CloneR™2

Defined supplement for improving survival of human ES and iPS cells in single-cell workflows

Catalog #100-0691 25 mL

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Product Description

CloneR™2 is a defined, serum-free TeSR™ supplement designed to increase the cloning efficiency and single-cell survival of human embryonic stem (ES) cells and induced pluripotent stem (iPS) cells. CloneR™2 facilitates the generation of clonal ES and iPS cell lines whether using low-density seeding (< 25 cells/cm^2) or FACS-sorting cells at 1 cell/well. Additionally, CloneR™2 improves survival of ES and iPS cells when plating as single cells, and supports the post-thaw survival of cryopreserved ES and iPS cell lines.

Properties

Storage: Store at -20°C.

Shelf Life: Stable for 1 year from date of manufacture (MFG) on label.

Materials Required but Not Included

PRODUCT NAME	CATALOG #
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™-E8™	85850 OR 100-0276 OR 100-0401 OR 05990

Preparation of Reagents and Materials

Coating Cultureware

Coat cultureware with matrix; use Vitronectin XF™, CellAdhere™ Laminin-521, or Corning® Matrigel® hESC-Qualified Matrix.

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.

1. Thaw matrix according to the applicable Product Information Sheet (PIS) or manufacturer's instructions.
2. Dilute matrix according to Table 1.

Table 1. Dilution and Incubation of Matrix

MATRIX	DILUENT	FINAL CONCENTRATION OF MATRIX	INCUBATION CONDITIONS
Vitronectin XF™	CellAdhere™ Dilution Buffer	10 µg/mL	Room temperature (15 - 25°C) for at least 1 hour
CellAdhere™ Laminin-521	CellAdhere™ Dilution Buffer	10 µg/mL	2 - 8°C overnight
Corning® Matrigel®	DMEM/F-12 with 15 mM HEPES	Refer to manufacturer's instructions for reconstitution	Room temperature (15 - 25°C) for at least 1 hour

3. Add diluted matrix to cultureware as indicated in Table 2. For other cultureware, use 150 µL/cm^2.

Table 2. Volume of Diluted Matrix for Various Cultureware

CULTUREWARE	SURFACE AREA (cm^2)	VOLUME OF DILUTED MATRIX
96-well plate	0.32	50 µL/well
12-well plate	3.5	0.5 mL/well
6-well plate	9.6	1 mL/well
10 cm dish	56.7	6 mL

4. Tilt plate back and forth to distribute matrix solution evenly.
5. Incubate cultureware as indicated in Table 1.

Cloning Medium

The following example is for preparing 25 mL of cloning medium. If preparing other volumes, adjust accordingly.

1. Thaw CloneR™2 at room temperature (15 - 25°C).

   NOTE: If not used immediately, store at 2 - 8°C. Do not exceed the shelf life of the supplement. Alternatively, aliquot 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™-E8™, or TeSR™-AOF).

   NOTE: For complete instructions on media preparation, refer to the applicable PIS.
3. Add 2.5 mL of CloneR™2 to 22.5 mL of complete culture medium. Mix thoroughly by inverting. Do not shake.

   NOTE: If not used immediately, store cloning medium 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
III. Thawing Cryopreserved Cells

I. CLONING

A. Preparing Cultureware for Cloning

1. Aspirate matrix from coated plates (see Preparation of Reagents and Materials).
2. Add cloning medium to cultureware as indicated in Table 3 (initial seed volume). For other cultureware, use 200 µL/cm^2.

Table 3. Volume of Cloning Medium for Various Cultureware

CULTUREWARE	SURFACE AREA (cm^2)	VOLUME OF CLONING MEDIUM
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 ES or iPS cells cultured in complete culture medium (mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF) when they are 60 - 80% confluent.

1. Remove ES/iPS cell 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 (15 - 25°C) at 1 mL per 10 cm^2 surface area. Incubate at 37°C for 5 minutes.

   NOTE: Optimal dissociation time may be cell line-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 conical tube (e.g. Catalog #38009) containing cloning medium. If seeding a high-density single-cell culture, quench the dissociation reagent by diluting the cell suspension 1 in 2 with 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 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 cloning medium (e.g. add 50,000 cells to the conical tube and top up the volume to 5 mL with cloning medium).
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 mosiac colonies, seed cells at ≤ 50 cells/cm^2.
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 cloning medium.
2. If the cell suspension contains large aggregates of cells, pass the single-cell suspension through a 37 µm reversible strainer (e.g. Catalog #27215/27250) 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. **Days 2 and 3:** Perform a full-medium change with cloning medium. Incubate at 37°C for 24 hours.
2. **Day 4+:** Perform a full-medium change with mTeSR™1, mTeSR™ Plus, TeSR™-AOF, or TeSR™-E8™ (without CloneR™2) daily or according to the medium PIS until colonies are ready to be picked.

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

CloneR™2 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. THAWING CRYOPRESERVED CELLS

1. Ensure that all tubes, cloning medium (room temperature [15 - 25°C]), and coated cultureware (see Preparation section) are ready before starting the protocol. Prepare complete culture medium (mTeSR™1, mTeSR™ Plus, TeSR™-E8™, or TeSR™-AOF) and warm to room temperature.

   NOTE: Do not warm media 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. Proceed to section A for cell aggregates or section B for single cells.

A. Cell Aggregates

1. Add ~5 mL of complete culture medium to a 15 mL conical tube (e.g. Catalog #38009).
2. 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.
3. Centrifuge cells at 300 x *g* for 5 minutes at room temperature.
4. Aspirate the medium, leaving the cell pellet intact. Using a 2 mL serological pipette, gently add 1 mL of cloning medium and gently pipette up and down 2 - 3 times to break up the pellet. Take care to maintain the cells as aggregates.
5. Plate cell aggregates into prepared cultureware containing cloning medium.

   NOTE: The number of wells plated might need to be adjusted depending on how many cell aggregates were cryopreserved. Typically, more aggregates will need to be plated after thawing than during routine passaging.
6. 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 human ES and iPS cells.
7. Perform medium changes with complete culture medium (without CloneR™2) daily or according to the medium PIS. Visually assess cultures to monitor growth until the they are ready to be passaged (i.e. 60 - 80% confluent).

   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.

B. Single Cells

1. Add ~5 mL of complete culture medium to a 15 mL conical tube. Use a 1 mL pipettor to slowly transfer the contents of the cryovial to the tube.
2. Centrifuge cells at 300 x *g* for 5 minutes at room temperature.
3. Carefully remove the supernatant with a pipette and flick tube to break up the cell pellet.
4. Add 1 mL of cloning medium to the tube. Mix gently.
5. Plate cells onto coated cultureware.

   NOTE: In general, one frozen cryovial containing 1 x 10^6 cells can be thawed and plated into 2 - 4 wells of a 6-well plate.
6. 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.
7. Perform medium changes with complete culture medium (without CloneR™2) daily or according to the medium PIS. Visually assess cultures to monitor growth until the they are ready to be passaged (i.e. 60 - 80% confluent).

   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.