CloneR™3

Animal origin-free (AOF) cell seeding supplement to support cell survival

Catalog #100-2280 0.3 mL

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Product Description

CloneR™3 is an animal origin-free (AOF), chemically defined supplement designed to support human pluripotent stem cells (hPSCs) during periods of cellular stress, such as cryopreservation and electroporation. CloneR™3 promotes single-cell survival during cloning and efficient cell seeding for both hPSC maintenance and differentiation workflows. In addition, CloneR™3 supports the growth, successful adaptation, and expansion of cells in three-dimensional (3D) suspension culture.

Properties

Storage: Store at -20°C.

Shelf Life: Stable until expiry date on label.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
15 mL conical tube	e.g. 38009
2 mL serological pipettes	e.g. 38002
37 µL reversible strainer	e.g. 27215/27250
CellAdhere™ Dilution Buffer OR DMEM/F-12 with 15 mM HEPES	07183 OR 36254
CellAdhere™ Laminin-521 OR Corning® Matrigel® hESC-Qualified Matrix OR Vitronectin XF™	200-0117 OR Corning 354277 OR 07180
D-PBS (Without Ca++ and Mg++)	37350
Enzymatic dissociation reagent (e.g. ACCUTASE™ or TrypLE™ Express)	e.g. 07920 or Thermo Fisher 12605028
mTeSR™1 OR mTeSR™ Plus OR TeSR™-AOF OR TeSR™-AOF 3D	85850 OR 100-0276 OR 100-0401 OR 100-0720

Preparation of Reagents and Materials

Coating Cultureware

Prepare matrix-coated plates according to the applicable Product Information Sheet (PIS) for the matrix of choice (e.g. CellAdhere™ Laminin-521, Corning® Matrigel® hESC-Qualified Matrix, STEMmatrix™ BME, or Vitronectin XF™).

NOTE: For Vitronectin XF™, use non-tissue culture-treated cultureware; for the other matrices, use tissue culture-treated cultureware.

NOTE: If using CellAdhere™ Laminin-521, coat cultureware the day before cloning, as overnight incubation is required.

Preparation of Culture Medium Supplemented with CloneR™3

The following example is for preparing 25 mL of culture medium supplemented with CloneR™3. If preparing other volumes, adjust accordingly.

1. Thaw CloneR™3 at room temperature (15 - 25°C).

   NOTE: If not using immediately, aliquot into working volumes and store at -20°C. Once aliquots are thawed, use immediately. Do not re-freeze.
2. Prepare complete culture medium (mTeSR™1, mTeSR™ Plus, TeSR™-AOF, or TeSR™-AOF 3D).

   NOTE: For complete instructions on medium preparation, refer to the applicable PIS.
3. Add 25 µL of CloneR™3 to 25 mL of complete culture medium. Mix thoroughly by inverting. Do not shake.

   NOTE: If not using immediately, store culture medium supplemented with CloneR™3 at 2 - 8°C for up to 1 week.

Directions for Use

Please read the entire protocol before proceeding.

Use sterile technique when performing the following protocols:

I. Cloning

II. Single-Cell Seeding (Non-Clonal Density)

III. 3D Suspension Culture in TeSR™-AOF 3D Medium

IV. Thawing and Recovering Cryopreserved Cells

I. CLONING

A. Preparing Cultureware for Cloning

1. Aspirate matrix from coated plates (see Preparation of Reagents and Materials).
2. Add culture medium (mTeSR™1, mTeSR™ Plus, or TeSR™-AOF) supplemented with CloneR™3 to cultureware as indicated in Table 1 (initial seed volume). For other cultureware, use 200 µL/cm^2.

Table 1. Volume of Culture Medium Supplemented with CloneR™3 for Various Cultureware

CULTUREWARE	SURFACE AREA (cm^2)	VOLUME OF CULTURE MEDIUM SUPPLEMENTED WITH CLONER™3
96-well plate	0.32	0.1 mL/well
12-well plate	3.5	1 mL/well
6-well plate	9.6	2 mL/well
10 cm dish	56.7	10 mL

B. Preparing a Single-Cell Suspension

Use hPSCs cultured in complete culture medium (mTeSR™1, mTeSR™ Plus, or TeSR™-AOF) when they are 60 - 80% confluent.

1. Remove hPSC culture from incubator.
2. Under the microscope, mark regions of differentiation using a marker pen.
3. Remove regions of differentiation by aspiration. Rinse with D-PBS (Without Ca++ or Mg++) and aspirate.
4. Add enzymatic dissociation reagent (e.g. TrypLE™ Express) at room temperature at 1 mL per 10 cm^2 surface area. Incubate at 37°C for 5 minutes.

   NOTE: Optimal dissociation time may be cell line- or matrix-dependent.
5. Rinse cells from cultureware by pipetting the dissociation reagent up and down, dispensing onto the colonies and breaking them up.

6. Dilute cell suspension 1 in 4 by adding to a 15 mL conical tube containing culture medium supplemented with CloneR™3. If seeding a high density single-cell culture, quench the dissociation reagent by diluting the cell suspension 1 in 2 with a trypsin inhibitor solution (e.g. Catalog #07457) or with complete culture medium.
7. Create a single-cell suspension by flicking the tube 3 - 5 times or until suspension contains no large cell clumps.
8. Count cells using a hemocytometer (e.g. Catalog #100-1181) or other cell-counting method. Proceed to section C.

C. Plating Cells (Day 0)

Refer to the protocols below for plating at clonal density (section i) or for single-cell sorting (section ii).

i. Plating at Clonal Density

1. Dilute the single-cell suspension (prepared in section B) to 10 cells/µL in culture medium supplemented with CloneR™3 (e.g. add 50,000 cells to the conical tube and top up the volume to 5 mL with culture medium supplemented with CloneR™3).
2. Add desired number of cells to coated cultureware (prepared in section A).

   NOTE: The dissociation reagent should be sufficiently diluted so that it is less than 1% of the final volume when cells are added to the cultureware.

   NOTE: To minimize the probability of generating mosaic colonies, seed cells at ≤ 50 cells/cm^2 and use Vitronectin XF™ matrix.
3. Swirl the plate and rock back-and-forth and side-to-side 4 - 5 times to distribute cells evenly. Incubate at 37°C for 2 days, then proceed to section D.

ii. Single-Cell Sorting (96-well plates)

1. Centrifuge the single-cell suspension (prepared in section B) at 300 x g for 5 minutes. Aspirate supernatant and resuspend cells at 1 x 10^6 cells/mL in culture medium supplemented with CloneR™3.
2. If the cell suspension contains large aggregates of cells, pass the single-cell suspension through a 37 µm reversible strainer to remove any large clumps.
3. Sort cells into individual wells of a coated 96-well plate (prepared in section A) using a fluorescence-activated cell sorter (FACS) (as low as 1 cell/well).
4. Incubate at 37°C for 2 days, then proceed to section D.

D. Feeding Cells

1. **Day 2:** Perform a full-medium change with culture medium supplemented with CloneR™3. Incubate at 37°C for 2 days.
2. **Day 4+:** Perform a full-medium change with complete culture medium (without CloneR™3) daily or according to the medium PIS until colonies are ready to be picked.

II. SINGLE-CELL SEEDING (NON-CLONAL DENSITY)

CloneR™3 can also be used as a seeding supplement for additional single-cell applications, including passaging and seeding cells for differentiation, as follows:

1. Centrifuge the single-cell suspension (prepared in section I-B) at 300 x g for 5 minutes. Aspirate supernatant and resuspend cells at a concentration of at least 3 x 10^6 cells/mL in complete culture medium.
2. Count cells using a hemocytometer or other cell-counting method.
3. Add desired number of cells to coated cultureware (prepared in section I-A).
4. Place the cultureware in a 37°C incubator. Move the cultureware in several quick, short, back-and-forth and side-to-side motions to evenly distribute the cells across the surface. Do not disturb the plate for 24 hours.
5. Change medium after 24 hours, according to the protocol of choice.

III. 3D SUSPENSION CULTURE IN TESR™-AOF 3D MEDIUM

The following example is for preparing 100 mL of medium. If preparing other volumes, adjust accordingly.

1. Thaw CloneR™3 at room temperature (15 - 25°C).

   NOTE: If not using immediately, aliquot into working volumes and store at -20°C. Once aliquots are thawed, use immediately. Do not re-freeze.
2. Prepare complete culture medium (TeSR™-AOF 3D Seed Medium).

   NOTE: For complete instructions on medium preparation and storage, refer to the applicable PIS.

3. Add 0.1 mL of CloneR™3 to 100 mL of complete culture medium. Mix thoroughly by inverting. Do not shake.

   NOTE: If not using immediately, store complete culture medium supplemented with CloneR™3 at 2 - 8°C for up to 1 week.

   NOTE: Supplementation with CloneR™3 replaces the need to add Y-27632 (Dihydrochloride) to support cells in 3D suspension culture.

For more information about initiating 3D cultures from small clumps of cells, refer to the Technical Manual: Expansion of Human Pluripotent Stem Cells as Aggregates in Suspension Culture Using TeSR™-AOF 3D (Document #10000010775), available at www.stemcell.com, or contact us to request a copy.

For more information about initiating 3D cultures as single cells, refer to the web protocol Single-Cell Passaging of 3D hPSC Suspension Cultures, available at www.stemcell.com.

IV. THAWING AND RECOVERING CRYOPRESERVED CELLS

1. Ensure that all tubes, coated cultureware (see Preparation of Reagents and Materials), and culture medium supplemented with CloneR™3 (room temperature [15 - 25°C]) are ready before starting the protocol. Prepare complete culture medium (mTeSR™1, mTeSR™ Plus, or TeSR™-AOF) and warm to room temperature.

   NOTE: Do not warm medium in a 37°C water bath.

2. Wipe the outside of the vial of cells with 70% ethanol or isopropanol.
3. Quickly thaw cells in a 37°C water bath by gently swirling the vial. Remove the vial when a small frozen cell pellet remains. Do not vortex cells. Do not submerge the vial cap.
4. Wipe the outside of the vial with 70% ethanol or isopropanol. Add ~5 mL of complete culture medium to a 15 mL conical tube.
5. Use a 2 mL serological pipette to transfer the contents of the cryovial to the tube prepared in step 1.

   NOTE: Using a 2 mL serological pipette instead of a 1 mL pipettor will minimize breakage of cell aggregates.

6. Centrifuge cells at 300 x g for 5 minutes at room temperature.
7. Aspirate the medium, leaving the cell pellet intact. Using a 2 mL serological pipette, gently add 1 mL of prepared medium supplemented with CloneR™3 and gently pipette up and down 2 - 3 times to break up the pellet. Take care to maintain the cells as aggregates.
8. Plate cell aggregates into prepared two-dimensional (2D) or 3D cultureware containing prepared medium supplemented with CloneR™3.

   NOTE: Typically, more cells will need to be plated after thawing than during routine passaging.

9. Place the plate in a 37°C incubator. Move the plate in several quick, short, back-and-forth and side-to-side motions to distribute the cell aggregates. Do not disturb the plate for 24 hours.

   NOTE: Uneven distribution of aggregates may result in increased differentiation of hPSCs.

10. Perform medium changes with complete culture medium (without CloneR™3) daily or according to the medium PIS. Visually assess cultures to monitor growth until they are ready to be passaged at 60 - 80% confluence.

    NOTE: The time required to reach optimal confluency may vary when using different cell lines; cultures should be monitored under the microscope until the optimal passaging time is determined.