

Development and evaluation of DNA amplicon quantification. Case study: UV cabinets for PCR operations UVC/T-M-AR and Class II Biological Safety cabinets

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Introduction

Personal and product safety during clinical and laboratory studies have stimulated the development of sterile cabinets and special laboratory safety techniques, to protect the environment, operator, and product. Monitoring DNA/RNA amplicon concentration in laboratory air in sterile cabinets has become topical as PCR and isothermal amplification technologies have developed along with wide spread mass analyses.

Development of methods for repeatable DNA/RNA amplicon detection in air samples is now a reality. Recent research "Behaviour of aerosol particles in fibrous structures" (Igor Agranovsky's PhD thesis, 2008, Novosibirsk, Russia) describes the development of samplers and monitoring of DNA/RNA amplicon concentration in the air from sterile cabinets, microbial quantitative analyses.

Aim of the study

The aim of this study is to evaluate the efficiency of UV cabinets produced by BioSan (Latvia) in comparison to Class II BioSafety cabinets.

UV air treatment

More than a century has passed since the germicidal effect of UV light was recognized by Niels Ryberg Finsen — a Nobel Prize winner in physiology or medicine in 1903 [5], and many researches have been performed on UV induced destruction of DNA and microorganisms.

Low pressure germicidal UV lamps characteristically emit monochromatic low intensity radiation principally at 253.7 nm, within the germicidal wavelength range as defined by the DNA absorbance spectrum. The germicidal UV dose LP-UV lamps is calculated as the product of the volume averaged incident irradiance (E, mW/cm²) and the time of exposure (t, seconds) resulting in units of mJ/cm² for UV dose [1] (Fig. 1).

Air flow organization through HEPA filter

HEPA is an acronym for "high efficiency particulate absorbing" or "high efficiency particulate arrestance" or, as officially defined by the Department of Energy (DOE) "high efficiency particulate air".

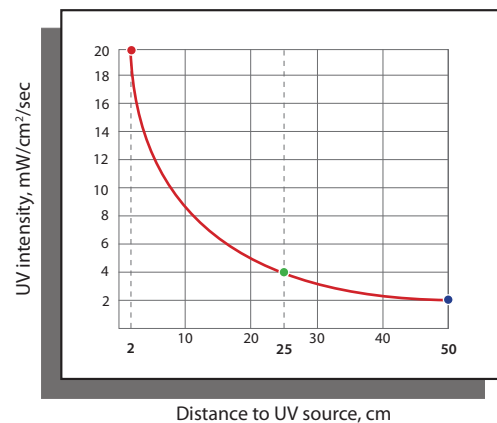
The first HEPA filters were developed in the 1940's by the USA Atomic Energy Commission to fulfil an efficient, effective way to filter radioactive particulate contaminants. HEPA filter technology was declassified after World War 2 and then allowed for commercial and residential use [6].

This type of air filter can theoretically remove at least 99.97% of dust, pollen, mold, bacteria and any airborne particles with a size of 0.3 μm at 85 litres per minute (l/min). In some cases, HEPA filters can even remove or reduce viral contamination. The diameter specification of 0.3 responds to the most penetrating particle size (MPPS). Particles that are smaller or larger are trapped with even higher efficiency [7] (Fig. 2).



UVC/T-M-AR,
UV cabinet for
PCR operations

Fig. 1, Germicidal, shortwave (254 nm) ultraviolet energy is used for complete destruction of various biological agents



UV intensity, mW/cm ² /sec	Distance, cm
20	2
4	25
2	50

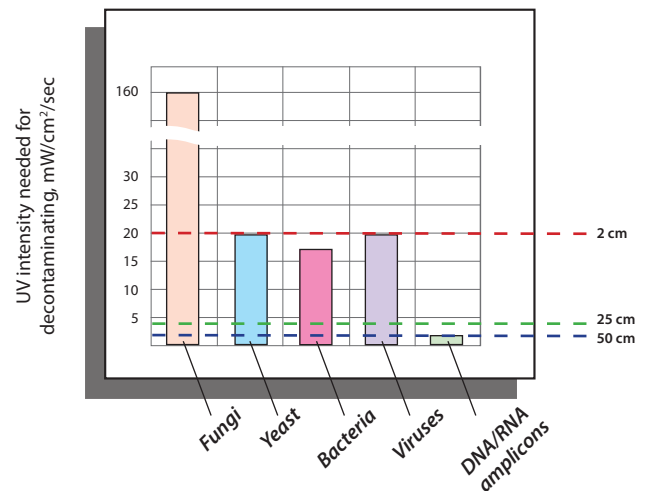
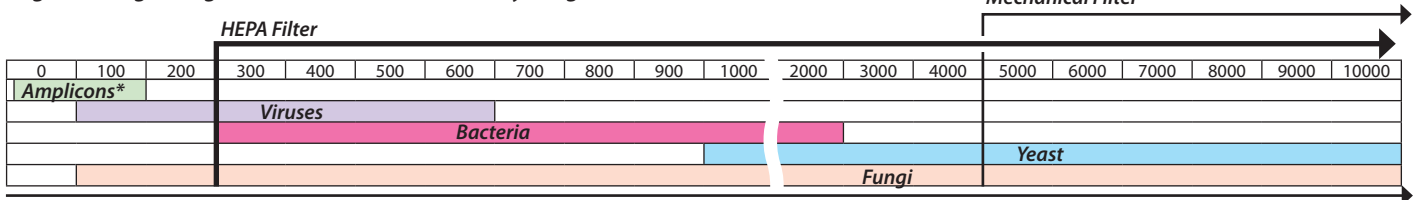


Fig. 2, Biological agent sizes and filters effectivity range, nm



* —DNA/RNA amplicons

Biological agent sizes, nm

Colony forming units (CFU) test

Media

LBA media was prepared using Standard Methods Agar (Tryptone Glucose Yeast Extract; Becton, Dickinson and Company) and dissolved in 1 litre of purified water. 7.5 grams of Yeast Extract (Biolife S.r.l.) and 5 grams of Tryptone (Difco laboratories) were added to enrich the media. The media was autoclaved at 121°C for 15 minutes. Media control samples were taken to check for presence/absence of colony forming units in media itself and the results were negative (0 CFU per 3 plates).

Experimental setup:

Impaction aerobiocollector airIDEAL 3P (bioMérieuxSA, France) was used to take air samples to test for the presence of colony forming units (CFU). Each sample was exposed to 500 litres of air. Aerobiocollector was set in the middle of the sterile cabinets for test samples and negative control samples, and in specific places in the middle of the laboratory room for positive control. The negative control was taken in Microflow ABS Cabinet Class II. This was repeated three times, the number of colony forming units was counted manually on each plate. Reading tables provided in airIDEAL 3P (bioMérieuxSA, France) The most probable number (MPN) of microorganisms collected per plate was estimated with respect to the number of agglomerates of colonies counted on the plate. (MPN was calculated from the CFU count using FELLER's law). Subsequently results were converted to CFU per m³.

Mechanical contamination test

Instrument:

Laser particle counter (produced by Met One, USA) was used to determine mechanical contamination in the sterile cabinets and laboratory air as positive control.

Method:

Average amount of particles per litre of air were measured in sterile cabinet/laboratory air. Measurements were performed 9 times and the average value presented in the results as number of particles per m³ of air.

Two channels were used to measure amount of particles of different size: 5 µm and 0.3 µm. Mechanical filter stops particles larger than 5 µm while HEPA filter larger than 0.3 µm.

DNA Amplicon test

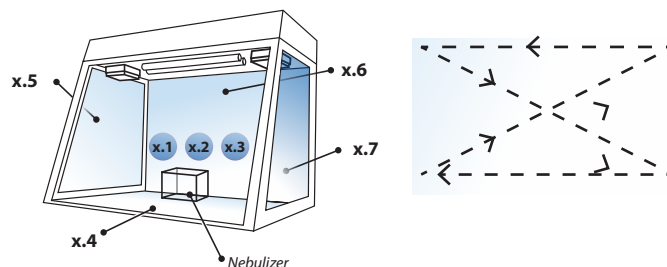
Instruments:

- Nebulizer, BioSan
- Shaker OS-20, BioSan
- Mini-Centrifuge/Vortex FV-2400, BioSan
- Centrifuge Pico 17, Thermo Electron Corp.
- Centrifuge-Vortex MSC 6000, BioSan
- Real-Time PCR cycler Rotor Gene 3000, Corbett Research

Reagents:

- Lambda DNA, Thermo Fisher Fermentas
- GeneJet Plasmid Miniprep Kit, Thermo Fisher Fermentas
- Real Time PCR reagents, Central Research Institute of Epidemiology.

Fig. 3, Air and surface samples and surface sample taking path



Samples taken from:

- x.1, x.2, x.3 : Air (Syringes)
- x.4 : Working surface (Smears)
- x.5, x.7 : Side walls (Smears)
- x.6 : Back wall (Smears)

Experiment setup:

- Sampling was performed as shown on Fig. 3
- Extraction and analyses were performed as shown on Fig. 4
- Quantitative PCR (Polymerase Chain Reaction):
DNA amplicon quantification in sterile cabinets was performed by qPCR. Controls and standards were set in each experiment:
 - » 4 standards of Lambda DNA of different concentration prepared in 10 fold dilution: starting concentration 0.6 ng/µl or ≈1000000 copies/µl
 - » 2 NTC (no template control- sterile H₂O), experiment was considered successful only if control was negative.

After samples were taken and extracted as mentioned above, qPCR reaction master mix was prepared by adding the following components for each 25 µl of reaction mix to a tube at room temperature:

PCR mix:

- 2-FL : 7 µl; dNTP's : 2.5 µl; Forward Primer : 1 µl;
- Reverse Primer : 1 µl; DNA probe : 1 µl; Template DNA : 10 µl;
- Water, nuclease-free to : 25 µl; Total volume : 25 µl

Table 1, Cycling protocol

Three-step cycling protocol Steps	Temperature, °C	Time	Number of cycles
Initial denaturation	95	5 min	1
Denaturation	95	5 sec	42
Annealing	60	20 sec	
Extension	72	15 sec	

Detection Channel: FAM

Fig. 4, DNA extraction, samples analyses and result detection

A Air / B Surface samples

DNA extraction:

A From Air Samples :

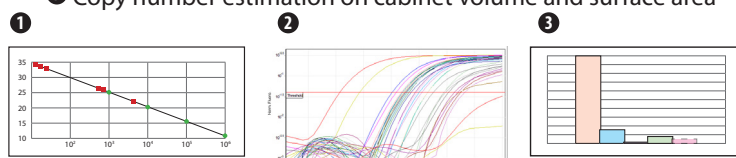
- Incubation on Shaker OS-20 180 rpm 15'
- Spin columned (GeneJet Plasmid Miniprep Kit, Thermo Fisher Fermentas)

B From Surface Samples:

- Vortex 2-3"
- Centrifuge at 13 300 rpm for 2'

Isolated DNA:

- 1 Real time PCR amplification (Fig. 7)
- 2 Detection of Ct values and normalization of data (Fig. 8)
- 3 Copy number estimation on cabinet volume and surface area

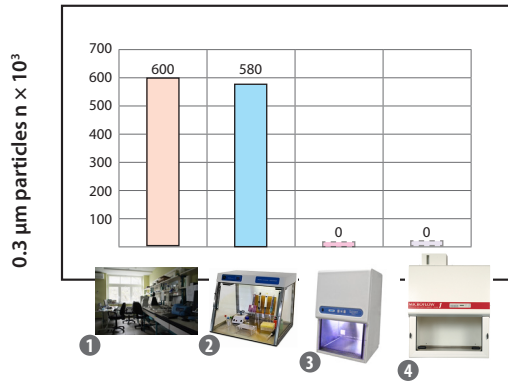


Results:

Mechanical contamination

Results of mechanical air contamination in cabinets of two types: PCR cabinet (UVC/T-M-AR, BioSan) and laminar flow cabinets (BioSafety class II cabinet prototype by BioSan and BSC II cabinet ABS Cabinet Class II by Microflow) as the positive control laboratory air samples were taken (Fig. 5).

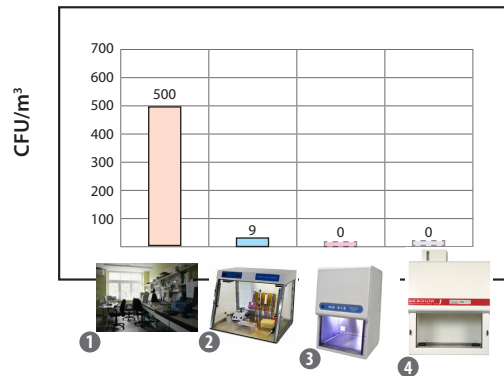
Fig. 5, Mechanical contamination, 0.3 μm particles



Microbial contamination

Microbial contamination in laboratory air and sterile cabinets. Quantitative results of microbial air contamination in cabinets of two types: PCR cabinet (UVC/T-M-AR, BioSan) and laminar flow cabinets (BioSafety class II cabinet prototype by BioSan and BSC II cabinet ABS Cabinet Class II by Microflow) as the positive control laboratory air samples were taken (Fig. 6).

Fig. 6, Microbial contamination



- 1 Positive control (laboratory air)
- 2 UV Cabinet (UVC-T-M-AR, Biosan, Latvia)
- 3 Laminar flow cabinet (HEPA BSC II Cabinet prototype, Biosan, Latvia)
- 4 BSC II Cabinet (ABS Cabinet Class II, Microflow, UK)

Amplicon contamination-inactivation efficiency:

Results analysis:

Real time PCR ensures product quantification using four standards of different Lambda phage DNA concentration and comparing Ct/Cq values of samples to those of concentration standards, based on standard curve (Fig. 8) (see Corbett Research Rotor Gene 3000 manual for more information) Following the amplification Lambda DNA copy number values were estimated for cabinet volume and surface area, results presented in (Fig. 9).

Inactivation efficiency was calculated as ratio of DNA amplicons before and after treatment: direct and indirect UV treatment for 15 and 30 minutes, presented in percents in table 2.

Fig. 7, Effect of UV irradiation on Ct/Cq values (raw results)

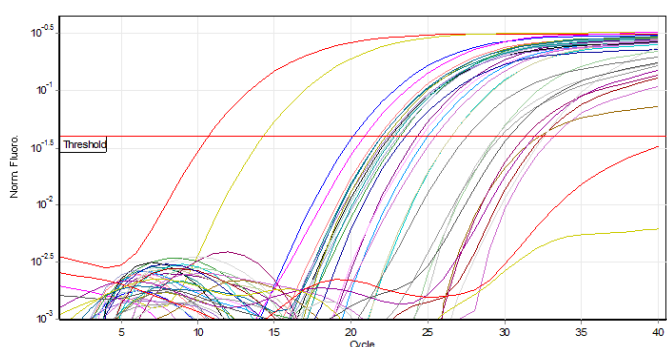


Fig. 8, Standard curve, influence of direct and indirect UV irradiation on lambda phage DNA copy number

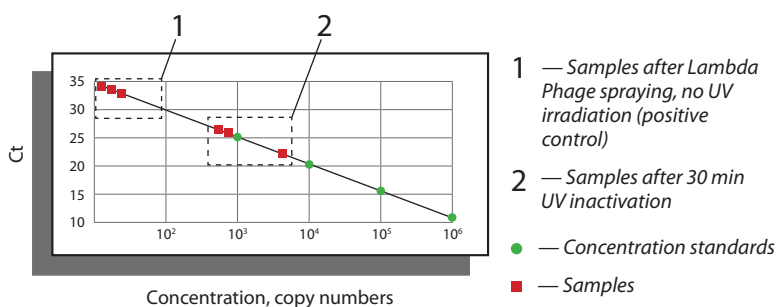
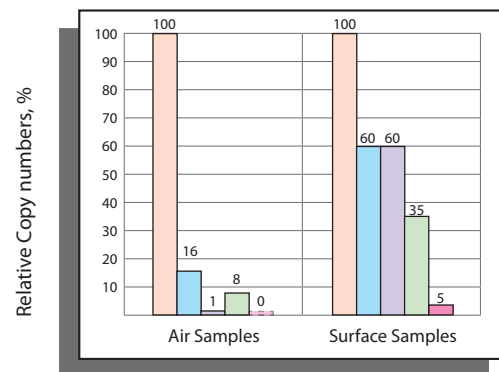


Fig. 9, Effect of direct and indirect UV irradiation on the amplicon concentration inside PCR cabinet UVC/T-M-AR, Biosan, Latvia



The horizontal axis show: air or surface samples, along with the relative copy number presented on vertical axis. Four series represent inactivation techniques and time of treatment, open UV light and UV air recirculator treatment kinetics are presented in the graph.

Table 2. DNA amplicon inactivation efficiency in PCR cabinet UVC/T-M-AR, Biosan, Latvia

		Inactivation efficiency			
Sample	Inactivation method	15 min UV/AR	30 min UV/AR	15 min Open UV	30 min Open UV
		Air Samples	84%	99%	92%
Surface Samples		40%	40%	65%	95%

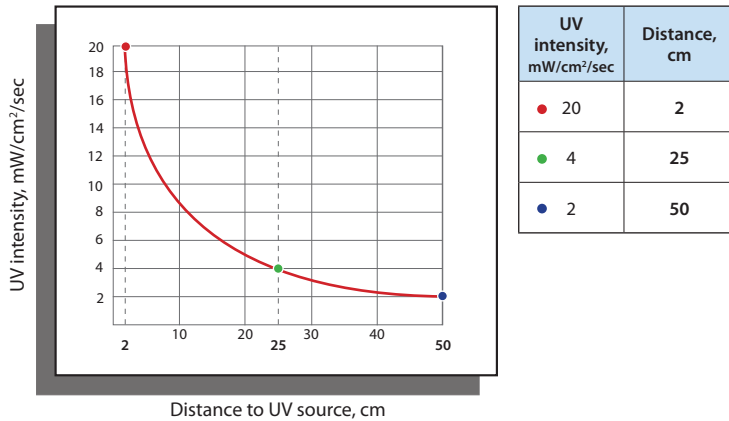
Calculation of UV dose for each treatment

Direct UV Irradiation

Cabinet's air treatment

BioSan's cabinet features a single open UV lamp 25 Watt, germicidal UV irradiation (253.7 nm) measurements have been performed and UV intensity were recorded at the level from 20 mW/sec/cm² to 2 mW/sec/cm² at distance to UV source from 2 cm to 50 cm respectively. [2] In PCR cabinet volume following UV intensity gradient is formed: from 20 mW/cm² to 2 mW/cm² (Fig. 10).

Fig. 10, UV intensity dependence on distance to UV tube (measured by radiometer VLX 254, Vilber Lourmat, France)



UV dosage during treatment = UV intensity at specific distance (mW/cm²/sec) × time of irradiation (sec)
 UV dosage during 15 min: gradient from 1800-18000 mW/cm²
 UV dosage during 30 min: gradient from 3600-36000 mW/cm²

Cabinet's Surface treatment:

Distance to UV source ranges between surfaces and consequently the UV intensity (table 3):

Table 3. Average dosage for different surfaces

Surface	Distance	Dosage after 15 min	Dosage after 30 min
Working surface	40-60 cm	1800-2700 mW/cm ²	3600-5400 mW/cm ²
Side walls	10-60 cm	1800-5400 mW/cm ²	3600-9000 mW/cm ²
Front window	10-60 cm	1800-5400 mW/cm ²	3600-9000 mW/cm ²

UV air recirculation:

Cabinet's Air treatment

BioSan PCR cabinets feature UV air recirculator. Recirculator consists of a fan, dust filters and closed UV-lamp (25W) installed in a special aluminium casing, which is located in the upper hood. Fan's air flow speed is 14 m³/hour, which processes 1.3 cabinet volumes per minute. Distance from closed the UV lamp to recirculator's walls is 2 cm at which UV intensity level is 20 mW/sec/cm² (Fig. 10).

UV air recirculators are designed for constant air decontamination during operations.

Resulting in following UV dosage for cabinet's volume:

- During 15 min recirculation: 380 mW/cm²
- During 30 min recirculation: 780 mW/cm²

Cabinet's Surface treatment:

UV Air recirculator does not provide cabinet surface irradiation.

For deactivation of microorganisms and amplicons on the cabinet's surface additional open UV treatment is needed for protection against contamination

Conclusions

Air sampling methods developed by BioSan has been proven to be compatible with real time PCR detection of product. This method enables monitoring of laboratory air and sterile cabinet for presence of target DNA amplicons.

The research was designed to evaluate BioSan PCR cabinets' efficiency in comparison to Class II BioSafety cabinets. Based on the experiment results PCR cabinets prevent microbial contamination with inactivation efficiency up to 96%, but in comparison to Class II BioSafety cabinets do not provide protection against mechanical contamination.

UV air treatment in BioSan PCR cabinets for 30 min provides DNA amplicon deactivation efficiency:

- Combined UV treatment (Open UV and UV air recirculator) provides 100% efficiency
- UV air recirculation provides 99% efficiency
- Open UV irradiation provides 100% efficiency

Based on classification of BioSafety cabinets from European standard EN 12469 [3] and experiment results: BioSan PCR Cabinets and Class I, II, III BioSafety Cabinets were compared on product protection ability in table 4.

Table 4. Classification of sterile cabinets, based on protection against contamination

Contamination forming units	Microorganisms	Viruses	DNA/RNA Amplicons
	BioSafety cabinets		
Class I	pos	neg	neg
Class II (A1, A2, B1, B2)	pos	neg	neg
Class III	pos	neg	neg
BioSan PCR Cabinets	pos/neg	pos	pos

Further studies will be focused on:

- Development of high speed monitoring technology of RNA amplicon concentration in the laboratory air and in sterile cabinets.
- Investigation of Class II BioSafety cabinets efficiency against DNA amplicon contamination. Based on preliminary experiment results: DNA amplicon particles which are not stopped by HEPA filters (Fig. 2) can result in constant contamination of cabinets volume.

Acknowledgement

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