T.E.M.P.I. syndrome

Protocol: Collecting and freezing bone marrow aspirate samples

Editorial notes

1. The mononuclear cells need to be separated from the red blood cells, bony fragments, and other debris prior to freezing. This is done by layering the bone marrow aspirate over a density gradient (Ficoll-Paque Plus) and centrifuging. The cells can then be collected as they will form a layer and the more dense red blood cells and debris will form a pellet that can be discarded.
2. After washing the mononuclear cells, they can be frozen.
3. Most important to the freezing of the cells is to make sure that they are gently but thoroughly mixed into a single-cell suspension as clumps do not freeze and thaw well.
4. Also important to the freezing of the cells is to freeze them slowly using a controlled-temperature freezing chamber.

Materials

- Isotonic buffer options
  - RPMI 1640 / Normal saline / PBS
- Freezing media options
  - Freezing media (2x) = 80% FBS / 20% DMSO (sterile-filtered)
  - Commercial option: CryoStor CS10 (1x)
    - https://www.stemcell.com/cryostor-cs10.html
  - Commercial option: Recovery Cell Culture Freezing Medium (1x)
    - https://www.thermofisher.com/order/catalog/product/12648010
- Ficoll-Paque PLUS density gradient (1.077) (GE 17-1440-02)
  - https://www.gelifesciences.com/en/us/shop/cell-therapy/media/ficoll-paque-plus-density-gradient-media-p-05824 (ordering)
  - https://cdn.gelifesciences.com/dmm3bwsv3/AssetStream.aspx?mediaformatid=10061&destinationid=10016&assetid=12637 (instructions)
- 50 ml conical centrifuge tubes
- Cryogenic storage vials (1.8 ml - 2 ml), many available options:
  - https://ecatalog.corning.com/life-sciences/b2c/US/en/General-Labware/Cryogenic-Storage-and-Accessories/Cryogenic-Vials/Corning%C2%AE-External-Thread-Cryogenic-Vials/p/corningExternalThreadCryogenicVials
  - https://www.sigmaaldrich.com/catalog/product/sigma/v7884
- Mr. Frosty freezing chamber (very important for controlled freezing of cells)
  - https://www.thermofisher.com/order/catalog/product/5100-0001)
- Optional: Acridine orange

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Protocol: Ficoll-Paque Plus Density Gradient

1. Collect patient bone marrow aspirate.
    a. If possible, please include 1 ml of heparin from a heparin flush (100 U/ml) in the aspirate syringe to ensure that there is no clotting within the syringe.
    b. If possible, collect 10-20 cc of aspirate in 1-2 aspirations.
2. Prepare 2 x 50 ml conical tubes by adding 20 ml of Ficoll-Paque Plus to each tube.
3. Transfer the aspirate to a 3rd 50 ml conical tube.
    a. Bring the volume up to 40 ml using Isotonic buffer and mix well.
    b. Carefully layer 20 ml of diluted aspirate over each of the 20 ml of Ficoll-Paque Plus.
4. Centrifuge 400g x 25 minutes at RT per manufacturer's protocol.
5. Combine the mononuclear cell layer from each of the 2 Ficoll gradients to a fresh 50 ml conical.
    a. Bring the volume up to 50 ml with Isotonic buffer and mix well.
    b. Optional: Count the cells using Acridine Orange.
    c. (Acridine orange highlights nucleated cells and excludes debris and red blood cells.)
6. Pellet the cells at 200g x 10 minutes.

Protocol: Freezing cells

1. **Option 1**. Freezing media (2x) = 80% FBS / 20% DMSO (sterile-filtered)
    a. Gently and thoroughly resuspend the cells into a single-cell suspension in Isotonic buffer.
        i. Use 1 ml of buffer per 20-40 million cells.
        ii. If no count is available, use 2 ml of buffer.
    b. Chill cells on ice.
    c. While agitating gently, add 1 volume of the 2x Freezing media and mix well.
    d. Distribute 1 ml into each labeled cryovial.
    e. Freeze overnight with Mr. Frosty in -80-degrees freezer.
    f. Transfer the next morning to liquid N2 prior to shipping.

2. **Option 2**. Commercial freezing media (1x)
    a. Use pre-chilled freezing media.
    b. Use 1 ml of 1x freezing media per 10-20 million cells.
    c. Gently and thoroughly resuspend the cells into a single-cell suspension.
    d. Distribute 1 ml into each labeled cryovial.
    e. Freeze overnight with Mr. Frosty in -80-degrees freezer.
    f. Transfer the next morning to liquid N2 prior to shipping.

Questions?

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- https://www.hematopia.com/tempi