SignaGen® Laboratories

CalFectin™ Mammalian DNA Transfection Reagent

A Protocol for Helper-Free AAV Packaging in HEK293T Cells (15 cm Dish)

SKU # SL100478 | SignaGen Laboratories | For Research Use Only — Not for Diagnostic Purposes

Introduction

CalFectin™ is a chemically optimized calcium phosphate-based transfection reagent that generates uniformly sized DNA–calcium co-precipitate complexes, enabling efficient endocytosis with reduced cytotoxicity relative to classical calcium phosphate methods. This protocol describes a fully **helper-free** triple-plasmid system for recombinant AAV (rAAV) production in HEK293T cells, eliminating the requirement for live adenovirus or herpesvirus co-infection. The three-plasmid components are: (1) an ITR-flanked AAV transfer vector carrying the transgene of interest; (2) a serotype-specific Rep/Cap plasmid providing the AAV replication and capsid proteins; and (3) a *cis*-deleted adenoviral helper plasmid (e.g., pHelper or pAdDeltaF6) supplying E2A, E4orf6, and VA RNA functions without producing infectious adenovirus. This system yields replication-incompetent rAAV particles suitable for stable transduction of dividing and non-dividing target cells.

■ ***Note:*** *Recombinant AAV vectors are generally classified as BSL-1 agents; however, vectors carrying potentially oncogenic transgenes or pseudotyped with certain capsid serotypes may require BSL-2 containment. Consult your Institutional Biosafety Committee (IBC) prior to initiating production.*

Component	Volume	Storage
CalFectin™ Mammalian DNA Transfection Reagent	1.0 mL	+4 °C

Shelf life: Stable for up to 12 months at +4 °C. Do not freeze.

General Guidelines

- Always dilute plasmid DNA and CalFectin™ in **serum-free DMEM (High Glucose)** prior to mixing. Serum proteins inhibit DNA–calcium co-precipitate assembly. Do **not** use Opti-MEM; its buffering composition is incompatible with calcium phosphate complex formation.
- Add CalFectin™ directly into the diluted DNA solution (*single-tube format*); do not pre-dilute the reagent separately. Use a **3:1** CalFectin™ (µL) : DNA (µg) ratio.
- Target **70–80% confluency** at transfection. AAV assembly is intranuclear; sub-confluent cells maintain sufficient mitotic activity and nuclear capacity for productive replication. Overly dense monolayers reduce transfection uptake without increasing yield.
- Use HEK293T cells at ≤**25 passages** to preserve transfection competency and stable SV40 Large T-antigen expression, which drives episomal plasmid amplification.

Plasmid Composition (Per 15 cm Dish — Total 18 µg)

Plasmid	Amount	Function
ITR-flanked transfer vector	5 µg	Transgene cassette flanked by AAV2 ITRs
pAAV-RC (serotype-specific Rep/Cap)	5 µg	AAV Rep (replication/packaging) and Cap (capsid) proteins
pHelper / pAdDeltaF6 (adenoviral helper, replication-incompetent)	8 µg	E2A, E4orf6, VA RNA — helper functions without producing infectious adenovirus

The **5:5:8 mass ratio** (transfer vector : Rep/Cap : helper) reflects the stoichiometric requirements of the helper-free system: Rep/Cap are needed equimolarly relative to the ITR template, while adenoviral helper functions are rate-limiting and benefit from a twofold molar excess. Deviation from this ratio reduces encapsidation efficiency and viral yield.

Step 1 — Cell Seeding

Seed HEK293T cells in a **15 cm dish** containing 20 mL of complete DMEM (10% FBS, 1% penicillin/streptomycin) **18–24 hours prior to transfection**, targeting **70–80% confluency** at the time of transfection. Add 20 mL of fresh, pre-warmed (37 °C) complete medium 30–60 minutes before complex addition to stabilize pH and replenish nutrients.

■ ***Note:*** *Verify monolayer morphology by phase-contrast microscopy prior to transfection. Unhealthy or over-passaged cells are the most common cause of low viral yield. Serum and antibiotics in the culture medium do not inhibit CalFectin™-mediated transfection, as the complex is pre-formed in serum-free medium before addition to cells.*

Step 2 — Preparation of CalFectin™/DNA Complex

1. In a sterile 1.5 mL microcentrifuge tube, combine the **18 µg total plasmid DNA** (5 µg transfer vector + 5 µg Rep/Cap + 8 µg pHelper) and dilute into **1,000 µL serum-free DMEM (High Glucose)**. Mix gently by pipetting 3–4 times. Do *not* vortex.
2. Add 54 **µL CalFectin™ Reagent** (3:1 µL:µg ratio) directly into the diluted DNA solution. Mix immediately by gentle pipetting 3–4 times. Do *not* vortex after addition — vigorous mixing disrupts nascent DNA–calcium co-precipitate nucleation.
3. Incubate at **room temperature (18–25 °C) for exactly 10 minutes** to allow uniform co-precipitate formation. Do *not* exceed 20 minutes.
4. Add the 1,000 µL complex **dropwise and evenly** over the medium in the 15 cm dish. Distribute by gently rocking the plate in a cross-wise pattern. Return to the 37 °C / 5% CO_2 incubator immediately.

■ ***CRITICAL:*** *Do not incubate the CalFectin™/DNA complex for more than 20 minutes. Over-incubation causes uncontrolled particle aggregation, reducing cellular uptake, transfection efficiency, and cell viability. Never vortex after combining DNA and reagent.*

Step 3 — Medium Change (24 h Post-Transfection)

At **24 hours post-transfection**, carefully aspirate the medium and replace with **20 mL of fresh, pre-warmed (37 °C) complete DMEM**. This step removes residual CalFectin™/DNA complexes and cytotoxic by-products that accumulate during the initial transfection window, preserving cell viability through the peak production phase.

■ ***Note:*** *Unlike lentiviral vectors, which are constitutively shed into the supernatant, the majority of rAAV particles remain* ***cell-associated*** *and are released only upon lysis. Supernatant-based harvest captures only a minor fraction of total yield and is not the primary collection strategy in this protocol.*

Step 4 — AAV Harvest by Cell Lysis (72 h Post-Transfection)

Peak intracellular AAV titers are typically achieved at **72 hours post-transfection**, corresponding to maximal Rep-mediated genome replication, capsid assembly, and DNA packaging.

1. Inspect producer cells by phase-contrast microscopy. Cytopathic changes (rounding, granularity, partial detachment) are expected and indicate productive replication. Proceed with harvest regardless of the degree of cytopathic effect.
2. Dislodge adherent cells by scraping into the existing medium. Transfer the suspension to a **50 mL conical tube**. Pellet at **300 × g, 5 min, 4 °C**. Discard supernatant (or pool with cell pellet if maximal recovery is required).
3. Resuspend the cell pellet in **1 mL of lysis buffer per 15 cm dish** (150 mM NaCl, 50 mM Tris-HCl, pH 8.5). Lyse by **three freeze–thaw cycles**: snap-freeze in dry ice/ethanol (5 min), then thaw at 37 °C with vortexing. Repeat for a total of three cycles.
4. Add **Benzonase® nuclease** to 50 U/mL and $MgCl_2$ to 1 mM. Incubate at **37 °C for 30 minutes** with gentle agitation. This degrades residual plasmid DNA and unpackaged AAV genomes without affecting encapsidated particles, substantially improving preparation purity.
5. Clarify the lysate by centrifugation at **4,000 × g, 20 min, 4 °C**. Collect the clarified supernatant (crude viral lysate) into a fresh tube. Proceed to downstream purification immediately, or store at –**80 °C**.

■ ***CRITICAL:*** *Do not exceed three freeze–thaw cycles for viral stocks; repeated cycling significantly reduces infectious titer. Aliquot purified vector as single-use volumes and store at –80 °C. Avoid repeated freeze–thaw of working aliquots.*

Storage

- **CalFectin™ Reagent:** Stable for up to 12 months at +4 °C. Do not freeze.
- **Crude viral lysate:** Store at –80 °C; purify as soon as possible to minimize titer loss from proteolytic degradation.
- **Purified rAAV stocks:** Store as single-use aliquots at –80 °C in PBS or a low-protein formulation buffer.

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