ARTICLE

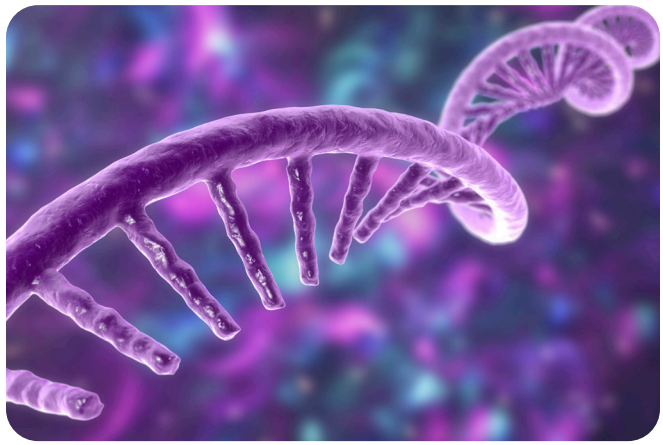
# Top tips for effective gene silencing in 3D cultures

Three-dimensional (3D) cell culture models, such as organoids and spheroids, provide a more accurate representation of human physiology compared to traditional 2D cultures. They are used in studying disease mechanisms, drug screening, and regenerative medicine. In particular, spheroids are becoming widely used to model solid tumors and the tumor microenvironment, which plays a role in conferring resistance to drugs. Like *in vivo* tumors, cancer cells grown in 3D tend to be more resistant to drugs compared to their 2D counterparts, mediated through distinct signaling pathways and gene activation.

Gene silencing through RNA interference (RNAi) is an effective method to understand gene function and relate cellular phenotypes to protein and gene function. RNAi technology is also very efficient in identifying or confirming novel drug targets in 2D cell models. However, only a few studies have successfully performed RNAi in complex 3D models to date. The main challenge is the lower penetration of molecules into 3D cultures or hydrogel-embedded cultures and the difficulty in targeting the central layers of these cultures. This may also lead to a heterogenous response, as not all cells of the culture have been silenced with the siRNA.

We have created Dharmacon™ Accell™ siRNA reagents with unique modifications that allow cell uptake without the need for lipid transfection reagents. This innovative delivery method permits repeated use of Accell siRNA reagents, ensuring prolonged gene knockdown while minimally impacting cell viability and the innate immune response. These features significantly expand the scope of biological questions and cell types that researchers can explore using RNAi.

In this article, we discuss four top tips for achieving efficient gene knockdown in 3D cell cultures. Data supporting our top tips can be found in our recent publication.[1]



## Top tips for gene silencing in 3D cultures:

1. **Empower researchers with efficient siRNA technology**

   Accell siRNA reagents allow cell uptake without the need for transfection reagents.

2. **Enhance effective knockdown by optimizing dosing and timing**

   3D cell cultures often require longer incubation times to allow for penetration of molecules and a more efficient gene knockdown.

3. **Use higher concentrations of siRNA in ECM models**

   Hydrogel-embedded 3D cultures have a higher overall volume that needs to be penetrated to ensure all cells have access to siRNA molecules.

4. **Optimize gene knockdown for your cell line**

   As with transfection in 2D, optimization is needed for each individual 3D culture.

revvity

**siRNA or CRISPR for 3D cultures?**

"Alternative genetic manipulation tools may be preferable to siRNAs in 3D culture. For example, CRISPR technology can be utilized to generate a genetic mutation that results in complete protein knockout whereas siRNA strongly reduces, but does not entirely disrupt, target mRNA and encoded protein expression. However, efficient delivery of both the Cas9 nuclease and the required sgRNA to target the Cas9 nuclease to a specific genetic locus remains a key challenge to successful genetic editing studies in 3D culture systems. For 3D culture models established from homogeneous cell lines, the desired genetic edit could instead be generated in 2D prior to seeding cells in 3D. Once a stable population of cells with the desired protein knockout is generated and characterized, these cells could then be seeded into a spheroid or organoid model to determine the functional role of the targeted gene in a more physiological 3D environment. Alternatively, an inducible Cas9 and sgRNA cassette could be transduced into a cell line in 2D and then seeded into a 3D environment. Upon spheroid or organoid formation, the inducible stimulus could then be added to the culture model to induce Cas9 expression and subsequent gene editing in intact 3D culture models."

*Josien Levenga, R&D Senior Scientist, Revvity, Lafayette, Colorado, USA*

**TOP TIP 1**

## Empower researchers with efficient siRNA technology

Traditional chemical transfection methods rely on complex formation of the siRNA with lipids or other molecules. However, large complexes are not readily taken up by 3D cell cultures and are less likely to reach the center of spheroids. Our Accell siRNA reagents work particularly well in 3D cell cultures. These reagents feature unique modifications that allows cell uptake without the need for lipid transfection reagents.

"Accell siRNA allows for uptake by the cells without the need for a transfection reagent. We were able to add this form of siRNA to our spheroid cultures in all of these different models, and it resulted in pretty potent knockdown of the genes of interest both at the RNA and protein level."

*Andrew Riching, R&D Senior Scientist, Revvity, Lafayette, Colorado, USA*

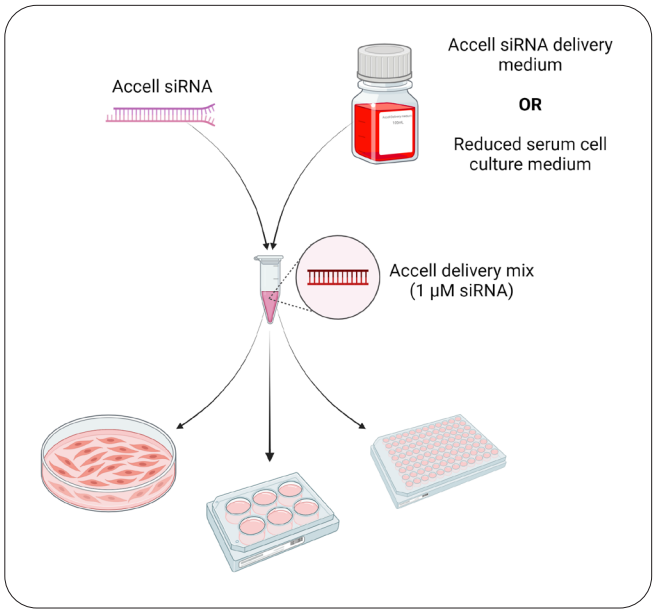
**TECHNOLOGY SOLUTION**

Accell siRNA promotes the delivery of siRNA without the need for viral vectors or lipid-based transfection reagents, a method known as 'passive delivery.' It can be used with virtually any cell type by incorporating a unique combination of chemical and bioinformatic enhancements. These enhancements increase functionality, stability, and enable lipid-independent delivery through endocytosis. They have proven to work efficiently in cancer spheroids grown in ULA plates, in Matrigel-embedded spheroids, and GrowDex®-embedded spheroids.[1]
Our transfection-free Accell siRNAs can be used in nearly any cell line, including difficult-to-transfect cells like neurons and primary cells.

Accell siRNA are available in two formats:

1. As an individual siRNA targeting a gene of interest
2. In SMARTpool™ format, an equimolar mixture of four siRNAs targeting the same gene of interest

Using the SMARTpool format typically results in knockdown efficiency comparable to the most active siRNA in the pool and results in fewer off-targets due to the lower effective concentration of each individual siRNA within the pool. Therefore, using the SMARTpool format for delivery can be advantageous, particularly in difficult or time-consuming 3D assays, as screening multiple individual siRNAs is not required.




Accell siRNA application protocol simplifies targeted gene knockdown. (1) Combine Accell siRNA with Accell delivery media (or other low- or no-serum media). (2) Add Accell delivery mix directly to cells and incubate for 72 hours.

**TOP TIP 2**

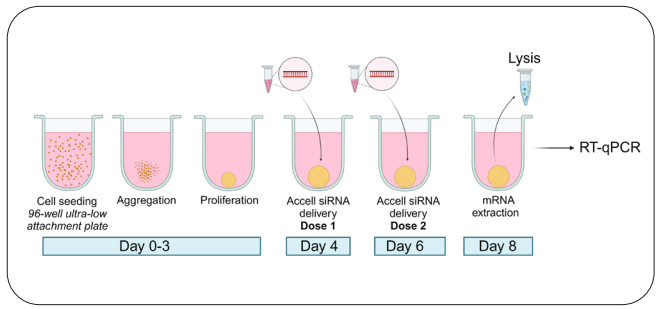
## Enhance effective knockdown by optimizing dosing and timing

Depending on the cell type, 3D cell cultures can be quite dense and difficult for molecules to penetrate. Therefore, they often require longer incubation times for the knockdown to become effective compared to 2D cultures, as well as repeated dosing of siRNAs. For long term knockdown and 3D cultures, Accell siRNAs should typically be replenished every 48 hours. A 96-hour treatment with Accell siRNA is usually enough to achieve target knockdown at the mRNA level, while protein knockdown is observable after 144 hours in the 3D cultures tested so far.

"Another challenge is timing. If you want to catch a really transient event with a transient delivery system, you have to optimize when you start adding your reagents to get a knockdown. We started seeing significant knockdown at 72 hours."

*Andrew Riching, R&D Senior Scientist, Revvity, Lafayette, Colorado, USA*

Timing siRNA dosing to the specific temporal event of interest should also be considered when optimizing gene knockdown studies. If researchers are specifically interested in catching a transient temporal event, they should consider the lag in time between the start of the delivery and the actual knockdown. This may require a time course to see what works best in the experiment.




Dosing timeline for Accell siRNA delivery on spheroids formed in a 96-well Ultra-Low Attachment (ULA) microplate. Accell siRNAs should be replenished every 48 hours.

**TOP TIP 3**

## Use higher concentrations of Accell siRNA in ECM models

Extracellular matrix (ECM) is commonly used for some types of 3D cultures, such as organoids or in studies of the tumor microenvironment, but it can add confounding variables to gene silencing experiments. By increasing the concentration of siRNA in hydrogel cultures or other ECM models, we can overcome this challenge and achieve higher and more efficient gene knockdown. While a final siRNA concentration of 1 µM is sufficient in ULA-based models, we found that a 2 µM siRNA concentration is more suitable for ECM-embedded cultures. This required increase in dosage should be considered when working with ECM models.

"Spheroids that are cultured in matrix are going to have different signaling because of the matrix, and there are different mechanical cues versus just floating in solution in a ULA plate. In terms of the siRNA delivery, we found that we had to go a little bit higher in our siRNA dosages for the ECM models than with the ULA models."

*Andrew Riching, R&D Senior Scientist, Revvity, Lafayette, Colorado, USA*

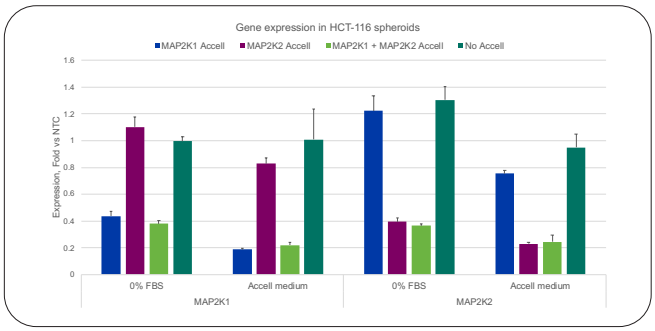
**TOP TIP 4**

## Optimize gene knockdown for your cell line

Serum and BSA can both inhibit uptake of Accell siRNA, so it's important to test whether the cell line being used for 3D culturing can tolerate serum starvation before optimizing siRNA delivery. Optimization with serum-free media formulations (Accell Delivery Media) or standard media with < 2.5% serum is recommended. Full-serum media may be added back after 48 hours of incubation. After optimizing siRNA delivery and the parameters to assess mRNA knockdown in a given cell type, researchers can confidently proceed to study the resulting biological phenotype.

"Make sure your cell model is really robust before you do any siRNA work because if your model is variable, your siRNA delivery is going to be even more variable."

*Andrew Riching, R&D Senior Scientist, Revvity, Lafayette, Colorado, USA*




High knockdown efficiency and target specificity in 3D cultures with Accell siRNA.

RT-qPCR data showing target specific knockdown of MAP2K1 and MAP2K2 genes with Accell siRNA in HCT-116 spheroids formed in a 96-well ULA microplate. Spheroids were treated with an Accell siRNA SMARTpool targeting MAP2K1 alone, MAP2K2 alone, or MAP2K1 and MAP2K2. As an additional control, gene expression in untreated spheroids (No Accell) are also shown. Delivery of Accell siRNA SMARTpool targeting MAP2K1 or MAP2K2 resulted in up to 80% knockdown efficiency in Accell medium.

Cell lines and types tested with Accell siRNA in 2D knockdown experiments. Note, not all cell lines and cell types listed were tested in 3D culture systems.

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|---|---|---|---|---|---|
| **Human adherent cell lines (24)** | | | | | |
| SH-SY5V | IMR-32 | LAN-5 | HEK293T | A549 | U-87 MG |
| LNCaP | A-375 | HeLa | HeLa S3 | MIA PaCa-2 | HEK293 |
| MCF7 | MCF 10A | SK-BR3 | OVCAR-3 | NCI/ADR-RES | U2OS |
| Huh7 | GTM-3 | DU 145 | HT-1080 | HepG2 | DLD-1 |
| **Mouse and rat cell lines (7)** | | | | | |
| NIH/3T3 | ES-D3 | PC-12 | 3T3-L1 | H9c2 | C2C12 |
| Rat2 | | | | | |
| **Human suspension cell lines (4)** | | | | | |
| Jurkat | IM-9 | THP-1 | PBMC | | |
| **Human differentiated stem cells (2)** | | | | | |
| Osteoblasts and adipocytes derived from hMSC | | | | | |
| **Human, mouse, and rat primary cells (9)** | | | | | |
| HUVEC | HUASMC | hMSC | Primary human astrocytes | Primary human keratinocytes | Primary human T-cells |
| Primary mouse hepatocytes | | Primary rat cortical neurons | | Primary rat striatum neurons | |
| **Cell types that failed Dharmacon Accell siRNA application (0)** | | | | | |

## References

1. Riching, A. S., Malloy, A., Anderson, E. M., Sheard, J., Mikkonen, P., van Brabant Smith, A., Strezoska, Z., & Levenga, J. (2024). A facile, transfection-free approach to siRNA delivery in *in vitro* 3D spheroid models. *Current Protocols*, 4, e1121. doi: 10.1002/cpz1.1121

Learn more about our 3D culture solutions: