# Streamlined production, application, and analysis of pooled genome-wide sgRNA lentiviral libraries

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## Abstract

Genome-wide loss-of-function genetic screens are a powerful way to identify novel protein functions and biological processes within a cell. A common approach in *in-vitro* loss-of-function screens is to knock out genes in a population of cells, apply selective pressure, and then identify mutations that are either enriched or depleted in the selected population relative to a control. The easy programmability and high knockout efficiency of the CRISPR/Cas9 system has helped researchers maximize the potential of this *in-vitro* screening method to identify genes responsible for a given phenotype of interest. Current methods using pooled sgRNAs in loss-of-function screens rely on lentiviral vector-based delivery followed by next-generation sequencing (NGS) to analyze the resulting distribution of sgRNA sequences in screened cell populations. Inherent challenges include maintaining sgRNA representation in lentiviral plasmids, achieving optimal titers upon scale-up of lentivirus production, and preparing high-quality NGS libraries that accurately reflect the distribution of sgRNA sequences.

Here we present a streamlined approach for producing Cas9+/sgRNA+ cell populations in sufficient quantities for a genome-wide screen, and for generating NGS libraries used to assess changes in sgRNA representation, using the Guide-It™ CRISPR Genome-Wide sgRNA Library System. Our methods enable even novice users to perform genome-wide phenotypic screens without concerns for sgRNA representation, low virus titer, or NGS library preparation.

## Methods

**Generation of sgRNA library- and Cas9-expressing lentivirus:** Xfect™ polymeric transfection reagent, Lenti-X™ packaging mix, and either Cas9 or sgRNA lentiviral vector library were combined into a transfection mix and lyophilized in a single-tube format. sgRNAs within the library were chosen from the Brunello sgRNA library (Doench et al. 2016) and target 19,114 genes with 76,610 unique guide sequences. Following reconstitution with water, the transfection mix was added to 293T cells to generate high-titer Cas9 and sgRNA lentiviral preparations.

**Quantification of sgRNA library lentivirus titers:** Libraries were quantified using the Lenti-X GoStix™ Plus lateral flow test for p24 quantitation. Following test completion, band density was quantified in the Lenti-X GoStix smartphone app and compared to a downloaded lot-specific standard curve.

**Validation of sgRNA activity:** Following the generation of a Cas9+/sgRNA+ A375 cell population using the method described above, random clones were selected, screened for corresponding sgRNA sequences, and cleavage of genomic targets assessed by resolvase assay. sgRNA activity was further demonstrated by performing a screen for resistance to the purine analog 6-thioguanine (6-TG). Finally, a complementary workflow for gDNA purification and sgRNA sequence amplification (the Guide-It CRISPR Genome-Wide Library NGS Analysis Kit) was developed to minimize issues with PCR primer design, gDNA isolation, and amplification of sgRNA sequences.

## 1 Issues with standard sgRNA library preparation

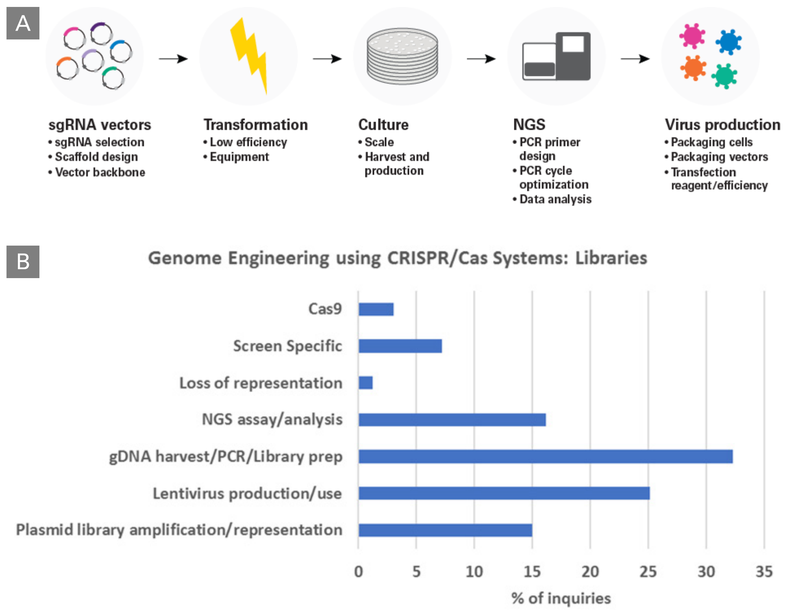


**Figure 1. Common challenges with lentiviral sgRNA library production. Panel A.** Schematic showing steps and common pain points during lentiviral sgRNA library production. **Panel B.** Data compiled outlining inquiries regarding issues associated with preparation and application of CRISPR/Cas9 sgRNA pooled libraries for phenotypic screens. Data from Google Groups: "Genome Engineering using CRISPR/Cas Systems" during the period of 12/30/15 to 12/21/17.

## Conclusions

- CRISPR/Cas9 knockout screens are a powerful way to identify genes involved in a phenotype of interest but require transduction with a representative lentiviral sgRNA library.
- Many find that the production of a representative sgRNA library is a time- and labor-intensive endeavor that can be very challenging.
- Simply with the addition of water, the Guide-it CRISPR Genome-Wide sgRNA Library System provides a simplified process for obtaining a representative, high-titer lentiviral sgRNA library with a high editing efficiency.
- NGS libraries were easily produced from 6-TG screen populations using the Guide-it CRISPR Genome-Wide Library NGS Analysis Kit.
- Editing activity of the Guide-it sgRNA library was confirmed in randomly selected clones as well as a 6-thioguanine screen.

**References**

Doench, J. G. *et al.* Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR-Cas9. *Nat. Biotechnol.* **34,** 184–191 (2016).

Li, W. *et al.* MAGeCK enables robust identification of essential genes from genome-scale CRISPR/Cas9 knockout screens. *Genome Biol.* **15,** 554–566 (2014).

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## 2 Guide-it CRISPR Genome-Wide sgRNA Library System

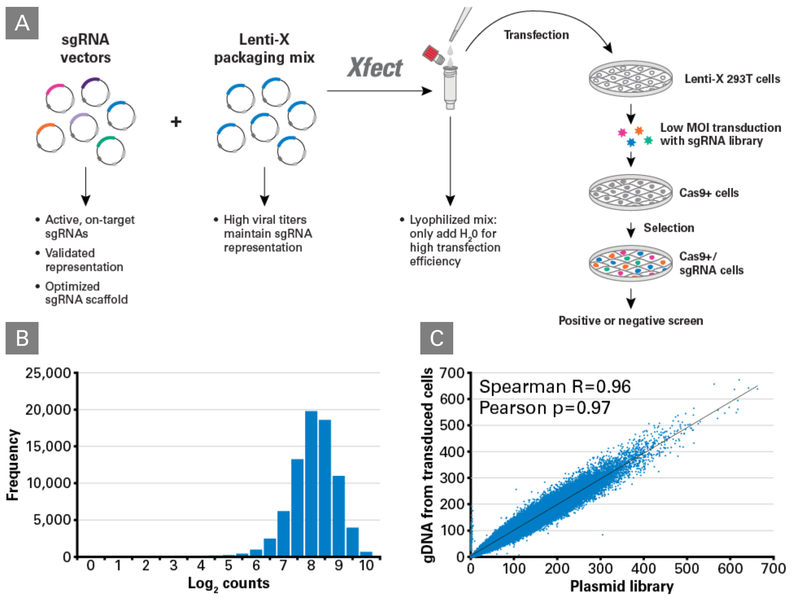



**Figure 2. A streamlined solution engineered to ensure full representation of sgRNAs throughout the entire lentiviral production process and enable rapid production of Cas9+/sgRNA+ cell populations. Panel A.** The library of previously-validated sgRNAs was sequenced to confirm full representation prior to formulation. Following addition of Lenti-X packaging mix and Xfect polymeric transfection reagent to the library, the library was lyophilized to create a one-step high-efficiency transfection mix. **Panel B.** Plasmid sgRNA library was sequenced on the Illumina® MiSeq® platform (20 million reads) to validate distribution of sgRNAs. **Panel C**. Correlation plot between read counts of sgRNAs integrated in the transduced cell population and corresponding read counts in the starting plasmid population. Strong Spearman and Pearson correlations indicate maintenance of sgRNA representation throughout transduction and target cell selection.

## 3 Cas9 lentivirus production and application

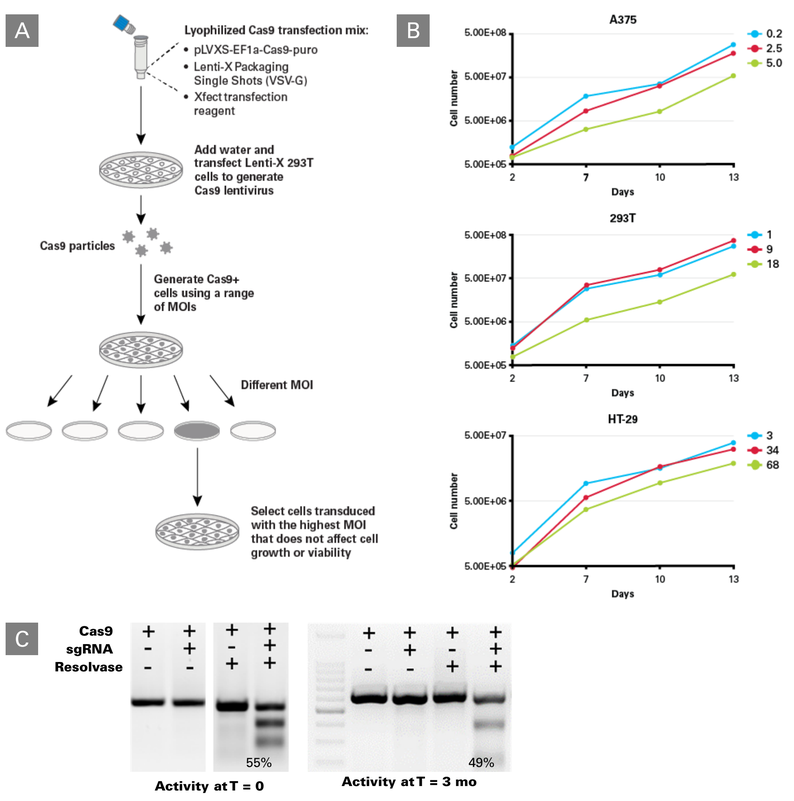
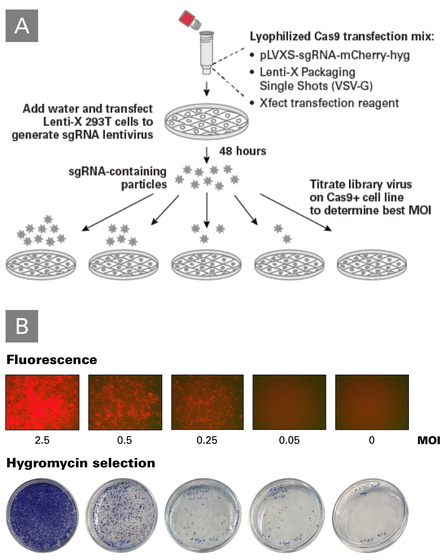



**Figure 3: Cas9 lentivirus production and application. Panel A.** Schematic of Cas9 virus production. Following addition of Lenti-X packaging mix and Xfect polymeric transfection reagent to the library, the library was lyophilized to create a one-step high-efficiency transfection mix for Cas9 lentivirus production. Target cells were transduced with a range of MOIs, selected, and analyzed for growth abnormalities caused by Cas9 overexpression. **Panel B.** Growth curves for HT-29, 293T, and A375 Cas9+ cell lines transduced with varying Cas9 lentiviral MOIs shows that certain cell targets are affected by Cas9 overexpression. **Panel C.** Duration of Cas9 expression. A375 cells were transduced with Cas9 at an MOI of 2.5, selected in puromycin and then assayed for Cas9 activity. These A375/Cas9+ cells were transduced at T=0 and after 3 months with an sgRNA targeting *CD81* and editing efficiency was assessed using the Guide-it Mutation Detection Kit one week post transduction. Editing efficiency was maintained throughout the duration of the culture period.

## 4 sgRNA lentivirus production and application

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| Parameter | Guide-it sgRNA library |
|---|---|
| Number of guides | 76,612 |
| Genes targeted | 19,114 |
| Cell number required for ~400X representation | ~3.1 x $10^7$ |
| Cell number to transduce | ~1.00 x $10^8$ |
| Transduction efficiency | 0.40 |
| Fold coverage/sgRNA (400–1,000 cells/sgRNA) | 400 |

**Number of cells needed to maintain sgRNA representation**

400–1,000 cells/sgRNA x 76,612 sgRNAs = 3.1 x $10^7$–7.6 x $10^7$ cells (after selection)

**Figure 4: sgRNA library lentivirus production and titration. Panel A.** sgRNA lentiviral vector library was added to Xfect polymeric transfection reagent and Lenti-X packaging mix, then lyophilized to create a one-step high efficiency transfection mix for sgRNA lentivirus production. Cas9+ target cells were transduced with a range of MOIs to determine the highest transduction rate that resulted in a single sgRNA per cell. **Panel B.** Transduction efficiency was determined by mCherry fluorescence or hygromycin selection of transduced cells as the vector expresses both mCherry and hygromycin markers. **Panel C.** Calculations for determining the number of cells required to maintain representation at a required transduction efficiency and sgRNA representation.

## 5 Library titration with Lenti-X GoStix Plus

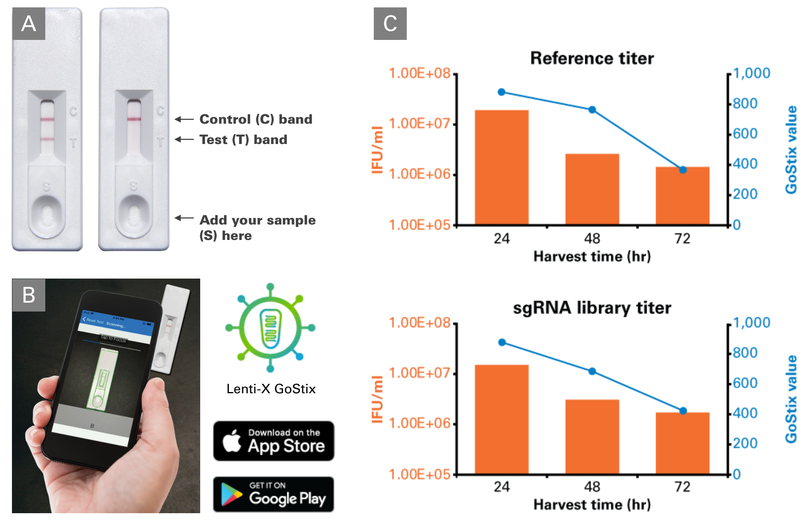


**Figure 5. sgRNA library lentivirus titration using Lenti-X GoStix Plus and the Lenti-X GoStix smartphone app. Panel A.** Lenti-X GoStix Plus lateral flow test for p24 quantitation. The T and C bands indicate sample presence and a correctly running test, respectively. The Lenti-X GoStix app (**Panel B,** available on iOS and Android™ platforms) scans the completed test and performs densitometric analysis to determine the p24 titer relative to a downloaded lot-specific standard curve. **Panel C.** Determination of reference and sgRNA library titers (IFU/ml) using the Lenti-X GoStix Plus quantified by the Lenti-X GoStix smartphone app.

## 6 Confirmation of editing activity

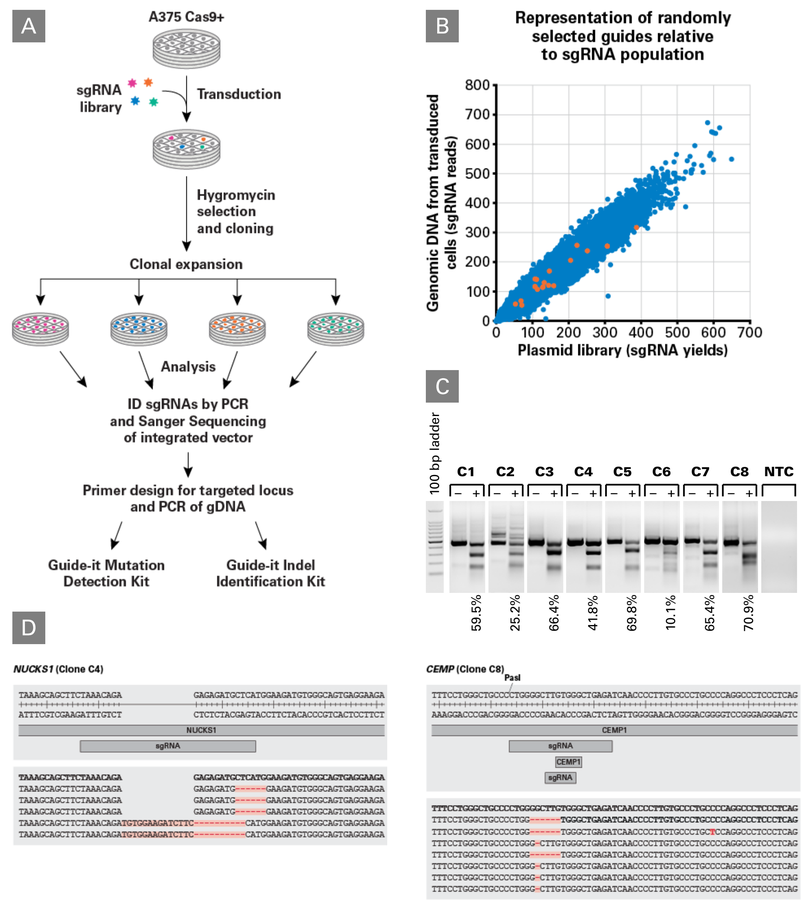


**Figure 6. Determination of editing activity of the Guide-it CRISPR Genome-Wide sgRNA Library in randomly selected clones. Panel A.** After transduction of Cas9+ A375 cells with the sgRNA library and hygromycin selection, twenty clones were selected randomly and expanded for analysis. **Panel B.** The sgRNA sequences from the twenty clones (orange dots) were mapped against the correlation plot of the plasmid library and transduced gDNA (blue dots). **Panel C.** Genomic DNA was isolated from each clone and analyzed for sgRNA activity using the Guide-it Mutation Detection Kit. The results of the resolvase assay for 8 representative clones are shown. NTC: non-transduced cells, (+) PCR products treated with Guide-it Resolvase, (–) untreated PCR products. **Panel D.** Indel identification in edited A375 clones. Sequencing data from two representative clones demonstrating typical indel patterns are shown.

## 7 6-Thioguanine resistance screen

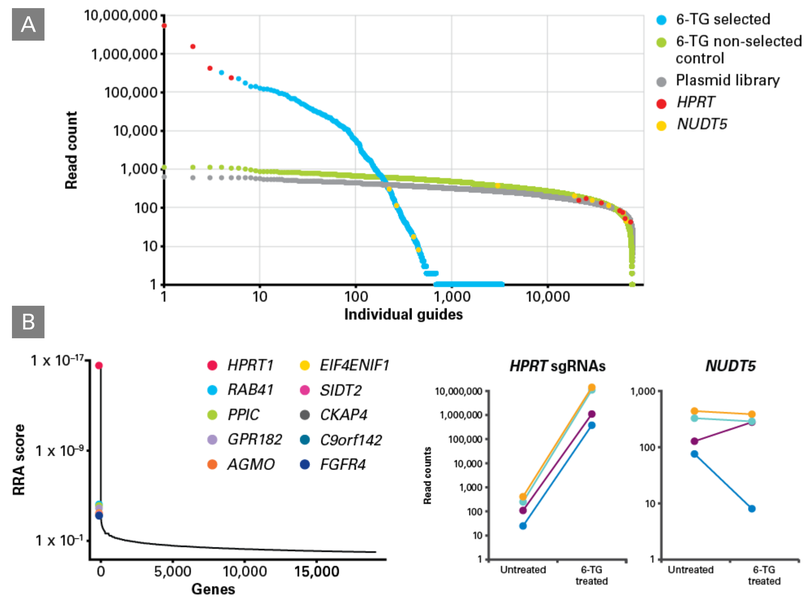


**Figure 7: Identification and analysis of sgRNAs isolated from cells after a 6-TG screen. Panel A.** sgRNA representation was compared between the original plasmid library and the transduced cell populations (6-TG selected and non-selected). First, the resulting transduced population demonstrated representation similar to the original plasmid population. Next, the sgRNA representation demonstrated a large shift in guide number in response to 6-TG (blue). Last, all four sgRNAs targeting *HPRT* (red) and *NUDT5* (yellow), respectively, were enriched in the 6-TG-selected population. **Panel B.** Analysis of the same 6-TG data using the MAGeCK algorithm (Li et al. 2014). Robust rank aggregation (RRA) returned *HPRT* as a significant hit (left panel), which demonstrated over 4 orders of magnitude of enrichment (right panel). Read counts of *NUDT5* was not enriched, but still had all four sgRNAs represented in the surviving population, consistent with previously published reports (Doench et al. 2016).