A high throughput screen with a clonogenic endpoint to identify radiation modulators of cancer

Nathan P. Gomes*[1], Barbara Frederick*[1], Jeremy R. Jacobsen[4], Doug Chapnick[4], Tin Tin Su[1,2,.3,5]

[1]SuviCa, Inc. PO Box 3131, Boulder CO, 80307-3131

[2] Department of Molecular, Cellular and Developmental Biology, University of Colorado Boulder, CO, 80309-0347

[3] Molecular and Cellular Oncology Program, University of Colorado Cancer Center, Aurora, CO 80045

[4]BioLoomics, 4665 Nautilus Ct. S., Ste. 501, Boulder, CO 80301

[5]Corresponding author tin.su@colorado.edu

*Equal Contribution

Running Title: A clonogenic HTS for radiation modulators

Abstract

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Clonogenic assays evaluate the ability of single cells to proliferate and form colonies. This process approximates the regrowth and recurrence of tumors after treatment with radiation or chemotherapy, and thereby provides a drug discovery platform for compounds that block this process. However, because of their labor-intensive and cumbersome nature, adapting canonical clonogenic assays for high throughput screening (HTS) has been challenging. We overcame these barriers by developing an integrated system that automates cell- and liquid-handling, irradiation, dosimetry, drug administration, and incubation. Further, we developed a fluorescent live-cell based automated colony scoring methodology that identifies and counts colonies precisely based upon actual nuclei number rather than colony area, thereby eliminating errors in colony counts caused by radiation induced changes in colony morphology. We identified 13 cell lines from 7 cancer types, where radiation is a standard treatment module, that exhibit identical radiation and chemoradiation response regardless of well format and are amenable to miniaturization into small-well HTS formats. We performed pilot screens through a 1584 compound NCI Diversity Set library using two cell lines representing different cancer indications. Radiation modulators identified in the pilot screens were validated in traditional clonogenic assays, providing proof-of-concept for the screen. The integrated methodology, hereafter 'clonogenic HTS', exhibits excellent robustness (Z' values >0.5) and shows high reproducibility (>95%). We propose that clonogenic HTS we developed can function as a drug discovery platform to identify compounds that inhibit tumor regrowth following radiation therapy, to identify new efficacious pair-wise combinations of known oncologic therapies, or to identify novel modulators of approved therapies.

40 **Introduction**

41 More than half of US cancer patients undergo radiation therapy (RT) alone, or in combination
42 with other oncologic therapies (*1*). Cancer recurrence following RT is common, resulting from
43 proliferation of tumor clonogens that survived initial RT modalities (*2*). Identification of therapies that
44 specifically inhibit clonogenic proliferation following radiation would be a significant advancement for
45 RT-based cancer treatment, likely to reduce recurrence rates, and improve overall outcomes. Several
46 therapeutic agents are approved for combinatorial use with RT for the treatment cancer, but none
47 were initially identified as specific inhibitors of clonogenic growth following RT. Rather, mostly
48 cytotoxic agents approved as discrete therapies are combined with RT empirically (*3*). It remains to be
49 determined if direct screening for compounds that inhibit clonogenic proliferation can objectively
50 identify novel and more potent radiation modulators. Advancements in cheminformatics, chemical
51 synthesis, and improved ability to extract natural product compounds provide abundant and diverse
52 chemical libraries from which to search. To systematically screen for inhibitors of clonogenic growth
53 following RT, canonical clonogenic assays would have to be adapted to high-throughput screen (HTS)
54 platforms, hereafter 'clonogenic HTS'.

55 The tradiational clonogenic assay is the only methodology that measures growth delay and
56 total clonogenic death, whether it be it by mitotic catastrophe, apoptosis, necrosis, terminal
57 differentiation, reproductive death, or other modes of cellular death (*4*). It is the assay of choice for
58 monitoring celluar growth in response to RT (*5*). Relative to simpler MTT-type HTS assays, clongenic
59 HTS assays, in principle, would be better suited for identifing novel radiation modulators, as well as
60 more accurately testing known-drug/RT combinations. Resulting hits from this methodology should
61 improve success in subsequent pre-clinical studies and accelerate translation into clinically relevant
62 therapeutics. However, traditional clonogenic assays are extremely labor-, resource- and time-
63 intensive. The traditional requirement to laboriously manually count a minimium of 50 well-seperated
64 colonies with greater than 50 cells per colony, for each treatment condidtion has traditionally
65 necessitated use of lager well formats, making the assay imcompatable with known HTS

methodolgies. To our knowledge no HTS system exists that follows the traditional clonogenic methodology, despite description of the original assay 6 decades ago (*6*).

Several recent papers describe methodologies that approximate clonongic assays in HTS formats by miniaturizing to small well formats and utilizing automated image analysis to score fixed and stained colonies (*7-11*). The closest approximation of a clonogenic HTS among this body of work is a 'High Content' screen of Non Small Cell Lung Cancer cells in 96-well plates (*10*). Cells were seeded at non-clonogenic desities that yield somewhat similar survival and radiation response as in traditional 6-well clonogenic assays. Cells were treated with radiation in the absence or presence of serially diluted drugs, and "colonies" were counted after 4 days. Assay processing was as for the conventional methodology: "colonies" were fixed, stained, destained, and scored by automatated microscopy aided image analysis. A subset of hits from the screen of 146 compounds were validated in conventional clonogenic assays, and xenograft models. While this work indicates that clongenic-like HTS screens are possible, the assay does not have time/growth periods representative of true clonogenic endpoints, and the protocol is as labor-intensive as conventional clonogenic assays, including sample processing, and the necessity for multiple drug and radiation doses. The data analysis methodology was identical to that of the conventional assay, and not amenable to large datasets produced from an HTS. In a related study, short term (5-day) survival at a single radiation dose and its modification by 18 drugs was assessed in 32 cancer cell lines (*12*). Survival was found to correlate with dose-modifying factors in traditional clonogenic assays, suggesting that a simpler, shorter-term assay in a high-throughput format may be used as the primary screen to identify potential radiation modulators, later to be validated with traditional clonogenic assays. We again note that this technology does not represent an HTS with a true clonogenic endpoint. A more recent paper reports a High Content Screen (HCS) based on the growth of cancer cells in 2D and in 3D (spheroids) assays (*8*). Colony growth of SW620 colorectal cancer cells was monitored following serial seeding dilutions to achieve 1-100 colonies per well, in the presence or absence of drug. This method allows for the testing of a small number of molecules at different plating densities, but which densities in 96-well

92 exhibits similar growth characteristics and drug-response as cells seeded as for traditional clonogenic
93 assays was not addressed.
94 The goal of HTS is to use automation to rapidly screen large numbers of compounds while
95 reducing time, reagents, and sample manipulation compared to conventional assays. Further,
96 automation inherently increases experimental consistency by reproducibly implementing standardized
97 protocols. Assay formats should be amenable to handling many-fold more compounds per unit time
98 than conventional assays. A survey of the literature indicates that most components of conventional
99 clonogenic assays have been successfully modified for use in HTS formats, and key technological
100 components for the assay are commercially available. However, a true clonogenic HTS remains
101 absent; its development required the functional combination of these known
102 methodologies/technolgoies, development of missing components, and integration into a discrete HTS
103 sytem, where scored hits validate in traditional assays. We have accomplished these tasks and
104 describe here an integrated high-throughput system with a clonogenic endpoint. Our system
105 integrates and automates cell- and liquid-handling, irradiation, dosimetry, drug-addition, and
106 incubation. We report a fluorescent live-cell based automated colony scoring methodology that
107 identifies and counts colonies based upon precise nuclei counting rather than colony area. We report
108 the identification of 13 cell lines, from 7 cancer types where radiation is a standard treatment module,
109 that exhibit equivalent radiation and chemoradiation response regardless of well format and are
110 amenable to miniaturization into small-well HTS formats. We performed pilot screens in two cell lines
111 representing two different cancer indications with a 1584 compound library, identifying radiation
112 modulators that validated in traditional clonogenic assays. Finally, our clonogenic HTS methodology
113 exhibits excellent robustness (Z'>0.5) and high reproducibility (>95%).
114
115 **Results**
116 *Identification of cell lines amenable to miniaturized clonogenic growth.*
117 Clonogenic assays are traditionally performed in 6-well plates or larger vessels, allowing for
118 growth of single cells into colonies without significant influence from neighboring clones. The large

119 growth area allows for well separated single cell seeding, while also achieving a minimum of 50
120 colonies per well, as is recommended in the literature (*13*). High throughput screens require assays to
121 be performed in smaller well formats to allow for many drugs to be screened simultaneously. To
122 develop a true clonogenic HTS, we set out to identify cell lines that: 1) could be grown in smaller well
123 formats, yielding well separated colonies that number >50/well over the traditional clonogenic growth
124 period of ~10 days and 2) exhibit equivalent radiation response between smaller well and traditional
125 6-well clonogenic formats. We focused on 7 cancer indications where radiation is a main treatment
126 modality: HNSCC, NSCLC, metastatic melanoma, glioma, soft tissue sarcoma, esophageal, and
127 rectal cancers. Not all cancer cell lines are expected to meet all two criteria. As shown in Figure 1A,
128 four different cell lines were seeded at clonogenic density in 6-well plates and allowed to grow for 10
129 days. Each colony contains >50 cells yet A2068 melanoma cells form large diffuse colonies while
130 SW837 rectal cancer cells form discrete compact colonies. An extensive literature search for
131 clonogenic images was performed to identified cell lines that display similar colony morphology to
132 SW837 and were commercially available with limited use restrictions. We obtained 2 to 5 cell lines for
133 each of the 7 cancer indications, seeded them at equivalent growth densities in 6-, 24- and 96-well
134 plates, and grew then over a period of 7-10 days to confirm the published colony morphology (Figure
135 1B). Of the four representative cell lines shown, the first two formed into well-separated colonies of
136 >50 per well in 24-well plates, while the final two did so in both 24- and 96-well plates. In general,
137 about half of the cell lines tested met this criterion in 24-well or smaller formats.

138 We next compared clonogenic radiation response between different well formats, using 3
139 doses of radiation as was done for validation of other high-content clonogenic assay formats (*10*).
140 Figure 1C-E shows representative data for two cell lines. Cells were seeded at identical density
141 (number of cells per growth area in the well) in the different well formats. In the traditional 6-well
142 clonogenic assay, cells are typically grown in 4-5 ml of media per well. This volume is equivalent to
143 1.7-2.2 ml per well in 24-well plates and 0.7-0.9 ml per well in 96-well plates. At these equivalent
144 media volumes, cells exhibit greater radiation resistance consistently in smaller well formats
145 compared to 6-well plates. For example, compare ‘6-well 4ml’ (red arrow) to ‘24-well 1.5 ml’ and ’24-

146 well 2 ml' (bracket) in Figure 1C-D. Systematic analysis shows that radiation survival is exquisitely
147 sensitive to culture volume regardless of well format, with smaller relative volumes correlated with
148 increased radiation sensitivity. We note that this is not an artifact of smaller well formats, as increased
149 radiation sensitivity in smaller culture volumes is also seen in the traditional 6-well plates for all cell
150 lines examined (Figure 1C, compare 6-well 2, 3, and 4 ml). One possible explanation is RT-induced
151 production of secreted growth inhibitory factors that would be, by definition, more concentrated in
152 smaller volumes. Importantly, as this parameter is typically not reported, variable culture volumes
153 could partially explain lab-to-lab clonogenic variability widely observed in literature (*14*).
154 Regardless, consistent variation of radiation survival relative to culture volume allowed for the
155 identification of conditions where survival in smaller well formats are equivalent to that of traditional 6-
156 well formats. Specifically, cells grown in 4 ml in 6-well plates, 1 ml in 24-well plates and 150 μl in 96-
157 well plates produced equivalent radiation survival curves, with 'equivalent' defined as average survival
158 from technical replicates that fall within one standard deviation at each radiation dose tested (red
159 arrows in Figure 1C-E). Similar results were obtained for all cell lines tested (thirteen total), suggesting
160 that this phenomenon is specific to well format and seeding volume but independent of the cell line
161 used. More specifically, nine of thirteen cell lines showed equivalent radiation survival in 6-well and
162 24-well formats when culture volumes were controlled while the remaining four showed equivalent
163 radiation survival in 6-well, 24-well and 96-well formats when culture volumes were controlled (Table 1
164 and Supplemental Figure 1). We identified two cell lines for each of the seven cancer indications of
165 interest, except for glioma where only one of more than ten cell lines we examined was amenable to
166 miniaturization.
167
168 *Confirmation of equivalent clonogenic chemo-radiation response regardless of plate format.*
169 The goal of the clonogenic HTS system is to identify compounds that modulate radiation
170 responses. Therefore, it was necessary to confirm that cell lines exhibiting equivalent radiation
171 responses in miniaturized formats also exhibit equivalent chemo-radiation responses. This was
172 assessed using chemotherapeutics that represent standard-of-care for each of four cancer

173 indications, specifically, Cisplatin for HNSCC; 5-FU, Oxaliplatin and Irinotecan for rectal cancer;
174 Dacarbazine for metastatic melanoma; and Temozolomide for glioma. Using clinically relevant doses
175 for each chemotherapeutic and the same 3-dose radiation design as in Figure 1, dose-modifying
176 factors (DMF's) were calculated using linear-quadratic plots for clonogenic survival for each well
177 format, cell line and appropriate chemo-radiation combinations (*15*). Importantly, the miniaturized
178 clonogenic formats exhibit equivalent chemo-radiation responses, in addition to radiation responses
179 (Figure 2 and Supplemental Fig. 2; 'equivalent' defined as averages that are within one standard
180 deviation from each growth condition).
181
182 *Automated colony scoring by nuclear position yields equivalent results as manual counting*
183 The most laborious and time-consuming step in clonogenic assays is the counting of colonies,
184 with discrimination between true clones and those that experienced clonogenic death (below the 50-
185 cell/colony threshold). While typically done manually, recent publications describe several automated
186 counting methodologies. A common feature amongst these is the use of area 'masks' to identify
187 colonies that have at least 50 cells (Figure 3A, pink circle). While likely accurate under control
188 conditions, this methodology is error-prone in the context of irradiation as radiation can induce cell
189 spreading in some cell lines, thereby changing the area occupied by 50 cells between control and
190 irradiated samples. The extent of cellular spreading is cell type and radiation dose-dependent and can
191 increase the area of a 50-cell colony by as much as 50% (Figure 3B). While this problem may be
192 partially addressed by quantifying the area of a 50-cell colony at different radiation doses and using
193 masks of differing sizes, this methodology would be further confounded by morphological changes
194 induced by drug and/or drug-radiation combinations in a compound library screen. A robust HTS
195 system must accommodate these variables; therefore, we employed an alternative method.
196 We developed a nuclear fluorescence-based, automated colony counting method that utilizes
197 cell line specific parameters to segment colonies and individual nuclei from images of live cells. It is
198 comprised of three steps: image preprocessing to eliminate background and spurious fluorescence,
199 colony segmentation (Figure 3C), and nuclei segmentation to determine nuclear number for each

200 colony (see Methods for details). A delimited text file is generated containing each colony's relative
201 position within the well along with the number of nuclei in it. A 50-nuclei threshold then identifies
202 clonogens. Fluorescent nuclear dyes are amenable to this methodology but, we found that these have
203 poor reproducibility, low signal to noise ratios, and require stain/de-stain steps (not shown). Instead,
204 we generated stable nuclear RFP (Nuclight Red)-expressing variants for cell lines in Table 1 and
205 validated their use by fixing and staining as in traditional clonogenic assays after live fluorescence
206 images have been acquired (Figure 3C). This allowed us to compare directly automated to manual
207 counts for the same wells, which were found to be equivalent (Figure 3D,F). Survival curves were also
208 equivalent between parental and nuclear RFP variants, suggesting that stable transfection of nuclear
209 RFP did not alter radiation responses (Figure 3E,G). Similar results were observed for all cell lines in
210 Table 1 (Supplemental Figure 3) but we chose to use CCL-30 and SW837 in pilot screens because of
211 their robust growth, well-defined and compact clonogens, and amenability for 96-well miniaturization
212 (Figure 1A-B, D-E and Supplemental Fig. 2).
213
214 *Automation of the clonogenic HTS screen*
215 With cell lines amenable to clonogenic miniaturization identified and a validated methodology
216 for automated clonogenic counting in place, we next developed a customized instrument that
217 integrates automated cell- and liquid-handling, irradiation with dosimetry monitoring, and cell plate
218 incubation (Supplemental Figure 4A). Automated seeding of SW837 cells at clonogenic density and
219 radiation survival following RT is consistent across 96-well plates (Supplemental Figure 4B-C).
220 Clonogenic survival with varying drug treatment volumes up to 25 μl is consistent, providing significant
221 flexibility for HTS drug administration (Supplemental Fig 4D). Clonogenic survival is unaffected at up
222 to 0.75% DMSO (drug solvent), more than an order of magnitude greater than the concentration
223 expected to be used in the clonogenic HTS (Supplemental Fig 4E). Similar results were observed
224 when using CCL-30 cells (data not shown). Finally, radiation dosing as measured via robotically
225 placed nanoDot (Landauer) dosimetry is consistent amongst plates (for example, 4.118±0.096 Gy
226 received by 6 plates irradiated with 4 Gy).

227 As stated previously, CCL-30 and SW837 cells were chosen for pilot clonogenic HTS screens,
228 with the overall goal to identify compounds that modulate clonogenic survival following RT. Therefore,
229 for the primary screen radiation doses that induce partial growth inhibition (30-40%; 3 Gy for CCL-30,
230 9Gy for SW837) were chosen as this provides conditions for identifying modulators that increase or
231 decrease clonogenic radiation survival. With these conditions, we performed the clonogenic HTS in
232 each cell line with the 1584 compound Diversity Set VI library from the Developmental Therapeutics
233 Program of the National Cancer Institute. The Diversity Set library is assembled from a larger
234 (140,000) molecule library by grouping chemicals according to structure and selecting representative
235 members. Thus, we would be sampling many structurally diverse chemical families with this library.
236
237 *Robustness of the clonogenic HTS screen*
238 Cells were seeded in 150 μl, irradiated 24 hr post-seeding, and then treated to a final drug
239 concentration of 1 μM in total volume of 175 μl, with a final DMSO concentration of 0.01%. Cells were
240 seeded to average clonogenic densities of 66.0±7.7 (CCL30) and 110.2±13.2 (SW837) per well.
241 Each screen was conducted in seventeen 96-well plates, with a minimum of six vehicle (DMSO)
242 controls per plate. Live fluorescent images were obtained 10-days post-treatment with an IncuCyte
243 S3. Automated clonogenic segmentation was performed for each cell line in batch, and average
244 growth and standard deviations (Z-score) for each cell line were calculated (Figure 4A-B). To assess
245 the dynamic range of the primary screen, we examined the responses between controls and
246 compounds exhibiting significant growth effects. Vehicle (DMSO) wells function as negative controls,
247 while for positive controls we selected compounds that are 4 (CCL-30) or 4.5 (SW837) Z-scores
248 below the mean. Z'-factor was computed from the average (μ) and standard deviation (σ) of the
249 positive (+) and negative (-) controls, according to the formula $Z' = 1-[(3\sigma_{c+} + 3\sigma_{c-})/|\mu_{c+} - \mu_{c-}|]$ exceed
250 0.5 in both cell lines (Figure 4C), indicative of an HTS with 'excellent' robustness and dynamic range
251 (*16*).
252

253 *Reproducibility of the clonogenic HTS hits*

254 A common threshold for calling hits in robust HTS screens are effects greater than 2 standard
255 deviations from the mean (*17*). In our primary clonogenic HTS screen, 54 compounds in SW837 and
256 62 compounds in CCL-30 increased the efficacy of radiation by greater than two standard deviations
257 (>2 Z-score), while no compounds for either cell line exhibited significant radiation protective or growth
258 enhancement effects (Figure 4A-B, shaded areas). Importantly, 41 compound hits were shared
259 between the two cell lines (Figure 5A). To access reproducibility of the identified shared hits, we
260 retested each under the same conditions as the primary screen (1 μM drug plus 3Gy in CCL-30, or
261 9Gy in SW837). Since the reproducibility test does not include the rest of the library as a control, we
262 assessed growth inhibition for both screens relative to vehicle (DMSO) controls. 39 of the 41 hits
263 (95%) reproducibly exhibited growth inhibition greater than 2 standard deviations from the control in
264 both cell lines (Figure 5B-C). The two compounds that did not reproduce were shared between the
265 cell lines and exhibited no activity in the re-test. Based upon these data we conclude that the
266 clonogenic HTS is highly reproducible.

267

268 *A secondary screen differentiates radiation modulators from single agent activity alone*

269 The clonogenic HTS screen was performed on irradiated cells, and it is therefore expected
270 that some hits will result from robust single agent activity alone rather than radiation modulatory
271 effects. Furthermore, radiation modulation could result in a simple sum of the drug and radiation
272 effects (additive interaction) or greater than the simple sum (synergistic interaction). To identify true
273 radiation modulators in the 39-compound hit pool, each compound was retested with and without
274 radiation (3Gy for CCL-30, 9Gy for SW837) at varying concentrations (10 nM, 0.1 μM, and 1 μM). In
275 SW837 cells, 6 compounds exhibited potent single agent activity, producing growth inhibition of
276 greater than 90% at 10 nM drug (for example NSC330770 in Supplemental Figure 5A). 29
277 compounds exhibited what is expected if combination with radiation is additive (for example,
278 NSC250594 in Supplemental Figure 5B). And 4 compounds exhibited greater than additive
279 interaction with radiation, which is what is expected of true radiation modulators (Figure 6A). Two of

280 these compounds (NSC177365 and NSC228155) exhibited greater than additive activity at 1 μM, the
281 drug concentration used in the primary screen. The other two compounds exhibited complete single
282 agent growth inhibition at 1 μM, but partial single agent activity when reduced to 10 nM (NSC94600
283 and NSC317003). And at 10 nM both showed greater than additive effects when combined with
284 radiation. Similar results were obtained with CCL30 cells; three of 39 compounds showing greater
285 than additive effects with radiation. All three showed robust single agent activity at 1 μM, with radiation
286 modulation becoming apparent at 1-100 nM depending on the compound (Figure 6B and
287 Supplemental Table 1A). Of these hits, two are shared between both cell lines, resulting in a total of 5
288 potential radiation modulators identified (Supplemental Table 1B).
289
290 *Validation of pilot HTS hits in traditional clonogenic assays*
291 To this point, the data from the primary clonogenic HTS and confirmatory experiments were
292 obtained using compounds supplied as 10 mM DMSO stocks in multi-well plates from the NCI. Our
293 previous experience indicates that plated compounds in solution can exhibit reduced activity relative
294 to freshly acquired and prepared solutions of the same compound. Therefore, we acquired from the
295 NCI vialed solid aliquots of the five potential radiation modulators and prepared fresh DMSO stocks.
296 As expected, freshly prepared compounds show varying degrees of activity as single agents relative
297 to plated drug, ranging from similar (NSC94600, NSC317003, NSC149286) to 10-fold more active
298 (NSC177365, NSC228155) (Supplemental Figure 5C). Therefore, to test for radiation modulation, we
299 titrated each fresh compound to induce 25-30% growth inhibition. We then used fresh dilutions at
300 these respective concentrations to generate survival curves with a broad range of 9 radiation doses,
301 and calculated the resulting DMF for each hit. These experiments were performed first in 96-well
302 plates as with the clonogenic HTS (Supplemental Figure 6), and then validated with traditional 6-well
303 clonogenic assays (Figure 7).
304 In the 96-well format, three of four hits found in the SW837 screen recapitulated radiation
305 modification with DMF's >1.4 (Supplemental Figure 6A-C), with the exception being NSC228155 (not
306 shown, see Discussion). In CCL-30 cells, all three hits recapitulated (Supplemental Figure 6E-G).

307 Interestingly, NSC149286, a hit in CCL-30 screen but not SW837 screen, was found also to modulate
308 radiation in SW837 when tested with 9 radiation doses; a possible explanation for this result is
309 provided in the Discussion. More important, all compounds that exhibited radiation dose modification
310 in miniaturized formats exhibited nearly identical modification in traditional formats, with DMFs ranging
311 from 1.375 to 1.581 in SW837 (Figure 7A-D) and from 1.220 to 1.411 in CCL-30 (Figure 7E-G). We
312 conclude that the clonogenic HTS methodology can robustly identify compounds that function as
313 radiation modulators in traditional clonogenic formats, and therefore can serve as a functional novel
314 drug discovery platform with a true clonogenic endpoint.
315

316 **Discussion**

317 *A validated HTS screen with a clonogenic endpoint*

318 We describe here an integrated system capable of performing HTS screens with a clonogenic
319 endpoint to identify radiation modulators. This system differs significantly from previously described
320 methodologies in key aspects: 1) while miniaturized, this is a true clonogenic assay wherein cells are
321 seeded at clonogenic densities and grown over traditional time periods, with traditional endpoints and
322 statistical practice; 2) the ability to perform clonogenic HTS in multiple cell lines for seven cancer
323 indications where RT is a standard treatment module; 3) automated colony scoring based upon
324 nuclei count rather than colony size masks, ensuring accurate colony counts regardless of
325 radiation/drug induced changes in colony morphology; and 4) integrated radiation dosimetry ensuring
326 consistency over HTS runs. Our data demonstrate that this clonogenic HTS system can identify
327 compounds that exhibit robust radiation Dose Modifying Factors (DMF) in traditional clonogenic
328 assays. Throughput is markedly increased due to miniaturization and automation of colony counting.
329 The significance of these innovations cannot be overstated: the traditional clonogenic assay is
330 laborious, including manual cell seeding, irradiation, drug treatment, fixing and staining, that ultimately
331 culminates in exceedingly tedious manual counting of colonies. Simple human limitations reduce
332 screening capability to no more than a dozen compounds per day. In contrast, our automated
333 clonogenic HTS is capable of processing over 2000 compounds a day, with throughput capacity that

334 can be scaled linearly with additional incubator space, off-the-shelf imaging units, and computer
335 processing.
336
337 *Clonogenic assay harmonization*
338 There is increasing recognition that disparate clonogenic assay protocols make comparing
339 data across research labs nearly impossible (*5, 18*). Worse, some agents identified as radiation
340 sensitizers appear not to be so, and vice versa depending upon the research group and assay
341 protocol used (*14*). The clonogenic HTS described here address several parameters of concern. We
342 assessed the role of plate format (well size) in relation to clonogenic survival and identified conditions
343 wherein clonogenic radiation survival is equivalent between miniaturized and traditional 6-well formats
344 (Figure 1). Our automated colony segmentation methodology controls for radiation induced variations
345 in cell and colony shape/size by assessing nuclear position/number rather than size masks (Figure 3).
346 The resulting positional identification of actual nuclear counts allows for the accurate quantification of
347 colonies greater than 50 cells, in agreement with the parameters of the original clonogenic assay
348 colony (*6*). We standardized protocols to achieve a minimum colony number of 50 per well, as
349 indicated by the literature (*13*), and exceed this parameter in the pilot screens (Figure 4C, the second
350 column). We validate candidate radiation modulators with a broad range of radiation doses (9 total),
351 allowing for monitoring of the initial "shoulder" and exponential portions of survival curves (Figure 7
352 and Supplemental Figure 6). We use DMF to quantify radiation modulation here but have developed
353 automated programs capable of computing additional parameters including, survival fraction at a
354 given radiation dose and α/β ratio from linear-quadratic plots. Finally, we address inter-person
355 variation by fully automating the clonogenic process, and conducting the pilot screens independently
356 with two researchers, in two different cell lines.
357
358 *Radiation modulators identified in the pilot screen*
359 The pilot screen and reproducibility tests identified five potential radiation modulators (Figure
360 5A): two shared between the SW837 and CCL-30 screens (NSC94600, NSC317003), two exclusive

361 to SW837 (NSC177365, NSC228115), and one exclusive to CCL-30 (NSC149286). Of these, only
362 NSC228155, an EGFR agonist (*19*), did not fully validate through subsequent experiments.
363 Specifically, radiation modulation was reproducibly observed with plated drug (Figure 6A), however
364 fresh compound exhibited 10-fold greater single agent activity (Supplemental Figure 5C) and showed
365 no modulation under any condition tested. The other four exhibited reproducible radiation modulation
366 in traditional 6-well clonogenic formats, regardless of variables between plated and fresh drug single
367 agent activity. Available data on these four compounds illustrate how our clonogenic HTS can identify
368 known as well as new radiation modulators.

369 One hit shared between the cell lines is Camptothecin (CPT, NSC94600) a well described
370 DNA intercalator and potent topoisomerase I inhibitor and a known radiation sensitizer (*20*). Clinically
371 relevant derivatives of Camptothecin include Irinotecan (Camptosar), a known radiation modulator
372 and standard of care for colorectal cancers which SW837 cells represent (*20, 21*). Our clonogenic
373 HTS was performed blind, using a library based on chemical structure and not for anti-cancer activity.
374 Therefore, identification of CPT is proof-of-concept that the screen is capable of accurately identifying
375 compounds that modulate radiation effects.

376 NSC317003, was shared between cell lines and is a structural analog of lucanthone and its
377 metabolite hycanthone (Supplemental Figure 8A). Lucanthone, a known DNA intercalator and
378 topoisomerase I/II inhibitor, was historically used to treat parasitic diseases, but more recently has
379 been studied as a radiation sensitizer (*22*). Lucanthone inhibits post-radiation DNA repair in human
380 cancer cells (*23*), accelerates the regression of brain metastases in patients receiving radiation
381 therapy (*24*), and has been in clinical trials as a radiation sensitizer for the treatment of Glioblastoma
382 (NCT01587144) and brain metastases of NSCLC (NCT02014545). Lucanthone and hycanthone are
383 not in Diversity Set VI but identification of a structural analog in our clonogenic HTS further validates
384 its ability to identify radiation modulators.

385 NSC149286 is an analog of curcumin (Supplemental Fig. 8B), which is has multiple reported
386 activities including radiosensitization of cancer cells and radio-protection of normal cells [(*25-27*);
387 reviewed in (*28*)]. NSC149286 exhibited slightly greater than additive effect with radiation in CCL-30

388 cells (Figure 6B) and an additive effect in SW837 cells (not shown). Fresh compound, however,
389 showed robust radiation modulation in both cell lines when tested with 7 radiation doses (Figure 7D
390 and G). The survival curves of SW837 suggest an explanation for the discrepancy between the
391 secondary screen and radiation dose range response data. Specifically, survival with or without drug
392 was equivalent at low doses of radiation, began to diverge at 9Gy, and increased significantly at
393 higher radiation doses (Figure 7D, Supplemental Fig. 6D). The level of divergence at 9Gy in the
394 secondary screen was considered to be within error and therefore scored as additive for SW837.
395 Greater divergence at >9Gy, however, resulted in a DMF>1. These data suggest that while use of a
396 single radiation dose in the pilot and secondary screens maximizes the number of compounds
397 assessed, we may miss modulators that show effect at other doses (false negatives). We believe this
398 tradeoff to be acceptable as compounds that modulate RT under many conditions are a preferred
399 outcome. While curcumin is not in the NCI Diversity Set VI library, identification of a closely related
400 analog further validates the ability of the clonogenic HTS to identify radiation modulators.
401 The pilot screens also identified a compound with known antineoplastic activity but previously
402 undescribed radiation modulation effects. This hit, specific to SW837 cells, was NSC177365 which
403 has been demonstrated to partially restore folding of the G-quadruplex in human telomerase reverse
404 transcriptase (hTERT) promoters, thereby silencing hTERT transcription and leading to cell death in
405 melanoma cells (*29*). Further, NSC177365 and related compounds have been demonstrated to inhibit
406 cystathionine β-synthase, reducing intratumor production of hydrogen sulfide which plays important
407 roles in cancer proliferation and migration [reviewed in (*30*)]. Finally, a patent for use of NSC177365
408 as a bone morphogenetic protein (BMP) signaling modulator has been granted (US-10434220-B2),
409 which has direct relation to cancer therapeutics [reviewed in (*31*)]. The ability of NSC177365 to
410 modulate radiation in SW837 cells significantly expands its potential role as a cancer therapeutic.
411 Interestingly, NSC177365 exhibited exquisitely robust single agent activity in CCL-30 cells even at the
412 lowest doses tested, to the point that no relevant compound/RT data could be obtained.
413
414 *Standard Operating Procedure and looking forward*

415 The streamlined clonogenic HTS procedure includes 4 critical steps: (1) a primary screen with
416 a single LD_{30-40} radiation dose for the cell line and 1 μM of drug library; (2) the use of Z>2 from the
417 population mean to call hits; (3) a secondary screen with 0 and LD_{30-40} Gy and each hit at 10 nM, 0.1
418 μM and 1 μM; and (4) validation in 6-well plates using fresh compound titrated to achieve 20-30%
419 growth inhibition on its own. We believe this methodology is a major step forward for the unbiased
420 identification of novel radiation modulators, but represents only the initial uses of this technology.
421 Clonogenic HTS may be adapted to screen for compounds that inhibit regrowth of tumors following
422 any standard therapies, as well as identify efficacious pair-wise combinations among existing
423 therapeutic agents. For example, an IC_{50} dose of a single agent may be applied to all wells, much like
424 single dose radiation, and used as the basis to screen for compounds that modulate that activity
425 towards a clonogenic endpoint. Further adaptions to this methodology in development include 3D
426 tumor growth, co-culture with somatic cells to recreate tumor microenvironments, and assays for
427 immune-cell infiltration in the context of irradiation.
428
429 **Materials and Methods**
430 *Cell culture*
431 All cell lines were obtained from ATCC (American Type Culture Collection) except for FLO-1,
432 A204 and KYSE270 which were obtained from Sigma-Aldrich. Cell line identities were confirmed by
433 STR fingerprinting at the University of Colorado Cancer Center Cell Technologies Shared Resource.
434 All cell lines were maintained in DMEM with 10% FBS supplemented with Penicillin (50 units/ mL) and
435 Streptomycin (50 µg/mL).
436
437 *Transduction with nucRFP lentivirus*
438 Cells with low passage number (20 or fewer) were labelled with Nuclight Red (Sartorius)
439 according to manufacturer's instructions. Briefly, cells were plated to 15-35% confluence and allowed
440 to adhere overnight. Nuclight Red lentivirus was added at 1 multiplicity of infection (MOI)/cell along

441 with Polybrene (8 μg/mL). After 24 hours, media was replaced with fresh media containing 1 μg/mL
442 puromycin. Media with puromycin was relaced every 48 hours until stable clones were selected.
443
444 *Plating efficiency, cell seeding and colony formation*
445 Cells were stained with trypan blue (1:5 in PBS) and manually counted with a
446 hematocytometer. Cell populations with less than 10% trypan blue-stained (dead) cells were serially
447 diluted in media and plated in 24 or 96 well plates to determine plating efficiency (PE) and optimum
448 cell seeding for clonogenic assays. PE varied widely among the 13 cell lines (>90% for A375 to <16%
449 for KYSE270) and were consistent in additional assays for each cell line. Colony formation was
450 monitored daily over 7-10 days to determine an endpoint where colonies were still distinguishable.
451
452 *Radiation survival curves of parental and nucRFP-labelled cells*
453 Parental and nucRFP cells were plated in 6, 24 or 96 well formats to give equivalent colony
454 densities and irradiated 24 hours later. After 10 days, parental cells were fixed and stained with 0.1%
455 crystal violet or 0.4% Sulforhodamine B for manual counting whereas nucRFP derivatives were
456 imaged live using the IncuCyte S3 for colony segmentation.
457
458 *High throughput screen*
459 The SuviCa high throughput system consists of 4 functional instruments: a 37°C CO_2 incubator
460 (LiCONiC), a Fluent 780 drug distribution workstation (Tecan), a TEMPEST Liquid Handler
461 (FORMULATRIX), and a custom irradiator (Hopewell Designs) calibrated to deliver 3.2 Gy/min. Cells
462 were counted using trypan blue exclusion described above and plated according to previously
463 determined cell seeding (250 cells/well for CCL30, 200 cells for SW837) in 150 μL/well in 96-well
464 plates. To prevent edge effects of prolonged incubation, outside wells were filled with 200 μL sterile
465 H_2O. Cells were incubated overnight before irradiation, and drugs were added immediately after
466 irradiation.

467 The NCI Diversity Set VI is arrayed in 96 well plates at 10 mM in 20 µL DMSO. Upon arrival,
468 compounds were partitioned into duplicate plates at 5 µL per drug and stored at -20°C. For all assays,
469 a replicate plate was thawed only once and diluted 1:40 in complete media 1-2 hours before
470 irradiation. 1.4 µL of diluted compound was added to 200 µL of complete media. The Fluent was
471 programmed to add 25 µL/well to give a final concentration of 1 µM in a total volume of 175 µL/well.
472 Drugs were added after irradiation to avoid X-ray damage to the drugs. Plates were incubated for 10
473 days before imaging in IncuCyte S3.
474
475 *Automated nuclear fluorescent based live-cell colony counting*
476 This is comprised of three steps: image preprocessing, colony segmentation, and nuclei
477 segmentation. Parameters for each step are empirically optimized for cell line and growth format
478 specific conditions, then subject to images from a given experiment in batch. Image preprocessing
479 involves reducing spurious signal from culture well walls (Crop Perc.) and defining the maximum and
480 minimum signal values within the image to be analyzed (Thresh min/max). Colony segmentation
481 involves implementing Gaussian blur functions to merge proximal nuclear signals into discreate
482 colonies (Blur Factor), followed by iterated erode functions to further refine Gaussian colonies and
483 remove other spurious signals (erode iter.), finally implementation of colony prominence thresholds
484 and seeded watershedding functions define actual colonies from the pool of putative Gaussian
485 colonies (colony prominence). Cell segmentation involves similar methodologies, wherein for each
486 identified colony individual nuclear signals are defined by prominence thresholds and seeded
487 watershedding functions (cell prominence). Further parameters allow for previewing of defined
488 portions of a given well image, allowing for optimization of the parameters for image processing and
489 colony/cell segmentation. Colony segmentation output for each well is provided in delimited text
490 format (CSV) containing each clonogens relative position within the well, and the nuclei count for all
491 colonies. Nuclei threshold (>50) is then applied to identify clonogens. A JAVA applet employing this
492 methodology can be obtained here: download link (to be available upon manuscript acceptance).

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497 grant R35 GM130374 to TTS.

498 **Figure and Table Legends**

499 **Figure 1. Identification of cell lines that are amenable to clonogenic growth in a small well**
500 **format.**

501 (A) Five cell lines that show the variation in colony size. Each was grown for 10 days to reach
502 colonies of 50 or more cells.

503 (B) Growth in 6, 24 and 96 well formats. 24 well images are shown at 2.3x magnification to
504 display the same final area as 6 well. 96 well growth is magnified 5.4x times to show equivalent area
505 as the 6 well format.

506 (C-E) A375 metastatic melanoma and SW837 rectal adenocarcinoma cells were seeded at the
507 same relative density (cells/surface area) but in different volumes per well in 6-, 24- or 96-well plates.
508 Cells were irradiated with 3, 6 or 9 Gy 24 hours after seeding. Clonogenic survival was quantified 10
509 days (SW837) or 7 days (A375) post radiation.

510

511 **Figure 2. Chemoradiation responses in 6-, 24- and 96-well plates.**

512 (A-D) CCL30 HNSCC and SW837 rectal adenocarcinoma cells were seeded at the same
513 relative density (cells/surface area) in 6-well (4 ml/ well), 24-well (1ml/well) and 96-well (150 µl/well)
514 plates and irradiated 24 hours after seeding and drugs were added immediately after irradiation.
515 Clonogenic survival was determined 10 days post radiation. Dose modifying factor (DMF) was
516 computed according to (*15*).

517 (E) Summary of additional known radiation sensitizers with SW837, A375 and LN-18 in
518 different plate formats.

519

520 **Figure 3. Colony segmentation allows automated colony counting.**

521 (A) A diagram to illustrate why a constant area mask cannot be used when cells spread after
522 irradiation.

523 (B) The area of a 50-cell colony increases with radiation for Cal27 HNSCC cells.

524 (C) The same well of a 6-well plate is shown stained for manual counting or imaged for nuclear
525 RFP. 'Colony segmentation' panel shows the RFP image after automated digital segmentation. Three
526 colonies are shown magnified to illustrate how the raw nuclear images are grouped or 'segmented'
527 into separate colonies based on the position of the nuclei.
528 (D-G) Manual counting and automated segmentation show similar results in clonogenic
529 survival after irradiation as do parental and RFP-expressing derivatives of CCL30 HNSCC and
530 SW837 rectal cancer cells.
531
532 **Figure 4. Results of NCI Diversity Set VI screen for two cell lines.**
533 (A-B) Data from the pilot screen through the Diversity Set of compounds using SW837 (A) and
534 CCL30 (B) cells. For both cell lines, the data for individual drugs are arranged along the x-axis in
535 same order that they occur on the plates of the drug library.
536 (C) Screen parameters and Z' table.
537
538 **Figure 5. Results of NCI Diversity Set VI screen for two cell lines.**
539 (A-B) Retest of 41 hits shared between the two cell lines from the Diversity set screen. Percent
540 (%) clonogenic survival relative to DMSO vehicle controls from the pilot screen (1st screen) are
541 plotted against the same values in the re-test (2nd screen) for SW837 (A) and CCL30 (B). Thirty nine
542 of 41 were within two STD from the vehicle average in both screens, demonstrating reproducibility.
543 Note that the data point at the coordinates 0,0 is comprised of multiple over-laid points; these are
544 wells with no growth in both screens.
545 (C) A summary of screening steps and the results.
546
547 **Figure 6. A secondary screen differentiates radiation modulators from cytotoxic agents.**
548 (A-B) Clonogenic survival after treatment with DMSO (control), radiation alone, drug alone or
549 the combination with drug with radiation in SW837 (A) and CCL30 (B) cells. Dashed lines show the

550 expected survival if the drug and radiation acted in an additive manner. Survival below the dashed
551 lines of combined treatments are likely caused by a synergistic interaction between the drug and
552 radiation. Control and radiation alone samples are an average of 6 replicate wells each. The others
553 are from one well per condition.
554
555 **Figure 7. Radiation modulators found in the HTS were validated in 6-well clonogenic assays.**
556 (A-D) Four compounds that showed greater than additive effect with radiation in the secondary
557 screen using SW837 cells were assessed in traditional 6-well clonogenic assays using 8 radiation
558 doses. Dose Modifying Factors indicate that four enhanced the effect of radiation. Drug
559 concentrations used with SW837 cells were 8 nM (NSC94600), 15 nM (NSC317003), 250 nM (NSC
560 177365) and 150 nM (NSC149286). Drug concentrations used with CCL-30 cells were 1 nM
561 (NSC94600), 10 nM (NSC317003) and 100 nM (NSC149286).
562 (E-G) Three compounds that showed greater than additive effect with radiation in the
563 secondary screen using CCL30 cells were assessed in traditional 6-well clonogenic assays using 7
564 radiation doses. Dose Modifying Factors indicate that three enhanced the effect of radiation.
565
566 **Table 1.** 13 cell lines that are amenable to miniaturization. Cancer type and available
567 information on mutation status and patient origin are shown. NSCLC = Non-Small Cell Lung Cancer;
568 HNSCC = Head and Neck Squamous Cell Carcinoma. The information is compiled from (*32-37*) and
569 the following online sources:
570 https://www.cbioportal.org/
571 https://www.atcc.org/
572 https://seandavi.github.io/UCCCReporter/reference/cell_line_ancestry.html
573 https://web.expasy.org/cellosaurus/

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660

Figure 1.

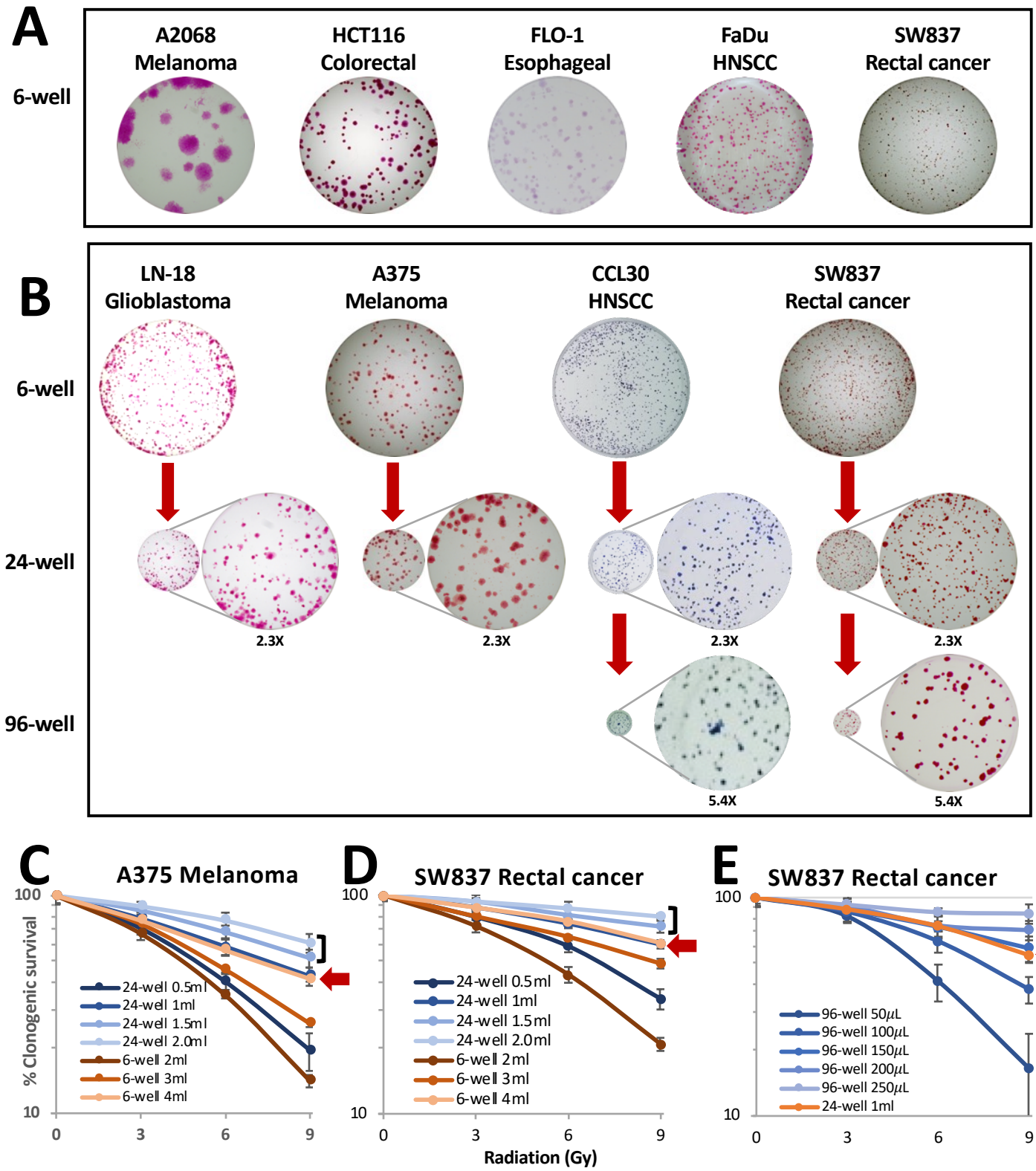
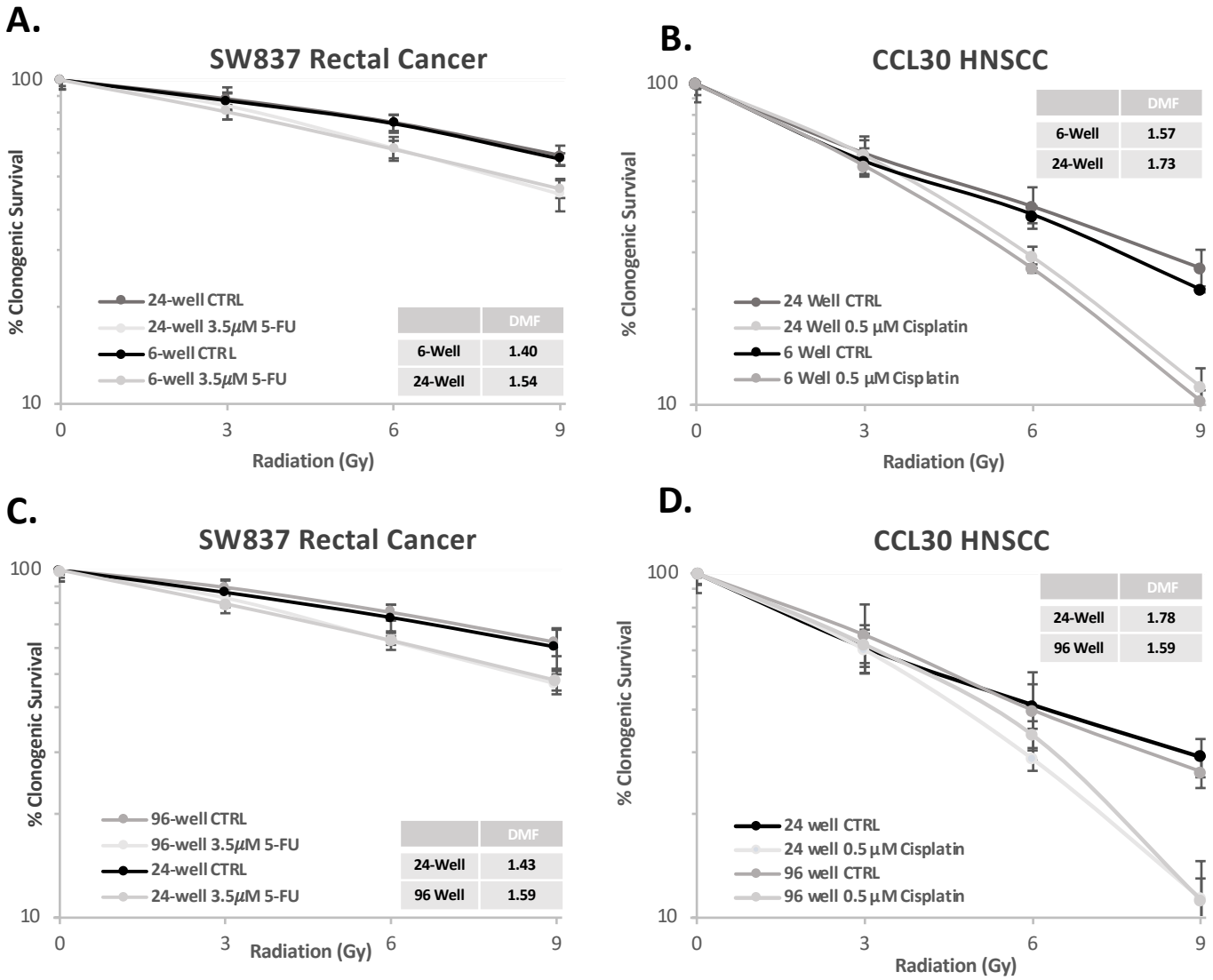

A
6-well
A2068
Melanoma
HCT116
Colorectal
FLO-1
Esophageal
FaDu
HNSCC
SW837
Rectal cancer
B
6-well
24-well
96-well
LN-18
Glioblastoma
A375
Melanoma
CCL30
HNSCC
SW837
Rectal cancer
2.3X
2.3X
2.3X
2.3X
5.4X
5.4X
C
A375 Melanoma
% Clonogenic survival
100
10
0
3
6
9
24-well 0.5ml
24-well 1ml
24-well 1.5ml
24-well 2.0ml
6-well 2ml
6-well 3ml
6-well 4ml
D
SW837 Rectal cancer
100
10
0
3
6
9
24-well 0.5ml
24-well 1ml
24-well 1.5ml
24-well 2.0ml
6-well 2ml
6-well 3ml
6-well 4ml
Radiation (Gy)
E
SW837 Rectal cancer
100
10
0
3
6
9
96-well 50μL
96-well 100μL
96-well 150μL
96-well 200μL
96-well 250μL
24-well 1ml

Figure 2.

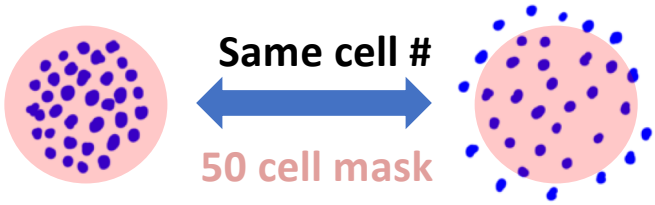


E.

Cell type	Cell line	Drug	Cell format	Ave DMF	Std Dev
Rectal Cancer	SW837	5-FU	6-well	1.379	0.153
			24-well	1.487	0.155
			96-well	1.494	0.133
		Irinotecan	6-well	2.222	0.574
			24-well	1.937	0.613
		Oxaliplatin	6-well	1.690	0.153
			24-well	1.523	0.187
Melanoma	A375	Dacarbazine	6-well	1.431	0.216
			24-well	1.624	0.384
Glioblastoma	LN-18	Temozolomide	6-well	0.921	0.168
			24-well	0.909	0.183

Figure 3.

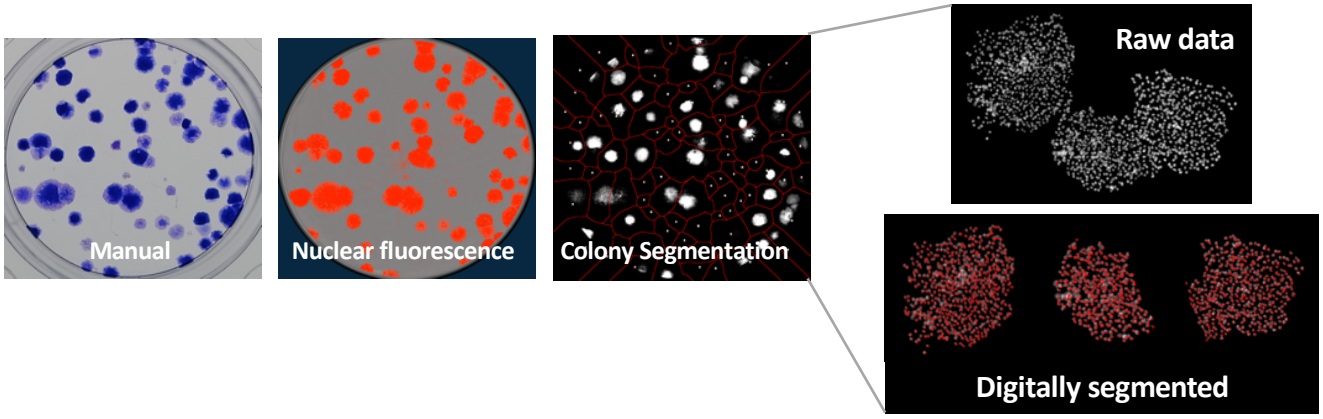
A.



B.

The area of a 50-cell colony of Cal27 cells in 6-well plates (X10⁴ µm²)				
	0 Gy	2 Gy	4 Gy	6 Gy
Avg	4.15	5.46	5.62	6.20
STD	1.29	0.95	0.82	1.34

C.



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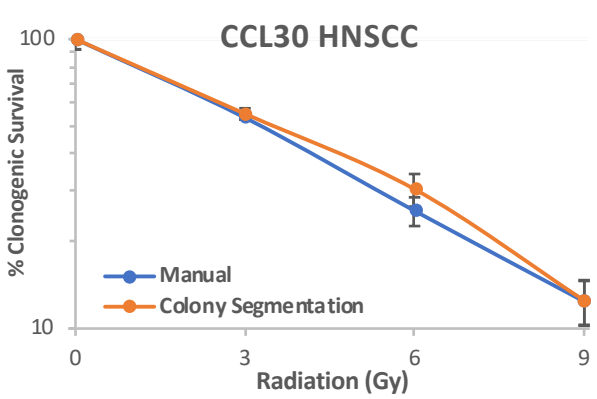

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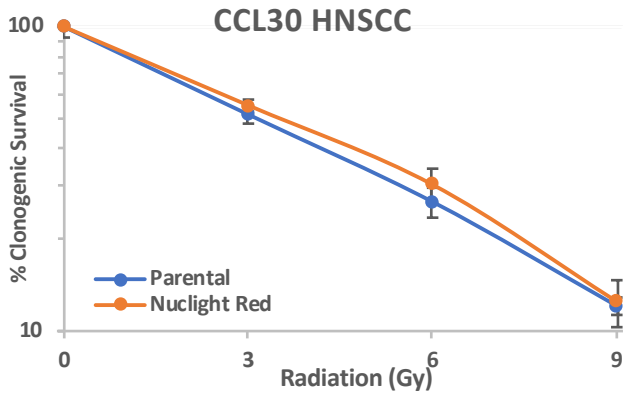

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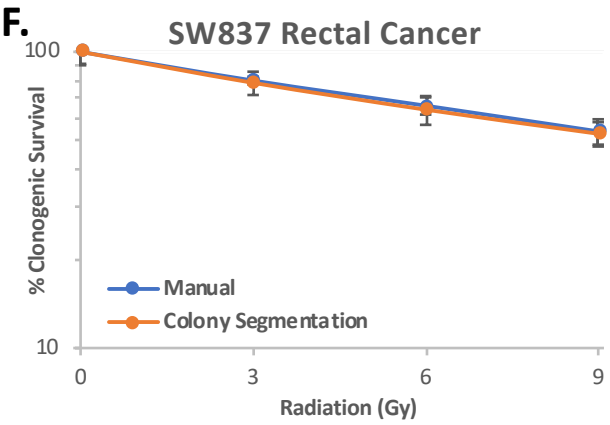


G.

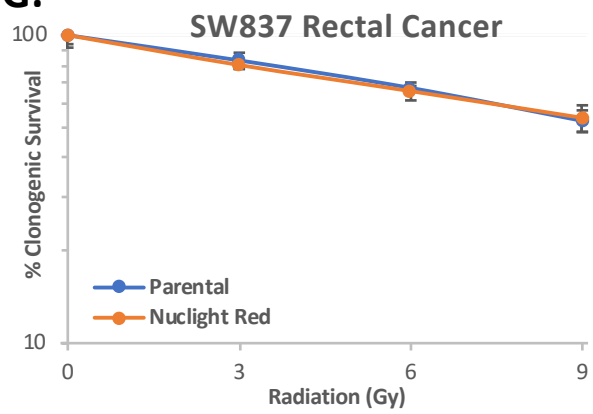
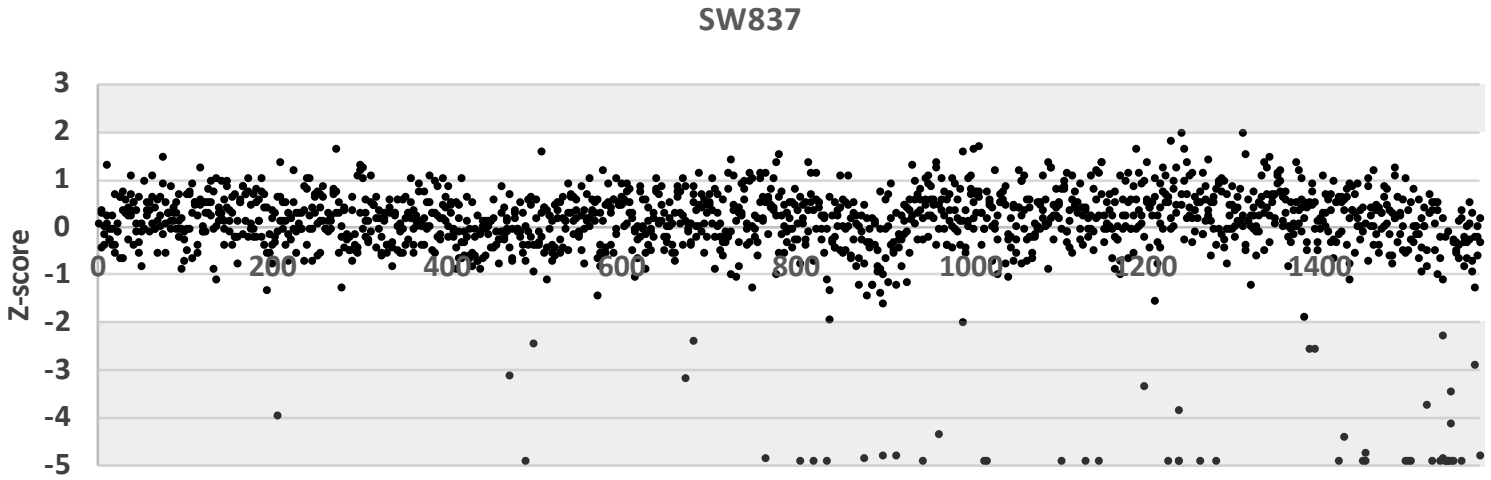
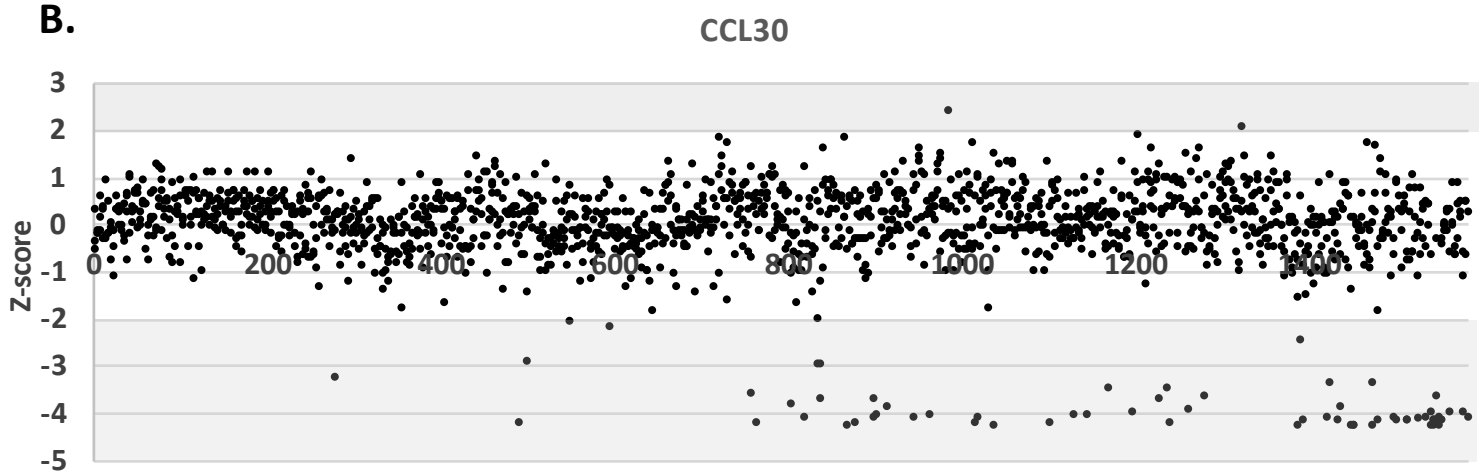


Figure 4.

A.



B.

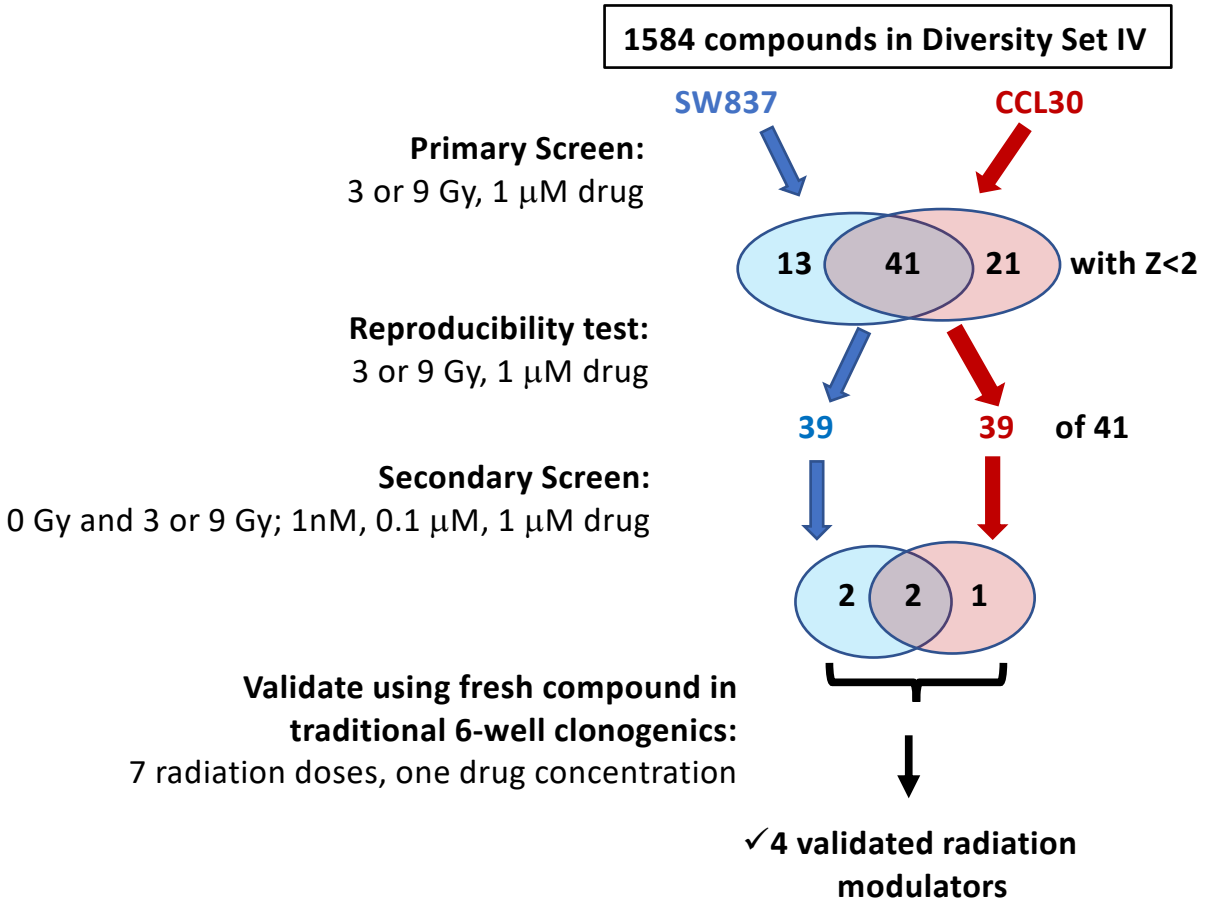


C.

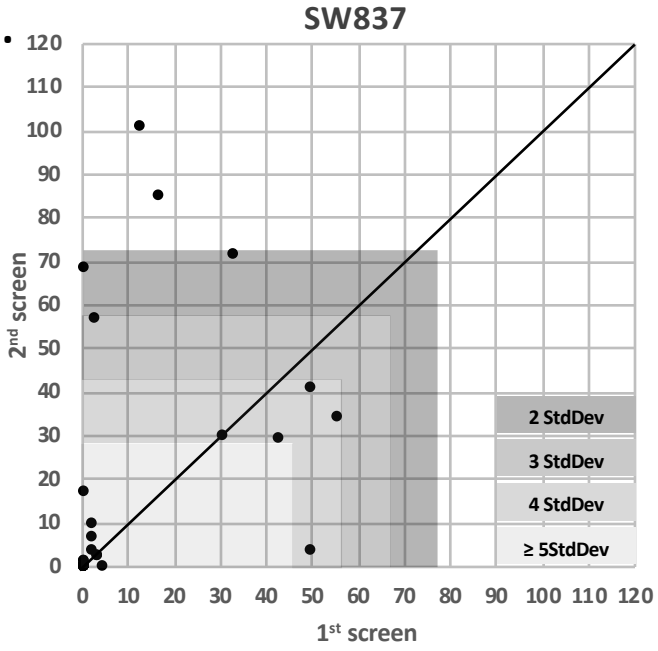
Cell line	m_{c-} (AVE -ctrl)	s_{c-} (STD -ctrl)	m_{c+} (AVE +ctrl)	s_{c+} (STD +ctrl)	+ctrl used	$Z' = 1 - \frac{(3\sigma_{c+} + 3\sigma_{c-})}{\lvert\mu_{c+} - \mu_{c-}\rvert}$
CCL30	66.0	7.7	1.2	0.9	Z -4 or less	**0.60**
SW837	110.2	13.2	0.5	1.1	Z -4.5 or less	**0.61**

Figure 5.

A.


1584 compounds in Diversity Set IV
SW837
CCL30
Primary Screen:
3 or 9 Gy, 1 µM drug
13
41
21
with Z<2
Reproducibility test:
3 or 9 Gy, 1 µM drug
39
39
of 41
Secondary Screen:
0 Gy and 3 or 9 Gy; 1nM, 0.1 µM, 1 µM drug
2
2
1
Validate using fresh compound in traditional 6-well clonogenics:
7 radiation doses, one drug concentration
✓4 validated radiation modulators

B.


SW837
2nd screen
1st screen
2 StdDev
3 StdDev
4 StdDev
≥ 5StdDev

C.

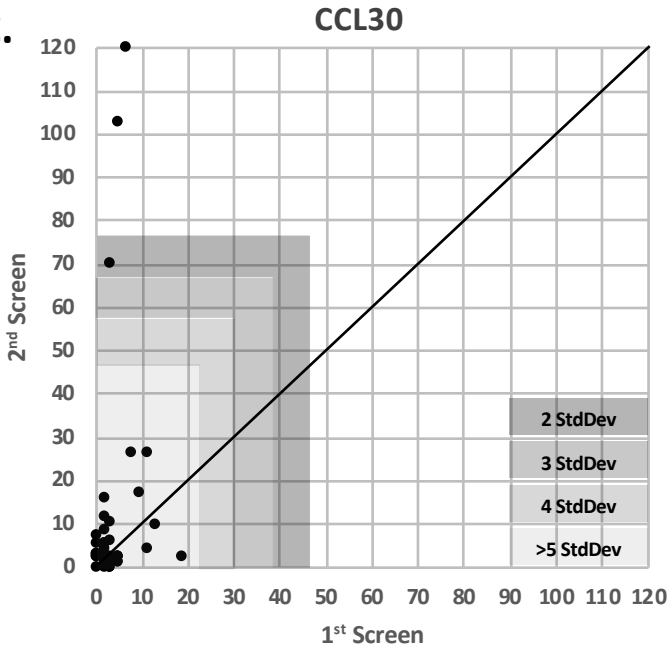
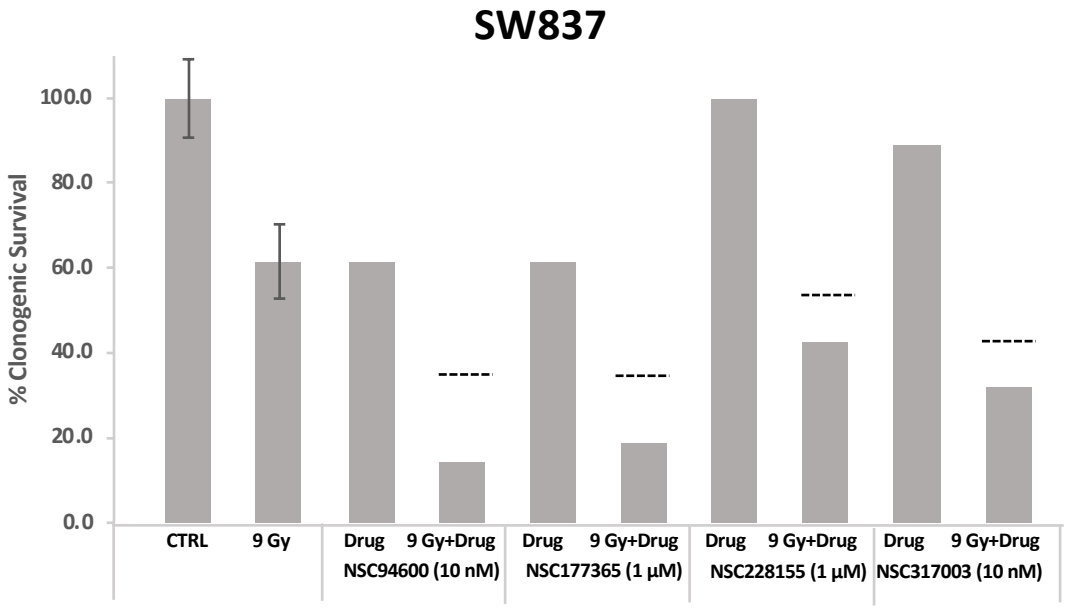

CCL30
2nd Screen
1st Screen
2 StdDev
3 StdDev
4 StdDev
>5 StdDev

Figure 6.

A.


SW837
% Clonogenic Survival
100.0
80.0
60.0
40.0
20.0
0.0
CTRL
9 Gy
Drug
9 Gy+Drug
NSC94600 (10 nM)
Drug
9 Gy+Drug
NSC177365 (1 µM)
Drug
9 Gy+Drug
NSC228155 (1 µM)
Drug
9 Gy+Drug
NSC317003 (10 nM)

B.

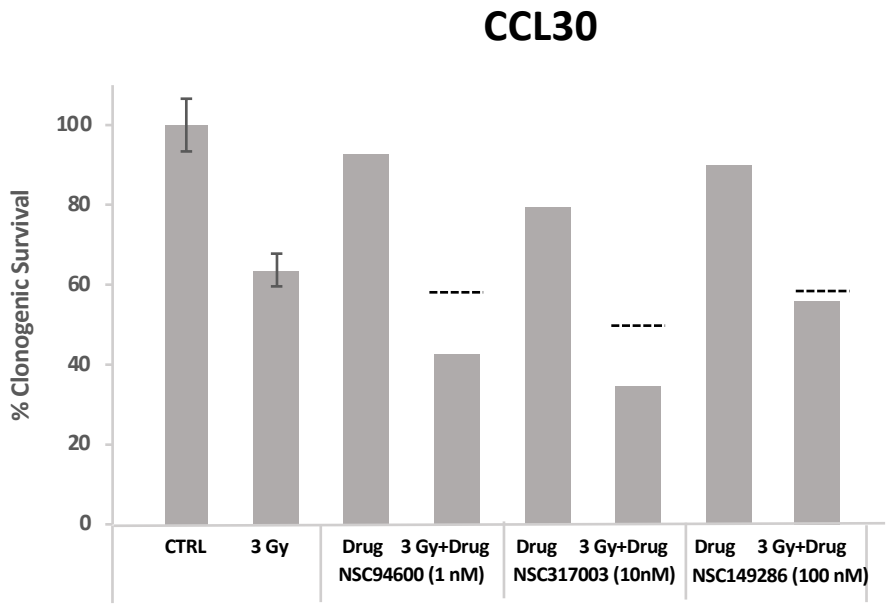

CCL30
% Clonogenic Survival
100
80
60
40
20
0
CTRL
3 Gy
Drug
3 Gy+Drug
NSC94600 (1 nM)
Drug
3 Gy+Drug
NSC317003 (10nM)
Drug
3 Gy+Drug
NSC149286 (100 nM)

Figure 7.

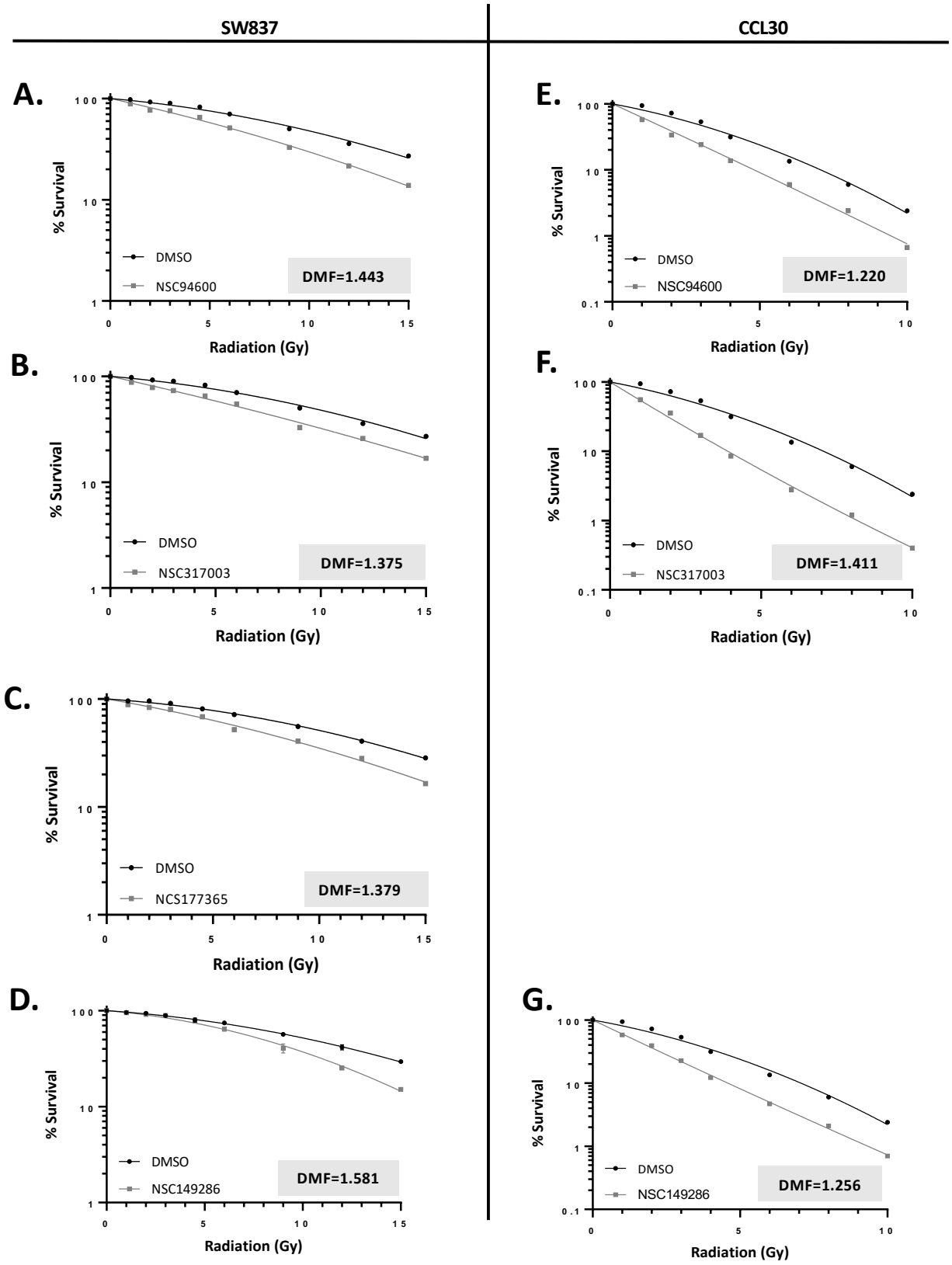

SW837
CCL30
A.
% Survival
Radiation (Gy)
DMSO
NSC94600
DMF=1.443
B.
% Survival
Radiation (Gy)
DMSO
NSC317003
DMF=1.375
C.
% Survival
Radiation (Gy)
DMSO
NCS177365
DMF=1.379
D.
% Survival
Radiation (Gy)
DMSO
NSC149286
DMF=1.581
E.
% Survival
Radiation (Gy)
DMSO
NSC94600
DMF=1.220
F.
% Survival
Radiation (Gy)
DMSO
NSC317003
DMF=1.411
G.
% Survival
Radiation (Gy)
DMSO
NSC149286
DMF=1.256

Table 1.

	Cancer type	Cell lines	Plate format	Mutations	Origin
1	NSCLC	NCI-H358	24 96	TP53 homozygous del, KRAS G12C	Caucasian male, metastatic site before chemotherapy
		NCI-H460	24	TP53 WT, CDKN2A del, KRAS G61H, PIK3CA 1633G>A	Male of European ancestry, Lung effusion
2	HNSCC (HPV^-)	CCL30	24 96	STK11 A205T, BRD4-NUTM1 fusion	Caucasian male, nasal septum, metastatic pleural effusion
		Cal27	24	TP53 H193L, CDKN2A del E69*	Caucasian male, tongue
3	Esophageal cancer	FLO1	24	TP53 C277F	Caucasian male, primary oesophageal adenocarcinoma
		KYSE270	24	EGFR L861Q, TP53 X187_splice, CDKN2A del	Japanese male, primary oesophageal squamous cell
4	Soft tissue sarcoma	A204	24	Dip/tetraploid, EGFR L747_S752del, TP53 WT	Female of European ancestry, rhabdomyosarcoma, muscle
		SW872	24	Hyper-triploid, BRAF V600E, CDKNA R80*, TP53 I251N	Caucasian male, liposarcoma
5	Metastatic melanoma	A375	24	EGFR L858R D256Y, TERT AMP, TP53 WT	Female of European ancestry, malignant melanoma
		G-361	24	BRAF V600E, MET AMP, CDKN2A del	Caucasian male, malignant melanoma
6	Rectal cancer	SW837	24 96	TP53 R248W, KRAS G12C, APC R213* R1450*	Caucasian male, grade IV, adenocarcinoma
		SW1463	24 96	TP53 743G>A, APC V452fs*7, FBXW7 1436G>A, KRAS 34G>T	Caucasian female, adenocarcinoma
7	Glio-blastoma (only 1 fits criteria)	LN-18	24	TP53 C238S, PIK3CB E1051K, PTEN WT, CDKN2A del	Caucasian male, right temporal lobe