Study Execution Guide v1

Dual-Platform mRNA Therapy for X-Linked Adrenoleukodystrophy (ALD)
LNP + EV, Peripheral-First → CNS

Modeling-Integrated Study Manual Aligned with Stage-1 Architecture

Method2Model

Abstract

This Study Execution Guide describes, in practical and sequential terms, how to conduct the dual-platform mRNA ALD programme in a way that is fully aligned with the Stage-1 architecture. It is written for medical and biomedical researchers (wet-lab and clinical/translational teams) and is intended to serve as an operational manual: it specifies what must be done, in what order, which data must be collected, how those data will feed the model blocks (A–G), and which minimal quality checks are required. No simplifying assumptions (e.g. single-compartment PK, purely linear dose–response) are imposed on the experimental design; instead, the protocol is structured to support the richer architecture that has already been defined.

The Guide is organized by workstream (in vitro, in vivo, CMC/analytics, and modeling) and by decision-relevant outputs (VLCFA, ALDP targeting, CNS exposure, safety, and robustness under scenarios). It is designed to be self-contained for study execution, while referencing the Stage-1 Architecture Specification for formal model details.

Contents

1 Purpose and Scope

1.1 Purpose

The purpose of this document is to transform the initial proposals and the Stage-1 architecture into a concrete, step-wise Study Execution Guide that:

- Can be understood and executed by medical and biomedical researchers without prior expertise in mathematical modeling.
- Specifies, for each experimental component, the required measurements, timing, and minimal quality criteria.
- Clarifies how each data element will be used within the multi-block model (Blocks A–G), without forcing the experimental team to adopt oversimplified assumptions.
- Makes explicit the coordination between laboratory work, analytics, and modeling so that Go/No-Go and Pivot decisions are traceable and defensible.

1.2 Scope

The Guide covers:

1. **In vitro workstream (Workstream 1)**: ALDP expression and peroxisomal targeting, and VLCFA reduction in patient-derived cells (Aim 1).
2. **In vivo workstream (Workstream 2)**: efficacy, CNS effect, and safety in mouse models (Aim 2).
3. **CMC and analytics workstream (Workstream 3)**: production and characterization of LNP and EV batches (potency, quality, stability).
4. **Modeling workstream (Workstream 4)**: data ingestion, parameterization, scenario definition, and verification checks, aligned with the Stage-1 architecture.

Human clinical trials are *not* part of the practical execution described here; however, the in vivo and modeling components are structured to generate human-projected insights (e.g. via NfL and Loes-based projections) as described in the architecture.

2 Programme Overview and Objectives

2.1 Clinical and Biological Context

X-linked adrenoleukodystrophy (ALD) arises from loss-of-function mutations in the *ABCD1* gene, leading to deficiency of ALDP (ATP-binding cassette subfamily D member 1) at peroxisomal membranes. The resulting defect in peroxisomal β-oxidation causes accumulation of very-long-chain fatty acids (VLCFA), notably C26:0, in plasma and CNS. This accumulation is associated with progressive neurological damage. The programme aims to restore ALDP function via mRNA delivery using two complementary platforms:

- Lipid nanoparticles (LNP), primarily for systemic/peripheral correction.
- Extracellular vesicles (EV), with routes (IN, IV+FUS) designed to enhance CNS targeting.

2.2 High-Level Objectives

The integrated study has three interlinked objectives:

1. **Aim 1 (In vitro)**: Demonstrate that mRNA delivery restores ALDP at peroxisomal membranes, normalizes peroxisomal import, and reduces VLCFA levels in patient-derived cells, with an acceptable innate immune profile.
2. **Aim 2 (In vivo)**: Demonstrate that, in relevant mouse models, the chosen regimens produce:
   - substantial reduction of VLCFA in plasma and CNS,
   - improvements in functional and imaging endpoints (e.g. Rotarod, DTI),
   - and do so within an acceptable safety window.
3. **Aim 3 (Translational modeling)**: Using the data from Aims 1–2, populate and run the Stage-1 architecture so that CNS exposure limits, peroxisomal targeting windows, robustness to variability, and candidate Go/No-Go/Pivot decisions are quantified.

3 Workstreams and Roles

3.1 Workstream 1: In Vitro Cellular Studies (Aim 1)

Lead profile: medical/biological researcher with expertise in cell culture and peroxisomal biology, supported by an analytical chemist for VLCFA measurements.

Core responsibilities:

- Establish and maintain ALD patient-derived fibroblast (or relevant neural cell) lines and healthy controls.
- Perform mRNA transfection or exposure using LNP and/or EV formulation prototypes.
- Quantify:
  - ALDP expression and localization (peroxisomal vs mislocalized),
  - peroxisomal import (PTS1/PTS2 reporters),
  - VLCFA levels and ratios,
  - innate immune readouts (e.g. interferon-stimulated gene expression, cytokines where feasible).
- Prepare structured data tables for the modeling team, following the Input Spec Sheet.

3.2 Workstream 2: In Vivo Mouse Studies (Aim 2)

Lead profile: preclinical neuroscientist or translational researcher with access to an ALD mouse model (e.g. *Abcd1*$^{-/y}$) and facilities for MRI and behavioral assays.

Core responsibilities:

- Design and conduct dosing regimens with LNP and EV routes (IV, IN, IV+FUS, EV+FUS) in ALD mice and controls.
- Collect:
  - PK/exposure samples for mRNA and carrier,
  - VLCFA levels in plasma and CNS,
  - DTI/MRI data,
  - behavioral readouts (e.g. Rotarod),
  - histology for myelin, axons, and microglia,
  - safety data (clinical observations, ALT/AST, cytokines).
- Format data according to the Input Spec Sheet.

3.3 Workstream 3: CMC & Analytics

Lead profile: formulation scientist / CMC lead with nanoformulation and EV expertise.

Core responsibilities:

- Produce and characterize LNP and EV batches, including: size, PDI, encapsulation efficiency, mRNA integrity, endotoxin, potency, and stability.
- Maintain a batch registry with unique IDs and measured attributes.
- Provide CMC data to the modeling team for Block E.

3.4 Workstream 4: Modeling and Data Integration

Lead profile: quantitative modeler familiar with PBPK/PD, systems pharmacology, and the Stage-1 architecture.

Core responsibilities:

- Implement the architecture (Blocks A–G) as software modules.
- Ingest data from Workstreams 1–3 according to the Input Spec Sheet and Assumptions Map.
- Define and run baseline and stress scenarios (Scenario Pack v1).
- Generate robustness curves, first-break maps, and Go/No-Go/Pivot analyses.
- Coordinate verification with experimental team using the Verification Plan.

4 Input–Output Map and Data Flows

4.1 Conceptual Overview

The data flows can be summarized as follows:

1. Workstream 3 defines batch-specific attributes (Block E: CMC / Batch Variability).
2. These attributes modulate effective doses and exposure patterns in Workstreams 1 and 2 (Block A: Delivery & Exposure).

3. Workstream 1 provides detailed ALDP expression and targeting data (Block B) and cell-level VLCFA dynamics (Block C, in vitro).
4. Workstream 2 provides in vivo exposure, VLCFA, and functional endpoints (Blocks A, C, D).
5. All streams feed into the modeling workstream, which uses Blocks F and G to explore uncertainty and generate decision-ready outputs.

4.2 Data Structure Expectations

For practical purposes, the experimental teams should aim to provide data in tabular form (e.g. spreadsheets or CSV files) with:

- One row per experimental unit (e.g. well, animal) per time point.
- Columns for:
  - IDs (subject, batch, route, regimen).
  - Timestamps (absolute and relative to dosing).
  - Dose and formulation details (including batch IDs).
  - Measured endpoints (e.g. VLCFA, ALDP IF scores, DTI metrics, ALT, NfL, etc.).
- Explicit codes for missing values and known reasons (e.g. sample loss, assay failure).

The modeling team will map these columns onto the specific variables of the Stage-1 architecture; the execution guide focuses on ensuring that the necessary variables are actually measured.

5 Workstream 1: In Vitro Execution Plan (Aim 1)

5.1 Cell Models and General Setup

Cell sources:

- ALD patient-derived fibroblasts (or induced pluripotent stem cell-derived neural cells with documented *ABCD1* mutations).
- Healthy control cells (matched where possible).

Inclusion criteria (for ALD cells):

- Genetically confirmed *ABCD1* loss-of-function mutation.
- Documented VLCFA elevation at baseline (e.g. C26:0/C22:0 above a predefined threshold).

Culture conditions:

- Use standardized media, passage ranges, and seeding densities across experiments.
- Record passage number and culture duration at the time of transfection.

5.2 Experimental Design

Groups: For each cell type (ALD and control), include at minimum:

- Untreated baseline.
- Vehicle control (LNP empty, EV without *ABCD1* mRNA).
- LNP-mRNA variants (e.g. two dose levels).
- EV-mRNA variants (e.g. two dose levels).

Replicates and time points:

- At least three biological replicates per group.
- Time points:
  - For expression & targeting: e.g. 24, 48, 72 hours.
  - For VLCFA dynamics: 48, 72, 96 hours and possibly later time points if feasible.

5.3 Measurements and Procedures

ALDP expression and localization (Block B).

- Immunofluorescence: stain for ALDP and peroxisomal markers (PEX14, PMP70); quantify colocalization (e.g. Pearson or Manders coefficients).
- Protease protection: isolate peroxisomal fractions and perform protease-protection assays to confirm membrane insertion.
- Western blot: total versus fractionated ALDP to approximate $P_{\mathrm{ALDP,tot}}$ and $P_{\mathrm{ALDP,peri}}$.

Peroxisomal import (PTS1/PTS2).

- Introduce PTS1/PTS2 reporter constructs (if not constitutive).
- Quantify peroxisomal localization of fluorescent or enzymatic reporters, serving as a functional readout of import.

VLCFA levels (Block C, in vitro).

- Measure C26:0 and relevant VLCFA species by GC–MS or LC–MS/MS.
- Report both absolute levels and ratios (e.g. C26:0/C22:0).

Innate immune readouts (Block D, in vitro slice).

- If feasible, measure expression of interferon-stimulated genes and key cytokines (e.g. IL-6, TNF-α) in treated cells.
- At minimum, record qualitative signs of stress or toxicity at the cellular level.

5.4 Data Formatting for Modeling

For each experiment, generate tables with columns including:

- Cell line ID, genotype (ALD vs control), passage number.
- Treatment ID (batch of LNP/EV, dose, exposure duration).
- Time point (hours after treatment).
- ALDP IF scores and derived colocalization metrics.
- PTS1/PTS2 import metrics.
- VLCFA concentrations and ratios.
- Innate immune indicators (if measured).

5.5 Minimal Quality Criteria

- Confirm baseline elevation of VLCFA in ALD cells versus controls.
- Verify that at least one dose level produces a detectable increase in ALDP expression and peroxisomal localization.
- Ensure that analytical methods (VLCFA, IF quantification) have documented limits of detection and reproducibility.

6 Workstream 2: In Vivo Execution Plan (Aim 2)

6.1 Animal Model and Grouping

Animal model:

- Male *Abcd1*$^{-/y}$ mice (ALD model) and wild-type controls, age ranges appropriate for phenotype (to be specified in protocol).

Groups: At minimum, for ALD mice:

- Untreated ALD baseline.
- LNP-mRNA peripheral regimen(s) (e.g. IV weekly, IV Q2W).
- EV-mRNA CNS-targeted regimen(s) (IN, EV+FUS).
- Combination regimen (peripheral LNP + CNS EV) if feasible.

  Wild-type groups can be used to anchor baseline ranges and safety comparisons.

6.2 Dosing Regimens

Schedule:

- Define exact timing of doses (weekly or Q2W), duration (e.g. 4–8 weeks).
- Record administered dose per kilogram, batch ID, and route for each injection.

FUS parameters (if applicable):

- Document sonication parameters, contrast agent use, and timing relative to EV or LNP administration.

6.3 Endpoints and Time Points

At each planned time point (e.g. weeks 2 and 4, plus follow-up if possible) collect:

Exposure and distribution (Block A).

- Plasma samples for mRNA/carrier levels where feasible.
- Tissue collection (liver, spleen, adrenal, CNS regions) for biodistribution assays and mRNA/protein expression if possible.

VLCFA and PD (Block C).

- Plasma VLCFA (C26:0, ratios).
- CNS VLCFA from predefined brain regions.

Functional and imaging endpoints (Block C).

- Rotarod performance (with standardized training procedures).
- DTI/MRI with metrics (FA, MD, RD) in relevant regions of interest.

Safety endpoints (Block D).

- Clinical observations (body weight, activity, visible signs).
- Plasma ALT and AST at baseline and key time points.
- Cytokine panels (if feasible).
- For FUS arms: MRI sequences sensitive to microbleeds or edema.

Histology.

- Myelin staining, axonal markers, and microglial activation in selected animals at endpoint.

6.4 Data Formatting for Modeling

For each animal and time point:

- Animal ID, genotype, group, regimen ID.
- Dosing history (dates, doses, route, batch IDs).
- PK/biomarker values (VLCFA, ALT/AST, cytokines).
- Functional scores (Rotarod).
- DTI metrics per region (FA, MD, RD).
- Safety flags (any observed adverse events).

6.5 Minimal Quality Criteria

- Documented randomization and blinding where appropriate.
- Minimum group sizes justified by variance estimates or prior data (even if final power analysis is Stage 2/3 work).
- Adequate completion of planned time points to assess trends in VLCFA and function.

7 Workstream 3: CMC and Analytics Execution Plan

7.1 LNP Batches

For each LNP batch:

- Assign a unique batch ID.
- Measure and record:
  - Particle size and PDI (e.g. by DLS).
  - Encapsulation efficiency and mRNA loading.
  - RNA integrity (e.g. RIN-like score).
  - Endotoxin levels.
  - Osmolality and pH.
  - In vitro potency (e.g. reporter expression or VLCFA rescue in a standard cell line).
- Perform stability assessments:
  - Potency as a function of storage time.
  - Effect of freeze–thaw cycles.

7.2 EV Batches

For each EV batch:

- Assign a unique batch ID.
- Measure and record:
  - Particle count and size distribution.
  - mRNA copies per fixed number of EV (e.g. per 10^{10} particles).
  - EV markers and purity.
  - Translation competency in a reporter assay.
- Conduct analogous stability checks as for LNP where feasible.

7.3 Data Formatting and Minimal Criteria

Provide the modeling team with a batch registry including:

- Batch ID, date of production, storage conditions.
- CQA values and in vitro potency measurements.
- Flags indicating in-spec, borderline, or out-of-spec status according to predefined thresholds.

8 Workstream 4: Modeling and Integration Execution Plan

8.1 Pre-Execution: Version Lock and IDs

Before data ingestion:

- Confirm the architecture ID (e.g. ARCH-ALD-mRNA-v1.0) and link it to the Stage-1 Architecture Specification.
- Register a formula/model specification ID (FORM-ALD-PD-v1.0).
- Reserve a code ID for the implementation (CODE-ALD-CORE-v0.x).

8.2 Data Ingestion and Mapping

- Define input schemas (column names and types) for: in vitro tables, in vivo tables, and CMC tables.
- Map each column to the corresponding model variable:
  - e.g. "C26_plasma" $\rightarrow C_{26}^{\text{plasma}}(t)$,
  - "ALDP_IF_coloc" $\rightarrow S_{\text{target}}$ components,
  - "ALT" $\rightarrow \text{ALT}(t)$ in Block D, etc.
- Document any transformations (e.g. unit conversions, log transforms) used for modeling.

8.3 Scenario Pack v1

Using the architecture, define:

- A baseline scenario:
  - representative batch quality,
  - reference regimen (e.g. one LNP regimen + one EV regimen),
  - typical ALD phenotype (e.g. AMN or early cALD).
- Stress scenarios:
  - low CNS uptake,
  - low batch potency,
  - immune-hot background,
  - FUS-disabled vs FUS-enabled,
  - AMN vs very-early cALD.

Each scenario explicitly specifies parameter ranges and which outputs (VLCFA, Rotarod, DTI, safety) will be used for evaluation.

8.4 Verification Plan (Stage-3 Focus, Stage-1 Definition)

Although full verification will occur in later stages, the Study Execution Guide must already define:

- Which unit tests will check that Block A, B, C, D, and E operate as described in the architecture.
- Which acceptance tests will be used to confirm that the code reproduces simple, manually-computable scenarios (e.g. no-treatment baseline, constant-dose toy scenario).
- How scenario equivalence will be checked: ensuring that the scenario definitions used in analysis match the definitions in the Study Execution Guide.

9 Minimal Coordination and Timeline

9.1 Sequential Dependencies

- Workstream 3 (CMC) should generate at least one in-spec batch per platform before large-scale in vitro and in vivo experiments.
- Workstream 1 (in vitro) can start once initial batches are available, and preliminary ALDP targeting and VLCFA data can be used early to constrain Block B and in vitro aspects of Block C.
- Workstream 2 (in vivo) should begin after basic in vitro feasibility is established, but does not need to wait for full in vitro completion.
- Modeling work can begin in parallel, implementing the architecture and ingesting early data as they become available.

9.2 Checkpoints

At minimum, define the following checkpoints:

- Checkpoint 1: confirmation of at least one in vitro condition with: ALDP peroxisomal localization + significant VLCFA reduction.
- Checkpoint 2: availability of initial in vivo VLCFA and safety data sufficient to run preliminary scenarios.
- Checkpoint 3: completion of Stage-1 modeling runs (baseline + key stress scenarios) to generate a first decision-support report.

10 Documentation and Versioning

10.1 Document Control

For each of the following, maintain a version-controlled record:

- Study Execution Guide (this document).
- Stage-1 Architecture Specification.
- Assumptions Map / Log (MRR).
- Input Spec Sheet (ISS).

- Scenario Pack v1.
- Verification Plan.

10.2 Traceability

All data files and analyses should reference:

- The architecture ID (ARCH-...),
- The formula/model specification ID (FORM-...),
- The code implementation ID (CODE-...),
- The Study Execution Guide version (SEG-v1.x).

This ensures that, at any later point, reviewers can trace decisions and results back to the exact study design, assumptions, and architecture in force at the time.

11 Conclusion

This Study Execution Guide provides a detailed, step-wise description of how to execute the dual-platform mRNA ALD study in a way that is fully consistent with the Stage-1 architecture. It translates a high-level proposal and a technical architecture into practical instructions that a medical research team can follow:

- Every critical experimental component (in vitro, in vivo, CMC) is linked explicitly to model blocks and decision-relevant outputs.
- Data collection and formatting requirements are clear, enabling seamless integration into the model.
- No oversimplifying assumptions are imposed on the biology; the protocol is designed precisely so that the richer model (with separate blocks for delivery, targeting, PD, safety, and CMC) can be informed and challenged by real data.

In subsequent stages, this Guide can be updated as necessary to reflect refinements in the architecture, new empirical insights, and feedback from early runs of the integrated model.