

## ORIGINAL ARTICLE

# Detection of adeno- and lentiviral (HIV1) contaminations on laboratory surfaces as a tool for the surveillance of biosafety standards

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**Abstract**

**Aims:** As a biosafety laboratory, we survey the handling of adenovirus type 5 (Ad5) and HIV1-derived lentivirus in contained-use facilities in Switzerland to identify insufficiencies of the safety precautions taken by the laboratories.

**Methods and Results:** In the past 9 years, we took 430 swab samples from various types of surfaces in research laboratories. Samples were examined for Ad5 contaminations by real-time PCR and infectivity assay or for the presence of lentivirus (HIV1) nucleic acids by real-time (RT) PCR. Samples collected from centrifuges did not only contain Ad5 DNA more frequently but also exhibited higher numbers of Ad5 and lentiviral (HIV1) DNA copies than swabs from any other area of sampling. Five of ten samples containing infectious Ad5 particles or lentivirus (HIV1) RNA were found in samples taken from centrifuges. Ad5 contamination rates were higher in the tube holder and lower on the inner wall of the rotor chamber in centrifuges that were fitted with aerosol tight covers compared to centrifuges without covers.

**Conclusions:** Our results allowed the comparison of hygiene standards of different laboratories and lead to the identification of centrifuges as hotspots for contaminations.

**Significance and Impact of the Study:** Based on our results, we propose to use the collected data as a tool for rating future swab results. Furthermore, the amount of Ad5 and HIV1-derived lentivirus DNA could serve as an indicator of the level of good laboratory practice in contained-use laboratories handling these viral vectors.

**Introduction**

Genetically modified adenovirus type 5 (Ad5) and lentivirus of human (HIV1) origin are widely used as vectors for gene expression *in vitro* and for gene therapy (Bouard *et al.* 2009; Howarth *et al.* 2010). For safety reasons as well as to improve transduction capacity and to decrease the immunogenic response, parts of the original viral genome have continuously been modified. The first and second generations of Ad5 vectors exhibit deletions of 'early' genes in the genome regions E1 and E3 as well as E2 and E4, respectively (Bett *et al.* 1994; Engelhardt *et al.* 1994; Dedieu *et al.* 1997). In some of these adenoviral vector systems, homologous recombination after transfection was still observed, thus holding the risk of creating repli-

cation-competent Ad5 particles (Lochmuller *et al.* 1994; Hehir *et al.* 1996). More advanced systems have since been introduced involving gutless adenoviral vectors that only contain viral DNA sequences indispensable for DNA packaging and replication. They require the addition of helper plasmids carrying the structural genes of Ad5 (Kochanek *et al.* 1996; Parks *et al.* 1996). The use of newly developed helper cell lines has decreased the risk of homologous recombination between vector and cellular DNA and in consequence the probability of generating replication-competent adenoviruses (Fallaux *et al.* 1998; Schiedner *et al.* 2000). In contrast to adenovectors, lentiviral vectors stably integrate into the genome and have become very popular for the production of recombinant proteins in mammalian cells and for gene therapeutic

applications (Bouard *et al.* 2009; Pauwels *et al.* 2009). Because of their high pathogenicity, first and second generations of HIV1-derived lentivectors already consist of multiple plasmid systems to minimize the danger of emerging replication competent lentiviruses (Naldini *et al.* 1996; Zufferey *et al.* 1997; Dull *et al.* 1998). The possible activation of cellular oncogenes at the site of vector integration and the possible vector mobilization can be inhibited by using self-inactivating lentiviral vectors (Miyoshi *et al.* 1998).

The intentional handling of viral vectors demands to take appropriate safety measures and to apply good microbiological practice, including the use of state of the art equipment. This is generally regulated by national or supranational (such as EU directives) biosafety regulations for the work in enclosed systems. As an enforcement authority, we survey research facilities, biotechnology companies and diagnostic laboratories subjected to the Swiss Ordinance on the Contained Use of Organisms (1999b) and the Ordinance on Occupational Safety in Biotechnology (1999a). The purpose of surveillance is to detect disruption of containment of relevant viral material. It is well documented that disregarding safety precautions when operating centrifuges can lead to the formation of aerosols (Rutter and Evans 1972; Harper 1981; Bennett and Parks 2006) and to surface contaminations (Stern *et al.* 1974). Further causes for accidental exposure to viral material include carry-overs and improper hygiene. This has been widely shown for hospital environments and to some extent in public and household settings where transmission of viruses and diseases by handshaking or by contacting infrastructural surfaces such as door handles was observed (Rheinbaben *et al.* 2000; Boone and Gerba 2007; Weber *et al.* 2010).

In Switzerland, biosafety inspections are selectively supplemented by a surveillance programme using analytical means of swabbing. For this purpose, we have been taking swab samples from a specific range of laboratory surfaces and analysed them for the presence of contaminations by organism most frequently handled in Swiss laboratories. This has recently been reported for bacterial pathogens handled in diagnostic facilities (Schmidlin *et al.* 2010). In this work, we present the development of specific methods for the detection of Ad5 and HIV1-derived lentiviral contaminations in swab samples taken from laboratory surfaces and instrumentations. We summarize the data collected for both viruses each in 22 laboratories in Switzerland over the past 9 years. The samples were examined for the presence of nucleic acid containing virus-specific sequences. This included genomic as well as plasmid DNA of Ad5. Human lentiviral sequences were analysed in RNA as well as DNA originating from either plas-

mids or sequences integrated in cellular DNA of cell lines used for virus production. Lentiviral (HIV1) RNA was used as an approximation for the presence of structurally intact potentially infectious viruses, infectious Ad5 were identified by bioassay. The results were compared to identify locations in the laboratories that were frequently subjected to surface contaminations. We discuss the practicability of the data to assess biosafety standards of individual laboratories by means of their swab results and to define a critical limit for good microbiological practice based on the extent of viral contaminations.

## Materials and methods

### Plasmids, viral specimens and cell lines

Adenovirus Type 5 (Ad5) constructs Ad5 $\Delta$ E1GFP [E1 region of Ad5 replaced by a green fluorescent protein (GFP) expression cassette; replication deficient] and Ad5PacIGFP ( $\Delta$ E3) [E3 region of Ad5 replaced by a GFP expression cassette, replication competent] (Qu *et al.* 1998; Ehrenguber *et al.* 2000) were a kind gift from Markus Ehrenguber, Department of Biology, Kantonsschule Hohe Promenade, Zürich, Switzerland. Lentiviral (HIV1) vector construct pLL4.0-PKG [containing the phosphoglycerate kinase promoter and EGFP (enhanced green fluorescent protein) and lentivirus preparations thereof as described in Osinde *et al.* (2008)] was kindly provided by Maribel Osinde and Kumlesh K. Dev, Novartis Institutes for BioMedical Research, Novartis Pharma, Basel, Switzerland and Cambridge, USA, respectively. The pLL3.7 lentiviral vector as described in (Rubinson *et al.* 2003) as well as the helper plasmids pMD2.G, pRSV-Rev and pMDLg/pRRE (15) was obtained from Addgene Inc. (Cambridge, MA, USA). HEK-293 (ACC 305), HEK-293T (ACC 635) and HELA (ACC 57) cells were purchased from DSMZ (Braunschweig, Germany).

### Preparation of lentiviral stock solutions of pLL3.7

HEK-293T cells grown to 80% confluency in a 75-cm<sup>2</sup> tissue culture flask were transfected with pMD2.G, pRSV-Rev, pMDLg/pRRE (3.5  $\mu$ g each) and pLL3.7 (10  $\mu$ g) using 100  $\mu$ l FuGENE<sup>®</sup> (Roche Diagnostics, Rotkreuz, Switzerland) according to the manufacturer instructions. The virus containing supernatant of the cells was harvested and pooled after 24, 48 and 72 h of transfection. After filtration through a 0.45- $\mu$ m Ultrafree-MC membrane (Millipore, Zug, Switzerland), the titre was determined by GFP inspection ( $5 \times 10^4$  infectious particles per ml) and aliquots of the supernatant were stored at  $-70^{\circ}\text{C}$ .

### Swabbing from laboratory surfaces

Sterile cotton swabs soaked in 1 ml of swabbing buffer were used to wipe off viral particles from approx. 100 cm<sup>2</sup> laboratory surfaces. The swabbing buffer consisted of 1× PBS, 0.1% BSA, 0.05% Tween 80 (all from Sigma-Aldrich, Buchs, Switzerland) for the detection of Ad5. When used for swabbing lentiviruses, the buffer was supplemented with RNAlater<sup>®</sup> (Ambion; Applied Biosystems, Rotkreuz, Switzerland) to a final concentration of 50%. After wiping, the head of the cotton stick was broken off and plunged into the remaining buffer until further analysis. For transportation from the surveyed laboratory to the State Laboratory, the samples were placed in a cool box with a temperature logger (Hotdog; Elpro, Buchs, Switzerland). Temperature during transport ranged from 4 to 12°C. For the validation of the procedure, pieces of Kelco<sup>®</sup> standard laboratory surface (Vosseler, Therwil, Switzerland) were used to streak serial dilutions of virus suspensions that were dried for 2 h before swabbing. Transport was mimicked by placing the samples at 4°C for 2 h. To determine the recovery rate, the dried and wiped samples were compared to the corresponding untreated samples. All samples were further processed within 1 week of swabbing and after a single freezing/thawing cycle, which did not affect the subsequent analyses (data not shown).

### DNA and RNA extraction

DNA extraction from samples containing Ad5 was performed from 200 µl of the respective virus-containing solutions using the QIAamp DNA Mini kit according to the manufacturer protocol (Qiagen, Basel, Switzerland). Before extraction, samples were spiked with 1 µg of sonicated herring sperm DNA (Promega, Wallisellen, Switzerland). Elution of the viral DNA from the QIAamp spin columns was performed with 50 µl of elution buffer. Nucleic acid extraction from samples containing lentiviral (HIV1) vectors was performed from 140 µl of the swab sample using the QIAamp Viral RNA Mini kit procedure (Qiagen). Elution of the viral nucleic acids from the QIAamp spin columns was performed with 60 µl of the elution buffer. For the analysis of DNA, the samples were used without further treatment. For the analysis of RNA, the extracts were additionally subjected to a DNase digestion step by applying the RNase-free DNase set from Qiagen. The resulting RNA solution was then purified by a second run of RNA extraction including elution identical to the first. The effectiveness of the DNase digestion step was verified in each sample by performing a respective real-time PCR (data not shown).

### Quantitative detection of the virus-specific genes in DNA and RNA extracts

All primers and probes were designed using PRIMER EXPRESS<sup>®</sup> Software (Applied Biosystems) and tested *in silico* for specificity and cross-reactivity using BLAST from the National Center for Biotechnology Information (Altschul *et al.* 1990). For the detection of Ad5 DNA, a fragment of the fibre gene [GenBank<sup>®</sup> (Benson *et al.* 2008) Acc. no. M73260] was amplified in a standard 25 µl real-time PCR (Holland *et al.* 1991) containing 5 µl template DNA, 1× TaqMan<sup>®</sup> Universal PCR Master Mix (Applied Biosystems) and by using the following primers and probe: Ad5fiber-F (5'-aag cta gcc ctg caa aca tca-3', at a final concentration of 100 nmol l<sup>-1</sup>), Ad5fiber-R (5'-ccc aag cta cca gtg gca gta-3', at 300 nmol l<sup>-1</sup>) and Ad5fiber-FAM (FAM-5'-cct cac cac cac cga tag cag tac cct tac-3'-TAMRA, 100 nmol l<sup>-1</sup>). All primers and probes were purchased from Microsynth (Balgach, Switzerland). Quantification of fibre gene copies was performed using serial dilutions of a reference plasmid. Lentiviral (HIV1) vector copies (DNA) and genomes (RNA) were detected by performing real-time PCR and one-step RT-PCR, respectively, using the QuantiTect Probe RT-PCR kit (Qiagen) according to the manufacturers protocol using the following primers and probe: HIV1-PSS-F (5'-cgc agg act cgg ctt gct-3', 400 nmol l<sup>-1</sup>), HIV1-PSS-R (5'-gac gct ctc gca ccc at-3', 400 nmol l<sup>-1</sup>) and HIV1-PSS-Pr (5'-FAM-ccy ctc gcc tct tgc ygt gyg crc-TAMRA-3', 150 nmol l<sup>-1</sup>). The amplified fragment contains part of the packaging signal sequence Ψ (PSS, GenBank<sup>®</sup> Acc. no. AF033819). All samples were amplified and analysed on the 7500 System (Applied Biosystems) with the following parameters: (for Ad5 samples) initial heating to 95°C for 10 min followed by 45 cycles of 95°C for 15 s and 60°C for 60 s; (for lentiviral samples) initial heating of 50°C for 30 min (RT reaction only) followed by 95°C for 15 min before performing 45 cycles of 94°C for 15 s and 60°C for 60 s. For quantification of the human lentivirus, the pLL3.7 vector was used without further manipulation. Serial dilutions of viral stocks and reference plasmids were used to determine the limit of detection (Currie 1995) of both systems, which was 100 fibre gene copies per swab for Ad5, 260 HIV1-Ψ gene copies per swab (lentiviral RNA) and 120 HIV1-Ψ gene copies per swab (lentiviral DNA; data not shown). The quantitative significance level (limit of quantification, LOQ; Currie 1995) was ten times higher.

### Production of reference plasmids for the real-time PCR of Ad5

The reference standard DNA was produced by subcloning a 350-bp fragment containing the target region of the

fibre gene (M73260: 31450–31799) using the pGEM-T vector system (Promega).

### Bioassay for infectious Ad5 viral particles

For the detection of infectious Ad5, 100  $\mu\text{l}$  of serial dilutions of an adenoviral stock of  $1.5 \times 10^8$  PFU  $\text{ml}^{-1}$  (as controls and for the validation) or 100  $\mu\text{l}$  of a swab sample (during routine surveillance), each diluted one-to-one with standard culture medium (Dulbecco's modified Eagle's medium) supplemented with 10% foetal bovine serum (Sigma-Aldrich) and 27  $\mu\text{g ml}^{-1}$  geneticin (Gibco, Invitrogen, Basel, Switzerland), was equally distributed in 24-well culture plates. HEK-293 and HELA cells were routinely cultivated at 37°C with 5% CO<sub>2</sub> using standard culture medium. For setting up the bioassay, cells of one 75-cm<sup>2</sup> cell culture flask at a confluency of 70% were detached using 3 ml of 1× trypsin/EDTA solution for cell culture (Sigma-Aldrich). One-third of the trypsinized cell suspensions was diluted to a total volume of 50 ml using standard culture medium. Two millilitre of the diluted HEK-293 and HELA cells, respectively, was added to the wells of each of the 24-well culture plates, already containing the swab samples or respective controls. After overnight incubation, 1.8 ml supernatant of the infected HEK-293 and HELA cultures were withdrawn and replaced with 2 ml fresh standard culture medium and incubated further for up to 14 days. For the validation, 1 h after medium replacement, and on days 3, 9 and 14 postinfection, the cell cultures were analysed microscopically (Axiovert 25 CFL with digital camera AxioCam; Carl Zeiss, Feldbach, Switzerland) for the presence of cytopathic effects (CPE) and expression of GFP. In addition, 200  $\mu\text{l}$  of the supernatant was sampled and analysed for the presence of fibre gene copies by real-time PCR. When swab samples taken from laboratory surfaces during routine surveillance were tested, 200  $\mu\text{l}$  of the supernatant was analysed identically after one and 14 days.

## Results

### Detection of viral DNA/RNA

To detect Ad5 DNA, a real-time PCR system was developed, which amplifies a fragment of the adenoviral fibre gene. Situated on the 3' end of the Ad5 genome (late gene region L5), it is neither affected by the insertion of transgenes nor by deletions of the early genes E1–E4 (Weinberg and Ketner 1986). The amplified sequence would therefore be present in the majority of Ad5 expression vectors except for the generations of gutless vectors (Graham 1991; Alba *et al.* 2005), which were not used in the laboratories tested. For the analysis of HIV1-derived

lentiviral vectors, a real-time RT-PCR system was developed, which detected the region of the packaging signal sequence  $\Psi$ . This sequence was chosen because it is present even in minimal lentivectors (HIV1) and it is unchanged by the integration of transgenes (Sinn *et al.* 2005; Howarth *et al.* 2010). The  $\Psi$  primers and probe system were also used in a real-time PCR to detect lentivector (HIV1) DNA sequences in plasmids or as part of viral integrations in cellular DNA present in viral stocks from virus production (own observations; Zufferey 2002). Both detection systems recognized wild-type as well as recombinant viral nucleic acids.

To test the efficiency of swabbing, contaminations with viral particles were mimicked by streaking viral solutions of serial dilutions on test plates. After drying, the plates were swabbed and DNA (adenoviral, human lentiviral) or RNA (human lentiviral) was extracted from the samples. The number of genome copies was determined by real-time PCR and real-time RT-PCR, respectively, and compared to the corresponding control samples, which were not dried. The recovery rate of the procedure ranged from 74 to 103% (median 89%) for Ad5 DNA and from 15 to 23% (median 19%) for lentiviral (HIV1) RNA representing lentiviral particles (Table 1). The results obtained for the analysis of human lentivector DNA demonstrated that a considerable amount of HIV1- $\Psi$  sequences containing lentivector DNA was also recovered from the lentiviral solutions. The recovery rate varied from 34 to 52% (median 38%). The swabbing efficiencies were higher for DNA detection compared to RNA, but independent of the titre within the test range.

### Detection of infectious Ad5

Biologically active Ad5 particles were identified by an infectivity assay using HEK-293 and HELA cells. Serial dilutions of Ad5 $\Delta$ E1GFP virus containing 10–10<sup>5</sup> PFU were used to infect HEK-293 cells. After various time points (1, 3, 9 and 14 days), an aliquot of the supernatant was removed and the amount of Ad5-specific fibre gene copies was determined by in-culture real-time PCR (Fig. 1). Simultaneously, the cell monolayer was screened microscopically for CPE and expression of the GFP transgene (Fig. 2). A significant increase in genome copies in the culture medium after 3, 9 and 14 days could be detected for cells infected with 100 PFU of Ad5 or higher. When 10 PFU of Ad5 were added, the increase was only apparent after 9 and 14 days because the amount of fibre gene copies was not yet measurable at days 1 and 3 postinfection. Results obtained by microscopic observation to monitor infection were less evident. When using 10<sup>5</sup> PFU of Ad5 to infect cells, we could observe CPE and confirm by GFP expression of the cells

Dilution step	Gene copies per control sample	Gene copies per swab sample	Recovery rate (%)*
<i>Ad5 DNA</i>			
10 <sup>-1</sup>	3.19 × 10 <sup>5</sup> ± 5.04 × 10 <sup>3</sup> †	3.30 × 10 <sup>5</sup> ± 6.15 × 10 <sup>4</sup> †	103 ± 18
10 <sup>-2</sup>	5.55 × 10 <sup>4</sup> ± 3.10 × 10 <sup>4</sup>	3.39 × 10 <sup>4</sup> ± 4.06 × 10 <sup>3</sup>	74 ± 34
10 <sup>-3</sup>	3.12 × 10 <sup>3</sup> ± 80	2.59 × 10 <sup>3</sup> ± 430	83 ± 12
10 <sup>-4</sup>	446 ± 112‡	270 ± 137‡	n.d.
10 <sup>-5</sup>	n.d.	n.d.	n.d.
<i>Lentiplasmid DNA</i>			
10 <sup>-1</sup>	2.55 × 10 <sup>7</sup> ± 5.45 × 10 <sup>6</sup>	1.28 × 10 <sup>7</sup> ± 1.02 × 10 <sup>6</sup>	52 ± 15
10 <sup>-2</sup>	3.13 × 10 <sup>6</sup> ± 1.60 × 10 <sup>5</sup>	1.13 × 10 <sup>6</sup> ± 2.27 × 10 <sup>5</sup>	36 ± 5
10 <sup>-3</sup>	3.07 × 10 <sup>5</sup> ± 3.16 × 10 <sup>4</sup>	1.12 × 10 <sup>5</sup> ± 2.50 × 10 <sup>4</sup>	37 ± 12
10 <sup>-4</sup>	3.99 × 10 <sup>4</sup> ± 1.34 × 10 <sup>4</sup>	1.31 × 10 <sup>4</sup> ± 2.40 × 10 <sup>3</sup>	34 ± 5
10 <sup>-5</sup>	3.35 × 10 <sup>3</sup> ± 173	1.14 × 10 <sup>3</sup> ± 166	n.d.
10 <sup>-6</sup>	250 ± 99‡	204 ± 85‡	n.d.
<i>Lentivector RNA</i>			
10 <sup>-1</sup>	2.54 × 10 <sup>6</sup> ± 8.01 × 10 <sup>5</sup>	5.80 × 10 <sup>5</sup> ± 2.38 × 10 <sup>5</sup>	23 ± 2
10 <sup>-2</sup>	3.62 × 10 <sup>5</sup> ± 1.72 × 10 <sup>5</sup>	5.60 × 10 <sup>4</sup> ± 3.32 × 10 <sup>4</sup>	15 ± 2
10 <sup>-3</sup>	3.90 × 10 <sup>4</sup> ± 1.06 × 10 <sup>4</sup>	7.41 × 10 <sup>3</sup> ± 2.03 × 10 <sup>3</sup>	21 ± 11
10 <sup>-4</sup>	5.34 × 10 <sup>3</sup> ± 111	734 ± 507‡	n.d.
10 <sup>-5</sup>	655‡§	537‡	n.d.

n.d., Not detected.

\*The recovery rate was determined by the ratio of gene copy numbers of dried and swabbed virus (swab) divided by the equivalent amount of untreated virus (control).

†Numbers represent the mean of triplicates ± SD.

‡Values below the limit of quantification were not considered for the calculation of the recovery rate.

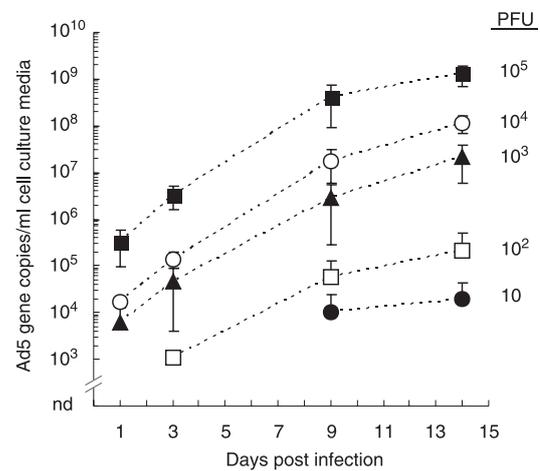
§One detectable sample only.

after 3 days (Fig. 2, panel III and IV), while adding 100 PFU resulted in very limited CPE (example given in Fig. 2, panel III). After 14 days postinfection, most cells including the noninfected controls were shed (data not shown) and CPEs could not be identified. By real-time PCR analysis, an increase in virus titre in the supernatant of the cells could still be monitored but levelled off because of cell death (Fig. 1). The in-culture real-time PCR has thus demonstrated to be more robust and more sensitive compared to microscopic screening. In parallel, these experiments were performed using HELA cells. In this cell type, the infections with Ad5ΔE1GFP never gave rise to CPE or generated adenoviral genomes in the cell supernatants in contrast to when the replication-competent Ad5PacIGFP was used in a parallel set-up (data not shown). Thus, HELA cells were successfully employed to differentiate between replication-competent and replication-deficient adenoviral constructs.

### Swab samples from laboratory surfaces

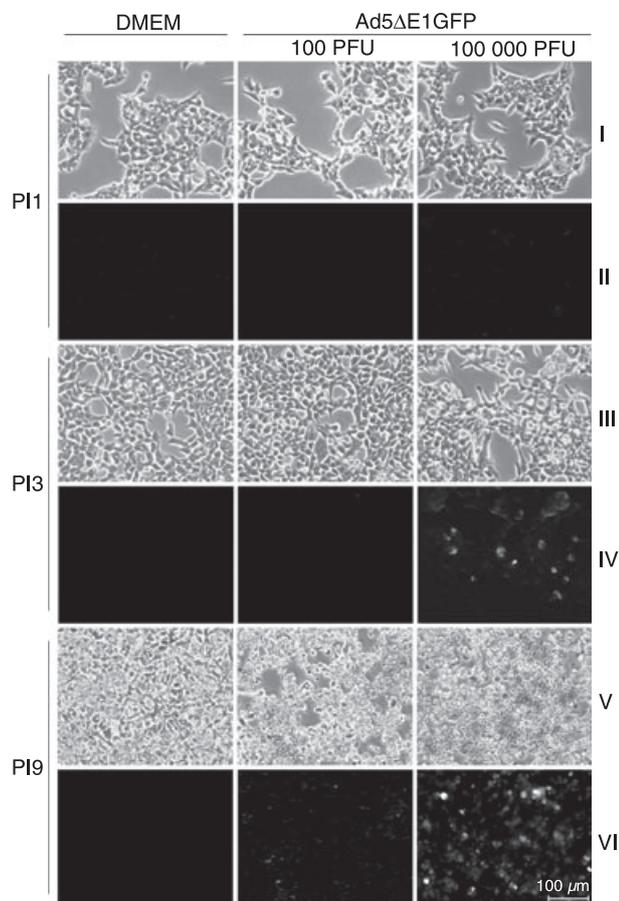
Over the past 9 years, we took swab samples from laboratory surfaces of numerous contained-use facilities to analyse them for potential contaminations with Ad5 or lentivirus (HIV1) handled in the laboratories. The vast

**Table 1** Recovery rates for swabbing of Ad5 and lentivirus after drying



**Figure 1** In-culture real-time PCR analysis of HEK-293 cells infected by Ad5. Numbers of Ad5-specific gene copies in the supernatant of HEK-293 after infection with 10, 100, 1000, 10<sup>4</sup> and 10<sup>5</sup> PFU of Ad5ΔE1GFP (as indicated). At day 1, 3, 9 and 14 postinfection, aliquots of cell culture medium were removed, and the amount of fibre gene copies was measured by real-time PCR.

majority (21 of 22 of each Ad5 and lentivirus (HIV1) survey) were research laboratories or diagnostic facilities with a research unit handling predominantly recombinant



**Figure 2** Microscopic analysis of uninfected and Ad5-infected HEK-293 cells. Phase-contrast (uneven-numbered panels) and GFP fluorescence (even-numbered panels) microscopy were used to document HEK-293 cells infected with two different titres of Ad5ΔE1GFP (100 and 100 000 PFU) and Dulbecco's modified Eagle's medium control HEK-293 cells (uninfected) at day 1, 3, 9 days postinfection (PI). The scale bar in panel VI applies to all illustrations. GFP, green fluorescent protein.

viral vectors. All samplings were announced beforehand. Generally, ten samples were collected per laboratory. Sites of sampling included the following locations: centrifuge (inner wall of the rotor chamber and the interior of the tube holder), operating surface of devices, general laboratory surfaces, microscope, work surface of biosafety cabinets as well as personal equipment and locations outside of the laboratory as exemplified in Table 2. The swab samples were analysed for the presence of the respective DNA and RNA sequences of the viral vectors. In total, 193 samples were analysed for the presence of Ad5 DNA and 237 samples for lentivirus (HIV1) DNA by real-time PCR (Fig. 3, Table 3), and selected samples were further subjected to the infectivity assay (Ad5) or for the presence of lentivector (HIV1) RNA (Table 4).

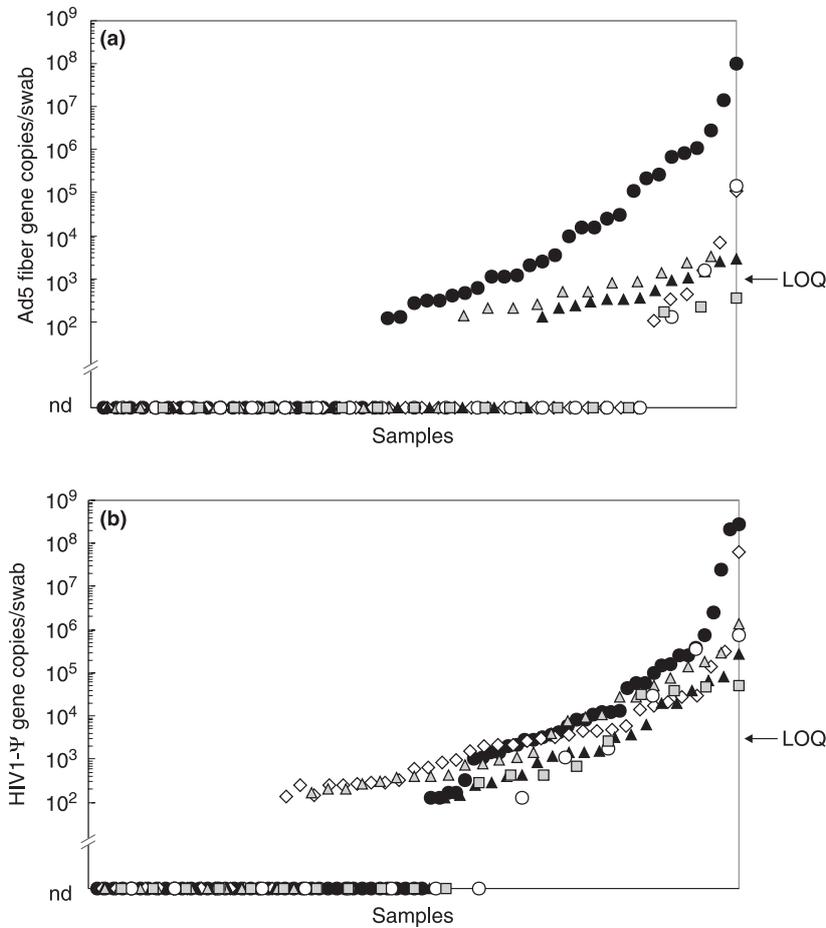
**Table 2** Areas of sampling in BSL2 laboratories in Switzerland taken from 2001 to 2009

Area of sampling	Sampling site in the laboratory
Centrifuge	Centrifuge: internal wall of rotor chamber Centrifuge: interior of tube holder
Controls of devices	Door handle of incubator Door handle of fridge or freezer Door handle of centrifuge Control panel of incubator Control panel of biosafety cabinet Control panel of autoclave Control panel of centrifuge
General laboratory surfaces	Deposit space next to devices Wall next to devices Floor next to devices Surface of devices Surface of shelf/bench
Personal equipment/outside containment	PC accessories Telephone receiver Door handle of laboratory Surfaces outside the lab
Microscope	Adjustment knob Surface of stage Eyepiece Stage knob
Biosafety cabinet	Work surface of biosafety cabinet

### Samples analysed for Ad5 contaminations

The majority of all samples analysed for Ad5 contained no detectable amounts of virus-specific DNA (128 of 193 samples, 66.3%, Table 3). This was also observed for five of the six areas with the exception of the centrifuge where only 44% (22 of 50) of the samples collected exhibited no detectable Ad5 DNA. For further examination, the data were arranged in order of increasing amounts of genome copies per swab (Fig. 3). This allowed a direct comparison of the number and level of contaminations found per sampling location. Because not the same number of samples was taken at the indicated locations, the data points were equally distributed on the x-axis. As the resulting figure illustrates, samples collected from centrifuges did not only contain Ad5-specific DNA more frequently (40% of all centrifuge samples above the LOQ vs 0–15% of samplings from other areas; Table 3, Fig. 3a) but also comprised a higher number of fibre gene copies than samples from any other area with the highest number being  $10^8$  copies per sample. Thus, a significant difference was observed between the centrifuge and all other areas of sampling with respect to specific Ad5 DNA contamination on these surfaces.

By detecting infectious viral particles in the swab samples, we wanted to get a supplementary assessment of the



**Figure 3** Presence of viral-specific DNA sequences in swab samples taken from laboratory surfaces. Number of (a) Ad5- and (b) lentivirus-specific DNA gene copies per swab taken from laboratory surfaces. Each symbol represents one sample taken from one of the areas of sampling that was analysed for the Ad5 fibre gene and the HIV1-Ψ gene, respectively, by real-time PCR: centrifuges [●, (a)  $n = 50$ , (b)  $n = 76$ ], general laboratory surfaces [◇, (a)  $n = 39$ , (b)  $n = 46$ ], personal equipment/outside containment [▲, (a)  $n = 40$ , (b)  $n = 42$ ], controls of devices [△, (a)  $n = 26$ , (b)  $n = 38$ ], microscopes [○, (a)  $n = 20$ , (b)  $n = 15$ ], biosafety cabinets class 2 [■, (a)  $n = 18$ , (b)  $n = 20$ ]. The data are arranged according to the number of gene copies per swab sample and equally distributed on the x-axis with the lowest number on the left and the highest on the right. Samples with no detectable specific viral DNA (nd) are placed on the x-axis. LOQ corresponds to 1000 (Ad5) and 1200 (lentivirus) gene copies per swab. LOQ, limit of quantification.

Area of sampling	Samples with no detectable specific DNA		Samples with specific DNA > LOQ	
	Number/total	% of total	Number/total	% of total
<i>Ad5</i>				
Centrifuge	22/50	44	20/50	40
Controls of devices	14/26	54	4/26	15
General laboratory surfaces	33/39	85	3/39	8
Personal equipment/outside containment	27/40	68	4/40	10
Microscope	17/20	85	2/20	10
Biosafety cabinet	15/18	83	0/18	0
Total	128/193	66	33/193	17
<i>Lentivirus</i>				
Centrifuge	39/76	51	30/76	39
Controls of devices	12/38	32	13/38	34
General laboratory surfaces	13/46	28	20/46	43
Personal equipment/outside containment	21/42	50	14/42	33
Microscope	9/15	60	4/15	27
Biosafety cabinet	11/20	55	5/20	25
Total	105/237	44	86/237	36

\*Detected contaminations below LOQ are not listed.

**Table 3** Number of samples with Ad5- and lentivirus-specific DNA below the limit of detection and above the limit of quantification (LOQ)\*

**Table 4** Number of Ad5- and lentivirus-specific gene copies of swab samples exhibiting biological activity of Ad5 and detectable amounts of lentiviral RNA, respectively

Area of sampling	Ad5	Lentivirus	
	Fibre gene copies per swab	RNA: $\Psi$ gene copies per swab	DNA: $\Psi$ gene copies per swab
Centrifuge	$1.8 \times 10^{8**\dagger}$	747‡	$2.5 \times 10^5$
		$5.5 \times 10^3‡$	$1.2 \times 10^4$
		$2.2 \times 10^4‡$	$2.8 \times 10^8$
		$8.6 \times 10^4‡$	$2.5 \times 10^6$
Controls of devices	–	$8.8 \times 10^3§$	$2.8 \times 10^4$
General laboratory surfaces	$1.1 \times 10^5¶$	$1.5 \times 10^3**$	$3.1 \times 10^5$
		$2.6 \times 10^3††$	$6.4 \times 10^7$
Personal equipment/outside containment	–	–	–
Microscope	–	–	–
Biosafety cabinet	–	$2.9 \times 10^4$	$4.0 \times 10^4$

\* Values correspond to the original DNA analysis of the swab samples.

†Internal wall of centrifuge.

‡Interior of tube holder.

§Door handle of incubator.

¶Wall behind waste basket.

\*\*Floor next to waste basket.

††Laboratory surface adjacent to centrifuge.

biosafety standard of particular facilities. For this purpose, selected samples containing virus DNA higher than the LOQ (with the exception of a set of samples originating from one campaign for which only Ad5 DNA was measured) were further analysed for the presence of infective Ad5. Of 15 samples analysed with the bioassay, two swabs, one taken from within a centrifuge (internal wall of rotor chamber) and one from a general laboratory surface (wall behind waste bin), revealed infectivity in HEK-293 but not HELA cells (Table 4). Both swabs contained the highest number of Ad5-specific genome copies of the respective area of sampling taken from the original DNA analysis of the swabs. They originated from the same laboratory.

#### Samples analysed for lentivirus (HIV1) contaminations

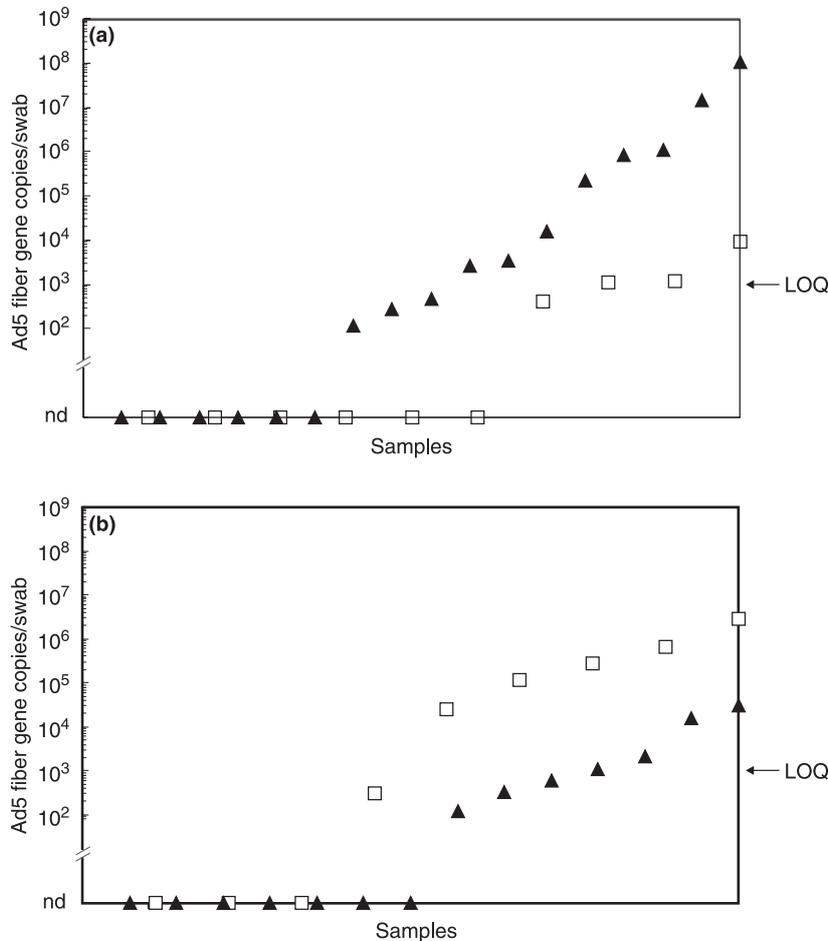
Of a total of 237 swab samples taken in laboratories handling lentiviral (HIV1) vectors, a minority of 105 samples were found to contain no detectable lentiviral DNA (44%, Table 3). On general laboratory surfaces and controls of devices, negative results of human lentivirus-specific DNA were observed less frequently compared to other areas of sampling (28% and 32 vs 50% and higher, Table 3). In contrast to the general laboratory surfaces that also include work benches, the controls of devices are surfaces where no intentional handling of viral material is carried out. Similar to the results for Ad5, samples from the centrifuge contained higher number of HIV- $\Psi$  gene copies than samples from any other area with the

strongest contamination exhibiting  $2.8 \times 10^8$  gene copies per swab (Fig. 3).

To get an indication of the presence of structurally intact lentiviral (HIV1) particles in the swabs, real-time RT-PCR was performed to detect lentiviral RNA. In 164 samples analysed, eight samples were found positive (Table 4). The majority of these samples, which were taken from five different laboratories, originated again from the two areas of sampling, namely centrifuges (four) and general laboratory surfaces (two). With two exceptions, these samples belonged to the group of 10% of samples containing the highest number of HIV- $\Psi$  DNA copies.

#### Surface contaminations of centrifuges

For a more detailed analysis of the data obtained from centrifuges, the results from the inner wall of the rotor chamber and the interior of the tube holder were separated. The data points were again plotted in order of increasing amounts of genome copies per swab and equally distributed on the x-axis analogous to Fig. 3. There were fewer swab samples from the internal wall of centrifuges with aerosol lids containing detectable levels of Ad5 DNA, and these displayed relative lower numbers of Ad5 fibre gene copies than samples from centrifuges without aerosol cover (Fig. 4a). The opposite effect was observed when plotting the amount of specific DNA per swab from the interior of the tube holder (Fig. 4b), thus illustrating the effect of aerosol being largely contained



**Figure 4** Presence of Ad5 DNA in swab samples taken from centrifuges. Number of Ad5 fibre gene copies per swab taken from the internal wall of rotor chamber (a) and the tube holder (b) of centrifuges operated with [□, (a)  $n = 10$ , (b)  $n = 17$ ] or without [▲, (a)  $n = 9$ , (b)  $n = 14$ ] aerosol covers. Each symbol represents one sample taken from one of the indicated area of sampling that was analysed for the Ad5 fibre gene by real-time PCR. The data are arranged according to the number of gene copies per swab sample and equally distributed on the x-axis with the lowest number on the left and the highest on the right. Samples with no detectable specific viral DNA (nd) are placed on the x-axis. LOQ corresponds to 1000 (Ad5). LOQ, limit of quantification.

within the tube holder if tight covers were used. In the case of lentiviral (HIV1)-containing samples, this result could not be confirmed because of too little data points for no aerosol covers (data not shown).

#### Surface contaminations of individual laboratories

We surveyed 22 laboratories for Ad5 and 22 laboratories for lentiviral (HIV1) contaminations. An average of 9 samples (Ad5, median 10) and 11 samples (lentivirus, median 10) were collected per laboratory. Individual laboratories did not contribute the same number of samples to the overall results in each sampling area. We examined whether a particular laboratory was contributing more contaminations of very high levels of Ad5 or lentivirus (HIV1) plasmid DNA than others. For this reason, all swab results within those 10% of samples containing the highest number of viral genome copies in each area of sampling were closer examined by laboratory. Of the overall 24 (Ad5 DNA) and 27 samples (lentiviral HIV-Ψ

DNA) within this category, seven and 17, respectively, originated from one facility (data not shown). The contaminations were equally distributed between all sampling areas in this laboratory (data not shown). Although this laboratory did contribute a higher than average number of samples to the study, the proportion of samples with high DNA contaminations was still elevated compared to any other laboratory (47%, Ad5 and 40%, HIV1-lentiplasmid *vs* <29% for all other laboratories). Furthermore, three of eight samples positive for lentiviral (HIV1) RNA (38%) originated from this facility.

#### Discussion

Inspections are the general tool to control biosafety standards of laboratories handling Ad5 and lentiviral (HIV1) vectors. Biosafety standards are defined by the quality and effectiveness of infrastructural, technical and organizational safety measures taken by the facility leading to or preventing surface contaminations. In our laboratory, we

have been complementing the inspections by analytical means of swab taking from laboratory surfaces, as previously reported for bacterial pathogens (Schmidlin *et al.* 2010). The use of this concept has also just recently been reported in a study on the surveillance of genetically modified organisms on surfaces of three laboratories (Rutjes *et al.* 2011).

To survey laboratory surfaces for contaminations with viral vectors, in the past 9 years, we took 193 samples for the analysis of Ad5 and 237 samples to be examined for lentivirus (HIV1). The samples were tested for the presence of viral sequences. In the case of Ad5, DNA represents plasmids and viral particles. Lentiviral samples were analysed for the presence of lentiviral (HIV1) DNA, because lentivector preparations from cell lines regularly contain plasmids and viral integrates in cellular DNA from virus production. This previous observation was confirmed by the presented experiments of recovery of lentiviral (HIV1) RNA and DNA from dried lentivector stock solutions. At places where no plasmid DNA was handled, the detection of lentiviral (HIV1)-specific DNA sequences therefore serves as an indirect indication of the presence of infectious lentiviruses. In addition, to get a more conclusive evidence of the presence of infectious viral particles in the swabs, lentiviral (HIV1) particles were detected on the basis of their RNA by real-time RT-PCR. Because of the instability of RNA outside the viral particle, RNA levels served as an approximation of the number of structurally intact potentially infectious particles. For the identification of infectious Ad5, we developed a bioassay in HEK-293 and HELA cells.

The results presented in this study show that compared to the other tested laboratory areas, the swabs collected from centrifuges contained not only the overall highest numbers of copies of Ad5 fibre gene and lentivirus (HIV1) plasmid, but they also accounted for the highest number of samples containing Ad5 DNA. Furthermore, 50% of the samples containing infectious Ad5 and lentivirus RNA were attributed to centrifuges (one of two and four of eight, respectively). Deduced from a study by Higashikawa and Chang (2001) and own recent observations, it was most likely that not all of the lentivector RNA positive swabs contained infectious HIV1-derived lentivirus. In standard virus preparations, only a minor portion of viral particles was found to be structurally intact as judged by electron microscopy. Nonetheless, these results were consistent with previous reports, showing that centrifuges pose a biohazard. Bennett and Parks used a suspension of *Bacillus atrophaeus* spores to demonstrate that a leakage during centrifugation because of unsealed buckets generated at least as much aerosol as, for example, dropping a large bottle (Bennett and Parks 2006). Whereas most studies reported aerosol formation

within the context of centrifuge accidents or broken tubes (Rutter and Evans 1972; Harper 1984; Bennett and Parks 2006; Gilman Duane and Fink 2006), the indispensability of using tightly sealed rotors or buckets even with intact vials was demonstrated likewise (Harper 1981). We could clearly confirm the effect of aerosol tight covers for Ad5 contaminations when analysing the samples collected from the inner wall of the rotor chamber separately from the samples taken from the tube holder. Adenovirus-containing samples from the wall of the rotor chamber of centrifuges fitted with aerosol protection contained less viral-specific DNA than samples drawn at the same location in centrifuges without tight covers. The examination of the samples from the tube holders leads to reverse results. This demonstrated that the aerosol covers held back residues of viral vectors within the tube holder, which were otherwise distributed along the wall of the rotor chamber or potentially further.

The exposure of laboratory staff to aerosols created during centrifugation occurs in two ways: through respirable airborne particles and via deposition of larger droplets onto surfaces (Stern *et al.* 1974; Harding and Brandt Byers 2006). The latter can also be a cause of carry-overs. Contaminated nonporous surfaces have been shown to efficiently transfer micro-organisms to hands (Rusin *et al.* 2002). Further, Rheinbaben *et al.* (2000) demonstrated that door handles once contaminated with a test virus could pass on the contamination as far as to 14 volunteers that were consecutively contacting it. Therefore, it is noteworthy that in our study, 50 and 38% of swabs containing infectious Ad5 and lentiviral (HIV1) RNA, respectively, were wiped from general laboratory surfaces and operating surfaces of devices. These data were analogous to the findings of lentiviral (HIV1) DNA in samples from these two areas. At both locations but particularly at control panels, there is no intentional handling of virus DNA carried out. Because lentiviruses do not belong to the natural flora of the human skin, these contaminations cannot be caused by natural deposition by the laboratory staff as we have recently shown for *Staphylococcus aureus* (Schmidlin *et al.* 2010). These findings suggested a carry-over from other locations in the laboratory. It could be assessed as an indication of inappropriate laboratory practices.

As Ad5 and lentiviruses (HIV1) are structurally different organisms, they are most likely to exhibit different abilities to adhere and survive in dried condition on variable surfaces (Boone and Gerba 2007). Enveloped viruses such as lentivirus are more susceptible to environmental influences. Nevertheless, the available data vary substantially because of the different experimental set-ups with respect to type of surface, medium, temperature or humidity, which all affect the survival rate (Kramer *et al.*

2006; Valtierra 2008). This parameter will undoubtedly affect the numbers of swab samples containing infectious viruses. As we did not monitor laboratory contaminations over a period of time, the samplings represent snapshots of the current situation in the laboratory. Therefore, the chance of detecting structurally intact or infectious viral particles depended not only on the stability of the dried virus in its environment but also largely on the temporal proximity of the sampling to the laboratory activity. However, the aim of the samplings and this study was to compare contamination rates for Ad5 and separately for lentivirus (HIV1) among different areas of sampling as well as among individual laboratories. As adenoviral and lentiviral (HIV1) DNA, the latter often being found concomitantly in viral preparations, could be detected after a longer drying time than structurally intact and infectious viruses, their results were less affected by these variables. Thus, a negative finding with respect to infectious Ad5 or to lentivirus (HIV1) RNA does not necessarily rule out the occurrence of an earlier contamination with viral particles if vector DNA was detected concurrently, in particular, because all samples from within the laboratory containment were collected in BSL2 facilities rather than laboratories used purely for DNA work. In addition, this study has given evidence of the consistency of DNA data with results of Ad5 infectivity or of lentiviral (HIV1) RNA. This was particularly evident for centrifuges. They accounted for the highest number of Ad5-containing samples compared to any other location and exhibited the highest levels of Ad5- and lentiviral (HIV1)-specific gene copies as well as comprised half of all samples containing infectious Ad5 and lentiviral RNA.

Taken together, we have shown that the amount of Ad5- and lentivirus (HIV1)-specific DNA in swabs from laboratory surfaces can be implemented as an indicator for biosafety standards and the level of good laboratory practice as has been suggested for bacterial contaminations (Schmidlin *et al.* 2010; Rutjes *et al.* 2011). For this purpose, the plotted data as it is presented in Fig. 3 could be used to assess the collected data and future swab results. Thereby, individual laboratories were identified that showed a low compliance to biosafety standards compared to the majority of the laboratories tested. In a further step, the data might serve as a tool for defining a critical limit for good laboratory practice. The critical value may be comprehended as a value that will not be exceeded when good laboratory practice is applied similar to what has been defined for genetically modified plant ingredients in foods (EU, 2003; Waiblinger *et al.* 2007). We suggest to set the cut-off at 90%. Because of the different nature of micro-organisms and the different degree of exposure of the locations to the biological material, we propose to base critical values on the collected data for

each micro-organism and location individually. This leads to six critical values per micro-organism.

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