4D-Nucleofector CRISPR–Cas9 RNP Delivery to T and NK Cells

Dillon Corvino

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Author: Dillon Corvino

Purpose

This protocol provides a unified workflow for CRISPR–Cas9 ribonucleoprotein (RNP) delivery into T and NK cells using the Lonza 4D-Nucleofector system with 20 µL Nucleocuvette™ strips. It harmonises RNP preparation, cell handling, nucleofection, and recovery steps across:

- Primary mouse CD8 T cells (naïve and activated)
- Primary mouse NK cells (activated)
- Human CD8 T cells (activated)
- NK-92 cells

Cell-type–specific parameters (nucleofection buffer, pulse program, and recommended RNP load) are summarised in a lookup table.

Critical notes (read before starting)

- This protocol is specific to the **4D-Nucleofector X unit with 20 µL Nucleocuvette™ strips**.
- Always use the **cell-type–specific buffer and pulse code** from the lookup table; do not interchange programs between cell types without re-optimisation.
- Nucleofector solutions (P3, P4, SE) are **toxic** to cells; minimise exposure time and handle rapidly once cells are resuspended.
- Residual culture medium or serum will impair nucleofection efficiency; remove supernatants completely after the final PBS/DPBS wash.
- Assemble RNP at a defined gRNA:Cas9 molar ratio (2–3:1) and incubate **15 min at 37 °C** before mixing with cells.

- Use **pre-warmed rescue medium** and allow cells to recover in the cuvette for 10–25 min after nucleofection before transfer to culture plates.
- Perform all steps under appropriate biosafety conditions (typically BSL2), as cells and reagents may be of human origin.

> ⚠ Warning
>
> Do not leave cells in nucleofector solution for extended periods. Prepare RNP, wash cells, resuspend in buffer, mix, load cuvette, and pulse within a tightly controlled time window to preserve viability.

> 💡 Tip
>
> Prepare a simple "nucleofection map" for each run, listing wells/strips, cell types, buffers, programs, gRNAs, and replicates. This reduces labelling errors and simplifies downstream analysis.

Approximate timing

- RNP preparation and incubation: 20–30 min
- Cell harvest and washing: 30–60 min (depending on source and activation protocol)
- Nucleofection (loading strips and pulsing): 15–30 min
- Post-nucleofection recovery and transfer to culture: 30–45 min

Total hands-on time (per run): ~2–3 hours (excluding prior activation and downstream readouts).

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Procedure

Step 1 – Cell-type–specific lookup

Use the table below to select the appropriate **buffer**, **4D-Nucleofector program**, and starting **RNP load** for each cell type. All reactions use **20 µL total nucleofection volume** in 20 µL Nucleocuvette™ strips.

1.1 Cell-type–specific nucleofection settings

Cell type	Activation state	Buffer (solution)	Program	Cells per reaction (20 µL)	Suggested gRNA per reaction[1]	Suggested Cas9 per reaction[1]	Post-nucleofection cytokines (examples)
Mouse CD8 T (naïve)	IL-7, no TCR stimulation	P4	DS-137	1.0 × 10	~150 pmol	~60 pmol	IL-7 (e.g. 5–10 ng/mL)
Mouse CD8 T (activated)	TCR/peptide stimulated	P4	CM-137	1.0 × 10	~150 pmol	~60 pmol	IL-2 ± IL-7 (lab-specific)
Mouse NK (primary)	Pre-activated (e.g. NK1.1 + IL-15/IL-2)	P3 or P4[2]	EN-138	0.5–1.0 × 10	~150 pmol	~60 pmol	IL-15 + IL-2 (as per NK culture SOP)
Human CD8 T (activated)	TCR/anti-CD3/CD28, IL-2	P3	EH-115	1.0 × 10	~120–150 pmol	~60 pmol	IL-2 (e.g. 100–200 IU/mL)
NK-92	NK-92 medium, log-phase	SE	CM-137	0.5–1.0 × 10	~150–180 pmol	~60 pmol	IL-2 as per NK-92 culture SOP (if used)

[1]Values are per 20 µL reaction and are starting points based on existing optimisation; adjust per guide, locus, and experimental needs.
[2]If both P3 and P4 have been tested for mouse NK, use the solution that gave the best balance of viability and editing in your prior experiments.

> **Note**
>
> This unified protocol describes a common workflow. Cell-type–specific pre-activation (e.g. NK priming, T-cell stimulation) and culture conditions should be followed according to the relevant cell culture SOPs and are not exhaustively detailed here.

Step 2 – gRNA duplex and RNP preparation

This section assumes use of **crRNA + tracrRNA** duplexes and recombinant Cas9 protein.

2.1 crRNA:tracrRNA duplexing

1. Thaw crRNA and tracrRNA aliquots on ice.
2. Mix crRNA and tracrRNA at **equimolar concentrations** (e.g. each at 100 µM) to generate a duplex:
   - Example: 5 µL crRNA (100 µM) + 5 µL tracrRNA (100 µM) → 10 µL duplex at 100 µM.
3. Incubate at **95 °C for 5 min**, then allow to cool to room temperature (bench-top) for at least 10 min, or follow vendor instructions.
4. Store duplexed gRNA on ice during RNP preparation.

2.2 RNP assembly

1. Thaw Cas9 protein aliquots on ice.
2. For each nucleofection reaction, prepare RNP mix in a low-bind tube:
   - Example per reaction (starting point, adjust using Table 1):
     – 1.5 µL gRNA duplex (100 µM) → 150 pmol gRNA
     – 3.0 µL Cas9 (20 µM) → 60 pmol Cas9
     – Optional: add nuclease-free water to increase handling volume, if needed.
3. Gently mix by pipetting; avoid bubbles.
4. Incubate the RNP mix at **37 °C for 15 min**.
5. After incubation, keep RNP at room temperature or on ice and use within **1–2 hours** for nucleofection.

> ⚠ Warning
>
> Do not repeatedly freeze–thaw Cas9 or gRNA aliquots. Prepare small working aliquots and discard thawed leftovers at the end of the day to preserve activity.

Step 3 – Cell preparation

The steps below describe the unified handling for all T and NK cell types. Follow cell-type–specific culture SOPs for prior expansion and activation.

3.1 Harvesting cells

1. Harvest cells from culture flasks or plates at the desired time point (e.g. 2–3 days post activation for T cells; 3 days post priming for NK).

2. For suspension cultures (mouse NK, NK-92, activated T cells): gently resuspend by pipetting up and down.

3. For mixed cultures (e.g. mouse spleen-derived T or NK), process according to the relevant isolation SOP (e.g. magnetic separation) before nucleofection.

3.2 Washing and counting

1. Transfer cells to 15 mL conical tubes.

2. Centrifuge at **300–400 g for 5 min** at room temperature.

3. Carefully aspirate supernatant.

4. Resuspend the pellet in **10 mL** DPBS or PBS and mix gently.

5. Centrifuge again under the same conditions.

6. Aspirate the supernatant completely.

7. Take an aliquot and count cells (e.g. trypan blue exclusion).

8. Calculate total cell number and plan **0.5–1.0 × 10 cells per nucleofection reaction**.

> ⚠ Warning
>
> Residual serum or culture medium in the pellet will reduce nucleofection efficiency and viability. After the final wash, remove supernatant carefully and completely before adding nucleofector solution.

Step 4 – Resuspension in nucleofector solution and mixing with RNP

4.1 Preparation of nucleofector cell suspension

1. Pre-equilibrate nucleofector solutions (P3, P4, SE) to **room temperature** as recommended by the manufacturer.

2. For each cell type, prepare enough nucleofector solution for all planned reactions, following the kit instructions (e.g. supplementing solution with additive as required).

3. Resuspend washed cells in the appropriate nucleofector solution at a concentration of:

- **5 × 10 cells/mL** for 1.0 × 10 cells in 20 µL
- **2.5 × 10 cells/mL** for 0.5 × 10 cells in 20 µL

4. Gently mix and keep at room temperature. Avoid leaving cells in nucleofector solution for longer than **15–20 min** before pulsing.

4.2 Mixing cells with RNP

For each reaction:

1. Aliquot the appropriate volume of RNP mix into a fresh low-bind tube or directly into the well/strip position (e.g. 4.5 µL per reaction).
2. Add nucleofector cell suspension to reach a **total volume of 20 µL per reaction**:
   - Example: 4.5 µL RNP + 15.5 µL cell suspension.
3. Gently pipette up and down to mix; avoid introducing air bubbles.
4. Immediately proceed to loading the Nucleocuvette™.

Step 5 – Loading Nucleocuvette™ and running the 4D program

5.1 Loading Nucleocuvette™ strips

1. Place the 20 µL Nucleocuvette™ strip into its holder.
2. Carefully pipette **20 µL** of the RNP–cell mix into each well, aiming the pipette tip to the side of the well to minimise bubbles.
3. Visually inspect each well for uniform filling and absence of air pockets.
4. Insert the holder into the 4D-Nucleofector X unit.

5.2 Selecting and running programs

1. On the 4D-Nucleofector control unit, select the **nucleofection program** corresponding to the cell type (see lookup table in Step 1).
2. Confirm that the selected program and kit (P3, P4, SE) match the cells in the strip.
3. Start the nucleofection run.
4. When complete, remove the Nucleocuvette™ strip promptly and place it in a biosafety cabinet.

> **Note**
>
> If running multiple cell types in parallel, clearly separate strips by cell type and program to avoid mismatches. Only one program can be used per strip/run.

Step 6 – Immediate post-nucleofection rescue

6.1 Addition of rescue medium in cuvette

1. Immediately after nucleofection, add **60–100 µL** of **pre-warmed culture medium** directly into each Nucleocuvette™ well:
   - Use the appropriate base medium (e.g. T-cell medium, NK medium, or NK-92 medium).
   - Cytokines may be omitted from this initial rescue step if preferred (see cell-type notes below).
2. Gently pipette up and down once to mix, without generating bubbles.
3. Place the Nucleocuvette™ strip into a 37 °C incubator (inside a sterile container) or keep at 37 °C in the biosafety cabinet if feasible.
4. Allow cells to recover in the cuvette for **10–25 min**.

6.2 Transfer to culture plates

1. After the recovery period, gently resuspend each Nucleocuvette™ well by pipetting up and down.
2. Transfer the entire volume (typically 80–120 µL) into the corresponding well of a pre-warmed 96- or 48-well plate or into a culture tube containing:
   - **Final volume**: 200–1000 µL per well/tube.
   - **Appropriate cytokine supplementation**, for example:
     - Naïve mouse T: IL-7 ± low IL-2.
     - Activated T (mouse/human): IL-2 ± IL-7.
     - Mouse NK: IL-15 + IL-2 as per NK culture SOP.
     - NK-92: NK-92 medium ± IL-2 as per NK-92 culture SOP.
3. Adjust final cell density to **0.5–1.0 × 10 cells/mL** for standard culture.
4. Incubate plates at **37 °C, 5% CO** .

> ⚠ Warning
>
> Do not leave nucleofected cells in undiluted nucleofector buffer longer than necessary. Rescue medium should be added within minutes after the pulse and cells should be transferred to standard culture conditions within 30–40 min.

Step 7 – Recommended timelines for analysis

Although the exact timepoint will depend on the target locus and downstream readouts, the following windows are commonly used:

- **Mouse NK cells:** assess viability and KO efficiency at **day 3** and **day 7**.
- **Mouse T cells (naïve/activated):** assess between **day 3–6**, with extended phenotyping up to **day 10–15** when needed.
- **Human T cells (activated):** assess at **day 3–5** post nucleofection.
- **NK-92:** assess between **day 3–5** post nucleofection.

Choice of timepoint should balance maximal editing efficiency with sufficient recovery and expansion.

Buffers Required

Full composition, preparation, storage, and safety information for each buffer lives in its own linked buffer document — only a concise per-buffer summary is reproduced here, generated automatically from that document.

NK Cell Culture Medium

Component	Final concentration / amount
RPMI 1640	to 500 mL
Fetal calf serum (FCS)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)
HEPES	10 mM (~1% v/v)
Sodium pyruvate	1 mM (1% v/v)
Non-essential amino acids (NEAA)	1% (v/v)
beta-mercaptoethanol	50 uM

- **Final volume:** 500 mL
- **Preparation note:** Mix gently by inversion; do not vortex. IL-15 is added fresh (100 IU/mL) immediately before plating cells and is not part of the stored base medium.
- **Storage:** 4C, protected from light, 4-5 weeks; do not freeze
- **Caveat:** IL-15 must not be stored in the base medium - add fresh immediately prior to culture.

Full composition, preparation steps, storage, and safety information: `BUF-NK-MED`.

Materials

Reagents

Reagent	Supplier	Cat. #	Notes
4D-Nucleofector™ X Unit + 20 µL Nucleocuvette™ strips	Lonza	—	Core instrument and plasticware
Nucleofector™ P3 Primary Cell kit	Lonza	—	For mouse NK (option), human activated T cells
Nucleofector™ P4 Primary Cell kit	Lonza	—	For mouse CD8 T cells (naïve and activated), mouse NK (option)
Nucleofector™ SE Cell Line kit	Lonza	—	For NK-92 cells
crRNA and tracrRNA (or sgRNA)	IDT / similar	—	Target-specific; duplexed prior to RNP assembly
Cas9 nuclease (recombinant)	IDT / similar	—	High-purity Cas9 for RNP formation
DPBS / PBS, 1×	Various	—	For washing cells before nucleofection
T-cell culture medium	In-house / various	—	e.g. RPMI + 10% FCS + Pen/Strep + supplements
NK culture medium	In-house / various	—	e.g. NK medium (BUF-NK-MED)
NK-92 culture medium	In-house / various	—	Defined NK-92 medium formulation
Recombinant cytokines (IL-7, IL-2, IL-15)	PeproTech / similar	—	For pre-activation and post-nucleofection culture
Trypan blue or viability dye	Various	—	For viability and counting

Disposables

Item	Specification
15 mL and 50 mL tubes	Sterile, conical
96-well or 48-well culture plates	Tissue culture-treated
Low-bind microcentrifuge tubes	For RNP assembly
Serological pipettes (5–25 mL)	Sterile
Pipette tips (10–1000 µL)	Sterile, filter tips recommended
Reservoirs for media and buffers	Sterile

Equipment

Equipment	Specification / Notes
4D-Nucleofector™ X Unit	With 20 µL Nucleocuvette™ strips
Biological safety cabinet	Class II, for sterile and safe handling
CO incubator	37 °C, 5% CO
Benchtop centrifuge	For 15/50 mL tubes (300–400 g)

Equipment	Specification / Notes
Water bath	37 °C, for reaction and thawing
Cell counter or hemocytometer	For counting and viability assessment

Troubleshooting

Problem	Possible cause	Suggested solution
Low viability after nucleofection	Prolonged exposure to P3/P4/SE; incorrect program	Shorten handling time in nucleofector solution; verify program; reduce cell density per reaction
Poor editing efficiency (low KO)	Insufficient gRNA/Cas9; residual serum; incorrect guide	Increase gRNA (within range); ensure complete washes; verify guide design
High variability between replicates	Uneven cell counts; pipetting error in RNP addition	Standardise counting; use multichannel pipettes or master mixes where possible
Clumping after nucleofection	High cell density; inadequate resuspension	Reduce cells per reaction; gently resuspend and filter if necessary
Delayed recovery or poor proliferation	Inadequate cytokine support; suboptimal post-nucleofection density	Optimise cytokine cocktail; adjust seeding density; ensure fresh medium
Apparent toxicity of RNP mix	Old or degraded Cas9/gRNA; repeated freeze–thaw cycles	Use fresh aliquots; avoid refreezing; check storage conditions

Safety (brief)

- Handle all primary cells and cell lines, especially human-derived material, under **BSL2** conditions according to institutional guidelines.
- Wear appropriate PPE (lab coat, gloves, eye protection) throughout the procedure.
- Dispose of cell culture waste, nucleofection strips, and contaminated plastics as biohazardous waste.
- Follow manufacturer safety data sheets (SDS) for nucleofector solutions, Cas9, and any chemical reagents used.

Version history

Version	Date	Author	Changes
v1.0	2025-11-21	Dillon Corvino	First unified 4D-Nucleofector protocol for T and NK cell CRISPR–Cas9 RNP.
v1.1	2026-09-18	Dillon Corvino	Replaced `# Buffers used` (bare `library(tidyverse)`, leaked R package-startup warnings) with an auto-generated `# Buffers Required` section; moved it from after Materials to before Materials