invitrogen

LentiPool v2 Human CRISPR Libraries

USER GUIDE

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Revision A.0

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Revision history: Pub. No. MAN0017461

Revision	Date	Description
Rev A.0	15 September 2021	New document for the use of LentiPool v2 Human CRISPR Libraries

Contents

1 Product information

Product description

CRISPR-Cas9 is an established method for genome editing and engineering, including the inducement of total gene expression knockout following imperfect repair of a specific genomic cleavage event. Invitrogen™ LentiPool v2 Human CRISPR Libraries have been developed for loss-of-function studies to identify the role of genes in the regulation of cellular processes and cellular responses to compounds, drugs or any perturbant that may impact these processes. The CRISPR-Cas9 guide RNAs (gRNAs) included in the LentiPool v2 Human CRISPR Libraries are designed based on proprietary gRNA design algorithm, which selects guides for maximum knockout efficiency without sacrificing specificity. Each library contains up to four sequence-verified distinct gRNA vector constructs per gene, packaged as lentiviral particles. LentiPool v2 Human CRISPR Libraries consist of gRNAs assembled in custom or pre-defined collections targeting particular gene families for functional genomics screening in a lentiviral pooled format, see page 29.

Contents and storage

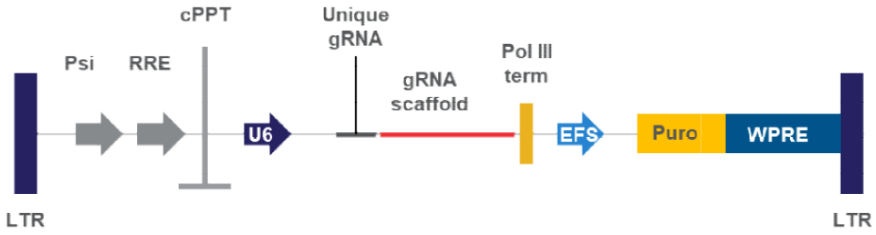
Contents	Amount	Storage [1]
LentiPool v2 Human CRISPR Library	50 µL x 6 aliquots (Druggable and Human Genome)	-80°C.
	50 µL x 4 aliquots (all other libraries)	
LentiPool CRISPR Negative Control	50 µL x 2 aliquots	

[1] Avoid repeated freeze/thaw cycles, which will severely reduce functional viral titer. All components are stable for at least 12 months after receipt when stored as directed.

Lentivirus characteristics

Characteristic	Description
Viral titer	Titers are typically >1x10^8 TU/mL by puromycin antibiotic-selection. Druggable and Human Genome are typically >1x10^9 TU/mL. Refer to the Certificate of Analysis provided with your library.
Gene number	See page 29, or see the corresponding CoA
sgRNA number	See page 29, or see the corresponding CoA

(continued)

Characteristic	Description
Lentiviral map	

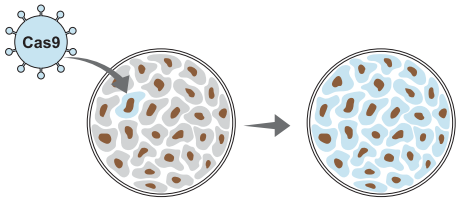
Workflow for functional screening

A Cas9-expressing stable cell line is generated with LentiArray™ Lentiviral Cas9 Nuclease utilizing selection by blasticidin resistance. These Cas9-expressing cells are then transduced with the LentiPool sgRNA library at the appropriate MOI and undergo selection for puromycin resistance. The transduced population then undergoes some selective pressure or treatment (drug, chemical perturbant, etc.). Genomic DNA is then isolated and the sgRNA inserts amplified by PCR. The amplicons then are sequenced to determine which genes were enriched/depleted in response to the treatment.

Workflow

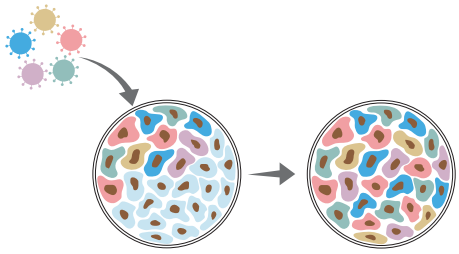
Generate Cas9-expressing cells

1. Lentiviral transduction of cells with LentiArray™ Cas9 lentiviral particles
2. Selection with blasticidin
3. Expansion of resistant cells



Transduction with pooled sgRNA library

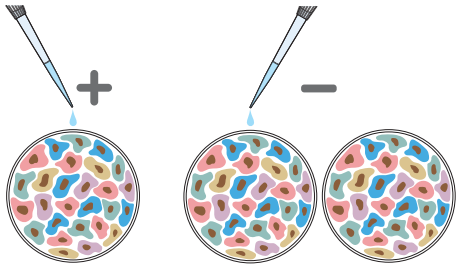
1. Lentiviral transduction of Cas9-expressing cells with LentiPool sgRNA library
2. Selection with puromycin
3. Expansion of resistant cells



Primary screening: two approaches

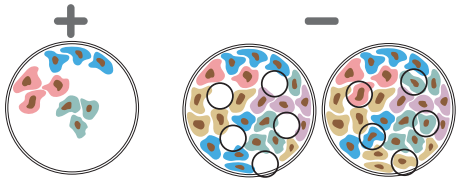
1. Positive selection (+): Apply treatment to cells (e.g. drugs, chemical perturbant)
2. Negative selection (-): Divide cells into reference and experimental samples

   Apply selective pressure or treatment only to experimental sample



Hit identification: NGS

- High-throughput sequencing analysis of enriched [positive selection (+)] or depleted [negative seletion (-)] sgRNA from genomic DNA



2 Methods

Procedural guidelines

Lentiviral pooled screening

Pooled screening is a widely adopted technique for identification of genes whose loss of function plays a role in drug responses, cellular viability, and other phenotypes that can be distinguished by cell survival, death, or expression of a fluorescent reporter.

Although the workflow is relatively straightforward, proper planning and care must be taken at each step to maintain proper representation of the sgRNAs.

Considerations for assay development prior to performing a pooled screen

- Before using the Invitrogen™ LentiPool v2 Human CRISPR libraries for screening, determine the growth kinetics, puromycin sensitivity, Polybrene™ tolerance, and transduction efficiency for your cell line.
- We recommend using cell lines stably expressing Cas9 nuclease for LentiPool v2 Human CRISPR library screening. Cas9 stable cell lines can be rapidly created using LentiArray™ Cas9 Lentivirus (Cat. No. A32064). For protocols to create Cas9 stable cell lines, see the *Invitrogen™ LentiArray™ Cas9 Lentivirus User Guide*.
- We recommend the use of LentiArray™ positive and negative control lentiviral particles (Cat. No. A32060 and A32063) to optimize the transduction and puromycin-selection conditions for your cells of interest.
- If possible, we strongly recommend using the LentiPool negative control library spiked with gRNAs against positive control genes to optimize the conditions for your screen. Individual gRNAs packaged as lentivirus for use as positive controls can be purchased from the Invitrogen™ LentiArray™ CRISPR library collection at **www.thermofisher.com/lentiarraygrna**.

Optimize conditions for LentiPool v2 Human CRISPR libraries

MOI determination

- Multiplicity of infection (MOI) is the ratio of the number of viral particles (or transduction units = TU) to the number of target cells. The optimal MOI for lentiviral delivery must be determined empirically for each cell line.
- The nature of each cell line affects the optimal MOI for successful transduction. For example, HT1080 cells are readily transducible, and an MOI of 1 (1 TU/cell) provides transduction efficiencies of around 90%. In some cell types a 5-fold or higher MOI may be needed to obtain the same transduction efficiency. We recommend using LentiArray™ CRISPR Positive Control Lentivirus, human HPRT, with GFP (Cat. No. A32060) to help determine the optimal MOI for use with your cell line. For protocols to determine MOI for your cell line, see the *Invitrogen™ LentiArray™ CRISPR Control Lentivirus Particles User Guide*.
- The LentiPool library should be applied at an MOI which provides a transduction efficiency of 25–30% to ensure that transduced cells are receiving no more than one gRNA. For example, if using a cell line such as HT1080 where an MOI of 1 results in 90% transduction efficiency, you would apply the LentiPool library at an MOI between 0.2 to 0.5 TU/cell. However, if using a cell type that requires a MOI of 10 for 90% transduction efficiency, you may need to apply the LentiPool library at 2 to 5 TU/cell to achieve transduction efficiency of 25–30%.

Experimental setup for your pooled screens

Loss-of-function screening using LentiPool v2 Human CRISPR libraries will require empirical assay development that will depend on the phenotype and downstream assay, for example, cell viability, proliferation, surface marker expression followed by cell sorting, etc.

The following are some general considerations during assay development:

- Optimize the concentration and duration of compound or perturbant being added to cells based on the desired phenotype being studied.
- In all situations, it is critical to have a control that lacks compound treatment, this is especially true for a negative selection screen, in which the treatment causes cell death in response to knockout of certain genes.
- In a positive selection screen (cells survive or are enriched after compound treatment), a control to compare the basal gRNA profile to the experimental sample will significantly improve the quality and interpretation of results.

Additional considerations

- Addition of Polybrene™ reagent (hexadimethrine bromide) at concentrations up to 8 µg/mL to the cell media can enhance lentiviral transduction efficiency by 2 to 10-fold; we recommend the use of Polybrene™ reagent when transducing the LentiPool libraries. However, not all cell types are tolerant of Polybrene™ reagent. A test for tolerance (i.e. minimal toxicity) with a serial dilution in the range of 1–8 µg/mL is suggested. If your cells are not tolerant of Polybrene™ reagent it may be excluded.
- Puromycin selection is used to eliminate non-transduced cells. Before performing your screen, the optimal puromycin concentration for selection must be determined empirically for your cell line. Antibiotic lot, cell type, cell growth kinetics, and cell culture conditions, including cell density and tissue culture vessel, may impact the amount of puromycin required for selection. See the LentiArray™ positive control (Cat. No. A32060) user guide for protocols to determine the optimal puromycin concentration for your cell type.
- If you have no previous experience with pooled screening it may be helpful to utilize the LentiPool negative control library spiked with positive control gRNAs to perform a mock screen to optimize the conditions. For the experimental details of this type of pilot screen, see “A positive selection screen of 6-thioguanine (6-TG) resistance gene using LentiPool negative control library spiked with HPRT1 gRNAs” on page 19.

Transduction protocol

The following procedure describes the suggested transduction protocol using the Invitrogen™ LentiPool Human CRISPR lentivirus particles in 6-well plates. Transduction may be scaled to larger wells and petri dishes depending on the size of the library and the number of cells required for the screen.

Fold coverage

A critical key to success for any lentiviral pooled screen is the degree of representation of any given construct within the screen, specifically, how many cells contain an integration of a given sgRNA. This is known as fold coverage, or fold representation. Sufficiently high sgRNA fold coverage improves the reproducibility between biological replicates and ensures a sufficient window for detecting changes in sgRNA abundance. It is recommended to perform calculations based on 500 to 1,000 independent integrations per sgRNA (i.e., 500- to 1,000-fold coverage). Higher fold coverage is easier to achieve with smaller libraries. See Table 1 for an example calculation using the human kinase LentiPool library with 500-fold coverage and 25–30% transduction efficiency in a cell line with an MOI = 1.

Table 1 Calculate the starting number of cells needed

Description	Amount
Number of positive control gRNAs[1]	3
Number of negative control gRNAs[2]	142
Number of gRNAs in the LentiPool Human CRISPR Kinase Library	3008
Total number of gRNAs	3153
Fold coverage of virus particles[3]	500
Viral particle number (= Total gRNA number x fold coverage)	1,576,500
MOI for 90% transduction efficiency[4]	1
TU/cell for 25 to 30% transduction efficiency	0.3
Total cells needed for transduction (=Viral particle number ÷ TU/cell)	5,255,000

[1] Positive control gRNAs in the same lentiviral vector may be used to spike into the pooled library for phenotypic selection. Inclusion of a positive control gRNA is optional but recommended.

[2] 142 gRNAs are contained in the LentiPool negative control library. Negative control gRNAs are designed to have no homology to the human genome.

[3] Fold coverage may be driven by the size of the gRNA library (500-1000 fold is recommended).

[4] Determined empirically, using positive control.

Day 1: Seed cells (use 6-well plate)

Note: Cells may be seeded in larger petri dishes depending on the size of the LentiPool Human CRISPR library.

Description	Amount
Number of cells to seed[1]	2,627,500
Seeding 2 × 10^5 cells/well of 6-well plate (20-25% confluence)	200,000
Number of wells for LentiPool Human CRISPR Library	14
Number of wells for mock control[2]	1

[1] 50% of total cells required for transduction if doubling time is 24 hours
[2] Mock control without virus is recommended to monitor the completion of puromycin selection

Incubate the cells at 37°C in a humidified 5% CO_2 incubator for 24 hours.

Day 2: Transduction

Description	Amount
Expected cell confluence	40–50%
Approximate total cells in LentiPool library wells on Day 2 (if doubling time is 24 hours)	5,600,000
Viral titer (TU/µL) (Kinase pooled library is 3 × 10^8 TU/mL [3 × 10^5 TU/µL], refer to CoA for viral titer of the library you will be using)	300,000
Volume (µL) of virus (3 × 10^8 TU/mL) required	5.6
Polybrene™ reagent (8 µg/mL) + 3% FBS complete medium for virus[1] (mL).	28
Polybrene™ reagent (8 µg/mL) + 3% FBS complete medium for mock control (mL)	2

[1] Do not add virus into the mock control well

1. Add virus (5.6 µL) to prepared medium (28 mL).
2. Remove the culture medium from cells.
3. Add 2 mL medium containing the virus to each well containing cells (do not add virus to the mock control).
4. (Optional) Centrifugation at 800 × *g* at room temperature for 20 minutes after adding the virus to the cells may enhance the viral infectivity.
5. Incubate the cells at 37°C overnight in a humidified 5% CO_2 incubator.

Day 3: Change medium and start antibiotic selection

1. Remove the transduction medium from cell cultures.
2. Add 2 mL/well of the complete medium with 10% FBS and the optimized concentration of puromycin.

Day 5 and after

1. Change medium every other day until all cells in mock control well are dead, typically 7–10 days.
2. (Optional) Expand cells transduced with gRNA library before performing screens. If expansion is required, it is recommended to consider co-transduction of Cas9 or using an inducible Cas9 system to limit gRNA cleavage activity to the initial stages following transduction.

Post-screen hit identification

Isolation of genomic DNA

1. Following completion of the screen isolate genomic DNA (gDNA) from your samples using the Invitrogen™ JetQuick™ Blood and Cell Culture DNA Midi Kit (Cat. Nos. A30703, A30704) or JetQuick™ Blood and Cell Culture DNA Maxi Kit (Cat. Nos. A30705, A30706). For protocol details, see the JetQuick™ Blood and Cell Culture DNA Maxi Kit user guide.
2. (Optional) Size fractionate gDNA via Thermo Scientific™ SdaI (SbfI) (10 U/µL) (Cat. No. ER1192) restriction enzyme digestion followed by gel purification. This is recommended for large cell samples (>10 million). Sample loss is expected when following this optional step.
3. Follow manufacturer's recommended protocol for SdaI digestion. Run digest overnight at 37°C.
4. For gel purification of digested samples, use a large well size (1–2 mL) to reduce number of gels required to run samples. Excise gel between 100 bp–600 bp and proceed with gel purification using Thermo Scientific™ GeneJET™ Gel Extraction Kit (Cat. Nos. K0691, K0692) or similar product following manufacturer's instructions.

Relative quantitation of sgRNA from gDNA samples by using PCR and next generation sequencing (NGS) analysis

Two critical points:

- Amplify the low abundant gRNAs to the level that can be detected by NGS.
- Keep PCR reaction in the linear range to prevent over-amplification of the PCR product.

1. In a pooled screen, only one sgRNA is introduced per cell therefore the relative amount of sgRNA within the isolated gDNA is very low and multiple PCR reactions are required to enrich for sgRNA content. The number of PCR reactions needed will be defined by the size of the pooled library, the fold coverage used in the experiment, and the relative efficiency of your PCR reaction.

   To calculate the amount of the input gDNA to be processed we use the estimate that 1 genome contains 6.6 pg of genomic DNA. Therefore, if one utilizes a library of 1,000 sgRNAs to maintain 500X coverage you would need to amplify the sgRNAs from 500,000 cells or 3.3 µg of gDNA. Scale the amount of input DNA according to your library size and library coverage used in your experiment. The number of PCR reactions to be performed to process this amount of gRNA will be determined by the efficiency of your PCR reaction.

2. The other critical point is to keep the PCR amplification reaction in the linear range and never over-amplify your PCR product. We suggest starting with 15–20 cycles for the 1st PCR and 5–10 cycles for the 2nd PCR, as we tested in the following example.

Note: You may also try performing one round (20–30 cycles) of PCR using primers of 2nd PCR or the primers adapted with the sequences from other NGS platform as long as your PCR product is a single band at ~155 bp.

1st PCR primers	2nd PCR primers[1]	Expected nested PCR product size
Primer-F1: 5′ CCCATGATTCCTTCATATTTGCATA 3′	Primer-F2: 5′ PHO-GGACTATCATATGCTTACCGTA 3′	155 bp
Primer-R1: 5′ TGCCATTTGTCTCAAGATCTAG 3′	Primer-R2: 5′ PHO-TTCAAGTTGATAACGGACTAGC 3′	

[1] 5′ phosphate (5′PHO) primers are used to facilitate downstream next generation sequencing (NGS) adaptor ligation for Ion Torrent™ NGS. You will need to modify the nested primers to facilitate NGS library sample preparation if you use a different NGS platform.

Note: The use of a high-fidelity Taq polymerase such as Phusion™ Green High-Fidelity DNA Polymerase (Cat. No. F534L) is recommended to maintain sgRNA sequence integrity when enriching sgRNAs from gDNA samples.

We recommend performing a serial dilution test of gDNA sample input to determine the optimal gDNA concentration to use for each reaction of the sgRNA enrichment PCR. A suggested range for this dilution test is 50 to 1000 ng/reaction in 50–100 ng increments (this may be limited by purified gDNA concentration). For this test, use the 1X reaction conditions (see Table 2). Once an optimal amount of gDNA/reaction is selected, continue to process up to the total amount of gDNA necessary for your experiment.

Table 2 1st PCR reaction to enrich sgRNA from gDNA

Reagent	Volume
5X Phusion™ Green HF Buffer	20 µL
10 mM dNTP	2.5 µL
Primer-F1 (10 µM)	2 µL
Primer-R1 (10 µM)	2 µL
DMSO (100%)	3 µL
Phusion™ DNA Polymerase (2 U/µL)	1 µL
gDNA	50–1000 ng
DNA/RNA-free water	to 100 µL
Total volume	**100 µL**

Table 3 PCR conditions

Temperature	Time	Cycles
98°C	20 sec	1 cycle
98°C	10 sec	20 cycles
62°C	20 sec	
72°C	20 sec	
72°C	1 min	1 cycle

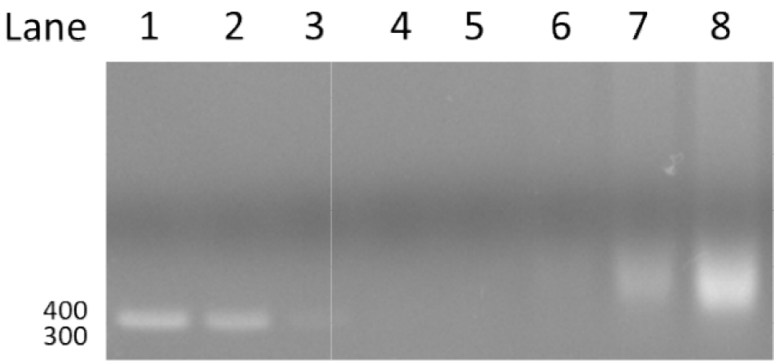


Figure 1 The results from first-round PCR testing gDNA sample input of 50 ng to 1.0 µg gDNA. The amplicon is detected in lanes 1–3 that correspond to 50–200 ng/reaction. There is reduced amplicon at increasing concentrations of gDNA and at 750 ng/reaction and above, there is an obvious loss of amplicon specificity. In this example, the user would want to use 50–100 ng of gDNA template. To complete our example from above, utilizing 100 ng of gDNA as the reaction input, a user would need to perform 33 PCR reactions to process the 3.3 µg of gDNA required to analyze their experiment.

① 50 ng gDNA
② 100 ng gDNA
③ 150 ng gDNA
④ 200 ng gDNA
⑤ 250 ng gDNA
⑥ 500 ng gDNA
⑦ 750 ng gDNA
⑧ 1000 ng gDNA

Prepare sgRNA-enriched PCR sample for NGS analysis

Note: This procedure describes how to prepare the enriched sgRNA sample for analysis on the Ion Torrent™ NGS platform. Please refer to manufacturer protocols for NGS library preparation for use on NGS systems other than the Ion Torrent™ NGS platform.

1. Pool first-round PCR samples and mix well. Perform PCR purification (use a column or bead based clean up kit, such as Invitrogen™ PureLink™ Pro 96 PCR Purification Kit (Cat. No. K310096A) and ethanol precipitation to concentrate PCR product. The length of the first-round fragment is about 350 bp (see Table 4) and 1 ng of dsDNA is about 2.6×10^9 amplicons. In general, 2 ng of the sgRNA enriched sample is considered sufficient to cover >1000X gDNA integrations for LentiPool libraries.

Table 4 2nd (Nested) PCR using 5′PHO-U6 / 5′PHO-Lenti-R2

Reagent	Volume
5X Phusion™ Green HF Buffer	10 µL
10 mM dNTP	1.25 µL
PHO-primer-F2[1] (10 µM)	1 µL
PHO-primer-R2[1] (10 µM)	1 µL
DMSO (100%)	1.5 µL

Table 4 2nd (Nested) PCR using 5′PHO-U6 / 5′PHO-Lenti-R2 *(continued)*

Reagent	Volume
DEPC-H_2O	29.75 µL
Phusion™ DNA Polymerase (2 U/µL)	0.5 µL
1st round PCR product	5 µL
Total volume	**50 µL**

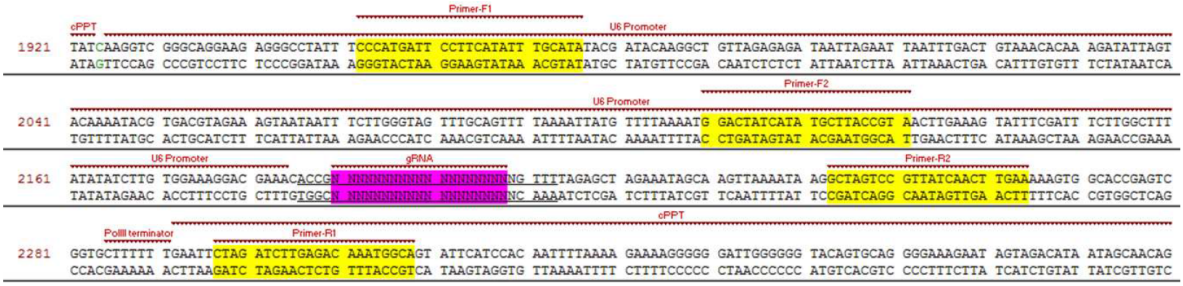
[1] *5′ Phosphate primers are used to facilitate the downstream Ion adaptor ligation.*

Table 5 PCR conditions

Temperature	Time	Cycles
98°C	20 sec	1 cycle
98°C	10 sec	10 cycles
62°C	20 sec	
72°C	20 sec	
72°C	1 min	1 cycle

2. Confirm PCR product by loading 5 µL of the nested PCR product on 2% E-Gel™ EX Agarose Gel (Cat. No.: G402022). The reaction should result in a clean band with an expected size of 155 bp.

3. Refer to the following sequence alignment for the first-round and second-round PCR primer positions relative to gRNA position.



NGS and data analysis for identification of hits

Note: You may use Ion Torrent™ or other NGS platforms for identification of the hits of gene/gDNA involved in your loss-of-function screening.

Purify the nested PCR product using PureLink™ PCR Micro Kit (Cat. No. K310250) following standard protocol.

Perform adaptor ligation

Reagents	Volume (1X reaction)
Water	13 µL
5x ligase buffer	5.0 µL
Adaptor P1	0.5 µL
dNTP mix (10 µM)	0.5 µL
HC DNA ligase (5 u/µl)	0.5 µL
Platinum™ Tfi Exo(-) DNA Polymerase	2 µL
PHO-PCR product (Total: 10–20 ng)	5 µL
Barcode A adaptor[1]	1 µL
Total:	**25 µL**

[1] Samples may be barcoded using the Barcode adaptor (Cat. No. 4474517) when interrogating multiple samples.

1. Incubate reaction at 16°C for 30 minutes, 25°C for 30 minutes, and 72°C for 30 minutes and hold at 4°C.

2. Pool all barcoded samples at this stage and purify the mixed sample using AMPure XP beads (Beckman Coulter™; A65880, A65881).

Cleanup ligation reaction

Table 6 Purification using AMPure XP beads

Reagent	Volume (1X reaction)
Nest PCR-5P product	20 µL
Beads (1.5X sample volume)	30 µL
Total volume	**50 µL**

1. Mix 10 times by pipetting. Separate the beads using magnetic separator for 1–2 minutes, and remove the supernatant.

2. Wash the beads twice with 200 µL of 70% ethanol by gentle pipetting. Remove residual 70% ethanol completely after the second wash.

3. Elute DNA in 20 µL of DNA/RNA-free water by pipetting 10 times. Separate the beads using a magnetic separator for 1–2 minutes and collect the supernatant containing DNA in a fresh tube.

Amplify ligated PCR product

Table 7 Amplification of adaptor-ligated PCR product

Reagent	Volume (1X reaction)
Platinum™ PCR Supermix High Fidelity	100 µL
Ion Torrent™ amplification primer mix	5 µL
Pooled sample	5 µL
Total volume	**110 µL**

Table 8 PCR conditions

Temperature	Time	Cycles
95°C	5 min	1 cycle
95°C	15 sec	10 cycles
58°C	15 sec	
70°C	30 sec	
70°C	3 min	1 cycle
4°C	Hold	—

Cleanup PCR reaction

Table 9 Purification using AMPure XP beads

Reagents	Volume
Barcoded A/P1 PCR product	50 µL
Beads (1.5X sample volume)	75 µL
Total	**120 µL**

1. Mix 10 times by pipetting beads using magnetic separator for 1–2 minutes and remove supernatant.
2. Wash the beads twice with 200 µL of 70% ethanol by gentle pipetting. Remove 70% ethanol residue completely after second wash.
3. Elute DNA in 20 µL of DNA/RNA-free water by pipetting 10 times, separate beads using magnetic separator for 1-2 minutes and collect the supernatant containing DNA in a fresh tube.
4. Check the PCR product on a gel. The expected size should be about 240 bp. Make sure no small size adaptors or primer dimers (<100 bp) are visible on gel. Repeat cleanup if there is any visible small size adaptor.

5. Measure DNA concentration using Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851)

6. Calculate concentration in picomolar (pM) for final PCR product that is going to be used for NGS emulsion reaction using Ion Chef™ platform:

Description	Value
Qubit™ dsDNA concentration	1 ng/µL
Length of insert	155 bp
MW per base pair in case of double strand DNA	660
MW of 155 bp double strand DNA	102300
pM concentration = (dsDNA conc. x 10^9)/ insert MW	9775
Fold to dilute DNA to 100 pM if using PGM™ sequencing platform	98
Fold to dilute DNA to 200 pM if using S5 sequencing platform	49

7. Use Ion Chef™ system for NGS emulsion reaction. Use 50 µL of diluted DNA sample for each chip with Ion PI™ Hi-Q™ Chef Kit (Cat. No. A27198).

8. Run sequencing on PGM™ platform following corresponding manual.

9. Use "Coverage Analysis" plug-in from Torrent Suite™ or corresponding analysis tools if using other NGS platforms for read counting against the gRNA sequence reference.

A positive selection screen of 6-thioguanine (6-TG) resistance gene using LentiPool negative control library spiked with HPRT1 gRNAs

- 6-TG is a purine analog that is used for the treatment of leukemia in adults and children as well as the treatment of inflammatory diseases. The enzyme encoded by *HPRT1* converts 6-TG to 6-thioguanine monophosphate, which is then converted to 6-thioguanine triphosphate (6-TGTP). 6-TGTP and deoxyguanosine triphosphate (dGTP) have similar structures and are interchangeable in several processes, including DNA replication. If 6-TGTP is incorporated into genomic DNA (gDNA) during DNA replication instead of dGTP, the resulting base pair mismatches trigger cell cycle arrest and cell death. Disruption of the *HPRT1* gene blocks the conversion of 6-TG to 6-TGTP, which prevents 6-TG-induced cell death.

  The aim of this study was to demonstrate the identification of *HPRT1*-knockout cells in the context of a positive selection pooled screen.
- Four gRNAs were designed to target *HPRT1* (see Table 10) and cloned into the lentivirus vector for LentiPool libraries. A cassette encoding Green Fluorescent Protein (GFP) was inserted between the gRNA sequence and the puromycin resistance gene to monitor dynamic changes in cells with incorporated *HPRT1*-targeting gRNAs during the screening process.

Table 10 CRISPR-Cas9 guide RNAs designed to target the *HPRT1* gene

Guide RNA	Target sequence
HPRT-T1	CTGTCCATAATTAGTCCATG
HPRT-T2	TCTTGCTCGAGATGTGATGA

Table 10 CRISPR-Cas9 guide RNAs designed to target the HPRT1 gene *(continued)*

Guide RNA	Target sequence
HPRT-T3	CATACCTAATCATTATGCTG
HPRT-T4	ATTATGCTGAGGATTTGGAA

Procedural guidelines

The following screening procedure was used to screen HT1080 and HEK293 cells. You may use different cells according to your particular needs.

1. Perform a kill curve experiment by treating the HT1080 and HEK293 cells with 6-TG at concentrations of 0.5 µM, 1 µM, 5 µM, 10 µM, 20 µM and 50 µM. The calculation example (see Table 11) was performed for both cell lines treated with 10 µM 6-TG. Calculations for the negative control library (NCL) were based on the LentiPool NCL.

   Table 11 General NCL + HPRT calculation

Description	Value
Number of *HPRT1* gRNAs	4
Number of NCL gRNAs	142
Total number of gRNAs	146
Target coverage	1,000
Viral particle number (total gRNA coverage)	146,000
Target multiplicity of infection (MOI)	0.3
Total cells needed for transduction (viral particle total/MOI)	486,666

2. To spike the NCL with *HPRT1*-targeting gRNAs (NCL + HPRT), mix *HPRT1* gRNA viral particles into the NCL with an equal ratio of viral particles and calculate the final viral titer. The final titer of the mixed library may vary depending on the titer of each lot. The titers of all *HPRT1* positive controls and the NCL in this work were 7×10^5 TU/µL, and the titer of the mixed control library was the same.

Day 1: Seed cells in a 6-well plate

Description	Value
Seed 2.43 × 10^5 cells per well[1] (20–25% confluence)	243,333
Number of wells for LentiPool Human CRISPR Library[2]	6
Number of wells for mock control[3]	1

[1] 50% of total cells required for transduction if the doubling time is 24 hours.
[2] See "Six-well plate layout for seeding the cells".
[3] Including a mock control without the virus is recommended for monitoring puromycin selection.

Six-well plate layout for seeding the cells

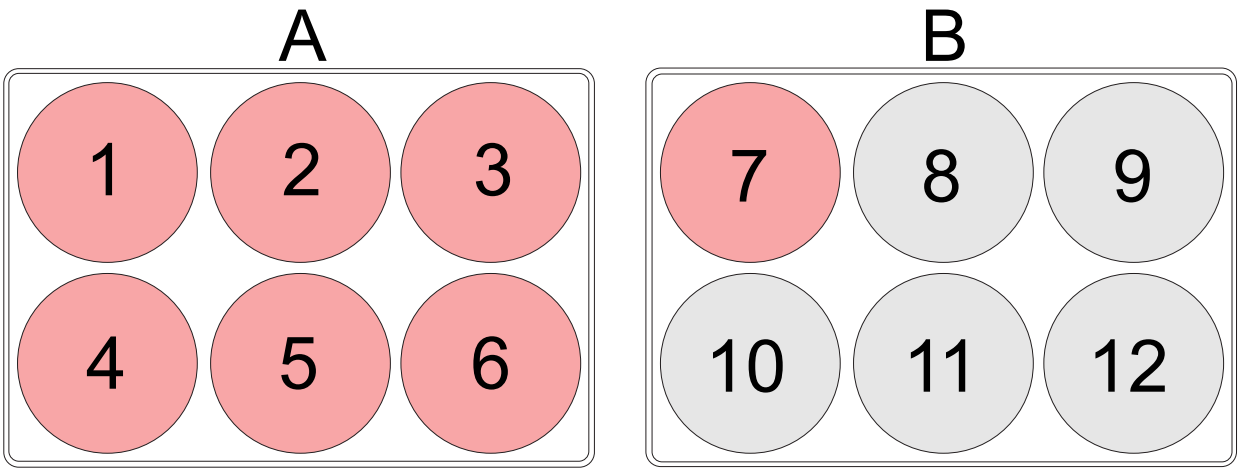


Figure 2 Six-well plate layout

A. Experimental plate; **B**. Untreated plate

① Harvest Day 5 Cells + lentivirus
② Harvest Day 5 Cells + lentivirus + 6-TG
③ Harvest Day 7 Cells + lentivirus + 6-TG
④ Harvest Day 5 Cells + lentivirus
⑤ Harvest Day 5 Cells + lentivirus + 6-TG
⑥ Harvest Day 7 Cells + lentivirus + 6-TG
⑦ Cells only
⑧ –
⑨ –
⑩ –
⑪ –
⑫ –

Day 2: Transduction

Description	Value
Expected cell confluence	40–50%
Approximate number of cells on Day 2 with a doubling time of 24 hours	486,666
Viral titer (TU/µL)	50,000
Volume (µL) of virus required for 1000-fold coverage (146 guides × 1000 = 146,000 viral particles)[1]	2.92
Volume (mL) of complete medium containing Polybrene™ reagent (8 µg/mL) and 3% FBS to prepare for the virus	12
Volume (mL) of complete medium containing Polybrene™ reagent (8 µg/mL) and 3% FBS to prepare for the mock control	2

[1] Add virus (2.92 µL) to 12 mL of the prepared medium. Do not add virus to the mock control.

1. Remove the culture medium from the wells.
2. Add 2 mL of the medium containing the virus to each well that contains cells.

   Centrifuging the plate at 800 × *g* at room temperature for 20 minutes after adding the virus to the cells may enhance the viral infectivity (*optional*).
3. Incubate the cells at 37°C overnight in a humidified incubator with 5% CO_2.

Day 3: Cell treatment with puromycin and 6-TG

1. Remove the transduction medium from the cell cultures.
2. Add 2 mL of the complete medium prepared with 10% FBS, 0.75 µg/µL puromycin, and 10 µM 6-TG to each well.

Day 6 (Day 3 post 6-GT treatment)

Check the cells for GFP expression and replace the medium containing 10% FBS, puromycin, and 6-TG with fresh medium.

Day 8 (Day 5 post 6-GT treatment)

Check for GFP expression and harvest cells five days after 6-TG treatment. For the cells that will be harvested 7 days after 6-TG treatment, replace the medium containing 10% FBS, puromycin, and 6-TG with fresh medium.

Day 10 (Day 7 post 6-GT treatment)

Check for GFP expression and harvest cells seven days after 6-TG treatment. For images used to monitor cell viability and GFP expression at all time points, see Figure 3.

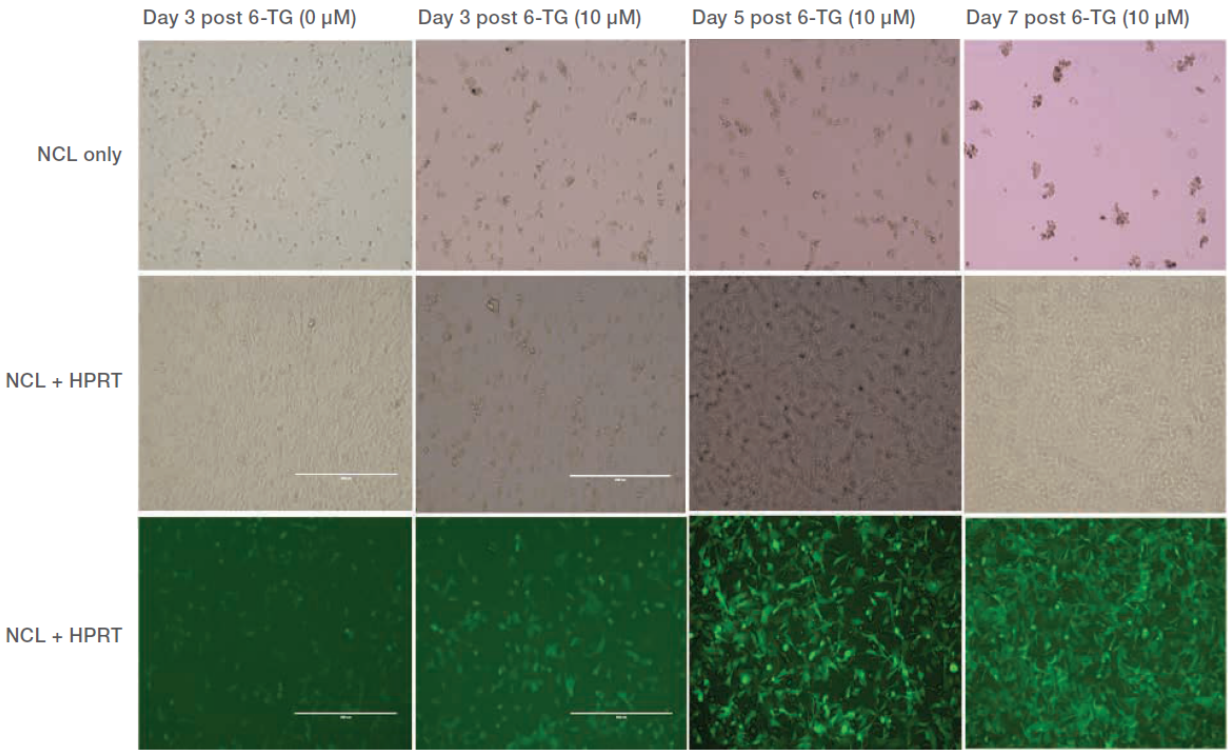


Figure 3 Positive selection screen for 6-TG resistance gene in HT1080 cells transduced with LentiPool NCL spiked with HPRT1 gRNAs

Bright-field images of cells transduced with the LentiPool NCL as a negative control are shown in the top row. Bright-field images of cells after transduction with the LentiPool NCL spiked with four *HPRT1* gRNAs (NCL + HPRT) are shown in the middle row, and GFP screening images are shown in the bottom row. Images were collected 3, 5, and 7 days after treatment with 10 µM 6-TG. Cell death was observed 3 days after treatment among both the negative control cells (NCL only) and the NCL + HPRT cells treated with 10 µM 6-TG (column 2), but cell death was not observed in samples that were not subjected to 6-TG treatment (column 1). NCL + HPRT cells transduced with the *HPRT1* gRNA were alive 3 days after 6-TG treatment and were enriched 5 and 7 days after treatment. Cells in the NCL-only samples were dead by day 7.

NGS for hit identification

1. Use the PureLink™ Genomic DNA Mini Kit (Cat. No. K1820-00) to isolate genomic DNA (gDNA) from the cells harvested on Day 5 and Day 7 following 6-TG treatment.

2. Perform two consecutive PCR reactions using these primers and the Phusion™ Green High-Fidelity DNA Polymerase (Cat. No. F534L).

1st PCR primers	Primer-F1: 5′ CCCATGATTCCTTCATATTTGCATA 3′
	Primer-R1: 5′ TGCCATTTGTCTCAAGATCTAG 3′
2nd PCR primers	Primer-F2: 5′ PHO-GGACTATCATATGCTTACCGTA 3′
	Primer-R2: 5′ PHO-TTCAAGTTGATAACGGACTAGC 3′

Table 12 1st PCR

Reagent	Volume (1X reaction)
5X Phusion™ Green HF Buffer	5.0 µL
10 mM dNTP	0.6 µL
F1: U6 primer 2 (10 µM)	0.5 µL
R1: Lenti-R1 (10 µM)	0.5 µL
DMSO (100%)	0.75 µL
DNA/RNA-free water	14.9 µL
Phusion™ DNA Polymerase (2 U/µL)	0.25 µL
gDNA (10 ng/µL)	2.5 µL
Total volume:	**25 µL**

Table 13 Conditions for 1st PCR

Temperature	Time	Cycle
98°C	20 sec	1 cycle
98°C	10 sec	20 cycles
62°C	20 sec	
72°C	20 sec	
72°C	1 min	1 cycle

Table 14 2nd PCR with 5′ PHO-U6 and 5′ PHO-Lenti-R2 nested primers

Reagents	Volume (1X reaction)
5X Phusion™ Green HF Buffer	10 µL
10 mM dNTP	1.25 µL
F1: PHO-U6* (10 µM)[1]	1 µL

Table 14 2nd PCR with 5′ PHO-U6 and 5′ PHO-Lenti-R2 nested primers *(continued)*

Reagents	Volume (1X reaction)
R1: PHO-Lenti-R2* (10 µM)	1 µL
DMSO (100%)	1.5 µL
DEPC-H_2O	32.75 µL
Phusion™ DNA Polymerase (2 U/µL)	0.5 µL
1st round PCR product	2 µL
Total volume	**50 µL**

[1] 5' phosphate (5' PHO) primers are used to facilitate downstream next generation sequencing (NGS) adaptor ligation for Ion Torrent™ NGS. You will need to modify the nested primers to facilitate NGS library sample preparation if you use a different NGS platform.

Table 15 Conditions for 2nd PCR

Temperature	Time	Cycles
98°C	20 sec	1 cycle
98°C	10 sec	10 cycles
62°C	20 sec	
72°C	20 sec	
72°C	1 min	1 cycle

3. To visualize the PCR product, load 5 µL of the nested PCR product onto an E-Gel™ EX agarose gel (2%). The expected size should be around 155 bp.

4. Purify the PCR product using PureLink™ PCR Micro Kit (Cat. No. K310250) following the standard protocol.

5. Perform NGS adaptor ligation using the following protocol:

Table 16 Adaptor ligation and nick repair

Reagent	Volume (1X reaction)
Water	13 µL
10X ligase buffer	2.5 µL
Adaptor P1	0.5 µL
dNTP mix	0.5 µL
T4 DNA ligase (5 U/µL)	0.5 µL
Platinum™ *Tfi* Exo⁻ DNA polymerase	2 µL
PHO-PCR product (Total: 10–20 ng)	5 µL
Barcode A adaptor[1]	1 µL
Total volume:	**25 µL**

[1] Samples may be barcoded using barcoded adaptors when examining multiple samples.

6. Incubate at 16°C for 30 minutes, 25°C for 30 minutes, and 72°C for 30 minute then hold at 4°C.

7. Pool all barcoded samples at this stage and purify the mixed sample using AMPure XP beads (Beckman Coulter™, A65880 or A65881).

Cleanup ligation reaction

Table 17 Purification using AMPure XP beads

Reagent	Volume (1X reaction)
Nest PCR-5P product	20 µL
Beads (1.5X sample volume)	30 µL
Total volume	**50 µL**

1. Mix 10 times by pipetting. Separate the beads using magnetic separator for 1–2 minutes, and remove the supernatant.

2. Wash the beads twice with 200 µL of 70% ethanol by gentle pipetting. Remove residual 70% ethanol completely after the second wash.

3. Elute DNA in 20 µL of DNA/RNA-free water by pipetting 10 times. Separate the beads using a magnetic separator for 1–2 minutes and collect the supernatant containing DNA in a fresh tube.

Amplify ligated PCR product

Table 18 Amplification of adaptor-ligated PCR product

Reagent	Volume (1X reaction)
Platinum™ PCR Supermix High Fidelity	100 µL
Ion Torrent™ amplification primer mix	5 µL
Pooled sample	5 µL
Total volume	**110 µL**

Table 19 PCR conditions

Temperature	Time	Cycles
95°C	5 min	1 cycle
95°C	15 sec	10 cycles
58°C	15 sec	
70°C	30 sec	
70°C	3 min	1 cycle
4°C	Hold	—

Cleanup PCR reaction

Table 20 Purification using AMPure XP beads

Reagents	Volume (1X reaction)
Barcoded A/P1 PCR product	50 µL
Beads (1.5X sample volume)	75 µL
Total	**120 µL**

1. Mix 10 times by pipetting. Separate the beads using a magnetic separator for 1–2 minutes, and remove the supernatant.
2. Wash the beads twice with 200 µL of 70% ethanol by gentle pipetting. Remove residual ethanol completely after the second wash.
3. Elute DNA in 20 µL of DNA/RNA-free water by pipetting 10 times. Separate the beads using a magnetic separator for 1–2 minutes and collect the supernatant containing DNA in a fresh tube.
4. Check the PCR product on a gel. The expected size should be ~240 bp. See Figure 4. Make sure no small size adaptors or primer dimers (<100 bp) are visible on the gel. Repeat cleanup if any adaptor is detected.

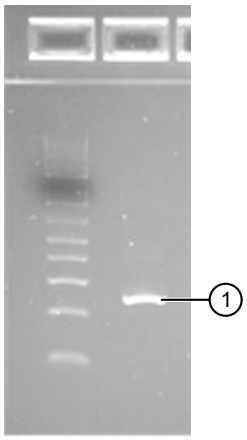


Figure 4 Ion Torrent™ adaptor-ligated PCR product and purified by AMPure XP beads.
A single band at approximately 240 bp was observed. No small size adaptor was detected.

5. Measure DNA concentration using Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851)

6. Calculate the concentration of the final PCR product that will be used to purify the NGS emulsion reaction on an Ion Chef™ instrument. See the following table:

Description	Value
dsDNA concentration	4.96 ng/µL
Length of insert	155 bp
MW per base pair of double-stranded DNA	660
MW of 155 bp double-stranded DNA	102,300
Concentration (pM) = (ng/µL × 10^9)/MW	48,485
Dilution factor to get100 pM DNA using Ion PGM™ Hi-Q™ Chef Kit	484

7. Use Ion Chef™ instrument for NGS emulsion reaction. Use the Ion PGM™ Hi-Q™ Chef Kit (Cat. No. A25948) to obtain 50 µL of diluted DNA for each chip.

8. Perform sequencing on the Ion PGM™ system following the instructions in the manual.

9. Use the Coverage Analysis plug-in in the Torrent Suite™ software package.

10. Download the .csv data file and save it in Microsoft™ Excel™ format.

11. Normalize the read number for each gRNA to reads per million (RPM) to more easily compare different samples and NGS runs.

12. Plot the values against each gRNA to identify potential *HPRT1* gRNA hits. See Figure 5.

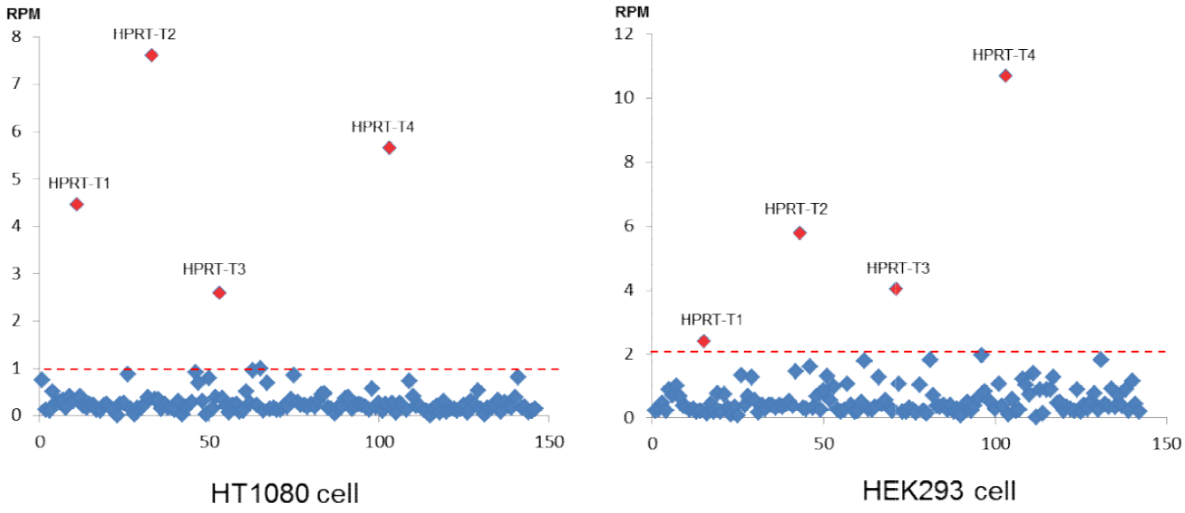


Figure 5 Positive selection screen for the 6-TG resistance gene in HT1080 and HEK293 cells using the LentiPool NCL spiked with HPRT1 gRNAs.

The RPM of each *HPRT1* gRNA 7 days after 6-TG treatment was plotted against those of 142 negative controls and four HPRT1 positive controls.The RPMs of the four HPRT1 gRNAs were significantly higher than the RPM of any negative control.

A

Pre-defined libraries

Pre-defined LentiPool v2 Human CRISPR libraries

Invitrogen™ LentiPool v2 Human CRISPR libraries	Gene #[1]	gRNA #
Human Kinases	752	3008
Human GPCRs	376	1504
Human Phosphatases	277	1108
Human Cancer Biology	495	1980
Human Drug Transport	96	384
Human Tumor Suppressors	689	2756
Human Proteases	458	1832
Human Nuclear Hormones	45	180
Human Epigenetics	383	1532
Human Ion Channels	315	1260
Human DNA Damage Response	541	2164
Human Membrane Trafficking	136	544
Human Cell Surface	748	2992
Human Transcription Factors	1983	7932
Human Cell Cycle	1444	5776
Human Apoptosis	879	3516
Human Ubiquitin	943	3772
Human Druggable Genome	11,891	47,564
Human Whole Genome	18,278	73,112
Negative Controls[2]	0	142
Human HPRT Positive Control	1	4

[1] Total gene and gRNA counts are subject to change.

[2] Negative Controls are non-targeting gRNAs that do not match any sequence in the human genome.

B

Safety

WARNING! GENERAL SAFETY. Using this product in a manner not specified in the user documentation may result in personal injury or damage to the instrument or device. Ensure that anyone using this product has received instructions in general safety practices for laboratories and the safety information provided in this document.

- Before using an instrument or device, read and understand the safety information provided in the user documentation provided by the manufacturer of the instrument or device.
- Before handling chemicals, read and understand all applicable Safety Data Sheets (SDSs) and use appropriate personal protective equipment (gloves, gowns, eye protection, and so on). To obtain SDSs, see the “Documentation and Support” section in this document.

Chemical safety

WARNING! GENERAL CHEMICAL HANDLING. To minimize hazards, ensure laboratory personnel read and practice the general safety guidelines for chemical usage, storage, and waste provided below. Consult the relevant SDS for specific precautions and instructions:

- Read and understand the Safety Data Sheets (SDSs) provided by the chemical manufacturer before you store, handle, or work with any chemicals or hazardous materials. To obtain SDSs, see the "Documentation and Support" section in this document.
- Minimize contact with chemicals. Wear appropriate personal protective equipment when handling chemicals (for example, safety glasses, gloves, or protective clothing).
- Minimize the inhalation of chemicals. Do not leave chemical containers open. Use only with sufficient ventilation (for example, fume hood).
- Check regularly for chemical leaks or spills. If a leak or spill occurs, follow the manufacturer cleanup procedures as recommended in the SDS.
- Handle chemical wastes in a fume hood.
- Ensure use of primary and secondary waste containers. (A primary waste container holds the immediate waste. A secondary container contains spills or leaks from the primary container. Both containers must be compatible with the waste material and meet federal, state, and local requirements for container storage.)
- After emptying a waste container, seal it with the cap provided.
- Characterize (by analysis if needed) the waste generated by the particular applications, reagents, and substrates used in your laboratory.
- Ensure that the waste is stored, transferred, transported, and disposed of according to all local, state/provincial, and/or national regulations.
- **IMPORTANT!** Radioactive or biohazardous materials may require special handling, and disposal limitations may apply.

WARNING! HAZARDOUS WASTE (from instruments). Waste produced by the instrument is potentially hazardous. Follow the guidelines noted in the preceding General Chemical Handling warning.

WARNING! 4L Reagent and Waste Bottle Safety. Four-liter reagent and waste bottles can crack and leak. Each 4-liter bottle should be secured in a low-density polyethylene safety container with the cover fastened and the handles locked in the upright position.

Biological hazard safety

WARNING! Potential Biohazard. Depending on the samples used on this instrument, the surface may be considered a biohazard. Use appropriate decontamination methods when working with biohazards.

WARNING! BIOHAZARD. Biological samples such as tissues, body fluids, infectious agents, and blood of humans and other animals have the potential to transmit infectious diseases. Conduct all work in properly equipped facilities with the appropriate safety equipment (for example, physical containment devices). Safety equipment can also include items for personal protection, such as gloves, coats, gowns, shoe covers, boots, respirators, face shields, safety glasses, or goggles. Individuals should be trained according to applicable regulatory and company/ institution requirements before working with potentially biohazardous materials. Follow all applicable local, state/provincial, and/or national regulations. The following references provide general guidelines when handling biological samples in laboratory environment.

- U.S. Department of Health and Human Services, *Biosafety in Microbiological and Biomedical Laboratories (BMBL)*, 6th Edition, HHS Publication No. (CDC) 300859, Revised June 2020
  https://www.cdc.gov/labs/pdf/CDC-BiosafetymicrobiologicalBiomedicalLaboratories-2020-P.pdf
- Laboratory biosafety manual, fourth edition. Geneva: World Health Organization; 2020 (Laboratory biosafety manual, fourth edition and associated monographs)
  www.who.int/publications/i/item/9789240011311

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  Note: For SDSs for reagents and chemicals from other manufacturers, contact the manufacturer.

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