**Rhesus Monkey Instillation Protocol – STUDY 2**

**STUDY 2: Delivery of a base-editor RNP + peptide to target editing of the CCR5 gene (n=4 animals).**

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| --- | --- | --- |
| **Animal** | **Shuttle** | **Cargo** |
| Monkey #3 | FS66d2 (S10) | Cas9-ABE RNP |
| Monkey #4 | FSD315 | Cas9-ABE RNP |
| Monkey #5 | FSD315 | Cas9-ABE RNP |
| Monkey #6 | - | Cas9-ABE RNP |

Experimental Plan:

1. Prepare the “gRNA duplex”.
	1. Thaw all materials on ice.
	2. Add the components in the order listed (critical) in the Table 1 to prepare a master mix for 4 monkeys. Mix with P1000 pipette tip, avoid forming bubbles, do not vortex.
	3. Incubate gRNA duplex at 95 degrees Celsius for 10 minutes
	4. Cool to room temperature.

Table 1. “gRNA duplex” preparation, master mix for 4 monkeys.

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| crRNA (CCR5)  | 96 |
| tracrRNA (IDT)  | 96 |
| Duplex Buffer  | 768 |
| Total Volume “gRNA duplex” | 960 |

1. Prepare the “RNP mix”.
	1. Add the components in the order listed (critical) in the Table 2 to prepare a master mix for 4 monkeys. Mix with P1000 pipette tip, avoid forming bubbles, do not vortex.
	2. Incubate the “RNP mix” at room temperature for a minimum of 15 min (maximum 4 hours)

Table 2. “RNP mix” preparation, master mix for 4 monkeys.

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| DPBS | 1,083 |
| “gRNA duplex” | 912 |
| Cas9-ABE | 285 |
| Total Volume “RNP mix” | 2,280 |

1. Prepare the Instillation Solution.
	1. Prepare the Instillation Solution for each monkey separately as indicated in the Table 3 – 6 below.
	2. Add the components in the order listed (critical). Mix with P1000 pipette tip, avoid forming bubbles, do not vortex.

Warm up to RT for 30 min before instillation, and use no later than 4 hours after preparation.

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| DPBS | 374 |
| Shuttle FS66d2 (S10) | 176 |
| “RNP mix” (Cas9-ABE/gRNA duplex) | 550 |
| Total Volume “Instillation Solution” | 1,100 |

Table 3. “Instillation Solution” preparation for Monkey #3

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| DPBS | 374 |
| Shuttle FSD315 | 176 |
| “RNP mix” (Cas9-ABE/gRNA duplex) | 550 |
| Total Volume “Instillation Solution” | 1,100 |

Table 4. “Instillation Solution” preparation for Monkey #4

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| DPBS | 374 |
| Shuttle FSD315 | 176 |
| “RNP mix” (Cas9-ABE/gRNA duplex) | 550 |
| Total Volume “Instillation Solution” | 1,100 |

Table 5. “Instillation Solution” preparation for Monkey #5

|  |  |
| --- | --- |
| **Component** | **Volume (µl)** |
| DPBS | 550 |
| “RNP mix” (Cas9-ABE/gRNA duplex) | 550 |
| Total Volume “Instillation Solution” | 1,100 |

Table 6. “Instillation Solution” preparation for Monkey #6

1. Sedate the animals as per established protocol (animals approximately 3 months of age; n=4 animals).
2. Collect the bodyweight, body temperature, and respiration rate.
3. Place the animal in a dorsal recumbency on a procedure table and intubate the trachea as per established protocol.
4. Remove the atomizer from the original sealed pouch and insert the atomizer into the trachea so that the atomizer’s tip is positioned below the cricoid cartilage and at least 1 cm above the bifurcation.
5. Pre-fill 1,000 ul of the instillation solution into 1 ml syringe.
6. Connect the pre-filled 1 ml syringe to the proximal end of the atomizer.
7. Instill the solution by placing fast and firm pressure on the syringe pestle. (Instillation as fast as possible is needed to generate an aerosol from the atomizer’s tip).
8. While keeping the atomizer in place in the trachea, disconnect the emptied syringe, connect a new 1 ml syringe pre-filled with 400 ul of ambient air, and expel the air by placing fast and firm pressure on the syringe pestle in the same manner as in previous point. (This will empty the instillation solution trapped in the dead space of the atomizer’s tubing, which is about 230 ul).
9. Remove the atomizer and intra-tracheal canula, if any.
10. Proceed with the animal recovery as per established protocol.
11. Tissue collection will be performed 7-9 days after instillation.

**Rhesus Monkey Tissue Sampling – STUDY 2**

**STUDY 2:** Delivery of a base-editor RNP + peptide to target editing of the CCR5 gene (n=4 animals).

**Organ collection after delivery of a base editor – 7-9 days after instillation**

1. Open the chest cavity and allow the lungs to collapse.
2. Cut the large vessels at the level of diaphragm.
3. Perfuse the vasculature of the lungs: use a 50 mL syringe with a 20 - 22 G needle to inject between 20 - 100 ml of 1X PBS with 10U/mL Heparin into the right ventricle of the heart until the lungs are white.
4. Remove the lungs en bloc (including the heart).
5. Cut off the left lower lobe at the proximal end of the major lobar airway. Dissect the lobar airway into up to 5 (five) sections, each about 0.5 cm long, and place into 3 ml of Trizol, each. Dissect 5 (five) pieces of lung tissue adjacent to the proximal major lobar airway, each size of 0.5 cm3 and place into 3 ml of Trizol, each. Keep the tubes at 4dg C.
6. Place the lungs and the remaining left lower lobe in a triple ziplock bag. Expel as much air from the bags before closing without compromising the lungs tissue.
7. Place the bagged lungs and tubes with tissues in Trizol in a box with cold packs. Package and ship as per the applicable guidelines overnight to the University of Iowa (shipping address below).

University of Iowa shipping address:

McCray Laboratory

Attn: Katarina Kulhankova, Soumba Traore

6320 PBDB

169 Newton Rd
Pappajohn Biomedical Institute

Carver College of Medicine

University of Iowa

Iowa City, IA 52242

Email: katarina-kulhankova@uiowa.edu, soumba-traore@uiowa.edu

24-hour phone numbers: (319) 855-8541, (319) 471-6370

**Analysis of Editing Efficiency**

Prior to euthanasia the clinical parameters and blood was collected. Following euthanasia, the chest cavity was opened, the large vessels were cut at the diaphragm, and the lung vasculature was perfused with PBS and 10 U/ml Heparin via the right heart ventricle.

The animals treated with the gene editor were processed as follows: At necropsy the lungs were removed from the chest cavity, the right lower lobe was separated and used for cryobanking of fresh tissue in RNALater. The remaining lungs were placed on ice, and along with the tissues in RNALater shipped chilled to the UI. Upon receipt, the left and right mainstem bronchus was dissected from the trachea at the bifurcation and from the lobes before the first major airway branching. Each lung lobe was separated at the main lobar airway. Trachea and each mainstem bronchus separately were brushed with a 4 mm diameter cytological brush (Ref 25-2199, Puritan Medical Products Company LLC, Guilford, ME); and each main lobar bronchus was brushed with another individual brush depending on the size of the airway (FLOQSwabs, Copan Diagnostics, Inc, Murrieta, CA; ConMed Disposable Bronchial Cytology Brush, ConMed Corporation, Utica, NY). Left lower lobe was perpendicular to the main axis at approximately half way, and the major airway of the distal part of the lobe was brushed. Brushings were collected into 5 ml PBS in 15 ml conical tubes placed on ice and centrifuged at 200xG for 5 min. The cell pellets were reconstituted in 500 µl of PBS, and the cell suspensions divided into 2 aliquots – one for DNA extraction and one for cryopreservation. The tissues containing a large airway and a small airways were dissected, placed in RNALater or Trizol and cryopreserved. The selected remaining lung tissue was preserved in 4% PF.

Next Generation Sequencing and analysis.

Genomic DNA was isolated from the cells isolated by airway brushing using QuickExtract (catalog #QE09050, Lucigen, Middleton, WI) according to the manufacturer’s protocol. The target locus was PCR amplified (KAPA DNA polymerase, Roche, Basel, Switzerland)) using primers designed to the rhesus CCR5 locus and the appropriate Illumina forward and reverse adaptors as described (table below). Unique Illumina barcoding primer pairs were then added to each sample in a second PCR reaction. Purified PCR products next quantified and sequenced using a single-end read of 200-250 bases on the Illumina MiSeq instrument using the manufacturer’s protocols. Following high throughput sequencing (HTS), the sequencing reads were demultiplexed using MiSeq Reporter (Illumina) and aligned to the appropriate reference genome as previously reporter. Base substitution and indel frequencies were assessed using the software package CRISPResso2, which counts indels of ≥1 base occurring in a 30-base window around the ABE nicking site. Indels were defined as detectable if there is a significant difference (Student’s two-tailed t-test, P < 0.05) between indel formation in the treated sample and untreated control. Base editing frequencies were further assessed using a previously described MATLAB script. For each CCR5 modification we determined the target base editing frequencies, bystander edits, and indel frequency. The allelic editing frequency was calculated by the following equation: (% editing-50)/50.

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| --- | --- | --- | --- | --- |
| Target Gene | gRNA sequence | PAM | Forward Primer\* | Reverse Primer\* |
| CCR5 | GAG**A**GTTTCTTGTAGGGGAA | CGG | 1: ACTGTCTATATGATTGATTTGCACAACT2: CGCCTTCAATACTCAATTATTTAACTC | 1: CCTTTAAAGTCTTTCACTCACAATC2: GTCAGAGTTTAGAATGAGTTTCAGATT |

\* Adapters were added to the primer in the following format:

ACACTCTTTCCCTACACGACGCTCTTCCGATCTNNNN-Forward Primer Sequence

TGGAGTTCAGACGTGTGCTCTTCCGATCT-Reverse Primer Sequence