Video Article

Production of Chick Embryo Extract for the Cultivation of Murine Neural Crest Stem Cells

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Abstract

The neural crest arises from the neuro-ectoderm during embryogenesis and persists only temporarily. Early experiments already proofed pluripotent progenitor cells to be an integral part of the neural crest[1]. Phenotypically, neural crest stem cells (NCSC) are defined by simultaneously expressing p75 (low-affine nerve growth factor receptor, LNGFR) and SOX10 during their migration from the neural crest[2,3,4,5]. These progenitor cells can differentiate into smooth muscle cells, chromaffin cells, neurons and glial cells, as well as melanocytes, cartilage and bone[6,7,8,9]. To cultivate NCSC *in vitro*, a special neural crest stem cell medium (NCSCM) is required[10]. The most complex part of the NCSCM is the preparation of chick embryo extract (CEE) representing an essential source of growth factors for the NCSC as well as for other types of neural explants. Other NCSCM ingredients beside CEE are commercially available. Producing CCE using laboratory standard equipment it is of high importance to know about the challenging details as the isolation, maceration, centrifugation, and filtration processes. In this protocol we describe accurate techniques to produce a maximized amount of pure and high quality CEE.

Video Link

The video component of this article can be found at https://www.jove.com/video/2380/

Protocol

The authors state that experiments on animals were performed in accordance with the European Communities Council Directive (86/609/EEC), following the Guidelines of the NIH regarding the care and use of animals for experimental procedures and the regulations set forth by the Institutional Animal Care and Use Committee (IACUC) at the University of Duisburg-Essen (Germany).

Part 1: Setting Up (not Shown on Video)

PREPARING MATERIALS AND SOLUTIONS (prior to taking the eggs out of the incubator)

- Eggs have to be incubated in a humidified incubator for 11 days at 37°C (100°F).
- 70% ethanol spray, clean plates, precise clean forceps, 4°C (40°F) cold sterile DMEM on ice and 60 mL sterile syringes
- 50 mL Sterile corning tubes have to be half-filled with 4°C cold DMEM to mix the macerated embryos at a ratio of 1:1 (macerated mass: DMEM). As soon as the eggs are taken out of the incubator dissection of the chick embryos has to be started.
- Sterilize dissection tools in the autoclave or on the day of dissection, immerse the tools in 70% ethanol for 20 minutes.

Part 2: Dissection of the Chick Embryos (demonstrated on Video)

1. Wet the incubated eggs with a 70% ethanol spray for at least 30 seconds and dry them carefully with clean soft paper towels while not shaking the eggs excessively.
   Note: We recommend not dissecting more than 60 chick embryos simultaneously.
2. Open the eggs carefully by dashing against a sharp edge and take out the embryo with caution not destroying the yolk sack or the chick embryo itself.
3. Search for the beginning of the umbilical cord quickly and open the clear wrapping without destroying the yolk sack or any little vessels. Then dissect the umbilical cord with precise clean forceps.
4. Take the embryo out of the yolk sack.
   Note: Embryos should be decapitated quickly (not shown in the video).

5. Collect the embryos in minimal essential medium at 4°C.

Part 3: Maceration of the Chick Embryos

1. Take the plunger out of a 60 mL sterile syringe.
2. Transfer approximately 10 embryos into one 60 mL syringe.
3. Put the plunger carefully back into the syringe in order not to risk losing macerated material.
4. Macerate by pushing down the plunger.
5. Collect the macerated mass in the prepared corning tubes half-filled with DMEM at a ratio of 1:1 (macerated mass: DMEM). Using 10 embryos a volume of approximately 25 mL is produced.
6. Place the mixture on a shaker for 45 min at 4°C.

Part 4: Centrifugation of the Chick Embryo - DMEM Suspension

1. Transfer the chick embryo - DMEM suspension into the centrifugation tubes.
2. Add sterile hyaluronidase at a concentration of 1 mg per 25 mg of embryo.
3. Tare the centrifugation tubes very precisely.
   Note: This step should be performed with caution as centrifugation is run at very high g-force and damage of material or centrifuge could occur if using imbalanced tubes. With a high accuracy weighing machine accordance regarding the three positions after decimal point for all tubes should be received by adjusting the missing amount with DMEM.
4. Reduce the temperature of the centrifuge to 4°C.
   Note: Also the centrifugation rotor should be stored in a cooling chamber or fridge over night.
5. Centrifuge 6 hours at least for 180.000 x g x h. Note: For superior results regarding the separation of the single phases use 266.000 x g x h. If possible, use the vacuum adjustment.

Part 5: Filtration of the Chick Embryo Extract

After the centrifugation, three phases can be noticed: The upper dingy liquid phase with fatty fragments, the clear liquid phase in the middle and the pellet at the bottom of the centrifugation tube. **Note:** Shaking or brisk moving of the centrifuged mixture has to be strictly avoided, as this would destroy the result of the centrifugation step by losing the clear central liquid phase.

Note: All following steps have to be performed under sterile conditions using a laminar air flow bench:

1. Pipette the clear liquid phase into a filter system with pores of 0.45 µm. Then switch on the connected vacuum pump.
   Note: Do not touch the pellet at the bottom. This would lead to a contamination of the extract and a blockage of the filter system.
2. Transfer the pre-extract into a filter system with pores of 0.22 µm and switch on the connected vacuum pump again.
3. Aliquot the attained chick embryo extract in sterile 15 mL corning tubes.
4. Quickly store the aliquots at -80°C (-62°F). Note: The CEE last for up to two years if stored at -80°C.

Discussion

This protocol is based on modifications of procedures that have been described in the past[10,11]. In addition to former publications we here show the individual different steps of the process more expanded. This supports the experimenter with important details assuring correct proceedings and reliable outcomes. Further, as the dissection process is an essential part of CEE extraction we describe the procedure of taking the embryos out of the yolk sack in every detail. There are several important points to note as discussed below.

The dissection of the chick embryos should be started as soon as possible after the incubation has ended. Embryos being dead prior to dissection should not be used for any steps of this protocol as enzymatic activation and protein degradation might influence the result adversely.

Before getting started some hand-on training should be performed as all steps of the dissection process have to be carried out fast and accurately. It is important for the success of the whole procedure, to avoid contamination with egg yolk which was accompanied by a noteworthy amount of negatively interfering proteins. After the embryos were taken out of the yolk sacks they should be decapitated quickly (not shown in the video).

Temperature as well as celerity of the rotor are very important, too. In order to avoid enzymatic activation and a reduction of quality and quantity of the factors needed for the NCSCM the temperature of the centrifuge and the rotor should be reduced to 4°C. After the centrifugation step the clear central liquid phase is the important pre-extract containing the essential growth factors for the NCSCM. To achieve a considerable separation of the different fractions and an enrichment of the factors needed we recommend a celerity not lower than 180.000 x g x h.

As the difference in density between the clear liquid phase and the dingy liquid phase at the top of the centrifugation tube does not prevent a remixing of the two parts any shaking has to be avoided. The components of the dingy liquid phase cannot be filtrated through a 0.45 µm filter system, as the fatty parts of it would very soon lead to a clogging of the filter system. Also the pellet at the bottom of the centrifuge should not be touched at all, as parts of it would lead to clogging of the filter system.

In a final step the potential of the CEE for cultivation of NCSCs has to be validated. This multipotent transient population of stem cells loses its viability rapidly in media without having added adequate produced CEE. Therefore a transgenic mouse line (JoMa1), which expresses early

NCSC markers in a transgene-activity dependent manner and is used to study NCSC biology is cultivated in every newly obtained charge of CEE[10]. The successful cultivation of NCSCs represents an appropriate validation of the CEE.

Disclosures

No conflicts of interest declared.

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