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ADSI –CCO-SOP-6.0

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Issued:
28/03/2019
Revised:

INTESTINAL ORGANOID DRUG SCREENING

Purpose

The SOP-ADSI-6.0 was issued to describe the timeline, workflow and procedure of anti-tumor activity testing of drugs on wild-type versus tumor organoids.

Scope

SOP-ADSI-6.0 is intended to cover all resources, personnel and equipment needed to investigate the response (viability) of wild-type as well as tumor organoids on treatment with anti-tumor drugs by using the RealTime-Glo MT Cell Viability Assay.

Introduction

Therapeutic strategies that are based on the individual genetic profile of a patient represent a new frontier of applied cancer research. Especially in cancer research, standard cell culture conditions fail to proper mimic the parental tumor architecture and microenvironment. In this context, tumor-organoids are of special relevance. Tumor-organoids are three-dimensional in vitro cultures derived from the cells obtained from the patient tumor. Tumor-organoids keep the special properties of the original patient's tumor with its microenvironment and mirror the key-features. Thus, tumor-organoids are an ideal tool to identify patient-specific therapies by performing drug-screenings on this primary patient material. The aim of this SOP is to provide protocols for the analysis of tumor-organoids and organoids viability in response to anti-cancer drugs.

1. Equipment, cell culture media, reagents and solutions

1.1 Equipment

- Incubator Binder APT.line™ (150CE2)
- Laminar Air-Flow Labculture Plus ESCO, Class II BSC

- Micro Plate Reader (Mithras LB940)
- Centrifuge (4˚C) Hettich Zentrifugen ROTIXA A50 RS
- ImageXpress Micro XLS Widefield High-Content Analysis System
- Table centrifuge VWR Mini Star
- Analog Vortex Mixer (VWR)
- Waterbath

1.2 Materials

- 24 well Culture plates (Greiner bio-one #662160)
- 96-well-plate (Corning #3603)
- Sterile Erlenmeyer flask (200 ml, 500ml)
- Sterile 15 ml and 50 ml tubes
- Sterile 0,5 ml, 1,5 ml, 2 ml and 5 ml tubes
- Set of pipettes 10 µl, 20 µl, 200 µl, 1000 µl and pipette tips
- Pipette Aid and pipettes (10 ml, 25 ml, 50 ml)
- Box with crashed ice

1.3 Chemicals

- Advanced DMEM/F-12 (Gibco-Thermo Fisher #12634028)
- GlutaMax 100 x (GIBCO #35050038)
- HEPES (1 M) (GIBCO #15630080)
- Pen/Strep 100 x (GIBCO #15070063)
- 0.25 % Trypsin-EDTA (Sigma-Aldrich #T4049-100ML)
- WCM* (Broutier 2016)
- RCM **(Broutier 2016)
- NCM***(Broutier 2016)
- Matrigel (Corning #356231)
- DMSO Hybri-Max (Sigma #D2650)
- Nicotinamide (Sigma-Aldrich #N0636-100G)
- N-Acetylcyteine (Sigma-Aldrich #A9165-25G)
- B-27 (Thermo Fisher #17504044)

* Wnt3a conditioned medium was obtained from L-Wnt3a cells. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

** R-spondin1-conditioned medium was produced from 293T-HA-Rspol-Fc producer cell line; MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

*** Noggin-conditioned medium from HEK293-mNoggin-Fc cell line. MTA for the use of producer cell line was obtained from the Hubrecht Institute, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands

- A83-01 (R&D Systems Europe #2939)
- SB 202190 (Sigma Aldrich S7067-5MG)
- Primocin – 500 mg (InvivoGen Cat. Code: ant-pm-1)
- *R-Spondin (REPROTECH #120-38) optional instead of RCM (100 ng/ml)*
- *Noggin (REPROTECH #250-38) optional instead of NCM (1000 ng/ml)*
- *Wnt3a – if required 100 ng/ml*
- m-EGF (REPROTECH #315-09)
- Real Time – Glo™ MT Cell Viability Assay (Promega #G9711)
- Gastrin (R&D Systems Europe #3006/1)
- Prostaglandin E2 (Sigma-Aldrich #P0409)
- STEMPRO hESC (Fisher A1000701)

1.4 Buffer and Solutions

Buffer preparation for organoids see ADSI – CCO – SOP-1

2. Reagent preparation

2.1 Media preparation for intestinal organoids

Medium preparation for organoids see SOP-ADSI-1.0.

2.2 Preparation of cells

One well of organoids from a 24 well plate is needed per drug testing condition.

2.2.1 Harvest

- Remove medium from the wells (check if the matrigel is not loose!)
- Add 500 µl cold GF- to every well
- Detach matrigel drop with 1000 µl tip
- Transfer to 15 ml tube containing (keep on ice)
- Wash wells with 500 µl cold GF-
- Add GF- up to 10-14 ml
- Centrifuge for 3 min 800 rpm at 4˚C
- Remove supernatant (matrigel pellet with the organoids should be visible by eye).

- If the matrigel pellet is very big, remove the supernatant and resuspend the pellet with 3ml cold GF-. Pipette up and down. Add 7ml of ice cold GF- and centrifuge again for 3 minutes at 1200 rpm at 4°C.

2.2.2 Trypsinisation

- Add 200 to 500 µl 0.25% Trypsin-EDTA (depending on the size of the pellet)
- Pipette up and down
- Incubate 7 min at 37°C (water bath)
- Pipette up and down with a 100 µl pipette tip (if cells will not get loose either extend the incubation time or attach a 200µl pipette tip to the 1000µl pipette tip)
- Check repeatedly under the microscope (in the tube).
- Repeat incubation cycles until the majority of cells are single cells
- If single-cells still do not appear after 3 times of checking under the microscope, add 200 µl 0.25% Trypsin-EDTA and extend incubation cycles at 37°

2.2.2 Seeding

- When single cell suspension is obtained, add 10 ml GF-
- Centrifuge for 5 min, 1500 rpm at 4°C
- Remove the supernatant and resuspend in 1ml of GF- and count the cells.
- Add 10 ml GF-
- Centrifuge for 5 min, 1500 rpm at 4°C
- Resuspend the cells in GF- at $1.5x10^6$ cells/ml in the required amount GF- (example: 3,3 µl GF- and 6,7 µl matrigel per well in 96 well plate) resulting in 5000 cells/ well.
- Seed 10 µl droplets per well. Pipette fast to avoid cooling of the pre-warmed plate. (See ***seeding scheme***)
- Incubate for 10-15 min at 37°C (5% CO2)
- Carefully add 150 µl pre-warmed medium (M2 - M6) per well.
- Incubate at 37°C (5% CO2) for 3 days. (See ***time schedule***).
- After 3 days, organoids are ready for the drug screening (Real Time-Glo Viability assay).

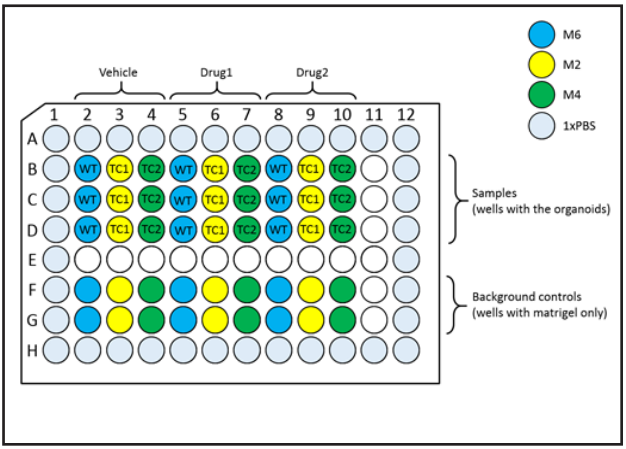

Figure 1: Seeding scheme for WT and tumor-organoids on a 96 well plate. Assay to investigate the viability of wild-type and tumor organoids upon drug treatment. Seed 10 µl droplets of matrigel mixed with GF- and 5x10^3 cells per wells. WT… wild type organoids, TC1… Tumoroids clone 1, TC2… Tumoroids Clone 2. The wells F2 to G10 – with matrigel only. Empty wells E2-11 prevent from crosstalk between samples and background controls. Outer wells (indicated with light blue wells) are filled with 150 µl PBS to prevent evaporation.

Time schedule

Weekday	Day	Media	RTG	Images
Friday	d0	seed in respective EM		
Saturday	d1			
Sunday	d2			
Monday	d3	drug/ vehicle + RTG	RTG timepoint 1h, 2h	+
Tuesday	d4		RTG timepoint 24h	+
Wednesday	d5		RTG timepoint 48h	+
Thursday	d6		RTG timepoint 72h	+

3. Procedures for viability assay conduction

This SOP shows a simple example with 2 different drugs + 1 control (vehicle) tested at 3 different batches of organoid cultures (1x healthy WT organoids + 2 x Tumor organoids in different media –from the same patient). The experimental setup allows the comparison of the anti-tumor activity of a drug on two different organoid clones compared to organoids from healthy tissue.

3.1 Preparatory work

- Put the Real Time-Glo (RTG) Kit at RT, vortex and centrifuge down.
- Prepare 1,25 x of the respective media (#2, #3, #4, #5, or #6). The medium is stable up to 2 weeks at 4°C.
- Table 1 shows media preparation for 4,4 ml 1,25x medium.

- A 5x stock solution of the RTG enzyme and substrate is prepared in GF-. This is essential to guarantee an equally amounts of Real Time-Glo throughout the entire assay.
- The 5x RTG in GF- is than used to prepare the 5x drugs/ vehicle stock solutions (*The 5x solution mixed with the 1,25x medium results in a 1x medium in the well*). The amount of stock solution required depends on how many wells per 96 well plate is to be tested (*always work in triplicates + duplicates for the blank control*). In total a 96 well contains 80 µl of the 1,25 x media solution + 20 µl of the 5 x stock solution.

Media		**Expansion Media**		**M2+ Y**	**M3 + Y**	**M4+ Y**	**M5+Y**	**M6+Y = WT+Y**
Compound	**stock conc.**	final conc.	**dilution**	**µl**	**µl**	**µl**	**µl**	**µl**
Final volume				5500.00	5500.00	5500.00	5500.00	5500.00
GF-				3379.20	3376.82	3377.37	3374.98	624.43
WCM	1x	50%	2 x	0	0	0	0	2750.00
RCM	10x	10%	10 x	550.00	550.00	550.00	550.00	550.00
NCM	20x	5%	20 x	275.00	275.00	275.00	275.00	275.00
mEGF	10000x	50 ng/ml	10000 x	0	0.55	0	0.55	0.55
Nicotinamide	1M	10 mM	100 x	55.00	55.00	55.00	55.00	55.00
N-acetyl*	500mM	1.25 mM	400 x	13.75	13.75	13.75	13.75	13.75
B27	50x	1 x	50 x	110.00	110.00	110.00	110.00	110.00
Y-27632 (ROCK-inh)	10mM	10 µM	1000 x	5.50	5.50	5.50	5.50	5.50
A83-01 (TGFß-inh)	1,5mM	500 nM	3000 x	0	0	1.83	1.83	1.83
SB202190 (P38 inh)	30mM	10 µM	3000 x	0	1.83	0	1.83	1.83
Primocin	50mg/ml	100 µg/ml	500 x	11.00	11.00	11.00	11.00	11.00
Gastrin	100µM	10 nM	10000 x	0.55	0.55	0.55	0.55	0.55
PGE2	100µM	10 nM	10000 x					0.55
Volume without GF-				1020.80	1023.18	1022.63	1025.02	3775.57
1,25x stock medium				4400.00	4400.00	4400.00	4400.00	4400.00

*N-acetyl needs to be added last because of it's low pH!

3.2 Drug treatment

- Remove medium from the wells of 96 well plate (do not touch the matrigel drop. Use P1000+P10 tip).
- Carefully add 80 µl of pre-warmed 1,25x media (Medium #2, #3, #4, #5, #6)
- Add 20 µl of pre-warmed 5x RTG in GF- containing drugs or vechicle, according the pipetting scheme.
- Incubate at 37°C (5% CO2).
- Perform luminescence measurement at time points: 1h, 4h, 24h, 48h, and 72h.
- Take pictures of every well with organoids at time points: 1h, 24h, 48, 72h, (optionally also 96h, 120h, and 144h).
- If you plan to image longer than 72h, than refresh medium after 72 hours to medium without enzyme and substrate, but with the drug.

3.3 Measurement

- Switch on the luminometer (Mitras) at least 1 hour before the measurement.
- Turn on the PC and open the program MikroWin 2000.
- Open program to measure luminescence for 1 and 0.5 s according to your instrument setting. Make sure thate the luminometer is at 37°C
- Use a filter-set allowing luminescence measurement
- The measurement is very temperature sensitive. Keep the plates in a styrofoam box for the transfer from the incubator to the luminometer. Try to be as fast as possible to prevent the plate from cooling down.
- Export the data as EXCEL file for further calculations.

4. References

Broutier L, Andersson-Rolf A, Hindley C J, Boj S F, Clevers H, Koo B K, Huch M. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. Nat Protoc. 2016 Sep;11(9):1724-43. doi: 10.1038/nprot.2016.097. Epub 2016 Aug 25.

5. Applicable references

ADSI – CCO - SOP-1.0, ADSI – CCO - SOP-2.0, ADSI – CCO - SOP-3.0