Chapter 17

Screening Strategies for TALEN-Mediated Gene Disruption

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Abstract

Targeted gene disruption has rapidly become the tool of choice for the analysis of gene and protein function in routinely cultured mammalian cells. Three main technologies capable of irreversibly disrupting gene-expression exist: zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system. The desired outcome of the use of any of these technologies is targeted insertions and/or deletions (indels) that result in either a nonsense frame shift or splicing error that disrupts protein expression. Many excellent do-it-yourself systems for TALEN construct assembly are now available at low or no cost to academic researchers. However, for new users, screening for successful gene disruption is still a hurdle. Here, we describe efficient and cost-effective strategies for the generation of gene-disrupted cell lines. Although the focus of this chapter is on the use of TALENs, these strategies can be applied to the use of all three technologies.

Key words TALEN, CRISPR, T7E1, Gene disruption, Gene editing, Indels, Screening

1 Introduction

Targeted gene disruption or genome editing has revolutionized our ability to assess protein function in mammalian cells. Unlike other technologies that modulate gene expression (e.g., RNAi), programmable nucleases result in complete and permanent loss of protein expression. Three main gene-editing technologies have emerged over recent years: zinc-finger nucleases, transcription activator-like effector nucleases (TALENs), and the CRISPR/Cas9 system [1–12]. These methodologies differ in both their mechanism of action and ease of construct assembly, but the desired result is the same: on-target double-stranded breaks (DSBs) at a desired locus, usually within the coding sequence of a gene of interest [13]. In most cell lines, DSBs are inaccurately repaired by nonhomologous end-joining (NHEJ), leading to insertions or deletions (indels) that disrupt protein expression. While it is feasible to exploit the other DSB repair mechanism, homology directed

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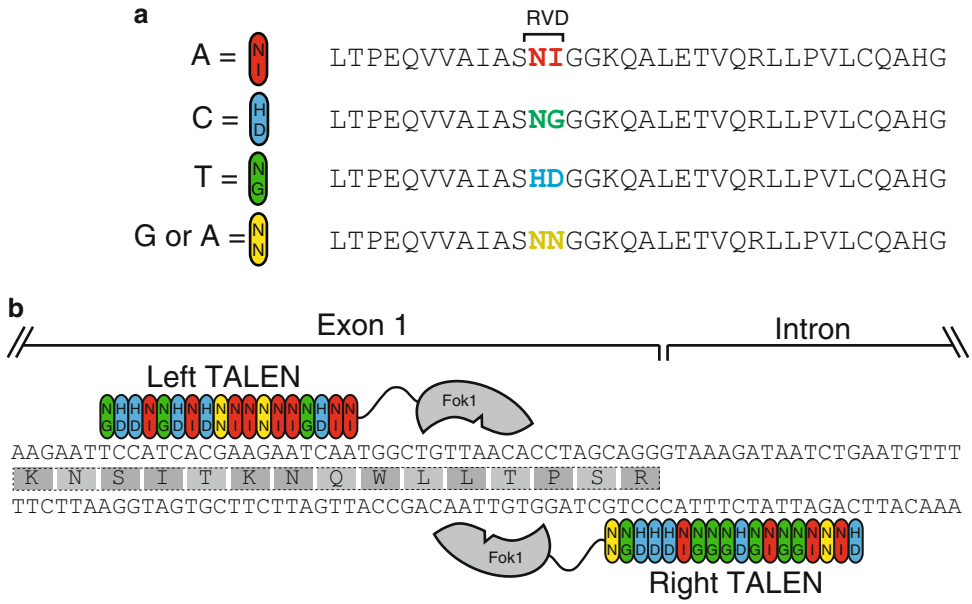

Fig. 1 (**a**) Schematic representation of a transcription activator-like effector (TALE) showing the four different repeat variable diresidues (RVDs). (**b**) Simplified illustration depicting a pair of TALENs targeting exon 1 of *GHITM*

repair (HDR) to make precise edits, this application of gene editing involves specific screening methodologies and thus will not be covered here.

TALENs consist of two domains, a DNA sequence-specific transcription activator-like effector (TALE) which is fused to the nuclease Fok1 to yield a TALEN [7–11]. TALEs can be further broken down to ~15–20 repeat variable diresidue domains (RVDs). There are four commonly used RVDs (NI, HD, NG, NN) named according to the two variable amino acids that define the domain's nucleotide specificity (Fig. 1a) [14, 15]. Fok1 is a nonspecific nuclease that is only active upon dimerization; therefore pairs of TALENs are constructed to promote dimerization over the desired DSB site (Fig. 1b). Construction of TALEN pairs is not trivial, and their efficient and cost-effective assembly is the major barrier to their use, especially when considering the low costs of entry of other systems such as CRISPR/Cas9. However, TALENs may have a specificity advantage over the CRISPR/Cas9 system due to their comparably large binding site (~60 nt vs. ~20 nt for CRISPRs; [13]) and thus may be more suitable for certain applications where low off-target activity is required. A number of companies design and assemble custom TALEN pairs or make available pre-existing TALEN pairs derived from large-scale libraries for a fee. However, most users choose to assemble TALENs in-house and several groups have developed well-documented systems for TALEN

assembly and have made these available as kits through the non-profit plasmid repository Addgene (http://www.addgene.org).

TALEN design is a critical step and in our hands, target site selection is most efficiently achieved through web-based design tools (Table 1). While these tools assist the user in obeying TALEN design rules (e.g., rules specific to the TALE:DNA interaction), there are a number of additional considerations to be made specific to the gene being targeted (Fig. 2a). To ablate expression of a protein-coding gene, a common strategy is to target the translation initiation ATG. Care must be taken, however, as translation can alternately start from subsequent in-frame ATGs within the same exon [16]. Several alternate approaches are (1) target the most 3′ in-frame ATG within the first exon, (2) target an exon–intron boundary such that mRNA splicing will be disrupted, or (3) target a motif critical for the protein's biogenesis and function (e.g., a targeting signal or transmembrane domain). We have observed loss of protein function through all of these approaches [16–20]. Another consideration is the existence of different splice variants where translation is initiated from different coding exons (Fig. 2a, lower panel). In this case, we have had success targeting the first common coding exon [17, 20] subject to the considerations discussed above.

The repair of TALEN generated DSBs is a somewhat random event, with deletions (or less commonly insertions) ranging in size from a single to hundreds of base pairs. Each allele will often receive a different indel, and this is further complicated by the high frequency of aneuploidy in cultured cell lines. Characterization of all alleles present within the cell is therefore necessary to avoid non-protein function destroying mutations such as in-frame deletions. This is typically achieved through the generation of single cell-derived clonal populations in which all indels have been characterized. Typically researchers (and reviewers) demand two to three unique clones to control for clonal differences as well as potential off-target effects. At the commencement of a project we normally generate a clonal cell line in which all gene disruptions will be made (Fig. 2b). TALEN pairs are introduced into this line by transient transfection and single cells are derived through fluorescence activated cell sorting (FACS). To ensure efficient sorting of transfected cells, a fluorescent marker is co-transfected with the TALEN constructs using a limiting amount of DNA. In our hands, co-transfection of 1/10th the amount of a green fluorescent protein (GFP)-tagged construct relative to each TALEN construct is sufficient to allow FACS sorting of a majority of cells also harboring the TALEN constructs (data not shown). Following sorting, 10–20 single cell clones are expanded over the course of several weeks to cell numbers permissive of screening. If the TALENs are functional, at least ~10–50 % of clones should contain indels.

Table 1
List of the currently known TALEN targeting and design tools

Tool	Website URL	Usage	Kit specificity	Genome-wide off-target analysis.	TALENs scored/ ranked.	Detects restriction sites for screening.	Can specify target site class (e.g. UTR, 1st exon, splice site).	Outputs primers for screening and/ or sequencing.
ZiFiT [27]	http://zifit.partners.org/ZiFiT/	ZFN/TALEN/CRISPR	REAL/REAL-Fast/FLASH	no	no	no	no[a]	no
TALE-NT [28]	https://tale-nt.cac.cornell.edu/	TALEN	Any	yes	no	yes	no	no
Mojo Hand [29]	http://www.talendesign.org/	TALEN/CRISPR	Any	no[b]	yes[c]	yes	no	no
E-TALEN [30]	http://www.e-talen.org/E-TALEN/	TALEN	Major kits only	yes	yes	yes	yes	no
SAPTA [31]	http://bao.rice.edu/	TALEN	Any	no[d]	yes	yes	no	no
CHOPCHOP [22]	https://chopchop.rc.fas.harvard.edu/	TALEN/CRISPR	Any	yes	yes	yes	yes	yes

[a]Gene sequence entered manually by user
[b]*Danio rerio* only
[c]Single search outputs and ranks both TALEN and CRISPR guides
[d]Companion site "PROGNOS" permits off-target analysis

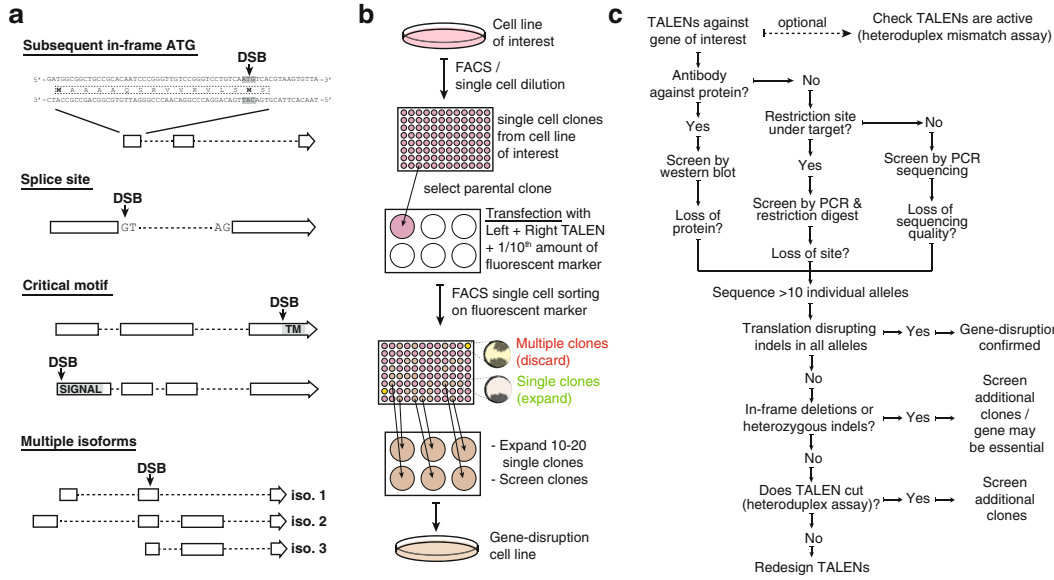

Fig. 2 (**a**) Example DSB targeting strategies for gene disruption. (**b**) Gene disruption workflow. A single-cell cloned cell line of interest is established as a parental line for gene disruption. The cell line is cotransfected with both TALEN pairs and a limiting amount (1/10th) of a cotransfection marker (e.g., cytosolic expressed GFP) to enable transfected single cells to be sorted by FACS into 96-well plates. Single cell colonies are expanded and screened for gene disruption. (**c**) Screening strategy decision tree

The choice of screening strategy largely depends on the reagents and equipment available to the researcher. Screening by Western blotting for loss of protein expression is the simplest and most robust approach, and the existence of a quality antibody usually directs our screening workflow (Fig. 2c). If an antibody is not available, we utilize one of two approaches that employ amplification of the targeted region by polymerase chain reaction (PCR): (1) mutation of a unique restriction site present at the target site, or (2) by sequencing the PCR product to detect indels. Once candidate clones are identified, sequencing of the individual alleles covering the target site in each clone is necessary to exclude non-translation disrupting as well as heterozygous indels.

2 Materials

The following protocols are intended for use with common lab cell lines cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS). We have successfully used these protocols to generate gene disruptions in HEK293T cells [16, 19, 20], HeLa cells, mouse embryonic fibroblasts (MEFs) [18] and HCT116 cells [17] (*see* **Note 1**). All reagents for cell culture should be sterilized by autoclave, or

prepared aseptically by passage through a 0.22 μm filter. Possession of basic tissue culture equipment (e.g., electronic pipette fillers, laminar flow hoods, CO_2 incubators) and consumables (e.g., pipette tips, 100 mm dishes, T75 flasks) is assumed, as is access to a staffed flow cytometry facility with 96-well plate single cell sorting capability. Reagents for screening should be prepared with autoclaved ultrapure water and reactions conducted in fresh, sterile plastic ware. For reagents commonly obtained from an international vendor, we have indicated the vendor's name to aid identification of the reagent. Substitutions can usually be made; however, the alternate vendor's product information sheets should be consulted to confirm parameters such as reagent concentration.

2.1 Maintenance of Cells in Culture, Transfection of TALENs, and Sorting of Transfected Cells

1. Sterile, phosphate buffered saline (PBS).
2. Incomplete DMEM: DMEM, high glucose (4.5 g/L glucose).
3. Transfection media: DMEM, high glucose (4.5 g/L glucose) supplemented with 10 % fetal bovine serum (FBS) (*see* **Note 2**).
4. Growth media: DMEM, high glucose (4.5 g/L glucose) supplemented with 10 % FBS and penicillin/streptomycin.
5. Freezing media: DMEM, high glucose (4.5 g/L glucose) supplemented with 20 % fetal bovine serum (FBS) and penicillin/streptomycin, 10 % Dimethyl sulfoxide (DMSO).
6. Cell sorting media: PBS supplemented with 10 % FBS and 1 mM ethylenediaminetetraacetic acid (EDTA).
7. 0.25 % Trypsin, phenol red.
8. Lipofectamine® 2000.
9. TALEN constructs: known concentration of plasmids encoding left- and right-TALEN constructs, suspended in sterile, ultrapure water.
10. Transfection marker, for example a known concentration of plasmid pEGFP-N1 (Clontech; *or see* **Note 3**) suspended in sterile, ultrapure water.
11. 70 μm cell strainer.
12. Multichannel pipette for 200 μl tips.
13. Preracked or sterilized 200 μl pipette tips.
14. 20 ml reservoir suitable for use with multichannel pipettes.
15. 96-well and 6-well tissue culture plates.

2.2 General Molecular Biology Reagents (Required for All Strategies)

1. SDS/proteinase K lysis buffer: 100 mM Tris–HCl (pH 8.8), 20 mM ammonium sulfate, 5 mM β-mercaptoethanol, 10 mM $MgCl_2$, 5 μM EDTA, 2 μM sodium dodecyl sulfate (SDS), 20 μg/ml proteinase K. Store in aliquots at –20 °C.

2. Robust proofreading DNA polymerase, e.g., Q5® High-Fidelity DNA Polymerase and programmable PCR thermocycler.
3. PCR purification kit, e.g., QIAquick PCR Purification Kit or Wizard® SV Gel or PCR Clean-Up System or equivalent.
4. Miniprep kit, e.g., QIAprep Spin Miniprep Kit or PureYield™ Plasmid Miniprep system or equivalent.
5. Sterile lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl).
6. LB Agar plates supplemented with the appropriate antibiotic.
7. Competent *E. coli* cells for transformation (*see* **Note 4**).
8. pGEM®-4Z vector (Promega) (or *see* **Note 5**).
9. Restriction enzymes as needed, with compatible 10× buffers (*see* **Note 6**).
10. Oligonucleotides as needed (desalt purified by the manufacturer).
11. Alkaline Phosphatase, Calf Intestinal (10,000 U/ml).
12. T4 DNA Ligase (2,000,000 U/ml).
13. 2× QLB (Quick Ligation Buffer): 132 mM Tris–HCl (pH 7.6), 20 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 15 % PEG 6000.
14. DNA ladder, e.g., 1 kb DNA Ladder.
15. 6× DNA loading buffer: 30 % (v/v) glycerol, 20 mM Tris–HCl (pH 8.0), 60 mM EDTA, 0.5 % (w/v) SDS, 0.02 % (w/v) Orange G, 0.02 % (w/v) Xylene cyanol FF.
16. TAE buffer: 0.1 M acetic acid, 1 mM EDTA, 40 mM Tris–HCl (pH 8.0).
17. Agarose, low electroendosmosis (EEO).
18. Generic agarose gel casting, electrophoresis, and blue-light imaging system.
19. Blue-light compatible DNA stain, e.g., 1000× SYBR® Safe or equivalent (*see* **Note 7**).

2.3 Heteroduplex Mismatch Assay

1. T7 Endonuclease I (New England Biolabs), Guide-it™ Resolvase (Clontech), or CELII (sold as Surveyor™ Nuclease S by Transgenomic). Selection of appropriate reagent is discussed in Subheading 3.2.

2.4 SDS-PAGE and Western Blotting

1. Precast or self-made (*see* **Note 8**) SDS-PAGE and Western blotting system selected based on the requirements of your protein of interest.
2. 4× SDS sample buffer: 200 mM Tris–HCl (pH 6.8), 8 % (w/v) sodium dodecyl sulfate (SDS), 40 % (v/v) glycerol, 0.04 % (w/v) bromophenol blue. Can be stored in aliquots at –20 °C or at room temperature (*see* **Note 9**).

3. 1 M dithiothreitol (DTT). Stored in single-use aliquots at −20 °C.
4. Enhanced Chemiluminescence (ECL) reagent and compatible imaging system.

3 Methods

3.1 Primer Design and Basic PCR Amplification of the Target Site

All screening strategies converge with the sequencing of individual alleles (*see* Fig. 2c) therefore it is imperative that early in the project, oligonucleotide primers are designed that permit efficient amplification of the target region from genomic DNA. As this step requires cloning of the amplified products, we find it convenient to include restriction site overhangs—in our hands this does not affect the PCR efficiency or the PCR product's function in the other screening assays. Primers should be designed to yield a PCR product of ~500 bp, centered on the target site, with a T_m for the complementary region of 60–65 °C. We typically use the web-based "Primer-BLAST" tool [21] to search for specific primer pairs that yield no or few off-target products. The excellent TALEN design tool "CHOPCHOP" [22] is also capable of designing specific primers centered on the chosen target site. Restriction sites should be added to the primers such that they are unique (i.e., not present in the insert) and compatible with the vector chosen for downstream analysis. Overhang sequences should not impact on the primer T_m calculations. For synthesis, only desalt purification is required.

1. Harvest approximately 1×10^6 cells (equivalent to a confluent well of a 6-well dish) of the cell line of interest and transfer to a sterile 1.5 ml microcentrifuge tube. Centrifuge for 3 min at 300 × *g*.
2. Wash cells with PBS and centrifuge for 3 min at 300 × *g*.
3. Aspirate PBS and solubilize cells in 100 µl of SDS/proteinase K lysis buffer. Incubate for 1 h at 37 °C, followed by 10 min at 80 °C.

   Optional: Sonicate crude extract in a ultrasonic water bath for 5 min to reduce viscosity.
4. Centrifuge crude extract for 5 min at 16,000 × *g* and transfer 90 µl of the supernatant to a new tube being careful not to disturb the pellet.
5. The amount of crude DNA extract to use in a PCR reaction needs to be titrated. Assemble the PCR reactions (*see* **Note 10**) and cycle as described in Table 2.
6. Cast a 2 % (w/v) agarose gel in TAE, supplemented with 1× SYBR Safe DNA stain.

Table 2
Protocol for generic PCR amplification using crude genomic DNA extract as template

	Increasing amounts of crude genomic DNA extract (μl)					Cycle parameters		
Reagent	**1**	**2**	**3**	**4**	**5**	**Step**	**Temperature (°C)**	**Time**
5× reaction buffer	5	5	5	5	5	Denaturation	98	30 s
10 mM dNTPs[a]	0.5	0.5	0.5	0.5	0.5	35 cycles	98	10 s
10 μM Forward primer	1.25	1.25	1.25	1.25	1.25		55[b]	30 s
10 μM Reverse primer	1.25	1.25	1.25	1.25	1.25		72	30 s[c]
Crude genomic DNA extract[d]	0.25	0.5	1	1.5	2.5	Final extension	72	2 min
High-fidelity DNA polymerase	0.25	0.25	0.25	0.25	0.25	Hold	4	–
Nuclease-free water	16.5	16.25	15.75	15.25	14.25			

[a]Consisting of 2.5 mM each dNTP
[b]We find 55 °C to strike a good balance between specificity and efficiency for most amplicons
[c]Increase if product is >1000 bp.
[d]If excessively viscous, perform optional sonication step. Use of high amounts of crude DNA extract can inhibit PCR performance due to detergent present in the crude extract buffer

7. Combine 5 μl of each PCR reaction with 1 μl 6× DNA loading dye. Load onto agarose gel along with a suitable DNA ladder and perform electrophoresis at 150 V for ~15–30 min. Visualize using a blue-light transilluminator, or image using appropriate equipment.

3.2 Heteroduplex Mismatch Assay

The heteroduplex mismatch assay is a simple and time saving means to assess activity of TALEN pairs following their cotransfection but prior to single cell cloning and screening. Crude genomic DNA is extracted from cells, and the target region amplified by high-fidelity PCR. The PCR product, which will consist of a mixture of wild-type alleles from untransfected cells and mutant alleles from gene-disrupted cells, is melted and rehybridized generating mismatched heteroduplex DNA—mismatches resulting from as little as a single missing or mutated base are detected by the addition of a T7E1 resolvase or S1 endonuclease, both of which cleave mismatched DNA. The ratio between cleaved and uncleaved products can be compared by DNA gel electrophoresis (Fig. 3). There is no difference in experimental output between T7E1 resolvases and S1 endonucleases, although the former has been reported as being

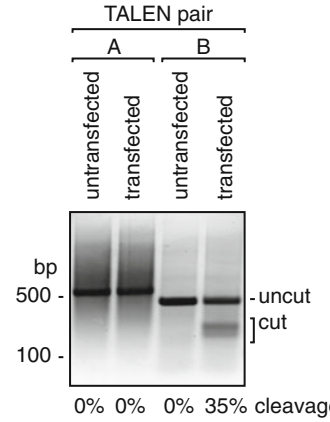

Fig. 3 Example results from a heteroduplex mismatch assay using nonfunctional (*A*) and functional (*B*) TALEN pairs. *Lanes 1* and *2* show the results following transfection of a nonfunctional TALEN pair. *Lanes 3* and *4* show the results from a functional TALEN pair with a cutting activity of 35 %, calculated by comparing the density of the cut bands with the density of both uncut and cut bands within a particular lane

more sensitive to low efficiency cutting, and the latter better for detecting single base changes [23]. In our hands, the Guide-it™ Resolvase (Clontech) has given the most robust results.

1. For each TALEN pair being tested, seed two wells of cells so as to achieve 70–90 % confluency on the day of transfection. Only one well will be transfected and the other will serve as an untransfected control. For HEK293T cells, we typically seed 500,000 cells per well of a 6-well dish the day prior to transfection.
2. In a sterile 1.5 ml microcentrifuge tube, combine 1.5 μg each TALEN construct and dilute with 500 μl incomplete DMEM (*see* **Note 11**).
3. Add 9 μl Lipofectamine® 2000, briefly vortex and incubate at room temperature for 5 min.
4. Aspirate media from one of the wells, carefully wash with PBS and overlay cells with 1.5 ml transfection media. Carefully add the transfection solution by pipette. Incubate cells overnight at 37 °C under 5 % CO_2.
5. Wash cells with PBS, add 500 μl 0.25 % trypsin and incubate for several minutes at 37 °C.
6. Detach cells with 500 μl growth media and transfer to sterile 1.5 ml microcentrifuge tube. Centrifuge for 3 min at 300 × *g*.
7. Prepare crude genomic DNA extract as described in Subheading 3.1.

8. Using the optimum amount of crude genomic DNA extract determined in Subheading 3.1, amplify the targeted region from both untransfected and transfected samples. Analyze 10 % of the reaction on a 2 % agarose gel. A clean, single PCR product is expected with an intensity of 1:1 with DNA ladder loaded such that each band represents 100 ng DNA (*see* **Note 12**).
9. In most cases, the PCR product can be used directly in the heteroduplex mismatch assay. Check the buffer composition used in the DNA polymerase buffer; the heteroduplex mismatch assay requires ≥1.5 mM $MgCl_2$ and ~50 mM salt (NaCl/KCl) at final concentration. If the buffer is incompatible or cannot be supplemented with additional $MgCl_2$, purify the PCR product with a commercial PCR purification kit. Elute the product in 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, and 50 mM KCl aiming for a concentration of ~25–50 ng/μl.
10. Transfer 10 μl of the PCR product to a new tube. Perform a melt-hybridization in a programmable thermocycler (*see* **Note 13**) as follows: (1) 95 °C, 5 min, (2) ramp 95–85 °C at 2 °C/s, (3) ramp 85–25 °C at 0.1 °C/s, hold at 4 °C.
11. Add 1 μl of T7E1 (10 U), 1 μl Guide-it™ Resolvase, or 1 μl each Surveyor® Nuclease S and Enhancer S (*see* **Note 14**).
12. If using T7E1 or Guide-it™ Resolvase, incubate samples for 15 min at 37 °C. For Surveyor® incubate at 42 °C for 60 min.
13. Add 2 μl 6× DNA loading dye and run the entire reaction on a 2 % agarose gel.
14. Cutting efficiency can be quantified with appropriate software by comparing the density of the cut band(s) with the density of both uncut and cut band(s) within a particular lane.

3.3 Transfection of TALENs and Sorting of Transfected Cells

1. Parental cells should be seeded so as to achieve 70–90 % confluency at the time of transfection. For HEK293T cells, we typically seed 500,000 cells per well of a 6-well dish 24 h prior to transfection.
2. In a sterile 1.5 ml microcentrifuge tube, combine 1.5 μg each TALEN construct with 150 μg pEGFP-N1 (*see* **Note 15**) and dilute with 500 μl incomplete DMEM.
3. Add 1.5 μl Lipofectamine 2000 (*see* **Note 16**), briefly vortex and incubate at room temperature for 5 min.
4. Aspirate media from the cells to be transfected, carefully wash with PBS and overlay cells with 1.5 ml transfection media (*see* **Note 17**). Carefully add the transfection solution by pipette. Incubate cells overnight at 37 °C under 5 % CO_2.

5. Prepare 96-well plates for cell sorting by adding 200 μl growth media into each well using a multichannel pipette and buffer reservoir.
6. Aspirate transfection media and wash cells with PBS. Trypsinize using 200 μl 0.25 % trypsin for 5 min at 37 °C. Block trypsin using 2 ml cell sorting media and transfer cells through a 70 μm cell strainer to a tube suitable for FACS analysis.
7. Sort single cells into wells of the pre-prepared 96-well plate based on a medium level GFP fluorescence (*see* **Note 18**). Typically we sort a single plate based on recovery of ~20–50 single cell populations/plate, with a view to expanding and screening 12–24 clones. Recovery is highly dependent on both the cell type and proper use and calibration of the FACS, therefore additional 96-well plates may need to be sorted should recovery be less than expected.

3.4 Expansion of Candidate Gene Disruption Clones

1. Plates should be examined after 3–5 days for evidence of single cell populations. Mark wells containing single cell populations and exclude those containing more than one population—these should occur infrequently at the rate of 1–2 per 96-well plate.

   Optional: 7 days after sorting, change media using a multichannel pipette and two buffer reservoirs, one for waste and one for new media. Take care not to cross-contaminate clonal populations whilst using multichannel pipettes. Presterilized filter pipette tips are recommended.
2. Depending on the cell type, clonal populations will be clumped and overgrown 10–15 days post sorting. Mark 12–24 clonal populations for further screening (*see* **Note 19**).
3. Aspirate media from marked wells, and using single channel pipettes carefully wash with PBS. Add 20 μl of 0.25 % Trypsin and incubate for several minutes at 37 °C.
4. Detach cells with 180 μl of culture media. Using a pipette set to 100 μl, move half the cells from each well to a new well in a new 96-well plate prepared with 100 μl media per well. Supplement the original well with 100 μl media for freezing in Subheading 3.5. If performing SDS-PAGE and western blotting analysis in Subheading 3.6, a third plate should be assembled and the culture split to accommodate this. This is an opportune time to update well numbering for ease of culturing and to conserve reagents (as only one or two rows of the 96-well plate are required per gene disruption). Since the cells in the original plate will be subjected to cryo-storage, care should be taken to update the new numbering on this plate so a specific clone can later be revived.

5. Continue culturing clonal populations on the new plate until confluent. Repeat **step 3**, and using 180 µl media move the cells to a marked well of a 6-well plate. Supplement with 1.8 ml of growth media.
6. The clones growing in 6-well plates are used in the following sections, however, may be expanded further for additional analyses and/or freezing as per the needs of the researcher.

3.5 Freezing Candidate Clones in a 96-Well Plate

1. The original 96-well plate can be frozen as a backup, or for later analysis by carefully aspirating the media using a multichannel pipette.
2. Using the multichannel pipette, wash cells with PBS and add 20 µl 0.25 % trypsin. Incubate for several minutes at 37 °C.
3. Resuspend the cells in 180 µl freezing media.
4. Carefully (as the plate is not sealed) wrap the plate in paper towel and store at −80 °C (*see* **Note 20**).

To thaw cells: Swab with 70 % ethanol and place in a 37 °C incubator for 20 min. In a swinging centrifuge with microtiter plate adaptor, centrifuge for 5 min at 500×*g*. Slowly aspirate 180 µl using a multichannel pipette and replace with 180 µl fresh culture media.

3.6 Screening by SDS-PAGE and Western Blot

The duplicate 96-well plate from Subheading 3.4 should reach 80–90 % confluency in about 3–7 days. Cells will be directly solubilized in the plate and analyzed by SDS-PAGE without normalization for protein concentration. While most populations will have similar cell numbers, there will no doubt be outliers. Despite such variability, we find this approach rapid and accurate enough for routine screening provided a loading control (e.g., actin, or Hsp70, *see* Fig. 4a) is used. *Aseptic technique is not required for the following steps.*

1. Note down on each well the approximate cell confluence as a factor of 100 (100 being the average density).
2. Aspirate media and wash cells by carefully adding 200 µl PBS to the side of each well, rocking the plate by hand and aspirating with the same tip.
3. Solubilize cells in 30 µl 1× SDS-PAGE sample buffer supplemented with 50 mM DTT by slowly pipetting up and down. *Optional:* Heat plate to 95 °C for 5 min with gentle shaking. Allow plate to cool down and centrifuge briefly.
4. Prepare a suitable SDS-PAGE gel (*see* **Note 8**) for electrophoresis and load 15 µl of each sample with a confluence factor of 100 as determined in **step 1**. Using this factor, load appropriate amounts of the remaining samples to a minimum of 5 µl and a maximum of 30 µl.

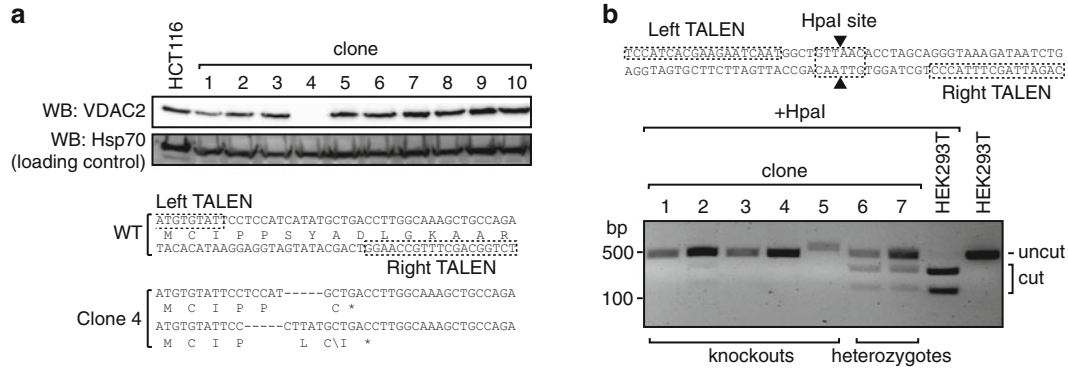

Fig. 4 (**a**) Example results of screening by SDS-PAGE and western blot. *Upper panel*, protein extracts from control (HCT116 parental line) and candidate clonal populations analyzed by SDS-PAGE and western blotting with antibodies against human VDAC2 and loading control Hsp70. *Lower panel*, targeted alleles present in clone 4 (*see* Subheading 3.9) (**b**) Example results following screening by loss of restriction sites. *Upper panel*, targeting strategy for gene disruption of the *GHITM* gene, showing targeting centered over the HpaI restriction site. *Lower panel*, the targeted region was amplified from control HEK293T cells and indicated clonal populations, and digested with the restriction enzyme HpaI. Gene disruption is evident from the loss of digested PCR products. Clones 6 and 7 are heterozygous for loss of the restriction site

5. Perform electrophoresis and western blotting as described by the manufacturer/literature for the gel and western blotting system being used.

3.7 Screening by Restriction Digestion

Screening by loss of restriction site is an effective means for identifying clones having undergone gene disruption. This strategy must be considered during the design process such that a unique (within the PCR of the target region) restriction site is located within the target site. In this assay, wild-type alleles (or those present in negative clones) will be cut by the restriction enzyme, however, alleles having undergone gene disruption will not be digested due to mutations in the restriction site (*see* Fig. 4b). This assay has the advantage of being the only strategy capable of revealing true heterozygotes DSB events (e.g., the TALENs only having generated DSBs on one allele) early in the screening process (Fig. 4b, compare clones 6 and 7 with clones 1–5).

1. Generate crude genomic DNA extracts (*see* Subheading 3.1) of clonal populations in the 6-well plates seeded in Subheading 3.4.
2. Using the optimum amount of crude genomic DNA extract determined in Subheading 3.1, amplify the targeted region from both untransfected and transfected samples. Analyze 10 % of the reaction on a 2 % agarose gel.
3. Purify the PCR product with a generic PCR purification kit, eluting in 22.5 μl nuclease free water.

4. To each sample, add 2.5 μl appropriate 10× restriction enzyme buffer and 0.2 μl (*see* **Note 21**) of the required restriction enzyme.
5. Incubate samples for 1 h at 37 °C or as is appropriate for the restriction enzyme being used.
6. Add 5 μl of 6× DNA loading buffer to each sample and resolve on a 2 % (w/v) agarose gel in TAE, supplemented with 1× SYBR Safe DNA stain. Visualize using a blue-light transilluminator, or image using appropriate equipment.

3.8 Sequencing the PCR Product as a Screening Strategy

In the absence of other available screening methodologies, we have been able to identify candidate clones with gene disruptions by direct sequencing of the PCR products amplified from individual clonal populations. This method relies on DSB repair rarely resulting in identical indels on all alleles (Fig. 5a). The mixed population of PCR products is sequenced by standard Sanger sequencing primed with one of the oligonucleotides used in its original amplification—the sequencing read will begin as normal but due to the mixture of alleles with different indels, the sequencing quality will deteriorate at the site of DSBs (*see* Fig. 5b, upper panel). Depending on the local costs associated with sequencing, this can be a reliable and cost effective means to isolate positive clones for further analysis.

1. Generate crude genomic DNA extracts (*see* Subheading 3.1) of clonal populations in the 6-well plates seeded in Subheading 3.4.
2. Using the optimum amount of crude genomic DNA extract determined in Subheading 3.1, amplify the targeted region

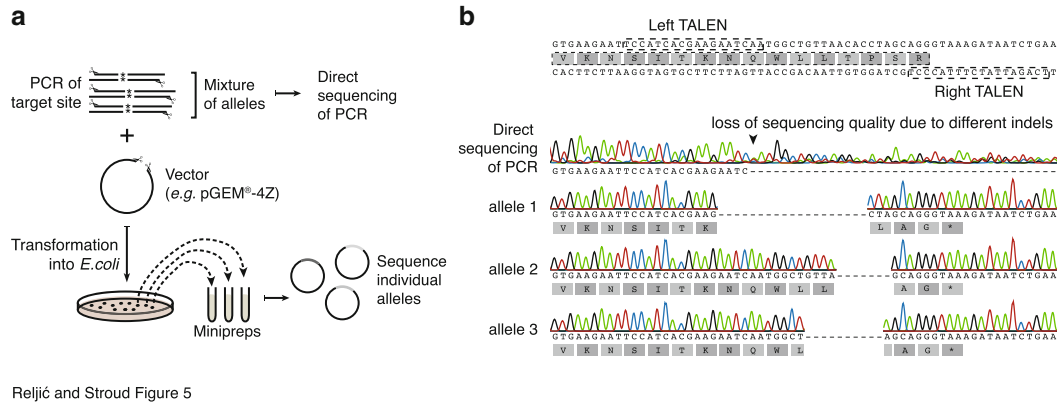

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Fig. 5 Screening by sequencing. (**a**) Schematic representation of the screening strategy. (**b**) *Upper sequencing chromatogram,* example result following direct sequencing of a mixed PCR product amplified from a single clone having undergone the above targeting strategy. *Lower chromatograms*, example results from sequencing individual alleles as described in (**a**). A total of 12 bacterial clones were sequenced, resulting in three unique indels

from both untransfected and transfected samples. Analyze 10 % of the reaction on a 2 % agarose gel.

3. Purify the PCR product with a generic PCR purification kit, eluting in a minimum volume of nuclease free water. Calculate the DNA concentration using its absorbance at 260 nm.
4. Using the forward primer designed in Subheading 3.1, assemble the sequencing reaction as per the requirements for sequencing a PCR product set by the local sequencing provider. A 10 μl reaction generally consists of 10–20 ng of DNA and 10 ρmol primer diluted in nuclease free water.

3.9 Sequencing of Individual Alleles

The final step regardless of initial screening strategy is sequencing of the individual alleles present in a number of short-listed clones. The step is absolutely essential, as no other technique can fully exclude nontranslation disrupting indels such as in-frame deletions. The mixed population of PCR products representing all alleles (*see* **Note 22**) in a given clone is purified and the digested restriction sites introduced as overhangs to the primers (*see* Subheading 3.1) are used to ligate the mixture into a standard bacterial cloning vector. Individual alleles are isolated through transformation into competent *E. coli* and culturing of individual bacterial clones, each colony representing a unique allele. Plasmid DNA is isolated and the isolated alleles are sequenced using standard Sanger sequencing (*see* Fig. 5a). Given that cells in culture are typically diploid or triploid at a certain locus, the inevitable problem is deciding how many bacterial clones (and therefore representative alleles) to sequence. Our typical workflow involves the initial sequencing of eight clones. Sequences are aligned to the reference sequence (*see* Fig. 5b, lower panels) and duplicate reads are discarded. For example, when working with HEK293T cells we are usually satisfied upon detection of two or three unique alleles in a given clone.

1. Generate crude genomic DNA extracts (*see* Subheading 3.1) of clonal populations in the 6-well plates seeded in Subheading 3.4.
2. Using the optimum amount of crude genomic DNA extract determined in Subheading 3.1, amplify the targeted region from both untransfected and transfected samples. Analyze 10 % of the reaction on a 2 % agarose gel.
3. Purify the PCR product with a generic PCR purification kit, eluting in 25 μl of nuclease free water.
4. Assemble the following restriction enzyme digestions (*see* **Note 23**):

Clone specific PCR product(s)	Plasmid
21.5 µl purified PCR product	1 µg pGEM®-4Z or appropriate cloning vector
2.5 µl 10× buffer compatible with enzyme A and B	2.5 µl 10× buffer compatible with enzyme A and B
0.5 µl enzyme A	0.5 µl enzyme A
0.5 µl enzyme B	0.5 µl enzyme B
	Nuclease free water to 25 µl

5. Incubate the reactions for 2 h at 37 °C.
6. To the plasmid digest only, add 10 U (1 µl of 10,000 U/ml) alkaline phosphatase. Incubate both digests for an additional 30 min at 37 °C.
7. Purify digests with a generic PCR purification kit, eluting in 25 µl of nuclease free water.
8. For each clone, assemble the following ligation reaction (*see* **Note 24**):

Ligation/clone
2 µl digested/alkaline phosphatase treated plasmid
2 µl digested PCR product
1 µl T4 DNA ligase
5 µl 2× QLB buffer

9. Incubate ligations for 15 min at 37 °C.
10. In a sterile 1.5 ml microcentrifuge tube, combine 5 µl each ligation with 50 µl chemically competent *E. coli* (*see* **Note 25**) and incubate on ice for 10 min.
11. Heat shock at 42 °C for 90 s.
12. Immediately return transformation to ice and incubate for 2 min.
13. Add 500 µl LB and incubate for 60 min at 37 °C with shaking.
14. Plate ½ the suspension on LB Agar plates supplemented with the appropriate antibiotic for the cloning vector in use.
15. Incubate inverted plates at 37 °C overnight.
16. Using a sterile pipette tip, the following morning transfer eight colonies to pre-assembled 10 ml culture vials containing 5 ml LB supplemented with the appropriate antibiotic.
17. Incubate overnight at 37 °C with shaking.

18. Prepare miniprep scale plasmid DNA isolations using a generic miniprep kit. Calculate the DNA concentration using its absorbance at 260 nm.
19. Using the forward primer designed in Subheading 3.1, assemble sequencing reactions as per the requirements for sequencing a plasmid based template set by the local sequencing provider. A 10 μl reaction generally consists of 1 μg of DNA and 10 ρmol primer diluted in nuclease free water.

4 Notes

1. While we have successfully made gene disruptions in HCT116 cells cultured in DMEM supplemented with 10 % FBS, optimal growth is achieved using McCoy's 5A (Modified) Medium (Life Technologies) supplemented with 10 % FBS and 1 mM sodium pyruvate.
2. Note omission of penicillin/streptomycin. As we have observed toxicity following high levels of TALEN expression, we avoid using commercial transfection enhancers such as Opti-MEM (Life Technologies) and directly replicating manufacturer's protocols as these have been optimized for high levels of expression. Cells used in the T7E1 assay will not be expanded therefore in this case the optimized protocols may be applied.
3. Can be replaced with any suitable marker for transfection according to the needs of the researcher and capabilities of the FACS; e.g., should cells already express a GFP tagged protein, another fluorophore should be utilized. Transient expression of the fluorophore should be nontoxic and should not overtly alter cellular function, yet should be bright enough for detection by FACS. Many empty vectors designed for C-terminal expression of GFP produce cytosolic localized protein driven by the strong CMV promoter, and these are highly suitable as transfection markers. Some TALEN constructs also express their own fluorophores in which case a transfection marker can be omitted.
4. These can be obtained commercially, however, as high transformation efficiency is not particularly important, competent cells may be made in-house using published protocols. We suggest using the excellent "one-step" method for generation of competent *E. coli* published by Chung et al. [24].
5. Can be replaced by any plasmid suitable for routine cloning using chemically competent *E. coli*.
6. Chosen as per the researcher's screening strategy and obtained from the preferred supplier.

7. May be substituted with ethidium bromide at 1 μg/ml final concentration (we typically use a 1000× working stock of 1 mg/ml). Note, ethidium bromide requires an ultraviolet transilluminator for visualization unlike other stains, which can be used with blue-light systems.
8. May be substituted with a self-made system. We commonly use the Tricine-SDS-PAGE and semidry electroblotting system developed by Hermann Schägger for which several detailed methods publications exist [25].
9. Alternatively, 200 mM DTT can be added to the 4× SDS sample buffer and aliquots for single-use stored at −20 °C.
10. Although in principle any high-fidelity DNA polymerase may be used, we have observed large differences in the quality and quantity of PCR product depending on polymerase. If feasible, multiple DNA polymerase systems should be tested to achieve optimal purity and amplification. For most downstream applications, a single strong band of the correct size will be required.
11. Cells will not be expanded and thus transfection toxicity will not be a problem, so reagents and protocols may be substituted with manufacturer recommended enhancers such as Opti-MEM® (Life Technologies) and more powerful transfection reagents such as Lipofectamine® LTX with PLUS™ Reagent (Life Technologies).
12. If a single PCR product cannot be obtained, gel purification of the correct band is possible. Elute the DNA in a buffer containing 10 mM Tris–HCl (pH 8.8), 1.5 mM $MgCl_2$, and 50 mM KCl at a concentration of ~25–50 ng/μl.
13. In the absence of a ramp function, melt-hybridization can be performed by incubation of the sample in a 95 °C water bath for 5 min, following which the water bath is turned off and allowed to cool to room temperature. Samples should be placed at 4 °C until use.
14. For the Surveyor assay, the manufacturer recommends to add additional $MgCl_2$ to a final concentration of 15 mM.
15. In order to sort only cells that have been transfected with the TALEN constructs, we co-transfect with a limiting amount (typically 1/10th each TALEN plasmid concentration) of pEGFP-N1. As discussed above, this may be replaced with any suitable fluorescent marker for cotransfection. Our experience is that the majority of cells with high level of GFP fluorescence also carry both TALEN plasmids.
16. We have optimized our transfection protocol to achieve low toxicity but good level of TALEN activity using the JDSx series of plasmids in HEK293T cells as described by Reyon et al. [26]. The amount of transfection reagent may need to be opti-

mized depending on the TALEN constructs used, as well as the parental cell types being studied.

17. Normal culture media lacking penicillin/streptomycin. We have found that omission of antibiotics results in higher numbers of single cell populations recovering after cell sorting.
18. We have found that gating on the highest level of fluorescence results in low numbers of single cell populations recovering after cell sorting. Best results are achieved by gating on average levels of fluorescence and excluding the highest quartile from single cell deposition.
19. While it is tempting to select slow or fast growing populations based on predicted gene disruption phenotypes, in our experience clonal populations with average growth rates tend to yield gene disruptions. This being the case, there are many examples where gene disruption has indeed impaired growth so our advice is to randomly select 12–24 clonal populations for an initial screen.
20. For a longer term solution, plates can be sealed using adhesive foil and stored inside a cryobox in liquid nitrogen vapor phase.
21. This amount of restriction enzyme is based on following assumptions, the enzyme is concentrated at 10 U/μl, and the PCR reaction yielded ~1 μg of DNA.
22. Although most lab cell lines are polyploid, we have found most loci in HEK293T and HeLa cell lines to be either diploid or triploid. We have observed up to five unique alleles in transformed MEFs, whilst the human colorectal cancer cell line HCT116 has been reliably diploid as reported.
23. Depending on the restriction enzymes chosen in Subheading 3.1, buffer compatibility may decree that two separate digests must be performed. If this is the case, following the initial digest using enzyme A purify the DNA with a generic PCR purification kit, eluting in 25 μl of nuclease free water, and repeat the reaction with enzyme B buffer conditions.
24. The insert:plasmid ratios indicated here have been optimized based on the average yield of a 25 μl PCR (25–50 ng/μl prior to purification), and both PCR and plasmid not having been gel extracted. Typically we ligate 150 fmol insert:50 fmol alkaline phosphatase treated plasmid, and achieve >80 % efficiency without using screening techniques such as colony PCR. Optimization may be required depending on experience and quality of reagents in use.
25. If using commercially prepared cells, refer to manufacturers for specific instructions regarding transformation.

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