CELLECTA

USER MANUAL

LINE-1 Mobile Element NGS Assay

v1 — Last update: 2018/07/04

Cellecta, Inc.

Table of Contents

LINE-1 Mobile Element NGS Assay

Background

Two-thirds of the human genome is made up of mobile elements comprising *Long Interspersed Nuclear Elements* (LINEs, such as LINE-1). Members of the L1H subfamily of LINE-1 are active transposons that induce genetic rearrangements throughout the genome. These transpositions contribute to genetic diversity in tumor cell populations and have also been implicated in schizophrenia, Alzheimer's, and other neurodegenerative diseases. Cellecta's LINE-1 Mobile Element NGS Assay provides a convenient, efficient, and targeted NGS-based approach to identify *de novo* polymorphic insertions of LINE-1 in the human genome. The assay uses specially-designed primers to selectively amplify only the transposable elements of the youngest L1H family of LINE-1 that are still active in the genome, as opposed to the ancient elements fixed in the population. The amplified fragments are then characterized by NGS to assess the prevalence and diversity in the genome. This approach enables detection and identification of all the 3' L1H variations for a sample in a single reaction, enabling rapid analysis of multiple samples.

Intended Use:

The LINE-1 Mobile Element NGS Assay kit is designed to profile L1H insertion sites in human DNA. This kit is for research use only.

Please read the entire user manual before proceeding with your experiment.

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Download the PDF Version of the LINE-1 Mobile Element NGS Assay User Manual here

LINE-1 Kit Components

Components

Description	Cap Color	No. of Vials	Concentration	Volume
Taq DNA Polymerase	pink	1	100X	15 µl
Taq DNA Polymerase Buffer	pink	1	10X	150 µl
GSP1* Primer	yellow	1	10 µM	55 µl
dNTP Mix	white	1	50X	105 µl
PCR-Grade Water	white	2	NA	3,600 µl
DNA Ligase	purple	1	100X	15 µl
DNA Ligase Buffer	purple	1	10X	130 µl
T-Adapter	yellow	1	10 µM	55 µl
HF DNA Polymerase	light blue	1	100X	40 µl
HF DNA Polymerase Buffer	light blue	1	5X	780 µl
Adapter Primer	yellow	1	10 µM	55 µl
GSP2 Anchor	yellow	1	10 µM	30 µl
P5 Primer	yellow	1	10 µM	30 µl
Read 1 Seq Primer	blue	1	100 µM	30 µl
Index 1 Seq Primer	blue	1	100 µM	30 µl
Read 2 Seq Primer	blue	1	100 µM	30 µl
P7 Indexed Primer 1	white	1	10 µM	5 µl
P7 Indexed Primer 2	white	1	10 µM	5 µl
P7 Indexed Primer 3	white	1	10 µM	5 µl
P7 Indexed Primer 4	white	1	10 µM	5 µl
P7 Indexed Primer 5	white	1	10 µM	5 µl
P7 Indexed Primer 6	white	1	10 µM	5 µl
P7 Indexed Primer 7	white	1	10 µM	5 µl
P7 Indexed Primer 8	white	1	10 µM	5 µl
P7 Indexed Primer 9	white	1	10 µM	5 µl

P7 Indexed Primer 10	white	1	10 µM	5 µl
P7 Indexed Primer 11	white	1	10 µM	5 µl
P7 Indexed Primer 12	white	1	10 µM	5 µl
P7 Indexed Primer 13	white	1	10 µM	5 µl
P7 Indexed Primer 14	white	1	10 µM	5 µl
P7 Indexed Primer 15	white	1	10 µM	5 µl
P7 Indexed Primer 16	white	1	10 µM	5 µl
P7 Indexed Primer 17	white	1	10 µM	5 µl
P7 Indexed Primer 18	white	1	10 µM	5 µl
P7 Indexed Primer 19	white	1	10 µM	5 µl
P7 Indexed Primer 20	white	1	10 µM	5 µl
P7 Indexed Primer 21	white	1	10 µM	5 µl
P7 Indexed Primer 22	white	1	10 µM	5 µl
P7 Indexed Primer 23	white	1	10 µM	5 µl
P7 Indexed Primer 24	white	1	10 µM	5 µl

*GSP = Gene-Specific Primer

Note: Primer sequences can be accessed on the Cellecta website.

Storage Conditions:

Store kits and all components at -20°C until ready for use.

Additional Necessary Materials

Additional Reagents and Apparatus:

Name	Recommended Manufacturer	Catalog #	Description
Paired-End Cluster Generation Kit for HiSeq or NextSeq Instruments	Illumina	PE-402-4002	NGS of libraries
QIAquick Gel Extraction Kit	QIAGEN	28704	DNA extraction from gel
QIAquick PCR Purification Kit	QIAGEN	28701	PCR purification
Semi-skirted 96-well PCR plates	Eppendorf	951020303	PCR amplification
Clear Adhesive Film for 96-well plates	Bio-Rad	MSB1001	PCR Plate seal

Required Instrumentation:

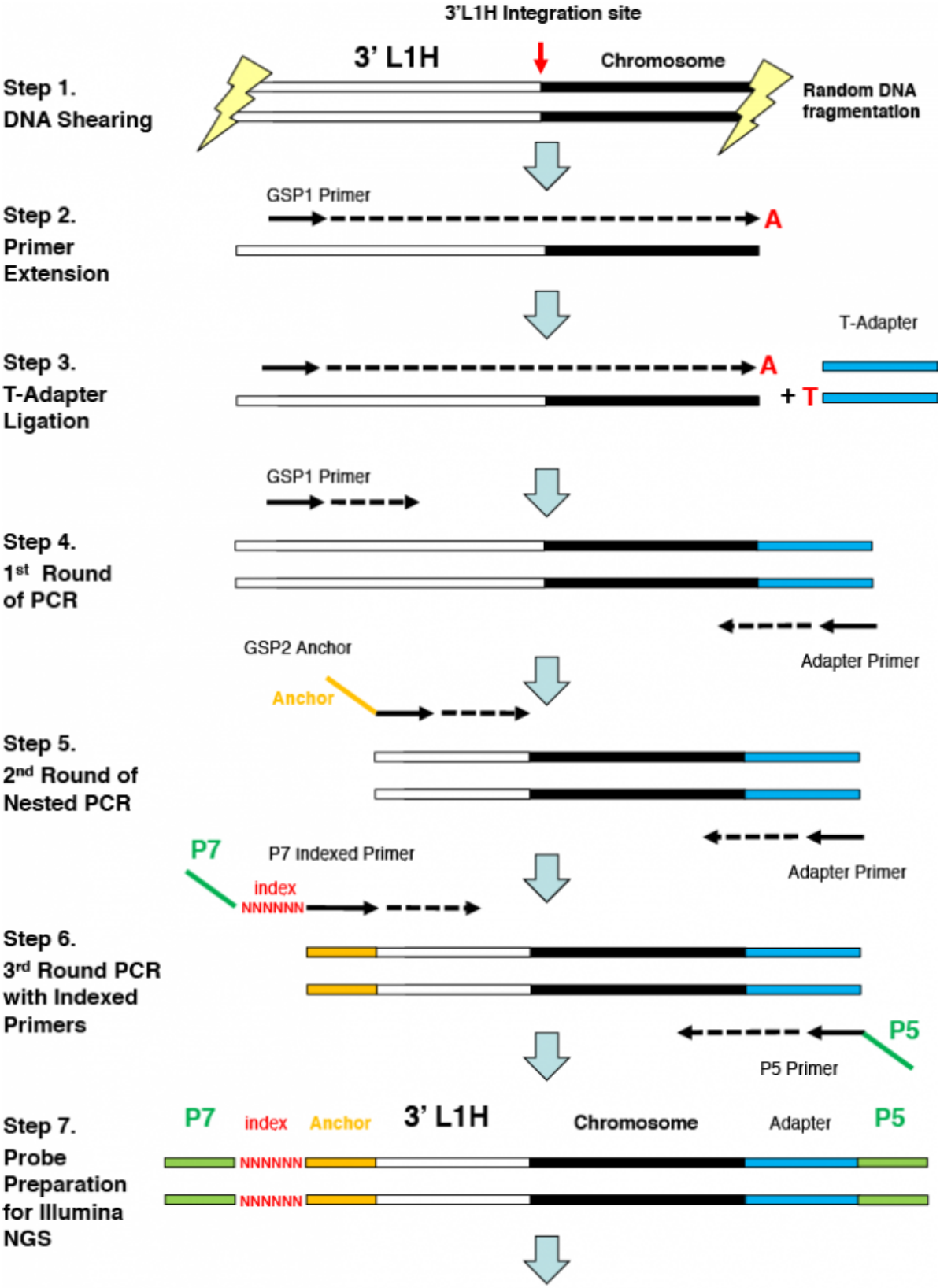
The protocols were optimized using the specific instrument specified. Some modifications and optimization to the protocol may be necessary if using different models or instruments from other manufacturers.

Name	Manufacturer	Model
Sonicator	Covaris	E-series
96-Well Thermal Cycler (or equivalent)	Applied BioSystems	Veriti® 96-well
Next-Gen Sequencing (NGS) Platform	Illumina	HiSeq®, NextSeq®

Other than the specific reagents and instruments above, the protocol assumes the user has access to standard materials (e.g., polypropylene tubes, pipette tips), equipment (table top centrifuges, pipettes, scales), and common reagents (e.g., TE buffer, ethanol) and buffers used in a typical life science laboratory.

Protocol Overview

The LINE-1 Mobile Element NGS Assay uses primers specifically designed to amplify the 3' L1H mobile genetic elements in randomly-sheared DNA (Step 1) to detect novel L1H genomic insertions. An L1H 3'-UTR-specific primer (GSP1) is used for a single round of extension into the chromosome (Step 2), followed by ligation of a T-Adapter to generate the template for PCR amplification (Step 3). Two rounds of amplification follow synthesis of the 3' L1H templates (Steps 4 & 5). The first round uses GSP1 and the Adapter Primer. The second round of PCR uses a nested GSP2 Anchor Primer with the Adapter Primer. A third round of amplification with primers containing the Illumina P5 and P7 adapter sequences (Step 6) prepares the samples for sequencing on the Illumina HiSeq or NextSeq instrument (Step 7).



Step 8. Illumina NGS and Data Analysis

Step 1. DNA Shearing

Genomic DNA from the biological samples first needs to be sheared by sonication into small enough fragments for subsequent denaturation, primer extension, and addition of the T-Adapters. Random fragmentation of DNA by sonication does not depend on specific DNA sequences, so that the unique ends at random DNA breaks allow for detection of NGS reads coming from independent ligations to the T-Adapter which ensures reliable detection of L1H integration sites. For this reason, we do not use restriction enzymes or other sequence-dependent nucleases techniques.

> **NOTE:** The protocol below was developed using the Covaris E-series sonicator. Other sonicators or mechanical techniques (e.g., shearing through a syringe) may be used to generate randomly-sheared DNA in the size range of 750-1200 bp. DNA shearing conditions need to be optimized for the specific instrument used to generate fragments of 750-1200 bp. Regardless of the instrument used for this step, fragmentation must be random. Do not use nucleases or other enzymatic approaches for the reasons noted above.

For each DNA sample to be sequenced, prepare one library.

1. Dilute 1 µg of high-quality genomic DNA (OD 260/280 ratio within a 1.8 to 2.0 range) with 1X Low TE Buffer (10:0.1) in a 1.5-ml LoBind (or similar) tube to a total volume of 50 µl.

2. Randomly fragment DNA to a size range of 750-1200 bp. For the Covaris E-series or S-series instruments, follow the manufacturer's instructions. An example of parameter settings from the Covaris E instrument are the following:

Parameters	Settings
Duty factor	0.2
Cycles per Burst	200
Treatment Time	6 minutes
Bath Temperature	4 – 8° C

3. Slowly transfer sheared DNA into a PCR tube or well of the 96-well plate.

Step 2. Primer Extension

This step primes and elongates the 3’ regions of L1 elements (3’ L1) using the GSP1 primer which sits ~100 bp from the start of the L1 element (Figure 2A). The Taq DNA Polymerase needs to be used in this reaction since it adds a 3’-adenine overhang to the ends of the extended double-stranded fragments. Do not use the HF DNA Polymerase, as it does not have terminal transferase activity.

1. Prepare the following reaction mix on ice in a 50 µl thin-wall PCR tube:

Component	Volume
Sheared DNA sample from Step 1 (200 ng)	10 µl
Taq DNA Polymerase Buffer (10X)	5 µl
dNTP Mix (50X)	1 µl
GSP1 Primer	1 µl
PCR-Grade Water	32.5 µl
Taq DNA Polymerase (100X)	0.5 µl
Total	**50 µl**

2. Mix gently, and centrifuge briefly to collect droplets.

3. Perform primer extension using the following program:

Temperature	Time
95°C	1 minute
62°C	5 minutes
72°C	5 minutes
4°C	∞

4. Purify the extended 3’ L1 product using the QIAquick PCR Purification Kit. Elute DNA in 30 µl of QIAGEN EB buffer.

Step 3. T-Adapter Ligation

This step ligates a phosphorylated T-Adapter with a 3'-T overhang to the ends of the DNA fragments from primer extension 3' L1H products that have 3'-A overhangs generated at Step 2. The reaction adds a specific adapter sequence to the 3' ends of the genomic fragment.

1. Prepare the reaction mix on ice in the following order:

Component	Volume
Extended 3' L1H products from Step 2	30 µl
T4 DNA Ligase Buffer (10X)	5 µl
T-Adapter	2 µl
PCR-Grade Water	12.5 µl
T4 DNA Ligase (100X)	0.5 µl
Total	**50 µl**

2. Incubate the reaction mix for 30 minutes at 37°C.

3. Follow the instructions in the QIAquick PCR Purification Kit to purify the reaction on one QIAquick column, eluting in 30 µl of QIAGEN EB buffer.

Step 4. 1st Round of PCR

The First Round of PCR selectively amplifies DNA fragments that have the T-Adapter on one end and the 3' L1-specific sequences on the other. These fragments are amplified by PCR using the HF (High Fidelity) Polymerase for 15 cycles in 50 µl using the GSP1 primer and T-Adapter primer.

1. Use half (15 µl) of the T-Adapter-ligated 3' L1H DNA sample from Step 3. Save the other half in case you may need to repeat the experiment due to potential problems during the next steps of the protocol. Prepare the following reaction mix in a 50 µl thin-wall PCR tube:

Component	Volume
T-Adapter-ligated DNA from Step 3	15 µl
HF DNA Polymerase Buffer (5X)	10 µl
dNTP Mix (50X)	1 µl
GSP1 Primer	1 µl
Adapter Primer	1 µl
PCR-Grade Water	21.5 µl
HF DNA Polymerase (100X)	0.5 µl
Total	**50 µl**

2. Mix gently and centrifuge briefly to collect droplets.

3. Amplify adapter-ligated 3' L1H DNA products using the following PCR program:

98°C, 60 seconds	**1 cycle**
98°C, 15 seconds 62°C, 15 seconds 72°C, 30 seconds	**15 cycles**
72°C, 60 seconds	**1 cycle**
4°C, ∞	**1 cycle**

Step 5. 2nd Round of Nested PCR

The second PCR amplification step uses the nested GSP2 Anchor Primer to ensure the specific amplification of the correct 3' L1H-specific fragments and add an additional anchor sequence to the ends of the amplified DNA.

1. Dilute the reaction from the First Round of PCR (Step 4 above) 10-fold with water. Use 2 µl of the diluted First Round PCR reaction for the Second Round of Nested PCR.

2. Prepare the Second Nested PCR Reaction Mix as follows on ice in a 50 µl thin wall PCR tube:

Component	Volume
10-fold diluted DNA from 1st PCR (Step 4)	2 µl
HF DNA Polymerase Buffer (5X)	10 µl
dNTP Mix (50X)	1 µl
GSP2 Anchor	1 µl
Adapter Primer	1 µl
PCR-Grade Water	34.5 µl
HF DNA Polymerase (100X)	0.5 µl
Total	**50 µl**

3. Mix gently and centrifuge briefly to collect droplets.

4. Amplify the Second Nested PCR Reaction Mix using the following PCR program:

98°C, 60 seconds	**1 cycle**
98°C, 15 seconds 62°C, 15 seconds 72°C, 30 seconds	**15 cycles**
72°C, 60 seconds	**1 cycle**
4°C, ∞	**1 cycle**

Step 6. 3rd Round PCR with Indexed Primers

In this amplification step, flanking sequences for the P5 and P7 Illumina adapters are added to the 3' L1 DNA product using the Adapter P5 Primer and one (from the 24) P7 Indexed Primers. Samples with different P7 Indexed Primers can be deconvoluted based on the unique index sequence when loaded in the same lanes of the Illumina Sequencing Cell (see Appendix B: Structure of the Adaptor Sequences of the 3'-Amplicon). As a result, the DNA products with different indexes can be mixed together for cluster generation on the Illumina NGS Sequencing Cell.

1. Use 2 µl of the Second Nested PCR product from Step 5 for the final P5/P7 PCR reaction below.

2. For each indexing reaction, use the appropriate Indexing Primer (one of the P7 Ind1-24 primers). Samples that will be sequenced in the same lane of the Illumina cell must have different P7 Index Adaptors. Be certain to keep track of which P7 Index Adaptor was used to amplify each sample so the sequencing results can be deconvoluted to assess the specific reads for each sample.

3. Prepare the reaction mix on ice in a 50 µl thin wall PCR tube:

Component	Volume
DNA sample from 2nd PCR (Step 5)	2 µl
HF DNA Polymerase Buffer (5X)	10 µl
dNTP Mix (50X)	1 µl
P7 Indexed Primer 1-24 (as appropriate)	1 µl
P5 Primer	1 µl
PCR-Grade Water	34.5 µl
HF DNA Polymerase (100X)	0.5 µl
Total	**50 µl**

4. Mix gently and centrifuge briefly to collect droplets.

5. Amplify Indexed-3'L1H-DNA products using the following PCR program:

98°C, 60 seconds	**1 cycle**

98°C, 15 seconds 62°C, 15 seconds 72°C, 30 seconds	**10 cycles**
72°C, 60 seconds	**1 cycle**
4°C, ∞	**1 cycle**

6. Analyze the P7-Index-3'L1H-DNA-P5 product on a 3% Agarose TAE gel. Cast a 3% agarose gel with 1X TAE buffer and 400 ng/ml EtBr (60 µg). Mix 5 µl of each PCR product from the Step 6 amplification with 5 µl of 2X loading buffer (e.g. 50% sucrose with Bromophenol Blue tracking dye) and load onto the gel. In at least 1 well, include a 100-bp DNA ladder.

7. Run gel at 5 V/cm until the 100-bp band is about an inch from the bottom. PCR products should appear as a smear of approximately 250-500 bp with the brightest region around 350-450 bp (see Example Gel Data image).

Optional Analysis Step: To validate the quality of the Indexed-3'L1H-DNA, use the "Library Validation for Illumina NGS by TA Cloning" procedure described in Appendix A. This may be useful to check if you are having trouble getting good sequencing runs.

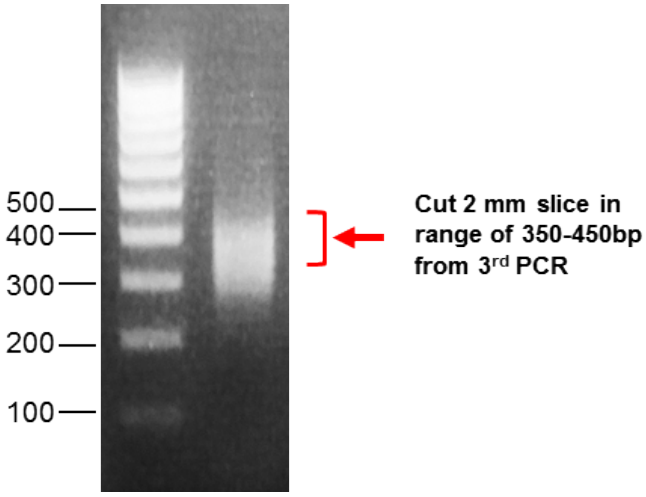
Step 7. Probe Preparation for Illumina NGS

1. Cast a 150 ml 3% agarose gel with 1X TAE buffer and 400 ng/ml EtBr (60 µg) using a gel comb that can accommodate approximately 60 µl in each well. *Recommended well size:* 1 mm (length) x 8 mm (width) x 7 mm (height).

2. Mix 30 µl of each PCR product from the Step 6 amplification with 3 µl of 10X loading buffer (e.g. 50% sucrose with Bromophenol Blue tracking dye) and load onto the gel. In at least 1 well, include a 100-bp DNA ladder.

3. Run gel at 5 V/cm until the 100 bp band is about an inch from the bottom (usually about 1 hour), then using a UV Transilluminator and clean scalpel, excise a 2 mm narrow band from the brightest region in the 350-450 bp size range (see Example Gel Data image).

> ! **CAUTION!** Be sure to use UV safety glasses to protect your eyes when viewing and excising DNA from the gel on the UV Transilluminator.



Example Gel Data

4. Isolate each PCR product from the gel fragment using the QIAquick Gel Extraction kit and following the manufacturer's protocol. Elute in 30 µl of QIAGEN EB Buffer.

5. Measure concentration of each purified P7-Index-3'L1H-DNA-P5 product using a NanoDrop 2000 (or similar spectrophotometer) at OD260. Combine different Index-tagged DNA samples together at

equimolar amounts into the final sequencing sample pool. Dilute the combined DNA samples to 10nM (approximately 2 ng/µl).

Step 8. Illumina NGS and Data Analysis

1. Follow the standard Illumina procedures for Cluster Generation starting with 10 nM of the purified PCR product from Step 6. The final samples can contain up to 24 indexed libraries for sequencing on either the Illumina NextSeq or HiSeq instrument.

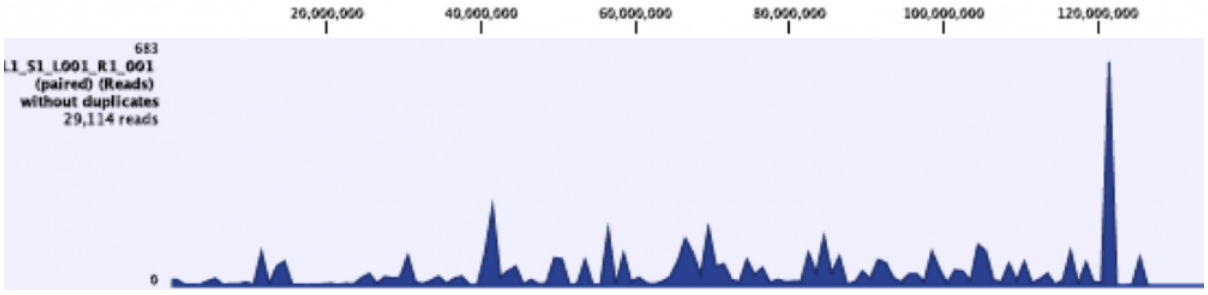
2. Add 6 µl of each of the custom sequencing primers into the sequencing mix as directed by the Illumina NGS protocol:
    - Read 1 Seq Primer
    - Index 1 Seq Primer
    - Read 2 Seq Primer

3. Perform Illumina NGS Paired-End (PE) sequencing (75 cycles) using the Illumina NextSeq or HiSeq. Proceed to cluster amplification using the Illumina Paired-End Cluster Generation Kit; refer to the manufacturer's instructions for this step. The optimal seeding concentration for cluster amplification of DNA libraries is approximately 6 to 8 pM.
    - Program:

Step	Primer	Cycles
Read 1:	Read 1 Seq Primer	60
Index 1:	Index 1 Seq Primer	6
Index 2:	Read 2 Seq Primer	6
Read 2:	Read 2 Seq Primer	13

4. NGS FASTQ sequencing files should be analyzed for quality using FastQC. Remove PCR duplicate reads, then map sequences to the appropriate reference genome. During development of this assay, we used reference repetitive element annotation files from the UCSC genome browser and annotated the GRCh38 (hg38) reference genome in CLC Genomics Workbench (CLC Bio/QIAGEN, Cambridge MA). Generate sequencing profiles of L1 insertions from cancer samples and matched normal controls as the reference to isolate all *de novo* cancer-specific L1 insertions.

Representative Results



Distribution of L1 elements along the chromosome. The position of each element is covered by several hundred reads on average.

Appendices

Appendix A: Library Validation for Illumina NGS by TA Cloning (Optional Procedure)

This procedure can be used to validate the structure of the amplified fragments and ensure they contain intact P5/P7 sequences for Illumina cluster generation, correct adapter sequences, as well as defined mobile element-chromosome junction regions.

1. Prepare the following reaction mix on ice in a 50 µl thin-wall PCR tube:

Component	Volume
DNA sample from 3rd PCR (Step 6)	2 µl
Taq DNA Polymerase Buffer (10X)	5 µl
dNTP Mix (50X)	1 µl
P7 Indexed Primer 1 (as appropriate)	1 µl
P5 Primer	1 µl
PCR-Grade Water	39 µl
Taq DNA Polymerase (100X)	0.5 µl
Total	**50 µl**

2. Mix gently and centrifuge briefly.

3. Amplify using the following PCR program:

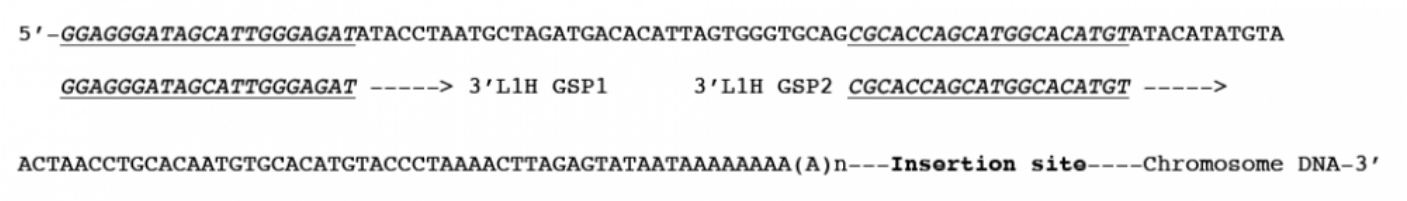
98°C, 60 seconds	**1 cycle**
98°C, 15 seconds 62°C, 15 seconds 72°C, 30 seconds	**5 cycles**
72°C, 60 seconds	**1 cycle**
4°C, ∞	**1 cycle**

4. Follow the instructions in the TA PCR Cloning Kit (Invitrogen/ThermoFisher) to clone 1 µl of the PCR reaction into the TA cloning vector and transform the TA cloning mix into the appropriate bacterial strain.

5. Pick 20 colonies at random from the plate. For colony PCR, use the T3/T7 PCR protocol. Sequence the 20 random colonies with T3/T7 primers. If the 3' L1 DNA was prepared correctly, the amplified fragments should contain intact P5 and P7 sequences for Illumina cluster generation and correct adapter sequences, as well as defined mobile element-chromosome junction regions as shown on Figure 2.

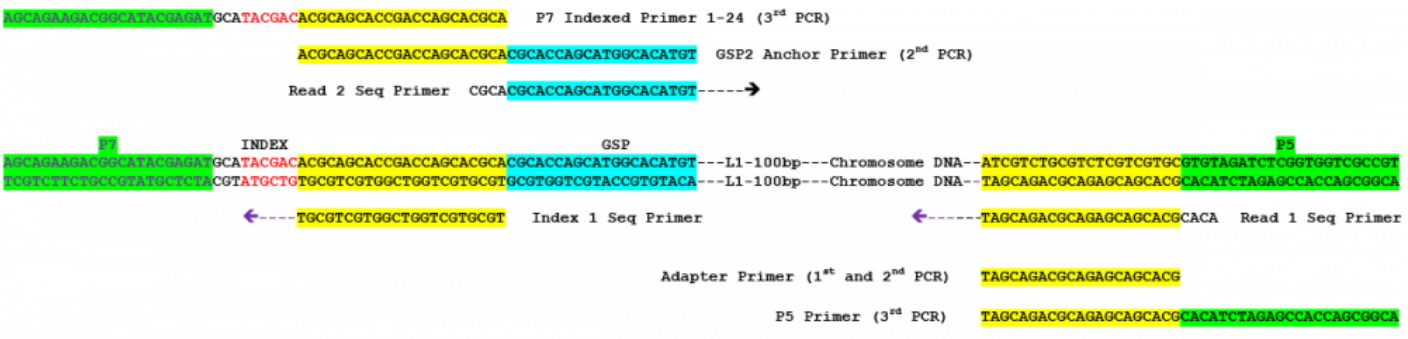
Appendix B: Structure of the 3' L1H Amplicon and Primer Locations

A. Consensus Sequence of L1H 3' UTR



(A) Consensus sequence of the L1H 3' UTR portion amplified in the protocol.

B. 3' L1H Amplicon Structure



(B) 3'L1H amplicon structure.

Structure of 3' L1H Amplicon. (A) Consensus sequence of the L1H 3'UTR portion amplified in the protocol. Primers specific for L1 sequences are used for amplification of only the youngest families of L1 (L1H) elements from the human genome. Two primers (Gene-Specific Primers, or GSP1 and GSP2) are designed for 3' ends of the elements. For L1H, 3' L1H UTR-specific primers bind within the UTRs of the L1 and extend away from the element and into the chromosome insertion sites. The primers are designed in such a way that they specifically amplify only the youngest subfamilies of polymorphic transposable elements which are still active in the genome, as opposed to the ancient elements fixed in the population. **(B) 3' L1H amplicon structure.** 3' L1H fragments are amplified by 1st Round PCR followed by 2nd Round Nested PCR in Step 5. Illumina NGS P5 and P7 adapter sequences (green) are introduced during the 3rd PCR in Step 6. The library is then sequenced by Illumina NGS in Step 8 using the Read 1 Seq Primer to read 13 nt of the L1H, and 3' L1H Read 2 Seq Primer to read the corresponding chromosome junction portion as well as the Index 1 Seq Primer to read the multiplexing index shown in red.

Appendix C: Index Sequences

Index Sequences for P7 Indexed Primers 1-24

Index	Sequence
1	CGTGAT
2	ACATCG
3	GCCTAA
4	TGGTCA
5	CACTGT
6	ATTGGC
7	GATCTG
8	TCAAGT
9	CTGATC
10	AAGCTA
11	GTAGCC
12	TACAAG
13	TTGACT
14	GGAACT
15	TGACAT
16	GGACGG
17	CTCTAC
18	GCGGAC
19	TTTCAC
20	GGCCAC
21	CGAAAC
22	CGTACG
23	CCACTC
24	GCTACC

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