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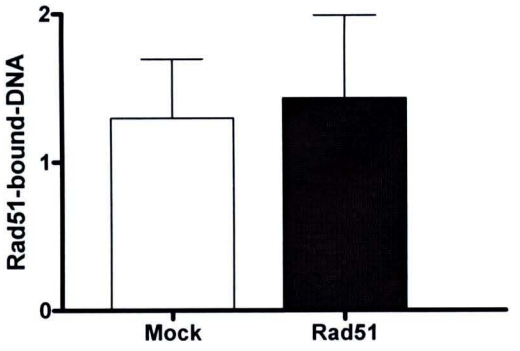
APPENDICES

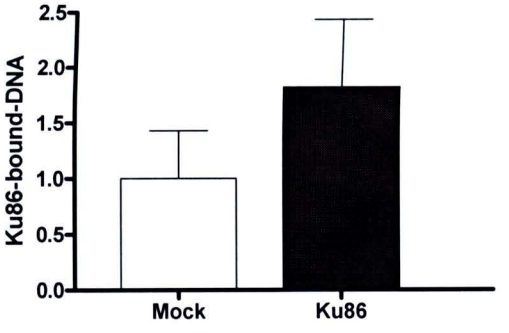
APPENDIX A

Detect Ku86 and Rad51 bound DNA by ChIP assay

Homologous recombination (HR) and Non-homologous end joining (NHEJ) are the main DSB repair pathway in cell. Rad51 and Ku86 are the key protein in HR and NHEJ, respectively. To mature DSB repair in each pathway, we detected the quantity of Rad51-bound and Ku86-bound DNA by Chromatin immunoprecipitation (ChIP) assay. Subsequently, precipitated Rad51 and Ku86-bound DNA was detected the quantity by realtime PCR with 5´L1 primers, termed realtime 5´L1PCR. From this data show that DNA precipitated by Rad51 or Ku86 was not 2 fold greater than DNA precipitated by normal mouse IgG antibody like γ-H2AX-bound DNA in previous study. Suggesting that Rad51 and Ku86-bound DNA shorter than γ-H2AX-bound DNA. Therefore the possibility to precipitate Rad51 and Ku86-bound may lower too.



Level of Rad51-bound DNA in HeLa cells.



Level of Ku86-bound DNA in HeLa cells.

Chromatin immunoprecipitation (ChIP)

The ChIP assay was performed essentially as previously described with some modifications. Histone cross-linking to DNA was induced by adding formaldehyde directly to culture medium at a final concentration of 1%, with incubation at 37 ^{o}C for 10 min. After stopping the reaction with glycine (0.125 M final concentration) and incubation for 5 min at room temperature, adherent cells were washed twice with ice-cold PBS, and then scraped into ice-cold PBS containing protease Inhibitor. Non-adherent cells were collected by centrifugation for 4 min at 510 g at 4 ^{o}C and washed as above. Nuclei were isolated by resuspending the cell pellet in cell lysis buffer (5 mM PIPES pH 8.0, 85 mM KCl, 0.5% Nonidet P-40, containing protease Inhibitor) and incubated on ice for 20 min. Intact nuclei were collected by centrifugation at 3,210 g for 5 min at 4 ^{o}C, resuspended in nuclear lysis buffer (1% SDS, 50 mM Tris-HCl pH 8.1, 10 mM EDTA, containing protease Inhibitor), and incubated on ice for 10 min. Chromatin was sheared with an Ultrasonics sonicator at 30% power output for four 30 s intervals on ice to an average size of 500–1000 bp. After centrifugation at 21,720 g for 10 min at 4 ^{o}C, the chromatin solution was diluted 10-fold with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, containing protease Inhibitor) and then precleared for 30 min at 4 ^{o}C with protein G Plus-Agarose with rotation. The agarose beads were pelleted for 1 min at 180 g and the chromatin fragments were immunoprecipitated overnight at 4 ^{o}C with Rad51 monoclonal antibody, Ku86 monoclonal antibody or normal mouse IgG antibody as a negative control on a rotator. Protein-DNA-antibody complexes were isolated by the addition of protein G Plus-Agarose. After 2 h, agarose beads were collected by centrifugation at 120 g for 1 min, washed once each in 500 mM, 550 mM and 600 mM high-salt wash buffers (0.1% SDS, 1% Triton X-100, 20 mM Tris-HCl pH 8, 2 mM EDTA, 500–600 mM NaCl), and twice in wash buffer (100 mM Tris-HCl pH 8, 500 mM LiCl, 1% Nonidet P-40, 1% deoxycholic acid). Complexes were eluted with elution buffer (50 mM NaHCO3, 1% SDS) for 15 min at room temperature. Cross-links were reversed by adding NaCl (200 mM final concentration) and RNA was removed by adding 10 mg/ml of RNase A, followed by incubation for 4 h at 65 ^{o}C, and then precipitated overnight with ethanol. Samples were deproteinized with proteinase K. After phenol/chloroform extraction, the DNA was

precipitated with ethanol. The precipitated DNA was subjected to realtime 5´L1PCR and COBRA-L1.

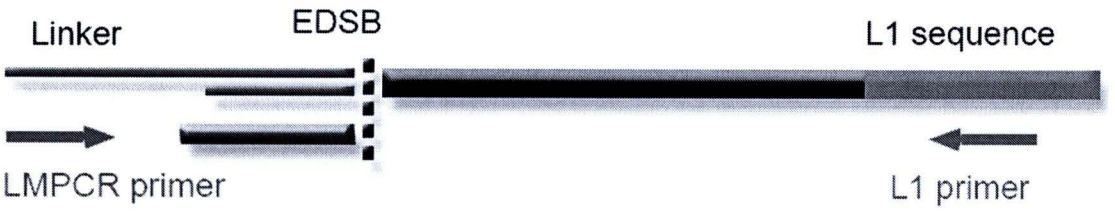
Realtime 5´ L1 PCR

Quantification of the amount of immunoprecipitated DNA was carried out by realtime PCR using SYBR Green according to the manufacturer's instructions. Briefly, 1x QuantiTect SYBR Green PCR Master Mix, 0.2 µM forward primer (L1.2HpaIIRFLPF: 5´-CTCCCAGCGTGAGCGAC-3´), and 0.2 µM reverse primer (5´LIDSIP1st: 5´-ACTCCCTAGTGAGATGAACCCG-3´) were used for each PCR assay. The PCR program was initiated at 95 ^{o}C for 15 min to activate the HotStarTaq DNA polymerase, followed by 50 thermal cycles of 15 s at 95 ^{o}C, 20 s at 57 ^{o}C and 20 s at 72 ^{o}C. A melting curve test (68 ^{o}C) was always carried out after the final reaction step to confirm that appropriate amplification products were obtained. Each sample was analyzed in triplicate PCR reactions. One sample with less than double the amount of the mock control was excluded.

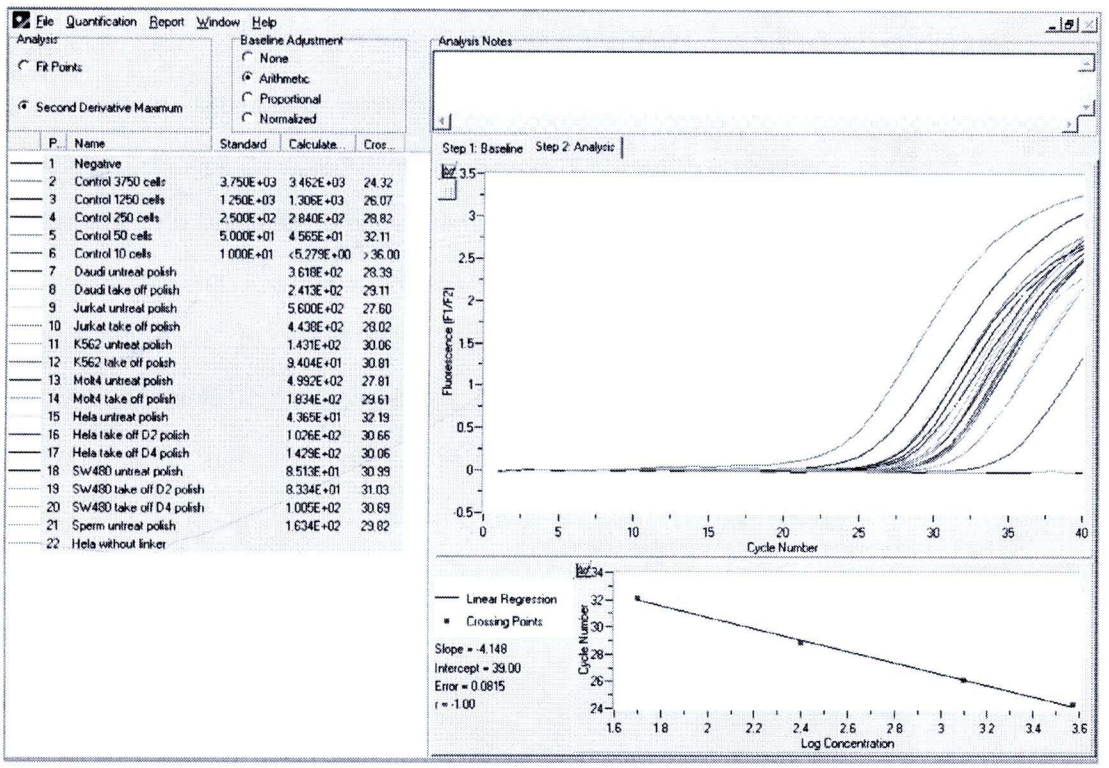
APPENDIX B

LM-PCR and COBRA-L1 EDSB method

In order to determine quantity of EDSB, we performed a technique for the detection of EDSBs, called L1-EDSB-LMPCR. Locus-specific EDSBs can be detected using ligation-mediated polymerase chain reaction (LMPCR) (20), a commonly used PCR technique designed for the analysis of EDSBs during lymphoid development, such as V(D)J recombination and somatic hypermutation (21). Since general EDSBs are believed to occur rarely and randomly throughout the genome, repetitive sequences that widely intersperse in the human genome can be applied in a similar assay for the detection of EDSBs in their proximity, which would represent genome-wide EDSBs. Therefore, we combined LMPCR with interspersed repetitive sequence PCR (IRSPCR) (22) using LINE-1 (L1) human retrotransposons (54) (L1-EDSB-LMPCR). In this assay, linker oligonucleotides are ligated to EDSBs in high molecular weight DNA preparation and quantitatively analyzed by realtime PCR using an L1 primer and a Taqman probe complementary to the linker. The figure 3 showed an example of results of L1-EDSB-LMPCR by using realtime PCR. It indicated that EDSBs could be quantitated by this technique.



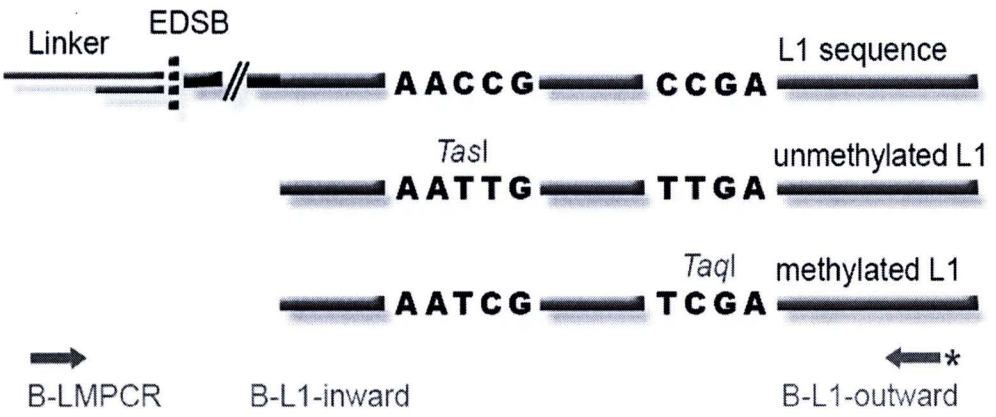
Schematic Illustration of L1-EDSB-LMLPCR. L1 sequence ligated by linker at EDSB. The white rectangle is Taqman probe complementary to LMPCR linker. Arrows are PCR primers.



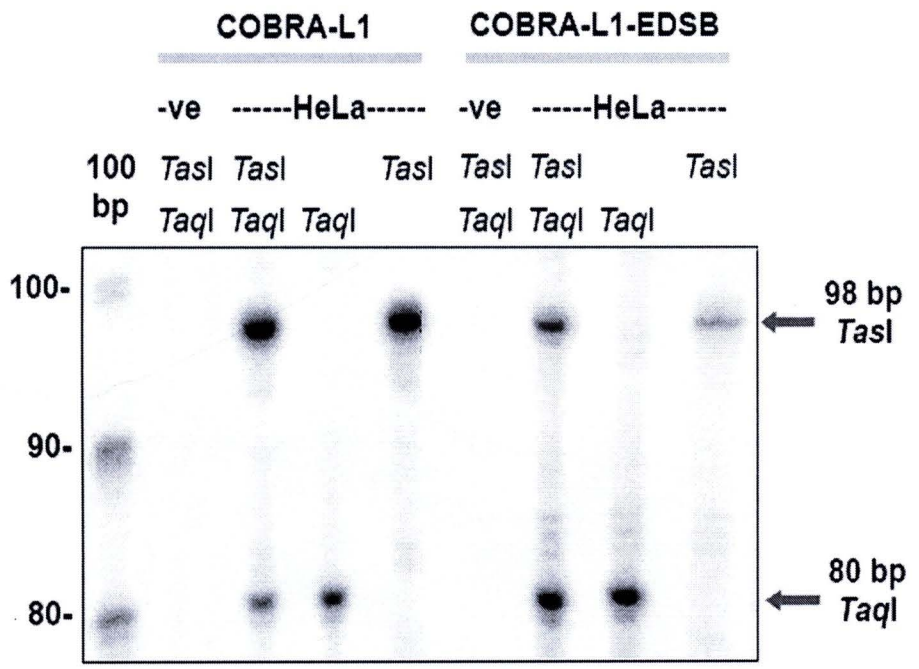
P...	Name	Standard	Calculate...	Cros...
1	Negative			
2	Control 3750 cells	3.750E+03	3.462E+03	24.32
3	Control 1250 cells	1.250E+03	1.306E+03	26.07
4	Control 250 cells	2.500E+02	2.840E+02	28.82
5	Control 50 cells	5.000E+01	4.565E+01	32.11
6	Control 10 cells	1.000E+01	<5.279E+00	>36.00
7	Daudi untreat polish		3.618E+02	28.39
8	Daudi take off polish		2.413E+02	29.11
9	Jurkat untreat polish		5.600E+02	27.60
10	Jurkat take off polish		4.438E+02	28.02
11	K562 untreat polish		1.431E+02	30.06
12	K562 take off polish		9.404E+01	30.81
13	Molt4 untreat polish		4.992E+02	27.81
14	Molt4 take off polish		1.834E+02	29.61
15	Hela untreat polish		4.365E+01	32.19
16	Hela take off D2 polish		1.026E+02	30.66
17	Hela take off D4 polish		1.429E+02	30.06
18	SW480 untreat polish		8.513E+01	30.99
19	SW480 take off D2 polish		8.334E+01	31.03
20	SW480 take off D4 polish		1.005E+02	30.69
21	Sperm untreat polish		1.634E+02	29.82
22	Hela without linker			

An example of results of L1 PCR by using realtime PCR

We assessed the amount of EDSBs and their methylation status in G0. We performed PCR combined with bisulfite restriction analysis (COBRA) of L1s (COBRA-L1) (3). To compare percentage of methylation level between genomic DNA and EDSBs, matched pair degree of methylation between L1 and L1-EDSB sequences was examined by COBRA-L1 and COBRA-L1-EDSB, respectively. COBRA-L1 was performed as previously described to quantify genome-wide methylation status. For COBRA-L1-EDSB, all ligated HMW DNA samples were chemically modified by bisulfite. Treatment with bisulfite converts unmethylated cytosines, but not methylated cytosines, to uracils and then thymines after PCR. Subsequently, the PCR products of bisulfite 5'L1 sequences were digested with *Taq*I and *Tas*I as restriction enzymes. While *Taq*I detected methylated L1 sequences, *Tas*I detected nonmethylated L1 sequences. The percentage of *Taq*I digestible amplicon was measured as COBRA-L1 methylation level by α-^{32}P-labeled-bisulfite-L1-outward, B-L1-outward, radiation intensity. For COBRA-L1-EDSB, the same protocol was adopted but the B-L1-inward primer was replaced by a linker primer, B-LMPCR.



Schematic Illustration of COBRA-L1 and COBRA-L1-EDSB. L1 sequence ligated by linker at EDSB. Arrows are PCR primers, with star indicating 5' labeled primer with ^{32}P for COBRA. AACCG and CCGA are L1 sequences; when treated with bisulfite and PCR, unmethylated AACCG will be converted to AATTG (*Tas*I site) and methylated CCGA to TCGA (*Taq*I site).

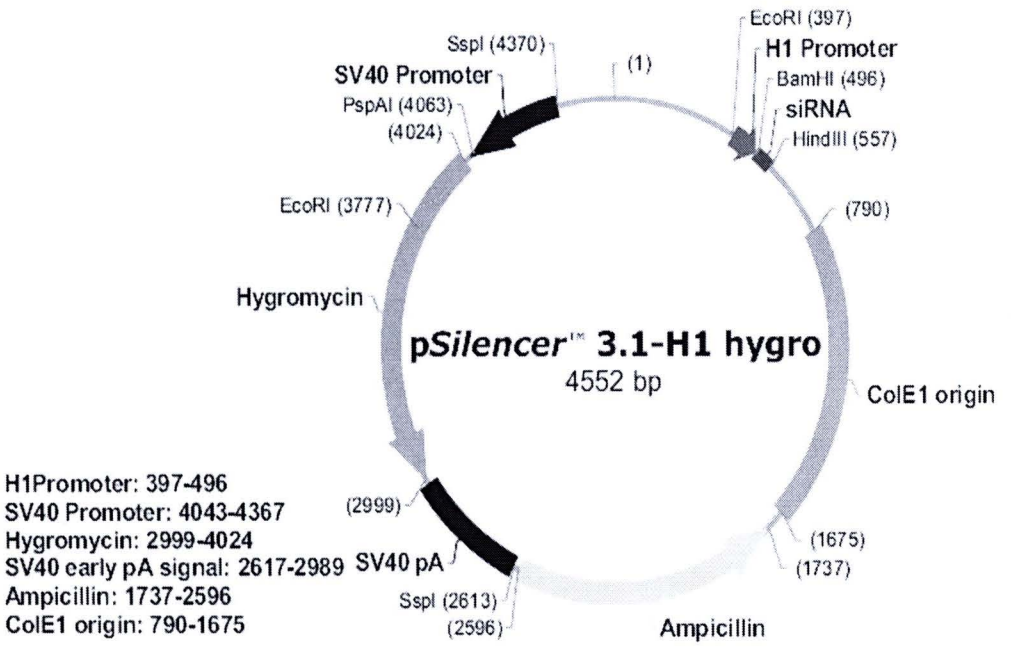


A Typical Example of Results form COBRA-L1 and COBRA-L1-EDSB Experiments. The arrow at 98 bp indicates *Tas*I digested unmethylated L1 sequences and the arrow at 80 bp indicates *Taq*I digested methylated L1 sequences –ve is dH_2O for COBRA-L1 and nonligated HMW DNA for COBRA-L1-EDSB. *Tas*I and *Taq*I are enzymes added in each experiment.

APPENDIX C

PLASMID AND siRNA SEQUENCES

1. p*Silencer*™ 3.1-H1 hygro



2. siRNA sequences

Negative siRNA from p*Silencer*™ kit (Ambion, Cat # 5760) was used as control

Ku86 siRNA	5'-GACGGTGTGCTCATGCGGC-3'
ATM siRNA	5'-GGGCGCUAAUCGUACUGAA-3'
DNA-PKcs siRNA	5'- GCACCAGUCCAGUAUUGGC-3'
Rad51 siRNA	5'-GAGCUUGACAAACUACUUC-3'

APPENDIX D

BUFFER AND REAGENT

1. Lysis I buffer

Sucrose	109.54	g
1 M Tris-HCl (pH 7.5)	10	ml
1M $MgCl_2$	5	ml
Triton X-100	10	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at 4°C.

2. Lysis II buffer

5 M NaCl	15	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

4. Proteinase K

Proteinase K	20	mg
Distilled water to volume	1	ml

Mix the solution and store at -20°C.

5. 1 M Tris-HCl (pH 7.5)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

6. 0.5 M EDTA (pH 8.0)

Disodium ethyleneidamine tetraacetate	18.66	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1 M $MgCl_2$

$MgCl_2.6H_2O$	20.33	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

8. 5 M NaCl

NaCl	29.25	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

9. 10X TBE buffer

Tris-base	108	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml
Distilled water to volume	1,000	ml

Mix the solution and store at room temperature.

10. 6X loading dye

Ficoll 400	15	g
Bromphenol blue	0.25	g
Xylene cyanol	0.25	g
1 M Tris (pH 8.0)	1	ml
Distilled water to volume	100	ml

Mix well and store at room temperature.

11. 10 M NH4OAc

NH4OAc	77.08	g
Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

12. 25:24:1 (v/v) phenol : chloroform : isoamyl alcohol

Saturated phenol	50	ml
Chloroform	48	ml
Isoamyl alcohol	2	ml

Mix the reagent vigorously, cover with TE buffer and store at 4°C.

13. TE buffer (pH 8.0)

1 M Tris-HCl (pH 8.0)	10	ml
0.5 M EDTA (pH 8.0)	2	ml
Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

14. HMW digestion buffer

1 M Tris-HCl (pH 8.0)	50	µl
20 mg/ml proteinase K	50	µl
10 % Sodium lauryl sarcosine	100	µl
0.2 M EDTA (pH 8.0)	100	µl
Distilled water	500	µl

Mix the solution and use 400 µl per agarose plug.

15. 10% Sodium lauryl sarcosine

Sodium lauryl sarcosine	10	g
Distilled water to volume	100	ml

Mix the solution and store at room temperature.

16. 20mg/ml glycogen

Glycogen	200	mg
Distilled water to volume	10	ml

Sterilize the solution by filter through 0.2 μm membrane, aliquot and store at -20°C.

17. 200 mM Thymidine

Thymidine	48.44	mg
Distilled water to volume	1	ml

Sterilize the solution by filter through 0.2 μm membrane and store at 4°C.

18. 10X PBS

NaCl	80	g
Na2HPO4	2	g
KCl	14.4	g
KH2PO4	2.4	g
Distilled water to volume	1,000	ml

Mix to dissolve and adjust pH to 7.4

Sterilize the solution by autoclaving and store at room temperature.

19. 10X TBS

Tris base	61	g
NaCl	90	g
Distilled water to volume	1,000	ml

Mix to dissolve and adjust pH to 7.6

Sterilize the solution by autoclaving and store at room temperature.

20. 1X SDS Buffer (Lysis Buffer)

1 M Tris-HCl pH 8.8	6.25	ml
10% SDS	20	ml
Glycerol (87%)	11.5	ml
Distiled water to volume	100	ml

21. 6X Sample loading buffer

1 M Tris-HCl pH 6.8	3	ml
SDS	1.2	g
Glycerol (100%)	6	ml
Bromphenol blue	30	mg
Distiled water to volume	10	ml

Add 24% β-mercaptoethanol before using (stock 14.2 M and working 864 nM)

22. 8% SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Resolving gel (10 ml)

40% Acrylamide:Bis (37.5:1)	2	ml
1 M Tris-HCl (pH 8.8)	2.5	ml
10% SDS	100	µl
10% $(NH_4)_2S_2O_8$	50	µl
TEMED	5	µl
Distilled water	5.5	ml

Stacking gel (4 ml)

40% Acrylamide:Bis (37.5:1)	0.4	ml
0.5 M Tris-HCl (pH 6.8)	1	ml
10% SDS	40	µl
10% $(NH_4)_2S_2O_8$	20	µl
TEMED	4	µl
Distilled water	2.6	ml

23. 1 M Tris-HCl (pH 8.8)

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 8.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

24. 0.5 M Tris-HCl (pH 6.8)

Tris base	6.055	g

Dissolve in distilled water and adjusted pH to 6.8 with HCl (conc)

Distilled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

25. 10X Tris-glycine (pH 8.3)

Tris base	6.055	g
Glycine	147.1372	g

Dissolve in distilled water and adjusted pH to 8.3

Distilled water to volume	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

26. Running Buffer

10X Tris-glycine (pH 8.3)	100	ml
10% SDS	10	ml
Distilled water	890	ml

Mix the solution and store at room temperature.

27. Transfer buffer

10X Tris-glycine (pH 8.3)	100	ml
Methanol	200	ml
Distilled water	800	ml

Mix the solution and store at room temperature.

28. Cell Lysis buffer (Lysed Buffer)

1M PIPES (pH 8.3)	500	µl
KCl	0.634	g
100% NP40	500	µl
Distiled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

29. Nuclei Lysis buffer (Lysis Buffer)

1 M Tris-HCl (pH 8.1)	5	ml
0.5 M EDTA (pH 8.0)	2	ml
10% SDS	10	ml
Distiled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

30. 2.5 M Glycine

Glycine	9.384	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH to get to dissolve

Distiled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

31. IP Dilution buffer (ChIP Buffer)

10% SDS	100	µl
100% Triton X 100	1.1	ml
0.5 M EDTA (pH 8.0)	240	µl
1 M Tris-HCl (pH 8.1)	1.67	ml
5 M NaCl	3.34	ml
Distiled water to volume	100	ml

Sterilize the solution by autoclaving and store at room temperature.

32. High Salt 500 mM

10% SDS	2	ml
100% Triton X 100	2	ml
0.5 M EDTA (pH8.0)	800	µl
1 M Tris-HCl	4	ml
5 M NaCl	20	ml
Distiled water to volume	500	ml

Sterilize the solution by autoclaving and store at room temperature.

33. High Salt 550 mM

10% SDS	2	ml
100% Triton X 100	2	ml
0.5 M EDTA (pH8.0)	800	µl
1 M Tris-HCl	4	ml
5 M NaCl	22	ml
Distiled water to volume	500	ml

Sterilize the solution by autoclaving and store at room temperature.

34. High Salt 600 mM

10% SDS	2	ml
100% Triton X 100	2	ml
0.5 M EDTA (pH8.0)	800	µl
1 M Tris-HCl	4	ml
5 M NaCl	24	ml
Distiled water to volume	500	ml

Sterilize the solution by autoclaving and store at room temperature.

35. IP Wash buffer

1 M Tris-HCl (pH8.0)	50	ml
LiCl	10.6	g
100% NP40	5	ml
Deoxycholic acid	5	g
Distiled water to volume	500	ml

Sterilize the solution by autoclaving and store at room temperature.

36. RNaseA

RNaseA	100	mg
Distiled water to volume	10	ml

Heat at 100°C for 20 minutes and store at -20°C.

37. Elution buffer

NaHCO3	0.21	g
10% SDS	5	ml
Distiled water to volume	50	ml

Sterilize the solution by autoclaving and store at room temperature.

38. 5X PK buffer

1 M Tris-Hcl (pH 7.5)	0.5	ml
0.5 M EDTA (pH 8.0)	0.5	ml
10% SDS	1.25	ml
Distiled water to volume	10	ml

Mix the solution and store at -20°C

APPENDIX E

SEQUENCE OF LINE-1

>gi|339773|gb|M80343.1|HUMTNL22 Human transposon L1.2

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AATAAAAAAAAAAAAAAAAAAAAAAAAA

BIOGRAPHY

Miss Wanpen Ponyeam was born in Bangkok in 1982. In 2004, she graduated from faculty of Science, Chulalongkorn University in Biochemistry program and then attended to particulate in Medical Science program in Faculty of Medicine for her master degree.