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Genuine 3D cell culture - Simply and Routinely

alvetex 

**Technology for Routine
Three Dimensional (3D) Cell Culture**

Product Information Booklet

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Technology for Routine Three Dimensional (3D) Cell Culture

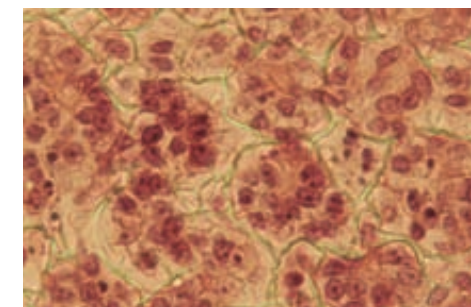
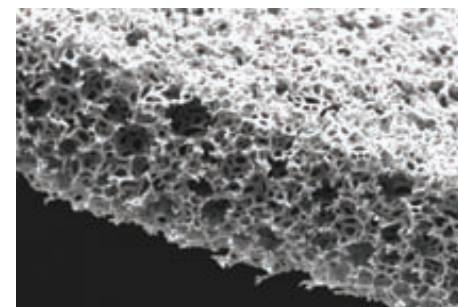
1.0 What is 3D cell culture?

3D cell culture is about creating suitable surroundings for optimal cell growth, differentiation and function by:

- Allowing individual cells to maintain their normal 3D shape and structure with minimal exogenous support and interference,
- Encouraging cells to form complex interactions with adjacent cells and receive and transmit signals,
- Enabling a more natural environment to foster the creation of native architecture found in tissue structures,
- Reducing stress and artificial responses as a result of cell adaptation to flat, 2D growth surfaces.

2.0 What is alvetex®?

Alvetex® is a highly porous, cross-linked polystyrene scaffold, which has been sectioned into 200 µm thick membranes (below left). The resulting material is inert and does not degrade during normal use. It has been adapted to fit a variety of conventional cell culture plastic-ware formats. Alvetex® provides a suitable 3D structure in which cells can proliferate, migrate, differentiate and function in an appropriate niche environment. Cells maintain a 3D shape and form close interactions with adjacent cells (below right, TERA2.cl.SP12 cells maintained for 12 days).



The product has been terminally sterilised by gamma irradiation and remains sterile until opened. Alvetex® requires an ethanol (EtOH) wash prior to use to render it hydrophilic. The material is compatible with a broad range of standard molecular, cellular and histological techniques (visit www.amsbio.com for further details).

FOR IN VITRO RESEARCH USE ONLY: NOT FOR CLINICAL, DIAGNOSTIC, OR THERAPEUTIC PROCEDURES. NOT FOR USE IN HUMANS. PATENT PENDING

3.0 Choosing the right alvetex® format

Alvetex® is available in a variety of formats, each specifically designed for different 3D cell culture applications. In the 12-well plate (AVP002) alvetex® is located at the bottom of each well, whereas in well-inserts (AVP004-3 and AVP005-3) it is suspended. The well inserts are easy to use and are highly versatile. They fit a range of culture plates from different manufacturers as well as Reinnervate's custom-made "Well insert holder in a deep Petri dish" (AVP015).

In deciding which alvetex® format to use, the following factors should be considered in combination:

- Cell type and duration of culture,
- The desired depth of cell penetration in the 3D cell culture,
- The type of assay to be performed or application to be used for.

The rate of cell growth in alvetex® is affected by cell metabolism, proliferation rate, motility and cell size. It is therefore important to choose the alvetex® format that will achieve optimal growth for the chosen cell type.

For example, in Figure 1. two different cell types were grown for the same time period using alvetex® 12-well plates (AVP002), resulting in two cultures with different characteristics:

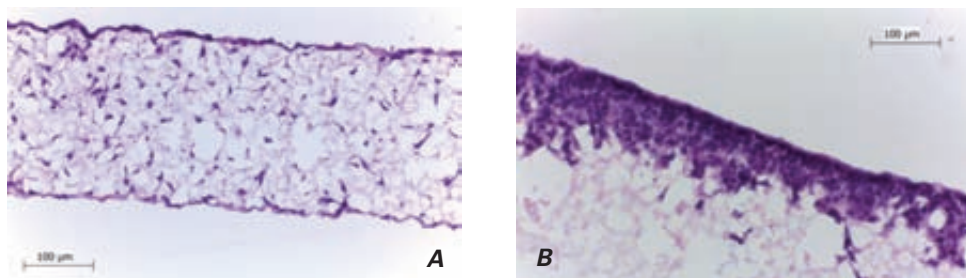


Figure 1. 3T3 (A.) and HepG2 (B.) cultures grown on alvetex® in 12-well plate format for 7 days. 3T3 cells are small, highly proliferative and invasive, and therefore readily penetrated through the entire scaffold. In contrast, HepG2 cells are slower growing cells that have a high tendency for cell-cell attachment and consequently penetrated only the top 30% of the alvetex® membrane. In both cases 3D cell culture was achieved. Micrographs taken at 20x magnification.

Alvetex® formats have been specifically designed to enable optimal 3D cell culture in both short and long term experiments. The 12-well plate format is ideal for short term cell cultures where the medium is replaced every 1 to 2 days. The alvetex® membrane sits at the bottom of each well and the cells are exposed to the medium only from above. This is desirable for studies where easy access is required to the cells as they predominantly reside in the top portion of the

scaffold (e.g. for transfection studies). Alternatively a shorter experiment in well inserts is also suitable, as the cells will not have had time to penetrate the scaffold.

The well inserts are capable of supporting cell growth for up to 3 weeks for assays and applications where higher cell numbers are desirable (Table 1.). The design of the well insert allows for greater cell penetration into the scaffold and higher cell yields because alvetex® is suspended in the medium such that cells receive nutrients from above and below. Therefore, they sustain optimal growth for longer and achieve greater differentiation creating a cultured tissue that more closely resembles the growth of cells in the body. Well inserts can be used to create co-cultures either in plates or in the well insert holder in the Petri dish.

Application / alvetex® format	AVP002 (12-well plate)	AVP004-3 (6-well insert)	AVP005-3 (12-well insert)
Histology (10 µm sections)	+++	+++	+++
Immunohistochemistry	+++	+++	+++
Confocal microscopy	+++	+++	+++
Light microscopy (e.g. phase contrast)*	N/A	N/A	N/A
Viability assay	++	+++	+++
Toxicity assay	++	+++	+++
Proliferation assay	++	+++	+++
Metabolic activity assay	++	+++	+++
Gene expression / Microarray	++	+++	+++
Protein expression (e.g. Western Blotting)	++	+++	+++
Air-liquid interface differentiation	N/A	+++	+++
Cell signalling assay	++	+++	+++
Permeability assay	N/A	+++	+++
Transfection	+++	++	++
Co-culture	+	+++	+++
Invasion assay	+	+++	+++
Migration assay	+	+++	+++

Table 1. Suggested guidelines for the use of alvetex® formats for cell applications and assays. +++ most suitable, ++ suitable, + least suitable, N/A= not applicable.

Ranking is based on alvetex® disc format suitability, the likely cell yields and therefore signal generation, and whether exogenously added chemicals/cells can be contained to only one side of the membrane.

*The growth of cells cannot be followed by traditional light microscopy as in 2D, but as with ex vivo tissues, 3D structures have to be evaluated using histology or confocal microscopy. Alternatively cell proliferation can be monitored using a viability assay such as the MTT.

The well insert holder in a deep Petri dish (AVP015) is designed to hold well inserts in a large volume Petri dish reducing the need for frequent media changes. This format should be used for prolonged cell growth of highly proliferative and demanding cell types (Figure 2.). The well inserts can be positioned at three different levels in the insert holder to allow for cultures to be raised to the air liquid interface (for air liquid interface differentiation) and subsequent permeability / barrier testing (Table 1.). 3D co-cultures can also be set-up in one or two of the well inserts within the same Petri dish (see section 7. for full description of versatility).

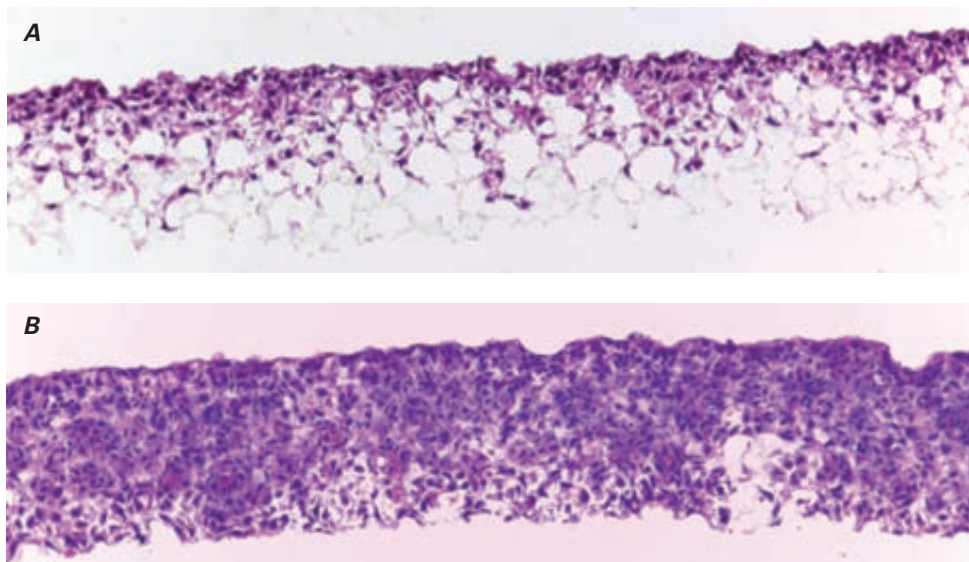


Figure 2. HaCaT cells grown on alvetex® in 12 well insert (AVP005-3) in a 6-well plate (A) and (B) in well insert holder in deep Petri dish (AVP015). Note significantly more proliferation and cell invasion in the cultures grown in the well insert holder system, where more nutrients were available. Micrographs taken at 20x magnification.

Thus, alvetex® products provide cell biologists with a broad range of choice and great flexibility when designing their cell culture experiments.

4.0 Handling alvetex®:

All procedures concerning the handling of alvetex® should be performed wearing gloves according to standard aseptic methods required for cell culture in a Class I/II cabinet.

- When dry, alvetex® is reasonably fragile with a wafer-like consistency; however once rehydrated the discs become much more robust. Therefore handle the material carefully when performing any manipulation including media changes, transferring the discs for analysis, fixing and embedding for histology, etc. When using forceps, exercise care as manipulating the scaffold can damage its structure. Try to handle the alvetex® discs around the edges only.
- When dispensing liquids (e.g. 70% EtOH, PBS and medium) over alvetex®, place the end of the pipette tip towards the wall of the culture vessel avoiding touching the scaffold. If using the 12-well plate format, retain cylindrical clip in place. Well inserts should be fed from the outside: place the end of the pipette tip towards the wall of the culture vessel (either by going through the window of the well insert or beside it). Let the liquid rise gently to touch the base of the well insert and if required dispense the rest of the solution into the well insert to prevent it from floating. Seed cells on the middle of the disc without touching the membrane itself.

In the 12-well plate format, the alvetex® is held in place by a polystyrene clip that can easily be removed to release the disc for analysis of the cultured cells (Figure 3.)



Figure 3. Alvetex® discs can easily be removed from the 12-well plate using flat ended forceps (Fisher Scientific, MNK-155-M).

In well insert formats, the alvetex® membrane can be removed using forceps (Figure 4. A & B) or can be cut out using a scalpel with a size 11 blade (C & D).

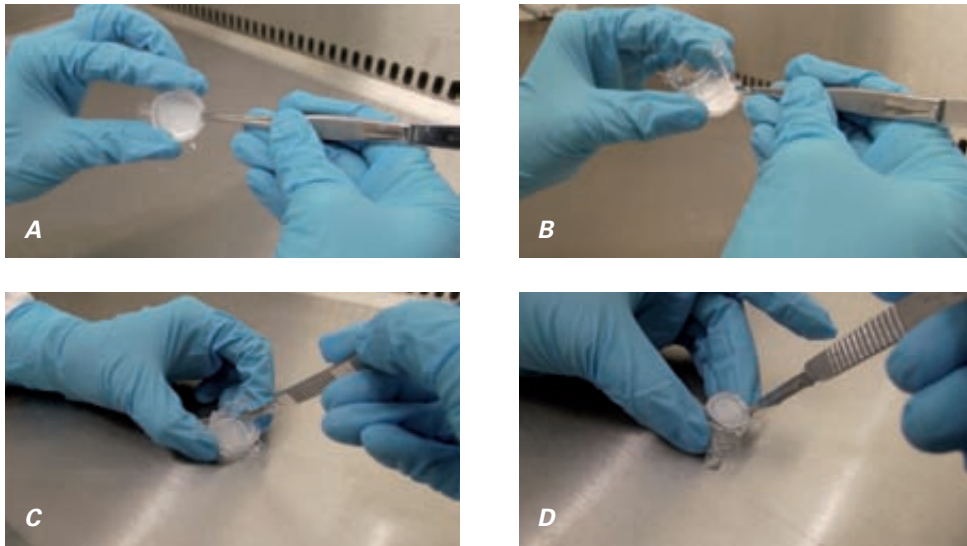


Figure 4. Removal of alvetex® disc from well-insert. Unclip the base of the well insert all the way around (A), and pull down to release the disc (B). Alternatively the disc can be cut out by holding the well insert upright (C) or upside down (D)

5.0 Use of alvetex® in 12-Well Plate Format (AVP002)

5.1 Alvetex® 12-well plate format

The 12-well plate format is a simple presentation of alvetex® technology: it comprises a single loose disc and clip per well (Figure 5.). The clip holds alvetex® in position during transit and use. The clip is made from polystyrene, it is sterile and inert, and can easily be removed to release the alvetex® disc. The 12-well plate format is primarily suitable for short term culture experiments where the medium is replaced every 1-2 days.



Figure 5. Presentation of alvetex® in 12-well plate format.

5.2 Preparing alvetex® (in 12-well plates) for first use and cell seeding

- Open the 12-well plate carefully to ensure that the clips holding the alvetex® discs are not displaced.
- Add approximately 2 ml of 70% EtOH to each well to pre-treat the alvetex® disc in preparation for incubation in aqueous solutions (e.g. PBS, culture medium).
- Carefully aspirate the EtOH solution and immediately wash the alvetex® disc in ~2-3 ml of appropriate medium for ~1 min.
- Carefully aspirate medium wash and replace with final wash medium (use same type of medium as for cell seeding). The alvetex® disc is now ready for cell seeding: aspirate medium just before application of cells. If preparation of cell suspension is delayed, incubate plate with medium at 37 °C with 5% CO₂ until further use.
- Similarly to 2D culture, if using serum-free medium, consider the use of coating agents to enhance cell attachment.

- Prior to cell seeding, alvetex® can also be pre-coated with standard cell culture reagents such as collagen, fibronectin, laminin, poly-D/L-lysine, poly-L-ornithine and Matrigel™ to encourage cell adhesion, differentiation and optimise function. Perform this step after the EtOH treatment and appropriate buffer wash steps instead of medium.

5.3 Optimisation of seeding and 3D cell culture using the alvetex® 12-well format

3D cell culture is different to conventional 2D cell culture and as such requires optimisation according to cell type:

- For most applications initial cell seeding densities of $0.5\text{--}2.0 \times 10^6$ cells in 100-150 μl per disc are recommended. Seeding in a low volume enables cells to attach predominantly to the alvetex® disc and avoids cell loss on other surfaces.
- When inoculating, aspirate washing medium thoroughly from the plate and carefully dispense cells on the middle of the discs. Replace lid and incubate in a humidified incubator at 37 °C with 5% CO₂ for 30 minutes to 3 hours to facilitate cell attachment.
- After this time gently flood the wells with medium by dispensing up to 4 ml of medium per well.
- With 3D cell culture there will be many more cells growing per unit volume of medium. Therefore, users must refresh media more frequently; ideally once a day, however this will also depend on the population doubling rate and nutrient demands of the cell type cultured.

6.0 Use of alvetex® in well insert formats

6.1 Alvetex® 6-well insert (AVP004-3) and 12-well insert (AVP005-3)

The presentation of alvetex® in well insert formats is versatile (Figure 6), enabling long term 3D culture as cells can receive nutrients from media above and below the membrane, sustaining optimal 3D cell growth.

Currently there are two well insert sizes available: AVP004-3 (22 mm diameter, A) and AVP005-3 (15 mm diameter, B). Both are supplied in blister packs with three individually sealed inserts containing alvetex®. The 6- and 12-well inserts are designed to fit into most 6-well plates or Reinnervate's custom-made 'Well Insert Holder in Deep Petri Dish' (AVP015). Snapping the extended wings of AVP005-3 will also enable it to fit into a 12-well plate. Note that plates and well insert holders are not supplied with the product and have to be sourced separately.

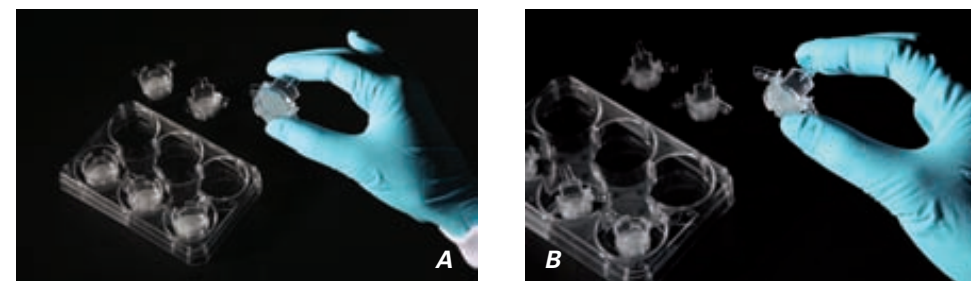


Figure 6. Presentation of alvetex® in 6- and 12-well formats.

6.2 Preparing alvetex® (6-well and 12-well inserts) for first use and cell seeding

- Open the required number of blister packs carefully and pick up the well insert(s) using forceps.
- Immersion in 70% EtOH will instantly pre-treat alvetex® in preparation for incubation in aqueous solutions (e.g. PBS, culture medium). This can be done by dipping the well insert into a beaker containing 70% EtOH before placing it into the chosen holder vessel. Gently shake or tap the well insert to remove excess ethanol.
- Alternatively EtOH treatment can be performed *in situ*, once the well insert is positioned in the plate. Add sufficient 70% EtOH to the well so that the level of the liquid rises above the membrane (for 6-well plates add approximately 5 ml/well, for 12-well plates add approximately 2 ml/well).
- Carefully aspirate to waste, leaving no excess liquid and immediately wash alvetex® in an appropriate medium (for 6-well plates use 7 ml/well, for 12-well plates use 2.5 ml/well) for ~1 min.

- Aspirate and replace with final wash medium (use same type of medium as for cell seeding). The scaffold is now ready for cell seeding: aspirate medium just before application of cells. If preparation of cell suspension is delayed, incubate plate with medium at 37 °C with 5% CO₂ until further use.
- Similarly to 2D culture, if using serum-free medium, consider the use of coating agents to enhance cell attachment. Prior to cell seeding, alvetex® can also be pre-coated with standard cell culture reagents such as collagen, fibronectin, laminin, poly-D/L-lysine, poly-L-ornithine and Matrigel to encourage cell adhesion, differentiation and optimise function. Perform this step after the EtOH treatment followed by an appropriate buffer wash step instead of medium.

6.3 Optimisation of seeding and 3D cell culture using the alvetex® 6-well and 12-well insert formats

3D cell culture is different to conventional 2D and as such requires optimisation according to cell type, assay being performed and insert configuration used:

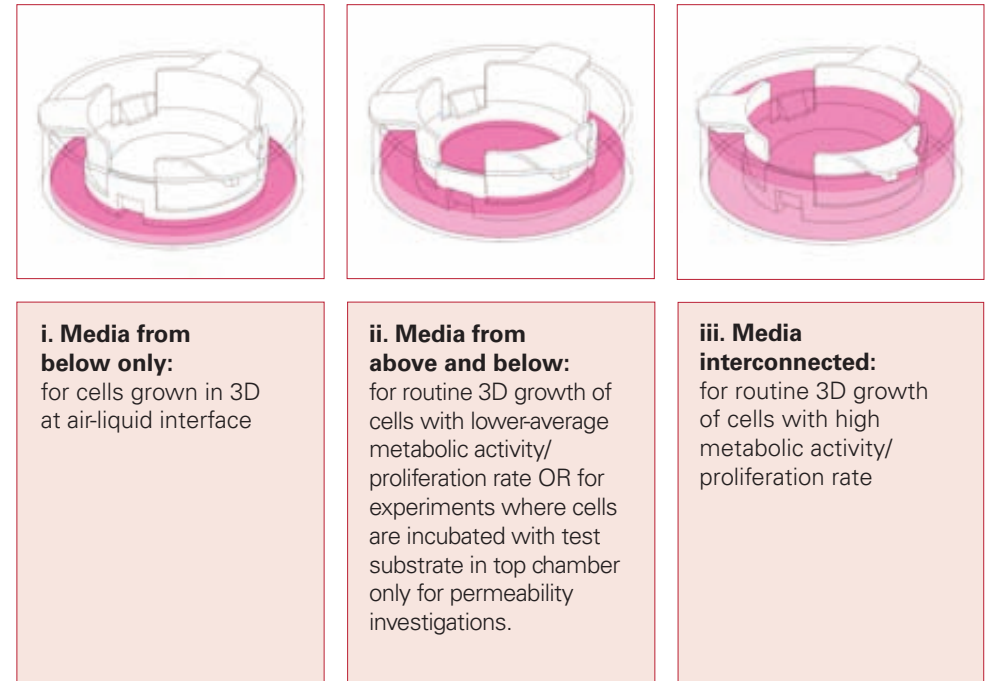


Figure 7. Media filling levels and well insert configurations.

6.3.1 6-well insert in 6-well plate:

- For most applications initial cell seeding densities of $0.5\text{-}2.0 \times 10^6$ cells in 100-150 μl per disc are suitable. Seeding in a low volume enables cells to attach predominantly to the disc and avoids cell loss on other surfaces.
- When inoculating, aspirate washing medium thoroughly from the plate and carefully dispense cells on the middle of the discs without touching the membrane. Replace lid and incubate in a humidified incubator at 37 °C with 5% CO₂ for 30 to 90 minutes to facilitate cell attachment.
- After this time gently flood the wells with medium by dispensing 3.0-10.5 ml of medium per well: Fill up the wells carefully beside the insert, so the medium comes up from the bottom to gently contact the cellularised alvetex[®] disc and gradually floods the insert itself. The volume of medium required will depend on user requirements and recommendations are outlined in Table 2 below.

6.3.2 12-well insert in 6-well plate:

- For most applications initial cell seeding densities of $0.25\text{-}1.0 \times 10^6$ cells in 50-75 μl per disc are suitable. Seeding in a low volume enables cells to attach predominantly to the disc and avoids cell loss on other surfaces.
- When inoculating, aspirate washing media thoroughly from the plate and carefully dispense cells on the middle of the discs without touching the membrane. Replace lid and incubate in a humidified incubator at 37 °C with 5% CO₂ for 30 to 90 minutes to facilitate cell attachment.
- After this time gently flood the wells with media by dispensing 3.0-10.5 ml of medium per well. Fill up the wells carefully beside the insert, so the medium comes up from the bottom to gently contact the cellularised alvetex[®] disc and gradually floods the insert itself. The volume of medium required will depend on user requirements and recommendations are outlined in Table 2 below.

6.3.3. 12-well insert in 12-well plate:

- For most applications initial cell seeding densities of $0.25\text{-}1.0 \times 10^6$ cells in 50-75 μl per disc are suitable. Seeding in a low volume enables cells to attach predominantly to the disc and avoids cell loss on other surfaces.
- When inoculating, aspirate washing media thoroughly from the plate and carefully dispense cells on the middle of the discs without touching the membrane. Replace lid and incubate in a humidified incubator at 37 °C with 5% CO₂ for 30 to 90 minutes to facilitate cell attachment.
- After this time gently flood the wells with media by dispensing 1.4-4.0 ml of medium per well: Fill up the wells carefully beside the insert, so the medium comes up from the bottom to gently contact the cellularised alvetex[®] disc and gradually floods the insert itself. The volume of medium required will depend on user requirements and recommendations are outlined in Table 2.

Well insert and holder type	Feeding volumes		
	Below only ⁱ	Above and below separately ⁱⁱ	Above and below interconnected ⁱⁱⁱ
6-well insert in a 6-well plate	3.5 ± 0.5 ml/well	7 ± 1 ml/well	10 ± 0.5 ml/well
12-well insert in a 6-well plate	3.5 ± 0.5 ml/well	7 ± 1 ml/well	10 ± 0.5 ml/well
12-well insert in a 12-well plate	1.6 ± 0.2 ml/well	2.4 ± 0.2 ml/well	3.8 ± 0.2 ml/well

Table 2. Feeding volumes for the different well insert configurations

- In 3D cell culture there will be more cells per unit volume of medium. Therefore, users must refresh medium more frequently typically every 2±1 days, however this will also depend on the population doubling rate, nutrient demands of the cell type cultured and the volume of medium used as described above.
- If any signs of cell attachment and growth are evident on the bottom of the plate, transfer the well inserts into a new plate, re-feed and then incubate as usual.

7.0 Use of Well Insert Holder in Deep Petri Dish (AVP015)

A single well insert holder capable of housing up to three well inserts (6- or 12-well inserts, Figure 8.) in a deep Petri dish is supplied in a pack. The Petri dish itself is not tissue culture treated. The whole product has been terminally sterilised by gamma irradiation and remains sterile until opened.

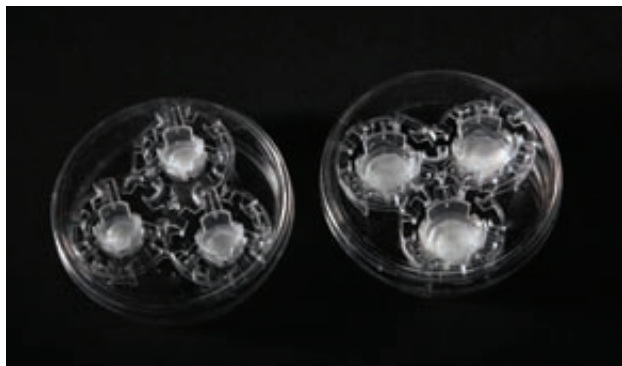
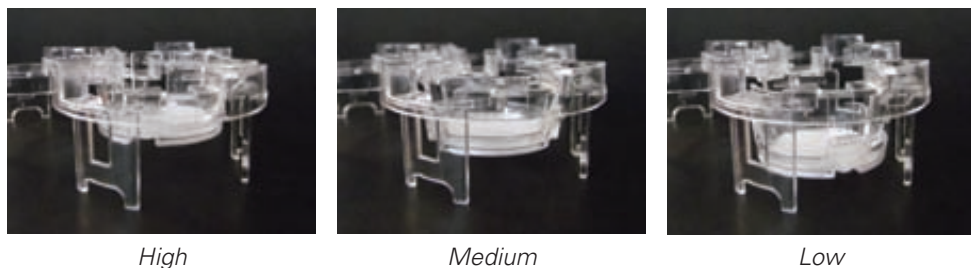


Figure 8. AVP005-3 (left) and AVP004-3 (right) in well insert holder system in deep Petri dishes.

7.1 Alvetex® well insert formats and their use in the well insert holder

The deep Petri dish enables users to grow their 3D cultures in larger volumes of media compared to an ordinary multiwell plate. Up to 95 ml of media can be used in the deep Petri dish and is therefore capable of sustaining long term 3D culture experiments (3-4 weeks) and reducing the frequency of medium exchanges. If required, a magnetic stirrer bar can be placed in the bottom of the dish to circulate media and facilitate exchange.

The well insert can be positioned at three different levels in the insert holder: high, medium and low.



High

Medium

Low

Figure 9. Well insert settings within the well insert holder (AVP015).

This feature allows cultures to be raised to the air liquid interface by moving the insert to a different level within the same holder.

Positioning the well inserts at different levels may be used to conserve expensive media or allow for increasing media volumes for demanding cell types over the course of a long term experiment.

Additionally, cultures can be fed from below the well insert only (i.), media from above and below separately (ii.) and media interconnected (iii.) through the windows at the side of the well inserts:



i.

ii.

iii.

Figure 10. Media filling levels and well insert configurations.

Recommended volumes for AVP004-3 (6-well insert):

Well insert setting within holder	Feeding Volume		
	Below only (i.)	Above and below separately (ii.)	Above and below interconnected (iii.)
Low	20ml ± 1ml	40ml ± 3ml	70ml ± 5ml
Medium	34ml ± 2ml	50ml ± 3ml	80ml ± 3ml
High	48ml ± 2ml	70ml ± 5ml	92ml ± 3ml

Recommended volumes for AVP005-3 (12-well insert):

Well insert setting within holder	Feeding Volume		
	Below only (i.)	Above and below separately (ii.)	Above and below interconnected (iii.)
Low	20ml ± 1ml	40ml ± 3ml	72ml ± 5ml
Medium	34ml ± 2ml	52ml ± 3ml	82ml ± 3ml
High	48ml ± 2ml	70ml ± 5ml	92ml ± 3ml

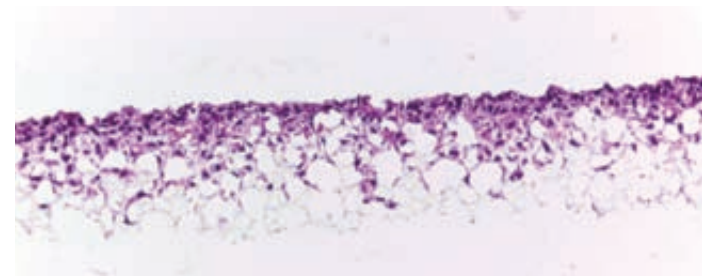
The well insert holder system also allows for the 3D co-culture of more than one cell type by seeding different cells in one or two of the well inserts within the same Petri dish. 3D co-cultures can also be set up within the same well insert. Alternatively, a support cell line can be cultured at the base of the Petri dish in 2D and another in 3D in the well inserts.

As the Petri dishes are untreated, coat with poly-L-lysine, or similar, to facilitate cell attachment. Ensure that suitable media is chosen that will simultaneously support the growth of both cell types cultured. The well insert holder will fit into deep Petri dishes with approximate dimensions of 86 mm (internal diameter) x 25 mm (height).

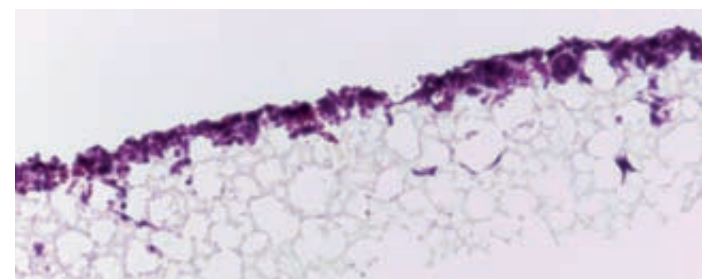
8.0 Examples of 3D cell cultures on alvetex® presented in different formats

8.1. Comparison of 3D cell growth patterns on alvetex® 12-well plate format (AVP002)

Alvetex® in the 12-well plate format (AVP002) was treated with EtOH and washed with complete medium prior to cell seeding. [Complete medium consisted of: DMEM, 10% FBS, 2 mM L-glutamine and 100 U/ml Penicillin & Streptomycin]. HaCaT cells (a human keratinocyte cell line) were plated at a density of 0.5×10^6 cells in 150 µl per well while HepG2 cells (a human liver cell line) were seeded at 2×10^6 cells in 150 µl per well. Plates were incubated for three hours before flooding with further media and maintained for 7 days. After preserving in Bouin's fixative the discs were paraffin embedded, sectioned (10 µm) and counterstained with Haematoxylin and Eosin. HaCaT cultures demonstrated significant cell invasion into the matrix, while HepG2 cells remained resident in the top 25% of the matrix.



HaCaT cells



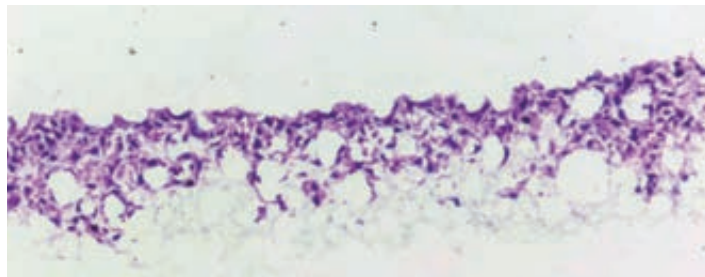
HepG2 cells

Figure 11. Comparison of 3D cell growth patterns on alvetex® 12-well plate format (AVP002). Micrographs taken at 20x magnification

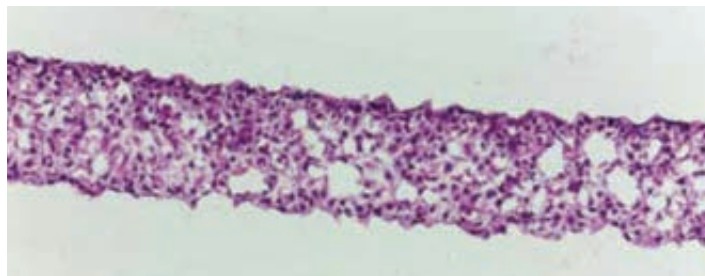
8.2. Comparison of 3D cell growth patterns of HaCaT cells on alvetex® presented in various formats

HaCaT cells (a human keratinocyte cell line) were seeded (0.5×10^6 cells in 150 μ l per well) on EtOH-treated and complete medium washed alvetex® scaffolds in the following formats: 12-well plate (AVP002), 6-well inserts (AVP004-3) in 6-well plate and 12-well inserts (AVP005-3) in well insert holder in deep Petri dish (AVP015). Cultures were maintained for 7 days. [Complete medium consisted of: DMEM, 10% FBS, 2 mM L-glutamine and 100 U/ml Penicillin & Streptomycin]. After preserving in Bouin's fixative the discs were paraffin embedded, sectioned (10 μ m) and counterstained with Haematoxylin and Eosin.

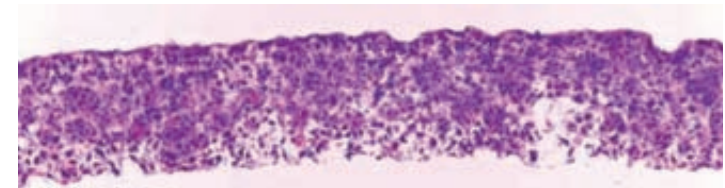
Note significantly more proliferation and cell invasion into the alvetex® scaffold in cultures grown in well-inserts, (media interconnected feeding regime) due to nutrient availability from above and below the scaffold. Thus, as media volume and availability increased so did cell proliferation and scaffold penetration. In the case of well inserts contained in a well insert holder in a deep Petri dish, this resulted in the formation of a slab of tissue-like material.



12 well plate (AVP002)



6-well insert (AVP004-3) in a 6-well plate



12-well inserts (AVP005-3) in well insert holder in deep Petri dish (AVP015)

Figure 12. Comparison of 3D cell growth patterns of HaCaT cells grown on various alvetex® formats. Micrographs taken at 20x magnification.

9.0 Additional Information

9.1. Recent Publications:

1. Schutte et al (2011). Rat primary hepatocytes show enhanced performance and sensitivity to acetaminophen during three-dimensional culture on a polystyrene scaffold designed for routine use. *Assay Drug Dev. Technol.* Epub ahead of print.
2. Neofytou et al (2011). Adipose tissue-derived stem cells display a proangiogenic phenotype on 3D scaffolds. *J. Biomedical Materials Research.* Epub.
3. Rajan et al (2011). Dysregulated TRK signalling is a therapeutic target in CYLD defective tumours. *Oncogene.* Epub ahead of print.
4. Fox et al (2010). Validation of reference gene stability for APAP hepatotoxicity studies in different in vitro systems and identification of novel potential toxicity biomarkers. *In Vitro Toxicology*, 24 (7), 1962-1970.
5. Barbetta et al (2005). Porous polymers by emulsion templating. *Macromolecular Symposia*, 226, 203-211.
6. Carnahan et al (2006). Tailoring the morphology of emulsion-templated porous polymers. *Soft Matter*, 2, 608-616.
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