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A long downstream probe-based platform for multiplex target capture

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ABSTRACT

A simple and rapid detection platform was established for multiplex target capture through generating single-strand long downstream probe (ssLDP), which was integrated with the ligase detection reaction (LDR) method for the purpose of multiplicity and high specificity. To increase sensitivity, the ladder-like polymerase chain reaction (PCR) amplicons were generated by using universal primers that complement ligated products. Each of the amplicons contained a stuffer sequence with a defined yet variable length. Thus, the length of the amplicon is an index of the specific suppressor, allowing its identification via electrophoresis. The multiplexed diagnostic platform was optimized using standard plasmids and validated by using potato virus suppressors as a detection model. This technique can detect down to 1.2×10^3 copies for single or two mixed target plasmids. When compared with microarray results, the electrophoresis showed 98.73–100% concordance rates for the seven suppressors in the 79 field samples. This strategy could be applied to detect a large number of targets in field and clinical surveillance.
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The circulation of the novel H7N9 avian influenza A virus spreading from one country to another within a short span in 2013 [1] and the outbreak of plant viruses [2] are a serious threat to human health and plant production. To take measures promptly, a rapid yet efficient method to detect the pathogen types at the very early stage of the virus outbreak is in great demand [3]. Multiplex target capture technology can meet this type of diagnostic requirement. It can enrich the low level of the target pathogens in a multiplex fashion, followed by a detection strategy. Considerable effort has been devoted to augment the targets for multiplexing [4]. Nonetheless, it is still a great challenge to achieve a streamlined approach while bearing the advantages of specificity, sensitivity, and cost efficiency at the same time.

Within the multiplex capturing strategy, DNA microarray technology can detect thousands of templates in a single assay. However, it has limited specificity and sensitivity during hybridizations of DNA samples to the respective capture probes [5–7]. Multiplex polymerase chain reaction (PCR) has greatly improved this situation, but it could create spurious amplicons, uneven or nonexistent amplification, despite decades of effort [8–10]. To improve the specificity, Schouten and coworkers [11] developed a method by hybridizing the probes adjacent to the target DNA for ligation and only the ligated probes, not the original target DNAs, are amplified by PCR. However, this method requires producing long downstream ligase detection reaction (LDR) probes. The procedure is laborious. It includes the initial multistep conventional cloning, cell amplification, DNA extraction, double-stranded DNA modification, and so on [12,13].

To overcome these issues, we developed a novel yet low-cost approach that is called multiplex LDR–PCR (MLP) (Fig. 1). This method generates ladder-like single-strand long downstream probes (ssLDPs) with lengths between 163 and 754 bases. The conventional asymmetric PCR produces a sufficient amount of ssLDPs that are used as the downstream LDR probes. Each pair of ssLDP and the upstream LDR probes are pathogen specific, containing two universal tags and one variable long stuffer spacer. The platform combines the specificity of the probe-mediated ligation and the advantage of homogeneous amplification for multiplex targets. A test set of seven suppressors was selected as a model assay [14–18]. The assay platform was also tested on a set of 79 field samples, and the results were compared with those of the microarray method, demonstrating a great potential for this assay platform as a tool in surveillance and diagnostics.

Abbreviations: PCR, polymerase chain reaction; LDR, ligase detection reaction; MLP, multiplex LDR–PCR; ssLDP, single-strand long downstream probe; $P0^{BWYV}$, beet western yellow virus P0; $2b^{CMV}$, cucumber mosaic virus 2b; $P0^{PLRV}$, potato leafroll virus P0; $P25^{PVX}$, potato virus X P25; $HcPro^{PVY}$, potato virus Y help component proteinase; $16kDa^{TRV}$, tobacco rattle virus 16-kDa protein; $19kDa^{TRSV}$, tobacco ringspot virus 19-kDa protein.

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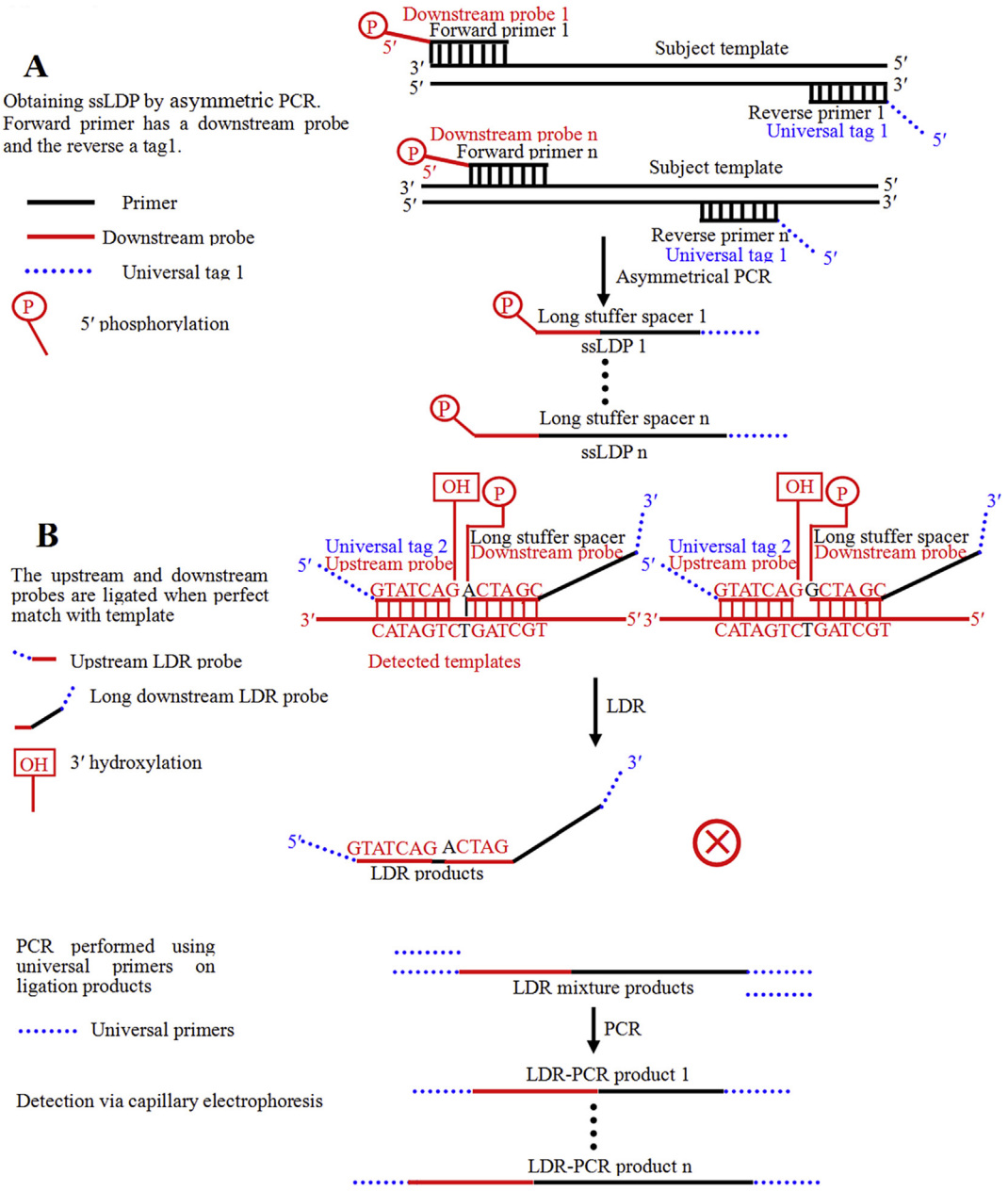

Fig.1. Schematic representation of MLP assay. (A) Preparation of the asymmetric PCR-derived ssLDP oligonucleotides. Each ssLDP has a defined stuffer length (163–754 nt). The subjective template (*Hickory Apetala 1* gene) and two primers are used to generate the stuffer sequence with different lengths via asymmetric PCR. The forward primer (left) has a downstream probe adaptor (red) with the 5′ end phosphorylated. The reverse primer (right) has a universal tag 1. The AP1 template has no sequence homology with the detected targets. (B) Depiction of the essential components of an MLP assay. Ligation is achieved by using one synthesized upstream LDR probe and one asymmetric PCR-derived ssLDP. The upstream LDR probe (left) is composed of target-specific sequence complementary to the detected region (red) with the 5′ universal tag 2 (blue), which is hydroxylated on its 3′ end. The ssLDP (right) includes a target-specific sequence complementary to the detected region (red), a stuffer (black), and a universal 3′ tag 1 tail sequence, which is phosphorylated on its 5′ end. In the case of a perfect match, the set of upstream LDR probe and ssLDP will be hybridized adjacently to each other by DNA ligase (left). In the case of a mismatch, no ligation will occur (right). The ligation products are further amplified in a PCR by the universal primers. The amplicons are loaded onto gel electrophoresis for separation and identification. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.).

Materials and methods

Materials

Seven suppressor genes used in this study were *beet western yellows virus* P0 ($P0^{BWYV}$), *cucumber mosaic virus* 2b ($2b^{CMV}$), *potato leafroll virus* P0 ($P0^{PLRV}$), *potato virus X* P25 ($P25^{PVX}$), *potato virus Y* help component proteinase ($HcPro^{PVY}$), *tobacco rattle virus* 16-kDa protein (16 kDa^{TRV}), and *tobacco ringspot virus* 19-kDa protein (19 kDa^{TRSV}). The viruses (infected potato leaves) were purchased from Bioreba (Switzerland) and stored at −80 °C. Two nontarget viruses, *wheat yellow mosaic virus* VPg protein region and *Chinese wheat mosaic virus* W19K protein region, were used to determine the specificity of the assay. *Hickory Apetala 1* gene (GenBank: KF918309, *AP1*), which shares no homology with any of the targets, was used as an initial subjective template to generate different sizes of ssLDPs.

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