Journal of Visualized Experiments

Detection of Retrotransposition Activity of Hot LINE-1s by Long-Distance Inverse PCR

--Manuscript Draft--

Article Type:	Methods Article - Author Produced Video
Manuscript Number:	JoVE59880R1
Full Title:	Detection of Retrotransposition Activity of Hot LINE-1s by Long-Distance Inverse PCR
Keywords:	LINE-1; retrotransposons; transposable elements; mobile elements; genetics; PCR; Nanopore sequencing; cancer; Cancer diagnosis; biomarkers; TTC28; MCF7 cell line
Corresponding Author:	Liisa Kauppi FINLAND
Corresponding Author's Institution:	
Corresponding Author E-Mail:	liisa.kauppi@helsinki.fi
Order of Authors:	Barun Pradhan, M.S.
	Liisa Kauppi
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)

TITLE:

Detection of Retrotransposition Activity of Hot LINE-1s by Long-Distance Inverse PCR

AUTHORS AND AFFILIATIONS:

Barun Pradhan[1,2], Liisa Kauppi[1,2]

[1]Systems Oncology Research Program, University of Helsinki, Helsinki, Finland
[2]Department of Biochemistry and Developmental Biology, Medicum, University of Helsinki, Helsinki, Finland

Email address of co-author:
Barun Pradhan (barun.pradhan@helsinki.fi)

Corresponding author:
Liisa Kauppi (liisa.kauppi@helsinki.fi)

KEYWORDS:

PCR, inverse PCR, retrotransposons, LINE-1, retrotransposition, structural variation, cancer, DNA, mobile DNA, genomics

SUMMARY:

This article outlines a simple PCR-based assay to monitor the activity of an active LINE-1 retrotransposon and to map *de novo* retrotranspositions in a given genome. Using the MCF7 cell line, we demonstrate herein how this method can be applied to detect activity of a LINE-1 located at 22q12.1.

ABSTRACT:

Long interspersed nuclear elements 1 (LINE-1s) are the only family of mobile genetic elements in the human genome that can move autonomously. They do so by a process called retrotransposition wherein they transcribe to form an mRNA intermediate which is then consequently inserted into the genome by reverse transcription. Despite being silent in normal cells, LINE-1s are highly active in different epithelial tumors. *De novo* LINE-1 insertions can potentially drive tumorigenesis, and hence it is important to systematically study LINE-1 retrotransposition in cancer. Out of ~150 retrotransposition-competent LINE-1s present in the human genome, only handful of LINE-1 loci, also referred to as “hot” LINE-1s, account for majority of *de novo* LINE-1 insertion in different cancer types. We have developed a simple polymerase chain reaction (PCR)-based method to monitor retrotransposition activity of these hot LINE-1s. This method, based on long-distance inverse (LDI)-PCR, takes advantage of 3´ transduction, a mechanism by which a LINE-1 mobilizes its flanking non-repetitive region, which can subsequently be used to identify *de novo* LINE-1 3´ transduction events stemming from a particular hot LINE-1.

INTRODUCTION:

Long interspersed nuclear elements (LINE-1s) are a family of mobile genetic elements called

retrotransposons that can independently move from one place to another via a copy-and-paste mechanism called retrotransposition. Over evolutionary time, the human genome has accumulated more than 500,000 copies of LINE-1 repeats[1]. However, most of the LINE-1 copies present in the genome are mutated and hence cannot move via retrotransposition; only ~150 copies have intact copy of DNA sequence necessary for them to move[2]. In normal somatic cells, the mobility of these LINE-1s is restricted by different host factors[3]. These restrictions are relieved in different epithelial tumors, causing LINE-1s to be derepressed and resulting in many *de novo* insertions in the tumor genome[4]. Some of these tumor-associated *de novo* insertions have been shown to cause insertional mutagenesis in genes, hence driving tumor progression[5,6]. Therefore, it is important to be able to map novel insertions in the tumor genome.

Existing methods to detect *de novo* LINE-1 insertions use (1) whole-genome sequencing approach[4,7,8], where different computational algorithms are used to find *de novo* LINE-1 insertions from the WGS data, or (2) next-generation sequencing that targets the 3´ end of young, potentially active LINE-1s[9-13]. However, finding novel insertions among several thousand near-identical copies with these methods is far from trivial, and the challenge is further aggravated by tumor heterogeneity and genomic alterations associated with LINE-1 insertion[4].

Studies using these existing methods showed that just a few LINE-1s contribute to the majority of *de novo* LINE-1 insertions observed in tumors[7,8]. Therefore, to answer whether or not a particular tumor sample displays LINE-1 activity, it suffices to map retrotransposition events caused by this handful of highly active LINE-1 loci. In this article, we describe a simple polymerase chain reaction (PCR)-based method[14] that can be used to monitor the activity of a particular LINE-1 locus in the first intron of the *TTC28* gene at 22q12.1 that is highly active in colorectal cancer[7,8]. This LINE-1 locus will be referred to as *TTC28*-LINE-1 throughout the article. This assay specifically identifies *de novo* LINE-1 retrotransposition events that mobilize non-repetitive sequence on the 3´ flanking region of the source LINE-1 by a mechanism called 3´ transduction[15]. 3´ transduction occurs due to the weak LINE-1 polyadenylation signal (PAS) that causes the transcriptional machinery to skip it and to instead terminate transcription at the stronger PAS downstream, thus capturing the flanking non-repetitive sequence (henceforth referred to as the “unique tag”) which is then inserted into the target location alongside LINE-1. Philippe et al.[16] recently showed that different cell types can express different LINE-1 loci. In light of this finding, this method can be applied to monitor the activity of the most highly expressed LINE-1 that mobilizes its unique tag in the cancer type of interest.

The first step in LDI-PCR is digestion of genomic DNA with a restriction enzyme that generates a restriction fragment containing the LINE-1 being assayed (here, *TTC28*-LINE-1) and its unique tag (**Figure 1**). Digested DNA are then circularized by self-ligation and PCR amplified using inverse primers located within the unique tag. By doing so, the full-length source LINE-1 at its “native” location is always amplified and alongside it, offspring LINE-1 insertions at different target loci containing the unique tag will also be amplified (**Figure 1**), thus reporting retrotransposition activity of the LINE-1 in question.

PROTOCOL:

This research was approved by the Institutional Review Board and Ethics committee of Helsinki University Hospital. Signed informed consent was obtained from the subject for the blood sample used to demonstrate this protocol.

1. Designing inverse primers and selecting restriction enzymes (bioinformatics)

1.1. Determining a LINE-1 associated unique tag

1.1.1. Download the *TTC28*-LINE-1 sequence in FASTA format from a LINE-1 database such as L1Base[17]. The L1base ID for *TTC28*-LINE-1 is 135.

1.1.2. Include 5 kb sequence flanking both 5´ and 3´ ends of the LINE-1 sequence and annotate it in a word processor.

NOTE: Here the LINE-1 flanking sequence is annotated in brown font, and LINE-1 sequence is in grey font (**Supplemental File**).

1.1.3. Enter 1 kb sequence downstream of selected LINE-1's cognate PAS into a PAS prediction tool such as polyadq[18] or Dragon PolyA spotter[19] and annotate all the polyadenylation signal in this 1 kb window.

NOTE: If there is no PAS in the 1 kb window, search for PAS in next 1 kb window downstream. *TTC28*-LINE-1's own weak PAS is highlighted in pink and all the other PAS in 1 kb window downstream is highlighted in red (**Supplemental File**).

1.1.4. Annotate the sequence between the end of LINE-1's cognate PAS and the strongest PAS downstream as "unique tag".

NOTE: The "unique tag" of *TTC28*-LINE-1 is highlighted in yellow (**Supplemental File**).

1.2. Designing inverse primers

1.2.1. Design inverse PCR primers by entering the "unique tag" sequence into a web-based primer-designing tool like Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/) or NCBI's primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/). Since primer pairs designed by these tools face each other facilitating conventional PCR, use the reverse-complement function for the primer pair to perform inverse PCR.

NOTE: As LINE-1 transductions are heavily truncated at their 5´ end and the size of the transduced region is highly variable, aim to keep the distance between the two inverse primers minimal, by setting the "PCR product length" parameter in NCBI primer-BLAST to the minimum. In the case of multiple PAS within the unique tag, design several primer pairs that correspond to different PASs. Design primers close to the PAS, as LINE-1 insertions initiate from the 3´end of the RNA

intermediate and the 5′ end is variably truncated. Here three primer pairs were designed, highlighted in teal and green, corresponding to three strong polyadenylation signals in the unique tag of TTC28 LINE-1 (**Supplemental File**).

1.3. Selecting restriction enzymes

1.3.1. Digest the LINE-1 sequence along with its 5 kb upstream and downstream flanks *in silico* using web-based tool such as RestrictionMapper. This will give a comprehensive list of restriction enzymes that digests this region, generating different restriction fragments.

1.3.2. Select restriction enzymes that cut the native locus of LINE-1 as follows: at the 5′ end, either upstream of the LINE-1′s 5′end or far 5′end of the LINE-1 itself, and at the 3′ end, downstream of the LINE-1's unique tag.

NOTE: The selected restriction enzyme should be insensitive to DNA methylation, should be heat-inactivatable, and should generate staggered "sticky" ends that are complementary to each other. In order to demonstrate LDI-PCR, *Sac*I restriction enzyme that cuts DNA at GAGCTC sites, highlighted here in light green, is used (**Supplemental File**).

1.3.3. Take note of the restriction fragment size made by selected restriction enzymes. This should not be longer than 12 kb as it might not be efficiently amplified by PCR.

2. Making circular DNA templates for long-distance inverse PCR

2.1. DNA extraction and quality assessment

2.1.1. Extract genomic DNA from samples (tumor or blood) using commercially available DNA extraction kits that can extract good quality, high molecular weight DNA necessary for LDI-PCR according to the manufacturer's instructions. Alternatively, high molecular weight DNA can also be extracted by phenol:chloroform[20].

2.1.2. Measure the DNA concentration using a fluorometer according to the manufacturer's instructions, and run 100 ng of DNA on 1% (w/v) agarose gel containing ethidium bromide (0.5 µg/mL) in 1x Tris-acetate-EDTA (TAE) buffer at 4.5 V/cm[21] alongside λ-HindIII DNA molecular weight markers to check DNA quality and quantity.

2.2. Digesting genomic DNA

2.2.1. Make a digestion reaction mix (final volume of 50 µL) by adding 20 units *Sac*I restriction enzyme, 5 µL of 10x reaction buffer (**Table of Materials**), 100 ng of DNA (up to 44 µL) in a 0.2 mL PCR tube on ice (1 µL of restriction enzyme from most manufacturers is sufficient to completely digest 100 ng of genomic DNA). Mix the solution by flicking the tube, and centrifuge briefly.

2.2.2. Use a thermal cycler to incubate the reaction mix at 37 °C for 1 h, followed by heat

inactivation at 65 °C for 5 min.

2.3. Self-ligating the digested genomic DNA

2.3.1. To the 50 µL digestion mix (after step 2.2.2), add 8 µL of 10x T4 DNA ligase buffer, 1 µL (5 units) of T4 DNA ligase and 21 µL of ultrapure water to make a final reaction volume of 80 µL. Mix the solution by flicking the tubes, and centrifuge briefly.

2.3.2. Incubate in a thermal cycler at 22 °C for 10 min, terminating with a heat inactivation step at 65 °C for 10 min.

3. Long-distance inverse PCR

3.1. Determining primer annealing temperature by gradient PCR

3.1.1. Set an annealing temperature gradient of (A-4) °C, (A-2) °C, A, (A+2) °C, (A+4) °C where A is the theoretically annealing temperature of the primer pair calculated using the commercial vendor's web-based tool.

NOTE: Thermal cyclers from some manufacturers may not allow setting the temperature gradient manually. In that case, the automatic gradient setting can be used with a temperature range of (A-4) °C to (A+4) °C.

3.1.2. Prepare a master mix for the PCR by combining and mixing the following components in a 1.5 mL microcentrifuge tube: 4 µL of 5x reaction buffer, 0.4 µL of 10 mM dNTP, 5 µL of 2 µM PCR primer (forward and reverse, designed in step 1.3.1), 0.2 µL (0.1 U) of DNA polymerase per reaction. Set up one reaction for each annealing temperature in the gradient.

3.1.3. For each reaction, aliquot 19 µL of the master mix into 0.2 mL PCR tubes and add 1 µL (1.25 ng) of circular DNA template made in section 2.

NOTE: Use circular self-ligated DNA generated from normal blood DNA as template so as to avoid consuming potentially precious tumor DNA for this optimization step.

3.1.4. Run gradient PCR program on a thermal cycler as described below: (i) one cycle of 30 s at 98 °C (denaturation); (ii) 35 cycles of (10 s at 98 °C [denaturation], 20 s at the temperature gradient of [A-4] to [A+4] °C [Annealing] and 1−6 min [30 s per kilobase of expected PCR product] at 72 °C [polymerization]); (iii) one cycle of 10 min at 72 °C (final polymerization).

3.1.5. Run 6 µL of the PCR product in 1% agarose gel[21] prepared in 1x TAE buffer at 4.5 V/cm and analyze the PCR products yielded at different annealing temperatures.

3.1.6. Select the annealing temperature that yields a PCR product that corresponds to the expected size.

3.2. Detecting *de novo* LINE-1 retrotransposition activity in the tumor genome

3.2.1. Perform LDI-PCR using the inverse PCR primer pair on circular DNA templates generated from tumor samples (section 2). Follow same instructions as for gradient PCR (section 3.1), but this time replacing the temperature gradient with the optimal annealing temperature.

3.2.2. Analyze the PCR products by agarose gel electrophoresis as done in step 3.1.5. PCR product of known size or the "native" PCR product corresponding to the LINE-1 at its native locus should be visible for each reaction. *De novo* LINE-1 3′ transduction in the tumor sample assayed is detectable as PCR products of different sizes, along with the native PCR product in the agarose gel.

4. Sequencing LDI-PCR products to reveal the identity of target sites for LINE-1 3′ transduction

4.1. Perform single-molecule long read sequencing of all the PCR amplicons generated in each LDI-PCR reaction to identify the target integration sites of these LINE-1 3′ transduction events.

NOTE: Cloning and Sanger sequencing of the LDI-PCR products is also a possible, albeit cumbersome, approach.

4.2. Align the reads produced by single-molecule long read sequencing platforms to the reference genome using standard sequence alignment pipelines. Analyze the aligned reads using LDI-PCR software[14] to identify *de novo* LINE-1 insertions and its target sites.

REPRESENTATIVE RESULTS:

In case of *TTC28*-LINE-1, there is more than one PAS within a 1 kb window downstream of its cognate PAS, hence the region between the *TTC28*-LINE-1 PAS and strongest PAS at 811 bp downstream was considered as the unique tag for *TTC28*-LINE-1. Three inverse PCR primer pairs were designed at this unique tag that correspond to different PASs present[14]. We selected three restriction enzymes: (i) *Nsi*I that cuts 5′ upstream of *TTC28*-LINE-1 and 3′ outside the unique tag, and (ii) *Sac*I and (iii) *Pst*I that cut 5′ within the LINE-1 in its far 5′ end, and 3′ outside the unique tag. These generate restriction fragments of 10,288 bp, 5,699 bp, and 6,305 bp respectively.

In order to demonstrate this method, we performed LDI-PCR on DNA extracted from MCF7 cell line. This breast cancer cell line has been previously reported to display *TTC28*-LINE-1 activity[16]. For simplicity, we made a circular DNA template using one restriction enzyme, *Sac*I, out of three and performed an LDI-PCR using one primer pair out of three (**Table 1**) to detect *de novo* LINE-1 insertions stemming from *TTC28*-LINE-1.

Good quality of DNA extracted from MCF7 cell line was ensured by agarose gel electrophoresis (**Figure 2**). Intact high molecular weight DNA shows that the genomic DNA is of optimum quality for this assay. If a smear is visible instead, this indicates poor quality of the extracted DNA, which in turn will hamper downstream procedures.

Figure 3 shows a representative result of a gradient PCR experiment, aimed at determining the optimal annealing temperature of the *TTC28*-LINE-1 inverse primer pair. Blood genomic DNA digested with *Sac*I, followed by self-ligation to form a circular DNA template, was used for this reaction. A highly specific PCR product of expected size (5,649 bp) at 62, 64 and 66 °C shows that the optimum annealing temperature for this primer pair lies within the range of 62−66 °C.

We generated a circular DNA template by digesting MCF7 genomic DNA with the *Sac*I restriction enzyme followed by self-ligation. **Figure 4** shows that *TTC28*-LINE-1 3′ transduction occurs in MCF7 cell lines: *de novo* insertions can be detected as LDI-PCR products of varying sizes along with a native PCR product of known size (5,649 bp). To identify genomic coordinates of the *de novo* target sites, PCR amplicons can be sequenced (see protocol section 4).

FIGURE AND TABLE LEGENDS:

Figure 1: Overview of LDI-PCR to detect LINE-1 3′ transduction. A circular DNA template is generated by first digesting (I) it with a restriction enzyme and self-ligating (II) it. This step is followed by inverse PCR (III) with inverse PCR primers targeted to the unique tag of the LINE-1 of interest (sequence between LINE-1's own weaker PAS, in pink, and stronger PAS downstream, in red).

Figure 2: Quality assessment of extracted DNA. 100 ng of DNA extracted from the MCF7 cell line and blood from a normal individual, which will be used as a control sample, were run alongside 1 µL and 2 µL of λ DNA/HindIII marker labelled as M.

Figure 3: Gradient PCR to determine optimal annealing temperature for inverse PCR primers (Table 1). LDI-PCR using inverse primer pairs at annealing temperature ranging from 56 to 66 °C shows a distinct PCR product at 62−66 °C. Green arrow indicates the selected annealing temperature for future experiments. Circular DNA template generated by digesting blood genomic DNA from a normal individual with *Sac*I followed by self-ligation was used for this optimization step. M, marker (1 kb plus DNA ladder).

Figure 4: LDI-PCR to identify LINE-1 3′ transduction stemming from *TTC28*-LINE-1. Circular DNA templates generated by digesting MCF7 and blood (from normal individual) genomic DNA with *Sac*I followed by self-ligation were amplified by inverse primers in optimum annealing temperature. "Native" PCR product, marked with asterisk, of expected size (5,649 bp) was detected in both MCF7 DNA and normal blood DNA, while MCF7 also produced additional PCR products of varying sizes, indicating *de novo* LINE-1 retrotransposition. M, marker (1 kb DNA ladder).

Table 1: Inverse PCR primer pair designed for the unique tag of *TTC28*-LINE-1[14].

DISCUSSION:

Here we describe a method that can be used to identify *de novo* LINE-1 insertions stemming from

any active LINE-1 of interest. We have optimized this method for a highly active LINE-1, located at 22q12.1, and previously demonstrated it to be highly sensitive in detecting sub-clonal insertions in colorectal cancer[14].

Success of LDI-PCR depends on the quality of genomic DNA. Therefore, we have included an additional quality control step to ensure that at the start of the protocol, high-molecular weight DNA is present (step 2.1.2). We recommend storing genomic DNA at -20 °C for long term storage, and to prepare aliquots in order to avoid cycles of freezing and thawing. Using genomic DNA from blood or patient-matched normal tissue is highly recommended to distinguish whether the LINE-1 retrotransposition detected is a germline or a somatic event. Since cut sites for restriction enzymes are stochastic in the genome, it is possible that a particular *de novo* LINE-1 insertion site might not harbor any cut sites for the restriction enzyme being used in its vicinity. Hence to increase the likelihood of detecting the majority of *de novo* LINE-1 insertions in tumor DNA, more than one restriction enzyme should be used in separate reactions to generate different libraries of circular DNA template. Furthermore, if the unique tag of the LINE-1 of interest has more than one PAS, then using primer pairs adjacent to each PAS improves the chances of detecting heavily truncated transductions.

Although elegant methods for genome-wide detection of *de novo* LINE-1 insertions exist, they can be overwhelming if the aim is to probe the retrotransposition competence of a particular LINE-1 in a specific cellular context. For this purpose, LDI-PCR can be an inexpensive and simple yet robust approach to visualize LINE-1 retrotransposition events. The targeting approach used in this method is similar to TS-ATLAS[22]; however, LDI-PCR avoids using linker oligonucleotides and can amplify both 5′ and 3′ junctions of *de novo* LINE-1 insertion simultaneously. Information regarding both 5′ and 3′ junctions of the LINE-1 insertion, the target site of integration, polyA tail and target-site modifications, all of which are hallmarks of LINE-1 retrotransposition, can be obtained by coupling LDI-PCR with single-molecule long-read sequencing technologies. Long reads thus generated contain the inserted LINE-1 sequence, its unique tag and the target sequences in one single read, circumventing difficulties of mapping short reads in the repetitive region.

There are two major limitations to using LDI-PCR method for detection of LINE-1 activity. The first is inherent to PCR: it can only reliably amplify fragments up to 10 kb in size. This should be considered while selecting restriction enzyme(s), as the native fragment should not exceed this limit. Secondly, this method can only detect retrotransposition events that mobilize the LINE-1's 3′ flanking region by 3′ transductions. Hence, activity of those LINE-1s that do not exhibit 3′ transduction will not be detected using this method. Additionally, despite of being amplified by LDI-PCR, some LINE-1 retrotransposition events that (a) generate a PCR target of similar size as the "native" location or other retrotranspositions or (b) are rare or subclonal, may not be detected by agarose gel electrophoresis. Such LINE-1 retrotransposition events can be captured by sequencing the LDI-PCR product using single molecule long-read sequencing technologies[14].

The workflow described here can be easily modified to detect the activity of other "hot" LINE-1s by using a suitable restriction enzyme and by designing inverse primers targeting these LINE-1s.

In addition to detection of LINE-1 mediated 3´ transduction, this method can be adapted to detect less frequent LINE-1 mediated 5´ transductions[23]. Similar method have been used to identify the integration site of LINE-1 reporters in cell-based assays[24] and proviral integration sites in cancer[25]. Besides LINE-1 insertions, this method can also be utilized to detect other genomic aberrations, such as DNA rearrangements, where information regarding the rearrangement-prone region pre-exists[26].

ACKNOWLEDGMENTS:

We would like to thank all our co-authors in the article where this method was first described[14], especially Tatiana Cajuso, Kimmo Palin, Outi Kilpivaara and Esa Pitkänen for valuable discussions while developing the method. L.K. is funded by the Academy of Finland (grant numbers 25996, 292789, 306026 and 314394), the Sigrid Juselius Foundation, and the Finnish Cancer Society. B.P. is recipient of University of Helsinki Research Foundation PhD studentship, a Finnish Cancer Society dissertation grant, and a doctoral research grant from Ida Montinin Säätiö. We also thank Teemu Masalin (University of Helsinki) and Kul Shrestha from L.K.'s research group for assisting us with the video production.

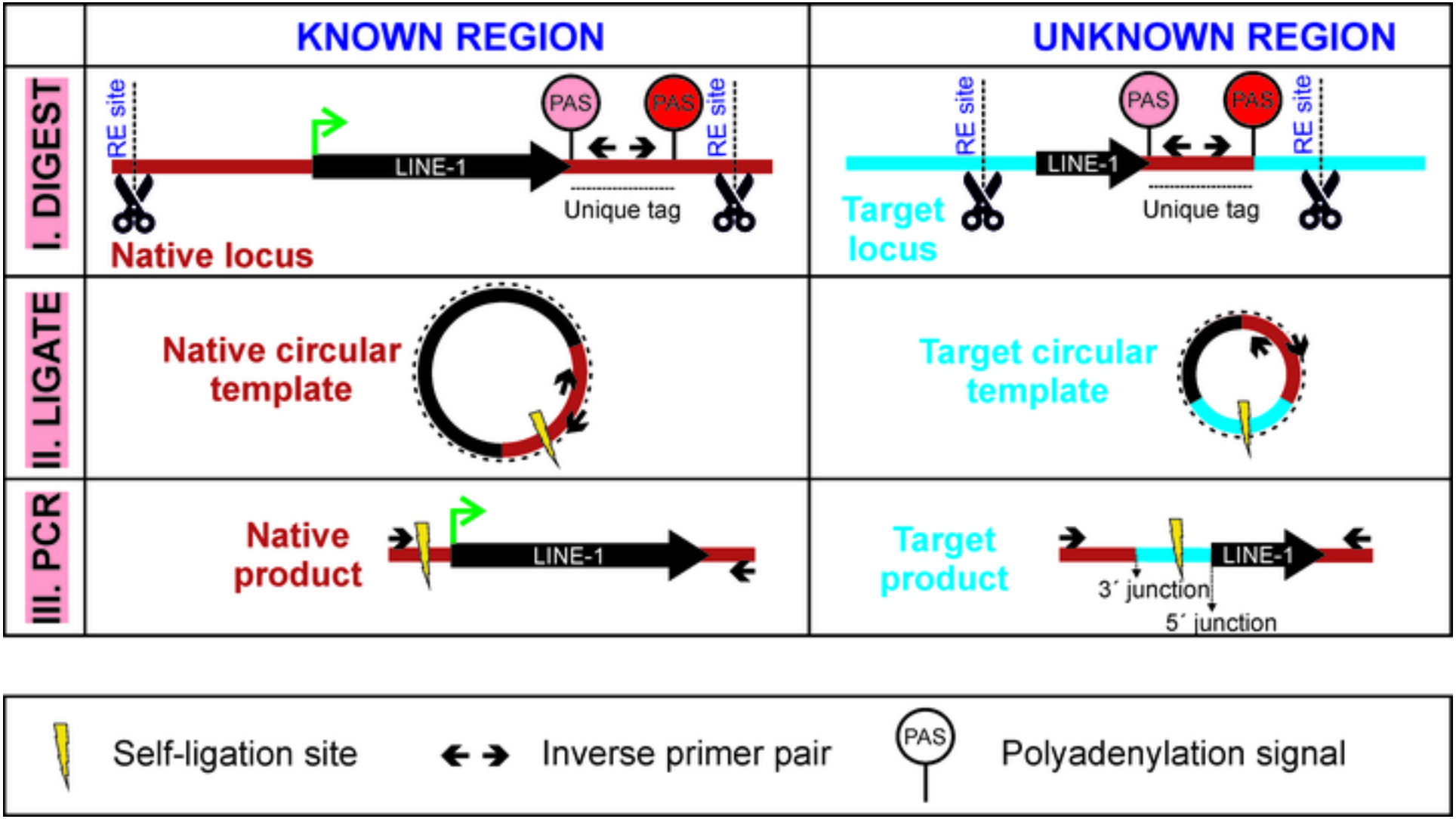
DISCLOSURES:

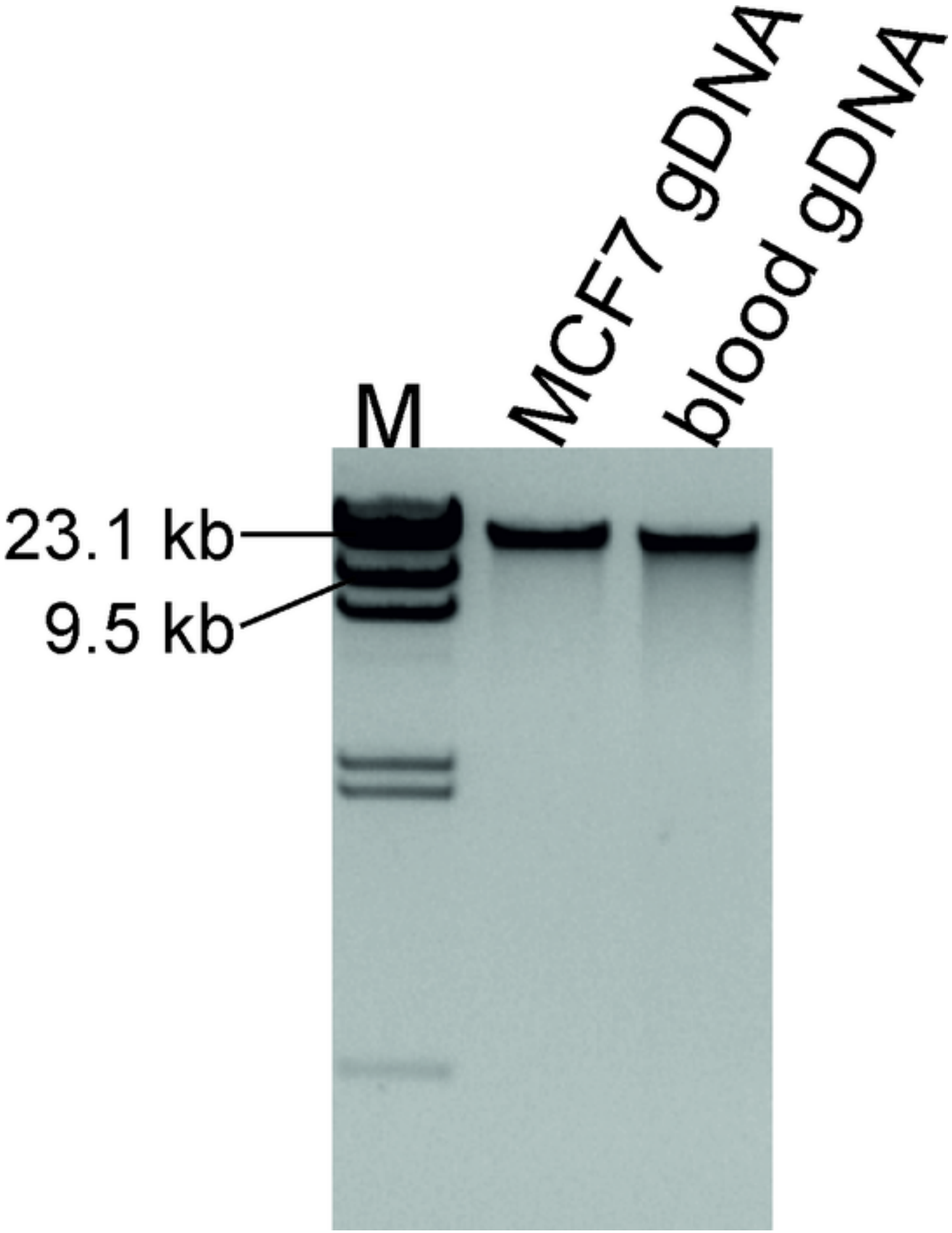
The authors have nothing to disclose.

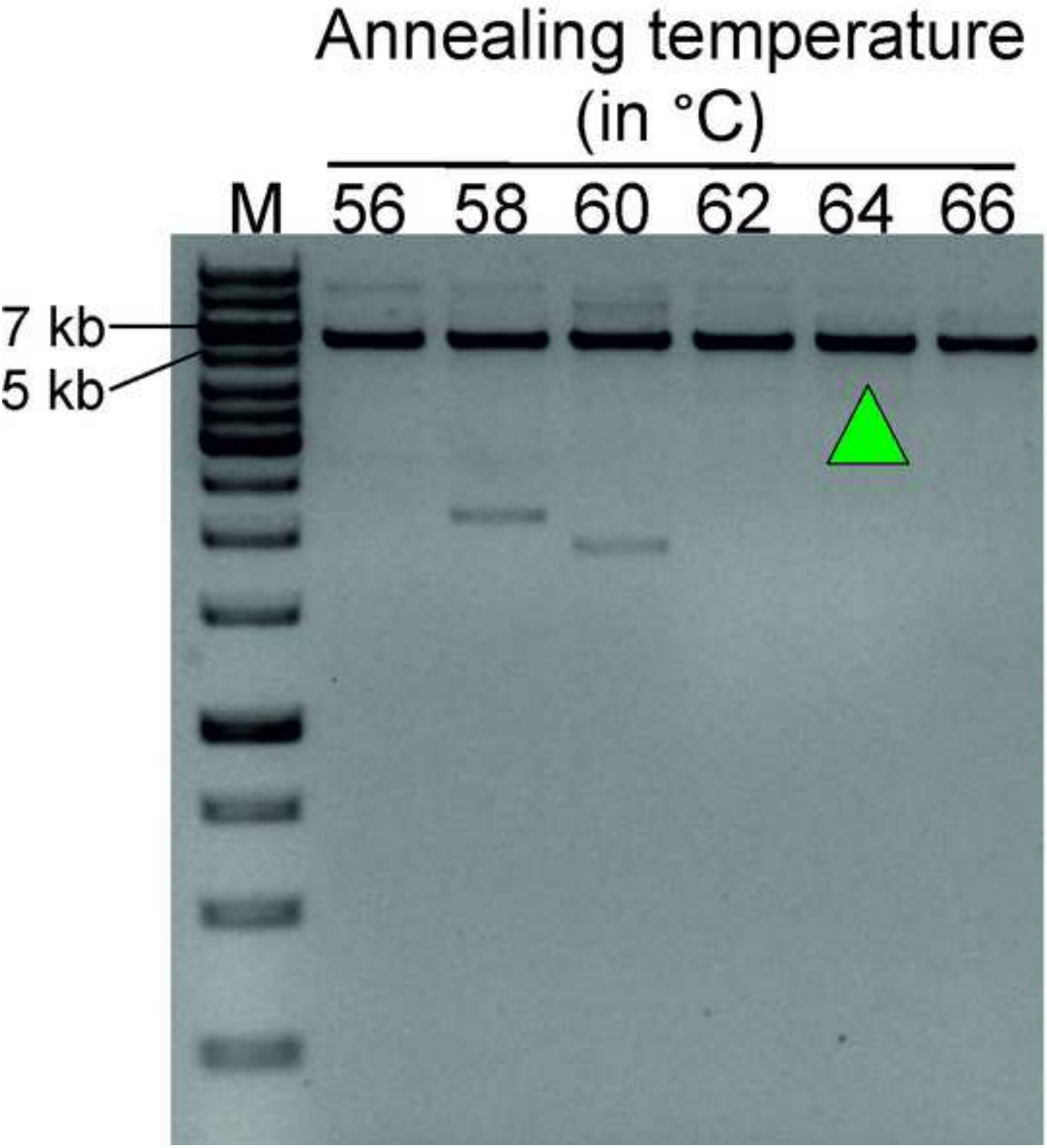
REFERENCES:

1. Lander, E. S. et al. Initial sequencing and analysis of the human genome. *Nature.* **409** (6822), 860-921 (2001).
2. Brouha, B. et al. Hot LINE-1s account for the bulk of retrotransposition in the human population. *Proceedings of the National Academy of Sciences of the United States of America.* **100** (9), 5280-5285 (2003).
3. Goodier, J. L. Restricting retrotransposons: a review. *Mobile DNA.* **7**, 16 (2016).
4. Lee, E. et al. Landscape of somatic retrotransposition in human cancers. *Science.* **337** (6097), 967-971 (2012).
5. Miki, Y. et al. Disruption of the APC gene by a retrotransposal insertion of L1 sequence in a colon cancer. *Cancer Research.* **52** (3), 643-645 (1992).
6. Scott, E. C. et al. A hot L1 retrotransposon evades somatic repression and initiates human colorectal cancer. *Genome Research.* **26** (6), 745-755 (2016).
7. Pitkanen, E. et al. Frequent L1 retrotranspositions originating from TTC28 in colorectal cancer. *Oncotarget.* **5** (3), 853-859 (2014).
8. Tubio, J. M. et al. Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. *Science.* **345** (6196), 1251343 (2014).
9. Badge, R. M., Alisch, R. S. & Moran, J. V. ATLAS: a system to selectively identify human-specific L1 insertions. *American Journal of Human Genetics.* **72** (4), 823-838 (2003).
10. Rodic, N. et al. Retrotransposon insertions in the clonal evolution of pancreatic ductal adenocarcinoma. *Nature Medicine.* **21** (9), 1060-1064 (2015).
11. Ewing, A. D., Kazazian, H. H., Jr. High-throughput sequencing reveals extensive variation in human-specific L1 content in individual human genomes. *Genome Research.* **20** (9), 1262-1270 (2010).

12. Sanchez-Luque, F. J., Richardson, S. R., Faulkner, G. J. Retrotransposon Capture Sequencing (RC-Seq): A Targeted, High-Throughput Approach to Resolve Somatic L1 Retrotransposition in Humans. *Methods in Molecular Biology.* **1400**, 47-77 (2016).
13. Zhao, B. et al. Somatic LINE-1 retrotransposition in cortical neurons and non-brain tissues of Rett patients and healthy individuals. *PLOS Genetics.* **15** (4), e1008043 (2019).
14. Pradhan, B. et al. Detection of subclonal L1 transductions in colorectal cancer by long-distance inverse-PCR and Nanopore sequencing. *Scientific Reports.* **7** (1), 14521 (2017).
15. Moran, J. V., DeBerardinis, R. J., Kazazian, H. H., Jr. Exon shuffling by L1 retrotransposition. *Science.* **283** (5407), 1530-1534 (1999).
16. Philippe, C. et al. Activation of individual L1 retrotransposon instances is restricted to cell-type dependent permissive loci. *Elife.* **5**, (2016).
17. Penzkofer, T. et al. L1Base 2: more retrotransposition-active LINE-1s, more mammalian genomes. *Nucleic Acids Research.* **45** (D1), D68-D73 (2017).
18. Tabaska, J. E., Zhang, M. Q. Detection of polyadenylation signals in human DNA sequences. *Gene.* **231** (1-2), 77-86 (1999).
19. Kalkatawi, M. et al. Dragon PolyA Spotter: predictor of poly(A) motifs within human genomic DNA sequences. *Bioinformatics.* **29** (11), 1484 (2013).
20. Sambrook, J., Russell, D. W. Purification of nucleic acids by extraction with phenol:chloroform. *Cold Spring Harbor Protocols.* **2006** (1), (2006).
21. Green, M. R., Sambrook, J. Agarose Gel Electrophoresis. *Cold Spring Harbor Protocols.* **2019** (1), pdb prot100404 (2019).
22. Macfarlane, C. M. et al. Transduction-specific ATLAS reveals a cohort of highly active L1 retrotransposons in human populations. *Human Mutation.* **34** (7), 974-985 (2013).
23. Beck, C. R., Garcia-Perez, J. L., Badge, R. M., Moran, J. V. LINE-1 elements in structural variation and disease. *Annual Review of Genomics and Human Genetics.* **12**, 187-215 (2011).
24. Morrish, T. A. et al. DNA repair mediated by endonuclease-independent LINE-1 retrotransposition. *Nature Genetics.* **31** (2), 159-165 (2002).
25. Li, J. et al. Leukaemia disease genes: large-scale cloning and pathway predictions. *Nature Genetics.* **23** (3), 348-353 (1999).
26. Pradhan, B. et al. Detection and screening of chromosomal rearrangements in uterine leiomyomas by long-distance inverse PCR. *Genes, Chromosomes and Cancer.* **55** (3), 215-226 (2016).







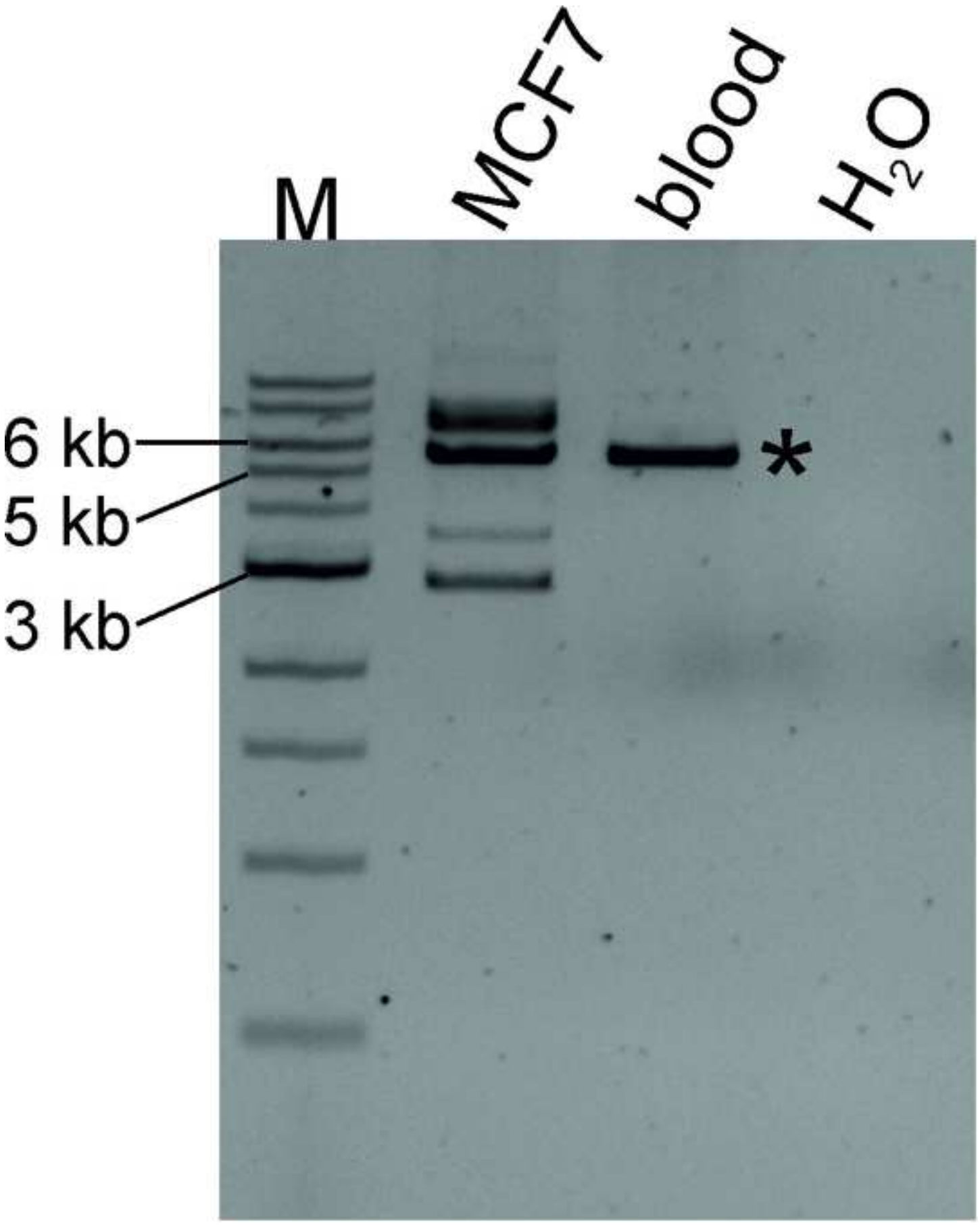


Table 1: Primer pair used for LDI-PCR to detect 3´ transductions stemming from *TTC28* -LINE-1

Primer name	Sequence (5´→ 3´)
L1_001 (rev)	TTCACTAAGCATGTATGTGGAAAAC
L1_002 (fwd)	CCCAAAATATACCCAATTACTGGCA

Table of Materials

Click here to access/download;Table of Materials;JoVE_Table_of_Materials_revised.xlsx

Name of Material/Equipment	Company	Catalog Number	Comments/Description
1 Kb DNA Ladder	New England Biolabs	N3232L	
10 mM dNTP	ThermoFisher Scientific	18427013	
Acetic acid	ThermoFisher Scientific	64-19-7	Used to make TAE buffer
Agarose	BioNordika	BN-50004	
Blood sample (frozen)			Blood sample from a healthy individua
ChemiDoc XRS+ System	Bio-rad	1708265	
DNA Gel Loading Dye (6X)	ThermoFisher Scientific	R0611	
DNeasy Blood & Tissue Kits	Qiagen	69504	
Ethidium Bromide	Bio-rad	161-0433	
Ethylenediaminetetraacetic acid (EDTA)	ThermoFisher Scientific	25102-12-9	Used to make TAE buffer
FastDigest buffer	ThermoFisher Scientific	B64	
FastDigest SacI	ThermoFisher Scientific	FD1133	
Generuler 1 Kb plus DNA Ladder	ThermoFisher Scientific	SM1331	
Generuler Lambda DNA/HindIII Marker, 2	ThermoFisher Scientific	SM0103	
Mini-Sub Cell GT Cell	Bio-rad	1704406	
Phusion Green Hot Start II High-Fidelity DNA Polymerase	ThermoFisher Scientific	F537L	
PowerPac Basic Power Supply	Bio-rad	1645050	
Quantus Fluorometer	Promega	E6150	
T4 DNA Ligase	ThermoFisher Scientific	EL0011	
Tear-A-Way 96/8, 96 Well PCR Plate	4titude	4ti-0750/TA	
Tris(hydroxymethyl)aminomethane	ThermoFisher Scientific	77-86-1	Used to make TAE buffer
Veriti Thermal Cycler	Applied Bioscience	4375786	

al for control PCR

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: DETECTION OF RETROTRANSPOSITION ACTIVITY OF HOT LINE-1s BY LONG-DISTANCE INVERSE PCR

Author(s): BARUN PRADHAN, LIISA KAUPPI

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	LIISA KAUPPI
Department:	GENOME-SCALE BIOLOGY RESEARCH PROGRAM
Institution:	UNIVERSITY OF HELSINKI
Title:	DR.
Signature:	
Date:	20th December 2018

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Dear Editor,

We are pleased to have our manuscript thoroughly peer-reviewed. We have now addressed all the editorial/ production and peer-review comments in our revised manuscript/video. You can find our response to each comments below them in *italics*. All the changes made in the manuscript text are highlighted in yellow. We thank you and the peer-reviewers for all the valid suggestions which has definitely added more value and clarity to the manuscript. We look forward to hearing from you.

Best regards,
Liisa Kauppi

Editorial and production comments:
Changes to be made by the author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. You may use the generic term followed by “(Table of Materials)” to draw the readers’ attention to specific commercial names. Examples of commercial sounding language in your manuscript are: Oxford Nanopore Technologies, Pacific Biosciences, PacBio, Generuler, ThermoFisher Scientific, New England Biolab, etc.

We have removed all the commercial language from the manuscript and video and replaced it with generic terms.

3. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

We have removed all the personal pronouns in the protocol text.

4. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

5. Section 2.1: Please do not generalize the protocol and be as specific as you can with respect to your experiment providing all the details. For instance, how to extract genomic DNA? How to measure the DNA concentration? What are the parameters for running agarose gel electrophoresis? Alternatively, relevant references can be provided here.

We have corrected this issue and have provided the instructions we used in the protocol and included relevant references

6. 2.1.1: Will tumor or blood samples be used for demonstration in the video? Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee or animal care guidelines.

Only blood samples and MCF7 cell line were used to demonstrate the protocol in this video. We have included the ethics statement before the numbered protocol steps.

7. 2.2.1: What are the reaction buffer and restriction enzyme used here? Please provide product information in the Table of Materials.

The table of materials is updated with the reaction buffer and the restriction enzyme used.

8. 2.2.2: Please specify the heat inactivation parameters.

Heat inactivation parameters are now included in the manuscript.

9. 2.3.1: How is the solution mixed?

This part of the text has been updated to match with the protocol step in the video.

10. 3.1.1: How to calculate the annealing temperature?

We have added a mention of how to calculate annealing temperature of primer pairs.

11. 3.2.2: Please describe how agarose gel electrophoresis is done.

We now refer to the previous step (step 3.1.5) where the process of agarose gel electrophoresis is described in detail and added a relevant protocol reference.

12. Section 4: Please write the text in the imperative tense. Any text that cannot be written in the imperative tense may be added as a "NOTE".

We have updated the text in this section to imperative tense.

13. Please provide information for the supplemental file and reference it in the manuscript.

Supplemental File is now mentioned in section 1.1

14. Table of Materials: Please remove trademark (™) and registered (®) symbols. Please sort the items in alphabetical order according to the name of material/equipment.

Table of Materials file is now updated by removing the trademark symbols and items are sorted in alphabetical order.

15. References: Please do not abbreviate journal titles.

We have updated the journal titles. (JoVE endnote style (downloaded from JoVE website) abbreviated journal titles in our initial submission)

Changes to be made by the author(s) regarding the video:

All of the suggested edits are now incorporated in the revised video. In order to maintain homogeneity between the video and text protocols we have revised the text protocol by replacing "L1" by "LINE-1".

1. Please update the video according to the revised manuscript.

2. Please increase the homogeneity between the written protocol and the narration in the video. It would be best if the narration is a word for word from the written protocol text.

3. 1:45-5:52: This part of the video is hard to follow with the written manuscript. Many details in the video are not mentioned in the written manuscript. Please also remove the introduction/discussion about the protocol from the Protocol section.

4. 07:54-08:00: The mixing step is not mentioned in the written manuscript. Please include it in the manuscript.

5. 06:48, 09:22, 10:30: Please move the results to the results section.

6. The video must have a representative results section following the protocol. This section must have voice-over describing the results being shown.

7. 11:38 - It sounds like the last word of this statement is cut off slightly. This should be corrected.

8. Please upload a revised high-resolution video here:
https://www.dropbox.com/request/H5IfIrdrxor6wnOb3xzz?oref=e

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript applied a physical mapping and cloning technique to detect a specific LINE-1 retrotransposition. Although the author successfully cloned retrotrasposed fragments from a cancer celline, I found it is difficult for this technique will be of interest by other researchers.

Major Concerns:
1. The method is specific to one LINE-1 locus. Most techniques in monitoring LINE-1 retrotransposition have been designed to explore genome wide. A new technique studying a single locus is a regression of science.

We acknowledge the utility of whole-genome sequencing (WGS) approach to detect the activity of all young LINE-1s (discussed in the third paragraph of the discussion section). However, this is a rather expensive and bioinformatics-heavy approach if the question is simply to know whether LINE-1 retrotransposons are active or not in a given tumor. As only few LINE-1 contribute to majority of somatic LINE-1 retrotransposition (Brouha et al. 2003; Tubio et al. 2014; Philippe et al. 2016), it can be sufficient to monitor the activity of handful of LINE-1s to show LINE-1 retrotransposition activity in a given tumor sample. For that purpose, LDI-PCR is a simple, inexpensive and faster alternative to genome-wide approach. The LINE-1 locus assayed in this protocol is reported to be highly active in different tumor types (as also pointed out by Reviewer#2). Furthermore, compared to 40x WGS, LDI-PCR has far superior sensitivity in detecting subclonal retrotransposition events: in colon cancer samples, WGS detected only 15 de novo insertions, while LDI-PCR detected 39 (Pradhan et al. 2017).

2. Efficiency of the test depends on random locations of selected restriction enzyme, circulalization of DNA, heterogeneity of the retrotranspose cells and efficiency of PCR. Therefore it is the test that if the test is negative, the study cannot be conclusive that there was no LINE-1 retrotransposition. This problem will be more troublesome with apply to another locus.

To circumvent the problem of random locations of selected restriction enzyme we have suggested to make a circular DNA library using multiple enzymes. By doing so in our original publication of this method (Pradhan et al. 2017), we were able to detect subclonal

LINE-1 insertions which were not detected by 40x whole genome sequencing. An advantage of this method is that it has an internal control (the "native" circular PCR product) for the self-ligation event . This PCR product (ranging from 5 kb to 10 kb) containing LINE-1 sequence at its native genomic location is always expected to amplify in both normal as well as tumor samples if the self-circularization step is successful.

Minor Concerns:
Incomplete citation

The references has been updated.

Reviewer #2:
Manuscript Summary:
Pradhan and Kauppi describes a long-distance inverse PCR-based method to detect de novo L1 insertions with 3' transduction from a specific source L1. The authors demonstrated the feasibility of the method by tracing a hot source L1 in an intron of TTC28. The source L1 has been reported to be highly active and to generate frequent 3' transduction. Using the unique 3' transduction sequence as a target for primers in inverse PCR, the authors successfully amplified and subsequently identified the genomic locations of de novo L1 insertions with 3' transduction of the source L1 in MCF7 cell line. Overall, the work proposed a useful protocol for tracing a specific source L1 with 3' transduction, but to generally apply this approach to trace other source L1s, several points need to be addressed/discussed.

Major Concerns:
1. To detect both source and offspring L1 insertions events, genomic DNA needs to be cut at the downstream of the 3' transduction sequence. They selected one restriction enzyme (RE) that might work for the source L1, but the RE might not work for offspring L1 sites with a different genomic context. In other words, the method sensitivity would depend on the efficiency of REs to create proper DNA fragment from offspring L1 sites for inverse PCR. The authors need to describe this concern and might consider followings:

* The combination of multiple REs to improve sensitivity.
* Transposase tagmentation reaction might be an alternative way to generate long genomic fragments and circularization? An example is the method described in Illumina's Nextera Mate Pair Library Preparation Kit, which can randomly generate long cyclized fragments.
* Sonic fragmentation, end repair, and circularization might be an alternative way to generate the template for inverse PCR?

We agree that the sensitivity of the method is limited when only one restriction enzyme is used. Therefore we have selected three restriction enzyme that makes suitable cut as

discussed in the "Representative Result" section of the manuscript and in the original description of this method (Pradhan et al. 2017). As discussed in response to Reviewer#1's second comment above, using additional restriction enzymes improved the sensitivity of our method. We appreciate the reviewer's suggestion of using transposase tagmentation and sonication to generate template for inverse PCR. These methods of random shearing should improve the sensitivity of our assay even further when sequenced using single molecule sequencing technologies. However the LDI-PCR product from such templates generated by random fragmentation of genomic DNA will be uninterpretable in the agarose gel image, as there are no means to differentiate the native PCR product from the target PCR product. Although beyond the scope of this manuscript, we consider running pilot experiments with the suggested approaches in the future.

2. As authors described in the Discussion, the method can detect insertions with targeted 3' transduction sequences only. Since there is no evidence that all hot L1s show 3' transduction, the authors should revise the Title, Abstract, and Introduction to make this point clear. For example, the last sentence in the Abstract should be revised.

We have revised the abstract and introduction to emphasize that we detect 3´ transduction events specifically. However, for ease of readership for a broad audience, we prefer to not edited the title. By detecting 3´ transduction in the genome we are detecting retrotransposition activity of hot LINE-1s, however we agree this will not capture all of the retrotransposition events stemming from the LINE-1 that is assayed for (as already discussed on lines 274-276).

Minor Concerns:
1. Since the method uses conventional inverse PCR rather than nested PCR, offspring L1s with very low allele frequency would be undetectable with the method or invisible on the gel. Describing limitation in detecting somatic offspring L1s would be informative for readers.

We have described this limitation in the fourth paragraph of discussion section (page 7, line 276 onwards). As described in manuscript this limitation can be resolved by sequencing the LDI-PCR product using single molecule sequencing. By using Nanopore sequencing we previously showed that this approach can detect even low-frequency subclonal L1 retrotransposition events (Pradhan et al. 2017).

2. Although the frequency is much lower than 3' transduction, ~0.1% of L1 insertions are known to have 5' transduction (i.e., L1 transcription starts at the upstream of the canonical L1 transcription start site). In theory, the method can be used to detect 5' transduction, so I'd like to suggest the authors discuss this possibility.

We have included this suggestion in the discussion section (page 7, line 283 onwards) with relevant reference.

3. For the limited number of source L1s with 3' transduction, add Jung et al., Genome Research 2018, and for targeted L1 sequencing, cite additional methods: L1-seq (Ewing Genome Research 2010), RC-seq (Methods Mol Biol.), SLAV-seq (Erwin, Nat. Neuroscience 2016) and HAT-seq (Zhao, bioRxiv).

The first reference suggested has already been cited as Lee et al., Science 2012 where the method was first published. We have added the other suggested references to the manuscript.

4. In abstract, 'out of 150 retrotransposition competent' needs to be 'out of ~150...' since we do not know the exact number.

We have made this correction in the abstract.

Reviewer #3:
The method mentioned in the current paper "Detection of retrotransposition activity of hot LINE-1s by Long-Distance Inverse PCR" has been previously published by the authors and their colleagues in 2017 as "Detection of subclonal L1 transductions in colorectal cancer by long-distance inverse-PCR and Nanopore sequencing. Scientific reports Sci Rep 7, 14521, 307 doi:10.1038/s41598-017-15076-3 (2017)" but in this paper their attempt is to generalized the use of their technique for unknown regions of retrotransposition activity of LINE-1. I found their technique interesting and useful for researchers who are dealing with the same issue. Their video is also useful.

Reviewer #4:
Manuscript Summary:
Enclosed are my comments regarding the manuscript entitled, " Detection of retrotransposition activity of hot LINE-1s by Long-Distance Inverse PCR." The manuscript provides a detailed description of a useful and inexpensive invPCR methodology to detect a locus specific retrotransposon that has mobilized to a new location, by utilizing the genomic sequence information of the flanking 3' sequence that was residing with the retrotranspositionally initially active locus specific "hot" LINE-1. Below are minor comments that should be addressed regarding the manuscript

Major Concerns:
1. A similar methodology has been used to examine LINE-1 insertions in genomes that were not fully characterized using the cell culture assay for LINE-1 insertions and also for examining viral insertions. These earlier papers should be referenced. These papers should also be included in the discussion as to how there is potential of modifying this methodology has the potential to examine insertions using less characterized sequence information,

especially for active LINE-1s that might be polymorphic and/or not represented in the human genome.

Morrish TA1, Gilbert N, Myers JS, Vincent BJ, Stamato TD, Taccioli GE, Batzer MA, Moran JV.Nat Genet. DNA repair mediated by endonuclease-independent LINE-1 retrotransposition. 2002 Jun;31(2):159-65.
Li, J. et al. Leukaemia disease genes: large-scale cloning and pathway predictions. Nature Genet. 23, 348-353 (1999).

We have discussed the possibility of using this method to identify the integration site of different sequence elements and added the relevant references (page 7, lines 284 onwards)

2. Please explain why the ligation reactions are not being done using unimolecular ligation conditions, to minimize re-ligation of more than one genomic fragment.

We modified the manufacturer's protocol for self-circularization of linear DNA by using 100 ng of digested DNA instead of recommended 10-50 ng. Simultaneously, the reaction volume was increased to 80 µl (recommended ligation reaction volume=50µl), to use the digested DNA product directly after digestion reaction. Using 100 ng of genomic DNA from tumor samples potentially also increases the chances of rare subclonal LINE-1 insertions to be detectable in our experiments.

3. Figure 2: needs in the legend to comment the blood gDNA, which will be used as the control. Also please include or comment if this is freshly isolated blood or how stored (heparin-EDTA. Etc), which should also be included in the materials Table. The manuscript should also emphasize that normal blood or tissue is an important control.

The Table of Material is now updated to have frozen blood samples used, and the figure legend includes comment of the blood gDNA. We have added a mention of normal blood or tissue being an important control to the discussion section (page 7, line 249)

Minor Concerns:
4. The FASTA file needs a key that indicates the coloring that shows the RE site and the primer sequences and the other highlighted characteristics.

We have described the color keys in the FASTA file now.

5. The Tables needs headings and should appear as a table rather than a list.

We have updated the Table 1 with headings.

Video Placeholder

Click here to access/download

Video Produced by Author: Less than 50 MB

Video Placeholder.mp4

>hg38_dna range=chr22:28658284-28674315 5'pad=5000 3'pad=5000 strand=+ repeatMasking=none

Brown fonts = 5kb flanking region

Grey fonts = LINE-1 sequence

Light Green Highlight = SacI restriction site

Pink Highlight = LINE-1's cognate polyadenylation signal

Red Highlight = Polyadenylation signals 1 kilobase downstream of LINE-1

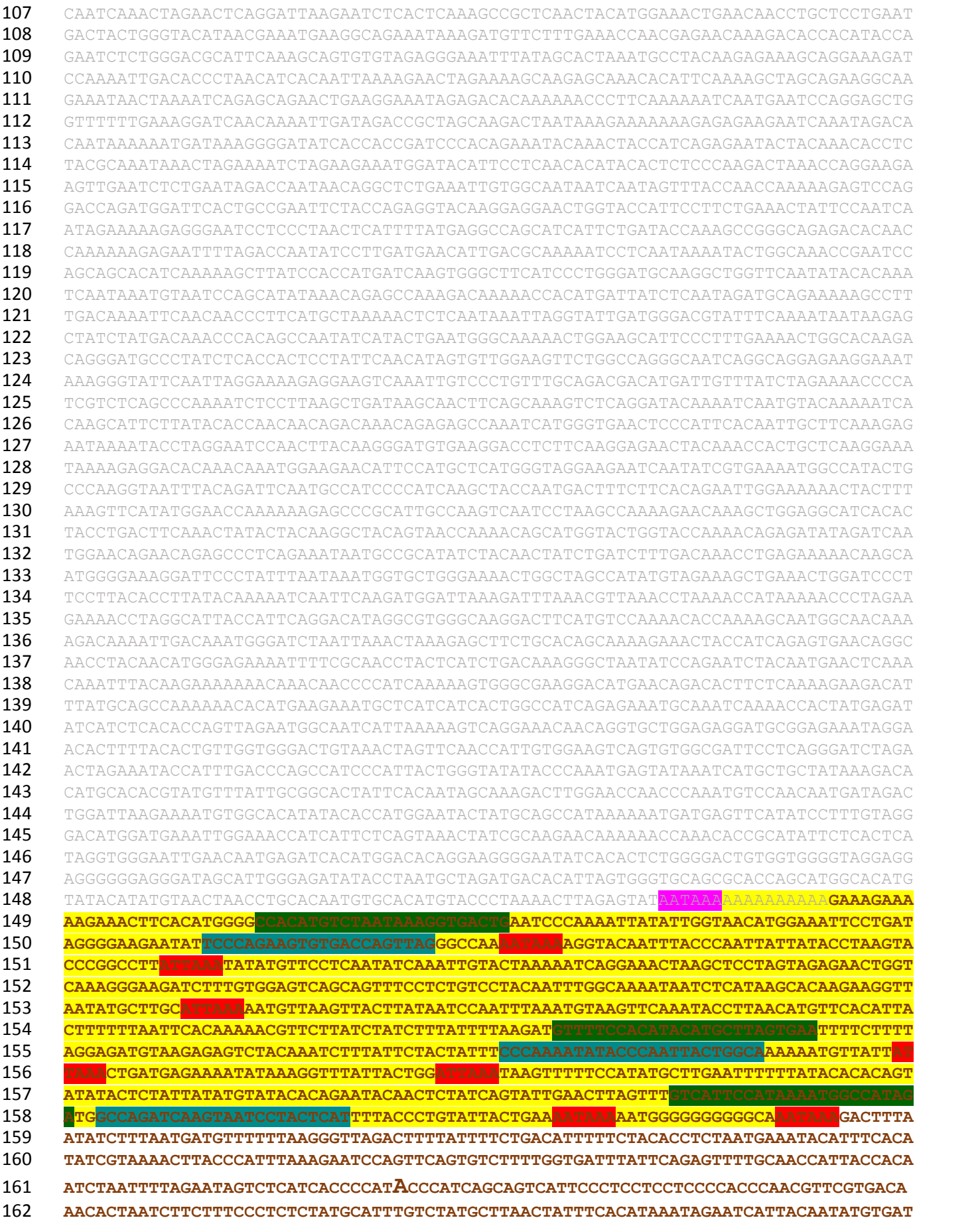
Green Highlight = Reverse Primer

Teal Highlight = Forward Primer

Yellow Highlight = Unique tag of *TTC28*-LINE-1

AGTAAATATATGAAATAATTCTAACTCTTCCAACTTCAATTAGAAATATGTACTCTAAAGCAGAAGAGTAATCATATTTC
ATTATAGCTGCTAACATGTTTACAACCCATCTTGCAAGGAGTAAAAAATCTTCATTAAAAATTTTTCTCTAAATTGCTTT
GGGTTCACAAACAGACATGATTTGAAACATGGCAGCATAGTCAAAATCATCGAGATGGAAAATAGAATGGTGGCTGCCAG
AGGCTGGAGAGAGGGAATAGGGATTTTATTGTTTAGTGGATAGAGTTTCACTTTTACAAGATGAAGAGTTATAGAGATGG
ATAATGGTGATAGTTGCACAACATCATGAATGTATTTAATTCCACTTAACTGTACACATAAAAATGATTAAGATGGTAAT
TTGACATACATTTTACAATTTAAAAATTGAGTGCCAGGCGCAGTGGCTCATGCCTGTAATGCCAGCACTTTGGGAGGCCA
AGGCAGGTGGATCACCTGAGGTCAGGATTTCCAGATCAGCCTGGCCAACATGGTAAAACCTCGTCTCTACTAAAAATACA
AAAATTAGCCGGACATGGTGACGCACATCTGTAATCTCAGCTAGTCAGGAGACTGAGGCAGGAGAATTGCTTGAGCCTGG
GAGGTGGAGGTCACAGTGAGCCAAGATCATGCCACTGCACTCTAGCCTGGGTGACAGAGTGAGATTCTGTCTCAAAAAAA
GAAAAAAACAATTGAAGAAAAAAAGAGATGATCTCCCTATCATGTAAGAAAACAGCAGGAAGGTGTCTATCTGCAAAGCA
GACAGTGGGCCCTCACCAGAAGCAAACTGACTGGCACCTTGACGTTGAACTTCCTAACCTCCAGAACTGTGAGAAATAAA
TGTTTGTTGCTAAAAACAAGAACAAACAAAAAATAACTCCATCACAGTATAAAAATGTCTCTAAACATTCAACTGTTTCA
ACTCTTACAAGGGTAAGAAACGGACTCAAATTAATTTTTTTTTGAGACAGAGTCTTGCTGTGTTGCCCAGGATGAAGTGC
AGTGGCACGATCTTGGCTCATTGCAACCTCCGCCTCCCAGGTTCAAGTGATTCTCATGCCTCTGCCTCCCAAGTAGCTGG
GCCTACAGGTTCATGCCACCACGTCCAGCTAATTTTTGTGTTTTTAGTGGAGATGGGGTTTTACCAGTTGGCCAGGCTAG
TCTCAAACTCCTGAACTCAAGTGATCCACCTGCCTCAGCCTCCCAAAGTGCCAGGATTACAGGTCAAAATTACTATTTAT
TAATAACTACATTTCTAGCTTCACTAGAAATTCCTGCTCAAATAAAGAATATGCCTGGTGTGGTGGCTCATGCCTGCAAT
CCCAACTACTTAGGAGGCTGAGGCAAGAGAATCCCTTGAGCCTAGGAGTCCGAGGCTGGGGTGAGCTATAAGAGCACCAA
TGAACTCCAGCCTGGGTGACAGGGCCAGACACTGTCTCTAAATCTTTTATTTTAATTTAAAAATAAAATCTGAAATGCAT
TTTTTTTTTTCTTGAGACAAGGTCTCGCTCTGTCACCCAGGCTGGAGTGCAGTGGCATGATCATGGCTCACTGCAACCTC
AACCTCCTGGGCCGAACTGATCCTCCCACCTCAGCCTCCCAAGTAGCTGGGACTACAGACACACCACCACGCCCAACT
AATTTTTGTATTTTGGAGATGGGGTTTCATCATGTTGCCCAGGCTGACCTTGAACTCCTGGGCTCAGGCGATCCTCTCAT
CTTGGCCTCCCAAAGTACTAGGATTACATGTGTGAGCCACCACACACAGCCTGAAATGCATTTTTTTTAACAGCAAAAAA
ATATTTTCAGGAAATTATATCTTTATTTCACAAACCTGGTCTAAAACTAAAATTCTGAATCAAAACACAACTTATAGTAC
TAACACATTTTCTCTTTAACTTTTATTTGTACTATGAAAATTGATTCTTCCTCATCAATAACCAAGGAATATACAAGTAG
TGATTTTCTTTATCATTTCTTTCATCAGCACAGTAAGAAAAGGAAATTTTTTTTCTTTTAGAGACGGAGTCTCGCTCTGT
TGCCCAGACTGGAATGCAGTGGCACCATCTCAGCTCACTGCAACCTCTGCCTCCCAGGTTCAAGTGATTCTTCTGCCTCA
GCCTCCCAAGTAGCTGGGACTGCAGGCACACACCACCACACCCGGCTAATTTTTTGTTTTTGTTTTGTTTTGATTTGGTT
TGGTTTGGTTTGGTTTTTTGAGATGGAGTCTCACTCTGTCACTCAGACTGGAGTACAGTGGCGCTATTCGGCTCACTGCA
ACCTCCACCTCCTGGTTTCAAGAGATTCTTCTGTCTCAACCTCCCGAGTAGCTGATACTACAGGCATGTGCCACCACACC
CGGCTAATTTTTGTGTTTTCAGTAGAGACGGGGTTTCACAATGTTAGCCAGGCTGGTCTCGAACTCCTGACCTCAGGTGA
TCCACCCACGTTGGCTTCCCAAAGTGCTGGGATTACAGGCATGAGCCACCGCGGCTGGCCAATTTTTGTATTTTTAGTAG
AGATGGGTTTCACCATATTGGCCAGAATGGTCTCGAACTCCTGACCTCATGATCTGAGAAAAGGAAAAAAAAAATTTTAA
ATATATAATTCCCTTTATTTCAAAGTTGCATAGGAAATTAGAATCATTCTTTTGCTATCTTAAAAACTACTGCTTTTAAG
GCCAGCCGCAGTGGCTTACACCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGAGCGGATCACCTGAGGTCGGGAGTTCG
AGACCAGCCTGACCAACATGGAGAAACCCCGTCTCTACTAAAAATACAAAATTAGCCGTGTGTGGTGGCACATGCCTGTA
ATCCCAGCTACTTGGGAAGCTAAGGCAGGAGAATCCCTTGAACCCAGGAGGTGGAGGTTGCGGTGAGTCGAGATCGTGCC
ATTGTACTCCAGCCTGGGCAACAAGTACGAAACTCCATCTCAGAAAAAAAACAATTGTTGTTATATAGCCTCATTTCATA
TAATCCCTTCTTCATTATTTAATTTCCATCATCCAAGAAACATTTATATCAGGTACTACATTAAGTATTAGTTTAATAAC

50 AGCTTTATAACATTCATTTATTCATTTATTTTTAGAGACTGGGTTTCATTCTGTGGCCCAGGCTACAGTGCAGTGGTGCC
51 AACATAGCTCACTGTAGCTTGGAACTACTGGGTTCAAGGGGTCTTCCTGCCTCAGCCTCCTGAGTAGCTGGGACTACAGG
52 TACATGCCACCATACCCGGCTAATTTGACTGATTGATAGACAGATAGTTTTGTTTTTTTTTTTGAGACAGAGTCTCACTC
53 TGTCACCCAGGCTGGAGTGCAGTGGCACGATCTCAGCTCACTGCAACCTCTGCCTCCCAGGTTCAAGCAATTATCCTACC
54 TCAGCTTCCCAAGTAGCTGGGATTACAAGGGCATCACCACACCCAGCTAATTTTTGTATTTTTAGTGGAGATGGGGTCTC
55 ACCATGTTGGCAAAGCTGGTCTCGAACTCCTGACCTCAGGTGATCCACCTGCCTCGGCCTCCCAAAGTTCTGGGATTACA
56 GGCATGAGCCACTGCACCTGGCCTAAAAAATATATTTTAGAGACTGGGTCTCACACTATGTCACCCAGGCTGGTCTTGAA
57 CTCCTGACCTCAAGCAATCCTCCTACTTCAGCCTCCCAAATAGCTGAAATACAGAAAGGAACAACCATGCCTGGCTCAGT
58 TTTGTAACTTTCAATACATAAAGAAATTACACTGCTCACATTGTAATTAATTTCATCTTTTCATTAAATTAATAGATAAT
59 AAAATTTGATAATAAACTTAATGTCAAATACTGCTTGATCAGTATTATAGAGACAAATTCCAGAATGGTGTGCCAACATA
60 ACCTTAAACCTCACAATAAAAACTAGGTTTTAACAATATCCACAGGGAAATTATGTTATTTTCTGGCAACAAGTCTAGCG
61 TTTAGTAGCAAAAGGTAGTTATAATTGATCACTATTTGCTTAGTGATCATACAAATCAAAGACAGTCTAAGGCAAATGGT
62 TAAGAACAGATTCCAGAGCCAGGCTGCTAAATTAGAATCCAAGGCAATTCAGTTTCCTAGCTGGATGGTATTGATAAAGT
63 TACTTAACCTCTCTGTACTACACTTGTCTCATATGTAAGATGGGGATAATAGTGCCTACTTCATAAAGTTGTGAGAATTA
64 AATTAGTTAATATATGTAAAGTATTTAAACAGTACTGACACAGAGTACACACAATATAAATGCTAGTCTAGCTATAATAA
65 TCTCAAACACCTATACTTACAGAAGGAAGAGTGCTTCCACACAAATACTCTATAATTTATATGGTTCTTCCAAATAAGCC
66 TAATGAAAATTTTATTTTTCATAAAATATATTTGGAATTTGTGGCCAATTTCTTGAACAATTATTATTAAATGGTTTTCA
67 TAAATCTTTGTGAATATTAGGAAGCAATGGATTCAACAAAAGCATTATGTGACAGTGGAGCAATATTCAAAACTCCTGTT
68 GCTACACAAGCCAAAAATGACAGGGATCCAAGACAACTCAAAACAAAAATATTACACACAGAACAGAAGCAACTGTGCAG
69 TATCAGGAAATGCAATACACTTTTTCTAGTGATAAGAAACTTCCACCGGGCGCGGTGGCTCACACCTGTAATCCCAGTAC
70 TTTGGGAGGCTGAGGCAGGCTGATCTCGAGGTCAGGAGATCGAGACCATCCTGGCTAACACAGTGAAACCCCGTCTGTAC
71 TAAAAATACAATAAATTAGCCAGGCGTGGTGGCGAACGCCTGTAGTCCCAGCTACTCGGAAGGCTGAGATAGGAGAATGG
72 CGTGAACCCAGGAGGCAGAGATTGCAGTGAGCCGAGATAGTGCCGCTGCACTCCAGCCTGGGAGACAGAGCGAAACTCCG
73 TCTCAAAAAAAAAAAAAAAAGAAAGAAAGAAAGAAAAAGAGAGGGAGGAGCCAAGATGGCCGAATAGGAACAGCTCCGGT
74 CTACAGCTCCCAGCGTGAGCGACACAGAAGACGGGTGATTTCTGCATTTCCATCTGAGGTACCGGGTTCATCTCACTAGG
75 GAGTGCCAGACAGTGGGCGCAGGCCAGTGTGTGTGCGCACCGTGCGCGAGCCGAAGCAGGGCGAGGCATTGCCTCACCTG
76 GGAAGCGCAAGGGGTCAGGGAGTTCCCTTTCCGAGTCAAAGAAAGGGGTGACGGACGCACCTGGAAAATCGGGTCACTCC
77 CACCCGAATATTGCGCTTTTCAGACCGGCTTAAGAAACGGCGCACCACGAGACTATATCCCACACCTGGCTCAGAGGGTC
78 CTACGCCCACGGAATCTCGCTGATTGCTAGCACAGCAGTCTGAGATCAAACTGCAAGGCGGCAGCCAGGCTGGGGGAGGG
79 GCGCCCGCCATTGCCCAGGCTTGCTTAGGTAAACAAAGCAGCCGGGAAGCTCCAACTGGGTGGAGCCCACCACAGCTCAA
80 GGAGGCCTGCCTGCCTCTGTAGGCTCCACCTCTGGGGGCAGGGCACAGACAAACAAAAAGACAGCAGTAACCTCTGCAGA
81 CTTAAGTGTCCCTGTCTGACAGCTTTGAAGAGAGCAGTGGTTCTCCCAGCACGCAGCTGGAGATCTGAGAACGGGCAGAC
82 TGCCTCCTCAAGTGGGTCCCTGACCCCTGACCCCCGAGCAGCCTAACTGGGAGGCACCCCCCAGCAGGGGCACACTGACA
83 CCTCACACGGCAGGGTATTCCAACAGACCTGCAGCTGAGGGTCCTGTCTGTTAGAAGGAAAACTAACAACCAGAAAGGAC
84 ATCTACACCGAAAACCCATCTGTACATCACCATCATCAAAGACCAAAAGTAGATAAAACCACAAAGATGGGGAAAAAACA
85 GAACAGAAAAACTGGAAACTCTAAAACGCAGAGCGCCTCTCCTCCTCCAAAGGAACGCAGTTCCTCACCAGCAACAGAAC
86 AAAGCTGGATGGAGAATGATTTTGACGAGCTGAGAGAAGAAGGCTTCAGACGATCAAATTACTCTGAGCTACGGGAGGAC
87 ATTCAAACCAAAGGCAAAGAAATTGAAAACTTTGAAAAAAATTTAGAAGAATGTATAACTAGAATAACCAATACAGAGAA
88 GTGCTTAAAGGAGCTGATGGAGCTGAAAACCAAGGCTCGAGAACTACGTGAAGAATGCAGAAGCCTCAGGAGCCGATGCG
89 ATCAACTGGAAGAAAGGGTATCAGCAATGGAAGATGAAATGAATGAAATGAAGCGAGAAGGGAAGTTTAGAGAAAAAAGA
90 ATAAAAAGAAATGAGCAAAGCCTCCAAGAAATATGGGACTATGTGAAAAGACCAAATCTACGTCTGATTGGTGTACCTGA
91 AAGTGATGTGGAGAATGGAACCAAGTTGGAAAACACTCTGCAGGATATTATCCAGGAGAACTTCCCCAATCTAGCAAGGC
92 AGGCCAACGTTCAGATTCAGGAAGTACAGAGAACACCACAAAGATACTCCTCGAGAAGAGCAACTCCAAGACACATAATT
93 GTCAGATTCACCAAAGTTGAAATGAAGGAAAAAATGTTAAGGGCAGCCAGAGAGAAAGGTCGGGTTACCCTCAAAGGAAA
94 GCCCATCAGACTAACAGCGGATCTCTCGGCAGAAACCCTACAAGCCAGAAGAGAGTGGGGGCCAATATTCAACATTCTTA
95 AAGAAAAGAATTTTCAACCCAGAATTTCATATCCAGCCAAACTAAGCTTCATAAGTGAAGGAGAAATAAAATACTTTATA
96 GACAAGCAAATGCTGAGAGATTTTGTCACCACCAGGCCTGCCCTAAAAGAGCTCCTGAAGGAAGCGCTAAACATGGAAAG
97 GAACAACCGGTACCAGCCGCTGCAAAATCATGCCAAAATGTAAAGACCATCGAGACTAGGAAGAAACTGCATCAACTAAT
98 GAGCAAAATCACCAGCTAACATCACAATGACAGGATCAAATTCACACATAACAATATTAACTTTAAATATAAATGGACTA
99 AATTCTGCAATTAAAAGACACAGACTGGCAAGTTGGATAAAGAGTCAAGACCCATCAGTGTGCTGTATTCAGGAAACCCA
100 TCTCACGTGCAGAGACACACATAGGCTCAAAATAAAAGGATGGAGGAAGATCTACCAAGCCAATGGAAAACAAAAAAAGG
101 CAGGGGTTGCAATCCTAGTCTCTGATAAAACAGACTTTAAACCAGCAAAGATCAAAAGAGACAAAGAAGGCCATTACATA
102 ATGGTAAAGGGATCAATTCAACAAGAGGAGCTAACTATCCTAAATATTTATGCACCCAATACAGGAGCACCCAGATTCAT
103 AAAGCAAGTCCTCAGTGACCTACAAAGAGACTTAGACTCCCACACATTAATAATGGGAGACTTTAACACCCCACTGTCAA
104 CATTAGACAGATCAACGAGACAGAAAGTCAACAAGGATACCCAGGAATTGAACTCAGCTCTGCACCAAGCAGACCTAATA
105 GACATCTACAGAACTCTCCACCCCAAATCAACAAAATATACATTTTTTTCAGCACCACACCACACCTATTCCAAAATTGA
106 CCACATAGTTGGAAGTAAAGCTCTCCTCAGCAAATGTAAAAGAACAGAAATTATAACAAACTATCTCTCAGACCACAGTG



163 CTCTTGTGACTGGTTTCTTCCGCTTAGAATGTTTTCAAGGTTCATCCATGTTGTATCATGCCTCAGTACTTCTTCCTTTT
164 ATGGCAGAATAATACTCCACTGGAAATAATACTCCACTGGAAAAAAATATTTTGTTTATCCAGTCAACAGTTAGTTGTAC
165 TTGTGGCTGTTTTCACTTTTTGGCTTTTATAAATAATATAGCAATGAACATTCATGTTCACACAAGTTTTTGTGTGAACA
166 ATTGTTTTGATGTCTTTAGGGCATATATATATATATATATATGAGTGGAATTACTGGGCCATATGGCAAGTCTATGTTTA
167 ACATTTTGAGGAGATGCCAAAGCATTTTCTAAGACAGCTGCACCACTTTACATTCCAGCAATGTATGAGAGCTCCAATTT
168 CTCCACATCCTCACCAGTACTTGTTATTGTCTATCTTTTTAACTTTAGCCACCCCAGTTGATGTAAAGTGGATCTCATTG
169 TGGTTTTGATTGCATGACTGATGACTAACGATGTTGCATACCTTTTCATGTTATTGGCCATTTGTATATCTTTAGGAAAT
170 GTCTACTTTTTTGGTTTCACTCTGTCACCCAGGCTGGAGTGCAGCAGTGTAATCACTGCTCACTGCAGCTGCGATTTCCC
171 AGGCTCAAGTGATCACGGGCTAACATTTTTTTCTATTTTTTTTTGTAGAGAAGAGGTGTTACTATGTTGCCCAGGCTGATC
172 TCAAACTCCTGGGCTCAAGTGATCCTCCCACCTTGGCCTCCCAAAGTGCTAGGATTACAGGCATGAACCACCTCAACCGG
173 CCTGTTTATTTTTAAATTCGGTTGTCCTTTTATCATTGCATAGTAAGTTATTAATATATTCTGAATAATAAGTCCTTTAA
174 CAAATATATATTTGCAAATATTTTCTTCCTTGGCCGGGCGCGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGACCA
175 AGGCAGGTGGATCACGAGGTCAGGAGATCGAGACCATCCTATCTAACACAGTGAAACCCCATCTCTACTAAAAATACAAA
176 AAATTAGCCAGGCGTGGTGGCGGGAGCCTGTAGTCCCAGCTACTCAGGACACTGAGGCAGGAGAATGGCGTGAACCCGGT
177 AGGCAGAGCTTGCAATGAGCCGAGATCGCACCACTGCACTCCAGCCTGGGCGACAGAACAAGACTCCATCTCAAAAAAAA
178 AAAAAAAAAAAATTTCTTCCATTATGTGGATTTTGTTTTCATTTTTTTTTTGAGACAGAGTCTTGCTTTGTCGCCCAGGC
179 TGGAATGCAGTGGCCCAATCTCAGCTCATTGCAACCTCCACCTCCCAGGTTCACATGATTCTCCTGCCTCAGCCACCCAA
180 GTAACTGGGATTACAGGCGCCTGCCACCATGCCCGGCTAATTTTTGTGTTTTTAGTAGAGACAGGGTTTCACCATGATGC
181 CCAGGCTGGTCTCGAACTCCTGACCCCAAGTCATCTGCCCACCTCGGCCTCCCAAAGTGCTGGGATTACAGGTATGAGCC
182 ACTGCACCTGGACAAAATAAGGCTTTTATCAGAAAATTAAAATAGAAATAAACCATTTAAAACTAAAACACTTTTTTTTT
183 TTGAGACAGAGTCTCGCTCTGTCACCCAGGCTGGAGTGCAGTGGTGTGATCTCAGCTCACTGCAACATCCGCCTCCTGAG
184 TTCAAGTGATTCTCCTTCCTCAGCCTCCCAAGTAGATGGGACTATAGGCTAATTTTTTGGGTTGTTTTGTTTTGAGACGG
185 AGTCTTACTCTCTCGCCCAGGCTGGAGGCCATCTCGGCTCACTGCAAGCTCCGCCTCCCGGGTTCAAGCAGTTCTCTGCC
186 TCAGCCTCTGGAGTAGCTGGGATTACAGGCGCCTGCCACCATGCACAGCTACTTTTTGTATTTTTAGTAGAGACAGGGTT
187 TCATCATATTGGCCAGGCTGGTCTTGAACTCCTGACCTCGTGATCCACCCACCTCGGCCTCCCAAAGTGCTGGGATTACA
188 GGCATGAGCCACCGCGCCCGGCCAATTTTTTGTATTTTTATTAGAGACATGGTTTCACCCTGTTGGTCAGGCTGGTCTCA
189 ATCTCCTAACCTCATGATCCACCCGTCTCGGCCTCCTAAAATGCTGGGATTACAGGCATGACCCACCACACCTGGCCTAA
190 AATATTTATTACTAAAAATCAATAATATTTTCTTGCTGTTGAGAAAGAGAAAGCAACAATAATGTCCTTCCACTCTTTGA
191 CTAAATACCAGGAATTATTTTGCTAGACACCAAAAGTCATATTTGGAGAACTGAAGAGGTAATAATAGTAACTGGAGAGT
192 AGAACAGAAAAAGAGGGCAAGTAGGTGTTGTAGTATGGCTGTAATTGGCCTCACTGTGACCACTACAGAAAAAACCCTTT
193 TTCATCATAGCTATACTGCCCCTAGGCAAGGACGTGAAGAACATACCTTATTTTACAAGATTAATAGCGTAAAACTAACA
194 TATAGTAATCTGGAATGTGATTTTTTGAGATCCCACTTGATAAATAATTGATTGTAATGAGGATGTGGTTTTGTGGATAA
195 AGAGTAGGATTGCTAAGAAATAGCAAATGGTCTAAGCACAACAAACCTGACTTTGTGTTGTGGTTTTGCAGGAGTTAAAA
196 AAAGAAGTTGGGCCGGGAGCGGTGGCTCACACCTGTAATCCCAGCACTTTGGGAGGCTGAGGCGGGCGGATCACGAGGTC
197 AGGAGATCGAGACCATCCTGGCTAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTTAGCTAGACTTGGTGGC
198 AGGCGCCTGTAGTCCCAGCTACTTGGGAAACTGAGGCAGGAGAATGGCGTGAACCCAGAAGGCGGAGCTTGCAGTGAGCA
199 GAGATCGCGCCACTGCACTCCAGCCTGGGCGACAGAGACTCCGTCTCAAAAAAAAAAGTTAACTGCGAATGTTAAATAAG
200 AGAATGCATTATTATTGTATGTAGCTTCCCCCCCTTACCTGGTACGTACTTGGCATTTCTATGCCATAATGGAAGTCAAA
201 AATCACCATGATATTGTTTCAACATACTATGGACGTAGTTCAGAAAATGTTAGGACAAATTTGATATCAACATTCTTTGC
202 ACTCACAGAATTGCATAAAGAGTATCACTTTGGTAAAGATGAAAATTACTAAGATTATTGTCCTCTAATGTCAATCATCA
203 TAAAATGAATATCAAATTAGCATTGATACTTATATTTTGCTAAAACATGCCTATAGAAAACTGGTTAGTGTTTGTTTTTG
204 GAAGACTATAGATAATACAGTGTGAATATACAAGAAACATGAGATGTTCCAAGCATCATATTTGTATCATACTATGTCTC
205 TAGTTTCTCTTCTTAGAAAATAACAATGCACCCAGCTGCTGAAGAGACACCAAAATAAATGTTCTACCAAGTTTAAGTTC
206 AAACATCAGGCTTCCCGCTAACAGTTTTTAAAATAGCAATAATTTAAAGGAAGAAAGATTGTAACCATGAAATTTAATGA
207 AAAAATATTGTTTCTGCTTTCAGGAAAAAAAGTAGTGAAAAAACAGCACACAAAACAAGAATCATAGAGTAAAAATAAAG
208 AGCTTTCAATTATACTTTTATTATTTTTAATATTTAAAATTCACTGTCAAATATTCTATATAGCAACCTTGATTTCACAT
209 GCATTTGCAATACAACTATGTGGTATGAGAGACTTGTCTTCTTTAGTGGTTCCTCAAGCGCTCACAGGGATTTCTTGGGT
210 CGTAGAAAGGTGATGGGGACAGGAAGAGGCTTGGGGTTTCTGTTTGTGGTTTTTTCTTTGTGGTTTTTTTTTTTTTTTT
211 TTTGGTCTGTGGTTTTGGAGTTTTTGTTTTTCT