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LEAP: L1 Element Amplification Protocol

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Summary

Long Interspersed Element-1 (LINE-1 or L1) retrotransposons encode two proteins (ORF1p and ORF2p) that are required for retrotransposition. The L1 Element Amplification Protocol (LEAP) assays the ability of L1 ORF2p to reverse transcribe L1 RNA *in vitro*. Ultracentrifugation or immunoprecipitation is used to isolate L1 ribonucleoprotein particle (RNP) complexes from cultured human cells transfected with an engineered L1 expression construct. The isolated RNPs are incubated with an oligonucleotide that contains a unique sequence at its 5′ end and a thymidine-rich sequence at its 3′ end. The addition of dNTPs to the reaction allows L1 ORF2p bound to L1 RNA to generate L1 cDNA. The resultant L1 cDNAs then are amplified using polymerase chain reaction (PCR) and the products are visualized by gel electrophoresis. Sequencing the resultant PCR products then allows product verification. The LEAP assay has been instrumental in determining how mutations in L1 ORF1p and ORF2p affect L1 reverse transcriptase (RT) activity. Furthermore, the LEAP assay has revealed that the L1 ORF2p RT can extend a DNA primer with mismatched 3′ terminal bases when it is annealed to an L1 RNA template. As the LINE-1 biology field gravitates toward studying cellular proteins that regulate LINE-1, molecular genetic and biochemical approaches such as LEAP, in conjunction with the LINE-1 cultured cell retrotransposition assay, are essential to dissect the molecular mechanism of L1 retrotransposition.

Keywords

LINE-1; reverse transcriptase; ribonucleoprotein particle (RNP); L1 Element Amplification Protocol (LEAP)

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Conflict of Interest

J.V.M. is an inventor on the patent: “Kazazian, H.H., Boeke, J.D., Moran, J.V., and Dombrowski, B.A. Compositions and methods of use of mammalian retrotransposons. Application No. 60/006,831; Patent No. 6,150,160; Issued November 21, 2000.” J.V.M. has not made any money from this patent and voluntarily discloses this information.

1. Introduction

Long Interspersed Element-1s (LINE-1s or L1s) are the only active, autonomous transposable elements in the human genome (1, 2). L1s mobilize through an RNA intermediate by a “copy and paste” process termed retrotransposition (reviewed in (3)). An average individual human genome contains ~80–100 active (*i.e.*, retrotransposition-competent L1s (RC-L1s) (4, 5). RC-L1s are ~6 kb in length and encode two proteins (ORF1p and ORF2p) that are required for their retrotransposition (1, 6–8). L1 ORF1p is an approximately 40 kDa RNA binding protein (9–15) with nucleic acid chaperone activity (16, 17). L1 ORF2p is an approximately 150 kDa protein (18–21) with both endonuclease (EN) (6) and reverse transcriptase (RT) activities (22).

Although L1 retrotransposition has had a dramatic impact on the structure of the human genome, the detailed molecular mechanism of L1 retrotransposition requires elucidation (reviewed in (3)). Assays using Ty1/L1 ORF2p expression constructs (22, 23), as well as L1 ORF2p produced in recombinant expression systems (24–26), have revealed that L1 ORF2p contains reverse transcriptase activity. Similarly, a recombinant L1 ORF2p EN domain produced in *E. coli* contains DNA endonuclease activity that can generate single-strand DNA breaks at thymidine-rich sequences in double-stranded DNA, leading to nicks that contain a 5′ phosphate and 3′ hydroxyl group (6).

The above experiments, in conjunction with the LINE-1 cultured cell retrotransposition assay (7) and molecular genetic approaches (reviewed in (3)), have demonstrated that L1 ORF1p and ORF2p preferentially associate with their encoding L1 RNA *in cis* (27–29), leading to the formation of a ribonucleoprotein particle (RNP) that is a necessary intermediate for retrotransposition (18, 21, 30). Components of the L1 RNP are thought to gain access to the nucleus, where the L1 ORF2p EN activity generates a single-strand endonucleolytic nick in genomic DNA at a thymidine-rich consensus sequence (*e.g.*, 5′-TTTT/A-3′, 5′-TTTC/A-3′, 5′-TTTA/A-3′ *etc.*, where “/” denotes the scissile bond) (31–35). This endonucleolytic activity liberates a 3′-hydroxyl group, which can serve as a primer for the L1 ORF2p RT activity to initiate the reverse transcription of L1 RNA by a process termed target site-primed reverse transcription (TPRT) (Figure 1) (6, 24, 36). However, the difficulty in purifying high levels of recombinant L1 ORF2p *in vitro*, and the fact that L1 ORF2p is made in low quantities *in vivo* (as few as one molecule of L1 ORF2p is made per L1 RNA (37)), has hampered efforts to study L1 TPRT at the molecular level.

Epitope and RNA tagging strategies have been used to detect L1 ORF1p, L1 ORF2p, and L1 RNA from engineered L1 expression constructs (18, 20, 21, 28, 30). Importantly, these technologies also have allowed the discrimination of the L1 proteins and RNA produced from transfected L1 expression constructs from the proteins and RNAs expressed from endogenous L1s. In 2006, we developed an assay, termed the L1 Element Amplification Protocol (LEAP), that allows the detection of ORF2p reverse transcriptase activity in RNP preparations derived from cultured human cells transfected with engineered L1 expression constructs (28). The LEAP assay uses a similar rationale that has been used to detect RT activity from mitochondrial plasmids of *Neurospora crassa* (38) and is similar to the strategy

employed to detect telomerase activity using the telomere repeat amplification protocol (TRAP) assay (39).

For the LEAP assay, HeLa-JVM cells are transfected with engineered L1 constructs that express versions of ORF1p and ORF2p that contain different epitope tags at their respective carboxyl termini (Figure 2) (18, 28, 30). Hygromycin B is used to select for HeLa-JVM cells containing the engineered L1 constructs. Cellular RNP complexes then are isolated from hygromycin B-resistant HeLa-JVM cells using differential centrifugation through a sucrose cushion (Figure 2B) (28, 30). Alternatively, L1 RNPs can be isolated from hygromycin B-resistant HeLa-JVM cells by immunoprecipitation, using an antibody directed against the L1 ORF2p carboxyl terminal epitope tag (Figure 2C) (18). The resultant RNP then is incubated with an oligonucleotide (*i.e.*, a LEAP adapter) to prime cDNA synthesis (Figure 3A) (28). The LEAP adapter contains a unique sequence at the 5′ end (RACE) followed by 12 thymidines (dT_{12}) and ends with VN nucleotides (where V represents adenosine (A), guanosine (G), or cytidine (C), and N represents any nucleotide). The L1 cDNAs are PCR-amplified using oligonucleotide primers to the RACE sequence and the engineered L1 construct. The LEAP PCR products then are visualized by gel electrophoresis and can be subsequently cloned and sequenced to characterize the products (Figure 3B).

Data obtained from the LEAP assay have revealed that, unlike retroviral reverse transcriptases (*e.g.*, Moloney murine leukemia virus (MMLV)-RT), the L1 RT can initiate reverse transcription from a DNA primer with mismatched 3′ terminal bases when it is annealed to an L1 RNA template (28). Indeed, the ability of L1 RT to extend mismatched DNA primer/RNA template duplexes explains how certain genomic DNA sequences (*e.g.*, 5′-TTTA/A or 5′-TTTC/A, where '/' corresponds to the L1 EN nick) can serve as primers to initiate TPRT *in vivo* (31, 35, 40). That being stated, increasing the length of DNA primer 3′ terminal mismatches to 4 mismatched nucleotides decreases the efficiency of reverse transcription (28, 41). Notably, the presence of different VN dinucleotide pairs and poly (A) tail lengths in the resultant LEAP products allows independent products to be distinguished from one another in a single LEAP reaction (28). Finally, the use of gene specific primers has allowed the identification of cellular mRNAs that can be reverse transcribed at low levels by L1 RT *in trans* (28, 42–44).

LEAP adapters also have been designed to mimic genomic integration sites observed in cultured cell experiments. For example, the LEAP assay was used to examine how endonuclease-deficient L1s are able to integrate at dysfunctional telomeres in Chinese hamster ovary (CHO) cell lines that are defective in components required for the non-homologous end-joining (NHEJ) pathway of DNA repair (45, 46). Here, the LEAP adapter was modified to mimic potential telomere ends (*e.g.*, 5′-RACE-$(TTAGGG)_3$-3′, 5′-RACE-$(TTAGGG)_3$TT-3′, 5′-RACE-$(TTAGGG)_3$TTAG-3′, *etc.*). A LEAP adapter with a telomeric repeat ending in 5′-$(TTAGGG)_3$TT-3′ was more efficient to prime first strand L1 cDNA synthesis than an adapter ending in 5′-$(TTAGGG)_3$-3′ (45). Characterization of the LINE-1 cDNA/primer junctions of these LEAP products revealed that they generally contained a perfect telomere repeat followed by a poly (T) sequence that resulted from the reverse transcription of the L1 poly (A) mRNA; hence, the LEAP products recapitulated the structure of endonuclease-deficient LINE-1 retrotransposition events observed in NHEJ-

deficient CHO cells (33, 45, 46). Indeed, these data further demonstrated that L1 RNP preparations are associated with a nuclease activity that can process the oligonucleotide adapter prior to its use as a primer in the LEAP reaction (45).

The LEAP assay also has been used to determine whether missense mutations in L1 ORF1p or ORF2p affect L1 RT activity (18, 28, 45). For example, LEAP reactions revealed that missense mutations in ORF1p that adversely affect L1 retrotransposition by decreasing the ability of ORF1p to bind L1 RNA retain LEAP activity (18, 28). Similarly, missense mutations in the EN or cysteine-rich domain of L1 ORF2p adversely affect L1 retrotransposition but retain LEAP activity (18, 28, 45).

A number of cellular proteins that interact with L1 RNPs recently have been identified and the overexpression of a subset of these proteins can adversely affect L1 retrotransposition in cultured human cells (21, 47, 48). The LEAP assay can be used to determine if these cellular proteins affect L1 RT activity. For example, the LEAP assay revealed that the L1 mRNA template remains annealed to the L1 cDNA in an RNA/DNA hybrid after reverse transcription, protecting the L1 cDNA from APOBEC3A-mediated cytidine deamination (49). However, the addition of RNase H (an enzyme that degrades RNA in an RNA/DNA hybrid) to the LEAP reaction renders the L1 cDNA susceptible to cytidine deamination (49). These data, in conjunction with cultured cell based experiments, revealed that APOBEC3A inhibits L1 retrotransposition, in part, by deaminating the transiently exposed single-strand L1 cDNA generated during TPRT (49).

In sum, the LEAP assay has been used to elucidate mechanistic details of TPRT. The following protocol has been adapted from previously published studies and is optimized for HeLa cells (18, 28). Important experimental controls and technical tips are highlighted in the Notes section of this protocol.

2. Materials

Special care should be taken when preparing materials for L1 RNP isolation and RT-PCR reactions. Prepare all solutions in DNase/RNase-free water. All lab instruments, consumables (*e.g.*, pipettor, pipets, conical tubes, and microcentrifuge tubes) and solutions should be RNase-free and used only for RNA work. Work areas should be cleaned regularly with RNase*Zap*® (Life Technologies #AM9786) or other similar cleaning reagent.

2.1 Cell Culture Medium and Transfection Reagents

1. HeLa cells: we typically use HeLa-JVM cells for our assays (7, 28, 50).
2. Dulbecco's Modified Eagle Medium (DMEM) (with 4.5 g/L D-glucose, Life Technologies #11960-051) containing 10% fetal bovine serum (FBS) (Sigma #F2442 or Life Technologies #26140-079), and 1X Pen Strep Glutamine (100 U/mL penicillin, 100 μg/mL streptomycin, and 292 μg/mL glutamine, Life Technologies #10378-016). This is called HeLa-JVM DMEM growth medium in the protocol below.
3. 1X phosphate-buffered saline (PBS), pH 7.4, sterilized

4. L1 expression plasmid constructs (*e.g.*, pJM101/L1.3 (Figure 2B; (5)) or pES2TE1 (Figure 2C; (18))
5. A cell counter (*e.g.*, Countess® Automated Cell Counter, Life Technologies #C10227) or hemocytometer
6. Tissue culture T-25, T-75, or T-175 flasks (Corning #353108, #353136, or #353112, respectively)
7. FuGENE® 6 (Promega #E2691)
8. Opti-MEM® I (Life Technologies #31985-062)
9. Hygromycin B (Life Technologies #10687-010)

2.2 Lysis Buffer

1. 1.5 mM KCl (Sigma #P3911)
2. 2.5 mM $MgCl_2$ (Sigma #M2670)
3. 5 mM Tris-HCl, pH 7.5 (Life Technologies #15567-027)
4. 1% deoxycholic acid (Calbiochem #264103)
5. 1% Triton X-100 (Sigma #T8787)
6. 1X Complete Protease Inhibitor Cocktail, EDTA-free (Roche #11873580001, add fresh)
7. UltraPure™ DNase/RNase free water (Life Technologies #10977-015)

2.3 Sucrose Stock Solution, 47%

1. 80 mM NaCl (Sigma #S9888)
2. 5 mM $MgCl_2$
3. 20 mM Tris-HCl, pH 7.5
4. UltraPure™ sucrose (Life Technologies #15503-022), 47% weight to volume (w/vol) in UltraPure™ DNase/RNase free water
5. 1 mM dithiothreitol (DTT) (Fisherbrand™ #BP172, add fresh)
6. 1X Complete Protease Inhibitor Cocktail, EDTA-free (add fresh)
7. UltraPure™ DNase/RNase free water
8. Sterile Filter System, 0.22 μm CA 500 mL bottle (Corning #430769)

2.4 Sucrose dilution buffer

1. 80 mM NaCl
2. 5 mM $MgCl_2$
3. 20 mM Tris-Cl, pH7.5
4. 1 mM DTT (add fresh)

5. 1X Complete Protease Inhibitor Cocktail, EDTA-free (add fresh)
6. UltraPure™ DNase/RNase free water

2.5 IP Flag buffer

1. 100 mM KCl
2. 20 mM Tris-HCl pH 8.0 (Life Technologies #15568-025)
3. 1 mM DTT (add fresh)
4. 10% UltraPure™ Glycerol (Life Technologies #15514-011)
5. 0.1% IGEPAL CA-630 (Sigma #I8896)
6. 1X Complete Protease Inhibitor Cocktail, EDTA-free (add fresh)
7. UltraPure™ DNase/RNase free water

2.6 Flag elution buffer

1. IP FLAG buffer (see 2.5 above)
2. 200 μg/mL 3X FLAG peptide (Sigma #F4799)

2.7 LEAP reaction buffer (L1 reverse transcriptase reaction)

1. 50 mM Tris-HCl, pH 7.5
2. 50 mM KCl
3. 5 mM $MgCl_2$
4. 10 mM DTT (add fresh)
5. 0.2 μM 3′ RACE Adapter (see Note 1)
6. 20 U recombinant RNasin® ribonuclease inhibitor (Promega #N2511)
7. 0.2 mM dNTPs (Life Technologies #18427-013)
8. 0.05% Tween®-20 (Fisherbrand™ #BP337)
9. UltraPure™ DNase/RNase free water

2.8 LEAP PCR reaction mix

1. 1X Pfu reaction buffer (Agilent #600320)
2. 0.2 mM dNTPs
3. 0.4 mM SENSE primer (see Note 2)

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1. The LEAP adapters for the RT reaction work best if they are HPLC-purified (Integrated DNA Technologies (IDT, Iowa, USA)). LEAP Adapter sequence: 5′-GCGAGCACAGAATTAATACGACTCACTATAGGTTTTTTTTTTTTVN-3′, where V is A, G, or C and N is any nucleotide (28).
2. PCR primers of standard purity are obtained from IDT (Iowa, USA).

a. SENSE: 5′-GGGTCCGAAATCGATAAGCTTGGATCCAGAC-3′. This “SENSE” primer is specific to the transfected engineered L1 construct and does not amplify endogenous L1s (28).

4. 0.4 mM OUTER primer (see Note 2)
5. 2.5 U PfuTurbo Hotstart DNA Polymerase (Agilent #600320)
6. UltraPure™ DNase/RNase free water

2.9 Other

1. RNase*Zap*® RNase decontamination solution (Life Technologies #AM9786)
2. Cell scrapers, small or large (*e.g.*, Fisherbrand™ #08-100-241 or #08-100-242, respectively)
3. Conical tubes, 15 mL (*e.g.*, Nunc™ #362694)
4. Barrier pipette tips (*e.g.*, Neptune #BT1250.N, #BT200, #BT20, and #BT10XL)
5. M-MLV Reverse Transcriptase (MMLV-RT) (Promega #M1701)
6. RNeasy Mini Kit (Qiagen #74104)
7. Agarose and low-melt agarose (Life Technologies #16500-500 and #16520-100, respectively) with 1X TAE
8. Ethidium bromide (Sigma #E8751), GelRed™ Nucleic Acid Stain (Biotium #41003-1) or SYBER® Safe DNA gel stain (Life Technologies #S33102)
9. EZview™ Red ANTI-FLAG® M2 Affinity Gel (Sigma #F2426) (see Note 3)
10. Bradford reagent (Bio-Rad Protein Assay, #500-0006)
11. PCR machine (*e.g.*, Applied Biosystems 2720 Thermal Cycler, Bio-Rad iCycler, or Eppendorf Mastercycler proS)
12. Thin-walled PCR 96-well plate (Fisherbrand™ #14230232) and adhesive PCR film (Thermo Scientific™ #AB-0558) or 0.2 mL tubes (Corning #3745)
13. Refrigerated centrifuge that can accommodate conical tubes (*e.g.*, Eppendorf 5702R)
14. Thermo Scientific Sorvall MTX 150 Micro-Ultracentrifuge

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b. OUTER: 5′-GCGAGCACAGAATTAATACGACT-3′. This “OUTER” primer is depicted as the RACE primer in Figure 3A (28).

3. EZview™ Red ANTI-FLAG® M2 Affinity Gel is the anti-FLAG M2 antibody covalently linked to agarose beads. Anti-FLAG beads should be equilibrated according to the manufacturer’s instructions. To equilibrate the anti-FLAG beads, spin down the affinity gel solution at 8200xg at 4°C for 30 seconds and aspirate the supernatant being careful not to disturb the beads. Resuspend the beads in at least 10 times the packed bead volume in IP Flag buffer. Repeat this wash 4 more times. Spin the beads a final time and resuspend the beads in an equal volume of the packed bead volume in IP Flag buffer. For example, if your packed bead volume is 200 μL, resuspend the beads in 200 μL IP Flag buffer. This 50% slurry of anti-FLAG beads is the working stock of equilibrated EZview™ Red ANTI-FLAG® M2 Affinity Gel.

15. Micro-ultracentrifuge tubes (Thermo Scientific #45237)
16. Nucleic acid electrophoresis system (*e.g.*, Bio-Rad Sub-Cell GT cell #170-4401)
17. QIAquick Gel Extraction Kit (Qiagen #28706)
18. PCR product cloning kit (*e.g.*, Zero Blunt® PCR cloning kit, Life Technologies #K2700-20)

3. Methods

Detailed below is a scaled-down version of the original LEAP assay (28) as well as an alternative protocol using immunoprecipitation (18) instead of ultracentrifugation to isolate L1 RNPs. This protocol is optimized for HeLa cells but has also been used for other human cell lines (21, 42–44). Hygromycin B concentrations and transfection protocols should be optimized for any different cell lines used for this assay.

3.1 LINE-1 RNP isolation using ultracentrifugation (Figure 2A and 2B)

1. Day 1 – Plate cells: Seed 2×10^6 HeLa-JVM cells in HeLa-JVM growth medium in a T-75 tissue culture flask. Cells are grown in a humidified incubator at 37°C with 7% CO_2 (see Note 4).
2. Day 2 – Transfect cells: Cells typically are transfected 14 to 16 hours post-plating, day zero (d0) (Figure 2A), using the FuGENE® 6 transfection reagent following the manufacturer's instructions. The LEAP assay should include the following transfection conditions: 1) an empty vector (*e.g.*, pCEP4, Life Technologies); 2) a wild type LINE-1 expression plasmid (*e.g.*, pJM101/L1.3 (5)); and 3) an RT mutant LINE-1 plasmid (*e.g.*, pJM105/L1.3, which has a D702A mutation in the ORF2 RT domain (29)) (see Note 5). Prepare a transfection mix in a 1.5 mL microcentrifuge tube containing 8 μg of the pCEP4 or LINE-1 expression plasmid and 32 μL FuGENE® 6 and 500 μL Opti-MEM® I. Incubate the solution at room temperature for 20 minutes. Add the transfection mix to the growth medium of one flask of cells.
3. Day 3 – Stop the transfection: Approximately 16 to 24 hours post-transfection, one day post-transfection (d1) (Figure 2A), aspirate medium from the cells and add fresh DMEM growth medium to the cells.

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4. HeLa-JVM cells can also be seeded at 2×10^5 cells per T-25 cell culture flask or 6×10^6 cells per T-175 cell culture flask. For cells in a T-25 flask, prepare a transfection mix in a 1.5 mL microcentrifuge tube containing 1 μg of the pCEP4 or LINE-1 expression plasmid, 4 μL FuGENE® 6, and 100 μL Opti-MEM® I. For cells in a T-175 flask, prepare a transfection mix in a 1.5 mL microcentrifuge tube containing 20 to 30 μg of the pCEP4 or LINE-1 expression plasmid, 80 to 120 μL FuGENE® 6 and 1mL Opti-MEM® I. Incubate the solution at room temperature for 20 minutes. Add the transfection mix to the growth medium of one flask of cells.
5. Transcription of L1 elements being tested in the LEAP assay should be driven by the CMV promoter and have the pCEP4 backbone, which allows for episomal replication of the plasmid and hygromycin B-selection of cells containing the L1 expression construct. It is difficult to detect LEAP activity from L1 expression constructs where L1 transcription is driven by the promoter activity in the L1 5′UTR.

4. Days 5–11 – Select for transfected cells: Begin drug selection 3 days post-transfection (d3) and continue until 9 days post-transfection (d9). Grow cells in DMEM growth medium containing 200 μg/mL hygromycin B. Change the hygromycin B-containing media every day until 9 days post-transfection (d9) (Figure 2A).

5. Day 11 – Harvest the cells for ultracentrifugation: Rinse the cells once with cold 1X PBS. Aspirate the 1X PBS. Add 5 mL cold 1X PBS and scrape the cells from the flask using a cell scraper. Pellet the cells by centrifugation at 3,000xg at 4°C for 5 minutes. Transfected cells from one T-75 flask can be divided into 2 aliquots and can be stored at −80°C for at least one month without affecting reproducibility (see Note 6).

6. Lyse cells: Add 250 μL Lysis Buffer (Materials 2.2) to the cell pellets. Pipette up and down to resuspend the pellets. Let the cells sit on ice for 15 minutes. Centrifuge the cell lysates at 3,000xg at 4°C for 10 minutes. Transfer the supernatants to new clean tubes and keep on ice. A small aliquot of the whole cell lysates should be flash frozen in an ethanol/dry ice bath and stored at −80°C (see Note 7).

7. Prepare the sucrose cushion: Using the Sucrose Dilution buffer (Materials 2.4), dilute the 47% sucrose stock solution (Materials 2.3) to 8.5% and 17% sucrose solutions. Prepare 500 μL of the 17% sucrose solution and 250 μL of the 8% sucrose solution per sample. Carefully layer the sucrose solutions such that 500 μL of the 17% sucrose solution is at the bottom and 250 μL of the 8% sucrose solution is on top in a 1 mL micro-ultracentrifuge tube. On top of the 8% sucrose, gently layer 150 to 200 μL of the cell lysate. If using a fixed angle rotor, leave an empty space between samples or use sealable tubes to minimize possible cross-contamination during the spin.

8. Run the micro-ultracentrifuge: Spin the cell lysates at 168,000xg at 4°C for 2 hours.

9. Resuspend the RNP: After centrifugation, a pellet should be visible at the bottom of the tube. Aspirate off the sucrose solution, being careful not to disturb the pellet. Resuspend the pellet in 50 to 100 μL 1X Complete Protease Inhibitor Cocktail, EDTA-free (in DNase/RNase-free water) by gently pipetting up and down. Quantitate protein concentrations of RNPs using the Bradford protein assay reagent. If needed, dilute RNPs with 1X

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6. For ultracentrifugation, the entire cell pellet from a T-25 flask should be used for ultracentrifugation. The cell pellet from one T-175 flask can be divided into 4 equal aliquots and stored at −80°C.

7. MMLV-RT and western blot analyses should be done to show comparable RNA and protein levels, respectively, of the isolated L1 RNP. Similarly, RNA and L1 proteins can be analyzed from whole cell lysates and compared to the isolated L1 RNP fractions. RNA is purified from RNPs using the Qiagen RNeasy kit. The purified, DNA-free RNA is reverse transcribed and PCR amplified using the same adapters in the LEAP reactions but with MMLV-RT instead of L1 RNPs. The L1 cDNAs are PCR amplified using the same primers and conditions as listed for the LEAP PCR but for 25–30 cycles. Control cellular RNAs (*e.g.*, GAPDH) are PCR amplified using gene-specific primers (28). Western blots of RNPs expressed from pES2TE1 are performed using anti-T7 antibody (Millipore #69522-3) to detect L1 ORF1p or anti-HA (3F10 clone, Roche #11867423001) to detect L1 ORF2p (18, 30). Untagged L1 ORF1p can be detected from L1 RNPs and cell lysates using an anti-ORF1p antibody (30, 48, 51).

Complete Protease Inhibitor Cocktail, EDTA-free to a concentration of 1.5 to 2 μg/μL of total protein. To preserve the enzymatic activity upon storage, dilute the RNPs to a concentration of 0.75 to 1 μg/μL of total protein with glycerol (final 50% volume/volume glycerol concentration). Divide the RNPs into 25 to 50 μL aliquots, flash freeze in an ethanol/dry ice bath, and store the RNPs at −80°C (see Note 7).

3.2 LINE-1 RNP isolation using immunoprecipitation (Figures 2A and 2C)

1. Day 1 – Plate cells: Seed 6×10^6 HeLa-JVM cells in HeLa-JVM DMEM growth medium in a T-175 tissue culture flask.
2. Day 2 – Transfect cells: Cells typically are transfected 14 to 16 hours post-plating, day zero (d0) (Figure 2A) using the FuGENE® 6 transfection reagent following the manufacturer's instructions. The LEAP assay using RNPs isolated by immunoprecipitation should include the following transfection conditions: 1) an empty vector (*e.g.*, pCEP4, Life Technologies); 2) an epitope-tagged wild type LINE-1 plasmid (*e.g.*, pES2TE1 (18)); and 3) a non-tagged LINE-1 plasmid (*e.g.*, pJM101/L1.3 (5), which does not have any epitope tag on ORF1p or ORF2p) (see Note 5). Prepare a transfection mix in a 1.5 mL microcentrifuge tube containing 20 to 30 μg of the pCEP4 or LINE-1 expression plasmid, 80 to 120 μL FuGENE® 6, and 1 mL Opti-MEM® I. Incubate the solution at room temperature for 20 minutes. Add the transfection mix to the growth medium of one flask of cells.
3. Day 3 – Stop the transfection: Approximately 16 to 24 hours post-transfection, one day post-transfection (d1) (Figure 2A), aspirate the medium from the cells and add fresh DMEM growth medium to the cells.
4. Days 5–11 – Select for transfected cells: Begin drug selection 3 days post-transfection (d3) and continue until 9 days post-transfection (d9). Grow cells in DMEM growth medium containing 200 μg/mL hygromycin B. Change the hygromycin B-containing media every day until 9 days post-transfection (d9) (Figure 2A).
5. Day 11 – Harvest the cells for immunoprecipitation: Rinse the cells once with cold 1X PBS. Aspirate the 1X PBS. Add 10 mL cold 1X PBS and scrape the cells from the flask using a cell scraper. Pellet the cells by centrifugation at 3,000xg at 4°C for 5 minutes. Transfected cells from one T-175 flask can be divided into 2 aliquots and can be stored at −80°C for at least one month without affecting reproducibility.
6. Lyse the cells: Add IP Flag buffer (Materials 2.5) three times the volume of the cell pellet. For example, if the volume of the cell pellet is approximately 100 μL, add 300 μL of the buffer. Pipette up and down to resuspend the pellets. Let the cells sit on ice for 15 minutes. Centrifuge the cell lysates at 3,000xg at 4°C for 10 minutes. Transfer the lysates to new clean tubes and keep on ice. A small aliquot of the whole cell lysates

should be flash frozen in an ethanol/dry ice bath and stored at −80°C (see Note 7).

7. Determine the cell lysate protein concentration using the Bradford protein assay reagent.

8. Prepare immunoprecipitation: For each immunoprecipitation, incubate 3 mg of protein from the cell lysate with 20 μL of equilibrated EZview™ Red ANTI-FLAG® M2 Affinity Gel (see Note 3) at 4°C overnight on a rotating wheel or nutator (see Note 8). Collect the beads by centrifugation at 3,000xg at 4°C for 10 minutes. Resuspend the beads in 1 mL cold IP Flag buffer. Wash the beads 4 more times with 1 mL cold IP Flag buffer.

9. Elute the RNPs: Incubate the beads with 50 μL IP Flag buffer containing 200 μg/μL of 3XFLAG peptide at 4°C for 1 hour on a rotating wheel or nutator. Spin down the beads at 6,000xg at 4°C for 5 minutes and transfer the eluates to new tubes (see Note 7).

3.3 LEAP Reactions and PCR

The following reactions are performed the same for RNPs prepared by ultracentrifugation or immunoprecipitation. Reactions are prepared in a PCR workstation equipped with HEPA-filtered air circulation and UV-sterilization.

1. L1 reverse transcription reaction: Incubate 1 μL L1 RNP [0.75 to 1.0 μg] or IP sample with the LEAP reaction buffer (Materials 2.7) in a total volume of 50 μL at 37°C for 1 hour. LEAP cDNAs can be stored at −20°C.

2. PCR amplification of L1 RT-synthesized cDNA: Add 1 μL of LEAP cDNA to the LEAP PCR reaction mix (Materials 2.8) in a total volume of 50 μL (see Note 9).

3. The LEAP PCR program is as follows:

1 cycle	94°C for 3 minutes;
35 cycles	94°C for 30 seconds,
	58°C for 30 seconds,
	72°C for 30 seconds;
1 cycle	72°C for 7 minutes;
	4°C hold

4. LEAP products are visualized by electrophoresis on a 2% agarose gel (equal parts agarose to low-melt agarose) in 1X TAE stained with ethidium bromide, GelRed™, or SYBER® Safe nucleic acid stains (Figure 3B). LEAP bands can be excised, extracted using the QIAquick Gel Extraction Kit, and cloned into a commercial sequencing vector (*e.g.*, Zero

8. Cell lysates should be pre-cleared with agarose beads if immunoprecipitation is done with anti-FLAG-coupled agarose beads. Other resins, such as Dynabeads (Life Technologies), may have less non-specific protein binding and may not require pre-clearing.

9. LEAP cDNAs may also be subjected to qPCR and quantified as detailed in Kulpa and Moran, 2006 and Doucet *et al.*, 2010 (18, 28).

Blunt® PCR Cloning Kit). These plasmids can then be sequenced for characterization of individual LEAP products within one reaction.

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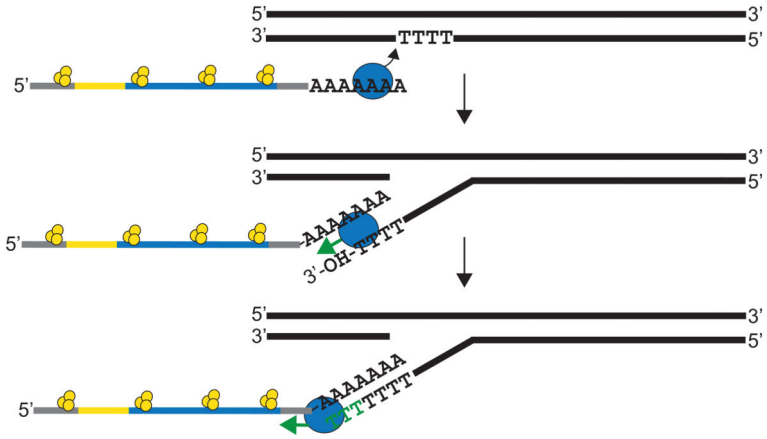

Figure 1. Target site-primed reverse transcription (TPRT)

The L1 RNA (grey-yellow-blue-grey line), ORF1p (yellow circles), and ORF2p (blue circle) minimally constitute the L1 RNP. The L1 RNP targets genomic DNA (black bold lines) and nicks one T-rich strand exposing a free 3′OH. L1 ORF2p RT uses the T-rich DNA strand as a primer for reverse transcription of the L1 RNA template. After initial priming, L1 ORF2p RT synthesizes a complementary L1 cDNA strand (green arrow) using L1 RNA as a template. Second strand synthesis and completion of integration remain to be elucidated from L1 retrotransposition but may follow the *Bombyx mori* R2 retrotransposon model of integration (52).

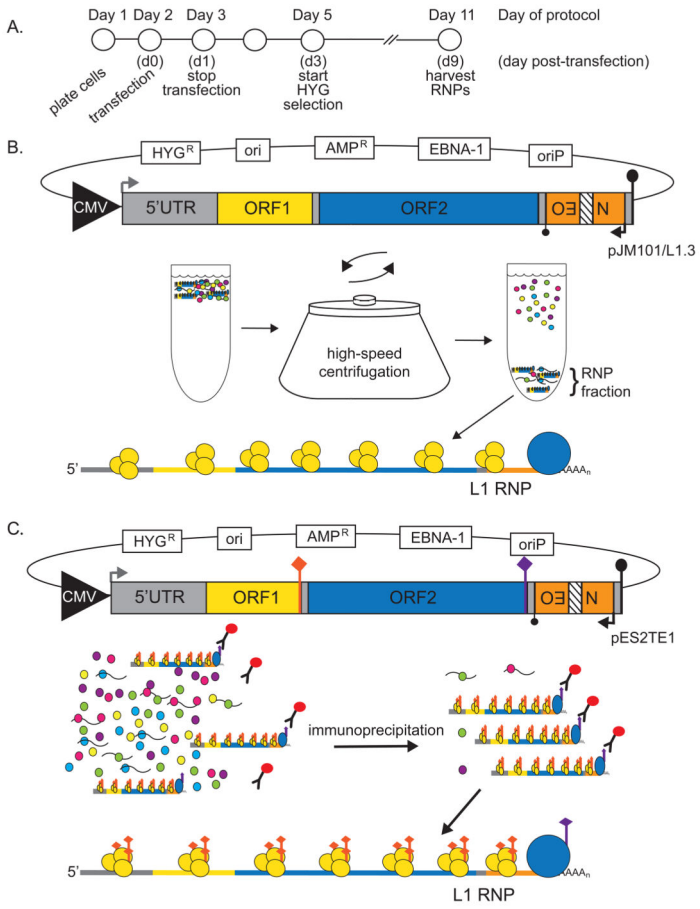

Figure 2. Enrichment of the L1 RNP

(A) A timeline of the LEAP assay is depicted and described in the Methods. Days of the protocol are noted above and the corresponding days post-transfection (d0-14) are noted below. **(B)** The pJM101/L1.3 L1 construct contains the L1.3 element (accession no. L19088). The pCEP4 plasmid (Life Technologies) backbone encodes for the EBNA-1 (EBNA-1) viral protein and contains an origin of viral replication (oriP), a hygromycin B-resistance gene (HYG^R), the cytomegalovirus (CMV) promoter (large black triangle) and an SV40 polyadenylation signal (large black lollipop) for plasmid replication, hygromycin-selection, and transcription, respectively, in mammalian cultured cells. The pCEP4 backbone also has a bacterial origin of replication (ori) and an ampicillin-resistance gene (AMP^R) for replication and ampicillin-selection (respectively) in *E.coli*. (For details of the *mneoI* reporter cassette, please see the "LINE-1 Cultured Cell Retrotransposition Assay" chapter in this volume.) After transfection and hygromycin B-selection in HeLa-JVM cells, the cells are lysed and subjected to high-speed centrifugation through a sucrose cushion. After centrifugation, cellular RNPs are enriched in the pellet fraction. This fraction contains the L1 RNA bound by L1 ORF1p and ORF2p, which minimally constitutes the L1 RNP (see Figure 1). **(C)** The pES2TE1 L1 construct (18), like pJM101/L1.3, contains the L1.3 element in a pCEP4 (Life Technologies) backbone. Unlike pJM101/L1.3, the pES2TE1 construct encodes a T7-tagged (orange diamond) ORF1p and a FLAG-HA-tagged (purple diamond) ORF2p (18). After transfection and hygromycin B selection, HeLa cells are lysed

and subjected to immunoprecipitation using an anti-FLAG antibody conjugated to beads (red circle with black “Y”). Immunoprecipitated complexes contain L1 ORF1p and ORF2p bound to its encoding RNA, which minimally constitutes the L1 RNP.

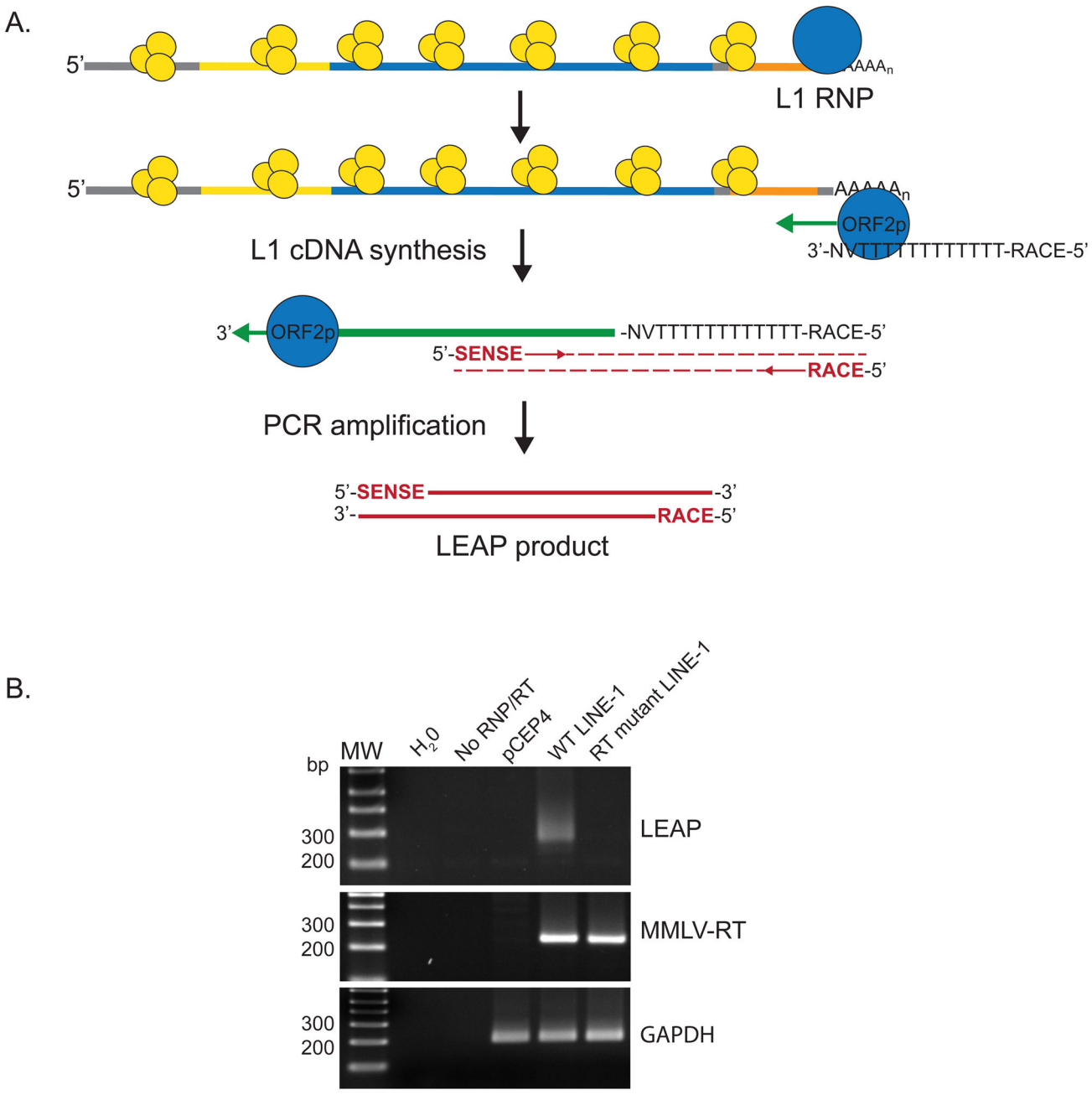

Figure 3. The LEAP Assay

(A) The L1 RNP minimally consists of L1 ORF1p (yellow circles) and ORF2p (blue circle) bound to the L1 RNA (multicolored line). The L1 RNP is incubated with a 5′-RACE-T_{12}VN-3′ primer and dNTPs. The L1 RT activity (green arrow) of ORF2p initiates L1 cDNA (green line) synthesis. Subsequently, the L1 cDNA is amplified using an engineered LINE-1 construct-specific primer (SENSE) and a RACE primer (red arrows), resulting in the LEAP product (double red line). **(B)** LEAP products can be resolved by electrophoresis and visualized by staining. LEAP products (top panel), L1 RNA present in RNPs (MMLV-RT; middle panel), and GAPDH RNA levels (GAPDH; lower panel) are shown. A standard

LEAP assay includes a water control PCR reaction (H_20); a control reaction without any RT (No RNP/RT); a reaction using RNPs from empty vector-transfected cells (pCEP4); a reaction with wild type L1 RNPs (WT LINE-1); and a reaction with RT-mutant L1 RNPs (RT mutant LINE-1). The molecular weight (MW) ladder sizes are shown in base pairs (bp). L1 ORF1p and ORF2p protein levels can also be detected by western blot analyses (not shown) (18, 28).