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## Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection

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**October 14, 2019**

**To the editors of *JoVE***

Dear Dr. Steindel,

Please consider our revised manuscript entitled "***Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection"*** by Andersen, *et al.* for publication in *JoVE*.

We sincerely appreciate the interest that you and the reviewers have taken to our manuscript. We have addressed all critiques and questions and as a consequence our revised manuscript is significantly improved. We also address all points from the reviewers in our attached point-by-point response-to-reviewers document. Changes to the manuscript are highlighted in the compare document included with our resubmission.

Overall, we removed any mention of this technique as a substitute for BLT mice, because we believed this focused the reviewers on comparisons to BLT mice which we did not intend and do not have any data to address.

To accommodate the requests from the editor and reviewers, we have significantly expanded the Table of Materials and Reagents, and removed any mention of tradenames etc. in the protocol text. In addition, we have added three additional tables that detail the flow cytometry antibody panels and mouse cART chow diet. We hope that these changes will aid in achieving a comprehensive overview of the used reagents.

In response to Reviewer 2 we have included references of appropriate literature detailing the utilities of these mice and added key details they requested for completeness of the protocol.

We greatly appreciate your time and your consideration of our revised manuscript for publication. Please know that I am available and willing to take any questions you may have about this resubmission.

Sincerely,

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**TITLE:**

# Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection

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**KEYWORDS:**

immunology and infection, NOG mouse, humanized mice, HIV, cART, CCR5, stem cells, ddPCR

**SUMMARY:**

We have developed a protocol for the generation and evaluation of a humanized and human immunodeficiency virus-infected NOG mouse model based on stem cell transplant, intravaginal human immunodeficiency virus exposure, and droplet digital PCR RNA quantification.

**ABSTRACT:**

Humanized mice provide a sophisticated platform to study human immunodeficiency virus (HIV) virology and to test antiviral drugs. This protocol describes the establishment of a human immune system in adult NOG mice. Here, we explain all the practical steps from isolation of umbilical cord blood derived human CD34+ cells and their subsequent intravenous transplantation into the mice, to the manipulation of the model through HIV infection, combination antiretroviral therapy (cART), and blood sampling. Approximately 75,000 hCD34+ cells are injected intravenously into the mice and the level of human chimerism, also known as humanization, in the peripheral blood

is estimated longitudinally for months by flow cytometry. A total of 75,000 hCD34+ cells yields 20%–50% human CD45+ cells in the peripheral blood. The mice are susceptible to intravaginal infection with HIV and blood can be sampled once weekly for analysis, and twice monthly for extended periods. This protocol describes an assay for quantification of plasma viral load using droplet digital PCR (ddPCR). We show how the mice can be effectively treated with a standard-of-care cART regimen in the diet. The delivery of cART in the form of regular mouse chow is a significant refinement of the experimental model. This model can be used for preclinical analysis of both systemic and topical pre-exposure prophylaxis compounds as well as for testing of novel treatments and HIV cure strategies.

**INTRODUCTION:**

Human immunodeficiency virus (HIV) is a chronic infection with more than 37 million infected individuals worldwide[1]. Combination antiviral therapy (cART) is a life-saving therapy, but a cure is still warranted. Thus, there is a need for animal models that mirror the human immune system and its responses in order to facilitate continued research in HIV. Multiple types of humanized mice that are capable of supporting cell and tissue engraftment have been developed by transplanting human cells into severely immunodeficient mice[2]. Such humanized mice are susceptible to HIV infection and provide an important alternative to nonhuman primate simian immunodeficiency virus models, as they are cheaper and simpler to use than nonhuman primates. Humanized mice have facilitated research in HIV viral transmission, pathogenesis, prevention, and treatment[3–11].

We present a flexible humanized model system for HIV research developed by transplanting cord blood derived human stem cells into mice of the NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Sug}$/JicTac (NOG) background. Besides being of non-fetal origin, the practical bioengineering of these mice is less technically demanding compared to the microsurgical procedures involved in the transplant of the blood-liver-thymus (BLT) construct.

We show how to establish HIV infection through intravaginal transmission and how to monitor the plasma viral load with a sensitive droplet digital PCR (ddPCR)-based setup. Subsequently, we describe the establishment of standard cART given as part of the daily mouse diet. The aim of these combined methods is to reduce stress to the animals and facilitate large-scale experiments where time spent handling each animal is limited[12].

In humans, a *CCR5*$^{\Delta 32/wt}$ or *CCR5*$^{\Delta 32/\Delta 32}$ genotype causes reduced susceptibility to HIV infection with transmitter/founder viruses[13], and some precautions must be taken when bioengineering humanized mice with stem cells for the purpose of HIV studies. This is especially true in our region because naturally occurring variants in the *CCR5* gene, particularly Δ32 deletions, are more prevalent in Scandinavian and Baltic native populations compared to rest of the world[14,15]. Thus, our protocol includes an easy, high-throughput assay for screening donor hematopoietic stem cells for CCR5 variants prior to transplantation.

For the intravaginal exposure we chose the transmitter/founder R5 virus RHPA4259, isolated from a woman in an early stage of infection who was infected intravaginally[16]. We exposed the

mice to a viral dose that was sufficient to yield successful transmission in the majority of mice, but below a 100% transmission rate. Choosing such a dose enables a sufficient dynamic range in transmission rate such that antiviral effects of a drug candidate can result in protected animals in HIV prevention experiments and decreased viral load for treatment studies.

**PROTOCOL:**

All cord blood samples were obtained in strict accordance with locally approved protocols, including informed consent of anonymous donation by the parents. All animal experiments were approved and performed in strict accordance with Danish national regulations under the license 2017-15-0201-01312.

CAUTION: Handle HIV exposed mice and blood with extreme caution. Decontaminate all surfaces and liquids that have been in contact with HIV with a confirmed HIV disinfectant (**Table of Materials**).

**1. Isolation of human CD34+ stem cells**

1.1. Collect cord blood samples in EDTA-coated blood collection tubes after planned Caesarean sections or vaginal births and according to local ethical approvals.

1.2. Isolate PMBCs from cord blood by density-gradient separation according to the manufacturer's protocol.

1.3. Isolate CD34+ cells from the PBMC population by first pre-enriching with antibodies against common markers for mature cells, which induces crosslinking of cells of undesired lineages with red blood cells. This is followed by CD34+ cell enrichment using magnetic beads, according to the manufacturer's protocol.

1.3.1. Determine live cell count by standard trypan blue exclusion by resuspending 10 µL of cell suspension in 90 µL of trypan blue. Add 10 µL of this solution to a hemacytometer and count non-blue cells, according to the manufacturer's protocol.

1.3.2. Viably cryopreserve CD34+ cells in 1 mL of 10% DMSO in fetal bovine serum (FBS) until the day of mouse transplantation.

1.3.3. Viably cryopreserve a small fraction of both isolated (CD34+) and flow-through cells (CD34-) separately for assessing CD34+ stem cell purity (~30,000 cells of each sample). Alternatively, test the purity on freshly enriched cells (see step 2 below).

1.3.4. Freeze a fraction of non-pelleted flow-through (CD34-) for determination of *CCR5*Δ32 status. Cells can be frozen directly without conditioned freezing solution, but the presence of red blood cells in the pellet can inhibit the subsequent PCR if the flow-through is pelleted.

**2. Assessing CD34+ stem cell purity via flow cytometry**

2.1. Thaw the isolated cells (CD34+) and flow-through cells (CD34-). Wash the cells by resuspending the cells from each vial in 9 mL of room temperature (RT) FACS buffer, consisting of 2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS).

2.2. Centrifuge for 5 min at 300 x *g* at RT to pellet cells.

2.3. Pour off the supernatant, resuspend the cells in the remaining liquid and transfer to FACS tubes. Repeat the washing step with 3 mL of FACS buffer. After the second centrifugation, pour off the supernatant and resuspend the cells in the remaining liquid.

2.4. Add 5 µL of Fc Receptor blocking solution (**Table of Materials**) and leave for 10 min at RT. Do not wash off the Fc Receptor blocking solution.

2.5. Add the mix containing predetermined volumes of antibodies against human CD3 (clone SK7) BUV395, CD34 (clone AC136), FITC, and CD45 (clone 2D1) APC (**Table 1**). Leave the cells for 30 min at RT in the dark. The fluorophores must be chosen based on parameters that can be assessed with available flow cytometers without requiring a compensation matrix.

2.6. Wash the cells with 3 mL of FACS buffer.

2.7. Centrifuge for 5 min at 300 x *g* at RT to pellet the cells.

2.8. Pour off the supernatant and resuspend the cells in the remaining liquid.

2.8.1. Repeat this washing step 2x to ensure all unbound antibodies have been removed.

2.9. Record the samples on the flow cytometer (**Table of Materials**) and perform data analysis with appropriate software. The gating strategy is presented in **Figure 1A–F**.

**3. Genetic screening for *CCR5*Δ32 variants in cord blood**

3.1. Incubate 1.25 µL of non-pelleted flow-through with 11.25 µL of PCR mix containing 200 µM of dNTP mix, 0.01 U/µL high fidelity DNA polymerase, and the forward and reverse primers detailed in **Table 2**.

3.1.1. Adjust the volume with nuclease-free water to approximately 12.5 µL for each PCR reaction.

3.2. Amplify the genomic fragments with the PCR cycling program detailed in **Table 3**.

3.3. Separate the PCR products on a 2% agarose gel[13].

3.3.1. PCR products from the wild type alleles and the Δ32 alleles yield PCR fragments of 196 base pairs and 164 base pairs bands respectively, making them easily distinguishable by gel electrophoresis[13] (**Figure 1G**).

**4. Intravenous stem cell transplant**

NOTE: Having one person prepare the cells in the laboratory and another person prepare the mice and workspace for transplants is an efficient approach.

4.1. In an animal facility, 4–6 h before the planned transplantation of the stem cells, irradiate 6–7 week-old female NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Sug}$/JicTac (NOG) mice (**Table of Materials**) with 0.75 Gy with a $Cs^{137}$ source. The best preconditioning dose may vary based on mouse age, source of radiation, and other factors. This process conditions the animals for successful engraftment with human stem cells.

4.2. In an animal facility, prepare the flow bench workspace and all reagents before bringing the mice or cells into the workspace.

4.2.1. Place a sterile blue pad to cover the working surface of the flow bench. Prepare sterile gauze and a sharps container.

4.2.2. Place a heating lamp disinfected with 70% ethanol in the flow bench with an empty sterile mouse cage underneath the heat.

4.3. In the laboratory, thaw isolated CD34+ cells and dilute them in 9 mL of 37 °C plain RPMI.

4.4. Centrifuge the cells at 350 x *g* for 5 min at RT, discard the supernatant by aspiration, and resuspend the pellet in 1 mL of plain RPMI at 37 °C.

4.5. Determine the cell count by trypan blue exclusion, and adjust the volume to 200 µL per mouse. Make extra to take into account possible loss due to the subsequent handling steps.

4.5.1. Prepare to transplant 75,000 CD34+ cells per 200 µL into each mouse.

4.5.2. The cells can be kept at 4 °C during transport to the animal facility before the transplant. Avoid keeping the cells on ice to reduce aggregation or clumping.

4.6. In the animal facility, bring the cage with the mice into the flow bench and transfer the mice to the cage under the heating lamp to dilate the blood vessels. Leave one end of the cage away from the heat source so that the mice can move away from the heat upon becoming warm. Mice that have moved to the end of the cage, away from the heat source, are sufficiently warm for a successful tail vein injection.

4.7. Load a 1 mL lubricated syringe to above the 800 µL mark with suspended CD34+ cells. Using a lubricated 1 mL syringe will dramatically ease the intravenous injection and increase the precision of this technique.

4.8. Attach a 30 G 13 mm needle and prepare the needle and syringe for injection. This order of operation allows for the syringe to be loaded quicker while protecting the integrity of the cells to be transplanted given the possible damage that can occur during the rapid aspiration of cells through such a small gauge needle. Fill the needle hub with liquid by pressing the plunger and remove liquid down to the 800 µL mark of the needle (800 µL is an appropriate volume for one cage housing four animals).

4.9. Place a heated mouse (step 4.6) in a restrainer used for giving IV injections. Carefully inject 200 µL of the cell suspension into the tail vein of the mouse. Spend 2 s performing the plunge and keep the needle inserted for approximately 2 s after completion of the injection. This ensures that the cells have migrated adequately far from the injection site prior to removal of the needle.

4.10. As necessary, wipe the mouse tail with sterile gauze to remove any visible blood. Put the mouse back into its non-heated home cage.

4.11. Repeat the injection procedure with the remaining mice. It is not necessary to change the needle between the different mice unless the needle becomes dull, which is possible after 8–12 attempted tail vein injections.

**5. Blood collection and processing for analysis**

NOTE: Human cell engraftment in the peripheral blood can be evaluated via flow cytometry 3–5 months after human stem cell transplantation.

5.1. Draw blood samples from the mice using local IACUC-approved techniques.

5.2. Collect a maximum of 70–100 µL of total blood into sterile PCR microcentrifuge tubes containing 10 µL of 0.5 M EDTA (pH = 8.0) to avoid coagulation of blood.

**6. Evaluation of human engraftment via flow cytometry**

6.1. Transfer 40–50 µL of blood to FACS tubes.

6.2. Add 5 µL of Fc Receptor blocking solution to prevent nonspecific binding of antibodies and leave for 10 min at RT.

6.3. Add the mouse anti-human antibody mix containing CD4 (clone SK3) BUV 496, CD8 (clone RPA-T8) BV421, CD3 (clone OKT3) FITC, CD19 (clone sj25c1) PE-Cy7, CD45 (clone 2D1) APC (**Table 4**) and leave to stain in the dark at RT for 30 min. Fluorophores must be chosen based on

parameters that can be assessed with the available flow cytometers without requiring a compensation matrix.

6.4. Add 2 mL of an appropriate red blood cell lysing buffer to each tube to lyse the red blood cells. Use a lysis buffer specifically formulated for antibody staining prior to red blood cell lysis (a suitable example is given in the **Table of Materials**). Vortex briefly to ensure equal distribution of the cells in the lysing solution and leave for 10 min at RT. Equal distribution of the cells is important.

6.5. Add 2 mL of FACS buffer to stop the lysis reaction.

6.6. Centrifuge for 5 min at 300 x *g* at RT to pellet the cells.

6.7. Pour off the supernatant and vortex gently until the cells are resuspended.

6.8. Add 3 mL of FACS buffer and centrifuge for 5 min at 300 x *g* at RT.

6.9. Pour off the supernatant and resuspend the cells.

6.10. Record the samples on an appropriate flow cytometer and analyze using appropriate software (**Table of Materials**). Representative analysis and results are depicted in **Figure 2** and **Figure 3**.

## 7. Intravaginal HIV exposure

NOTE: The virus used for intravaginal exposure of the mice can be produced using previously published protocols[17]. The virus is kept at -80 °C and transported between locations while stored on dry ice following locally approved protocols. The virus is stored on dry ice until immediately before the exposure of the mice. The virus can be diluted into plain RPMI (avoid RPMI that has antibiotics or serum additives) to achieve the appropriate concentration immediately prior to exposure (21,400 IUs were used for this IVAG exposure). Once generated, keep the diluted stock on wet ice throughout the procedure to avoiding freeze-thaw cycles that would occur if the diluted virus was placed back on dry ice once thawed.

7.1. Prepare all the equipment and flow bench workspace as presented in **Figure 4** before bringing the mice or virus into the flow bench (similar to step 4.2).

7.1.1. Place the heating lamp focus in the center of the workspace where the mouse will be located during the HIV exposure procedure. The heating lamp will ensure no decrease in body temperature of the mice. Other equipment that controls temperature can also be used, (e.g. a heated gel pad or a circulating warm water blanket, according to local IACUC regulations[18]).

7.1.2. Bring sterile 20 µL pipette tips and an appropriate pipette into the bench. Place a container with liquid disinfectant (**Table of Materials**) in the bench for immediate inactivation of materials and liquids that have been in contact with the virus.

7.2. Place a mouse into a chamber with 3% isoflurane gas and paper towels. This percentage of gas will anesthetize the animals within 2–4 min. As with all other materials used with immunodeficient mice, the anesthesia apparatus must be properly disinfected prior to use.

7.3. Once anesthetized, transfer the mouse to a sterile blue pad under the heating lamp. Insert the mouse snout into a mask supplying continuous 3% isoflurane gas to maintain anesthesia. Hold the mouse at the base of the tail, stomach facing up, with your hand supporting the mouse back as depicted in **Figure 4**.

7.4. With a sterile pipette tip, stimulate the genital area by gently stroking upwards towards the anus to induce emptying of the rectum, relieving pressure on the vagina.

7.5. Carefully bare the vaginal opening by wrapping the mouse tail across your fingers such that the vulva naturally opens, perhaps with slight nudging using a sterile pipette tip.

7.6. Change the pipette tip and pipette 20 µL of virus atraumatically into the mouse vagina without creating bubbles. Do not insert the tip deep into the vagina. Rather, with the vulva opened, place the pipette tip at the level of the vaginal opening, avoid going deeper to eliminate the potential for abrasions during the inoculation process, release the virus, and allow gravity to pull the virus into the vagina. Alternatively, use a 22 G 1.25 mm blunt-end, straight needle, as described in Veselinovic et al.[6].

7.7. Retain the mouse in this position with the vagina facing up for 5 min after exposure to avoid gravity-induced leakage of the virus suspension.

7.7.1. Carefully place the mouse into the home cage, taking care to place the mouse on its back.

**8. Processing of blood samples prior to viral load analysis**

8.1. Collect blood as described in section 5 above.

8.2. Centrifuge the blood samples for 5 min at 500 x *g* at RT to separate the plasma and cells.

8.3. Collect 40 µL of plasma for viral load measurement into a new sterile PCR-approved microcentrifuge tube and store at -80 °C for at least 1 h until further processing. It is important to freeze all samples before RNA extraction to avoid the risk of bias from comparing RNA levels in samples that have not been frozen prior to RNA isolation to samples frozen prior to RNA isolation.

8.4. Adjust the volume of blood back to the original volume by adding 40 µL of suspension media (PBS with 2.5% bovine serum albumin, 50 U/mL penicillin G and streptomycin, and 10 U/mL DNase, sterile-filtered at 0.22 µm).

8.5. Transfer 15 µL of the adjusted blood volume to a new PCR-approved microcentrifuge tube.

8.6. Add 1 mL of 1x RBC lysis solution (**Table of Materials**), vortex, and incubate for 10 min at RT.

8.7. Centrifuge for 1 min at 9,600 x *g* at RT to pellet cells.

8.8. Aspirate supernatant and leave only the tiny white cell pellet, because red blood cell contamination can inhibit PCR.

8.9. Store pellet at -80 °C for at least 1 h until further processing.

NOTE: Optionally, any remaining blood from step 8.4 can be used for flow cytometry analysis, as described above in step 6.

**9. DNA extraction using a proteinase K extraction method**

9.1. Extract DNA from peripheral cell pellets (generated in step 8.8) using a proteinase K extraction method as described below. This method has been demonstrated to extract the highest DNA yield from a small volume of blood such as that required for the serial blood collections utilized in this study[19].

9.2. Add 25 µL of proteinase K (20 µg/mL) to 1 mL of 0.1M Tris buffer.

9.3. Vortex the proteinase K solution briefly.

9.4. Add 50 µL of the proteinase K solution to each cell pellet to be digested.

9.5. Mix by pipetting up and down and confirm the resuspension of the cell pellet.

9.6. Shake on a thermoshaker (**Table of Materials**) at 400 rpm (depending on the instrument) at 56 °C for 1 h. Tape tubes down to hold them in place, if necessary.

9.7. Immediately and in the same thermoshaker, inactivate proteinase K with a temperature shift to 95 °C while the shaking continues for an additional 20 min.

9.8. Vortex each sample.

9.9. Place each sample at -80 °C for a minimum of 30 min.

9.10. Thaw, then centrifuge the samples at 17,000 x *g* for 1 min at RT to pellet unwanted cellular fragments.

9.11. Place the DNA-containing supernatant into a new microcentrifuge tube.

9.12. The DNA template is ready for PCR. The DNA templates can be stored at -80 °C.

**10. RNA extraction, cDNA synthesis, and ddPCR quantification of viral RNA**

10.1. Isolate RNA from thawed mouse plasma with a virus RNA isolation kit following the manufacturer's protocol (**Table of Materials**).

10.2. After addition of the sample to the column, add an on-column DNase treatment step to ensure removal of all DNA in the plasma sample.

10.2.1. For each sample, add 95 µL of RNase-free DNase solution (mix 2 µL RNAse-free DNase and 98 µL reaction buffer) to the column and incubate for 15 min at RT.

10.3. Store the RNA samples at -80 °C for at least 1 h before further processing. It is important to freeze all samples after RNA extraction do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen.

10.4. Synthesize cDNA using a reverse transcriptase step using reagents as described previously[20]. Make sure to add 0.5 µL of an RNase inhibitor to the cDNA reaction to avoid degradation of the RNA.

10.4.1. Perform cDNA synthesis with the program detailed in **Table 5**.

10.5. Store the cDNA samples at -80 °C for at least 1 h. It is important to freeze all samples after cDNA synthesis do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen.

10.6. Prepare samples for ddPCR as follows[20]:

10.6.1. Mix 3 µL of the cDNA sample with 11 µL of the ddPCR probe mixture (no dUTP)[20], 250 nM minor groove-binding probe, and 900 nM of each of the forward and reverse primers as detailed in **Table 6**.

10.6.2. Adjust the total PCR volume to 22 µL with nuclease-free water.

10.7. Emulsify the PCR mixes with Droplet Generation Oil for Probes on a droplet generator according to the manufacturer's protocol and previous descriptions[20].

10.8. Run the PCR program as detailed in **Table 7**.

NOTE: The primer/probe sequences and PCR programs displayed here have been specifically designed and optimized for sensitive detection of the HIV strain RHPA4259. Primer and probe sequences can easily be adjusted to detect any other HIV strain of choice.

10.9. Detect droplet fluorescence from the samples on a droplet reader, and analyze the results with appropriate software according to the manufacturer's protocol.

**11. Treatment with cART-containing chow**

11.1. Feed mice with pellets containing a standard cART regimen containing 4,800 mg/kg raltegravir (RAL), 720 mg/kg tenofovir disoproxil fumarate (TDF), and 520 mg/kg emtricitabine (FTC)[21] (**Table 8**). These doses were determined assuming that a mouse weighs 25 g and eats 4 g of chow per day. This corresponds to a daily dose of 768 mg/kg RAL, 2.88 mg/kg TDF, and 83 mg/kg FTC[21].

11.2. Use a cART diet that has been prepared by an external vendor (See **Table of Materials**) of prescription drugs. Other companies could potentially also produce this regimen. Use a cART diet colored red to easily distinguish it from ordinary mouse chow.

11.2.1. Produce a control chow diet without cART in a standard brown color for easy distinction.

11.3. For initiation of cART, prepare sterile mouse cages with the addition of cART-containing chow diet, and then transfer the mice from the old cage to the new cage.

11.3.1. Monitor the weights of the mice and consumption of cART-containing chow by visual inspection to ensure that the mice are adjusting to the change.

**REPRESENTATIVE RESULTS:**

The gating strategy for the analysis of stem cell purity is depicted in **Figure 1**. **Figure 1A–C** shows the purified CD34+ population and **Figure 1D–F** the CD34- flow-through used to illustrate that the minimal amount of the CD34+ population is lost in the isolation process. The purity of isolated CD34+ stem cells was between 85%–95% with less than 1% T-cell contamination. **Figure 1G** depicts CCR5 bands from one adult human control donor with the *CCR5*$^{\Delta 32/wt}$ genotype, followed by bands from two *CCR5*$^{wt/wt}$ and one *CCR5*$^{\Delta 32/wt}$ stem cell donors. The frequency of the genotype *CCR5*$^{\Delta 32/wt}$ in a group of 19 donors was 15.8% (**Figure 1H**). This is in agreement with larger epidemiological studies[14,15] reporting the genotype in up to 23.6% of investigated persons in Denmark.

Human CD45+ levels in mice peripheral blood was assessed via flow cytometry 3–5 months after transplantation of human CD34+ stem cells. The gating strategy is presented in **Figure 2A–E**. **Figure 3A** and **Figure 3B** illustrate the variability between 10 and 16 individual mice receiving stem cells from two different donors. Transplantation of 75,000 hCD34+ cells yielded 20%–50% human CD45+ in the peripheral blood. All mice developed human B and T cells, including both

CD4- and CD8+ T cells.

For atraumatic intravaginal exposures, the setup depicted in **Figure 4** was used. Mice were anaesthetized in a closed chamber and kept under anesthesia during the exposure. Mice were held with the vagina facing up for 5 min after exposure to ensure virus solution engagement with mucosal surfaces.

**Figure 5A** shows the 64% HIV transmission success rate observed using this model. Mice were challenged with 21,400 infectious units (IU) of RHPA4259 intravaginally. This dose resulted in 64% of mice becoming HIV infected following vaginal exposure. For comparison, data from two different cohorts of mice exposed through an intravenous route are included. As expected, 100% of the mice became HIV+ with similar doses of RHPA and an additional strain (YU2) using this route.

**Figure 5B** depicts representative results from three mice that were infected with HIV and switched to a diet containing standard cART. Mice were switched back to regular mouse chow after 40 days of cART. In this assay setup, the limit for viral load detection was 725 copies/mL. Viral loads were all below the detection limit after 4 weeks of cART. After cessation of cART, the virus rebounded, mirroring clinical data[22]. Mice on cART tolerated the change in diet well as indicated in **Figure 5C**.

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Representative flow cytometry gating strategy for validation of stem cell purity and CCR5 donor variant status.** (**A**–**C**) The gating strategy used for the isolated CD34+ cell population. Doublets and debris are excluded in panel **A** and **B** respectively (FSC-A vs. FSC-H and FSC-A vs. SSC-A). (**C**) The frequency of CD34+ stem cells and CD3+ T cell contamination. (**D**–**F**) The CD34- flow-through gating strategy. Percentages in gates are calculated as a fraction of the parent population. (**G**) The results of a *CCR5*$^{\Delta 32/wt}$ PCR analysis. Lane 1: DNA from a human *CCR5*$^{\Delta 32/wt}$ donor, lanes 2 and 3: two *CCR5*$^{wt/wt}$ human stem cell donors, lane 4: A *CCR5*$^{\Delta 32/wt}$ human stem cell donor. (**H**) Frequency of the genotype *CCR5*$^{\Delta 32/wt}$ in our group of 19 stem cell samples is 15.8%.

**Figure 2: Flow cytometry gating strategy for validation of human cell engraftment and differentiation.** The total mononuclear cell population from humanized mice was analyzed via flow cytometry. (**A**) The percentage of human CD45+ cells was determined as a fraction of the total recorded events. (**B**) Doublets were subsequently excluded based on FSC-A/FSC-H gating. (**C**) The true lymphocyte population was defined based on size and granularity. (**D**) Lymphocytes were then characterized as either CD3+ (T cells) or CD19+ (B cells). (**E**) CD3+ T cells were either CD4+ T cells or CD8+ T cells. Percentages in gates were calculated as a fraction of the parent population.

**Figure 3: Representative humanization levels 4–5 months after stem cell transplantation with cell subtype fractions for 10 and 16 mice generated from two different human donors.** (**A**) The mononuclear cell population (MNC) from 10 and (**B**) 16 humanized mice were analyzed via flow

cytometry and gated as presented in **Figure 2**. The fraction of human CD45+ cells is presented as %hCD45 (of total MNC), and %B and %T cells as a fraction of hCD45. T cells were subsequently divided into %CD4 and %CD8. Each data point represents one mouse. Data is presented as mean ± SD.

**Figure 4: Experimental lab bench setup for intravaginal exposure of mice.** Experimental setup for HIV exposure of humanized mice through the intravaginal route. The procedure is performed in a flow bench where all reagents and surfaces have been sterilized prior to use.

**Figure 5: Rate of HIV strain transmission through different exposure routes and efficacy and safety of cART-containing chow in viral suppression.** (**A**) Humanized NOG mice were successfully infected with two different strains of HIV through either the intravaginal or the intravenous route. Mice were exposed with 21,400 IUs of RHPA4259 intravaginally, 5,157 IUs IV with RHPA4259, or 3,000 IUs IV with YU2. Details regarding IV exposure of humanized mice are not included in this protocol. HIV infections were successfully treated with a cART regimen delivered through mouse chow. (**B**) The viral load decreased to below detection for all three mice on cART and rebound emerged after the cessation of cART. The dotted line indicates limit of quantification at 725 copies/mL. Mice fed with cART chow had similar weight development as mice housed on non-cART chow during the same time period, indicating no taste-preference or side effects of the cART diet. (**C**) Weights are presented as fold change compared to the start of cART. Each data point represents the mean of three animals ± SD.

**Table 1: Antibodies used for determination of stem cell purity.** Suggested multicolor flow cytometry panel for evaluation of stem cell purity. Listed are the antibody target, the clone, and the fluorophore.

**Table 2: CCR5Δ32 variant detection PCR primers.** Forward and reverse primers used for detection of the 32 bp deletion in the CCR5 gene.

**Table 3: CCR5Δ32 variant detection PCR program.** PCR cycling program used for amplification of the CCR5 gene.

**Table 4: Antibodies used for determination of mouse humanization.** Suggested multicolor flow cytometry panel for humanization. Listed are the antibody target, the clone, and the fluorophore.

**Table 5: cDNA amplification program.** Program used for amplification of complementary strand DNA to the viral RNA.

**Table 6: HIV ddPCR primers.** Primers and probes used for ddPCR amplification of viral cDNA.

**Table 7: HIV ddPCR program.** PCR cycling program used for amplification of viral RNA.

**Table 8: Mouse cART chow diet.** Mouse chow diet was formulated as previously published[21]. The chow diet was made on a base of standard mouse chow, and after production, the food was γ-

irradiated with 25 kGy and double-bagged. The chow was stored at -20 °C until use.

**DISCUSSION:**

The severely immunocompromised mouse strain NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Sug}$/JicTac (NOG) is extremely well suited for transplantation of human cells and tissues. Both innate and adaptive immune pathways in these mice are compromised. NOG and NSG mice harbor a Prkdc$^{scid}$ mutation that results in defective T and B cell function. Furthermore, these mice lack a functional interleukin-2 receptor γ-chain (common gamma chain, IL2rg) which is indispensable in the binding complexes of many key cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21. Immunodeficient mice, such as the NOG, transplanted with a human immune system are a powerful tool for the study of HIV transmission and immunology. Contributions in these fields made using humanized mice have been extensively reviewed[2,23–26]. The use of these mice to study human innate immune responses are also gaining increased attention[27,28].

The aim of this manuscript is to supply a comprehensive protocol of mouse and ddPCR procedures to go from a naive mouse to HIV transmission and treatment data. Our system utilized ddPCR for quantification of viral RNA and DNA. In a ddPCR reaction, the reactants are partitioned into up to 20,000 droplets, each containing a single, separate micro PCR reaction. The amplification of a target inside a droplet leads to a positive fluorescent signal for that droplet. Thus, the readout is binary and by applying Poisson statistical analyses, the number of positive reactions can be directly translated to a number of template copies in the original sample. The benefit of ddPCR lies in its ability to directly quantify a target independent of a standard curve. This is particularly attractive when analyzing RNA samples that are challenging to utilize as PCR standard curves due to their labile nature[29]. Moreover, by analyzing multiple replicas of the same sample and merging the individual data points for the final sample quantification, the binary nature of ddPCR makes it possible to lower the detection limit of template copies per mL of sample[29]. This is especially important in a humanized mouse setting, where only limited sample material is available and high sensitivity is required.

Administration of cART to humanized mice can be done either by oral gavage or intraperitoneal injections with solutions of cART[30–32], and as shown recently, by formulation into the diet[21]. One of our major aims was the implementation of a cART regimen in the mouse diet to reduce potential stress on the animals due to the extra handling steps inherent in other drug delivery methods. The dose of medicine that a mouse will eat can be accurately estimated based on the average daily food intake of the mice[33]. Oral delivery through the diet serves as the easiest delivery route with both minimal stress for the animal and minimal workload for the handler. We based our combination of antiviral drugs on previous published studies in humanized mice[21, 30]. Furthermore, our cART strategy is clinically relevant given that the drug combination utilized clinically is orally administered by patients around the globe.

Certain limitations are noted regarding the use of NOG mice. Importantly, human T cells in these mice are cultivated in a mouse thymic environment, as opposed to a human environment. The recent focus is on generating xenorecipient strains that have a favorable environment for the development of robust human immune responses. These new strains include immunodeficient

mice that are transgenic for human MHC molecules, such as A2. These models enable HLA-restricted antigen T-cell responses that result in better maturation and effector functions of the adaptive immune system[34]. Another approach is to replace mouse genes with key human cytokines for IL-3/GM-CSF[35], IL-6[36], IL-15[37], TPO, M-CSF[38], and IL-7/TSLP[31]. Such models have gained increased attention for their ability to generate better differentiation of innate cell types. Our protocol is easily adaptable for the humanization and HIV infection of mice using any such enhanced genetic background immunodeficient strain.

In summary, the ease and utility of the described approach facilitates research in HIV-related fields in vivo. Humanized mice can be a very powerful tool in guiding research towards generating better research hypotheses. Along with the generation of more "human" humanized mice with human transgenes, we believe our standardized protocol will contribute to the streamlining of experimental procedures across different research environments.

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The authors would like to thank the Biomedicine Animal Facility staff at Aarhus University, particularly Ms. Jani Kær for colony maintenance efforts and for tracking mouse weights. The authors would like to thank Professor Florian Klein for developing standard-of-care cART and for guidance.

**DISCLOSURES:**

The authors declare no conflicts of interests.

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Figure 1



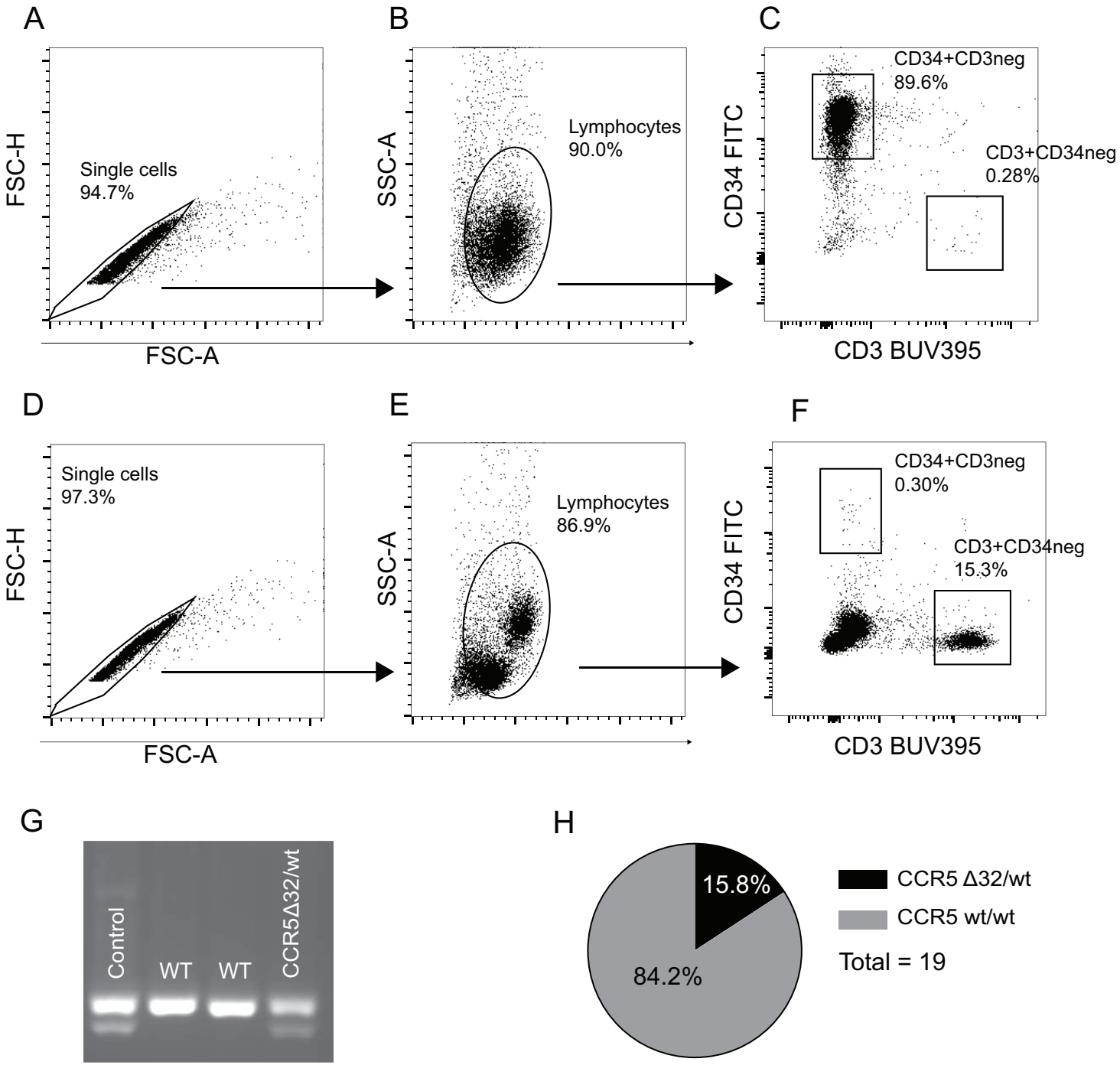

Figure 2

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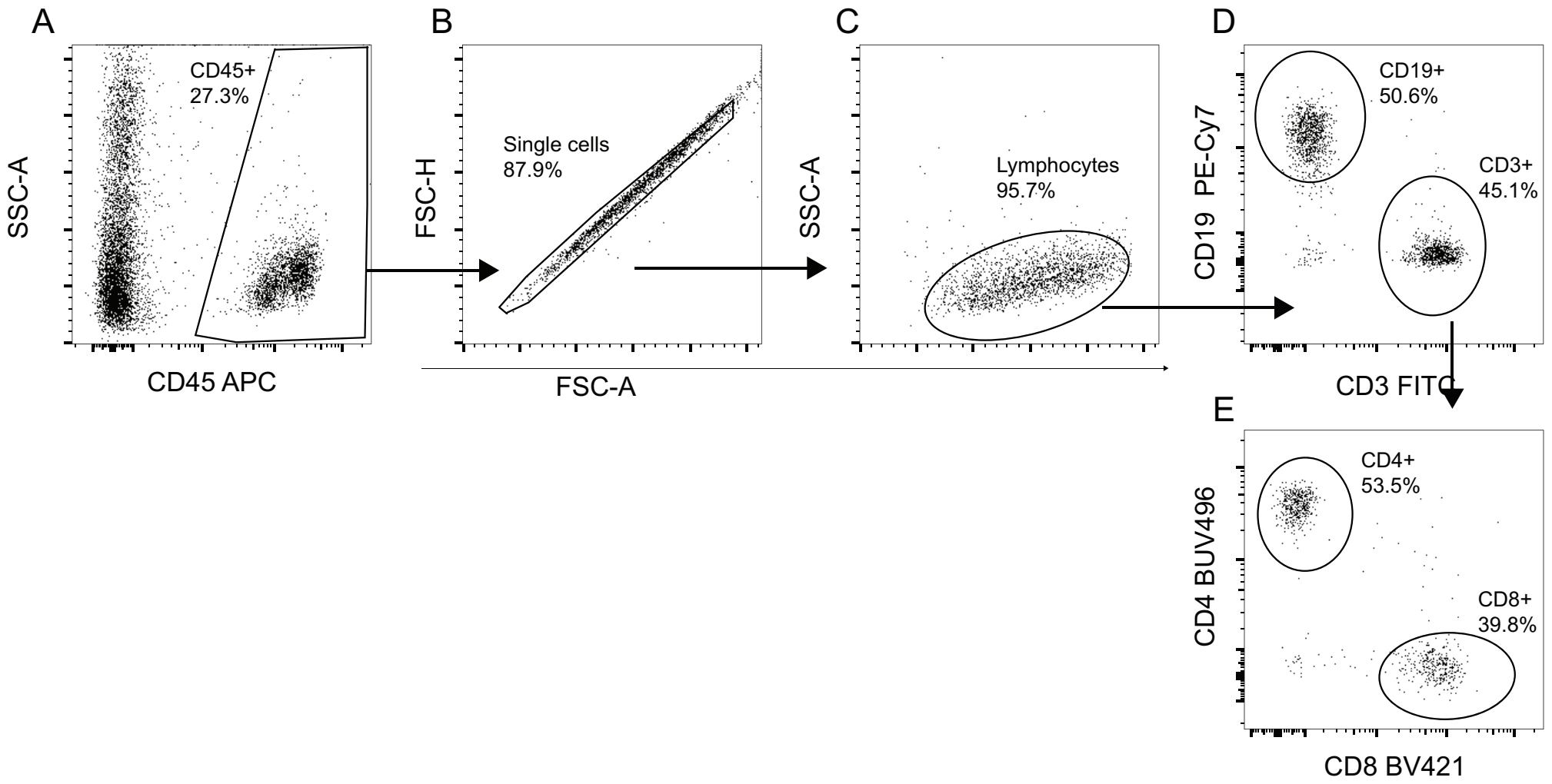

Figure 3

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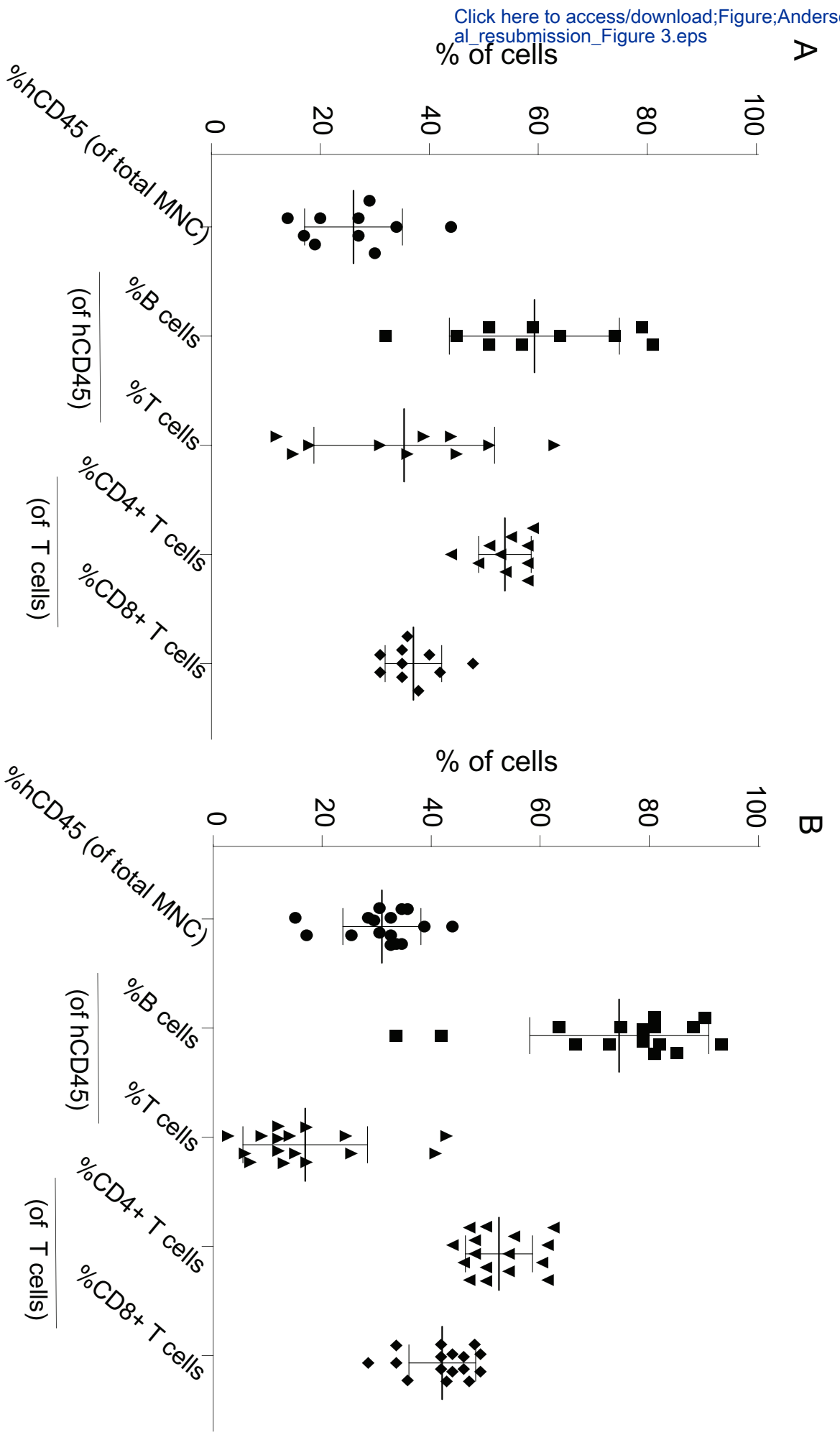

Figure 4

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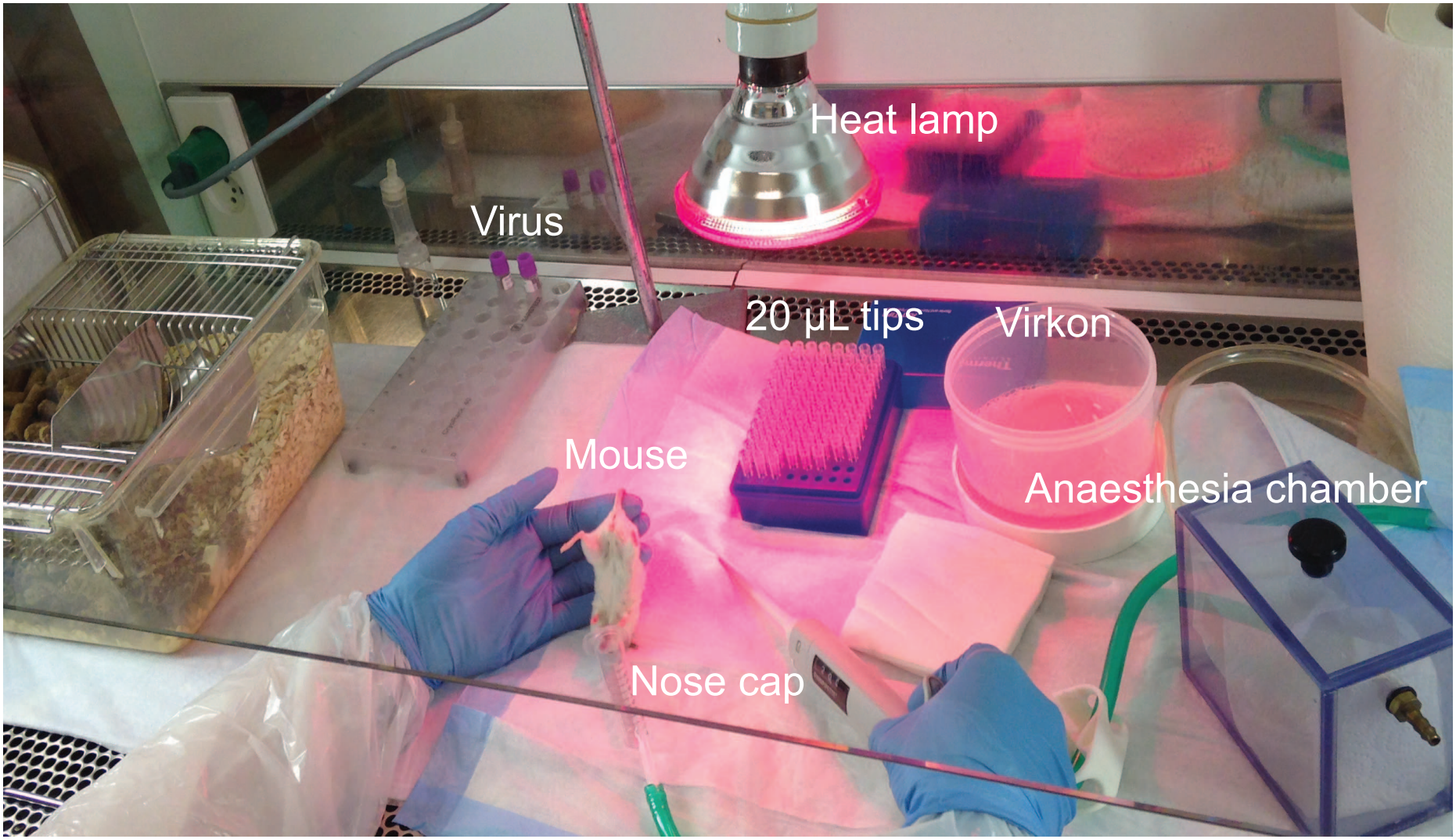

Figure 5



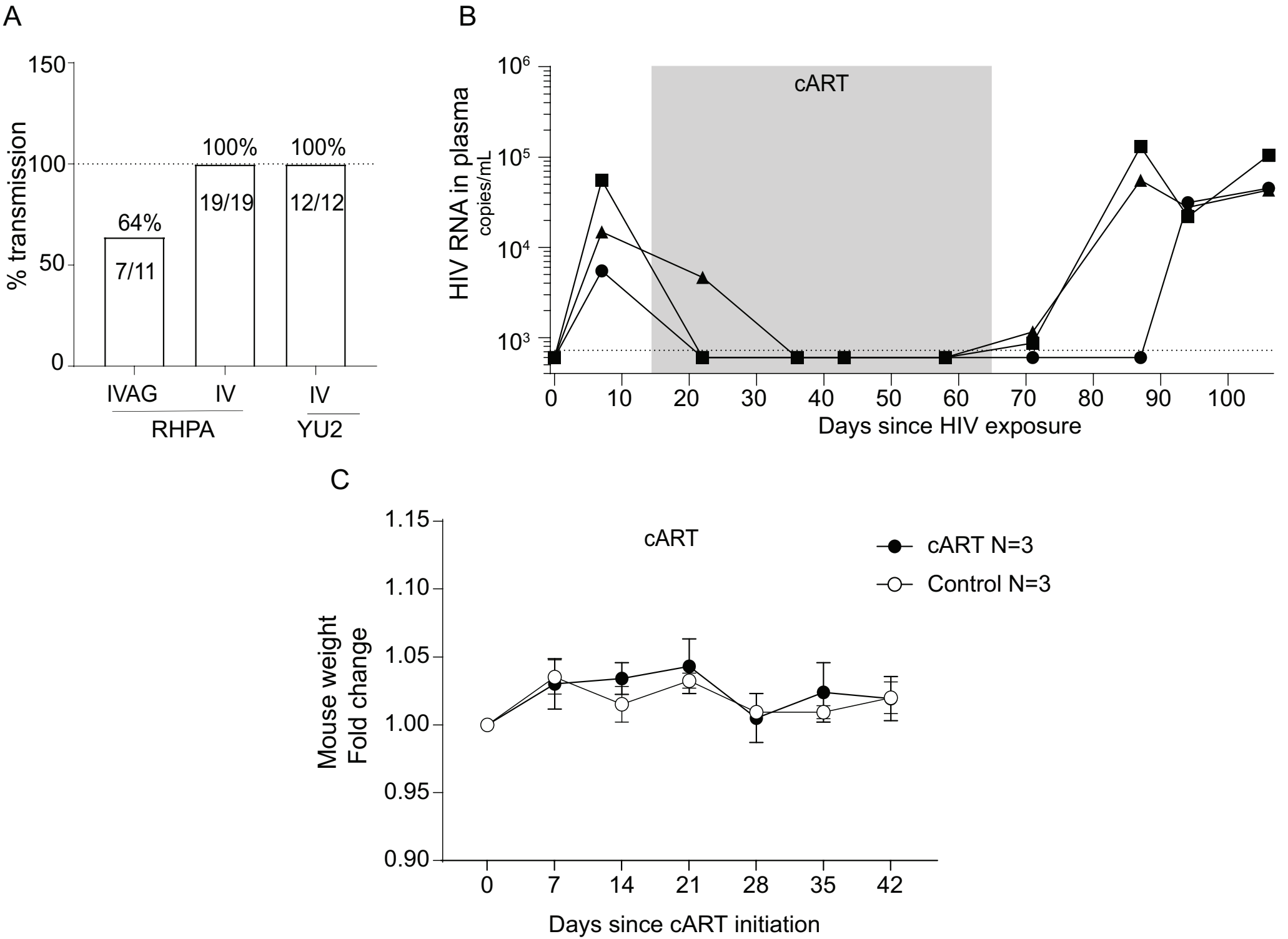

Table 1

Click here to access/download;Table;Table 1 Antibodies used for.xlsx

| Antibody target | Clone | Fluorophore |
|---|---|---|
| CD3 | clone SK7 | BUV395 |
| CD34 | clone AC136 | FITC |
| CD45 | clone 2D1 | APC |

Table 2



| CCR5Δ32 detection | Primers |
|---|---|
| Forward primer | 5'CTTCATTACACCTGCAGCT'3 |
| Reverse primer | 5'TGAAGATAAGCCTCACAGCC'3 |

Table 3



| | | | | |
|---|---|---|---|---|
| No. of Cycles | 1 | 45 | 1 | ∞ |
| Temperature (°C) | 98 | 98/63/72 | 72 | 10 |
| Time | 30 s | 10 s/30 s/15 s | 5 min | ∞ |

Table 4



| Antibody target | Clone | Fluorophore |
|---|---|---|
| CD4 | SK3 | BUV 496 |
| CD8 | RPA-T8 | BV421 |
| CD3 | OKT3 | FITC |
| CD19 | sj25c1 | PE-Cy7 |
| CD45 | 2D1 | APC |

Table 5

Click here to access/download;Table;Table 5 cDNA amplification program.xlsx

| | | | |
|---|---|---|---|
| No. of Cycles | 1 | 1 | ∞ |
| Temperature (°C) | 51 | 80 | 4 |
| Time | 45 min | 15 min | ∞ |

Table 6

Click here to access/download;Table;Table 6 HIV ddPCR primers.xlsx

| HIV quantification | Primers |
|---|---|
| Forward primer | 5’AGGGCAGCATAGAGCAAAAA’3 |
| Reverse primer | 5’CAAAGGAATGGGGGTTCTTT’3 |
| FAM probe | 5’ATCCCCACTTCAACAGATGC’3 |

Table 7

Click here to access/download;Table;Table 7 HIV ddPCR program.xlsx

| No. of Cycles | 1 | 39 | 1 | ∞ |
|---|---|---|---|---|
| Temperature (°C) | 95 | 95/54.5 | 98 | 4 |
| Time | 10 min | 30 s/1 min | 10 min | ∞ |

Table 8



| | |
|---|---|
| Raltegravir (RAL) | 4800 mg/kg |
| Tenofovir disoproxil fumarate (TDF) | 720 mg/kg |
| Emtricitabine (FTC) | 520 mg/kg |

Table of Materials

Click here to access/download;Table of Materials;Table of Materials and Reagents.xlsx

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Blue pad | VWR | 56616-031 | Should be sterilized prior to use |
| Bovine serum albumin (BSA) | Sigma | A8022 | |
| CD19 (clone sj25c1) PE-Cy7 | BD Bioscience | 557835 | |
| CD3 (clone OKT3) FITC | Biolegend | 317306 | |
| CD3 (clone SK7) BUV395 | BD Bioscience | 564001 | |
| CD34 (clone AC136) FITC | Miltenyi | 130-113-740 | |
| CD4 (clone SK3) BUV 496 | BD Bioscience | 564652/51 | |
| CD45 (clone 2D1) APC | Biolegend | 368511/12 | |
| CD8 (clone RPA-T8) BV421 | BD Bioscience | 562428 | |
| ddPCR Supermix for probes (no dUTP) | Bio-Rad | 1863025 | |
| DMSO | Merck | 10,029,521,000 | |
| DNAse | Sigma | D4263 | For suspension buffer |
| dNTP mix | Life Technologies | R0192 | |
| Dulbeccos phosphate-buffered saline (PBS) | Biowest | L0615-500 | |
| EasySep Human Cord Blood CD34 Positive Selection Kit II | Stemcell | 17896 | |
| EDTA | Invitrogen | 15575-038 | |
| FACS Lysing solution 10X | BD | 349202 | Dilute 1:10 in dH20 immediately before use |
| FACS tubes (Falcon 5 mL round-botton) | Falcon | 352052 | |
| Fc Receptor blocking solution (Human Trustain FcX) | Biolegend | 422302 | |
| Fetal bovine serum | Sigma | F8192-500 | |
| Ficoll-Paque PLUS | GE Healthcare | 17144002 | |
| Flowjo v.10 | | | |
| Gauze | Mesoft | 157300 | Should be sterilized prior to use |
| Heating lamp | | | Custom made |
| Hemacytometer (Bürker-Türk) | VWR | DOWC1597418 | |
| Isoflurane gas | Orion Pharma | 9658 | |
| LSR Fortessa X20 flow cytometer | BD | | |
| Microcentrifuge tubes, PCR-PT approved | Sarstedt | 72692405 | |

| | | | |
|---|---|---|---|
| Mouse cART food | ssniff Spezialdiäten GmbH | Custom made product | |
| Mouse restrainer | | Custom made product | |
| Needle, Microlance 3, 30G ½" | BD | 304000 | |
| NOG mice NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac | Taconic | NOG-F | |
| Nuclease-free water | VWR chemicals | 436912C | |
| Nucleospin 96 Virus DNA and RNA isolation kit | Macherey-Nagel | 740691 | |
| PCR-approved microcentrifuge tubes | Sarstedt | 72.692.405 | |
| Penicillin-Streptomycin solution 100X | Biowest | L0022-100 | |
| Phusion Hot Start II DNA polymerase | Life Technologies | F549S | |
| Pipette tips, sterile, ART 20P Barrier | ThermoScientific | 2149P | |
| Proteinase K | NEB | 100005398 | |
| QuantaSoft software | Bio-Rad | | |
| QX100 Droplet Generator | Bio-Rad | 1886-3008 | |
| QX100 Droplet Reader | Bio-Rad | 186-3003 | |
| RBC lysis solution | Biolegend | 420301 | |
| RNase-free DNAse size F + reaction buffer | Macherey-Nagel | 740963 | |
| RNAseOUT Recombinant Ribonuclease inhibitor | ThermoScientific | 10777-019 | |
| RPMI | Biowest | L0501-500 | Dissolve in H20 |
| Softject 1 mL syringe | Henke Sass Wolf | 5010-200V0 | |
| Superscript III Reverse Transcriptase | ThermoFisher Scientific | 18080044 | |
| Thermoshaker | VWR | 89370-910 | |
| Trypane blue | Sigma | T8154 | |
| Ultrapure 0.5 EDTA, pH 8.0 | ThermoFisher Scientific | 15575-020 | |
| Virkon S (virus disinfectant) | Dupont | 7511 | |

Author response to editor and review comments

**Editorial comments:**
General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- **In the revised manuscript we have ensured proper quality of the language.**

2. Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.

- **We have ensured that our manuscript is formatted according to the JoVE guidelines.**

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript/figures and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Virkon S, Ficoll, RosetteSep, EasySep, TruStrain FcX , BD Fortessa X20, FlowJo, Phusion Hot Start II, Softject, Nucleospin, Superscript, QX100, QuantaSoft, Emtricitabine, ssniff Spexialdiäten

- **We have replaced all trade names from the text with generic terms and listed trade named in the materials section. We have left emtricitabine in the text, since this is not a license tradename, but a generic name similar to raltegravir.**

Protocol:

1. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

- **In the revised manuscript, we have included a statement about the ethics and approvals used for this protocol. Parts of this information were previously embedded in the "protocol-headline" (page 3, lines 99-100 original manuscript) but can now be found on page 3 lines 94-98 in the revised manuscript.**

2. There is a 10 page limit for the Protocol, but a 2.75 page limit for filmable content. If revisions cause the highlighted portion to be more than 2.75 pages, please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- **We have noted these requirements and have made certain that the highlighted portion is equal to or less than 2.75 pages**

3. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

- **In the revised manuscript we have made a great effort to ensure the clarity of the description on actions.**

Specific Protocol steps:
1. 4.1: Where do these mice come from? Also, how exactly are mice irradiated?

- **In the revised manuscript, we have now included a description of the acquisition of the mice as well as the source of irradiation, along with the specification that we used female mice for this particular experiment. The details are also included in Materials and Equipment.**

2. 10.9: Please provide more details about this step if it is to be filmed

- **In the revised manuscript, this particular step has been omitted from the part which needs visualization. We believe that this step needs to be optimized for each laboratory and too time consuming for a video representation.**

Figures and Tables:
1. Please remove the embedded table(s) from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

- **All tables are removed from the revised manuscript and uploaded separately. The revised manuscript now includes a detailed description of each table (page 11-12, lines 480-506 lines).**

2. Please remove 'Figure 1' etc. from the figures themselves.

- **The requested changes have been made.**

3. Figure 5C: What are the error bars here?

- **The error bars are the standard deviation based on the data from three animals. We have included this important detail in the figure legend for Figure 5 (page 11, lines 477-478)**

References:

1. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. *Source*. **Volume** (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

- **The reference list have now been formatted to accommodate JoVEs requirements.**

2. Please do not abbreviate journal titles.

- **This issue has been corrected in the revised manuscript.**

Table of Materials:

1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

- **In the revised manuscript we have ensured that all reagents, materials and equipment used in the protocol are listed.**

2. Please remove trademark (™) and registered (®) symbols from the Table of Materials.

- **In the revised manuscript, we have removed all trademark and registered symbols.**

**Reviewers' comments:**
Reviewer #1:

Manuscript Summary:
Reconstitution of immune deficient mice with CD34+ cord blood cells is not a new procedure neither are the virological assays described novel. There are additional concerns in providing cART in a diet rather than ensuring garage or injectable dosing as drug levels would be highly variable. There are no antiretroviral drug levels provided in the plasma of these animals. Additional concerns are the adequate human cell reconstitution in the genitourinary system in mice reconstituted mice with CD34+ cord blood. This is known to be highly variable and inferior to what is seen in replicate BLT transplanted animals.

Major Concerns:
Novelty of CD34+ humanization using cord blood and approach limitations in establishing intravaginal infections with these animals compared to more conventional BLT mice.

Minor Concerns:
None

- **We appreciate the reviewer's perspectives. Regarding cART delivery in food, we agree that we have not performed a definitive head-to-head test of delivering cART via food**

**pellets, gavage and injection. We are unaware of any study that has shown this comparison in humanized mice. Thus, we cannot speak to the potential for drug level variability that is suggested. However, it is clear that there is effective drug delivery via food pellets. This is reflected both in our own data regarding the ability of cART in food pellets to maintain sustained suppression of HIV viremia in HIV infected mice for the duration of the treatment period as well as in the data from the originally published work using food for cART delivery (ref# 21). Simply replacing the food in a cage leads to sustained viral suppression in a manner that is far less invasive for the animals than either gavage or injection. Thus, we are utilizing this drug delivery method without any claims about its superiority for drug delivery versus other methods. We claim it as a refinement of the drug delivery method simply because it is less invasive to the mice while being very effective at viral suppression.**

- **We are unsure what published data the reviewer is referencing when they claim that the genitourinary system of mice reconstituted mice with CD34+ cord blood is highly variable and inferior to what is seen in replicate BLT transplanted animals. In contrast to this statement, there are many papers that have used CD34+ transplanted animals (multiple mouse strains) to conduct vaginal HIV studies (one review on this topic is by us and found in Chapter 19 (pg. 235-245) of the book: Humanized Mice for HIV Research, ISBN 978-1-4939-1655-9). In fact, the ability to utilize so many strains of mice transplanted with only hCD34+ cells (no thymic implant) is why we submitted this video protocol for publication. We are not emphasizing novelty. Rather we are making a video protocol available to increase accessibility to a very useful technique (as noted by Reviewer 2). Perhaps the reviewer is mixing their comment with the fact that human reconstitution of the intestinal tract is indeed variable between humanized mouse strains. This is something that we have provided a mechanistic explanation for in our papers. Specifically, the immune inductive sites of the intestine are not formed when gamma chain signaling is absent (PMID: 22569301 and 23791525). This is not dependent upon the human transplant technique (stem cells only versus BLT), but it is driven by the strain of mouse that is being humanized.**

Reviewer #2:

Manuscript Summary:

In the manuscript "Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection", Andersen and colleagues focused on describing the methodology to establish HIV-1 infection via vaginal route in a humanized mouse model. The hu-mouse model is prepared by using human cord blood CD34 cells. A detailed protocol to detect the CCR5Δ32/WT or CCR5Δ32/ Δ32 genotypic deletions in the human CD34+ cord blood cells was included in addition to assaying the viral loads in plasma by a very sensitive ddPCR. Viral control by using cART treatment via drug incorporated feed pellets was described.

All the methods described in this manuscript were already published in the literature including some by the present authors' themselves and thus there is not much new here. However, the idea of putting together many previously described methods in one setting in the JoVE journal is a good one and will be helpful to the investigators that are newly entering the field. Using the drugs in a feed is a very good idea to reduce mouse stress and technician time.

The paper is primarily about describing the hu-HSC humanized mouse model as a substitute for BLT mouse model for HIV-1. The present manuscript starts well in description in the beginning but fades and becomes diffuse towards the end wherein cART was discussed. Many manuscripts using the hu-HSC model for vaginal HIV-1 transmission were previously published by other groups particularly from the Akkina group and this was not sufficiently acknowledged in the references section which needs to be rectified. The following points need to be addressed to improve and make this paper acceptable.

Major Concerns:

1. The literature review and references are not inclusive of previous important contributions that used hu-HSC mice for HIV-1 vaginal transmission. A methods paper by M. Veselinovic et al 2016, Methods in Molecular Biology, 1354:203 would need to be cited. A review by Akkina 2013, Virology 435;14-28 that compares hu-HSC and BLT models for mucosal transmission need to be cited as well in the background section.

- **We thank the reviewer for reminding us of these important pieces of literature. We have ensured that these and other relevant references are now properly cited.**

2. The viral infecting dose (5,000 infectious units) described here as a low dose is not really a low dose for a mouse. Other investigators described 100% of mice being infected (Berges et al 2008 ref) with a lower R5 viral vaginal exposure (200TCID/50). Is it because here only 75,000 CD34 cells from cord blood cell source were used resulting in lower vaginal mucosal engraftment compared with other Hu-HSC mucosal transmission reports? This needs to be clarified.

- **Because it is virtually impossible to compare an inoculum reported as a TCID50 with a TCIU(IU) as we report here, we have removed the language that we had included regarding "low" inoculum to eliminate confusion. We now state the TCIU values as fact without qualifiers. We would like to note that this question lead us to discover an omission in the previously submitted version of our manuscript. Specifically, we had omitted the inoculum dose for vaginal exposures of HIV. We have now included the TCIU for both intravenous and vaginal exposures of in the revised where appropriate (vaginal exposure: page 6, line 256, page 10, line 417 and page 11, line 469, and intravenous exposure: page 11, line 470). Regarding the impact that the number of CD34 cells transplanted may have on vaginal engraftment, we are not aware of any published data that directly addresses this question. The relatively limited number of studies that have performed head to head comparisons of chimerization strategies in humanized mice have not examined the female reproductive tracts in a comparative manner. In this void of data, we are uncomfortable making any claims regarding relative measures between models. This is a second reason for simply stating our TCIU without qualifies.**

3. The authors describe using a 20ul pipette tip (Page 6, 7.7) for vaginal delivery of the virus. In practice, the pipette tips can be sharp and can cause abrasions in the mucosa. To overcome this, Berges et al used pipette tips that had been previously heated over a flame to smooth out any

abrasive surfaces and then were used to deliver the virus. Alternatively a 22 gauge 1.25mm straight gave needle was used to avoid abrasion as described by M. Veselinovic et al 2016, Methods in Molecular Biology, 1354:203. These methods need to be pointed out and referenced for the benefit of the readers to avoid any possible abrasions.

- **We appreciate the concern for reproducibility displayed by the reviewer. While it is possible that plastic pipette tips may have the capacity for abrasion – that is extremely unlikely in our approach to vaginal HIV exposure. This is because we never actually insert the pipette tip into the vagina. Rather, as will be clearly shown in the video protocol, the mouse is held (while anesthetized) upside down in such a manner that the vulva opens up and a 20ul aliquot of inoculum can be dripped (via gravity) into the vagina with the tip of the pipette even with the vulva. This means that the pipette barely touches the mouse, if at all, during this technique. If an investigator were to be placing the tip of the pipette deep in the vagina (e.g. next to the cervix of the mouse) prior to releasing the inoculum, then we would fully agree with the reviewer's point that plastic tips may lead to some abrasion. This is not the case in our technique, but in such an instance we would be very uncomfortable heating pipette tips before use because of the high potential for dramatic changes in the volume of aspiration between tips that may have been differentially heated which could lead to incorrect dosing. With regards to the alternative suggestion using a 22-gauge 1.25 mm straight needle, we can say anecdotally that we have tried this approach. We found it unwieldy and impossible to accurately transfer 20µl. (The inoculum volume is less than ½ of the dead volume in a syringe hub.) The tolerances for accuracy are severely impacted in such a scenario of low volume transfer. Therefore, to keep the inoculum as consistent as possible, we utilize a precision P20 pipette in the 20 µL range with high quality, precision, PCR-grade pipette tips (not the cheapest option that is more likely to have jagged edges) and we avoid touching the mouse in a manner that is likely to lead to any abrasion. Alongside our explanation for why we took the approach we have taken, we have now noted that other methods have been successfully utilized and provide the indicated citations (page 7, line 292).**

4. In page 6, it is not clear why heat lamp is used during vaginal infections. Please clarify.

- **The heat lamp is used in this setting to ensure the body temperature of the mice remain stable during the procedure under anesthesia. The body temperature of anaesthetized animals can decrease as much as 5° C during a 30 min period under anesthesia (ref #18 in the revised manuscript). Such changes can influence the overall health of the mice. Other measures such as a heated gel pad or circulating-warm-water-blanket could also be used with similar effects, although many animal facilities have banned electric heating pages because of uneven temperatures and the potential for "hotspots" that can seriously harm a mouse. Heat lamps are a very effective strategy for creating a properly warmed workspace for mice undergoing anesthesia. We have elaborated on this step in the revised manuscript and included an appropriate reference (page 6-7, lines 263-267 in the revised manuscript).**

5. In the abstract, the last sentence "the model can be used for preclinical analysis of systemic and topical pre-exposure prophylaxis compounds……" Since no evidence for this is presented in this

paper using this cord blood hu-HSC model, it is misleading. Therefore this sentence need to be removed from the abstract and shifted to the discussion section and citing examples done with hu-HSC models by the Akkina group wherein both topical and systemic prophylaxis were demonstrated (Veselinovic et al, Virology. 2012, 432:505, Neff et al, 2010, PLoS One 5:e15257, Neff et al 2011, PLoS One 6:e202209, Veselinovic et al, 2014, Virology 464-465:253-263

- **We respectfully disagree with the reviewer regarding the last sentence. The purpose of the last sentence of an abstract is to highlight the perspectives and potential of the described protocol, and thus we believe that this concept definitely belongs in our manuscript. In the introduction in the original manuscript, the following sentence ends the first paragraph: "Humanized mice have facilitated research in HIV viral transmission, pathogenesis, prevention, and treatment". To this statement we have included appropriate references. In the revised manuscript, we have ensured citation of the mentioned pivotal papers (page 2, line 64).**

6. The authors do not talk about Figure 5 anywhere in the protocol or text, which is extremely important given that it contains the data for half of the manuscript regarding the ddPCR experiments showing detection of plasma viral loads as well as the HIV-1 infected humanized mouse response to cART.

- **We have the figure and table call outs highlighted in gray to draw attention to them because they are very important, as pointed out by the reviewer. In this case, the reviewer unfortunately missed these call outs in the originally submitted text. They were found under "Representative results" on page 10, lines 386, 392 and 397 in the original manuscript (page 10, lines 416, 423, 428 in the revised manuscript).**

7. Under "4. Intravenous stem cell transplant," the authors make no mention of how old these NOG mice are that are being irradiated and engrafted with human CD34+ stem cells.

- **We thank the reviewer for the attention to detail. In the revised manuscript, we have included the very important information regarding the age of the animals as well as the gender used for this particular protocol (page 4, lines 171-174).**

8. In the 7. Intravaginal HIV exposure" protocol section, there is no indication of the viral titer used to inoculate these humanized mice intravaginally.

- **In the revised manuscript, we have now included the viral titer under this protocol step (page 6, line 256). The inoculums are references again under the "Representative results" section (page 10, line 417) and in the legend for Figure 5 (page 11, line 469-470)**

Minor Concerns:

1. The "RosetteSep" and "EasySep Human Cord Blood CD34 Positive Selection kit II (line 110)," which are crucial to the isolation of CD34+ cells from cord blood are not listed in the materials and

equipment section.

- **These reagents are now included in the revised Table of Materials and Equipment section.**

2. The clones for the antibody panel used to verify CD34+ purity are listed on lines 133-134; however, the manufacturer is not. This also applies to the antibodies listed on lines 211-213, which were used to check the engraftment of the humanized mice.

- **All antibodies including information on clone and fluorochrome are now listed in two new tables, (Table 1 and Table 4) and in the Table of Material and Equipment.**

2. The materials and equipment section appears to be lacking some crucial pieces of materials/equipment that are necessary to complete this protocol such as a heating lamp (line 167, Figure 4), anesthesia chamber (line 237, Figure 4), sterile PCR-approved microcentrifuge tubes (lines 203-204), 3% isoflurane gas (line 237), 1x RBC lysis solution (line 269), QX100 Droplet Generator (line 319), QX100 Droplet Reader (line 324), QuantaSoft software (line 325), and mouse cART chow diet. Please add these to the materials and equipment section.

- **The revised manuscript includes a more detailed description of materials and equipment used.**

4. The "suggested multicolour flow cytometry panel for humanization" listed the antibodies and clones used. When completing a general search on these antibodies and clones, multiple companies appear to make these antibodies. Please provide the company they were purchased from.

- **Please find in the revised manuscript all details including catalogue numbers and vendor is supplied for the antibodies used in Table 1 and Table 4**

5. Language can be improved overall in some places. For example, page 12 line 459, a "pristine" mouse can be corrected as "naïve" mouse.

- **In the revised manuscript, we have strived to improve the overall language and change the wording. This specific example has been addressed and changed to the word suggested by the reviewer (revised manuscript page 12, lines 523).F**

6. It is unclear what the yellow highlighted text in pages 8 and 9.

- **JoVE specifically requests that authors highlight selected sections of the manuscripts that would be suitable for video commentary in the submission file.**

Biography:

Anna Halling Folkmar Andersen (ahfa@clin.au.dk) studied Molecular Medicine at Aarhus University, Denmark, and obtained her Master of Science in 2016 with a strong focus on *in vivo* pharmacology and infectious diseases. From there, she moved onto pursue a PhD degree within the field of HIV-infected humanized mice at Institute of Clinical Medicine at Aarhus University. The PhD study is being concluded end of 2019.

Anna has a strong interest in development and use of novel rodent models, particularly the use of humanized mice for the study of infectious diseases and immunology.

**TITLE:**
Humanized NOG mice for intravaginal HIV exposure and treatment of HIV infection

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**KEYWORDS:**
Immunology and infection, NOG mouse, Humanized mice, HIV, cART, CCR5, Stem cells, ddPCR

**SUMMARY:**
We have developed a protocol for the generation and evaluation a humanized and HIV-infected NOG mouse model, based on stem cell transplant, intravaginal HIV exposure and ddPCR RNA quantification.

**ABSTRACT:**
Humanized mice provide a sophisticated platform to study human immunodeficiency virus (HIV) virology and to test antiviral drugs. This protocol describes the establishment of a human immune system in adult NOG mice. Here, we explain all practical steps from isolation of cord-blood derived human CD34+ cells and their subsequent intravenous transplantation into the mice, to the manipulation of the model through HIV infection, combination antiretroviral therapy (cART) and blood sampling. Approximately 75,000 hCD34+ are injected intravenously into the mice and the level of human chimerism, also known as humanization, in the peripheral blood is estimated longitudinally for months by flow cytometry. 75,000 hCD34+ cells yield 20-

50% human CD45+ cells in the peripheral blood. The mice are susceptible to intravaginal infection with HIV and blood can be sampled once weekly for analysis, and twice monthly for extended periods. This protocol describes an assay for quantification of plasma viral load using droplet digital PCR (ddPCR). We show how the mice can be effectively treated with a standard-of-care cART regimen in the diet. The delivery of cART in the form of regular mouse chow is a significant refinement of the experimental model. This model can be used for preclinical analysis of both systemic and topical pre-exposure prophylaxis compounds as well as for testing of novel treatment and HIV cure strategies.

**INTRODUCTION:**

Human immunodeficiency virus (HIV) is a chronic infection with more than 37 million infected individuals worldwide[1]. Combination antiviral therapy (cART) is a life-saving therapy, but a cure is still warranted. Thus, there is a need for animal models that mirror the human immune system and its responses in order to facilitate continued research in HIV. Multiple types of humanized mice have been developed by transplanting human cells into severely immunodeficient mice, that are capable of supporting cell and tissue engraftment[2]. Such humanized mice are susceptible to HIV infection and provide an important alternative to nonhuman primate SIV models, as they are cheaper and simpler than nonhuman primates. Humanized mice have facilitated research in HIV viral transmission, pathogenesis, prevention, and treatment[3–11].

We present a flexible humanized model system for HIV research developed by transplanting cord-blood derived human stem cells into mice of the NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Sug}$/JicTac (NOG) background. Besides being of non-fetal origin, the practical bioengineering of these mice is less technically demanding compared to the microsurgical procedures involved in the transplant of e.g. the blood-liver-thymus (BLT) construct.

We show how to establish HIV infection through intravaginal transmission, and how to monitor the plasma viral load with a sensitive droplet digital PCR (ddPCR)-based setup. Subsequently we describe the establishment of standard cART given as part of the daily mouse diet. The aim of these combined methods is to reduce stress to the animals and facilitate large-scale experiments where limited time can be spent handling each animal[12].

In humans, a *CCR5*$^{\Delta 32/wt}$ or *CCR5*$^{\Delta 32/\Delta 32}$ genotype causes reduced susceptibility to HIV infection with transmitter/founder viruses[13], and some precautions must be taken when bioengineering humanized mice with stem cells, with the purpose of HIV studies. This is especially true in our region because naturally occurring variants in the *CCR5* gene, particularly Δ32 deletions, are more prevalent in Scandinavian and Baltic native populations compared to rest of the world[14, 15]. Thus, our protocol includes an easy, high-throughput assay for screening donor hematopoietic stem cells for CCR5 variants prior to transplantation.

For the intravaginal exposure herein we chose the transmitter/founder R5 virus RHPA4259, isolated from a woman in an early stage of infection who was infected intravaginally[16]. We exposed the mice to a viral dose that was sufficient to yield successful transmission in the

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majority of mice, but below a 100% transmission rate. Choosing such a dose enables a sufficient dynamic range in transmission rate such that antiviral effects of a drug candidate can result in protected animals in HIV prevention experiments and decreased viral load for treatment studies.

## STATEMENTS OF ETHICS

All cord bloods were obtained in strict accordance following local approved protocols, including informed consent of anonymous donation by the parents. All animal experiments were approved and performed in strict accordance with Danish national regulations under the license 2017-15-0201-01312.

**PROTOCOL:**

A list of all reagents and catalogue numbers can be found at the end of this protocol along with a detailed description of relevant PCR programs and primer sequences.

CAUTION: handle HIV exposed mice and blood with extreme caution. Decontaminate all surfaces and liquids that have been in contact with HIV with a confirmed HIV-disinfectant (Table of Materials and Reagents).

1. **Isolation of human CD34+ stem cells**
   - 1.1. Collect cord blood samples in EDTA-coated blood-collection tubes. (After planned caesarean sections or vaginal births and according to local ethical approvals)
   - 1.2. Isolate PMBCs from cord blood by density-gradient separation, according to manufacturer's protocol.
   - 1.3. Isolate CD34+ cells from the PBMC population by first pre-enriching with antibodies against common markers for mature cells that which induces crosslinking of cells of undesired lineages with red blood cells. This is followed by CD34+ cell enrichment using magnetic beads, according to manufacturer's protocol.
     - 1.3.1. Determine live cell count by standard trypane blue exclusion: Briefly, resuspend 10 µL of cell suspension in 90 µL of trypane blue. Add 10 µL of this solution to a hemacytometer and count non-blue cells, according to manufacturer's protocol.
     - 1.3.2. Viably cryopreserve CD34+ cells in 1mL 10% DMSO in fetal bovine serum (FBS) until day of mouse transplantation.
     - 1.3.3. Viably cryopreserve a small fraction of both isolated (CD34+) and flow-through cells (CD34neg) separately for assessing CD34+ stem cell purity (Approximately 30,000 cells of each sample). (Alternative: Test purity on freshly enriched cells: Step 2 below.)
     - 1.3.4. Freeze a fraction of non-pelleted flow-through (CD34neg) for determination of *CCR5*Δ32 status. (Cells can be frozen directly without conditioned freezing solution, but note that the presence of red blood cells in the pellet can inhibit the subsequent PCR if the flow-through is pelleted.)

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**2. Assessing CD34+ stem cell purity via flow cytometry**

2.1. Thaw the jsolated cells (CD34+) and flow-through cells (CD34-). Wash cells by resuspending cells from each vial in 9 mL of room temperature (RT) FACS buffer [2% fetal bovine serum (FBS) in phosphate-buffered saline (PBS)].

2.2. Centrifuge for 5 min at 300 x g, at RT to pellet cells.

2.3. Pour off supernatant, resuspend cells in remaining liquid and transfer to FACS tubes, repeat washing step with 3 mL of FACS buffer. After completion of second centrifugation, pour off supernatant and resuspend cells in remaining liquid.

2.4. Add 5 µL of Fc Receptor blocking solution (Table of Materials and Reagents) and leave for 10 min at RT (Do not wash off Fc Receptor blocking solution).

2.5. Add mix containing pre-determined volumes of antibodies against human CD3 (clone SK7) BUV395, CD34 (clone AC136) FITC and CD45 (clone 2D1) APC (Table 1). Leave cells for 30 minutes at RT in the dark. (Fluorophores must be chosen based on parameters that can be assessed with the available flow cytometers without requiring compensation matrix)

2.6. Wash cells by addition of 3 mL of FACS buffer.

2.7. Centrifuge for 5 min at 300 x g, RT to pellet cells.

2.8. Pour off supernatant and resuspend cells in remaining liquid.

2.8.1. Repeat this washing step twice to ensure all non-bound antibodies have been removed)

2.9. Record samples on flow cytometer (Table of Materials and Reagents) and perform data analysis with appropriate software. (Gating strategy presented in Figure 1A-F)

**3. Genetic screening for *CCR5*Δ32 variants in cord bloods.**

3.1. Incubate 1.25 µL of non-pelleted flow-through with 11.25 µL PCR mix containing: 200 µM of dNTP mix, 0.01 U/µL high fidelity DNA polymerase, forward and reverse primers detailed in Table 2.

3.1.1. Adjust volume with nuclease-free H2O to approximately 12.5 µL for each PCR.

3.2. Amplify genomic fragments with the PCR cycling program detailed in Table 3.

3.3. Separate PCR products on a 2% agarose gel[13].

3.3.1. PCR products from the wild type alleles and the Δ32 alleles yield PCR fragments of 196 base pairs and 164 base pairs bands respectively, making them easily distinguishable by gel electrophoresis[13]. (Figure 1G)

**4. Intravenous stem cell transplant** (when possible having one person preparing cells in the laboratory and one person preparing the animal mice and workspace for transplants is an efficient approach)

4.1. In animal facility: 4-6 hours before planned transplantation of stem cells, female NOD.Cg-Prkdc$^{scid}$ Il2rg$^{tm1Sug}$/JicTac (NOG) mice (Taconic) of 6-7 weeks of age, should be irradiated with 0.75 Gy with a Cs$^{137}$ source (the best preconditioning dose may vary based on mouse age, source of radiation etc.). This process conditions the animals for successful engraftment with human stem cells.

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4.2. In animal facility: Prepare flow bench workspace and all reagents before bringing mice or cells into the workspace.

4.2.1. Place sterile blue pad to cover working surface of the flow bench. Prepare sterile gauze and sharps container.

4.2.2. Place a heating lamp disinfected with 70% ethanol in the flow bench with an empty sterile mouse cage underneath the heat.

4.3. In laboratory: Thaw isolated CD34+ cells and dilute them in 9 mL of 37 °C plain RPMI.

4.4. In laboratory: Centrifuge cells at 350 x g for 5 minutes at RT, discard supernatant by aspiration and resuspend pellet in 1 mL of plain RPMI at 37 °C.

4.5. In laboratory: Determine cell count by trypan blue exclusion, and adjust volume to 200 µL per mouse. (Make extra to take into account possible loss due to subsequent handling steps)

4.5.1. Plan to transplant 75,000 CD34+ cells in 200 µL into each mouse.

4.5.2. The cells can be kept at 4 °C for during transport to the animal facility before the transplant. (Avoid keeping the cells on ice, to reduce aggregation/clumping)

4.6. In animal facility: Bring cage with mice into the flow bench and transfer mice to the cage under the heating lamp to dilate vessels. Leave one end of the cage away from the heat source so that mice can move away from the heat upon becoming warm. Mice that have moved to the end of the cage away from the heat source are sufficiently warmed for a successful tail vein injection.

4.7. In animal facility: Load 1 mL pre-lubricated syringe to above the 800 µL mark with suspended CD34+ cells. (Using a lubricated 1 mL syringe will dramatically ease the intravenous injection and increase the precision of this technique.)

4.8. In animal facility: Attach 30-gauge 13 mm needle and prepare needle and syringe for injection. This order of operation allows for the syringe to be loaded more quickly while protecting the integrity of the cells to be transplanted given the possible damage that can occur during rapid aspiration of cells through such a small gauge needle. Fill the needle hub with liquid by pressing the plunger and remove liquid down to the 800 µL mark of the needle (800 µL is an appropriate volume for 1 cage that houses 4 animals)

4.9. In animal facility: Place a heated mouse (Step 4.6) in a restrainer used for giving IV injections. Carefully inject 200 µL of cell suspension into the tail vein of the mouse. Spend 2 seconds performing the plunge and keep the needle inserted for approximately 2 seconds after completion of injection. (This ensures cells have migrated adequately far from the injection site prior to removal of the needle.)

4.10. In animal facility: As necessary, wipe the mouse tail with sterile gauze to remove any visible blood. Put the mouse back into their non-heated home cage.

4.11. In animal facility: Repeat injection procedure with the remaining mice. It is not necessary to change the needle between the different mice unless the needle becomes dull (possible after 8-12 attempted tail-vein injections.)

## 5. Blood collection and processing for analysis

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5.1. 3-5 month after human stem cell transplantation, human cell engraftment in the peripheral blood can be evaluated via flow cytometry.

5.2. Draw blood samples from the mice using local IACUC-approved techniques.

5.3. Collect a maximum of 70-100 µL of total blood into sterile PCR-approved microcentrifuge tubes containing 10 µL 0.5 M pH 8.0 EDTA to avoid coagulation of blood.

**6. Evaluation of human engraftment via flow cytometry**

6.1. Transfer 40-50 µL of blood to FACS tubes

6.2. Add 5 µL of Fc Receptor blocking solution to prevent non-specific binding of antibodies and leave for 10 min at RT.

6.3. Add mouse anti-human antibody mix containing CD4 (clone SK3) BUV 496, CD8 (clone RPA-T8) BV421, CD3 (clone OKT3) FITC, CD19 (clone sj25c1) PE-Cy7, CD45 (clone 2D1) APC (Table 4) and leave to stain in the dark at RT for 30 min. (Fluorophores must be chosen based on parameters that can be assessed with the available flow cytometers without requiring compensation matrix)

6.4. Add 2 mL of appropriate red blood cell lysing buffer to each tube to lyse red blood cells. Use a lysis buffer is one that is specifically formulated for antibody staining prior to red blood cell lysis (one suitable example is given in the Table of Materials and Reagents). Vortex briefly to ensure equal distribution of cells in the lysing solution (important) and leave for 10 min at RT.

6.5. Add 2 mL of FACS buffer to stop lysis reaction.

6.6. Centrifuge for 5 min at 300 x g at RT to pellet cells.

6.7. Pour off supernatant and vortex gently until cells are resuspended.

6.8. Add 3 mL of FACS buffer, centrifuge for 5 min at 300 x g at RT.

6.9. Pour off supernatant and resuspend cells.

6.10. Record samples on appropriate flow cytometer and analyze using appropriate software (Table of Materials and Reagents) (Representative analysis and results are depicted in Figure 2 and Figure 3)

**7. Intravaginal HIV exposure**

7.1. Virus used for intravaginal exposure of mice can be produced using previously published protocols[17]. Virus is kept at -80 °C and transported between locations while stored on dry ice following local approved protocols. Virus is stored on dry ice until immediate before exposure of the mice. Virus can be diluted into plain RPMI (avoid using RPMI that has antibiotics or serum additives) to achieve the appropriate concentration immediately prior to exposure. (21,400 IUs were used for this IVAG exposure). Once they are generated, keep diluted stock on wet ice throughout procedure (to avoiding freeze-thaw cycles that would occur if diluted virus was placed back on dry ice once thawed.

7.2. Prepare all equipment and flow bench workspace as presented in Figure 4 before bringing mice or virus into the flow bench (similar to step 4.2.)

7.2.1. Place heating lamp focus in the center of the workspace where the mouse will be located during the HIV exposure procedure. The heating

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lamp will ensure no decrease in body temperature of the mice. (Other equipment that controls temperature can also be used, e.g. a heated gel pad or a circulating-warm-water blanket, according to local IACUC regulations[18].)

7.2.2. Bring sterile 20 µL pipette tips and appropriate pipette into the bench. Place a container with liquid disinfectant (Table of Materials and Reagents) in the bench for immediate inactivation of materials and liquids that have been in contact with virus.

7.3. Place a mouse into a chamber supplied with 3% isoflurane gas and enriched with paper towels. This percentage of gas will take the animals into the plane of anesthesia within 2-4 minutes. As with all other materials that immunodeficient mice encounter, the anesthesia apparatuses must be properly disinfected prior to use in this protocol.

7.4. Once anesthetized, transfer the mouse to a sterile blue pad under the heating lamp. Insert the mouse snout into a mask supplying continuous 3% isoflurane gas to maintain anesthesia. Hold the mouse at the base of the tail, stomach facing up, with your hand supporting the mouse back as depicted in Figure 4.

7.5. With a sterile pipette tip, stimulate genital area by gently stroking upwards towards the anus to induce emptying of the rectum, relieving pressure on the vagina.

7.6. Carefully bare vaginal opening by wrapping the mouse tail across your fingers such that the vulva naturally opens, perhaps with the slightest nudging using a sterile pipette tip.

7.7. Change pipette tip and pipette 20 µL of virus a-traumatically into the mouse vagina without creating bubbles. Do not insert the tip deep into the vagina. Rather, with the vulva opened, place the pipette tip at the level of the vaginal opening (avoid going deeper) to eliminate the potential for abrasions during the inoculation process, release the virus and allow gravity to pull the virus into the vagina (Alternative: use a 22G 1.25 mm blunt-end, straight needle, as described in[6])

7.8. Retain the mouse in this position with the vagina facing up for 5 minutes after exposure to avoid gravity-induced leakage of virus suspension.

7.8.1. Carefully place the mouse into the home cage, taking care to place the mouse on its back.

**8. Processing of blood samples prior to viral load analysis**

8.1. Collect blood as described in step 5 above.

8.2. Centrifuge blood samples for 5 min 500 x g at RT to separate plasma and cells.

8.3. Collect 40 µL plasma for viral load measurement into a new sterile PCR-approved microcentrifuge tubes and store at -80 °C for at least 1 hour until further processing. (It is important to freeze all samples before RNA extraction do avoid the risk of bias from comparing RNA levels in samples that have not been frozen prior to RNA isolation to samples frozen prior to RNA isolation.)

8.4. Adjust the volume of blood back to the original volume by adding 40 µL suspension media (PBS with 2.5% bovine serum albumin (BSA), 50 U/ml penicillin G

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and streptomycin, and 10 U/mL DNase and sterile-filtered at 0.22 µm) and vortex briefly to mix.

8.5. Transfer 15 µL of adjusted blood volume to a new PCR-approved microcentrifuge tube.

8.6. Add 1 mL of 1X RBC lysis solution (Table of Materials and Reagents), vortex and incubate for 10 min at RT.

8.7. Centrifuge 9,600 x g for 1 min at RT to pellet cells.

8.8. Aspirate supernatant and leave only the tiny white cell pellet as red blood cell contamination can inhibit PCR.

8.9. Store pellet at -80 °C for at least 1 hour until further processing.

8.10. Optional: any remaining blood from step 8.4 can be used for flow cytometry analysis, as described above in step 6.

**9. DNA extraction using a proteinase K extraction method**

9.1 Extract DNA from peripheral cell pellets (generated in step 8.8) using a proteinase K extraction method as described below. This method has been demonstrated to maintain the highest DNA yield from a small volume of blood such as is required for serial blood collections utilized herein[19].

9.2 Add 25µL of proteinase K (20 µg/ml) to 1ml of 0.1M TRIS buffer.

9.3 Vortex proteinase K solution briefly.

9.4 Add 50 µL proteinase K solution to each cell pellet to be digested.

9.5 Mix by pipetting up and down – watch to ensure resuspension of the cell pellet.

9.6 Shake on a thermoshaker (Table of Materials and Reagents) at 400 rpm (depending on instrument) at 56 °C for 1 hour. (Tape tubes down to hold them in place, if necessary.)

9.7 Immediately and in the same thermoshaker, inactivate proteinase K with a temperature shift to 95 °C while shaking continues for an additional 20 min.

9.8 Vortex each sample.

9.9 Place each sample at -80 °C for a minimum of 30 min.

9.10 Thaw, then centrifuge samples at 17,000 x g for 1 min at RT to pellet unwanted cellular fragments.

9.11 Place the DNA-containing supernatant into a new microcentrifuge tube.

9.12 The DNA template is ready for PCR. The DNA templates can be stored at -80 °C.

**10. RNA extraction, cDNA synthesis and ddPCR quantification of viral RNA.**

10.1. Isolate RNA from thawed mouse plasma with a virus RNA isolation kit following manufacturer's protocol (Table of Materials and Reagents).

10.2. After addition of sample to the column, add an on-column DNase treatment step to ensure removal of all DNA in the plasma sample.

10.2.1. For each sample, 95 µL of RNase-free DNase solution to the column and incubate for 15 min at RT. (mix 2 µL RNase-free DNase and 98 µL reaction buffer)

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10.3. Store RNA samples at -80 °C for at least 1 hour, before further processing. (It is important to freeze all samples after RNA extraction do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen)

10.4. Synthesize cDNA using a reverse transcriptase step using reagents as described previously[20]. Important: Add 0.5 µL of an RNase inhibitor to the cDNA reaction to avoid degradation of RNA.

10.4.1. Perform cDNA synthesis with the program detailed in Table 5.

10.5. Store cDNA samples at -80 °C for at least 1 hour. (It is important to freeze all samples after cDNA synthesis do avoid the risk of bias when comparing samples that have not been frozen to samples that were frozen)

10.6. Prepare samples for ddPCR as follows[20]:

10.6.1. Mix 3 µL cDNA sample with 11 µL ddPCR probe mixture (no dUTP)[20], 250 nM minor groove-binding probe and 900 nM of each of the forward and reverse primers as detailed in Table 6.

10.6.2. Adjust total PCR volume to 22 µL with nuclease-free water.

10.7. Emulsify PCR mixes with Droplet Generation Oil for Probes, on a droplet generator according to manufacturer's protocol and described previously[20].

10.8. Run PCR program as detailed in Table 7. Note: The primer/probe sequences and PCR programs displayed here have been specifically designed and optimized for sensitive detection of the HIV strain RHPA4259. Primer and probe sequences can easily be adjusted to detect any other HIV strain of choice.

10.9. Detect droplet fluorescence from samples on a droplet reader, and analyze results with appropriate software, according to manufacturer's protocol.

**11. Treatment with ART containing chow**

**11.1.** Mice can be fed with pellets containing a standard cART regiment containing 4800 mg/kg raltegravir (RAL), 720 mg/kg tenofovir disoproxil fumarate (TDF) and 520 mg/kg Emtricitabine (FTC) [21]. (Table 8).

11.2. The doses were determined assuming that a mouse weighs 25 g and eats 4 g of chow per day. This corresponds to a daily dose of 768 mg/kg RAL, 2.88 mg/kg TDF and 83 mg/kg FTC[21].

11.3. cART diet was prepared by an external vendor (See Table of Materials and Reagents) from prescription drugs. Other companies could potentially also produce this regimen. cART diet was produced with a red color to easily distinguish it from ordinary mouse chow.

11.3.1. Control chow diet without cART can be produced in a standard brown color for easy distinction.

11.4. For initiation of cART, sterile mouse cages are prepared with the addition of cART-containing chow diet, and then mice are simply transferred from the old cage to the new cage.

11.4.1. Monitor weights of the mice and consumption of cART-containing chow by visual inspection to ensure that the mice are adjusting to the change.

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<#>Add 0.5 µL RNAseOUT RNAse inhibitor to the cDNA reaction.¶

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**Deleted:** <#>Emulsify PCR mixes with Droplet Generation Oil for Probes, on a QX100™ Droplet Generator according to manufacturer's protocol. ¶
<#>Run PCR program as detailed in Table 5.

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**Table 1: *CCR5*Δ32 variant detection PCR primers** ¶
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**Table 2: CCR5Δ32 variant detection PCR**

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**REPRESENTATIVE RESULTS:**

Gating strategy for analysis of stem cell purity is depicted in Figure 1. Figure 1A-C show the purified CD34+ population and Figure 1D-F the CD34neg flow-through used to illustrate that minimal amount of the CD34+ population is lost in the isolation process. Purity of isolated CD34+ stem cells was between 85-95% with less than 1% T-cell contamination. Figure 1G depicts CCR5 bands from one adult human control donor with the *CCR5*$^{\Delta 32/wt}$ genotype, followed by bands from two *CCR5*$^{wt/wt}$ and one *CCR5*$^{\Delta 32/wt}$ stem cell donors. The frequency of the genotype *CCR5*$^{\Delta 32/wt}$ in a group of 19 donors was 15.8% (Figure 1H). This is in agreement with larger epidemiological studies[14, 15] reporting the genotype in up to 23.6% of investigated persons in Denmark.

3-5 months after transplantation of human CD34+ stem cells, human levels in peripheral blood was assessed via flow cytometry. The gating strategy is presented in Figure 2A-E. Figure 3A and Figure 3B illustrates the variability between 10 and 16 individual mice receiving stem cells from two different donors. Transplantation of 75,000 hCD34+ cells yielded 20-50% human CD45+ in the peripheral blood. All mice developed human B and T cells, including both CD4- and CD8-positive T cells.

For atraumatic intravaginal exposures, the setup depicted in Figure 4 was used. Mice were anaesthetized in a closed chamber and kept under anesthesia during the exposure. Mice were held vagina facing up for 5 minutes after exposure to ensure virus solution engagement with mucosal surfaces.

Shown in Figure 5A is the 64% HIV transmission success rate observed using this model. Mice were challenged with 21,400 infectious units (IU) of RHPA4259 intravaginally. This dose resulted in 64% of mice becoming HIV infected following vaginal exposure. For comparison, data from two different cohorts of mice, both exposed through intravenous route, are included. As expected, 100% of the mice became HIV+ with similar doses of RHPA and an additional strain, YU2, using this route.

Figure 5B depicts representative results from 3 mice which have been infected with HIV and switched to a diet containing standard cART. Mice were switched back to regular mouse chow after 40 days of cART. In this assay setup, the limit for viral load detection was 725 copies/mL. Viral loads were all below the detection limit after 4 weeks of cART. After cessation of cART, virus rebounded, mirroring clinical data[22]. Mice on cART tolerated the change in diet well as indicated in Figure 5C.

**FIGURE AND TABLE LEGENDS:**

**Figure 1A-H Representative flow cytometry gating strategy for validation of stem cell purity and CCR5 donor variant status.**

Panel A-C depicts the gating strategy used for the isolated CD34+ cell population. Doublets and debris are excluded in panel A and B respectively (FSC-A vs FSC-H and FSC-A vs SSC-A). Panel C shows the frequency of CD34+ stem cells and CD3+ T cell contamination. Similarly, the CD34neg



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flow-through gating strategy is presented in Panel D-F. Percentages in gates are calculated as a fraction of the parent population. The results of a *CCR5*$^{\Delta 32/wt}$ PCR analyses are presented in Panel G. Lane 1: DNA from a human *CCR5*$^{\Delta 32/wt}$ donor, lane 2+3: two *CCR5*$^{wt/wt}$ human stem cell donors, lane 4: A *CCR5*$^{\Delta 32/wt}$ human stem cell donor. Frequency of the genotype *CCR5*$^{\Delta 32/wt}$ in our group of 19 stem cell samples is 15.8% (Panel H).

**Figure 2A-E Flow cytometry gating strategy for validation of human cell engraftment and differentiation**

The total mononuclear cell population from humanized mice were analyzed via flow cytometry. The percentage of human CD45+ cells was determined as a fraction of the total recorded events (Panel A). Doublets were subsequently excluded based on FSC-A/FSC-H gating (Panel B). Based on size and granularity the true lymphocyte population was defined (Panel C). Lymphocytes were then characterized as either CD3+ (T cells) or CD19+ (B cells) (Panel D). CD3+ T cells were either CD4+ T cells or CD8+ T cells (Panel E). Percentages in gates were calculated as a fraction of the parent population.

**Figure 3A-B Representative humanization levels 4-5 months after stem cell transplantation with cell subtype fractions for 10 and 16 mice generated from two different human donors**

The mononuclear cell population (MNC) from 10 (Panel A) and 16 (Panel B) humanized mice were analyzed via flow cytometry and gated as presented in Figure 2. The fraction of human CD45+ cells is presented as %hCD45 (of total MNC), and %B and %T cells as a fraction of hCD45. T cells were subsequently divided into %CD4 and %CD8. Each data point represents one mouse. Data is presented with mean ± S.D.

**Figure 4 Experimental lab bench setup for intravaginal exposure of mice**

Experimental setup for HIV exposure of humanized mice through the intravaginal route. Procedure is performed in a flow bench where all reagents and surfaces have been sterilized prior to use.

**Figure 5A-C Rate of HIV strain transmission through different exposure routes and efficacy and safety of cART-containing chow in viral suppression**

Humanized NOG mice were successfully infected with two different strains of HIV through either the intravaginal or the intravenous route (Panel A) Mice were exposed with 21,400 IUs of RHPA4259 intravaginally, 5157 IUs IV with RHPA4259 or 3000 IUs IV with YU2 (Protocol details regarding IV exposure of humanized mice are not included in this protocol). HIV infections were successfully treated with a cART regimen delivered through mouse chow. Viral load decreased to below detection for all three mice on cART, and rebound reemerged after cessation of cART. The dotted line indicates limit of quantification at 725 copies/mL (Panel B). Mice fed with cART chow had similar weight development as mice housed on non-cART chow during the same time period, indicating no taste-preference or side effects of the cART diet. Weights are presented as fold change compared to start of cART. Each data point represents the mean of three animals ± standard deviation (Panel C).

**Table 1: Antibodies used for determination of stem cell purity**

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Suggested multicolor flow cytometry panel for evaluation of stem cell purity. Listed are the antibody target, the clone and fluorophore.

**Table 2: CCR5Δ32 variant detection PCR primers**
Forward and reverse primers used for detection of the 32 bp deletion in the CCR5 gene.

**Table 3: CCR5Δ32 variant detection PCR program**
PCR cycling program used for amplification of the CCR5 gene.

**Table 4: Antibodies used for determination of mouse humanization**
Suggested multicolor flow cytometry panel for humanization. Listed are the antibody target, the clone and fluorophore.

**Table 5: cDNA amplification program**
Program used for amplification of complementary strand DNA to the viral RNA.

**Table 6: HIV ddPCR primers**
Primers and probes used for ddPCR amplification of viral cDNA.

**Table 7: HIV ddPCR program**
PCR cycling program used for amplification of viral RNA.

**Table 8: Mouse cART chow diet**
Mouse chow diet was formulated as previously published[21]. The chow diet was made on a base of standard mouse chow, and after production, the food was γ-irradiated with 25 kGy and double-bagged. The chow was stored at -20 °C until use.

**DISCUSSION:**

The severely immunocompromised mouse strain, NOD.Cg-*Prkdc*$^{scid}$ *Il2rg*$^{tm1Sug}$/JicTac (NOG) is extremely well suited for transplantation of human cells and tissues. Both innate and adaptive immune pathways in these mice are compromised. NOG (and NSG) mice harbor a Prkdc$^{scid}$ mutation that results in defective T and B cell function. Furthermore, these mice lack a functional interleukin-2 receptor γ-chain (common gamma chain, IL2rg) which is indispensable in the binding complexes of many key cytokines such as IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21. Immuno-deficient mice such as the NOG mouse, transplanted with a human immune system are a powerful tool for the study of HIV transmission and immunology. Contributions in these fields made using humanized mice have been extensively reviewed by us and others[2, 23–26]. The use of these mice to study human innate immune responses are also gaining increased attention[27, 28].

The aim for this manuscript was to supply a comprehensive protocol of mouse and ddPCR procedures to go from a naïve mouse to HIV transmission and treatment data. Our system utilized ddPCR for quantification of viral RNA and DNA. In a ddPCR reaction, the reactants are

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partitioned into up to 20,000 droplets, each containing a single, separate micro PCR reaction. The amplification of a target inside a droplet leads to a positive fluorescent signal for that droplet. Thus, the readout is binary and by applying Poisson statistical analyses, the number of positive reactions can be directly translated to a number of template copies in the original sample. The benefit of ddPCR lies in its ability to directly quantify a target, independent of a standard curve. This is particularly attractive when analyzing RNA samples that are challenging to utilize as PCR standard curves due to their labile nature[29]. Moreover, by analyzing multiple replicas of the same sample and merging the individual data points for the final sample quantification, the binary nature of ddPCR makes it possible to lower the detection limit of template copies per mL of sample[29]. This is especially important in a humanized mouse setting, where only limited sample material is available and high sensitivity is required.

Administration of cART to humanized mice can be done either oral gavage or intraperitoneal injections with solutions of cART[30–32], and as shown recently by formulation into the diet[21]. One of our major aims was the implementation of a cART regimen in the mouse diet to reduce potential stress on the animals due to extra handling steps inherent in other drug delivery methods. The dose of medicine that a mouse will eat can be accurately estimated based on the average daily food intake of mice[33]. Oral delivery through the diet serves as the easiest delivery route with both minimal stress for the animal and minimal workload for the handler. We based our combination of antiviral drugs on previous published studies in humanized mice[21, 30]. Furthermore, our cART strategy is clinically relevant given that the drug combination utilized herein is orally administered by patients around the globe.

Certain limitations are noted regarding the use of NOG mice. Importantly, human T cells in these mice are educated in a mouse thymic environment, as opposed to a human environment. Recent focus is on generating xenorecipient strains that have a favorable environment for the development of robust human immune responses. These new strains include immune-deficient mice which are transgenic for human MHC molecules such as A2. These models enable HLA-restricted antigen T-cell responses, that result in better maturation and effector functions of the adaptive immune system in these mice[34]. Another approach is to replace mouse genes with key human cytokines for IL-3/GM-CSF[35], IL-6[36], IL-15[37], TPO[38], M-CSF and IL-7/TSLP[31]. Such models have gained increased attention for their ability to generate better differentiation of innate cell types. Our protocol will be easily adaptable for the humanization and HIV infection of mice using any such enhanced-genetic background immunodeficient strain.

In summary, the ease and utility of the described approach facilitates research in HIV-related fields *in vivo*. Humanized mice can be a very powerful tool in guiding research towards generating better research hypotheses. Along with the generation of more “human” humanized mice with human transgenes, we believe our standardized protocol will contribute to the streamlining of experimental procedures across different research environments.

**ACKNOWLEDGMENTS:**
The authors would like to thank the Biomedicine Animal Facility staff at Aarhus University, particularly Ms. Jani Kær for colony maintenance efforts and for tracking mouse weights. The



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authors would like to thank Professor Florian Klein for developing standard-of-care cART and for guidance.

**DISCLOSURES:**

The authors declare no conflicts of interests.

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**Suggested multicolour flow cytometry panel for humanization (for use on BD Fortessa X20)**
CD4 (clone SK3) BUV 496, CD8 (clone RPA-T8) BV421, CD3 (clone OKT3) FITC, CD19 (clone sj25c1) PE-Cy7, CD45 (clone 2D1) APC

**Table 1: *CCR5*Δ32 variant detection PCR primers**

| CCR5Δ32 detection | Primers |
|---|---|
| Forward primer | 5'CTTCATTACACCTGCAGCT'3 |
| Reverse primer | 5'TGAAGATAAGCCTCACAGCC'3 |

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**program**

| No. of Cycles | 1x | 45x | 1x | ∞ |
|---|---|---|---|---|
| Temperature | 98° | 98°/63°/72° | 72° | 10° |
| Time | 30 Sec | 10sec/30sec/15sec | 5min | ∞ |

**Table 3: cDNA amplification program**

| No. of Cycles | 1x | 1x | ∞ |
|---|---|---|---|
| Temperature | 51° | 80° | 4° |
| Time | 45 min | 15min | ∞ |

**Table 4: HIV ddPCR primers**

| HIV quantification | Primers |
|---|---|
| Forward primer | 5'AGGGCAGCATAGAGCAAAAA'3 |
| Reverse primer | 5'CAAAGGAATGGGGGTTCTTT'3 |
| FAM probe | 5'ATCCCCACTTCAACAGATGC'3 |

**Table 5. HIV ddPCR program**

| No. of Cycles | 1x | 39x | 1x | ∞ |
|---|---|---|---|---|
| Temperature | 95° | 95°/54.5° | 98° | 4° |
| Time | 10min | 30 sec / 1 min | 10min | ∞ |

**Detailed description of mouse cART chow diet**
Mouse chow diet contained 4800 mg/kg raltegravir (RAL) (Isentress®, MSD), 720 mg/kg tenofovir disoproxil fumarate (TDF) (Viread®, Gilead) and 520 mg/kg Emtricitabine (FTC) (Emtriva®, Gilead)[16]. The chow was produced by ssniff Spezialdiäten GmbH, Ferd.-Gabr.-Weg 16, DE-59494 Soest. The chow diet was made on a base with PS PicoLab Mouse 20 5058, γ-irradiated with 25 kGy and double-bagged.

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