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Cancer research

Engineering patient-derived tumoroids to help advance cancer research

Keywords
Tumoroids, cancer organoids, genetic engineering, electroporation, lentiviral transduction, lipofection

Introduction

Tumoroids are *in vitro* 3D cell cultures established from patient-derived cancer tissue. They are emerging as next-generation models for cancer research because of their ability to capture the cellular heterogeneity and spatial organization of a tumor *in vitro* [1]. The response of patient-derived tumoroids to chemotherapeutic drugs is often comparable to clinical responses observed *in vivo*, which could aid in the advancement of personalized medicine [2,3].

For a better understanding of cancer biology, or to assess the effects of certain drugs on tumor cells, it is often useful to engineer the tumoroids to either overexpress or downregulate specific genes. For example, in a recent study, healthy colon organoids engineered to express colorectal cancer–specific mutations helped elucidate the progression of cancer [4].

Here we outline methods for overexpressing and downregulating proteins of interest in primary colorectal cancer tumoroids via lipofection, electroporation, and lentivirus-based transduction, and provide recommendations to achieve desirable results. Each method has its own strengths and limitations (Table 1). Although the efficiency of payload uptake by the different methods is cell line–specific, similar approaches can be employed for tumoroids derived from other tissues.

Table 1. Strengths and limitations of methods available for engineering tumoroids.

	Nonviral		Viral
Method	**Lipofection**	**Electroporation**	**Lentivirus-based transduction**
Strengths	• Easy to use and inexpensive; no additional equipment required • Suitable for many cell types	• Higher transfection efficiency than lipofection • Efficient for difficult-to-transfect cells • Can deliver larger constructs than lipofection and lentivirus-based transduction • Rapid processing and preoptimized protocols (for Neon NxT Electroporation System)	• Highest efficiency relative to lipofection and electroporation • Effective payload delivery to nondividing cells • Stable integration of payload into host genome
Limitations	• Lower transfection efficiency than electroporation and lentivirus-based transduction • Transient expression of the delivered genetic material • Potential cytotoxicity due to lipid reagents	• Requires specialized equipment • Potential cytotoxicity at high voltages	• Payload capacity is limited to ~9 kb • Biosafety considerations for lentivirus production • More time-consuming than electroporation and more costly than lipofection • Potential for insertional mutagenesis

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Overview of delivery methods

Electroporation of tumoroids is performed using the Invitrogen™ Neon™ NxT Electroporation System (Figure 1). The system has 24 built-in protocols incorporating various voltages, pulses, and times to help determine optimal transfection parameters for a cell model. For any new cell type, payload delivery by electroporation involves three steps: conducting an optimization experiment on the Neon NxT system, choosing the optimal conditions for maximum transfection efficiency and cell viability, and scaling up the transfection reaction as required for downstream assays.

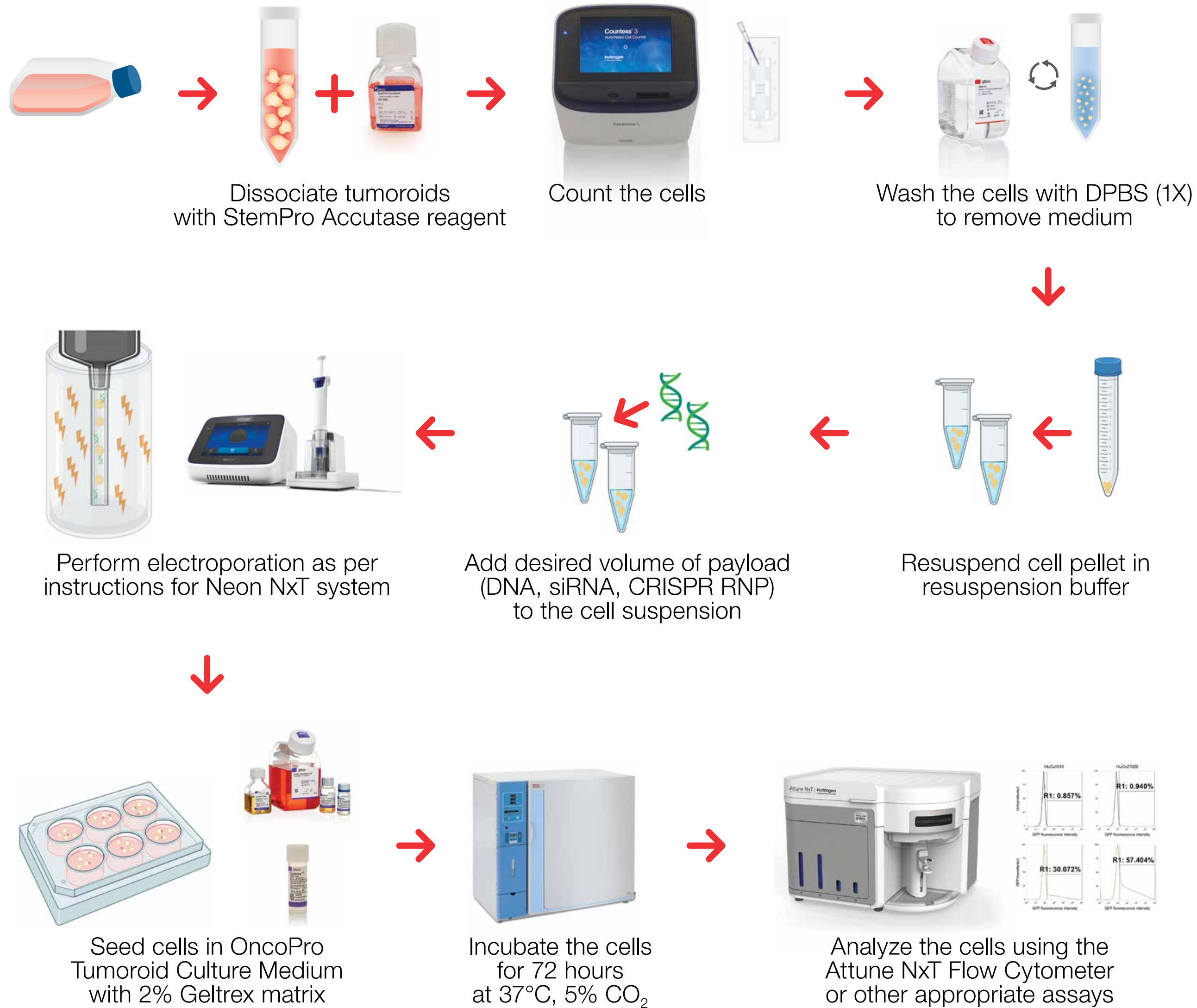

Figure 1. Guidance for transfecting tumoroids by electroporation.

Lentivirus-based transduction involves packaging the payload into lentiviral particles followed by transducing the tumoroids with the lentivirus (Figure 2). As a best practice, tumoroids are transduced at various multiplicities of infection (MOIs) and then assessed for transduction efficiency and cell viability. The MOI resulting in maximum transduction efficiency and cell viability is considered for follow-up experiments. See Table 2 for cell numbers and volumes recommended for lentivirus-based transduction in tumoroids. Typical workflows involve combining tumoroids in suspension with diluted lentivirus on the first day of transduction, centrifugation of the plate to aid lentiviral delivery to the cells, and a media change to remove lentiviral particles at 24 hours after initial exposure. Following transduction, positive clones are selected using antibiotics. It is necessary to construct an antibiotic kill curve prior to lentivirus-based transduction so as to optimize the appropriate dose for selecting positive clones.

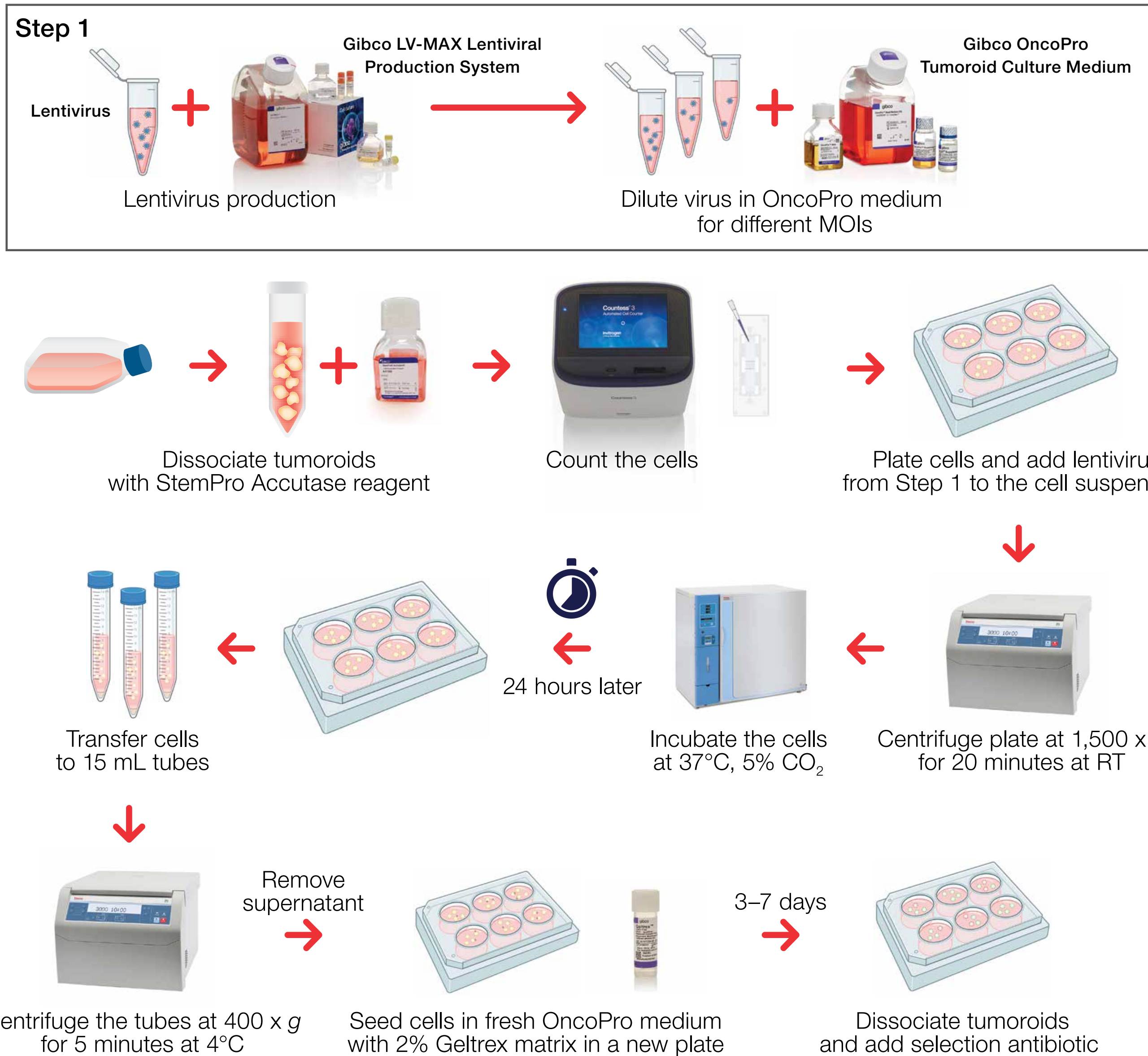

Figure 2. Guidance for payload delivery by lentivirus-based transduction.

Table 2. Cell numbers and volumes recommended for lentivirus-based transduction of tumoroids.

	24-well plate	12-well plate	6-well plate
Tumoroid cells	0.1×10^6	0.2×10^6	0.4×10^6
OncoPro medium	0.25 mL	0.5 mL	1 mL
Lentivirus in OncoPro medium	0.25 mL	0.5 mL	1 mL
OncoPro medium resuspension on day 2	0.5 mL	1 mL	2 mL

Lipofection involves packaging nucleic acids in lipid complexes for cell delivery (Figure 3). Lipofection is typically performed using Gibco™ Opti-MEM™ I Reduced Serum Medium to avoid potential interference of serum with the lipid–nucleic acid complexation process. For tumoroid workflows, dissociated tumoroid cells are resuspended in Opti-MEM I Reduced Serum Medium in 1.5 mL or 15 mL sterile microcentrifuge tubes.

In parallel, nucleic acids and Invitrogen™ Lipofectamine™ 3000 Transfection Reagent are complexed. See Table 3 for example target cell numbers and nucleic acid amounts. Tumoroids and nucleic acid–lipid complexes are then combined and incubated overnight, prior to collecting tumoroids and seeding in fresh Gibco™ OncoPro™ Tumoroid Culture Medium. Tumoroids can then be cultured and interrogated at different time points to assess transfection efficiency.

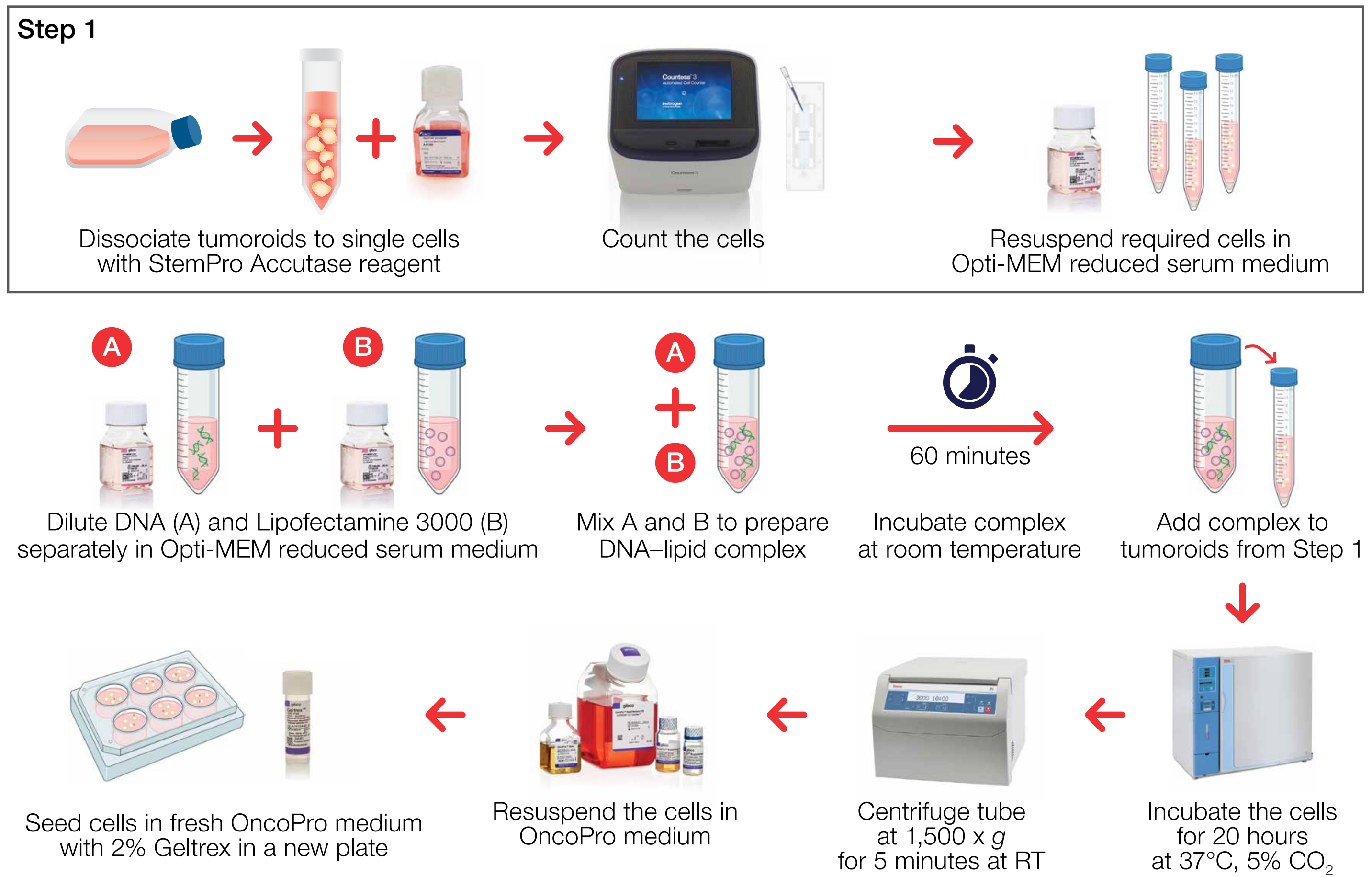

Figure 3. Guidance for payload delivery by lipofection.

Table 3. Recommended volumes for lipofection in tumoroids.

Tube	Complexation component	Amount per well (24-well plate)
Tube A	Opti-MEM I medium	To 25 µL*
	DNA (0.5–5 µg/µL)	2.4 µg
Tube B	Opti-MEM I medium	21 µL
	Lipofectamine 3000 reagent	4 µL

* Bring volume to 25 µL after calculating the volume of DNA to be added.

Table 4 summarizes the experimental parameters for electroporation and lentivirus-based transduction of tumoroids. Conditions are compared to those for lipofection using Lipofectamine 3000 Transfection Reagent.

Table 4. Experiment parameters and results for delivery of payload to colorectal cancer tumoroids. Parameters can be scaled to process higher numbers of cells.

	Electroporation			Transduction (<10 kb DNA)	Lipofection (<10 kb DNA)
	DNA (<10 kb)	siRNA	CRISPR		
Product	Neon NxT Electroporation System			LV-MAX Lentiviral Production System	Lipofectamine 3000 Transfection Reagent
Cells (per well of 24-well plate)	0.1 x 10^6	0.1 x 10^6	0.1 x 10^6	0.1 x 10^6	0.2 x 10^6
Payload	300 ng–1 µg	30 nM	Cas9: 1 µg gRNA: 200 ng	25 MOI	2.4 µg
Processing time	~2 hr			~24 hr (without antibiotic selection)	~24 hr
Expected efficiency of transfection or transduction	20–70%	60–80%	~40%	60–95%*	5–15%
Stable cell pool** generation?	No	No	No	Yes	No

* Slow-growing lines may show lower transduction efficiency.

** Clonal selection after delivery of payloads via electroporation or lipofection is not advised, as clonal expansion and/or low efficiencies may lead to loss of heterogeneity in tumoroid populations. Transduction is recommended for stable cell pool generation with retention of genetic and transcriptomic fidelity to the parental tumoroid population.

Representative results

Figures 4–8 show example results for each of the three delivery methods.

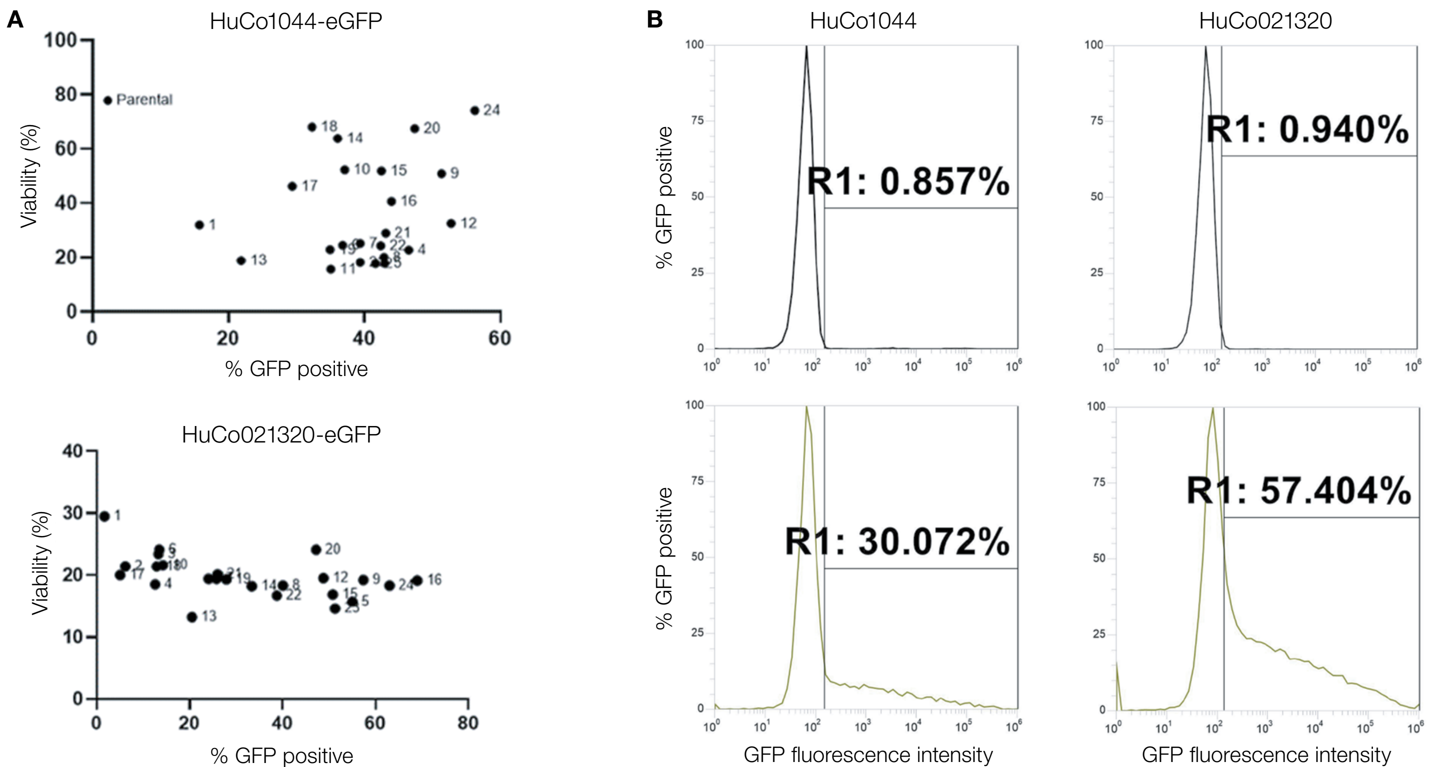

Figure 4. Electroporation of plasmid DNA into colorectal tumoroids for transient expression. Electroporation parameters were tested using the Neon NxT 24-well optimization protocol and then applied in a larger 6-well format. **(A)** Flow cytometry was performed using the Invitrogen™ Attune™ NxT Flow Cytometer, and data were analyzed using GraphPad™ Prism 9.0 software 72 hours after electroporation. **(B)** 3 µg of eGFP plasmid DNA was used to electroporate 0.5 x 10^6 cells, which were then seeded in a 6-well plate. Flow cytometry histograms show transfection efficiency in HuCo1044 (left) and HuCo021320 (right) colorectal tumoroids using protocol number 16 selected from the 24-well optimization experiment and analyzed 72 hours post-electroporation. Top graphs show untransfected cells and bottom graphs show transfected cells. Region 1 (R1) is the GFP-positive gate.

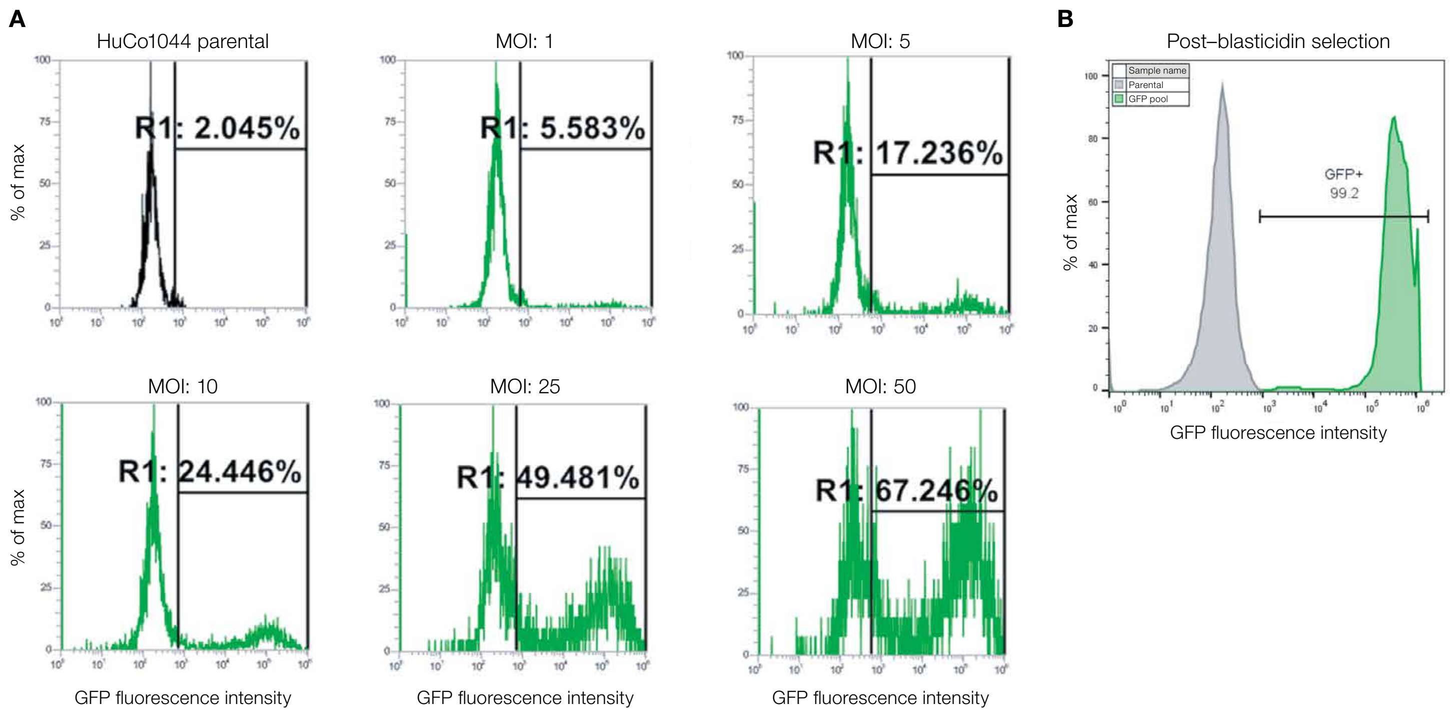

Figure 5. Representative flow cytometry histograms for HuCo1044 tumoroid cells transduced with an eGFP lentivirus at the indicated MOIs. **(A)** Before selection and **(B)** after 4 weeks of selection with 5 µg/mL Gibco™ Blasticidin S HCl. 0.4 x 10^6 cells were seeded per well on a 6-well plate for transduction. Tumoroids were dissociated to a single-cell suspension and passed through a 35 µm cell strainer before analysis with Invitrogen™ Attune™ NxT Software.

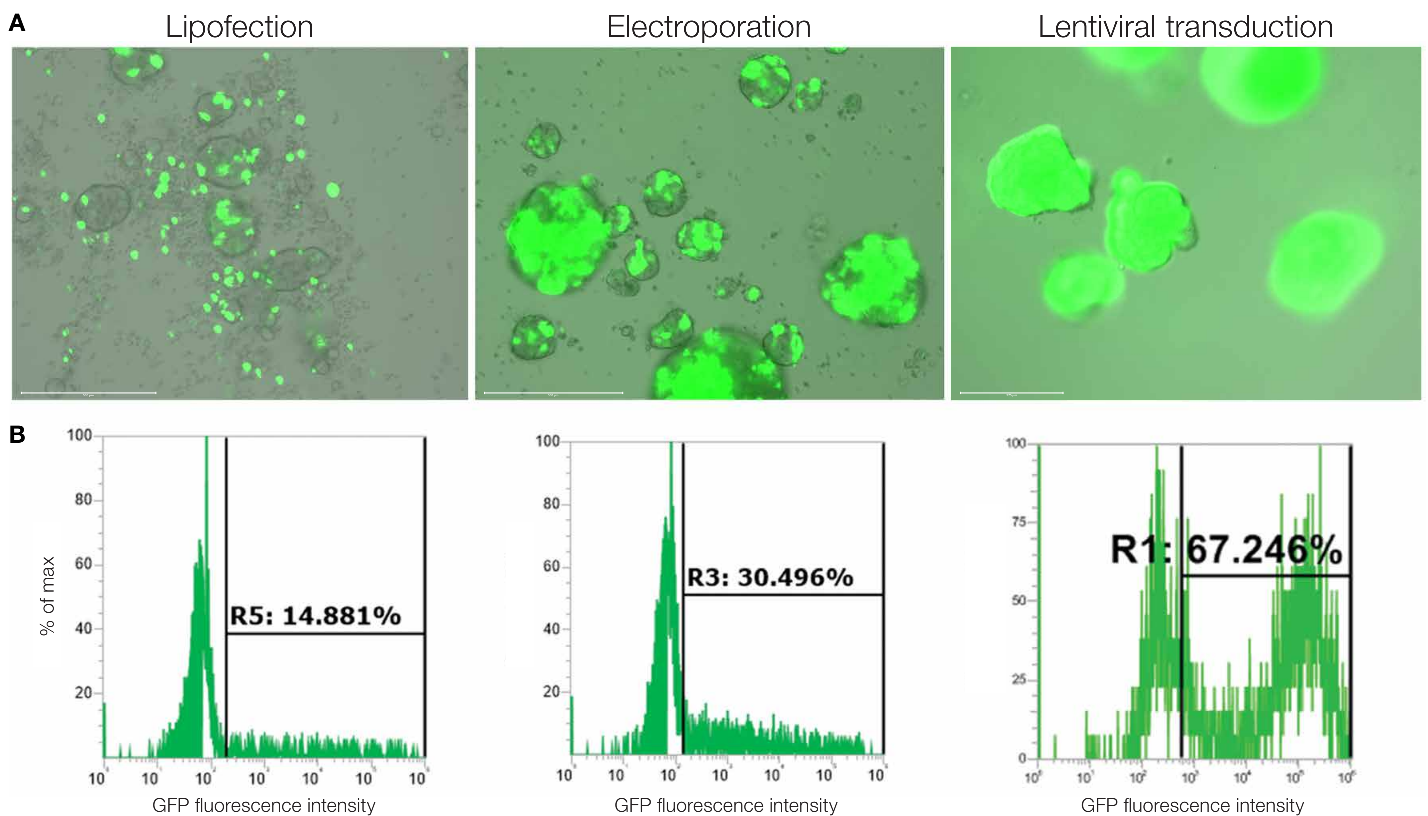

Figure 6. Colorectal cancer tumoroid (HuCo1044) transfection or transduction with an eGFP construct using the three methods discussed above. (A) Representative images obtained 7 days after engineering and **(B)** flow cytometry analysis of gene delivery. Images were captured using the Invitrogen™ EVOS™ M7000 Imaging System. Tumoroids were dissociated to single cells using Gibco™ StemPro™ Accutase™ Cell Dissociation Reagent 3 days after engineering, and flow cytometry was performed on the Attune NxT Flow Cytometer.

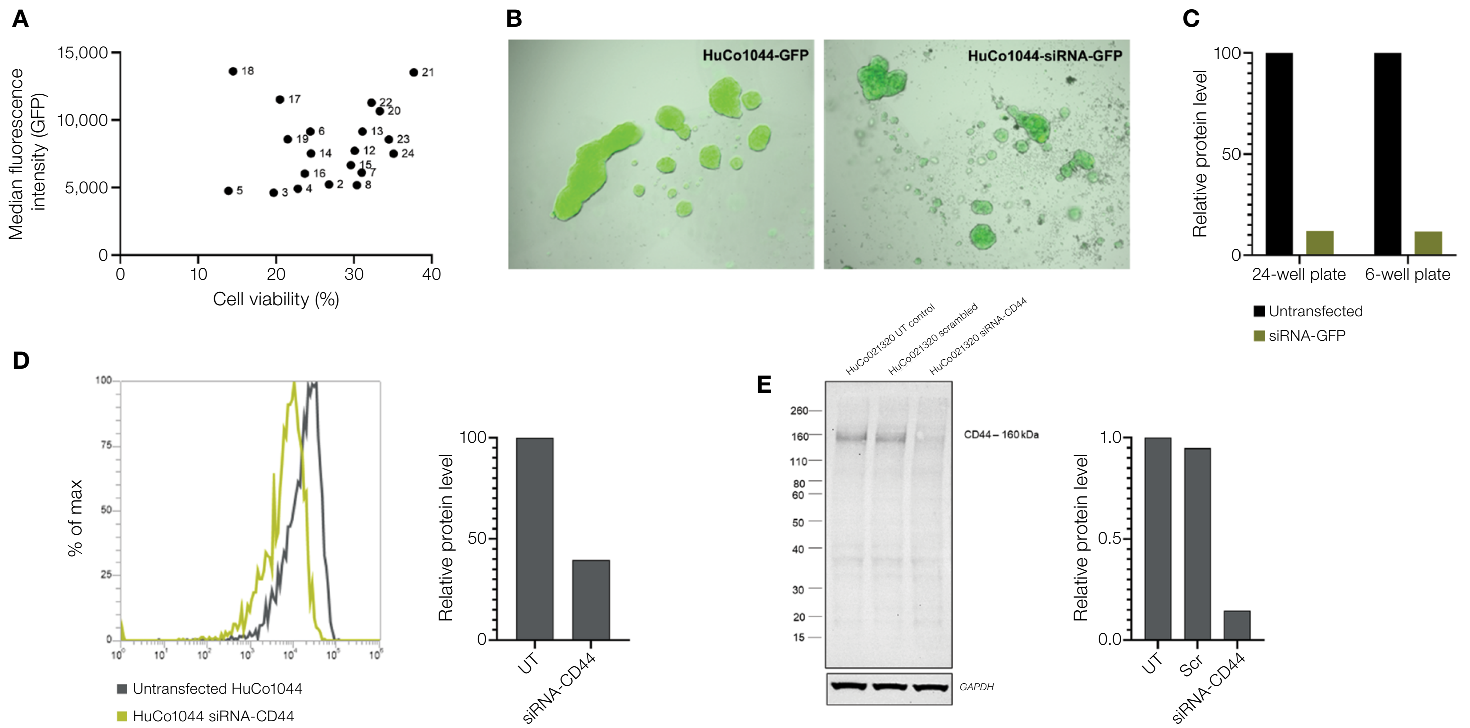

Figure 7. Knocking down the protein of interest in tumoroids. (A) Representation of 24-well optimization protocol for siRNA-mediated knockdown of protein expression in HuCo1044 tumoroids. HuCo1044 tumoroids expressing GFP (HuCo1044-GFP) were transfected with siRNA against eGFP and analyzed using flow cytometry 72 hours post-transfection. Numbers within the graph denote the protocol numbers on the Neon NxT Electroporation System. **(B)** Representative images of untransfected (left) and siRNA-transfected (right) HuCo1044-GFP tumoroid cells. 72 hours post-transfection, cells were plated in a 24-well Thermo Scientific™ Nunc™ non-treated plate. **(C)** Relative levels of GFP in untransfected and siRNA-transfected HuCo1044-GFP cells. Transfection was performed in 24-well and 6-well plates with 30 nM siRNA against CD44 using protocol 4 (1,600 V, 20 ms, 1 pulse) on the Neon NxT Electroporation System; flow cytometry analysis was performed 72 hours post-transfection. **(D)** Flow cytometry analysis for siRNA-mediated CD44 knockdown in HuCo1044 cells using protocol 4. **(E)** Western blot analysis of CD44 knockdown in HuCo021320 tumoroids. UT: untransfected cells; Scr: cells transfected with scrambled siRNA; siRNA-CD44: cells transfected with siRNA against CD44.

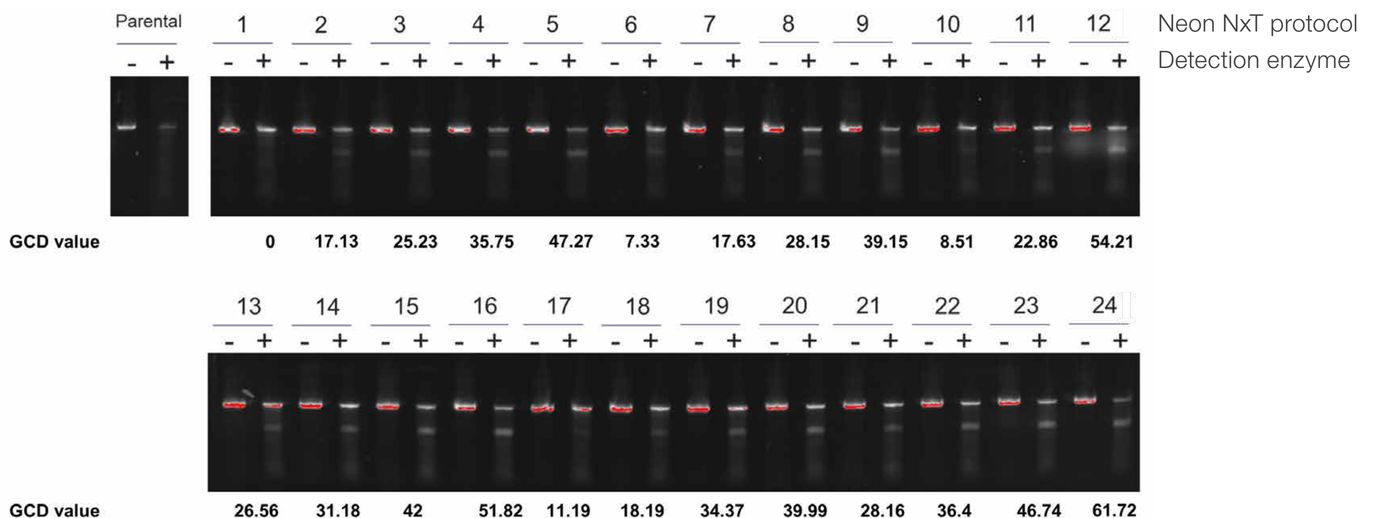

Figure 8. Knocking out the gene of interest in tumoroids. HuCo1044 tumoroids were co-transfected with Invitrogen™ TrueCut™ Cas9 Protein v2 and gRNA against the *HPRT* gene using the Neon NxT Electroporation System with the Neon NxT Resuspension R buffer. The 24-well optimization protocol was used to find the optimal conditions for performing the gene knockout, and 0.1 x 10^6 dissociated tumoroid cells were used per well of a 24-well plate, along with 200 ng of gRNA and 1 µg of TrueCut Cas9 Protein v2. Genome cleavage was performed using the Invitrogen™ GeneArt™ Genomic Cleavage Detection Kit as per the user guide with *HPRT*-specific PCR primers at 72 hours post-electroporation. Following the cleavage reaction and gel electrophoresis, the gel was imaged using the Invitrogen™ iBright™ FL1500 Imaging System, and the cleavage efficiency (GCD value) was calculated using Alpha Innotech™ AlphaImager™ software as per the user guide of the GeneArt Genome Cleavage Detection Kit. Electroporation parameters of 1,600 V, 10 ms, and 3 pulses resulted in the highest cleavage efficiency (62%, lane 24).

Performance of transfection of intact tumoroids

While it would be ideal to perform transfection on intact tumoroids, we have observed lower transfection efficiency in them compared to dissociated tumoroids. For example, 1 µg of eGFP plasmid DNA was transfected into 0.1 x 10^6 HuCo021320 cells in the following formats: 7-day-old intact tumoroids, 4-day-old intact tumoroids, and freshly dissociated tumoroids (Figure 9). Transfection was performed using the Neon NxT Electroporation System with the following parameters: 1,400 V, 20 ms, 2 pulses. The percentage of GFP-positive cells was analyzed by flow cytometry at 72 hours post-transfection. Transfection efficiency reached nearly 80% when dissociated tumoroids were electroporated, but dropped to 35–40% when processing intact 4-day-old and 7-day-old tumoroids.

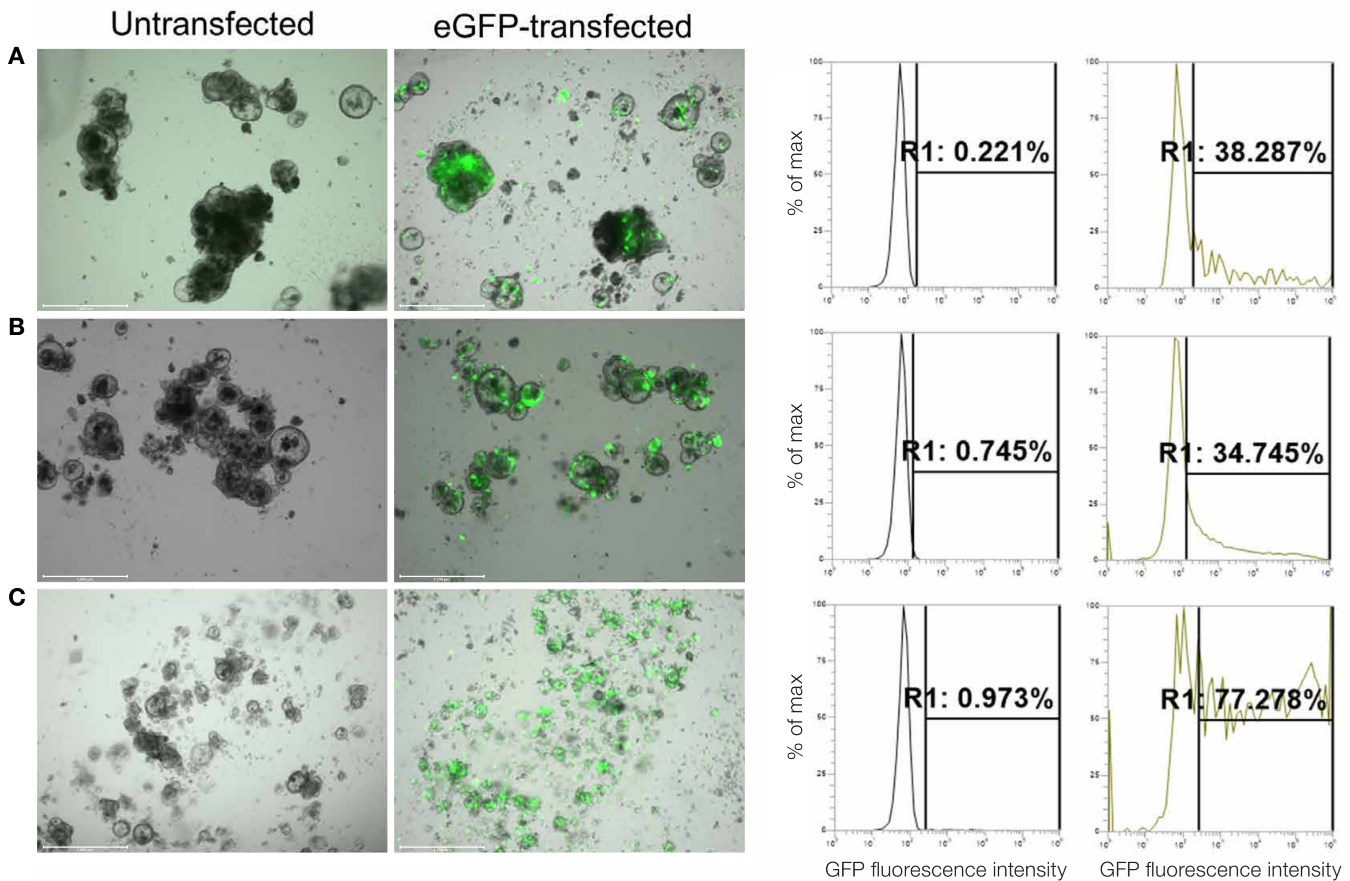

Figure 9. Representative images of untransfected and eGFP-transfected HuCo021320 colorectal cancer tumoroids on different days. Transfection formats were **(A)** 7-day-old intact tumoroids, **(B)** 4-day-old intact tumoroids, and **(C)** freshly dissociated tumoroids. Cells were analyzed by flow cytometry on the Attune NxT Flow Cytometer 72 hours post-transfection, and data were analyzed using the Attune NxT Software. Note: scale bar = 1,000 µm.

FAQs and tips

Frequently asked questions about the delivery technologies are answered in Table 5. For recommendations to optimize results, see Table 6.

Table 5. Commonly asked questions concerning the delivery technologies.

Question	Response
How do I assess transfection efficiency?	The detection method depends on the payload transfected or transduced. For example, if the payload contains a fluorophore probe such as eGFP, transfection efficiency can be determined using flow cytometry. If the payload is shRNA or siRNA against a protein, quantitative assessment of transfection efficiency can be performed using flow cytometry or western blotting. For assessing genetic modification like gene knockout, editing efficiency can be quantified using the GeneArt Genomic Cleavage Detection Kit followed by qPCR, western blotting, flow cytometry, Sanger sequencing, or next-generation sequencing (NGS).
How do I assess cell viability post-transfection?	If using flow cytometry for cell viability assessment, a fixable viability dye can be added to the cells to assess the percentage of viable cells. Cell counters like the Invitrogen™ Countess™ 3 Automated Cell Counter can also be used to assess cell viability.
Should I perform transfection/ transduction in intact tumoroids or dissociated tumoroids?	To maximize efficiency, tumoroids dissociated into small clumps are the best choice for performing transfection or transduction. The smaller size of the aggregates improves uptake of the payload (see Figure 9).
How long after transfection/ transduction should I assess the efficiency?	Efficiency of transfection or transduction can be assessed 24–72 hours post-experiment. For edited pool selection, antibiotics are added 72–96 hours after transfection or transduction and it takes about 2–4 weeks to obtain the selected pool.
What is the optimal strategy for clone selection using antibiotics and clonal expansion following transduction?	Tumoroids consist of a heterogeneous population of cell types, representative of the tumor in the patient. Thus, pool selection would be an optimal strategy for selecting an engineered cell population post-transduction. The transduction method needs to be optimized to ensure uptake of the payload by the maximum number of cells before antibiotic selection, to maintain cellular heterogeneity. Single-cell clonal expansion is not recommended following antibiotic selection, so that the heterogeneity of the cell population in tumoroids can be maintained.

Table 6. Recommendations to maximize payload uptake and maintain good cell viability post-transfection.

Observation	Possible cause	Action
Excessive cell death following electroporation	Excessive dissociation of cells	Perform a protocol optimization to determine the best dissociation conditions to maximize cell viability and transfection efficiency.
	Excessive pipetting during processing	Pipette gently during cell processing.
	Protocol used caused stress on cells	Perform electroporation in antibiotic-free medium.
	Use of higher-voltage program	Ensure use of optimal electroporation program.
Low transduction efficiency using lentivirus, or cells not recovering after antibiotic selection	Low virus titer	Increase virus titer or MOI for transduction.
	Antibiotics were added too soon after transduction	Add antibiotic selection after cells have recovered from transduction.
	Cell line is not a good transduction host	Use a compatible transduction host.
Low transfection efficiency during electroporation protocol optimization	Further optimization is required	Further optimize payload amount.
	Air bubbles in tip.	Ensure there are no air bubbles in the electroporation tip.
	Cell line is not a good transfection host	Consider stable pool generation with lentivirus.
Low transfection efficiency using lipid-mediated DNA transfection	Low uptake of DNA by cells.	Incubate tumoroids with lipid-containing DNA for 4–24 hours in Gibco™ Opti-MEM™ I Reduced Serum Medium to enhance payload uptake.

Conclusion

Based on our results, electroporation is the preferred method for transient transfection of various payloads, and lentivirus-based transduction is the method of choice for stable cell line generation. Moreover, dissociated tumoroids showed higher transfection efficiency than intact tumoroids. The engineered tumoroids can be utilized for further downstream assays like drug screening and immuno-oncology, as well as understanding the role of cancer-driving genes. They can also be used to study cell–cell interactions in the tumor microenvironment (TME). For instance, a constitutively GFP-expressing tumoroid line can be distinguished from other cells when co-cultured with cells belonging to the TME.

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Ordering information

Description	Cat. No.
Plastics	
Nunc Non-Treated Multidishes	144530
	150200
	150239
Nunc Non-Treated Flasks	169900
Nunclon Sphera Dishes	174932
	174930
Nunc Serological Pipettes	170356
	170357
Media, supplements, and antibiotics	
OncoPro Tumoroid Culture Medium Kit	A5701201
DMEM/F-12	11320082
Blasticidin S HCl	A1113903
Penicillin-Streptomycin (10,000 U/mL)	15140122
Geltrex LDEV-Free, hESC-Qualified, Reduced Growth Factor Basement Membrane Matrix	A1413302
DPBS, no calcium, no magnesium	14190144
StemPro Accutase Cell Dissociation Reagent	A1110501

Description	Cat. No.
Transfection reagents/kits	
Lipofectamine 3000 Transfection Reagent	L3000015
Opti-MEM I Reduced Serum Medium	31985062
Neon NxT Electroporation System 10-µL Kit	N1096
Neon NxT Electroporation System 100-µL Kit	N10096
LV-MAX Lentiviral Production System	A35684
Silencer Select CD44 siRNA	4390824
Silencer Select Negative Control No. 1 siRNA	4390843
TrueCut Cas9 Protein v2	A36498
GeneArt Genomic Cleavage Detection Kit	A24372
Silencer GFP (eGFP) siRNA	AM4626
TrueGuide sgRNA Positive Control, HPRT1 (human)	A35524
Instruments	
Neon NxT Electroporation System	NEON18SK
Countess 3 Automated Cell Counter	A49865
EVOS M7000 Imaging System	AMF7000
Attune NxT Flow Cytometer	A29004

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