Protocol for Establishment of Organoids(Mouse Intestinal Organoids is an example)

A.Establishment of Organoids from Primary Mouse Intestinal

1. Equipment, reagents and consumables

1.1 Equipment: Biosafety cabinet, pipette, carbon dioxide incubator, inverted microscope, centrifuge(Low-speed).

1.2 Reagent:Matrigengel(Cat:082703/082755) Mouse Intestinal Organoid Kit（Mogengel Cat：MA-0817H006L）,DPBS,Anti-Adherence Rinsing Solution(Mogengel Cat：MB-0818L03L)，Penicillin-Streptomycin Solution，DPBS containing 0.1%BSA，0.5M EDTA Solution（pH=8.0）

1.3 Consumables: sterile pipette tips; cell culture plate（48-well for example in this protocol）;Cell culture dish (diameter:3.5 cm, 6 cm and 10 cm),Sterile forceps, sterile tissue scissors, 70 μm strainer, and surgical blade, Sterile EP tube and other consumables. (Or be adjusted according to the experimental design).

2. Preparation before Experiment:

2.1 Put the Matrigengel in the ice box and put it in the refrigerator at 4℃ so that the Matrigengel can slowly melt overnight; (Do not allow this product to warm up above 4℃ during manipulation. Keep the product on ice and dilute using ice-cold solutions or cell suspensions.)

2.2 Prepare Mouse Intestinal Organoid Complete Medium as directed.

2.3 Prepare plenty of DPBS containing 0.1%BSA.

3.Experimental operating procedures

3.1Sacrifice mice according to the experimental animal ethics and operating norms approved by the unit.

3.2 Prepare 6 cm dishes and add ice-cold DPBS containing 0.1%BSA for later use.(kept on ice)

3.3 Under sterile conditions,remove 3~5cm intestinal tissue near the gastric end and put into the“3.2”pre-cold DPBS ..

3.4 Cut the intestinal cavity lengthwise, gently scrape off the surface villi, wash twice，cut to a 2mm wide intestinal segment, wash twice, and transfer to pre-cooled DPBS containing 5mM EDTA，wait 20min for digestion（kept on ice）.

3.5 After digestion, transfer the tissue fragments to a new dish containing DPBS to wash, and repeat twice to remove EDTA.

3.6 Rinse the 5 mL pipette tip by Anti-Adherence Rinsing Solution.Resuspend intestinal fragment with DPBS containing 0.1% BSA，pipetting the mixture up and down 3~4times，collect the suspension and filter it with a 70 μm strainer. and repeat this step once more time.

3.7 Centrifuge 300 g for 3 min to collect crypts, resuspend using 1mL of DPBS containing 0.1% BSA, take 20 μL of suspension for microscopic examination and crypt counting.

3.8 Counting is completed, aspirate the suspension containing the required amount of crypts, centrifuge 300g for 3 min 。

3.9 Aspirate the supernatant and resuspend the crypts in Matrigengel. The Matrigengel should be kept on ice to prevent it from solidifying. Perform the process as quickly as possible. The volume of Matrigengel used depends on the size of the pellet. Approximately 70~100 crypts should be plated in 10 μL of Matrigengel.

CRITICAL: Do not overly dilute the Matrigengel (Matrigengel ratio should be >70% (Matrigengel vol/Total vol)), as this may inhibit the proper formation of the solid droplets.

3.10 Plate the Matrigengel containing crypts at the bottom of 48-well cell culture plates in droplets of 12 ~20μL each around the center of the well.proceed with plating as quickly as possible, as the Matrigengel may solidify in the tube or pipette tip. Do not let the Matrigengel touch the wall of wells.

The amount of crypts-Matrigengel mixture added for different plates is shown in the table below:

Number of wells	96	48	24

Volume of crypts-Matrigengel mixture（μL）	3~8	12~20	20~30

3.11 Place the culture plate into carbon dioxide incubator at 37 °C for 15 min to let the Matrigengel solidify.

3.12 After Matrigengel was completely solidified, the prepared complete medium of mouse intestine organoid was added to a 48-well plate at 250uL per well.

Note: Please add slowly along the wall to avoid damaging the solidified structure.

3.13 The 48-well plate was incubated in a carbon dioxide incubator at 37℃.

3.14 The medium should be changed every 3 days to avoid damaging Matrigengel when changing the medium.

3.15 Closely monitor the growth status of organoids. Ideally, mouse intestinal organoids should be established within 5 to 7 days.（Changes in mouse intestinal organoids can be observed by taking pictures daily.）

B.Splitting and Passaging of Mouse Intestinal Organoids

4. Equipment, reagents and consumables

4.1 Equipment: Same as 1.1.

4.2 Reagent:Melted Matrigengel, Mouse Intestinal Organoid Kit, Organoid Dissociation Solution (Mogengel Cat：MB-0818L01L), Epithelial Organoid Basal medium(Mogengel Cat：MB-0818L07)

4.3 Consumables: Sterile pipette tips; 48-well cell culture plate;Sterile EP tube (Or be adjusted according to the experimental design).

5. Experimental operating procedures

5.1 Keep the original culture medium, gently scrape off the extracellular Matrigengel and organoid mixture with a pipette tip, transfer to a 1.5mL， Pipette the organoid suspension up and down to mix thoroughly by pipetting against the bottom of the tube to create pressure, which will aid the removal of Matrigengel。

5.2 Centrifuge with 300g at 4℃ for 3min, discard the supernatant,Aspirate the supernatant and split organoids using either Organoid Dissociation Solution or by mechanical disruption. For Organoid Dissociation Solution-based cell dissociation, resuspend the pellet in 0.2 mL of Organoid Dissociation Solution, pipette up and down and incubate at 37 °C until organoids fall apart（Do not dissociate in Organoid Dissociation Solution for >7 min）. For mechanical disruption-based resuspended the bottom organoid precipitation with Epithelial Organoid Basal medium, Pipette the organoid suspension up and down 5~10 times，centrifuge again with 300g at 4℃ for 3min,Clean and place on ice.

5.3 After shearing is complete, wash twice with 1 mL of Epithelial Organoid Basal medium, and centrifuge at 300g for 3 min.

5.4. Organoid precipitation was resuspended with appropriate amount of Matrigengel, then placed on ice after resuspended, and the resuspended time should not be more than 30s to avoid premature solidification of Matrigengel.

Note: The dilution ratio of Matrigengel should be above 70% to ensure the structural stability of Matrigengel during culture.

5.5. Place the mixed suspension of Matrigengel and organoid into the center of the bottom of the 48-well cell culture plate, avoiding the suspension from contacting the side wall of the plate, with about 15uL for each well.

Note: This step should be completed as soon as possible to prevent Matrigengel from solidifying at room temperature.

5.6. Put the inoculated culture plate into 37℃ carbon dioxide incubator and incubate for about 15min until Matrigengel solidified.

5.7. After Matrigengel was completely solidified, the prepared complete medium of mouse small intestine organoid was added to a 48-well cell culture plate of 250μL per well.

5.8. The 48-well cell culture plate was placed in a humidified incubator at 37 °C and 5% (vol/vol) CO_2.