MADE SCIENTIFIC

DEVELOPMENT WORK PACKAGES

Design of Experiments Plans

for the RTX-001 Pivotal Process (CP-2)

Eight plans, one per development area, for the changes proposed between the phase 1/2 process and the pivotal process

Program	RTX-001, autologous IL-10 and MMP-9 mRNA-engineered macrophages for decompensated cirrhosis; pivotal process CP-2
Prepared for	Resolution Therapeutics
Companion to	The Resolution Therapeutics and Made Scientific pivotal process workshop, 28 September 2026, East Norriton, Pennsylvania
Prepared by	Made Scientific, Inc.
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PLAN	DEVELOPMENT AREA	RTX QUESTION	DONOR MATERIAL
D-1	Controlled-Rate Freezer Thermal Mapping and Cassette Configuration	Q2a exemplar; change 8	Surrogate loads; 3 or more paired donors
D-2	Process-Related Impurity (Interleukin) ELISA: Development, Qualification and Clearance	Q2a exemplar; Q2b	Spike and matrix; 3 process runs
D-3	Post-Electroporation Recovery Window and mRNA Dose	Q1 IL-10 secretion; change 6	3 or more donors, then 3 or more
D-4	Day 4 versus Day 5 Maturation Plateau: Phenotype and Potency Time Course	Change 4; Q4	3 or more donors, D3 to D6
D-5	MACS1000 Bag Culture, Prodigy Formulation-Bar Seeding and Incubator Fit	Changes 2 and 3; Q1 facility fit	Measurements; 2 to 3 donors
D-6	Kytopen Flowfect Operating Range and Comparability to the Nucleofector LV	Change 5; Q4	2 screening; 3 or more paired
D-7	Ares X20 Fill Uniformity, QC-Vial Representativeness and Pre-Freeze Hold Times	Changes 1 and 7; Q2b	Surrogate; 3 or more donors
D-8	CP-1 versus CP-2 Comparability Statistics and the Proposed Study 5	Q4; Q3; Study 5	4 to 5 paired runs and up

How to read these plans. Each page states the objective, the published and shared record that shapes the design, the factors and levels, the responses with acceptance criteria, the design and analysis, and the decision rule that closes the study. The factors, levels, sample sizes, durations, acceptance targets and decision rules are Made Scientific's proposed study designs: they are confirmed in a joint protocol with Resolution Therapeutics before the first run and are not acceptance criteria for any gate. Durations marked as planning figures are estimates to be confirmed against equipment, cassette, material and assay readiness. Sample sizes are counted in healthy-donor leukapheresis units; a unit enters a split design only when the CD14-positive yield covers every arm, and when material is short the arms are prioritized in the order the plan states. Where a figure is quoted from a Resolution Therapeutics document it is quoted as shared and remains subject to Resolution's final data review before process lock in December 2026. Bracketed numbers are citations to the reference list on the final pages.

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Work Package D-1: Controlled-Rate Freezer Thermal Mapping and Cassette Configuration

Development work package (Q2a exemplar); proposed route WO 4.0 / WO 5.0 · Surrogate mapping from Q4 2026; matched-product arm once recipe and cassette are confirmed; 8 to 12 weeks after equipment, cassette, material and assay readiness

Donor material. Surrogate loads for mapping; at least 3 paired healthy-donor leukapheresis units for the matched-product arm

OBJECTIVE

Show that the selected CryoMed LN2 controlled-rate freezer and the proposed metal cassette, at the intended minimum, nominal and maximum loads, reproduce the RTX001 freezing profile across the usable chamber, and that product frozen in the proposed configuration is comparable post-thaw to product frozen in the reference configuration, in both the 10 mL dose bag and the 1 mL QC vial.

WHAT THE RECORD SAYS

- RTx has not started temperature mapping or equipment suitability for the pivotal freezer, the cassette is changing, and no controlled-rate freezer is owned in-house; the development laboratory uses an electric ViaFreeze and EMERALD manufacturing used an LN2-driven freezer, so CryoMed makes a three-way matrix. [19, 21]
- Drug product is 10 mL in CryoMACS 50 bags at 1 x 10^7 cells/mL, 4 to 9 bags per batch, plus about 20 x 1 mL QC vials filled after the bags, so vials see a different fill volume, surface-to-volume ratio and CS10 exposure than the bags. [20, 21]
- Frozen monocytes failed in the MATCH GMP development (macrophage viability 45.5% from thawed monocytes vs 75.2% from fresh; CD206 and CD163 lost, CCR2 gained), whereas cryopreserved differentiated, IL-4/IL-13-conditioned human macrophages recovered 87% viable post-thaw vs 95% fresh with markers unchanged and fresh equivalent to thawed in vivo: the biology allows freezing the differentiated product, and the profile, container geometry and thaw are what this plan bridges. [1, 5]
- Optimal DMSO for monocyte cryopreservation was 7.5 to 10%; macromolecular cryoprotectants with ice nucleators doubled post-thaw monocyte recovery, so controlled nucleation is the phase-2 lever if cassette geometry proves sensitive. [10, 11]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Freezer platform	Reference: current Planer configuration (RTx to confirm) and the EMERALD LN2 profile; proposed: MADE CryoMed LN2 CRF; ViaFreeze data from the RTx laboratory as the third reference set	The platform is the change RTx asked about; the reference must be the one the EMERALD data sit on.
Cassette	Current cassette vs proposed metal cassette	Cassette mass and contact area change the product cooling curve independently of the freezer.
Load	Minimum, nominal and maximum intended loads (RTx currently shows 4 to 9 cryobags per batch; confirm the loading strategy and whether vials share the run)	Chamber uniformity degrades with load; the operating range must be mapped, not assumed.
Container	10 mL fill in a CryoMACS 50 bag; 1 mL QC vial	Vial-to-bag representativeness is a Q2b question and is answered in the same runs.
Probe position	Eight product-simulating and air probes: upper, center, lower, edge, and door-adjacent zones	Finds the warm and cold locations that define the load diagram.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Chamber and product-simulating temperature profile	Calibrated multichannel logger; probes in surrogate bags and vials	Within recipe tolerance at every mapped position on 3 repeat runs per load; no unexplained outliers, alarms or data gaps
Nucleation and end-point temperatures, cooling rate through the phase change	In-bag and in-vial probes	Reference and proposed configurations agree within a pre-set margin; bag and vial curves reported side by side
Post-thaw viability and viable recovery	NC-202 at 0 h and at the in-use hold	Paired differences within EMERALD-derived margins; both configurations meet the release criterion
Macrophage identity; transgene expression	Flow (BD FACSLyric); RT-qPCR or dPCR at 0 h	No meaningful adverse shift vs the reference configuration
IL-10 and MMP-9 secretion; MMP-9 activity	ELLA; FRET, post-thaw culture	Paired differences within EMERALD-derived margins
Container and cassette condition	Visual inspection, leak and damage check, handling observations	No leakage or damage at minimum and maximum loads

DESIGN AND EXECUTION

- Stage 1, readiness review: IQ/OQ, calibration, alarms and data capture on the selected CryoMed; recipe, cryobag, cassette, fill volume, batch load, CQAs and acceptance criteria confirmed in writing before any run.
- Stage 2, surrogate thermal mapping: product-simulating loads at minimum, nominal and maximum, 3 repeat runs each (9 runs), eight probes per run; output is the warm and cold locations, repeatability and a provisional load diagram. Needs no cells; can start before process lock.
- Stage 3, cassette engineering assessment: fit, orientation, bag protection, labeling and overwrap, cryogenic handling, storage and thaw interface, supplier readiness.
- Stage 4, matched-product comparability: one formulated drug substance per donor split into bags and vials and frozen side by side in the reference and proposed configurations; preferred design is the 2 x 2 of freezer x cassette so the two effects are separated; fallback is complete current vs complete proposed configuration, which cannot assign the effect to one component. At least 3 paired healthy-donor runs; Biofreeze and BioGenics 2101 as backups.
- Stage 5, confirmation and implementation: final load diagram, recipe, operating range, cassette orientation, handling instructions and change-control inputs.

DECISION RULE AND DELIVERABLE

The proposed configuration is adopted when the mapped profile is within tolerance at every position across the load range on repeat runs and the paired post-thaw differences for viability, recovery, identity, transgene expression and IL-10/MMP-9 sit inside the pre-declared EMERALD-derived margins in both bag and

vial on at least 3 donors. If the vial diverges from the bag, mini-bags become the QC container for viability and potency. Mapping alone is not evidence that the change preserves product quality.

Deliverable. Mapping and product-verification protocols; raw temperature files and run records; comparability report; selected freezer and cassette configuration, load diagram, recipe, operating range and manufacturing instructions; change-control recommendation and BOM update.

References [1, 5, 10, 11, 19, 20, 21, 31, 35], see the reference list at the end of this document.

Work Package D-2: Process-Related Impurity (Interleukin) ELISA: Development, Qualification and Clearance

Development work package (Q2a exemplar); proposed route WO 3.0 / WO 6.0 or a new work order if development is needed · From Q1 2027 in parallel with the analytical transfer; MADE planning figure 14 to 18 weeks from kit or cartridge receipt to the AD qualification report; clearance runs ride on the pilot runs

Donor material. Cell-free spike and matrix work first; banked clearance samples from RTx, then 3 process runs (pilot or engineering) for the clearance study

OBJECTIVE

Develop and qualify an immunoassay that quantifies a residual ancillary-material interleukin from the culture or recovery medium in RTX001 drug substance and drug product, with a limit of quantitation well below a safety-based limit, and show consistent clearance across medium, Rotea, drug substance and drug product on three runs.

WHAT THE RECORD SAYS

- RTx lists "develop and qualify an ELISA based method to quantify process related impurities (for example an interleukin in growth media)" as an ongoing development item and a potential CDMO development project; the analyte, the medium level and any RTx data are not yet shared. [19]
- The MATCH process used serum-free TexMACS with 100 ng/mL M-CSF; the RTx patent adds an overnight conditioning medium containing 20 ng/mL IL-4 and 20 ng/mL IL-13 before cryopreservation. M-CSF is a cytokine rather than an interleukin, so the interleukins in a conditioning or recovery medium are the most likely analytes; RTx confirms which one and whether the conditioning step is in CP-2. [1, 4]
- The product itself secretes IL-10 (the payload) and constitutively high MMP-9, so the impurity assay must target a molecule the macrophage does not secrete at a comparable level, or cell-free spike runs must isolate carry-over from secretion. [1, 4]
- Phase-appropriate qualification follows ICH Q2(R2); ancillary-material control follows USP <1043> and specification setting ICH Q6B; consistent clearance on multiple runs is the usual basis for moving an impurity test from release to characterization. [24, 25]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Analyte and limit	The interleukin RTx names; medium level and a safety-based limit agreed at knowledge transfer	Sets the required LOQ and whether a plate ELISA or ELLA cartridge exists.
Platform	ProteinSimple ELLA cartridge if one exists for the analyte; otherwise plate ELISA read on the VarioSkan LUX with the 96-well washer	RTx did not accept a manual ELISA in place of ELLA for the payload assays; for an impurity assay either platform is acceptable if qualified.
Matrix	Culture medium; post-Rotea buffer; DS formulation buffer; CS10 drug product	Dilution, spike recovery and parallelism differ by matrix; the DP matrix contains DMSO.
Minimum required dilution and spike level	MRD screen; spikes at 3 levels spanning the expected range and the limit	Accuracy and range are demonstrated where the decision is made.
Carry-over vs secretion	Cell-free spiked buffer vs cell-containing samples; mock-EP and non-engineered cells as baselines	Isolates process carry-over from anything the cells make.
Analyst and day	2 analysts x 3 days	Intermediate precision per ICH Q2(R2).

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Specificity	Blank, CS10, related cytokines	No interference
Accuracy	Spike recovery at 3 levels in each matrix	80 to 120% recovery
Precision	Repeatability and intermediate precision	CV at or below 15%; at or below 20% at the LOQ
Range and linearity	Back-calculated standards and diluted samples	Within plus or minus 20%; parallelism demonstrated
LOQ	Lowest standard meeting accuracy and precision	Below the proposed limit
Robustness and sample stability	Incubation time, temperature, freeze-thaw, hold at 2 to 8 C	Within the accuracy limits
Clearance	Medium, post-Rotea, DS, DP on 3 runs	Consistent log reduction; DP below the limit on all 3 runs

DESIGN AND EXECUTION

- Stage-gated with RTx agreement at each gate: (1) knowledge transfer of analyte, medium level and any RTx data; (2) gap assessment of kit or cartridge, matrix and standards; (3) BOM and acquisition; (4) feasibility on MRD, spike recovery and parallelism with a go/no-go; (5) development in the DS and CS10 matrices; (6) AD qualification to an ICH Q2(R2) protocol agreed with RTx and an AD report; (7) clearance study across medium, Rotea, DS and DP on 3 runs; (8) QC transfer with protocol, training, GMP SOP and transfer report.
- Resources: an AD scientist with AD lead oversight, a QC analyst at transfer, QA review; ELLA and VarioSkan LUX; from RTx, media lots, the reference standard and banked clearance samples.
- All data shared with RTx weekly; next-stage work agreed before it starts.

DECISION RULE AND DELIVERABLE

The method is declared qualified when every ICH Q2(R2) target above is met in the DS and DP matrices and the AD report is approved by RTx. It enters release testing at the engineering runs; consistent clearance on 3 runs, with DP below the limit on all three, supports a proposal to move the assay from release to characterization before the pivotal.

Deliverable. Development plan; qualification protocol and AD qualification report; clearance study report; QC transfer protocol, GMP SOP and forms, QC transfer report; method status entry for the analytical transfer plan.

References [1, 4, 19, 24, 25, 35], see the reference list at the end of this document.

Work Package D-3: Post-Electroporation Recovery Window and mRNA Dose

Development work package; biology may run at RTx or MADE, the GMP time-and-motion study runs at MADE; proposed route WO 4.0 · Before process lock where possible (screening on RTx development-scale material), confirmation at MADE during the training and pilot runs; MADE planning figure 10 to 12 weeks for screening plus confirmation

Donor material. At least 3 healthy-donor leukapheresis units for screening (matched split-donor); at least 3 further donors for confirmation with GMP processing-time excursions

OBJECTIVE

Define the shortest post-electroporation recovery hold, and the mRNA dose, that maintain pre-defined post-thaw quality and functional criteria for RTX001, and define the four-hour window (T0, final Flowfect step complete, to T4, CRF cycle initiated) that manufacturing must execute.

WHAT THE RECORD SAYS

- RTx has shortened the recovery after electroporation from overnight to about 4 h (target window 2 to 6 h) so that mRNA-driven IL-10 and MMP-9 expression is used before the mRNA decays; RTx observed variability in IL-10 secretion with a risk of OOS at the lower limit and found no single cause, with stress, electroporation parameters, hold times and mRNA as candidate factors. RTx calls this one of the riskiest changes; small-scale work is complete and at-scale characterization is ongoing on healthy donors. [19, 21]
- The RTx patent quantifies expression 16 to 24 h after transfection on differentiated macrophages, at 8 micrograms mRNA per 10^6 cells for the IL-10-MMP9 construct and 50 to 200 x 10^6 cells/mL at electroporation; the patent also claims an overnight IL-4/IL-13 plus M-CSF conditioning immediately before cryopreservation. The new window is therefore shorter than the rest at which RTx defined its potency targets, and shorter than its own cryoresilience step. [4]
- Membranes stay leaky for a short time after electroporation, Annexin V and DRAQ7 rise transiently, mitochondrial membrane potential falls and reactive oxygen species rise within hours, and HSP70 rises after 2 to 6 h; viability should be read after recovery, and the post-EP samples must be compared at matched recovery times. [35]
- Innate activation after mRNA transfection of primary monocytes and macrophages depends on carrier and nucleotide modification, so mRNA dose is a lever on both expression and activation; a higher input dose may not give a proportional increase in secreted IL-10. [7, 8]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Recovery hold (T0 to freeze)	2 h, 4 h, 6 h, overnight	Brackets the RTx target window and includes the CP-1 overnight rest as the reference.
mRNA dose	Low, current reference, high (RTx defines the actual levels around its reference)	Tests whether dose moves median IL-10 and its spread without an unacceptable viability cost.
Fixed	Same donor split, same Flowfect configuration and settings, same cell concentration, same formulation and fill process, same freezer configuration	Isolates the two questions from every other CP-2 change.
Readout time	Fresh (pre-freeze) at end of hold; post-thaw at 0 h and after post-thaw culture	The release readout is post-thaw; the fresh readout explains it.
Processing-time excursion (confirmation only)	Realistic Day 4 queue times at each hand-off	Confirms the selected combination survives the site workflow.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Viability and viable recovery	NC-202; Annexin V / DRAQ7 by flow	Post-thaw viability meets the release criterion; no downward trend with shorter hold
Intracellular transgene mRNA	RT-qPCR or dPCR	Reported per condition; used to explain secretion, not as a criterion
Intracellular IL-10	IL-10 capture assay by flow	Reported per condition
Secreted IL-10 and MMP-9, normalized to viable cells	ELLA on post-thaw culture supernatant	Meets the RTx acceptance limit at every donor; median and spread compared with the overnight reference
MMP-9 activity	FRET	At least the reference condition
Phenotype and stress panel	Flow: CD14, HLA-DR, CD163, CD206, CD80/CD86; JC-1 or TMRM, CellROX, HSP70	No activation signal (CD80/CD86 up) at the selected dose; stress markers recovered before freeze

DESIGN AND EXECUTION

- Study 1, recovery hold: matched donor material electroporated once and split into the four hold conditions with mRNA dose, cell concentration and Flowfect settings constant; fresh and post-thaw readouts as above; at least 3 donors. Decision: the shortest hold that meets the quality and functional criteria on every donor.
- Study 2, mRNA dose: at the selected hold, three doses on matched donor material, fresh and post-thaw; secreted IL-10 normalized to viable cells is the primary response. Decision: the dose that improves IL-10 output without an unacceptable cell-quality cost.
- Confirmation: the selected hold and dose across at least 3 further donors with realistic GMP processing-time excursions and the site workflow, run as the MADE time-and-motion study so the batch record windows (T0 to T4) are set on data.
- Analysis on within-donor paired differences against the overnight reference; margins pre-declared from the EMERALD CQA spread; donors as blocks.

DECISION RULE AND DELIVERABLE

The recovery hold and mRNA dose are locked for the pivotal process when the selected combination meets the post-thaw viability and IL-10/MMP-9 criteria on every confirmation donor and the time-and-motion study shows the T0-to-T4 window is executable within the staffing model with defined holds at each hand-off. If 2 h fails and 4 h passes, 4 h is the minimum, not the target; if only overnight passes, the change is not adopted and RTx decides on data.

Deliverable. Study 1 and Study 2 reports; confirmation and time-and-motion report; the agreed four-hour definition with T0 to T4 hold windows for the batch record; recommendation on hold and dose for process lock.

References [1, 4, 7, 8, 19, 21, 34, 35], see the reference list at the end of this document.

Work Package D-4: Day 4 versus Day 5 Maturation Plateau: Phenotype and Potency Time Course

Development work package; RTx-led with a MADE column, or executed at MADE on request; proposed route WO 4.0 · Before process lock in December 2026 if RTx material allows; otherwise on the first MADE training runs; MADE planning figure 8 to 10 weeks

Donor material. At least 3 healthy-donor leukapheresis units, each run as a D3 to D6 time course (D3, D4, D5 and D6 where material allows)

OBJECTIVE

Show whether the differentiation markers, yield and potency of monocyte-derived macrophages have reached a plateau by Day 4, so that the 5-to-4-day compression can be justified as a change that does not alter the product, and identify which markers move if it has not.

WHAT THE RECORD SAYS

- RTx gives "plateau in differentiation markers; reduced facility time" as the rationale for the 4-day conversion, with ongoing CQA and molecular-pathway (omics) data collection; RTx itself classes a shorter maturation as a major change. The MADE question is whether the plateau starts at D4 or D5, which needs a time course, not two time points. [19, 21]
- The RTx patent claims both a "day5" (3 to 5 or 4 to 5 day) and a "day7" differentiation method with TexMACS plus 100 ng/mL M-CSF, a direct precedent for the compression; the MATCH GMP process used 7 days with 50% feeds on days 2 and 4, and the CP-2 narrative does not state the feed schedule for a 4-day culture. [1, 4]
- The MHRA-accepted MATCH release identity was CD45+/CD14+ with 25F9 and CD206 geometric MFI at least 5-fold the day-0 monocyte value and viability above 80%; CD14 selection was highly reproducible across donations but CD14-to-macrophage conversion efficiency was highly variable, so the plateau must be shown per donor, not on a pooled mean. [1, 2]
- Expected direction at D4 vs D5: CD163, CD206, CD16 and 25F9 lower; CCR2-positive unconverted monocytes and CD3/CD19/CD56 carry-over higher; CD45, CD11b, CD64, CD14 and HLA-DR comparable. IL-10 from the payload lowers HLA-DR and raises CD163, so post-electroporation samples are compared at matched recovery time. [35]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Culture day at harvest	D3, D4, D5 (and D6 where material allows)	A plateau is a slope, so at least three points are needed around the change.
Donor	At least 3 healthy donors as blocks	Conversion variability is donor-driven; the plateau is shown per donor.
Post-harvest arm	Non-engineered vs electroporated at the CP-2 dose, read at the same recovery time	Potency of the engineered product is the attribute the change must not move.
Fixed	Same seeding density, medium, M-CSF, bag format and feed schedule (RTx to state the CP-2 feed)	Isolates culture duration from the other CP-2 changes.
Bag sampling	Per-bag count and viability at harvest	Gives the yield trajectory and the bag-to-bag consistency figure for D-5 at no extra donor cost.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Maturation markers	Flow: 25F9, CD206, CD163, CD16 (MFI fold over day-0 monocytes)	D4 vs D5 paired difference inside the pre-declared margin; 25F9 and CD206 fold-change at D4 meets the identity criterion on every donor
Purity markers	Flow: CCR2, CD3/CD19/CD56, CD14, HLA-DR, CD45/CD11b/CD64	Residual monocytes and lymphoid carry-over at D4 not meaningfully higher than D5
Yield and viability	NC-202; TruCount by flow	Macrophages per input CD14+ at D4 vs D5; viability above the release criterion
Potency of the engineered arm	IL-10 and MMP-9 secretion (ELLA), MMP-9 activity (FRET), transfection efficiency	Paired D4 vs D5 differences inside EMERALD-derived margins
Function	pHrodo Red Zymosan phagocytosis kinetics with a non-engineered contrast	Reported; kinetic readout, not a single end-point uptake
Molecular	RTx omics panel if available	Reported; supports the totality-of-changes argument

DESIGN AND EXECUTION

- Each donor is seeded as one culture and harvested in parallel aliquots (bags) on D3, D4, D5 and D6, so the time course is within-donor; each harvest is split into a non-engineered arm and an electroporated arm processed through the CP-2 recovery and freeze.
- Readouts pre-freeze and post-thaw at matched recovery time; analysis as within-donor differences (D4 minus D5) with 90% confidence intervals against margins declared in the joint protocol; a slope across D3 to D6 is fitted per marker to show where the plateau begins.
- The same runs supply the D-5 per-bag consistency data and can supply retained samples for the D-8 comparability battery.

DECISION RULE AND DELIVERABLE

The 4-day conversion is supported for process lock when, on every donor, the D4-to-D5 paired differences for identity, purity, yield and engineered-arm potency sit inside the pre-declared margins and the D3-to-D6 slope has flattened by D4. If the maturation markers are still rising at D4 on any donor, the report states which markers, by how much, and whether a D4 harvest with a defined identity criterion or a return to D5 is the lower-risk path; RTx decides on data.

Deliverable. Time-course report with per-donor marker trajectories, yield and potency tables, the identity-criterion assessment at D4, and the recommended culture duration and feed schedule for the pivotal process.

References [1, 2, 3, 4, 19, 21, 22, 35], see the reference list at the end of this document.

Work Package D-5: MACS1000 Bag Culture, Prodigy Formulation-Bar Seeding and Incubator Fit

Site feasibility review with a confirmation study if needed; proposed route WO 2.0 / WO 4.0 · Shelf-fit measurement at the 28 September 2026 workshop; seeding and harvest-recovery arms on the first MADE training runs; MADE planning figure 6 to 8 weeks

Donor material. Engineering measurements need no cells; 2 to 3 healthy-donor leukapheresis units for the per-bag consistency and harvest-recovery arm (shared with D-4 where possible)

OBJECTIVE

Confirm that up to 10 MACS1000 differentiation bags seeded from the CliniMACS Prodigy through the formulation bar fit the incubator configuration at working volume, that bag position does not change yield or phenotype, and that harvest recovery from the larger bag is at least that of the MACS500 format, so the incubator count per batch and the harvest time can be set on data.

WHAT THE RECORD SAYS

- CP-2 seeds monocytes into up to 10 MACS1000 bags through the Prodigy formulation bar (about 1 h, to be confirmed) in place of up to 18 to 20 MACS500 bags filled by CellConnect or Cue; a custom application from Miltenyi is required, the bag number per run is limited and filtered air is required. RTx reports no CQA issues so far with the larger bag and is testing more robust harvest methods before the Rotea; the 7-hour harvest-and-wash figure was MACS500 time and is expected to fall with MACS1000. [19, 20]
- Incubator fit is unmeasured: one estimate assumes 6 bags per shelf and 3 shelves (one incubator per batch), another 4 bags per shelf and 2 shelves per unit (1.5 incubators per batch). The consumable itself could not be verified in any public source; the MATCH consumable was the MACS GMP Differentiation Bag seeded at 2 x 10^6 monocytes per cm2 and per mL in TexMACS plus 100 ng/mL M-CSF, and the RTx patent seeds at the same density. [1, 4, 35]
- In MATCH, removing serum made macrophages less adherent to the bag and improved harvest recovery; medium depth, adherence and harvest method interact, so bag size and harvest method are studied together. [1]
- A closed CliniMACS Prodigy process for monocyte-derived dendritic cells is published and is the closest closed-system myeloid analogue for the formulation-bar seeding step. [18]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Bag format	MACS1000 (CP-2) vs MACS500 (reference), same seeding density	The change under assessment; the reference gives the harvest-recovery baseline.
Bags per shelf and shelves per unit	Measured at working volume with port clearance and flatness, 4 to 6 bags per shelf, 2 to 3 shelves	Sets incubators per batch (1 vs 1.5) for the capacity model.
Shelf position	Upper, middle, lower; front vs back	Tests for a position effect on gas exchange and temperature.
Seeding route	Prodigy formulation bar per the Miltenyi custom application vs manual reference	Fill accuracy per bag and closed-fill time are the bar-specific outputs.
Harvest method	RTx current method vs the more robust pre-Rotea method RTx is testing	Harvest recovery is the yield-critical response.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Incubator fit	Physical measurement at working volume; door closure; gas and humidity recovery after loading	Bags per shelf and shelves per batch recorded; incubator count per batch fixed
Fill accuracy per bag	Gravimetric, formulation bar vs target	Within the tolerance in the custom application; closed-fill time per 10 bags recorded
Per-bag count and viability at harvest	NC-202	Bag-to-bag CV reported; no shelf-position effect beyond the pre-declared margin
Per-bag identity	Flow: 25F9, CD206, CD163, CD14	No position effect; meets the identity criterion in every bag
Harvest recovery	Cells recovered / cells seeded, and cells recovered / cells in bag before harvest	MACS1000 at least equal to MACS500 on matched donor material
Harvest and pooling time	Time-and-motion on 10 bags with 2 operators	Recorded; feeds the Day 4 schedule

DESIGN AND EXECUTION

- Engineering arm (no cells): the MACS1000 vs MACS500 shelf-fit comparison at working volume in the incubator model planned for the suite, repeated on 2 units; formulation-bar fill accuracy with surrogate fluid once the custom application is available.
- Biological arm: 2 to 3 donors, each seeded into both bag formats at the same density (split-donor), bags distributed across shelf positions by a randomized plan; harvest on D4 by the agreed method with per-bag sampling; the electroporated pool follows the CP-2 process so the fill and freeze data can serve D-7.
- Analysis on within-donor differences (MACS1000 minus MACS500) and a position-effect model with bag as the unit; donors as blocks.

DECISION RULE AND DELIVERABLE

The MACS1000 format and formulation-bar seeding are confirmed for the site when the bag count per incubator is measured and recorded, per-bag count, viability and identity show no position effect beyond the margin, harvest recovery is at least the MACS500 reference on every donor, and the closed-fill time is inside the Day 0 window. If a position effect is found, the load diagram restricts positions rather than the format being rejected.

Deliverable. Incubator load diagram with bags per shelf and incubators per batch; formulation-bar fill and time record; per-bag consistency and harvest-recovery report; inputs to the capacity model and the Day 0 and Day 4 batch-record steps.

References [1, 4, 17, 18, 19, 20, 35], see the reference list at the end of this document.

Work Package D-6: Kytopen Flowfect Operating Range and Comparability to the Nucleofector LV

Development work package; screening at RTx or with Kytopen before the MADE unit is installed, confirmation at MADE; proposed route WO 4.0 · Operating-range screening before process lock (RTx-led, MADE column); site comparability on the first MADE runs after installation; planning figure 8 to 10 weeks

Donor material. At least 3 healthy-donor leukapheresis units for the split-donor platform comparison; screening on surplus macrophages from D-4 or D-5 runs

OBJECTIVE

Define the Flowfect operating range (energy, cell concentration, mRNA dose, flow condition) inside which transfection efficiency, viability and IL-10/MMP-9 expression meet the RTx criteria, and show on matched split-donor material that Flowfect-transfected RTX001 is comparable to Nucleofector LV-transfected RTX001, so the device change carries into the CP-1 to CP-2 comparability argument.

WHAT THE RECORD SAYS

- RTx intends to progress with the Kytopen Flowfect (to be confirmed) for faster throughput, qualification and customization; the concept is demonstrated and optimization is pending. Continuous flow replaces the Lonza 4D-Nucleofector LV; the electroporation itself is about 5 minutes with a 1.75 h transfection block. [19, 20]
- The RTx patent names the Lonza Nucleofector (pulse code CM-137), the Nucleofector LV and the CliniMACS Electroporator, at 50 to 100 x 10^6 cells/mL (200 x 10^6/mL for large-scale fluidics) and 8 micrograms mRNA per 10^6 cells for the IL-10-MMP9 construct, read at 16 to 24 h; Kytopen is not in the patent, so the device change is itself a comparability event on the potency-critical step. [4]
- Kytopen reported over 90% delivery efficiency and over 90% viability in monocyte-derived macrophages, with the protocol moved to the Flowfect Tx GMP platform in under three weeks; peer-reviewed benchmarks for mRNA electroporation of primary myeloid cells range from 45 to 72% to platform claims of 80 to 99%, mRNA outperforms plasmid (89% vs 40%), and a continuous-flow platform for cell therapy manufacturing is published. [6, 13, 14, 15, 16]
- Innate activation after mRNA transfection depends on carrier and nucleotide modification; the energy-versus-viability trade-off is the usual axis of an electroporation design. [7]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Energy setting	RTx program center point plus or minus the range Kytopen recommends (3 levels)	The broad parameter space is the stated MADE concern; the range must be bounded.
Cell concentration	50, 100 and 200 x 10^6 cells/mL (patent levels)	Concentration drives cartridge load, mRNA consumption and run time.
mRNA dose	Reference and one lower level (coordinated with D-3)	Dose interacts with energy on viability and activation.
Flow condition and cartridge	Per the Flowfect configuration RTx selects (Discover for screening, Tx for GMP); vendor center-point flow rate	Recorded, not varied beyond the vendor envelope.
Platform (comparability arm)	Nucleofector LV (CP-1 reference) vs Flowfect at the selected operating point, split donor	The bridge the comparability package needs.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Transfection efficiency	RT-qPCR or dPCR transgene copies; intracellular IL-10 by flow	Meets the RTx criterion across the operating range; Flowfect vs LV paired difference inside the margin
Viability and viable recovery	NC-202 immediately post-EP and after the CP-2 recovery hold	Above the release criterion after recovery; no energy level with a viability cliff inside the range
IL-10 and MMP-9 secretion	ELLA at 16 to 24 h (patent reference) and at the CP-2 recovery time	Meets the acceptance limit; spread across donors reported (does Flowfect reduce the IL-10 variability seen with the LV?)
MMP-9 activity	FRET	At least 1.5 x untransfected (patent reference)
Innate activation	CD80/CD86 and HLA-DR by flow; TNF-alpha, IL-6, IL-12 by MSD or ELLA	No activation signal beyond the LV reference
Run time, closure and hold-up	Observation; volume recovered	Closed bag to bag; cartridge cell loss recorded

DESIGN AND EXECUTION

- Stage 1, operating-range screen (RTx or Kytopen laboratory, MADE column): a 3 x 3 grid of energy x cell concentration at the reference dose on surplus D4 macrophages from 2 donors, randomized run order; ranks the settings and bounds the range.
- Stage 2, split-donor platform comparability at MADE once the Flowfect is installed and qualified: each donor harvest is divided so the LV reference and the Flowfect operating point run on the same cells the same day, followed by the CP-2 recovery, formulation and freeze; at least 3 donors; readouts pre-freeze and post-thaw.
- Analysis on within-donor paired differences (Flowfect minus LV) with 90% confidence intervals against margins declared in the joint protocol and derived from the EMERALD CQA spread; the operating range is reported as the region of the grid inside which every criterion is met on both donors.
- Precondition: RTx finalizes the program and operating range with Kytopen before transfer; MADE confirms facility fit, training, disposables, integration and qualification.

DECISION RULE AND DELIVERABLE

The Flowfect operating point and range are accepted for process lock when every criterion is met inside the range on both screening donors and the paired Flowfect-vs-LV differences on at least 3 donors sit inside the pre-declared margins pre-freeze and post-thaw. If the range is narrower than the run-to-run variation of cell concentration at Day 4, the cell-count IPC before transfection becomes a gating IPC with an action limit. If comparability fails, the LV remains the CP-2 device and the throughput argument is re-costed.

Deliverable. Operating-range report with the bounded grid; platform comparability report on split-donor material; recommended program and IPC limits for transfection set-up; input to the comparability package (D-8).

References [4, 6, 7, 13, 14, 15, 16, 19, 20, 22, 35], see the reference list at the end of this document.

Work Package D-7: Ares X20 Fill Uniformity, QC-Vial Representativeness and Pre-Freeze Hold Times

Development work package; proposed route WO 4.0 / WO 5.0, feeding the QC fill and retains philosophy (Q2b) · Water and surrogate runs as soon as the MADE Ares X20 is installed; cell runs on the training and pilot runs, co-scheduled with D-1; MADE planning figure 6 to 8 weeks after installation

Donor material. Surrogate fluid and cell-free formulation for the engineering runs; at least 3 healthy-donor leukapheresis units for the bag-versus-vial and hold-time arms (shared with D-1)

OBJECTIVE

Show that the Ares X20 fills 4 to 20 dose bags and about 20 QC vials from one formulated drug product with uniform dose from first to last container, that the 1 mL QC vial is representative of the 10 mL dose bag post-thaw, and how long the formulated product can be held in CS10 before the freeze starts without a change in post-thaw quality.

WHAT THE RECORD SAYS

- CP-2 formulates drug substance and then drug product (CryoStor CS10 addition) on the Xiogenix Ares X20 and fills bags 1 to 20 followed by the QC vials; drug product is 10 mL in a CryoMACS 50 bag at 1 x 10^7 cells/mL, 4 to 9 bags per batch, with up to about 20 x 1 mL QC vials. RTx has completed water and buffer runs and is testing cells; the 2.5 h formulation time is "TBC for Ares"; the narrative asks whether visual inspection can run concurrently with the fill. [20, 21]
- Dose is set on drug substance from the TruCount result, which gates the fill; the release and characterization samples are taken at DS/FDP formulation; the QC vials are filled after the bags and therefore see a longer CS10 exposure before freezing and a different surface-to-volume ratio, so freeze and thaw kinetics differ. [20, 35]
- RTx asks how MADE has filled QC and stability units alongside the dose container and how it showed they were representative, and what the philosophy on retains and stability sampling is when there is essentially nothing to spare on an autologous product. [19]
- An illustrative allocation of about 20 vials per batch is post-thaw release tests 6, tier 3 information-only assays 4, retest reserve 4, retain 4 and investigation spare 2, with the final split set from validated sample volumes and stability pulls taken only from engineering and PPQ lots. [31, 35]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Number of containers	4, 9 and 20 bag positions plus 20 vials	Brackets the RTx range and the Ares capacity; uniformity is tested at the extremes.
Fill order	Bags then vials (current) vs vials interleaved or first	Changes the CS10 exposure of the vial relative to the bag.
Pre-freeze hold in CS10	0, 30, 60 and 90 min from CS10 addition to CRF start (MADE planning levels; RTx to confirm the range)	Sets the DP-before-freeze hold window in the batch record.
Container	10 mL fill in CryoMACS 50 bag vs 1 mL vial (and a mini-bag if vials diverge)	The representativeness question.
Concurrent visual inspection	Sequential vs concurrent with fill	Time-and-motion for the T3-to-T4 leg.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Fill volume and weight per container	Gravimetric, every container	First-to-last difference and CV inside the tolerance agreed with RTx; no trend with position
Viable cell dose per container	TruCount by flow on first, middle and last bag and on vials	Inside the dose tolerance; bag and vial concentrations agree
Cell recovery through the Ares	Cells in / cells filled; hold-up volume	Recorded; loss inside the tolerance
Post-thaw viability, viable recovery, identity, IL-10/MMP-9, MMP-9 activity, transgene expression	NC-202; flow; ELLA; FRET; RT-qPCR or dPCR	Bag-vs-vial paired differences inside the pre-declared margin on at least 3 runs; no trend with pre-freeze hold up to the selected window
Closure and aseptic integration	Observation; APS coverage review	Closed from DS bag to sealed container; open steps identified for the aseptic process simulation
Fill and inspection time	Time-and-motion, 2 operators	Recorded; feeds the four-hour window (D-3) and the Day 4 schedule

DESIGN AND EXECUTION

- Engineering arm: water and surrogate runs at 4, 9 and 20 positions plus vials to set volume accuracy and hold-up; then cell-free formulation (buffer plus CS10) to confirm the CS10 mixing and the fill sequence.
- Cell arm: for each of at least 3 donors, one formulated drug product filled into bags and vials under the planned fill order, with aliquots held in CS10 for 0, 30, 60 and 90 min before entering the same CRF run (co-scheduled with the D-1 matched-product freezes so bag and vial are frozen with product probes in both); readouts post-thaw at 0 h and at the in-use hold.
- Analysis on within-run paired differences (vial minus bag; hold minus 0 min) with 90% confidence intervals against margins from the EMERALD CQA spread; the fill-order arm is decided on the vial-minus-bag difference.
- If the vial diverges from the bag beyond the margin, mini-bags are evaluated as the QC container for viability and potency, and the vial is kept for identity, safety and retains.

DECISION RULE AND DELIVERABLE

The Ares X20 fill is accepted for the site when dose uniformity meets the agreed tolerance from first to last container at the maximum position count, the bag-versus-vial post-thaw differences sit inside the margin on at least 3 runs, and the pre-freeze hold window is set at the longest hold with no trend. The QC-vial allocation and the retains philosophy are then written against validated sample volumes; patient batches give up only what release, retest and retain need.

Deliverable. Fill uniformity and recovery report; bag-versus-vial representativeness report (shared with the D-1 comparability report); DP-before-freeze hold window and fill-order instruction for the batch record; QC-vial allocation table and retains policy; time-and-motion record for the fill and inspection leg.

References [1, 9, 19, 20, 21, 31, 35], see the reference list at the end of this document.

Work Package D-8: CP-1 versus CP-2 Comparability Statistics and the Proposed Study 5

Comparability design and statistics; proposed route WO 9.0 (comparability report) with runs under WO 7.0 / WO 8.0 · Protocol and margins fixed before the first MADE pilot run; Study 5 runs alongside the RTx Study 3 and Study 4 material in 2027; N confirmed with FDA at pre-IND

Donor material. RTx Studies 2 and 3: split healthy-donor leukapheresis in RTx laboratories. Study 4: healthy-donor units at MADE. Proposed Study 5: matched split-donor units, one arm CP-2 at RTx laboratory scale and one arm CP-2 at MADE at full scale; 4 to 5 runs are under discussion, and the arithmetic below shows what N buys

OBJECTIVE

Fix the statistical design, the acceptance margins and the run count for the CP-1 to CP-2 and site comparability so that a 4-to-5-run study can reach a conclusion, and add a Study 5 that ties the RTx laboratory CP-2 data to the MADE at-scale CP-2 data on the same donors.

WHAT THE RECORD SAYS

- RTx plans four studies: Study 1 tests MS-2 mRNA against the RTX001_01 specification; Study 2 splits healthy-donor leukapheresis to make drug product with MS-1 vs MS-2 in RTx laboratories; Study 3 splits healthy-donor leukapheresis across CP-1 and CP-2 in RTx laboratories; Study 4 runs CP-2 at the CDMO against specification, in-process action limits and defined operating ranges. A direct site-to-site comparison is not feasible because neither process transfers to the other site, clinical data are not pooled, and RTx is seeking FDA guidance. [19]
- RTx is discussing 4 or 5 comparability runs, to be confirmed at pre-IND, with equivalence acceptance criteria set from the spread of the EMERALD CQAs; RTx states that the main gap is time and getting enough N, with an N of 3 to 4 in hand and a wish for 10. Safety and dose data carry over from EMERALD; phase 1/2 efficacy is presented at pre-IND but the BLA rests on pivotal efficacy only. [21]
- CD14 selection performance was highly reproducible across donations in MATCH but CD14-to-macrophage conversion efficiency was highly variable, so between-donor spread is large and a paired (within-donor) design is far more powerful than a two-group comparison. Frozen monocytes as a starting material failed in the MATCH development, which constrains how a cross-site split can be executed. [1, 2]
- ICH Q5E and the FDA 2023 draft guidance on manufacturing changes and comparability for cellular and gene therapy products frame the argument: an analytical battery sensitive to the change, potency included, with pre-set criteria; methods must be shown comparable first, or an assay difference reads as a process difference. RTx has published its own view of the FDA draft potency guidance for an mRNA gene-modified cell therapy. [22, 23, 27]

FACTORS AND LEVELS

FACTOR	LEVELS	WHY IT IS IN THE DESIGN
Comparison	CP-1 vs CP-2 (Study 3); MS-1 vs MS-2 (Study 2); CP-2 at RTx laboratory scale vs CP-2 at MADE at scale (proposed Study 5); CP-2 at MADE vs specification (Study 4)	Each is a separate paired contrast; Study 5 is the bridge that lets Studies 3 and 4 be read together.
Design	Within-donor paired (split leukapheresis) wherever the starting material allows; two-group otherwise	Pairing removes donor variance, the dominant term.
Starting-material format for a cross-site split	Fresh split with both arms at one site vs cryopreserved leukapheresis split across sites	Frozen monocyte starting material is itself a change; a fresh split within the 48 h shelf life is only practical with both arms at MADE (RTx scientists execute the laboratory-scale arm in the MADE PD laboratory).
Attribute tiers	Tier 1 release attributes on bridged methods at both sites; tier 2 comparability-only assays in one laboratory on cryo-retained samples from both arms; tier 3 information-only	Keeps assay variance out of a 4-to-5-run comparison.
Margin	Pre-declared from the EMERALD CQA spread, expressed in units of the within-donor paired standard deviation	The margin, not the N alone, decides feasibility.

RESPONSES AND ACCEPTANCE

RESPONSE	METHOD	ACCEPTANCE / TARGET
Tier 1: viability, viable macrophage dose, identity (25F9, CD206, CD163), transgene identity and efficiency, IL-10 and MMP-9 secretion, MMP-9 activity, endotoxin, impurity	Bridged release methods	Paired differences (post minus pre) with 90% CI inside the margin (TOST); safety-relevant attributes included in tier 1
Tier 2: collagen degradation, IL-10 activity, myofibroblast deactivation, phagocytosis kinetics, recruitment, polarization	One laboratory, cryo-retained samples from both arms	Descriptive with paired plots; no equivalence claim
Two methods for one attribute (TE by RT-qPCR or dPCR)	Both methods on every sample	Bland-Altman bias and limits of agreement; conversion fixed before the study
Method transfer (site)	At least 3 lots across the range plus reference standard; 2 laboratories, at least 2 analysts, at least 3 runs each	TOST equivalence, 90% CI inside the margin, before any product comparison is read
Process performance (Study 4 and Study 5)	In-process controls and operating ranges	Within action limits and defined ranges on every MADE run

DESIGN AND EXECUTION

- Paired TOST planning arithmetic (alpha 0.05 one-sided, 80% power, normal approximation; a t-based correction adds about one to two runs): required pairs n is approximately 2 x (1.645 + 0.842)^2 / delta^2, where delta is the margin in units of the within-donor paired standard deviation. This gives about 13 pairs for a margin of 1.0 SD, about 6 pairs for 1.5 SD and about 4 pairs for 2.0 SD. Read the other way, 4 runs can show equivalence to a margin of about 1.8 SD, 5 runs to about 1.6 SD and 10 runs to about 1.1 SD. The paired SD is estimated first from the method-transfer and Study 3 data, so the margin each attribute can actually support is known before pre-IND.
- Proposed Study 5: matched split-donor material, one arm executed as CP-2 at RTx laboratory scale and one arm as CP-2 at MADE at full scale, on the same day at the same site where a fresh split is used; N of 4 to 5 donors to match Study 3, with the option to extend to the N RTx wants once the FDA position is known. Its purpose is to connect the RTx laboratory dataset (Studies 2 and 3) to the MADE at-scale dataset (Study 4) on a common donor axis, so the totality of changes can be argued as a chain of paired contrasts rather than as unlinked datasets.

- Sequence: methods shown comparable (transfer, bridging) and locked with the reference standard; Study 5 and Study 4 material generated on the MADE pilot and engineering runs; tier 2 testing in one laboratory on retained cryo samples from both arms; comparability report with the pre-set criteria, then the pre-IND package.
- Where post-EP rest data are healthy-donor only, patient-derived material is added if available; where the split-pack design depends on starting-material format, the fresh-split-at-one-site option is preferred over a cryopreserved leukapheresis split.

DECISION RULE AND DELIVERABLE

Comparability is concluded, attribute by attribute, when the 90% confidence interval of the within-donor paired difference sits inside the pre-declared margin on bridged methods, with tier 2 attributes reported descriptively and safety-relevant attributes inside tier 1. The run count is set from the paired SD estimated on the transfer and Study 3 data against the margins RTx derives from EMERALD; if the supportable margin at N of 4 to 5 is wider than the clinically justified margin for an attribute, that attribute is flagged for FDA discussion at pre-IND with the N needed to close it. Study 5 is proposed, not assumed; RTx decides whether to add it.

Deliverable. Comparability statistical analysis plan with margins per attribute and the N-versus-margin table; Study 5 protocol outline; method-comparability and reference-standard prerequisites; inputs to the comparability report and the pre-IND briefing package.

References [1, 2, 19, 21, 22, 23, 24, 27, 32, 35], see the reference list at the end of this document.

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