## Use of alvetex® in well insert formats

### Alvetex® 6-well insert (AMS.AVP004-32) and 12-well insert (AMS.AVP005-34)

The presentation of alvetex® in well insert formats is versatile (Figure 1), 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: AMS.AVP004-32 (22 mm diameter, A) and AMS.AVP005-34 (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' (AMS.AVP015-2). Snapping the extended wings of AMS.AVP005-34 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.

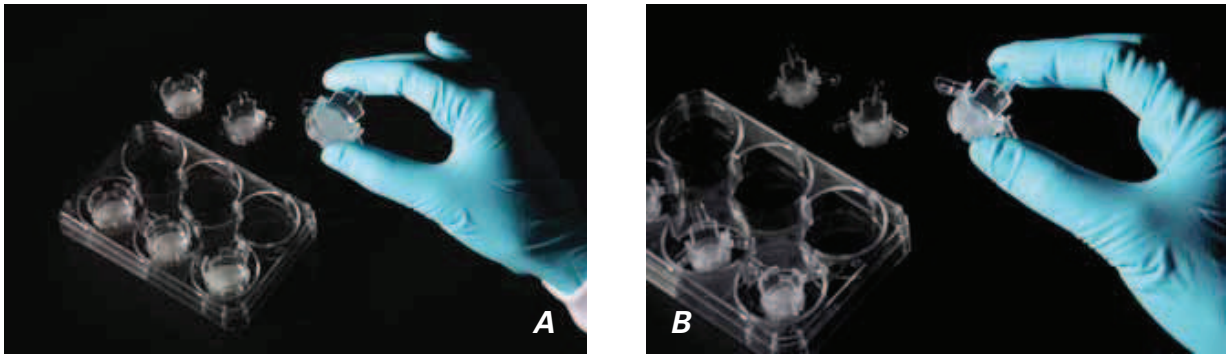


***Figure 1.*** *Presentation of alvetex® in 6- and 12-well formats.*

### 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.

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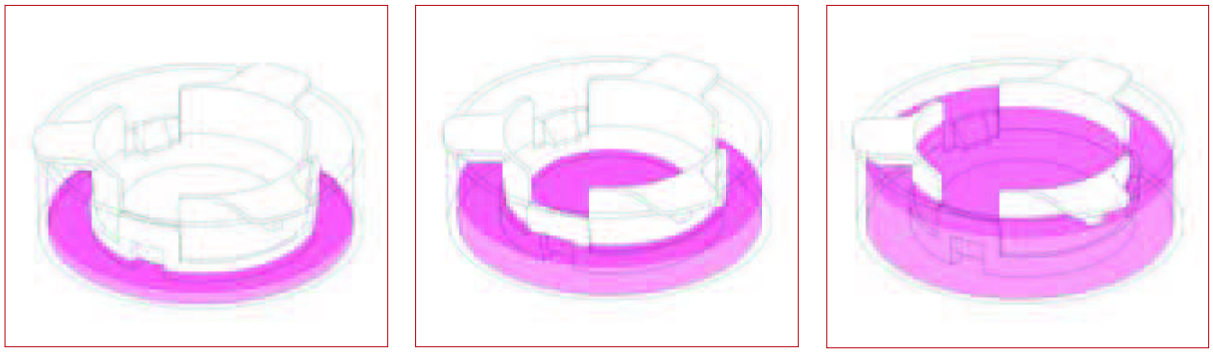
- Aspirate and replace with final wash medium (use same type of medium as for cell seeding). The scaffold is now ready for cell seeding: aspirate medium just before application of cells. If preparation of cell suspension is delayed, incubate plate with medium at 37 ℃ with 5% $CO_2$ until further use.
- 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.

## 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 2):



| i. Media from below only: for cells grown in 3D at air-liquid interface | ii. Media from above and below: for routine 3D growth of cells with lower-average metabolic activity/ proliferation rate OR for experiments where cells are incubated with test substrate in top chamber only for permeability investigations. | iii. Media interconnected: for routine 3D growth of cells with high metabolic activity/ proliferation rate |
|---|---|---|

***Figure 2.*** *Media filling levels and well insert configurations.*

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### 6-well insert in 6-well plate:

- For most applications initial cell seeding densities of 0.5-2.0x$10^6$ 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% $CO_2$ 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 1 below.

### 12-well insert in 6-well plate:

- For most applications initial cell seeding densities of 0.25-1.0x$10^6$ 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% $CO_2$ 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 1 below.

### 12-well insert in 12-well plate:

- For most applications initial cell seeding densities of 0.25-1.0x$10^6$ 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% $CO_2$ 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 1.

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| 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 1.*** *Feeding volumes for the different well insert configurations (i-iii, see also Figure 2.)*

- 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.