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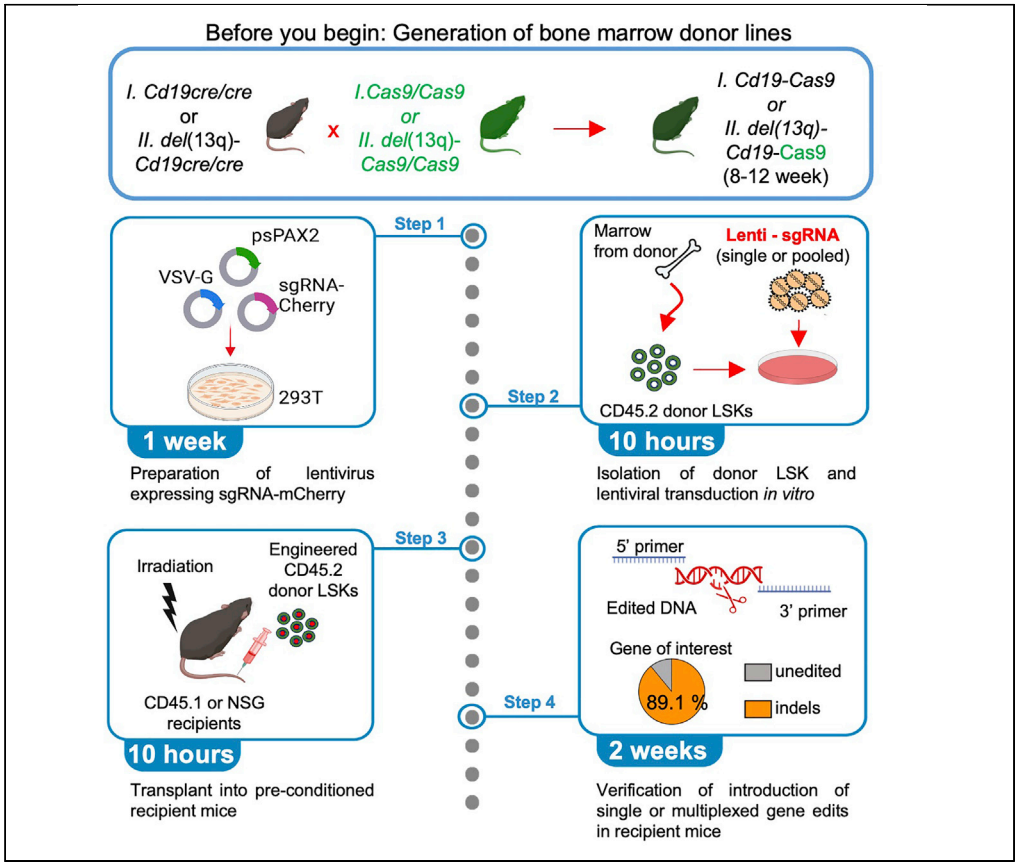
Protocol

Generation of mouse models carrying B cell restricted single or multiplexed loss-of-function mutations through CRISPR-Cas9 gene editing



Here, we present a protocol to generate B cell restricted mouse models of loss-of-function genetic drivers typical of lymphoproliferative disorders, using stem cell engineering of murine strains carrying B cell restricted Cas9 expression. We describe steps for preparing lentivirus expressing sgRNA-mCherry, isolating hematopoietic stem and progenitor cells, and *in vitro* transduction. We then detail the transplantation of engineered cells into recipient mice and verification of gene edits. These mouse models represent versatile platforms to model complex disease traits typical of lymphoproliferative disorders.

Elisa ten Hacken, Michaela Gruber, María Hernández-Sánchez, ..., Kendell Clement, Kenneth Livak, Catherine J. Wu

elt4010@med.cornell.edu (E.t.H.)
cwu@partners.org (C.J.W.)

Highlights

CRISPR-Cas9 engineering of murine stem cells via multiplexed lentiviral transduction

Steps to isolate, transduce, and transplant engineered stem cells into recipient mice

Rapid generation of B cell restricted mouse models of loss-of-function drivers

Facile translatability to the modeling of lymphoproliferative disorders

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Generation of mouse models carrying B cell restricted single or multiplexed loss-of-function mutations through CRISPR-Cas9 gene editing

Elisa ten Hacken,[1,2,10,11,12,*] Michaela Gruber,[3,10] María Hernández-Sánchez,[4] Gabriela Brunsting Hoffmann,[1] Kaitlyn Baranowski,[1] Robert A. Redd,[5] Kendell Clement,[6,7] Kenneth Livak,[1,8] and Catherine J. Wu[1,2,6,9,13,*]

[1]Department of Medical Oncology, Dana Farber Cancer Institute, Boston, MA, USA

[2]Harvard Medical School, Boston, MA, USA

[3]CEMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

[4]Department of Biochemistry and Molecular Biology, Pharmacy School, Universidad Complutense de Madrid, Madrid, Spain

[5]Department of Data Science, Dana Farber Cancer Institute, Boston, MA, USA

[6]Broad Institute of MIT and Harvard, Cambridge, MA, USA

[7]Molecular Pathology Unit, Center for Cancer Research and Center for Computational and Integrative Biology, Massachusetts General Hospital, Charlestown, MA, USA

[8]Translational Immunogenomics Laboratory, Dana Farber Cancer Institute, Boston, MA, USA

[9]Department of Medicine, Brigham and Women's Hospital, Boston, MA, USA

[10]These authors contributed equally

[11]Present address: Department of Medicine, Division of Hematology and Medical Oncology, Weill Cornell Medicine, New York, NY, USA

[12]Technical contact

[13]Lead contact

*Correspondence: elt4010@med.cornell.edu (E.t.H.), cwu@partners.org (C.J.W.)
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SUMMARY

Here, we present a protocol to generate B cell restricted mouse models of loss-of-function genetic drivers typical of lymphoproliferative disorders, using stem cell engineering of murine strains carrying B cell restricted Cas9 expression. We describe steps for preparing lentivirus expressing sgRNA-mCherry, isolating hematopoietic stem and progenitor cells, and *in vitro* transduction. We then detail the transplantation of engineered cells into recipient mice and verification of gene edits. These mouse models represent versatile platforms to model complex disease traits typical of lymphoproliferative disorders.

For complete details on the use and execution of this protocol, please refer to ten Hacken et al.,[1] ten Hacken et al.,[2] and ten Hacken et al.[3]

BEFORE YOU BEGIN

This protocol outlines steps required to generate B-cell restricted mouse models carrying single or combinatorial loss-of-function mutations achieved via CRISPR-Cas9 gene editing. The protocol includes lentiviral preparation and handling, and those experiments should be performed in appropriate biosafety level conditions.

Institutional permissions

All animal care and use protocols require approval from the Institutional Animal Care and Use Committee. For the presented studies, all protocols have been approved by the Dana Farber Cancer Center Institutional Animal care and use committee (DFCI IACUC) prior to experimentation.

Cell line culture and expansion for lentiviral preparation

Timing: 1 week

1. Culture HEK293T cells in DMEM supplemented with 10% FBS and 1% Penicillin/Streptomycin (Pen/Strep) in T175 ventilated flasks.
2. Passage cells at least twice post-thaw and never allow them to reach >70% confluency to best preserve cellular morphology.

Generation of mouse models to serve as bone marrow donor line

Timing: 6–8 months

3. Singly-edited B-cell restricted mouse models: generate *Cd19*-Cas9 marrow donors by intercrossing homozygous *Cd19*-cre/cre mice with homozygous Cas9/Cas9 mice.
4. Multiplexed mouse models of chronic lymphocytic leukemia (CLL): generate *del*(13q)-*Cd19*cre/cre mice by interbreeding MDR mice with *Cd19*cre/cre mice.

Note: MDR represents the minimally deleted region of murine chr14, syntenic to human *del*(13q).

5. Similarly, generate *del*(13q)-Cas9/Cas9 by interbreeding MDR mice with homozygous Cas9/Cas9 mice.
6. Finally, breed *del*(13q)-*Cd19*cre/cre with *del*(13q)-Cas9/Cas9 to obtain *del*(13q)-*Cd19*Cas9 [i.e., expressing B-cell restricted *del*(13q) and Cas9-GFP] bone marrow donors.

Design of sgRNAs to generate loss-of-function lesions and validation of targeting efficiency

Timing: 2–3 weeks

7. Design single guide RNAs (sgRNAs) against the genes of interest to introduce loss-of-function mutations via the online GFP sgRNA Designer (CRISPick) design tool (https://portals.broadinstitute.org/gppx/crispick/public).

Note: Choose sgRNAs based on the highest editing efficiency and the lowest predicted off-targeting activity.

8. Choose position of the sgRNA predicted cut site to lie within early exonic regions according to the UniProt database (https://www.uniprot.org), to facilitate early protein truncation. For more information regarding criteria for sgRNA design, please refer to Hanna and Doench.[4]
   a. Clone sgRNAs into the pLKO5- mCherry expressing plasmid (please refer to https://media.addgene.org/data/plasmids/52/52961/52961-attachment_B3xTwla0bkYD.pdf for graphic examples of cloning strategy and detailed protocol).
   b. To clone the sgRNAs into the pLKO5 vector, design two complementary oligos for each sgRNA including two 4-nt-overhang sequences.
   c. Digest pLKO-mCherry plasmid with Esp3I (BsmBI) for 30 min at 37°C.
   d. Denature the two complementary oligos at 95°C for 5 min, ramp cool to 25°C over a period of 45 min to allow annealing and ligate with the linearized pLKO5 plasmid via Quick Ligase.
   e. Transform competent bacteria (Stbl3 cells) with 2 μL of the ligated plasmid.
   f. Pick and expand single bacterial colonies from ampicillin-containing LB agar plates and extract DNA from them via QIAprep Spin Miniprep Kit.
   g. Confirm correct insertion of the sgRNA sequences by Sanger sequencing.

9. Obtain lentiviral vectors containing the selected mCherry-expressing plasmids (see below part 1 of protocol for details on lentiviral preparation).
10. Transduce a Cas9-expressing BaF3 cell line (see below part 2 and ten Hacken et al.[1] for transduction details) and confirm mCherry$^+$ expression by flow cytometric analysis 3 days post-transduction.

Note: Use plasmids expressing non-targeting control (i.e., scramble) sgRNAs in parallel, as negative controls.

11. Perform DNA targeted sequencing (see below part 4 for details) on mCherry$^+$ cells from targeting and non-targeting sgRNA-expressing cells and move forward onto *in vivo* studies when sgRNAs show >70% editing efficiency.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Pacific Blue anti-mouse Lineage (1:200 dilution)	BioLegend	Cat#133310; RRID: AB_11150779
PE anti-mouse Sca-1 (1:100)	BioLegend	Cat#108108; RRID: AB_313345
APC anti-mouse c-Kit (1:100)	BioLegend	Cat#105812; RRID: AB_313221
BV711 anti-mouse CD45.2 (1:200)	BioLegend	Cat#109847; RRID: AB_2616859
Pacific Blue anti-mouse B220 (1:200)	BioLegend	Cat#103227; RRID: AB_492876
PerCPCy5.5 anti-mouse Cd11b (1:200)	BioLegend	Cat#101228; RRID: AB_893232
PE/Cy7 anti-mouse CD3 (1:200)	BioLegend	Cat#100220; RRID: AB_1732057
APC anti-mouse CD5 (1:200)	BioLegend	Cat#100626; RRID: AB_2563929
FcR blocking agent (1:100)	BioLegend	Cat#101302; RRID: AB_312801
Bacterial and virus strains		
Stbl3 cells	Thermo Fisher Scientific	Cat#C737303
Chemicals, peptides, and recombinant proteins		
Murine TPO	PeproTech	Cat#315-14
Murine SCF	PeproTech	Cat#250-03
StemSpan	StemCell Technologies	Cat#09600
Polybrene	Santa Cruz Biotechnology	Cat#SC-134220
Parafolmaldehyde	Electron Microscopy Services	Cat#15710
DMEM	Gibco (Thermo Fisher)	Cat#11995065
GlutaMAX	Gibco (Thermo Fisher)	Cat#35050061
MEM-NEAA	Gibco (Thermo Fisher)	Cat#11140050
RPMI	Gibco (Thermo Fisher)	Cat#11875119
Pen/Strep	Gibco (Thermo Fisher)	Cat#15140122
FBS	Gibco (Thermo Fisher)	Cat#10437028
PBS	Corning	Cat#21040CV
EDTA	Thermo Fisher	Cat#15575-038
ACK lysis buffer	Gibco (Thermo Fisher)	Cat#A1049201
OPTI-MEM	Gibco (Thermo Fisher)	Cat#31985062
Lenti-X	Takara	Cat#631232
TrasIT-LT1	Mirus	Cat#MIR 2300
LB Agar	Sigma-Aldrich	Cat#L2897
Ampicillin	Sigma-Aldrich	Cat#59349
Bleach	Of choice	
Critical commercial assays		
EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit	StemCell Technologies	Cat#19856
QIAamp DNA Microkit	Qiagen	Cat#56304
QIAprep Spin Miniprep Kit	Qiagen	Cat#27104

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
DNA, RNA and protein purification kit	Macherey-Nagel	Cat#74060920
Phusion high-fidelity PCR master mix	New England BioLabs	Cat#M0531S
Esp3I (BsmBI) restriction enzyme	New England Biolabs	Cat#ER0451
Quick Ligase	New England Biolabs	Cat#M2200S
Experimental models: Cell lines		
BaF3-Cas9 expressing cell line (up to passage 20)	Our lab	
HEK 293T (up to passage 10)	Takara	Cat#632180
Experimental models: Organisms/strains		
CD19cre/cre mice (B6 strain, female and male, up to 8 months)	Jackson Laboratories	Cat#06785
Cas9/Cas9 mice (B6 strain, female and male, up to 8 months)	Jackson Laboratories	Cat#026179
CD19-Cas9 (B6 strain, female and male, 8–12 weeks of age)	Our lab	
del(13q) (i.e., MDR mice) (B6 strain, female and male, up to 8 months)	Jackson Laboratories	Cat#017642
del(13q) with CD19cre/cre (B6 strain, female and male, up to 8 months)	Jackson Laboratories	Cat#37070
del(13q) with Cas9/Cas9 (B6 strain, female and male, up to 8 months)	Jackson Laboratories	Cat#37071
del(13q) with CD19-Cas9 (B6 strain, female and male, 8–12 weeks of age)	Our lab	
Oligonucleotides		
Trp53 forward primer	Ten Hacken et al., Genome Biol 2020	5′-aagacgtgccctgtgcagtt-3′
Trp53 reverse primer	Ten Hacken et al., Genome Biol 2020	5′-gcaagaataagtcagaagccggg-3′
Mga forward primer	Ten Hacken et al., Genome Biol 2020	5′-gatgcatcaaccagtgtctttcta-3′
Mga reverse primer	Ten Hacken et al., Genome Biol 2020	5′-tctgaatgttctgataagctgttactg-3′
Chd2 forward primer	Ten Hacken et al., Genome Biol 2020	5′-ccactgctgtggggatgtcattat-3′
Chd2 reverse primer	Ten Hacken et al., Genome Biol 2020	5′-ctgccttttgggagcttcatcat-3′
Birc3 forward primer	Ten Hacken et al., Genome Biol 2020	5′-aacatggttcaagacagcgcc-3′
Birc3 reverse primer	Ten Hacken et al., Genome Biol 2020	5′-cacttgaccttgtcattggcac-3′
Samhd1 forward primer	Ten Hacken et al., Genome Biol 2020	5′-agcagccagctaagcgac-3′
Samhd1 reverse primer	Ten Hacken et al., Genome Biol 2020	5′-gctacctctgaagatgtccagca-3′
Atm forward primer	Ten Hacken et al., Genome Biol 2020	5′-acgtctttgtttctcctctcgaa-3′
Atm reverse primer	Ten Hacken et al., Genome Biol 2020	5′-caacagcattctggttctctccat-3′
Trp53 sgRNA	Ten Hacken et al., Genome Biol 2020	5′-gagcgctgctccgatggtga-3′
Mga sgRNA	Ten Hacken et al., Genome Biol 2020	5′-aagtaaaagtatgaacaccg-3′
Chd2 sgRNA	Ten Hacken et al., Genome Biol 2020	5′-aagcaacctaagattcagcg-3′
Birc3 sgRNA	Ten Hacken et al., Genome Biol 2020	5′-aaagtcatacttcaactcaa-3′
Samhd1 sgRNA	Ten Hacken et al., Genome Biol 2020	5′-cctccgaacctgggaaccgg-3′
Atm sgRNA	Ten Hacken et al., Genome Biol 2020	5′-tggatcacggagtacatcca-3′
Recombinant DNA		
psPAX2 plasmid	Addgene	Cat#12260; RRID: Addgene_12260
VSV-G plasmid	Addgene	Cat#8454; RRID: Addgene_8454
pLKO5- sgRNA Cherry	Our lab	
Software and algorithms		
UniProt	Open source	https://www.uniprot.org
CRISPick	Open source	https://portals.broadinstitute.org/gppx/crispick/public
CRISPResso2	Open source	https://github.com/pinellolab/CRISPResso2
Other		
Tools for mouse dissection	Of choice	N/A
Mortar and pestle	Of choice	N/A
Cell counting chamber	Of choice	N/A
Filters, pore size 40 micron (for bone marrow cell filtering)	Corning	Cat#352340

(*Continued on next page*)

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Syringe, 10 mL (for viral harvest)	Fisher Scientific	Cat#14955459
Filters, pore size 0.45 micron (for viral harvest)	Thermo Scientific	Cat#723-2545
Serological pipette 10 mL	Corning	Cat#357551
P1000 tips	Genesee Scientific	Cat#26-430
P200 tips	Genesee Scientific	Cat#26-412
P20 tips	Genesee Scientific	Cat#26-404
P10 tips	Genesee Scientific	Cat#26-401
T175 cell culture flask	Corning	Cat#431080
5 mL polystyrene FACS tube	Corning	Cat#352054
5 mL polystyrene FACS tube (with filter)	Corning	Cat#352235
50 mL Falcon tube	Corning	Cat#352070
15 mL Falcon tube	Corning	Cat#352096
1.5 mL safe-lock tube	Eppendorf	Cat #022363204
EDTA-coated tubes for blood collection	BD	Cat#365974
6-well plate	CellTreat	Cat#229105
48-well plate	CellTreat	Cat#229147
10-cm^2 plates for cell culture	Corning	Cat#353003
10-cm^2 plates for bacteria culture	Corning	Cat#351029
Gammacell irradiator	Of choice	N/A
Cell counting chamber	Of choice	N/A
Centrifuge	Of choice	N/A
Cell sorter	Of choice	N/A
Flow cytometer	Of choice	N/A

MATERIALS AND EQUIPMENT

FACS Buffer

Reagent	Final concentration	Amount
EDTA 0.5 M	2 mM	2 mL
FBS	2%	10 mL
PBS	N/A	4 mL
Total	**N/A**	**500 mL**

Store at 4°C for a maximum of one month.

STEP-BY-STEP METHOD DETAILS

Part 1. Lentiviral preparation

Timing: 1 week total; 1 h for Day 1, 3 h for Day 2, 2 h for Day 3, 2 h for Day 4, and 6 h for Day 5

This section details methods and tips for preparation of high-titer lentivirus.

1. Day 1 – Plating 293T.
   a. Seed 1.8–2.0 × 10^6 HEK293T cells per 10 cm^2 plate in 10 mL DMEM + 10% FBS (no antibiotics).
   b. Add cells dropwise and gently swirl to mix evenly.
   c. Incubate (37°C, 5% CO_2) until the following afternoon until ~60%–70% confluence (Figure 1).
2. Day 2 – Transfection of 293T cells.
   a. In a biosafety cabinet, set up 2 sterile 1.5 mL tubes per plate. Perform the below steps for each plate.

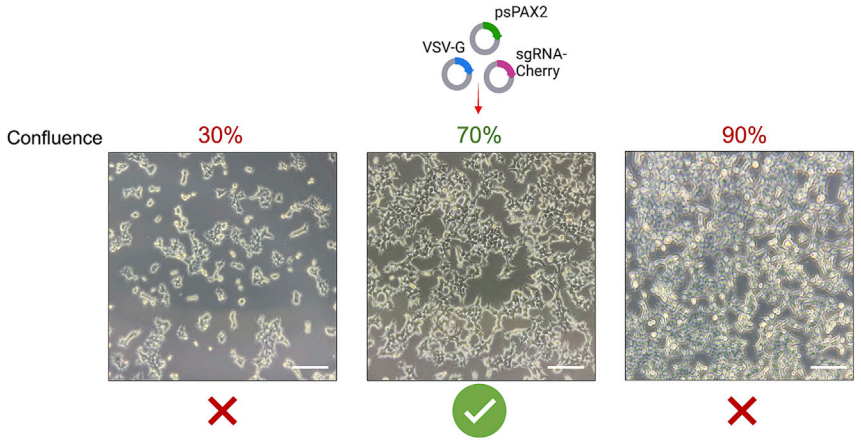

Figure 1. Example of suboptimal (left and right), and optimal (middle) confluence for HEK293T cells at time of transfection

Scale bar: 100 μm.

b. In one 1.5 mL tube, prepare a mixture of the 3 transfection plasmids: psPAX2 packaging plasmid (9 μg), VSV-G envelope plasmid (0.9 μg), pLKO5 vector expressing sgRNA of interest and mCherry (9 μg), and add OPTI-MEM to reach a total volume of 225 μL.

Reagent	Amount
psPAX2 plasmid	9 μg
VSV-G	0.9 μg
pLKO5-sgRNA	9 μg
OPTI-MEM	up to 225 μL

c. In second 1.5 mL tube, prepare a dilution of TransIT-LT1 transfection reagent: 36 μL OPTI-MEM, 54 μL TransIT-LT1 added dropwise.
   i. Mix by swirling the tip or gently flicking the tube (but ***not*** pipetting or vortexing).
   ii. Incubate for 5 min at room temperature (20°–25°C).
d. Add the diluted TransIT-LT1 mix to the 3 plasmid mix dropwise and mix by swirling the tip or gently flicking the tube.
e. Incubate the transfection mix for 30 min at room temperature (20°–25°C).
f. Carefully add the transfection mix dropwise to the plate of HEK293T cells. Be careful not to dislodge cells.
g. Move cells to lentivirus room BSL2 incubator and incubate for 18 h or until the following morning (37°C, 5% CO_2).

3. Day 3 – Media change.
   a. Use a 10 mL serological pipet to remove starting media.
   b. Then, using a 10 mL serological pipet, add 10 mL of virus harvest media (DMEM with 20% FBS, 1% GlutaMAX, 1% MEM-NEAA) *very slowly* (suggestion: 1 mL/10 s) against side of plate to reduce dislodging of cells.
   c. Incubate cells 24 h (37°C, 5% CO_2).
4. Day 4 – Virus harvest 1 and media change.
   a. Using a 10 mL syringe, carefully remove virus-containing media from the plate.
   b. Attach a 0.45 μm filter to syringe and filter into a 50 mL tube.

Note: Viral harvest from plates containing the same virus may be pooled. The first- and second-day's viral harvest will also be pooled. Do not fill over 40 mL per Falcon tube and keep on ice or at 4°C.

c. Using a 10 mL serological pipet, add 10 mL of virus harvest media *very slowly* (suggestion: 1 mL/10 s) against side of plate to reduce dislodging of cells.
d. Incubate cells 24 h in lentivirus room (37°C, 5% CO_2).
e. Store virus harvest at 4°C (in cold room) overnight (12–16 h).

5. Day 5 – Virus harvest 2, Lenti-X concentration, and preparing virus aliquots.
   a. Repeat viral harvest with syringe and filter, pooling as desired (but keep tube volume under 40 mL).
   b. Add 1:3 dilution of Lenti-X Concentrator, for example: 10 mL Lenti-X to 30 mL virus, 13.3 mL Lenti-X to 40 mL virus. Mix by inverting 10 times.
   c. Incubate at 4°C for 2–4 h.
   d. Spin in refrigerated centrifuge at 1,500 × *g* for 45 min.
   e. Decant off supernatant in a container with 100 mL bleach (to inactivate any residual viral particles).
   f. Resuspend pellet(s) in 50–100 μL single-use aliquots.
   g. Store at −80°C until use.

⚠ CRITICAL: Do not freeze-thaw viral preparations as it considerably reduces viral titers.

Pause point: Virus can be stored at −80°C for up to 6 months without substantial loss of titer.

Part 2. Hematopoietic stem and progenitor isolation and lentiviral transduction

Timing: 12 h

This section details methods for isolation of hematopoietic stem and progenitor cells [i.e., Lineage^{neg} Sca1^{pos} c-Kit^{pos} (LSK) cells] and *in vitro* transduction with high-titer lentivirus.

6. Day 1(morning)- Isolation of total bone marrow cells.
   a. Euthanize donor mouse of interest (see 'before you begin' for choice of strain) according to approved methods, collect spine and complete lower limbs.
   b. Remove all muscle from bones and smash spine, femur, tibia, and fibula from each of the lower limbs with mortar and pestle in RPMI + 10% FBS media. Use approx. 40 mL of media (4 rounds of smashing with 10 mL media each).
   c. Filter media through a 40-μm filter placed on a Falcon 50 tube.

   Note: Cut spine into pieces once in the mortar to smash more effectively and use pestle to prevent any pieces from falling into the filter when decanting off. It takes approximately 15 min to process one bone marrow.

   d. Count cells in a standard cell counting chamber before proceeding with hematopoietic progenitor enrichment.

   Note: Expected total cell count: 180 to 250 million cells per mouse.

   e. Spin down (400 g, 5 min at 4°C) and resuspend in FACS buffer (2% FBS, 2 mM EDTA in PBS) using 1 mL of buffer per 100 million cells.
   f. Enrich hematopoietic progenitors using EasySep Mouse Hematopoietic Progenitor Cell Isolation Kit according to the manufacturer's instructions (https://cdn.stemcell.com/media/files/pis/10000003753-PIS_02.pdf?_ga=2.128784460.1514010518.1675898534-1149677530.1675898534).

Note: when pouring enriched cell suspension into a 50 mL collection tube, hold and pour from magnet with one hand and collect excess cell suspension at the tip of the flow tube using a P1000 tip.

g. Count after EasySep isolation (expected count: 15 to 20 million cells per mouse).

7. Day 1 (afternoon)- Sorting of Lineage-/Sca-1+/c-Kit+ (LSK) cells by flow cytometry.
   a. After EasySep isolation, spin down (400 g, 5 min at 4°C) and resuspend in sterile FACS buffer for sort using approximately 1 mL FACS buffer per 20 × 10^6 cells.
   b. Remove supernatant gently using a P1000 tip to prevent cell loss and proceed with staining using the same 50 mL Falcon tube.
   c. Block Fc receptors by incubating cells with 1 μL purified anti-CD16/32 (1:100 dilution) per 1 million cells on ice for 10 min.

Note: during incubation, start thawing Stemspan media for collection and culture.

d. Stain cells with the following antibodies and incubate on ice for 15 min in the dark:

Reagent	Amount
Pacific Blue anti-mouse Lineage	100 μg/10^6 cells
Phycoerythrin (PE) anti-mouse Sca1(Ly-6^a/E)	200 μg /10^6 cells
Allophycocyanin anti-mouse c-Kit (APC):	200 μg /10^6 cells

   e. After staining, wash by adding ~10 mL FACS buffer and spinning down (a high volume wash helps minimize cell loss).
   f. Discard supernatant using P1000 and resuspend in FACS buffer using approximately 1 mL/ 20 × 10^6 cells.
   g. Right before sorting, filter using a 40-μm filter cap polystyrene 5 mL FACS tube.
   h. Sort Lineage$^-$ cells that are Sca-1^+ and c-Kit^+ (sorting strategy in Figure 2) into StemSpan media with murine cytokines thrombopoietin (TPO) and stem cell factor (SCF) at 50 ng/mL; expect a LSK population frequency of around 2%–3%.

Note: Expected output per each bone marrow is 180–250,000 LSKs.

8. Day 1 (late afternoon) - LSK Transduction.
   a. Gently resuspend sorted cells in a 50 mL Falcon tube at 20,000 LSKs/mL in pre-warmed media (StemSpan with 50 ng/μL TPO and 50 ng/μL SCF) and gently invert tube for homogenous resuspension.
   b. Plate cells according to downstream application (48-well plate for titration vs. 6-well plate for *in vivo* transplantation) at 20,000 LSKs/mL.

Note: In a 48-well plate add 1 mL per well, in 6-well plates add maximum 5 mL per well.

   c. Incubate plate for 20–30 min at 37°C, 5% CO_2.
   d. During this time, make sure virus and polybrene are thawing on ice and centrifuge for spinfection is pre-warmed at 37°C.
   e. For transduction: first add polybrene 1 μL/mL (concentration = 10 μg/μL stock aliquots) then 10 μL virus added dropwise.
   f. Resuspend gently using a P1000.
   g. Spin for 2 h, 900 g at 37°C.

⚠ CRITICAL: make sure centrifuge spinning temperature is stable throughout the run as this may largely impact transduction efficiency.

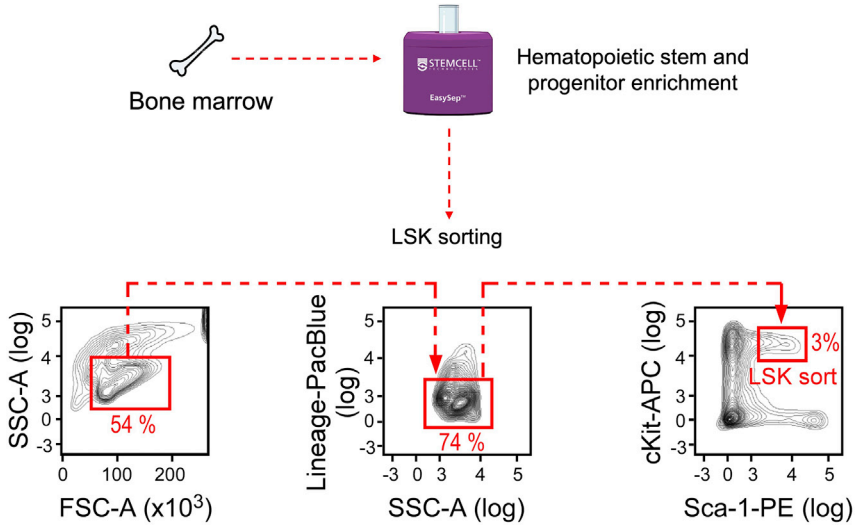

Figure 2. Schematic representation of steps required to extract LSKs via cell sorting
Image adapted from Figure S1A of ten Hacken et al.[3] with permission from the journal.

h. Remove plate and incubate plate at 37°C overnight (12–16 h).

The below steps highlight procedures required to: assess viral transduction efficiency *in vitro* (step 9) and *in vivo* transplantation of engineered LSKs (part 3).

9. Lentiviral transduction read out *in vitro*:
   a. Change LSK media after overnight (12–16 h) incubation: spin plate for 5 min at 400 g and exchange media extremely gently.
   b. Be careful when moving plate from incubator to hood to avoid dislodging cells.
   c. Incubate at 37°C for 72 h post transduction, then assess mCherry positivity by flow cytometric analysis.

Note: an optimal viral titer yield is 60% transduction rate achieved after transducing LSKs with 10 μL virus per mL (Figure 3). Virus volume is proportionally scaled up when utilizing plates of different size (e.g., 6 mL plates allow transduction of up to 5 mL cells, with 50 μL virus/well).

⚠ CRITICAL: individual titers should be very high (>50% transduction rate with 10 μL virus) to ensure efficient multiplexed transduction of LSKs.

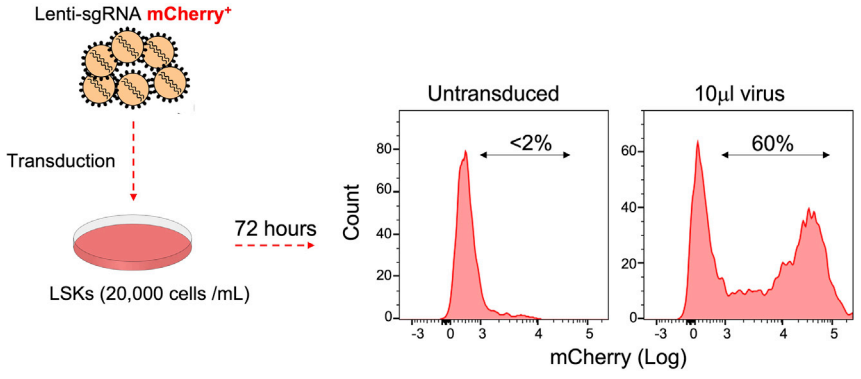

Figure 3. Example of successful viral titration, as identified by presence of >60% mCherry+ LSKs at 72 h post-transduction

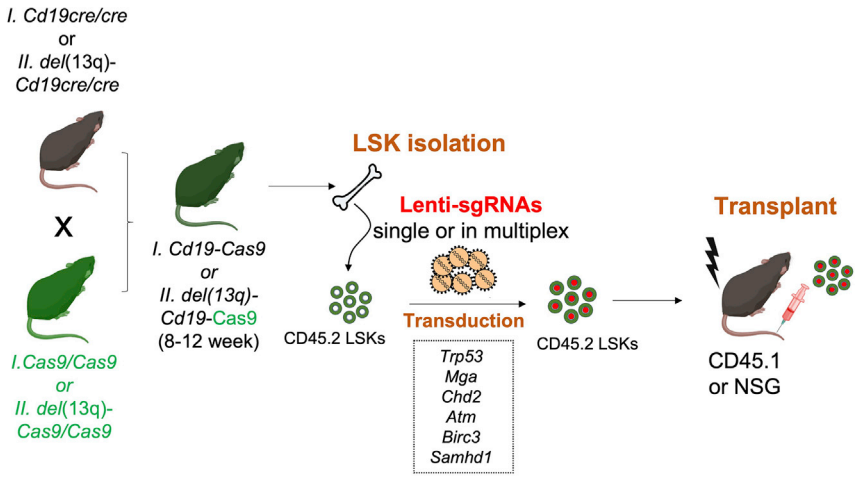

Figure 4. Schematic transplant workflow for single and multiplexed B-cell edited mouse models
Image adapted from Figure 1A of ten Hacken et al.[2] with permission from the journal.

Part 3. Transplant of lentivirally-engineered LSKs into recipient mice

Timing: 4 h Day 1, 6 h Day 2

This section describes procedures for transplant of engineered LSKs into pre-conditioned immunocompetent or immunodeficient recipient mice.

10. Pre-condition 8–12 week old CD45.1 (JAX ID: B6.SJL-*Ptprc*^{a} *Pepc*^{b}/BoyJ) recipient mice with 800 rads split dose irradiation (2 doses of 400 rads each), one day prior to injection. If recipient strain is NSG (JAX ID: NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ), 250 rads split dose (2 doses of 125 rads each) is sufficient (Figure 4).
11. On the day of transplant, prior to collecting the transduced LSKs, isolate hematopoietic stem and progenitors from the bone marrow of a separate donor mouse.

Note: these isolated cells will serve as 'bone marrow' helper cells (200,000 unsorted hematopoietic stem and progenitors to be co-injected with transduced LSKs per recipient mouse) to facilitate immune reconstitution of recipient mice.

12. Collection and transplant of lentivirally-transduced LSK cells.
    a. Spin plate containing transduced LSKs at 400 g for 5 min at room temperature (20°–25°C) to allow cell adhesion to bottom of plate.

⚠ CRITICAL: Open plate adapter very gently inside lentiviral hood to avoid cells returning in suspension.

    b. Thoroughly collect all cells and wash with PBS containing 20% FBS (PBS- 20% FBS) and transfer to 15 mL Falcon tubes.
    c. Spin for 5 min at 400 g at room temperature (20°–25°C).
    d. Add 1 mL PBS- 20% FBS to well.
    e. Gently collect by pipetting 3–4 × against the side and bottom of well to wash cells off the surface.

⚠ CRITICAL: Be extremely gentle to avoid lysing cells, minimize bubbles, and try to use a circular motion with pipet to dislodge cells at edges.

f. Collect cells into a 15 mL tube and repeat one wash with 5 mL PBS.

Note: Samples of the same condition should be pooled.

g. Spin tubes at 400 g for 5 min at room temperature (20°–25°C).
h. Remove supernatant with P1000 until about 1 mL remains, then use P200 to remove supernatant until ~100–150 μL remains.

⚠ CRITICAL: Be careful not to disturb pellet.

i. Gently resuspend samples in a total of 600–650 μL PBS per cells for every 5 mice.
j. Split sample into 1 eppendorf per cage with 600–650 μL resuspended cells – 100 μL injection per mouse plus extra volume to account for syringe dead volume.
k. Inject 100 μL cells into the tail vein of recipient mice.

Part 4. Verification of single or multiplexed gene edits

Timing: 2 weeks total, 4–6 h for Day 1, 5 h Day 2, 3 h for Day 14

These steps describe procedures necessary for assessment of efficacy of gene editing in mature B cells in transplanted mice *in vivo*.

13. Isolate B cells from the peripheral blood of animals at 4 months post-transplant by cell sorting.
    a. To do so, draw 200 μL peripheral blood from the submandibular vein, and lyse erythrocytes by incubating blood with 1 mL Ammonium-Chloride-Potassium (ACK) lysis buffer for 5 min.
    b. Wash cells with FACS buffer.
    c. Stain cells with 100 μg each of the following antibodies:

Reagent	Marker for
BV711 anti-mouse CD45.2	Donor-derived cells
Pacific Blue anti-mouse B220	B cells
PerCPCy5.5 anti-mouse Cd11b	Monocytes
PE/Cy7 anti-mouse CD3	T cells
APC anti-mouse CD5	T cells/CLL

    d. Sort edited ($GFP^+mCherry^+$) B cells according to sorting strategy in Figure 5, with an expected sorting output of 5–10,000 cells per sample.
14. Perform multiplexed PCR assessment of gene edits on DNA isolated from edited B cells.
    a. Isolate DNA from sorted cells via the QIAamp DNA Microkit and amplify 5–10 ng by multiplexed PCR, as previously described[1] on Day 2.

⚠ CRITICAL: to design primers for the multiplexed PCR, optimize primer design by targeting a 200bp region flanking the sgRNA cut site, ideally with identical or ±1°C melting temperature, to facilitate optimal concomitant amplification of all targets.

    b. Purify PCR product using a DNA, RNA and protein purification kit and submit for targeted sequencing (10–35 ng/μL concentration).
    c. Sequence at 50–80,000 reads per sample (MiSeq is generally sufficient).
    d. On Day 14, process FASTQ files by CRISPResso2 software analysis[5] to assess presence of gene edits quantitatively and qualitatively in individual samples (Figure 6).

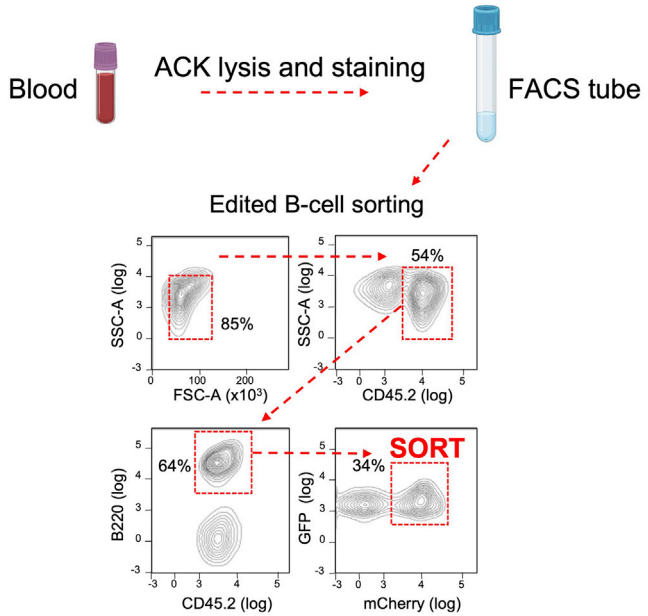

Figure 5. Schematic workflow for isolation of edited B cells from peripheral blood via cell sorting

Donor-derived (CD45.2$^+$) lymphocytes, which express B220 (i.e., B cells) and are double positive for GFP (i.e., Cas9) and mCherry (i.e., lentivirus) are selected for sorting.

e. Install CRISPResso2 using the instructions here: https://github.com/pinellolab/CRISPResso2#installation using Bioconda or Docker. Amplicons can be analyzed by running the command "CRISPResso –fastq_r1 {fastq read 1} –fastq_r2 {fastq read 2} –amplicon_seq {amplicon_sequence} –guide_seq {guide sequence}".

f. Read out editing rates in the file "CRISPResso_quantification_of_editing_frequency.txt" under the columns "Unmodified%" and "Modified%". Runs should have at least 10,000 reads aligned as reported in the "Reads_aligned" column.

g. Edited/unedited rates as well as abundance of frameshift vs. in-frame mutations is provided by the CRISPResso software output.

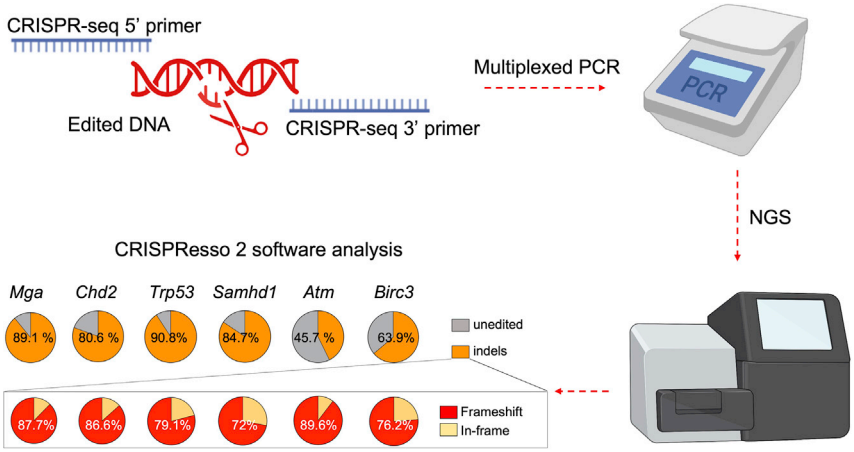

Figure 6. Multiplexed PCR-based assessment of CRISPR-Cas9 introduced gene edits

Indels, insertions-deletions. CRISPResso2 analysis results are also depicted in Figure 1A of ten Hacken et al.,[2] with permission from the journal.

EXPECTED OUTCOMES

Co-occurrence and co-selection of combinatorial disease drivers is a key oncogenic event in most cancers. We have recently reported the generation of single and multiplexed gene edited mouse models reflective of the genetic heterogeneity of chronic lymphocytic leukemia (CLL) and reliably recapitulating transformation into Richter's syndrome (RS).[3]

In this protocol, we provide step-by-step guidance on the various aspects to be taken into consideration when attempting to reproduce this strategy. We would like to point out that while our initial system is B cell restricted (i.e., *Cd19*-cre driven) and carrying a *del*(13q) background (as our initial intent was to model CLL, and *del*(13q) represents the most common genomic alteration of human disease),[6] the platform is largely applicable across cancers of hematologic origin. Examples of aspects to modify include: (i) conditional expression of Cas9 in cell lineage of interest (e.g., *Aid*-Cre for GC-derived B cell lymphomas); (ii) disease-predisposing trait (e.g., Cyclin-D1-amp for mantle cell lymphoma); (iii) pool of defining loss-of-function drivers (e.g., *Kmt2d*, *Crebbp*, *Cdkn2a/b* for transformed follicular lymphoma).

Whereas *S.Piogenes* Cas9-methods allow introduction of point mutations upon co-delivery of homology-directed-repair (HDR) templates, this process is largely inefficient,[7] and such strategy is complicated by the challenge of potential co-presence of cellular populations carrying both loss-of-function of the target allele and point mutations of the same gene. Thus, our reported methods can most reliably be utilized for LOF modeling, whereas novel base editing approaches remain more suitable for introduction of point mutations.[8] One additional caveat of utilizing Cas9-based approaches is represented by the inability to control zygosity of the introduced traits, given the random bi-allelic insertion of indels driven by presence of Cas9; this aspect should be taken into consideration when modeling drivers that exclusively appear as heterozygous variants. We point out that our methods are also amenable for LOF multiplexing,[3] with careful consideration about number of introduced lesions (optimal if <10), since the more chromosomal breaks are generated, the higher the chance of unwanted chromosomal translocations between Cas9-edited sites.[9] Overall, our strategy largely overcomes hurdles of conventional multiplexed genetic engineering achieved through complex breeding strategies, providing an affordable and versatile approach to LOF modeling *in vivo* with applicability to other hematologic cancers.

LIMITATIONS

The presented strategy is based on introduction of single or multiple sgRNAs in stem cells, with loss-of-function mutations then expressed in mature B cells. Such an approach does not allow the modeling of mutational acquisitions over time since all lesions are introduced concomitantly. As pointed out in the above section, since our strategy is based on the *S.Piogenes* Cas9 variant, it also preferably introduces loss-of-function mutations rather than point mutations, which remain potentially achievable through base-editing approaches.[8] Because of the stochastic nature of stem cell repopulation and differentiation into B cells, it is also not possible to exactly predict B cell editing efficiency based on LSK titration values, although we have reliably obtained high levels of B cell editing (20%–50% edited B cells) when high-titer lentiviral particles were utilized for LSK transduction.

TROUBLESHOOTING

Problem 1

Low number of retrieved LSKs post-sort (<200K/bone marrow) in step 7.

Potential solution

- Verify gating strategy. LSKs should account for ~2%–3% of enriched hematopoietic precursors.
- Verify that your collection media contains the murine cytokines SCF and TPO, which are essential to preserve cellular viability.

Problem 2

Low lentiviral titer at time of LSK transduction (<40% mCherry$^+$ LSK) in step 9.

Potential solution

The main reason for low lentiviral titers is either the low quality of the HEK293T cells, lack of activity of polybrene or reduced lentiviral titer due to temperature fluctuations in −80°C storage freezers when viral particles are stored for prolonged periods of time. We recommend:

- Utilize early passages HEK293T (up to P10) and avoid using >70% confluent plates at time of transfection.
- Store virus in small 'one-time use' aliquots and avoid freeze-thaw.
- Do not store viral preps to be used for *in vivo* transplant experiments for >6 months.
- Utilize 'one-time' aliquots of polybrene, carefully stored at −20°C.

Problem 3

Limited mCherry$^+$ engraftment rates in step 14.

Limited presence of mCherry$^+$ B cells in transplant recipients may occur because of several reasons: low number of LSKs injected at time of transplant, incomplete LSK transduction, issues with intravenous injection.

Potential solution

- Inject a minimum of 20–25,000 cells per recipient in addition to 200,000 bone marrow helper cells, considering some cellular loss during sample prep and/or intravenous injection.
- Incomplete transduction: low amounts of mCherry$^+$ cells may stem from transduction with low titer lentivirus. Please refer to 'problem 1' solution.
- Particularly when using CD45.1 immunocompetent strains, successful intravenous injection may be more challenging in smaller (e.g., 8-week old) mice. We thus recommend the use of 12-week recipients.

Problem 4

Limited presence of gene edits in mature B cells in step 14.

Assuming the PCR targeted sequencing protocol was previously tested on a BaF3-Cas9 cell line, possible reasons for low number of gene edits are:

- one or more of the introduced lesions is detrimental to mature B cell development;
- low lentiviral titers.

Potential solution

If the modeled gene lesion is detrimental to B cell development, we suggest the use of inducible approaches (e.g., Tet-inducibility), which allow to 'turn-on' expression of a certain sgRNA (and thus introduction of the gene edit) later during the life of the animal. To improve lentiviral titers, please refer to problem 2 potential solution.

Problem 5

Low co-occurrence rates of gene edits in step 13.

A possible reason for low number of gene edit co-occurrences is that lentiviral titers of individual viruses are not comparable, thus favoring transduction of a specific sgRNA-containing virus/es over the rest.

Potential solution

Try to standardize lentiviral prep procedures as much as possible, preferentially preparing multiple viruses at the same time, to reduce batch effects.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Catherine J. Wu (cwu@partners.org).

Materials availability

All reagents are commercially available or can be provided by the technical and/or lead contact, upon reasonable request.

Data and code availability

Data related to CRISPR-seq results of the single or multiplexed lines is included in previous publications.[2,3] No code was generated in this study.

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AUTHOR CONTRIBUTIONS

E.t.H., M.G, M.H.S., and K.L. designed and optimized methods; R.R. and K.C. analyzed sequencing data; G.B.H., J.H., and K.B. performed experiments; and C.J.W. provided overall supervision to the study.

DECLARATION OF INTERESTS

C.J.W. is an equity holder of BioNtech, Inc. and receives research funding from Pharmacyclics.

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