Development and evaluation of DNA amplicon quantification

Case study: UV–Cabinet with UV Air Recirculator 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.

UVC/T-M-AR, UV–Cabinet for PCR operations

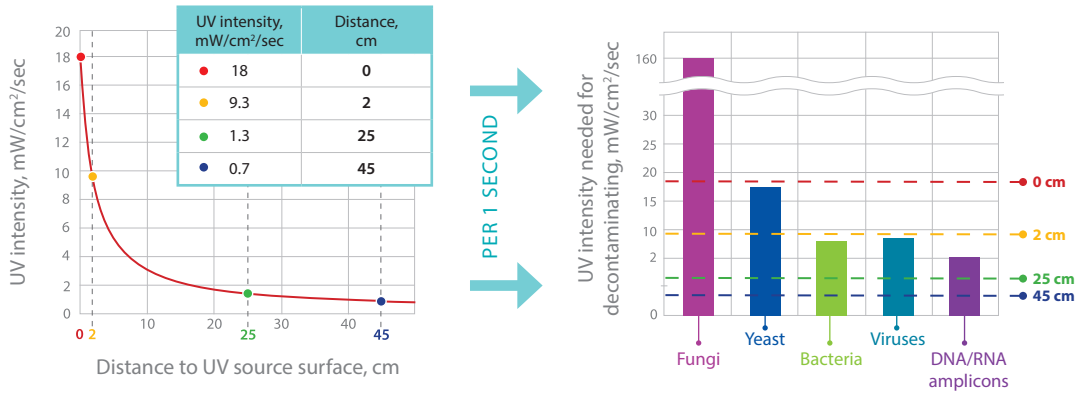


Fig. 1, Germicidal, shortwave (254 nm) ultraviolet energy is used for complete destruction of various biological agents

Aim of the study

The aim of this study is to evaluate the of 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/cm^2) and the time of exposure (t, seconds) resulting in units of mJ/cm^2 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 a 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)*.

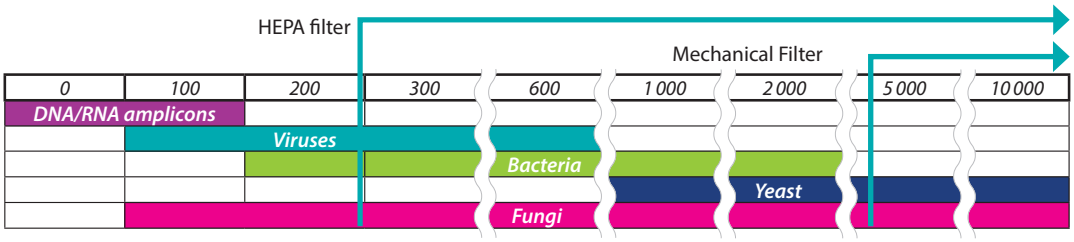


Fig. 2, Biological agent sizes and filters effectivity range, nm

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 m^3.

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 then 0.3 µm.

DNA Amplicon test

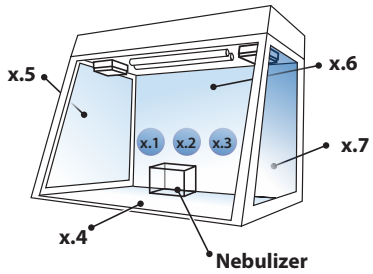
Instruments:

- 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** (Swab)
x.5, x.7	: **Side walls** (Swabs)
x.6	: **Back wall** (Swab)

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 ≈1,000,000 copies/µl

  » 2 NTC (no template control- sterile H_2O), 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

Sample taking path

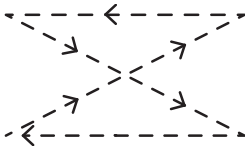


Table 1, Cycling protocol

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	42
Extension	72	15 sec	42

Detection Channel: FAM

Ⓐ Air / Ⓑ Surface samples

DNA extraction:

Ⓐ From Air Samples :

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

Ⓑ From Surface Samples:

- Vortex 2-3″
- Centrifuge at 13,300 rpm for 2′

Isolated DNA:

❶ Real time PCR amplification *(Fig. 7)*
❷ Detection of Ct values and normalization of data *(Fig. 8)*
❸ Copy number estimation on cabinet volume and surface area

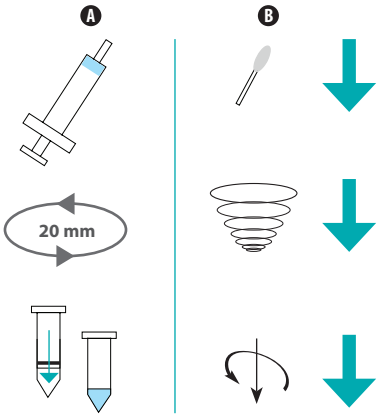
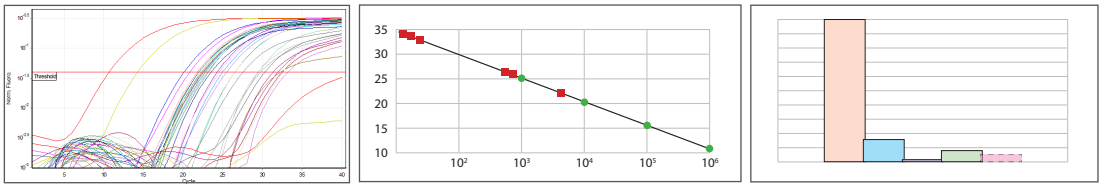


Fig. 4, DNA extraction, samples analyses and result detection

❶ ❷ ❸



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*).

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. 5, Mechanical contamination, 0.3 µm particles

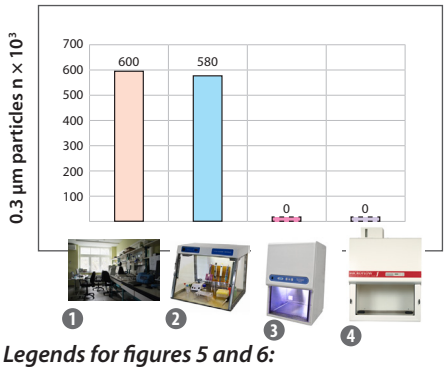
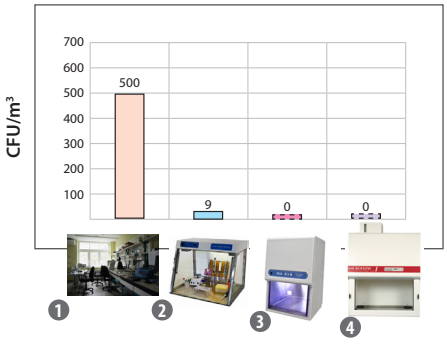


Fig. 6, Microbial contamination



Legends for figures 5 and 6:

❶ Positive control (laboratory air)
❷ UV Cabinet (UVC-T-M-AR, Biosan, Latvia)
❸ Laminar flow cabinet (HEPA BSC II Cabinet prototype, Biosan, Latvia)
❹ BSC II Cabinet (ABS Cabinet Class II, Microflow, UK)

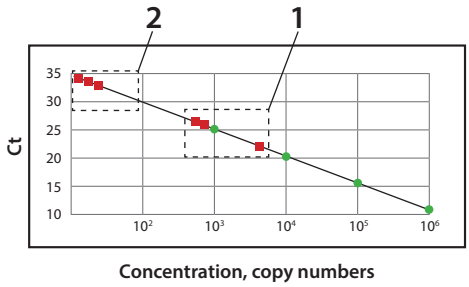
Amplicon contamination-inactivation efficiency:

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



1 — *Samples after Lambda Phage spraying, no UV irradiation (positive control)*

2 — *Samples after 30 min UV inactivation*

— *Concentration standards*

— *Samples*

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

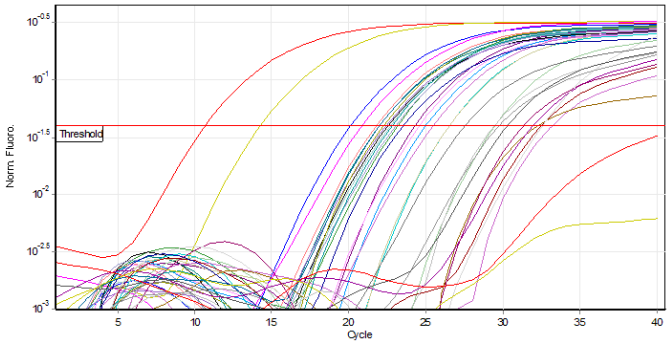
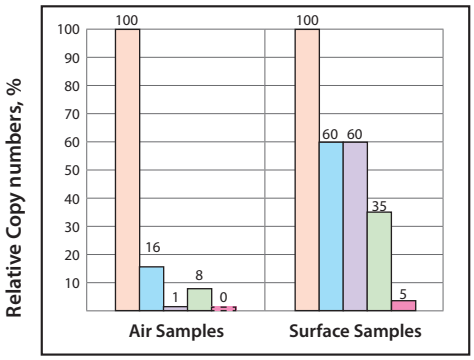


Fig. 9, Effect of direct and indirect UV irradiation on the amplicon concentration inside PCR cabinet UVC/T-M-AR, Biosan, Latvia



After Lambda phage DNA spraying

UV Air Recirculator for 15 min (Closed UV light irradiation, 25 W)

UV Air Recirculator for 30 min (Closed UV light irradiation, 25 W)

Open UV light (25 W) irradiation for 15 min

Open UV light (25 W) irradiation for 30 min

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

Sample	Inactivation method efficiency			
	15 min of UV Air Rec.	30 min of UV Air Rec.	15 min of Open UV + UV Air Rec.	30 min of Open UV + UV Air Rec.
Air Samples	84%	99%	92%	100%
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 18 mW/sec/cm^2 to 0.7 mW/sec/cm^2 at distance to UV source surface from 0 cm to 45 cm respectively. [2] In PCR cabinet volume following UV intensity gradient is formed: from 0.7 mW/cm^2 to 18 mW/cm^2 *(Fig. 10)*.

UV dosage during treatment = UV intensity at specific distance (mW/cm^2/sec) × time of irradiation (sec)

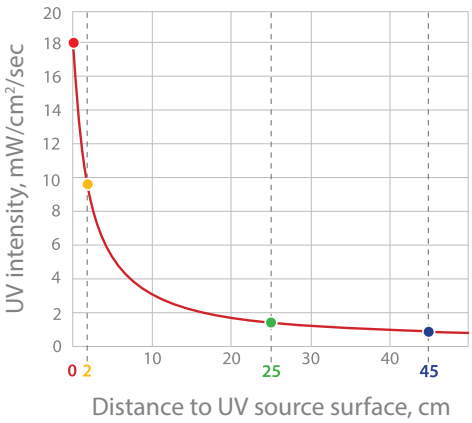
UV dosage during 15 min: gradient from 570-15,700 mW/cm^2

UV dosage during 30 min: gradient from 1,140-31,400 mW/cm^2

Cabinet's Surface treatment:

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

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



UV intensity, mW/cm^2/sec	Distance, cm
18	**0**
9.3	**2**
1.3	**25**
0.7	**45**

Table 3. Average dosage for different surfaces

Surface	Dosage after 15 min	Dosage after 30 min
Working surface (40-50 cm)	570-680 mW/cm^2	1,140-1,360 mW/cm^2
Side walls (10-50 cm)	570-2,500 mW/cm^2	1,140-5,000 mW/cm^2
Front window (10-50 cm)	570-2,500 mW/cm^2	1,140-5,000 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 (25 W) installed in a special aluminium casing, which is located in the upper hood. Fan's air flow speed is 14 m^3/hour, which processes 1.3 cabinet volumes per minute. Distance from closed UV lamp to recirculator's walls is less than 2 cm at which UV intensity level is 9.3-18 mW/sec/cm^2 *(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: 180 mW/cm^2
- During 30 min recirculation: 360 mW/cm^2

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 recirculation) 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*.

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.

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

BioSafety cabinets	Protection against contamination forming units		
	Microorganisms	Viruses	DNA/RNA Amplicons
Class I	+	–	–
Class II (A1, A2, B1, B2)	+	–	–
Class III	+	–	–
BioSan PCR Cabinets	+ / –	+	+

Table 5. Relation of risk groups to biosafety levels, practices and equipment (source: Laboratory biosafety manual, Third edition)

Risk Group	Biosafety Level	Laboratory Type	Laboratory Practices	Safety Equipment
1	Basic — Biosafety Level 1	Basic teaching, research	GMT	None; open bench work
2	Basic — Biosafety Level 2	Primary health services; diagnostic services, research	GMT plus protective clothing, biohazard sign	Open bench plus BSC for potential aerosols
3	Containment — Biosafety Level 3	Special diagnostic services, research	As Level 2 plus special clothing, controlled access, directional airflow	BSC and/or other primary devices for all activities
4	Maximum Containment — Biosafety Level 4	Dangerous pathogen units	As Level 3 plus airlock entry, shower exit, special waste disposal	Class III BSC or positive pressure suits in conjunction with Class II BSCs, double-ended autoclave (through the wall), filtered air

BSC, biological safety cabinet; GMT, good microbiological techniques

Table 6. Summary of biosafety level requirements (source: Laboratory biosafety manual, Third edition)

	Biosafety Level			
	1	2	3	4
Isolation [a] of laboratory	No	No	Yes	Yes
Room sealable for decontamination	No	No	Yes	Yes
Ventilation:				
— Inward airflow	No	Desirable	Yes	Yes
— Controlled ventilating system	No	Desirable	Yes	Yes
— HEPA-filtered air exhaust	No	No	Yes/No [b]	Yes
Double-door entry	No	No	Yes	Yes
Airlock	No	No	No	Yes
Airlock with shower	No	No	No	Yes
Anteroom	No	No	Yes	—
Anteroom with shower	No	No	Yes/No [c]	No
Effluent treatment	No	No	Yes/No [c]	Yes
Autoclave:				
— On site	No	Desirable	Yes	Yes
— In laboratory room	No	No	Desirable	Yes
— Double-ended	No	No	Desirable	Yes
Biological safety cabinets	No	Desirable	Yes	Yes
Personnel safety monitoring capability [d]	No	No	Desirable	Yes

[a] Environmental and functional isolation from general traffic.
[b] Dependent on location of exhaust (see Chapter 4 of Laboratory Biosafety Manual).
[c] Dependent on agent(s) used in the laboratory.
[d] For example, window, closed-circuit television, two-way communication.

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