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ProMDCK™ Cell Growth Medium for MDCK in 2D and 3D Culture

Instructions for use

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I. Introduction

ProMDCK™ 2D and ProMDCK™ 3D are serum-free media for the growth of Madin-Darby Canine Kidney Cells (MDCK) culture**.** ProMDCK™ Media is optimized for expansion and virus infection of MDCK cells in planar culture (2D) or on microcarriers (3D). Cells can be directly transitioned from ProMDCK™ 2D Cultures onto microcarriers with ProMDCK™ (3D) Medium. In addition, 3D subculture can be accomplished by adding fresh microcarriers to microcarrier-expanded MDCK cells already in culture. Both version s of the medium have been demonstrated to support expansion of influenza virus (H1N1, H3N2, and B).

For answers to frequently asked questions and citations regarding these products, please visit our knowledge center: **https://knowledge.lonza.com**

II. Storage instructions

Upon arrival, store ProMDCK™ Media at 4 - 8°C, protect from light, do not freeze.

III. Suggested materials

- MDCK Cells (ATCC CCL-34 or other)
- L-Glutamine (Lonza P/N 17-605E, BE17-605E, BEBP17-605E)
- Corning® Tissue Culture Flasks
- Phosphate Buffered Saline (Lonza P/N 17-516F, BE17-516F, BEBP17-516Q)
- Trypsin-EDTA (Lonza P/N 17-161E, BE17-161E)
- Corning® Untreated Microcarriers (Corning® 3772)
- Corning® 125 mL Disposable Spinner Flasks (Corning® 3152)
- Cell Strainers (Corning® Falcon® 352340)
- TPCK-Trypsin (Thermo Fisher Scientific™ 20233)

IV. Instructions for use

Preparation of media

1. Completely thaw L-glutamine (Lonza P/N 17-605E).
2. Decontaminate the external surfaces of the L-glutamine and ProMDCK™ 2D & 3D basal medium bottles with 70% v/v ethanol or isopropanol and place in sterile field.
3. Supplement the ProMDCK™ 2D & 3D Basal Media with L-glutamine for a final concentration of 2mM.

a. For 1L bottle, add 10.1 mL of 200 mM L-glutamine

4. Store at 2 - 8°C, protect from light.

Thawing of cells / initiation of culture process

Note: Cells can be directly transitioned from serum-containing medium to ProMDCK™ (2D) with little or no adaptation.

1. Initiate cell cultures in 2D with ProMDCK™ 2D Medium (with 2 mM L-glutamine) using Corning® T-flasks.
2. The recommended seeding density for MDCK in 2D culture is 5,000 – 20,000 cells per cm^2.
3. To set up cultures, calculate the number of vessels needed based on the recommended seeding density and the surface area of the vessels being used. Add the appropriate amount of medium to the vessels (0.2 - 0.4 mL per $cm^{2)}$ and allow the vessels to equilibrate in a 37°C, 5% CO_2 humidified incubator for at least 30 minutes.
4. Wipe cryovial containing the cells with ethanol or isopropanol before opening. In a sterile field, briefly twist the cap a quarter turn to relieve pressure, and then retighten. Quickly thaw the cryovial in a 37°C water bath.
5. Watch your cryovial closely; when the last sliver of ice melts remove it. Do not submerge it completely. Thawing the cells for longer than 1 ½ minutes may result in less than optimal cell viability.
6. Remove the cryovial immediately from the water bath and wipe dry. Spray the cryovial with 70% alcohol, and then wipe to remove excess.
7. Transfer the cryovial to a sterile field. Using a micropipette, gently add the thawed cell suspension to 5 - 10 mL of room temperature ProMDCK™ (2D).
8. Centrifuge the cell suspension at 200 x g for 5 minutes at room temperature. Aspirate the supernatant and resuspend the cell pellet with 1-2 mL of ProMDCK™ (2D).
9. Gently mix the cells by pipetting up and down. Count the cells with a hemacytometer or cell counter and calculate the total number of viable cells.
10. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

11. Use the following equation to calculate the volume of cell suspension to seed into your flasks. Determine the volume of ProMDCK™ (2D) to add to each flask so the final culture volume is 0.2 – 0.4 mL per cm^2.

$$\text{Seeding Volume} = \frac{\text{Growth area x Seeding Density}}{\text{Viable cells/mL}}$$

12. Add the calculated volume of cell suspension to each prepared flask and gently rock to disperse the cell suspension over the growth surface.
13. Incubate at 37°C, 5% CO_2 and 90% humidity.
14. Forty-eight to seventy-two hours after seeding, completely remove the medium. Replace with an equal volume of ProMDCK™ Medium (2D).
15. Subculture the cells at ≤ 90% confluence. Cultures reach 90% confluence between day 3 and day 4.

Subculturing in 2D

1. For further expansion, MDCK cells may be subcultured in T-flasks. Passage the cells before they reach 90% confluence. Over-confluent cultures are difficult to dissociate and may require longer exposure to trypsin or the use of an alternate enzyme and may damage the cells.
2. Aseptically remove and discard all of the spent medium from the flasks.
3. Wash the attached cell monolayer with PBS (Lonza P/N 17-516F, BE17-516F or BEBP17-165Q). Add the wash solution to the side of the flask opposite the attached cell layer. Rinse by rocking the flask back and forth several times. Allow the PBS wash to remain on cells for two to three minutes. Aseptically remove and discard the wash solution.
4. Add 1 mL/25 cm^2 Trypsin-EDTA (Lonza P/N 17-161E, BE17-161E), or a NAO cell dissociation reagent to cover the cell layer. Gently rock the flasks to ensure that the cells are covered by the solution. Incubate at 37°C for eight minutes, and then observe under a microscope. If the cells are less than 95% detached, continue incubating and observe every three minutes. Tapping the flask before fifteen minutes of incubation time may cause clumping of the cells. Cultures may take up to twenty minutes for dissociation with trypsin.

5. Once ≥95% of the cells are rounded and detached, stand the flasks on end for a minimal length of time to allow the cells to drain.
6. Dilute the cell/trypsin suspension with
   a. 1:10 with ProMDCK™ (2D).
   or
   b. 2 mL/25 cm^2 of a soybean trypsin inhibitor (125 mg/L)
7. Disperse the cell suspension by pipetting over the cell layer surface several times. Then pipette into a sterile 15 mL or 50 mL conical tube.
8. Centrifuge cells at 200 - 300 x g for five to six minutes at room temperature.
9. Resuspend the cell pellet in a minimal volume of temperature-equilibrated ProMDCK™ (2D) and remove a sample for counting.
10. Count the cells with a hemacytometer or cell counter and calculate the total number of cells. Make a note of your cell yield for later use.
11. If necessary, dilute the suspension with ProMDCK™ (2D) to achieve the required counting range and re-count the cells.
12. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

13. Determine the total number of flasks to inoculate by using the following equation. The number of flasks that can be seeded depends upon cell yield and seeding density. Determine the volume of ProMDCK™ (2D) to add to each flask so that the final culture volume is 0.2 – 0.4 mL per cm^2. The recommended seeding density for 2D culture of MDCK cells is 5,000 – 20,000 cells per cm^2.

$$\text{Total \# of Flasks to innoculate} = \frac{\text{Total \# of viable cells}}{\text{Growth area} \times \text{Rec. Seeding Density}}$$

14. Use the following equation to calculate the volume of cell suspension to seed into your flasks.

$$\text{Seeding Volume} = \frac{\text{Growth area x Seeding Density}}{\text{Viable cells/mL}}$$

15. Prepare flasks by labeling each with the cell line, passage number, seeding density, date and other pertinent information.
16. Add the appropriate volume of temperature equilibrated ProMDCK™ (2D) as determined in step 13 and the appropriate cell suspension as determined in step 14.
17. Incubate at 37°C, 5% CO_2 and 90% humidity.
18. Forty-eight to seventy-two hours after seeding, completely remove the medium. Replace with an equal volume of ProMDCK™ (2D).
19. Subculture the cells at ≤ 90% confluence. Cultures reach 90% confluence between day 3 and day 4.

Transitioning from 2D into 3D

Corning® Untreated Microcarriers and disposable spinners are recommended. Alternative microcarriers and spinners can be used but processes must be optimized for each microcarrier and spinner type.

1. Prepare microcarriers according to manufacturer's product insert.
2. Aliquot the desired volume of sterile microcarriers that will deliver the required final surface area into a sterile vessel, such as a bottle, or centrifuge tube.
3. After microcarriers have settled, aseptically remove the excess liquid slowly with a pipette. Avoid disturbing the settled beads during aspiration. Set the microcarriers aside until cells are ready for seeding.
4. Cells should be approximately 80 - 90% confluent. Over-confluent cultures are difficult to dissociate and may require longer exposure to trypsin or the use of an alternate enzyme.
5. Aseptically remove and discard all of the spent ProMDCK™ (2D) Medium from the flasks.
6. Wash the attached cell layer with PBS (Lonza 17-516F, BE17-516F or BEBP17-516Q). Add the wash solution to the side of the flask opposite the attached cell layer. Rinse by rocking the flask back and forth several times. Allow the PBS wash to remain on cells for two to three minutes. Aseptically remove and discard the wash solution.
7. Add 1 mL/25 cm^2 Trypsin-EDTA (Lonza 17-161E or BE17-161E), or a NAO cell dissociation reagent to cover the cell layer. Gently rock the flask(s) to ensure that the cells are covered by the solution. Incubate at 37°C for eight minutes, and then observe under a microscope. If the cells are less than 95% detached, continue incubating and observe every three minutes. Tapping the flask before fifteen minutes of

incubation time may cause clumping of the cells. Cultures may take up to twenty minutes to for dissociation with trypsin.

8. Once ≥95% of the cells are rounded and detached, stand the flasks on end for a minimal length of time to allow the cells to drain.
9. Dilute cell/trypsin suspension with
   a. 1:10 with ProMDCK™ (3D).
      or
   b. 2 mL/25 cm2 of a soybean trypsin inhibitor (125 mg/L)
10. Disperse the cell suspension by pipetting over the cell layer surface several times. Then transfer into a sterile 15 mL or 50 mL conical tube.
11. Centrifuge cells at 200 x - 300 x g for five minutes at room temperature.
12. Resuspend the cell pellet in a minimal volume of temperature-equilibrated ProMDCK™ (3D) and remove a sample for counting.
13. Count the cells with a hemacytometer or cell counter and calculate the total number of viable cells. Make a note of your cell yield.
14. If necessary, dilute the suspension with ProMDCK™ (3D) to achieve the required counting range and re-count the cells.
15. Use the following equation to determine the total number of viable cells.

$$\text{Total \# of Viable Cells} = \frac{\text{Total cell count} \times \text{percent viability}}{100}$$

16. Determine the total number of spinner flasks that can be inoculated by using the following equation. The number of spinner flasks that can be seeded depends upon cell yield and the total surface area of microcarriers in each spinner. Corning® Untreated Microcarriers at a minimum density of 0.1 grams per 15 mL medium with a seeding density of 20,000 cells per cm^2 is recommended. For other manufacturers' microcarriers follow manufacturers' recommendation on specific microcarrier density and cell seeding densities. Optimization may be required

$$\text{Total \# of spinners to innoculate} = \frac{\text{Total \# of viable cells}}{\text{Total cm2/spinner} \times \text{Rec. Seeding Density}}$$

17. Resuspend microcarriers in ProMDCK™ (3D) and add to each spinner flask so the volume is half maximal volume. Follow manufacturers' recommendations for each spinner flask for final volume. A final volume of 60 – 100 mL in a Corning® 125 mL spinner flask is recommended.
18. Use the following equation to calculate the volume of cell suspension required to seed each spinner flasks.

$$\text{Seeding Volume} = \frac{\text{Desired seeding density x total cm2}}{\text{Viable cells/mL}}$$

19. Label each spinner with the cell line, passage number, seeding density, lot numbers, date and/or other pertinent information.
20. Add the appropriate volume of cell suspension as determined in step 18.
21. Incubate spinners at 37°C, 5% CO_2 and 90% humidity overnight **with <u>no</u> stirring**. Following overnight incubation, add ProMDCK™ (3D) to spinner flask final volume per manufacturers' recommendations.
22. Return to incubator at 37°C, 5% CO_2 and 90% humidity. A stir speed of 35 rpm for Corning® microcarriers and 125 mL spinner flasks is recommended. Spinner speed should be adjusted so cells and microcarriers remain in homogenous suspension and do not settle to bottom of spinner flask.
23. MDCK cells in microcarrier culture should reach confluence in four to five days. Cultures at this time can be used for virus propagation or to seed subsequent spinner flask microcarrier cultures.

Sampling microcarrier cultures from 3D (spinners)

1. Aseptically using a 5 or 10 mL serological pipette, stir the microcarrier cell suspension similar to that of the impeller speed to achieve a homogenous suspension. While in motion, slowly aspirate 5 mL of suspension and add to a 15 mL conical tube.
2. Allow microcarriers to settle to bottom of tube for a minimum of 10 minutes. A longer settling time may be necessary.
3. Carefully and aseptically remove and discard excess spent media from the conical tube.
4. Add 5 mL of PBS (Lonza 17-516F) to the conical tube. Rinse by rocking the conical tube back and forth several times. Allow the PBS wash to remain on cells for two to three minutes.
5. Centrifuge the microcarriers at 200 x g for 5 minutes at room temperature. Carefully aspirate the supernatant.

6. Resuspend the microcarrier/cell pellet in 1-2 mL Trypsin-EDTA (Lonza 17-161E), or a NAO cell dissociation reagent. Gently rock the tube to ensure that the cells are covered by the solution. Incubate at 37°C for ten minutes rocking, and then observe under a microscope. If the cells are less than 95% detached, continue incubating and observe every 5 minutes. Gentle vortexing or triturating for a short period of time (~10 sec) may aid in dissociation of the cells from the microcarriers. Cultures may take up to 20 minutes for dissociation with trypsin.
7. Once cells are dissociated from microcarriers, pass the suspension through a 40 µm cell strainer to separate the cells from the microcarriers and collect the cells in a fresh conical tube.
8. Rinse the original tube with 5 mL PBS (Lonza 17-516F) and then pass through same cell strainer.
9. Centrifuge the cell suspension at 200 x g for 5 minutes at room temperature.
10. Carefully aspirate or decant the supernatant and resuspend cell pellet in 0.5-1 mL ProMDCK™ (3D).
11. Count the cells with a hemacytometer or cell counter and calculate the total number of viable cells. Make a note of your cell yield.

Subculturing microcarrier cultures from 3D to 3D

Follow procedure for "Sampling microcarrier cultures from 3D" to determine cell concentration.

1. Determine volume required to seed spinner flask at desired concentration. Using the following formula

$$\text{Seeding Volume} = \frac{\text{Growth area x Seeding Density}}{\text{Viable cells/mL}}$$

2. Aliquot new microcarriers. Be sure to account for microcarriers being transferred from the original culture in the calculation of the final surface area to be seeded.
3. To a new spinner flask add microcarriers and ProMDCK™ (3D) to final volume per manufacturers' recommendations.
4. Aseptically, using a serological pipette, stir the microcarrier cell suspension similar to that of the impeller speed to achieve a homogenous suspension. While in motion, slowly aspirate the volume required for seeding as determined in step 1 and transfer to a conical tube.
5. Allow microcarriers to settle to bottom of tube for a minimum of 10 minutes. Longer settling time may be necessary.
6. Carefully and aseptically remove and discard excess spent medium from the conical tube.
7. Resuspend microcarrier/cell pellet in minimal volume of ProMDCK™ (3D) and add to the new spinner flask.

Infecting microcarrier cultures

NOTE: Viral infections should be carried out using a Biological Safety Cabinet and all necessary safety precautions.

Follow procedure from "Sampling microcarrier cultures from 3D" to determine cell concentration (cells/mL). Multiply by total spinner volume to obtain Total Cell number.

1. Determine amount of virus required for infection using the following formula. When using Influenza virus a multiplicity of Infection of 0.01 is recommended.

$$Virus\ Stock\ Required\ (mL) = \frac{Total\ Cell\ \#\ x\ \ MOI}{Virus\ Titer\ \left(\frac{pfu}{mL}\right)}$$

2. Place spinner flask in laminar flow hood. Allow microcarrier cell suspension to settle to the bottom of the spinner flask for approximately 10-15 minutes.
3. Aseptically remove 70 - 80% of the spent medium volume carefully avoiding any microcarriers. The volume of medium remaining in the spinner flask should be just sufficient for the impeller to stir the microcarrier/cell mixture.
4. Inoculate the spinner flask with the appropriate volume of virus stock. If using Influenza Virus, trypsin is required for hemagglutinin (HA) viral protein cleavage for attachment and entry into the cells. TPCK-Trypsin at 2 ug/mL is recommended.
5. Incubate spinner flask at 37°C, 5% CO_2 and 90% humidity for 1 hour. Stir at half the speed of a full volume spinner flask. For a Corning 125 mL spinner flask, 15-17 rpm is recommended.
6. After 1 hr. incubation, add ProMDCK™ (3D) containing trypsin to a final volume as previously determined in step 17 of "Transitioning from 2D into 3D".
7. Return spinner flask to the incubator. Temperature may vary depending on virus strain used. For Influenza A viruses, 35 - 37°C and for Influenza B viruses 33 - 35°C is recommended.

Incubation time for Influenza virus ranges from 24-72 hrs. Virus can be quantitated by TCID50 or Plaque Assay.

V. Ordering information

Cat. no.	Product	Size
BE12-924Q	ProMDCK™ 2D	1 L
BE12-925Q	ProMDCK™ 3D	1 L
17-605E* BE17-605E* BEBP-17-605E	L-glutamine 200 mM	100 mL
17-161E BE17-161E	Trypsin-EDTA	100 mL
17-516F* BE17-516F* BEBP17-516Q	Phosphate Buffered Saline without Calcium and Magnesium	500 mL

Product use statement

GMP PRODUCTS ARE INTENDED FOR RESEARCH OR FOR FURTHER MANUFACTURING USE ONLY. This product is not intended for direct therapeutic use in humans.

***THESE PRODUCTS ARE FOR RESEARCH USE ONLY.** Not approved for human or veterinary use, for application to humans or animals, or for use in clinical or *in vitro* diagnostic procedures.