditional PCR and Unidel-PCR. (A) There are two significant waves in the traditional PCR product. (B) Only the indel product could be found after direct sequencing in the Unidel-PCR product. Compared to the sequencing result of traditional PCR, there is a deletion a.a. L747_A750>P mutation occurring in the site indicated by the arrow in both techniques, but the wild sequence can not be observed in the Unidel-PCR.

that the Unidel-PCR is a sequence-independent assay and can exclusively amplify other types of EGFR exon 19 deletions.

Moreover, we used Unidel-PCR to discover five types of EGFR exon 19 deletions, which were not detected by traditional PCR with sequencing technologies (Table 1). The results reveal that not only the common deletion variants (variant 1 and 2), but also the complex gene alteration variants or rare variant (variant 3, 4 and 5) can be selectively amplified even using pleural effusion specimens.

Discussion

Indels can be germline or acquired in disease development and progression. For some genes there are various types of microindels which in some instances cluster in the hot spot area. Indels can be found in conventional human cancer, and some of them become critical genomic events leading to cancer initiation, progression or survival. Trying to detect all types of microindels within cancer tissue is always difficult even within a predefined genomic region. The normal cells usually outnumber cancer cells and mask cancer genotypes in direct sequencing, and lead to a “wild-type” result. This could

mislead clinic physicians when choosing cancer-targeted drugs, particularly in regards to the drugs’ effect on the mutant signal transmitting proteins.

Various methods are used to increase detection sensitivity of indels. In identifying EGFR exon 19 indels in lung adenocarcinoma, there are three highly sensitive diagnostic methods: the high resolution melting technique [1]; scorpions ARMS detecting method [2] and the PNA-LNA clamp method [3]. The high resolution melting technique can detect various known and unknown indel variants in tissue sample, but cannot provide direct evidence for the exact nucleotide changes. The scorpions ARMS detecting method has been commercialized into an IVD diagnostic kit. It can only determine known genetic changes. PNA and LNA are modified deoxyriboses which are applied to inhibit wild-type amplification and magnify indels signals. The PNA-LNA clamp design is excellent but is expensive and requires a high-precision temperature-controlled thermocycler.

Our novel universal insertion/deletion-enrich PCR (Unidel-PCR) technique is simple and easy, cheap and user-friendly. Tissue samples should first be analyzed on a conventional PCR thermocycler, results obtained from agarose gel

Table 1

Five variants of EGFR exon 19 deletions were discovered by Unidel-PCR from EGFR “wild-type” pleural effusion specimens. These variants include the most common types, c.2235_2249del15 and c.2236_2250del15, two rare variants with complex gene alterations, c.2239_2247del10;2248G>C and c.2237_2256del20insTC, and one rare variant c.2240_2254del15.

Variant	Nucleotide number and sequence	a.a. sequence
1	c.2235_2249del GGAATTAAGAGAAGC	E746-A750
2	c.2236_2250del GAATTAAGAGAAGCA	E746-A750
3	c.2239_2247del TTAAGAGAAC; 2248G>C	L747-P749, A750P
4	c.2237_2256del AATTAAGAGAAGCAACATCT insTC	E746-S752>V
5	c.2240_2254del TAAGAGAAGCAACGT	L747-T751 del

electrophoresis, and the changes in the indels nucleotide determined by direct sequencing. Unidel-PCR has ultra-high sensitivity and can be used to screen for known and unknown indels in a predefined area. Unidel-PCR can also detect any known and unknown indels in a wild-type background as the sensitivity is 1:10000. No wild type will be detected via Unidel-PCR and subsequent direct sequencing, and the power of inhibition is up to 10^6 wild type. We believe Unidel-PCR is currently the most sensitive, specific and wide-covered method for any known and unknown micro-indels detection within a predefined genomic area. The application strength will be the highest in detecting circulating tumor cells, tumor cells within body fluid, and diffuse and scanty tumor cells infiltrating in solid tissue. The universal detecting character can also contribute to infectious diseases

and the identification of minor populations of alternative splicing variants of RNA transcribed.

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