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# A simple filtration device for separating nonadherent microorganisms from mammalian cells in in vitro studies on microbial adherence

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**Abstract** In adherence studies, the removal of nonadherent microorganisms is essential for the valid enumeration of microorganisms that adhere to host cells. Although filtration devices are available commercially for the removal of nonadherent microorganisms, these are expensive and not reusable. In this article, we describe a simple, inexpensive, and reusable filtration device composed of two chambers of nylon, a nylon membrane of desired pore size, a rubber washer, and supporting stainless steel mesh. The device was effective in in vitro adherence assays for removing nonadherent endospores of *Rhinosporidium seeberi* from human buccal epithelial cells, providing valid counts of adherent microorganisms.

**Key words** Adherence assays in vitro · Filtration device · *Rhinosporidium seeberi*

Assay of the capability of microorganisms to adhere to host cells is a common method for investigating the initial events in the pathogenesis of disease caused by microorganisms (Ellen and Gibbons 1974; Beachey 1981; Ray et al. 1984). The validity of the enumeration of adherent microorganisms in in vitro adherence assays depends on the absence of nonadherent microorganisms in the field under observation. If nonadherent microorganisms are not removed, the count could be inflated by counting microorganisms that are merely deposited on the target cell surface but are not truly adherent.

In vitro mixtures of host cells and the microorganism under study have most commonly been filtered through commercially available plastic or stainless steel devices that generally use polycarbonate filter pads of appropriate pore size (Kimura and Pearsall 1978), and the suspending fluid is extracted by suction. Although commercially available devices such as multiple manifold filters are effective for the removal of nonadherent microorganisms, these are expensive and require an aspirator for use. A simpler method is repeated differential centrifugation of the cell–microorganism mixture at low speed; e.g., 75 *g* (S.N. Arseculeratne, personal observation). By centrifugation at low speed, nonadherent microorganisms are retained in the supernatant, while the target cells with adherent microorganisms are spun down as the sediment. The disadvantage of the centrifugation method is that it is tedious, with the loss of target cells, especially in small volumes of cell suspensions, through pipetting for the removal of the supernatant containing the nonadherent microorganisms. Thus, the development of a simpler and less expensive device has been necessary. This article describes a simple, inexpensive, easily crafted, two-chambered filter that could be used with a nylon filter membrane of appropriate pore size in adherence assays.

The filtration device is composed of upper and lower chambers, a stainless steel mesh, nylon filter membrane, and a flat rubber washer (Figs. 1, 2). The upper and lower chambers of the filter (outer diameter, approximately 20 mm) (Figs. 1, 2) were lathed from a nylon rod. The two sections fit sufficiently tightly into each other, but allow dismantling by hand to retrieve the filter membrane. A mark on each chamber is made for alignment. A disk (diameter, 17 mm) of stainless steel mesh (pore size, 35 μm; available commercially) to provide support for the nylon filter membrane is inserted into the lower chamber (Fig. 1). The membrane is placed on the mesh, followed by the thin flat rubber washer (diameter of inner hole, 13 mm; thickness, 0.5 mm). The pore size of the membrane could be selected depending on the sizes of the organism and the cell under study; e.g., 8- to 12-μm filtration membranes or pads are used for separating nonadherent *Candida albicans* (Sobel et al. 1981; Ray et al. 1984; Sandin 1987). The upper chamber is inserted into the lower chamber with the washer, membrane, and mesh, and the assembled device is tapped on a hard surface to ensure adequate seating of the upper chamber against the washer on the lower chamber to prevent leakage of cells. The height of the assembled apparatus is 26 mm. These dimensions permit a volume

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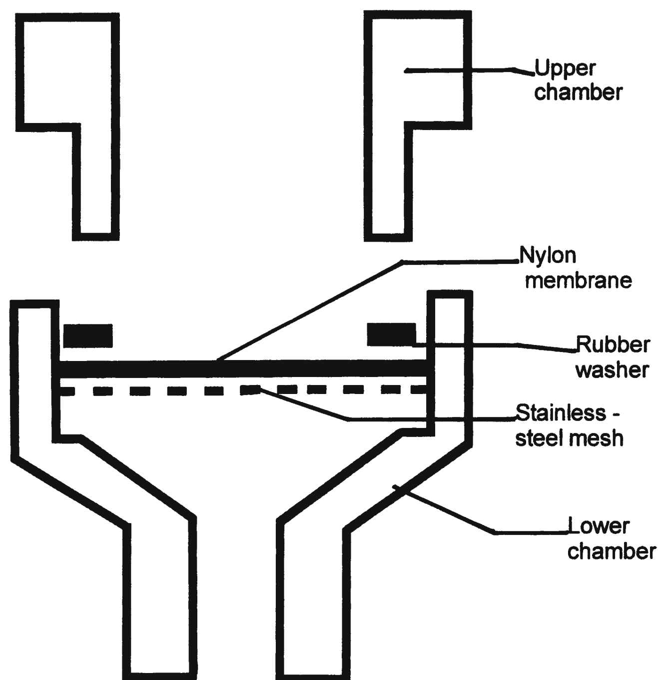


**Fig. 1.** Diagram of the filtration device (not to scale)

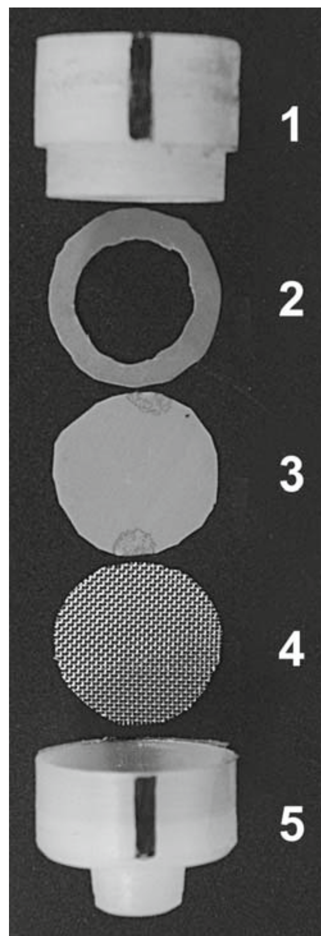


**Fig. 2.** Dismantled filtration device. *1* Upper chamber. *2* Rubber washer. *3* Filtration membrane. *4* Stainless steel mesh. *5* Lower chamber. Marks on *1* and *5* indicate alignment marks

of 300–500 μl of the cell–microorganism mixture to be filtered; these dimensions could be altered for the use of different volumes of reactants.

For successive use of the device, it should be washed sufficiently. After each filtration, the filter is dismantled and the components are washed in several changes of distilled water, with jets directed at the filter membrane and mesh. After use with viable organisms, to sterilize the filter, it is dismantled and immersed in 10% formalin in physiological saline, followed by rinses in water. To clean the filter membrane, its pores are examined microscopically at 100× for blockage after repeated use of the membrane. The filter membrane is cleaned with a jet of compressed air or by brief exposure to ultrasound.

For this article, we evaluated the efficiency of the device by using endospores of *Rhinosporidium seeberi* (Wernicke) Seeber and human buccal epithelial cells (BEC). *Rhinosporidium seeberi* causes rhinosporidiosis in humans and animals. This disease has been found in 70 countries, especially in Sri Lanka and India (Arseculeratne 2002); in order of frequency of incidence, the upper respiratory tract, eye, and urethra are the principal sites with rare dissemination and fatality. The human BEC is a widely used target cell for adherence assays with microorganisms, especially *Candida* spp. Endospores from a patient with nasal rhinosporidiosis and BEC were incubated with gentle agitation at 37°C for 1–4 h as described by Ray et al. (1984) and Sandin (1987).

To standardize the method, the minimum number of rinses required to eliminate the nonadherent microorganisms, and the effect of the number of rinses on the endospore : cell adherence ratios, were examined. This step was performed because, although usually three rinses are arbitrarily used (S. Jayatilleke, personal communication), there are no available data for the number of rinses that are necessary for the total removal of nonadherent (free) microorganisms, even with commercially available, manifold filtration devices. In our experiments, a membrane of pore size 25 μm was selected in view of the endospore diameter of approximately 15 μm and the approximate diameter of 50 × 70 μm of a BEC; the volumes of the cell–endospore mixture were 300 μl cells ($7–10 \times 10^5$/ml) in 0.1 M phosphate buffered saline (PBS, pH 7.4), 50 μl endospore suspension in PBS ($1–2 \times 10^6$ endospores/ml), i.e., an endospore : cell ratio of approximately 1.2 : 5.2. The mixtures were incubated at 37°C for 2–3 h, with gentle intermittent agitation.

Rinses with squirts of 1 ml PBS, through a Pasteur pipette, were used to flush out and remove nonadherent endospores. We tapped the device to allow the suspending fluid containing nonadherent endospores to escape easily through the filter without the use of suction. After rinses with PBS, the filter was dismantled, and the nylon membrane was placed face down on a microscope slide. Two smears were made successively, of decreasing cell density, for selection of an appropriate density for easy counting; the second smear was generally preferable. The smears were stained with polychrome methylene blue for microscopy, which easily differentiated the BEC (stained

pale blue) from the endospores (purple-blue). The final assessment of adherence was based on the counts after the final rinse.

As a result, the endospore : cell ratios were not significantly altered by the successive rinses. In triplicate experiments, the original (pre-rinse) ratios were 1.12, 1.10, and 0.80, and these were 1.00, 1.00, and 0.95 after three, five, and five rinses, respectively; the pooled values before and after the rinses showed no statistically significant change ($P = 0.463$; paired Student's *t* test), indicating that the rinsing procedure did not dislodge the endospores that showed true adherence. The initial count of apparently adherent endospores in the basal smear decreased slightly after the first rinse; this was probably caused by the removal of nonadherent endospores. The higher basal count before rinsing probably included free endospores that were passively lodged or nonspecifically (electrostatically) and reversibly attached on the BEC surface. The nonreduction of the adherent endospore : BEC ratios with successive rinses indicated that the adherence was probably a specific, irreversible event between the microbial adhesions and the cellular receptors (Beachey 1981; Sandin 1987).

The numbers of free (nonadherent) endospores per field in quadruplicate experiments (pooled) were reduced significantly ($P = 0.024$, paired Student's *t* test) from an original (pre-rinse) mean value of $0.98 \pm 0.41$ to a mean value of $0.08 \pm 0.03$; five rinses would therefore be sufficient to reduce the numbers of nonadherent endospores to a minimum or zero (in one experiment) with the volumes and concentrations of each reactant used in this study.

In conclusion, this filtration device was useful and practical in in vitro studies on microbial adherence. The device is inexpensive and easily crafted, and its membrane filter is reusable, in contrast to commercial polycarbonate filter pads, which are expensive and are discarded after a single use. In addition, our filtration device does not require suction to remove the suspending fluid containing nonadherent microorganisms. Our results indicate that five rinses are sufficient to reduce, significantly, the numbers of nonadherent endospores to a minimum or zero level.

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