Appendix A

Protocol: Generating libraries using the LGN cell line

Materials required

LGN cells ready for transfection on six well cell culture plate(s).

Feeder plates resistant to both G418 and puromycin.

PBC expression plasmid Qiagen maxi prep or similar, at least 1 mg/ml. PBC = pCMV-hyPBase-hCDT1. PBC mRNA can also be used.

4-hydroxytamoxifen 1 mM solution in ethanol.

M15 medium supplemented with 100 U/ml LIF, and derivatives below (all media contain LIF):

HGF medium M15 medium supplemented with 200 μg/ml G418, 1X HAT, 200 nM FIAU.

HTG medium M15 medium with 200 μg/ml G418 and 1X HT supplement (Invitrogen).

DBL medium M15 with 200 μg/ml G418 and 3 μg/ml puromycin.

Protocol

1. Wash cells twice with PBS and add 500 μl trypsin solution. Incubate at 37C for 10 minutes.
2. Quench trypsin with 500 μl M15 medium, pipette to break up cell clumps. Wash cells with PBS and resuspend in 900 μl PBS with 15 μg PBC plasmid. Transfer to an electroporation cuvette (0.4 cm BioRad).
3. Electroporate (230 V, 500 μF). Incubate at room temperature for five minutes. Transfer cells to a 10 cm feeder plate with M15 medium. Plate 1/100 of each electroporation to a six well plate to estimate the number of new insertions obtained (select in HGF until colonies are visible, stain and count). Alternatively, use Qiagen Transmessenger reagent to transfect 1–2 μg capped *in vitro* transcribed mRNA (Use Ambion mMessage mMachine T7 kit with *Avr*II-linearised pCMV-hyPBase or pCMV-PBCDT1).
4. The next day, change medium to **HGF**. Change medium daily. Passage cells at a ratio of 1:2–1:4 if they become confluent, maintaining selection at all times.
5. After eight days of selection, change medium to **HTG** for two days.
6. (Day 10) Change medium to M15, supplemented with G418 alone (200 μg/ml).
7. Cells can be expanded further if required. I typically expand until day 12–14, as cells grow more slowly than normal in HAT-containing medium.
8. Once the required expansion has been reached, harvest the cells with trypsin as above. Ensure colonies are effectively dispersed by pipetting. Count the cells and record the number (this can be used to estimate the number of cells per mutant clone). Plate the entire culture onto 10 cm plates containing M15 supplemented with 1 μM 4-OHT, at no more than 5×10^6 cells per plate. Incubate overnight.
9. The next day, change medium to M15. After two days, trypsinise the cells and replate half of each culture to a 10 cm plate containing **DBL** medium. Again, plating some cells at low density is useful to estimate the number of double-resistant clones in the library. A small number of cells can be plated in puromycin to check the induction of Cre was efficient.
10. Change medium daily and select for at least 10 days. Passage cells under selection at least once, or more if they become confluent. When passaging, plate some at low density to pick clones for analysis[1].
11. Freeze the enriched library. As the library has been expanded by over 1000× since beginning DBL selection, small aliquots can be used without affecting representation. Colonies can be picked from the low density plate and analysed by Southern blot to determine the complexity of the library.

[1]There is no need to replate the entire culture every time—for every 2–3 days of growth, the proportion to replate can be reduced by 1/4–1/2. As long as the representation of clones is retained, it is not necessary to retain all cells from every clone.

Appendix B

Primer sequences

Name	Sequence (5′ to 3′)	Purpose
ActB-F	ATGGGTCAGAAGGACTCCTA	Beta actin RT-PCR primer
ActB-R	CAACATAGCACAGCTTCTCT	Beta actin RT-PCR primer
Ccdc107-FseI-f	GCATTTAGGCCGGCCGAGCCAAGGAGACAG- -ACTGG	Primers to amplify Ccdc107 mutagenic exons
CCdc107-AscI-r	GGAATCGGCGCGCCTTTATTTCCCCACTGG- -ATCTT	—
Dom3z-f	GCATTTAGGCCGGCCCCAAGTCCTCAGACC- -CAGTG	Primers to amplify Dom3z mutagenic exons
Dom3z-r	GGAATCGGCGCGCCGCCAGCCTCTACACCC- -AGTA	—
XhoI-H3-adaptA	TCGAGATCGATACATGTA	adaptor oligo - goes into XhoI + HindIII, adds PciI and ClaI
XhoI-H3-adaptB	ACGTTACATGTATCGATC	rev comp to above
PacI-PmeI-palilinker	GTTTAAACAT	Goes into PacI site (3' AT OH), adds a PmeI site. Palindromic oligo
g1101a-a1103t	GAATGACCGAGAAGGCTGAATTCCCTCTGT--GTGCATGAA	Site directed mutagenesis primers for mutagens
g1101a-a1103t-antisense	TTCATGCACACAGAGGGAATTCAGCCTTCT--CGGTCATTC	—
c2379a-c2381a-c2388t-a2394t	GTTTTGTGTCTAGAAGTTCCATATGGGTTT--CAACCTAAGTCGTCACCCTGTAGAAA	—
c2379a-c2381a-c2388t-a2394t-antisense	TTTCTACAGGGTGACGACTTAGGTTGAAAC--CCATATGGAACTTCTAGACACAAAAC	—
HmSpAa-SPCR	CGAAGAGTAACCGTTGCTAGGAGAGACCGT--GGCTGAATGAGACTGGTGTCGACACTAGTG- -G	Splinkerette linker
HmSpBb-SPCR-GATC	GATCCCACTAGTGTCGACACCAGTCTCTAA--TTTTTTTTTTCAAAAAAA	Splinkerette linker with GATC overhang (for Sau3AI)
HmSpBb-SPCR-TA	TACCACTAGTGTCGACACCAGTCTCTAATT--TTTTTTTTCAAAAAAA	Spinkerette linker with TA overhang
HMSp1-SPCR	CGAAGAGTAACCGTTGCTAGGAGAGACC	Primer to Splinkerette linker
HMSp2-1-SPCR	GTGGCTGAATGAGACTGGTGTCGAC	—
HMSp2-2-SPCR	ATGAGACTGGTGTCGACACTAGTG	—
PB5-1-SPCR	TAAATAAACCTCGATATACAGACCGATAAA	Primers for SPCR of PB5
PB5-2-SPCR	ATATACAGACCGATAAAACACATGCGTCAA	—
PB5-seq-SPCR	TTTTACGCATGATTATCTTTAACGTACGTC	—
PB3-1-SPCR	CAAAATCAGTGACACTTACCGCATTGACAA	Primers for SPCR of PB3
PB3-2-SPCR	CTTACCGCATTGACAAGCACGCCTCACGGG	—
PB3-seq-SPCR	TTAGAAAGAGAGAGCAATATTTCAAGAATG	—
Neo-SV40-F-AscI	ATAGGCGCGCCTTGGAGGCCTAGGCTTTTG	Amps neo-SV40pA from pcDNA3 with AscI and SfiI (specific site in YTC85) for cloning
Neo-SV40-R-RCSfiI-KpnI	TTAGGCCTGATCGGCCGGTACCTGTGGAAT--TGTGAGCGGATA	—
Dym-insertion-F	AGCATAGAGGAGGAGATAAGCACTC	gPCR primers 288bp w/PB5
Dym-insertion-R	GTTTTGGGCTCTACCATTATTTATTTT	gPCR primers - 234bp w/F
PB5-R	taaataaacctcgatatacagaccgata	gPCR primers
Ddt-insertion-F	AGGTGGCTCTGTTTTTCCCTCT	gPCR primers 169 w/PB5
Ddt-insertion-R	GTATCTTAGGGACCAGAGAGAGATG	gPCR primers 232 w/F
Picalm-F	CGCAATGGATTGTCACATTTTT	TN is -, use with PB5-R, 151bp
Picalm-R	CACCTGGACTGTGAGTGAAGAC	use with Picalm-R, 220bp
Arrb2-F	TGTTAGGGTCTTCAAGAAGTCGAG	use with PB5-R, 259bp
Arrb2-R	AAGCTTGCTTAGGAACCCAGAC	use with Arrb2, 227bp
Arrb2-e1-F	gcaccatgggagaaaaacc	RT-PCR primers
Arrb2-e5-R	cttcttcagcagtcggtcct	—
Dym-e1-F	tgacctacggaaccatggag	—
Dym-e3-R	gaaatggttgcctcttccaa	—
TNP100-F3-F	TTTAGGATGGGCTTCCCTTT	Genotyping primers for TNP100 insertions

Name	Sequence (5′ to 3′)	Purpose
TNP100-F3-R	AAGACCACCGTTTTCCCTCT	—
TNP100-G10-F	AGGGCAGCTGAGTTTAAGCA	—
TNP100-G10-R	GGCAGGAAACAGGTAGGACA	—
TNP100-H10-F	AGAAACCACCCACAAAAACG	—
TNP100-H10-R	AGGGGGTTAGCCACAAGTTT	—
TNP100-D1-F	AATCTGGTGATGGCCTTCTG	—
TNP100-D1-R	AGAGCCCTGACACTCTTCCA	—
TNP100-C5-F	CACCTGCAACCATCAAACAC	—
TNP100-C5-R	TCTGCACTGGGAGAAGGTCT	—
TNP100-B9-F	TTGCCGCATTGTCTCTATTG	—
TNP100-B9-R	CCAAACCTTTGTGAAGTCGAA	—
PB5-gPCR	taaataaacctcgatatacagaccgata	—
TNP100-C8-F	TGCAGGCAAAATTCTTTTATTG	—
TNP100-C8-R	TCTCCATATGTATTCAATTACAATTTCTC	—
TNP100-F3-probe-F	TTACGGTCTGTCCCAAGGTC	Locus-specific Southern probes for TNP100 insertions
TNP100-F3-probe-R	AATGAGGCTGCAAGAGGAAA	—
TNP100-B9-probe-F	AAAATCAGTGTGTTGTCACTACCTC	—
TNP100-B9-probe-r	CCAACAAACAAAGCCAAAAA	—
TNP100-C5-probe-F	CAGTCTTAAAAATCAAGGCTGACC	—
TNP100-C5-probe-r	CCTTTACCAGGTCTTTTCAAGC	—
TNP100-C8-probe-F	AGAAAAGGGAACCGAAAGGA	—
TNP100-C8-probe-r	AGACAGGATGGAAGCCATTG	—
TNP100-H10-probe-f	GAAGGATGGAGAGGAAGGGTA	—
TNP100-H10-probe-r	CACAGCTCCCTAACCTATAACACA	—
Myo5a-RT-F	GGCAGCCCTATGATAGAAGG	RT-PCR primers
Myo5a-RT-R	TTGTGCAGCTGTCTGAATCC	—
iCre-target-1	CCTAAAGAAGAGGCTGTGCTTTGG	Rosa26:ERT2-iCre-ERT2 primers (J. Takeda, K. Yusa)
iCre-target-2	CATCAAGGAAACCCTGGACTACTG	—
iCre-popout-1	TAAGGGATCTGTAGGGCGCAGTAGTCCAGG	—
iCre-popout-2	TAAGCTAGCTTGGGCTGCAGGTCGAGGGAC	—
AseClaNsi-linker-A	AGCTAATCGATTAATCGCATTCAATGCATG- -CGTCAATTTTACGCAGACTATCTTTCTAGG- -GTTAA	Linker to reconstruct PB3 up to NsiI site
AseClaNsi-linker-B	AGCTTTAACCCTAGAAAGATAGTCTGCGTA- -AAATTGACGCATGCATTGAATGCGATTAAT- -CGATT	—
FL2-B4-F	CCCTGTCCTTGGTTTATGGA	Genotyping primers for further clone-by-clone enrichment experiments
FL2-B4-R	TACCGCCCTTAAAGAACCAG	—
FL2-C1-F	CTCTGGGATCCCTCCTCTTC	—
FL2-C1-R	CCCAAGACTGAGTGCCATCT	—
FL2-C4-F	AACCCAGGCCTCTGAAGTTT	—
FL2-C4-R	CTCTGCCTCTGAGTGCCTTT	—
FL2-A7-F	AAGCATGGGCTACTTCTCCA	—
FL2-A7-R	ATGCAGTGTCCAGTGCTGAG	—
FL2-C6-F	AGAGACCATGGATGCCAGAC	—
FL2-C6-R	GGTATTTTGGTGGTGGTGGT	—
FL2-C9C10-F	ACTCTGCACATGGCACACAT	—
FL2-C9C10-R	GGAGGCTCCTTCCTCATTCT	—
FL2-A3-F	CGTTTGTTCTGCAGGTCTGA	—
FL2-A3-R	CAACTGAGGAGTGTGGCAGA	—
FL2-A4-F	TTTCCGGGCACATCTTTATC	—
FL2-A4-R	ATGATCCCAGATGCCTTCAG	—
FL2-A11-F	GTGGGGCTCATGTAGGAAGA	—
FL2-A11-R	GTAGCTGCCTCCCAAGACTG	—
FL2-B9C7-F	AATAGCCGCATACCTGCATC	—
FL2-B9C7-R	CGGAGCTGTTCCTTGTCATT	—
PB5-gPCR	taaataaacctcgatatacagaccgata	—
Sall1-e1-F	ACCCGGAAGAGGGAGTACAG	—
Sall1-e3-R	GGCATCCTTGCTCTTAGTGG	—
Acpp-e2-F	TTCCTACCGACCCCATTACA	—
Acpp-e4-R	ATCCCCTCTGGAGGAAACAG	—
TNP100-B6-F	GCTCTGAGCCTGGGAGATTA	—
TNP100-B8-F	ATCTTGTGGGATGGCATAGC	—
TNP100-B11-F	CCACAGCCTGGGAAACTATT	—
PB3-gPCR	acggattcgcgctatttaga	—

Name	Sequence (5′ to 3′)	Purpose
XX-Tmp-F	TGGATCAACAGAACAAGGAAA	Primer to amplify tag insertion in bleomycin-sensitive pool
TV28-3-geno-F	TAAACCTCGATATACAGACCGATAAAACAC	TV28 targeting genotyping, by long range PCR
TV28-3-geno-R	CTACCTCACACCATGCACAAAAATAAAT	—
TV28-probe-F	TGATTTAATACCAGCACATCCAAATTAT	TV28 Southern probe
TV28-probe-R	ACCTTTCCAGTTAAAGTTGAGAGATCAT	—
FL2-E7G7-F	AACCCAGGCCTCTGAAGTTT	Genotyping primers for further clone-by-clone enrichment experiments
FL2-E7G7-R	GACCAGGATCCTTGGACTCA	—
FL2-D6-F	ATGTCCCTCTCCTGTGTTGG	—
FL2-D6-R	CCTCGCTTCACCTCTGAGAC	—
FL2-F11-F	AGGGTGGGGATAGAGCAGAT	—
FL2-F11-R	CTTGCTCTTGGCAACTTGTG	—
HmSpBb-NcoI-CATG	CATGCCACTAGTGTCGACACCAGTCTCTAA--TTTTTTTTTTCAAAAAAA	Splinkerette linker with CATG overhang (for NcoI)
TV28-jump-F	CTGGTCAAGGAAATGGTGCT	Primers for amplifying Hprt donor locus after transposon jumping
TV28-jump-R	CACCAACACACCAGCTCAAC	—
CDT1-R	CTCTAGCATTTAGGTGACACTATAGAATAG--GGCCCTCTAGATGCATGCTCTCATTACAAC--TCCCCAGCATCCTGGGCACT	Amplify human CDT1 fragment plus homology arms to hyPBase plasmid
CDT1-F-AscI	AGAAGGTCATCTGCCGGGAGCACAACATCG--ACATGTGCCAGAGCTGTTTCgggcgcgccC--CCAGCCCCGCCAGGCCCGCACTCCGCGCC	—
LGNL1-A1-F	CGAACTCAGAGATCTGCTTGTCT	Genotyping primers for LGNL1 allelic clones (used with PB5-gPCR primer)
LGNL1-A1-R	GAAGGTGAGGTCACTCTGAGCTA	—
LGNL1-A2-F	CCCAAGTCCTCTGTAATTCCTCT	—
LGNL1-A2-R	TGTTTTACAGACTGGATGGCTTT	—
LGNL1-A3-F	CTGATGACATTACACCTGCGTTA	—
LGNL1-A3-R	GAGAGATGGCTCAGTGGTTAAGA	—
LGNL1-A4-F	CTCAAAAGCCTTTCTCTCCTTTC	—
LGNL1-A4-R	CTCCTTTCTCACCTCAGTAGCAA	—
LGNL1-A8-F	TGGCTTCTATCTACCCACAGCTA	—
LGNL1-A8-R	CCATCACATGTGGCCTATATTTT	—
LGNL1-B11-F	TTATGATTGCCTCAGGATCATCT	—
LGNL1-B11-R	AGCAACTCACTGCAAGAGAGAAC	—
LGNL1-B1-F	GAACCAAAGGGTAAAAGGAGAGA	—
LGNL1-B1-R	CCCAGAGCATTTTACATTTCAAG	—
LGNL1-B2-F	AAGGAAACCTGAAGAAACCAGTC	—
LGNL1-B2-R	CTAGTCAGCAGTGCCCAATATCT	—
LGNL1-B5-F	CTGGCTCTGCTGAAGATAAACAT	—
LGNL1-B5-R	CATCAGATCCCATTACAGATGGT	—
LGNL1-B6-F	TAGGGTTTCTCTGTGTAGCCTTG	—
LGNL1-B6-R	TTCTCCATGCTCAGTCACACTTA	—
LGNL1-B7-F	CCCCATCTTCTGAGACTAAAGGT	—
LGNL1-B7-R	GTGTGTTACAAGGCAAGCTCTCT	—
LGNL1-B8-F	AGTGTGTCCAAAAAGATCAAGGA	—
LGNL1-B8-R	GGTTCTAATGCCTTGGAGAAGAT	—