Primary Cancer Culture System

Instruction Manual

Product	Size	Catalog Number
Primary Cancer Culture System consists of:		C-28081
▪ Primary Cancer Cell Medium D-ACF	250 ml	C-28080*
▪ Primary Cancer Cell Medium D-ACF SupplementMix	for 250 ml	C-39880*
▪ NCCD-Reagent	2 ml	C-43080

not available as single items

Recommended for

- Tumor tissue samples
- Malignant/cancerous cells
- Established primary tumor cell cultures

Product Description

This advanced culture system, consisting of the Primary Cancer Cell Medium D-ACF and the NCCD-Reagent, was designed to be the first universally applicable, cost-effective solution for in vitro isolation of long-term primary cultures of human malignancies, e.g. from patient tumor samples or patient-derived xenografts (PDX). The selection process dispenses with the use of toxic agents and provides a defined and animal-component free culture environment. Since on the cellular level malignancy itself is the only selection criterion, the cell diversity of the cancerous subpopulations of the original tumor is preserved. One bottle of medium is typically sufficient for 3 to 5 primary isolations.

Our Primary Cancer Culture System is a complete culture system consisting of a bottle of Basal Medium, one vial of SupplementMix and one vial of NCCD-Reagent. Adding the SupplementMix to the Basal Medium results in the complete Primary Cancer Cell Medium D-ACF. The NCCD-Reagent is intended for treatment of the culture surface. Do not add the NCCD-Reagent to the medium.

The Primary Cancer Culture System can also be used for other applications, e.g., enriching malignant subpopulation(s) in established cell lines or depleting of stromal cells and other non-cancerous cells from established primary cancer cell cultures. For more detailed information, please refer to the corresponding Application Note on our website www.promocell.com.

Supplementation Details

Our Primary Cancer Cell Culture Medium D-ACF contains all the growth factors and supplements necessary for the optimal culture of cancerous/malignant cells.

Note: The Primary Cancer Cell Medium D-ACF does not contain antibiotics or antimycotics and is formulated for use in an incubator with an atmosphere of 5% CO_2.

NCCD-Treatment of the Culture Surface

The treatment of the culture surface with the NCCD-Reagent provided with the Primary Cancer Culture System is indispensable for successful isolation and maintenance of cancer cells. Please refer to the instructions for a detailed protocol.

Note: The NCCD-Reagent (C-43080) is also available separately.

Preparation of the supplemented Medium for Use

Thaw the SupplementMix in a 37°C water bath with occasional swirling. Do not incubate longer than necessary. Then, transfer the entire content of the SupplementMix to the Basal Medium. Close the bottle and swirl gently until a homogenous mixture is formed.

Storage and Stability

Store the Basal Medium at 4 to 8°C in the dark and the SupplementMix at -20°C immediately after arrival. Do not freeze the Basal Medium. If stored properly, the products are stable until the expiry date stated on the label. After the supplements are added to the basal medium, store at 4–8°C and use within 8 weeks. Do not freeze the complete medium. For use, pre-warm only an aliquot of the complete medium and keep the remaining medium refrigerated at 4 to 8°C.

Quality Control

All lots and components of the PromoCell Primary Cancer Cell Culture Kit are subjected to comprehensive quality control tests using a human cancer cell line. Each lot of the Primary Cancer Culture System is checked for growth promoting activity for malignant cells, adherence rate, and typical morphology of the test cell line.

In addition, all lots of media have been tested for the absence of microbial contaminants (fungi, bacteria, mycoplasma).

Intended Use

The products are for in vitro use only and not for diagnostic or therapeutic procedures. For safety precautions please see appropriate MSDS.

Primary Cancer Culture System Protocol

I. Tumor Cell Isolation Procedure

Use aseptic techniques and a laminar flow bench.

Materials

- Fresh tumor tissue (0.2 – 3 grams; ≥ 1 gram is optimal)
- Hanks Balanced Salt Solution (HBSS) with Ca^{2+}/Mg^{2+} w/o phenol red
- Primary Cancer Culture System (C-28081)*
- Gentamicin (50 mg/ml stock)
- Phosphate buffered saline (PBS) w/o Ca^{2+}/Mg^{2+} (C-40232)
- Accumax (e.g., Sigma #A7089) – tissue digestion/isolation
- Accutase (C-41310) – passage/subcultivation of established culture
- Scalpel/forceps/scissors
- Cell strainers of descending size down to 40 µm (e.g. 400/100/40 µm)
- Tilt-roll-shaker, rotary mixer or comparable
- Tissue culture flasks and dishes

**consists of the Primary Cancer Cell Medium D-ACF and 2 ml of NCCD-Reagent (C-43080; also available separately)*

1

NCCD Treatment of the Plasticware (Day 0 or earlier)

The use of the NCCD-Reagent provided with the Primary Cancer Culture System is indispensable for successful isolation and maintenance of cancer cells. Dilute the thawed NCCD-Reagent stock solution 1:20 with PBS. Use 100 µl per cm^2 of culture surface to treat the tissue culture vessel with the diluted NCCD-Reagent and leave the closed vessel for at least 1 hour at room temperature.

Make sure that the NCCD covers the entire vessel surface. Aspirate the NCCD solution just before seeding the cells.

Note: Unless used immediately, the sealed vessel containing the NCCD-Reagent may be stored for up to 3 months at 2 to 8°C for later use. Diluted NCCD-Reagent solution may be stored for up to 4 weeks at 2 to 8°C protected from light.

2

Wash and weigh the tumor tissue (Day 0)

Remove visible residues of healthy tissue from the tumor. Place the tumor sample in a tube and wash twice with a generous amount of PBS and vigorous shaking. Then weigh the tumor tissue in a pre-tared sterile petri dish.

Note: The tumor tissue should be as fresh as possible and stored in HBSS at 2 to 8°C immediately after surgical removal. Tissue up to 6 hours old is optimal for isolation purposes. However, successful isolations have been accomplished from tumor samples as old as 24 hours. Keep in mind that recently applied chemical or radiation therapy may affect the isolation results.

3

Homogenize the tumor tissue (Day 0)

Place the washed tumor sample on the lid of a petri dish. Add a small volume (1–2 ml) of Primary Cancer Cell Medium D-ACF to the tumor tissue and dissect it into small pieces using a scalpel. Homogenize the tissue to a "slurry" or into small pieces of approximately 1 mm^3 by additionally mincing the tissue chunks using the scalpel. Avoid attrition of the tissue.

4

Wash the homogenized tumor tissue (Day 0)

Transfer the homogenized tumor tissue to a 50 ml tube using forceps. Add 10x the volume (w/v) of PBS and vortex or mix vigorously. Let the tissue pieces settle for 2 minutes and then aspirate the supernatant. Repeat if there is still a lot of blood/debris observable. Aspirate as much as possible of the PBS without losing the tissue.

Note: If there is floating homogenized tissue, use a sieve, e.g., 400 µm, for separating the washed, homogenized tissue from the washing buffer.

5

Perform the enzymatic digest of the tumor tissue (Day 0)

Resuspend the tissue pellet in Accumax solution at a concentration of 20 ml per gram of tumor tissue. Incubate at room temperature (20-25°C) with gentle but constant mixing, e.g., by a tilt-roll mixer at 50 rpm. Digest until the solution becomes distinctly turbid. Depending on the type of tissue, this is typically the case after approximately 30–60 minutes. A 45-minute incubation is a good starting point.

Note: Do not digest the tissue longer than necessary and never digest for longer than 60 minutes since this may significantly compromise cell viability. Always perform the digestion reaction at room temperature and consult the Accumax manual for instructions on proper storage and handling.

6

Remove tissue residues from the sample (Day 0)

Let the remaining tissue pieces settle down for 2 minutes. In order to obtain a single-cell suspension, progressively filter the turbid supernatant using cell strainers of descending pore size down to 40 µm, e.g., 400 µm to 100 µm to 40 µm.

Note: Discard the remaining tissue pieces.

7

Dilute and pellet the sample with medium (Day 0)

Dilute the single-cell suspension at least 1:1 with Primary Cancer Cell Medium D-ACF. Use a higher dilution ratio if the solution is still viscous. Pellet the cell suspension for 10 minutes at 240 x g at room temperature and carefully aspirate the supernatant without disturbing the cell pellet.

8

Determine the number of viable nucleated cells (Day 0)

Resuspend the cell pellet in 5 ml of Primary Cancer Cell Medium D-ACF. Determine the number of viable nucleated cells using an appropriate method.

Note: In case of cell clumps, which cannot be resuspended, filter the cell suspension once more through a 40 µm cell strainer before counting. The expected yield is 1–3 million viable nucleated cells per gram of tumor tissue. If it is not possible for any reason to determine the viable nucleated cell count in the primary isolate, continue with step 9 and refer to the Note in step 9. Keep in mind that omitting cell counting may lead to suboptimal seeding densities.

9

Wash and plate the cells (Day 0)

Pellet the cell suspension for 10 min at 240 x g at room temperature and carefully aspirate the supernatant without disturbing the cell pellet. Finally, resuspend the cell pellet in 1 ml of Primary Cancer Cell Medium D-ACF. Plate 100.000 to 200.000 viable nucleated cells per cm^2 in the prepared NCCD-treated tissue culture vessel(s).
Use approximately 200 µl of medium per cm^2 for vessels ≤25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for >25 cm^2. Add 50 µg/ml of Gentamicin to the final volume and incubate at 37°C with 5% CO_2.

Example: Plate 2.5–5 million nucleated viable cells per T-25 flask using 5 ml of medium. Plate 7.5–15 million nucleated viable cells per T-75 flask using 10 ml of medium.

Note: If the viable nucleated cell count was not determined in step 8, then plate the primary isolate from up to 2 grams of tumor tissue in a T-25 flask using 5 ml of Primary Cancer Cell Medium D-ACF.

II. Primary Cancer Cell Culture

Use aseptic techniques and a laminar flow bench.

Note: Depending on the tissue quality, type, and malignancy stage of the tumor sample, obtaining a homogeneous primary culture may require 4–8 weeks.

Materials: See page 2.

1

Initiate of the primary tumor cell culture (Day 0)

Incubate the culture for a total of 10 to 14 days to let the primary tumor cell culture begin, but proceed with step two on day 6 after plating (see II. step 2).

Note: Typically, adherent and non-adherent cells as well as formation of multicellular primary suspension aggregates can be observed during the first two weeks of culture.

2

Add fresh medium (Day 6)

On days 5to 7, add an additional volume of the initial culture volume of fresh medium (w/o antibiotics) to the cells. Do not change the medium, simply add more fresh medium. Continue incubation until the culture reaches the stage described in step 3.

Example: For an existing culture with a volume of 5 ml of medium, add another 5 ml of fresh medium. The resulting total culture volume is then 10 ml.

Note: If the medium turns orange-yellow due to high metabolic activity of the isolated cells before day 6, the fresh medium should be added sooner. A slightly orange color is noncritical, however. If significant media exhaustion is still observed before the culture is ready for step 3, increasing the total culture volume by adding more fresh medium is recommended.

3

Initiate a separate secondary suspension culture (Days 10 to 14)

The primary culture is ready for step 3 as soon as sufficiently large floating multicellular aggregates (i.e., ≥70 µm in diameter) have developed or small aggregates of fewer than 10 cells appear. This is usually the case after 10 to 14 days. Perform step 3 no later than day 14. Depending on the suspension cell pattern of your primary culture, continue with step 3a or 3b, whatever is more appropriate.

Performing step 3 results in two separate culture vessels: the original primary flask containing the residual adherent cells and a new secondary flask containing the suspension cell fraction.

3.A

Separation of larger aggregates (≥70 µm or ≥10 cells)

To separate the established primary tumor cell aggregates, collect the used medium (containing the aggregates) in a separate 15 ml conical tube. Wash the remaining adherent cells twice with PBS, immediately add an appropriate amount of fresh medium (see below) and put them back into the incubator.

Let the tube with the suspension aggregates stand upright for 15 minutes at room temperature to allow gravity sedimentation of the primary aggregates. Then gently aspirate the supernatant, leaving back 0.5–1 ml. Plate the sedimented primary aggregates with an appropriate amount of fresh medium (see below) in a separate new culture vessel treated with the NCCD-Reagent.

3.B

Separation of small aggregates and single suspension cells

Collect the used medium containing the small aggregates/single suspension cells in a separate 15 ml conical tube. Wash remaining adherent cells twice with PBS, immediately add an appropriate amount of fresh medium (see below) and return them to the incubator.

Centrifuge the suspension cell sample for 10 minutes at 240 x g at room temperature. Leave 200 µl of the supernatant behind while gently aspirating the spent medium, since the pellet may be quite loose. Resuspend and plate the cells in an appropriate amount of fresh medium (see below) in a separate new culture vessel treated with the NCCD-Reagent.

Recommended media volume: Use approx. 200 µl of medium per cm^2 for vessels with ≤25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for >25 cm^2. Continue incubation of the cultures at 37°C and 5% CO_2.

3.C

Add fresh medium

Add another volume of fresh medium to each culture 7 days after separating the adherent/suspension cells.

Note: The residual adherent cell fraction in the original primary culture flask contains stromal cells and other types of unwanted benign cells but may still contain cancer cells. Therefore, closely monitor the culture of the adherent cell fraction for significant sustained proliferation, e.g., colony formation.

"Budding" of new aggregates or suspension cells from existing adherent cell clusters may be observed during up to 4 weeks. Combine newly formed aggregates/cells with the separate secondary suspension culture during the regular medium changing interval (refer to step 4).

Note that separated suspension aggregates may revert to an adherent or intermediate growth pattern in the further course of the isolation process.

4

Change medium during cancer cell selection (every 10 to 14 days)

After successfully completing step 3, completely replace the medium of all samples every 10 to 14 days as described in steps 4a-4d. The appropriate media change technique will depend on the growth pattern of the corresponding primary isolate. Methods for each of the possibilities are described in steps 4a-4c.
Adherent cultures remain in the same culture vessel until the first passage (see step 7). In the case of suspension cultures, replacing the used vessel with a new one when regularly changing the medium is optional but not mandatory in most cases. Always keep flasks with adherent cells for at least 4 weeks or until you are absolutely sure they do not contain cells of interest.

Note: Make sure to prevent extensive medium exhaustion (indicated by an orange-yellow color; a slightly orange hue is still acceptable). Isolations in which no viable primary culture has become successfully established within 6 weeks after initial plating are not promising and can be discarded.

4.A

Change medium for adherent cultures

Aspirate the used medium of adherent cells, wash the culture twice with PBS and add an appropriate amount of fresh medium (see below) to the cells. If the spent medium contains significant amounts of suspension cell aggregates or viable single cells, use the passage techniques described in 4b and 4c to recover these cells.

4.B

Change medium for large cell aggregates (≥70 µm)

Collect the used medium containing the suspension aggregates in a separate 15 ml conical tube. If applicable, wash remaining adherent cells twice with PBS, immediately add an appropriate amount of fresh medium (see below) and return them to the incubator.

Let the tube with the suspension aggregates stand upright for 12 minutes at room temperature for gravity sedimentation of larger cell aggregates. Then gently aspirate the supernatant while leaving 0.5–1 ml behind. Carefully transfer the sedimented aggregates with a serological pipet into a NCCD-treated culture vessel containing an appropriate amount of fresh medium (see below).

Note: In contrast to other sphere culture techniques, disaggregation of the primary cell aggregates is neither recommended nor necessary.

4.C

Change medium for small aggregates and single suspension cells

Collect the used medium containing the small aggregates/single suspension cells in a separate 15 ml conical tube. If applicable, wash remaining adherent cells twice with PBS, immediately add an appropriate amount of fresh medium (see below) and return them to the incubator.

Centrifuge the suspension cell sample for 10 minutes at 240 x g at room temperature. Leave 200 µl of the supernatant behind when gently aspirating the spent medium, since the pellet may be quite loose. Resuspend the pellet in fresh medium and use a serological pipette to transfer the cell suspension into a NCCD-treated culture vessel containing an appropriate amount of fresh medium (see below).

Recommended media volume: Use approximately 200 µl of medium per cm^2 for vessels with ≤25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for >25 cm^2. Continue incubation of the cultures at 37°C and 5% CO_2.

4.D

Add fresh medium (Day 6 after changing the medium)

Add another total culture volume of fresh medium to each culture after another 5 to 7 days.

Example: For a 5 ml culture, add 5 ml of fresh medium for a total culture volume of 10 ml.

5

Determine the growth pattern of the isolated cancer cells (Weeks 2 to 4)

Adherently growing cancer cells can typically be identified within 2 to 4 weeks after plating, since they are present as slow-growing colonies. Follow the regular medium changing schedule for adherent cells without passaging the cells until they reach an adequate confluency level (see step 7).

It is more difficult to assess the traits of isolated primary cancer cells that exhibit a suspension growth pattern, in other words as cell aggregates or single cells. Typically, they proliferate very slowly and intrinsically lack morphological information on the level of single cells. It is therefore recommended to wait for the numbers of cancer cells to increase sufficiently to permit further characterization and in-depth analysis (see also the Note in step 6).

It is highly advisable to keep all the primary cultures until you have unequivocally identified the growth pattern of your isolated primary cancer cells.

Note: Depending on the properties of the primary tumor and the doubling times of primary cancer cells in vivo [2 – 4], the selective culture conditions may result in slow growth of the isolated cancer cells with doubling times ranging from a couple of days to several months (near-quiescent state). In addition, the initial cell loss caused by successive depletion of non-cancerous cells from the primary isolate may also slow down expansion of the isolate, especially in the first weeks after initial plating.

In general, primary cancer cell isolates that exhibit an adherent growth pattern tend to proliferate significantly faster than their counterparts growing in suspension.

6

Use the purified malignant cells for your experiments (Week 4+)

After making sure that all unwanted benign cells have been eliminated, you can set up your experiments with the isolated cancer cells. Alternatively, the cells may be passaged and expanded further (see step 7).

Note: We strongly recommend keeping the stock culture in the Primary Cancer Cell Medium D-ACF in combination with NCCD-treated culture vessels to ensure long-term maintenance of the unaltered primary cancer cell isolate.

If necessary to enhance proliferation at this point, it is possible to increase the growth factor supplementation for a fraction of the culture. However, avoid using serum and/or other non-defined supplements. Alternatively, move part of the culture to a suitable expansion medium of your choice, e.g., the PromoCell Cancer Stem Cell Medium (C-28070) in NCCD- treated vessels.

Always keep in mind, however, that changing the culture conditions may also pose the risk of irrecoverable loss of the original primary cancer cells as a result of "differentiating proliferation" like that witnessed in most primary cancer cells cultured in established standard media. This may require you to identify specific culture conditions for your cancer cell isolate that will induce proliferation without terminal differentiation.

7

Passage the tumor cell primary culture

Passaging the cells before they proliferate to a high confluence level is not recommended. Until they do, continue changing the medium as described for step 4.

7.A

Passage the suspension cultures

Increase the total culture volume by adding fresh medium and split it in two (or more) fresh NCCD-treated vessels (see below for the recommended amounts of medium). Use gravity sedimentation for the regular medium changes to reduce the amount of debris in the culture.

Note: IIn contrast to other types of sphere cultures, it is not necessary to disaggregate cell clusters because aggregates of malignant cells propagate autonomously under these culture conditions.

7.B

Passage the adherent cells

Prepare new NCCD-treated culture vessels (see I. step 1). Depending on the overall confluence, perform a 1:1 or 1:2 split of the culture using Accutase (not Accumax). Wash the culture twice with ambient tempered PBS w/o Ca^{2+}/Mg^{2+} and then incubate the cells for 5–10 minutes with 150 µl/cm2 Accutase at 37°C. After the first 5 minutes of incubation, monitor the detachment process visually. When the cells start to detach, facilitate their complete dislodgement by tapping the flask. Add the same volume of Primary Cancer Cell Medium D-ACF to the detached cells and spin down for 5 minutes at 300 x g at room temperature. Carefully aspirate the supernatant and gently resuspend the cell pellet in an appropriate amount of Primary Cancer Cell Medium D-ACF (see below). Seed the cells into new NCCD-treated vessels and incubate them further at 37°C and 5% CO_2.

Recommended media volume: Use approximately 200 µl of medium per cm^2 for vessels with ≤25 cm^2 of culture surface and approximately 130 µl medium per cm^2 for >25 cm^2. Continue incubation of the cultures at 37°C and 5% CO_2.

7.C

Add fresh medium

Add another volume of fresh medium to each flask after 5 to 7 days (or earlier if required) and continue incubating at 37°C and 5% CO_2.

III. Depleting Contaminating Non-Cancerous Cells in Established Primary Cancer Cell Cultures

Use aseptic techniques and a laminar flow bench.

Materials

- Existing culture containing malignant cells, e.g., cell line or primary isolate
- Primary Cancer Culture System (C-28081)*
- Phosphate buffered saline (PBS) w/o Ca^{2+}/Mg^{2+} (C-40232)
- Accutase (C-41310)
- Tissue culture treated cell culture vessel

**consists of the Primary Cancer Cell Medium D-ACF and 2 ml of NCCD-Reagent (C-43080; also available separately)*

1

NCCD-treatment of plasticware with TC surface

Dilute the thawed NCCD-Reagent stock solution 1:20 with PBS w/o Ca^{2+}/Mg^{2+}. Use 100 µl per cm^2 of culture surface to treat the tissue culture vessel with the diluted NCCD-Reagent and leave the vessel closed for at least 1 hour at room temperature. Make sure that the NCCD solution covers the entire vessel surface. Aspirate the NCCD solution just before seeding the cells.

Note: Unless the sealed vessel containing the NCCD-Reagent will be used right away, it can be stored for up to 3 months at 2–8°C for later use. Diluted NCCD solution may be stored for up to 4 weeks at 2–8°C protected from light.

2

Determine the growth pattern of the malignant cells

Passage your established culture containing the malignant cells as usual. Plate a sample of the cells in a NCCD-treated vessel containing an appropriate amount of Primary Cancer Cell Medium D-ACF. Grow the cells as described in protocol II. 1–7. Monitor the culture for proliferation of the malignant cells that interest you and determine their growth pattern (see II.5).

Note: The malignant cells may grow adherently and/or as spheres in suspension. During the induction phase, they may proliferate more slowly than under your established standard culture conditions. However, the culture will recover as soon as the non-malignant cells have been substantially depleted (in passage 2 and 3) and the cultured cells have fully adapted to the new conditions.

3

Clean up your culture

After you have identified the growth pattern of the malignant cells that interest you under these selective culture conditions, passage the culture into Primary Cancer Cell Medium D-ACF. Expand and passage the cells as required (see II.7) for 2 to 3 times in order to deplete the culture of non-malignant cells.

If you require special media modifications, we offer a custom media service starting at 10 bottles per order.
Contact us at info@promocell.com to find out more.

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