AXIS™: *AX*on *I*nvestigation *S*ystem-Plasma Bonded Tissue Culture Dish

For Catalog Numbers: AX15005PBC, AX45005PBC, and AX50005PBC

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Intended Use

Millipore's AXon Investigation System (AXIS™) is an advanced platform designed specifically for neuronal cell culture. Plasma Bonded AXIS products are AXIS devices that have been permanently sealed to microscope slides using plasma gas exposure. This format essentially eliminates issues with leakage or cell penetration under the device and should facilitate researchers with confocal imaging. **This kit is for research purposes only**.

Introduction

Millipore now offers its most advanced tool for the isolation, visualization, and characterization of neurite outgrowth, the AXon Investigation System (AXIS). AXIS is a unique, easy to use, slide-mounted microfluidic platform that allows culture, regulation, and directional differentiation of neuronal cells via a system of growth chambers and interconnected channels. AXIS devices physically isolate developing neurites from each other and their respective neural cell bodies (Figure 1). In addition, the use of hydrostatic pressure by filling the chambers with different volumes during analysis provides researchers with an opportunity for targeted exposure of neuronal parts to growth factors, drug compounds, or other reagents of biological interest. This ability to selectively control exposure of neuronal outgrowth makes AXIS a powerful platform for any researcher interested in understanding axons, dendrites, somas, and their roles in synaptic formation, neural cell development, differentiation, regeneration, degeneration, and trauma.

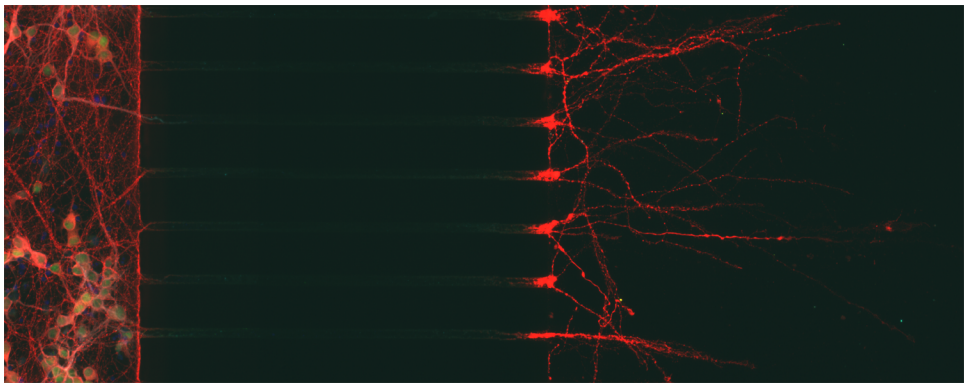


Figure 1: E18 rat hippocampal primary cells were loaded and cultured for six days in an AXIS Plasma Bonded device (AX50005PBC). Cells were fixed and staining was performed using NS420 and DAPI. NS420 is a proprietary blend of antibodies that shows staining of specific neuronal sub-architecture. The axonal marker is depicted in red and shows numerous prominent extensions of axons through the microgrooves into the channel on the right. Whereas the nuclear (blue), neuronal nuclear (green), and dendritic (cyan) staining is all contained in the channel on the left where the cells were loaded.

Plasma Bonded AXIS products are AXIS devices that have been permanently sealed to glass bottom tissue culture dishes using plasma gas exposure. Plasma Bonded AXIS devices are offered in three different formats to provide test flexibility during investigation (Figure 2). The first two formats (AX1505PBC and AX4505PBC) each have the same basic design consisting of four wells, two channels, and a set of microgrooves. Two of the wells are interconnected by a channel on each side of the device and constitute a chamber. The microgrooves are located in the area between the two channels and their length varies for each of the device formats; 150 µm for AX1505PBC, and 450 µm for AX4505PBC (Figure 3). Each device has approximately 120 microgrooves which are extremely small in size (approximately 5 µm in height by 10 µm in width). These microgroove dimensions were selected because they prevent cell bodies from flowing through while allowing neurite passage. The third AXIS device format that is offered contains 6 wells, 3 channels, and two separate sets of microgrooves (AX5005PBC). The AX5005PBC is similar to the previous ones except a 500 µm wide

channel has been added in the middle and lies between the two sets of microgrooves which are each 500 µm in length (Figure 2).

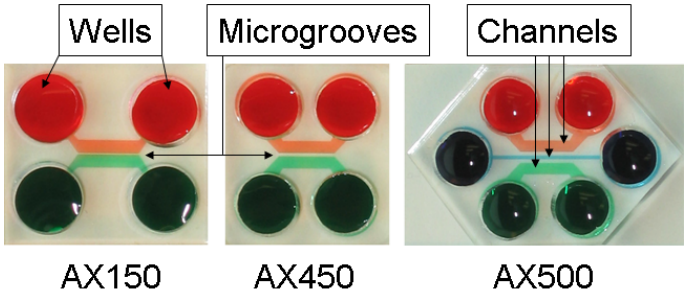
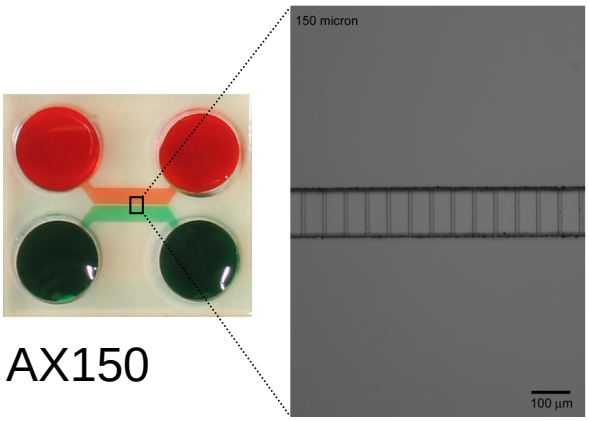


Figure 2: Pictures of the three different AXIS plasma bonded devices are shown. Examples of the three main features of these devices (wells, microgrooves, and channels) are identified. Please note that a chamber is composed of two wells with an interconnected channel and are depicted with red, green or blue coloring.

AXIS devices are fabricated from a polymer (PDMS) that is inert, non-toxic and optically clear[6,7]. This allows cells cultured in them to a) be propagated within the devices for long periods of time (2-3 weeks routinely) and b) get immunostained and imaged with high resolution microscopy, including live cell imaging, fluorescence microscopy, confocal microscopy, and differential interference microscopy[1-5] without requiring transfer or special handling.

AXIS devices were designed to take advantage of the microfluidic flow of liquids. One benefit of this is the ability to use AXIS devices to fluidically isolate a drug or chemical to a single chamber. This is accomplished when the volume in one chamber is kept greater than that in the other causing a flow of liquid from high to low. The hydrostatic pressure caused by the flow isolates the fluid on the low side. Since the fluid in the low volume chamber will not flow against the pressure coming from the high volume chamber, it remains almost exclusively on that side.[2] Thus, a compound of interest can be exposed to specific cellular structures provided a hydrostatic gradient is established and maintained. A second benefit of the microfluidic design of AXIS devices is their ability to create chemical gradients in one of two different ways. The first gradient can be established by simple passive diffusion if equal volumes in the chambers are used yet only 1 side contains the compound of interest. A second type of gradient can be formed if the compound of interest is added to a chamber with a higher total volume. Over time, it will diffuse across the microgrooves and a gradient from high concentration to low will form. The options for testing using AXIS are extensive and should be carefully considered prior to starting any experimentation.



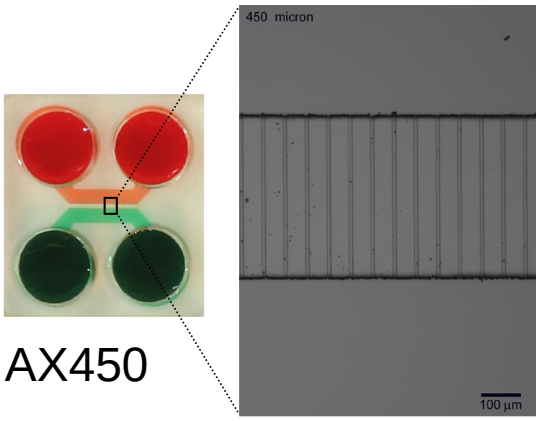


Figure 3: A higher magnification image of a portion of the microgroove area for two AXIS plasma bonded devices is shown. On the left is the AX150 device which has microgrooves that are 150µm in length. On the right is the AX450 device and its microgrooves are 450µm in length.

For additional technical publication go to **Error! Hyperlink reference not valid.**http://www.millipore.com and search for PB2876EN00 and PR3682EN00

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Kit Components (Note: each kit is sold separately)

AX15005PBC- AXIS™ Axon Isolation Device, 150µm, plasma bonded to tissue culture dishes.Contains 5 AX150 devices plasma bonded to glass bottom tissue culture dishes.

AX45005PBC- AXIS™ Axon Isolation Device, 450µm, plasma bonded to tissue culture dishes. Contains 5 AX450 devices plasma bonded to glass bottom tissue culture dishes.

AX50005PBC- AXIS™ Axon Isolation Device, 500µm, plasma bonded to tissue culture dishes Contains 5 AX500 (6-well) devices plasma bonded to glass bottom tissue culture dishes.

Materials Not Supplied

1. Tissue culture instruments/supplies (37ºC incubator with CO_2, laminar flow/tissue culture hood, growth media, flasks, etc.)
2. Sterile 1X PBS (Millipore Cat No BSS-1005-B)
3. Sterile Milli-Q™ water (Millipore Cat No TMS-006-B)
4. Human Collagen Type II (Millipore Cat No CC052), Laminin (Millipore Cat No CC095), Poly-D-Lysine(Millipore Cat No A-003-E), or any other cell attachment matrix
5. 70% ethanol
6. 4% paraformaldehyde
7. Sterile plastic petri dishes (100 X 15 mm or larger)
8. Light Diagnostic Mounting Media (Millipore Cat No 5013)
9. DAPI (Millipore Cat No S7113)

Storage

The AXIS plasma bonded devices should be stored at room temperature in a clean dry area away from any chemicals or direct light. When stored properly the devices are stable for 6 months from date of receipt. Discard any remaining reagents after the expiration date.

Assay Instructions

Listed below are detailed instructions about how to prepare the AXIS plasma bonded devices for use, maintaining neuronal cell cultures within them, and processing of them once an experiment has been completed. While these protocols have been extensively tested and validated to work with many cell types and culture conditions, some optimization of testing may be necessary as not all cells have the same characteristics.

Please note that the AXIS plasma bonded devices provided are clean but not sterile. They must be sterilized prior to culturing cells in them to prevent any bacterial or fungal contamination. Detailed protocols are given below for preparation of the devices, adding the cells, and subsequently culturing them during testing.

Cell Preparation

AXIS devices have been functionally validated with numerous different cell types[1-5]. Given the diversity of culture conditions and cell types it has been successfully tested with, it would be expected that most any neuronal cell type that can be grown in culture would be compatible for use with AXIS.

Furthermore, the offering of different microgroove formats should enable testing of any neurite producing cell.

The media and cell culture conditions standard for each specific cell type should also be employed during experimentation with AXIS plasma bonded devices. Cells to be utilized in testing can be prepared from actively growing cultures, frozen stocks, or freshly dissected tissues. Simply prepare the cells following standard protocols and then resuspend at a concentration of ~5 million cells per mL in their normal growth/differentiation media (higher or lower concentrations may be required depending upon the cell type utilized). Although a high concentration of cells is recommended the total number needed per device is only about twenty five thousand cells (5 µL of the cell suspension). The timing of when to begin preparing the cells is dependent upon how long the protocol for resuspension is and how long they can be incubated without side affects prior to use. Therefore, cells can be prepared prior to starting the standard protocol, during the two hour cell matrix coating at step 17, or once the device is nearly ready for use at step 22.

Standard Protocol for Plasma Bonded AXIS Devices

All steps detailed below should be performed within a laminar flow hood using aseptic technique and sterile reagents.

1. Remove from the plastic bag the number of tissue culture devices containing plasma bonded AXIS devices necessary for testing and place them in the hood.
2. Please note the orientation of the devices and their well designations as depicted in Figure 4 below. All subsequent instructions will refer to these descriptions and care should be taken to follow them precisely.

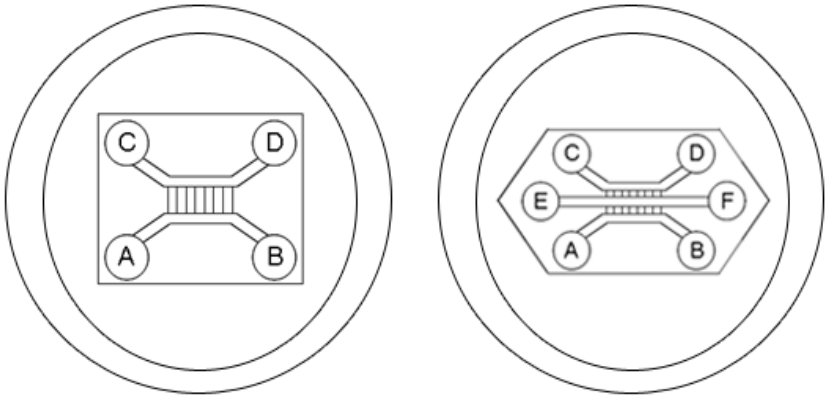

Figure 4: Models of AX15005PBC and AX45005PBC (left), and AX50005PBC (right) with designated well nomenclature. Please refer to these well designations for all protocol instructions.

3. For all devices being tested, add 200 µL of 70% ethanol to well A for AX15005PBC and AX45005PBC or to well E if using AX50005PBC. The ethanol should immediately flow into and down the channel attached to the well. If it does not, pipet some of the ethanol up and down at the base of the opening of the channel to force fluid flow into it.
4. Add 200 µL of 70% ethanol to well B (for AX15005PBC and AX45005PBC) or to well F (AX50005PBC).

5. Wait for approximately 1-2 minutes to allow the ethanol to flow through the microgrooves. The flow of ethanol can be visually checked by looking at the slides using an inverted microscope.

6. Once the ethanol has flowed through the microgrooves, add 200 µL of 70% ethanol to well C (for AX15005PBC or AX45005PBC) or wells A and C (for AX50005PBC). The ethanol should immediately flow into and down the channel attached to the well. If it does not, pipet some of the ethanol up and down at the base of the opening of the channel to force fluid flow into it

7. Add 200 µL of 70% ethanol to all remaining wells.

8. Spray top of Axis device and inside of tissue culture dish with 70% ethanol. The surface should be completely coated with ethanol solution.

Note: *Do not skip steps 3-7 as air bubbles may get trapped in the microgrooves possibly affecting results.*

9. Incubate at room temperature for 5 minutes.

10. Aspirate the ethanol from the top of the Axis device and from the tissue culture dish using a vacuum or pipet. Then aspirate the ethanol from the wells, but do not remove it from the channels. Removal of liquid from the channels may lead to the formation of air bubbles in the channels or microgrooves which could affect results.

11. Add 200 µL of sterile Milli-Q water to well A (for AX15005PBC and AX45005PBC) or to well E (for AX50005PBC) for all devices being tested.

12. Add 200 µL of sterile Milli-Q water to all remaining wells.

13. Incubate at room temperature for 1 minute.

14. Aspirate the water from all the wells, but do not remove it from the channels.

15. Add 200 µL of cell attachment matrix solution (poly-D-lysine, laminin, collagen, poly-L-ornithine, polyethyleneimine, etc.) to wells A and B (for AX15005PBC and AX45005PBC) or to wells E and F (for AX50005PBC).

16. Add 100 µL of the cell attachment matrix solution to all remaining wells.

17. Incubate for 2 hours at 37℃ or overnight at 4° C. Store at 4° for up to 2 weeks prior to use.

18. Aspirate the coating solution from all wells but do not remove it from the channels.

19. Add 200 µL of sterile Milli-Q water to all wells.

20. Aspirate the water from all wells, but do not remove it from the channels.

21. Add 200 µL of sterile cell culture media to all wells.

22. Incubate AXIS plasma bonded devices with media until the cells are ready for loading. See cell preparation section above for more information.

23. Aspirate media from all wells, but do not remove it from the channels.

24. Load 5 µL of the cell suspension (~5 million cells per mL) directly into the opening of the channel in a well. Pipet up and down 3 or 4 times to help induce flow of the cells into the channel. It may help to tilt the slide at an angle to aid loading. For the AX15005PBC or AX45005PBC devices cells are typically loaded into the channel opening in well A. For AX50005PBC the cells can be loaded in the middle channel between wells E and F or they can be loaded in both channels between A/B and

C/D. If necessary, 5 µL of the cell suspension can be loaded into the opposite opening of a channel to facilitate even loading.

25. Replace the lid on the tissue culture device and observe the flow of cells into the channel using an inverted microscope. Depending upon how fast you check it after loading the cells they should either be flowing through the channel or starting to settle down in the channel. If the cells are not in the channel check to verify cells were actually loaded or are in the well but did not enter the channel. If the cells did not enter the channel pipet up and down at the opening of the channel to induce flow into it.

26. Place the tissue culture dish with cells loaded into the Axis device into a tissue culture incubator for ~15 to 30 minutes to allow cell attachment to the coated glass surface. Make sure the device is lying flat during this time to limit fluid flow.

27. After the incubation check the cells again using a light microscope. At this point the cells should be attached down on the glass in the channel. If the number of cells present is acceptable, proceed to the next step. If the cell number is low, repeat the cell loading steps using 5 µL of additional cell suspension. If there are too many cells loaded, reduce the number of cells per mL in future experiments.

***Note**: The optimal amount of cells is user defined and is dependent upon variables such as cell type, viability, media, extent of differentiation, etc.*

28. In rapid succession add 50 µL of media to the two wells on either side of the channel with cells (for AX15005PBC and AX45005PBC it would be wells A and B). Repeat this step until both wells have a total of 200 µL of media each.

***Note**: Adding media to both wells in tandem will limit the amount of liquid flow through the channel, thus minimizing cell displacement. Some media flow in the channel may be observed due to the microfluidic balancing of the volume between wells.*

29. Add 200 µL of media to all other wells in the device. If fluidic isolation experiments are being performed, add 100 µL of media to “low” volume wells and 200 µL to “high” volume wells.

30. Culture the AXIS plasma bonded devices in a tissue culture incubator at the appropriate temperature to promote cell growth and/or differentiation.

The cells and the media in the AXIS device should be monitored daily. The extent of cell differentiation and axonal extension into the microgrooves should be tracked microscopically. The volume and condition of the media must be observed and periodically changed. For most cell types, it is ideal to remove and replace half of the media every three to four days. However, the frequency of media changes is dependent upon both the cell type and the tissue culture incubator, and it will need to be determined empirically. Ideally, the number of media changes should be kept to a minimum to limit cell disruption. Media volumes should remain fairly constant but need to be monitored as excess evaporation can cause increases in salt concentration impacting cell health.

Researchers will have several options regarding cell culture conditions during testing with AXIS devices. Cells can be cultured from beginning to end in differentiation media. Alternatively, cells can be loaded into AXIS devices with growth media, which is subsequently changed to differentiation media once the optimal cell density is obtained. Furthermore, chemotaxis agents can also be added and they can be uniformly distributed or microfluidically isolated on either the axonal or cellular side of the device. All the various cell culture options will have to be considered and decided upon by the researcher prior to initiating testing.

Microscopy

The ability to visualize and capture high resolution confocal images of neurons that have differentiated through the microgrooves of the AXIS device is of great importance and is easily attainable using this system. Because the AXIS device is optically transparent, it is possible to monitor and capture cell differentiation in real time using live cell imaging. Furthermore, it is possible to carryout all steps of the immunostaining protocol with the device still in place. A validated protocol is provided below, but some optimization of the procedure may be necessary depending upon the cell type and the antibodies being utilized.

1. Carefully aspirate the media from the wells, but do not remove it from the channels.
2. Wash the cells by adding 200 µL of 1X PBS.
3. Incubate for 5 minutes at room temperature.
4. Carefully aspirate the 1X PBS from the wells, but do not remove it from the channels. Repeat the 1X PBS wash two additional times.
5. Add 200 µL of 4% paraformaldehyde to wells A and B of the AXIS device (for AX15005PBC and AX45005PBC) or into wells E and F (for AX50005PBC).
6. Add 100 µL of 4% paraformaldehyde to all other wells.

***Note**: These unequal volumes will induce flow through the micro-grooves aiding testing. Addition of these exact volumes into the respective wells should be maintained for all fluid additions in the subsequent steps unless noted otherwise. Unequal staining of axons in the microgroove may be observed due to reduced fluid flow.*

7. Incubate for 20-30 minutes at room temperature.
8. Wash with 1X PBS three times as detailed above.
9. Add 200 µL of blocking buffer (typically PBS with 1% BSA, 5% serum, and 0.2% Triton X-100) to wells A and B of the AXIS device (for AX15005PBC and AX45005PBC) or into wells E and F (for AX50005PBC).
10. Add 100 µL of blocking buffer to all other wells.
11. Incubate for 1 hour at room temperature.
12. Aspirate the blocking buffer from the wells, but do not remove it from the channels.
13. Add 200 µL of primary antibody diluted in blocking buffer to wells A and B of the AXIS device (for AX15005PBC and AX45005PBC) or into wells E and F (for AX50005PBC).
14. Add 100 µL of primary antibody diluted in blocking buffer to all other wells.
15. Incubate at room temperature for 2 hours or overnight at 4℃.
16. Wash with 200 µL of 1X PBS four times.
17. Add secondary antibody diluted in blocking buffer and incubate at room temperature for 2 hours or overnight at 4℃.
18. Wash with 200 µL of 1X PBS four times.
19. *Optional Step:* If desired add DAPI solution and incubate for 5-10 minutes.
20. Wash with 1X PBS two times as described in step 4, and fill all four wells with 200 µL of 1X PBS.

For best results the immunostained cells should be imaged as soon as possible. If necessary they can be stored temporarily at 4℃ for a short period of time (~1 week) prior to analysis. The plasma bonded AXIS device cannot be easily removed from the glass but because it is transparent microscopic imaging can still be done.

References

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** The AXIS product line is covered by US Patent 7,419,822. *

Cat #s: AX15005PBC, AX45005PBC, and AX90005PBC

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