Takara Bio USA

Universal GenomeWalker™ 2.0 User Manual

Cat. No. 636405

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I. Introduction

DNA walking is a simple method for finding unknown sequences adjacent to a known genomic DNA sequence. For example, you can find regulatory sequences upstream of a gene, or determine integration sites for transposons or viruses. The **Universal GenomeWalker 2.0** kit (Cat. No. 636405) is designed with this in mind, enabling researchers to apply this powerful method of DNA walking to genomic DNA from any species.

Applications

- The Universal GenomeWalker 2.0 kit enables researchers to create uncloned libraries from any gDNA for walking by PCR in any genomic DNA. In less than a week, the method provides access to the genomic DNA sequences adjacent to a known DNA sequence in any species. Using both the **SMARTer® RACE 5′/3′ Kit** (Cat. No. 634858) and the Universal GenomeWalker 2.0 kit, you can clone full-length cDNAs and the surrounding genomic sequences without ever screening a library.
- In addition to obtaining promoters or regulatory sequences, GenomeWalker DNA walking can also be used to map intron/exon junctions and to walk bidirectionally from any sequence-tagged site (STS) or expressed sequence tag (EST).
- The Universal GenomeWalker 2.0 kit can also be used to confirm genome modifications performed using zinc finger nucleases, TAL effector nucleases, or other methods.
- Although individual steps are limited to about 6 kb, multiple steps can be strung together to create longer walks. Consequently, this method is useful for filling in gaps in genome maps, particularly when the missing clones have been difficult to obtain by conventional library screening methods.
- In all applications, GenomeWalker PCR products are generally pure enough to allow restriction mapping without cloning. Nevertheless, a discussion of cloning PCR products and testing them for promoter activity is included at the end of this manual.

Protocol Overview

- Using your genomic DNA of interest, the first step is to construct pools of uncloned, adaptor-ligated genomic DNA fragments, which, for convenience, are referred to as GenomeWalker “libraries.”
- The starting genomic DNA must be very clean and have a high average molecular weight—so the Universal GenomeWalker 2.0 kit includes a NucleoSpin Tissue kit, as well as controls for comparison.
- Separate aliquots of DNA are completely digested with different restriction enzymes that leave blunt ends. The Universal GenomeWalker 2.0 kit comes with a set of four such restriction enzymes; however, alternative blunt end cutters may be substituted. Each batch of digested genomic DNA is then ligated separately to the GenomeWalker Adaptor.
- After the libraries have been constructed, the protocol takes just two days and consists of two PCR amplifications per library (Figure 1).
    - The first or primary PCR uses the outer adaptor primer (AP1) provided in the kit (see Figure 5 in Appendix B) and an outer, gene-specific primer (GSP1) provided by the researcher.
    - The primary PCR product mixture is then diluted and used as a template for a secondary or “nested” PCR with the nested adaptor primer (AP2) and a nested gene-specific primer (GSP2). This generally produces a single, major PCR product from at least three of the four libraries (and often in all four; Figure 1).
    - Each of the DNA fragments—which begin in a known sequence at the 5’ end of GSP2 and extend into the unknown adjacent genomic DNA—can then be sequenced, cloned, and further analyzed.

Long-distance PCR with the Advantage® 2 PCR Kit

GenomeWalker reactions should be performed with the included Advantage 2 PCR Kit (Cat. No. 639207), which contains a polymerase mix suitable for long-distance PCR (LD PCR)—a combination of two thermostable DNA polymerases that increases the range and accuracy of PCR amplification. Most of the extension is carried out by a primary polymerase, while a secondary polymerase provides the critical 3’ to 5' exonuclease or "editing" function that corrects misincorporated nucleotides. This protocol is optimized for Advantage 2 Polymerase Mix—we do not recommend using any other enzyme with this kit. Using LD PCR in the GenomeWalker protocol extends the range of possible PCR products to about 6 kb.

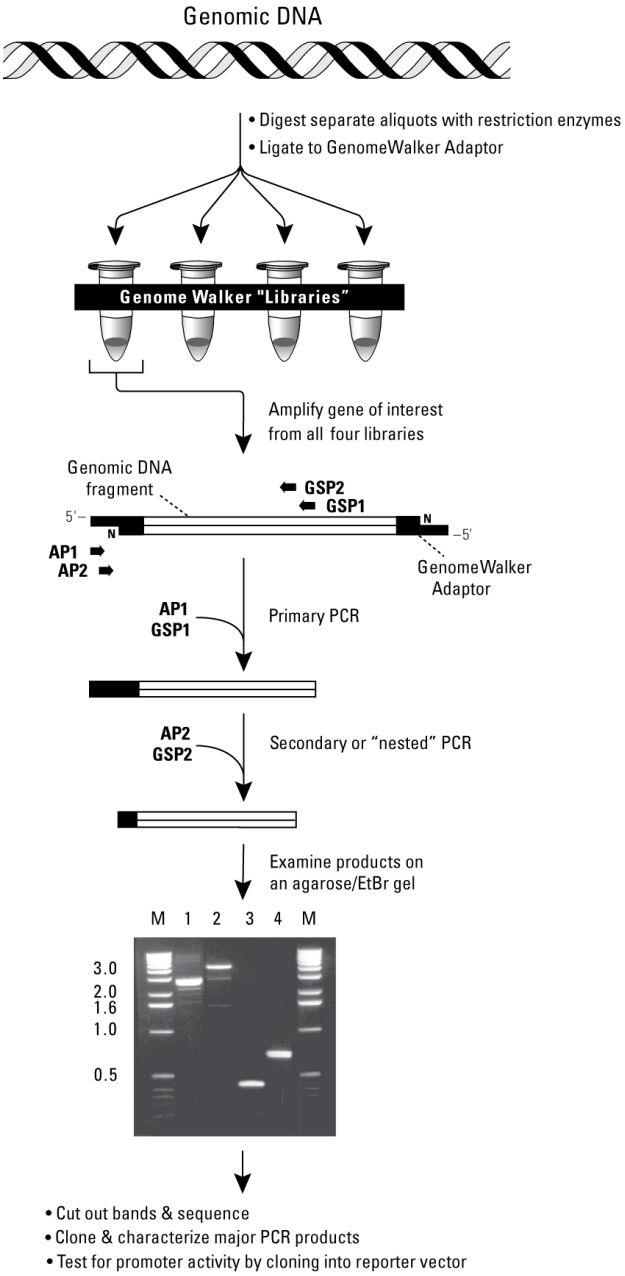

Figure 1. Flow chart of the GenomeWalker protocol. The gel shows a typical result generated by walking with GenomeWalker human libraries and gene-specific primers. Lane 1: EcoRV Library. Lane 2: DraI Library. Lane 3: PvuII Library. Lane 4: Ssp I Library. Lane M: DNA size markers. The absence of a major product in one of the libraries is not unusual. In our experience, there is no major band in one or more lanes in approximately half of the GenomeWalker experiments. As explained in the Expected Results and Troubleshooting Guide (Section VI), this is usually because the distance between the primer and the

upstream restriction site is greater than the capability of the system. N: Amine group that blocks extension of the 3' end of the adaptor-ligated genomic fragments. AP: Adaptor primers. GSP: Gene-specific primers.

II. List of Components

Universal GenomeWalker 2.0 contains sufficient reagents for construction three sets of four GenomeWalker libraries. Each library construction is sufficient for 80 rxns.

Table 1. Universal GenomeWalker 2.0 components.

Universal GenomeWalker 2.0	636405 (Each)
Univeral GenomeWalker Components (Cat. No. 636406)	
Package 1 (Store at –20°C)	
Dra I (10 units/µl)	30 µl
10X Dra I Restriction Buffer	100 µl
EcoR V (10 units/µl)	30 µl
10X EcoR V Restriction Buffer	100 µl
Pvu II (10 units/µl)	50 µl
10X Pvu II Restriction Buffer	100 µl
Stu I (10 units/µl)	30 µl
10X Stu I Restriction Buffer	100 µl
Control Human Genomic DNA (0.1 µg/µl)	75 µl
T4 DNA Ligase (6 units/µl)	10 µl
10X Ligation Buffer	40 µl
GenomeWalker Adapter	40 µl
Adaptor Primer 1 (AP1; 10 µM)	250 µl
Nested Adaptor Primer 2 (AP1; 10 µM)	250 µl
Positive Control GenomeWalker Human Library	10 µl
Positive Control tPA Primer (PCP1; 10 µM)	50 µl
Positive Control tPA Nested Primer (PCP2; 10 µM)	50 µl
NucleoSpin Tissue (Cat. No. 740952.10)	10 preps
NucleoSpin Gel and PCR Clean-Up (Cat. No. 740609.50)	50 preps
Advantage® 2 PCR Kit (Cat. No. 639207)	30 rxns
Package 1 (Store at –20°C)	
50X Advantage 2 Polymerase Mix	100 µl
10X Advantage 2 PCR Buffer	600 µl
10X Advantage 2 SA PCR Buffer	600 µl
50X dNTP Mix (10 mM each)	120 µl
Control DNA Template (100 ng/µl)	100 µl
Control Primer Mix (10 µM each)	100 µl
PCR-Grade Water	4 x 1.25 ml

III. Additional materials required (Not Provided)

- 1. 5 ml microcentrifuge tubes
- 96–100% ethanol
- 0.5X TBE Buffer or 1X TAE Buffer (see Note in Section VII.C.1)
- PCR reaction tubes
- Deionized H_2O (Milli-Q-filtered or equivalent)
- 1 kb ladder DNA size markers

- 1X TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5)

IV. Construction of GenomeWalker Libraries

A. General Considerations

- Construction of GenomeWalker DNA libraries should begin with very clean, high-molecular weight genomic DNA. This requires a higher quality preparation than the minimum suitable for Southern blotting or conventional PCR. For mammalian samples, a NucleoSpin Tissue kit has been included. However, keep in mind that methods vary for different species. To ensure that your genomic DNA is of adequate quality, follow the procedure described in Section IV.C.
- Work in an area away from all PCR products. Use only equipment that is not exposed to PCR products.
- For PCR, use only deionized H_2O (Milli-Q or equivalent). Do not use DEPC-treated or autoclaved H_2O.
- Human genomic DNA and positive control gene-specific primers (PCP1 and PCP2) are provided to test the system. They are designed to walk upstream from exon I of the human tissue-type plasminogen activator gene.
- The following protocol is designed for the construction of four libraries from experimental genomic DNA and one PvuII library from positive control human genomic DNA (provided in the kit).

B. Controls

Two types of controls are provided with the Universal GenomeWalker 2.0 kit:

- **Control Human Genomic DNA:** purified undigested high molecular weight genomic DNA that serves as a control for the entire process of restriction digestion and adaptor ligation (Section IV).
- **Positive Control GenomeWalker Human Library:** purified restriction-digested high molecular weight genomic DNA ligated to adaptors that serves as a control for PCR-based DNA walking in GenomeWalker libraries (Section V).

Figure 2 shows typical results of primary and secondary PCR with these positive controls. Amplification of the PvuII GenomeWalker human library with the adaptor primers and primers derived from exon 1 of the human tissue-type plasminogen activator (tPA) gene (PCP1 and PCP2) should generate a single major 1.5 kb product.

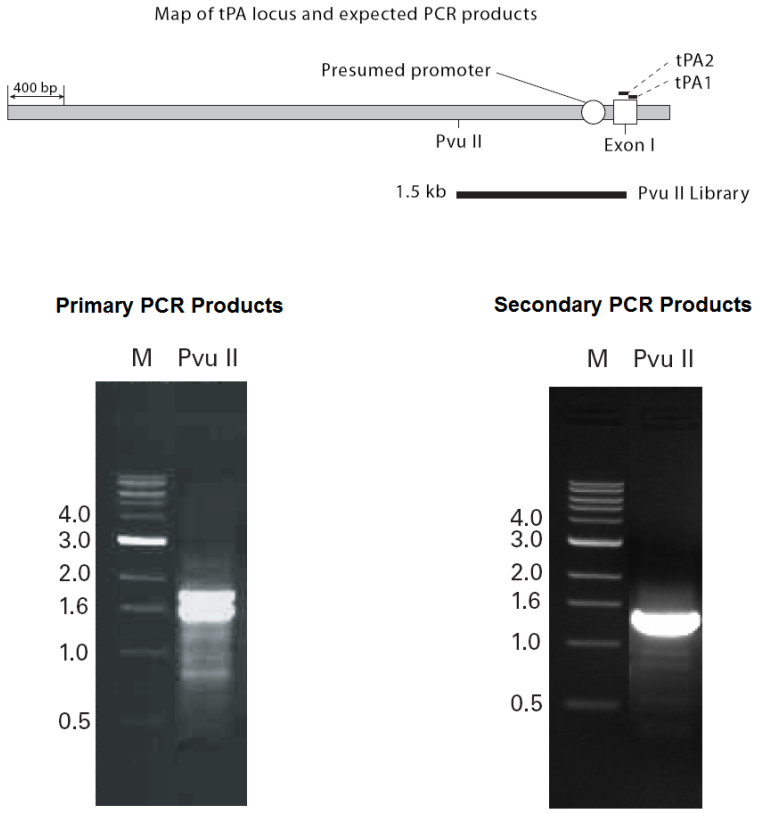

Figure 2. Map of the human tissue-type plasminogen activator (tPA) locus (Friezner-Degen *et al*., 1986) and results of primary and secondary GenomeWalker PCR using tPA primers. Primary and secondary (nested) PCR was performed using the included Advantage 2 Polymerase Mix and the cycling parameters described in the protocol. The tPA primers used in this experiment are the positive control primers PCP1 and PCP2 provided with the kit. Lane M: 1 kb ladder of DNA size markers.

C. Quality of Genomic DNA

1. Isolate genomic DNA from tissue or cultured cells using the procedure outlined in the NucleoSpin Tissue Genomic DNA Purification User Manual, Section 5. Check the size of your purified experimental genomic DNA on a 0.6% agarose/EtBr gel as follows:

   Load 1 µl of experimental genomic DNA (0.1 µg/µl) and 1 µl of control genomic DNA (0.1 µg/µl) on a 0.6% agarose/EtBr gel in 1X TAE, along with DNA size markers, such as a 1 kb ladder or λ/Hind III digest. Genomic DNA should be bigger than 50 kb with minimum smearing. This analysis can be done in parallel with the digestion in Step 2.

2. Check the purity of your experimental genomic DNA by DraI digestion.

   a. In a 0.5 ml reaction tube, combine the following:

5 µl	Experimental genomic DNA
1.6 µl	DraI (10 units/µl)
2 µl	10X DraI Restriction Buffer
11.4 µl	Deionized H_2O
20 µl	Total volume

   Also set up a control digestion of your experimental genomic DNA without any enzyme.

   b. Mix gently by inverting tube. Do not vortex; vigorous mixing will shear genomic DNA.

   c. Incubate at 37°C overnight.

d. Run 5 µl of each digestion reaction and 0.5 µl of the undigested experimental genomic DNA from Step 1 on a 0.6% agarose/EtBr gel. Only your DraI-digested experimental genomic DNA should produce a smear, indicating that it can be digested by restriction enzymes.

D. Digestion of Genomic DNA

For each library construction, you should set up a total of five reactions. For your experimental genomic DNA, set up four blunt-end digestions—one for each blunt-end restriction enzyme provided. Additionally, set up one PvuII digestion of human genomic DNA as a positive control.

1. Label five 1.5 ml tubes as follows :
   DL-1, DL-2, DL-3, DL-4, and PvuII Positive Control

   DL-1: experimental genomic DNA digested with DraI
   DL-2: experimental genomic DNA digested with EcoRV
   DL-3: experimental genomic DNA digested with PvuII
   DL-4: experimental genomic DNA digested with StuI

   (DL = DNA Library)

2. For each reaction, combine the following in a separate 1.5 ml tube:

25 µl	Genomic DNA (0.1 µg/µl)
8 µl	Restriction enzyme (10 units/µl)
10 µl	Restriction enzyme buffer (10X)
57 µl	Deionized H_2O
100 µl	Total volume

   Mix gently by inverting tube. Do not vortex. Vigorous mixing will shear genomic DNA.

3. Incubate at 37°C for 2 hr.

4. Vortex the reaction at slow speed for 5–10 sec. Return to 37°C overnight (16–18 hr).

5. From each reaction tube, remove 5 µl and run on a 0.6% agarose/EtBr gel to determine whether digestion is complete. You may wish to save an additional aliquot of each sample to run on the gel used in Step E.5 (see below).

E. Purification of DNA

Use the reagents from the included NucleoSpin Gel and PCR Clean-Up kit in the following protocol:

1. **Adjust DNA binding conditions**
   Mix remaining sample from Section D (~90 µl) with 200 µl of Buffer NT1.

2. **Bind DNA**
   a. Place a NucleoSpin Gel and PCR Clean-Up Column into a Collection Tube (2 ml) and load diluted sample from Step 1.

   b. Centrifuge for 30 sec at 11,000 x g. Discard flowthrough and place the column back into the collection tube.

3. **Wash silica membrane**
   Add 700 µl Buffer NT3 to the NucleoSpin Gel and PCR Clean-Up Column. Centrifuge for 30 sec at 11,000 x g. Discard flowthrough and place the column back into the collection tube.

4. **Dry silica membrane**
   Centrifuge for 1 min at 11,000 x g to remove Buffer NT3 completely. Make sure the spin column does not come in contact with the flowthrough while removing it from the centrifuge and the collection tube.

   NOTE: Residual ethanol from Buffer NT3 might inhibit enzymatic reactions. Total removal of ethanol can be achieved by incubating the columns for 2–5 min at 70 °C prior to elution.

5. **Elute DNA**
   Place the NucleoSpin Gel and PCR Clean-up Column into a new 1.5 ml microcentrifuge tube (not provided). Add 20 µl Buffer NE (prewarmed to 70°C) and incubate at room temperature (18–25 °C) for 1 min. Centrifuge for 1 min at 11,000 x g.

6. **Estimate DNA Yield**
   From each reaction tube, remove 1 µl and run on a 0.6% agarose/EtBr gel to determine the approximate quantity of DNA after purification.

F. Ligation of Genomic DNA to GenomeWalker Adaptors

For each library construction, you should set up a total of five ligation reactions. You will have four blunt-end digestions of your experimental genomic DNA and one positive control PvuII digestion of human genomic DNA.

1. From each tube (labeled DL-1, DL-2, DL-3, DL-4, and Positive Control Library), transfer 4.8 µl of digested, purified DNA to a fresh 0.5 ml tube. To each, add the following:

   1.9 µl GenomeWalker Adaptor (25 µM)
   0.8 µl 10X Ligation Buffer
   0.5 µl T4 DNA Ligase (6 units/µl)

2. Incubate at 16°C overnight.

   NOTE: A PCR thermal cycler holds a very constant temperature and is recommended in place of a water bath for this reaction.

3. To stop the reactions, incubate at 70°C for 5 min.
4. To each tube, add 32 µl of 1X TE Buffer (10 mM Tris, 1 mM EDTA, pH 7.5)—40 µl total.
5. Vortex at slow speed for 10–15 sec.

V. GenomeWalker DNA Walking

A. Primer Design

You will need to design two gene-specific primers—one for primary PCR (GSP1) and one for secondary PCR (GSP2), according to the following guidelines:

1. Primer Location Guidelines

- The nested PCR primer (GSP2) should anneal to sequences beyond the 3’ end of the primary PCR primer (GSP1) (i.e., upstream of the primary PCR primer when walking upstream and downstream of the primary PCR primer when walking downstream—see Figure 3).
- Whenever possible, the primary and secondary (nested) primers should not overlap; if overlapping primers must be used, the 3’ end of the nested primer should have as much unique sequence as possible.

Figure 3. Locations of GenomeWalker primers.

- In general, the gene-specific primers should be derived from sequences as close to the end of the known sequence as possible.
  - For walking upstream from cDNA sequence, the primer should be as close to the 5' end as possible. Ideally, the primers should be derived from the first exon of the gene.
  - If primers are derived from downstream exons, the resulting PCR products are less likely to contain the promoter, particularly if the intervening intron(s) and exon(s) comprise more than a few kb.

2. Primer Sequence Guidelines

- **Length:** Gene-specific primers should be 26–30 nucleotides in length and have a GC content of 40–60%. (Even if the T_m's seem high, do not design primers shorter than 26 bp. This will ensure that the primers will effectively anneal to the template at the recommended annealing and extension temperature of 67°C.
- **Secondary structure:** Primers should not be able to fold back and form intramolecular hydrogen bonds, and sequences at the 3' end of your primers should not be able to anneal to the 3' end of the adaptor primers.
- **GC Content:** no more than three G's and C's in the last six positions at the 3' end of the primer.
- **Cloning Considerations:**
  - Five restriction sites have been incorporated into the GenomeWalker Adaptor—Sal I, Mlu I, Xma I, and overlapping Srf I (cohesive ends), and Sma I (blunt ends) (see Figure 5 in Appendix B). These sites allow easy insertion of PCR products into commonly used promoter reporter vectors. If you wish to use other restriction sites, these should also be designed into the 5' end of GSP2 (i.e., the nested gene-specific primer used for secondary PCR.)
  - Alternatively, GenomeWalker PCR products can be cloned into a general purpose cloning vector using restriction sites, or into a TA-type cloning vector using the A overhang left by *Taq* DNA polymerase.

B. General Considerations

1. Cycling parameters

The cycling parameters in this protocol have been optimized using Advantage 2 Polymerase Mix, and the reagents and positive control primers provided in the GenomeWalker Kit. The optimal cycling parameters may vary with different gene-specific primers and thermal cyclers. Refer to the Troubleshooting Guide (Section VI.B) for suggestions on optimizing PCR conditions.

2. Use some form of hot start PCR

It is advantageous to use some form of hot start in PCR. The Advantage 2 Polymerase Mix provided in this kit includes TaqStart® Antibody for this purpose. We do not recommend that you use any other PCR mix.

3. Touchdown PCR

The PCR cycling parameters in Section V.C (Primary PCR, Step 8 and Secondary PCR, Step 8) are for "touchdown" PCR—which involves using an annealing/extension temperature several degrees higher than the T_m of the primers during the initial PCR cycles. Although primer annealing (and amplification) is less efficient at this higher temperature, it is much more specific. The higher temperature also enhances the suppression PCR effect with AP1 (see Appendix B and Figure 6), allowing a critical amount of gene-specific product to accumulate. The annealing/extension temperature is then reduced to slightly below the primer T_m for the remaining PCR cycles, permitting efficient, exponential amplification of the gene-specific product. As noted above, we recommend using primers with T_m's greater than 68°C to allow you to use the touchdown cycling programs given in this protocol.

4. Use of the positive controls

In each experiment, we suggest that you include a positive control in which you amplify the supplied control library using the positive control primers (PCP1 and PCP2). This will confirm that your DNA polymerase mix is functional and thermal cycling parameters are compatible with the GenomeWalker protocol.

5. Amplify all four libraries with your gene-specific primers

To make sure that you obtain at least one PCR fragment to sequence, we recommend that you amplify all four libraries with your gene-specific primers.

6. Use the recommended amounts of enzymes

These have been carefully optimized for the GenomeWalker amplification protocol and reagents.

C. Procedure for PCR-based DNA Walking in GenomeWalker Libraries

The GenomeWalker DNA walking protocol consists of eight primary and secondary PCR amplifications: four experimental libraries, two positive controls (GenomeWalker Human Positive Control Library and one positive control library constructed from Control Human Genomic DNA), and two negative controls (without templates). For both positive controls, use the positive control gene-specific primers, PCP1 and PCP2 (provided). For primary PCR, use 1 µl of each library. For secondary PCR, use 1 µl of a 50X dilution of the primary PCR product.

All GenomeWalker PCR steps have been optimized with the Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR.

Primary PCR

1. Label eight 0.5 ml PCR tubes. For convenience, we suggest using the plan in Table 2 (GSP1 indicates your primary gene-specific primer):

Table 2. Suggested Tube Labeling Plan for Primary PCR

Tube Label	DNA template	Forward Primer	Reverse Primer
1A	DL-1 (DraI)	AP1	GSP1
2A	DL-2 (EcoRV)	AP1	GSP1
3A	DL-3 (PvuII)	AP1	GSP1
4A	DL-4 (StuI)	AP1	GSP1
5A	None	AP1	GSP1
6A	Human Control Library (PvuII)[a]	AP1	PCP1
7A	None	AP1	PCP1
8A	Preconstructed Human Control Library[b]	AP1	PCP1

[a] Positive control for library construction. Construct this library from the Control Human Genomic DNA provided in the kit (see Section IV).
[b] Positive control for PCR. This Positive Control Human GenomeWalker Library is included in the kit.

2. Prepare enough **primary PCR master mix** for all eight reactions plus one additional tube. Combine the following reagents in a 0.5 ml tube:

per rxn	9 rxns	
19.5 µl	175.5 µl	deionized H_2O
2.5 µl	22.5 µl	10X Advantage 2 PCR Buffer
0.5 µl	4.5 µl	dNTP (10 mM each)
0.5 µl	4.5 µl	AP1 (10 µM)
0.5 µl	4.5 µl	Advantage 2 Polymerase Mix (50X)
23.5 µl	211.5 µl	Total volume

Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

3. Add 23.5 µl of the primary PCR master mix to the appropriately labeled tubes.
4. For reactions 1A through 5A, add 0.5 µl of GSP1 to each tube. For reactions 6A through 8A, add 0.5 µl of PCP1 to each tube (see Table 2).
5. Add 1 µl of each DNA library (including the positive control library) to the appropriately labeled tubes. **Do not add any library DNA to the negative controls (Tubes 5A and 7A in Table 2).**
6. Add 1 µl of H_2O to each negative control.
7. Briefly spin tubes in a microcentrifuge.
8. Commence thermal cycling using the following two-step cycle parameters:

   7 cycles:
   94°C 25 sec
   72°C 3 min

   32 cycles:
   94°C 25 sec
   67°C 3 min

   67°C for an additional 7 min after the final cycle.
9. Analyze 5 µl of the primary PCR products on a 1.5% agarose/EtBr gel, along with DNA size markers such as a 1 kb ladder. If you do not see any product, perform five additional cycles.

Expected results of primary PCR: In all lanes except for negative controls, you should observe your predicted banding patterns. Be aware, however, that there may be smearing in some lanes, and you may observe a multiple banding pattern, ranging in size from about 500 bp to 5 kb. See Figure 2 for a sample gel showing products of primary PCR.

- If you obtain any bands or smearing with your gene-specific primer, continue with secondary PCR as described below in Steps 1–9 (even if your products are weaker than the positive control or the bands in Figure 2).
- If you do not observe any product or smear with your gene-specific primers, consult the Troubleshooting Guide (Section VI).

Secondary PCR (Nested PCR)

1. Label eight 0.5 ml PCR tubes. For convenience, we suggest using the plan in Table 3 (GSP2 indicates your gene-specific primer):

Table 3. Suggested Tube Labeling Plan for Secondary PCR

Tube Label	DNA Template (taken from primary PCR)	Forward Primer	Reverse Primer
1B	1A	AP2	GSP2
2B	2A	AP2	GSP2
3B	3A	AP2	GSP2
4B	4A	AP2	GSP2
5B	5A	AP2	GSP2
6B	6A[a]	AP2	PCP2
7B	7A	AP2	PCP2
8B	8A[b]	AP2	PCP2

[a] Positive control for library construction. Construct this library from the Control Human Genomic DNA provided in the kit (see Section IV).
[b] Positive control for PCR. This Positive Control Human GenomeWalker Library is included in the kit.

2. Using a clean 0.5 ml tube for each sample, dilute 1 µl of each primary PCR (including positive and negative controls) into 49 µl of deionized H_2O.
3. Prepare **enough secondary PCR master mix** for all eight reactions plus one additional tube. Combine the following reagents in an 0.5 ml tube:

per rxn	9 rxns	
19.5 µl	175.5 µl	deionized H_2O
2.5 µl	22.5 µl	10X Advantage 2 PCR Buffer
0.5 µl	4.5 µl	dNTP (10 mM each)
0.5 µl	4.5 µl	AP2 (10 µM)
0.5 µl	4.5 µl	Advantage 2 Polymerase Mix (50X)
23.5 µl	211.5 µl	Total volume

Mix well by vortexing (without introducing bubbles) and briefly spin the tube in a microcentrifuge.

4. Add 23.5 µl of the secondary PCR master mix to the appropriately labeled tubes (Table 3).
5. For reactions 1B through 5B, add 0.5 µl of GSP2 to each tube. For reactions 6B through 8B, add 0.5 µl of PCP2 to each tube (see Table 3).

6. Add 1 µl of each diluted primary PCR product (from Step 2) to the appropriately labeled tubes. Be sure to include the positive and negative controls.
7. Briefly spin tubes in a microcentrifuge.
8. Commence thermal cycling using the following two-step cycle parameters:

   - 5 cycles:
     94°C 25 sec
     72°C 3 min
   - 20 cycles:
     94°C 25 sec
     67°C 3 min
   - 67°C for an additional 7 min after the final cycle.

9. **Analysis of Secondary PCR Products:**
   a. Analyze 5 µl of the secondary PCR products on a 1.5% agarose/EtBr gel, along with DNA size markers such as a 1 kb ladder or λ/Hind III digest. If you do not see any product, perform four additional cycles.
   b. Store the unused portion of each secondary PCR at 4°C until you have confirmed that the procedure has been successful. At that point, proceed with analyzing and cloning the fragments of interest (e.g., putative promoter fragments), as described in Section VII.

VI. Expected Results and Troubleshooting Guide

A. Expected Results

1. Primary PCR

For primary GenomeWalker PCR gel analysis results, see Figure 2. In general, primary PCR should produce multiple fragments, ranging in size from ~500 bp–5 kb. There may be smearing in some lanes. You should continue with secondary PCR if you obtain any bands or smearing with your gene-specific primer.

2. Secondary PCR

a. Positive control primers (PCP1 and PCP2)

The expected size of the band amplified from both the human positive control library and the library you constructed using the positive control human genomic DNA should be 1.5 kb (see Figure 2).

b. Experimental PCR primers

In approximately half the cases, single major bands will be observed with each of the four libraries. The exact size of the major bands will depend on the positions of restriction sites in your gene. Typically, products of secondary PCR will range from 0.2 to 6 kb.

- Fragments generated from nested gene-specific primers that are less than 0.4 kb from one of the restriction sites represented in the GenomeWalker libraries may appear as a low molecular weight smear on a 1.5% agarose/EtBr gel. If this occurs with one or more of the GenomeWalker libraries, run the particular PCR product(s) on a 2% agarose/EtBr gel.
- In our experience, no product is observed in one or more of the libraries in approximately half the cases—usually because the distance from the primer to the restriction site is greater than the capability of the system (~6 kb). This limit reflects the diminished

suppression PCR effect as template size increases (see Appendix B and Figure 6). Targets greater than ~6 kb often become indistinguishable in a smear of high molecular weight material. Such smearing may also occur in lanes that do contain major bands, but should not affect the major bands. The absence of a major band in one or more of the libraries does not mean that products obtained with other libraries are not correct, since redundancy is a part of the assay.

B. Troubleshooting Guide

Table 4. Troubleshooting Guide

Problem	Possible Explanation	Solution
1. No products are obtained with the positive control primers	Annealing/extension temperatures are too high	Reduce all annealing/extension temperatures by 2°C (i.e., 72°C to 70°C and 67°C to 65°C).
	Incubation at 94°C is too long	Reduce the length of the incubation at 94°C.
	50X polymerase mix is inactive	Check your 50X polymerase mix by PCR using two specific primers and a 1–10-kb template that has previously been successful.
2. Expected products are observed with positive control primers, but no product is observed either from library positive control or from your experimental libraries	Ligation failed	Check the ligation step. If the PCR positive control produces the expected PCR product, but the control library and your experimental libraries do not, it is probably due to failure of your ligation. In this case, repeat the adaptor-DNA ligation step.
	Loss of DNA during purification steps following restriction enzyme digestion	Check the digestion and purification steps by running samples of the DNA on an agarose gel before and after purification. If the intensity of EtBr staining is two-fold less after purification, you should concentrate the DNA—either by ethanol precipitation or placing tubes in a rotating evaporator (e.g., Savant SpeedVac), and resuspending the DNA in a lower volume.
3. Expected products are observed with positive control primers (for both the PCR positive control and the library control), but no product is observed with your gene-specific primers ***(continued on next page)***	Annealing/extension temperatures are too high	Try decreasing the temperature for annealing and extension to 65°C or lower.
	Primers were not designed or synthesized correctly	Check the design of your primers. • If the positive control PCP primers produce the expected PCR products, but your gene-specific primers do not produce major PCR products with any of the libraries, you will probably need to redesign your primers. • If your primer sequence was derived from cDNA sequence information, the primary or secondary PCR primer may cross an exon/intron junction. If this is the case, it will be necessary to redesign one or both gene-specific primers. Remember that all primers should be able to

		anneal efficiently at 70°C (i.e., have a T_m ≥70°C). • If you are sure your primers do not cross intron/exon boundaries, recheck the sequence of your primers. In some instances, primers will fail to produce any products due to a mistake in primer design or synthesis. • If possible, redesign your GSPs to have T_m's greater than 70°C. For this purpose, GSPs should be 26–30 bp in length, with a GC content of 40–60%. Do not design primers shorter than 26 bp. • If it is impossible to redesign your GSPs, try a touchdown PCR cycling program. For primary PCR, start with an annealing temperature of 72°C and decrease it by 1°C every second cycle to a "touchdown" at 67°C. Keep the annealing temperature at 67°C for the remaining 32 cycles. For secondary PCR, follow the same procedure, but use only 20 cycles after the annealing temperature reaches 67°C.
4. Nonspecific PCR products are observed with your gene-specific primers	Your primers are not specific enough or have annealing temperatures that are too low	• If possible, redesign your GSPs to have T_m's greater than 70°C. For this purpose, GSPs should be 26–30 bp in length, with a GC content of 40–60%. Do not design primers shorter than 26 bp. • If it is impossible to redesign your GSPs, try a touchdown PCR cycling program. For primary PCR, start with an annealing temperature of 72°C and decrease it by 1°C every second cycle to a "touchdown" at 67°C. Keep the annealing temperature at 67°C for the remaining 32 cycles. For secondary PCR, follow the same procedure, but use only 20 cycles after the annealing temperature reaches 67°C.
	Restriction digestion of genomic DNA may be incomplete	Repeat to ensure that digestion is complete. Normally, if the DNA is completely digested, a single major band should be observed after secondary PCR. However, multiple bands may result from

		the species used (e.g., some plants are multiploid) or from genes that belong to multi-gene families.

VII. Suggestions for Characterizing GenomeWalker Products

A. Restriction Mapping of GenomeWalker PCR Products

GenomeWalker PCR products are generally clean enough to allow simple restriction mapping without cloning. An example of such an experiment is shown in Figure 4.

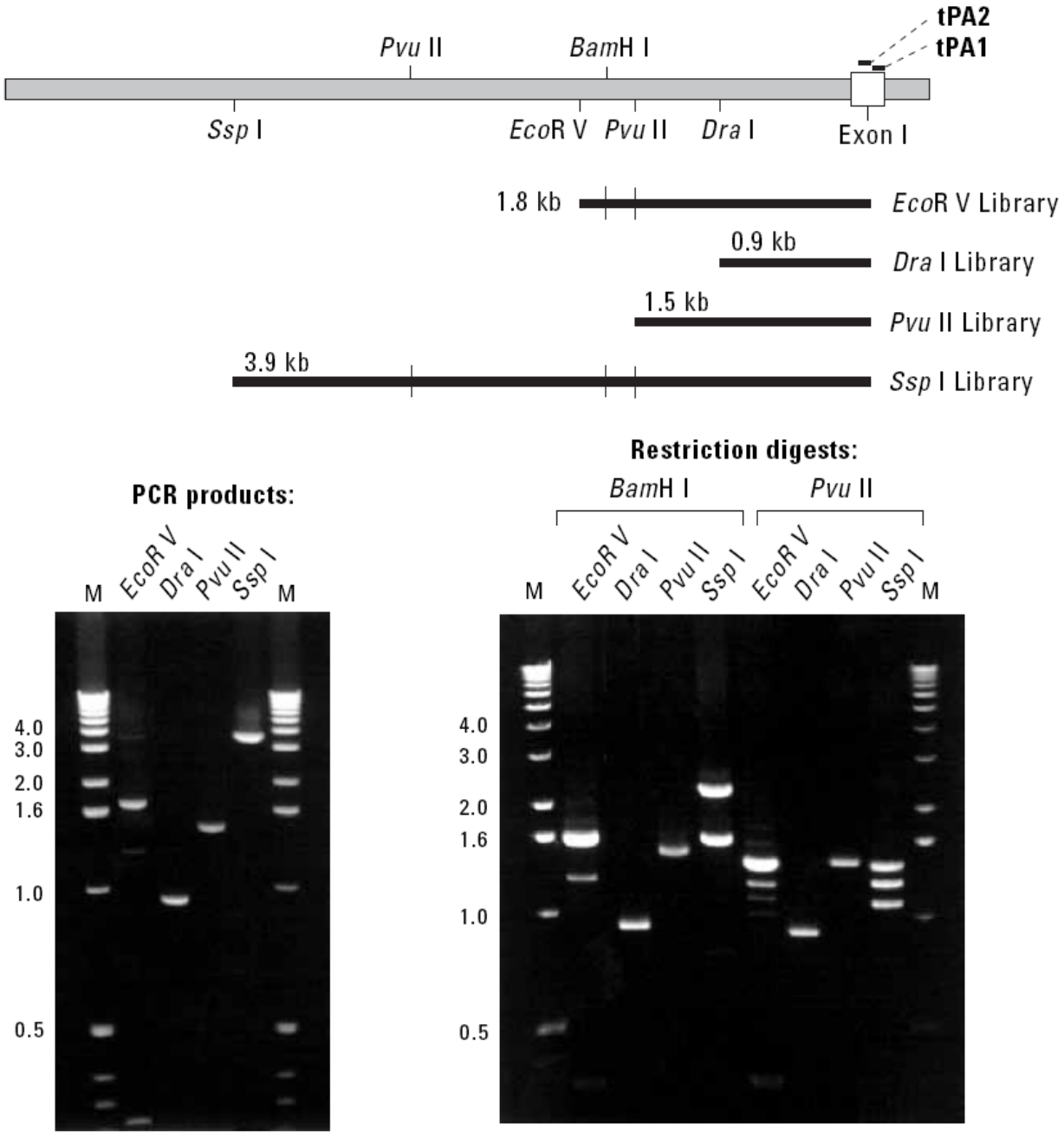

Figure 4. Simple restriction mapping of GenomeWalker PCR products from the human tPA locus. The map shows the positions of the relevant restriction sites in the genomic DNA and in the predicted GenomeWalker PCR products. The gel on the left shows the products of GenomeWalker PCR. The gel on the right shows the pattern of restriction fragments generated by digestions of each PCR product with either BamH I or PvuII. Lane M: DNA size markers.

B. Direct Sequencing of PCR Products

Major bands observed in secondary PCR may be excised from the gel and purified using the included NucleoSpin Gel and PCR Clean-Up kit. Follow the protocol outlined in the NucleoSpin Gel and PCR Clean-Up User Manual, Section 5.2.

- **Sequencing primers:** Purified PCR products may be sequenced using primers AP2 and GSP2.
- **Sequence data analysis:** Use web-based bioinformatics sites such as BLAST at NCBI (www.ncbi.nlm.nih.gov), or ENSEMBL (www.ensembl.org).

C. Cloning GenomeWalker Products and Testing for Promoter Activity

1. Cloning GenomeWalker products

Once you have obtained major bands using your gene-specific primer, you may want to clone the fragments into a general purpose cloning vector using restriction sites, or into a TA-type cloning vector using the A overhang left by *Taq* DNA polymerase. In some cases, you may wish to clone directly into a promoter reporter vector (See Section C.3 below).

- **Direct cloning:** If your secondary PCR produces a single, major band with little background and no minor bands, you may be able to clone the fragment directly.
- **Gel purification:** If the product of your secondary reaction has significant background, you will need to gel-purify the desired band. We recommend the included NucleoSpin Gel and PCR Clean-Up kit (Cat. No. 740609.50) for gel-purifying PCR products.

NOTES:

- **TAE vs. TBE gels:** We recommend that you use Tris-Acetate-EDTA (TAE) buffer instead of Tris-Borate-EDTA (TBE) buffer in your agarose gels when purifying DNA fragments for cloning. In our experience, DNA purified from TBE gels is more difficult to clone than DNA purified from TAE gels.
- **EtBr and UV damage to DNA:** Minimize the exposure of your DNA to UV light.

2. Sequencing and scanning for regulatory elements

Prior to testing GenomeWalker products for promoter activity, most researchers will want to sequence at least part of their clones and look for common regulatory sequence motifs such as promoters or enhancers.

3. Testing for promoter activity

GenomeWalker products can be cloned into a promoter reporter vector to test for the presence of a promoter. Cloning in both orientations will provide a positive and negative control. Suitable promoter-cloning vectors from Clontech include the following:

- **pSEAP2-Basic** is sold separately (Cat. No. 631715) and as a component in the chemiluminescent Great EscAPe™ SEAP Reporter System 3 (Cat. No. 631736; Yang *et al*, 1994). This kit also includes pSEAP2-Control and reagents necessary for 100

chemiluminescent assays. The reporter molecule in the Great EscAPe system is a secreted form of alkaline phosphatase (SEAP), which can be conveniently measured directly in the culture medium using a sensitive chemiluminescent assay.

- **pβgal-Basic** is sold separately (Cat. No. 631707) and as a component in the Luminescent β-gal Reporter System 3 (Cat. No. 631713; Sinai *et al*., 1994). This kit also includes pβgal-Control Vector and reagents necessary for 100 chemiluminescent assays.
- **Promoterless Fluorescent Protein Vectors** (pAcGFP1-1, Cat. No. 632497, pDsRed-Express-1, Cat. No. 632413, pZsGreen1-1, Cat. No. 632473) use flourescent proteins as reporters to monitor transcription from different promoters or promoter/enhancer combinations.

All of these vectors have large multiple cloning sites to facilitate cloning.

Note on ATG start codon: If your gene-specific primer was downstream of the ATG start codon in your gene of interest, then you may wish to eliminate the ATG from your promoter reporter construct(s). This may prevent a possible false negative result due to the expression of a bicistronic message (See Section 4 below).

4. Explanation of possible results of tests for promoter activity

Some GenomeWalker products will have no promoter activity when cloned in both orientations in a promoter reporter vector. There are several possible explanations.

- **None of the fragments contains the promoter.**
  - Your primer may be several kb from the promoter and/or there may be intervening restriction sites between the primer and the promoter. This may also be an indication that the primer does not fall within the first exon (or within a downstream exon that is within 6 kb of the promoter).
  - If this is the case, you may need to obtain sequence data from closer to the 5' end of the transcript. Alternatively, you can "walk another step" by sequencing the distal end of the GenomeWalker product(s), designing a new gene-specific primer, and repeating the amplification protocol.
- **The promoter is present, but the reporter is not expressed.**
  - There are several possible reasons why you might not detect promoter activity even if your promoter-reporter construct contains the promoter. These include the following:
    - **The fragment is cloned in the wrong orientation.**
      - Reclone and test in the opposite orientation.
    - **The promoter is too weak to be detected in your assay.**
      - If this is the case, it may be possible to add an enhancer to your construct or reclone your fragment(s) in a vector that has an enhancer.
    - **The promoter needs to be induced (and you have no means to induce it).**
      - Again, recloning into a vector that has a strong enhancer may allow you to detect promoter activity.
    - **The promoter is tissue- or stage-specific.**
      - Again, recloning into a vector that has a strong enhancer may allow you to detect promoter activity. Alternatively, it may be possible to demonstrate the presence

of a promoter by testing the construct in another host cell or in the whole organism.

- **Reporter construct makes a bicistronic message.**
  - The cloned fragment contains the ATG and some portion of the open reading frame from the gene of interest. This results in a bicistronic message in which two ORFs may compete for translation; the downstream ORF (i.e., the reporter) may not be efficiently translated.
  - If you suspect this to be the case, test for promoter activity at the RNA level by performing RT-PCR. (Reporter expression can be assayed by Northern blot; however, RT-PCR is much faster and more sensitive if suitable primers are available.)
- **The cloned fragment(s) contains a strong negative enhancer.**
  - There are numerous instances of so-called "negative enhancers" that prevent transcription of a functional promoter. If you suspect this to be the case, try recloning in the presence of a known strong enhancer, or testing subclones in which upstream sequences have been deleted.

5. Deletion analysis of promoter

After finding fragments that have promoter activity, you may want to perform a deletion analysis to define the minimal promoter. **In-Fusion® Snap Assembly** (Cat. Nos. 638947, 638948 & 638949) is ideal for performing these types of manipulations.

D. Other Applications of the GenomeWalker Method

Other possible applications of the GenomeWalker DNA walking method include:

- Mapping intron/exon boundaries.
- Walking short distance upstream or downstream in genomic DNA from known sequences (e.g., expressed sequence tags [EST] or other sequence tagged sites [STS]). Although individual steps are limited to ~6 kb, multiple steps can be strung together to create longer walks.
- Walking from 5' or 3' ends generated by RACE using the **SMARTer RACE 5′/3′ Kit** (Cat. No. 634858).
- Confirmation of genomic modifications from the use of zinc finger nucleases, TAL effector nucleases, or other homologous recombination-based methods
- Gap filling in BAC and YAC clones
- Identification of lentiviral or retroviral integration sites using the **Lenti-X™ Integration Site Analysis Kit** (Cat. No. 631263) or **Retro-X™ Integration Site Analysis Kit** (Cat. No. 631467)
- Mapping of transposable elements used in insertional mutagenesis techniques

VIII. References

Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T. & Tumer, D. H. (1986) Improved free-energy parameters for predictions of RNA duplex stability. *Proc. Natl. Acad. Sci. USA* **83:**9373–9377.

Friezner-Degen, S. J., Rajput, B. & Reich, E. (1986) Structure of the human tissue-type plasminogen activator gene. *J. Biol. Chem.* **261:**6972–6985.

Appendix A: Sequences of the Positive Control Primers

The positive control primers in the GenomeWalker Universal Kit are derived from exon 1 of the tissue-type plasminogen activator (tPA) cDNA.

PCP1 (tPA1):
5'-AGA AAC CCG ACC TAC CAC GGC TTG CTC CTT-3'

PCP2 (tPA2):
5'-CCC TTT CCT CGC AGA GGT TTT CTC TCC AGC-3'

Appendix B: Design of the GenomeWalker Adaptor

The GenomeWalker Adaptor has the following three design features that are critical to the success of GenomeWalker DNA walking, which can be seen schematically in Figure 1. (See Figure 5 for adaptor and primer sequences and locations.)

1. The use of a 5'-extended adaptor that has no binding site for the AP1 primer used in primary PCR. An AP1 binding site can only be generated by extension of the gene-specific primer.
2. Blocking of the exposed 3' end of the adaptor with an amine group to prevent extension of the 3' end (which would create an AP1 binding site).
3. The use of an adaptor primer that is shorter than the adaptor itself ("suppression PCR"). As shown in Figure 6, the suppression PCR effect prevents amplification of templates where the 3' end has been extended to create an AP1 binding site. Though rare, such extension does occur, presumably due to incomplete amine modification or incomplete adaptor ligation. Given the exponential nature of PCR amplification, such events would lead to nonspecific amplification and unacceptable backgrounds in the absence of suppression PCR.

Each of these features helps eliminate nonspecific amplification among the general population of DNA fragments. In combination with touchdown PCR and nested PCR, these innovations allow amplification of a specific target from a very complex mixture of DNA fragments—all of which have the same terminal structure—using a single set of gene-specific primers. Of the three features, suppression PCR is the most critical.

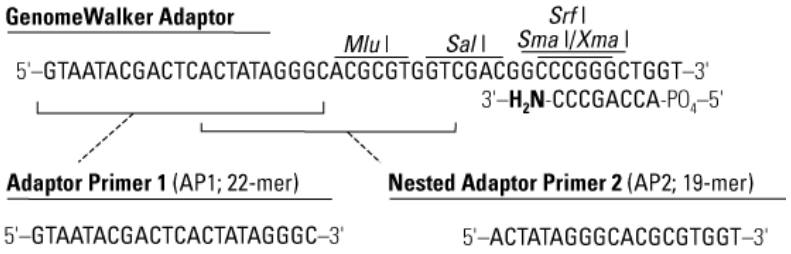

Figure 5. Structure of the GenomeWalker adaptor and adaptor primers. The adaptor is ligated to both ends of the genomic DNA fragments to create GenomeWalker libraries. The amine group on the lower strand of the adaptor blocks extension of the 3' end of the adaptor-ligated genomic fragments, and thus prevents formation of an AP1 binding site on the general population of fragments. The design of the adaptor and adaptor primers is critical for the suppression PCR effect (Figure 6). The T_m's of AP1 and AP2 are 59°C and 71°C, determined by nearest neighbor analysis (Freier *et al.*, 1986).

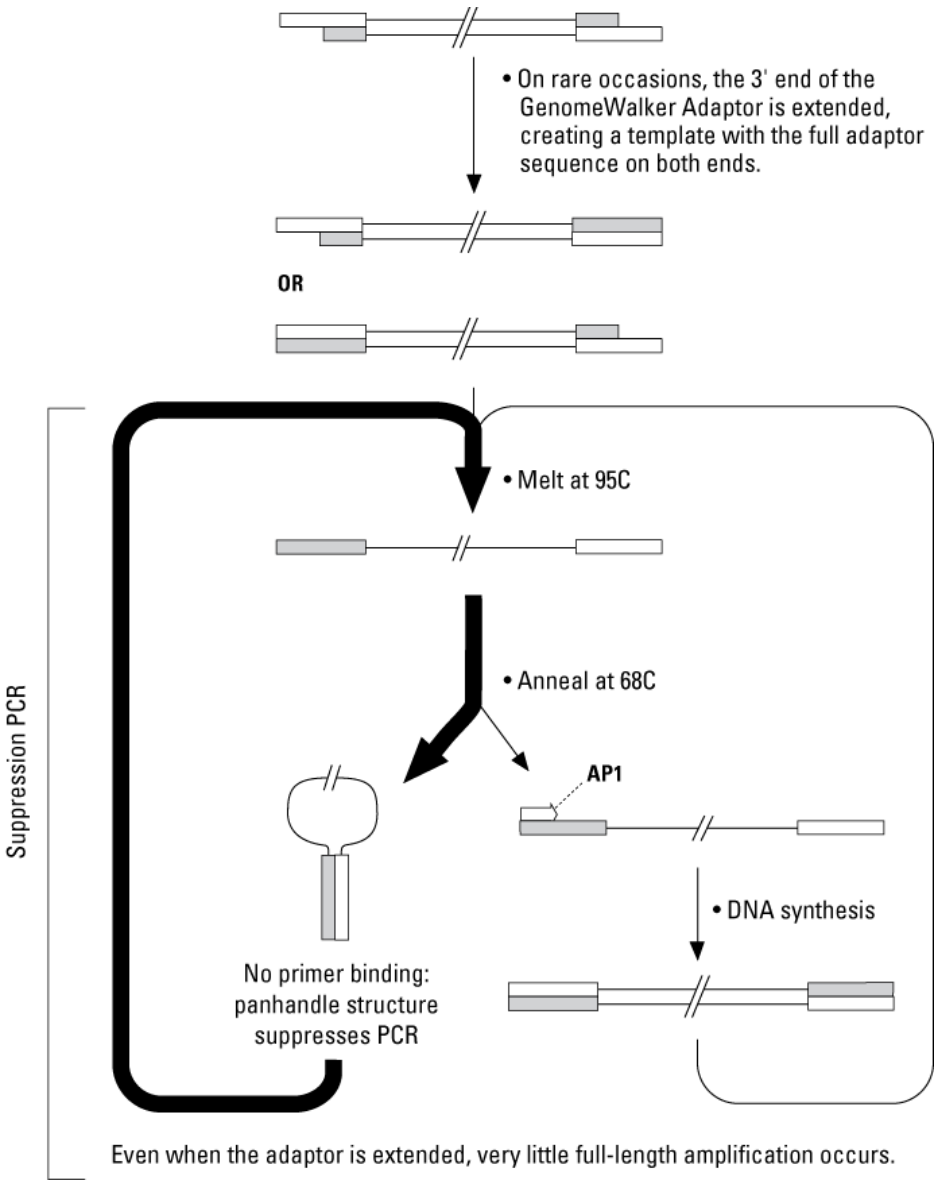

Figure 6. The suppression PCR effect. In rare cases, the 3' end of the GenomeWalker Adaptor gets extended. (Though rare, such extension does occur, presumably due to incomplete amine modification during oligonucleotide synthesis or incomplete adaptor ligation.) This creates a molecule that has the full-length adaptor sequence on both ends and can serve as a template for end-to-end amplification. Without suppression PCR, these rare events would lead to unacceptable backgrounds due to the exponential nature of PCR amplification. However, in suppression PCR, the adaptor primer is much shorter than the adaptor itself. Thus, during subsequent thermal cycling, nearly all the DNA strands will form the "panhandle" structure shown above, which cannot be extended. At the appropriate annealing/extension temperature, this intramolecular annealing event is strongly favored over (and more stable than) the intermolecular annealing of the much shorter adaptor primer to the adaptor. The suppression PCR effect will be reduced or lost if you use an annealing temperature lower than 60–65°C. The upper limit of the suppression PCR effect is about 6 kb.

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