One-week 96-well soft agar growth assay for cancer target validation

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Soft agar growth, used to measure cell anchorage-independent proliferation potential, is one of the most important and most commonly used assays to detect cell transformation. However, the traditional soft agar assay is time-consuming, labor-intensive, and plagued with inconsistencies due to individual subjectivity. It does not, therefore, meet the increasing demands of today's oncology drug target screening or validation processes. This report describes an alternative 96-well soft agar growth assay that can function as a replacement for the traditional method and overcomes the aforementioned limitations. It offers the following advantages: a shortened assay duration (1 week instead of 4 weeks) that makes transient transfection or treatment possible; plate reader quantification of soft agar growth (measuring cloning efficiency and colony size); and a significant reduction in required labor. Higher throughput also makes it possible to process large numbers of samples and treatments simultaneously and in a much more efficient manner, while saving precious workspace and overall cost.

INTRODUCTION

Anchorage-independent growth, the cell's ability to proliferate without attachment to, or spreading onto, a substratum, is one of the hallmarks of transformation and the most accurate in vitro indication of tumorigenicity (1). It is, therefore, the most commonly used criteria for the validation of oncology targets. The primary method of monitoring anchorage-independent growth is the detection of soft agar colony formation, which measures proliferation by manual counting of colonies in semisolid culture media. This traditional assay, though effective, is rather laborious in the initial setup and final quantification stages. For even a limited number of samples, the experiment is quite cumbersome, demanding a great deal of incubator space due to the traditional method's rather bulky scale. Also, the duration of the assay is long; 3 or 4 weeks are usually required to observe the indicated phenotype. Furthermore, the necessarily long culture period significantly increases the chance of contamination and reduces productivity. Lastly, the traditional counting of colonies by eye is subject to bias, and colony size is difficult, if not impossible, to determine meaningfully.

In an attempt to reconcile these limitations, anchorage-independent growth in microplates was assessed using liquid culture poly (2-hydroxyethylmethacrylate) (poly-HEMA; Sigma, St. Louis, MO, USA)-coated plates, in which anchorage-independent growth or survival can be measured (2). However, this method has certain limitations: (*i*) it is usually rather difficult to coat the plate homogeneously (which is important to ensure consistent results); (*ii*) the variant growth assay is not widely accepted as a substitute for measuring growth in semisolid medium (perhaps explaining why there are no such commercially coated plates available); and (*iii*) evidence suggests that this method may not measure the exact same oncological properties as the traditional soft agar assay. In contrast to these limiting factors, the 96-well semisolid culture media growth assay has enjoyed remarkable success in the examination of hemopoietic progenitor cell growth (3).

Target discovery equipped with current genomics tools (i.e., global gene expression profiling, etc.) identifies many candidates as potential drug targets. However, the majority of these are false positives with very few remaining candidates actually possessing disease-modifying properties. Phenotypic validation of these candidates has become the bottleneck in identifying drug targets and thus requires a more efficient, more reliable screening method. A high-throughput soft agar growth assay, such as the one described here, may prove to be a solution. We have tested an alternative soft agar growth assay in a 96-well microplate using fluorimetric excitation and emission as a readout of cell proliferation in semisolid media. In this variant method, both colony number and colony size are contributors to an aggregate measure of viable cell proliferation. Because quantification is accomplished using a plate reader, determinations previously made manually (and thus subject to individual bias) are rendered obsolete. The plate reader's ability to "see" colonies that remain invisible to the naked eye results in extremely accurate, unbiased measurements, thereby tremendously improving the quantitative quality of the system. In addition, a short incubation time can be utilized since one does not need to wait for visible colonies to form, making it possible to validate targets using transient transfection and/or treatment, further increasing productivity. Using a small assay format is advantageous in that it allows the researcher to screen nearly one hundred times the number of samples in a given volume with decreased use of reagents and preparatory work. In this report, we tested a higher throughput variant assay against the traditional assay in two respects: (*i*) soft agar growth of cells with different anchorage-independent growth potentials and (*ii*) transient transfection versus stable transduction of small interfering RNA (siRNA) expression as validation methods.

MATERIALS AND METHODS

Cells

HeLa is a cervical carcinoma cell line, and HeLaHF is a HeLa revertant with a loss-of-transformation phenotype (4). DLD-1 is a human colon carcinoma cell line. These cells were cultured in 1× Dulbecco's modified Eagle's medium (DMEM), 10% fetal bovine serum (FBS), 2 mM L-Glutamine (L-Glu), 1× nonessential amino acids (NEAAs), and 1% sodium pyruvate (all from Invitro-

Table 1. Target Sequences and Primers

Targets for In Vitro Transcribed siRNA	
Name	**Sequence**
$Kras^{D13}$	5′-GTTGGAGCTGGTGACGTAG-3′
CNTL	5′-GCGCGCTTTGTAGGATTCG-3′
PLK	5′-GAGACCTACCTCCGGATCA-3′
Targets for Lentiviral-Expressed siRNA	
Name	**Sequence**
$Kras^{D13}$	5′-GTTGGAGCTGGTGACGTAG-3′
CNTL	5′-GTGCGCTGCTGGTGCCAACCC-3′
Primers for Amplification of U6-siRNA Cassettes	
Name	**Sequence**
U6-$Kras^{D13}$ siRNA for	5′-GAACTAGTGGATCCGACGCC-3′
U6-$Kras^{D13}$ siRNA rev	5′-GGATCCAAAAAAGTTGGAGCTGGTGAC GTAGTCTCTTGAACTACGTCACCAGCTCCAA CAAACAAGGCTTTTCTCCAAGGG-3′
U6-luc siRNA for	5′-GGATCCAAGGTCGGGCAGGAAGAGGG-3′
U6-luc siRNA rev	5′-GGATCCAAAAAAGTGCGCTGCTGGT GCCAACCCTCTCTTGAAGGGTTGGCA CCAGCAGCGCACGGTGTTTCGTCCTTTCCAC-3′

CNTL, control; PLK, polo-like kinase; for, forward; rev, reverse; luc, luciferase.

gen, Carlsbad, CA, USA) in a humidified incubator (Ultra-Tech WJ301D; Baxter Scientific Products, West Chester, PA, USA) with 5% CO_2 at 37°C.

Measurement of Cell Growth

Anchorage-dependent growth (normal liquid cell culture) in 96-well plates. Cell suspensions (100 µL) containing 0–2 × 10^3 cells were plated into each well of a 96-well flat-bottom microplate. The cells were allowed to grow for 1–3 days before the cell growth was measured using alamarBlue™ staining (1:10 volume reagent; Biosource International, Camarillo, CA, USA), according to the manufacturer's instructions.

Anchorage-independent growth (soft agar cell culture) in 96-well plates. A mixture of 25 µL prewarmed (37°C) 2× Iscove's modification of Dulbecco's medium (IMDM) containing 20% FBS, 4 mM L-Glu, 2× NEAA, 0.6% sodium bicarbonate, 2% sodium pyruvate, 200 U/mL penicillin/streptomycin (Invitrogen), and 25 µL prewarmed (56°C) 1.2% Bacto™ Agar Select (BD Biosciences, San Jose, CA, USA) were plated onto each well of a 96-well microplate to serve as a prelayer for the assay. Ten microliters of cell suspensions containing 0–2 × 10^3 cells were mixed with 20 µL 2× IMDM and 30 µL 0.8% Bacto Agar Select in a 96-well round-bottom polypropylene microplate and transferred to the 96-well microplate containing the solidified prelayers. Semisolid feeder layers were then prepared by mixing 25 µL 2× IMDM and 25 µL 1.2% Bacto Agar Select and layered on top of the solidified cell layers. The cells were allowed to grow in the humidified 37°C incubator with 5% CO_2 for 1–2 weeks before cell proliferation and viability were scored using the recommended alamarBlue assay methods. Cell growth was measured using a CytoFluor® Series 4000 Multi-Well Plate Reader (PerSeptive Biosystems, Framingham, MA, USA), with excitation at 530 nm and emission at 590 nm.

Anchorage-independent growth (soft agar cell culture) in a 10-cm plate. A mixture of 2 mL of prewarmed (37°C) 2× IMDM and 3 mL prewarmed (56°C) 0.8% Bacto Agar Select per plate (0.4% final agar) were mixed with 1 mL cell suspension and seeded over a 0.6% agar/IMDM prelayer (8 mL) in a 10-cm dish. Semisolid 0.6% feeder layers (6 mL) were overlayed on top of the solidified cell layers. The cells were allowed to grow in the humidified 37°C incubator with 5% CO_2 for 21–28 days. Colony numbers were determined by Qcount™ (Spiral Technology, Norwood, MA, USA).

SiRNA Transfection and Lentiviral Vector Transduction of siRNA Expression Vector

Transient transfection of in vitro transcribed siRNA. siRNA against target $Kras^{D13}$, polo-like kinase (PLK), and a nonspecific sequence control siRNA were prepared using the GeneSilencer™ siRNA Construction Kit (Ambion, Austin, TX, USA), following the manufacturer's instructions. Target sequences are listed in Table 1. HeLa or DLD1 cells (5 × 10^3 or 1 × 10^4, respectively) were seeded on day 1 and transfected on day 2 with 10 nM of siRNA using Oligofectamine™ transfection reagent (Invitrogen), according to the manufacturer's recommendations. On day 3 (24 h posttransfection), the cells were trypsinized and a desired number seeded into either liquid culture or soft agar culture.

Transduction of cells with lentiviral vector expressing siRNAs. The pHIV-7 vector was a gift from M. Li, J.-K. Yee, and J. Rossi (City of Hope, Duarte, CA, USA). This vector was constructed from pHIV-7-GFP (5,6) by digestion with *Pst*I to remove the cytomegalovirus (CMV) promoter/enhanced green fluorescent protein (EGFP) cDNA cassette and religation of the 530- and 5.7-kb fragments. A 1.4-kb *Pvu*II/*Bam*HI fragment comprising the simian virus 40 (SV40) promoter/$puro^r$ cassette was isolated from the pPUR vector (BD Biosciences Clontech, Palo Alto, CA, USA), treated with Klenow to repair the *Bam*HI-digested 3′ overhang, and ligated into the *Sma*I site of pHIV-7 to yield pHIV-7-puro. Forward orientation of the SV40 promoter/$puro^r$ cassette was confirmed by digestion with *Bam*HI and *Xba*I to yield a 1.2-kb fragment.

Target sequences for the $Kras^{D13}$ (7) and control (luciferase) siRNAs are

listed in Table 1. Coding sequences for hairpin siRNAs were appended to U6 promoters by PCR amplification. The U6 promoter sequences in pSilencer (Ambion) and pTZ U6+1 (8) were used as templates for the construction of expression cassettes for $Kras^{D13}$ (7) and control (luciferase) siRNAs, respectively. PCR primers comprising the siRNA coding sequences are given in Table 1. PCR was performed using Platinum PCR SuperMix High Fidelity (Invitrogen). The PCR products were ligated into the pCR Blunt® II-TOPO® vector (Invitrogen), sequenced in both directions, digested with *Bam*HI, and ligated into *Bam*HI-digested pHIV-7-puro. The reverse orientation (i.e., U6 and SV40 promoters facing in opposite directions) was selected for both vectors.

Vesicular stomatitis virus G glycoprotein (VSV-G) pseudotyped lentiviral vector was packaged in 293FT cells using the ViraPower™ Lentiviral Packaging Mix (both from Invitrogen). DLD1 cells were transduced using standard methods (9) and subjected to selection with puromycin for 7–10 days. A desired number of cells was seeded into either the traditional or the 96-well soft agar culture for the assay.

RESULTS AND DISCUSSION

Anchorage-Independent Growth in 96-Well Soft Agar Plates

HeLa cells are known to form colonies in soft agar media and form tumors in nude mice, while the nontransformed derivative HeLaHF does not (4). The growth of HeLa and its nontransformed derivative HeLaHF was therefore compared in both normal liquid media and semisolid soft agar media in 96-well microplates. Our traditional soft agar assay indicated that HeLa shows a 50% cloning efficiency in soft agar, while HeLaHF shows approximately 1% cloning efficiency (data not shown). Varying numbers of cells ($0–2 \times 10^3$) were plated in each well and allowed to grow in their respective cultures. Growth in liquid culture represents anchorage-dependent growth and was measured 24 h later with alamarBlue staining, which contains an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. Growth in soft agar media represents anchorage-independent growth and was measured after 7 days. In liquid culture, a linear relationship between alamarBlue readings and cell number was observed for both HeLa and HeLaHF in the range from $0–2 \times 10^3$ cells (Figure 1A). The revertant HeLaHF cells showed slightly slower growth in liquid culture compared to the transformed HeLa cells. A linear relationship for anchorage-independent growth was observed for HeLa in the range from 6×10^2 to 2×10^3 cells/well, while the reading for HeLaHF was not above the background for up to 1.4×10^3 cells (Figure 1B). These results are consistent with the distinct anchorage-independent growth potential of each member of the isogenic cell pair, as observed in the traditional soft agar colony assay. Our observations have demonstrated for the first time that we can measure anchorage-independent growth reliably in a higher throughput system and, at the same time, reduce incubation times to just 1 week. In addition, these results provided us with a wide working range of cell densities (6×10^2 to 1.4×10^3 cells/well) that could be used to distinguish cells with different anchorage-independent growth potential.

We next compared the incubation times of samples receiving the same soft agar culture condition. Similar observations were found for 2-week incubations with the same cell plating densities (data not shown), except that higher alamarBlue values were achieved due to increased cell proliferation. Incubation for 3 weeks resulted in a significant increase in background readings and sample-to-sample variation. Therefore, incubation for longer

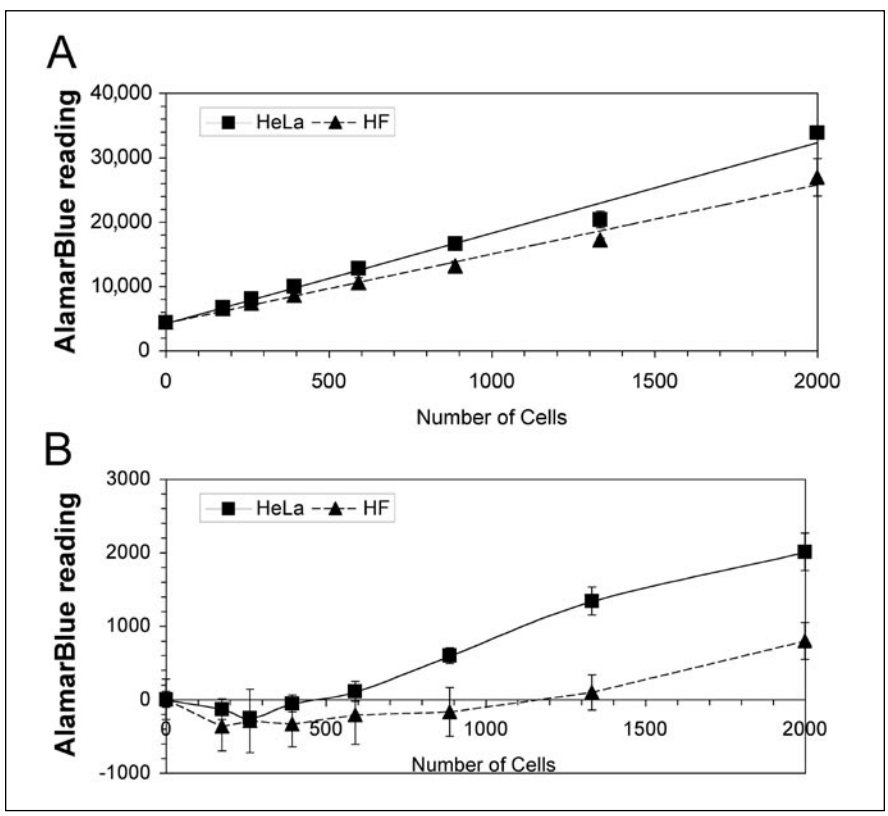

Figure 1. Standard curve of alamarBlue reading and cell numbers plated. HeLa or HeLaHF cells (0, 176, 263, 395, 593, 889, 1333, or 2000) were plated into each well of a 96-well microplate in either (A) liquid or (B) soft agar media and allowed to grow for 1–7 days. Growth in liquid culture was scored after 1 day by alamarBlue staining at $\lambda = 590$ nm. Growth in soft agar culture was scored after 7 days. Each sample was in triplicate. The x-axis represents the cell number plated, and the y-axis represents the alamarBlue reading corrected for background. Error bars indicate standard deviations. When not shown, the standard deviation is within the size of the symbols.

Table 2. Linear Range of Cell Plating Density by AlamarBlue Staining

Cancer Cell Lines Tested	Effective Cell Plating Density
A431	1000–5000
A549	1000–5000
DLD1	0–3000
DU145	0–4000
HCT116	1000–4000
HeLa	0–2000
MCF7	0–1400
U87	0–500

than 2 weeks is not recommended (data not shown). Our observations indicated that incubation for 1 week is adequate for HeLa cells. We also compared alamarBlue staining with tetrazolium salt WST-1 staining (Roche Applied Science, Mannheim, Germany) and found that alamarBlue reagent was more desirable for quantitative interpretation due to reduced background in soft agar, ultimately increasing the working range and consistency of the assay.

We have assayed several other cancer cell lines that grow in soft agar media using this higher throughput format, including A431 (epidermoid cancer), A549 (lung cancer), DLD-1 (colon cancer), DU145 (prostate cancer), HCT116 (colon cancer), MCF7 (breast cancer), and U87 (glioma). Similar observations were obtained with variation in optimal seeding density (Table 2). Our experience strongly indicates that the assay could be applied to many cell systems, following optimization of conditions.

Soft Agar Growth of DLD-1 Cells Stably Expressing Kras^{D13} siRNA in Both Traditional and 96-Well Formats

One of the goals in developing the 96-well soft growth assay was to establish a highly efficient and reliable method to screen for putative oncogenes. First, we tested K-RAS in the colon carcinoma cell line DLD-1 to demonstrate the utility of this assay. K-RAS belongs to the RAS family of GTPases and is a classical dominant oncogene. Its activation, usually via point mutations around the GTP binding site, causes cell transformation (e.g., elevated anchorage-independent growth). siRNA has recently been shown to down-regulate gene expression effectively, including Kras^{V12} mutant allele in pancreatic cancer cells CAPAN-1 cells (7,8,10–12). We constructed a siRNA lentiviral vector against mutant K-RAS^{D13} and delivered the vector into DLD-1 cells by transduction. Cells stably expressing the siRNA constructs were then subjected to TaqMan® real-time reverse transcription PCR (RT-PCR) analysis (Roche Applied Science). Our results showed that the targeted message is down-regulated (Figure 2B). The soft agar growth assays for the stable siRNA expression cells were set up in both traditional 10-cm dishes and 96-well plates. The 10-cm dishes were cultured for 3 weeks and the colonies were quantitated by Qcount to avoid human counting subjectivity, and the 96-well cells were cultured for 1 week and the cell growth were as measured by alamarBlue staining described as above. The cells transduced with lentiviral vector expressing siRNA against mutant K-RAS were shown to be significantly lower in anchorage-independent growth in both 96-well plates (alamarBlue staining) and 10-cm dishes (number of colony) (both by 60%; Figure 2A), due to the down-regulation of this activated oncogene (Figure 2B). This result demonstrated the correlation of 96-well soft agar growth to the traditional soft agar growth in monitoring real oncogene transformation potential.

Target Validation Through Transient Transfection of siRNA in 96-Well Soft Agar Assays

Traditional soft agar growth is difficult to adapt for transient expression experiments or treatment due to the assay's long incubation time. However, the 96-well assay, with an incubation time of just 1 week, could make just such adaptation possible. We have shown that transient transfection of siRNA or siRNA expression vectors can mediate siRNA effects for up to 1 week (X. Hu et al., unpublished data). We tested siRNA transient transfection for oncogene validation in this higher throughput system. A Kras^{D13} siRNA corresponding to the hairpin siRNA expressed from the lentiviral vector was transcribed in vitro, along with a nonspecific control siRNA, and delivered into DLD-1 cells by transient transfection. The transfected cells were plated into soft agar media 24 h after transfection. Similar to the stable siRNA expression experiments described above (Figure 2), transiently introduced Kras^{D13} siRNA caused 30%

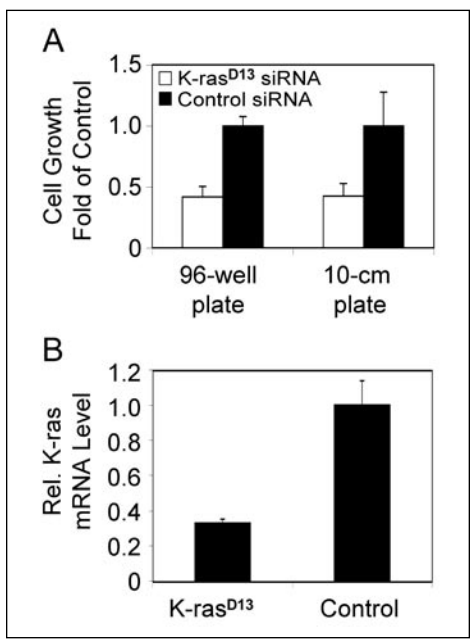

Figure 2. Soft agar growth for DLD-1 cells stably expressing short interfering RNA (siRNA) against Kras^{D13}. (A) DLD-1 cells containing a lentiviral vector stably expressing hairpin siRNA against K-RAS^{D13} or nonspecific control siRNA were obtained by transduction. For 10-cm dish cultures, 5000 cells were plated in soft agar and allowed to grow for 3 weeks before being scored by Qcount; for 96-well cultures, 1000 cells per well of each type were seeded into soft agar and allowed to grow for 1 week before being scored by alamarBlue staining. Each sample was performed in triplicate. Error bars indicate standard deviations, $P = 0.03$ for the 10-cm plate and $P < 0.01$ for the 96-well assays. (B) Total RNA was prepared from the stable DLD-1 cells containing either Kras^{D13} or control siRNA, and RNA was subjected to the real-time reverse transcription PCR (RT-PCR) analysis to detect Kras mRNA levels. Each sample was in triplicate. Error bars indicate standard deviations.

reduction in anchorage-independent growth as well (Figure 3). It is worth noting that because the transfection efficiency is about 50% for DLD-1 cells in this experiment, the phenotype is not as strong as in the stable expression cell line in which 100% of the cells express the siRNA.

Next, we tested another more recently recognized oncogene, PLK (13,14). PLK is a serine/threonine protein kinase belonging to a subgroup of protein kinases, whose members include PLK1, PLK2, and PLK3. PLK plays multiple roles during the cell cycle, especially in M phase progression and cytokinesis and has been implicated as an oncogene (13,14). PLK is expressed at a higher level in transformed HeLa than in nontransformed revertant HeLaHF, based on real-time PCR (data not shown). We were interested in examining whether PLK contributes to HeLa cell growth in soft agar. We also wanted to test the robustness of our 96-well format soft agar method using in vitro transcribed siRNA in the validation of additional oncogene targets.

The in vitro transcribed siRNA against PLK was generated, and the transient transfection and 96-well soft agar growth assay were carried out in HeLa cells. The results demonstrated that the down-regulation of PLK caused reduction of HeLa cell soft agar growth by 50% (Figure 3). Therefore, this observation not only confirms the oncogenic properties of PLK, but also, together with above K-Ras data, demonstrates the general utility of the 96-well soft agar assay in oncology target validation. It also shows the utility of 96-well soft agar assay in oncology target validation using transient transfection of siRNA. Transient transfection has very important implications in large-scale target screening due to the difficulty and expense of constructing siRNA expression vectors in large numbers by cloning. Thus, a convenient alternative is to generate siRNAs in parallel by chemical synthesis or in vitro transcription, which would require transient transfection for the cell-based assay.

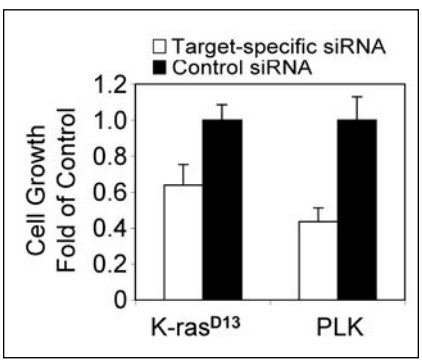

Figure 3. Soft agar growth in 96-well plates in cells transiently transfected with short interfering RNA (siRNA) targeted against oncogenes. DLD-1 or HeLa cells were transiently transfected with 10 nM of in vitro transcribed siRNAs against Kras^{D13} (DLD-1) or polo-like kinase (PLK; HeLa) and control siRNA using Oligofectamine reagent. After transfection (24 h), the cells were trypsinized, and 1000 cells/well were seeded into soft agar media and allowed to grow for 7 days before being scored by alamarBlue staining. Each sample was in triplicate. Error bars indicate standard deviations. $P = 0.01$ for Kras^{D13} in DLD-1 cells, and $P < 0.01$ for PLK in HeLa cells.

In summary, the 96-well soft agar assay has several advantages: (*i*) it shortens incubation time, making transient transfection possible; (*ii*) plate readers offer automatic quantification and analysis, providing reliable results and eliminating experimental bias; (*iii*) parallel operations increase validation speed and overall productivity; (*iv*) assays may be applied to many different cancer cell lines; (*v*) the assay may be applied to validate tumor suppressor-based increase of soft agar growth; and (*vi*) assays may be adapted for use in drug screening efforts.

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