

# Pooled CRISPR Screens in *Drosophila* Cells

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High-throughput screens in *Drosophila melanogaster* cell lines have led to discovery of conserved gene functions related to signal transduction, host-pathogen interactions, ion transport, and more. CRISPR/Cas9 technology has opened the door to new types of large-scale cell-based screens. Whereas array-format screens require liquid handling automation and assay miniaturization, pooled-format screens, in which reagents are introduced at random and in bulk, can be done in a standard lab setting. We provide a detailed protocol for conducting and evaluating genome-wide CRISPR single guide RNA (sgRNA) pooled screens in *Drosophila* S2R+ cultured cells. Specifically, we provide step-by-step instructions for library design and production, optimization of cytotoxin-based selection assays, genome-scale screening, and data analysis. This type of project takes ~3 months to complete. Results can be used in follow-up studies performed in vivo in *Drosophila*, mammalian cells, and/or other systems. © 2019 by John Wiley & Sons, Inc.

**Basic Protocol:** Pooled-format screening with Cas9-expressing *Drosophila* S2R+ cells in the presence of cytotoxin

**Support Protocol 1:** Optimization of cytotoxin concentration for *Drosophila* cell screening

**Support Protocol 2:** CRISPR sgRNA library design and production for *Drosophila* cell screening

**Support Protocol 3:** Barcode deconvolution and analysis of screening data

Keywords: CRISPR • *Drosophila* • functional genomics • high-throughput screening • pooled-format screening

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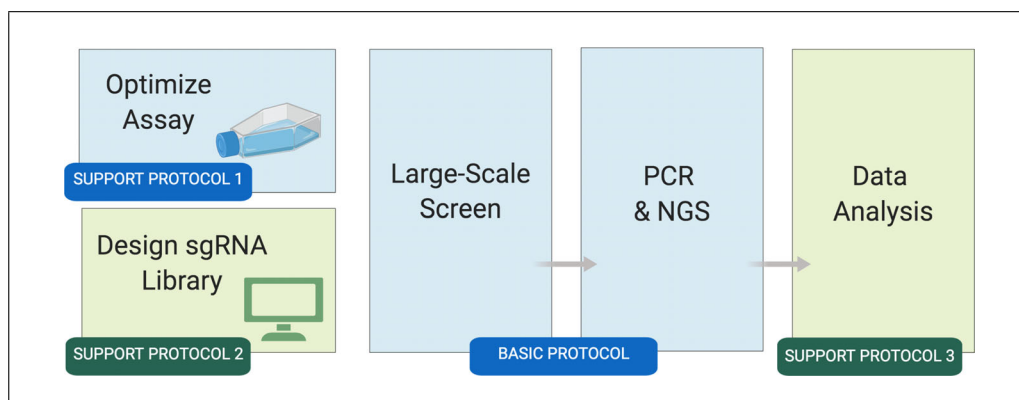
## INTRODUCTION

*Drosophila melanogaster* is a well-established model for large-scale in vivo and cell-based screens. The relatively low-redundancy *Drosophila* genome, uniformity of cultured cells, and availability of transcriptomics and other large-scale data make fly cultured cells particularly useful for large-scale functional genomics studies. Impactful findings from large-scale screens in *Drosophila* cells done using RNA interference (RNAi) include the identification of a mediator of hypercapnic immune suppression (Helenius et al., 2016), new drug targets for the development of therapeutic treatments for the

proliferative disorder tuberous sclerosis complex (Housden et al., 2015), genes relevant to nucleolar size (Neumuller et al., 2013), genes relevant to host-pathogen interactions (e.g., Akimana, Al-Khodor, & Abu Kwaik, 2010; Sessions et al., 2009), and genes encoding conserved ion transporters, such as LETM1 (Jiang, Zhao, & Clapham, 2009) and ORAI1/CRACM (Feske et al., 2006; Vig et al., 2006; Zhang et al., 2006) (additional examples at <https://fgr.hms.harvard.edu/publications-data>). These findings underscore the power of the *Drosophila* cultured cell system for gene function discovery. Nevertheless, the array-format RNAi screening approach has limitations, including a need for specialized equipment and the potential for reagent-specific off-target effects.

Pooled-format screens using CRISPR technology provide an attractive alternative. Pooled-format approaches, in which reagents are introduced in bulk and at random, eliminate the need for liquid-handling automation equipment. Moreover, CRISPR-Cas9 activity can result in knockout, rather than knockdown, of a gene and is associated with lower off-target potential. Pooled-format CRISPR screening was reported in 2014 as an approach to mammalian cell screening (Shalem et al., 2014; Wang, Wei, Sabatini, & Lander, 2014). With CRISPR pooled-format cell-screening technology, tens of thousands of CRISPR reagents are delivered to millions of isogenic cells simultaneously such that each cell receives a single reagent targeting a single locus. This is followed by outgrowth of the cells in the presence or absence of selection, such as with a drug (protocols include Joung et al., 2017; Piccioni, Younger, & Root, 2018). Technical comparisons of CRISPR- and RNAi-based perturbations in mammalian cells have concluded either that the approaches are complementary in large-scale screens or that CRISPR outperforms RNAi (Evers et al., 2016; Morgens, Deans, Li, & Bassik, 2016).

We have established CRISPR pooled-format screening as an additional functional genomics approach for interrogation of gene function and drug target discovery using *Drosophila* cultured cells (Viswanatha, Li, Hu, & Perrimon, 2018). In mammalian cells, the introduction of sgRNAs relies on lentiviral transduction and integration, a step that makes it possible to later track which sgRNAs are associated with positive results. We use bacterial site-specific recombination-based plasmid transfection to deliver comparably large CRISPR reagent libraries to *Drosophila* S2R+ cells (Fig. 1). We have showed that pooled screening resulted in highly accurate identification of ~1300 cell-essential genes. Further, we applied the procedure to gene-drug interaction screening (Viswanatha et al., 2018). This method has also been used to identify new components of the insect molting hormone pathway (Okamoto et al., 2018). The approach is extensible to other *Drosophila*



**Figure 1** Overview of the *Drosophila* cell CRISPR pooled screen workflow. Wet-bench activities are shown in blue and bioinformatics steps in green. Once the cell-based assay is optimized (Support Protocol 1) and a library is obtained or built (Support Protocol 2), the large-scale screen (Basic Protocol) can begin; this takes ~45 days to complete. After the screen, the resulting next-generation sequencing (NGS) data are analyzed to identify screen “hits” (positive results) at the reagent and gene levels (Support Protocol 3).

cell lines. The *Drosophila* Genomics Resource Center (DGRC) in Bloomington, Indiana, maintains a large catalog of *Drosophila* cell lines (<https://dgrc.bio.indiana.edu>).

Here, we provide detailed protocols for conducting and evaluating *Drosophila* pooled CRISPR screens performed in the presence of a cytotoxin (i.e., cytotoxin sensitivity and resistance screen). An overview is shown in Figure 1. Before screening is performed, an appropriate Cas9-positive cell line should be obtained from the DGRC or established, the cell assay should be optimized (Support Protocol 1), and a large-scale sgRNA library in an appropriate vector should be obtained or generated (Support Protocol 2). A cell line tailored for the assay (Viswanatha et al., 2018) is available from the DGRC (cat. no. 268). The screen itself comprises three main phases: library transfection, library integration, and selection (Basic Protocol). Completion of these phases takes ~45 days. At the end of the screening period, sgRNA reagents present at the start and end of the assay are amplified, the resulting PCR fragments are purified, and the PCR fragments are subjected to next-generation sequencing (NGS; Basic Protocol). Screen data analyses include the identification of sgRNAs that are enriched or depleted under cytotoxin treatment conditions versus control conditions, followed by gene-level analyses (Support Protocol 3).

The screen can be done in the absence or presence of a cytotoxin. In the absence of a cytotoxin, a simple outgrowth assay is used to separate sgRNAs that confer cell lethality when knocked out (i.e., sgRNAs that target essential genes) from sgRNAs targeting nonessential genes. Such a screen can be performed to establish the set of essential genes in a given cell line (Viswanatha et al., 2018) or can be performed in parallel in wild-type and mutant cells, for example to identify genes that are synthetically lethal in conjunction with the knockout of a given gene. In the presence of a cytotoxin, CRISPR pooled screening can be used to identify sgRNAs that target genes whose knockout confers sensitivity or resistance to the cytotoxic treatment, which could be a cytotoxic drug such as a chemotherapeutic (e.g., see Viswanatha et al., 2018), a biomolecule that induces cell death (e.g., see Okamoto et al., 2018), an environmental toxin, an infectious pathogen, etc. Additional types of assays are compatible with the CRISPR pooled format, as discussed in the Commentary.

## STRATEGIC PLANNING

### Getting Started

The Basic Protocol takes ~45 days to complete. Before this, the cells must be expanded to ensure that there are enough for the screen. A variety of simple tests can be used to make sure that the cells have the appropriate attributes. For example, integration can be tested using the empty pLib6.4 vector (as demonstrated in Figure 1 of Viswanatha et al., 2018). In addition, Cas9 activity can be confirmed, for instance by transfection with an sgRNA targeting mCherry followed by FACS (if using the mCherry-positive S2R<sup>+</sup>-MT::Cas9 cell line) or by using a T7 endonuclease activity assay to assess cutting at a given sgRNA-targeted locus.

### Setup for sgRNA Library Transfection into Cas9-Expressing S2R<sup>+</sup> Cells

The amount of the CRISPR library to transfect depends on the number of sgRNAs in the library, the desired coverage, and the integration efficiency. You should transfect the library in units of 6-well-dish wells to contain potential contamination. Determine the number of wells needed using the following formula:

$$\text{No. of wells} = \frac{\text{no. of sgRNAs in library} \times \text{coverage}}{\text{integration efficiency} \times \text{cells per well}}$$

At least 400× coverage of the library is recommended in the final integrated pool. Integration efficiency is usually >30%. The optimal density of S2R+ cells at the time of transfection is  $3 \times 10^6$  per well. Using these parameters, for the genome-wide *Dsg\_group1+2+3* library containing 86,364 sgRNAs (Viswanatha et al., 2018), ~40 wells must be transfected to cover the library, whereas for the *Dsg\_group1+2* library containing 26,566 sgRNAs (Okamoto et al., 2018), ~11 wells must be transfected to cover the library.

Integration efficiency depends on transfection efficiency, which varies between experiments. It is therefore necessary to empirically evaluate the integration efficiency within each experiment. In our hands, transfection efficiency (with Effectene and measured by flow cytometry) after transfection with pLib6.4 is >50%, and integration efficiency is ~30%. Integration efficiency can be evaluated by including the controls listed below during library transfection.

	Amount of pBS130	Amount of CRISPR library	Passage in Puro?
Well 1	0.2 µg	0.2 µg	No
Well 2	–	0.4 µg	No
Wells 3- <i>n</i>	0.2 µg	0.2 µg	Yes

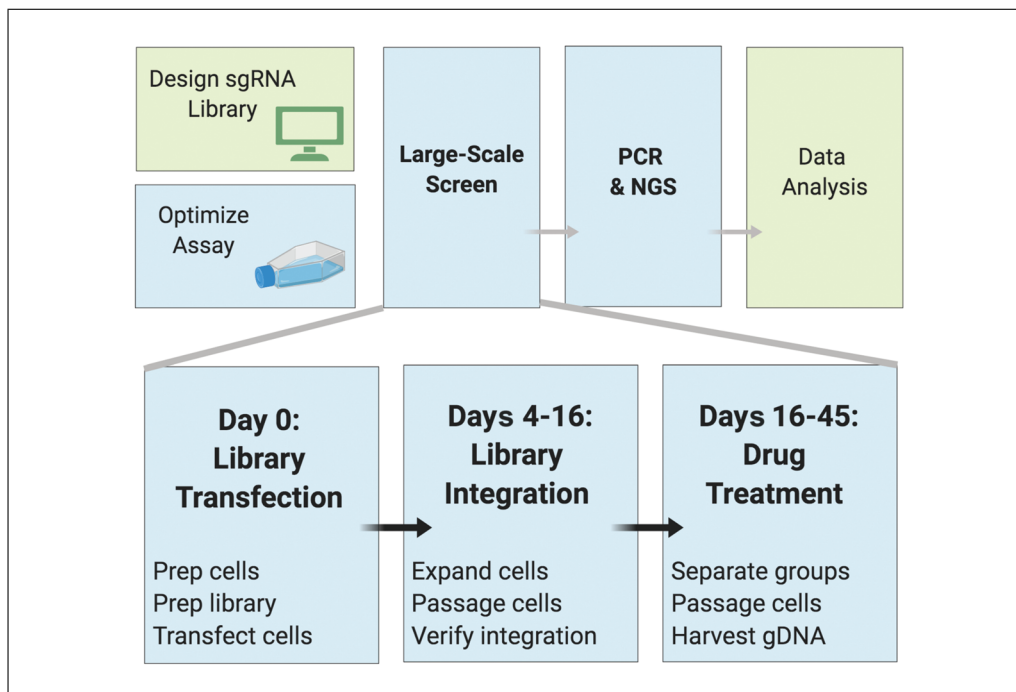
Integration efficiency is approximately equal to the number of stable GFP-positive cells derived from Well 1 minus the number from Well 2. Note that this value can only be determined after transiently delivered (nonintegrated) plasmids have been effectively diluted out of the population, which takes ~3 weeks. We recommend evaluating after 3 weeks of passaging using a BD LSR II flow cytometer. If integration efficiency is <30%, discard the CRISPR cell pool and repeat the procedure.

### Thinking Ahead

Large-scale screens typically result in the identification of a large number of “hits,” or positive results. Often, these can be subcategorized using secondary cell-based or in vivo assays (a) to determine detailed *Drosophila* cell phenotypes; (b) to explore in vivo relevance in *Drosophila*, either in general or in a specific tissue, stage, or cell type; and/or (c) to understand the relevance of the finding to mammalian orthologs, e.g., by asking whether a similar phenotype is observed upon perturbation of orthologous genes in mammalian cultured cells. Secondary assays should be considered and developed before the large-scale cell-based screen; knowing what follow-up assays you will do after the screen will be relevant to the analysis of screen data, as that analysis can be adjusted with end goals in mind. If the follow-up assay is particularly time or resource intensive, for example, you might only want to test high-confidence hits. Thus, you would set a high statistical cut-off and/or look for other types of supporting evidence in the published literature or other datasets at a screen data integration stage. If, conversely, there are one or more relatively easy and inexpensive secondary assays that can be applied to a large number of primary screen hits, you might “cast a broad net” by setting a lower statistical threshold, and plan to test not only high-confidence hits but also moderate- and low-confidence hits from the primary screen in the secondary assay.

## **POOLED-FORMAT SCREENING WITH CAS9-EXPRESSING DROSOPHILA S2R+ CELLS IN THE PRESENCE OF A CYTOTOXIN**

This protocol (Fig. 2) assumes that the goal of the screen is to identify genes whose knockout confers sensitivity or resistance to a cytotoxic treatment. Support Protocol 1 describes how to identify an optimal concentration of a cytotoxin for screening. Additional potential alternative protocols include a “drop-out” screen to identify genes that are essential in a given cell line, or separation via flow cytometry–based cell sorting based



**Figure 2** Overview of the Basic Protocol for *Drosophila* cell CRISPR pooled screening. Workflow shown for a selection screen using a cytotoxic treatment at day 16. For a “drop-out” screen to identify genes that are essential, omit drug selection in Basic Protocol and passage the cells without drug.

on a fluorescent marker or antibody. In addition, the Basic Protocol can be performed in parallel with both wild-type and mutant cells, e.g., to use a drop-out approach to identify synthetic lethal interactions detected in mutant but not wild-type cells (see Commentary for additional discussion on assay types).

This Basic Protocol also assumes the use of a Cas9<sup>+</sup> S2R<sup>+</sup> cell line with an *attP* integration site together with an existing sgRNA library, such as the library described in Viswanatha et al. (2018) and available from Addgene (cat. nos. 134582 to 134584). The specific strategy for integration-based insertion of sgRNAs in the library into cell genomes is shown in Figure 3. New libraries can be developed as described in Support Protocol 2. Depending on the vector and library design, some modifications might be necessary to the protocol for data analysis, as presented in Support Protocol 3.

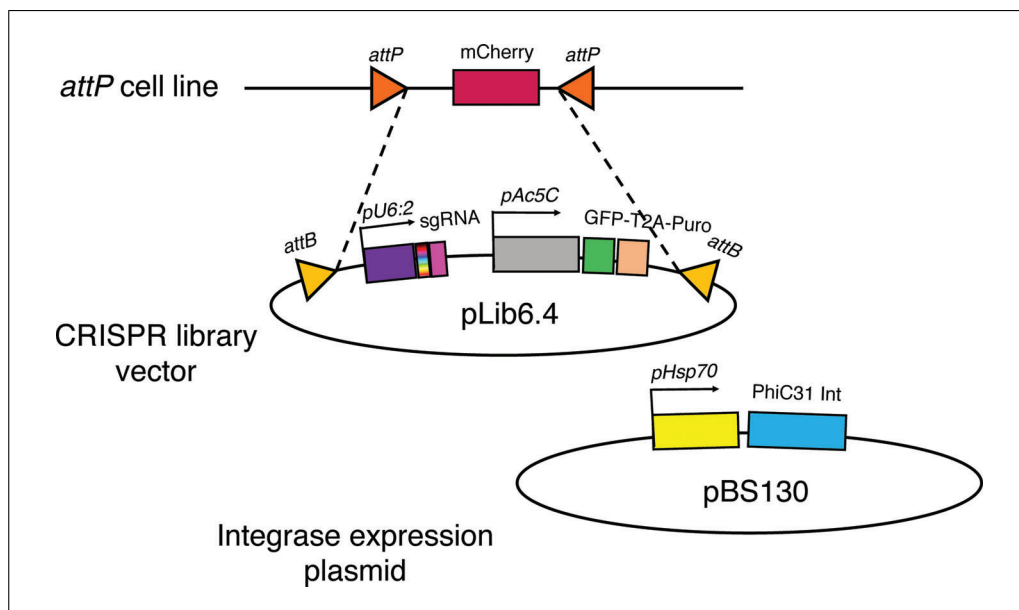
### Materials

- S2R<sup>+</sup>-MT::Cas9 cultured cells (*Drosophila* Genomics Resource Center stock no. 268)
- Drosophila* cell culture medium (see recipe)
- CRISPR plasmid sgRNA library for *Drosophila* gene knockout, such as the library described in Viswanatha et al. (2018; Addgene cat. nos. 134582-134584) or developed as described in Support Protocol 2
- pBS130 plasmid (pHSP70-PhiC31 integrase; Addgene 26290)
- Qiagen Effectene transfection reagent (cat. no. 301427)
- Puromycin selection medium (see recipe)
- Screen-specific cytotoxin (Support Protocol 1)
- Zymo<sup>®</sup> gDNA Miniprep Kit (D3025)
- Phusion polymerase kit (polymerase, dNTPs, buffer)
- PCR1 and PCR2 primers for sgRNA detection from cells (Table 1)
- Kapa Biosystems Library Quantification kit (KK4835)
- 2% agarose SYBR<sup>™</sup> Safe<sup>™</sup> gel
- Qiagen Gel Extraction Kit (cat. no. 28704)

Hemocytometer  
 6-well tissue culture treated dishes (Corning 3516)  
 10-cm tissue culture treated dishes (Corning 430164)  
 Plastic wrap, such as Reynolds 900 Food Service Film (Careforde Scientific G1682075)  
 Corning Cell Lifter 3008 (cat. no. 3008)  
 250-ml medium bottle (Nalgene 2019-0250)  
 Qubit fluorometer  
 Multichannel pipette  
 Illumina Next Seq 500 instrument (e.g., as accessed through a core facility)  
 Illumina Next Seq 500 v2 75-cycle High Output cartridge

### Transfection of Library—Day 0

1. Determine the number of cells needed to obtain the appropriate integration level for a given CRISPR plasmid library (see Strategic Planning).
2. Grow cultured cells in a T75 flask, and verify that they are doubling approximately once per day prior to transfection.
3. Dislodge by tapping flask and pipetting. To eliminate clumps and generate a single-cell suspension, withdraw medium and cells into a Pasteur pipet and press the tip of the pipet against a flat surface, such as the side of the flask, to narrow the opening. Dispense the medium with cells through narrow opening to generate a spray of cells. Repeat once or twice and confirm that most cells are now in a single-cell suspension.
4. Count cells with a hemocytometer and adjust concentration to  $1.5 \times 10^6$  cells/ml.
5. Dispense 2 ml cell suspension per well into a 6-well tissue culture dish and allow to attach.
6. Prepare transfection mixtures according to the Qiagen Effectene Handbook, using the base protocol. Briefly, first prepare 0.4  $\mu\text{g}$  DNA/well and then add 3.2  $\mu\text{l}$  Enhancer solution/well. Vortex and spin down. Incubate 5 min. Add 10  $\mu\text{l}$  Effectene reagent. Vortex. Incubate 15 min. Add complexes dropwise to cells.



**Figure 3** Plasmid-based library integration strategy for pooled CRISPR screening in *Drosophila* S2+ cells. Co-transfection of cells with pLib6.4 mixture containing diverse sgRNAs and pBS130 PhiC13 integrase vector causes a portion of the transfected cells to integrate the sgRNA, GFP, and puromycin-resistance expression cassette.

**Table 1** PCR Primers Used in the Basic and Support Protocols

Example of primer name	Primer sequence
PCR1fwd	5'- <b>CTTCCCTACACGACGCTCTTCCGATCT</b> NNNTAGCTTgtttcctcaatactcgttcg-3' Partial Read1 sequence; fingerprint; barcode Drosophila U6:2 promoter sequence
PCR1rev	5'-tttgtgttttagaatatagaattgcatgctgggtaccc-3' Complementary to pLib6.4 vector
PCR2fwd	5'- <b>AATGATACGGCGACCACCGAGATCTACACTCTTTCCTACACGACGCTCTTCCGATCT</b> -3' P5 sequence; spacer (required for NextSeq but not for HiSeq platforms); complete Read1 sequence
PCR2rev	5'- <b>CAAGCAGAAGACGGCATACGAGAT</b> tttgtgttttagaatatagaattgcatgctgggtaccc-3' Reverse complement of P7; complementary to PCR1

\*Each PCR1 primer varies based on the length of the random nucleotide fingerprint region and the identity of the 6-bp barcode. In a typical experiment, it is necessary to order at least four distinguishable PCR1 primers.

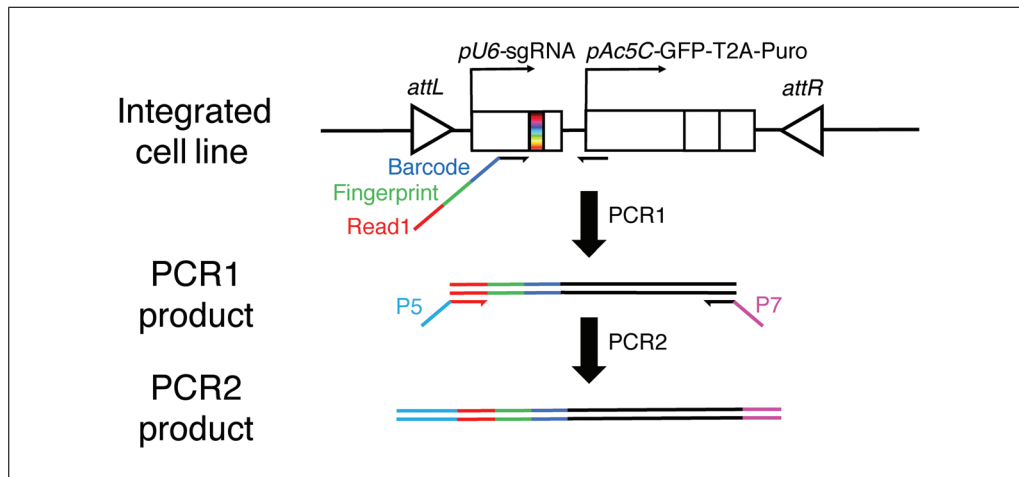
- Wrap stacks of 6-well dishes in plastic wrap to prevent evaporation and incubate 4 days at 25°C.

#### Library Expansion and Maintenance—Days 4-16

- Expand the transfected cells from 6-well dishes to 10-cm plates:* First, dislodge the cells from the 6-well plates by lifting up cells from each well individually using a Corning Cell Lifter 3008 with the flat side facing up. Eliminate clumps (see step 3). Transfer cells and medium together into a 10-cm tissue culture dish containing 10 ml puromycin selection medium. Stack and completely seal 10-cm dishes in plastic wrap and incubate 4 days at 25°C.
- CRISPR screen pool maintenance:* Determine the number of cells needed in each passage to adequately maintain library diversity (see Strategic Planning). Dislodge cells from each dish using cell lifter. Dislodge cells from all 10-cm dishes and combine cell suspension into a single 250-ml medium bottle. Eliminate clumps (see step 3). Invert bottle containing combined cell suspension two to four times to mix. Count cells and dispense the number of cells needed at a maximum density of  $1 \times 10^7$  cells/10 cm dish into puromycin selection medium.
- Repeat passaging procedure every 4 days until Day 16.

#### Cytotoxin Treatment and Outgrowth—Day 16 to End of Screen (~Day 45)

- From the remaining cell suspension, distribute integrated cell population into a minimum of four groups: two groups as untreated controls and two groups treated with the cytotoxin. We recommend continuing screen at 400× coverage (at least 400 cells/sgRNA per replicate). Note that the number of cells per replicate is critical to prevent population bottlenecks during passages (see Strategic Planning). For example, for a library containing ~100,000 sgRNAs, at least four 10-cm dishes are needed per replicate. Plate  $1 \times 10^7$  cells/10 cm dish and allow them to grow for 4-5 days.
- Dislodge cells and combine together cells from the same replicates into one bottle. Invert two to four times to mix. Distribute  $1 \times 10^7$  cells per dish to the same number of 10-cm dishes. This procedure maintains 400× coverage throughout the screen. Record cell density after each passage for each group, as these data can be used to verify that the cytotoxin caused the expected reduction of cell proliferation.



**Figure 4** PCR strategy for amplifying sgRNA sequences from cell pools. To amplify sgRNAs expression cassette from S2R<sup>+</sup> cells, a two-step PCR strategy is used. In PCR1, barcoded primers are used to attach sample-barcodes to sgRNA sequences in each cell pool. The PCR1 forward primer also contains a fingerprint (or stagger) sequence that allows cluster distinction during Illumina sequencing and a primer binding site for PCR2, which is a portion of the Illumina Read1 primer binding sequence. In PCR2, a second round of tailored PCR adds Illumina P5 and P7 sites for Illumina Flow Cell attachment.

13. Continue high-coverage passaging until you reach the planned end date for exposure to the cytotoxin. It is critical to maintain as many cells as possible (at least 400× coverage, see above) during the passages to avoid population bottlenecks. We recommend passaging cells for at least 30 days in selective medium for the initial screen.
14. Once the desired cytotoxin exposure period is accomplished, pellet cells in 50-ml aliquots. Aspirate supernatant and store pellets at  $-80^{\circ}\text{C}$  to await genomic DNA (gDNA) extraction.

### Sample Preparation for Next-Generation Sequencing

The goal of this part of the procedure is to extract gDNA from the cells and then amplify the sgRNAs that have integrated into the gDNA. The specific sgRNAs present in gDNA from experimental and control cells (before and after an experimental manipulation) will later be identified using NGS and then compared to identify positive results. A barcode strategy is applied during amplification of sgRNAs and used in analysis steps (Fig. 4).

#### *gDNA extraction*

15. Thaw pelleted cells from the final passage (Day 45) on ice for  $\sim 10$ -15 min or until the pellet liquifies. To reduce thawing time, remove cells from ice and let sit at room temperature.
16. Using Zymo<sup>®</sup> gDNA Miniprep Kit (D3025), set up six collection tubes with collection columns per pellet.
17. When pellet is thawed, resuspend in 5 ml gDNA Lysis Buffer by triturating and letting sit for 5 min.
18. Dispense into each of the six collection columns and then centrifuge 30 s at  $10,000 \times g$ , room temperature.
19. Follow the rest of the manufacturer's protocol.
20. Pool each of the six Eppendorf tubes containing the DNA eluted in Elution Buffer coming from a single pellet.



### PCR1: Amplification of sgRNAs from S2R+ cell line genome

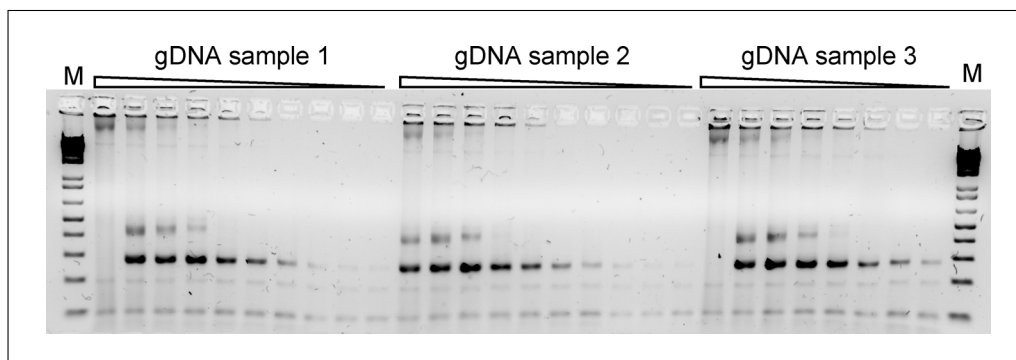
21. To verify gDNA integrity and determine amount of PCR needed to provide appropriate coverage, prepare twofold serial dilutions of gDNA preps and perform test amplifications using 2× Phusion PCR master mix. To do this, set up the following PCR mix:

Component	Volume (μl)
Nuclease-free water	4
5× Phusion HF buffer	4
2.5 mM dNTPs	1.6
100 μM PCR1fwd	0.1
100 μM PCR1rev	0.1
Template gDNA	10
Phusion DNA polymerase	0.2
Total volume	20

Perform PCR1 using the following amplification reaction conditions:

Initial denaturation	2 min	98°C
23 cycles	15 s	98°C
	45 s	59°C
	30 s	72°C
Elongation	10 min	72°C
Hold		4°C

22. Resolve each reaction on a 2% agarose gel (see example in Fig. 5). Identify the highest concentration of gDNA that still permits the reaction to yield a single band of ~280-300 bp. Using a Qubit fluorometer, determine the concentration of DNA in this sample. You now have enough information to calculate the minimum required PCR volume for this sample. Based on the assumption that the diploid mammalian genome weighs 6.6 pg (Chen et al., 2015), the tetraploid S2R+ genome, which is ~1/11 the size, should weigh 0.60 pg. PCRs should contain at least 400× coverage (Wang et al., 2014). For instance, using these parameters, for the genome wide *Dsg\_group1+2+3* library containing 86,364 sgRNAs (Viswanatha et al., 2018), a minimum of 21 μg gDNA is needed. Typically, >700 μl of PCR are needed per sample.



**Figure 5** Small-scale verification of genomic DNA (gDNA) integrity. After gDNA extraction, we perform twofold serial dilutions of gDNA to determine highest concentration of gDNA that will still allow detection of sgRNA cassette by PCR. In the example shown, PCRs containing indicated gDNA prep are serially diluted and then each is added to 2× Phusion PCR master mix and subjected to 23 cycles of PCR before being resolved on 2% agarose SYBR Safe DNA gel (image inverted). Expect a 280-300 bp band depending on PCR1 primer used. Marker, 1 kb plus DNA Ladder (Thermo 10787018). Result indicates that gDNA samples 1 and 3 must be diluted twofold before PCR to detect the sgRNA cassette, whereas dilution is not necessary for gDNA sample 2.

23. Using a multichannel pipette, aliquot the pooled reaction in 20  $\mu$ l aliquots into a 96-well plate and perform scaled-up PCR (using the same PCR master mix and amplification reaction conditions as described in step 22) to optimize coverage as required. After cycling, pool the reaction together and run 50  $\mu$ l of the pooled reaction on a 2% agarose SYBR Safe DNA gel.
24. Cleanly excise the 280- to 300-bp band from the gel.
25. Gel-purify DNA using Qiagen Gel Extraction Kit and proceed to PCR2.

***PCR2: Addition of P5, read1, and P7 to PCR1***

26. Attach the barcodes to each reaction individually by performing a second PCR using primers that attach P5, Read1, and P7 sequences required for downstream NGS. To maintain representation, 40 ng of purified PCR1 is used as template in each reaction. To do this, set up the following PCR mix:

Component	Volume ( $\mu$ l)
Nuclease-free water	Variable
5 $\times$ Phusion HF or GC buffer	40
2.5 mM dNTPs	16
100 $\mu$ M PCR2fwd	1
100 $\mu$ M PCR2rev	1
Template DNA	Variable (40 ng)
Phusion DNA polymerase	2
Total volume	200

Perform PCR2 using the following amplification reaction conditions:

Initial denaturation	30 s	98°C
17 cycles	8 s	98°C
	45 s	57°C
	1 min	72°C
Elongation	1 min	72°C
Hold		4°C

27. After cycling, pool the reactions together and run approximately half on a 2% agarose SYBR Safe gel.
28. Cleanly excise the  $\sim$ 350-bp band.
29. Gel-purify DNA using Qiagen Gel Extraction Kit and proceed to sample pooling for NGS.

***Pooling of samples for next-generation sequencing***

30. Measure the concentration of each PCR2 product using Qubit fluorometer and Qubit dsDNA HS Assay Kit. Using starting library diversity, derive the sgRNA concentration and normalize all samples by sgRNA concentration.
31. Pool together equal volumes of all samples.
32. Use Kapa Biosystems Library Quantification kit (KK4835) or equivalent to more accurately determine library concentration.

***Next-Generation Sequencing***

33. Load pooled PCR2 directly on an Illumina Next Seq 500 v2 75-cycle High Output cartridge. Request 91 cycles from manufacturer-provided Read1 primer without Phi-X spike-in and without additional indexing reads.
34. Collect data as raw FASTQ file. For analysis, proceed to Support Protocol 3.

## OPTIMIZATION OF CYTOTOXIN CONCENTRATION FOR *DROSOPHILA* CELL SCREENING

The Basic Protocol describes a method for screening with a specific and predefined concentration of a cytotoxin. Optimization of the cytotoxic treatment is aimed at providing a robust assay (i.e., concentration is not too low) while at the same time giving cells a chance to survive (i.e., concentration is not too high). The protocol uses a readout of total ATP levels as a proxy for both cell number and viability. Tests are first performed in a microwell plate to determine an optimal range, and then performed in flasks to more closely mimic the screen conditions.

### Materials

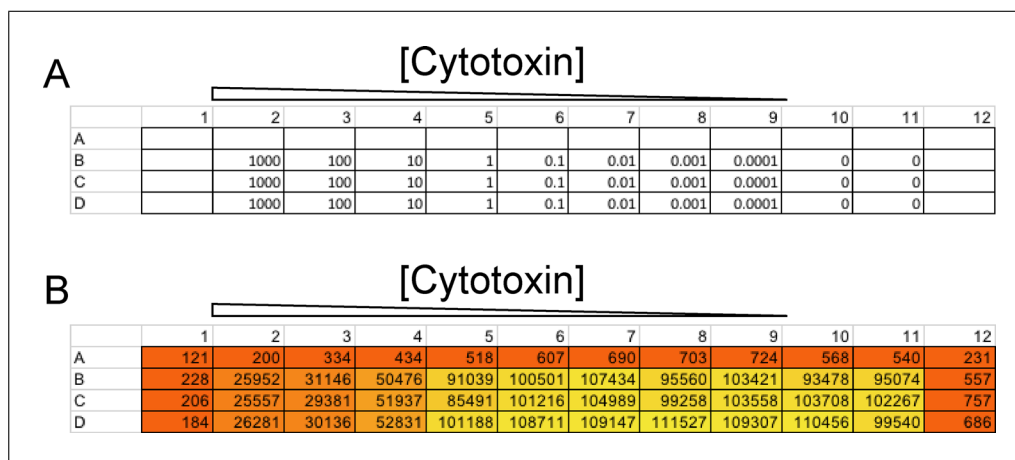
S2R+-MT::Cas9 (DGRC stock no. 268, <https://dgrc.bio.indiana.edu/>)  
Hygromycin selection medium (200 ng/ml; see recipe)  
Cell Titer-Glo (Promega G7573)  
Cytotoxin: for example, rapamycin (LC Laboratories R-5000), trametinib (Selleck Chemicals S2673), or 20-hydroxecdysone (Sigma H5142)  
  
Flat-bottom 96-well plate (Corning 3799)  
T75 flasks  
Plastic wrap, such as Reynolds 900 Food Service Film (Careforde Scientific G1682075)  
Luminometer (plate reader).  
0.22- $\mu$ m-pore-size filter

### Broad-range optimization using 96-well plates

1. Obtain S2R+-MT::Cas9 from DGRC. When the cells arrive, follow DGRC recommendations for thawing cells (see [https://dgrc.bio.indiana.edu/include/file/Thawing\\_Cells.pdf](https://dgrc.bio.indiana.edu/include/file/Thawing_Cells.pdf)), and passage for ~2 weeks in 200 ng/ml hygromycin selection medium until cells are actively growing and doubling approximately once per day.
2. Perform the assay in a flat-bottom 96-well plate. Put cells in medium in all wells, but use only non-edge wells as experimental wells to avoid the potential impact of 'edge effects' due to differences in well microenvironments (see example in Fig. 6). Prepare tenfold serial dilutions of desired cytotoxin(s) in a total volume of 50  $\mu$ l in experimental wells (or 50  $\mu$ l vehicle control) and add to cells.
3. Dislodge actively growing S2R+-MT::Cas9 cells from a T75 flask and adjust concentration to 600,000/ml. Serially dispense 50  $\mu$ l cell suspension into each well (30,000 cells/well), bringing the total volume in every well to 100  $\mu$ l.
4. Wrap plate in plastic wrap and incubate 5 days at 25°C.
5. Add 50  $\mu$ l Cell Titer Glo assay reagent (Promega) to each well, mix, and measure luciferase activity using a luminometer (plate reader).
6. Compare treated samples to vehicle controls and determine a cytotoxin concentration range for which cell growth is impeded but not completely halted. Typically, luminescence readings should be 10%-50% of untreated value, indicating partial perturbation in cell growth.
7. To further narrow the concentration range, repeat steps 1-4 with a twofold dilution around the effective concentration range.

### Narrow-range optimization in T12.5 flasks

8. In a laminar-flow tissue culture hood, dilute cytotoxin to concentrations identified from the 96-well assay in 10 ml growth medium. Filter-sterilize the



**Figure 6** Determining an optimal concentration of a cytotoxin for a selection-based screen. Shown is an example layout for a 96-well plate of cells used to determine the optimal concentration range of a cytotoxin. **(A)** Trametinib at the indicated concentration (in nM) was first added to the plate in 50  $\mu$ l of growth medium. The same number of cells (50  $\mu$ l of a cell suspension in fresh growth medium at  $2 \times 10^5$  cells/ml, or 10,000 cells per well) was then added to each well. **(B)** Cells were grown for 5 days and then subjected to Cell-Titer Glo assay. Arbitrary luminometry values from corresponding wells are shown. 50 nM was ultimately chosen as an optimal concentration for the screen.

cytotoxin-containing medium by attaching a 0.22- $\mu$ m filter and dispensing it into a new 15-ml Falcon tube. Store at 4°C for  $\leq 2$  weeks.

- Add 500,000 cells to 2.5 ml medium with cytotoxin per T12.5 flask and passage 3-5 days depending on growth rate. Count cells and seed  $1 \times 10^7$  every passage.
- Identify a cytotoxin concentration that consistently gives  $\sim 50\%$  fewer cells on every passage. This should be useful for screening.

## SUPPORT PROTOCOL 2

### CRISPR sgRNA LIBRARY DESIGN AND PRODUCTION FOR *DROSOPHILA* CELL SCREENING

One option for screening is to use ready-made CRISPR sgRNA libraries for *Drosophila* cell screening as reported in Viswanatha et al. (2018), which are available from Addgene (cat. nos. 134582 to 134584). Another option is to generate a new custom library with genome-wide or more limited coverage, using the plasmid vector pLib. This support protocol is based on use of Find CRISPR3.

Typically, there are many potential sgRNA target sites per gene. It is important to select sgRNA designs with high specificity as well as high efficiency. The Find CRISPR tool implemented by the DRSC allows you to find sgRNA sites and information such as predicted efficiency score, predicted likelihood of frameshift mutation, and potential off-target sites (Housden et al., 2015; Ren et al., 2013). An improved version, Find CRISPR3 (<https://www.flyrnai.org/crispr3/web/>), provides efficiency scores predicted using different algorithms and includes an efficiency score calculated using a machine learning approach (unpublished) based on the genome-wide pooled screen reported in Viswanatha et al. (2018) and a specificity score calculated based on potential off-target sites and annotation of protein domains. The sgRNAs reported in Find CRISPR3 (v3.0) were designed using the FlyBase reference genome. The tool also provides annotation of genome variants detected in the S2R+ cell line and protein domain annotations. Designs targeting within protein domains can be prioritized, which is useful given that even the in-frame mutations in these regions are more likely to impact the function of the protein (Shi et al., 2015). An overview of a workflow for choosing sgRNAs for inclusion in a library is provided in Mohr et al. (2016).

Find CRISPR3 provides a batch search option. You can thus search all sgRNA designs for a set of genes, download the list of sgRNAs, then select designs for inclusion in your library based on efficiency scores, specificity scores, SNP annotations, and/or protein domain annotations.

### Materials

Phusion polymerase kit (polymerase, dNTPs, buffer)  
Dialout primers (see step 3)  
2% and 1% agarose gels  
Qiagen Gel Extraction Kit (cat. no. 28704)  
*Bpi*I restriction enzyme (Thermo ER1012)  
Qubit dsDNA HS Assay Kit (Thermo Q32854)  
20% TBE Novex Gel (Thermo EC63152BOX)  
O'Range 10-bp marker (Thermo SM1313)  
SYBR Safe DNA gel stain (Thermo S33102)  
pLib6.4 plasmid vector (Addgene 133783)  
T4 DNA ligase buffer (New England Biolabs B0202S)  
T7 DNA ligase (Enzymatics L6020L)  
Electrocompetent *Escherichia coli* such as Ecloni 10GF' Electrocompetent cells (Novagen)  
LB agar plates with ampicillin  
LB broth with ampicillin  
PCR Purification Kit (Qiagen 28106)  
Plasmid Miniprep kit (Qiagen)  
  
Dark Reader (Bioexpress U-2235-18)  
Plastic pestle (VWR KT749521-1590)  
Spin-X column (Corning CLS8161)  
Qubit fluorometer (Thermo Q33238)  
Corning Cell Lifter 3008 (cat. no. 3008)  
0.45- $\mu$ m-pore-size cell strainer

### Custom sgRNA library design and synthesis

1. Use the “Find CRISPR3” batch design tool from DRSC/TRiP Functional Genomics Resources ([flyrnai.org/crispr3](http://flyrnai.org/crispr3)) to obtain CRISPR sgRNA designs targeting a set of genes that you custom input. You should note the Find CRISPR3 version number and FlyBase release number (Find CRISPR version 3.0 is based on FlyBase genome release r6.24). In choosing sgRNAs, exclude from the library sgRNA designs that target a region with genome variants in S2R+ compared with this reference genome. SNPs for S2R+-pMT::Cas9 cells are indicated in the results output at Find CRISPR (version 3; <https://www.flyrnai.org/crispr3/web/>). In addition, you can choose to prioritize designs targeting within protein domains, as this may increase knockout efficiency (Shi et al., 2015; also indicated in Find CRISPR version 3).
2. Eliminate and manually replace any sgRNAs containing the U6 terminator (TTTT) or a *Bbs*I or *Bpi*I site (GAAGAC or TGCTTC).
3. “Dialout” primers are used to retrieve classes of oligos from oligo pools. Append dialout primers, *Bpi*I/*Bbs*I recognition sites, U6:2 promoter sequence, and *trans*-activating CRISPR RNA (tracrRNA) backbone sequence to each sgRNA sequence as follows:

5'-ctataatatagccagctcagctctgggggtGAAGACgcGTCGACTGCCGATTC  
GATTCGAACGTTTcgGTCCTCgtttatccaggcgaggggctgacaggggaatt-3'  
(red, dialout fwd primer; green, *Bpi*I/*Bbs*I site; blue, sgRNA sequence;

brown, reverse *BpiI/BbsI* site; violet, dialout rev primer —order reverse complement)

4. Order the resulting list of sequences as 109-mer oligo pools from a gene synthesis company such as GenScript ([www.genscript.com](http://www.genscript.com)).

#### ***Amplification and cloning of sgRNAs into the vector***

5. Use Phusion HF polymerase (from kit) to amplify the library using the following PCR parameters. Note that you should divide the reaction into  $\sim 10 \times 20\text{-}\mu\text{l}$  reactions.

Component	Volume ( $\mu\text{l}$ )	
ddH <sub>2</sub> O	138	
5 $\times$ buffer	40	
2.5 mM dNTPs	16	
100 $\mu\text{M}$ dialout fwd primer	1	
100 $\mu\text{M}$ dialout rev primer	1	
Library at 50 ng/ $\mu\text{l}$	2	
Phusion HF Polymerase	2	

Initial denaturation	30 s	98°C
12 cycles	8 s	98°C
	45 s	65°C
	1 min	72°C
Elongation	1 min	72°C
Hold		4°C

6. Gel-purify 109-mer fragments from a 2% agarose gel.
7. Use two Qiagen Gel Extraction Kit columns to purify 109-mer amplicons. Elute each in 20  $\mu\text{l}$  and pool. Add 10  $\mu\text{l}$  Buffer G (supplied with *BpiI* restriction enzyme) and 5  $\mu\text{l}$  *BpiI*. Digest overnight at 37°C.
8. Run a 20% TBE Novex Gel for  $\sim 1$  hr at 180 V along with O'Range 10-bp marker. Post-stain with SYBR Safe DNA gel stain. Visualize in dark reader, and cut out the 24-nt band representing the double-stranded sgRNA fragments and transfer to a clean 1.5-ml microcentrifuge tube.
9. Using a plastic pestle, crush band into powder and reconstitute in 100  $\mu\text{l}$  nuclease-free water. Soak pulverized gel fragments with gentle shaking or end-over-end rotation overnight at 37°C.
10. Resuspend slurry and transfer to a Spin-X column.
11. Spin 1 min in a tabletop centrifuge at maximum speed ( $\sim 17,000 \times g$ ), room temperature.
12. Use Qubit or equivalent fluorometer to determine DNA concentration.
13. Linearize pLib6.4 empty vector (Addgene 133783) with *BpiI* in Buffer G for 2 hr (5  $\mu\text{g}$  plasmid + 3  $\mu\text{l}$  FastAP + 3  $\mu\text{l}$  *BpiI* in a 60- $\mu\text{l}$  reaction).
14. Resolve restriction digest reaction on a 1% agarose gel and purify the  $\sim 8$ -kb band containing linearized pLib6.4.
15. Mix pLib6.4 with all eluted DNA from step 10 at a 1:2 molar ratio. Add 1/9 volume  $10\times$  T4 DNA ligase buffer. Add 1/20 volume T7 DNA ligase. Incubate overnight at 16°C.
16. Use a Qiagen PCR Purification Kit column to purify ligation reaction and elute in 20  $\mu\text{l}$  ddH<sub>2</sub>O.

17. Perform test electroporation from 1  $\mu$ l of the resulting reaction into 25  $\mu$ l highly competent *E. coli* cells and use the provided recovery medium to recover reaction for 1 hr at 30°C with shaking. Plate 1  $\mu$ l bacterial culture onto LB agar plate with ampicillin. Count resulting colonies.
18. Scale electroporation to cover expected library diversity. Specifically, perform enough 25- $\mu$ l electroporation reactions that colony number = 100  $\times$  sgRNA number. Plate resulting bacterial cells onto enough 150-mm ampicillin LB agar plates to cover the library. To ensure proper separation of colonies, do not exceed  $2 \times 10^5$  CFU/plate. Thus, a  $\sim$ 100,000-guide library should result in  $1 \times 10^7$  CFU and be plated onto at least 50 plates. Incubate plates overnight at 30°C. As soon as visible colonies are detected, proceed to step 19.
19. Harvest plates into liquid LB medium by pipetting  $\sim$ 10 ml LB medium directly into the middle of each plate and then brushing the plate with light downward pressure using a Cell Lifter 3008. This procedure will avoid scratching the plate and causing loss of LB to the plate bottom.
20. Pool LB resulting from all plate harvests and extensively vortex to mix colonies. Pass cell suspension through a 0.45- $\mu$ m cell strainer to clear chunks of agar.
21. Mix with an equal volume of 50% glycerol and freeze library in 1-ml aliquots at  $-80^\circ\text{C}$ .

#### ***Preparation of library DNA from an sgRNA aliquot***

22. Quick-thaw at 37°C and spin 1 min in a tabletop centrifuge at maximum speed ( $\sim$ 17,000  $\times$  *g*), room temperature, to harvest cell pellet.
23. Remove medium and wash with 1 ml PBS. Vortex and spin in a tabletop centrifuge at maximum speed ( $\sim$ 17,000  $\times$  *g*), room temperature.
24. Aspirate and discard PBS and replace with 250  $\mu$ L Qiagen Buffer P1. Proceed with Qiagen Plasmid Miniprep.

#### ***Library validation***

25. Perform 12 20- $\mu$ l, 15-cycle Phusion High Fidelity PCRs using PCR1fwd and PCR1rev primers and  $\sim$ 10 ng CRISPR plasmid library in each reaction.
26. Submit sample for NGS.
27. Proceed to Support Protocol 3 to determine sgRNA distribution in plasmid library.
28. Proceed to Basic Protocol to introduce the sgRNA library into S2R+-MT::Cas9 cells.

### **BARCODE DECONVOLUTION AND ANALYSIS OF SCREEN DATA**

Analysis of the NGS data sets resulting from a screen can be divided into three sections: de-multiplexing of the sample, determination of the number of counts obtained for each unique sgRNA, and gene-level analysis of screen results (Fig. 7). Note that in addition to analysis of sgRNAs amplified from genomic DNA in a screen (Basic Protocol), this procedure can also be applied to sgRNAs amplified from a newly generated library (Support Protocol 3) as a quality analysis step.

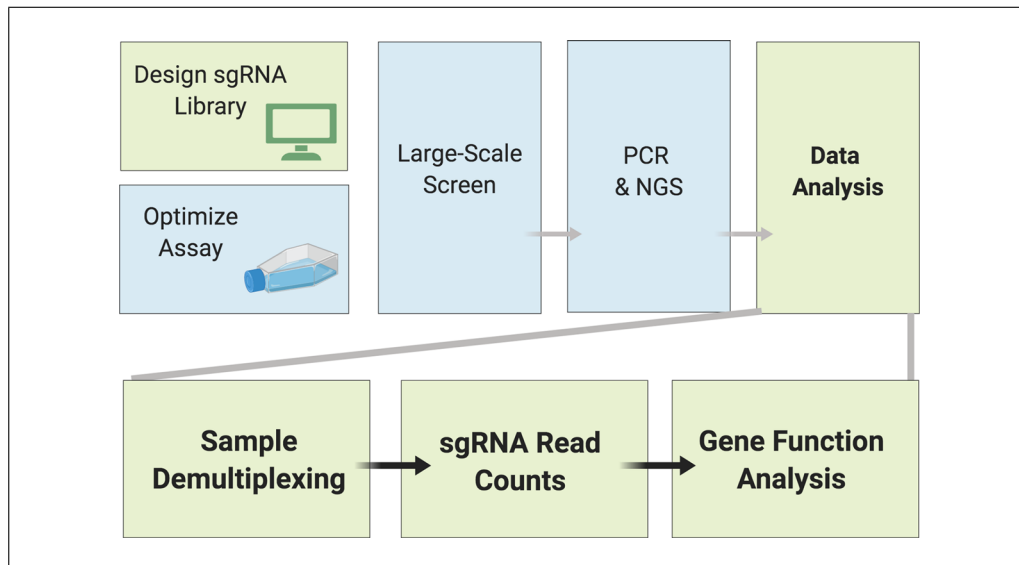
#### ***Materials***

Tagdust software (Lassmann, Hayashizaki, & Daub, 2009), <http://genome.gsc.riken.jp/osc/english/dataresource/>

### ***SUPPORT PROTOCOL 3***

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**Figure 7** Data analysis for *Drosophila* cell CRISPR pooled screening. Analysis of next-generation sequencing (NGS) data from a screen can be divided into three sections: de-multiplexing of the sample, determination of the number of counts obtained for each unique sgRNA, and gene-level analysis of screen results.

MAGeCK software (Li et al., 2014), <https://sourceforge.net/p/MAGeCK/wiki/Home/>

1. *Sample demultiplexing*: Process FASTQ file using TagDust (Lassmann et al., 2009) to demultiplex in-line barcodes. Below are example commands to demultiplex 3 samples from the same sequencing output file:

```
tagdust file.fastq -1 F:NNN -2 B: TAGCTT -3 R:N -o
  Sample1
tagdust file.fastq -1 F:NNNN -2 B: CAGTCC -3 R:N -o
  Sample2
tagdust file.fastq -1 F:NNNNN -2 B: ACATGA -3 R:N -o
  Sample3
```

Successful TagDust demultiplexing results in a new file that contains the sample name followed by the barcode (e.g., sample1\_BC\_TAGCTT.fq ...).

2. *sgRNA counting in each sample*: To count reads matching each sgRNA sequence, we use MAGeCK Count (Li et al., 2014). Because each PCR1 primer has 22 bp of homology to the U6:2 promoter, trimming the first 22 bp will yield the sgRNA sequence. Example demultiplexed sample files can be found at [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads/reads/](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads/reads/). Library files that relate the sgRNA sequences to the targeted gene must be provided for each library. Example MAGeCK-compatible library files can be downloaded at: [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads/library\\_files/](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads/library_files/). Use the following commands to obtain read-count files for each demultiplexed .fq file:

```
count -l library_file.txt -n sample1 --sample-label
  sample1 --trim-5 22 --fastq sample1_BC_TAGCTT.fq
count -l library_file.txt -n sample2 --sample-label
  sample2 --trim-5 22 --fastq sample2_BC_CAGTCC.fq
count -l library_file.txt -n sample3 --sample-label
  sample3 --trim-5 22 --fastq sample3_BC_ACATGA.fq
```



Successful read counting will result in a new file that contains the sample name followed by “.count.txt” (e.g., ...sample1.count.txt...). For our sample data, read counts can be found at [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads/read\\_counts/](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads/read_counts/).

3. *Gene-level statistics*: To determine *Z* scores at the gene level, at least two sets of read-count files containing the same sgRNA sequences must be compared. Statistical significance exist when multiple sgRNAs targeting the same gene show significant deviations between samples. For this analysis, we use MAGeCK MLE (using the maximum-likelihood estimate statistical approach; Li et al., 2014). Note that numerous analysis tools exist for CRISPR screening data, each employing different statistical approaches, and no consensus yet exists regarding best practices (Schuster et al., 2019). To use MAGeCK MLE, first use standard spreadsheet software to reorganize sample read counts into a single tab-delineated text file. In the example above, this can be done by sorting each file by sgRNA sequence (column 1) and then saving. Next, copy and paste the read-count column (column 3) from sample2.count.txt and sample3.count.txt to create new columns 4 and 5 within sample1.count.txt, and save the new file under the name “merged.count.txt”. Finally, create a so-called “design matrix file” to instruct the program to test every desired pairwise experimental combination of read-count files. For instance, the design matrix file below tests sample 1 against sample 2 and sample 1 against sample 3. The contents of this file are then saved as a new text file called “design\_matrix.txt”.

Samples	baseline	HL60	KBM7
sample1	1	0	0
sample2	1	1	0
sample3	1	0	1

Now run MAGeCK MLE as follows:

```
mageck mle --count-table merged.count.txt
--design-matrix design_matrix.txt
--permutation-round 100
```

For the example data set, a successful analysis using MAGeCK MLE to compare read-count files generated from the plasmid library versus cells outgrown for 45 days is available at [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads/results\\_mageck\\_mle/](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads/results_mageck_mle/).

## REAGENTS AND SOLUTIONS

### *Drosophila cell culture medium*

Schneider’s medium (Thermo 21720)  
1× Pen/Strep (Thermo 15070063) final  
10% fetal bovine serum (Thermo 16140071) final

To prepare the medium, add Pen/Strep, FBS, and if relevant, additional antibiotics to achieve final concentrations shown above into Schneider’s medium. Pass the medium through a 0.2-µm bottle-top filter (Nalgene 566-0020) or equivalent to sterilize. Keep refrigerated until use.

### *Hygromycin selection medium*

*Drosophila* cell culture medium (see recipe)  
200 ng/ml hygromycin B (Calbiochem 400051, 1:2,300 dilution of stock solution)

Add hygromycin B along with Pen/Strep when preparing the medium.

**Table 2** Example Pooled Screen Assays for Gene Function Discovery in *Drosophila* Cells

Cells	Assay	Outcome(s)	Example
Wild type	Treatment with a cytotoxic drug or compound	Genes whose knockout confers sensitivity Genes whose knockout confers resistance	Viswanatha et al. (2018)
Wild type	Treatment with a ligand or hormone that induces lethality	Genes whose knockout confers sensitivity Genes whose knockdown confers resistance	Okamoto et al. (2018)
Wild type	Outgrowth	Genes essential in the cell line Genes whose knockout confers a growth advantage	Viswanatha et al. (2018)
Wild type (control)	Outgrowth	Synthetic lethal genes (i.e., genes whose knockout causes lethality in mutant but not wild-type cells)	
Mutant (experimental)	Outgrowth	Genes whose knockout suppresses a growth disadvantage (i.e., growth advantage present in mutant but not wild-type cells)	
Wild type	FACS, TransWell, other method for physical separation of cell populations	Genes whose knockout results in a separable difference (e.g., fluorescence marker turned off or on, loss or gain in ability to migrate in response to a chemoattractant, etc.)	

***Puromycin selection medium***

*Drosophila* cell culture medium (see recipe)

5 µg/ml puromycin (Calbiochem 540411)

Add puromycin along with Pen/Strep when preparing the medium.

**COMMENTARY****Background Information**

This protocol presents a method for identifying genes whose knockdown confers sensitivity or resistance to a cytotoxin. CRISPR pooled-format screening is not limited to this type of assay; several other assay types are feasible and would allow the exploration of diverse gene functions (Table 2). Moreover, as noted in the introduction, this protocol is extensible to other cell lines. Notably, *Drosophila* cell lines have been derived from different stages or tissue, have different transcriptomes, and have different growth requirements (reviewed in Luhur, Klueg, and Zelhof, 2019). Thus, the same or similar assays could be applied to different cell lines to help uncover context-specific findings.

**Critical Parameters**

The following parameters are particularly critical for success: (1) optimization of the

concentration of cytotoxin (see Support Protocol 1), (2) library design (see Support Protocol 2), (3) efficient integration of the library into cells (see Basic Protocol) and (4) avoidance of bottlenecking the cell population during the screen (see Strategic Planning). Common issues with these steps are further addressed in Troubleshooting.

**Troubleshooting**

Common problems, causes, and solutions related to these procedures are presented in Table 3.

**Understanding Results**

At the end of a screen, the outcome is a set of log-fold changes for sgRNAs. But of course, the overall goal is to identify the relationship between the phenotype and specific genes or gene sets. To accomplish this, we use mean and variance modeling to identify positive hits

**Table 3** Common Problems Encountered, Potential Causes, and Proposed Solutions

Problem	Potential cause	Solution
Low integration efficiency	Low transfection efficiency	Ensure that cells are doubling approximately once per day prior to transfection.
Cell doubling rate does not consistently decrease after cytotoxin treatment	Damaged or degraded plasmid DNA	Perform fresh minipreps of pBS130 and plasmid library prior to transfection.
	Cytotoxin may be losing activity.	Use Support Protocol 1 to confirm cytotoxin effect on naïve cells periodically during cytotoxin exposure to the CRISPR library to ensure that it has not lost activity; replace as needed.
Cell lines crash during expansion	Cytotoxin treatment had too great an effect on doubling.	Lower cytotoxin level and repeat.
	Contamination occurred.	Discard and replace all cell culture reagents and repeat. For general advice about <i>Drosophila</i> cell culture, see Luhur et al. (2019).
A few sgRNAs targeting different genes dominate reads	Insufficient cell coverage of CRISPR library leading to artifactual “bottleneck” in cell population	Increase coverage of cells per passage.
	Insufficient template DNA during PCRs led to “bottleneck” in PCR products.	Increase total volume of PCRs used in PCR1 reaction.

at the gene level (see Support Protocol 3 and Viswanatha et al., 2018).

Ideally, this screening approach can be used to identify factors conferring either growth advantage (e.g., resistance to a cytotoxin) or growth disadvantage (i.e., genes essential in a particular context such as genotype or treatment with a cytotoxin). For the former, it is reasonable to expect a relatively small number of genes to be identified. These should be further tested experimentally. For the latter, which we refer to as “fitness genes” or identification of essential genes, you can expect a larger number. As a validation of screen results, the set of fitness genes can be compared to RNA sequencing results, as we reported, as a gene in this category should be expressed. Moreover, these data can be used to determine a false discovery rate, as described in Viswanatha et al. (2018). A single replicate of a genome-wide screen yields ~1000 essential genes at a false-discovery rate of 5%. For positive selection screens, the predictions made by the screens must be validated experimentally.

To assist with data interpretation, data from Viswanatha et al. (2018) have been made pub-

licly available for download and reanalysis at: [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads/](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads/)

### Time Considerations

Obtain or build sgRNA library, 0-2 weeks; thaw and revive S2R+-MT::Cas9 cells, 2 weeks; determine effective concentration of cytotoxin, 1 to 4 weeks; perform screen: 6 to 8 weeks.

The total time for a large-scale pooled format screen is ~9 to 14 weeks.

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### Conflicts of Interest

The authors declare that they have no conflicts of interest.

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### Internet Resources

- <https://www.flymai.org/crispr3/web/>.  
*sgRNA design resource for finding CRISPRs within DNA sequence.*
- [https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR\\_fitness\\_screen\\_reads](https://sharehost.hms.harvard.edu/genetics/?perrimon/CRISPR_fitness_screen_reads).  
*Screen data from Viswanatha et al. (2018).*
- <http://genome.gsc.riken.jp/osc/english/dataresource/>.  
*Tagdust software (Lassmann et al., 2009).*
- <https://sourceforge.net/p/mageck/wiki/Home/>.  
*MAGeCK software (Li et al., 2014).*