MILLIPORE

Protocol Note

Literature no: **PC1621EN00**

Title: **Millicell®-24 Cell Culture Plate: Fixation and Immunostaining Guidelines**

Date: **February 2006**

Introduction

MilliCell-24 Cell Culture Plate is a 24-well device designed to support cell attachment, growth and differentiation for many cell-based applications. The Millicell-24 is designed such that all fixation and immunostaining procedures can be carried out in a single device either manually or using automation. MilliCell® 24 is available with 0.4 µm PCF membrane or 1.0 µm PET membrane, which allows both live and fixed cells to be visualized by phase contrast or fluorescent methods.

Cell Fixation and Staining

The majority of immunohistochemical procedures employ a fixation step prior to incubation with primary antibody. Fixation is required to stabilize sub-cellular morphology and prevent degradation of antigens during subsequent staining procedures. Typically, cell preparations are submerged in a fixative solution (such as cold 90% methanol or 3.7% formaldehyde, etc.) or can be simply air-dried.

Procedure:

(Note: Reagents and buffers should be carefully pipetted down the side of the well in order to prevent disturbing cells adhered to the membrane)

1.) Aspirate all media from inside filter plate wells and from receiver wells (24 or single). Fill filter plate wells with 1 ml of washing buffer (typically PBS or HBSS). It is important to also fill the receiver wells (1 ml or 32 ml for 24 or single well, respectively) with wash buffer to properly wash the underside of the membrane.

2.) Incubate at room temperature for about 5 minutes and repeat wash two more times. Do not allow to dry

3.) Add 200ul of fixative solution to the inside of each well. It is not required to treat the underside of the membrane with fixative. Incubate according to protocol instructions. Generally treatment is for approximately 5 minutes. During this time, the solution should remain in the well and not leak through the membrane.

4.) After treatment, aspirate fixative and fill filter wells with 1 ml washing buffer. Repeat steps 1 and 2 in order to fully remove the fixative solution from both sides of the filter membrane. Do not allow cells to dry.

5.) Dilute primary antibody according to vendor recommendations. In order to obtain best results, it is recommended that optimal working dilutions be determined by the user. If permeabilization is required (such as for cytoplasmic or nuclear antigens), saponin can be added to the solution at a concentration of 0.1%.

6.) Add 100ul of antibody solution to each well, incubate at recommended temperature (typically room temperature or 4ºC) with mild shaking or rocking to assure that solution wets out entire filter surface. If antibody is fluorescently labeled (direct labeling), cover plate with foil to protect from light.

7.) Aspirate antibody solution and wash both sides of membrane as indicated in steps 1 and 2 to remove all unbound antibody

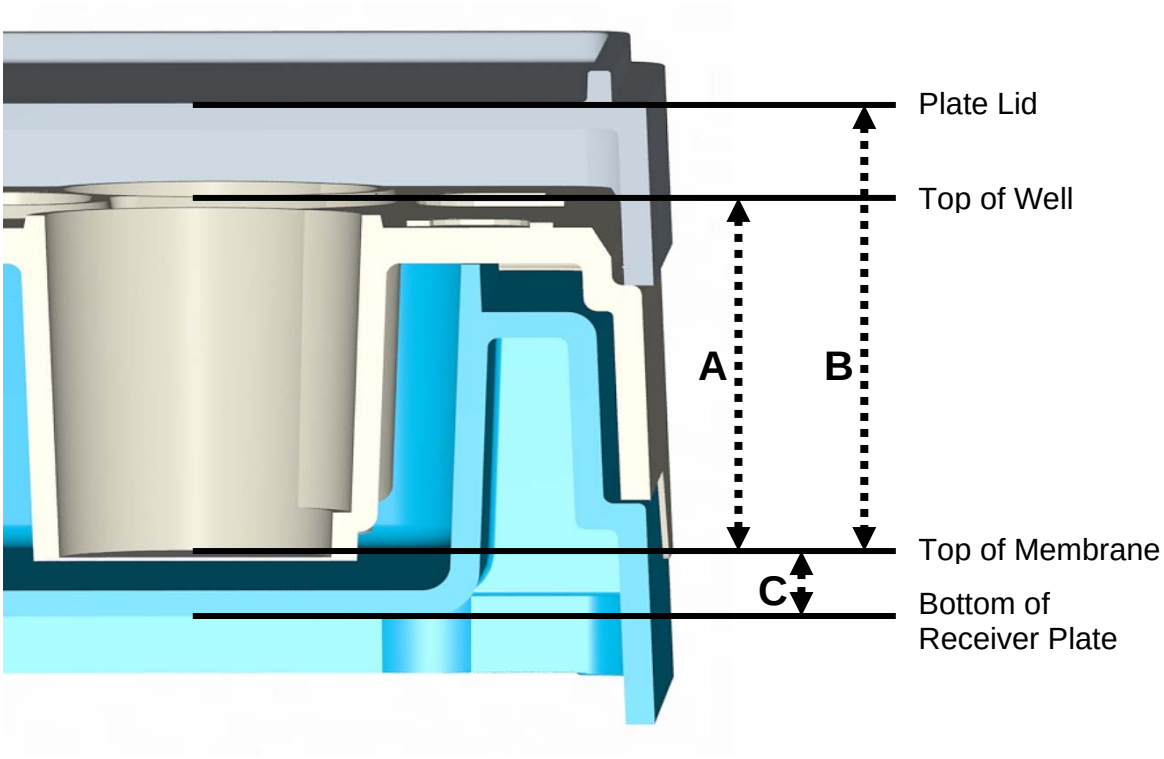
8.) If performing indirect labeling with a secondary antibody, repeat steps 5 through 7 with secondary antibody. For fluorescent antibodies continue to microscopy procedure. For enzyme linked assays (HRP, etc.), follow vendor procedures for developing using 100 ul per well in each step.

Microscopy Procedure

Microscopic examination of samples can be performed in two modes: directly in the plate under low magnification, or on microscope slides for higher magnification using removed membrane.

In-Plate Visualization Modes (lower magnifications)

Add 50 ul of mounting fluid to each sample well. If using fluorescence, it is recommended to use a mounting fluid that contains an anti-fade additive to prevent photobleaching



A = 13.6mm, B = 18.0mm, C = 2.2mm

Viewing from below the plate (through PET membrane):

The Millicell-24 device has been designed to allow visualization of cells from below using an inverted microscope. In order to focus on the cells with the receiver plate in place, a working microscope objective (typically 5-20X) must have a working distance (see below for finding objective specifications) of at least 2.2 mm to span from the bottom of the receiver plate to the top of the membrane (see C in diagram above). Fixed cells that do not require to be visualized through media can be viewed directly without the receiver plate but care should be taken not to contaminate the objective by liquid residue (media, mounting fluid) on membrane.

Viewing from above the plate (PET or PCF membrane):

Cells can be viewed in a conventional microscope directly from above. Cells can be visualized through the lid or with the lid removed. Working distances of the objective must be longer when reading from above compare to from below. Typically 5-10X objectives are used that

have at least a (A) 13.6 mm or a (B) 18.0 mm working distance when viewing without or with the lid, respectively.

Visualizing Membranes on Microscope Slides (for higher magnification or with objectives with short working distances)

The membrane can be removed from each well for microscopic evaluation. This allows for higher magnification examination and storage of the slides for future use.

Removal of membrane and mounting:

1.) With a sharp scalpel, make a small incision in the edge of the membrane and carefully cut along the well side approximately one quarter of the diameters of the well. Using forceps (Millipore cat# XX62 000 06), carefully hold the membrane while continuing to cut around well diameter to remove membrane. Use care to prevent membrane from curling.

2.) Place membrane disk, cells facing up, onto a microscope slide. Add 50ul mounting fluid to the membrane disk and allow to wet out in order to prevent bubbles under the disk. Slowly lower a cover slip at an angle to allow air bubbles to be removed.

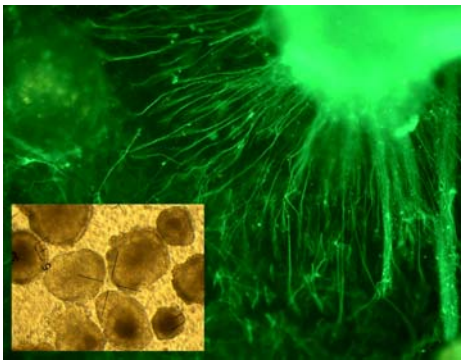
Microscope Objective Information

Information regarding microscope objective magnification power and working distances can be obtained from individual optical dealers or from the microscope vendors below

Nikon Instruments: http://www.nikon-instruments.jp

Olympus Corporation: http://www.olympusamerica.com

Carl Zeiss: http://www.zeiss.com



Neuron differentiation of embryonic stem cells in Millicell 24 1um PET filter plates. Murine embryonic stem cells were formed into suspended embryoid bodies (EBs) then transferred to 1um PET Millicell-24 Cell Culture Plate for attachment and differentiation (insert – inverted phase contrast through membrane of live EBs in media). Neural differentiation after retinoic acid treatment of attached EBs was confirmed by anti-neurofilament immunofluoresence.

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