Powerful loss-of-function screening with LentiArray CRISPR gRNAs: maximize knockout efficiency while maintaining specificity

Introduction

Genome-wide knockout screening that leads to novel druggable targets for therapeutic intervention remains a top priority for the pharmaceutical and biotech industry. Invitrogen™ LentiArray™ CRISPR libraries expand the application of CRISPR-Cas9 technology into high-throughput methods for functional genomics screening, and serve as a powerful platform for understanding complex biological pathways and identifying new therapeutic targets. Award-winning LentiArray CRISPR libraries from Thermo Fisher Scientific provide a flexible system that will allow you to achieve complete and permanent knockout of entire panels of genes in a one-gene-per-well format, increasing both the quality and the efficiency of your screening experiments. Critical to the functional efficiency of CRISPR libraries are the guide RNA (gRNA) designs. Our libraries are built using up-to-date genomic sequence information and gRNA rule sets that are critical for maintaining specificity while maximizing target gene knockout. In this application note, we demonstrate the robustness of our predesigned LentiArray CRISPR library gRNA designs using next-generation sequencing (NGS) to validate genomic DNA (gDNA) cleavage. We also demonstrate protein knockout efficiency using immunofluorescence and western blot assays. Together, these tools serve as a powerful screening platform for target discovery and verification [1,2].

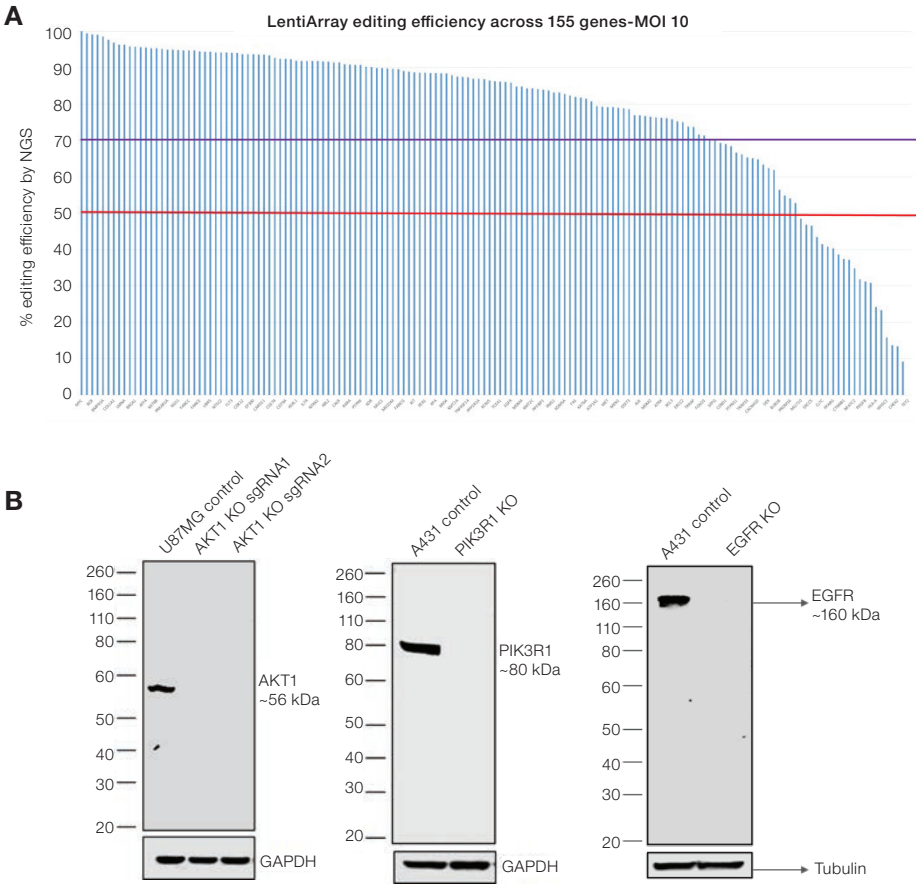

Figure 1. High editing efficiencies are obtained for a large percentage of targeted genes with predesigned LentiArray gRNAs. (A) Editing efficiency analysis by NGS. HT1080 cells stably expressing Cas9 were transduced with arrayed LentiArray gRNA lentiviral particles against a subset of genes found in the Invitrogen™ LentiArray™ Human Cancer Biology CRISPR Library, at a multiplicity of infection (MOI) of 10. Cells were harvested 5 days after puromycin selection. PCR amplicons spanning the genomic target regions were amplified after cell lysis and subsequently barcoded; these were then used for NGS analysis, which showed that 87% of the targeted genes were edited at >50% efficiency, and 77% of them were edited at >70% efficiency. **(B)** U87MG and A431 cells stably expressing Cas9 were transduced with individual lentiviral gRNAs, then harvested for western blot analysis using specific antibodies against corresponding target genes. The absence of detectable AKT, PIK3R1, and EGFR proteins demonstrates efficient protein knockout by this method.

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Results

High editing efficiency of LentiArray gRNAs

High-quality gRNAs for each gene target within the LentiArray CRISPR libraries were built using our proprietary CRISPR design algorithm, which takes into consideration the latest design rules and our extensive in-house verification experiments to ensure maximum knockout efficiency without sacrificing specificity. Up to four distinctive gRNAs for each target gene, mostly targeting 5′ protein-coding exons and having minimal overlapping sequences, were selected to ensure high-efficiency knockout of target genes across a wide array of cell types. In order to confirm the editing efficiency, gRNAs for a subset of LentiArray Human Cancer Biology CRISPR Library target genes were packaged into lentiviruses in arrayed 96-well format with one target per well, using our high-throughput process. The viruses were subsequently used to transduce a HT1080 cell line stably expressing Cas9, at a multiplicity of infection (MOI) of 10. Following puromycin selection for 5 days, the cells were harvested for detection of editing efficiency via NGS using the Ion Proton™ System. As shown in Figure 1A, 87% of the target genes tested showed over 50% knockout efficiency, and 77% of the genes tested had over 70% knockout efficiency.

To further verify functional knockout efficiencies, we tested protein expression for several gene targets posttransduction, using either western blot or immunocytochemistry. As demonstrated in Figure 1B, the protein expression levels of AKT1, PIK3R1, and EGFR after gRNA transduction were undetectable by western blot analysis, thereby suggesting efficient gene and protein knockout.

Functional verification of knockout using immunofluorescence staining and western blot assay

We further demonstrated the utility of the LentiArray gRNAs to interrogate the impact of individual gene knockout (KO) in a cell-signaling pathway. The transcription factor NF-κB plays a pivotal role in immune and inflammatory responses. In cell lines where the NF-κB pathway is active, stimulation by TNF-α activates NF-κB, which then rapidly translocates from the cytoplasm to the nucleus, where it activates specific genes. We first established a clonal Invitrogen™ CellSensor™ NF-κB ME-180 cell line (Cat. No. K1667) that stably expresses Cas9 protein following transduction with Invitrogen™ LentiArray™ Cas9 Lentivirus. The cells were then transduced with lentiviral gRNAs that target genes relevant to kinase pathways, such as *IKKα* and *TRADD*, or with no gRNA as a negative control. All cells (both control and KO) were then either left untreated or treated with TNF-α. As expected, activation of the pathway by TNF-α resulted in translocation of the NF-κB p65 subunit from the cytoplasm to the nucleus. In contrast, knockout of *IKKα* or *TRADD* impaired the translocation of the NF-κB p65 subunit, as these two genes play a significant role in the NF-κB signaling pathway (Figure 2A). Results from western blot analysis confirmed efficient knockout of the IKKα and TRADD proteins (Figure 2B).

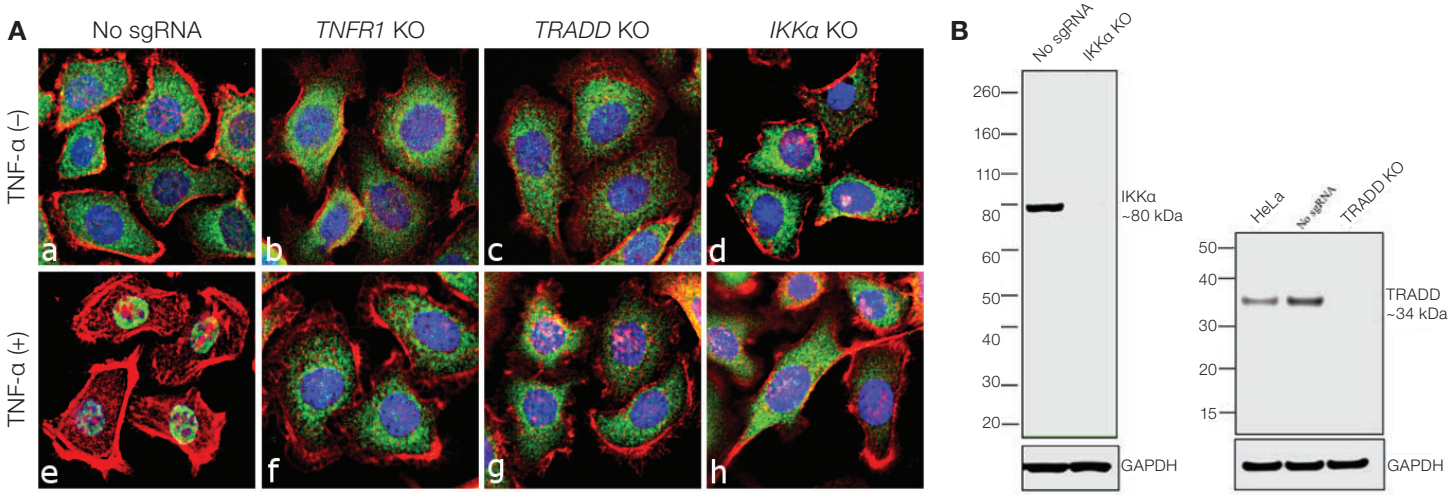

Figure 2. Functional validation of gene knockout using LentiArray CRISPR gRNAs. (A) Images of cells that were transduced with LentiArray gRNAs targeting *TNFR1*, *TRADD*, and *IKKα*, or no single-guide RNA (sgRNA) as negative control. NF-κB p65 (green) was stained using Invitrogen™ NF-κB p65 Polyclonal Antibody (Cat. No. 710048). Nuclei were stained with Invitrogen™ DAPI (blue), and actin was stained with rhodamine phalloidin (red). **(B)** Confirmation of knockout of IKKα and TRADD proteins by western blot analysis using IKKα- and TRADD-specific antibodies (Cat. No. PA517803 and PA517465).

Functional verification of signaling pathway using epidermal growth factor receptor (EGFR) KO cell line

EGFR is a transmembrane protein that is activated by the binding of specific ligands, including epidermal growth factor (EGF) (Figure 3A). Upon activation, EGFR dimerizes, autophosphorylates, and initiates a signaling cascade via phosphorylation of one or more downstream proteins. This cascade can mediate various cellular activities, including proliferation, survival, growth, and development. We first established a clonal EGFR KO A431 cell line using a LentiArray gRNA. The knockout of EGFR protein expression was confirmed using western blot analysis. We then investigated the effects of EGFR KO on the phosphorylation status of key downstream effectors, including SHC, MEK, and ERK, using corresponding antibodies. Phosphorylation of SHC, MEK, and ERK was detected in the A431 control cells and A431-Cas9 cells upon treatment of cells with 200 ng/mL of EGF for 10 min (Figure 3B). By contrast, the EGFR-KO A431 cell line failed to respond to EGF, which demonstrates the effective use of LentiArray CRISPR gRNAs for gene function analysis.

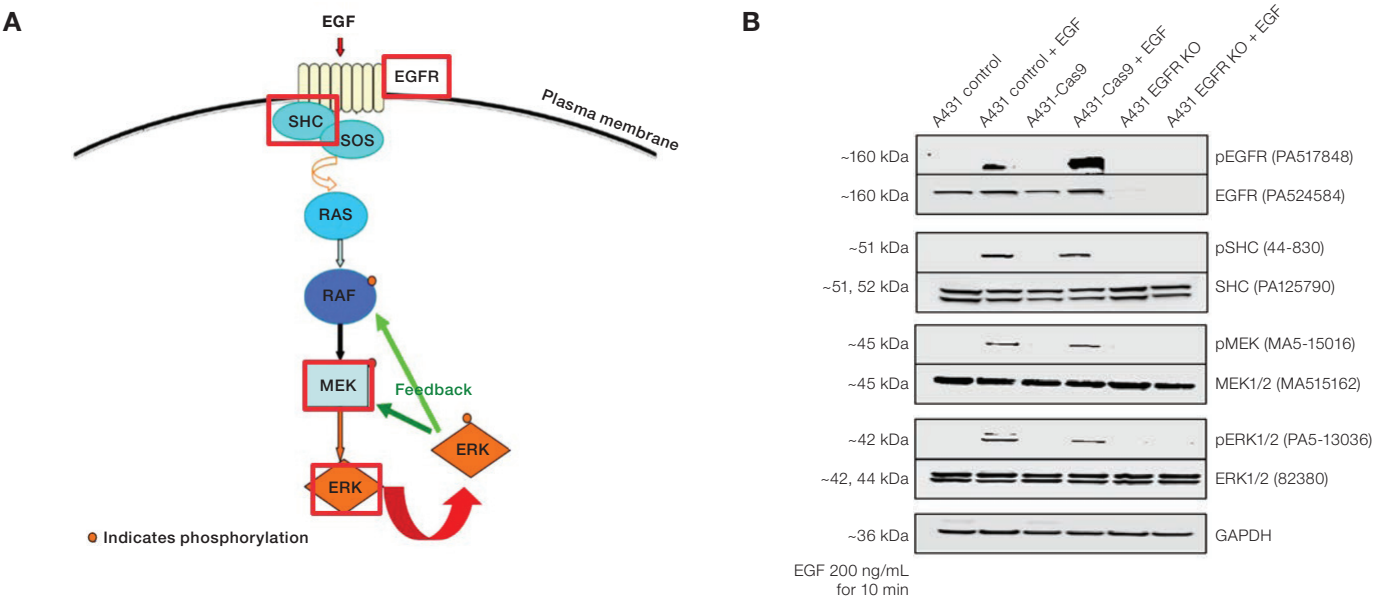

Figure 3. Western blot analysis of phosphorylation of EGFR and its downstream targets. (A) Schematic diagram of EGFR signaling pathway, which includes downstream proteins of the signaling cascade that are phosphorylated upon binding of EGF to EGFR. The targets that were investigated in this study are indicated with red boxes. **(B)** Western blot analysis of SHC, MEK, and ERK was performed using 30 µg of whole cell extract from the following samples: A431 control, A431 treated with EGF (200 ng/mL for 10 min), A431-Cas9, A431-Cas9 treated with EGF (200 ng/mL for 10 min), A431 EGFR KO, A431 EGFR KO treated with EGF (200 ng/mL for 10 min). Inactivation of the EGFR pathway by knocking out EGFR was confirmed via western blot using antibodies against EGFR, phosphorylated EGFR (pEGFR), and phosphorylated proteins of the downstream pathway of EGFR. No detectable EGFR was found in cells transduced with gRNA against EGFR (last two lanes on right, both containing A431 EGFR KO). Furthermore, the lack of phosphorylation of downstream pathway proteins also indicates complete knockout of the EGFR receptor. Using antibodies to pSHC, pMEK, and pERK, the parental A431 cell line and the A431 cell line stably expressing Cas9 were shown to have strong phosphorylation along the signaling cascade upon addition of EGF. In contrast, in cells that received the gRNA targeting EGFR, no downstream proteins of the signaling cascade were phosphorylated upon EGF addition. The ability to screen proteins in experiments where CRISPR is used to inhibit entire signaling pathways can, therefore, provide high-quality confirmation of knockout.

Conclusion

CRISPR-Cas9 technologies have revolutionized the entire drug discovery process from target identification to generation of disease models. The experiments presented here demonstrate the efficiency of our predesigned gRNAs and the robustness of our LentiArray CRISPR libraries to successfully disrupt genes of interest for functional knockout studies.

The LentiArray CRISPR libraries are available as either bacterial glycerol stocks containing sequence-verified lentiviral vectors, or premade ready-to-use lentivirus with functional titers of >1 x 10^8 TU/mL as measured by antibiotic selection. In addition, individual LentiArray CRISPR gRNAs are available to support assay development and downstream hit verification when performing high-throughput screening experiments. In contrast to lentiviral pooled libraries, screening assays using these libraries are not limited to detecting cell viability, specific markers, and reporters. They enable researchers to perform complex phenotypic readouts such as morphology changes, high-content imaging, and multiple gene knockouts in a single screen. With robust gRNA designs and high-titer viral particles, LentiArray CRISPR libraries are designed to enhance your target discovery screening and set you up for success.

Get additional information at **thermofisher.com/lentiarray**

Methods and materials

gRNA design, cloning, and lentivirus packaging

CRISPR gRNA sequences were generated using our proprietary design algorithm. The algorithm takes into consideration the latest findings on specificity and efficiency of gRNA binding, combined with extensive verification at the bench.

A subset of CRISPR gRNAs (T1 and T2) from the LentiArray Human Cancer Biology CRISPR Library was selected for this evaluation. All the LentiArray gRNA constructs were sequence-verified and arrayed as one gRNA per well in a 96-well plate. The plasmids were purified using the Invitrogen™ PureLink™ *Pro* Quick96 Plasmid Purification Kit (Cat. No. K211024A), and the concentration of DNA was measured using the Invitrogen™ Quant-iT™ dsDNA Assay Kit, Broad Range (Cat. No. 33130). The lentivirus was packaged using a proprietary automation process. The titers of the virus were determined using puromycin antibiotic selection.

Lentiviral transduction

The following protocol was used for lentiviral transduction in a 96-well plate:

Day 0. Seed HT1080 cells stably expressing Cas9 at 3,000 cells/well in 100 µL of complete growth medium in the 96-well plate.

Day 1. Replace the spent medium in the wells with fresh complete growth medium containing 8 µg/mL Polybrene™ reagent (Sigma Aldrich, Cat. No. H9268). To each well, add 6 µL of lentivirus and gently swirl the plate to evenly distribute the lentivirus across each well; centrifuge the plate at 800 x *g* for 30 minutes at room temperature.

Day 2. Remove the medium containing the lentivirus, and replace it with 100 µL/well of fresh complete culture medium.

Day 3. Replace the medium with fresh medium containing 1 µg/mL of puromycin for the selection of transduced cells.

Day 8. Harvest cells by removing the medium by aspiration, then adding 50 µL of cell lysis buffer to each well.

NGS

The genomic target regions were amplified from cell extracts using Thermo Scientific™ Phusion™ Green Hot Start II High-Fidelity PCR Master Mix (Cat. No. F566L). The PCR products were verified by electrophoresis on Invitrogen™ E-Gel™ *48* Agarose Gels, 2% (Cat. No. G800802), then barcoded using the Ion Torrent™ IonCode™ Barcode Adapters 1-384 Kit (Cat. No. A31173). Samples from each plate were pooled and prepared using the Ion Chef™ Instrument (Cat. No. 4484177). The amplified library was then sequenced using the Ion Proton™ System (Cat. No. 4476610). The reads were aligned to the corresponding reference sequences, and subsequently characterized and counted using proprietary NGS analysis software.

Western blot assay

Whole-cell protein extracts (for IKKα, TRADD, AKT1, EGFR, SHC, MEK, and ERK) and membrane extracts (for PIK3R1) were used for western blot analysis. Briefly, samples and the Invitrogen™ Novex™ Sharp Pre-Stained Protein Standard were run on an Invitrogen™ NuPAGE™ 4-12% Bis-Tris gel (Cat. No. NP0321BOX) using the XCell SureLock Mini-Cell. Proteins were then transferred to a nitrocellulose membrane using the iBlot 2 Gel Transfer Device and blocked with 5% skim milk for 1 hour at room temperature. Specific antibodies for target proteins were all obtained from Thermo Fisher Scientific. The following conditions were used for each antibody:

Invitrogen™ PI3K p85 alpha Monoclonal Antibody (Cat. No. MA1-74183) was diluted 1:500 in 5% skim milk. Invitrogen™ Goat Anti–Mouse IgG (H+L) Superclonal™ Recombinant Secondary Antibody conjugated to HRP (Cat. No. A28177) was used at 1:4,000 dilution.

Invitrogen™ rabbit polyclonal antibodies against IKKα (Cat. No. PA5-17803), TRADD (Cat. No. PA5-17465), and EGFR (Cat. No. PA5-24584) were diluted 1:1,000. The Invitrogen™ AKT1 Recombinant Polyclonal Antibody (Cat. No. 710005) was used at 2 µg/mL in 5% skim milk. Invitrogen™ Goat Anti–Rabbit IgG (H+L) Superclonal™ Secondary Antibody conjugated to HRP (Cat. No. A27036) was diluted 1:4,000.

Chemiluminescence detection was performed using the Invitrogen™ Novex™ ECL Chemiluminescent Substrate Reagent Kit (Cat. No. WP20005).

Immunofluorescence staining

An Invitrogen™ CellSensor™ NF-κB ME-180 cell line stably expressing Cas9 was transduced with lentiviruses expressing gRNAs targeting *IKKα* and *TRADD*, or no gRNA as negative control. The transduced cells were either mock-treated or treated with TNF-α (50 ng/mL for 20 min). Cells were then fixed with 4% paraformaldehyde for 15 minutes, permeabilized with 0.1% Triton™ X-100 surfactant for 10 minutes, and blocked with 1% BSA for 1 hour at room temperature. The cells were subsequently labeled with Invitrogen™ NF-κB p65 Rabbit Polyclonal Antibody (Cat. No. 510500) at 1:300 in 0.1% BSA for 3 hours at room temperature. Following washing, cells were then labeled with Invitrogen™ Goat Anti–Rabbit IgG (H+L) Superclonal™ Recombinant Secondary Antibody, Alexa Fluor™ 488 conjugate (Cat. No. A27034) at a dilution of 1:2,000 for 45 minutes at room temperature. Nuclei were stained with Invitrogen™ *SlowFade*™ Gold Antifade Mountant with DAPI (Cat. No. S36938). F-actin was stained with Invitrogen™ Rhodamine Phalloidin (Cat. No. R415). The images were captured at 40x magnification.

References

1. Sredni ST, Suzuki M, Yang JP et al. (2017) A functional screening of the kinome identifies the Polo-like kinase 4 as a potential therapeutic target for malignant rhabdoid tumors, and possibly, other embryonal tumors of the brain. *Pediatr Blood Cancer* 64(11). doi: 10.1002/pbc.26551.
2. Sredni ST, Bailey AW, Suri A et al. (2017) Inhibition of polo-like kinase 4 (PLK4): a new therapeutic option for rhabdoid tumors and pediatric medulloblastoma. *Oncotarget* 8:111190-111212.

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Ordering information

Product	Lentiviral stocks Cat. No.	Glycerol stocks Cat. No.
LentiArray Human Kinase CRISPR Library	A31931	A32167
LentiArray Human Phosphatase CRISPR Library	A31932	A32168
LentiArray Human Cancer Biology CRISPR Library	A31933	A32169
LentiArray Human Epigenetics CRISPR Library	A31934	A32170
LentiArray Human Ubiquitin CRISPR Library	A31935	A32171
LentiArray Human Cell Cycle CRISPR Library	A31936	A32172
LentiArray Human Membrane Trafficking CRISPR Library	A31937	A32173
LentiArray Human Transcription Factor CRISPR Library	A31938	A32174
LentiArray Human Nuclear Hormone Receptor CRISPR Library	A31939	A32175
LentiArray Human Apoptosis CRISPR Library	A31940	A32176
LentiArray Human Drug Transporter CRISPR Library	A31941	A32177
LentiArray Human Ion Channel CRISPR Library	A31942	A32178
LentiArray Human Cell Surface CRISPR Library	A31943	A32179
LentiArray Human Protease CRISPR Library	A31944	A32180
LentiArray Human Tumor Suppressor CRISPR Library	A31945	A32181
LentiArray Human DNA Damage Response CRISPR Library	A31946	A32182
LentiArray Human GPCR CRISPR Library	A31947	A32183
LentiArray Human Druggable CRISPR Library	A31948	A32184
LentiArray Human Whole Genome CRISPR Library	A31949	A32185
LentiArray Custom CRISPR Plate	A32045	
LentiArray Cas9 Lentivirus, 100 µL	A32064	
LentiArray Cas9 Lentivirus, 1 mL	A32069	
LentiArray CRISPR Positive Control Lentivirus, Human HPRT, 100 µL	A32056	
LentiArray CRISPR Positive Control Lentivirus, Human HPRT, 1 mL	A32829	
LentiArray CRISPR Positive Control Lentivirus with EmGFP, Human HPRT, 100 µL	A32060	
LentiArray CRISPR Positive Control Lentivirus with EmGFP, Human HPRT, 1 mL	A32830	
LentiArray CRISPR Negative Control Lentivirus, Human Non-Targeting, 100 µL	A32062	
LentiArray CRISPR Negative Control Lentivirus, Human Non-Targeting, 1 mL	A32327	
LentiArray CRISPR Negative Control Lentivirus with EmGFP, Human Non-Targeting, 100 µL	A32063	
LentiArray CRISPR Negative Control Lentivirus with EmGFP, Human Non-Targeting, 1 mL	A32831	
GeneArt Genomic Cleavage Detection Kit	A24372	
Puromycin Dihydrochloride (10 mg/mL)	A1113803	

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