Plate-bound stimulation of NLCs (anti-CD3e +/- anti-NK1.1 + soluble anti-CD28)

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Purpose

To stimulate sorted NLCs in 96-well plates using plate-bound anti-CD3e and/or anti-NK1.1 with soluble anti-CD28, then transfer cells to uncoated wells for IL-2-driven expansion.

Critical notes (read before starting)

- Use sterile technique throughout.
- Coat plates at 4 °C overnight for consistent binding.
- anti-CD28 is **soluble** (add in media; do not coat).
- After stimulation, transfer cells to **uncoated** wells to avoid continued plate-bound signaling.

Working concentrations

Stimulation phase (in culture)

Reagent	Final concentration	Notes
anti-CD3e	2 µg/mL	plate-bound
anti-NK1.1	10 µg/mL	plate-bound
anti-CD28	5 µg/mL	soluble (in media)
Mouse IL-2	1 ng/mL	during stimulation

Expansion phase (post-stimulation)

Reagent	Final concentration	Notes
Mouse IL-2	100 IU/mL	in fresh complete T cell medium

Plate and seeding

- Plate type: 96-well **U-bottom** or **flat-bottom**
- Seeding density: **10,000–50,000 cells per well**
- Coating volume: **200 µL/well PBS** (sterile)

Procedure

Plate coating (Day -1)

1. Prepare coating solutions in **sterile PBS** (200 µL per well):
    - anti-CD3e (as needed per condition)
    - anti-NK1.1 (as needed per condition)
      (Do not include anti-CD28.)
2. Add **200 µL** coating solution per well.
3. Incubate **overnight at 4 °C**.

Stimulation (Day 0)

1. Remove coating solution.
2. Wash wells **2×** with **sterile PBS**.
3. Add cells in complete T cell medium, seeding **10–50K cells/well**.
4. Add soluble reagents to culture medium (per condition):
    - anti-CD28 to **5 µg/mL**
    - mouse IL-2 to **1 ng/mL**
5. Incubate **3 days** at 37 °C, 5% CO2.

Transfer to expansion (Day 3 → Day 4)

1. Gently resuspend cells and transfer the full well contents to **uncoated** wells (new 96-well plate).
2. Culture in fresh complete T cell medium containing mouse IL-2 at **100 IU/mL**.

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.

T Cell Medium

Component	Final concentration / amount
RPMI 1640	base medium, to volume
Fetal calf serum (FCS)	10% (v/v)
Penicillin/Streptomycin	1% (v/v)
L-glutamine (or GlutaMAX)	2 mM
beta-mercaptoethanol	50 uM
Sodium pyruvate	1 mM (optional)
HEPES	10 mM (optional)
MEM NEAAs	1% (v/v) (optional)

- **Final volume:** 500 mL (scalable 10-500 mL; see preparation table)
- **Preparation note:** Add beta-mercaptoethanol fresh where possible; mix gently, do not vortex.
- **Storage:** 4C, protected from light; use within 4 weeks; do not freeze.
- **Caveat:** beta-mercaptoethanol is essential for mouse primary T cells/NLCs - do not omit. L-glutamine degrades at 4C over weeks; substitute GlutaMAX for medium stored more than 1-2 weeks.

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

Storage and stability

- Coated plates can be prepared the day before and kept at **4 °C overnight** until use.
- Prepare antibody working solutions fresh where possible.

Safety

- Handle all primary cells and culture waste using appropriate biosafety procedures.
- Dispose of liquid waste containing cells as biohazardous waste.

Version history

Version	Date	Author	Changes
v1.0	2026-03-02	Dillon Corvino	Initial simple stimulation protocol.

Version	Date	Author	Changes
v1.1	2026-09-18	Dillon Corvino	Replaced `# Buffers used` with an auto-generated `# Buffers Required` section (one heading + one small table per buffer, sourced from each buffer's own `summary:` data), moved to the correct position after Procedure, and fixed leaked R package-startup warnings.