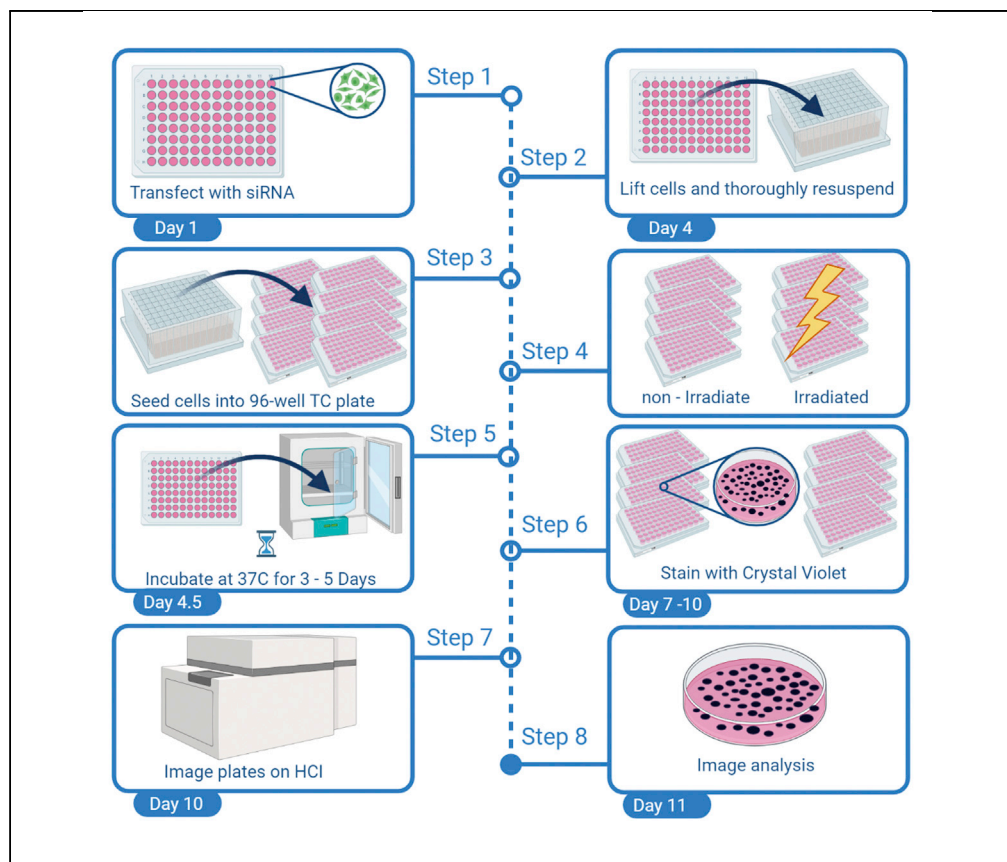


Protocol

Automated 96-well format high throughput colony formation assay for siRNA library screen



The colony formation assay is the gold-standard technique to assess cell viability after treatment with cytotoxic reagents, ionizing radiation, and cytotoxic combinatorial treatments. This protocol describes a high-throughput automated and high-content imaging approach to screen siRNA molecular libraries in HeLa cervical cancer cells in 96-well format. We detail reverse transfection of cells with siRNAs, followed by ionizing radiation, fixing, and staining of the plates for automated colony counting. This protocol can be used across a broad range of cell types.

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Highlights

High throughput
screening (HTS)
colony formation
assay using liquid
handling robots

Screens siRNA
molecular libraries in
HeLa cervical cancer
cells in 96-well format

Automated colony
counting and high
content imaging
readout approaches

Statistical analysis for
HTS quality control
and quantification

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Protocol

Automated 96-well format high throughput colony formation assay for siRNA library screen

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SUMMARY

The colony formation assay is the gold-standard technique to assess cell viability after treatment with cytotoxic reagents, ionizing radiation, and cytotoxic combinatorial treatments. This protocol describes a high-throughput automated and high-content imaging approach to screen siRNA molecular libraries in HeLa cervical cancer cells in 96-well format. We detail reverse transfection of cells with siRNAs, followed by ionizing radiation, fixing, and staining of the plates for automated colony counting. This protocol can be used across a broad range of cell types.

For complete details on the use and execution of this protocol, please refer to Tiwana et al. (2015).

BEFORE YOU BEGIN

This protocol describes a high throughput colony formation assay in which the HeLa cervical cancer cell line is used as the experimental cell type for all automated liquid handling steps. The assay measures the capacity of single cells to form colonies and so determines the capacity of a cell to divide indefinitely. HeLa cells form tight single colonies which are readily quantified using automated colony counting. The 22Rv1 prostate cancer cell line forms more diffuse colonies, so an alternative high content imaging protocol was employed to measure the total area of the colonies as the main readout (both methods are described).

In this experimental protocol HeLa cells are reverse transfected with a library of 960 siRNAs pools. Three days post-transfection, the cells are lifted from the plates by trypsinization, split into eight duplicate plates, and irradiated with 0 or 7 Gy. The cells are then allowed to form colonies before the final readout is performed using an automated colony counter or high content imaging.

Alternatives: There are several manufacturers of automated liquid handlers and plate washers. This protocol employs 50 μ l and 200 μ l 96-tip automated liquid handlers (Janus 96-Tip MDT), a FlexDrop dispenser (PerkinElmer, Waltham, MA, USA), and a BioTek Plate washer (Winooski, VT, USA), but alternative automated liquid handlers can be employed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dharmafect1 transfection reagent	Horizon Discovery	T-2001-03
5x siRNA buffer	Horizon Discovery	B-002000-UB-100

(Continued on next page)



<i>Continued</i>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
RNase-free water	Horizon Discovery	B-003000-WB-100
DMEM, high glucose, pyruvate, no glutamine	Thermo Fisher Scientific	21969035
Fetal Bovine Serum (FBS) Heat Inactivated	Merck Life Science	F9665-500ML
L-Glutamine Solution, 200 mM (100×)	Merck Life Science	G7513-100ML
Penicillin-Streptomycin Solution: 10,000 units penicillin and 10 mg streptomycin/mL (100×)	Merck Life Science	P4333-100ML
TRYPsin-EDTA Solution 1×	Merck Life Science	T3924-100ML
Crystal Violet	Merck Life Science	C0775-25G
<i>Experimental models: Cell lines</i>		
HeLa cervical carcinoma cell line	American Type Culture Collection	Cat# CCL-2
<i>Oligonucleotides</i>		
PLK1 siRNA pool	Horizon Discovery	L-003290-00-0005
DNA-PKcs (PRKDC) siRNA pool	Horizon Discovery	L-005030-00-0005
ON-TARGETplus Non-targeting pool	Horizon Discovery	D-001810-10
ON-TARGETplus siRNA library of siRNA pools (4 strands per gene)	Horizon Discovery	Various libraries available
<i>Software and algorithms</i>		
GelCount Software	Oxford Optronix	https://www.oxford-optronix.com/gelcount-cell-colony-counter
IN Cell Developer Toolbox	GE	N/A
<i>Other</i>		
Janus liquid handler with Modular Dispense Technology	PerkinElmer	N/A
FlexDrop non-contact reagent dispenser	PerkinElmer	N/A
BioTek ELx405 Plate washer	BioTek	N/A
Varian IX linear accelerator	Varian-Siemens	N/A
2 cm thick tissue-equivalent Plexiglas phantom	Dept. of Oncology Mechanical Workshop	N/A
GelCount colony counter	Oxford Optronix	N/A
IN Cell Analyzer 6000	GE	N/A
Robotic arm and AmbiStore plate stacker	HighRes	N/A
Scepter cell counter	Merck Millipore	PHCC20060
Scepter counting sensors	Merck Millipore	PHCC60050
175 cm ² TC-treated flasks	Greiner Bio-One	660175
200 μL 8-channel multichannel pipette	METTLER TOLEDO	17013805
1,000 μL 8-channel multichannel pipette	METTLER TOLEDO	17014496
P235 tips for the Janus MDT	PerkinElmer	Cat# 6001289
P50 tips for the Janus MDT	PerkinElmer	Cat# 6001294
96-well deep well plates (2 mL)	Corning	3960
96-well flat-bottom TC-treated microplates	Corning	3595
96-well v-bottom microplates	Corning	3896

Note: All liquid handling steps should be carried out under aseptic conditions.

Note: We recommend that this protocol is performed by at least two people - one person to handle the cells and manual dilution steps, and the other to handle the automation steps.

Note: Each plate of cells is transfected with one of 12 siRNA library plates, and then, after 3 days, the cells are split into 8 replicate plates, half of which are irradiated with 7 Gy. This gives a final total of ninety-six 96-well cell culture plates.

Alternatives: All stock volumes listed are for use of a siRNA library arrayed in the middle 10 columns of twelve 96-well plates. This can be scaled up or down, as long as an appropriate extra dead volume is added for pipetting accuracy.

MATERIALS AND EQUIPMENT

Automated liquid handling instruments/consumables

- Janus liquid handler with Modular Dispense Technology (PerkinElmer).
- FlexDrop non-contact reagent dispenser (PerkinElmer).
- BioTek ELx405 Plate washer (BioTek).
- P235 tips for the Janus MDT (PerkinElmer - Cat#6001289).
- P50 tips for the Janus MDT (PerkinElmer Cat#6001294).
- 96-well deep well plates (2 mL) (Corning Cat#3960).
- 96-well flat-bottom TC-treated microplates (Corning Cat#3595).
- 96-well v-bottom microplates (Corning Cat#3896).

Ionizing radiation

- Varian IX linear accelerator (Varian–Siemens).
- 2 cm thick tissue-equivalent Plexiglas phantom Dept. of Oncology Mechanical Workshop.

Colony formation counting

- GelCount colony counter (Oxford Optronix).

High content imaging

- IN Cell Analyzer 6000 automated microscope (GE).

Tissue culture

- Scepter cell counter (Merck Millipore Cat#PHCC20060).
- Scepter counting sensors (Merck Millipore Cat#PHCC60050).
- T-175 tissue culture flasks.
- 200 μ L 8-channel multichannel pipette (Anachem Cat#17013805).
- 1,000 μ L 8-channel multichannel pipette (Anachem Cat#17014496).

Cell culture medium

Reagent	Final concentration	Amount
DMEM, high glucose, pyruvate, no glutamine		440 mL
L-Glutamine Solution	2 mM	5 mL
Penicillin-Streptomycin Solution (Optional)	100 U penicillin and 0.1 mg/mL streptomycin	5 mL
Fetal Bovine Serum (FBS) Heat Inactivated	10%	50 mL
Total		500 mL

Note: Media should be warmed to 37°C before use and unused media should be kept at 4°C for up to 4 weeks.

Medium for reverse transfection of cells

Reagent	Final concentration	Amount
DMEM, high glucose, pyruvate, no glutamine		176 mL
L-Glutamine Solution	2 mM	2 mL
Fetal Bovine Serum (FBS) Heat Inactivated	10%	20 mL
Total		200 mL

Note: You will also need 41.6 mL of serum- and P/S-free media. Media should be warmed to 37°C before use and unused media should be kept at 4°C for up to 4 weeks.

STEP-BY-STEP METHOD DETAILS

Here, we describe the steps for performing a 12 siRNA Library Plate Screen (96 assay plates in total).

Dilute the siRNA library

⌚ **Timing:** Approximately 2 h, depending on the number of library plates diluted

This section describes how to dilute the siRNA library.

Example Protocol for RNAi transductions:

1. Dilute the siRNA library in siRNA buffer to a final concentration of 0.4 μ M. Mix well, by pipetting up and down multiple times.
2. Store at -80°C for long term storage.

⚠ CRITICAL: Avoid freeze/thaw cycles of siRNAs in solution.

Alternatives: All stock volumes listed are for use of a siRNA library arrayed in the middle 10 columns of twelve 96-well plates. This can be scaled up or down, as long as an appropriate extra dead volume of stock solutions is added for pipetting accuracy.

⏸ Pause point: The diluted siRNA library plates can be sealed and frozen, but avoid freeze/thaw cycles, if possible.

Thaw the HeLa cells

⌚ **Timing:** Approximately 30 min

This section describes how to revive and how to bulk-up the cells for the Reverse Transfection.

3. Five days before transfection thaw a vial containing 2 million cells and plate them in a 175 cm^2 TC-treated flask in 30 mL high glucose DMEM with 10% FBS (or as appropriate for other cell lines). Incubate the flask at 37°C 5% CO_2 for two days without medium change.
4. Three days before transfection, passage the cells, plating $\frac{1}{4}$ into each of 4 new 175 cm^2 TC-treated flasks. Incubate the flasks at 37°C 5% CO_2 for three days without medium change.

Alternatives: Any cell line of clinical interest can be used as long as the optimal transfection conditions have been predetermined.

⏸ Pause point: The cells can be passaged again, as long as there are enough cells on the day of transfection.

Note: It is always best to use the lowest passage number as possible of your cell line.

Note: There are no more pause points, besides the incubation times, until the plates have been fixed and stained on Day 9 (-IR) and Day 11 (+IR) in step #39.

⚠ CRITICAL: It is crucially important to maintain excellent aseptic tissue culture technique throughout the assay as the automated 96-well format high throughput colony formation assay proceeds for up to 10 days.

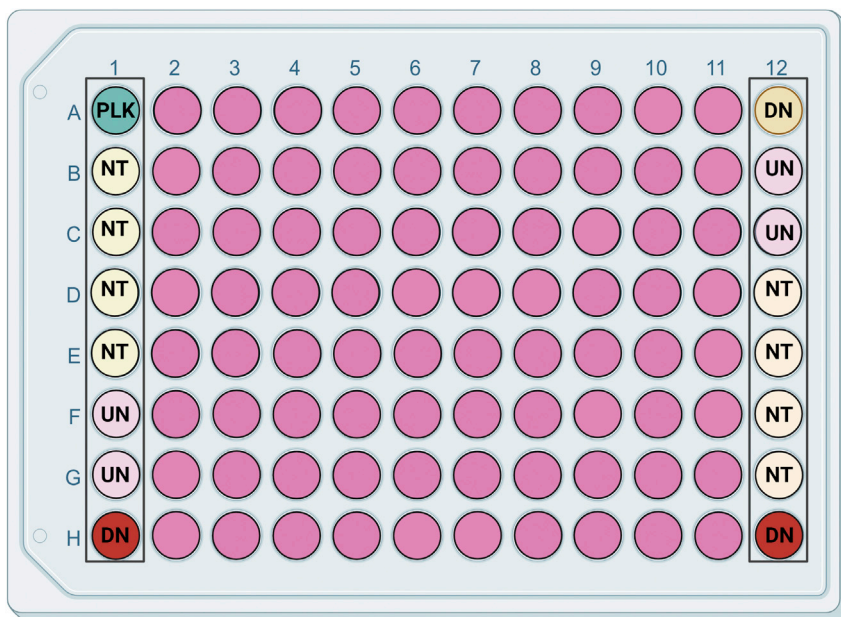


Figure 1. siRNA library plate layout

Controls are located on the edge columns, 1 and 12. A total of eight non-targeting (NT), one PLK1 siRNA (transfection control), three DNA-PKcs siRNA (DN; positive radiosensitization control), and four un-transfected (UN) wells are found in columns 1 and 12. Library siRNA are located in columns 2 through 11.

Reverse transfect cells

⌚ Timing: Approximately 6 h

This section describes the automated liquid handling siRNA Reverse Transfection protocol.

Library/Controls Plate and Transfection Reagent Plate Preparation:

5. Thaw the siRNA library, if frozen.
6. Label fourteen 96-Well V-bottom plates (Complex formation plates) as siRNA PLATE 1–12, the 13th as MOCK TRANSFECTED, and the 14th as the CONTROL PLATE. Label thirteen 96-Well Flat-bottom TC-treated plates as CELL ASSAY PLATE 1–12 and the 13th as MOCK TRANSFECTED. Label a 96-well deep-well block as the TRANSFECTION REAGENT PLATE.

⚠ **CRITICAL:** The cells in the MOCK TRANSFECTED plate will be essential for determining the volume of cells re-plated before irradiation.

7. Dilute the control siRNAs in 1 × siRNA buffer, in RNase-free 1.5 mL tubes and then add 150 μL of each to the first and last column of a V-bottom 96-well CONTROL PLATE following the plate map (Figure 1).

- a. 150 μL 0.4 μM PLK1 siRNA.

Reagent	Final concentration	Amount
20 μM PLK1 siRNA (PLK)	0.4 μM	3 μL
1 × siRNA buffer		147 μL
Total		150 μL

b. 450 μ L 0.4 μ M DNA-PKcs siRNA.

Reagent	Final concentration	Amount
20 μ M DNA-PKcs siRNA (DNA-PK)	0.4 μ M	9 μ L
1 \times siRNA buffer		441 μ L
Total		450 μL

c. 1,200 μ L 0.4 μ M NT siRNA.

Reagent	Final concentration	Amount
20 μ M Non-Targeting siRNA (NT)	0.4 μ M	24 μ L
1 \times siRNA buffer		1,176 μ L
Total		1,200 μL

d. 600 μ L siRNA buffer.

Note: Each of these solutions should be kept at room temperature (18°C–22°C), for the shortest time possible.

- Add 420 μ L Dharmafect1 to 41.58 mL of serum-free medium (minimum requirement is 41.3 mL) in a 50 mL tube and mix well. Using a reagent reservoir and a 1,200 μ L multichannel pipette, dispense 430 μ L/well into all wells of a deep well 96-well TRANSFECTION REAGENT PLATE (This provides a dead-volume of 40 μ L per well as the minimum requirement is $13 \times 30 \mu\text{L} = 390 \mu\text{L}$). See [troubleshooting 1](#).

Complex Formation:

- Using the Janus 96-Tip MDT, with P50 tips loaded, transfer 10 μ L of 0.4 μ M siRNA from each library plate to one 96-Well V-bottom plates labeled as siRNA PLATE 1–12. These will be the Library siRNA COMPLEX FORMATION PLATES.
- Next, transfer 10 μ L of dilution buffer into each well of the MOCK TRANSFECTED plate. While the 13 plates are being processed, store them in a plate stacker, or with lids.
- Remove the middle 10 columns of tips from one box of P50 tips, and using the Janus 96-Tip MDT, transfer 10 μ L of control siRNA from the CONTROL PLATE to the siRNA PLATES 1–12 and the MOCK TRANSFECTED plate. These will be the Library siRNA COMPLEX FORMATION PLATES plus Controls.

Alternatives: Use a manual multichannel pipette to add the controls to all 12 siRNA COMPLEX FORMATION PLATES.

- Form the siRNA complexes:
 - Using a fresh box of P50 tips on the Janus 96-Tip MDT, transfer 30 μ L of the diluted Dharmafect1 from the deep well 96-well TRANSFECTION REAGENT PLATE to the MOCK TRANSFECTION plate and mix three times, using a 15 μ L volume. Repeat with the 12 siRNA COMPLEX FORMATION plates, using a fresh box of tips for each.
 - Allow the complexes to form for 20 min at room temperature.

△ CRITICAL: Use a fresh box of tips for each siRNA COMPLEX FORMATION PLATE.

Reverse Transfection:

- Using the PE FlexDrop reagent dispenser, prepare the 13 flat-bottom TC-treated CELL ASSAY PLATES by adding 25 μ L of DMEM + 10% FBS (without P/S) to all wells.

14. Prepare HeLa cells for plating:
 - a. Trypsinize, resuspend, and count the cells from four 175 cm² flasks of HeLa cells.
 - b. Dilute cells to 80,000 live cells/mL in at least 150 mL of DMEM + 10% FBS P/S-free media in a 500 mL glass bottle.

Note: Although only 62.4 mL of cell suspension will be dispensed into the assay plates, a large excess is recommended for priming the dispenser tubing, to allow for test plates, and to ensure accurate dispensing by the FlexDrop.

Note: Trypan blue may be used to disregard any dead cells during cell counting.

△ CRITICAL: Lipid-based transfections should be performed in P/S-free media.

15. Add the siRNA complexes, and the cells, to the 96-well TC-treated plates:
 - a. Attach the 500 mL glass bottle containing the diluted HeLa cells to the FlexDrop and prime the dispenser. See [troubleshooting 2](#).
 - b. Using the Janus MDT, transfer 20 μL of the siRNA complexes to the 12 CELL ASSAY PLATES already containing 25 μL medium.
 - c. Do not add siRNA to the MOCK TRANSFECTION Plate. Move this plate directly to the next to step d. This is a control plate and will be used to calculate the optimized cell number calculation (see below).
 - d. Using the FlexDrop, dispense 50 μL of cell suspension to each cell plate (4,000 cells/well).
 - e. Incubate each cell plate at room temperature for 20 min to 1 h before placing it in the 37°C 5% CO₂ incubator.

Incubation time:

16. Incubate all cell plates for three days at 37°C 5% CO₂.

Split each of the transfected cell plates into eight 96-well plates (3 days after transfection)

⌚ **Timing:** Approximately 6 h for twelve Library plates

This section describes the automated liquid handling lifting/splitting protocol for seeding cells into 96-well assay plates for colony formation.

Note: Prepare a total of 2.5 L of cell culture media.

Plate Preparation:

17. Label twelve 96-well deep well plates, one per plate of transfected cells, and eight 96-well TC-treated flat bottom plates per plate of transfected cells – four plates (7 Gy or (+) IR) and four plates (0 Gy or (-) IR), for a total of ninety-six 96-well TC-treated Assay Plates.
18. Warm 2,500 mL of media and 200 mL trypsin in a water bath set at 37°C.
19. Using the FlexDrop, dispense 176 μL of DMEM + 10% FBS Pen/Strep to forty-eight 96-well TC-treated Assay Plates labeled (-) IR. Store the plates in a plate stacker, or with lids.

Knock-down Validation:

20. Visually inspect Well A1 (PLK1) in all plates. PLK1 is an essential gene in most immortalized cancer cell lines, therefore if no viable cells are observed, KO of the PLK1 gene was successful.

Optimized Cell Number Calculation:

21. Manually lift the cells (trypsinize) in the MOCK TRANSFECTED Cell Counting Control Plate containing the NT control-transfected cells as follows.
 - a. Using an 8-channel aspiration adapter and a manual multichannel pipette, aspirate the media from the cell plate and wash the wells once with 50 μL PBS.
 - b. Add 50 μL Trypsin per well and return the plate to incubator for 10 min.

△ CRITICAL: This time will vary for different cell lines. It is important to note that trypsinization of cells in a 96-well plate tends to take longer than for cells in a standard culture flask. It is crucial to confirm by visual inspection under the microscope that all cells have lifted before proceeding to the next step.

- c. Add 150 μL of medium to all wells, mix thoroughly and count the cells from several wells.
 - d. Calculate the volume of cells that yields 9,600 cells.
22. Using the FlexDrop, dispense 1,200 μL – minus the volume needed for 9,600 cells – into twelve 96-well deep well plates.

Automated Cell Lifting and Splitting procedure for one 96-well plate of transfected cells:

23. Lift the cells (Trypsinize) each plate of transfected cells using the Janus 96-Tip MDT with a fresh box of P235 tips loaded.
 - a. Aspirate the media from the cell plate at 1 mm above well bottom, 0% Liquid Level Tracking, at 10 $\mu\text{L}/\text{s}$.
 - b. Add 50 μL PBS at 1 mm above well bottom, 0% Liquid Level Tracking, at 10 $\mu\text{L}/\text{s}$.
 - c. Aspirate the PBS from the cell plate at 1 mm above well bottom, 0% Liquid Level Tracking, at 10 $\mu\text{L}/\text{s}$.

△ CRITICAL: Do not disturb the cell monolayer at this stage.

- d. Add 50 μL Trypsin per well and return the plate to incubator for 10 min.
 - e. Using the Janus MDT, add 150 μL of medium to each well and mix 125 μL three times at 1 mm above well bottom, with 100% Liquid Level Tracking, at 10 $\mu\text{L}/\text{s}$.
 - f. To thoroughly re-suspend and de-clump the cells, mix the wells four more times, once in each quadrant of the well (Figure 2) with four 125 μL mixes at 20 $\mu\text{L}/\text{s}$, aspirating at 4 mm above the well bottom and dispensing at 1 mm above the well bottom, with 100% Liquid Level Tracking.
24. Transfer the appropriate calculated volume for 9,600 cells (step 20) to the deep well plate containing 1,200 μL of media (minus the volume of cells), for a final concentration of 8,000 cells/mL.
25. To thoroughly re-suspend the cells in the deep well plate, mix the wells four times with four 200 μL mixes at 20 $\mu\text{L}/\text{s}$, aspirating at 4 mm above well bottom and dispensing at 1 mm above well bottom, with 100% Liquid Level Tracking.
26. Transfer 24 μL (192 cells) from the deep well plate to four 96-well TC-treated Assay Plates which already contain 176 μL medium/well (labeled 0 Gy or (-) IR).
27. Transfer 200 μL (1,600 cells) from the deep well plate to four 96-well TC-treated Assay Plates (labeled 7 Gy or (+) IR).
28. Incubate each cell plate at room temperature for 1 h before placing it in the incubator (Lundholt et al., 2003).
29. Repeat this process from step 22 above, for each plate of transfected cells.
30. Leave un-irradiated plates (labeled 0 Gy or (-) IR) in the incubator for the remainder of the experiment.
31. Incubate for at least 4 h at 37°C 5% CO₂ before irradiating the 7 Gy plates.



Figure 2. Mixing map showing quadrants of a single well in a 96-well cell plate

Irradiate half of the plates with 7 Gy

⌚ Timing: Approximately 2 h

This section describes how irradiate the assay plates.

32. Place 24 plates in a 2 cm thick tissue-equivalent Plexiglas phantom.
33. Irradiate with 6 MV photons using a Varian IX linear accelerator at gantry angles 0 and 180 degrees to a total dose of 7 Gy.
34. Repeat steps 31 and 32 for the remainder of the 7 Gy plates.
35. Incubate the un-irradiated (labeled 0 Gy or (-) IR) plates for a further 6 days and the (labeled 7 Gy or (+) IR) plates for 8 days.

Note: There are no media changes for the remainder of the experiment.

△ CRITICAL: The Plexiglas phantom should always contain 24 plates during irradiation. The number can be made up with plates containing PBS.

Fix, stain, and count colonies

⌚ Timing: Approximately 2 h

This section describes fixation and staining the 96-well Assay Plates for the colony formation readout, including instructions on how to use an automated colony counting instrument or high content imaging.

Note: All washing steps are carried out using a BioTek ELx405 Plate washer. Dispensing of the crystal violet solution is carried out by a Multidrop automated 8-channel dispenser or equivalent.

Reagent	Final concentration	Amount
Crystal Violet	5 mg/mL	2.5 g
Ethanol	10%	50 mL

(Continued on next page)

Continued

Reagent	Final concentration	Amount
Methanol	50%	250 mL
Water	40%	200 mL
Total		500 mL

Note: Store in the dark, at room temperature, tightly capped.

36. Prepare Crystal Violet Solution: 5 mg/mL crystal violet in 10% ethanol, 50% Methanol and 40 % water (total volume 500 mL).
37. Remove media from 96-well plates (no wash).
38. Add 20 μ L Crystal Violet Solution per well and leave at room temperature for 2 min.
39. Wash off the crystal violet using three cycles of 300 μ L of water per well, followed by a final aspiration. Tap plates upside-down to remove any residual liquid and leave the plates to dry.
40. Count colonies using a GelCount colony counter (Oxford Optronix Ltd, Abingdon, UK), using a 96-well plate mask at 1,200 dpi resolution and CHARM settings appropriate for the cell line used (Figure 3 illustrates the CHARM SETTINGS, while Figure 4 illustrates a matching pair of 96-well 0 Gy and 7 Gy plates). See [troubleshooting 4](#) and [5](#).

Alternative to step 40:

41. Image the plates using an IN Cell Analyzer 6000 automated microscope (GE Healthcare), or equivalent, fitted with a Nikon 2 \times objective (which captures one whole well of a 96-well plate per image), using the Red excitation laser (652 nm), 400 ms exposure time, and the Cy5 emission filter (670–742 nm). Use Laser-based hardware autofocus with an offset from the well bottom of 620 μ m to keep the colonies in focus. An example image is shown in [Figure 5A](#). Use a robotic arm and AmbiStore plate stacker (HighRes Biosolutions, Beverly, MA), or equivalent, to automatically load plates into the imager for imaging and unload plates on completion of imaging.
42. Analyze the images to generate total colony area per well. For this method we used a custom image analysis protocol (Developer Toolbox (GE Healthcare)). In step 1, the colonies are segmented using an intensity-based threshold (MIN 490 – MAX 65535 Grey levels, images are 16 bit). In step 2, the well is segmented, and in the final step colonies inside the well mask are identified (seen as red) (this enabled removal of out-of-well objects that were segmented such as the well rim and debris). Smaller non-colony cell fragments are removed using area (Area>5,000 μ m², seen in yellow). The total area of remaining colonies in the well is then calculated. The steps of the analysis protocol are shown in [Figure 5B](#). See [troubleshooting 6](#).

EXPECTED OUTCOMES

Upon completion of the screen there will be up to 100 distinct colonies in the Un-Transfected (UN) and Non-Targeting (NT) control wells ([Figure 4](#)), but none in the transfection efficiency control well (PLK1). The DNA-PKcs siRNA (DN) serves as positive radiosensitization control, leading to fewer colonies in the plates irradiated with 7 Gy than those that were unirradiated, after normalization (see [quantification and statistical analysis](#) below). In the library wells, known modulators of radiosensitivity, such as DNA-PKcs (PRKDC), CHEK1, ATM and ATR, should come up as “hits”, decreasing the surviving fraction in the irradiated plates to a greater degree than the unirradiated plates, as indicated by lower R-scores.

QUANTIFICATION AND STATISTICAL ANALYSIS

The plating efficiency (PE) for all wells is calculated by dividing the Average Colony Number for the two replicate wells by the number of Cells Plated. Wells that contain toxic siRNAs are excluded by

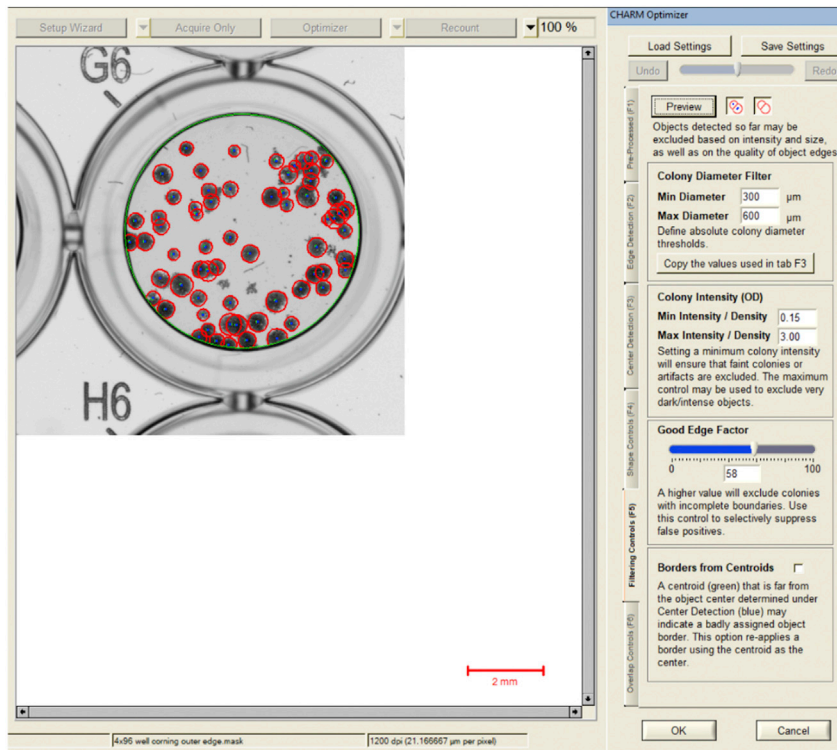


Figure 3. Screenshot of the CHARM settings used for colony counting (image has been enlarged and includes 2 mm scale bar).

removing any with a PE 0 Gy < 0.05. A Surviving Fraction (SF) is calculated for the remaining wells using the formula: $SF = (PE)0 \text{ Gy} / (PE)7 \text{ Gy}$. The Surviving Fraction for each library-treated well is then normalized to the average Surviving Fraction of the non-targeting (NT) control siRNA-transfected wells for each plate. A radiosensitization R-score is then calculated: $R\text{-score} = (\text{Normalized } SF - 1) / (\text{Mean absolute deviation of normalized NT } SF)$ (Tiwana et al., 2015).

To determine if the screen was of sufficient quality to identify radiosensitizers, z-factors should be calculated based on the normalized SF of the non-targeting (NT) negative control wells and the DNA-PK_{CS} positive control wells, using the formula below, where σ is the standard deviation and μ is the mean of the replicate control wells (Zhang et al., 1999). A z-factor between 0.5 and 1 is considered to denote a good assay window between the positive and negative controls.

$$Z - \text{Factor} = 1 - \frac{3(\sigma_{NTSF} + \sigma_{DNA - PK_{CSF}})}{|\mu_{NTSF} - \mu_{DNA - PK_{CSF}}|}$$

LIMITATIONS

This protocol can be adapted to most adherent cell lines, as long as cell density and the transduction conditions are determined in advance, although cells may be lost during fixation if the cells are only weakly adherent.

Twelve siRNA library plates, containing up to 960 siRNAs pools, generate ninety-six assay plates. Laboratory automation, and two people, are required to handle this number of plates. If a larger library is screened, or some steps will be done manually, we recommend splitting the screen into more manageable runs.

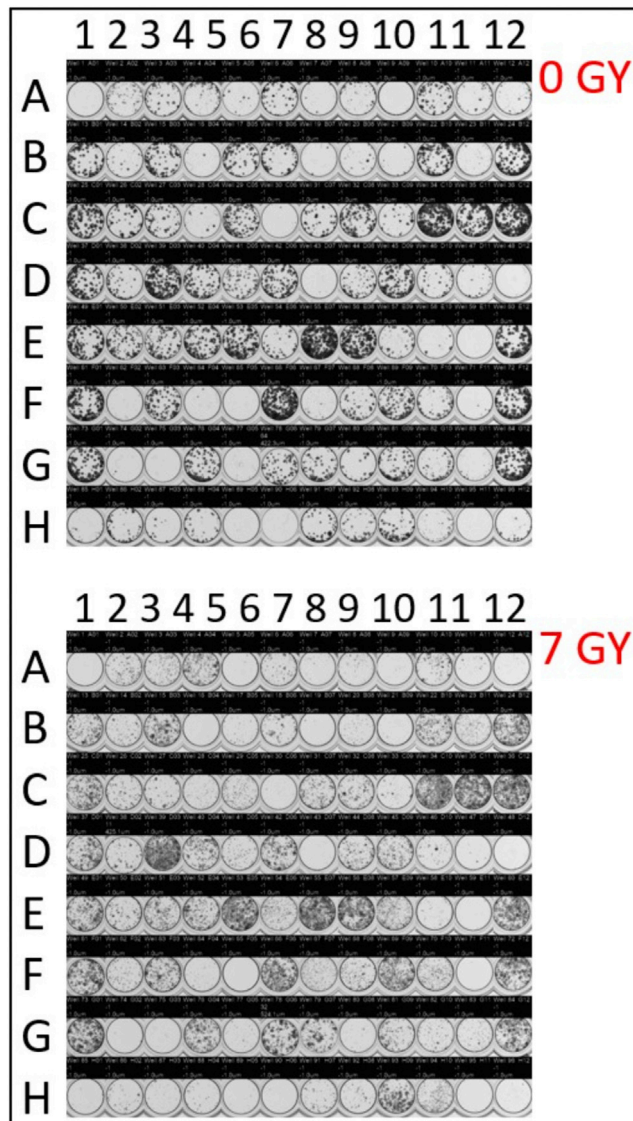


Figure 4. Illustration of a representational pair of 96-well 0 Gy and 7 Gy plates

TROUBLESHOOTING

Problem 1

Instability of diluted transfection reagent: As the transfection reagent diluted in medium will be kept at room temperature for the duration of the transfection procedure (around 6 h), it is important to confirm that it remains stable under these conditions. While we did not experience any issues using the transfection reagent described here (Dharmafect1) we have encountered stability issues with other transfection reagents when diluted in medium and kept at room temperature (for example Interferin; PolyPlus). This problem may start after step 8.

Potential solution

To solve this issue of transfection reagent instability, a fresh stock of transfection dilution can be prepared every hour, thus using a new stock for every three plates rather than a single batch for the entire procedure of twelve plates.

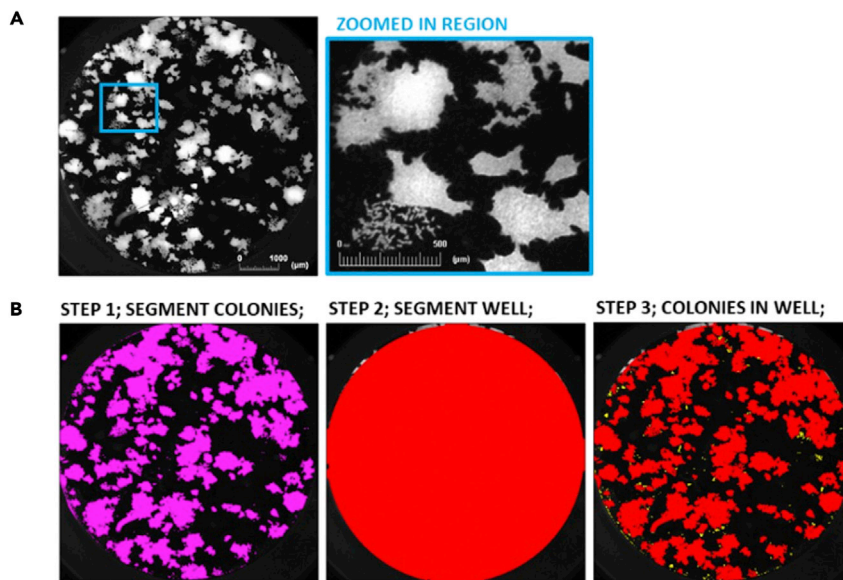


Figure 5. The 22Rv1 prostate carcinoma cell line forms more diffuse colonies than those formed by HeLa cells (the images have been enlarged and include a scale bar in 1000 and 500 μm).

(A) Example image of Crystal Violet-stained 22Rv1 cells.

(B) Image analysis steps.

Problem 2

Clumps of cells may clog the tips of the FlexDrop during plating, leading to less volume in one or more rows of the assay plates. This problem may occur during step 14.

Potential solution

Test more than one bank of valves before starting the protocol. As the cells are being dispensed, check the liquid levels in the assay plates periodically. If any tips appear to be clogged, stop the FlexPrep protocol, and change to another bank of valves. Alternatively, priming with air (by attaching an empty bottle) may clear the clogged tip. Test the valves by dispensing sterile PBS into a clear plate, before reattaching the bottle of cells and priming the tubing again.

Problem 3

During the Quantification and Statistical Analysis, low colony numbers across all plates (including non-irradiated) will make hit identification unreliable and can have several causes including:

- The transfection procedure (step 15) was too toxic. This will be apparent by low colony numbers across all library- and the NT siRNA-transfected wells but not the un-transfected wells.
- The lifting procedure (step 23) was not optimal. If trypsinization was not fully effective, not all cells will be transferred to the colony formation plates.

Potential solution

- During assay development, determine the lowest concentration of transfection reagent that causes sufficient knock-down.
- Careful visual inspection of every trypsinized plate prior to resuspension is recommended.

Problem 4

When using the GelCount colony counter, during step 39, too many colonies in a well, causing colonies to merge, will make colony identification unreliable.

Potential solution

In most cases, this will be caused by counting errors, resulting in too many cells being plated. Careful counting of several control wells is recommended to ensure the correct volume of cells is transferred from the transfection plates. Alternatively, high content imaging can be used to quantify the area of the colonies (see steps 41 and 42).

Problem 5

Irradiation can cause some cells to become permanently senescent. These cells often become enlarged and when clustered together can be falsely identified as a colony.

Potential solution

Careful optimization of the colony identification parameters (CHARM settings in the GelCount software) in step 39 is recommended to avoid counting false-positive colonies when these large senescent cells are present.

Problem 6

Bacterial contamination can cause wells to be densely stained with Crystal Violet, leading to full or near-full well coverage (step 39).

Potential solution

- Maintain aseptic conditions throughout the assay (up until Crystal Violet staining step).
- Remove any bacterially-contaminated wells from the final data analysis step.
- Other imaging artefacts caused by dirty plates can be avoided by cleaning the bottom of each plate with 70% Ethanol to remove any external Crystal Violet prior to imaging.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Daniel Ebner, daniel.ebner@ndm.ox.ac.uk.

Materials availability

No new materials generated.

Data and code availability

This Star Protocol does not contain any data or code.

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AUTHOR CONTRIBUTIONS

S.B.H., R.P., V.M., and D.E. wrote the manuscript. D.E. developed the high throughput liquid handling assays in collaboration with R.P. and G.H. D.E., T.C., R.P., and G.H. produced the high throughput screens. V.M. developed the high content imaging, image analysis and data processing. B.C. and G.H. initiated the research projects. D.E., G.H., and B.C. provided funding for the research. All authors edited the manuscript and gave final approval for publishing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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