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Purification of L1-ribonucleoprotein particles (L1-RNPs) from cultured human cells

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Abstract

Almost two third of the human genome is repetitive DNA, mostly derived from different kinds of transposon and retrotransposon sequences. Although most of these sequences are stable in the genome, one class called Long INterpersed Element (LINE1 or L1) is actively jumping in the human genome, particularly in brain, germ cells and certain types of cancer. Recent estimates predict that L1 activity combined with L1-mediated activity is responsible for a new insertion in 1 out of 25 newborns. In humans, more than 100 single-gene disease cases have been reported due to L1 activity. An active L1 encodes two proteins designated as ORF1p and ORF2p. L1 jumps by a target primed reverse transcription (TPRT) mechanism where L1 RNA forms L1-RNPs after binding with L1 proteins. L1-RNPs then enter into the nucleus where L1 RNA is converted to cDNA at the site of integration which subsequently integrates into the genome with the help of the L1 proteins (ORF1p and ORF2p) and other cellular factors. Although L1 is continuously jumping in the human genome the basic mechanism and requirement of other cellular factors in L1 retrotransposition is relatively unknown due to the difficulty in purifying intact L1-RNPs. Here we describe a detailed protocol for purification of L1-RNPs by an immunoaffinity method.

Keywords

Retrotransposons; L1 RNP; L1 RNA; L1ORF1p; L1ORF2p; LEAP

2. Introduction

Retrotransposons occupy the genomes of virtually all eukaryotes and are actively jumping in the genomes of human and mice (1, 2). In humans, the master retrotransposon is L1, while other elements Alu, SVA, and processed pseudogenes depend upon L1 retrotransposon machinery for their mobility. An active L1 is 6.0 kb in length, and contains two open reading frames (ORFs), designated ORF1 and ORF2, separated by a small inter-ORF spacer

sequence (Figure 1). ORF2 encodes a protein with reverse transcriptase (RT) (3) and endonuclease (EN) (4) activity whereas ORF1 encodes a protein with demonstrated single stranded RNA binding and nucleic acids chaperone activity (5). Although the functions of the ORF-encoded proteins are poorly understood, both proteins are critical for the process of L1 retrotransposition (6). L1 retrotransposition is highly tissue specific, while retrotransposition is observed in germ cells, neurons and in certain types of cancers (7–10).

It is believed that the process of retrotransposition starts with transcription followed by transport of L1mRNA into the cytoplasm where L1mRNA binds with its own encoded proteins (11) along with certain cellular proteins to form an L1-ribonucleoprotein particle (L1-RNP), the retrotransposition intermediate. L1-RNPs then enter into the nucleus where at the genomic target site L1cDNA is synthesized by the RT activity of ORF2p. Subsequently, with the help of L1 proteins (ORF1p and ORF2p) and other unknown cellular proteins a new L1 copy is inserted at the genomic target site. The process of reverse transcription and integration into the genome is named target primed reverse transcription (TPRT) (12, 13), and is not well understood due to a lack of knowledge of the participating proteins that complete the process.

Insertional mutagenesis occurs when a retrotransposon inserts into or near a gene and thus alters the gene's expression or results in the production of mutated proteins. Kazazian et al (14) first reported a human disease, hemophilia A, caused by *de novo* insertions of active L1s that disrupted the factor VIII gene. There are now ~100 cases in which L1 retrotransposon activity is responsible for single gene disease in human (15). Although L1 is continuously jumping in the human genome, many aspects of the basic mechanism of L1 retrotransposition remain unknown due to lack of knowledge concerning the components present in the L1-RNPs, the retrotransposition intermediate, and their action. Sucrose density gradient centrifugation was first used to purify mouse L1 RNPs from mouse cell line (16). Subsequently, the same technique was used to purify L1-RNPs from human cultured cells after transfecting an engineered disease-causing active full length L1 (17–19). Using this method, it was first demonstrated that basal L1 RNPs contain ORF1p, ORF2p and L1 RNA (19). Although informative, this method has a high likelihood of co-purifying other cellular particles that co-sediment with the L1 RNP during the process of centrifugation. Recently, immunoaffinity purification has been employed by several groups to purify L1-RNPs followed by mass spectrometry and RNA sequence analysis to identify the protein (20, 21) and RNA components of the complex (22). By using this method a few molecules with a role in L1 retrotransposition have been identified (20, 21). Here we describe the detailed protocol for purification of L1-RNPs by an immunoaffinity method.

3. Materials

3.1. Cell culture

1. High glucose Dulbecco's modified Eagle medium (DMEM) without pyruvate and glutamine.
2. L-Glutamine (200 mM) (Invitrogen)

3. Penicillin-Streptomycin (10,000 U/ml) (Gibco) (HEK 293T cells were maintained in a tissue culture incubator at 5% CO₂, 37°C in high glucose Dulbecco's modified Eagle medium (DMEM) without pyruvate (Invitrogen) supplemented with 10% fetal bovine calf serum, 2 mM L-glutamine and 100U/ml Penicillin-Streptomycin)

3.2. Components for transfection

1. Fugene 6 (Promega)
2. Lipofectamine (Invitrogen)
3. Opti-MEM reduced serum medium (Gibco)
4. Plasmid maxi kit (Qiagen)

3.3. Reagents for Immunoprecipitation, Western blotting and Northern blotting

1. Recombinant RNasin® Ribonuclease Inhibitor (20–40 U/ul) (Promega; Cat # N2511)
2. Protease inhibitor cocktail (Sigma; Cat # P8340)
3. Anti-FLAG agarose beads (Sigma; A2220)
4. 3X FLAG peptide (Sigma; F4799)
5. HEPES (1M) (Gibco; Cat # 15630-130)
6. KCl (2M) (Ambion; Cat # AM9640G)
7. MgCl₂ (1M) (Ambion; Cat # AM9530G)
8. NP-40
9. Dithiothreitol (DTT)
10. Glycerol
11. 4X Laemmli buffer (200 mM Tris-Cl (pH-6.8), 400 mM DTT, 8% SDS, 40% Glycerol, 0.08% (w/v) bromophenol blue).
12. NuPAGE® Novex® 4–12% Bis-Tris Protein Gels, 1.0 mm, 12 well (Cat # NP0321)
13. NuPAGE gel running buffer
14. PVDF membrane (Bio-rad; Cat # 162-0177)
15. Protein transfer buffer (39 mM Glycine, 48 mM Tris-base, 0.037% SDS, 20% methanol)
16. Non-fat dry milk (Bio-rad)
17. 1X Tris buffered saline with Tween-20 (TBST) [20 mM Tris-Cl (pH-7.5), 150 mM NaCl, 0.1% Tween-20].
18. West Pico Chemiluminescent Substrate (Thermo Scientific, Cat # 34080)

19. X-ray film
20. X-ray film developing reagent
21. Primary antibody to detect ORF2p (22)
22. Anti-FLAG antibody (Sigma; Cat# F1804)
23. Secondary antibody; ECL Rabbit IgG, HRP-Linked Whole Ab (from donkey) (GE healthcare; Cat # NA934)
24. Secondary antibody; ECL Mouse IgG, HRP-Linked Whole Ab (from sheep), (GE healthcare; Cat # NA931)
25. Anti-N terminus L1 ORF2p antibody (23)
26. SP6 RNA polymerase (Roche)
27. NorthernMax Gly Sample Loading Dye(Ambion; Cat # AM8551)
28. Northern Max Gly Kit (Ambion; Cat # AM-1946)
29. Nylon membrane, positively charged (Roche; Cat #11209299001)
30. DIG Easy Hyb Granules (Roche; Cat # 11796895001)
31. DIG RNA labeling mix (Roche; Cat # 11277073910)
32. DIG wash and block buffer set (Roche; Cat # 11585762001)
33. Anti-DIG -AP, Fab fragments (Roche; Cat # 11093274910)
34. CDP-Star ready to use (Roche; Cat# 12041677001).
35. 1X Dulbecco's Phosphate-Buffered Saline (DPBS) (2.67 mM KCl, 1.47 mM KH_2PO_4 , 138 mM NaCl, 8.06 mM $\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$)
36. DNaseI recombinant, RNase-free (Roche; Cat # 04716728001)
37. Anti-FLAG agarose beads wash buffer [100mM KCl, 5mM MgCl_2 , 10 mM HEPES (pH-7.0)]
38. Cell lysis buffer [100mM KCl, 5mM MgCl_2 , 10 mM HEPES (pH-7.0), 0.5% NP-40, 0.5mM DTT]
39. Wash buffer 1 [25 mM HEPES (pH-7.0) 250 mM KCl, 5mM MgCl_2 , 0.1% NP-40]
40. Wash buffer 2 [25 mM HEPES,(pH-7.0), 100 mM KCl, 5mM MgCl_2 , 0.1% NP-40]
41. Go Taq green master mix (Promega),
42. DIG RNA labeling mix (Roche)
43. 1X Glyoxal RNA loading buffer (Ambion)
44. 10X DNA gel loading buffer (Invitrogen; Cat # 10816-015)
45. Topo TA cloning Kit (Invitrogen; Cat # K4550-01)

46. RNP elution buffer (wash buffer 2 containing 150 µg/ml 3X FLAG peptide).

3.4. Primers for synthesizing DIG labeled L1 RNA probe

1. BGH-SP6rev: 5' -
ATTTAGGTGACACTATAGACTCATTTTATTAGGAAAGGACAG-3'
2. BGHfwd: 5' -ATCTCAGAAGAGGATCTGAATATGC-3' (Bold sequences denote SP6 promoter primer sequence).

3.5 Primers for LEAP reaction

1. LEAP primer: 5' -GCGAGCACAGAATTAATACGACTGGTTTTTTTTTTTTT-3'
2. L13' -Fwd: 5' -CCCGCTGATCAGCCTCGACTG-3'
3. LinkerRev: 5' -GCGAGCACAGAATTAATACGACT-3'

4. Methods

4.1. Transient transfection of epitope-tagged L1 construct and preparation of cell lysate

The procedure described here is used to purify L1-RNPs by an immunoaffinity technique following transient transfection of an epitope-tagged disease-causing L1 (Figure 1) into HEK293T cells. We used a single FLAG epitope which was cloned in the C-terminus of ORF1p in a full length L1 (Figure 1) (22). We choose HEK293T cells because this cell line grows very quickly, is very efficient in transient transfection and displays a high level of L1 retrotransposition. A 100 cm culture disc is sufficient to purify and detect the basal components in L1-RNPs, i.e., ORF1p and ORF2p, by Western blotting, transfected L1RNA by Northern hybridization, and ORF2p-mediated RT activity by the **LINE Element Amplification Protocol** (LEAP) assay (19). To purify other RNA and protein components of the L1 RNP, the procedure can be scaled up by growing cells in large vessels with increased surface areas.

1. Eight to 12 hours before transfection, plate 1.8×10^6 cells in a 100 mm disc containing 10 ml DMEM media to achieve ~50% confluency at the time of transfection.
2. Both Fugene 6 and Lipofectamine are equally effective in transfecting DNA into HEK293T cells. To use Lipofectamine as the transfecting reagent, in one tube place 600 µl of optimem reduced serum media and mix with 10 µg of DNA (The plasmid DNA should be in supercoiled form and freshly prepared using a Qiagen midi or maxi prep kit). Incubate the tube for 5 minutes.
3. In another tube, place 600 µl of optimem reduced serum media and mix with 20 µl lipofectamine reagent. Incubate the tube for 5 minutes.
4. Combine the diluted DNA and diluted lipofectamine and incubate for 20 minutes at room temperature (21°C).
5. Take the cells from the CO₂ incubator. Using a 1 ml pipette, add the DNA-transfection reagent mix to the cells drop by drop. (HEK293T cells are weakly

attached to the surface; care should be taken to add the DNA-transfection reagent mixture gently).

6. Swirl the disc to ensure distribution over the entire plate surface. Incubate cells in the presence of 5% CO₂ for 40–48 hours at 37°C. (To use Eugene 6 as transfection reagent, take 600 µl of serum free media in a 1.5 ml tube, add 18 µl Eugene 6, mix by pipetting 2–3 times and incubate for 5 minutes. After 5 minutes, add 8 µg DNA to the tube, pipette gently 2 to 3 times to mix all the components together and then incubate for another 15 minutes. After the final incubation, remove the culture vessel from the incubator and add the mixture drop by drop to the cells. Swirl the disc to ensure distribution over the entire plate surface. Incubate the cells in the presence of 5% CO₂ for 40–48 hours at 37°C).
7. After incubation, take the disc to the workbench, decant the media into a 500 ml plastic beaker which will be used for liquid disposal.
8. Wash the cells with cold 10 ml 1X Dulbecco's Phosphate-Buffered Saline (DPBS) solution once. Scrape cells by adding 5 ml cold 1XDPBS and transfer them to a chilled 15 ml tube. Harvest the cells by centrifugation at 2500 rpm at 4°C for 5 minutes.
9. Remove 1XDPBS solution without disturbing the pellet and add 1 ml cold lysis buffer [100mM KCl, 5mM MgCl₂, 10 mM HEPES (pH-7.0), 0.5% NP-40, 0.5mM DTT] containing RNase inhibitors (1unit/µl) and protease inhibitors cocktail (10 µl for 1 ml; Sigma). Mix cells with the lysis buffer by pipeting 6–8 times with a one ml pipette and transfer the entire volume to a chilled 1.5 ml tube.
10. Incubate the tube on ice for 15 minutes with intermittent mixing (by inverting the tube 2–3 times) for lysis of cells. Centrifuge the lysate at 12,000 rpm for 10 minutes at 4°C.
11. Collect the supernatant in a fresh chilled 1.5 ml tube without disturbing the pellet. For immediate use keep the tube on ice. If the supernatant is not be used immediately, flash freeze with liquid nitrogen and store at –70°C.

4.2. Preparation of Anti-FLAG agarose beads

1. We recommend using Anti-FLAG agarose beads from Sigma (Cat # A2220) for the purification of L1-RNPs. The Anti-FLAG agarose beads are supplied in 50% glycerol with buffer. Thoroughly resuspend the resin by gently inverting the container several times.
2. Transfer 40 µl Anti FLAG agarose gel suspension using a blunt cut 200 µl tip into a 1.5 ml tube. (40 µl bead suspension is equivalent to 20 µl packed gel beads which will efficiently bind ~2 µg FLAG-tagged protein).
3. Add 1 ml cold beads in wash buffer [100mM KCl, 5mM MgCl₂, 10 mM HEPES (pH-7.0)], mix with resin by inverting the tube several times and then centrifuge

at 2000 rpm for 1 min at room temperature (RT). Keep the tube on ice for 2 minutes to settle the beads at the bottom of the tube. Remove the wash buffer without disturbing the resin. Repeat the washing steps twice more.

4.3. Immunoprecipitation of L1-RNPs

1. Transfer 1 ml cell lysate to the washed resin. Incubate the tube at 4°C for one hour with gentle shaking either in a platform shaker or in an overhead mixing device (Beads should not settle during the incubation).
2. After incubation, centrifuge the tube at 2000 rpm for one min at 4°C, and incubate the tube on ice for 2 minutes to settle the beads. Discard the supernatant carefully without disturbing the beads.
3. Add 1 ml wash buffer 1 [25 mM HEPES (pH-7.0) 250 mM KCl, 5mM MgCl₂, 0.1% NP-40], mix with the beads gently by inverting several times. Repeat centrifugation to settle the beads and remove the supernatant. Wash an additional four times with wash buffer 1 and one time with wash buffer 2 [25 mM HEPES, (pH-7.0), 100 mM KCl, 5mM MgCl₂, 0.1% NP-40].
4. Remove wash buffer 2, add 100µl elution buffer (wash buffer 2 containing 150 µg/ml 3X FLAG peptide) and incubate the tube for 20 minutes with gentle shaking on a platform shaker.
5. Collect the supernatant which contains the L1-RNPs by centrifugation 2000 rpm for 1 minute and store in aliquots in 15% glycerol at -70°C if the RNPs are not to be used immediately (L1RNPs are stable for at least two months from the date of preparation).

4.4. Detection of ORF1p and ORF2p by Western blot analysis

1. To detect ORF2p, take 20 µl RNP elute, add 5 µl 4x Laemmli buffer and heat at 95°C for 5 minutes.
2. Centrifuge the tube at 12,000 rpm in a microcentrifuge for 30 seconds.
3. Resolve samples on NuPAGE 4–12 % Bis-Tris Polyacrylamide gel, 1 mm thick (Invitrogen) in an X-Cell Sure Lock mini cell following the manufacturer's instruction.
4. Following electrophoresis, transfer the proteins to a PVDF membrane (Bio-Rad) using a Xcell II blot module at 25 V for 1 hour.
5. Block the membrane in 1X Tris buffered saline with Tween-20 (TBST) containing 5% w/v non-fat dry milk at room temperature for 1 hour.
6. Dilute the primary antibody for ORF2p (23) in 1X TBST with 5% w/v non-fat dry milk and incubate overnight at 4°C.
7. Rinse the blot 3 times for 10 minutes with TBST.
8. Dilute the secondary antibody (GE healthcare) in 1X TBST with 5% w/v non-fat dry milk and incubate 1 hour at room temperature.

9. Rinse the blot 3 times for 10 minutes with 1X TBST.
10. Develop the blot using West Pico Chemiluminescent Substrate (Thermo Scientific).
11. Expose the blot to X-ray film or imaging system.
12. To detect ORF1p use 4 µl RNP elute. Use anti-FLAG antibody (Sigma) to detect ORF1p. Use the RNPs purified after transfecting with pcDNA6 (vector control) as a control.

4.5. Preparation of DIG labeled L1 RNA probe

1. PCR amplify the Bovine Growth Hormone (BGH) poly A signal sequence present in pcDNA6/myc-His B (Invitrogen) using primer pairs BGH-SP6 rev (5'-**ATTTAGGTGACACTATAG**ACTCATTTTATTAGGAAAGGACAG-3' and BGHfwd (5'-ATCTCAGAAGAGGATCTGAATATGC-3'). Bold sequence denotes SP6 promoter primer sequence. Note, SP6 promoter primer sequence is attached with reverse primer which will make antisense RNA complementary to BGH poly A sense signal sequence if the PCR DNA template is used to transcribe by SP6 RNA polymerase.
2. Set up the PCR reaction in a 25 µl reaction volume containing 1 µl pcDNA6 template (5–10 ng DNA), 12.5 µl 2X Go Taq green master mix (Promega), 1ul BGH fwd (20 µM), 1ul BGH-SP6rev (20 µM) and 9.5 µl water.
1. Use PCR conditions of one cycle at 94°C for 30 sec followed by 30 cycles at 94°C for 20 sec, 58°C for 20 sec and 72°C for 20 seconds and finally one cycle at 72°C for 2 minutes.
3. Resolve products in 2.0 % agarose electrophoresis, Excise the band and gel extract DNA using Qiagen gel extraction kit.
4. Take 100 ng purified PCR DNA template to generate digoxigenin (DIG)-11-UTP labeled antisense BGH poly A signal sequence RNA probe. Perform a transcription reaction in 20 µl volume containing 2 ul template DNA (100 ng), 2µl 10X DIG RNA labeling mixture (Roche), 2 µl 10X SP6 RNA polymerase buffer, 1 µl SP6 polymerase and 13 µl nuclease free water.
5. Incubate tube at 37°C for one hour.
6. Add 1 µl RNase free DNase (10 U/µl) to remove template DNA.
7. Incubate the tube for 15 minutes at 37°C.
8. Stop the reaction by adding 2 µl 0.2 M EDTA (pH 8.0).
9. Take 2 µl reaction and mix with 13 µl 1X DNA gel loading buffer. Incubate tube at 65°C for 15 minutes to denature RNA.
10. Chill the tube on ice for 5 minutes. Run the sample in a 1.5% neutral agarose gel to check the quality and quantity of the RNA probe (The yield of RNA should be around 200 ng/µl).

11. Store the DIG-labeled RNA probe at -70°C until use. Use 1 μl probe per 10 ml pre-hybridization solution.

4.6. Detection of engineered L1 RNA by Northern blot analysis

1. Take 50 μl of purified L1-RNP, increase its volume to 250 μl by adding RNase free water.
2. Add 250 μl trizol reagent, vortex for 15 seconds, and incubate at room temperature for 3 minutes.
3. Add 100 μl chloroform, vortex for 15 seconds and again incubate for 2 minutes at room temperature.
4. Centrifuge at 12,000 rpm for 5 min at 4°C .
5. Transfer upper aqueous phase to another tube, add 250 μl 100% isopropanol and 1 μl glycogen from 20 mg/ml stock (Roche). Incubate the tube at room temperature for 10 minutes.
6. Centrifuge at 12,000 rpm for 10 minutes at 4°C to precipitate RNA.
7. Wash the RNA pellet with 500 μl 70% ethanol by centrifugation at 12,000 x rpm for 5 minutes at 4°C .
8. Dry the RNA pellet in a 37°C incubator for 3–4 minutes. Dissolve RNA in 20 μl 1X NorthernMax Gly Sample Loading Dye (Ambion).
9. Denature the RNA by incubating at 68°C for 5 minutes. Chill the tube on ice for 5 minutes before separating RNA on a 0.8% denatured agarose gel. Use NorthernMax Gly Kit (Ambion) for running the gel and blotting the RNA onto a nylon membrane (Roche).
10. To UV-crosslink the RNA, place the membrane in a hybridization tube and add 10 ml preheated DIG Easy Hybe Granules (Roche). Rotate the tube in a hybridization oven at 68°C for one hour for prehybridization.
11. Hybridize overnight at 68°C with a BGH antisense RNA probe (150 nucleotides) labeled with digoxigenin (DIG)-11-UTP.
12. Wash membrane using DIG Wash and Block Buffer Set (Roche) as per manufacturer's instruction.
13. Immunodetect RNA with Anti-Digoxigenin-AP, Fab fragments (Roche).
14. Apply the chemiluminescent substrate CDP-Star ready to use (Roche) to the blot following manufacturer's suggestions.
15. Capture the chemoluminescent signals using X-ray film or imaging system (Figure 1B; panel 3).

4.7. Detection of ORF2p mediated Reverse Transcriptase (RT) activity in purified L1-RNP by LINE Element Amplification Protocol (LEAP Ref)

- 2 Set up reaction in a 20 µl reaction volume containing 1 µl L1-RNP, 4 µl 5X buffer [250 mM Tris-HCl (pH 8.3), 400 mM KCl, 20 mM MgCl₂], 1 µl dNTPs (10 mM), 1 µl LEAP primer (50 µM), 0.5 µl RNasein (20U/µl), 2 µl DTT (0.1M) and 10.5 µl nuclease free water.
- 3 Incubate tube at 37°C for 1 hour.
- 4 Perform PCR reaction in 25 µl total volume containing 1 µl RT reaction, 0.5 µl linker specific reverse primer (LinkerRev) (10 µM), 0.5 µl transcript specific forward primer (L13'-Fwd) (10 µM), 12.5 µl 2X Go Taq green master mix (Promega) and 10.5 µl water.
- 5 Use PCR conditions of one cycle at 94°C for 30 sec followed by 35 cycles at 94°C for 20 sec, 56°C for 20 sec and 72°C for 20 seconds and finally one cycle at 72°C for 2 minutes.
- 6 Resolve products in a 2.0% agarose gel (Figure 1B; panel 4).
- 7 Excise bands, gel extract, and clone in a TOPO TA cloning vector.
- 8 Check clones by colony PCR using M13F and M13R
- 9 Resolve colony PCR products in 1.2% agarose gel, band excise, and sequence using M13R primer.
- 10 Analyze LEAP product sequences that show L1 3' end with long poly A sequences (Figure 1c).

5. Notes

1. HEK293T cells should be healthy and 30–50% confluent before transfection.
2. The plasmid DNA used for transfection should be freshly prepared and in supercoiled form.
3. Anti-FLAG agarose beads need to be resuspended thoroughly. 20 ul packed beads is sufficient for cells obtained from one 100 mm disc. Use of more beads will show non-specific interaction.
4. For immunoprecipitation of L1-RNPs one hour incubation of cell lysate with Anti-FLAG agarose is sufficient. Longer incubation leads to non specific interaction and also has chance of RNA degradation.
5. It is recommended to use flat gel loading tip to remove buffer from anti-FLAG agarose beads (pore size of the tips are smaller than the size of agarose beads thus beads will not lose during repeated washing steps)
6. Never dry anti-FLAG agarose beads during purification of L1-RNPs.
7. It is recommended to use RNase free lab space and take extreme precaution to avoid any RNase contamination during the purification of L1-RNPs.

8. All chemicals and solution used to purify L1-RNps must be RNase free.

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Figure 1.

Epitope tag L1 for L-RNP purification. A) Disease causing full length L1RP (24) containing a FLAG epitope at the C-terminus of ORF1p (FL-O1F) was cloned between the CMV promoter and the BGH polyA signal sequence in pcDNA6 (Invitrogen). B) Detection of ORF1p, ORF2p, L1RNA and ORF2 RT activity in L1-RNPs. Panel 1: ORF2p was detected by anti-ORF2p N-terminal antibody (23). Panel 2: ORF1p was detected with anti-FLAG antibody. Panel 3: L1 RNA detected by Northern blot analysis -lanes containing 600 ng and 150 ng total RNA isolated from RNPs after transfecting FL-O1F and pcDNA6 into 293T cells, respectively. DIG-labeled BGH anti-sense RNA probe (200 bp) (marked thick line, Figure 1A) detects 6.2 kb L1 RNA transcribed from the engineered L1 construct (FL-O1F). Panel-4: L1 ORF2p reverse transcriptase activity on L1 RNA detected by the LEAP assay (19). L1-RNPs were incubated with LEAP RT primer that contains a unique 20 nt linker sequence at the 5'-end followed by a 12 nt poly T sequence. The resultant cDNA was then amplified by an L1 3'-end specific forward primer (L1 3'-Fwd) and a linker specific reverse primer (Linker Rev) and resolved on 2.0% agarose gel. C) Sequence of a LEAP clone.