Chapter 4

Lariat-Dependent Nested PCR for Flanking Sequence Determination

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Abstract

Methods detailed in this chapter relate to the use of Lariat-dependent Nested (LaNe) PCR to characterize unknown RNA or DNA sequence flanking known regions. A multitude of approaches designed to determine flanking sequences have been described in the literature. Variously, problems related to these approaches include lack of resolution or failure, depending on experimental context, and complex handling. LaNe-based methods are designed to harness "two-sided" gene-specific PCR with the option of nesting but without the requirement for inefficient and involved enzyme preprocessing steps.

Key words: Flanking sequence determination, LaNe, PCR walking

1. Introduction

While relatively recent higher throughput DNA sequencing is revolutionizing the way biological research is conducted, there remain applications for more focused analyses of flanking sequence. The size of the region to be resolved, number of specimens, complexity and duration of processing and bioinformatic analysis, intractability of sequence to methodology, and cost are among the factors influencing the choice of approach to the study of flanking sequence. Many methods for PCR-based flanking sequence determination have been described, such as 3′Rapid Amplification of cDNA Ends (3′RACE), 5′RACE, Rapid Amplification of Genomic DNA Ends, Inverse PCR, CapSelect, Ligase-mediated PCR, Splinkerette PCR, Thermal Asymmetric Interlaced (TAIL) PCR, and Universal Fast Walking (1–11). Tonooka and Fujishima have recently reviewed PCR methods for

Daniel J. Park (ed.), *PCR Protocols*, Methods in Molecular Biology, vol. 687,
DOI 10.1007/978-1-60761-944-4_4,

walking along genomic DNA (12). Methods for PCR-based flanking sequence determination exhibit various limitations, including a requirement for relatively large quantities of input material, complex processing steps relying on multiple handling steps, and dependence on efficient enzyme steps involving restriction endonucleases, ligases, and/or exonuclease processing. In most cases, there is a reliance on the so-called "one-sided" PCR in which extension is primed by a gene-specific primer and a "general" primer targeted to sites common across the genome or adapters present in most species of the template mix. These approaches work satisfactorily in many cases, but some can encounter difficulties in more challenging contexts. In particular, one-sided approaches often yield "smeared products" as visualized following gel electrophoresis such that desired products of interest are difficult to resolve.

The Lariat-dependent Nested (LaNe)-based methods 3′RACE LaNe, 5′RACE LaNe, and LaNe RAGE (13–15) are derived from the "panhandle" concept employed in Universal Fast Walking (UFW) in which a lariat structure is involved in self-priming to yield a template which enables "two-sided" and nested PCR to be applied to flanking sequence determination. LaNe-based methods streamline the UFW concept workflow and system by removing the requirement for multiple rounds of exonuclease addition and "end-fill" steps prior to initiating "PCR-proper." Instead, LaNe-based self-priming is facilitated by denaturation and annealing steps inherent to conventional PCR thermocycling (the reaction scheme for LaNe RAGE is depicted in Fig. 1).

In this chapter, LaNe-based protocols are presented for 5′ or 3′ oriented RNA flanking sequence determination (5′RACE LaNe and 3′RACE LaNe) and genomic DNA flanking sequence determination (LaNe RAGE). Further, special requirements and tips for customization of the approaches are presented in Subheading 4.

2. Materials

2.1. 3′RACE LaNe

1. 20 ng total RNA (see Note 1).
2. 10 mM dNTPs.
3. ThermoScript™ Reverse Transcriptase and accompanying buffers (Invitrogen) (see Note 2).
4. TTT3 primer: 5′-gene-specific sequence followed by TTTTTTTTTTTTTTTTV-3′. The 5-prime gene-specific region of this primer should be in the same orientation as GSP1 and GSP4 (see Subheading 2.1, item 7) in alignment with position "3" as depicted in Fig. 1.
5. RNaseH (Invitrogen).

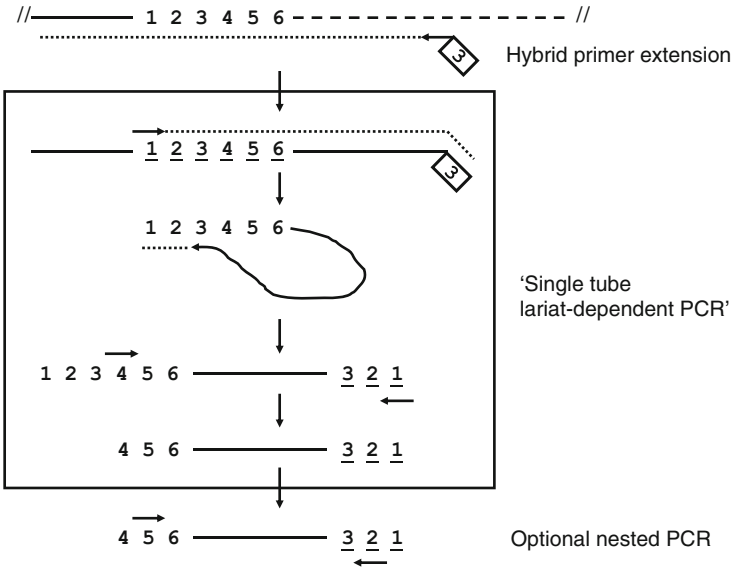

Fig. 1. Schematic illustration of the LaNe approach applied in LaNe RAGE. A hybrid primer containing gene-specific sequence at the 5′ end and nongene-specific sequence at the 3′ end is used to prime extension from the template (hybrid primer extension). The resulting product is then amenable to gene-specific second strand synthesis, followed by lariat structure formation and self-primed extension, to yield template which can be amplified by "two-sided PCR" using two gene-specific primers (Single tube lariat-dependent PCR). This can occur in a standard PCR thermocycling profile due to the multiple rounds of denaturation and annealing in the presence of the appropriate primers and thermostable DNA polymerase. The product of Single-tube lariat-dependent PCR can be applied as template in a nested PCR scheme (optional nested PCR). Numbers indicate features of a known sequence region. It is important to note that features 1–6 should always be oriented with feature 6 closest to the unknown sequence to be determined. The *dashed line* indicates the unknown sequence of the template which is to be determined. *Underlined numbers* indicate reverse complement sequences with respect to these features. Primers are represented by *horizontal arrows*. *Dotted lines* indicate polymerase extension.

6. *PfuTurbo*® DNA Polymerase and accompanying buffers and solutions (Stratagene) (see Note 3).
7. GSP1 and GSP4: Gene-specific primers 1 and 4 should be designed according to standard PCR primer conventions with respect to avoiding primer dimers and intraprimer annealing, attempting to prevent nonspecific priming, and ensuring that the T_m of the primers is a few degrees higher than the intended annealing temperature during thermal cycling *but* that both are in the same orientation as each other with the 3′ ends pointing towards the unknown region to be elucidated (refer to Fig. 1) (see Notes 4 and 5). GSP1 and GSP4 should be designed in alignment with positions "1" and "4," respectively, in Fig. 1.
8. GSP2 and GSP5: Gene-specific primers (optional – see Notes 6 and 7) should be designed in a similar manner to GSP1 and GSP4 but aligned with positions "2" and "5," respectively, in Fig. 1.

9. QIAEXII DNA purification system (Qiagen) (optional – see Note 8).
10. GSP6: Gene-specific primer (optional – see Note 8) should be designed in a similar manner to GSP1, GSP2, GSP4, and GSP5 but aligned with position "6" in Fig. 1.

2.2. 5′ RACE LaNe

2.2.1. First-Strand Intrastrand-Annealing 5′ RACE LaNe (FI RACE LaNe)

1. 200 ng total RNA (see Note 1).
2. 10 mM dNTPs.
3. ThermoScript™ Reverse Transcriptase and accompanying buffers (Invitrogen) (see Note 2).
4. RT primer: Reverse transcription primer should be oriented such that the 3-prime end is directed toward the unknown 5-prime mRNA region to be elucidated and reverse transcribed product includes features "1" to "6" of Fig. 1.
5. RNaseH (Invitrogen).
6. *PfuTurbo*® DNA Polymerase and accompanying buffers and solutions (Stratagene) (see Note 3).
7. GSP1 and GSP4: Gene-specific primers 1 and 4 (see Subheading 2.1, item 7 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.
8. GSP2 and GSP5: Gene-specific primers 2 and 5 (see Subheading 2.1, item 8 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.
9. QIAEXII DNA purification system (Qiagen) (optional – see Note 8).
10. GSP6: Gene-specific primer 6 (see Subheading 2.1, item 10 and Fig. 1) (optional – see Note 8). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.

2.2.2. Terminal Transferase-Mediated 5′ RACE LaNe (TT RACE LaNe)

1. 200 ng total RNA (see Note 1).
2. 10 mM dNTPs.
3. SuperScript™ II Reverse Transcriptase and accompanying buffers (Invitrogen) (see Note 9).
4. 25 mM manganese chloride.
5. 5 mg/ml bovine serum albumin.
6. RT primer: Reverse transcription primer should be oriented such that the 3-prime end is directed toward the unknown 5-prime mRNA region to be elucidated and reverse transcribed product includes features "1" to "6" of Fig. 1.
7. RNaseH (Invitrogen).
8. QIAEXII DNA purification system (Qiagen).

9. Terminal transferase (New England Biolabs).
10. 10 mM dATP.
11. *PfuTurbo*® DNA Polymerase and accompanying buffers and solutions (Stratagene) (see Note 3).
12. GSPT12G3 primer: 5′-gene-specific sequence followed by TTTTTTTTTTTTGGG-3′. The 5-prime gene-specific region of this primer should be in the same orientation as GSP1 and GSP4 (see Subheading 2.2.2, item 13) in alignment with position "3" as depicted in Fig. 1.
13. GSP1 and GSP4: Gene-specific primers 1 and 4 (see Subheading 2.1, item 7 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.
14. GSP2 and GSP5: Gene-specific primers 2 and 5 (see Subheading 2.1, item 8 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.
15. GSP6: Gene-specific primer 6 (see Subheading 2.1, item 10 and Fig. 1) (optional – see Note 8). In this case, 3-prime ends of primers are all oriented towards the unknown 5-prime region of mRNA to be elucidated.

2.3. LaNe RAGE

1. 300 ng genomic DNA (see Note 1).
2. 10 mM dNTPs.
3. *PfuTurbo*® DNA Polymerase and accompanying buffers and solutions (Stratagene) (see Note 3).
4. NNN3: First-strand synthesis primer 5′-gene-specific sequence followed by NNNNNCTCAC-3′ (see Note 10). The 5-prime gene-specific region of this primer should be in the same orientation as GSP1 and GSP4 (see Subheading 2.3, item 5) in alignment with position "3" as depicted in Fig. 1.
5. GSP1 and GSP4: Gene-specific primers 1 and 4 (see Subheading 2.1, item 7 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown region of genomic DNA to be elucidated.
6. GSP2 and GSP5: gene-specific primers 2 and 5 (see Subheading 2.1, item 8 and Fig. 1). In this case, 3-prime ends of primers are all oriented towards the unknown region of genomic DNA to be elucidated.
7. QIAEXII DNA purification system (Qiagen) (optional – see Note 8).
8. GSP6: Gene-specific primer 6 (see Subheading 2.1, item 10 and Fig. 1) (optional – see Note 8). In this case, 3-prime ends of primers are all oriented towards the unknown region of genomic DNA to be elucidated.

3. Methods

LaNe-based methods represent a suite of tools that are applicable to the elucidation of 5-prime and 3-prime mRNA flanking sequence (5′RACE LaNe and 3′RACE LaNe, respectively) and genomic DNA flanking sequence in either direction (LaNe RAGE). Theoretically, the latter could be applied to the determination of insertion elements such as transposons as part of mutagenesis screening or DNA fingerprinting strategies, although these applications remain to be tested. The examples that follow represent demonstration methods that have been tested. It should be noted that elements of the respective approaches could be applicable to alternative approaches within the suite of tools (see Notes 11 and 12).

3.1. 3′RACE LaNe

The reaction scheme for 3′RACE LaNe is similar to that depicted in Fig. 1 for LaNe RAGE, except NNN3 is swapped for the reverse transcription primer TTT3 in the first step.

1. TTT3 primer (1 μM final concentration) is used to prime ThermoScript™-catalysed reverse transcription of 20 ng total RNA at 55°C for 1 h in a total reaction volume of 20 μl.
2. Reverse transcription products are treated with 2 U RNaseH at 37°C for 20 min prior to heat inactivation at 85°C for 10 min.
3. Five microliters of this product are used to template a 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP1 and GSP4. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 50°C for 1 min, 72°C for 5 min (see Note 7).
4. Product can be analyzed by agarose gel electrophoresis at this stage (see Note 6). Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14). Optionally, a nested PCR can be performed, in which case, proceed to the next step.
5. Use 0.5 μl of product from the preceding step to template a nested 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP2 and GSP5. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 5 min (see Note 7).
6. Product can be analyzed by agarose gel electrophoresis at this stage. Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14).
7. Product can be subjected to direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).

8. Selected post-agarose gel electrophoresis resolved products can be gel-extracted using the QIAEXII gel extraction system, followed by direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).

3.2. 5′ RACE LaNe

Two example protocols are presented: FI RACE LaNe and TT RACE LaNe. The former uses a relatively streamlined workflow but a less "defined" mechanism for lariat structure formation (see Note 2). As such, FI RACE LaNe may be useful in some contexts but not others. The first step of FI RACE LaNe employs a non-hybrid reverse transcription primer instead of NNN3 depicted in Fig. 1 and relies on fortuitous lariat structure formation and extension by the first DNA strand. Ensure that features 1–6 with reference to Fig. 1 are oriented such that feature 6 is closest to the unknown 5-prime sequence. TT RACE LaNe is designed to control lariat structure formation to facilitate a more robust approach but uses a more cumbersome protocol to achieve this. Similarly, ensure that features 1 to 6 are oriented such that feature 6 is closest to the unknown 5-prime sequence. Reverse transcription and terminal transferase processing precede priming by GSPT12G3, which is analogous to NNN3 priming depicted in Fig. 1.

3.2.1. FI RACE LaNe

1. RT primer (1 μM final concentration) is used to prime ThermoScript™-catalyzed reverse transcription of 200 ng total RNA at 50°C for 1 h in a total reaction volume of 20 μl.
2. Reverse transcription products are treated with 2 U RNaseH at 37°C for 20 min prior to heat inactivation at 85°C for 10 min.
3. Two microliters of this product are used to template a 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP1 and GSP4. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min (see Note 7).
4. Product can be analyzed by agarose gel electrophoresis at this stage (see Note 6). Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14). Optionally, a nested PCR can be performed, in which case, proceed to the next step.
5. Use 0.5 μl of product from the preceding step to template a nested 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP2 and GSP5. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min (see Note 7).
6. Product can analyzed by agarose gel electrophoresis at this stage. Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14).

7. Product can be subjected to direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).
8. Selected post-agarose gel electrophoresis resolved products can be gel-extracted using the QIAEXII gel extraction system, followed by direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).

3.2.2. TT RACE LaNe

1. RT primer (1 μM final concentration) is used to prime SuperScript™ II-catalyzed reverse transcription of 200 ng total RNA in 1× buffer in the presence of 2 mM manganese chloride and 0.1 mg/ml bovine serum albumin at 42°C for 1 h in a total reaction volume of 20 μl (see Note 9).
2. Reverse transcription products are treated with 2 U RNaseH at 37°C for 20 min prior to heat inactivation at 85°C for 10 min.
3. Products are cleaned using the QIAEXII DNA purification system and eluted using 20 μl of elution buffer. 15 μl of eluate is collected, with care taken to avoid transfer of matrix slurry to the collection tube.
4. Five microliters of eluate is included in a 20 μl terminal transferase reaction in 1× buffer with the inclusion of 200 μM dATP and 20 U terminal transferase at 37°C for 20 min.
5. Two microliters of this product are used to template a 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, 100 nM GSPT12G3, and 250 nM GSP1 and GSP4. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min (see Note 7).
6. Product can be analyzed by agarose gel electrophoresis at this stage (see Note 6). Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14). Optionally, a nested PCR can be performed, in which case, proceed to the next step.
7. Use 0.5 μl of product from the preceding step to template a nested 50 μl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 μM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP2 and GSP5. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min (see Note 7).
8. Product can analyzed by agarose gel electrophoresis at this stage. Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14).
9. Product can be subjected to direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).
10. Selected post-agarose gel electrophoresis resolved products can be gel-extracted using the QIAEXII gel extraction

system, followed by direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).

3.3. LaNe RAGE

Figure 1 schematically depicts the steps in LaNe RAGE. It must be ensured that the orientation of features 1–6 are such that feature 6 is closest to the unknown region to be characterized.

1. 50 µl 1× *PfuTurbo*® buffer containing 300 ng genomic DNA and 250 nM NNN3 is heated to 94°C for 3 min and then placed on ice for 5 min prior to the inclusion of 3.75 U *PfuTurbo*® and dNTPs to achieve a concentration of 300 µM.
2. Heat the reaction tubes from 18°C to 72°C at a rate of 0.1°C per 5 s and hold at 72°C for 10 min prior to placing on ice (see Note 10).
3. Include GSP1 and GSP4 to achieve 250 nM concentrations of each and proceed immediately to thermocycling. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 2 min (see Note 7).
4. Product can be analysed by agarose gel electrophoresis at this stage (see Note 6). Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 7). Optionally, a nested PCR can be performed, in which case, proceed to the next step.
5. Use 0.5 µl of product from the preceding step to template a nested 50 µl *PfuTurbo*® hot start PCR in (see Note 13) 1× reaction buffer including 300 µM dNTPs, 3.75 U *PfuTurbo*® DNA Polymerase, and 250 nM GSP2 and GSP5. Thermocycling parameters used are: 40 cycles of 94°C for 1 min, 60°C for 1 min, 72°C for 2 min (see Note 7).
6. Product can analysed by agarose gel electrophoresis at this stage. Single band or multiple banding profiles might be expected depending on experimental conditions (see Note 14).
7. Product can be subjected to direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).
8. Selected post-agarose gel electrophoresis resolved products can be gel-extracted using the QIAEXII gel extraction system, followed by direct Sanger sequencing using GSP5 or GSP6 as the sequencing primer (see Note 8).

4. Notes

1. The integrity of the input nucleic acid specimen will have a considerable bearing on the potential for success using LaNe-based approaches, as with most molecular biology technologies. If the input material is significantly degraded or fragmented, only

relatively short regions of flanking sequence may be resolvable in any given "LaNe step." In this context, it is perhaps unwise to design primers and conditions in an attempt to yield long products.

2. ThermoScript™ Reverse Transcriptase is relatively thermostable, permitting relatively stringent priming of reverse transcription, if required. Alternative enzymes with similar properties should also be suitable.
3. Terminal transferase activity is undesirable in a thermostable DNA-dependent DNA polymerase employed in LaNe-based methods in which the lariat structure formation is facilitated by a gene-specific hybrid primer sequence incorporation event, e.g., via TTT3, GSPT12G3, or NNN3. Such activity would result in a majority of would-be lariat-primed events exhibiting mismatches at the 3-prime end, thus inhibiting lariat-primed extension dramatically. This does not apply to examples such as FI RACE LaNe or in other cases in which the principle of "fortuitous" lariat structure priming is applied (e.g., in principle, an iteration of LaNe RAGE could remove the requirement for NNN3-primed strand synthesis to simply employ nested PCR using GSP1 and GSP4, followed by GSP2 and GSP5 – see Notes 6 and 12). *PfuTurbo*® DNA Polymerase has been used effectively, but alternative enzymes with similar properties should also suit LaNe-based methods.
4. In the LaNe-based methods, for all primers used, gene-specific primer regions are always oriented with the 3-prime end towards the unknown sequence to be elucidated. In cases where hybrid primer is used, e.g., TTT3, GSPT12G3, and NNN3, the gene-specific region is always located at the 5-prime end of the primer, but the orientation of the gene-specific primer sequence is designed with its 3-prime end oriented towards the unknown sequence to be elucidated as with all other primers. This works because end-filling and subsequent lariat-based priming (as part of the LaNe mechanism) yield reverse complement forms of some sites at one end of the template, allowing primers to engage in two-sided PCR.
5. Primer design software such as OligoAnalyzer 3.1, Primer 3, and Primer-BLAST can be employed to design primers according to the desired melting temperature (aim for "typical" PCR primer properties) and to check for and avoid primers or primer combinations liable to form strong secondary structures (e.g., hairpins) or engage in primer dimer formation. PCR primers can be filtered against repetitive elements of reference genomes and checked for predicted off-target products. The scope for primer design is somewhat dependent, however, on the amount of known sequence available and the desire to design the primers to anneal close to the unknown region to be resolved if possible.

6. In some applications, just one round of PCR may be sufficient. This requires fewer primers and a shorter region of known sequence to design LaNe primers. There will be other cases where a single round of PCR is insufficient, and the added sensitivity and specificity afforded by a second nested PCR will resolve desired products.
7. The RT and PCR conditions detailed in this chapter represent proof-of-concept methodologies. These are most likely open to considerable optimization to suit requirements. For example, some applications may require greater or lower stringency necessitating higher or lower annealing temperatures, respectively. Some applications may require longer extension times during cycling to facilitate yield of longer products. Applications may require more or less enzyme or initial nucleic acid template. It is likely that fewer PCR cycles would be necessary than are detailed in this chapter. However, it should be noted that by their nature, LaNe-based protocols employ templates that form suppressive "panhandle" structures. As such, relatively high concentrations of primers GSP1 and GSP4 or GSP2 and GSP5 may be required to compete effectively with such structure formation for effective priming during thermocycling. Ideally, the primer regions should be relatively close together to minimize the relative stability and rate of formation of such structure formation (primer sites could even overlap to a limited extent). Conceivably, GSP1 and GSP4 or GSP2 and GSP5 could employ "heel clamps" to increase Tm and improve competitivity.
8. As a fast and convenient approach, products can be sequenced directly using GSP5 or GSP6 as the sequencing primer. In some cases, it may be desirable or necessary to isolate product bands from an agarose gel. Following gel extraction, products can be separately sequenced using GSP5 or GSP6 as the sequencing primer. Ideally, sequencing primers should be set back sufficiently from the unknown flanking sequence under study to allow verification of gene-specific amplicon. If the former is not possible due to design constraints or if preferred, product can be cloned into a plasmid and sequencing performed using plasmid-specific sequencing primers.
9. TT RACE LaNe makes use of the inherent terminal transferase activity of M-MLV-based reverse transcriptase, e.g., SuperScript™ II or Expand-RT, in the presence of manganese chloride as an additional divalent cation to magnesium chloride and in the presence of BSA as a stabilizing agent. Under these conditions, several cytosine residues are typically added in the presence of 5′ capped mRNA templates. In the presence of 5′-OH or 5′-phosphate termini, the activity is less pronounced and not cytosine-specific (3).

10. As a proof-of-concept utility, NNN3 was designed with a "NNNNNCTCAC" 3-prime terminus. However, any number of variations could be used to bias towards more frequent or less frequent hybridization sites to influence the tendency of product sizes. For instance, a longer "defined" region at the 3-prime end might tend towards the yield of longer products, since hybrid primer binding sites would be less frequent. The nature of the defined sequence at the 3-prime end could be varied to suit the experimental context. There is considerable scope to influence the performance of the system by adjusting hybrid primer sequence. Integrally related to this is the way in which annealing of NNN3 to denatured genomic DNA is conducted prior to extension. More stringent conditions should result in fewer hybrid primer binding events and fewer products, to enable the yield of longer products. For example, immediately following denaturation (in the presence of template, NNN3, *PfuTurbo*® DNA Polymerase, and dNTPs in reaction buffer) one might perform annealing at an elevated temperature, e.g., 45–55°C, for several minutes (without having cooled below this temperature) prior to slowly raising the temperature to 72°C (e.g., 1°C/20 s to balance proficiency of extension at suboptimal temperatures with maintenance of annealing), with all subsequent primers added at this temperature. Further, a reduction in concentration of NNN3 may contribute to less frequent hybrid primer binding events to permit "cleaner" product profiles and selection of longer products.
11. It is likely that LaNe RAGE would be useful in applications such as transposon insertional mutagenesis screening programs to identify insertion sites of mutants and in DNA fingerprinting, as has been demonstrated using Universal Fast Walking. However, these experiments have not been conducted.
12. One approach that may be valuable but has not been tested to date would combine aspects of CapTrapper (16) and 5′RACE LaNe, in which 5-prime capped mRNAs would be physically separated from noncapped mRNA species prior to utilization of a LaNe-based approach. Fortuitous lariat structure formation might be employed in LaNe RAGE without first-strand synthesis primed by NNN3, analogous to that used in TT RACE LaNe. This would result in a less-controlled but more streamlined protocol in which the user could proceed directly to a hot-start PCR templated with genomic DNA using primers GSP1 and GSP4, optionally followed by a nested PCR using GSP2 and GSP5.
13. In the proof-of-concept methods detailed, hot-start PCR was performed by separate addition of enzyme following thermal

denaturation. Alternatively (and preferably), chemical or antibody inhibition-based hot-start enzyme may be employed.

14. Product profiles vary considerably and are strongly influenced by hybridization conditions, primer design, and PCR parameters as discussed in this section.

Acknowledgments

This work was supported by the National Health and Medical Research Council of Australia grant no. 148630 to Professors J.A.M. Graves and M.B. Renfree, Associate Professor V.R. Harley and Dr. A.J. Pask.

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