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Title: Evaluating Cell Death Using Cell-Free Supernatant of Probiotics in Three-Dimensional Spheroid Cultures of Colorectal Cancer Cells

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hee Min Yoo**: This protocol has been optimized to make it easier to investigate the anti-cancer effects of *Lactobacillus* cell-free supernatant in multiple types of colon cancer cell spheroids **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Hee Min Yoo**: This technique is the first approach that investigates the anti-cancer effects of LCFS on colon cancer spheroids under in vivo mimicry conditions **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Introduction of Demonstrator on Camera

~~1.3. **Joo-Eun Lee**: Demonstrating the procedure will be Jina Lee, a graduate student researcher from my laboratory **[1][2]**.~~

~~1.3.1. INTERVIEW: Author saying the above~~
~~1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera~~

Protocol

2. Bacterial Cell Culture and *Lactobacillus* Cell-Free Supernatant (LCFS) Preparation

2.1. Begin by placing an autoclave-sterilized MRS (M-R-S) agar plate in a 37-degree Celsius hydrogen anaerobic chamber with 20 parts per million oxygen **[1-TXT]** and inoculating the plate with freshly thawed *Lactobacillus* bacterial stock **[2]**.

2.1.1. WIDE: Talent placing plate into chamber **TEXT: MRS: de Man, Rogosa, and Sharpe**

2.1.2. Talent adding bacteria to plate

~~2.2. **[1] [2]**.~~

~~2.2.1. Talent placing tube into chamber~~

~~2.2.2. Talent removing oxygen~~

2.3. Add 2-3 milliliters of MRS broth supplemented with L-cysteine to the tube **[1-TXT]** and seal the tube with a butyl rubber stopper and tube cap **[2]**.

2.3.1. Talent adding broth to tube **TEXT: See text for all agar, broth, medium, and solution preparation details**

2.3.2. Talent adding stopper and/or cap

2.4. Next, use a loop to collect a single colony **[1]** and place the colony into a 1.5-milliliter culture tube containing 500 microliters of PBS **[2]**.

2.4.1. Shot of plate, then colony being selected
2.4.2. Talent adding colony to tube

2.5. Resuspend the colony homogenously within the saline **[1]** and load the entire bacterial suspension into a 1-milliliter syringe **[2]**.

2.5.1. Colony being mixed
2.5.2. Talent loading suspension into syringe

2.6. Insert the needle into the center of the culture tube lid **[1]** and deliver the colony into the medium **[2]**.

2.6.1. Talent adding needle into tube lid
2.6.2. Colony being added to medium

2.7. Place the culture in a shaking incubator at 37 degrees, 5% carbon dioxide, and 200 revolutions per minute **[1]**.

2.7.1. Talent placing culture into incubator

2.8. After 2 days, measure the OD_{620} (O-D six-twenty) of the culture on a spectrophotometer **[1-TXT]**.

2.8.1. Talent loading sample onto spectrophotometer **TEXT: OD_{620}: optical density at 620 nanometers**

2.9. When the OD_{620} equals 2, sediment the bacteria by centrifugation **[1-TXT]** and wash the bacteria pellet with PBS **[2]**.

2.9.1. Talent adding tube(s) to centrifuge **TEXT: 15 min, 1000 x g, RT**
2.9.2. Shot of pellet, if visible, then PBS being added to tube, with PBS container visible in frame

2.10. After the second centrifugation, resuspend the bacteria in 4 milliliters of RPMI 1640 (R-P-M-I sixteen forty) supplemented with 10% fetal bovine serum without antibiotics **[1-TXT]** and place the bacteria in the shaking incubator for 4 hours at 100 revolutions per minute **[2]**.

2.10.1. Talent adding medium to tube, with medium container visible in frame *Videographer: Important step* **TEXT: RPMI: Roswell Park Memorial Institute**
2.10.2. Tube shaking in incubator *Videographer: Important step*

2.11. At the end of the incubation, pellet the bacteria by centrifugation **[1]** and sterile-filter the recovered supernatant through 0.22-micron strainer for minus 80 degree-Celsius storage **[2]**.

2.11.1. Talent placing tube(s) into centrifuge *Videographer: Important step*
2.11.2. Talent filtering supernatant *Videographer: Important step*

3. Colorectal Cancer Cell Line Preparation

3.1. To prepare colorectal cancer cell line cells for spheroid formation, culture the cells of

interest as monolayers in 100-millimeter Petri dishes in the cell culture incubator **[1-TXT]**.

3.1.1. WIDE: Talent placing plate(s) into incubator **TEXT: *e.g.*, DLD-1, HT-29, and WiDr cell lines**

3.2. When the cells reach a 70-80% confluency, wash the cultures two times with 4 milliliters of PBS per wash **[1]** before detaching the cells with 1 milliliter of 0.25% trypsin-EDTA per dish **[2]**.

3.2.1. Talent washing plate, with PBS container visible in frame
3.2.2. Talent adding trypsin-EDTA to container, with trypsin-EDTA container visible in frame

3.3. After two minutes, check for dissociation under a light microscope **[1]**. If they cells have detached, neutralize the enzymatic reaction with 5 milliliters of growth medium **[2]**.

3.3.1. Talent at microscope, checking dish
3.3.2. Talent adding medium to dish, with medium container visible in frame

3.4. Collect the cells by centrifugation in one 15-milliliter conical tube per cell culture **[1-TXT]** and gently resuspend the pellets with 3 milliliters of growth medium per tube **[2]**.

3.4.1. Talent placing tube into centrifuge **TEXT: 3 min, 300 x g, RT**
3.4.2. Shot of pellet(s) if visible, then medium being added to tube, with medium container visible in frame

3.5. Then count the number of viable cells by trypan blue exclusion **[1]**.

3.5.1. Talent at microscope, counting cells

4. Spheroid Formation

4.1. After counting, dilute the cells to a 1-2 x 10^5 cells/milliliter concentration in fresh medium **[1]** and add methylcellulose to the cells to a final 0.6% concentration **[2]**.

4.1.1. WIDE: Talent adding medium to tube, with medium container visible in frame *Videographer: Important step*
4.1.2. Talent adding methylcellulose to tube, with methylcellulose container visible in frame *Videographer: Important step*

4.2. Transfer the cells to a sterile reservoir **[1]** and use a multichannel pipette to dispense 200 microliters of cells to each well of an ultra-low attachment 96-well round bottom

microplate **[2]**.

4.2.1. Talent adding cells to reservoir *Videographer: Important step*
4.2.2. Talent adding cells to well(s), with reservoir visible in frame *Videographer: Important step*

4.3. Then place the plate in the cell culture incubator for 24-36 hours **[1]** before checking for the formation of spheroids by light microscopy **[2]**.

4.3.1. Talent placing plate into incubator
4.3.2. LAB MEDIA: Figure 2

5. 3D colorectal Cancer Cell LCFS Treatment

5.1. For LCFS (L-C-F-S) treatment, serially dilute the prepared, thawed LCFS stock solution in fresh growth medium to 25-, 12.5-, and 6% concentrations **[1]** and use a 200-microliter pipette to carefully remove as much supernatant as possible from each well of the plated colorectal cancer cell line cultures **[2]**.

5.1.1. WIDE: Talent adding LCFS to medium, with medium and LCFS containers visible in frame *Videographer: Important step*
5.1.2. Medium being aspirated from well(s) *Videographer: Important/difficult step*

5.2. Then gently add 200 microliters LCFS-supplemented growth medium in triplicate to each set of cancer cells **[1]** and return the plate to the incubator for an additional 24-48 hours **[2]**.

5.2.1. Talent adding medium to well(s), with medium container visible in frame *Videographer: Important/difficult step*
5.2.2. Talent placing plate into incubator *Videographer: Important step*

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?
2.10., 2.11., 4.1., 4.2., 5.1., 5.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?
5.1.,5.2. are the most difficult steps as they require gentle handling when removing the growth medium. Otherwise, the spheroids can break, making it impossible to observe the proper effects of LCFS.

Results

6. Results: Representative Effects of LCFS Co-Culture on Spheroid Formation and Viability

6.1. A methylcellulose concentration of 0.6% transforms the tested cancer cell line cells into compact spheroids **[1]**, indicating that spheroids can be generated from several types of colorectal cancer using this protocol **[2]**.

6.1.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize 0.6:HT-29 image*
6.1.2. LAB MEDIA: Figure 2

6.2. Spheroids cultured with 25% LCFS exhibit disrupted surfaces **[1]** and a decreased cell viability in a dose dependent manner **[2]**.

6.2.1. LAB MEDIA: Figures 3B and 3D *Video Editor: please emphasize 25 images*
6.2.2. LAB MEDIA: Figures 3B and 3D *Video Editor: please add descending arrow from dark blue data bar to lightest blue data bar in each graph*

6.3. Indeed, LCFS-co-cultured cells demonstrate higher levels of propidium iodide expression **[1]** compared to viable control cells **[2]**.

6.3.1. LAB MEDIA: Figure 4 *Video Editor: please emphasize red signal in LCFS images and black data bars*
6.3.2. LAB MEDIA: Figure 4 *Video Editor: please emphasize lack of signal in Control images and white data bars*

6.4. Reverse transcriptase-PCR analysis **[1]** … western blotting **[2]** … and flow cytometric Annexin five-7AAD (seven A-A-D) analyses can also be performed to assess the effects of LCFS on cancer cell apoptosis **[3]**.

6.4.1. LAB MEDIA: Figure 5A *Video Editor: please emphasize dark blue data bars*
6.4.2. LAB MEDIA: Figures 6A-6C *Video Editor: please emphasize bands in PARP1, Bcl-xl, and p-IkappaBalpha sections of LCFS lanes*
6.4.3. LAB MEDIA: Figure 6D *Video Editor: please emphasize cells in top right quadrant of LCSF plots*

Conclusion

7. Conclusion Interview Statements

7.1. **Hee Min Yoo**: Be sure to handle the spheroids gently when removing the growth medium, as the spheroids can easily break, making it impossible to observe the effects of the LCFS treatment **[1]**.

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (5.1., 5.2.)

7.2. **Hee Min Yoo**: Following this procedure, cell viability assays, quantitative real-time PCR, and western blotting and FACS analyses can be performed to characterize the anti-cancer effects of LCFS in greater detail **[1]**.

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera