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# A new simplified clarification approach for lentiviral vectors using diatomaceous earth improves throughput and safe handling

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ABSTRACT

Lentiviral vectors have proven their great potential to serve as a DNA delivery tool for gene modified cell therapy and gene therapy applications. The downstream processing of these vectors is however still a great challenge, particularly because of the low stability of the virus. Harvesting and clarification are critical and until now insufficiently characterized steps for lentivirus processing. To address this bottleneck, we analyzed whether lentiviral vectors produced by transient transfection of HEK293 T/17 SF suspension cells can be efficiently clarified with a lab-scale method with the filter aid diatomaceous earth (DE) and bioburden reducing membrane filters achieving high lentivirus recoveries.

Using a design of experiment approach we found that higher DE concentrations are advantageous for a higher turbidity reduction and shorter filtration times, but at the same time LV titer decreases with increasing DE concentration. A DE concentration of 9 g/L was identified with a DoE as a robust set-point. Clarification with DE was compared with for lab-scale traditionally employed centrifugation and subsequent bioburden reduction filtration of viral vectors. The use of DE allows to perform a harvest and clarification process, which does not only facilitate faster and safer virus handling, but enables a lower material consumption due to the extremely increased filter capacity, thus representing an efficient and robust lab-scale clarification process.

## 1. Introduction

Viruses are highly important tools in medicine and have become more relevant with the advent of gene therapy. Gene therapy has a significant therapeutic potential for the treatment of many hereditary as well as acquired diseases (Segura et al., 2011). In contrast to other retroviral vectors which can transduce only dividing cells, lentiviral vectors (LV) have the ability to transduce non-dividing cells as well (Mátrai et al., 2010), thus offering a wide range of applications for LV in gene modified cell therapy and gene therapy (Aiuti et al., 2013; Cartier et al., 2009; Cavazzana-Calvo et al., 2010; Mátrai et al., 2010; Maude et al., 2014; Milone and O'Doherty, 2018; Turtle et al., 2016). LV are widely used for cancer immunotherapies introducing genes for a chimeric antigen receptor (CAR) into T cells. This enhances the specificity of T cells to redirect them to target antigens overexpressed on cancer cells that are not recognized by their endogenous receptors. The patient's T cells are modified to express a CAR construct and after *ex vivo* cell expansion are re-infused back to the patient (Miliotou and Papadopoulou, 2018; Milone and O'Doherty, 2018).

Today, there are three thousand clinical trials with viral vectors registered as ongoing, among which about 10 % are conducted with lentiviral vectors (John Wiley and Sons, Inc et al., 2019). The promising prospect of the ongoing clinical trials on gene therapy (Hanna et al., 2017; Herzog et al., 2010) means that there is a strong demand for scalable and cost-effective methods for production and purification of viral vectors. Especially the downstream process is considered to be a major bottleneck for the commercialization of gene therapies (Ruscic et al., 2019). Lentiviral vectors are spherical, enveloped particles with a diameter of 80−120 nm and are extremely labile. Therefore, it is mandatory to design downstream processing (DSP) strategies that suit the physicochemical properties of the vector (Segura et al., 2006).

Lentiviral vectors are typically produced by transient transfection of HEK293 T cells (Merten et al., 2014) with multiple vector plasmids (Sakuma et al., 2012). The viral vector is released into the supernatant. The LV is an enveloped virus, which buds through the membrane of the host cell thereby acquiring their lipid bilayer. For the membrane fission

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step Gag molecules recruit factors of the cellular endosomal sorting complexes required for transport (ESCRT) pathway (Votteler and Sundquist, 2013). The first DSP step, is the separation from the cells of the cell culture broth and clarification of the supernatant (Rodrigues et al., 2007). At harvest the cells reach a density $1-5\cdot10^6$ cells/mL for a batch process and $10-20\cdot10^6$ cells/mL for a perfusion process with a viability of 60–80 % (Manceur et al., 2017; Merten et al., 2014; Segura et al., 2007). Clarification has so far received little consideration and is performed as an obligatory step before other LV DSP steps to reduce bioburden and to increase capacity of further downstream steps, which have gotten more attention in terms of process development and optimization, such as ultrafiltration (Cooper et al., 2011; Geraerts et al., 2005; Papanikolaou et al., 2013) or chromatography (Cheeks et al., 2009; Chen et al., 2010; Kutner et al., 2009; Mekkaoui et al., 2018; Olgun et al., 2019; Segura et al., 2005, 2007; Yu and Schaffer, 2006).

Currently there is no established method for LV clarification to be considered as the gold standard. Clarification in a laboratory scale is typically performed by centrifugation of the fermentation broth, complemented with subsequent microfiltration of the supernatant (Bauler et al., 2020; Rodrigues et al., 2007; Segura et al., 2011). For microfiltration of LV a pore sizes of 0.45 µm is typically used (Bauler et al., 2020; Reeves and Cornetta, 2000), since viral particle loss was reported with smaller pore sizes (Segura et al., 2013). For scales greater than 1 L, a single microfiltration step is preferred, because centrifugation is poorly scalable (Besnard et al., 2016). However, this can lead to rapid clogging of the membrane. A possible approach to perform microfiltration in a single step is the use of filter aids (Doran, 2013). Filter aids like diatomaceous earth (DE) are skeletal remains of diatoms with a highly porous structure. DE forms a nearly incompressible porous cake and its high porosity allows liquid to flow around the particles preventing filter clogging (Grauf et al., 2018). Besides its use in blood and plasma fractionation, DE was established for the clarification of protein expressing high-density mammalian cell broths (van der Meer et al., 2014). The process was not adapted for LV yet, which is crucial, since a 1:1 transfer is unlikely due to the distinct bio- and physicochemical properties of the molecules. To our knowledge, clarification of lentiviral vectors has not yet been analyzed with the use of the filter aid DE.

Although, comprehensive studies on the clarification of viral vectors are rarely published in literature (Besnard et al., 2016), there is a need to optimize this step to minimize loss of the viral vector from the beginning of the whole downstream process. Inappropriate clarification processes can rapidly result in filter clogging, which reduces the actual pore size of the filter leading to virus retention (Segura et al., 2011) and thus reduced product yields. This work investigates the effect of DE on clarification performance and whether the usage of DE can effectively provide a LV clarification process, which is both easy to handle and yields high LV titers.

## 2. Materials and methods

### 2.1. HEK293T/17 SF cell expansion

Suspension HEK293T/17 SF cells (ACS-4500, ATCC) were expanded in CD293 medium (Thermo Fisher Scientific) supplemented with 10 µL/mL Insulin-Transferrin-Selenium (ITS; Thermo Fisher Scientific) and 8 mM glutamine (Thermo Fisher Scientific) in disposable non-baffled shake flasks of a size range from 125 mL to 1000 mL (Corning). Cells were passaged in a 2–3 day rhythm when reaching a cell density above $1\cdot10^6$ cells/mL. Before and after passaging the cells, the cell density and viability was determined (see section 2.7). Shake flasks were inoculated with a viable cell density of $2–3\cdot10^5$ cells/mL in 37.5 mL medium for a 125 mL shake flask (75 mL medium for a 250 mL shake flask, 150 mL medium for a 500 mL shake flask and 300 mL medium for a 1000 mL shake flask). Cultivation was performed at 37.0 °C, 8 % $CO_2$ and 130 rpm in an orbital shaking incubator (Sartorius).

### 2.2. Lentiviral vector production

Third generation lentiviral vectors were produced by transient transfection of suspension HEK293 T/17 SF cells with multiple plasmids in a UniVessel® single-use (SU) 2 L bioreactor (Sartorius). One day before inoculation, the UniVessel® SU 2 L was filled with 1.25 L FreeStyle293 medium (Thermo Fisher Scientific) supplemented with 10 µL/mL ITS and antifoam C (Sigma Aldrich) at a final concentration of 0.025 %. Cultivation parameter set-points were 37.0 °C, 30 % dissolved oxygen, pH 7.1 and stirring speed of 190 rpm. Process control was performed with a BIOSTAT® DCU (Sartorius). The next day, the bioreactor was inoculated with 180 mL HEK293 T/17 SF cell solution to a final cell density of $1\cdot10^6$ cells/mL. Two 125 mL shake flasks were inoculated with the same cell density as a positive reference control (virus production in shake flask) and a negative control (30 mL each). 24 h later, transfection with PEIpro DNA transfection reagent (Polyplus) and four plasmids in a ratio of 4:1 was performed. 0.5 µg total plasmid amount per $1\cdot10^6$ cells in a ratio of 5:2.5:1:1 (pALD-Lenti-anti-CD19-CAR-EGFRt : pALD-GagPol-A : pALD-VSV-G-A : pALD-Rev-A, Aldevron) were prepared in 100 mL production medium (1.5 mL for the shake flask controls). In a separate reaction tube PEIpro was diluted in the same volume of production medium. Diluted PEIpro was added to the diluted DNA, gently mixed and incubated for 15 min at room temperature. The mixture was added to the cells. A negative control without using a transfection reagent was prepared and treated equally as the virus production cultures. 7–8 h after transfection anti clumping agent (Thermo Fisher Scientific) was added at a 1:500 (v/v) dilution and sodium butyrate (Sigma Aldrich) was added at a final concentration of 10 mM. Additionally, antifoam C was added to give a final concentration of 0.025 %.

### 2.3. Harvest and DNA digestion

The lentivirus was harvested 72 h post transfection. DENARASE® (c-Lecta) and $MgCl_2$ (Carl Roth) were added to the cell culture broth (37 °C) one hour before harvesting at a final concentration of 10 U/mL and 2 mM, respectively. After nucleic acid digestion, the lentivirus containing cell culture broth was directly clarified.

### 2.4. Clarification

Clarification of 50 mL LV culture broth samples was performed with Sartoclear Dynamics® Lab V50 0.45 µm polyethersulfone (PES) version (Sartorius) with DE concentrations between 5 g/L to 40 g/L according to manufacturer's instructions. The filtration setup is shown in Fig. 1. The detailed acceptance criteria for the DE material are listed in the validation guide, which can be found on the manufacturer's homepage. Additionally, clarification of 50 mL cell culture broth was performed without DE in a two-step method of 5 min centrifugation at 800 x g and subsequent filtration through a Sartolab® RF50 with 0.45 µm PES membrane (Sartorius). Two distinct 0.45 µm PES membranes were compared with each other; a symmetrical and an asymmetrical type. For filter capacity determination, clarification was performed until filter clogging occurred and filtrate volume was determined.

### 2.5. Design of experiment

A design of experiment (DoE) based approach was performed to analyze the effect of the factors DE concentration and contact time of the lentivirus containing cell culture with DE on the responses infective LV titer, turbidity and filtration time, respectively. To this end, the DE concentration was varied between 5 g/L, 12.5 g/L and 20 g/L and contact time between 0 min, 10 min and 20 min. The experiments were planned as a full factorial design and evaluated using the MODDE® software (Version 12.1, Sartorius) with four center points, resulting in a total of 12 experiments.

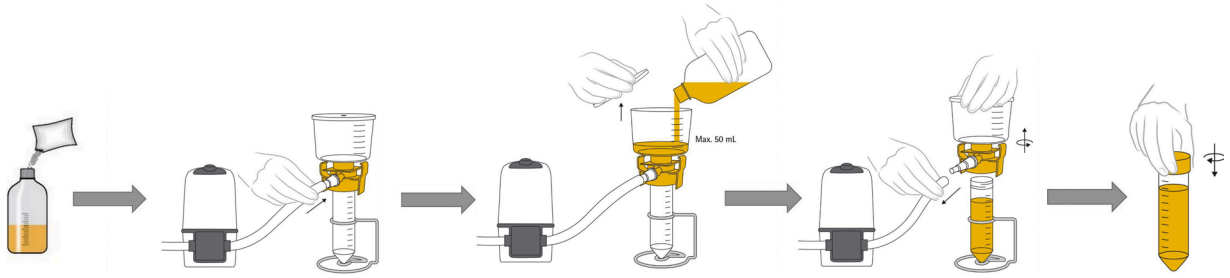


**Fig. 1.** Filtration setup with the filter aid diatomaceous earth. The filter aid is poured into the cell culture broth and mixed. The solution is filled into the funnel of the filtration device which is connected to a vacuum pump. After filtration is completed the vacuum tube is disconnected and the funnel unscrewed. The collection tube can be stored until further use.

### 2.6. Turbidity measurement

The turbidity of the bulk solution and the filtrates was measured using an Orion™ AQUAfast AQ3010 turbidity meter (Thermo Fisher Scientific) and is given in nephelometric turbidity units (NTU). The measurement was performed according to manufacturer's instructions.

### 2.7. Cell density and viability measurement

For determination of cell concentration and viability Cedex HiRes Analyzer (Roche) was used. Measurements were performed according to manufacturer's instructions.

### 2.8. Infectious LV titer determination

For quantification of the infectious lentiviral vector titer, adherent HEK293 T cells (ACC 635, DSMZ) were infected with serially diluted LV samples and the expression of the EGFRt-transgene-fusion protein was detected by flow cytometry. One day before infection $6{\cdot}10^4$ cells/well were seeded in 0.5 mL Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific) + 10 % fetal calf serum (FCS; Sigma Aldrich) (v/v) in a tissue culture (TC) treated 24-well plate (Greiner Bio-one). Cells were incubated at 37 °C and 5 % $CO_2$ in a static incubator (Sartorius). On the day of infection, culture medium was removed from the HEK293 T cells and the cells were transduced by transferring 0.5 mL of diluted virus solution. A negative control of the respective LV batch was analyzed as well. 8 µg/mL polybrene (Sigma Aldrich) were added to each well and the plate was incubated as before. 18 h post infection, the medium was removed and replaced with fresh culture medium without polybrene. 72 h post infection the expression of the gene-of-interest was analyzed by flow cytometry. First, the culture medium was removed from the cells, 200 µL trypsin-EDTA (Thermo Fisher Scientific) was pipetted to each well and incubated for 5 min at 37 °C. The enzymatic reaction was stopped by adding 500 µL culture medium, subsequently the plate was centrifuged at 300 x g for 5 min and the supernatant was removed. Cells were resuspended in 150 µL PBS and transferred to a non-TC 96-well plate with conical bottom (Sartorius). The 96-well plate was centrifuged and the supernatant was discarded. To discriminate viable and dead cells, 100 µL of a 1:1000 dilution (in PBS) of the fixable fluorescent dye Zombie NIR™ (BioLegend) was added to each well and incubated for 10 min in the dark. After another centrifugation step and removal of the supernatant, 100 µL Roti®-Histofix 4 % (Carl Roth) was added to the cells and incubated for 15 min. The supernatant was removed from the wells after centrifugation and cells were washed with 150 µL PBS. Hereafter, centrifugation of the plate was performed, the supernatant was removed and the cells were incubated for 30 min with an anti-human EGFRt phycoerythrin (PE) conjugated antibody (R&D Systems) at a 1:200 dilution in 40 µL staining buffer (1 % Bovine serum albumin (Carl Roth) in PBS). The cells were washed twice with 100 µL staining buffer. After each washing step the plate was centrifuged and the supernatant aspirated. Finally, the cells were resuspended in 40 µL staining buffer and flow cytometry was performed with an iQue ScreenerPlus flow cytometer (Sartorius). The obtained data was analyzed using the integrated ForeCyt 7.0 software. The percentage of EGFRt positive cells of viable single cells was determined. For calculation of the infective titer the virus dilution yielding between 5−20 % positive cells was selected. The functional lentiviral titer given in transducing units (TU) per mL was calculated using the following formula:

$$Infectious\ titer = \frac{P \cdot N \cdot D}{V \cdot 100}$$

With $P$ being the percentage of positive cells, $N$ being the number of cells at the time of transduction, $D$ is the dilution factor of the LV used for infection and the transduction volume $V$ in mL.

### 2.9. Total protein quantification

The Pierce™ Coomassie Bradford protein assay kit (Thermo Fisher Scientific) was used, according to the manufacturer's instructions, to quantify total protein concentrations. Standards and samples were analyzed in duplicates in transparent 96-well microtiter plates (Greiner Bio-one). The absorbance was read at 595 nm with a FLUOstar Omega plate reader (BMG Labtech). The obtained standard curve was fitted by linear regression.

### 2.10. Total dsDNA quantification

The dsDNA content was determined with the Quant-iT™ PicoGreen™ dsDNA assay (Thermo Fisher Scientific). The assay was performed according to manufacturer's instructions. Standards and samples were analyzed in duplicates. A 96-well black microplate (Corning) was used to perform the assay. Excitation was done at 480 nm and the fluorescence emission intensity was measured at 520 nm with a FLUOstar Omega plate reader. The obtained standard curve was fitted by linear regression.

## 3. Results

### 3.1. Evaluation of the impact of a filter aid on the processing time

Evaluation of the handling time for clarification of 50 mL lentivirus solution was compared by performing a single-step clarification with 5 g/L DE and a 0.45 µm PES filter or centrifugation with subsequent microfiltration of the supernatant through the same filter, which is often a lab-scale standard method. The entire processing time was broken down into preparation time (i.e. measuring culture volumes, removing supernatant after centrifugation, weighing of DE and addition of DE to the cell suspension), centrifugation time and filtration time and the results are shown in Fig. 2. The handling time for the conventional clarification method was about 8.5 min. During clarification of the centrifuged supernatant filter clogging occurred after approximately

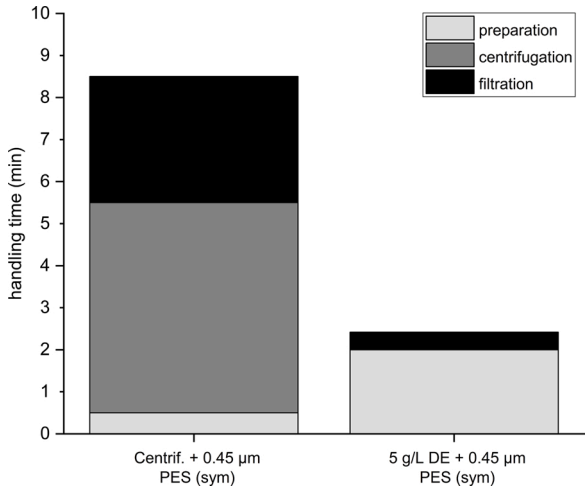


**Fig. 2.** Comparison of clarification methods regarding their processing time. The preparation, centrifugation and filtration time needed to clarify 50 mL mammalian cell culture containing lentiviral vectors with a total cell density of $1.1 \cdot 10^6$ cells/mL and a turbidity of 141 NTU using the conventional method of centrifugation combined with microfiltration or using 5 g/L diatomaceous earth (DE) and microfiltration for clarification. For both methods symmetrical (sym) 0.45 µm polyethersulfone (PES) membrane filters were used. Mean, N = 3.

25 mL. Hence, using a second microfilter was required and the remaining unfiltered solution had to be transferred to the second filter, which was laborious and complicated the overall handling. Using the same microfilter with 5 g/L DE reduced the total preparation time due to the lack of a need for a centrifugation step and resulted in a handling time of about 2.4 min. The use of 5 g/L DE reduced the handling time 3.5-fold compared to a standard centrifugation and microfiltration method.

### *3.2. Analysis of the effects of DE on lentivirus clarification performance by DoE*

The use of diatomaceous earth was hypothesized to lead to a higher turbidity reduction by removal of impurities due to retention by the porous structure of DE or adsorption of contaminants. With the intention of assessing potential adsorption and retention effects of the lentivirus by DE, filtrations with different DE concentrations were analysed. Preliminary experiments were performed to screen a wide range of DE concentrations from 5 to 60 g/L. Here, a DE concentration between 5–20 g/L, which is lower than the DE concentration of 40 g/L commonly used for protein clarification of a typical fed-batch high-density cell cultures, was identified as a suitable concentration range for further optimizations, due to a higher LV particle titer recovery (data not shown).

With the aim to investigate the effect of DE on LV clarification, the factors DE concentration (5−20 g/L) and contact time (0−20 min) with DE were selected for a Design of Experiment (DoE) approach. The experiments were planned as a full factorial design without replicates and evaluated using the MODDE® software. Contact time with DE could possibly be relevant if several samples of LV-containing broth have to be clarified at once and if they are e.g. filtered successively and not simultaneously and the operator prepares all samples in advance rather than one by one. The total cell density of the cell culture broth used for the filtrations of the DoE was $3.7 \cdot 10^6$ cells/mL with a turbidity of 382 NTU.

The DoE resulted in good models for each of the responses with R2 = 0.96 and Q2 = 0.88 for the turbidity, R2 = 0.82 and Q2 = 0.75 for the infective LV titer, R2 = 0.98 and Q2 = 0.97 for the filtration time. R2 indicates how well the model fits the data and Q2 how well the model predicts new data with a maximum value of 1 for both coefficients. The effect of each factor on the responses was assessed by analysis of the regression coefficient plot (Fig. 3A) and the response contour plot (Fig. 3B). The regression coefficient plots indicate how the two factors of the model influence the responses. The response contour plots show the dynamic of each response depending on the set-points of the factors. Fig. 3 shows that the responses infectious titer and filtration time are only affected by the factor DE concentration. With increasing DE concentration the infectious titer is reduced linearly. For the filtration time a quadratic term for the DE concentration exists, leading to a decrease of filtration time with increasing DE concentration of the tested concentration range. The turbidity was linearly reduced with increasing contact time of the sample with DE. Furthermore, with increasing DE concentration the turbidity was reduced (quadratic term), with the latter being the factor with the greater effect.

The design space is “the multidimensional combination and interaction of input variables (e.g., material attributes) and process parameters that have been demonstrated to provide assurance of quality” (U.S. Department of Health and Human Services et al., 2009). The design space of the process was estimated and is shown in Fig. 4. The green region of the graph has a low probability of failure and is named design space (Eriksson, 2008). The calculation for the design space is consider the model error as well as the precision of set-points (e.g. considering the calibration error). The response specifications were set to a moderate target turbidity (15 NTU) and filtration time (35 s) and to a high infectious LV titer ($1.9 \cdot 10^7$ TU/mL), which was our major interest for the clarification step. In Fig. 4 the green area indicates a probability of failure of 0.5 % for all responses. That means when selecting parameter combinations within the green area in less than 0.5 % of the process the specification will not be met for at least one of the responses. A DE concentration between 6−12 g/L yields acceptable process response values with a low probability of failure independent of the selected contact time. The optimizer function of MODDE® was used to find a robust set-point with the following criteria: Maximizing infective LV titer and minimizing turbidity and filtration time. The calculated robust set-point is located in the center of the design space with 9 g/L DE and an incubation time of 14.67 min. The robust set-point outlines, that a far lower DE concentration, compared to the DE concentration of 40 g/L recommended for protein clarification, is optimal to achieve a high LV titer.

### *3.3. Comparison of microfilter membrane structures and clarification methods*

Two 0.45 µm PES membrane microfilters were analyzed, having either a symmetrical or asymmetrical membrane structure. The two membranes were compared with each other to investigate whether the use of asymmetrical membranes increases filter capacity and LV titer recovery due to its higher permeability, which could potentially reduce virus entrapment. Clarification was performed either with 5 g/L DE or with the standard method by centrifugation of the cell culture broth and subsequent filtration. Resulting infectious LV titers, as well as turbidities of the filtrates are shown in Fig. 5. According to Fig. 5, no significant impact of a symmetrical and an asymmetrical membrane structure on the infectious LV titer could be observed for both of the methods. When comparing the two clarification methods, the two-step clarification method combining centrifugation and filtration leads to a significantly higher infectious LV titer. After clarification with DE, the infectious LV titer was about 65 % of the titer obtained with the standard method centrifugation and clarification. However, centrifugation and filtration results in a significantly higher final turbidity of 30–43 NTU depending on the membrane structure. This corresponds to a turbidity reduction of 89–92 % compared to the starting material, whereas with DE turbidity is reduced by 95 % down to 1820 NTU. When filtrating the centrifuged supernatant the turbidity is with a difference of 12.67 NTU significantly ($p \leq 1$ %) higher with the use of an asymmetrical membrane compared to the symmetrical membrane. When using 5 g/L DE the turbidity of the

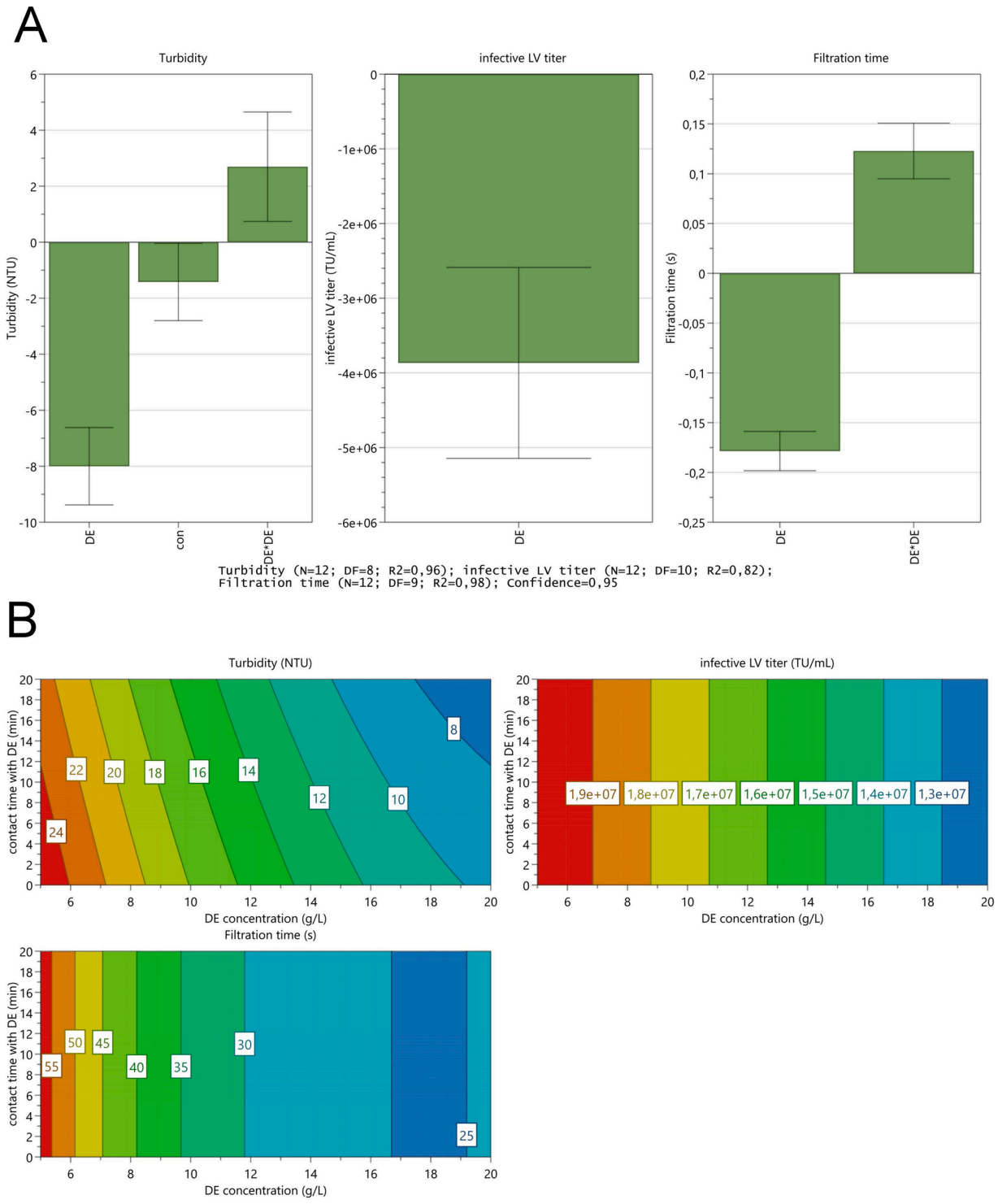


**Fig. 3.** Effects of diatomaceous earth (DE) on lentivirus clarification performance. Regression coefficient plot (A) and response contour plot (B) for the factors DE concentration (DE) and incubation time (time) on the responses turbidity, filtration time, and infectious lentiviral vector (LV) titer.

filtrates differs only by 2.55 NTU between the two membrane types, which is not a significant difference. This shows that there is no significant difference in final NTU using DE in combination with different membrane structures, whereas the latter has a great impact on final NTU when no DE is used. Moreover, filter clogging occurred after 15 mL for the symmetrical membrane and after 33 mL for the asymmetrical membrane when using it in combination with standard centrifugation method. This means, that four symmetrical or two asymmetrical filter devices would have been necessary to filtrate the target volume of 50 mL. Whereas, only one filter device was needed when using DE, since no filter clogging was observed when filtrating 50 mL.

### 3.4. Impurity removal capacity by different concentration of DE

The potential to remove process related impurities was analyzed by total protein and dsDNA quantification and the results are listed in Table 1. Several DE concentrations have been tested and compared to the standard method of centrifugation with subsequent filtration. It

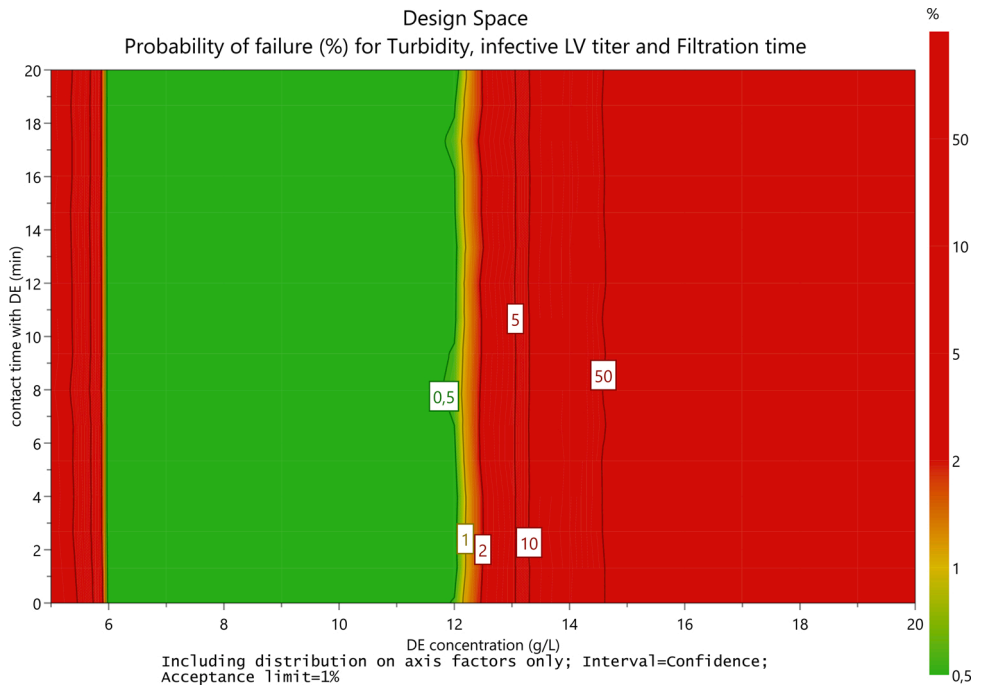


**Fig. 4.** Design space for clarification of lentiviral vectors (LV) with diatomaceous earth (DE). Design space showing the probability of failure in % to meet the response specifications for turbidity, filtration time and infectious LV titer.

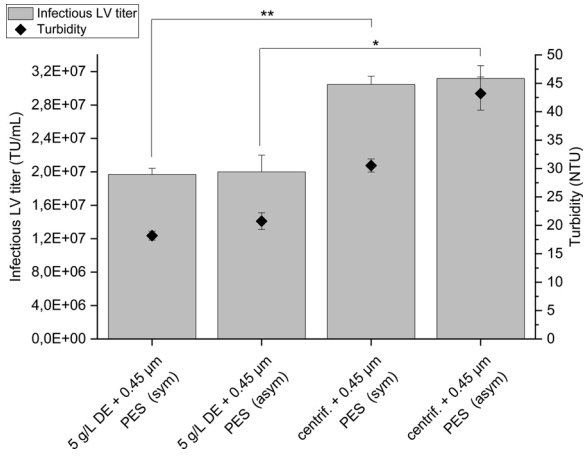


**Fig. 5.** Effects of clarification methods and membrane structures on turbidity and infectious lentiviral vector (LV) titer. Turbidity and infectious lentiviral vector titer after clarification with 5 g/L diatomaceous earth (DE) using either a symmetrical (sym) or asymmetrical (asym) 0.45 µm polyethersulfone (PES) membrane or by centrifugation of the culture broth and filtration through the same filter types. The LV containing cell culture broth had a cell density of $3.7 \cdot 10^6$ cells/mL and a turbidity of 398 NTU at the time of harvest. Mean ± standard deviation, N = 3, data was analyzed with unpaired *t*-test (* $p \leq 5$ %, ** $p \leq 1$ %).

should be investigated whether the use of DE has an advantage in terms of process related impurity removal towards the standard method and whether low DE concentrations yield a sufficient impurity reduction level.

According to Table 1, the use of 5 g/L DE with 0.45 µm PES (sym) membrane filter resulted in a lower impurity concentration in the filtrate compared to centrifugation combined with filtration with the same membrane. A DE concentration of up to 10 g/L DE resulted in reduced protein and dsDNA concentration, but higher DE concentrations between 10 g/L and 40 g/L did not lead to any further improvement of impurity removal.

**Table 1**
Impurity concentration and removal of different clarification methods. Analyzed clarification methods were clarification with DE and centrifugation with subsequent clarification of the supernatant. Microfilter PES membranes had a symmetrical (sym) structure. Total protein and dsDNA concentration are given as mean ± standard deviation, N = 3.

| Clarification method | Total protein concentration (µg/mL) | dsDNA concentration (ng/mL) | Removal of protein (%) | Removal of dsDNA (%) |
|---|---|---|---|---|
| 5 g/L DE +0.45 µm PES (sym) | 101.4 ± 8.1 | 144.7 ± 1.2 | 21.2 ± 6.3 | 26.3 ± 0.6 |
| 10 g/L DE +0.45 µm PES (sym) | 45.1 ± 6.7 | 116.9 ± 5.2 | 65.0 ± 5.2 | 40.4 ± 2.6 |
| 40 g/L DE +0.45 µm PES (sym) | 52.1 ± 6.3 | 114.0 ± 3.3 | 59.4 ± 5.0 | 41.9 ± 1.7 |
| Centrifugation +0.45 µm PES (sym) | 117.9 ± 6.3 | 151.0 ± 1.6 | 8.3 ± 4.9 | 23.0 ± 0.8 |

### 3.5. *Determination of filter capacities and the effects of the use of a filter aid*

Filter capacity is an important criteria when selecting the appropriate filter for a specific production process. A high filter capacity can reduce costs and foot-print of a viral vector downstream process. Filter aids can significantly increase filter capacities due to an increased porosity of the filter material. In this experiment filter capacities have been determined by using only a minimal DE concentration of 5 g/L for clarification with a 0.45 µm PES membrane filter (either symmetrical or asymmetrical membrane structure), or by performing centrifugation and subsequent filtration of the supernatant through the same membrane types. Filtration was performed until filter clogging occurred. The lentivirus containing cell culture broth used for filter capacity determination had a total cell density of $3.7 \cdot 10^6$ cells/mL and a turbidity of 398 NTU at the time of harvest. The filtrate volumes until filter clogging

occurred and respective volumetric flow rates are listed in Table 2.

Performing the conventional lab-scale method of centrifugation for cell removal and clarification of the supernatant resulted in rapid filter clogging. The used filtration device Sartolab® RF50 is designed to filtrate up to 50 mL. This volume was not reached with the conventional method. Using 5 g/L DE as a filter aid increased the maximal filtration volume immensely and exceeded the maximal filtration volume of the used filtration device more than 2-fold. Moreover, the asymmetrical structure of the microfilter allowed filtration of an even larger sample volume.

The filter capacities for the different clarification processes were calculated and are shown in Fig. 6. According to Fig. 6, filter capacities were low when performing conventional clarification by centrifugation and subsequent filtration with 7 L/m$^2$ for the symmetrical and 15 L/m$^2$ for the asymmetrical membrane. Filter capacities increased 8.5-fold when using 5 g/L DE in combination with the symmetrical membrane and 4-fold when using the asymmetrical membrane structure to about 60−63 L/m$^2$. The asymmetrical PES membrane structure had a significantly higher membrane capacity compared to the symmetrical PES membrane for both methods.

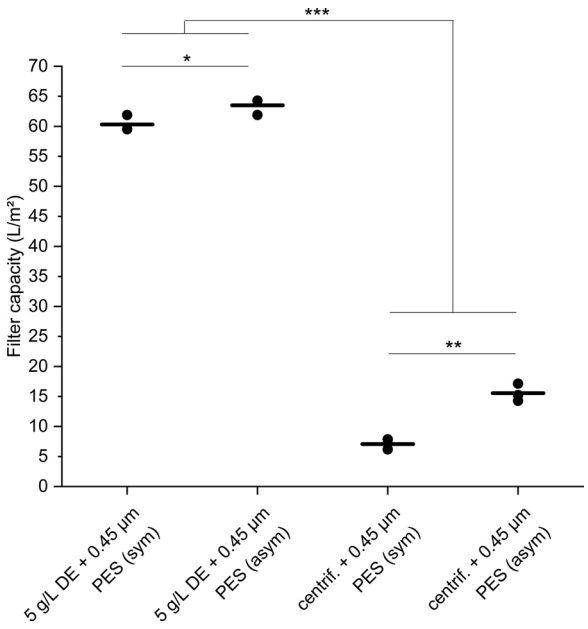


**Fig. 6.** Filter capacity determination for different clarification processes and membrane structures. Capacities of filters (in L /m$^2$) used for clarification either by 5 g/L diatomaceous earth (DE) and 0.45 µm polyetherfulfone (PES) (symmetrical or asymmetrical structure) or by centrifugation and subsequent microfiltration of the supernatant through the same membrane filters. N = 3, mean is shown as horizontal line, data was analyzed with unpaired *t*-test (* p ≤ 5 %, ** p ≤ 1 %, *** p ≤ 0.1 %).

## 4. Discussion

### 4.1. Evaluation of the impact of a filter aid on the processing time

The handling time is an important factor to obtain an efficient downstream process. Since rapid filter clogging occurred when performing the standard method of centrifugation and subsequent microfiltration, a second filter was required and the remaining solution had to be transferred to the second filter. This not only leads to a doubled material consumption, but also means complicated and unsafe handling for the user due to the risk of spillage of the lentiviral vector. In contrast, clarification with DE offered a safe and easy handling procedure for the user with less material consumption. Besides that, clarification is significantly accelerated when using DE compared to the two-step classical method of centrifugation and filtration. This is beneficial not only in terms of efficiency, but also to recover infectivity of the fragile lentiviral vector, which has a high decay rate (Higashikawa and Chang, 2001; Segura et al., 2005) and should therefore be processed in a short time frame at room temperature.

### 4.2. Analysis of the effects of DE on lentivirus clarification performance by DoE

With the DoE approach the DE concentration and incubation time were identified as factors affecting clarification performance in terms of turbidity reduction, filtration time and recovery of infectious LV titer. Although the design space is quite large, the operator should consider that higher DE concentrations reduce the LV titer. A reduced LV recovery may occur due to retention of the LV by the highly porous structure of DE containing up to 8090 % voids (Bakr, 2010) with pore sizes of about 1.522 µm (Wypych, 2016). With a size of about 80−120 nm (Segura et al., 2006) the lentiviral vector should be able to diffuse into the pores of DE. Another aspect to consider is the positive charge of DE which might result in adsorption of the negatively charged LV (Besnard et al., 2016). If the major aim of the operator is a short filtration time and high turbidity reduction, higher DE concentrations would provide this. This correlation can be associated to possible retention of impurities by the porous structure of DE (Bakr, 2010). The robust set-point identified with the Optimizer function of MODDE® software suggests to use an incubation, because the robust set-point is positioned in the center of the design space area with the lowest probability of failure and having the largest distances to its boundaries. The only positive effect of the incubation time on clarification performance was observed for the final turbidity. However, this effect was slight, therefore it is not recommended to let the lentiviral vector incubate with DE, if this can be avoided. Depending on the major interest of the operator (high reduction of turbidity, fast filtration or high lentivirus titer recoveries) a suitable DE concentration should be selected.

Moreover, the operator may have to adjust the DE concentration to the characteristics of his cell culture broth to be clarified. The HEK293 T cell density at time of transfection is typically between $8 \cdot 10^5$ cells/mL and $1.5 \cdot 10^6$ cells/mL (Merten et al., 2016). When producing the lentivirus in a classical batch process the cell density would reach approximately up to $5 \cdot 10^6$ cells/mL at time of harvest (Merten et al., 2014). These cell densities are far lower compared to the ones of classical protein production processes. Typical protein-producing cell cultures, like CHO cell lines, result in higher final cell densities of about $20 \cdot 10^6$ to $30 \cdot 10^6$ cells/mL for high density cell cultures in a 10–14 day fedbatch process (Yongky et al., 2019) for which the use of filter aids for clarification has great benefits. The wet cell weight (WCW) of a CHO cell culture of $25 \cdot 10^6$ cells/mL is about 8 % (w/v). A DE concentration of about 50 % of the wet cell weight is recommended to use (van der Meer

**Table 2**
Filtrate volumes until the occurrence of filter clogging and volumetric flow rates by performing different clarification strategies. Analyzed clarification methods were clarification with 5 g/L DE versus centrifugation and subsequent filtration of the supernatant. PES membranes had either a symmetrical (sym) or asymmetrical (asym) structure. Filtered volumes are given as mean ± standard deviation, N = 3.

| | 5 g/L DE +0.45 µm PES (sym) | 5 g/L DE +0.45 µm PES (asym) | Centrifugation +0.45 µm PES (sym) | Centrifugation +0.45 µm PES (asym) |
|---|---|---|---|---|
| Filtrate volume (mL) | 126.7 ± 2.9 | 133.3 ± 2.9 | 14.8 ± 1.8 | 32.7 ± 3.1 |
| Volumetric flow rate (L/m$^2$h) | 921.1 ± 61.3 | 1304.4 ± 286.6 | 631.5 ± 119.4 | 935.4 ± 112.4 |

et al., 2014), which would result in this case in a DE concentration of 40 g/L. The cell density and WCW was far lower for our lentiviral vector production protocol than for a typical therapeutic protein production process, with a maximum total cell density of $3.7 \cdot 10^6$ cells/mL and a WCW of 1.3 %. Hence, a lower DE concentration range appears to be more suitable for the lentivirus clarification. Half of the highest WCW obtained in this study would correspond to a DE concentration of 6.5 g/L. The experiment showed, that even a lower DE concentration of 5 g/L was sufficient to avoid filter clogging.

### *4.3. Comparison of membrane structures and clarification methods regarding lentivirus recovery, impurity reduction and filter capacity*

Comparison of the filter capacities of the different clarification methods highlighted the increase of filter capacity when using DE compared to a classical centrifugation plus microfiltration approach. Since the filter capacity highly depends on the characteristics of the cell culture broth, mainly cell density and viability (Immarino et al., 2007), results will vary from batch to batch. In our experiment the target volume for the filtration device of 50 mL was exceeded about 2.5-fold. This illustrates the robustness of the method, where a successful filtration of 50 mL culture volume with 5 g/L DE can be expected even with varying LV batches. The classical lab scale clarification method of centrifugation and filtration, however, seems to be more susceptible to batch variabilities, since filter clogging occurred after varying volumes depending on the LV batch. In contrast, clarification with 5 g/L DE was always successful. For a lentivirus containing cell culture with a low total cell density of $1.1 \cdot 10^6$ cells/mL and a turbidity of 141 NTU, filter clogging of the centrifuged supernatant already occurred after 25 mL, whereas for the two LV batches with approximately $3.7 \cdot 10^6$ cells/mL and a turbidity around 380–390 NTU, filter clogging occurred already after 15 mL using a symmetrical 0.45 µm PES membrane in both cases. Since the cells are completely removed during centrifugation, filter clogging depended on the turbidity of the supernatant with higher turbidities indicating more impurities that can potentially block the filter membrane. The robustness of the classical clarification method may therefore be rated as lower. On one hand, performing membrane filtration in a cross-flow filtration mode could possibly increase the filter capacity when filtrating the LV supernatant after centrifugation, since cross-flow filtration minimizes filter cake formation and consequently delaying filter blockade. On the other hand, cross-flow filtration increases shear stress which is a disadvantage when working with enveloped fragile viruses like LV. During dead-end filtration, exposure time to shear stress is short since the LV undergoes a rapid single pass (Besnard et al., 2016). Therefore, we assume, that dead-end filtration is a milder operation mode for the clarification process.

Impurity reduction increased with increasing concentration of DE, although between 10 g/L and 40 g/L there could not be observed any further improvement of impurity removal. Compared to centrifugation and microfiltration the use of DE resulted in a higher impurity removal, but with 5 g/L DE only slightly performing better. Hence, in order to achieve a sufficient impurity reduction level higher DE concentrations should be used.

Filter capacities were higher with an asymmetrical membrane structure. This can be explained by the depth filter-like structure of the membrane. The pore size at the surface site of the membrane is greater than 0.45 µm, but decreases towards the filtrate site where the pores have a size of 0.45 µm. In contrast, the symmetrical membrane has a consistent pore size from the surface site to the filtrate site. The asymmetrical membrane is more permeable overall and is known to have higher capacities (Gottschalk, 2009; Li et al., 2009), which was observed in this study as well. However, a disadvantage of a higher permeability of the asymmetrical membrane is that the degree of contaminant removal, e.g. turbidity, is lower, allowing more impurities to pass the membrane. The difference in filtrate turbidity between the two membrane structures is much more pronounced when filtrating the centrifuged supernatant (12.67 NTU difference) than with clarification using DE. For the latter method, the turbidities differ only by 2.55 NTU. This could be explained by the possibility that mainly DE is involved in depletion of impurities and the membrane structure effects are less visible than with the standard method. The final turbidity was lower when DE was used, although a turbidity below 10 NTU, which is commonly targeted, could not be achieved with low DE concentrations, like 5−10 g/L. Hence, to achieve this, a second microfiltration step would be necessary or the use of higher DE concentrations of about 20 g/L as predicted by the DoE model. At the same time LV titer would decrease with increasing DE concentration, thus the operator needs to consider his main objectives and find a compromise between these two conditions.

## 5. Conclusion

Suspension based upstream processes for the production of lentiviral vectors have become of high interest in the last few years. Although clarification has so far received little attention during LV DSP optimization, it is a critical step for the purification of viral vectors and has a strong impact on product yield and process reproducibility (Besnard et al., 2016). In this work, the main goal was to analyze the effect of applying a filter aid, like DE, on clarification of lentiviral vectors produced by transient transfection of HEK293 T suspension cells.

We could show that turbidity was reduced more efficiently with the use of DE. Higher DE concentrations are beneficial for a higher impurity reduction and to achieve shorter filtration times, but at the same time LV titer decreases with increasing DE concentration, thus the operator needs to consider his main objectives. A DE concentration of 9 g/L was identified as a robust set-point and incubation time with DE should be avoided. Besides an improved turbidity reduction, clarification with the filter aid DE has other advantages that may offset the virus titer loss. Clarification with DE allows the operator to perform a clarification process, which does not only facilitate faster and safer handling, but enables a lower material consumption due to the extremely increased filter capacity, thus representing an efficient and robust clarification process. The use of an asymmetrical membrane structure further increases filter capacity, but leads to a reduced removal of impurities. Usage of DE for the clarification of lentiviruses produced in suspension culture may become a commonly applied laboratory method in the future. In terms of scalability lab scale virus clarification can be covered with the Sartolab MultiStation which can hold six bottle top filters for filtration of 1 L virus solution each, allowing simultaneous clarification of 6 L. Currently the connection of 2 L bottle top filters is under development, which would then facilitate clarification of up to 12 L. Certainly vacuum filtration has its limitations here and for larger volumes other solutions must be found. An application for other viral vectors is conceivable and should be investigated.

## CRediT authorship contribution statement

**Jennifer J. Labisch:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing - original draft. **Franziska Bollmann:** Supervision, Writing - review & editing. **Michael W. Wolff:** Supervision, Writing - review & editing. **Karl Pflanz:** Supervision, Project administration.

## Declaration of Competing Interest

The authors report no declarations of interest.

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