

Chapter 2

Identification of Genes Required for Damage Survival Using a Cell-Based RNAi Screen Against the *Drosophila* Genome

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Abstract

Exposure to DNA-damaging agents invokes biological responses necessary for damage recovery and cell survival. Despite the presence of intact DNA repair pathways, lack of certain other biological pathways has been shown to sensitize cells to DNA-damaging agents' exposure. It is likely that following DNA damage a complex interplay between DNA repair pathways and other biological pathways might be required to ensure cell survival. In this chapter, we describe a high-throughput method for the identification of genes essential for cell survival following DNA damage by using a cell-based assay to measure viability in combination with an RNA interference-based genome-wide screening experiment.

Key words: DNA damage, RNAi, Genome-wide screening, *Drosophila*, Survival

1. Introduction

Although DNA is arguably the most biologically relevant target of exogenous DNA-damaging agents, other intracellular components such as RNA, protein, and lipids can also be chemically modified. As such, it is not surprising that a DNA-damaging agent invokes a pleiotropic biological response that is not just limited to DNA repair and damage response pathways. Similarly, defects in DNA repair pathways result in sensitivity to DNA damage, though decreased viability in response to damage exposure can also be observed with loss of certain biological pathways that have no apparent role in DNA repair. It is possible that such genes and pathways could play an ancillary role in the process of response to DNA damage and DNA repair. Understanding the overall coordinated response to damage exposure not only extends our existing

knowledge about DNA repair and damage survival, but also provides novel mechanisms that can be exploited in disease management, such as cancer treatment. Although microarray and proteomics are powerful exploratory tools for the analysis of genome-wide responses to DNA damage, understanding the role of such responses in terms of cell survival requires a cell-based assessment combined with loss of gene function, a reverse genetics approach. Since the discovery of RNA interference (RNAi) as a mechanism to specifically silence any gene's expression, this powerful technology has been utilized to determine loss of function phenotypes on a genomic scale. In conjunction with DNA damage, RNAi screening allows the rapid identification of genes that are essential for DNA damage survival. Although RNAi-based screens are performed with mammalian cells, such screening requires purchasing a genomic library of small interfering RNA (siRNA), which is relatively expensive. Frequently these libraries consist of combinations of four siRNAs for each target mRNA, any one of which may not be effective for the target sequence. In addition, further costs for mammalian screening result from the mode of siRNA introduction, usually lipid based, and this is often toxic to the cells itself. In contrast, RNAi with *Drosophila* cells is relatively inexpensive as these cells readily uptake long double-stranded RNA (dsRNA) from the medium by phagocytosis, a nontoxic procedure, followed by processing the dsRNA into siRNA internally within the cell effectively producing a large population pool of siRNA. This large population pool of siRNA induces a robust knockdown of the target mRNA. Since the implementation of this technology with *Drosophila* cells, there have been numerous investigations that resulted in finding novel disease mechanisms and it has facilitated the discovery of novel genes and biological pathways in various processes (1). We have utilized this technology to identify genes that are essential for cell survival following alkylation DNA damage induced by methyl methane sulfonate (MMS) and uncovered genes and pathways which were previously unknown to play a role in DNA damage response (2). *Drosophila*-based RNAi screening is a powerful tool for cell biologists aspiring to understand the complex biological response to DNA damage or any exogenous stimuli. However, it is important to consider that different types of DNA damage at different concentrations can alter cellular responses. Therefore, to obtain comprehensive knowledge about complex biology of cellular response to DNA damage, it might be necessary to conduct investigations with a variety of different DNA-damaging agents and at different concentrations using the methods described in this chapter.

2. Materials

2.1. High-Throughput Synthesis of dsRNA Library

1. Total RNA from whole flies or *Drosophila* cell line such as *Kc*₁₆₇ or S2.
2. Trizol.
3. *Reverse transcription*: Impromp-II Reverse Transcriptase system (Promega, WI).
4. *cDNA synthesis*:
 - (a) *Primers*: For synthesis and amplification of gene-specific cDNA in a high-throughput (HTP) scale, PCR primers and PCR reactions should be assembled in 96-well plates. To make use of the amplified cDNA for the synthesis of dsRNA, each gene-specific PCR primer (both forward and reverse) should include a T7 overhang at the 5' end (taatacgaactcactataggaga-5'-primer sequence-3') (see Note 1). Working stock of primers are prepared by combining forward and reverse primers in the same well and diluting the primers 1:5 with PCR grade water.
 - (b) PCR master mix (cDNA, dNTPs, MgCl₂, PCR buffer, Taq polymerase (ROCHE, IN), and nuclease-free water).
5. Thermal cycler.
6. Agarose gel (1 % in Tris–borate–EDTA (TBE) buffer).
7. TBE buffer.
8. Biorad horizontal gel electrophoresis tank and power supply.
9. Ethidium bromide (see Note 2).
10. UV transilluminator.
11. Sterile blades.
12. Deep well, 96-well plates.
13. Gel isolation kit (Qiagen or Invitrogen, CA).
14. Multiscreen HTS PCR purification plate (Millipore, MA).
15. Millipore vacuum manifold.
16. In vitro transcription kit (T7 Ribomax Express, Promega, WI).
17. Nuclease-free water.
18. 10 mM Tris–HCl, pH 7.5.
19. UV spectrophotometer (Spectramax M2, Molecular Devices, CA, multi-detection microplate reader).
20. 96-Well, UV transparent microplate (Corning).

**2.2. HTP Transfection
of dsRNA in
Drosophila Cells**

1. *Drosophila* cell line: *Kc₁₆₇*
2. Schneider's insect cell medium, serum free.
3. Schneider's insect cell medium, 10 % serum.
4. Schneider's insect cell medium, 15 % serum.
5. Fetal bovine serum (FBS) heat inactivated (55 °C, 10 min).
6. Antibiotics/antimycotics.
7. 384-Well plate, solid white (Costar).
8. Liquid handling system for aliquoting dsRNA (Precision XS Microplate sample processor (BioTek, Winooski, VT)).
9. Liquid dispensing system (Wellmate, Matrix Technologies/ Thermo Fisher Scientific, NH or Micro 10x, Hudson Robotics).
10. 70 % Ethanol.
11. PBS.
12. Counting chamber.
13. Centrifuge (Eppendorf 5810R).
14. Humid chamber.
15. *Drosophila* incubator, set at 25 °C.
16. Sterile 50 mL conical centrifuge tubes.
17. T75 tissue culture flask.
18. Tissue culture hood.

**2.3. Induction of DNA
Damage**

1. Damaging agent, such as MMS, Bleomycin, UV, Cisplatin, etc.
2. Sterile 2- or 16-well aspirating wand (V & P Scientific, CA).
3. Experimental plate.
4. Liquid dispensing system.
5. Centrifuge.
6. Humid chamber.
7. *Drosophila* incubator, set at 25 °C.
8. Tissue culture hood.

**2.4. Assessment
of Cell Viability**

1. Sterile 24- or 16-well aspirating wand.
2. Celltiter glo (Promega, WI).
3. 1× Phosphate-buffered saline (PBS).
4. Liquid dispensing system.
5. Experimental plate.
6. Centrifuge.
7. Plate reader (Spectramax M2, Molecular Devices, CA, multi-detection microplate reader).

3. Methods

3.1. HTP Synthesis of dsRNA Library

Drosophila-based genome-wide RNAi screen for DNA damage is performed using a library of long dsRNAs that are each approximately of 500 bp in length. The dsRNA for genomic screens is usually synthesized in an HTP scale by manipulating reactions in a 96-well PCR plate. First, total RNA from the *Drosophila* cells (such as *Kc₁₆₇* or S2 cell lines, or from whole flies) is isolated and used as template for generation of a cDNA pool through reverse transcription-based reaction. In the next step, the cDNA pool is used as template in the amplification of an individual cDNA, specific to a particular gene, by using gene-specific PCR primers. The amplified PCR products are then isolated individually after separation by agarose gel electrophoresis. Purified gene-specific cDNA products from this 1st round of PCR amplification are then re-amplified, purified, and used as templates for the synthesis of dsRNA by in vitro transcription. To facilitate in vitro transcription through T7 RNA polymerase-mediated catalysis, T7 overhangs are added to the 5' end of cDNA at the PCR amplification step by adding the T7 promoter sequence included as a part in the design of gene-specific primer. dsRNA library synthesized in this manner is used for genome-wide RNAi screening experiments. Steps involved in HTP synthesis of dsRNA are summarized in Fig. 1 (see Note 3).

3.1.1. First PCR

1. Array 10 μ L of primers from the working stock using multi-channel pipette and array 40 μ L of PCR master mix with repeater pipette into 96-well plates. The PCR master mix consists of 2 μ L of cDNA, 8 μ L of 1.25 mM dNTP mix, 2 μ L of 50 mM $MgCl_2$, 10 μ L of 10 \times PCR buffer, 17.58 μ L of nuclease-free water, and 0.5 μ L of Taq polymerase (5 U/ μ L).
2. Perform PCR reaction as follows: denaturation at 94 °C for 3 min, followed by two cycles of annealing at 58 °C for 30 s, extension at 72 °C for 1 min, and denaturation at 94 °C for 30 s, followed by 35 cycles of annealing at 60 °C for 30 s, extension at 72 °C for 45 s, and denaturation at 94 °C for 30 s. In the final step anneal at 56 °C for 30 s followed by an extension at 72 °C for 7 min and then the entire reaction is chilled to 4 °C.
3. Array 5 μ L of 10 \times gel loading dye to all wells using multichannel pipette, load the entire reaction mixture, and perform electrophoresis using 1 % agarose gel with ethidium bromide. Use 100 bp ladder marker to separate either rows or columns of a 96-well plate, while performing the gel electrophoresis.
4. Take a picture of the gel using a suitable gel documentation apparatus after completion of the electrophoretic separation, verify the band size, and note the band intensity.

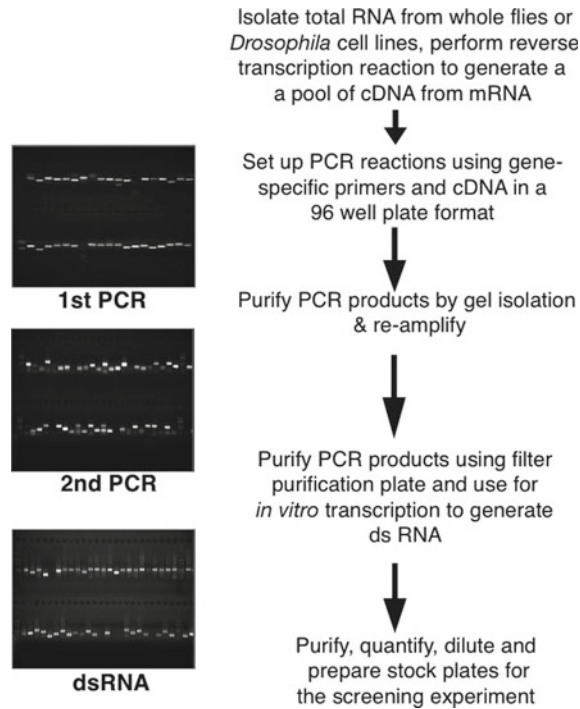


Fig. 1. Work flow for high-throughput synthesis of dsRNA. Following isolation of total RNA, reverse transcription is used to produce cDNA from which dsRNA template is amplified. Using primers specific to a target gene of interest, usually 500 bp apart, templates are PCR amplified twice, being gel purified each time, 96 targets at a time. As the primers also encode T7 primer sequences the amplified PCR products are then used for an *in vitro* transcription reaction to produce dsRNA, which is then purified, diluted, and aliquoted into stock plates ready for the screening experiment.

5. Equilibrate a water bath to 50 °C.
6. Slice out the area of the gel containing the amplified PCR product, by observing under UV transilluminator, and transfer into a pre-labeled microfuge tube or 96-deep-well plate. In order to minimize the risk of UV-induced DNA damage to PCR products, the agarose gel could be cut into small sections and processed separately (see Note 4).
7. Proceed to gel isolation of the PCR product.
8. For purifying the PCR products from the agarose gel slices in an HTP format, combination of PureLink HTP PCR purification kit and gel isolation buffer from Invitrogen Gel isolation kit will be useful (as described in the next step) (see Note 5).
9. Dispense 600 μ L (or three times the gel volume) of gel solubilization buffer (from Gel isolation kit) for a gel that approximately weighs 200 mg (see Note 6). Dispense the buffer using a repeater pipette to all wells, if all the gel slices are of uniform size (see Note 7).

10. Place the 96-well plate containing the gel slice and gel solubilization buffer into a water bath (or incubator) set at 50 °C, incubate for 10 min, and shake the plate occasionally to ensure dissolution of the gel. Gel slices appearing pink with gel solubilization buffer will become clear once the gel is completely dissolved. After the gel slice appears dissolved, continue incubation for an additional 5 min.
11. Add 200 µL of isopropanol to the dissolved gel slice that is approximately 200 mg by weight. Mix well thoroughly and proceed to next step.
12. Set up a vacuum manifold (Multiscreen vacuum manifold, 96 wells or equivalent) and place a 96-well PCR filter plate (PureLink HTP PCR purification kit) on top of the manifold.
13. Load isopropanol mixed solubilized gel solution to the PCR filter plate. Apply vacuum for 2–3 min or until the solution is drained off completely, and then release the vacuum.
14. Dispense 600 µL wash buffer (provided with PureLink HTP PCR purification kit) consisting of ethanol to the PCR filter plate. Apply vacuum for 1–2 min or until the solution is drained off completely, and then release the vacuum (see Note 8).
15. Repeat step 14 one more time, tap the filter plate on a stack of paper towels, and allow the plate dry for about 10 min.
16. Place a 96-well Elution Plate/Receiver (PureLink™ HTP PCR purification kit, Invitrogen) under the PCR filter plate, add 100 µL elution buffer to PCR filter plate, and allow the filter to soak at room temperature for 1 min. Centrifuge this setup for 2–3 min at $2,054\times g$ to elute DNA into the elution plate, label the plate, and seal and store the PCR products at –20 °C.

3.1.2. Second PCR

1. Thaw the primer plates and PCR products and centrifuge the plates at $2,683\times g$ for 5 min to get rid of air bubbles.
2. Array 10 µL of primers from the working stock using a multi-channel pipette, 38 µL of PCR master mix (minus 2 µL cDNA) as in 1st PCR step, instead of cDNA, array out 2 µL of purified 1st PCR products using a multichannel pipette.
3. Repeat PCR as in first PCR (see steps 2–4 in Subheading 3.1.1).
4. At the end of PCR, add 100 µL nuclease-free water to all wells, using repeater.
5. Transfer the entire volume using multichannel pipette to 96-well Multiscreen HTS PCR purification plate.
6. Turn on the vacuum (Multiscreen vacuum manifold, 96 wells) and set the pressure to 10 psi. To avoid damage to the filter plate, do not allow the vacuum to exceed 15 psi. Allow the wells to dry for 10–15 min until they appear shiny (see Note 9).

7. Once all the wells are dried, wash the wells with 100 μ L nuclease-free water/well and continue to drain with the vacuum.
8. When the wells are close to drying and appear shining, add 30 μ L nuclease-free water or 10 mM Tris-HCl pH 7.0 to the wells, seal the top and bottom of the plate with an aluminum foil, and shake it using a rocker or an Eppendorf mixer mate (800 rpm) for 10 min. Collect the cDNA elute using a multi-channel pipette and transfer into a new PCR plate labeled as 2nd PCR product. *Optional:* An additional elution may increase the yield of the PCR products, by repeating this with 10–15 μ L of nuclease-free water or buffer.
9. Run 1 % agarose gel, and load 3–4 μ L of the PCR product to confirm the amplification.

3.1.3. In Vitro Transcription

1. Thaw out the Ribomax express 2 \times buffer at 37–42 $^{\circ}$ C, mix using vortex, and ensure that the solution is clear; if not, continue to warm the solution and resuspend the buffer completely before proceeding to next step.
2. Aliquot 8 μ L of 2nd PCR product in the 96-well plate using multichannel pipette; keep the plate warm at room temperature (do not place the plate on ice). Of note, this reaction could be scaled up; however it is not recommended to scale the volume to more than two reactions/well because it could affect the downstream purification step if using Millipore multiscreen HTS plates.
3. Prepare a mix 10 μ L 2 \times buffer plus 2 μ L T7 Ribomax express mix, in bulk. Array using repeater pipette.
4. Spin the plate at 2,683 $\times g$ for 30 s, and proceed to the following PCR program: in vitro transcription at 37 $^{\circ}$ C for 16 h, denaturation at 80 $^{\circ}$ C for 1 min, and slow annealing (70 $^{\circ}$ C for 10 min, 65 $^{\circ}$ C for 1 min, 60 $^{\circ}$ C for 45 s, 55 $^{\circ}$ C for 30 s, 50 $^{\circ}$ C for 10 s).
5. Remove the plate from PCR machine and allow the plate to cool for 1 h at room temperature.
6. Warm nuclease-free water to 37 $^{\circ}$ C prior to the use in following steps (using warm water will enhance solubility of the salt precipitates formed in the reaction mixture, and might prevent clogging the pores present in the purification plate).
7. Add DNase 1 μ L/150 μ L nuclease-free water/well. Prepare the reagents in bulk and dispense using a repeater pipette.
8. Transfer the plate to 37 $^{\circ}$ C and incubate for 30 min.
9. Transfer the reaction mixture to 96-well Millipore Multiscreen HTS PCR purification plate and repeat the steps as described in 2nd PCR; for washing the wells, use 150 μ L of warm nuclease-free water.

10. For elution use 50 μL of 10 mM Tris-HCl, pH 7.0, seal the top and bottom sides of the plate with aluminum fold, and shake it on a rocker or microplate shaker (800 rpm for 10 min). A second elution with 25 μL Tris would improve the yield of dsRNA.
11. Store the dsRNA elutes in properly labeled and sealed 96-well plates at -70°C .

3.1.4. Quantification of dsRNA

1. Use single channel if possible or if using multichannel 10 μL pipette ensure that the volumes are accurate. Spin the plate to remove air bubbles.
2. Transfer 2 μL into 96-well UV plate (can increase to 2–3 μL but remember to change the calculation in the plate reader file later on, and keep the dilution 1:100).
3. Add 198 μL of 10 mM Tris-HCl pH 7.0 (dilution factor 1:100), mix well, and take reading at 260/280. Use $1 \text{ OD} = 45$ for dsRNA to calculate concentration (see Note 10).
4. Adjust the concentration (by using either nuclease-free water or preferably with 1 mM Tris-HCl, pH 7.0) of dsRNA to 0.08 $\mu\text{g}/\mu\text{L}$ or higher, if needed, and store it as a barcoded or labeled 96-well plate. It is important to perform a gel electrophoresis after diluting the dsRNA (using 1 % agarose and TBE buffer), with about 0.4 μg of dsRNA to check the quality and approximate uniformity in diluting dsRNA, before using in a screening experiment. By doing so, it is possible to identify those dsRNAs which failed or are insufficient in quantity for the experiment, and could be flagged to exclude from data analysis, which would otherwise become a source for false negative results.

3.2. HTP Transfection of dsRNA in *Drosophila* Cells

The genomic screening with dsRNA library involves transfection of dsRNA on Day 0, exposure with damaging agents on Day 3 allowing sufficient time for the RNAi to silence gene expression, and assessment of cell viability on Day 6. Steps involved in genomic screening with dsRNA are summarized in Fig. 2 and described in the following sections.

Transfection of dsRNA into *Drosophila* cells is performed by bathing *Drosophila* cells with dsRNA under serum deprived condition to facilitate the uptake of dsRNA from cell culture medium through phagocytosis. Using RNAi with *Drosophila* cells in this manner a genome-wide screen was conducted to identify essential genes required for cell viability (3). Subsequently this screening method was utilized to develop different types of HTP assays to study various cellular functions (1). We have used this screening method and the cell viability assay combined with DNA-damaging agents to identify genes essential for cell survival after MMS-induced DNA damage (2). RNAi libraries for the *Drosophila*

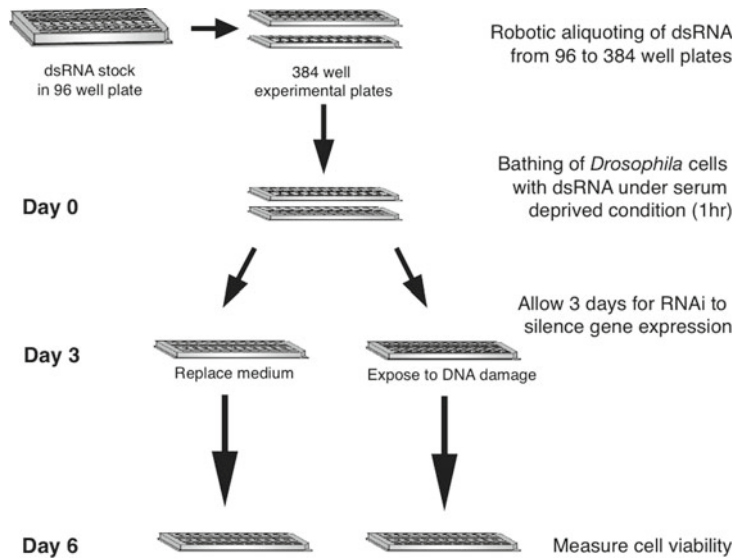


Fig. 2. Work flow for high-throughput screening with dsRNA library. Robotics are used to realiquot dsRNA from 96-well stock plates into 384-well experimental plates. *Drosophila* cells are aliquoted into the plates in the absence of serum to promote uptake of the dsRNA; serum-containing medium is then added after 1 h. Depending on the experiment, different timings can be used; here we incubate the cells for 3 days to achieve robust knockdown of target proteins. At that time either medium is exchanged for medium with or without DNA-damaging agent. Following 3 further days of incubation cell viability is then tested.

genome are available for screening from *Drosophila* RNAi Screening Center (DRSC), Harvard University (4), and commercial sources such as Invitrogen (formerly from Ambion) and Thermo Scientific (formerly from Open Biosystems). Initially the genomic screen for DNA damage is performed using the full library of dsRNA to target the knockdown of known and predicted mRNA encoded by the entire genome of *Drosophila*, and then a validation experiment is performed to confirm results obtained from the genomic screening experiment. Validation experiments are performed using a different set of dsRNAs which target a different region of mRNA than that targeted by the dsRNAs used in the genomic screening library. The experimental procedure for genomic screening and validation experiment is same, but the experimental design for validation experiment is more stringent because the objective of validation is to obtain high-quality data by reducing the rates of false positivity or false negativity from the genomic screening experiment.

1. dsRNAs used for screening or validation experiments—supplied in a 96-well plate—are aliquoted into 384-well plates for conducting the experiments with *Drosophila* cells.

(a) *Aliquoting dsRNA for the genomic screen*: About 22,915 individual dsRNAs are present in the original DRSC library

(version 1), and these stocks are maintained in 96-well plates, consisting of 92 unique dsRNA and 4 controls per plate. For the screening experiment, dsRNAs from four different 96-well plates are combined to fill one 384-well plate consisting of one dsRNA/well. Thus the entire DRSC library (22,915 individual dsRNAs) is represented on sixty-three 384-well plates. Each well (excepting empty control wells) contains, on average, 0.05 μg (0.01–0.43 μg) of dsRNA. The experimental plates with mapping for dsRNA in each plate for genomic screening are readily available from DRSC, while the commercial sources provide the dsRNA stock plates using a robotic liquid handling system dsRNA that can be aliquoted into 384-well plates. The limitation of this experimental plate format is the single representation of a unique dsRNA per 384-well plate such that replicates (duplicates of control or treatment with DNA-damaging agents) have to be performed in separate plates. This format can provide a source for interplate variations. It is therefore recommended to conduct duplicate experiments simultaneously or use the same batch of experimental plates for the duplicate experiments.

- (b) *Aliquoting dsRNA for validation experiment:* For validation experiments, a validation library could be either purchased from DRSC or synthesized by the end users in their own laboratory using primer sequences provided by DRSC and the method described in the section for HTP synthesis of dsRNA library. For the validation experiments, it is highly recommended that each dsRNA be represented in quadruplicates within a 384-well plate, and that 10 % of the wells of a plate be dedicated to control dsRNA. Also by using a standardized concentration of dsRNA, 0.4 μg /well, maximal RNAi efficiency can be achieved with the potential of eliminating false negative results as a consequence of incomplete target knockdown. The control dsRNAs are useful to normalize data and improve the robustness of validation analysis, further discussed in Subheading 3.4.2, and for additional reading refer to (5). The control dsRNAs suggested to use in validation experiments are the following: (a) control dsRNA (non-targeting dsRNA, such as dsRNA against luciferase, with 40 wells of a 384-well plate containing this dsRNA (see Note 11)); (b) positive control for dsRNA uptake (dsRNA against anti-apoptotic gene Thread (Th), which results in loss of cell viability in itself, in quadruplicates); (c) positive control for DNA damage (dsRNA against a gene known to result in loss of cell viability only in the presence of DNA damage, but not in itself, in quadruplicates); and (d) for background correction include high dose of a damaging

agent that would result in 100 % cell death, in quadruplicates. A suggested distributed pattern for aliquoting the dsRNA in quadruplicates is discussed in ref. 5 with the main intention being to offset edge effects, reduce interplate variations, and enhance the statistical reliability of the readout from the plate. The pattern described in ref. 5 is achievable only with certain types of robotic liquid handling systems; in the absence of availability of such instruments, users may define their own patterns and consider the points discussed here and in ref. 5.

2. Experimental plates should be equalized to room temperature and centrifuged at $110\times g$ for 1 min before proceeding to next step. If the plates are sealed, carefully peel the wrap and cover the plate with the lid.
3. (See Note 12) Harvest *Drosophila* cells such as *Kc₁₆₇* from a sub-confluent culture grown in a tissue culture flask (such as T175), transfer the cell suspension into a 50 mL conical tube, pellet down the cells, and resuspend in an appropriate volume of serum-free Schneider's medium. Count the cells, adjust the concentration of cells to $1.2\times 10^6/\text{mL}$, using serum-free Schneider's medium, and prepare the cell suspension in bulk for the required number of plates.
4. Dispense 10 μL (1.2×10^4 cells) of cell suspension to each well of 63 different 384-well plates, using a liquid dispensing system (similar to Wellmate, Matrix Technologies).
5. Centrifuge the plate at $110\times g$ for 1 min at room temperature.
6. Incubate for 1 h at 24 °C (or room temperature) to allow phagocytic uptake of the dsRNA, then dispense 20 μL of serum-containing medium (15 % heat-inactivated FBS), centrifuge the plate at $110\times g$ for 1 min at room temperature, and incubate the plates at 24 °C for a further 72 h. Since the medium present in the wells located at the edges of the plate tends to evaporate during the incubation, it is recommended to stack the plates in a loosely covered plastic container that has moist paper towels at the bottom and overlay few moist paper towels on the top of the plates that are above on the stack (see Note 13).

3.3. Induction of DNA Damage

Incubating the 384-well plates consisting *Drosophila* cells and dsRNA for 3 days should result in an efficient RNAi-mediated knockdown of the target mRNA and presumably depletion of the corresponding protein within the cell. This procedure in effect generates a library of *Drosophila* cells with one gene of the genome being silenced in each well by utilizing the RNAi technology mediated by a library of dsRNA. Applying DNA damage to these cells

allows the investigation of which proteins are essential for cell survival following damage exposure. Using this approach a genome-wide impact on cell survival could be analyzed using different types and different concentration of DNA-damaging agents to compare and contrast the mode of cellular recovery after such damages. Choosing a dose for DNA-damaging agent to use in the RNAi screening experiment depends upon the screeners' interest to measure decrease or increase (resistance) in viability in the absence of certain genes and in the context of the DNA-damaging agent. For example, to measure a decrease in cell viability following RNAi, screeners must choose a dose that would produce minimum but measurable effect on viability in the absence of any RNAi. Such a dose should then allow identification and quantification of further decrease in viability when the DNA-damaging agent is combined with an effective RNAi. In our genomic screening experiments we chose to use a concentration of DNA-damaging agent that would result in 70 % viability (in the absence of any RNAi) so that we could identify genes essential for cell survival following DNA damage in a robust manner (2). If the screen is intended to measure resistance, higher dose with stronger effect on viability could be selected for the screening experiment.

1. Remove the plates from the incubator, and centrifuge at $110\times g$ for 1 min at room temperature.
2. Prepare a stock of medium in bulk, with or without appropriate concentration of DNA-damaging agents sufficient for the number of plates to be used in the experiment (for example, 0.004 % (w/v) MMS in Schneider's medium consisting of 10 % heat-inactivated FBS for inducing DNA damage).
3. Aspirate the medium from the 384-well experimental plates carefully using a 16- or 24-channel wand (V & P Scientifics), under a low vacuum suction. Do not let the plate to dry out for long; proceed to next step as quickly as possible (see Note 14).
4. Dispense 40 μL /well of fresh (10 % serum containing) medium, with or without DNA-damaging agents, using a liquid dispensing system.
5. Centrifuge the plate at $110\times g$ for 1 min at room temperature.
6. Incubate the plates at 24 °C for an additional 72 h in the humid container as described before.

3.4. Assessment of Cell Viability

Following the knockdown of intended mRNA targets with RNAi and application of DNA damage, genes essential for cell survival could be surveyed on a genome level by using a cell viability assay. The cell viability assay described in this section quantifies the amount of live cells present based on the intracellular levels of ATP,

detected by using luciferin as a substrate and luciferase as an enzyme that utilizes ATP to produce luminescence. Therefore, the amount of luminescence detected is directly proportional to the amount of cells present in each well in these experiments. Using a commercially available assay (CellTiter-Glo, Promega) an RNAi-based genome-wide screen was originally performed to detect the genes essential for cell survival in *Drosophila* (3). This same method was then adapted to identify genes essential for cell survival following DNA damage (2). Using Celltiter-Glo provides a measure of viable cells in terms of luminosity values. These values are then used to compare damage-treated cells with untreated cells and the effect of RNAi-mediated knockdown on the loss of viability after DNA damage is determined. Therefore, in this section the experimental procedure and different methods to analyze the results are discussed.

3.4.1. Experimental Procedure

1. Following an additional incubation for 72 h, remove the plates from the incubator, and centrifuge at $110 \times g$ for 1 min at room temperature.
2. Aspirate the medium from the 384-well experimental plates carefully using a 16- or 24-channel wand (V & P Scientifics), under a low vacuum suction. Do not let the plate to dry out for long; proceed to next step as quickly as possible (see Note 15).
3. Dispense 40 μL /well of CellTiter-Glo reagent using a liquid dispensing system (see Note 16).
4. Centrifuge the plate at $110 \times g$ for 1 min at room temperature and proceed to read using a microplate reader capable of detecting luminescence (such as, Spectramax M5 plate reader, Molecular Devices).
5. Collect and map the results to identifiers by referring to the dsRNA used in these experiments to corresponding genes and proceed to data analysis.

3.4.2. Data Analysis

1. Results obtained from the genomic screen
Multiple methods are available for normalizing the data obtained from genomic screen; however we have tested the available methods and observed that a combination of background subtraction and scaling followed by quantile normalization to generate better quality data worked better than any single procedure (5). Therefore, the approaches for data normalization are discussed below.

Background subtraction and scaling: Background luminescence for each plate is calculated based on the median value of the plate and the value obtained for the well containing the lowest value. For example, the DRSC screening library has included a dsRNA targeting Thread (Th; CG12284), and an anti-apoptotic

gene is included in all experimental plate, as a positive control for RNAi. Silencing the expression of Th results in loss of cell viability and therefore, for background subtraction, the luminescence value of the well containing Th could be subtracted out from the value of each other well in the plate. Then the data could be scaled as follows. For untreated plates, the median value of each plate could be assumed to be equivalent to 100 % viability and the data should be scaled accordingly, as described in ref. 5. For damage-treated plates, a prior knowledge based on an estimate from cell viability (in the absence of any RNAi) with the amount of damaging agent used in the experiment should be used for scaling the data. For example, exposure with 0.004 % alkylating agent MMS results in 65 % viability and therefore, the median value of the plate treated with MMS could be assumed to be equivalent to 65 % viability of untreated cells.

Quantile normalization: Quantile normalization (6) is based on comparing quantiles between data sets, with no special allowance for outliers. Each of the 63 plate types following background subtraction and scaling could be quantile normalized between replicates of untreated and between replicates of damage treatment. Following quantile normalization, “M,” the moderated *t*-statistic in \log_2 fold change, could be calculated for each dsRNA, by which the genes could be ranked.

2. For results obtained from the validation experiments

Validation plates (described in Subheading 3.2) consist of 40 wells of non-targeting dsRNA against luciferase (Luc) to provide a control for the presence of dsRNA and 4 wells containing a high concentration of DNA-damaging agent (such as MMS (0.12 %, w/v) resulting in 99 % cell death) to determine the background luminescence in the absence of any viable cells. Luminescence values for each well could be scaled similar to the screen data, except the value for high concentration of DNA-damaging agent should be adjusted to 0 %. For the data analysis background subtracted well values should be normalized by dividing with median of scaled Luc values, and a *T-test* (two tailed, Type II) could be performed between normalized quadruplicates to determine if there is a difference between control and treatment. Since it is common in RNAi-based screens that removal of certain genes could result in decreased viability without any treatment, to account for reduced cell number under these circumstances and to determine if further reduction in cell viability with damage treatment is significant, a second *T-test* (two tailed, Type II) could be performed with Percent Control Viability (PCV) for each dsRNA targeting an mRNA against the PCV of dsRNA targeting Luc. To determine PCV, the data from normalized results could be scaled to 100 % based on values for Luc with untreated set, and to an appropriate percentage (based on previous estimate) for damage-treated

set. PCV could then be determined by comparing the normalized values for any gene with scaled percentage for Luc within untreated or damage-treated gene set. Statistically significant hits from these analyses could be selected to compare with results obtained from genomic screening. Values obtained based on PCV along with statistics could be used to rank hits to select candidate genes for follow-up studies, or the entire results could be combined to analyze for an interpretation to provide systems-level understanding on damage response.

4. Notes

1. To facilitate simultaneous PCR amplification of different genes in the HTP format, it is essential to maintain uniform conditions for all PCR reactions. For example, using working stock of primers prepared from a standardized concentration of primer stock will ensure uniform primer-to-template ratio in all PCR reactions. Therefore, it is recommended that forward or reverse primers be custom synthesized to a scale of 25 nmol, and then adjusted separately to yield 10 μ M stock when resuspended in a volume of 400 μ L.
2. *Caution:* Ethidium bromide is a carcinogen, so handle and dispose according to local regulations.
3. Genomic DNA could be used instead of reverse transcribed cDNA for gene-specific PCR amplification; however doing so introduces intronic sequences into the dsRNA and might increase off-target effects with RNAi. But if the cDNA for a particular gene of interest could not be amplified when the mRNA is used as a starting material then genomic DNA could be used as a substitute. In order to perform the reactions in HTP scale, it is necessary to prepare reagents at least in 10 % excess than needed so that reagent shortage while dispensing is avoided. For example, preparing reagents for 105 PCR reactions will ensure equal dispensing of PCR reagents to a 96-well plate.
4. *Caution:* UV exposure from transilluminator is harmful to eye and skin, so use appropriate personal protective equipment while handling the gel for this step.
5. Alternatively, Qiagen gel isolation kit could be used to isolate the individual PCR products by using 96 individual microfuge tubes and following manufacturer's recommendations; however such isolation of PCR products using individual tubes will be time consuming and laborious.
6. Making thin slice of gel would weigh approximately about 200 mg for all PCR products, if Subcell GT cell (Biorad) is used with a 20-well comb that is 0.75 mm thick.

7. The volume of the buffer can be adjusted based on the weight of the gel; accordingly it is necessary to adjust the volumes of other buffers to be used in the subsequent steps.
8. Wash buffer provided in the Invitrogen kit might require addition of ethanol.
9. Drying for extended period of time is undesirable for the elution of PCR products from the filter plate, if few wells were incompletely dried; keep the remaining wells hydrated by adding extra water or seal the plate with parafilm or saran wrap to reestablish the vacuum, and punch holes to selectively drain the incompletely dried wells.
10. Using nuclease-free water for quantification of dsRNA by this method has been observed to yield discordant results; therefore, it is highly recommended to use Tris buffer for the quantification of dsRNA instead of nuclease-free water.
11. dsRNA against GFP has off-target effects in *Drosophila*.
12. Important notes before proceeding with this step: The amount of *Drosophila* cells required for this step depends upon the number of experimental plates used for the screening or validation experiment. User may have to plan in advance and scale-up the numbers of flask to yield appropriate amounts of cells. In order to improve the efficiency of dsRNA uptake by phagocytosis avoid passaging or exchanging growth medium on the previous day of this step.
13. Using sterile water to moisten paper towels will reduce contamination issues.
14. *Drosophila* cell line such as *Kc167* is semi-adherent, so in order to avoid losing cells by vacuum suction, insert the tip of the wand along one corner of the well, gently slide it down towards the bottom of the well, and lift it up quickly.
15. Follow the precautions described in step 3, under Subheading 2.3.
16. Prepare Celltiter-Glo reagent in bulk for the required numbers of plates. The Celltiter-Glo reagent is supplied in 2× concentration and therefore, should be diluted 1:1 with PBS, prior to use.

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