Microbial evaluation of the Whitley A135 GMP workstation with *Bacillus atrophaeus* spore suspension

Charlotte Austin, Andrew Pridmore / Don Whitley Scientific Ltd / BD16 2NH, United Kingdom / Charlotte_Austin@dwscientific.co.uk

ABSTRACT

Particulate and microbial contamination of a product, and cross contamination between product batches, must be eliminated when performing manipulations in accordance with Good Manufacturing Practice (GMP). This is conventionally achieved using a pharmaceutical isolator or a Class II Microbiological Safety Cabinet (MSC). These allow an operator to perform manipulations within the workspace with low risk of particulate / microbial contamination but are limited to operation with an aerobic atmosphere. The manufacture of live biotherapeutic products has created a requirement for sterile manipulation of fastidiously anaerobic bacteria within an oxygen-free environment and has driven the development of the Whitley A135 GMP anaerobic workstation, which provides sterile laminar flow of an anaerobic atmosphere.

We validated the performance of a Whitley A135 GMP workstation with regard to protecting the workspace from spores of *Bacillus atrophaeus* ATCC 9372, using test methodology set out in BS EN 12469:2000. A suspension of *B. atrophaeus* spores was aerosolized within the workstation over a 5-minute period, using a 6 jet Collison nebulizer, to introduce > 2×10^7 spores into the chamber. Open 90 mm plates of Tryptone Soy Agar were placed in 8 positions over the workstation floor and were exposed to the circulating workstation atmosphere throughout the 5-minute nebulization period and for 1 minute afterwards. The experiment was conducted on 3 separate occasions; the number of spores nebulized always exceeded the density specified in BS EN 12469 by approximately tenfold.

Exposed agar plates were incubated at 37°C for 24h and inspected for bacterial colonies. No colonies were recovered from the workstation after 5 minutes of *B. atrophaeus* aerosolization. Control plates, exposed directly to the nebulizer outlet, yielded confluent growth of *B. atrophaeus* after 24h incubation. Thus, the A135 GMP workstation achieved rapid removal of *B. atrophaeus* spores to yield a sterile anaerobic atmosphere.

BACKGROUND

In recent years, the increase in the manufacturing of live biotherapeutic products under GMP regulations has stimulated development of the Whitley A135 GMP workstation. This novel device combines the sterility of unidirectional laminar flow with an oxygen free atmosphere and provides physical isolation with EU GMP Grade A air cleanliness. To verify maintenance of these conditions, we evaluated the removal of *Bacillus atrophaeus* ATCC 9372 spores from the internal work area, using methodology based on that set out in EN 12469:2000.

MATERIALS AND METHODS

A stock culture of *Bacillus atrophaeus* DSM 675 (ATCC 9382) was subcultured onto 15 plates of Campden Sporulating Agar[2], to encourage spore formation, and incubated for 24h at 37°C. Characteristic, orange pigmented colonies were removed from the agar using sterile spreaders, suspended in 100 ml of PBS and vortexed thoroughly to achieve a homogeneous suspension. This bacterial suspension was heat shocked at 65°C for 15 minutes in a water bath. The spore density of each suspension used in the investigation was determined by serial dilution and plating on Tryptone Soya Agar, which was incubated for 24h at 37°C. Spore counts determined in this way fell between 1.4×10^9 and 4.6×10^9 cfu per ml.

The Whitley A135 GMP workstation was used for all experiments. The workstation was tested on 3 separate occasions and was tested in Active mode (used when an operator is present in the workstation) and Eco mode (used when the workstation is not in use and the glove ports are closed). On each test day, the workstation fan was allowed to run for half an hour prior to the start of each experiment to ensure that stable operating conditions had been achieved.

Before the commencement of each test, *B. atrophaeus* spore suspension was diluted in 0.85% saline to achieve a theoretical density of 1.1×10^8 spores/ml, which exceeds the density of 5×10^6 to 8×10^6 spores/ml specified in EN 12469:2000. Diluted suspensions were enumerated (as described above) to confirm the spore density and were found to be in the range of 1.0×10^8 to 1.2×10^8 cfu per ml. In each test, 50 ml of diluted spore suspension was placed in a 6 jet Collison nebulizer supplied with compressed air at a pressure of 140 kPa. Each run consisted of the nebulizer being run for 5 minutes and ejected approximately 2 ml of aerosolized suspension (exact volume was determined by weighing the vessel before and after nebulization). On each occasion, 4 repeats of the test were carried out: 2 runs were performed with the nebulizer placed at the front of the cabinet facing the front grill (Active mode and Eco mode) and 2 runs were performed at the rear of the cabinet with the nebulizer facing the rear grill (Active mode and Eco mode). Throughout the 5-minute nebulization period, 8 TSA plates were positioned across the work area (floor tray) of the cabinet, as shown in shown in Figure 3, with lids removed to expose the agar surface to the downward atmospheric flow. During each run, 2 positive control samples were taken, after 2 minutes and 4 minutes of nebulization, by exposing an open agar plate to the nebulizer outlet for 10 seconds. After 5 minutes of nebulization, the nebulizer air supply was turned off and plates were left to stand for a further minute to allow complete circulation of atmosphere through the workstation's HEPA filter. Plate lids were then replaced. All plates were removed from the workstation and incubated at 37°C for 24h.

RESULTS AND DISCUSSION

For the three repeated experiments, the mean spore counts ejected into workstation during 5 minutes of nebulization were in the range 2.0×10^8 to 2.4×10^8 cfu. Recovery of *B. atrophaeus* colonies from the workstation interior (settle plates) is presented in Table 1 and Table 2. The consistent absence of colonies recovered after nebulization of the spore suspension indicates that the filter in the workstation was highly effective in the removal of spores from the atmosphere. Thus, we demonstrated that the A135 GMP workstation substantially eliminates bacterial and particulate contamination from the work area (as determined using *Bacillus spores*) and meets the requirements of EN 12469:2000. The workstation provides EU GMP Grade A cleanroom conditions appropriate for the manufacture and manipulation of live biotherapeutic products containing fastidious anaerobes, or other therapeutic products that require the absence of oxygen in their production.

REFERENCES

1. BS EN 12469:2000 Biotechnology – Performance criteria for microbiological safety cabinets. The British Standards Institution 92000.
2. Brown KL (1984) Spore studies in relation to heat processed food. Campden and Chorleywood Food Research Association, Chipping Campden, United Kingdom.

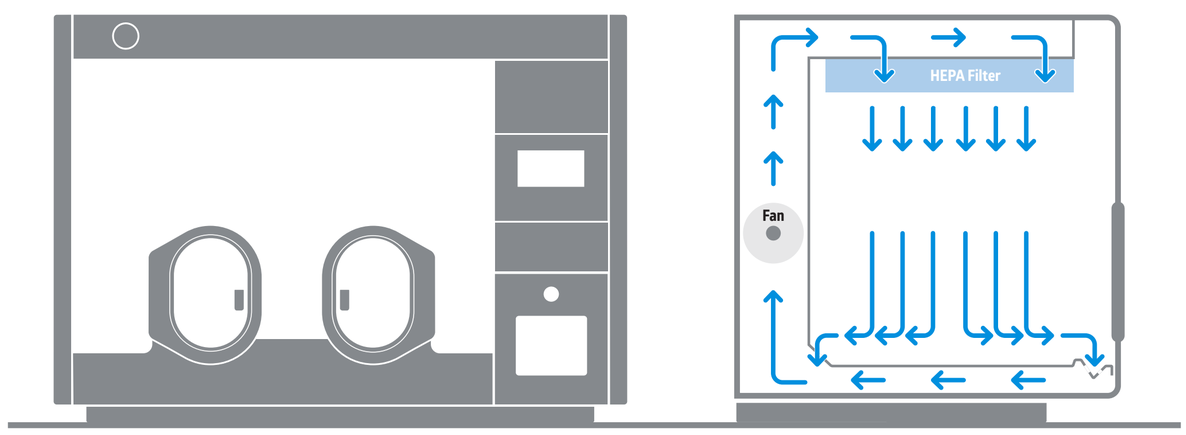

FIGURE 1: WHITLEY A135 GMP WORKSTATION

FIGURE 2: HEPA FILTRATION FLOW PATH

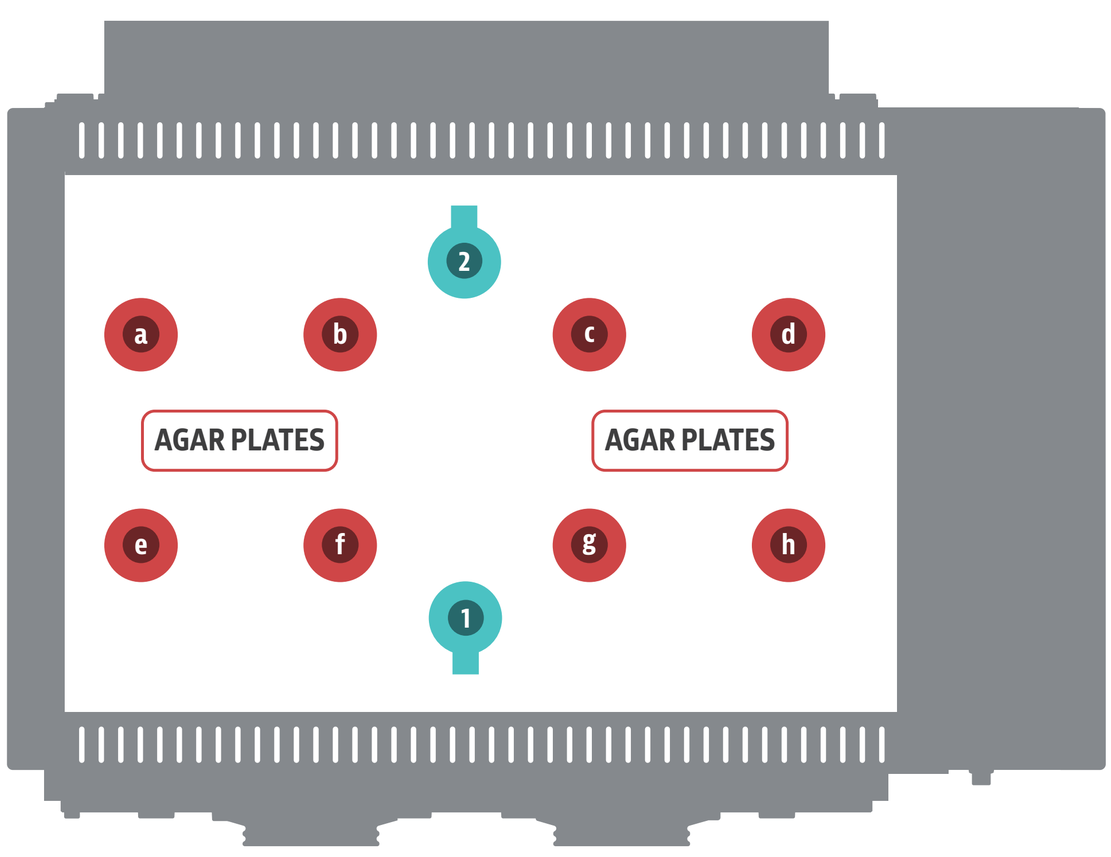

FIGURE 3: WHITLEY A135 GMP WORKSTATION (PLAN VIEW)

1. Nebulizer placed on the chamber floor facing the front of the cabinet ejecting spore suspension into the front grill
2. Nebulizer placed on the chamber floor facing the rear of the cabinet ejecting spore suspension into the rear grill

Table 1: Recovery of *B. atrophaeus* colonies on exposure plates after 24h incubation (workstation operation in Active mode)

Plate Identification	Run Number	
	1 (front)	2 (back)
a	0	0
b	0	0
c	0	0
d	0	0
e	0	0
f	0	0
g	0	0
h	0	0
Positive Control at 2 minutes	>300	>300
Positive Control at 4 minutes	>300	>300

Table 2: Recovery of *B. atrophaeus* colonies on exposure plates after 24h incubation (workstation operation in Eco mode)

Plate Identification	Run Number	
	3 (front)	4 (back)
a	0	0
b	0	0
c	0	0
d	0	0
e	0	0
f	0	0
g	0	0
h	0	0
Positive Control at 2 minutes	>300	>300
Positive Control at 4 minutes	>300	>300