A positive-selection screen for a 6-thioguanine resistance gene using the LentiPool lentiviral negative control CRISPR library spiked with *HPRT1* gRNAs

Introduction

Lentiviral pooled screening is a powerful tool for functional genomics. In contrast to screening with arrayed multi-well plate libraries, which may require robotics or high-throughput plate handling equipment, a lentiviral pooled screen for hundreds or thousands of genes can be carried out with just a few culture dishes.

Here we describe the use of the Invitrogen™ LentiPool™ Lentiviral CRISPR Library for a positive-selection screen. A positive-selection screen introduces a strong selective pressure, which gives a very low probability of selecting cells that have not acquired a mutation that can overcome the selection. These screens are often used to identify genes that confer resistance to drugs, toxins, and pathogens. In this study, we modeled a positive-selection screen using 6-thioguanine (6-TG) treatment as a selective pressure to demonstrate the power of the pooled screening approach.

Pooled lentiviral CRISPR gRNA libraries utilize the CRISPR-Cas9 system to create double-stranded DNA breaks in the coding regions of targeted genes. The resulting imperfect repair may generate a functional knockout, making these libraries powerful tools for loss-of-function studies. The LentiPool negative control library (NCL) is a collection of gRNA designs that have no targets in the human genome. In this application, we utilized the LentiPool NCL spiked with CRISPR-Cas9 guide RNAs (gRNAs) to target the *HPRT1* gene.

6-TG is a purine analog that is used for the treatment of leukemia in both 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* (Table 1) 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 1. CRISPR-Cas9 guide RNAs designed to target the *HPRT1* gene.

Guide RNA	Target sequence
HPRT-T1	CTGTCCATAATTAGTCCATG
HPRT-T2	TCTTGCTCGAGATGTGATGA
HPRT-T3	CATACCTAATCATTATGCTG
HPRT-T4	ATTATGCTGAGGATTTGGAA

Procedure

The following 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 shown below was performed for both cell lines treated with 10 µM 6-TG. Calculations for the NCL were based on the LentiPool NCL.

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 x 10^5 TU/µL, and the titer of the mixed control library was the same.

General NCL + HPRT calculation

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

Day 1: Seed cells in a 6-well plate

Seed 2.43 x 10^5 cells per well* (20–25% confluence)	243,333
Number of wells for the LentiPool Human CRISPR Library**	6
Number of wells for mock control†	1
Incubate cells at 37°C in a humidified incubator with 5% CO_2 for 24 hours.	

* 50% of total cells required for transduction if doubling time is 24 hours.

** See Figure 1.

† Including a mock control without the virus is recommended for monitoring puromycin selection.

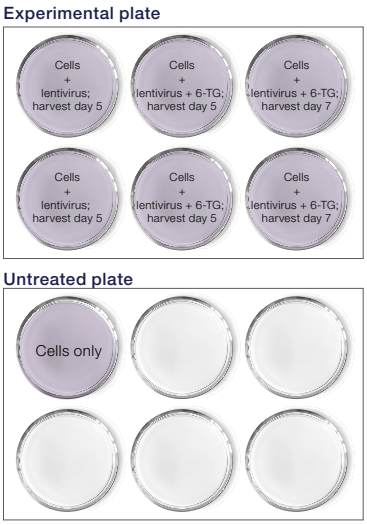


Figure 1. Six-well plate layout for seeding the cells.

Day 2: Transduction

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 1,000-fold coverage (146 guides x 1,000 = 146,000 viral particles)	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
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 x *g* at room temperature for 20 minutes after adding the virus may enhance 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

4. Remove the transduction medium from the cell cultures.

5. Add 2 mL of complete medium prepared with 10% FBS, 0.75 µg/µL puromycin, and 10 µM 6-TG to each well.

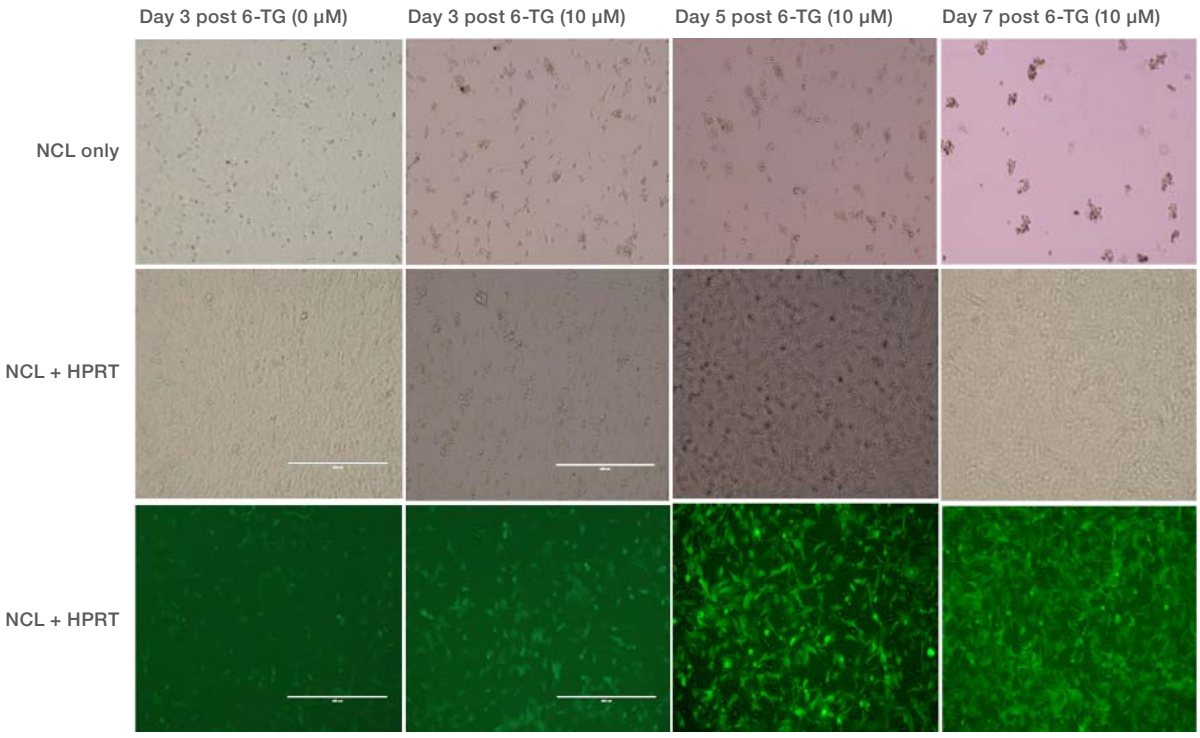


Figure 2. Positive-selection screen for 6-TG resistance in HT1080 cells transduced with the LentiPool NCL spiked with *HPRT1* gRNAs. Bright-field images of cells transduced with the LentiPool NCL as a negative control are shown in the top row. Bright-field images of cells after transduction with the 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.

Day 6 (day 3 post 6-TG treatment)

1. 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-TG treatment)

2. 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-TG treatment)

3. Check for GFP expression and harvest cells seven days after 6-TG treatment. Images used to monitor cell viability and GFP expression at all time points are shown in Figure 2.

NGS for hit identification

1. Use the Invitrogen™ PureLink™ Genomic DNA Mini Kit (Cat. No. K182000) to isolate genomic DNA (gDNA) from the cells harvested on days 5 and 7 following 6-TG treatment.

2. Perform two consecutive PCR runs using these primers and the Thermo Scientific™ Phusion™ Green High-Fidelity DNA Polymerase Kit (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′

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

Conditions for first PCR

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

Second PCR with 5´ PHO-U6 and 5´ PHO-Lenti-R2 nested primers	Each reaction (µL)
5X Phusion Green HF Buffer	10
10 mM dNTP mix	1.25
F1: PHO-U6* (10 µM)	1
R1: PHO-Lenti-R2* (10 µM)	1
DMSO (100%)	1.5
DEPC-treated water	32.75
Phusion DNA Polymerase (2 U/µL)	0.5
1st round PCR product	2
Total volume	**50**

* 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.

Conditions for second PCR

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

1. To visualize the PCR product, load 5 µL of the nested PCR product onto an Invitrogen™ E-Gel™ EX agarose gel (2%). The expected size is approximately 155 bp.
2. Purify the PCR product using the Invitrogen™ PureLink™ PCR Micro Kit (Cat. No. K310250) following the standard protocol.
3. Perform NGS adapter ligation using the following protocol:

Adapter ligation and nick repair	Each reaction (µL)
Water	13
10X ligase buffer	2.5
Adapter P1	0.5
dNTP mix	0.5
T4 DNA ligase (5 U/µL)	0.5
Platinum *Tfi* Exo⁻ DNA polymerase	2
PHO-PCR product (10–20 ng)	5
Barcode A adapter*	1
Total volume	**25**

* Samples may be barcoded using barcoded adapters when examining multiple samples.

- Incubate at 16°C for 30 minutes, 25°C for 30 minutes, and 72°C for 30 minutes, then hold at 4°C.
- Pool all barcoded samples at this stage and purify the mixed sample using Agencourt™ AMPure™ XP beads (Cat. No. A63880 or A63881).

Cleanup of ligation reaction using Agencourt AMPure XP beads

Purification using AMPure XP beads	Each reaction (µL)
Nest PCR-5P product	20
Beads (sample volume x 1.5)	30
Total volume	**50**

- Mix 10 times by pipetting, separate the beads using a magnetic separator for 1–2 minutes, and remove the supernatant.
- Wash the beads twice with 200 µL of 70% ethanol by gently pipetting. Remove residual 70% ethanol completely after the second wash.
- 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.

Amplification of ligated PCR product

Amplification of adapter-ligated PCR product	Each reaction (µL)
Invitrogen™ Platinum™ PCR SuperMix High Fidelity	100
Ion Torrent™ amplification primer mix	5
Pooled sample	5
Total volume	**110**

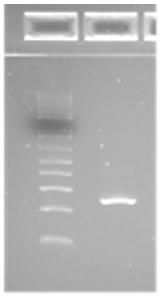
PCR conditions

95°C	5 min	1 cycle
95°C	15 min	10 cycles
58°C	15 min	
70°C	30 min	
70°C	3 min	1 cycle
4°C hold	Indefinitely	

Cleanup of PCR reaction using Agencourt AMPure XP beads

Purification using AMPure XP beads	Each reaction (µL)
Barcoded A/P1 PCR product	50
Beads (sample volume x 1.5)	75
Total	**125**

- Mix 10 times by pipetting, separate the beads using a magnetic separator for 1–2 minutes, and remove the supernatant.
- Wash the beads twice with 200 µL of 70% ethanol by gently pipetting. Remove residual ethanol completely after the second wash.
- 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.
- Check the PCR product on a gel. The expected band should be ~240 bp (Figure 3). Make sure no small adapters or primer dimers (<100 bp) are visible on the gel. Repeat cleanup if any adapter is detected.
- Measure the DNA concentration using the Invitrogen™ Qubit™ dsDNA HS Assay Kit (Cat. No. Q32851).
- Use the following table to calculate the concentration of the final PCR product that will be used to purify the NGS emulsion reaction on an Ion Chef™ Instrument.
- Use the Ion Chef Instrument for the NGS emulsion reaction. Use the Ion PGM™ Hi-Q™ Chef Kit (Cat. No. A25948) to obtain 50 µL of diluted DNA for each chip.
- Perform sequencing on the Ion PGM™ System following the instructions in the manual.
- Use the Coverage Analysis plug-in in the Torrent Suite™ software package.
- Download the .csv data file and save it in Microsoft™ Excel™ format.
- Normalize the read number for each gRNA to reads per million (RPM) to more easily compare different samples and NGS runs.
- Plot the RPM values against each gRNA to identify potential *HPRT1* gRNA hits (Figure 4).



NGS PCR product after bead purification (4.96 ng/µL)

Figure 3. Ion adapter-ligated PCR product purified with AMPure XP beads. A single band at approximately 240 bp was observed. No small adapter was detected.

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 x 10^9)/MW	48,485
Dilution factor to get 100 pM DNA using the Ion PGM™ Hi-Q™ Chef Kit	484

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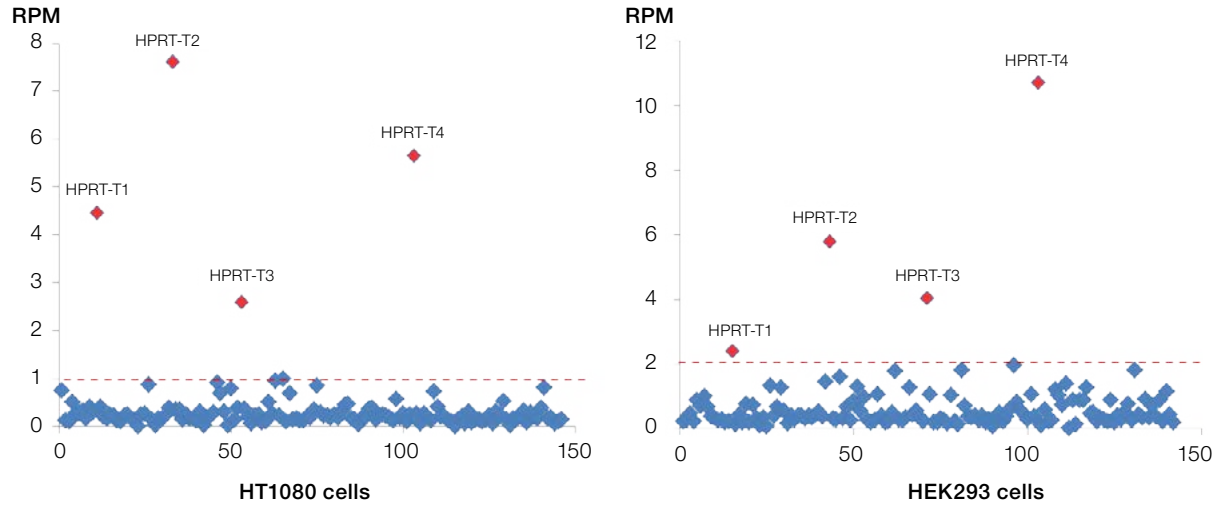

Figure 4. Positive-selection screen for the 6-TG resistance gene in HT1080 and HEK293 cells using the LentiPool NCL spiked with *HPRT1* gRNAs. The RPM of each *HPRT1* gRNA 7 days after 6-TG treatment was plotted against those of 142 negative controls and four *HPRT1* positive controls. The RPMs of the four *HPRT1* gRNAs were significantly higher than the RPM of any negative control.

Conclusion

This study successfully demonstrated the application of positive-selection pooled screening with the LentiPool lentiviral CRISPR negative control gRNA library spiked with four positive control *HPRT1* gRNAs. Cells transduced with these gRNAs to target *HPRT1* had a significantly enhanced survivability phenotype when treated with 6-TG compared to the remainder of the library. Potent target silencing can be achieved by utilizing the Invitrogen gRNA design and appropriate experimental parameters.

References

1. Koike-Yusa HL, Li Y, Tan E-P et al. (2013) Genome-wide recessive genetic screening in mammalian cells with a lentiviral CRISPR-guide RNA library. *Nature Biotechnology* 32:267–273.
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