

Selective *In Vitro* Culture of Primary Cancer Cells from Human Tumor Samples

Application Note

Background

Tumors consist of a heterogeneous mix of multiple interacting cell types organized in a complex hierarchy. Only a small subpopulation of the tumor cells are cancer cells capable of driving progression and ultimately the dissemination of the malignancy. The largest proportion of the cells contained in most tumors are non-tumorigenic, differentiated cells and benign cancer-associated cells such as cancer-associated fibroblasts (CAFs), tumor-associated macrophages (TAMs) and stromal cells.

Despite the unique cellular features of malignant cells – e.g. tumorigenicity, unlimited proliferation potential, self-renewal and resistance to cytotoxic drugs – the complex organization of tumors complicates the purification and characterization of the causative subpopulation of cancer driving cells.

The lack of specific markers as well as selective *in vitro* culture methods add to the difficulties.

Traditional culture systems for cancer cells based on classical media formulations lack specificity for malignant cells. These media predominately support the proliferation of benign cells, e.g. stromal cells, or differentiated (non-tumorigenic) cancer cells, thus leading to a gradual loss of the original malignant cell population. Only cells isolated from the most malignant types of tumors in these media, from which most traditional cancer cell lines have been established, have been successful. These cell lines, however, are model systems with a long history of selection and adaption in various ill-defined *in vitro* culture conditions and do not comprehensively reflect the behavior of primary tumors [1].

Mouse models have been developed for establishing cultures from tumors with a lower degree of malignancy, since direct *in vitro* isolation was not effective. After several rounds of serial *in vivo* transplantation of tumor tissue in severely immune-compromised mice, the cells of the primary tumor eventually develop into a stable tumor cell population. However, only a small fraction of these tumor cells is stable under traditional *in vitro* culture conditions. Most can only be maintained by serial *in vivo* transplantation in the mouse. These techniques are expensive, time-consuming and elaborate, and – most importantly – they induce major changes in the initial primary tumor cells inherent in a serial selection process in rodents. Consequently, direct *in vitro* isolation of patient-specific primary tumor cells in an unbiased defined culture environment is highly preferable.

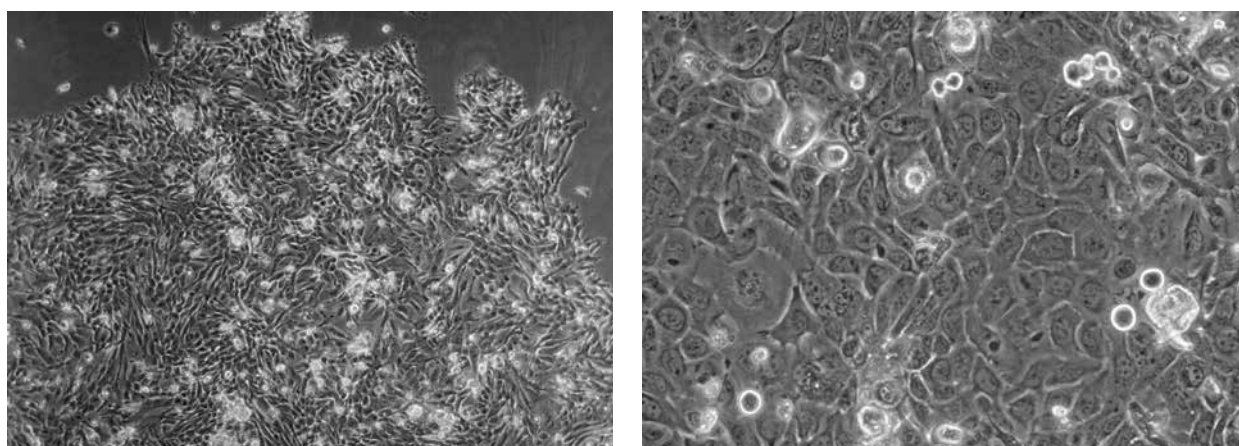


Fig. 1: Primary culture derived from a squamous cell carcinoma with the Primary Cancer Culture System. Left: The primary culture was obtained after 4 weeks as compact and heterogeneous adherent colonies formed by different types of epithelial-like cells. The culture proliferated in the Primary Cancer Cell Medium D-ACF with a population doubling time of approximately 7 days and could be serially passaged with no signs of growth inhibition. **Right:** Greater magnification reveals the cellular heterogeneity within the colony. See Tab. 1 for mutational analysis data on this primary culture.

The PromoCell Primary Cancer Culture System

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 cytotoxic agents in a defined and animal-free culture environment. Since on the cellular level malignancy itself is the only selection criterion, the cell diversity of the cancerous subpopula-

tions of the original tumor is preserved. One bottle of medium is typically sufficient for 3–5 primary isolations.

With regard to cancer cell primary isolation, traditional tumor cell media generally support the growth of benign cells, e.g. tumor stroma, CAFs and TAMs, but do not sufficiently support the cancer cell subpopulations that drive the progression of tumors *in vivo*. The vast majority of these *in vitro* primary cultures are therefore transient and exhibit a gradual loss of the original malignant cells that cause the disease.

In contrast, the Primary Cancer Culture System makes it possible to reliably deplete benign cells from the culture while supporting the maintenance of cancer

cells. Since malignancy itself serves as the sole functional selection criterion, the culture system is applicable to all types and entities of malignant cells, regardless of their origin and the stage of the tumor. Provisional enrichment techniques, e.g. cell sorting while relying on unproven markers, are therefore obsolete.

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.

Gene	Analyzed region	Transcript variant	DNA mutation	Protein mutation	Mutational load [#]
AKT1	Exon 4	ENST00000349310	-	-	-
ALK	Exon 22, 23, 25	ENST00000389048	-	-	-
BRAF	Exon 11, 15	ENST00000288602	-	-	-
CTNNB1	Exon 3	ENST00000396183	-	-	-
DDR2	Exon 5, 8, 12, 13, 14, 15, 17	ENST00000367921	-	-	-
EGFR	Exon 12, 18, 19, 20, 21	ENST00000275493	-	-	-
ERBB2	Exon 21, 22, 23	ENST00000406381	-	-	-
ERBB4	Exon 3, 4, 6, 7, 8, 9, 15, 23	ENST00000342788	-	-	-
FBXW7	Exon 5, 8, 9, 10, 11	ENST00000281708	-	-	-
FGFR1	Exon 4, 7	ENST00000447712	-	-	-
FGFR2	Exon 7, 9, 12	ENST00000358487	-	-	-
FGFR3	Exon 7, 9, 14, 16, 18	ENST00000340107	-	-	-
KRAS	Exon 2, 3, 4	ENST00000256078	-	-	-
MAP2K1	Exon 2	ENST00000307102	-	-	-
MET	Exon 2, 14, 16, 19	ENST00000318493	-	-	-
NOTCH1	Exon 26, 27	ENST00000277541	-	-	-
NRAS	Exon 2, 3, 4	ENST00000369535	-	-	-
PIK3CA	Exon 10, 14, 21	ENST00000263967	c.1633G>A	p.(E545K)	26%
PTEN	Exon 1, 3, 6, 7, 8	ENST00000371953	-	-	-
SMAD4	Exon 3, 5, 6, 8, 9, 10, 11, 12	ENST00000342988	-	-	-
STK11	Exon 1, 4, 5, 6, 8	ENST00000326873	-	-	-
TP53	Exon 2, 4, 5, 6, 7, 8, 10	ENST00000269305	c.298C>T c.586C>T	p.(Q100*) p.(R196*)	33% 64%

Tab. 1: Mutational analysis of the squamous cell carcinoma primary isolate depicted in Fig. 1. The tumor panel test detected three hotspot mutations: one in the pik3ca gene and two in tp53. The high mutational load is indicative of a selectively enriched culture of malignant cells, while the differing percentages of the individual mutations suggest the maintenance of cancer cell subpopulation heterogeneity *in vitro*.[#]Mutational load = percentage of mutated transcripts/total transcripts of the respective transcript variant.

Schematic Overview

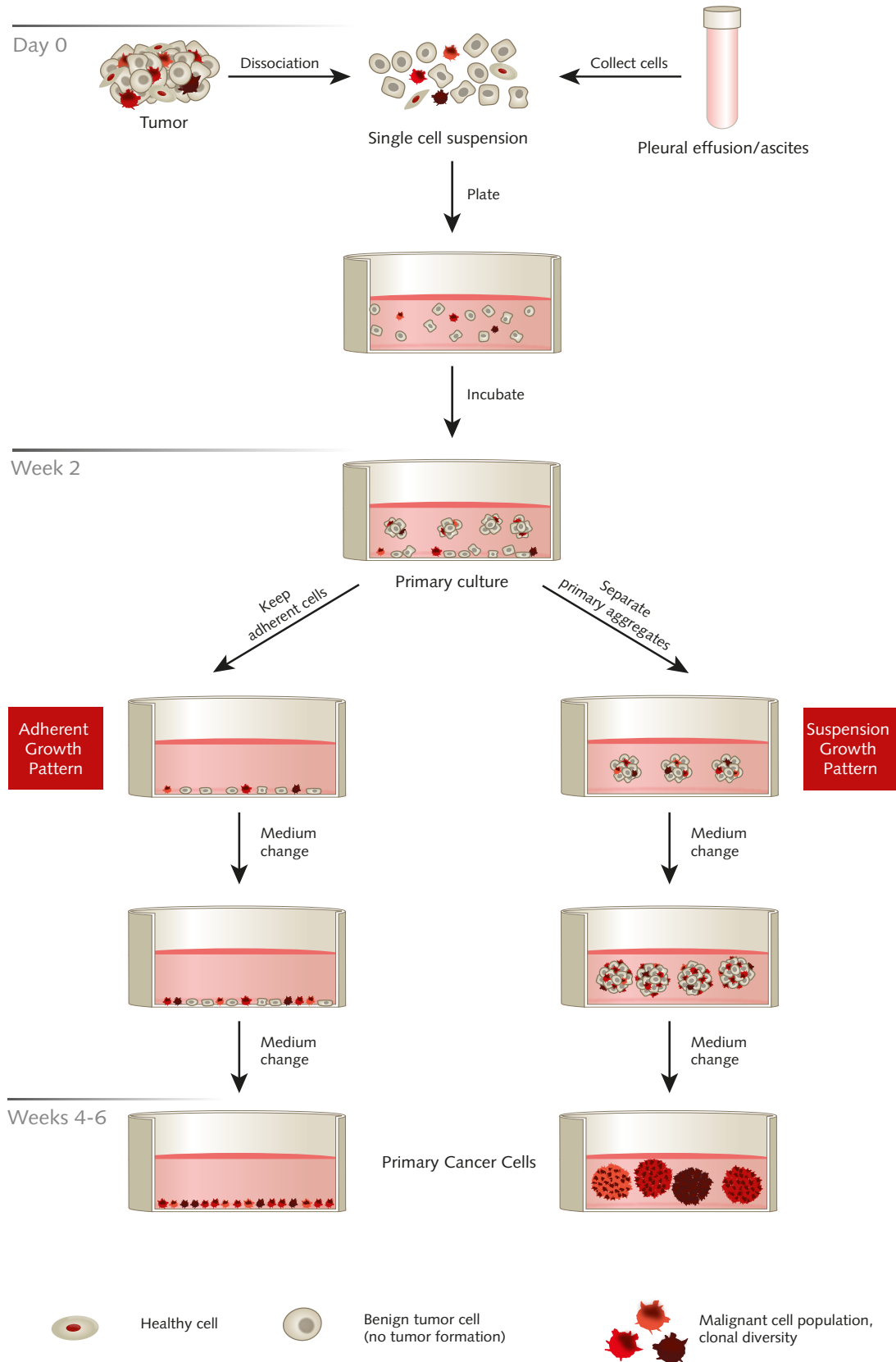


Fig. 2: Schematic overview of the course of the selective isolation of primary human cancer cells from tumor specimens or effusions using the Primary Cancer Culture System. Depending on the traits of the original tumor, the cancer cells typically exhibit an adherent or suspension growth pattern. However, note that the growth pattern of the primary culture may switch or adopt an intermediate state during the selection process.

Use aseptic techniques and a laminar flow bench.

A) Protocol for Establishment of Primary Cancer Cell Cultures

I. Materials

- Fresh tumor tissue (0.2–3 grams; ≥ 1 gram is optimal)
- Hanks Balanced Salt Solution (HBSS) with Ca²⁺/Mg²⁺ w/o Phenol Red
- Primary Cancer Culture System (C-28081)*
*consists of the Primary Cancer Cell Medium D-ACF and 2 ml of NCCD-Reagent (C-43080; also available separately)
- Gentamicin (50 mg/ml stock)
- Phosphate buffered saline (PBS) w/o Ca²⁺/Mg²⁺ (C-40232)
- Accumax (e.g. Sigma #A7098) – 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

II. 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 cm² 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 RT. 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.

III. Tumor Cell Isolation Procedure (day 0)

1. Wash and weigh the tumor tissue

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.

2. Homogenize the tumor tissue

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 approx. 1 mm³ by additionally mincing the tissue chunks using the scalpel. Avoid attrition of the tissue.

3. Wash the homogenized tumor tissue

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

*Primary Cancer
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*Primary Cancer
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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.

4. Perform the enzymatic digest of the tumor tissue

Resuspend the tissue pellet in Accumax solution at a concentration of 20 ml per gram of tumor tissue. Incubate at RT (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 min since this may significantly compromise cell viability. Always perform the digestion reaction at RT and consult the Accumax manual for instructions on proper storage and handling.

5. Remove tissue residues from the sample

Let the remaining tissue pieces settle down for 2 min. 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 \rightarrow 100 μm \rightarrow 40 μm .

Note: Discard the remaining tissue pieces.

6. Dilute the sample with medium

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.

7. Obtain the isolated single cells

Pellet the cell suspension for 10 min at 240 x g at RT and carefully aspirate the supernatant without disturbing the cell pellet.

8. Determine the number of viable nucleated cells

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 10. Keep in mind that omitting cell counting may lead to suboptimal seeding densities.

9. Wash the cells

Pellet the cell suspension for 10 min at 240 x g at RT 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.

10. Plate the cells

Plate 100.000 to 200.000 viable nucleated cells per cm^2 in the prepared NCCD-treated tissue culture vessel(s).

Use approx. 200 μl of medium per cm^2 for vessels \leq 25 cm^2 of culture surface and approx. 130 μl medium per cm^2 for $>$ 25 cm^2 . Add 50 $\mu\text{g}/\text{ml}$ 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.

Primary Cancer Cell Culture Establishment Protocol

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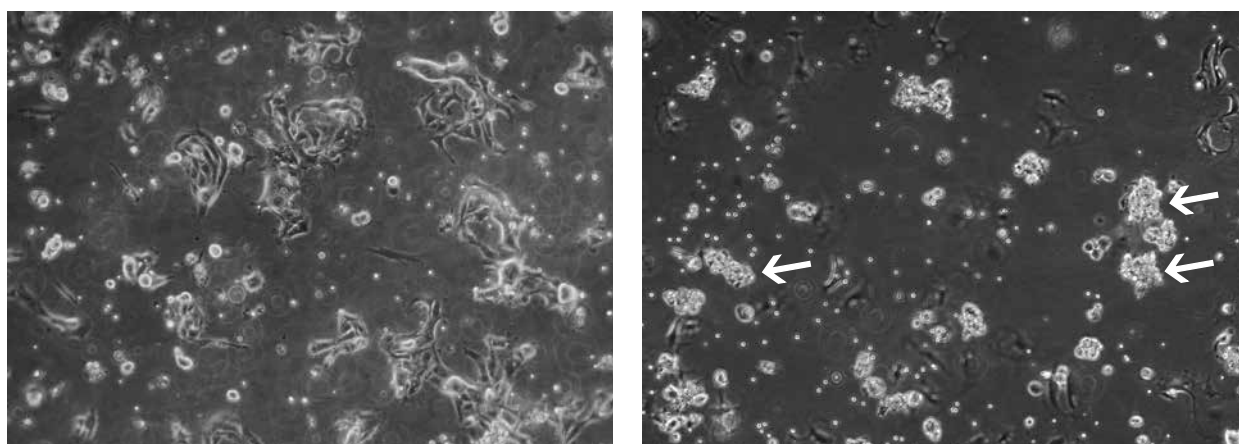


Fig. 3: General appearance of primary cancer cell cultures in the Primary Cancer Cell Medium D-ACF in early stages, in this example with lung squamous cell carcinoma cells. **Left:** Culture on day 2 after initial plating: a mix of residual erythrocytes, fibroblast- and epithelial-like adherent cells as well as floating single suspension cells can be observed. **Right:** On day 11, the formation of floating multicellular cell aggregates is already prominent (white arrows). The culture was used for primary aggregate separation on day 13 and was additionally cultured in a new flask parallel to the original sample containing the remaining adherent cell fraction (see Fig. 2 and protocol step IV.3).

IV. Primary Cancer Cell Culture

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

1. Initiate of the primary tumor cell culture (day 0)

Incubate the culture for a total of 10–14 days to let the primary tumor cell culture begin, but proceed with step two on day 6 after plating (see IV.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 5–7, 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 10–14)

The primary culture is ready for step 3 as soon as sufficiently large floating multicellular aggregates (i.e. $\geq 70 \mu\text{m}$ in diameter) have developed or small aggregates of fewer than 10 cells appear. This is usually the case after 10–14 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 (see Fig. 2).

Primary Cancer Cell Culture Establishment Protocol

Use aseptic techniques and a laminar flow bench.

3a) Separation of larger aggregates ($\geq 70 \mu\text{m}$ 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 RT 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.

3b) 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 $240 \times g$ at RT. 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 $\leq 25 \text{ cm}^2$ of culture surface and approx. 130 μl medium per cm^2 for $> 25 \text{ cm}^2$. Continue incubation of the cultures at 37°C and 5% CO_2 .

3c) 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 10–14 days)

After successfully completing step 3, completely replace the medium of all samples every 10–14 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.

Primary Cancer Cell Culture Establishment Protocol

Use aseptic techniques and a laminar flow bench.

4a) 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.

4b) Change medium for large cell aggregates ($\geq 70 \mu\text{m}$)

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 RT 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.

4c) 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 $240 \times g$ at RT. 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 approx. 200 μl of medium per cm^2 for vessels with $\leq 25 \text{ cm}^2$ of culture surface and approx. 130 μl medium per cm^2 for $> 25 \text{ cm}^2$. Continue incubation of the cultures at 37°C and 5% CO_2 .

4d) Add fresh medium (day 6 after changing the medium)

Add another total culture volume of fresh medium to each culture after another 5–7 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 2–4)

Adherently growing cancer cells can typically be identified within 2–4 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 of 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

Primary Cancer Cell Culture Establishment Protocol

Use aseptic techniques and a laminar flow bench.

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: PromoCell strongly recommends 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 3D Tumorsphere Medium XF (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.

7a) 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: In 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.

7b) Passage the adherent cells

Prepare new NCCD-treated culture vessels (see II). Depending on the overall confluence, perform a 1:1 or 1:2 split of the culture using Accutase (not Accutamax). 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 $\mu\text{l}/\text{cm}^2$ 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 RT. Carefully aspirate the supernatant and gently resuspend the cell pellet in an appropriate

Primary Cancer Cell Culture Establishment Protocol

Use aseptic techniques and a laminar flow bench.

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% CO₂.

Recommended media volume: Use approx. 200 µl of medium per cm² for vessels with ≤ 25 cm² of culture surface and approx. 130 µl medium per cm² for > 25 cm². Continue incubation of the cultures at 37°C and 5% CO₂.

7c) Add fresh medium

Add another volume of fresh medium to each flask after 5–7 days (or earlier if required) and continue incubating at 37°C and 5% CO₂.

Primary Cancer Cell Culture Establishment Protocol

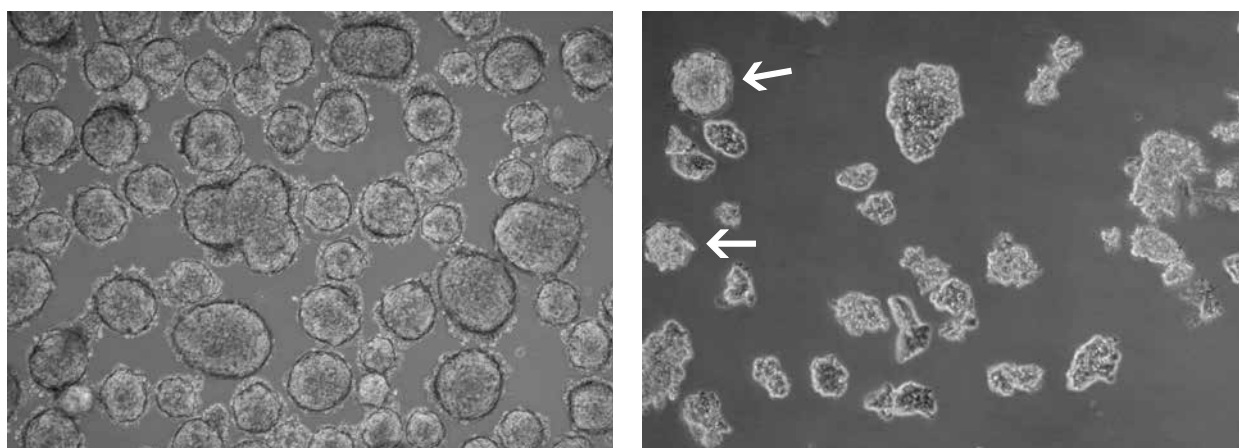


Fig. 4: Primary culture derived from a low-grade small cell lung cancer (SCLC) with the Primary Cancer Culture System. Left: The primary isolate was obtained after 4 weeks as a floating sphere-forming culture, which persisted in a near-quiet state even after 6 months. **Right:** Adding extra growth factors elicited significant expansion in the latent sphere culture with a doubling time of 3–4 weeks. Note that some spheres persisted under these modified culture conditions (white arrows), while the larger part of the culture proliferated as floating planar multicellular 2D sheets, which is a prototypical growth pattern for SCLC cells *in vitro*.

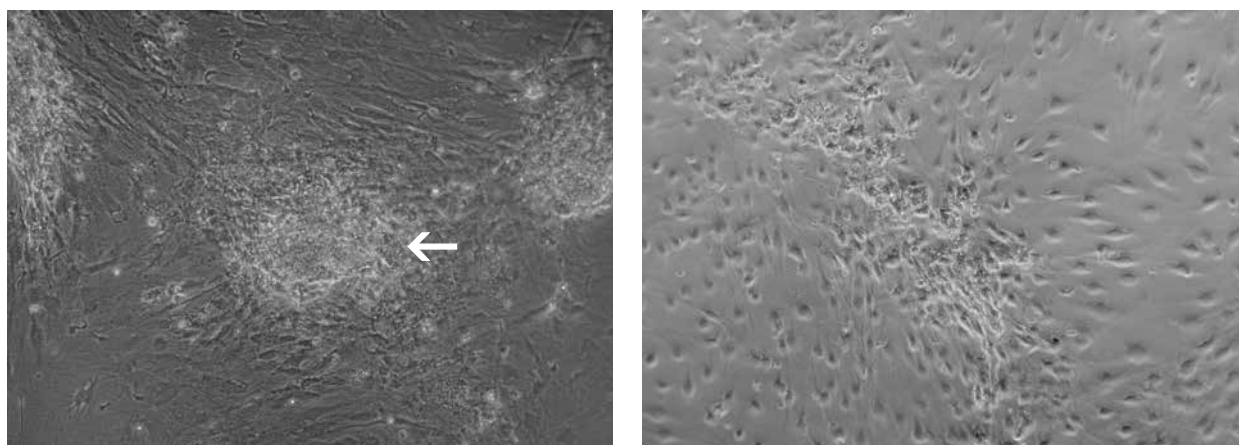


Fig. 5: Primary culture derived from an invasive adenocarcinoma with the Primary Cancer Culture System. Left: During the first two weeks of the isolation process, the cancer cells appeared as locally restricted, lightly-adherent convex cell clusters (white arrow) on top of the stroma layer. **Right:** After 4 weeks, highly motile cancer cells began migrating from their original locations to cover the whole culture surface. These cells proliferated as a homogeneous population in the Primary Cancer Cell Medium D-ACF.

Use aseptic techniques and a laminar flow bench.

B) Protocol for Depleting Contaminating Non-Cancerous Cells in Established Primary Cancer Cell Cultures

I. Materials

- Existing culture containing malignant cells, e.g. cell line or primary isolate
- Primary Cancer Culture System (C-28081)*
*consists of the Primary Cancer Cell Medium D-ACF and 2 ml of NCCD-Reagent (C-43080; also available separately)
- Phosphate buffered saline (PBS) w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$ (C-40232)
- Accutase (C-41310)
- Tissue culture treated cell culture vessel

II. Depletion Protocol

1. NCCD-treatment of plasticware with TC surface

Dilute the thawed NCCD-Reagent stock solution 1:20 with PBS w/o $\text{Ca}^{2+}/\text{Mg}^{2+}$. 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 RT. 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 IV. 1-7. Monitor the culture for proliferation of the malignant cells that interest you and determine their growth pattern (see IV.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 IV.7) for 2 to 3 times in order to deplete the culture of non-malignant cells.

*Depletion of
Non-Cancerous
Cells
Materials*

*Depletion of
Non-Cancerous
Cells
Protocol*



Products

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 Supplement Mix	for 250 ml	C-39880*
NCCD-Reagent	2 ml	C-43080

*not available as single item

Related Products

Product	Size	Catalog Number
Dulbecco's PBS, w/o Ca ⁺⁺ /Mg ⁺⁺	500 ml	C-40232
Accutase Solution	100 ml	C-41310
NCCD-Reagent	2 ml	C-43080
Cryo-SFM	30 ml	C-29910
3D Tumorsphere Medium XF	250 ml	C-28070

References

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