Published in final edited form as:

Curr Protoc Microbiol. 2012 August ; CHAPTER: Unit14D.2. doi:10.1002/9780471729259.mc14d02s26.

Gene Transfer in the Lung Using Recombinant Adeno-Associated Virus

Alisha M. Gruntman¹, Christian Mueller^{1,2}, Terence R. Flotte^{1,2,3}, and Guangping Gao^{1,3,4,*}

¹Gene Therapy Center, UMass Medical School, Worcester MA, 01605

²Department of Pediatrics, UMass Medical School, Worcester MA, 01605

³Department of Microbiology and Physiological Systems, UMass Medical School, Worcester MA, 01605

⁴State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu, Sichuan, People's Republic of China

Abstract

Adeno-associated virus (AAV) is a small replication deficient DNA virus belonging to the *Parvovirinae* family. It has a single-stranded approximately 4.7kb genome. Recombinant AAV (rAAV) is created by replacing the viral *rep* and *cap* genes with the transgene of interest along with promoter and polyadenylation sequences. The short viral inverted terminal repeats must remain intact for replication and packaging in production as well as vector genome processing and persistence in the transduction process. The AAV capsid (serotype) determines the tissue tropism of the rAAV vector. In this unit we will discuss serotype selection for lung targeting along with the factors effecting efficient delivery of rAAV vectors to the murine lung. Detailed procedures for lung delivery (intranasal, orotracheal, and surgical tracheal injection), sample collection and post-mortem tissue processing will be described.

Keywords

Adeno-associated virus; vector delivery; lung; gene therapy

Contact information, Guangping Gao, Gene Therapy Center, University of Massachusetts Medical School, 381 Plantation Street, Suite 250, Worcester MA 01605, Phone: (508) 856-3394, Fax: (508) 856-1552, Guangping.Gao@umassmed.edu.

Internet Resources

http://www.hallowell.com/index.php?doc=1&pr=Video_Presentations

Detailed procedural video demonstrating the orotracheal intubation technique. Note minor modification from technique described above. We do recommend the 20 gauge catheters listed in the materials list over those presented in the video because they are disposable and a new catheter can be used for each mouse. They are also a somewhat softer material and therefore less likely to induce trauma on placement.

http://www.kentscientific.com/products/productView.asp?productID=6354&Mouse_Rat=Respiratory&Products=Small+Animal+Intubation+Kit

Detailed procedural video demonstrating the BioLite intubation technique.

<http://www.jove.com/video/1941/direct-tracheal-instillation-of-solutes-into-mouse-lung>

Torres-Gonzalez, E., Helms, M. N., Goodson, P., Rojas, M. Direct Tracheal Instillation of Solutes into Mouse Lung. *J. Vis. Exp.* (42), e1941, DOI: 10.3791/1941 (2010).

Detailed procedural video demonstrating the surgical tracheal injection technique

http://www.medipoint.com/html/directions_for_use1.html

Video demonstrating blood collection from facial vein/artery using lancet

<https://www.aalaslearninglibrary.org/swfs/course2451/stabpst1.swf>

Video demonstrating blood collection from facial vein/artery using needle

INTRODUCTION

Adeno-associated virus (AAV) is a small, (20nm) replication deficient DNA virus belonging to the *Dependovirus* genus. It is a member of the *Parvovirinae* family with a single-stranded approximately 4.7kb genome. The wild-type AAV genome contains *rep* and *cap* genes flanked by short inverted terminal repeat (ITR) sequences. While AAV infections are common in humans, no clinical disease has been associated with AAV. Adeno-associated viruses have large capsid sequence variability with 6 clades of AAVs identified thus far (Gao, 2004). It is the AAV capsid (serotype) that determines the tissue specificity upon natural or experimental infection (Grimm, 2006). Recombinant AAV (rAAV), for use in gene transfer vectors, is created by replacing the *rep* and *cap* genes with the transgene of interest along with promoter and polyadenylation sequences. The ITRs must remain intact for replication and packaging in production as well as vector genome processing and persistence in the transduction process. The *rep* and *cap* genes are provided in a complementing plasmid, along with adenovirus or herpes simplex virus helper gene products, to successfully package the rAAVs. rAAV persists in non-dividing cells as an extra-chromosomal element (Flotte, 1994 and Afione, 1996).

The lung was the target of the first human rAAV trial (Flotte, 1996). In that trial a rAAV2 vector was used to deliver the CFTR gene to the lung of cystic fibrosis patients. rAAV has been used in multiple human trials subsequently, targeting the lung as well as other organ systems. While the safety of rAAV in human trials has been established, other challenges to efficient long-term gene transfer were encountered (Mueller, 2008 and High, 2011). We will discuss this further in the background section along with suggestions on serotype selection, expression analysis and study design.

This unit will cover methods of delivering rAAV to the murine lung (intranasal, orotracheal intubation and surgical tracheal injection) as well as evaluation of gene transfer (serum collection, bronchoalveolar lavage technique, and post-mortem collection and processing of lung tissue).

CAUTION: According to the NIH Guidelines for Research Involving Recombinant DNA Molecules (April 2000), adeno-associated virus is a Biosafety Level 1 (BSL-1) pathogen because both rAAV and wild-type AAVs are not known to cause disease in healthy human adults. BSL-1 status assumes that the rAAV construct does not encode a toxic or tumorigenic molecule and is produced without a helper virus. Biosafety protocols should be approved by the Institutional Biosafety Committee at the institution where the research is being conducted.

CAUTION: This experiment requires Animal Biosafety Level 1 (ABSL-1) conditions. Biosafety protocols should be approved by the Institutional Biosafety Committee at the institution where the research is being conducted. Protocols using live animals must first be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) or must conform to governmental regulations regarding the care and use of laboratory animals.

BASIC PROTOCOL

Intranasal Recombinant Adeno-associated Virus Vector Delivery

The intranasal route provides a straight-forward method of vector delivery with an affordable equipment list and a small learning curve. This leads to easily repeatable results in even the novice murine researcher. The downfall of nasal delivery is loss of vector in the upper airways (nasal passages, sinuses, and pharynx) and gastrointestinal tract rather than having the entire vector dose deposited within the lower airways. This may necessitate

larger vector doses to attain the same level of transduction compared to intra-tracheal delivery.

Materials

Xylazine (20mg/ml)

Ketamine (100mg/ml)

Ophthalmic ointment (such as Puralube, **Webster Item #: 07-888-2572**)

Webster Veterinary

137 Barnum Road

Devens, MA 01434

978-353-6000 (Phone)

800-225-7911 (Toll Free in the U.S.)

978-353-6016 (Fax)

www.webstervet.com

3/10 cc syringe with 31 gauge needle (8mm length needle) (such as: BD Ultra-Fine II Short Needle Insulin Syringes)

Or

100 or 200 microliter micro-pipette tip

Braintree Scientific:

PO Box 361

Braintree, MA 02185

Phone: 781-348-0768

Fax: 781-843-7932

<http://www.braintreesci.com>

Rodent Intubation Stand (RIS-100) or Rodent Work Stand (RW-A3467)

Place Velcro on under surface of work stand to allow incisor loop to be secured (Figure 1A)

Incisor loop (210A3490A)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent movement or sneezing/coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Inhalant halothane anesthesia also described. Ophthalmic lube can be applied to prevent corneal drying.

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand.
3. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure 1B).

A Velcro strip or tape can be used to gently secure the animal to the work stand to prevent instability while dosing the animal.

4. Slowly administer the vector solution via the nostril in a drop-wise fashion.

Allow the animal to inhale the previous drop prior to administering the next drop. A minimum volume of 50ul should be used to ensure maximum percent delivery to the lung. For larger volumes it is possible to deliver 75ul (37.5 per nostril) then allow respirations to return to normal and deliver another 75ul.

5. Gently remove the mouse from the rodent work stand and place in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).

This will insure optimal ventilation.

6. Monitor until recovered from anesthesia then return to home cage.

ALTERNATE PROTOCOL 1

Speculum/Otoscope Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

Orotracheal intubation provides the ability to directly instill vector into the lung without the risks of a surgical procedure or loss of vector in the upper airways. This means that the dose of vector reaching the lungs is more tightly controlled than with intranasal instillation because essentially the entire amount delivered reaches the lung. Orotracheal intubation does have an increased equipment investment and a steeper technique learning curve compare to intranasal instillation.

Additional Materials (also see Basic Protocol 1)

Braintree Scientific:

Mouse Intubation Pack (RW-A3746)

Includes: Incisor loop, intubation speculum (to use with otoscope), pointed cotton tipped applicators, mirror for verification of placement and tutorial video.

Welch Allyn Otoscope (NICAD (RW-A3749) or LI Ion (RW-A3754))

Lung inflation bulbs (LIB-03)

20 gauge 1.25 inch catheter (BD Angiocath, Becton Dickinson, Sandy Utah)

2% Lidocaine HCl Jelly (30 ml) (**Webster Item #:** 07-835-7610)

Webster Veterinary

137 Barnum Road

Devens, MA 01434

978-353-6000 (Phone)

800-225-7911 (Toll Free in the U.S.)

978-353-6016 (Fax)

<https://www.webstervet.com>

1 ml tuberculin slip tip syringe

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent movement during the intubation procedure and coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

All supplies should be set-up and within easy reach prior to anesthetizing the mouse (Figure 1A).

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand
 - a. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure 1).
 - b. The animal should be positioned so that its back is flat on the work stand and the chest is not leaning to either side. The animal should be secured in that position with tape or Velcro over the chest (the animal's front legs will also be under the tape/Velcro) (See Figure 1B).

3. Roll the tongue from mouth using pointed cotton-tipped applicator.
4. Gently insert intubation speculum (attached to otoscope) to the depth of the larynx (opening to the trachea) (approximately the depth of the front of the ears). The otoscope should be positioned such that the hand holding it is located to the side of the mouse's head and the intubation speculum has the concave side facing away from the tongue (See Figure 1C).
5. Gently move the otoscope toward you until the larynx can be visualized. It is often necessary to slightly rotate the speculum up to displace the soft tissues surrounding the larynx to obtain the best view.

This will be a very small movement; if the larynx is passed the operator will only see the back of the tongue. The speculum will have to be gently moved back again and the motion re-attempted.

6. Once the larynx is visualized, a very small drop of 2% lidocaine jelly can be applied using the lidocaine applicator for the intubation pack. The speculum is then removed and at least 30 seconds is allowed, for the larynx to be desensitized, before intubation is attempted.

During the 30 second wait, the stylet with catheter should be positioned for easy reach during the intubation procedure.

7. After 30 seconds repeat steps 3–5 to re-visualize the larynx.
8. Once the larynx is visualized, the stylet (with catheter attached) should be advanced into the tracheal opening. Once the stylet is in place the speculum can be removed and the catheter gently advanced down the stylet. The catheter should be placed such that the hub (pink portion) of the catheter just touches the incisors.

It is very important not to advance the stylet or catheter past the thoracic inlet. No more than 2 attempts to place the stylet/catheter should be made in a mouse in one setting or laryngeal swelling and death can occur.

9. Once the catheter is in place the stylet should be removed immediately to allow the animal to ventilate normally.

10. Correct placement of the catheter in the trachea must be determined prior to vector administration.

- a. Mirror: Hold the mirror at an angle in front of the tracheal catheter opening and observe clouding of the glass as the animal breaths.
 - i. Additional confirmation of correct placement can be obtained by gently covering the catheter and observing a change in breathing pattern that resolves once the finger is removed. Only occlude the catheter for 2–3 seconds.

The mirror must be ice cold to see breath condensate on the glass.

- b. Lung Inflation Bulb: Attach the inflation bulb gently to the catheter and gently squeeze. If the catheter is in the trachea the chest/ribs will expand as with a deep breath. If the catheter is in the esophagus you may see the abdomen expand.

If the catheter is in the esophagus you may see some abdominal expansion as air is deposited in the stomach when the bulb is squeezed.

- 11. The vector can then be administered in a 3ul/gram body weight volume using a 0.35ml syringe.
- 12. Immediately after vector administration instill 0.2ml of air slowly into the catheter 2–3 times to ensure that minimal vector remains in the catheter and to increase distribution throughout the lung. The air should be instilled using a clean 1.0 ml syringe without needle. Care should be taken when attaching the syringe to the catheter hub that any vector remaining in the hub is not forced out around the syringe.
- 13. Immediately following instillation of the air the catheter should be removed as any liquid remaining in the catheter will impede normal ventilation.
- 14. Gently remove the mouse from the rodent work stand and place in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).

This will insure optimal ventilation.

- 15. The mouse should be monitored until ambulatory.

A mild to moderate increase in respiratory effort is expected for the first 24–36 hours after dosing depending on the volume instilled.

ALTERNATE PROTOCOL 2

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery

Surgical injection of vector into the trachea has the advantage of improved lung vector delivery compared with intranasal instillation and decreased equipment costs and ease of learning compared with orotracheal intubation. There is increased risk associated with a surgical procedure including hemorrhage, incision dehiscence, infection, and longer recovery. Because of the small size of the murine trachea there is also a risk of injecting the vector through the trachea and targeting the peritracheal tissues or esophagus rather than the lung.

Materials (also see Basic Protocol 1)

#15 scalpel blade and handle or 4 ½ inch curved tissue scissors

(2) Adson tissue forceps (or other delicate tissue forcep)

4-0 monofilament absorbable suture on a cutting needle (such as PDSII) or tissue glue (such as Dermabond)

Needle holder (for suturing skin)

Depilatory cream (such as Nair), scissors, or clippers to remove hair from surgical sight

Chlorhexadine or betadine scrub and 70% alcohol

IV butterfly catheter with 23 or 25 gauge needle (alternative option)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent sensation on incising the skin and injecting the vector. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

2. Place the animal in dorsal recumbency (on back) and secure chin and front limbs with tape. The animal should be on an approximately 45 degree incline with the head elevated (Figure 1A).
3. Remove the hair from the ventral neck (throat to chest) using depilatory cream, scissors or clippers.
4. Clean the hair and depilatory cream from the area with a damp gauze sponge then scrub the skin using chlorhexadine solution followed by 70% alcohol.

Surgical preparation, including type of surgical scrub used, use of sterile surgical drape, sterile surgical gloves, surgical cap, lab coat, and surgical mask should be used according to your institution's animal care and use regulations.
5. Make a 4mm incision centered over the upper portion of the mouse's neck (Figure 2B).
 - a. Alternatively: Tent the skin in the upper portion of the neck and cut a 4mm length of skin with the long direction of the cut extending along the length of the neck (not across the neck).
6. Use the tissue forceps to gently dissect through the subcutaneous tissue down to the level of the strap muscles then overlay the trachea (Figure 3A).
7. Make an incision in the strap muscles to expose the trachea.
8. Once the trachea is visible the needle can be inserted parallel to the trachea with the bevel facing up and the point of the needle directed toward the lung. (Figure 2B). Withdraw needle.

Guidelines for volume of vector delivered are the same as for orotracheal intubation. The needle should be inserted at an angle pointing toward the lung in order to direct the vector toward the lung and not the upper airways. When drawing the vector into the syringe prior to administration an additional volume of air (0.2ml) should also be drawn into the syringe to force all vector from the hub of the syringe and the needle into the trachea.

Alternatively you can use a 23 or 25 gauge butterfly catheter, preloading the vector in the tubing and observe the column of fluid move with respirations, once the catheter is inserted in the trachea, to ensure proper placement.

9. Close the skin using 2–3 simple interrupted sutures or skin glue. To suture the skin the needle should be inserted at a 90 degree angle 1mm from the right side of the incision and exit the skin at a 90 angle 1mm from the left edge of the incision (Figure 2C). A forcep is used to stabilize the skin for needle insertion. A surgical knot is then tied (Figure 2D).
10. Place mouse in sternal recumbency (on chest) in a quiet cage with head and thorax slightly elevated (a nestlet or folded paper towel work well for this).
11. The mouse should be monitored until ambulatory.

ALTERNATE PROTOCOL 3

BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery

This protocol offers an alternative approach to orotracheal intubation to that described in Alternate Protocol 1. The equipment costs are less than those for speculum aided intubation (unless your laboratory already owns an otoscope) and the system boasts a smaller learning curve. The disadvantages include the lack of a magnifying glass associated with the system (this is present in the otoscope) which may increase the difficulty level in visualizing the larynx appropriately, especially in smaller mice, as well as the inability to move soft tissue out of the way (such as the soft palate) using the speculum blade in order to better visualize the larynx. The system consists of a fiber optic illuminator and fiber optic stylet rather than an otoscope with speculum and separate stylet used for Alternate Protocol 1. The fiber optic stylet allows simultaneous visualization and cannulation of the larynx, without magnification. The intubation catheter used is identical to that used in Alternate Protocol 1. See Alternate Protocol 1 for specific pros and cons of orotracheal intubation as a delivery route for viral vectors. See Internet Resources section for a video demonstrating the BioLITE procedure.

Additional Materials (also see Alternate Protocol 1)

Braintree Scientific:

BioLITE Mouse Kit (MI-Kit)

Includes: Fiber optic illuminator, fiber optic stylet, lung inflation bulbs, and 5 catheters

Rodent Intubation Stand (RIS-100) or Rodent Work Stand (RW-A3467)

1. Anesthetize using intraperitoneal ketamine and xylazine (see reagents and solutions section).

The animal must be at a deep enough plane of anesthesia as to prevent movement during the intubation procedure and coughing in reaction to the vector administration. This can be determined by a negative toe-pinch. Ophthalmic lube can be applied to prevent corneal drying.

All supplies should be set-up and within easy reach prior to anesthetizing the mouse.

2. Position the mouse in dorsal recumbency (on its back) on the rodent work stand
 - a. Place incisor loop over the upper incisors and attach the Velcro to the corresponding Velcro on the under surface of the rodent work stand (See Figure 1).
 - b. The animal should be positioned so that its back is flat on the work stand and the chest is not leaning to either side. The animal should be secured in

that position with tape or Velcro over the chest (the animal's front legs will also be under the tape/Velcro) (See Figure 1).

3. Roll the tongue from mouth using pointed cotton-tipped applicator.
4. Gently insert the BioLite stylet with catheter attached to the level of the larynx. Use light to visualize larynx.
5. Once the larynx is visualized gently pass the stylet into the trachea and slowly advance the catheter over the stylet into the trachea. At least 2–3 mm of the catheter should remain outside the mouse's mouth once the catheter is in position.

It is very important not to advance the stylet or catheter past the thoracic inlet. No more than 2 attempts to place the stylet/catheter should be made in a mouse in one setting or laryngeal swelling and death can occur.

6. Once the catheter is in place the stylet should be removed immediately to allow the animal to ventilate normally.
7. The remainder of the protocol is identical to that described in Alternative Protocol 1 Steps 10–15.

SUPPORT PROTOCOL 1

Preparing rAAV Vector for Delivery

The following are the steps we follow to prepare rAAV vectors for delivery to the mouse.

Materials

Pipette tips (200ul)

Pipette (20–200ul)

Parafilm or sterile petri dish

Ice

Delivery syringe or pipette (see particular delivery protocol)

1. Thaw rAAV vector on ice.
2. Calculate the volume to be delivered based on vector concentration and dose desired per mouse. If multiple groups are going to be injected (i.e. treatment and control) the same volume of vector should be delivered in each mouse, regardless of group. Therefore the most dilute vector will determine the volume and maximum dose possible.
3. If dilution of the vector is necessary it should be done using sterile saline.
4. Pipette amount of vector to be dosed per animal. Vector can be pipetted onto clean parafilm and drawn up into dosing syringe or pipetted directly into dosing syringe if using a syringe with a detachable needle.

Unlike intravenous preparation all the air does not need to be removed from the syringe prior to delivery.

5. Syringes should then be kept on ice until ready to dose mice.
6. Any unused, undiluted portion of thawed vector can be kept at 4 degrees Celsius. Refreezing rAAV vectors will result in reduction of infective titer.

SUPPORT PROTOCOL 2

Post-mortem Bronchoalveolar Lavage

Bronchoalveolar lavage collection allows for quantification of rAAV gene products within the distal airways. It is a relatively easy procedure to perform and requires minimal equipment.

Materials

Additional Materials (also see Alternate Protocol 2)

Iris scissors

Sterile Saline

3 ml syringe

1.5 ml eppendorf tube

1. Euthanize mouse according to your institutional animal care guidelines.
If cervical dislocation is used as part the euthanasia protocol be cautious not to damage the trachea.
2. Follow Alternate Protocol 2 Steps 5–7 to expose the trachea (Figure 3A).
3. Using forceps pull a 5–6cm length of suture material underneath the trachea (Figure 3B).
4. Make a small cut longitudinally across the trachea using iris scissors (or other fine tipped scissors) transecting approximately 1/3 of the tracheal diameter.
Be careful not to completely transect the trachea as this will make catheter placement extremely difficult.
5. Insert the 20 gauge catheter distally (toward the lung) into the trachea to the level of the carina (distal most portion of the lung, 2–3mm depending on the size of the mouse) and secure in place using previously placed suture (Figure 3C).
6. Instill 500–600 microliters into the lung using a 3ml syringe attached to the catheter.
7. Immediately use the 3ml syringe to draw the fluid back out of the lung.
It may be necessary to move the catheter slightly within the trachea to find the optimal location for fluid recovery.
8. Depending on fluid return from first instillation, a second 500 microliters can be instilled and collected.
9. The fluid can then be placed in an eppendorf tube and frozen or spun down and the supernatant collected and/or the pellet collected, depending on whether a cell-free or cell-rich fraction is desired.

SUPPORT PROTOCOL 3

Collecting Lung Tissue for Histology and/or Immunohistochemistry or Immunofluorescence

Proper removal of blood from the lungs will decrease autofluorescence when performing immunofluorescent staining. Fixing the lungs while inflated will aid in histologic evaluation

of the lung because the alveoli will have a more normal architecture rather appearing collapse, allowing identification of individual epithelial cells.

Materials

Additional Materials (also see Alternate Protocol 2 and Support Protocol 2)

5 ml syringe

27 gauge needle

10 ml conical tube

Neutral buffered formalin

1. Euthanize mouse according to your institutional animal care guidelines.
2. Follow Support Protocol 2 Steps 2–5 to place the tracheal catheter (Figure 3C).
3. Open the abdomen using tissue scissors then gently make a small cut in the diaphragm in order to collapse the lung (Figure 3D–E).
4. Open the chest cavity by cutting gently along the sternum, careful not to cut the heart or lungs (Figure 3F).
5. Flush the blood from the lungs by making a small cut in the left ventricle (lower left chamber of the heart (animal's left)) and flushing 5 ml of sterile PBS or saline through the right ventricle (lower right chamber of the heart (animal's right)) using a 5 ml syringe and 27 gauge needle (Figure 3F).

Adequate flushing of the lung will result in white lungs free of blood. Don't insert the needle too deeply into the ventricle or you will puncture into the atrium and adequate flushing of the lungs will not be achieved.

6. If the lungs are to be fixed, inflate the lungs with 1–2 mls of neutral buffered formalin using a 3ml syringe attached to the tracheal catheter (Figure 3G).
Infuse formalin until lungs are inflated, but not over inflated. Do not remove the syringe from the catheter or the formalin will leak out.
7. Tighten the suture around the catheter to seal the tracheal opening as you remove the syringe and catheter together.
8. Remove the lungs carefully from the chest cavity by cutting the trachea above the suture and carefully lifting the trachea as you cut behind it until the lungs are free from the chest cavity.

Be careful not to cut the lungs while removing or formalin will leak out and the lungs will not remain properly inflated.

9. Place lungs, with suture still closing trachea, into a 10ml conical tube filled with formalin.

The heart can be removed from the lungs before placing in formalin.

10. Allow the lungs to fix for 24 hours then replace formalin with sterile phosphate buffered saline.

REAGENTS AND SOLUTIONS

Ketamine/Xylazine Anesthesia

Xylazine (20mg/ml stock concentration)	0.25 ml (10 mg/kg dose to mouse)
Ketamine (100mg/ml stock concentration):	0.5 ml (100mg/kg dose to mouse)
<u>Sterile Isotonic Saline:</u>	<u>5 ml</u>
Total Cocktail:	5.75 ml

Dose of cocktail to mouse: 0.10 ml/10 g, intraperitoneally

Weigh all animals to determine anesthetic dose. Place the animal in a quiet cage following anesthetic administration and allow at least 5 minutes to pass before checking anesthetic depth by toe pinch. If adequate anesthetic depth is not present allow the animal another 5 minutes in the quiet cage. If still not sufficiently anesthetized a 50 mg/kg dose of ketamine alone should be administered. If that does not result in sufficient anesthesia then a 0.05 ml/10 g dose of the xylazine/ketamine cocktail can be re-administered.

COMMENTARY

Background Information

The lung was the first site for rAAV gene therapy in humans. The initial gene therapy trials involving the lung have shown rAAV to be a safe vector for human gene therapy, with only transient moderate adverse events being reported in any of the clinical trials published thus far. However, efficient gene therapy in the lung has encountered other obstacles. The original cystic fibrosis trial used rAAV2 to deliver the CFTR gene, with only low levels of expression seen within the lung. It was discovered that rAAV2 requires heparin sulfate proteoglycans (HSP) for efficient cell entry (Summerford, 1998). HSPs are only located on the basolateral surface of airway epithelial cells, not the apical surface. This necessitates disruption of the tight junctions between airway epithelial cells in order to allow the rAAV2 viral vector to gain access to the HSP receptors. Subsequently other serotypes have shown improved lung transduction, without the necessity for tight junction disruption, due to the presence of apical surface receptors. Currently the most promising vectors for lung-targeted gene therapy include AAV5, AAV6, AAV9 and AAV6.2. The choice of serotype should be based on the cell type that is being targeted (see Table 1). It should be noted however, that Table 1 focuses on transduction efficiency in murine models, it has been shown that murine and lower primate models may not reflect the transduction efficiency in higher primates, specifically chimpanzees and humans (Liu, 2007 and Flotte, 2010). Specifically, AAV5 showed higher efficiency in mouse and lower primate models, whereas AAV1 had improved efficiency in human airway epithelial cells and chimpanzees airways (Lui, 2007 and Flotte, 2010).

An additional problem encountered in rAAV mediated gene transfer to the lung is the lack of transgene persistence in the dividing epithelial cell population. This is due to the fact that, as mentioned above, rAAV is present in transduced cells as an extra-chromosomal element (Afione, 1996). This lack of persistence is compounded by the fact that repeated dosing of rAAV leads to inefficient transduction, depending on serotype, as a result of neutralizing antibodies directed against the AAV capsid (Sinn, 2009). Transgene expression in the murine lung following a single vector administration has been detected up to 217 days after dosing, with a decrease in transgene expression occurring between day 49 and 77 and vector genomes between day 28 and 90 (Limberis, 2006).

Neutralizing antibodies directed against AAV capsid are the main immune impediment to successful rAAV transduction, although T cell and innate immune responses, such as alveolar macrophages and certain pattern recognition receptors also pose a potential challenge (Zaiss, 2008 and High, 2011). Preexisting immunity to AAV is of particular concern when selecting a serotype for experimentation with future consideration of translation to human subjects. The prevalence of preexisting antibodies in humans is largely serotype dependent, although significant cross-reactivity exists between some serotypes (Boutin, 2010). Neutralizing antibodies in humans are most prevalent against AAV serotypes 1 and 2, with lower prevalence and titers for 5, 8, and 9 (Boutin, 2010). In cystic fibrosis patients, AAV2 neutralizing antibody titers were slightly higher than titers against AAV5 and AAV6 and adults demonstrated significantly higher titers than children (Halbert, 2006). It is also possible to have an immune response directed toward the delivered transgene (Moore, 2010 and Zaiss, 2008). This has further reaching implications for patient trials as it could preclude other treatments such as protein replacement therapy (Zaiss, 2008). Neutralizing antibody titers have also been shown to increase in patients following rAAV2 vector delivery to the lung, especially evident following repeat dosing (Moss, 2004 and 2007).

Physical barriers to efficient vector delivery also exist in the lung, including loss in the upper and conducting airways and gastrointestinal tract following intranasal delivery (Southam, 2002). Barriers within the lung include the mucociliary clearance system, the luminal epithelial glycocalyx, and as mentioned above, tight junction between airway epithelial cells (Kolb, 2006). There are also disease specific impediments to vector lung access, such as the viscous mucus layer that coats the respiratory epithelium in cases of cystic fibrosis (Hida, 2011).

All these factors must be taken into account when planning gene transfer experiments in the lung. When choosing a reporter or therapeutic rAAV gene construct a plan for determining transgene expression efficiency must be carefully designed. Some important factors to consider are whether the transgene is an intracellular or secreted protein. Are there diagnostic tests readily available to detect the transgene product such as ELISA, immunohistochemistry, Westernblot? It has been established that a significant amount of vector reaches the liver following respiratory tract delivery (Limberis, 2006, Mueller, manuscript in preparation). If the transgene product is secreted, is there a way to determine whether production is occurring due to lung transduction or transduction of another organ such as the liver? Immunohistochemistry or Westernblot of the lung to detect gene product within lung cells or detection of gene product within bronchoalveolar lavage fluid can aid in proving lung origin gene product. It is also important to determine whether gene product expression levels are due to serotype transduction efficiency versus gene or promoter efficiency. Determining vector genomes within the lung tissue by quantitative real-time PCR can help parse out this difference. For example, if vector genomes present are high, but gene product levels are low, the low level of gene product could be attributable to multiple factors that affect transgene expression, other than poor vector distribution, such as abnormalities in viral intracellular trafficking, nuclear targeting, uncoating, and DNA processing (Sanlioglu, 2001 and Ding, 2005).

In conclusion, several rAAV vectors have been shown to target the lung, but serotype specific, immune, and physical barriers to transduction should be considered when planning a lung gene transfer experiment. While a detailed description of transgene product detection procedures is beyond the scope of this unit, we hope that the above commentary will aid you in selecting an appropriate detection method.

Critical Parameters and Troubleshooting

Intranasal Recombinant Adeno-associated Virus Vector Delivery—The main pitfall encountered with intranasal delivery will involve poor distribution to the lung. Ensuring that vector is not lost by allowing adequate time for inhalation while delivering the vector solution is critical. Poorly anesthetized animals are also more likely to swallow the vector rather than inhaling it into the lungs (Southam, 2002). Poor lung deposition will also result if the volume delivered is less than 50ul total (Southam, 2002). If needle and syringe are used to deposit the vector, poor lung delivery could result if the vector is inadvertently injected into the nasal epithelium rather than into the nasal passage.

Speculum/Otoscope Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery—Inadvertent catheter placement into the esophagus is the most common mistake that occurs during the oro-tracheal intubation process. Quickly identifying that the catheter is in the wrong location will prevent inadvertent esophageal delivery of the vector. In order to avoid catheterizing the esophagus it is imperative to see the stylet entering the laryngeal opening. It may be necessary to bend or extend the stylet slightly in order to allow better visualization of entry. Repositioning the animal so that the head and neck are straight and in-line will also aid in adequate visualization of the larynx. Adequate anesthesia will also decrease swallowing and provide easier access to the larynx.

If the catheter is properly positioned within the trachea, not in the esophagus, it is normal to notice a change in breathing pattern when delivering the vector and subsequent air.

As mentioned in the protocol, death of the mouse can occur secondary to laryngeal trauma if more than 2 intubation attempts are made in one animal. If it is critical to dose that animal, it is advisable to allow 24 hours for recovery if any reddening or swelling is visualized.

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery—Potential problems associated with surgical vector delivery include trauma to critical structures in the neck including, carotid arteries, jugular veins, and esophagus. The carotid arteries and jugular veins are located along side the trachea and the esophagus is deep to it. Careful dissection down to the trachea should prevent inadvertent damage to any of these structures. Adherence to aseptic technique during the surgical procedure should prevent any infections. This involves proper hair removal, disinfection of the skin, and sterile surgical instruments. Some animal care and use committees may also recommend the use of a sterile drape, sterile surgical gloves, surgical mask, and preoperative antibiotics to prevent infection. Another common complication in any murine surgery is the animals removing the skin sutures. If this occurs it may be necessary to reanesthetize the animal and replace the sutures. If several days have passed since the surgery enough healing may have occurred such that the sutures will not need to be replaced. Some surgeons prefer tissue glue to prevent issue of suture removal, however some incision inflammation has been described with this product.

Problems of vector delivery could also occur. Ensuring that the needle is in the trachea can be done by drawing back on the syringe prior to and after delivery of vector to be sure that air is obtained. The animals should also be adequately anesthetized to ensure that the vector is inhaled and not coughed up and swallowed. Directing the needle toward the lung and having the animal positioned at an angle with the head up should also increase lung deposition.

BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery—See speculum/otoscope section for issues related to adequate visualization of the larynx.

Postmortem Bronchoalveolar Lavage—Suturing the catheter in place will prevent inadvertent catheter removal during delivery and removal of the fluid. Do not insert the catheter too far into the tracheal (no more than 2–3mm) or it can push through the distal trachea into the mediastinum resulting in no return of fluid.

Flushing Blood from Lungs—A poor flush of the lungs can result from inadvertently flushing through the left ventricle, pushing the needle too far into the heart, or delivering the PBS solution too slowly.

Inflating Lungs for Fixation—Poor inflation of the lungs with fixative will occur if the catheter is advanced too far and punctures the trachea, if the lungs are cut upon opening of the chest cavity, or the trachea is not closed well following catheter removal. Do not insert the catheter too far into the tracheal (no more than 2–3mm) to prevent tracheal puncture. Making a small nick in diaphragm before cutting diaphragm or chest wall further will allow the lungs to collapse and decrease chance of cutting the pulmonary tissue. Wait to inflate the lungs until after the thoracic cavity has been opened to prevent lung lacerations. Using blunt tipped scissors to open the thorax can also decrease the chance of puncturing the lungs.

If inflation and formalin fixation is not providing enough tissue fixation, such that the lungs still collapse when being sectioned, then 2.5% glutaraldehyde can be used in place of formalin for the procedure described above. If the lung tissue is going to be frozen in OCT, the lungs can be inflated with OCT instead of formalin.

Anticipated Results—All of the lung delivery techniques described above should result in widespread lung transduction, depending on serotype and promoter used. The duration to maximum expression of the delivered transgene will depend on multiple factors, including serotype, promoter, and whether vector is single stranded or self-complementary. Expression should be expected within 7 days with peak expression likely between 3–6 weeks.

Time Considerations

Intranasal Recombinant Adeno-associated Virus Vector Delivery—This procedure can be quickly learned and dosing an animal should take less than 1 hour, with 5–10 minutes for anesthesia, 5 minutes or less for dosing, and 20–30 minutes for anesthesia recovery. Multiple animals can be anesthetized and recovered simultaneously, meaning that the largely limiting factor for number of animals dosed per hour will be actual vector delivery time.

Speculum/Otoscope and BioLITE Assisted Orotracheal Recombinant Adeno-associated Virus Vector Delivery—Once this technique is learned dosing a single animal takes approximately 5–10 minutes for catheter placement and vector instillation. Times for anesthesia and recovery are similar to intranasal delivery. This technique can take several attempts, meaning multiple animals, before the catheter can be placed in the trachea successfully in less than 2 attempts. It is advised to learn the procedure on non-experimental animals.

Surgical Tracheal Recombinant Adeno-associated Virus Vector Delivery—Anesthesia and recovery times will be similar to intranasal delivery. Surgery and delivery time will depend on the operator's surgical experience. Inexperienced investigators may take as long as 20 minutes to complete the technique from incision to closure, while experienced investigators may be able to complete the entire technique in around 5 minutes. For longer surgical times it may be necessary to redo the anesthetic which will prolong the recovery time.

Literature Cited

- Afione SA, Conrad CK, Kearns WG, Chunduru S, Adams R, Reynolds TC, Guggino WB, Cutting GR, Carter BJ, Flotte TR. In vivo model of adeno-associated virus vector persistence and rescue. *J Virology*. 1996; 70(5):3235–3241. [PubMed: 8627804]
- Bell CL, Vandenberghe LH, Bell P, Limberis MP, Gao G, Van Vliet K, Agbandje-McKenna M, Wilson JM. The AAV9 receptor and its modification to improve in vivo lung gene transfer in mice. *The Journal of Clinical Investigation*. 2011; 121(6):2427–2435. [PubMed: 21576824]
- Boutin S, Monteilhet V, Veron P, Leborgne C, Benveniste O, Montus MF, Masurier C. Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors. *Hum Gene Ther*. 2010; 21(6):704–712. [PubMed: 20095819]
- Ding W, Zhang L, Yan Z, Engelhardt JF. Intracellular trafficking of adeno-associated viral vectors. *Gene Ther*. 2005; 12(11):873–880. [PubMed: 15829993]
- Flotte TR, Afione SA, Zeitlin PL. Adeno-associated virus vector gene expression occurs in nondividing cells in the absence of vector DNA integration. *American Journal of Respiratory Cell and Molecular Biology*. 1994; 11(5):517–521. [PubMed: 7946381]
- Flotte T, Carter B, Conrad C, Guggino W, Reynolds T, Rosenstein B, Taylor G, Walden S, Wetzel R. A Phase I Study of an Adeno-Associated Virus-CFTR Gene Vector in Adult CF Patients with Mild Lung Disease. *Human Gene Therapy*. 1996; 7(9):1145–1159. [PubMed: 8773517]
- Flotte TR, Fischer AC, Goetzmann J, Mueller C, Cebotaru L, Yan Z, Wang L, Wilson JM, Guggino WB, Engelhardt JF. Dual Reporter Comparative Indexing of rAAV Pseudotyped Vectors in Chimpanzee Airway. *Molecular Therapy*. 2010; 18(3):594–600. [PubMed: 19826405]
- Gao G, Vandenberghe LH, Alvira MR, Lu Y, Calcedo R, Zhou X, Wilson JM. Clades of Adeno-Associated Viruses Are Widely Disseminated in Human Tissues. *J Virology*. 2004; 78(12):6381–6388. [PubMed: 15163731]
- Grimm D, Pandey K, Nakai H, Storm TA, Kay MA. Liver transduction with recombinant adeno-associated virus is primarily restricted by capsid serotype not vector genotype. *Journal of Virology*. 2006; 80(1):426–439. [PubMed: 16352567]
- Halbert CL, Standaert TA, Wilson CB, Miller DA. Successful Readministration of Adeno-Associated Virus Vectors to the Mouse Lung Requires Transient Immunosuppression during the Initial Exposure. *J Virology*. 1998; 72(12):9795–9805. [PubMed: 9811715]
- Halbert CL, Allen JM, Miller AD. Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J Virol*. 2001; 75(14):6615–6624. [PubMed: 11413329]
- Halbert CL, Miller AD, McNamara S, Emerson J, Gibson RL, Ramsey B, Aitken ML. Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors. *Hum Gene Ther*. 2006; 17(4):440–447. [PubMed: 16610931]
- Hida K, Lai SK, Suk JS, Won SY, Boyle MP, Hanes J. Common Gene Therapy Viral Vectors Do Not Efficiently Penetrate Sputum from Cystic Fibrosis Patients. *PLoS ONE*. 2011; 6(5)
- High KA, Aubourg P. rAAV Human Trial Experience; Adeno-Associated Virus. *Methods in Molecular Biology*. 2011; 807:429–457. [PubMed: 22034041]
- Kolb M, Martin G, Medina M, Ask K, Gaudie J. Gene Therapy for Pulmonary Diseases. *Chest*. 2006; 130(3):879–884. [PubMed: 16963689]
- Limberis MP, Wilson JM. Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. *Proc Natl Acad Sci U S A*. 2006; 29;103(35):12993–12998. Epub 2006 Aug 22.
- Limberis MP, Vandenberghe LH, Zhang L, Pickles RJ, Wilson JM. Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro. *Mol Ther*. 2009; 17(2):294–301. Epub 2008 Dec 9. [PubMed: 19066597]
- Liu X, Luo M, Trygg C, Yan Z, Lei-Butters DCM, Smith CI, Fischer AC, Munson K, Guggino WB, Bunnell BA, Engelhardt JF. Biological Differences in rAAV Transduction of Airway Epithelia in

- Humans and in Old World Non-human Primates. *Molecular Therapy*. 2007; 15(12):2114–2123. [PubMed: 17667945]
- Moore MJ, Flotte TR. Autoimmunity in a Genetic Disease: A Cautionary Tale. *New England Journal of Medicine*. 2010; 363(15):1473–1475. [PubMed: 20925551]
- Moss RB, Rodman D, Spencer LT, Aitken ML, Zeitlin PL, Waltz D, Milla C, Brody AS, Clancy JP, Ramsey B, Hamblett N, Heald AE. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. *Chest*. 2004; 125(2):509–521. [PubMed: 14769732]
- Moss RB, Milla C, Colombo J, Accurso F, Zeitlin PL, Clancy JP, Spencer LT, Pilewski J, Waltz DA, Dorkin HL, Ferkol T, Pian M, Ramsey B, Carter BJ, Martin DB, Heald AE. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Hum Gene Ther*. 2007; 18(8):726–732. [PubMed: 17685853]
- Mueller C, Flotte TR. Clinical gene therapy using recombinant adeno-associated virus vectors. *Gene Therapy*. 2008; 15(11):858–863. [PubMed: 18418415]
- Pasquale GD, Davidson BL, Stein CS, Martins I, Scudiero D, Monks A, Chiorini JA. Identification of PDGFR as a receptor for AAV-5 transduction. *Nat Med*. 2003; 9(10):1306–1312. [PubMed: 14502277]
- Sanlioglu S, Monick MM, Luleci G, Hunninghake GW, Engelhardt JF. Rate limiting steps of AAV transduction and implications for human gene therapy. *Curr Gene Ther*. 2001; 1(2):137–134. [PubMed: 12108951]
- Southam DS, Dolovich M, O'Byrne PM, Inman MD. Distribution of intranasal instillations in mice: effects of volume, time, body position, and anesthesia. *American Journal of Physiology - Lung Cellular and Molecular Physiology*. 2002; 282(4):L833–L839. [PubMed: 11880310]
- Summerford C, Samulski RJ. Membrane-Associated Heparan Sulfate Proteoglycan Is a Receptor for Adeno-Associated Virus Type 2 Virions. *J Virology*. 1998; 72(2):1438–1445. [PubMed: 9445046]
- Walters RW, Yi SP, Keshavjee S, Brown KE, Welsh MJ, Chiorini JA, Zabner J. Binding of Adeno-associated Virus Type 5 to 2,3-Linked Sialic Acid Is Required for Gene Transfer. *Journal of Biological Chemistry*. 2001; 276(23):20610–20616. [PubMed: 11262413]
- Zabner J, Seiler M, Walters R, Kotin RM, Fulgeras W, Davidson BL, Chiorini JA. Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J Virol*. 2000; 74(8):3852–3858. [PubMed: 10729159]
- Zaiss AK, Muruve DA. Immunity to adeno-associated virus vectors in animals and humans: a continued challenge. *Gene Therapy*. 2008; 15(11):808–816. [PubMed: 18385765]

Figure 1. Speculum/Otoscope Assisted Orotracheal Vector Delivery

A: Set-up for speculum assisted intubation: incisor loop, pointed cotton-tipped applicator, stylet with catheter, chilled mirror, rodent stand with Velcro to secure incisor loop and chest restraint, syringe with vector, syringe to deliver air, otoscope with speculum properly positioned (left to right). **B.** Mouse positioned on rodent work stand with incisor loop and chest restraint. Tongue being rolled from mouth using cotton-tipped applicator. Roll tongue away from side where speculum will be held. **C.** Speculum in proper position to view larynx (held in dominant hand) and stylet with catheter in place positioned for passage into trachea.

Figure 2. Surgical Tracheal Delivery

A: Surgical set-up: Forceps (x2), scissors, needle holders, scalpel, surgical board on angle, syringe with vector, suture, surgical swab wipe, depilatory cream (left to right). **B:** Vector injection, note incision location and tracheal visibility. **C:** Demonstrating gripping needle and placement of suture. **D:** Properly closed incision.

Figure 3. Post-mortem Tissue Processing

A: Muscles overlying trachea, transect with scissors to better access trachea. **B:** Placement of suture used to secure catheter during BAL and close trachea following lung inflation. **C:** Catheter and suture properly placed. **D:** Small cut in diaphragm just under xyphoid to collapse the lungs. **E:** Careful cut down each side of diaphragm from initial opening. **F:** Chest opened and ribs retracted. Needle is pointing to the tip of the left ventricle of heart. For flushing blood from lungs make a small cut in left ventricle and insert needle into right ventricle (smaller than left). **G:** Lung inflated with fixative via tracheal catheter.

Table 1

Airway Serotype Specificity in Mice

Serotype	Surface Transduced	Transduction Level	Receptor	Reference
rAAV2	Basolateral >>>apical	A= +/-, AL= +/- B = +/-, ASm = ++	Heparin Sulfate	Halbert (2001 and 1998), Liberis (2006 and 2009) Zabner (2000)
rAAV5	Basolateral>apical	A= ++, AL= +++	PDGFR, 2–5-linked sialic acid	Limberis (2006) Zabner (2000) Pasquale (2003) Walters (2001)
rAAV6	Apical	A= ++, AL= +++ B = ++, ASm = +	Sialylated glycoproteins	Halbert (2001) Limberis (2009)
rAAV6.2	Apical	A= +++++, AL= +++	Unknown	Limberis (2009)
rAAV9	Basolateral and apical	A= +, AL= ++	Glycans with terminal galactose	Limberis (2006 and 2009) Bell (2011)

Key: A=Airway Cells, AL=Alveolar Cells, B=Bronchial Epithelium, ASm = Airway Smooth Muscle

Transduction Scoring: (–) No or minimal transduction, (+) Low Level Transduction, (++) Moderate Transduction, (+++) High Level Transduction, (+++++) Very High Level Transduction