PROTOCOL

PluriQ™ G9™ Cloning Medium

Cat. No. GSM-9020 (100 mL)

Culture Guidelines to Establish Single Cell-Derived Pluripotent Stem Cell Lines

Recommended Materials to Use with PluriQ™ G9™ Cloning Medium

Components	Catalog No.	Storage	Shelf Life
PluriQ™ G9™ Cloning Medium (1X); 100 mL	GSM-9020	-20°C	12 months
PluriQ™ G9™ Maintenance Medium (1 kit)	GSK-9001	2-8°C; -20°C	12 months
G9™ VTN 0.5 mg/mL (100X); 1 mL	GSR-2070	-80°C	12 months
G9™ Versene Solution (1X); 100 mL	GSM-1101	RT	12 months

Preparing the PluriQ™ G9™ Cloning Medium

NOTE: PluriQ™ G9™ Cloning Medium was optimized for use with human Pluripotent Stem Cell Lines (PSCs) grown in G9 Maintenance Medium, but it is also compatible with starting cultures grown in other media such as mTeSR1™ and Essential 8™.

1. Thaw frozen **PluriQ™ G9™ Cloning Medium** at room temperature. It is a complete medium and requires no additional supplements. **Do not re-freeze aliquots.**
2. Thawed **PluriQ™ G9™ Cloning Medium** should be stored at 2°C to 8°C in the dark and used within 14 days.
3. Take daily aliquots for use at room temperature. **Do not warm the entire bottle each day.**

Coating 96-well Plates with G9™ Vitronectin

NOTE: PluriQ™ G9™ Cloning Medium was optimized for use with vessels coated with G9™ Vitronectin, but it is also compatible with Matrigel®, Geltrex®, or Laminin-521. Plating efficiencies may vary on other substrates.

1. Thaw **G9™ VTN** at room temperature and add 50 µL of VTN (100X) stock solution in 5 mL of complete **PluriQ™ G9™ Maintenance Medium.** To each well of a 96-well plate add 50 µL of the diluted **G9™ VTN solution** (5 µg/mL). Let stand for at least 1 hour at room temperature. The remaining **G9™ VTN** (100X) stock should be stored at 2°C to 8°C in the dark and used within 14 days. **Do not refreeze G9™ VTN repeatedly.**
2. For convenience, the **G9™ VTN** solution can be left on 96-well plates that are wrapped in parafilm, stored in a sterile bag and held at 2°C to 8°C in the dark for up to two weeks.
3. Equilibrate a coated 96-well plate to room temperature, and aspirate **G9™ VTN** just prior to plating cells.

NOTE: It is not recommended to re-use diluted G9™ VTN, as the plating efficiency may decrease.

Preparing a Serial Dilution of PSCs to Plate Single Cells in PluriQ™ G9™ Cloning Medium on Vitronectin

NOTE: PluriQ™ G9™ Cloning Medium was optimized for use with G9™ Versene to gently dissociate human PSCs grown on G9™ VTN into a single-cell suspension. While the PluriQ™ G9™ Cloning Medium does not require the use of a Rho Kinase Inhibitor, the addition of 5 µM of Y-27632 during initial, overnight plating may be beneficial if the PSCs are prepared with an enzymatic passaging reagent such as Accutase. Plating efficiencies may vary with other passaging methods.

1. Prepare **PluriQ™ G9™ Maintenance Medium** (GSK-9001) or other medium for serial dilution and warm 30 mL to room temperature prior to use.
2. Aspirate the culture medium from a well of PSCs that has reached 75 to 90% confluency.
3. Rinse the starting well with an equivalent volume of Calcium and Magnesium-free PBS.
4. Add 0.5 mL or 1 mL of room temperature **G9™ Versene Solution (EDTA)** per well of a 24-well or 6-well plate and incubate at 37°C/5% CO_2 for 2-3 minutes.

CONTACT / ORDERING INFORMATION

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Note: Cultures that are confluent may require longer incubation of 3-5 minutes. PSCs growing on Matrigel® are more resistant to EDTA chelation and require additional trituration.

5. Check the cultures on an inverted microscope to confirm that individual PSCs have contracted and are clearly distinguishable but have not yet lifted off the plate and then aspirate the **G9™ Versene**.
6. Dissociate the cells from the well with 1 mL of complete pre-warmed **PluriQ™ G9™ Maintenance Medium** using a P1000 pipettor.
7. Triturate the cell suspension gently 3-5 times and confirm that cells have been dissociated into single cells.
   Note: If clusters of small cells persist repeatedly pipette again and/or pass the cell solution through a 40 µM Cell Strainer (BD Falcon) set into a 50 mL conical tube.
8. Perform an accurate cell count to determine the concentration of your starting cell suspension in **PluriQ™ G9™ Maintenance Medium.**
9. Prepare four additional 15 mL tubes for serial dilution and label as 100,000 cells/mL; 10,000 cells/mL; 1,000 cells/mL; and 100 cells/mL and add 5 mL of **PluriQ™ G9™ Maintenance Medium** to each.
10. Add the correct amount of your starting cell suspension to 5 mL of **PluriQ™ G9™ Maintenance Medium** to make an initial suspension of 100,000 cells/mL and mix.
11. Transfer 550 µL of cell suspension from the 100,000 cells/mL stock to the 10,000 cells/mL vial to make a 1/10 dilution. Pipette up and down to mix. Repeat to make 10,000 cells/mL; 1000 cells/mL and 100mL cell suspensions in 5 mL of **PluriQ™ G9™ Maintenance Medium**.
12. Take 550 µL of the 100 cells/mL suspension and transfer it into a 15 mL conical tube containing 5 mL of **PluriQ™ G9™ Cloning Medium** to make a final cell suspension containing 10 cells/mL.
13. Aspirate the **G9™ VTN** from the 96-well plate.
14. Transfer the 5 mL of **PluriQ™ G9™ Cloning Medium** containing on average 10 cells/mL to a sterile media trough and pipette 100 µL of the cell suspension into each well of a 96-well plate using a multichannel pipettor.
15. Transfer the 96-well plate to an incubator to culture at 37°C/5% CO_2 overnight.

Establishment of Clonal PSC Lines in PluriQ™ G9™ Cloning Medium (Day 2- to 12)

1. **Day 2**: Warm 5 mL of **PluriQ™ G9™ Cloning Medium** to room temperature prior to use.
   Do not aspirate any medium from wells but overlay 50 µL of fresh PluriQ™ G9™ Cloning Medium to each well.
2. **Day 3**: Warm 5 mL of **PluriQ™ G9™ Cloning Medium** to room temperature prior to use.
   Aspirate 50 µL of spent media from each well and add 50 µL of fresh **PluriQ™ G9™ Cloning Medium**.
3. **Day 4-6**: Warm 5 mL of **PluriQ™ G9™ Cloning Medium** to room temperature prior to use.
   Aspirate 50 µL of spent media from each well and add 50 µL of fresh **PluriQ™ G9™ Cloning Medium**.
   By Day 4-5 you should be able to see clusters of proliferating cells. Differentially mark wells containing single colonies and those containing 2 or more colonies that received more than one cell by chance.
4. **Day 7 to 10**: Warm 10 mL of **PluriQ™ G9™ Cloning Medium** each day to room temperature prior to use.
   Aspirate the majority of media from wells and add 100 µL of fresh **PluriQ™ G9™ Cloning Medium** each day. Continue to monitor colonies as they are expanding. Some wells may benefit from continued growth for 2-3 days.
5. **Day 11**: Wells can be passaged with **G9™ Versene** and re-plated in **PluriQ™ G9™ Cloning Medium** onto replicate 96-well **G9™ VTN**-coated plates for parallel cryo-preservation and screening or onto 24-well, **G9™ VTN**-coated plates for continued expansion in **PluriQ™ G9™ Maintenance Medium** or other medium.
6. **Day 12 onwards:** Aspirate the **PluriQ™ G9™ Cloning Medium** and add 100 µL/well for a 96-well plate of **PluriQ™ G9™ Maintenance Medium or** 0.5 mL/well in a 24-well plate or other medium each day and continue to expand clonal lines for banking, characterization and use.

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