Clontech® Laboratories, Inc.

Lenti-X™ Integration Site Analysis Kit User Manual

Cat. No. 631263
(040413)

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I. Introduction

DNA walking is a simple method used to identify unknown sequences adjacent to known genomic sequences. The Lenti-X Integration Site Analysis Kit (Cat. No. 631263) utilizes this method to determine the integration site(s) for lentiviral provirus in the genome of transduced cells. Since lentiviral vectors randomly insert into the host cell genome, both endogenous gene expression and vector gene expression patterns may be disrupted (Desfarges & Ciuffi, 2010). Therefore, it is often beneficial to characterize the viral insertion sites to better understand any potential effect they may have on your research.

NOTE: The Lenti-X Integration Site Analysis Kit has been optimized for the pLVX vector supplied with Lenti-X lentiviral expression systems but is compatible with most commercially available lentiviral vectors. However, check the vector sequence against the sequence of the LSP primers (Appendix A) prior to use.

A. Protocol Overview

The major steps in using the Lenti-X Integration Site Analysis Kit include construction of adaptor-ligated genomic DNA libraries, PCR to amplify the region of genomic DNA surrounding the viral integration site(s), and sequencing of the PCR products to identify the viral integration site(s) (Figure 1).

1. Construct Viral Integration Libraries

The first step in lentiviral integration site analysis is to construct pools of adaptor-ligated genomic DNA fragments from a transduced clone or population. For convenience, these genomic DNA fragments are referred to as viral integration "libraries."

- The starting genomic DNA must be very clean and have a high average molecular weight—so the Lenti-X Integration Site Analysis Kit includes a NucleoSpin Tissue kit, as well as controls for comparison.
- To construct a viral integration library, genomic DNA is first digested with blunt-end cutting restriction enzymes. Three viral integration libraries are created for each genome to be analyzed. The Lenti-X Integration Site Analysis Kit comes with a set of three different restriction enzymes; however, alternative blunt-end restriction enzymes may be substituted.
- The final step in viral integration library construction consists of a ligation reaction of the included GenomeWalker™ Adaptor to the digested genomic DNA fragments. This produces end-labeled DNA fragments that are ready for PCR.
- The creation of three different libraries increases the chances for identifying viral integration sites in the genome of the host cell, and this redundancy is an advantage of the system.

2. PCR of Viral Integration Libraries

After the libraries have been constructed, the protocol takes just two days and consists of two PCR amplifications per library.

- The first, or primary, PCR uses the outer adaptor primer (AP1) provided in the kit (see Figure 5 in Appendix B) and an outer, lentiviral specific primer (LSP1).
- 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 lentiviral-specific primer (LSP2). This generally produces a single, major PCR product from each of the three libraries (Figure 1).
- Each of the PCR products contain the region of genomic DNA adjacent to the 5' end of the viral insertion site and are bookended by known sequences corresponding to the AP2 and LSP2 primers.

3. Identification of Viral Integration Sites

The PCR products can be directly sequenced, or cloned and sequenced. The viral integration sites are identified by careful analysis of the sequencing data.

B. Long-distance PCR with the Advantage® 2 PCR Kit

Lenti-X Integration Site Analysis reactions should be performed with the included Advantage 2 PCR Kit (Cat. No. 639207). The Advantage 2 PCR Kit contains a combination of two thermostable DNA polymerases that increases the range and accuracy of PCR amplification, especially for long-distance PCR (LD PCR). Most of the extension is carried out by the primary polymerase, while a secondary polymerase provides the critical 3’ to 5' exonuclease or "editing" function that corrects misincorporated nucleotides.

NOTE: The Lenti-X Integration Site Analysis protocol is optimized for Advantage 2 Polymerase Mix—we do not recommend using any other enzyme with this kit. Using LD PCR in the protocol extends the range of possible PCR products to about 6 kb.

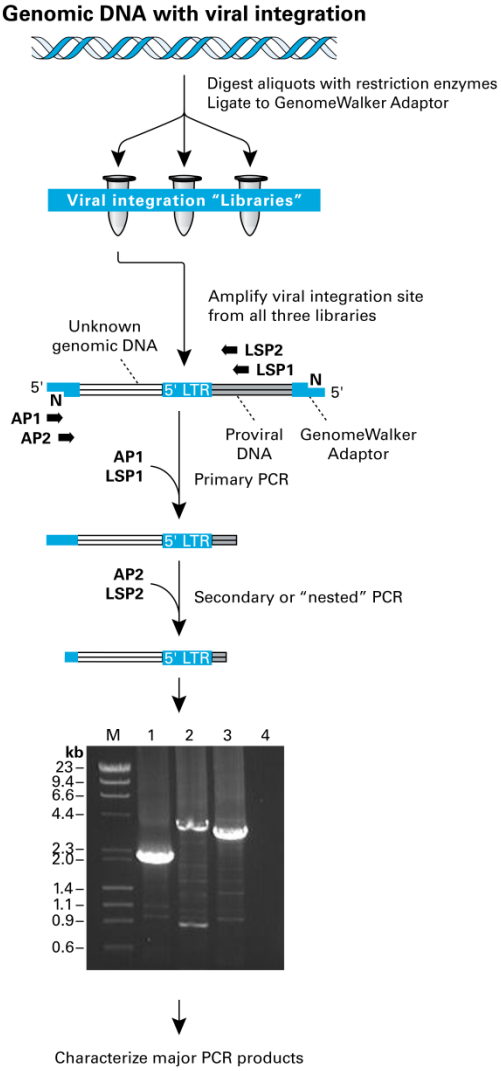

Figure 1. Flow chart of the Lenti-X Integration Site Analysis protocol. The major steps include construction of three viral integration libraries, PCR amplification of genomic DNA from the libraries, and analysis of PCR products. The gel shows a typical result. Lane 1: HpaI library. Lane 2: DraI library. Lane 3: SspI library. Lane 4: No template control. Lane M: DNA size markers. AP: Adaptor primer. LSP: Lentivirus-specific primer.

II. List of Components

Store all components at –20°C.

NOTE: These reagents are sufficient for constructing three sets of three viral integration libraries.

Lenti-X Integration Site Analysis Kit (Cat. No. 631263)

- 1 each **Lenti-X Integration Site Analysis Components** (Cat. No. 631264; Not sold separately)
  - 60 µl DraI (10 units/µl)
  - 100 µl 10X DraI Restriction Buffer
  - 30 µl SspI (10 units/µl)
  - 100 µl 10X SspI Restriction Buffer
  - 30 µl HpaI (10 units/µl)
  - 100 µl 10X HpaI Restriction Buffer
  - 75 µl Control Human Genomic DNA (Lentivirus; 0.1 µg/µl)
  - 10 µl T4 DNA Ligase (6 units/µl)
  - 40 µl 10X Ligation Buffer
  - 40 µl GenomeWalker Adaptor (25 µM)
  - 250 µl Adaptor Primer 1 (AP1; 10 µM)
  - 250 µl Nested Adaptor Primer 2 (AP2; 10 µM)
  - 250 µl Lentivirus Specific Primer 1 (LSP1; 10 µM)
  - 250 µl Nested Lentivirus Specific Primer 2 (LSP2; 10 µM)
- 10 preps **NucleoSpin Tissue** (Cat. No. 740952.10; Not sold separately)
- 50 preps **NucleoSpin Gel and PCR Clean-Up** (Cat. No. 740609.50)
- 30 rxns **Advantage 2 PCR Kit** (Cat. No. 639207)
  The Lenti-X Integration Site Analysis protocol has been optimized with Advantage 2 Polymerase Mix. Other PCR kits may not generate a band.

  NOTE: The supplied Advantage 2 Polymerase Mix is sufficient to test three cell clones (or 60 reactions at the indicated in Section V.B).

III. Additional Materials Required

The following materials are required but not supplied:

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

IV. Construction of Viral Integration Libraries

A. General Considerations

- You must begin library construction with very clean, high-molecular weight genomic DNA. A NucleoSpin Tissue kit has been included for this reason. 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. **Do not use equipment that is exposed to PCR products.**
- For PCR, use only deionized H_2O (Milli-Q or equivalent). Do not use DEPC-treated or autoclaved H_2O.
- This protocol is designed for the construction of three libraries from experimental genomic DNA and one positive control DraI library from the Control Human Genomic DNA (Lentivirus) provided in the kit.

B. Positive Control

A positive control is provided for use with restriction enzyme digestions, adaptor ligation reactions, and PCR. The Control Human Genomic DNA (Lentivirus) is purified and undigested high molecular weight genomic DNA that contains a single copy lentiviral provirus. A DraI library of this control produces a single major band of 700 bp after two rounds of PCR amplification (Figure 2).

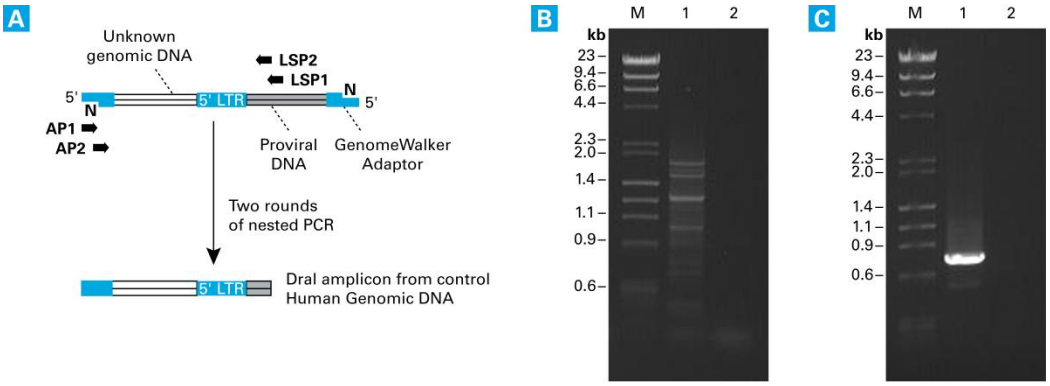

Figure 2. Primary and secondary PCR of the Control Human Genomic DNA. **Panel A**. Schematic of primer orientation on a relevant DNA fragment containing an integrated provirus. **Panel B.** Results of primary PCR with LSP1 and AP1 primers. Lane 1: Control Human Genomic DNA (Lentivirus) template. Lane 2: no template control. Lane M: DNA size marker. **Panel C**. Results of secondary (nested) PCR with LSP2 and AP2 primers and the diluted primary PCR product from panel B as template. PCR was performed using the included Advantage 2 Polymerase Mix with the cycling parameters described in the protocol. PCR of the DraI-treated control library produces a 700 bp secondary PCR product. Lane 1: Control Human Genomic DNA (Lentivirus). Lane 2: No template control. Lane M: DNA markers. AP: Adaptor primer. LSP: Lentivirus-specific primer.

C. Protocol: Isolation 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 (type "NucleoSpin Tissue" in the search box at www.clontech.com/manuals).
2. Check the integrity of your purified experimental genomic DNA on a 0.6% agarose/EtBr gel.
3. 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 20 kb with minimal smearing. **NOTE:** This analysis can be done in parallel with the digestion in Step 4.
4. Check the purity of your experimental genomic DNA by DraI digestion.
   a. Set up two 0.5 ml reaction tubes for experimental and control digestions of your genomic DNA:

Experimental Digestion		**Negative Control Digestion**	
5 µl	Experimental genomic DNA	5 µl	Experimental genomic DNA
1.6 µl	DraI (10 units/µl)		
2 µl	10X DraI Restriction Buffer	2 µl	10X DraI Restriction Buffer
11.4 µl	Deionized H_2O	13.0 µl	Deionized H_2O
20 µl	Total volume	20 µl	Total volume

   b. Mix each tube gently by inverting. 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 2, if not yet analyzed) on a 0.6% agarose/EtBr gel. Only DraI-digested genomic DNA should produce a smear, indicating that it can be digested by restriction enzymes (Figure 3, Lane 2).

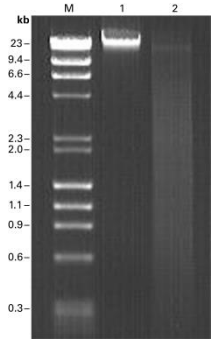

Figure 3. DraI restriction digest of the Control Human Genomic DNA. 5 µl of DraI digested Control Human Genomic DNA and 1 µl of undigested Control Human Genomic DNA was run on a 0.6% agarose/EtBr gel. Only the DraI-digested DNA should produce a smear, indicating that it is capable of being digested by restriction enzymes. Lane 1: undigested Control Human Genomic DNA. Lane 2: DraI digested Control Human Genomic DNA. Lane M: DNA size markers.

D. Protocol: Digestion of Genomic DNA

For each experimental genomic DNA sample, set up four restriction enzyme reactions: three blunt-end digestions—one for each restriction enzyme provided—and a positive control DraI digestion of the Control Human Genomic DNA.

1. Label four 1.5 ml tubes as follows :
   DL-1, DL-2, DL-3, and DraI Positive Control

   DL-1: experimental genomic DNA digested with DraI
   DL-2: experimental genomic DNA digested with SspI
   DL-3: experimental genomic DNA digested with HpaI
   DraI Positive Control: Control Human Genomic DNA digested with DraI

   (DL = DNA Library)

2. For each reaction, combine the following in the labeled 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 the 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. Check for complete digestion by running 5 µl of each digestion reaction on a 0.6% agarose/EtBr gel. Save an additional aliquot of each sample to confirm DNA yield in the next step (see Part IV.E.5).

E. Protocol: Purification of DNA

Use the reagents from the included NucleoSpin Gel and PCR Clean-Up kit. The protocol is listed in the NucleoSpin Gel and PCR Clean-up User Manual, Section 5 (type "NucleoSpin Gel and PCR Clean-up" in the search box at www.clontech.com/manuals) and below:

NOTE: Prepare Wash Buffer NT3 prior to DNA purification by adding the indicated volume of ethanol (96-100%) to Buffer NT3 concentrate. Mark the label of the bottle to indicate that ethanol was added.

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.

4. **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.

5. **Confirm DNA Yield**
   Remove 1 µl purified digested DNA from each reaction tube and run along with an aliquot of unpurified digested genomic DNA (from Step 5, above section) on a 0.6% agarose/EtBr gel to determine the approximate quantity of purified DNA.

F. Protocol: Ligation of Genomic DNA to GenomeWalker Adaptors

Set up four ligation reactions for each experimental sample: three for the blunt-end digestions of your experimental genomic DNA, and one for the DraI digestion of the Control Human Genomic DNA.

1. From each tube (labeled DL-1, DL-2, DL-3, 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 TE (10/1, pH 7.5)—40 µl total.
5. Vortex at slow speed for 10–15 sec.

V. PCR Amplification of Viral Integration Libraries

A. General Considerations

- **Sample considerations**
  The Lenti-X Integration Site Analysis Kit performs best with a single transduced clone containing a few defined integration sites. The kit can also be used to observe heterogeneity within a transduced population; however, gel analysis will be more difficult since the increased number of different integration sites can often appear as a smear.
- **Cycling parameters**
  The cycling parameters in this protocol have been optimized with the included Advantage 2 Polymerase Mix and the reagents, positive controls, and primers provided in the Lenti-X Integration Site Analysis Kit. Refer to the Troubleshooting Guide (Section VI.B) if you experience issues amplifying your library.
- **Use Advantage 2 Polymerase Mix**
  The Advantage 2 Polymerase Mix provided in this kit includes the TaqStart™ Antibody for hot start. Do not use any other PCR mix.

  NOTE: The supplied Advantage 2 Polymerase Mix is sufficient to test three cell clones (or 60 reactions at the indicated in Section V.B).
- **Use the positive control in every experiment**
  We suggest that you include a positive control in every experiment (i.e., amplify the library made with Control Human Genomic DNA). This will confirm that your DNA polymerase mix is functional and that your thermal cycling parameters are compatible with the protocol.
- **Amplify all three libraries**
  We recommend that you amplify all three libraries in order to ensure that you obtain at least one PCR product to sequence. We also recommend sequencing all bands that can be isolated cleanly. Redundancy is an advantage of the kit.
- **Follow the protocol**
  The amplification protocol and reagent volumes have been carefully optimized for use with the Lenti-X Integration Site Analysis Kit.
- **Lentiviral vector compatibility**

  The Lenti-X Integration Site Analysis Kit has been optimized for the pLVX vector supplied with Lenti-X lentiviral expression systems. The LSP primers are compatible with most commercially available lentiviral vectors, however, check the vector sequence with sequences of the LSP primers (Appendix A) prior to use.

B. Protocol: PCR-Based DNA Walking in Viral Integration Libraries

The DNA walking protocol consists of primary and secondary PCR amplifications of your three experimental viral integration libraries, one positive control library, and a no-template negative control.

All Lenti-X Integration Site Analysis PCR steps have been optimized with the Advantage 2 Polymerase Mix, which includes TaqStart Antibody for automatic hot start PCR.

Primary PCR

1. Label five 0.5 ml PCR tubes. For convenience, we suggest using the plan in Table 1 (LSP1 indicates your primary lentivirus-specific primer):

Table 1. Suggested Tube Labeling Plan for Primary PCR

Tube Label	DNA Template	Forward Primer	Reverse Primer
1A	DL-1 (DraI)	AP1	LSP1
2A	DL-2 (SspI)	AP1	LSP1
3A	DL-3 (HpaI)	AP1	LSP1
4A	DraI digested Control Human Genomic DNA	AP1	LSP1
5A	None	AP1	LSP1

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

per rxn	6 rxns	
19.5 µl	117 µl	deionized H_2O
2.5 µl	15 µl	10X Advantage 2 PCR Buffer
0.5 µl	3 µl	dNTP (10 mM each)
0.5 µl	3 µl	AP1 (10 µM)
0.5 µl	3 µl	LSP1 (10 µM)
0.5 µl	3 µl	Advantage 2 Polymerase Mix (50X)
24 µl	144 µl	Total volume

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

3. Add 24 µl of the primary PCR master mix to the appropriately labeled tubes.
4. Add 1 µl of the appropriate DNA library (including the positive control library) to the appropriately labeled tubes. **Do not add any library DNA to the negative control (Tube 5A in Table 1).**
5. Add 1 µl of H_2O to the negative control.
6. Briefly spin tubes in a microcentrifuge.
7. 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.

8. 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: You should observe banding patterns in all lanes except the negative control. There may be smearing in some lanes, and you may observe multiple banding patterns, 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 the virus-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, page 6).
   - If you do not observe any product or smear with your virus-specific primers, consult the Troubleshooting Guide (Section VI.B).

Secondary (Nested) PCR

1. Label five 0.5 ml PCR tubes. For convenience, we suggest using the plan in Table 2:

   Table 2. Suggested Tube Labeling Plan for Secondary PCR

Tube Label	DNA Template (from primary PCR)	Forward Primer	Reverse Primer
1B	1A	AP2	LSP2
2B	2A	AP2	LSP2
3B	3A	AP2	LSP2
4B	4A	AP2	LSP2
5B	5A	AP2	LSP2

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

per rxn	6 rxns	
19.5 µl	117 µl	deionized H_2O
2.5 µl	15 µl	10X Advantage 2 PCR Buffer
0.5 µl	3 µl	dNTP (10 mM each)
0.5 µl	3 µl	AP2 (10 µM)
0.5 µl	3 µl	LSP2 (10 µM)
0.5 µl	3 µl	Advantage 2 Polymerase Mix (50X)
24 µl	144 µl	Total volume

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

4. Add 24 µl of the secondary PCR master mix to the appropriately labeled tubes (Table 2).
5. 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.
6. Briefly spin tubes in a microcentrifuge.

7. 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.
8. Analyze 5 µl of each secondary PCR product 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.

   Store the unused portion of each secondary PCR product at 4°C until you have confirmed that the procedure has been successful. Then proceed with analysis, or clone the fragments of interest as described in Section VII.

VI. Expected Results and Troubleshooting Guide

A. Expected Results

Primary PCR

See Figure 2 for an example of the expected results from the primary PCR. 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 experimental samples.

Secondary PCR

Positive controls

The band amplified from the Control Human Genomic DNA (Lentivirus) should be 700 bp (Figure 2).

Experimental PCR Samples

In most cases, you will observe a single major band with each of the three libraries. There may be additional bands depending upon the number of integrants. The exact size of the major band(s) will depend on the positions of restriction sites near the site(s) of integration. Products of secondary PCR will generally range from 0.2 to 6 kb.

- Typically, the number of strong bands will increase as higher MOIs (multiplicity of infection) are used to transduce the target cells. This is due to an increase in integrated copy number with the population or cell clone. This kit performs best when used with a single transduced clone that contains a few defined integrations. When many integration sites are present, it may be more difficult to isolate individual bands during gel analysis.
- Avoid analyzing low molecular weight PCR products since these are primarily vector sequences and contain little information about the genomic integration site.
- On occasion, no PCR product is observed in one of the libraries. This is because of the diminished **suppression PCR effect** as template size increases (see Appendix B, Figure 6). Targets longer 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.

Note: 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 3. Troubleshooting Guide

Problem	Possible Explanation	Solution
No PCR product is observed from your experimental libraries and the positive control library	Thermal cycling parameters are incorrect	Use the recommended temperatures in the protocol
	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
	Ligation failed	Repeat the adaptor-DNA ligation step
	DNA was lost 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, concentrate the DNA—either by ethanol precipitation or placing tubes in a rotating evaporator (e.g., Savant SpeedVac)—and resuspend the DNA in a lower volume
No PCR product is observed from one or more of your experimental libraries even though the expected results are observed with the positive control	Distance from the primer to the restriction is greater than the capacity of the system (~ 6 kb)	Isolate as many bands as possible from positive libraries

VII. Suggestions for Characterizing Lenti-X Integration Site Analysis Products

A. 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. To obtain results in the shortest time, follow the protocol outlined in the NucleoSpin Gel and PCR Clean-Up User Manual, Section 5.2. To find the manual, go to www.clontech.com/manuals and type "NucleoSpin Gel and PCR Clean-Up" in the search box.

- **Sequencing primers:** Purified PCR products may be sequenced using primers AP2 and LSP2. We recommend using the AP2 primer as the primary source of sequence, since the LSP2 primer reads through the LTR and thus provides less genomic information (Table 4).
- **Sequence data analysis:** Use web-based bioinformatics sites such as BLAST at NCBI (www.ncbi.nlm.nih.gov), or ENSEMBL (www.ensembl.org).

B. Cloning Lenti-X Integration Site Analysis 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 a TA-type cloning vector and the A overhang left by *Taq* DNA polymerase.

- **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.

C. Sequencing Results and Analysis

See Figure 4 for an example of secondary PCR products from an experimental cell clone tested with the Lenti-X Integration Site Analysis Kit. See Table 4 for a summary of the sequencing results.
Key points to consider when analyzing your cell clone or population include:

- You can sequence PCR products using the AP2 or LSP2 primers. You will obtain higher query coverage from the AP2 primer, because LSP2-primed reads will contain lentiviral vector sequence.
- We recommend that you amplify all three libraries in order to obtain at least one PCR product to sequence. Redundancy is an advantage of this system.
- Amplifying all three libraries also means that you can distinguish between conclusive and inconclusive reads. In the example in Table 4, the 12q22 chromosomal integration site was identified in all three libraries, indicating a reliable result, while the weak coverage and representation of the HpaI minor PCR product indicates an inconclusive result.
- We recommend using NCBI BLAST (Altschul *et al*., 1990), or a similar sequence analysis algorithm, to analyze sequence reads. Considerations such as E-value and query coverage are important to a good analysis of the integration sites in your cell clone or population. A BLAST overview may be found here: http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs
- If you sequence the 700 bp secondary PCR product from the control library (Figure 2), the integration site for the Control Human Genomic DNA (Lentivirus) is on chromosome 2p13 within the TIA-1 gene.

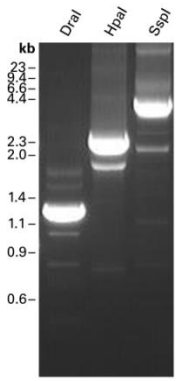

Figure 4. Secondary PCR products from an experimental HT1080 cell clone. Secondary PCR products from three viral integration libraries (as indicated above each lane) were visualized on an agarose gel. Five amplicons (the major bands in DraI, HpaI, and SspI libraries, and minor bands in the HpaI and SspI libraries) were purified and directly sequenced.

Table 4. Example of sequencing analysis

Chromosomal integration site	Sequenced band	Primer	Read length	Query coverage	E-value	Maximum identity
12q22[a]	DraI	AP2	136	97%	4e-50	100%
	HpaI	AP2	1000	98%	0	99%
	SspI	AP2	1000	94%	0	98%
	SspI (minor)	AP2	1000	99%	0	98%
	DraI	LSP2	1000	28%	1e-136	99%
	HpaI	LSP2	1000	28%	6e-134	98%
	SspI	LSP2	1000	29%	1e-137	99%
	SspI (minor)	LSP2	1000	28%	1e-125	98%
4q25	HpaI minor	AP2	1000	9%	0.018	92%
3p24.3	HpaI minor	LSP2	782	17%	1e-54	97%

Table 4. Sequencing Analyses of Secondary PCR Products. Five PCR products from the agarose gel in Figure 4 were sequenced with AP2 and LSP2 primers and analyzed by BLAST software. Four of the five PCR products corresponded to a lentiviral integration site at chromosome 12q22. The minor band in the HpaI library indicated two additional viral integration sites at chromosomes 4q25 and 3p24.3. However, these are not strong candidates for viral integration sites due to low query coverage. [a] Viral integration site at chromosome 12q22 is within an intron of the NR2C1 gene.

VIII. References

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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.

Appendix A: Primer Sequences

A. Lentivirus Specific Primer Sequences

The lentivirus specific primers (LSP) in the Lenti-X Integration Site Analysis Kit are located downstream of the 5' LTR within conserved sequences to avoid amplification of repeat sequences within the provirus. These primers are compatible with most available HIV-1 based vectors, however check your vector for compatibility before starting the protocol. Note that the LSP primers are reverse complements of the vector sequence.

LSP1: 5'-GCTTCAGCAAGCCGAGTCCTGCGTCGAG-3'
LSP2: 5'-GCTCCTCTGGTTTCCCTTTCGCTTTCAA-3'

B. Adaptor Primer Sequences

The Adaptor Primers (AP) contain sequence from the GenomeWalker Adaptor (Figure 5).

AP1: 5'-GTAATACGACTCACTATAGGGC-3'
AP2: 5'-ACTATAGGGCACGCGTGGT-3'

Appendix B: Design of the GenomeWalker Adaptor

The GenomeWalker Adaptor (Figure 5) contains three features designed to enhance the success of DNA walking:

- A 5'-extended primary PCR adaptor lacking AP1 binding sites. This ensures that an AP1 binding site can only be generated by extension of the lentivirus-specific primer.
- An amine group that blocks the exposed 3' end of the adaptor, thereby preventing the extension of the 3' end and the creation of an AP1 binding site.
- An adaptor primer that is shorter than the adaptor itself, for "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 features 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 lentivirus-specific primers. Of the three features, suppression PCR is the most critical.

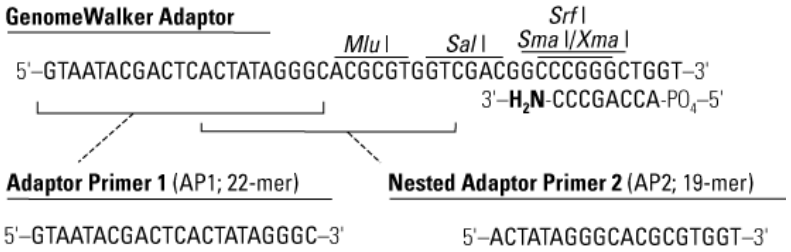

Figure 5. Structure of the GenomeWalker adaptor and adaptor primers. The adaptor is ligated to both ends of the genomic DNA fragments to create viral integration libraries. The amine group on the lower strand of the adaptor blocks extension of the 3' end of the adaptor-ligated genomic fragments, and 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_ms of AP1 and AP2 are 59°C and 71°C, as determined by nearest neighbor analysis (Freier *et al*., 1986).

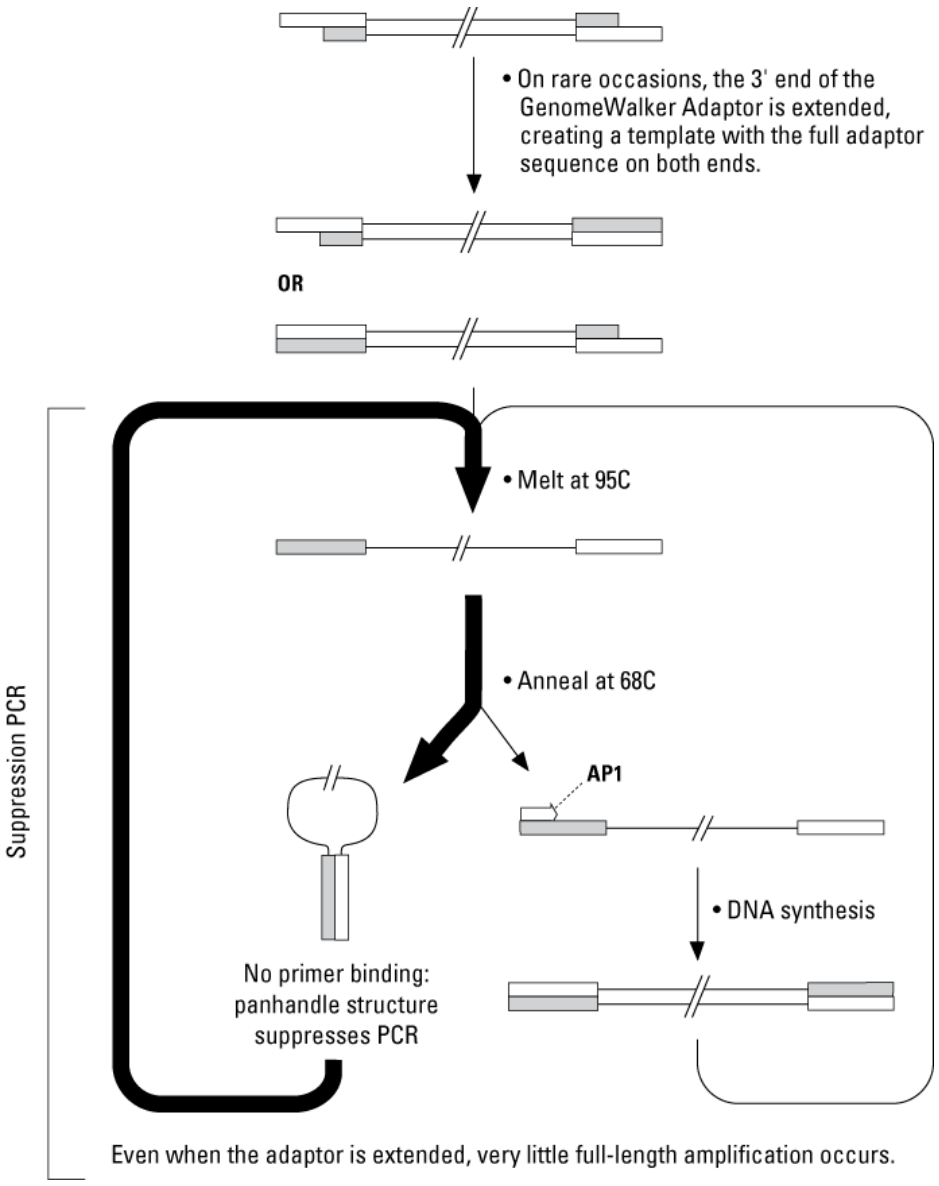

Figure 6. The suppression PCR effect. In rare cases, the 3' end of the GenomeWalker Adaptor gets extended, presumably due to incomplete amine modification during oligonucleotide synthesis or incomplete adaptor ligation. This creates a molecule that has the full-length adaptor sequence on both ends and 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 occurs at ~6 kb.

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