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Preparing EV-depleted media

Aizea Morales Kastresana[1], Bryce Killingsworth[1], Joshua A Welsh[2], Tim Traynor[1], Jennifer Jones[1]

[1]Translational Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health;

[2]Translataional Nanobiology Section, Laboratory of Pathology, Center for Cancer Research, National Cancer Institute, National Institutes of Health

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Translational Nanobiology Section

Joshua Welsh

ABSTRACT

This protocol describes a method to prepare bovine EV-depleted 10% FBS cell culture media by ultracentrifugation of 20% FBS media and subsequent dilution.

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GUIDELINES

This protocol is written specifically for a Type 45 Ti Rotor, using tubes holding 65 mL of 20% FBS media. The steps regarding preparation and ultracentrifugation of media in this protocol can be applied to different rotors and ultra centrifuges as long as all product manuals are consulted and potential modifications to centrifugation time is considered (based on k-factor differences between rotors.)

MATERIALS

NAME	CATALOG #	VENDOR
FBS		Invitrogen - Thermo Fisher
Polycarbonate bottle assembly (thick-walled tube red aluminum cap rubber stopper)	355622	Beckman Coulter
1L 0.2 µm PES Nalgene bottle-top filter		
1 L sterile cell culture bottle		

MATERIALS TEXT

Additional reagents
- Base media (ex: RPMI)
- Supplements (ex: L-Gutamine, sodium pyruvate)

Equipment
- Optima XE series ultracentrifuge
- Beckman Coulter metal rack to hold 355622 polycarbonate bottles
- Type 45 Ti Rotor

DISCLAIMER:

> This protocol summarizes key steps for a specific type of method, which is one of a collection of methods and assays used for EV analysis in the NCI Translational Nanobiology Section at the time of submission of this protocol. Appropriate use of this protocol requires careful, cohesive integration with other methods for EV production, isolation, and characterization.

BEFORE STARTING

- This protocol involves a 18 hour centrifugation so it is recmmended to start in the evening.

Setting up the centrifugation

1 Turn on and log into the ultracentrifuge using the account and password specific to the device.

2 Write your information on any sign-up sheets associated with the device.

3 Place 80 mL of FBS and one new 500 mL bottle of media in the 37 ˚C water bath and allow FBS to thaw.

4 While the FBS is thawing inspect the refrigerated rotor to ensure there are no cracks or other structural issues and that all o-ring seals are intact and greased with vacuum grease.

5 Obtain a set of six sterilized polycarbonate ultracentrifuge tubes with rubbers stoppers and red aluminum caps. Inspect all the tubes to ensure they look clean and there are no cracks. Generally the tubes will crack after 10 uses, due to stress induced by autoclaving.

6 Sterilize then transport the thawed FBS, media, and a ultracentrifuge tube rack into a sterile hood.

7 Pipette 52 mL of media into each tube, avoiding all bubbles and drips on the outside of the tubes (any should be quickly wiped up.)

8 Pipette 13 mL of FBS into each tube, avoiding bubbles and drips on the outside of the tubes.

9 Carefully inspect each tube to ensure the exact same volume is in each tube as even small differences can lead to severe rotor or centrifuge damage.

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10 Fasten the stoppers and caps onto the tubes very tightly.

11 Insert the tubes in the rotor and secure the rotor cap tightly, using a metal rod to tighten the top if necessary.

12 Transport the filled rotor to the centrifuge.

13 The vacuum button may need to be pressed to release pressure before the door of the centrifuge can be oppened.

14 Insert the filled rotor into the ultracentrifuge.

15 Select the rotor with its specific serial number (etched onto the rotor bottom) in the rotor selection menu.

16 Create a run at 100,000 x g for 18 hours at 4 ˚C and press vacuum.

17 After the device has sufficiently held a vaccum, press the green Start or Go button.

18 Stay with the ultracentrifuge until it reaches its final speed and stop the centrifuge if you have even a slight concern about an unusual sound or movement indicating an imbalance. The ultracentrifuge will hum as it reaches speed but not rattle.

Harvesting the media

19 Obtain a new bottle of media which can be cold unless you would like to use the EV-depleted media immediatley.

20 The moment that the rotor is done spinning, carefully remove the rotor and place it on the centrifuge or adjacent table. If more than 10 minutes elapses between when the rotor stops spinning and you are able to harvest the media you should consider re-centrifuging it as the supernatant may not be depleted of particles any longer.

Though the EV pellet is not visible you should handle the rotor and tubes with the understanding that there is a fragile pellet of EVs at the bottom of each of the tubes and any significant movement or josteling can result in part of the pellet becoming resuspended in the supernatant before it is harvested.

21 Use tweezers or other metal instrument to slowly and gently lift each ultracentrifuge tube from its chamber and place onto the metal rack, minimizing movement and bumping.

22 Inspect all tubes and tube slots to ensure none were cracked and had their sterility compromised.

23 Sterilize the outside of the tubes, the rack, the media bottles, and a 0.2 µm cell culture 1 L filter bottle and tranfer all to a hood.

24 In one motion, pipette off 50 ml from the 65 ml of media in each tube and transfer it on top of the filter

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25 Add 300 mL of media to the ultracentrifuged 20% FBS media, as well as any non-EV containing supplements

26 Filter the media into the bottle and store for later use

Cleaning

27 Remove all items from the hood

28 Add tap water to each tube and let sit for at least 30 minutes

29 Discard the tap water and refill with tap water for another 30 minutes

30 Discard the tap water and, using a centrifuge tube brush and 1% Alconox solution, scrub the insides of each tube and cap and stopper

31 Rinse the each tube and cap and stopper first with tap water and DI water. Then re-rinse each tube and cap and stopper with a small volume of pure water at least twice.

32 Allow to air-dry, covered to prevent dust accumulation.

33 Autoclave using the Gravity 2 setting and a sealable autoclave bag with indicator.

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