*Author manuscript*

# Optimization and Dose Estimation of Aerosol Delivery to Non-Human Primates

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Running title: Aerosol dosing in macaques

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## Abstract

***Background:*** In pre-clinical animal studies the uniformity of dosing across subjects and routes of administration is a crucial requirement. In preparation for a study in which aerosolized live-attenuated measles virus vaccine was administered to cynomolgus monkeys (*Macaca fascicularis*) by inhalation, we assessed the percentage of a nebulized dose inhaled under varying conditions.
***Methods:*** Drug delivery varies with breathing parameters therefore we determined macaque breathing patterns (tidal volume, breathing frequency and inspiratory to expiratory (I:E) ratio) across a range of 3.3 - 6.5 kg body weight, using a pediatric pneumotachometer interfaced either with an endotracheal tube or a facemask. Subsequently, these breathing patterns were reproduced using a breathing simulator attached to a filter to collect the inhaled dose. Albuterol was nebulized using a vibrating mesh nebulizer and the percentage inhaled dose was determined by extraction of drug from the filter and subsequent quantification.
***Results:*** Tidal volumes ranged from 24 to 46 ml, breathing frequencies from 19 to 31 breaths per minute and I:E ratios from 0.7 to 1.6. A small pediatric resuscitation mask was identified as the best fitting interface between animal and pneumotachometer. The average efficiency of inhaled dose delivery was 32.1% (standard deviation 7.5, range 24-48%), with variation in tidal volumes as the most important determinant.
***Conclusions:*** Studies in non-human primates aimed at comparing aerosol delivery with other routes of administration should take both the inter-subject variation and relatively low efficiency of delivery to these low body weight mammals into account.

# Introduction

Aerosol inhalation is used routinely in respiratory medicine for pulmonary drug delivery.[1] The most common application is treatment of chronic pulmonary diseases such as asthma and cystic fibrosis.[2, 3] In recent years aerosol inhalation has also been considered as an alternative route of vaccine delivery.[4-6] Vaccination is mostly performed in children, and this presents particular challenges for efficient pulmonary delivery and dose control.[7, 8] This is especially true in very young infants who have low tidal volumes[9] and variable breathing patterns.[10] In addition, small children often do not tolerate aerosol masks, and agitation may cause air leakage at the edges of the facemask.[11-13] As a result, the efficiency of lung deposition in infants may fall below 1%.[14, 15]

New drugs and biologicals for which human efficacy studies are not ethical or feasible may be evaluated according to the “animal rule”, which requires well-characterized animal models .[16] In some cases such studies need to be performed in non-human primates. Aerosol delivery to non-human primates meets with similar challenges as those seen for aerosol delivery to infants.[14, 17] Macaques have low body weights and hence low tidal volumes, they are obligate nose breathers, and are not cooperative. In many preclinical animal studies this has been solved in part by using exposure chambers that fit the complete head of the animal.[18, 19] However, this method has practical limitations for the administration of vaccines, where direct administration to the airways is preferable. We have previously used a nebulizer in combination with a facemask to deliver measles virus (MV) vaccine[20] or wild-type MV [21, 22] to sedated cynomolgus macaques (*Macaca fascicularis*). Although these studies were successful, they allowed limited control over the actual inhaled dose, amongst others because the facemask used was not tight fitting. In preparation for a measles vaccination study in macaques designed to compare different routes of vaccine administration, we set out to optimize aerosol delivery options and quantify inhaled doses of macaques. In this study, we describe a series of experiments performed to obtain basic information on respiration characteristics of sedated cynomolgus macaques, to identify an optimally-fitting facemask, and to perform dose-normalization studies.

# Materials and Methods

### *Ethics statement*

The animal studies were performed in subadult male cynomolgus monkeys (Table 1). Animal experiments were conducted in compliance with European guidelines (EU directive on animal testing 86/609/EEC) and Dutch legislation (Experiments on Animals Act, 1997). The protocol (nr. EMC2373) was approved by the independent animal experimentation ethical review committee DCC in Driebergen, The Netherlands. This committee is not affiliated to the Erasmus MC, where the experiments were performed. Animals were housed in groups, received standard primate feed and fresh fruit on a daily basis and had access to water *ad libitum*. In addition, their cages contained multiple sources of environmental enrichment in the shape of hiding places, hanging ropes, tires and other toys. Animal welfare was observed on daily basis, and animal handling and tidal breathing measurements were performed under light anesthesia using a cocktail of ketamine and medetomidine (intramuscular dose of 10 and 0.04 mg/kg bodyweight,

respectively). After handling, atipamezole was administered to antagonize the effect of medetomidine. No animals were sacrificed during this study.

***Measurement of macaque breathing patterns***

A pediatric pneumotachometer (MasterScreen Paed, Jaeger, CareFusion) in combination with JLAB 4.67 software was used to measure tidal volume ($V_t$), breaths per minute (BPM) and ratio of inspiratory to expiratory time (I:E ratio). Measurements consisted of duplicate or triplicate assessment of runs of approximately 20 breaths for each animal. The five lowest and highest values were automatically discarded to provide average values for the median ten measurements. Initially tidal breathing measurements were performed after intubation with a 3.5 mm or 5 mm endotracheal tube, subsequent measurements were performed using facemasks.

***Selection of macaque facemasks***

Several pediatric facemasks of different sizes and brands were evaluated for their potential fit on the faces of macaques. These masks were evaluated by comparing breathing parameters measured using the pneumotachometer through the endotracheal tube with those obtained through the facemask. The majority of facemasks resulted in considerable air leakage, resulting in invalid tidal breathing measurements. Based on this preliminary screening, two pediatric resuscitation masks were selected for further testing: LSR Silicone no. 0/1 (Laerdal 851600) and no. 0/0 (Laerdal 851500). Similar masks had been previously identified as highly efficient in achieving a tight seal in infants.[11]

***Breathing simulation: aerosol efficiency and mass balance measurement***

A breathing simulator (ASL5000, Ingmar Medical), programmed to simulate the recorded macaque breathing patterns, was attached to an absolute filter (Respirgard 303, Baxter). To determine delivered dose, a nebulizer (Aerogen Pro, Aerogen Ireland Ltd., mass median aerodynamic diameter 3.5 µm) with adapter (22 mm T-piece, Aerogen) was attached to the collecting filter (Figure 2E). A nominal dose of 0.5 ml (2.5 mg) albuterol sulphate (Ventolin, 5 mg/ml, Allen & Hansbury) was nebulized in each test run. Albuterol was eluted from filter or nebulizer components using a 1:4 mix of 96% (v/v) HPLC-grade ethanol in water. Albuterol mass, expressed as a fraction of the nominal dose, was determined using UV spectrophotometry (Biochrom UV Vis, Cambridge, UK) and interpolation on a standard curve at 276 nm. Albuterol was used as it is a commonly nebulized formulation used in the characterization of aerosol drug delivery systems, and is specified for use as a tracer aerosol in the international standard.[23] Previous comparison of nebulization of albuterol or measles vaccine using a similar vibrating mesh nebulizer had shown no differences in output rate or VMD.[24] Mass balances were also recorded for all four configurations. Mass balance characterization of configuration D with the breathing simulator is shown in Figure 2E. All dosing times were recorded. All test iterations were run in triplicate.

# Results

***Macaque breathing patterns***

Plethysmography was used to record the breathing parameters in subadult cynomolgus macaques. Sixteen valid measurements were obtained (Table 1): three by endotracheal intubation (A), four by using the larger facemask (B) and nine by

using the smaller facemask (C). Of the facemasks tested, the large resuscitation mask (Laerdal 0/1) showed significant air leakage in the majority of animals tested (Figure 1A), resulting in invalid measurements that were excluded from subsequent analysis. The small resuscitation mask (Laerdal 0/0) showed the best fit to the face of macaques. As macaques are obligate nose breathers, the best way to fit the mask proved to be placing the mask on the face of the animal, and subsequently move it to a slightly upward position (Figure 1B). Measurements obtained from a representative individual animal are shown as examples (Figure 1C and D). Data obtained with the smaller facemask were used for all further bench testing.

**Dose delivery**

A breathing simulator was used to characterize the dose efficiency of four nebulizer configurations (Figure 2 A-D). The first configuration (Figure 2A) was previously used for experimental MV infections of macaques.[21, 22] In a second configuration, the small connector between T-piece and breathing simulator was removed to prevent obstruction of airflow and reduce impactional aerosol losses exiting the nebulizer (Figure 2B). Subsequently, configurations C and D were prepared by using a neonate T-piece (C, diameter 12 mm) or an adult T-piece (D, diameter 22 mm) extended with a straight 22 mm tube. To compare the four configurations, the delivery efficiency was determined by measuring the percentage inhaled dose of albuterol sulphate as a tracer compound, using the average values obtained using the small resuscitation mask (Table 1, C-AVG) to program the breathing simulator.

The best performing configurations were D and B. The difference in respective inhaled doses for configurations D and B were not statistically significant ($p=0.443$), however, on the basis that configuration D did deliver a higher inhaled dose, with a low standard deviation between runs, it was decided to proceed with configuration D. Furthermore, configuration D delivered a significantly higher inhaled dose than both configuration A ($p<0.004$) and configuration C ($p<0.001$) (Table 2, see values shown in column 'Inhalation filter'). The mean time to delivery of the 0.5 ml dose was 75 ± 1 seconds (approximately 0.4 ml of albuterol sulphate/min) across all tests carried out.

**Mass balance measurements**

The results showed that for all configurations more than half the dose remained in the T-piece as condensate (Table 2 and Figure 2F). The highest levels of condensate in the T-piece were recorded in configurations A and C. The remaining fractions were detected in the inhalation filter, exhalation filter and nebulizer as residual mass. The standard deviations recorded for the test iterations were low, indicating good reproducibility of dosing between replicate measurements.

**Influence of tidal volume ($V_t$), respiratory rate and I:E ratio on inhaled dose efficiency**

At average $V_t$ values, the influence of maximal or minimal respiratory rate (BPM) and/or I:E ratios (Table 1) on dose efficiency was relatively limited (Table 3). However, at average BPM or I:E, the $V_t$ had a major effect on inhaled dose efficiency. These results are in agreement with the convention that larger animals will usually inhale a greater dose. Interestingly, with average BPM this was especially notable in combination with low I:E ratios (Table 3). A Pearson correlation coefficient analysis of all combinations of breathing parameters was carried out. Following this analysis, the only significant relationship identified was between $V_t$ and dose efficiency ($p = 0.002$).

The Pearson correlation coefficient was 0.786, confirming that a strong direct relationship exists between the two.

Further, a regression analysis was conducted in an attempt to identify a mathematical prediction model for dose efficiency. The resulting equation was as follows: [Dose efficiency = 6.28 + 0.537 $V_t$ + 0.290 BPM - 4.15 I:E], with a $R^2$ of 72.2 %. As expected from the previous analysis, the most significant variable impacting dose efficiency was confirmed to be $V_t$ (p=0.003). Whilst a $R^2$ of 72.2 % is relatively strong, it can predict only 72.2 % of the variability in this system. Means of improving this correlation include increasing animal numbers, increasing measurement sensitivity and other variations in the experimental setup.

# Discussion

The goals of this study were to obtain baseline data for breathing characteristics of sedated cynomolgus macaques, determine the best fitting facemask as optimal interface between animal and nebulizer and assess the efficiency of inhaled dose delivery over a range of representative macaque breathing parameters. To achieve these goals, a pediatric pneumotachometer was used for tidal breathing measurements in animals of different body weights, and the obtained values were used to program a pediatric breathing simulator and assess delivery efficiency using a commonly used “tracer” aerosol.

The tidal breathing measurements in macaques were not designed to compare baseline data obtained through endotracheal tube or resuscitation mask. Instead, they were intended to select the best fitting facemask in preparation of a subsequent vaccination study, and use this mask to collect baseline data for bench testing of dose delivery. This resulted in the unbalanced datasets presented in table 1. After three successful measurements of baseline characteristics through an endotracheal tube (dataset A) we saw no reason (and hence considered it unethical) to repeat these measurements in more animals. After several unsuccessful measurements with the large resuscitation mask (dataset B in table 1 only includes the measurements that resulted in valid data) we again considered it unethical to continue measurements in more animals. It was only with the small resuscitation mask (dataset C) that we were able to be able to reproducibly collect useful data from several different animals. The datasets for A and B are presented for information only in Table 1. By tilting the facemask upwards (Figure 1B), we were able to allow normal breathing with minimal air leakage. Breathing parameters showed substantial variation between animals (Table 1), of which some could be explained by differences in body weight (tidal volumes) while others seemed to be related to either individual differences or depth of anesthesia. Of note regarding the relevance of this study, the average tidal volume of 7 ml per kg body weight (Table 1) is almost identical to that of children.[25, 26] All tidal volume measurements were performed under anesthesia using ketamine and medetomidine, both agents known to potentially suppress respiration.[27, 28] However, in our experience animals this sedation protocol results in reproducible and stable breathing. Interestingly, tidal volume and respiratory rate data obtained were close to those previously reported for conscious cynomolgus macaques (26 ml and 30 BPM, respectively).[29]

With standard continuous jet nebulizers, inhaled dose is less associated with change in $V_t$ or BPM than I:E ratio.[30] However, with the vibrating mesh nebulizer used on our study, I:E ratio was found to be less of a factor than $V_t$. This may be a result of the lack of continuous flow generated with jet nebulizers and the bolus

inhalation facilitated by the T-piece used in conjunction with the vibrating mesh nebulizer. The generation of a bolus using jet nebulizers is not possible in this setup given that the driving gas will act to continually clear the T-piece.

The efficiency of dose delivery between four different nebulizer configurations was compared using the average $V_t$, I:E ratio and breathing frequency recorded for macaques. In all cases more than half the dose was deposited as condensate in the T-piece (Table 2). Configuration D showed the highest delivery efficiency, as determined by the highest percentage of the albuterol detected on the filter. We assume that the gains in efficiency in this configuration was due to the larger internal volume of the assembly, acting as a reservoir from which a larger bolus of aerosol could be inhaled with each breath. The internal volumes for each of Configurations A – D were 55.0, 59.5, 32.0 and 56.5 ml, respectively. Nevertheless, only 24-48 % of the nebulized dose was actually delivered to the inhalation filter, representing that dose available to the face of macaques. These values are in the same order of magnitude as the deposition efficiencies previously reported in cynomolgus macaques.[17] Of particular note, the waste residual dose left un-nebulized within the nebulizer was consistently low across all configurations (range 2.1 to 3.2 % of the nominal dose). In the selection of devices for delivery of high value therapeutics, this is an important point of comparison against alternate aerosol generator technologies with higher residual volumes, *e.g.* jet nebulizers.

Based on the tidal breathing measurements obtained, the impact of $V_t$, BPM and I:E ratios on dose efficiency was assessed. Using the average value for one parameter, the effect of minimal and maximal values of the other two was evaluated. Overall, $V_t$ was seen to have the greatest effect on delivery. However, it should be noted that the maximum $V_t$ (46 ml) was recorded in an animal with a body weight of 6.5 kg. Varying both BPM and I:E ratio whilst maintaining $V_t$ at the “average” value, was not associated with significant effects on dose efficiency (range 22.48 to 26.71 %). However, all other combinations of $V_t$, BPM and I:E were seen to result in much wider ranges of delivery efficiencies (Average BPM, range 20.42 to 39.70 %, Average I:E, range 21.30 to 38.70 %). We conclude that when using a vibrating mesh aerosol generator with connecting circuit that acts as a reservoir, and a properly fitting mask, we found less difference between delivery efficiency than anticipated, and recommend that in an aerosol-mediated delivery study in non-human primates standardized body weights should be used to ensure comparable dosing.

This study has some limitations. Practical and ethical limitations to working with non-human primates mean we were unable to obtain a larger number of tidal breathing measurements. However, the data provide a solid level of internal consistency. Although all subjects received moderate sedation, it is possible that animals responded differently. However, this same type of response is commonly observed in non-anesthetized infants during administration of aerosols *via* a facemask. Another limitation of our study is that we cannot distinguish between delivery to the upper or lower respiratory tract, and provide data on inhaled dose only. In anatomically correct infant airway models such as the SAINT,[(31)] it has been demonstrated that the inhaled dose found at the mouth does not have a linear correlation with the dose to the lungs. Therefore, the estimated efficiency of 24-48 % is only valid for inhaled delivery of the aerosol to the respiratory tract, with a fraction of this dose expected to be delivered to the lungs. Finally, use of albuterol solution as a tracer aerosol may not fully predict the aerosol performance of alternative formulations.

In conclusion, studies in non-human primates aimed at comparison of aerosol vaccination with other routes of vaccine delivery must take both the inter-subject variation and relatively low efficiency of delivery to these low body weight mammals into account. Especially important in the case of vaccines where a fixed dose is administered irrespective of body weight. Specifically, selection of body weight-matched subjects and an appropriate aerosol delivery system shall serve to effectively control inter and intra dose variation.

**Acknowledgements**

This work was funded by the Foundation for the National Institutes of Health (FNIH) through the Bill & Melinda Gates Foundation Grand Challenges in Global Health initiative (grant# DUPREX09GCGH0). The authors thank Skip Veugen (CareFusion), Andrew O'Sullivan (Aerogen Limited) and the members of the Scientific Advisory Board of our project for their contributions.

**Author Disclosure Statement**

Ronan MacLoughlin is employed by Aerogen Limited, James Fink is regularly contracted by Aerogen Limited as independent consultant. All other authors declare no conflicts of interest.

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**Table 1: Tidal breathing and inhaled doses.** Tidal volume measurements were performed by plethysmography in ten subadult male cynomolgus macaques, using either endotracheal intubation (A), Laerdal facemask 0/1 (B) or Laerdal facemask 0/0 (C) as interface.

| Interface | Animal ID # | Age (months) | BW (kg) | $V_t$[1] (ml) | BPM[2] | I:E ratio[3] | Calculated ml / kg | % Inhaled dose average (SD) |
|---|---|---|---|---|---|---|---|---|
| **A*** | 1 | 56 | 4.1 | 25.6 | 32.0 | 0.52 | 6.24 | NT |
| **A**** | 1 | 56 | 4.1 | 26.4 | 28.2 | 0.78 | 6.44 | NT |
| **A**** | 2 | 57 | 5.7 | 32.9 | 36.0 | 0.98 | 5.77 | NT |
| **B** | 2 | 57 | 5.7 | 34.4 | 17.8 | 1.36 | 6.04 | NT |
| **B** | 3 | 55 | 4.3 | 24.4 | 27.7 | 0.72 | 5.67 | NT |
| **B** | 3 | 55 | 4.3 | 27.3 | 26.2 | 0.84 | 6.35 | NT |
| **B** | 4 | 55 | 3.5 | 25.0 | 22.3 | 0.72 | 7.14 | NT |
| **C** | 1 | 56 | 4.1 | 26.9 | 20.7 | 0.80 | 6.56 | 26.67 (0.73) |
| **C** | 3 | 55 | 4.3 | 27.8 | 25.7 | 0.68 | 6.47 | 31.62 (1.15) |
| **C** | 4 | 55 | 3.5 | 28.5 | 19.4 | 0.73 | 8.14 | 26.48 (0.58) |
| **C** | 5 | 56 | 3.7 | 25.2 | 20.0 | 1.30 | 6.81 | 24.05 (0.92) |
| **C** | 6 | 57 | 6.5 | 27.4 | 28.4 | 1.15 | 4.22 | 29.10 (1.66) |
| **C** | 6 | 57 | 6.5 | 24.0 | 29.6 | 1.39 | 3.69 | 26.48 (0.97) |
| **C** | 7 | 54 | 6.5 | 46.0 | 28.7 | 0.86 | 7.08 | 48.05 (0.68) |
| **C** | 8 | 55 | 4.1 | 35.4 | 27.9 | 1.32 | 8.63 | 37.48 (1.05) |
| **C** | 9 | 55 | 3.3 | 24.2 | 31.1 | 1.61 | 7.33 | 30.19 (0.93) |
| **C-Min**[4] | -- | -- | -- | 24.0 | 19.4 | 0.68 | 3.69 | 24.05 |
| **C-Max**[5] | -- | -- | -- | 46.0 | 31.1 | 1.61 | 8.63 | 48.05 |
| **C-AVG**[6] | -- | -- | -- | 29.5 | 25.7 | 1.09 | 6.51 | 32.12 |
| **C-STD**[7] | -- | -- | -- | 7.1 | 4.5 | 0.33 | 1.64 | 7.46 |

[1] Vt = tidal volume; [2]BPM = breaths per minute; [3] I:E ratio = ratio of length of inspiration over expiration time; [4]minimal, [5]maximal and [6]average ([7]standard deviation) values measured for Vt, BPM and I:E using the small resuscitation mask (C); * = endotracheal tube 3.5 mm; ** = endotracheal tube 5 mm. NT: not tested.

**Table 2:** Mass distributions of albuterol sulphate after nebulization using different configurations (as shown in Figure 1 A-D). The configurations were connected to a breathing simulator (Figure 1E), which was programmed using the C-AVG values for tidal volume, I:E ratio and respiration rate (Table 1). Results are shown as percentages of nominal dose placed in the nebulizer that were recovered from each compartment, represented as means ± standard deviation of triplicate measurements. The percentage found in the inhalation filter represents the delivery efficiency.

| Config | Inhalation filter | Exhalation filter | Nebulizer | T-Piece | Sum |
|---|---|---|---|---|---|
| **A** | 20.48 ± 0.22 | 17.33 ± 3.82 | 3.24 ± 3.24 | 58.26 ± 1.59 | 99.31 |
| **B** | 24.95 ± 4.00 | 15.10 ± 0.73 | 2.81 ± 0.37 | 50.62 ± 0.72 | 93.48 |
| **C** | 22.14 ± 0.86 | 14.67 ± 0.54 | 2.05 ± 0.25 | 59.95 ± 0.75 | 98.81 |
| **D** | 26.71 ± 0.80 | 17.71 ± 0.80 | 2.21 ± 0.38 | 52.29 ± 0.61 | 98.92 |

**Table 3:** Delivery efficiency of configuration D across combinations of C-min, C-max and C-AVG values of Tidal Volume, I:E ratio and BPM.

| | Average | STDEV | MIN | MAX |
|---|---|---|---|---|
| **Average $V_t$** | | | | |
| min BPM min I:E | 22.48 | 1.06 | 22.48 | 26.71 |
| max BPM max I:E | 26.71 | 0.37 | | |
| max BPM max I:E | 23.52 | 2.38 | | |
| max BPM min I:E | 26.43 | 1.19 | | |
| **Average BPM** | | | | |
| min $V_t$ min I:E | 21.87 | 1.81 | 20.42 | 39.70 |
| max $V_t$ max I:E | 23.12 | 1.40 | | |
| min $V_t$ max I:E | 20.42 | 1.40 | | |
| max $V_t$ min I:E | 39.70 | 1.69 | | |
| **Average I:E** | | | | |
| min $V_t$ min BPM | 21.30 | 1.21 | 21.30 | 38.70 |
| max $V_t$ max BPM | 38.70 | 1.45 | | |
| min $V_t$ max BPM | 23.89 | 2.34 | | |
| max $V_t$ max BPM | 33.89 | 1.47 | | |

**Figure 1: Tidal breathing measurements.** Tidal volumes, BPM and I:E ratios were measured using a pediatric pneumotachometer, either by endotracheal intubation (not shown), large resuscitation facemask (A,) or a small resuscitation facemask (B). Patterns for inspiratory and expiratory flows and volumes for one of the measurements of (C, animal #1). Triplicate measurements of the same animal (D).

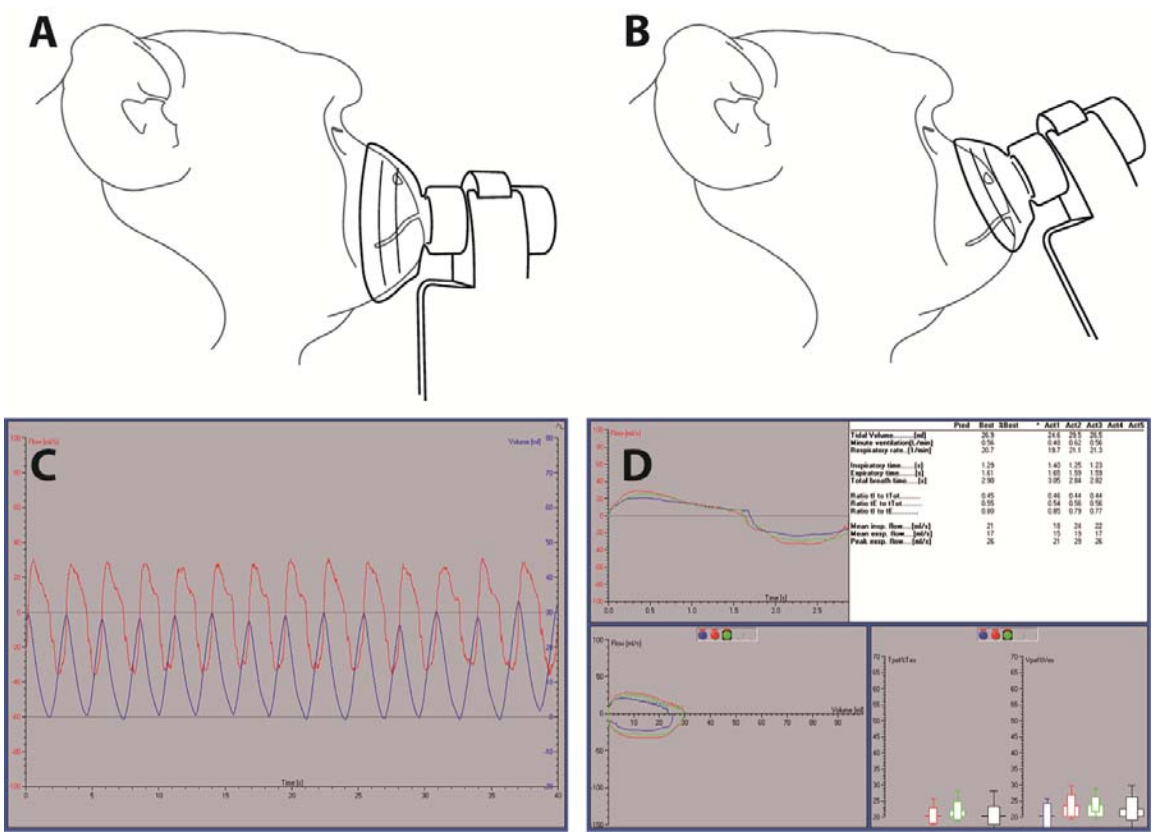

**Figure 2: Breathing simulator measurements.** Four different nebulizer configurations (A-D) were used to test the dose efficiency. Configuration D was used with a filter on the expiratory side for mass balance measurements (E). Significant losses were observed as rainout of the aerosol in the t-piece (F). NOTE: Configurations A and B differ only in the use of a 22 mm outer diameter connector between the T-piece and Filter for Configuration A. Configuration B incorporates a 22 mm inner diameter connector between the T-piece and Filter.