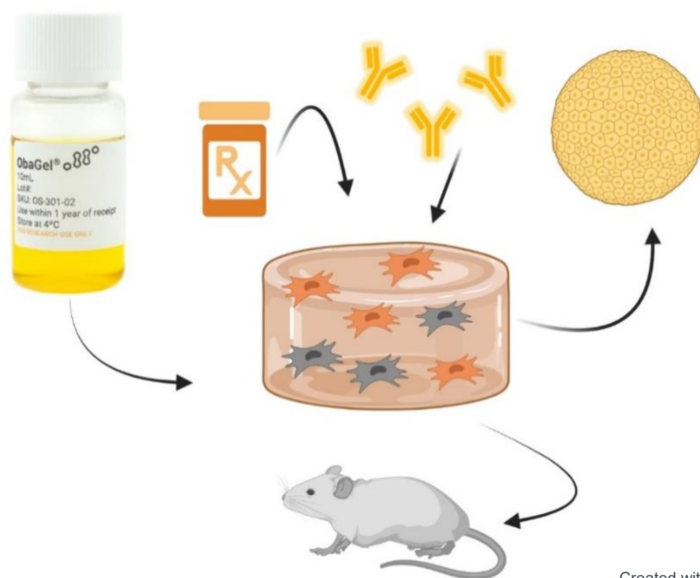
Obatala

ObaGel®

Human Derived Hydrogel 3D Culture Applications



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Handbook
Ver 1 May 2023

For Research Use Only. Not for Use in Diagnostic Procedures.

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Product Description and Specifications

ObaGel® is a human blood-derived 3D cell culture matrix that supports the development of robust human-like responses from multiple cell types. ObaGel® supports and maintains functional characteristics such as proliferation, differentiation, vascularization, and immunogenic responses that are superior to traditional culture methods. ObaGel® exhibits properties including lot-to-lot consistency, high protein content (>50 mg/mL), ease of cell recovery, fluorescent staining compatibility, and rapid gelation (~30 minutes). ObaGel® hydrogel kits include ObaGel® and ObaVate™, a companion product that enhances construct establishment and function.

Catalog No	***Product Name***	***Product Description***	***Storage Conditions***
OS-301	ObaGel®	Human-derived, 2D and 3D applications	<48 h at 4°C, >3 months at -20°C, 1 yr at -80°C
OS-302	ObaVate™	Human-derived solution for ObaGel® cultures	-20°C until prepared to use
OS-304	ObaFlow™	Solution for flow cytometry endpoint	4°C for 8 weeks
OS-303	ObaZolve™	Dissolution reagent for cell recovery	4°C for 8 weeks
OS-009	1X Phosphate Buffered Saline	Saline solution	4°C for 8 weeks
OS-011	Trypan Blue	Nuclear exclusion stain	4°C for 1 year
OS-101	Human Adipose-Derived Stromal/Stem Cells	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-107	Human Stromal Vascular Fraction	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-310	AdipoQual3D™	Adipogenic differentiation medium	4°C for 30 days
OS-309	StromaQual3D™	Pre-differentiation maintenance media	4°C for 30 days
OS-001	StromaQual™	Complete stromal pre-differentiation medium	4°C for 8 weeks
OS-007	Live-Dead Solution	Solution for validating cell viability	-20°C for 1 year

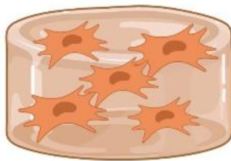
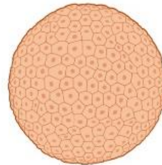
General Requirements for use of Obatala Sciences Products

- All personnel should be trained and certified by the Principal Investigator regarding Universal Precautions and Handling of Bloodborne Pathogens.
- All personnel should have access to relevant safety data sheets (SDS) pertaining to the use of materials.
- All procedures should be conducted by investigators using the appropriate personal protective equipment.
- Any waste materials should be decontaminated and disposed of using appropriate biohazard waste containers according to laboratory standard operating procedures.
- Wear protective eyewear during handling of cryopreserved vials of cells.

Use Cases with ObaGel® Hydrogels

The human derived ObaGel® hydrogel system versatility supports multiple use cases. ObaGel®, with its companion product, ObaVate™, can be used for as a "ready-to-use" kit for 3D cell culture.

To demonstrate the versatility and flexibility of our ObaGel® products, we list three of our most popular 3D *in vitro* and *in vivo* cell culture methods that can executed using our hydrogels: 3D culture, spheroid and organoid culture, and *in vivo* implantation of the ObaGel® encapsulated cultures.

3D Culture with ObaGel®	Spheroid and Organoid Culture with ObaGel®	*In vivo* implantation with ObaGel® 3D constructs
Culture cells in human-derived 3D microenvironment to promote cell-matrix and cell-cell interactions	Encapsulate cells in a human-derived cell for spheroid and organoid culture	Encapsulate cells in a human-derived 3D microenvironment to create an injectable construct for *in vivo* studies
		

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To learn more about ObaGel® culture methods, review our white papers and publications:

White Paper-Human Derived Hydrogel in a PDX Model
White Paper-Modeling Diseases with ObaCell Fat-On-A-Chip
Obatala Sciences Publications

Cell Culture Methods with ObaGel® Hydrogels

ObaGel® Culture Setup and Maintenance

Materials and Equipment

Catalog No	Product Name	Product Description	Storage Conditions
OS-001	StromaQual™	Complete stromal pre-differentiation medium	4°C for 8 weeks
OS-007	Live/Dead Assay Medium	Solution for validating cell viability	-20°C for 1 year
OS-011	Trypan Blue Exclusion Stain	Nuclear exclusion stain	4°C for 1 year
	Phosphate Buffered Saline	1X, Sterile	4°C for 8 weeks
OS-101	Human Adipose-Derived Stromal/Stem Cells	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-107	Human Stromal Vascular Fraction	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-310	AdipoQual3D™	Adipogenic differentiation medium	4°C for 30 days
OS-310	StromaQual3D™	Pre-differentiation maintenance media	4°C for 30 days
N/A	Well plate	Sterile, tissue culture plastic	
N/A	Tubes	Sterile 50 mL, 1.5 mL, 2 mL	
N/A	Micropipettes and tips	1000, 200, 100, and 10 µL	
N/A	Wet ice	For prolonged handling	
N/A	Benchtop centrifuge	For centrifugation step	
N/A	37°C, 5% CO_2 Incubator	For incubation steps	
N/A	BSL2 Biosafety Cabinet	For all culture procedures	

ObaGel® Culture Setup

1. Thaw ObaGel® and ObaVate™ overnight at 4°C until they are completely thawed. Do not warm to 37°C. Keep reagents cold while in use.
2. Warm growth medium of choice to 37°C.
3. Prepare a 50 mL conical tube with 4 mL of growth medium.
4. Thaw the cryovial of cells until the moment the ice crystals disappear from the vial.
5. Transfer the cryovial to a BSL2 BSC and add the contents of the cryovial dropwise to the 4 mL of growth medium in the conical tube.
6. Homogenize the cell suspension by pipetting several times.
7. Perform a cell count according to your laboratory's standard operating procedures.
8. Centrifuge the conical tube at 1200 RPM for 5 minutes at room temperature.
9. Aspirate the supernatant from the cell pellet and resuspend the cells in ObaVate™ at 1 million cells per mL.
10. From the 1 million cells per mL solution, calculate the total number of cells needed to create the seeding solution.
11. Calculate the volume of concentrated cell solution corresponding to the total cell number from above.
12. Subtract the volume of concentrated cell solution from the total volume of ObaVate™ needed for the seeding solution.[1]
13. Transfer the volume of concentrated cell solution to a new 50 mL conical tube.
14. Add the remaining volume of ObaVate™ needed for the seeding solution.
15. Pipette the cell solution multiple times to mix well.
16. Using a micropipette, vigorously pipette the ObaGel® prior to use.
17. Add the total volume of ObaGel® needed for the seeding solution to the 50 mL conical tube containing the ObaVate™ cell solution such that the seeding solution contains one-part ObaGel® to three-parts ObaVate™ (1:3 ratio). Pipette several times with a p1000 pipette to mix well.
18. Once the components are added, quickly transfer the necessary seeding volume of cell suspension onto the tissue culture plate growth surface. Pipette the solution between replicates to maintain homogeneous suspension.[2] [3]
19. After aliquoting, transfer the plate to a humidified 5% CO_2 incubator at 37°C. Observe after 1 hour for initial signs of gelation, and again after 24 hours to visualize cell growth. [4]

[1] Obatala recommends accounting for pipetting error by increasing the volume by 10%.
[2] Keep suspension on ice for a prolonged seeding period (>15 minutes).
[3] See Table 1 for recommended seeding volumes.
[4] Do not remove any liquid from the constructs after gelation.

ObaGel® Culture Maintenance

1. Place StromaQual3D™ and AdipoQual3D™ on a benchtop to equilibrate to room temperature.
2. Examine the ObaGel® culture under a brightfield microscope for confluency, network formation, and stability.
3. Transfer the plate and reagents to a BSL2 BSC.
4. Add the recommended volumes of media to the ObaGel® culture (refer to Appendix C). It is critical to add the media slowly and to the corner of each well. Adding media directly to the constructs could compromise their integrity.
5. Return the plate to a humidified 5% CO_2, 37°C incubator.
6. For subsequent media changes, remove approximately 50-60% of the spent media from each of the wells by tilting the plate and removing from the corner of the well.
7. Add recommended volume of fresh StromaQual3D™ or AdipoQual3D™ to their designated wells. It is critical to add the media slowly and to the corner of each well. Adding media directly to the constructs could compromise their integrity.
8. Steps (6) and (7) will occur 2x per week for 3 weeks. Culture time can be extended based on endpoints.

Figure 1: Workflow diagram outlining the steps required to create 3D cultures with ObaGel®. Steps for 3D culture with ObaGel®: Initial handling of ObaGel® and ObaVate™, seeding of the ObaCell® cultures, and maintenance of the established ObaCell® cultures.

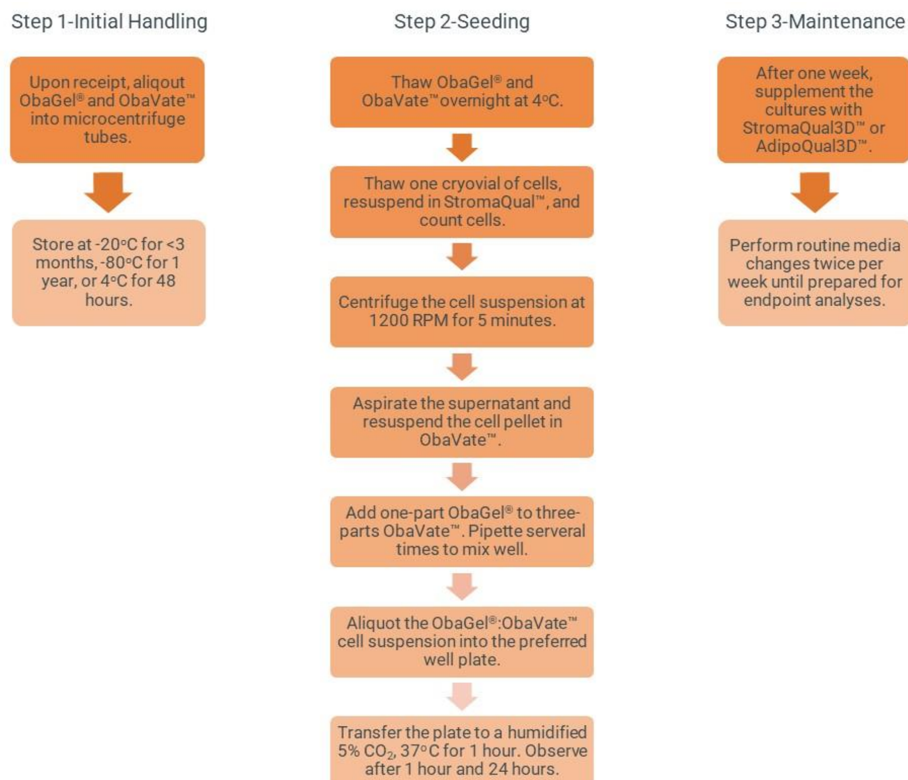

Table 1: Volumes of solution and medium and corresponding cell seeding density for 3D cell culture in 6-well, 24-well, and 96-well plates.

Reagent	6-well (5 mL)	24-well (1 mL)	96-well (100 µL)
ObaGel®	1.25 mL/well	250 µL/well	25 µL/well
ObaVate™	3.75 mL/well	750 µL/well	75 µL/well
Total Construct Volume	5 mL/well	1 mL/well	100 µL/well
hSVF cells	5,000,000 cells/well	1,000,000 cells/well	100,000 cells/well
hASC cells	500,000 cells/well	50,000-100,000 cells/well	5,000-10,000 cells/well
3D Medium Feedings	2.5 mL/well	500 µL/well	50 µL/well

ObaGel® Cultures for *In Vivo* Implantation

Materials and Equipment

Catalog No	Product Name	Product Description	Storage Conditions
OS-001	StromaQual™	Complete stromal pre-differentiation medium	4°C for 8 weeks
OS-007	Live/Dead Assay Medium	Solution for validating cell viability	-20°C for 1 year
OS-011	Trypan Blue Exclusion Stain	Nuclear exclusion stain	4°C for 1 year
	Phosphate Buffered Saline	1X, Sterile	4°C for 8 weeks
OS-101	Human Adipose-Derived Stromal/Stem Cells	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-107	Human Stromal Vascular Fraction	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-310	AdipoQual3D™	Adipogenic differentiation medium	4°C for 30 days
OS-310	StromaQual3D™	Pre-differentiation maintenance media	4°C for 30 days
N/A	Well plate	Sterile, tissue culture plastic	
N/A	Tubes	Sterile 50 mL, 1.5 mL, 2 mL	
N/A	Micropipettes and tips	1000, 200, 100, and 10 µL	
N/A	Parafilm	Retain moisture in plate	
N/A	Aluminum Foil	Protect the plate from light exposure	
N/A	Benchtop centrifuge	For centrifugation steps	
N/A	37°C, 5% CO_2 Incubator	For incubation steps	
N/A	BSL2 Biosafety Cabinet	For all culture procedures	

ObaGel® Culture Setup

1. Thaw ObaGel® and ObaVate™ overnight at 4°C until they are completely thawed. Do not warm to 37°C. Keep reagents cold while in use.
2. Warm growth medium of choice to 37°C.
3. Prepare a 50 mL conical tube with 4 mL of growth medium.
4. Thaw the cryovial of cells until the moment the ice crystals disappear from the vial.
5. Transfer the cryovial to a BSL2 BSC and add the contents of the cryovial dropwise to the 4 mL of growth medium in the conical tube.
6. Homogenize the cell suspension by pipetting several times.
7. Perform a cell count according to your laboratory's standard operating procedures.
8. Centrifuge the conical tube at 1200 RPM for 5 minutes at room temperature.
9. Aspirate the supernatant from the cell pellet and resuspend the cells in ObaVate™ at 1 million cells per mL.
10. From the 1 million cells per mL solution, calculate the total number of cells needed to create the seeding solution.
11. Calculate the volume of concentrated cell solution corresponding to the total cell number from above.
12. Subtract the volume of concentrated cell solution from the total volume of ObaVate™ needed for the seeding solution.[5]
13. Transfer the volume of concentrated cell solution to a new 50 mL conical tube.
14. Add the remaining volume of ObaVate™ needed for the seeding solution.
15. Pipette the cell solution multiple times to mix well.
16. Using a micropipette, vigorously pipette the ObaGel® prior to use.
17. Add the total volume of ObaGel® needed for the seeding solution to the 50 mL conical tube containing the ObaVate™ cell solution such that the seeding solution contains one-part ObaGel® to three-parts ObaVate™ (1:3 ratio). Pipette several times with a p1000 pipette to mix well.
18. Once the components are added, quickly transfer the necessary seeding volume of cell suspension onto the tissue culture plate growth surface. Pipette the solution between replicates to maintain homogeneous suspension.[6] [7]
19. After aliquoting, transfer the plate to a humidified 5% CO_2 incubator at 37°C. Observe after 1 hour for initial signs of gelation, and again after 24 hours to visualize cell growth.

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[5] Obatala recommends accounting for pipetting error by increasing the volume by 10%.
[6] Keep suspension on ice for a prolonged seeding period (>15 minutes).
[7] See Table 1 for recommended seeding volumes.

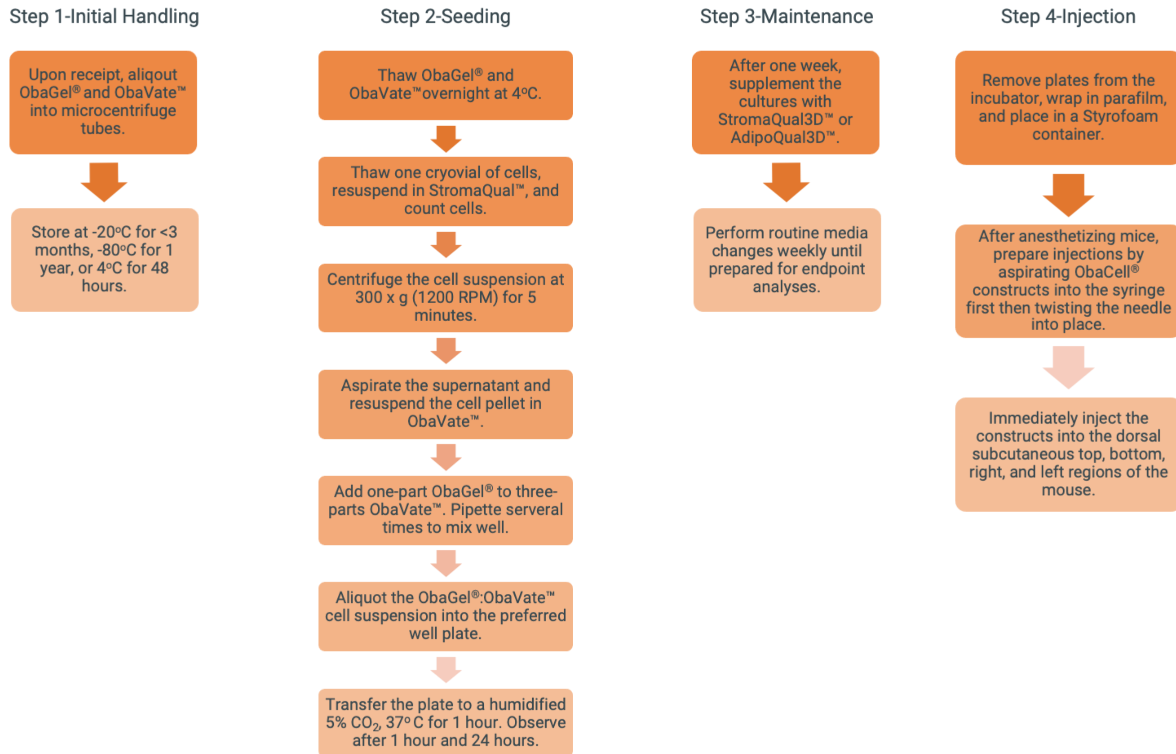
ObaGel® Culture Maintenance

1. Place StromaQual3D™ and AdipoQual3D™ on a benchtop to equilibrate to room temperature.
2. Examine the ObaGel® culture under a brightfield microscope for confluency, network formation, and stability.
3. Transfer the plate and reagents to a BSL2 BSC.
4. Add the recommended volumes of media to the ObaGel® culture (refer to Appendix C). It is critical to add the media slowly and to the corner of each well. Adding media directly to the constructs could compromise their integrity.
5. Return the plate to a humidified 5% CO_2, 37°C incubator.
6. For subsequent media changes, remove approximately 50-60% of the spent media from each of the wells by tilting the plate and removing from the corner of the well.
7. Add recommended volume of fresh StromaQual3D™ or AdipoQual3D™ to their designated wells. It is critical to add the media slowly and to the corner of each well. Adding media directly to the constructs could compromise their integrity.
8. Steps (6) and (7) will occur 2x per week for 3 weeks. Culture time can be extended based on endpoints.

Implantation of ObaGel® Constructs

1. When transporting ObaGel® constructs to the vivarium, do not place on ice. Wrap plates in aluminum foil or appropriately insulated material to aid in temperature maintenance. Place inside a Styrofoam container.
2. After anesthetizing the mice, prepare for injections.
3. Aspirate ObaGel® constructs from the individual wells into syringes by removing the needle from the syringe first, aspirating the ObaGel® construct, and finally twisting the needle back onto the syringe for injection.
4. Immediately inject the ObaGel® constructs into the dorsal subcutaneous top, bottom, left, and right locations (4 locations, 4 injections per mouse).

Figure 2: Workflow diagram outlining the steps required for *in vivo* implantation of ObaGel® cultures. Steps for *in vivo* implantation of ObaGel® cultures include: Initial handling of ObaGel®, seeding of the ObaGel® cultures, and maintenance of the established ObaGel® cultures, and implantation of the 3D constructs.



ObaGel® Cultures for Establishing Spheroids and Organoids

Materials and Equipment

Catalog No	Product Name	Product Description	Storage Conditions
OS-001	StromaQual™	Complete stromal pre-differentiation medium	4°C for 8 weeks
OS-007	Live/Dead Assay Medium	Solution for validating cell viability	-20°C for 1 year
OS-011	Trypan Blue Exclusion Stain	Nuclear exclusion stain	4°C for 1 year
	Phosphate Buffered Saline	1X, Sterile	4°C for 8 weeks
OS-101	Human Adipose-Derived Stromal/Stem Cells	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-107	Human Stromal Vascular Fraction	Primary cells isolated from human adipose tissue	Liquid Nitrogen
OS-310	AdipoQual3D™	Adipogenic differentiation medium	4°C for 30 days
OS-310	StromaQual3D™	Pre-differentiation maintenance media	4°C for 30 days
N/A	0.05% Trypsin/EDTA	For harvest of adherent ASCs	
N/A	T150 or T175 flask	Sterile, tissue culture plastic	
N/A	100 mm x 15 mm Petri Dish	Sterile, tissue culture plastic	
N/A	Tubes	Sterile 50 mL tubes	
N/A	Micropipettes and tips	1000, 200, 100, and 10 µL	
N/A	Wet ice	For prolonged handling	
N/A	Benchtop centrifuge	For centrifugation steps	
N/A	37°C, 5% CO_2 Incubator	For incubation steps	
N/A	BSL2 Biosafety Cabinet	For all culture procedures	

Harvesting Adherent Cells

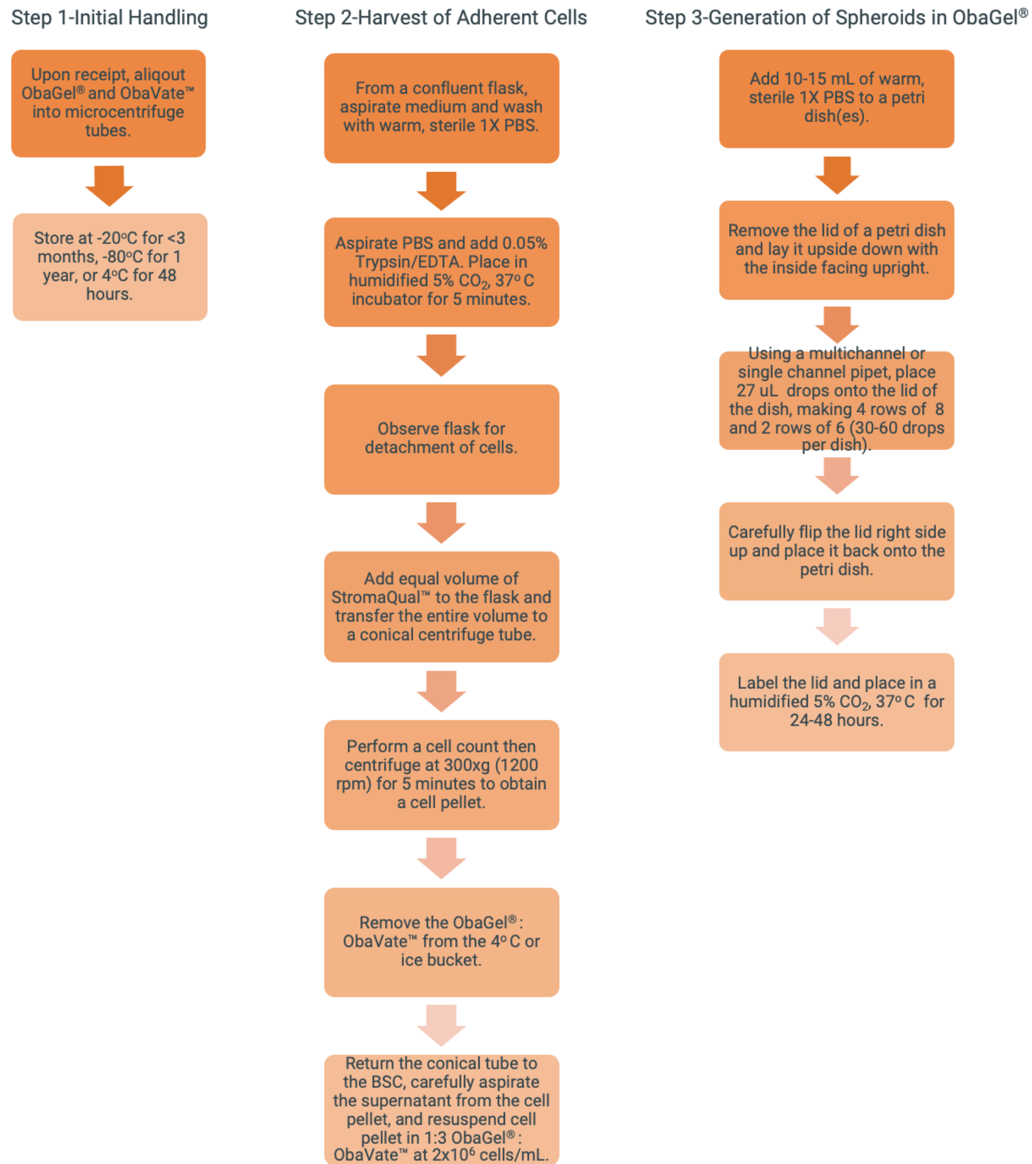
1. From a confluent T150 or T175 flask, aspirate medium and wash with warm, sterile 1X PBS.
2. Aspirate the PBS, add 0.05% Trypsin/EDTA, and place in the incubator at 37°C, 5% CO_2 for 5 minutes.
3. Add equal volume of StromaQual™ to the flask to neutralize the trypsin.
4. Transfer entire volume to a 50 mL conical tube.
5. Using an automated cell counter, hemacytometer, or other appropriate cell counting technique, determine the relative percentage of live cells and dead cells to determine total live cell count using the Trypan Blue Exclusion Stain.
6. Seal the cap on the conical tube. Transfer the conical tube to the bench top centrifuge and spin at 1200 RPM for 5 minutes at room temperature.
7. Retrieve ObaGel® and ObaVate™ from 4°C or ice bucket.
8. Return the conical tube to the biological safety cabinet and observe that a distinct and intact pellet is retrieved. Carefully aspirate the supernatant from the cell pellet.
9. Resuspend the cell pellet in one-part ObaGel® to three parts ObaVate™ for a final concentration of 2×10^6 cells per mL.

Hanging Drop Method for ObaGel® Spheroid Formation

1. Add 10-15 mL of pre-warmed, sterile 1X PBS to a petri dish.
2. Remove the lid of a petri dish and lay it upside down with the inside facing upright.
3. Using a multichannel or single channel pipet, place 27 µL drops onto the lid of the petri dish, making 4 rows of 8 and 2 rows of 6. [8]
4. Carefully flip the lid right side up and place it back onto the petri dish.
5. Label the lid and place at 37°C, 5% CO_2 incubator for 24-48 hours.
6. To visualize in the microscope, replace the bottom of the petri dish with a new one, invert the plate, and visualize under a microscope at 4X and 10X objectives.

[8] It is ideal to have ~30-60 drops per dish, depending on the size of the petri dish.

Figure 3: Workflow diagram outlining the steps required spheroid and organoid formation using ObaGel®. Steps for establishing spheroids and organoids in ObaGel® include: Initial handling of ObaGel®, harvesting of cells, and establishing spheroids and organoids in ObaGel®.



ObaGel® Culture Harvest

Note: This protocol is only suitable for cultures established with Obatala Sciences ObaGel® (Catalog #OS-301)

Materials

Catalog No	*Product Name*	*Product Description*	*Storage Conditions*
OS-304	ObaFlow™	Solution for flow cytometry endpoint	4°C for 8 weeks
OS-303	ObaZolve™	Dissolution reagent for cell recovery	4°C for 8 weeks
OS-009	1X Phosphate Buffered Saline	Saline solution	4°C for 8 weeks
N/A	Tubes	Sterile 15 mL, 1.5 mL, 2 mL	
N/A	Micropipettes and tips	1000, 200, and 100 µL	
N/A	Benchtop centrifuge	For centrifugation steps	
N/A	37°C, 5% CO_2 Incubator	For incubation steps	
N/A	BSL2 Biosafety Cabinet	For all culture procedures	

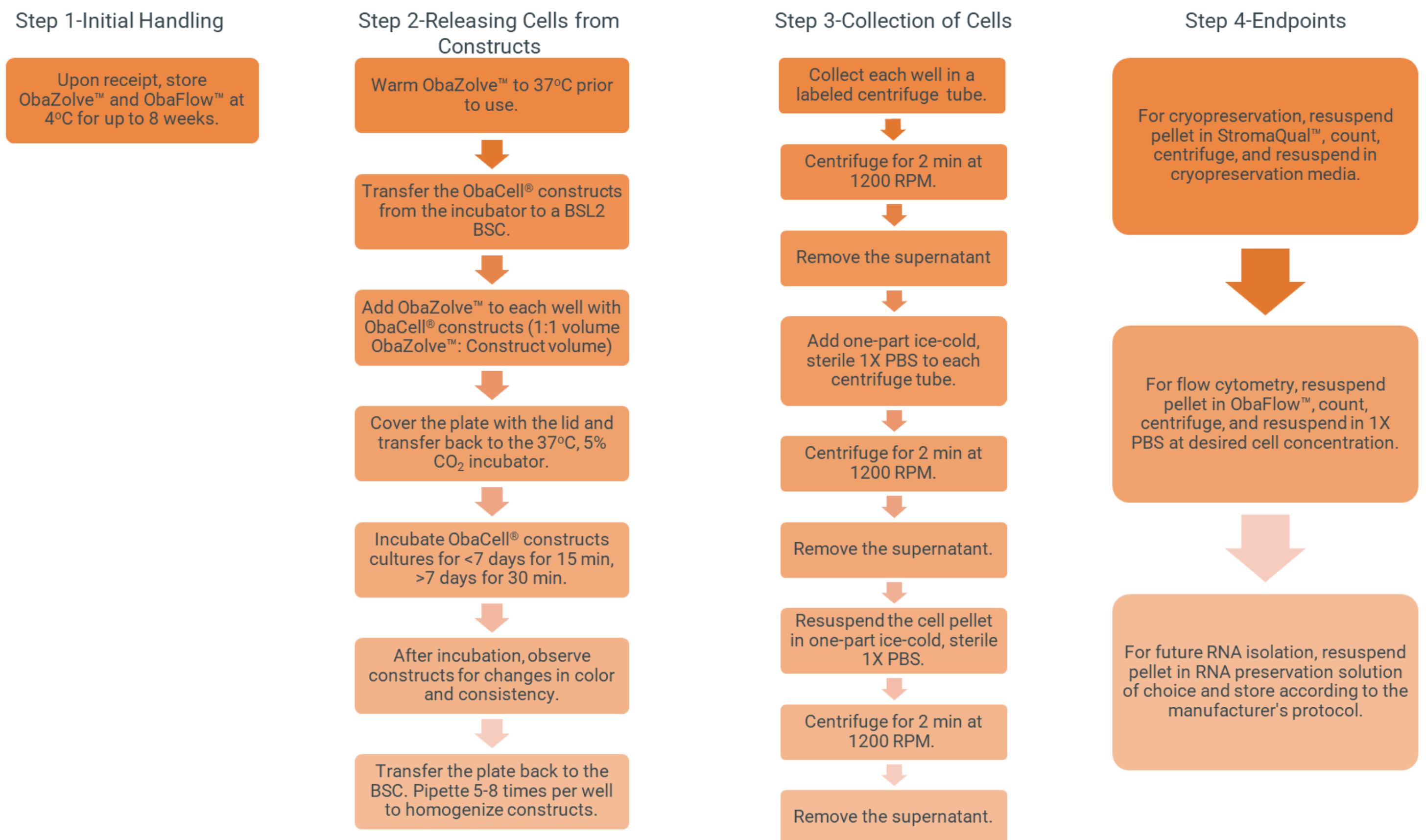
Harvesting of ObaGel® Cultures

1. Place ObaZolve™ in a water bath or equivalent piece of equipment to warm to 37°C prior to use.
2. Transfer the plate and reagents to a BSL2 BSC.
3. Carefully remove the spent media by tilting the plate and removing 50-60% of the media from each well.
4. Add approximately an equal volume of ObaZolve™ to each well of the plate for a 1:1 ratio v/v of ObaZolve™ solution to the construct volume.
5. Return the plate to a humidified 5% CO_2, 37°C incubator and incubate for 30 minutes.
6. Transfer the plate back to the BSL2 BSC and pipette each well to observe if the gel is dissolved. If resistance is observed when pipetting, incubate the plate for an additional 15 minutes.[9]
7. Collect each well in a tube.
8. Centrifuge each tube at 1200 RPM for 2 minutes.
9. Remove the supernatant with a pipette while also ensuring that the cell pellet remains intact.
10. Add one part of ice-cold, sterile 1X PBS to each tube.
11. Centrifuge each tube at 1200 RPM for 2 minutes.
12. Remove the supernatant with a p100 or p200 pipette.

[9] For constructs cultured for <7 days, incubate for 15 minutes. For constructs cultured for >7 days, incubate for 30 minutes. For cultures that appear especially dense, incubate for 30 minutes.

13. Add one-part ice-cold, sterile 1X PBS to each tube.
14. Centrifuge each tube at 1200 RPM for 2 minutes.
15. Remove the supernatant.
16. After resuspension of the cell pellet, proceed the following endpoints: cell passage and expansion, cryopreservation of the cells, flow cytometry setup, or preservation and storage of the cell pellet for RNA isolation.

Figure 4: Workflow diagram outlining the steps required to release cells from ObaGel® constructs. Steps for harvesting cells from ObaGel® constructs include: Initial handling of ObaGel® and ObaZolve™, harvesting of cells with ObaZolve™, and subsequent endpoints for recovered cells.



ObaGel® Culture Fixation and Staining

Note: This protocol is only suitable for cultures established with Obatala Sciences ObaGel® (Catalog #OS-301)

Catalog No	Product Name	Product Description	Storage Conditions
N/A	BODIPY 493/503	Neutral lipid stain	
N/A	Hoechst 33342 460/490	Dissolution reagent for cell recovery	4°C for 8 weeks
OS-009	1X Phosphate Buffered Saline	Saline solution	4°C for 8 weeks
N/A	10% Neutral Buffered Formalin	Fixative	
N/A	Tubes	Sterile 50 mL and 15 mL	
N/A	Parafilm	Retain moisture in plate	
N/A	Aluminum Foil	Protect the plate from light exposure	
N/A	Micropipettes and tips	1000, 200, and 100 µL	
N/A	BSL2 Biosafety Cabinet	For all culture procedures	

Reagent Preparation

1. Prepare working solutions for each fluorescent dye according to manufacturer's protocol.[10]

Fixation and Staining of ObaGel® Cultures

1. Transfer plate to a BSL2 biological safety cabinet to collect conditioned or spent media from each well. All other handling may be done on the benchtop.[11] [12]
2. Add 10% formalin to each well by pipetting slowly against the wall of the well to allow the liquid to flow across the surface of the constructs. [13]
3. Seal the plate with parafilm and incubate at 4°C for 24 hours.
4. Following fixation, wash the constructs 3x with 1X PBS or phosphate-free buffer of choice using the method detailed above to reduce shear stress on the constructs.
5. Add working solution of primary fluorescent dye to each well. Use volumes as recommended by manufacturer's protocol.

[10] Prepare any necessary dilutions in 1X PBS. Alternatively, use a phosphate-free buffer.

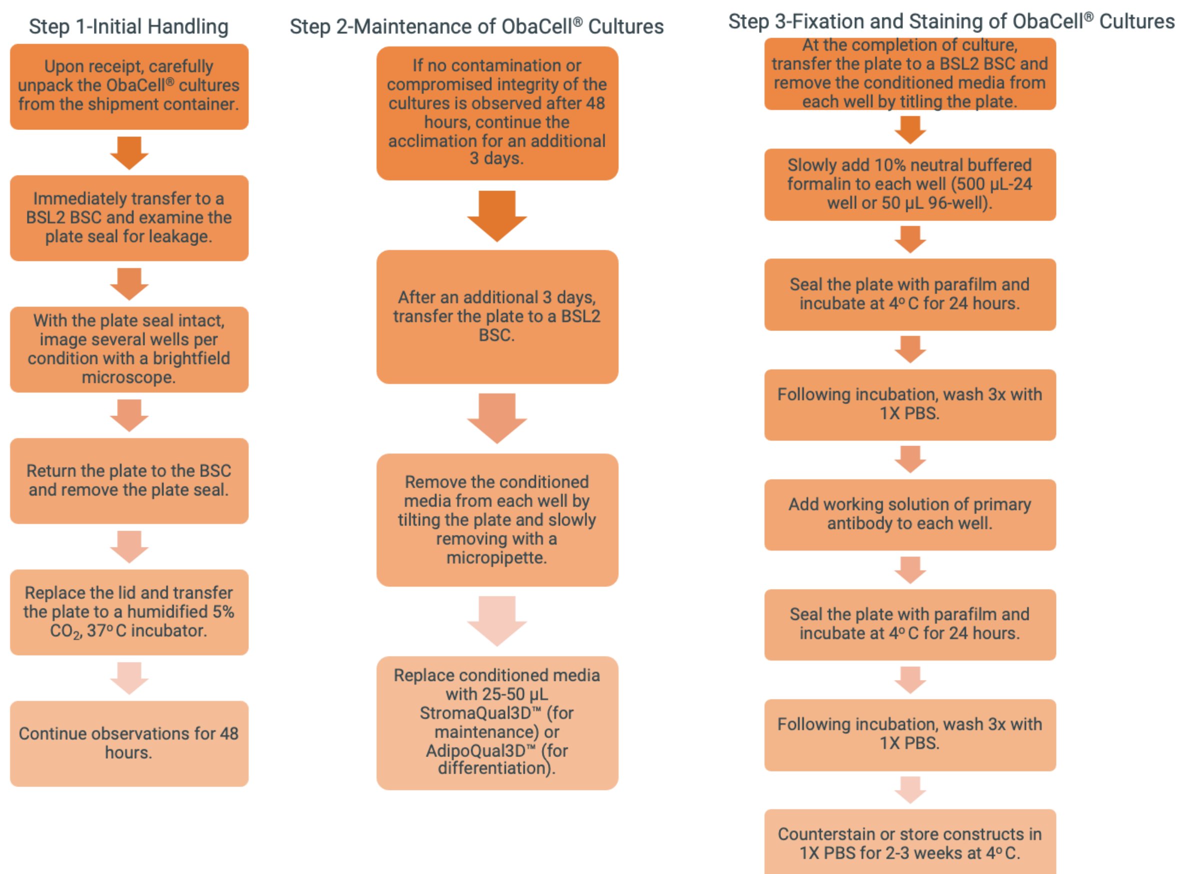
[11] Tilt the plate to allow liquid to collect at the base of the well. Use a micropipette to aspirate the volume slowly with consistent speed as to not disrupt the constructs.

[12] The constructs will appear as a dense region of gel at the base of the well and should appear distinct from the liquid interface when tilting.

[13] We recommend using 500 µL and 50 µL formalin for a 24-well plate and 96-well plate respectively. This volume will adequately submerge the constructs.

6. Seal the plate with parafilm, cover the plate in foil to minimize light exposure, and incubate at 4°C overnight.[14]
7. Following incubation, wash the constructs 3x with 1X PBS or phosphate-free buffer of choice using the method detailed above to reduce shear stress on the constructs.
8. Proceed with counterstaining or store constructs in 1X PBS at 4°C light-blocked with foil prior to imaging. [15]

Figure 5: Workflow diagram outlining the steps required to fix and stain ObaGel® cultures. Steps for fixation and staining of ObaGel® cultures include: Initial handling ObaGel® cultures, maintenance of the established ObaGel® cultures, fixation of ObaGel® cultures, staining of ObaGel® cultures, and storage of stained ObaGel® cultures.



[14] Overnight incubation is more than sufficient for most stains and can be reduced with experimentation. As a starting point, we recommend at least doubling the recommended incubation time from manufacturer's protocols for 2D cultures.
[15] Constructs should be stable at 4°C stored in 1X PBS for 2-3 weeks. It is not recommended that plates be stored for >6 weeks.

Appendix A: Troubleshooting

Problem	Reason	Solution
No gelation observed	ObaGel® pre-warmed at 37°C or equivalent prior to use	Do not heat ObaGel® prior to use, maintain on ice during use
	Repeated freeze-thaws	Aliquot ObaGel® into smaller volumes and thaw as needed
	Improper extended storage	Store at –20°C for up to one year from receipt date. Thaw and store at 4°C for no more than 48 hr prior to use.
Gelation observed during pipetting or handling	ObaGel® pre-warmed at 37°C or equivalent prior to use	Do not heat ObaGel® prior to use, maintain on ice during use
	ObaGel® not maintained at 4°C or on ice prior to use	Ensure ObaGel® is kept at 4°C or on wet ice prior to use
Contraction of 3D constructs	Physical disruption of the gel	Careful handling of the plates and manipulation of the constructs. Slowly remove and add media from edge of the well, tilt the plate to reduce shear stress at the gel-liquid interface.
	Inhomogeneous seeding solution	Pipette ObaGel®/ObaVate™ cell suspension vigorously immediately prior to seeding until solution appears homogenous. Pipette solution

		while seeding, every 3-6 replicates.
Rapid spheroid or organoid formation in 3D constructs	Too much media present at the gel-liquid interface	Collect conditioned media between feedings. Feed with recommended volumes.
	Initial seeding density of cells is too high	Reduce seeding density. Seeding density may need to be empirically determined for each cell type and lot and may depend on several factors, such as proliferation rate.
	Inhomogeneous seeding solution	Pipette several times to mix well and pipette several times between replicates
Spheroids do not remain intact	Insufficient incubation period	Allow the petri dishes to remain at 37°C, 5% CO_2 for 24-48 hr prior to manipulation
Constructs do not appear intact, or appear fragmented in wash solution	Constructs disrupted during pipetting	Pipette slowly and carefully when adding and removing reagents. Tilt the plate to collect solution from the gel-liquid interface. Use a wider-bore pipette tip to reduce shar stress on the constructs.
Increased background staining	Wash solution not fully penetrating the constructs	Repeat wash steps and consider incubating the constructs with the wash solution prior to removal. Note

		when the wash solution remains clear after incubation.
Gel does not appear dissolved following incubation	Incubation time not long enough	Incubate for a longer period of time, up to 1hr for extended cultures
	ObaZolve™ cell recovery solution not pre-warmed to 37°C prior to use	Warm ObaZolve™ to 37°C prior to use
	ObaZolve™ cell recovery solution improperly stored/handled	Store ObaZolve™ at 4C and use within 8 weeks of receipt. Purchase fresh reagents once expired.
No visible cell pellet	Cell pellet may have been disrupted during wash steps	Decant supernatant by inverting microtube, on final wash use a micropipette to remove any remaining volume
RNA yield – reduced quality and quantity	Not using ice-cold PBS following cell recovery	Use ice-cold PBS for wash steps
	Not devoting enough replicates per condition	Devote 3-6 replicate wells per experimental condition to yield enough quality RNA for endpoints

Appendix B: Pictorial Workflow Diagrams

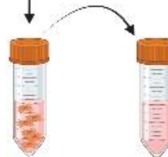
ObaGel® Culture Setup

Thaw ObaGel and ObaVate overnight at 4°C.

Thaw cells and resuspend in growth medium to remove DMSO.

Count cells and resuspend in ObaVate at 1x10^6 cells per mL.

Transfer volume of cell suspension to conical of ObaVate.



ObaVate cell suspension at 1x10^6 cells/mL

ObaVate only

Add the appropriate volume of ObaGel to the ObaVate cell suspension such that the solution is 1 part ObaGel and 3 parts ObaVate (1:3 ratio).

ObaVate cell suspension

ObaGel

Pipette the solution until it is homogenous with a p1000 pipette.

Pipette the solution with a pipette into a tissue culture plate, mixing the solution between replicates.

Transfer the plate to a humidified 5% CO_2 incubator at 37°C.

ObaGel® Culture Maintenance

Transfer StromaQual3D and AdipoQual3D to benchtop to warm to room temperature.

Examine the cultures for confluency, network formation, and stability.

Add recommended volume of StromaQual3D or AdipoQual3D to each well.
Media should be added slowly to the corner of each well.

Transfer the plate to a humidified 5% CO_2 incubator at 37°C.

Media changes will occur 2x per week for 3 weeks.
Spent media will be left in each well between feedings. Remove 50-60% of media prior to adding fresh media by tilting the plate and removing at the corner of the well, ensuring the constructs are not disturbed.

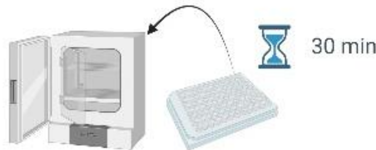
ObaGel® Culture Harvest

Warm ObaZolve to 37°C prior to use.

Carefully remove the media from all wells by tilting the plate and removing from the corner of each well.
~50-60% of the media volume will be removed from each well.

Add an equal volume of pre-warmed ObaZolve to each well for a 1:1 v/v of ObaZolve solution to construct volume.

Transfer the plate to a humidified 5% CO_2 incubator at 37°C for 30 minutes.



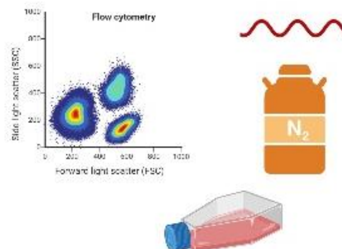
Pipette each well to observe if gel is dissolved.
If resistance is observed, incubate the plate for an additional 15 minutes.

Collect each well in a conical or microcentrifuge tube. Centrifuge each tube at 1200 RPM for 2 minutes.

Remove the supernatant with a pipette. Add one part ice-cold, sterile 1X PBS to each tube.

Centrifuge at 1200 RPM for 2 minutes. Remove the supernatant and repeat the PBS wash step an additional time.

Remove the supernatant. After resuspension of the cell pellet, proceed with the following endpoints: cell passage and expansion, cryopreservation of cells, setup for flow cytometry, or preservation and storage of cells for RNA isolation.



Appendix C: Tips for Success

1. Do not heat ObaGel® or ObaVate™ in a water bath or equivalent piece of equipment. Thaw both products overnight at 4°C.
2. Do not heat StromalQual3D™ or AdipoQual3D™ in a water bath or equivalent piece of equipment. Both products should be placed on a benchtop until they reach room temperature.
3. Do not centrifuge or vortex ObaGel® to remove precipitant. The precipitant is protein and will disperse upon pipetting.
4. You do not need to add culture media after initially establishing the ObaGel® culture. The ObaGel®: ObaVate™ mixture will support the cultures for one-week with no additional media.
5. When performing media changes, remove media by tilting the plate and removing media from the corner of the well. No more than 60% of spent media should be removed at a time. We suggest only removing 50% of spent media per media change.
6. The following calculations are based on the seeding of 60 wells of a 96-well plate at 5,000 cells per well. Each well has a 100 µL volume of ObaGel®: ObaVate™ cell mixture. The cell mixture is composed of a 1:3 ObaGel®: ObaVate™ ratio.
    - # Wells to be seeded = 60
    - Total mixture needed = 6 mL
    - ObaVate™ volume = 4.5 mL
    - ObaGel® volume = 1.5 mL
    - Cells per well = 5,000
    - Desired cell concentration of mixture= 50,000 per mL

   Add **300 µL** of 1 million per mL cell solution to **4.2 mL ObaVate™** and **1.5 mL ObaGel®**
7. When culturing in a 96-well plate, we recommend only using 60 of the 96 wells and filling the outer wells with sterile 1X PBS. The constructs in the outer 36 wells tend to contract and media evaporates at a quicker rate if not monitored closely.

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