COMPARATIVE ANALYSIS OF CD22 SCFV VARIANTS (M971 VS M972) IN CAR-T AND SYNNOTCH REPORTER SYSTEMS FOR ENHANCED TARGETING OF B-ALL

By

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EXECUTIVE SUMMARY

Chimeric antigen receptor T-cell (CAR-T) therapy targeting CD19 has transformed outcomes for pediatric B-cell acute lymphoblastic leukemia (B-ALL), but up to 50% of responding patients eventually relapse, often through antigen escape in which leukemic blasts lose CD19 surface expression. CD22, a B-cell-restricted surface glycoprotein frequently retained on CD19-negative relapse blasts, has emerged as the most clinically advanced alternative target. Two humanized anti-CD22 single-chain variable fragments (scFvs), m971 and m972, are the leading candidates for CD22-directed constructs; however, a paradox in the published literature complicates the selection. Despite approximately 10-fold higher binding affinity for CD22, m972 has shown inferior pre-clinical and clinical performance relative to m971. This paradox has direct design consequences for the Pulsipher Laboratory at Huntsman Cancer Institute, which is developing a prime-and-kill CAR-T architecture that requires scFvs optimized for two distinct functional roles: a SynNotch receptor gate that detects antigen and drives transcription, and a CAR effector that mediates cytotoxic killing.

This project systematically compared m971 and m972 in both functional contexts. Matched constructs were designed and cloned for each role; the m972 scFv required in-house gene assembly after two failed vendor synthesis attempts, and the resulting schedule compression required triage of several secondary objectives. The two scFvs were tested in cytotoxicity co-culture assays with primary human T cells and in a SynNotch transcriptional reporter assay. A five-round serial rechallenge assay was added as an orthogonal measure of sustained killing capacity. Two business deliverables were developed alongside the experimental work.

m971 outperformed m972 in every assay and condition tested. In the cytotoxicity timecourse, m971 produced lower target survival than m972 across all effector-to-target ratios and timepoints tested; at the highest dose (10:1, 72 hours), m971 reduced target cell survival to 19.6% compared to 38.5% for m972.

The serial rechallenge assay revealed a two-step exhaustion phenotype in m972-transduced T cells: killing capacity collapsed at round three despite retained CAR surface expression (functional anergy), followed by loss of the CAR-positive population itself by round five. m971 maintained effective killing through all five rounds. In the SynNotch BFP reporter assay, m971 produced 40.2% activation compared to 25.7% for m972 at equal target-to-effector numbers. Based on these findings, m971 is recommended as the lead anti-CD22 scFv for both the CAR effector and SynNotch gate roles in the Pulsipher Laboratory's prime-and-kill program, informing a therapeutic strategy aimed at patients who relapse after CD19-directed therapy.

All functional data was generated from a single biological replicate per condition, a constraint imposed by the schedule compression described above. Replication in additional donors is the recommended immediate next step before locking the scFv selection for clinical-trajectory work. Beyond the STEM findings, the project delivered two business deliverables for the Pulsipher Laboratory's screening pipeline: a standardized scFv A/B testing SOP with quality-control checkpoints at each stage, and a cost-per-construct analysis demonstrating that in-house production and testing is approximately half the cost of outsourced alternatives.

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1. Introduction and Background

Clinical Context: B-ALL and CD19-Directed CAR-T Therapy

B-cell acute lymphoblastic leukemia (B-ALL) is the most common pediatric malignancy, accounting for approximately 25% of childhood cancers (Hunger and Mullighan, 2015). Over the past decade, chimeric antigen receptor T-cell (CAR-T) therapy targeting the B-cell surface antigen CD19 has transformed treatment outcomes for patients with relapsed or refractory disease. CAR-T cells are autologous (derived from the patient's own blood) T cells that have been genetically engineered to express a synthetic receptor. The extracellular portion of the receptor is an antigen-binding domain, typically a single-chain variable fragment (scFv) derived from a monoclonal antibody. This binding domain is fused to intracellular signaling domains that activate T-cell cytotoxicity upon antigen recognition. Tisagenlecleucel, the first FDA-approved CAR-T product for pediatric B-ALL, achieved complete remission rates of approximately 81% in a pivotal trial (Maude et al., 2018), establishing CD19 CAR-T therapy as a standard-of-care option for patients who have failed conventional chemotherapy.

Despite these initial response rates, long-term outcomes remain a significant clinical challenge. Up to 50% of patients who initially respond to CD19 CAR-T therapy eventually relapse (Majzner and Mackall, 2019). A substantial proportion of these relapses involve antigen escape, in which malignant B cells downregulate or lose surface expression of CD19, rendering the CAR-T cells unable to recognize their target. Antigen-negative relapse has been documented following tisagenlecleucel, axicabtagene ciloleucel, and investigational CD19 CAR-T products across multiple institutions (Gardner et al., 2017; Majzner and Mackall, 2019). This failure mode is not a deficiency of the CAR-T platform itself but rather a consequence of targeting a single antigen; it has driven substantial interest in identifying alternative B-cell surface targets that can be used in combination with or as replacements for CD19.

CD22 as an Alternative Target

CD22 is a B-cell-restricted surface glycoprotein that is broadly expressed on B-ALL cells and, critically, is often retained on leukemic blasts even when CD19 expression has been lost (Haso et al., 2013). This expression pattern makes CD22 an

attractive alternative or complementary target for CAR-T therapy in the post-CD19 setting. Fry et al. (2018) demonstrated clinical proof-of-concept for CD22-directed CAR-T therapy, reporting complete remissions in patients with B-ALL that was naive to or had relapsed after CD19 CAR-T treatment. Updated clinical data from Shah et al. (2020) confirmed durable responses in a subset of patients, establishing CD22 as the most clinically advanced alternative target antigen for B-ALL immunotherapy.

The clinical success of CD22 CAR-T therapy has validated the target but also raised a design question: which anti-CD22 scFv should be used to build the next generation of CD22-directed constructs? The answer is not straightforward, because the two leading candidate scFvs show a paradoxical relationship between binding affinity and clinical efficacy.

The m971 vs. m972 scFv Question

The two humanized anti-CD22 scFvs at the center of this project, m971 and m972, were derived from the same parental murine antibody and engineered by Haso et al. (2013). Both use a VH-$(G_4S)_3$-VL single-chain architecture with a shared framework scaffold; they differ primarily in their complementarity-determining regions (CDRs), the segments of the antibody that directly contact the antigen and determine binding specificity and affinity. In preclinical binding assays, m972 demonstrated approximately 10-fold higher affinity for CD22 than m971 (Haso et al., 2013).

On the basis of affinity alone, m972 would be the expected lead candidate. However, the preclinical and clinical data tell the opposite story. Haso et al. (2013) reported that m971-based CARs produced more robust anti-leukemic activity than m972-based CARs in preclinical models, and the subsequent clinical development program selected m971 for advancement into human trials. The m971-based CD22 CAR-T product demonstrated clinically meaningful responses in both the Fry et al. (2018) and Shah et al. (2020) trials, while m972 has not been pursued clinically.

This paradox, in which the lower-affinity scFv outperforms the higher-affinity variant, is the core scientific question that this project addressed. At the outset, three hypotheses were on the table to explain the discrepancy:

Epitope accessibility. The m972 epitope on CD22 may be sterically hindered on the surface of B-ALL cells, reducing the effective binding frequency in a cellular context despite high affinity measured in solution-phase assays.

Binding kinetics affecting serial killing. If m972 binds CD22 so tightly that the CAR cannot efficiently release from a killed target to re-engage the next one, serial killing efficiency would be impaired despite (or because of) high affinity. This "affinity ceiling" phenomenon has been described for other CAR targets (Hudecek et al., 2013; Liu et al., 2015).

Tonic signaling differences. High-affinity scFvs can cluster spontaneously on the T-cell surface and trigger antigen-independent signaling, a phenomenon known as tonic signaling. Sustained tonic signaling through the CAR drives premature T-cell exhaustion, reducing long-term efficacy (Long et al., 2015). If m972's higher affinity lowers the threshold for spontaneous clustering, m972-transduced T cells may exhaust faster than their m971 counterparts.

These hypotheses are not mutually exclusive, and the published literature had not resolved which mechanism or combination of mechanisms accounts for the m971/m972 paradox. This gap in understanding has practical consequences: rational design of next-generation CD22-directed therapies requires knowing not just which scFv performs better overall, but whether the answer depends on the functional context in which the scFv is deployed. Resolving this question required evaluating both scFvs across multiple functional readouts, as described in the objectives and methods that follow.

SynNotch Receptors and Prime-and-Kill Architectures

The functional-context question is particularly relevant for an emerging class of engineered T-cell therapies that use combinatorial antigen recognition to improve tumor specificity. Synthetic Notch (SynNotch) receptors repurpose the Notch1 receptor's force-dependent cleavage mechanism: when the extracellular scFv engages its target antigen, mechanical force triggers proteolytic release of a synthetic transcription factor (typically Gal4-VP64) that drives expression of a user-defined payload gene from a matched upstream activation sequence (UAS) promoter (Roybal et al., 2016).

In a "prime-and-kill" architecture, the SynNotch receptor serves as a Boolean AND gate for T-cell activation. The SynNotch gate detects antigen A on the tumor surface and, upon activation, drives transcription of a CAR targeting a second antigen B. Cytotoxicity occurs only when both antigens are present on the same cell or in the same microenvironment, providing a layer of specificity that single-antigen CAR-T cells lack.

On-target, off-tumor toxicity is a major safety concern with conventional CAR-T therapy (Morgan et al., 2010); this AND-gate logic addresses it by requiring co-expression of two antigens for full T-cell activation (Roybal et al., 2016).

The prime-and-kill design creates two distinct functional roles for scFvs. In the gating role, the SynNotch receptor's scFv must engage antigen with sufficient affinity and kinetics to trigger Notch cleavage and transcriptional activation. In the killing role, the CAR's scFv must enable rapid target engagement, cytotoxicity, and serial killing. It was plausible at the outset of this project that these two roles would have different optimal scFv properties. In particular, the SynNotch gating role might favor a higher-affinity scFv like m972, because sustained antigen engagement drives the mechanical cleavage required for signal transduction. The CAR killing role, by contrast, might favor the lower-affinity m971 if rapid target disengagement is more important than peak binding strength (Figure 1, §3).

Sponsor Context: The Pulsipher Laboratory

This project was conducted at the Pulsipher Laboratory at Huntsman Cancer Institute, University of Utah. The laboratory is led by Dr. Michael Pulsipher, Division Chief of Pediatric Hematology and Oncology, and focuses on developing innovative cellular therapies for pediatric hematologic malignancies. Dr. Pulsipher is a co-author on the pivotal tisagenlecleucel trial (Maude et al., 2018), reflecting the laboratory's direct involvement in the clinical translation of CAR-T therapy for pediatric B-ALL. The laboratory has established in-house capabilities in lentiviral vector production, CAR-T cell manufacturing, and SynNotch receptor engineering, and is actively developing a prime-and-kill CAR-T program targeting B-ALL.

Within this pipeline, scFv selection for the CD22-targeting arm is a foundational design decision that affects both the SynNotch gate and the CAR effector components. Before this project, the laboratory had not performed a systematic head-to-head comparison of m971 and m972 in these two functional contexts, and no standardized protocol existed for conducting such comparisons. Each new scFv screening campaign was designed ad hoc, without documented decision criteria or cost benchmarks, making it difficult to plan timelines, allocate reagent budgets, or compare results across campaigns.

This gap motivated both the scientific objectives of the project (determine which scFv is optimal for each functional role) and the business objectives (develop a reusable, documented scFv A/B testing workflow with quality-control checkpoints, and conduct a cost-per-construct analysis comparing in-house screening against outsourced alternatives). Together, these deliverables were intended to provide the laboratory with both an evidence-based scFv recommendation and a repeatable operational framework for future screening decisions. The following section describes the four STEM and two business objectives that were designed to address these needs.

2. Objectives and Project Context

Given the clinical need for optimized CD22-directed constructs described in §1, this project established the following objectives to evaluate two candidate scFvs for the Pulsipher Laboratory's therapeutic pipeline.

The overall goal of this project was to systematically compare two humanized anti-CD22 scFvs, m971 and m972, to determine which variant is better suited for each functional role in a prime-and-kill CAR-T architecture. The two scFvs were tested in two complementary contexts: direct cytotoxicity via a second-generation BBz CAR construct, and antigen engagement via a SynNotch transcriptional reporter expressing BFP upon activation.

The objectives below were designed using the S.M.A.R.T. framework as specified in the project proposal. Several objectives were modified during execution due to the schedule compression described in §3.1.1; Table 5 (§5.5) provides a detailed comparison of planned versus actual execution.

STEM Objectives

Objective 1: Construct generation. Design and clone four lentiviral vectors (m971-BBz, m972-BBz, m971-SynNotch-BFP, m972-SynNotch-BFP), with successful cloning verified by restriction digest and nanopore sequencing, by Week 2 (February 9, 2026). *Met after four-week delay; six constructs cloned rather than four (§3.1.1, §4.1).*

Objective 2: Lentivirus production. Generate functional lentivirus for all constructs with titers confirmed by p24 ELISA, achieving minimum titers of 10^6 transducing units/mL, by Week 4 (February 23, 2026). *Met (§4.2).*

Objective 3: Transduction. Transduce primary human T cells with greater than 30% efficiency for all constructs, as measured by flow cytometry using APC-conjugated CD22 antigen and anti-G4S linker antibody, by Week 6 (March 9, 2026). *Modified and met; measurement changed from CD22-Fc staining to anti-G4S antibody, and all functional assays used magnetically enriched (~100% CAR+) populations (§3.5, §4.3).*

Objective 4: Functional comparison. Quantitatively compare m971 vs. m972 functional efficacy using (a) BFP reporter output in SynNotch constructs as a measure of antigen engagement and (b) NALM-6-GFP survival in co-culture with BBz CAR-T cells at multiple E:T ratios (1:1, 5:1, 10:1), with complete datasets generated by Week 8 (March 23, 2026). *Met with scope modifications and additions (§4.4, §4.5, §4.6).*

Business Objectives

Objective 5: Workflow development. Develop a documented, reproducible workflow for scFv A/B testing in CAR-T development contexts, including standard operating procedures, quality control checkpoints, and decision criteria for variant selection. *Met (Appendix E).*

Objective 6: Cost analysis. Conduct a cost-per-construct analysis comparing the in-house scFv A/B testing workflow against outsourced screening alternatives to inform future make-vs-buy decisions. *Met (Appendix F).*

Constraints and Project Context

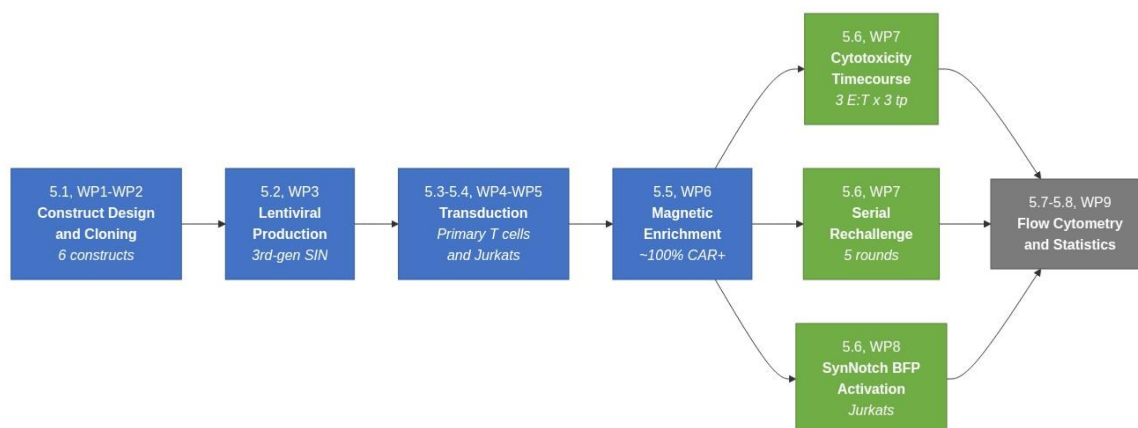
This project was executed by a single operator (Tyler Henderson) under the supervision of Dr. Candi Deimundo Roura over nine weeks (January 26 through April 1, 2026), (with the assistance and mentorship of lab personnel such as Payton Utzman and Dr. Candi Deimundo Roura) representing approximately 150 to 180 hours of bench work. The reagent budget of $2,000 to $3,000 constrained some methodological decisions, most notably the removal of CD22-Fc/APC staining from the transduction QC panel (Objective 3).

The critical path ran through construct cloning, lentivirus production, T-cell transduction, and functional assays, with limited opportunity for parallelization. The proposal had explicitly identified this dependency and placed contingency time in Weeks 7 and 8 to accommodate upstream delays. In practice, the IDT m972 gBlock synthesis failure (§3.1.1) consumed all built-in schedule contingency and compressed the downstream assay window into the final three weeks. The resulting scope triage prioritized the core m971-vs-m972 functional comparison over ancillary readouts: SynNotch testing was reduced to Jurkats only (§3.4), the Incucyte kinetic killing stretch objective was dropped (§3.6.4), and single biological replication was accepted as a documented limitation (§3.8, §5.3).

3. Methods / Approach

This section describes the methods used to address the project objectives defined in Section 2. Sections 3.1 through 3.4 address STEM Objective #1 (design and clone lentiviral vectors verified by restriction digest and nanopore sequencing). Section 3.2 additionally addresses STEM Objective #2 (generate functional lentivirus with titers confirmed by p24 ELISA at a minimum of 10^6 TU/mL). Sections 3.3 through 3.5 address STEM Objective #3 (transduce T cells with >30% efficiency, met through the combined transduction and magnetic enrichment pipeline). Sections 3.6 and 3.7 address STEM Objective #4 (quantitatively compare m971 and m972 functional efficacy by BFP reporter output and NALM-6-GFP survival at multiple E:T ratios). Section 3.9 describes the methods used to develop the SOP workflow (Business Objective #5, Appendix E) and cost-per-construct analysis (Business Objective #6, Appendix F). Figure 1 provides an overview of the experimental pipeline.

Figure 1. Experimental workflow pipeline. Six lentiviral constructs were designed and cloned (§3.1), packaged into lentivirus (§3.2), transduced into primary T cells or Jurkats (§3.3-3.4), and magnetically enriched to approximately 100% CAR-positive populations (§3.5). Three functional assays (green) were performed on the enriched populations, with all readouts acquired by flow cytometry and analyzed in R (§3.7-3.8).



3.1 Construct Design and Molecular Cloning

Six lentiviral constructs were designed and cloned to compare the m971 and m972 anti-CD22 scFv variants in two functional contexts: direct cytotoxicity via a second-generation BBz CAR, and antigen engagement via a SynNotch transcriptional reporter. The proposal specified four constructs; six were ultimately cloned because the

BBz CAR arm required two backbone versions per scFv to preserve the option of both a laboratory-standard promoter configuration (p65 backbone, p154/p155) and a clinically comparable promoter configuration (pELPS backbone, p156/p157). The six constructs are summarized in Table 1; a brief description of each follows.

Table 1. Construct summary.

ID	Name	scFv	Backbone	Functional Arm	Role in Project
p154	CD22-m971-41BBz	m971	p65 4-1BBz	BBz CAR	Precursor; transduced for validation only
p155	CD22-m972-41BBz	m972	p65 4-1BBz	BBz CAR	Precursor; transduced for validation only
p156	pELPS-CD22-m971-BBz	m971	pELPS	BBz CAR	Primary test construct (functional assays)
p157	pELPS-CD22-m972-BBz	m972	pELPS	BBz CAR	Primary test construct (functional assays)
p158	UAS-CD22-m971-CAR-BFP	m971	pHR-UAS	SynNotch-BFP	SynNotch reporter (Jurkats)
p159	UAS-CD22-m972-CAR-BFP	m972	pHR-UAS	SynNotch-BFP	SynNotch reporter (Jurkats)

The m971 and m972 scFv sequences were obtained from the parental humanized anti-CD22 antibody described by Haso et al. (2013) and verified against US Patent 8,591,889. Both scFvs use a VH-$(G_4S)_3$-VL architecture.

BBz CAR constructs (p154/p155 and p156/p157). Each scFv was incorporated into a second-generation CAR backbone containing a CD8α hinge and transmembrane domain, a 4-1BB costimulatory domain, and a CD3ζ signaling domain. Two backbone versions were cloned for each scFv (Table 1). Precursor constructs p154 and p155 were initially assembled into the p65 4-1BBz backbone by PaqCI restriction and NEB HiFi assembly. The final BBz CAR transfer vectors, p156 and p157, were generated by inverse PCR insertion of each scFv into the pELPS lentiviral backbone derived from the laboratory's existing CD19-BBz clinical construct (p3). The pELPS versions were selected for all downstream functional assays because their promoter configuration is more comparable to current clinical CAR-T constructs; p154/p155 were transduced in parallel for validation but are not included in the functional results.

SynNotch reporter constructs (p158, p159). Each scFv was fused to the Notch1 regulatory region (NRR) and linked to a Gal4-VP64 transcriptional activator. BFP expression was driven by an upstream activation sequence (UAS) response element encoded on the same transfer vector. The final SynNotch constructs, p158 (UAS-CD22-m971-CAR-BFP) and p159 (UAS-CD22-m972-CAR-BFP), were generated by inverse PCR insertion into pHR-UAS-CAR-BFP (p1). Notably, the SynNotch-BFP constructs did

not contain a constitutive mCherry transduction marker, despite the original design intent; implications for the readout are discussed in §3.4 and §5.3.

Assembly and verification. All constructs were assembled using NEB HiFi DNA Assembly, transformed into NEB Stable competent cells, and screened by restriction digest. Positive clones were verified by whole-plasmid nanopore sequencing through Plasmidsaurus, confirming complete scFv and backbone integrity with no deleterious mutations. Confirmed clones were archived as glycerol stocks and scaled by Qiagen EndoFree Plasmid Maxi Kit for lentiviral packaging.

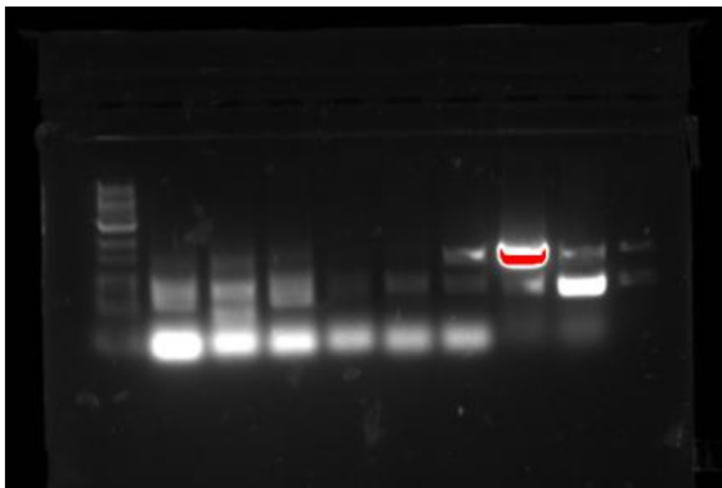
A CD19-BBz CAR construct (p3, pELPS backbone) served as the positive control for all BBz functional assays. The CD19 FMC63 scFv uses a VL-$(G_4S)_3$-VH architecture (the reverse orientation relative to the CD22 m971/m972 scFvs), which reflects the parental antibody conventions rather than a deliberate design choice.

3.1.1 IDT m972 gBlock Synthesis Failure and Staircase-PCR Recovery

The m971 scFv gBlock was synthesized successfully by Integrated DNA Technologies (IDT) on the first attempt. However, IDT was unable to synthesize the m972 scFv gBlock after two separate attempts. The root cause was consistent with sequence complexity in the $(G_4S)_3$ linker region: the canonical linker codon usage produces a 45-bp stretch at 93.3% GC content with 12 consecutive GGC codons, a known synthesis-killer motif. Computational analysis confirmed the problem and generated a codon-diversified linker sequence (73% GC) encoding the identical GGGGSGGGGSGGGGS amino acid sequence.

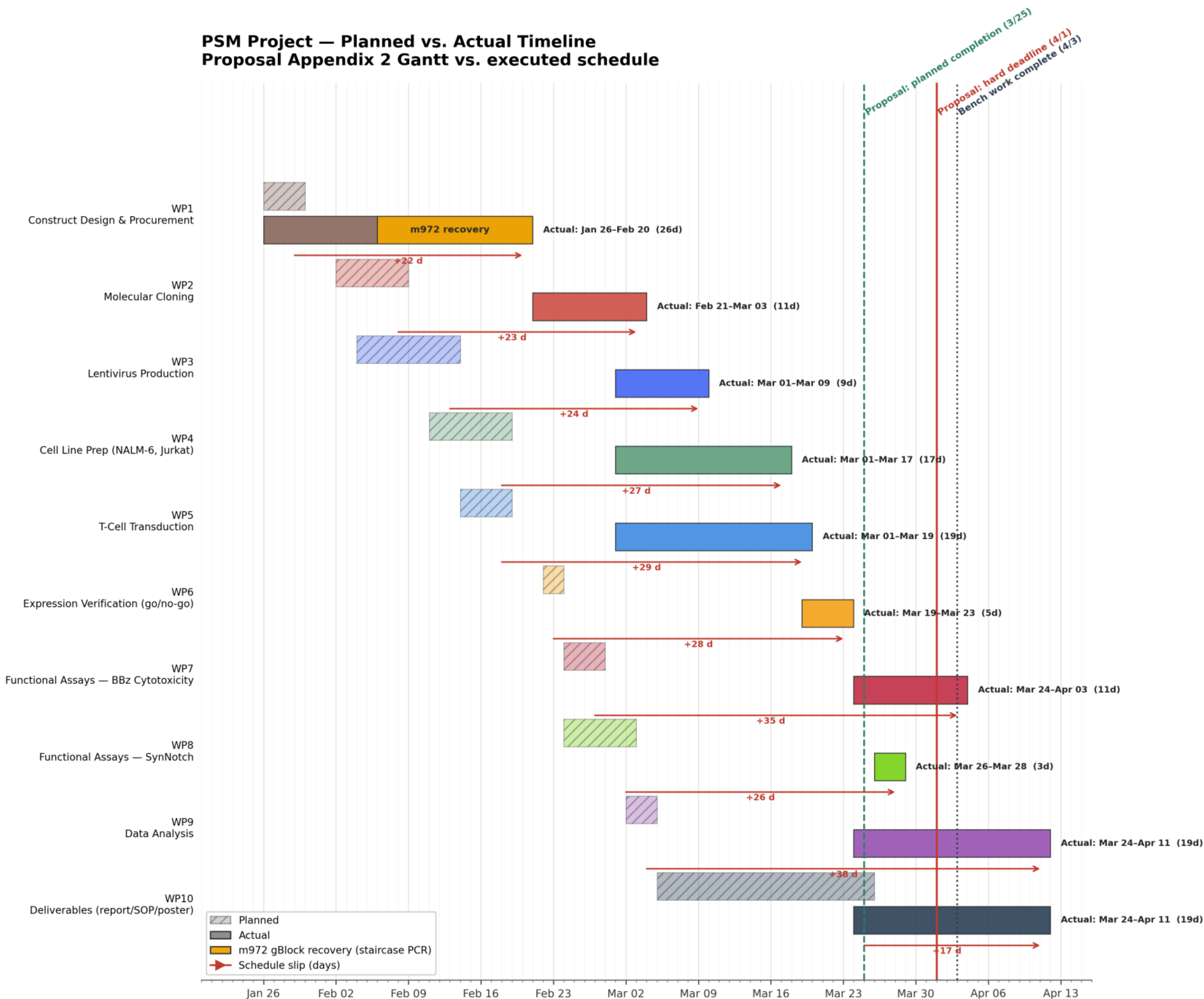
Recovery. The m972 scFv (720 bp) was synthesized de novo in-house using staircase PCR assembly, the same overlap-extension chemistry that commercial gene synthesis providers use internally. Twenty-four tiled 60-bp assembly oligonucleotides were designed with 30-bp overlap and assembled via a two-step protocol: primerless assembly followed by outer-primer amplification (full protocol in Appendix G). Gel electrophoresis confirmed a clean single band at the expected size (Figure 2). Oligo design and protocol execution were performed by Tyler Henderson and Payton Utzman.

Figure 2. Gel verification of m972 scFv staircase PCR assembly. 1% agarose gel, 150 V, 20 minutes. Lane 1: 100-bp DNA ladder. Lanes 7 and 8 (highlighted): full-length m972 scFv assembly product at the expected size of approximately 720 bp. The clean single band with no detectable off-target products confirmed successful de novo gene synthesis.



Schedule impact and project management response. The IDT synthesis failure and subsequent in-house recovery introduced an approximately four-week delay, consuming all downstream schedule float. Upon confirming the second IDT attempt had failed, the project team (Tyler Henderson, lab colleague Payton Utzman, and supervisor Dr. Candi Deimundo Roura) evaluated three options: (1) re-order from an alternative vendor with a 2-to-3 week lead time, (2) attempt in-house staircase PCR assembly, or (3) reduce the project to m971 only. Option 2 was selected because it preserved the core comparative design while offering the fastest recovery path, with option 1 as a fallback if the assembly failed. At the same time, the team identified which downstream scope items could be cut with the least impact on the primary m971-vs-m972 comparison. The resulting triage prioritized the functional comparison assays (cytotoxicity and rechallenge) over the Incucyte stretch objective and primary-T-cell SynNotch work, and accepted the loss of biological replicates as a documented limitation rather than delaying the project beyond the academic deadline. These decisions drove the subsequent scope adjustments detailed in §3.4 (SynNotch reduced to Jurkats), §3.6.4 (Incucyte dropped), and §3.8 (single biological replicate). Figure 3 shows the planned versus actual project timeline, with the m972 recovery period and resulting schedule compression visible across all downstream work packages.

Figure 3. Planned versus actual project timeline. Gantt comparison showing proposed schedule (hatched bars) and executed schedule (solid bars) for each work package. The m972 gBlock recovery period (orange, WP1) consumed all built-in contingency time, compressing WP5 through WP9 into the final three weeks. The dashed red line marks the original planned completion date; the dashed green line marks the actual completion date. Schedule slip in days is annotated for each work package.



3.2 Lentiviral Vector Production

Lentivirus was produced for all six constructs (p154 through p159) by transient transfection of HEK293T cells using a third-generation self-inactivating (SIN) lentiviral packaging system (Dull et al., 1998).

Transfection. Three-plasmid co-transfection (transfer vector, psPAX2 packaging plasmid, and pMD2.G envelope plasmid) was performed using polyethylenimine (PEI) in 10-cm plates, with two plates per construct (12 plates total). Detailed transfection parameters are provided in Appendix G.

Harvest and concentration. Viral supernatants were collected at 48 and 72 hours post-transfection, pooled, and concentrated approximately 100-fold using Takara Lenti-X Concentrator (detailed protocol in Appendix G). Aliquots were flash-frozen and stored at -80°C.

Titering. Lentiviral p24 capsid protein was quantified by ELISA (Takara Bio) at 1:10,000 dilution to confirm successful packaging. Functional titers in transducing units per milliliter (TU/mL) were independently derived from flow cytometry data acquired on March 19 (§3.5) via the Poisson single-hit approximation. All constructs exceeded the $\geq 10^6$ TU/mL threshold specified in STEM Objective #2: BBz constructs ranged from 2.49×10^7 to 3.10×10^7 TU/mL, and SynNotch-BFP constructs ranged from 5.86×10^6 to 7.56×10^6 TU/mL (Table 2, Figure 4).

3.3 Primary T-Cell Isolation, Activation, and Transduction (BBz CAR Arm)

Cell source and isolation. Cryopreserved peripheral blood mononuclear cells (PBMCs; STEMCELL Technologies) were thawed on March 1, 2026. T cells were isolated by negative magnetic selection using the BioLegend MojoSort Human T Cell Isolation Kit per the manufacturer's protocol, with magnetic separation performed using a STEMCELL Technologies EasySep magnet available in the laboratory. Post-isolation purity was not independently verified, which is standard practice in the Pulsipher Laboratory for experiments comparing constructs transduced from a shared PBMC preparation, as any residual non-T-cell contamination is constant across conditions and does not bias between-construct comparisons.

Activation. Isolated T cells were activated with Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher) at a 1:1 bead-to-cell ratio in R10 medium (RPMI 1640 + 10% FBS + 1% penicillin-streptomycin) supplemented with recombinant human IL-2 at

100 IU/mL. The proposal had specified plate-bound anti-CD3 (OKT3) with soluble anti-CD28; Dynabead activation was used instead as standard Pulsipher Laboratory practice. Dynabeads were magnetically removed on day 3 (March 4) and cells were rested in R10 + IL-2 for 8 days until transduction on March 12.

Transduction. On March 12, 2026, T cells were transduced on RetroNectin-coated plates (Takara Bio) by spinoculation (protocol details including g-force, temperature, and polybrene concentration in Appendix G). Four wells were plated per construct (2 × 10^6 cells total per construct). Four parallel transductions were performed from the same T-cell pool: m971-BBz (p156), m972-BBz (p157), CD19-BBz (p3, positive control), and untransduced (UT, negative control). Precursor constructs p154 and p155 were also transduced for validation purposes.

Post-transduction expansion. Medium was changed the following day and cells were maintained in R10 supplemented with IL-2 at 50 IU/mL (reduced from the 100 IU/mL activation concentration), with IL-2 supplementation every 3 days. After expansion, all CAR-T populations proceeded to magnetic enrichment (§3.5) before use in functional assays.

3.4 Jurkat Transduction (SynNotch Arm)

The SynNotch reporter arm was executed exclusively in Jurkat T cells (ATCC Clone E6-1), rather than in parallel with primary T cells as originally proposed. This scope reduction was one of the triage decisions made with Dr. Deimundo Roura following the m972 cloning delay (§3.1.1). The decision was scientifically defensible: Jurkats are clonal (eliminating donor-to-donor variability), SynNotch activation is a transcriptional readout that does not require the killing machinery of primary T cells, and a clonal population provides a more controlled baseline for comparing scFv-dependent activation differences.

Transduction. Jurkat cells were thawed on March 1, 2026, and maintained in R10 at log-phase growth with passaging every 3 days. On March 12, Jurkats were transduced with SynNotch constructs p158 (m971) and p159 (m972) using the same RetroNectin/spinoculation protocol described in §3.3. No IL-2 was supplemented, as Jurkats are IL-2-independent.

Positive activation control. A separate Jurkat population was co-transduced with p2 (pHR_PGK_antiCD19_synNotch_Gal4VP64) and p17

(pHR_Gal4UAS_tBFP_PGK_mCherry) as a functional positive control for the SynNotch arm. Together, p2 and p17 reconstitute a complete CD19-targeting SynNotch-BFP circuit: p2 encodes the SynNotch receptor, and p17 encodes the UAS-driven tBFP reporter plus a constitutive PGK-mCherry transduction marker. Because NALM-6 target cells are CD19-positive, this two-component control robustly activates BFP and confirms that the SynNotch circuit functions end-to-end. The p2 + p17 architecture is the two-plasmid design that p158/p159 consolidate into a single-vector format.

Magnetic enrichment. All SynNotch-transduced Jurkat populations were magnetically enriched to approximately 100% transgene-expressing cells using the anti-G4S/Dynabead protocol described in §3.5. The functional SynNotch BFP assay (§3.6.3) therefore used magnetically enriched, approximately 100% SynNotch-positive Jurkats as the effector population.

Absence of constitutive transduction marker. The p158/p159 constructs did not contain a constitutive mCherry transduction marker, despite the original design intent. This was confirmed by the absence of FL5/mCherry signal above autofluorescence in flow cytometry data across all SynNotch files, including the CD19 positive control. Because the Jurkats were magnetically enriched to approximately 100% positivity before the experiment, the impact on the readout is partially mitigated: %BFP-positive within the gated lymphocyte population primarily reflects SynNotch activation rate rather than (transduction × activation) jointly. However, there was no independent verification of transduction efficiency at the time of the BFP assay; the assumption is that magnetic enrichment held over the intervening culture period. This limitation is discussed further in §5.3, and next-generation constructs should include a constitutive marker.

3.5 CAR Expression Verification and Magnetic Enrichment

CAR detection. Surface expression of all four constructs was detected by flow cytometry using an anti-G4S linker (E7O2V) rabbit monoclonal antibody conjugated to Pacific Blue (Cell Signaling Technology, #44962). The $(G_4S)_3$ linker is a universal structural feature of scFv-based CARs present in all four project constructs, making this antibody applicable across both arms. Readout was acquired on the FL12 channel (V450-45) of the Beckman Coulter CytoFLEX LX. The proposal had additionally called for CD22-Fc/APC antigen staining to confirm antigen-binding competence; this was dropped due to reagent cost. The anti-G4S antibody confirms CAR surface expression

independently of antigen binding; it provides a clean expression QC, but not a functional-binding QC. This is a minor limitation given that m971 and m972 are literature-validated scFvs with well-characterized binding properties.

Magnetic enrichment. Following post-transduction expansion, all four construct populations (both BBz primary T cells and SynNotch Jurkats) were magnetically enriched for CAR-positive cells prior to the March 19 expression check. This procedure was not specified in the original proposal but is standard practice in the Pulsipher Laboratory. Cells were labeled with biotinylated anti-G4S antibody, incubated with Thermo Fisher Streptavidin Dynabeads M-280, and magnetically separated using a DynaMag magnet (full protocol with antibody volumes, bead ratios, and incubation times in Appendix G). This procedure yielded approximately 100% CAR-positive/SynNotch-positive populations for all four constructs.

Post-enrichment expression check. Flow cytometry was performed approximately 7 days post-transduction (March 19, 2026) to confirm maintained CAR surface expression in the enriched populations (Table 3, §4.3). These values represent post-selection confirmation of maintained expression, not raw transduction efficiency; the functional assays used the approximately 100% enriched populations from the magnetic enrichment step.

STEM Objective #3. The proposal set a target of >30% transduction efficiency. The pre-enrichment raw transduction percentages were below this threshold for some constructs; however, all functional assays used the post-enrichment approximately 100% CAR-positive populations. STEM Objective #3 was therefore met through the combined transduction and enrichment pipeline.

3.6 Functional Assays

Three functional assays were performed to compare m971 and m972 across complementary readouts: a cytotoxicity timecourse measuring target cell killing at multiple effector-to-target (E:T) ratios and timepoints; a serial rechallenge assay probing sustained killing capacity under repeated antigen exposure; and a SynNotch BFP reporter activation assay measuring antigen engagement in a transcriptional context. A fourth assay, the Incucyte S3 live-cell kinetic analysis, had been proposed as a stretch objective but was not performed because the cloning delay consumed all schedule float.

The Incucyte is discussed briefly in §3.6.4; its omission is addressed in §5.4 (Future Work).

3.6.1 BBz CAR Cytotoxicity Co-Culture (Primary Endpoint)

The cytotoxicity timecourse was the primary functional endpoint specified in the project proposal. Magnetically enriched CAR-T populations (approximately 100% CAR-positive; §3.5) were co-cultured with NALM-6-GFP target cells, a B-ALL cell line that constitutively expresses GFP and is positive for both CD22 and CD19, at three E:T ratios (1:1, 5:1, and 10:1) and harvested at 24, 48, and 72 hours for flow cytometric analysis. Co-cultures were set up on March 28, 2026 in 96-well flat-bottom plates under standard co-culture conditions (Appendix G). Four constructs were tested at each E:T ratio and timepoint: m971-BBz (p156), m972-BBz (p157), CD19-BBz (p3, positive control), and untransduced T cells (UT, negative control), yielding 36 experimental conditions plus session controls. Target cell survival was quantified as %GFP-positive cells within the singlet population; killing was calculated as the reduction in %GFP-positive relative to the UT control at the same E:T ratio and timepoint.

3.6.2 BBz CAR Rechallenge Assay (Added Readout)

A five-round serial rechallenge assay was performed to assess sustained killing capacity, an in vitro correlate of in vivo T-cell persistence and a more stringent test of long-term function than a single-timepoint co-culture. This assay was not in the original proposal but was added to probe a clinically relevant dimension of CAR-T function beyond the single-timepoint co-culture.

Design. On March 24, 2026, magnetically enriched CAR-T populations were co-cultured with NALM-6-GFP target cells at a 1:1 ratio (1×10^6 effectors : 1×10^6 targets) in 24-well plates under standard co-culture conditions (Appendix G). Four conditions were maintained across all five rounds: m971-BBz, m972-BBz, CD19-BBz (positive control), and UT (negative control), in single wells per condition. Every 2 days, an aliquot was removed for flow cytometric analysis, and 1×10^6 fresh NALM-6-GFP target cells were added to re-challenge the surviving effectors.

Schedule. Rechallenge rounds were analyzed on the following dates: RC-1 (March 26), RC-2 (March 28), RC-3 (March 30), RC-4 (April 1), and RC-5 (April 3, 2026). Two readouts were quantified per round: %GFP-positive of singlets (target cell survival,

reflecting killing capacity) and %CAR-positive of GFP-negative T cells (CAR surface retention, reflecting T-cell phenotype under antigen stress).

3.6.3 SynNotch BFP Reporter Activation Assay (Jurkats)

The SynNotch BFP assay measured antigen engagement by quantifying UAS-driven BFP reporter induction following co-culture of SynNotch-transduced Jurkats with NALM-6-GFP target cells.

Effector cells. Magnetically enriched Jurkat populations (§3.4, §3.5): m971-SynNotch-BFP (p158), m972-SynNotch-BFP (p159), the p2 + p17 two-component CD19-SynNotch-BFP positive control, and untransduced Jurkats (UT, BFP-negative baseline).

Target cells and co-culture conditions. NALM-6-GFP cells were co-cultured with Jurkats for approximately 48 hours at 37°C / 5% CO_2 in complete RPMI (RPMI 1640 + 10% FBS + 1% penicillin-streptomycin) without IL-2 supplementation (Jurkats are IL-2-independent, and IL-2 would be inappropriate for a transcriptional activation readout). Two target-to-effector (T:E) ratios were tested: 0.5:1 (0.5×10^6 NALM-6 : 1.0×10^6 Jurkats, effector excess) and 1.0:1 (1.0×10^6 NALM-6 : 1.0×10^6 Jurkats, equal numbers). The proposal had specified E:T ratios of 1:1, 5:1, and 10:1 for SynNotch activation, matching the BBz cytotoxicity arm. The experiment used T:E 0.5:1 and 1.0:1 instead, as effector excess is the biologically informative condition for a transcriptional reporter readout. The ratio convention is therefore T:E rather than E:T; both conditions used the same target dose, varying only the effector count.

Format and replication. Co-cultures were plated in 24-well format, one well per condition. No biological or technical replicates were performed; the limited number of transduced cells available after the compressed timeline (§3.1.1) precluded plating in triplicate. This was therefore a single-replicate, single-timepoint experiment; the resulting limitation is discussed in §5.3, and results are exploratory.

Readout. BFP induction was measured as %BFP-positive within the gated lymphocyte population on the FL12-A channel (V450-45). The BFP-positive threshold was set from the UT Negative sample (untransduced Jurkats co-cultured with NALM-6-GFP). GFP-positive population on FL1-A was used to confirm NALM-6 target cell presence in each co-culture well.

3.6.4 Incucyte Kinetic Assay (Not Performed)

The Incucyte S3 live-cell kinetic killing assay was proposed as a stretch objective to generate real-time killing curves and EC50 values for each scFv variant. This assay was not performed because the m972 cloning delay (§3.1.1) consumed all schedule float. The proposal had explicitly designated this objective as contingent on time and "not required for the primary comparative analysis." The potential value of kinetic killing data is discussed in §5.4 (Future Work).

3.7 Flow Cytometry Acquisition and Analysis

All flow cytometry data were acquired on a Beckman Coulter CytoFLEX LX spectral cytometer using CytExpert software (version 2.3.1.22; Beckman Coulter, 2023) at the Huntsman Cancer Institute. Samples were preserved in formalin prior to acquisition (post-fixation analysis).

Staining panel. Two fluorescence channels carried biology-relevant signal across all assays: FL1 (FITC/GFP, 525/40 filter) for NALM-6-GFP target cell identification, and FL12 (V450-45/Pacific Blue) for either CAR detection via anti-G4S Pacific Blue antibody (rechallenge and cytotoxicity timecourse assays) or BFP reporter induction (SynNotch assay). All other fluorescence channels were present in the acquisition template but unused for this project (Appendix B).

Gating strategy. Manual gating was performed in Floreada (web-based flow cytometry analysis software; Floreada, 2025). The gating hierarchy was: singlets (FSC-H versus FSC-A), lymphocytes (FSC-A versus SSC-A), then endpoint-specific gates (%GFP-positive of singlets for target survival; %CAR-positive of GFP-negative T cells for CAR retention; %BFP-positive of lymphocytes for SynNotch activation). Representative gating walkthroughs are shown in Figure 6 (BBz CAR assays) and Figure 9 (SynNotch BFP assay); full gating-course displays for all constructs are provided in Appendix C. The original proposal specified FlowJo for flow cytometry analysis; Floreada was used instead as it produces identical Gating-ML output and is freely available.

Programmatic data export. Per-sample gated population frequencies were exported programmatically using a custom Python script (apply_floreada_gates.py) that parsed Floreada's Gating-ML exports and applied them to raw FCS files by reimplementing the Gating-ML polygon and range gate logic in Python. This ensured

consistent gate application across all samples and enabled direct input to the downstream R statistical pipeline.

3.8 Statistical Analysis

All statistical analyses were performed in R (version 4.3.x; R Core Team, 2024) using the following packages: flowCore (Hahne et al., 2009), flowWorkspace, ggcyto (Van et al., 2018), and xml2 (Bioconductor packages for flow cytometry data handling and visualization) for FCS file parsing, gate application, and gating-course displays; ggplot2, patchwork, and scales for figure generation; rstatix (Kassambara, 2023) for pairwise statistical comparisons; and tidyr, dplyr, and readr for data manipulation. The analysis pipeline was designed to be fully reproducible from raw FCS files to final figures and statistical output.

Gated proportions and confidence intervals. Gated population proportions (%GFP-positive of singlets for target survival, %CAR-positive of GFP-negative T cells for CAR retention, and %BFP-positive of singlets for SynNotch activation) are reported as point estimates with Wilson binomial 95% confidence intervals computed from event counts within each sample. Wilson intervals were chosen over the normal-approximation Wald interval for their superior coverage properties at extreme proportions (near 0% or 100%) and small effective sample sizes, following the recommendations of Brown, Cai, and DasGupta (2001).

Pairwise comparisons. Within-experiment pairwise construct comparisons were evaluated by Fisher's exact test on 2×2 contingency tables of positive versus negative event counts within the same experimental condition (same co-culture day, same cell lot, same instrument run). Because each gated event represents an independent observation within the parent gate, and the conditions being compared share identical internal controls, Fisher's exact test is appropriate for testing whether the proportions differ within this experiment. A total of 91 pairwise comparisons were performed across all experiments and conditions. No family-wise multiple-testing correction (e.g., Bonferroni) was applied because the comparisons are structured within distinct experimental contexts (cytotoxicity timecourse, rechallenge, SynNotch activation), each with its own hypothesis, and the primary inferential goal is to characterize effect direction and magnitude rather than to declare significance against a global threshold. Unadjusted

p-values are reported throughout §4; results that depend on borderline significance are interpreted conservatively.

Killing normalization (rechallenge series). For the rechallenge assay, killing was normalized to the per-round untransduced (UT) effector baseline using the formula: killing = 1 - (%GFP-positive in sample / %GFP-positive in same-round UT co-culture). Of note, no UT co-culture was acquired in rechallenge round 3; the UT baseline for RC-3 was linearly interpolated between RC-2 and RC-4 values.

Important limitation: N(biological) = 1 per condition. The schedule compression from the m972 cloning delay (§3.1.1) limited all functional assays to a single biological replicate per condition. The Wilson CIs and Fisher p-values reported throughout §4 therefore reflect measurement precision within this experiment, not biological reproducibility. The full interpretive implications of this constraint are discussed in §5.3; replication is the highest-priority future work item (§5.4).

Tooling deviation. The original proposal specified GraphPad Prism for statistical analysis with two-way ANOVA and Bonferroni correction. The project used R instead, which produces equivalent statistical output with the additional benefit of a fully archivable and reproducible analysis script (Appendix D). The shift from ANOVA to event-level Fisher's exact tests was driven by the N(biological) = 1 constraint: cross-replicate ANOVA is not appropriate when only a single biological replicate exists per condition, whereas event-level Fisher tests provide defensible within-experiment inference. Full statistical outputs are provided in Appendix D, including 158 gated proportions with Wilson 95% CIs and 91 pairwise Fisher tests.

3.9 Business Deliverable Methods

The two business objectives (SOP workflow, Business Objective #5; cost-per-construct analysis, Business Objective #6) required their own methodological approaches distinct from the bench science described above. The full deliverables are provided in Appendix E and Appendix F, respectively; this subsection describes how they were developed.

3.9.1 SOP for scFv A/B Testing Workflow (Business Objective #5, Appendix E)

The SOP was developed retrospectively from the m971/m972 project by systematically reviewing all lab notebook entries (January 26 through April 3, 2026),

identifying the sequence of decision points, QC checkpoints, and protocol parameters at each stage, and generalizing project-specific details into reusable workflow guidance. The document follows a six-stage structure (Design, Cloning, Lentiviral Production, Transduction and Enrichment, Functional Screening, Data Analysis and Go/No-Go Decision) that mirrors the experimental pipeline in §3.1 through §3.8. Each stage includes explicit QC checkpoints with pass/fail criteria (e.g., minimum titer thresholds, minimum transduction efficiency before enrichment) and decision criteria for variant selection (e.g., what magnitude of functional difference justifies selecting one scFv over another). Lessons learned from the m972 synthesis failure and the resulting scope triage (§3.1.1) are incorporated as standing guidance for future campaigns, including contingency planning for vendor synthesis failures and criteria for when to attempt in-house gene assembly versus re-ordering from an alternative provider. The SOP was reviewed by Dr. Deimundo Roura for accuracy against the executed protocols.

3.9.2 Cost-Per-Construct Analysis (Business Objective #6, Appendix F)

The cost analysis used a bottom-up accounting approach to quantify the true cost of in-house scFv A/B testing and compare it against outsourced alternatives. In-house costs were catalogued from 76 individual reagent and consumable line items tracked in the Pulsipher Laboratory's internal purchasing system, with each item allocated to one of nine workflow stages (Design/Synthesis through General Consumables) and assigned a fractional usage rule based on actual consumption during the m971/m972 project. Items not in the lab catalog (IDT direct orders, vendor-direct purchases) were priced from institutional rate cards or distributor list prices and accounted for separately.

Outsourced pricing was collected at two comparison scopes: (1) plasmid construction only, benchmarked against commercial gene synthesis and cloning services (VectorBuilder, GenScript, Twist Bioscience), and (2) the full A/B testing workflow, benchmarked against contract research organization (CRO) quotes and academic vector core pricing. All costs were normalized to a per-construct basis to enable direct comparison across pathways. The analysis also assessed non-dollar factors including timeline control, intellectual property considerations, iteration speed, failure-recovery flexibility, and capability building within the laboratory. The IDT synthesis failure provided a concrete, project-specific data point for quantifying the hidden costs of vendor dependencies.

4. Results / Outputs

This section presents the results of each experimental stage described in Section 3, organized by STEM objective. Subsections 4.1 through 4.3 address Objectives #1 through #3 (construct generation, viral titers, and transduction efficiency). Subsections 4.4 through 4.6 address Objective #4 (functional comparison of m971 and m972) across three complementary readouts: cytotoxicity timecourse, serial rechallenge, and SynNotch BFP reporter activation. Section 4.7 provides a cross-readout summary, and Section 4.8 briefly addresses Business Objectives #5 and #6. The statistical framework used throughout this section, including the N(biological) = 1 limitation, is described in Section 3.8.

4.1 Construct Generation and Sequence Verification

All six lentiviral constructs were successfully cloned and verified by whole-plasmid nanopore sequencing (Plasmidsaurus), confirming complete scFv and backbone integrity with no deleterious mutations (Table 1; plasmid maps in Appendix A). The four constructs used in functional assays (p156, p157, p158, p159) and the two precursor constructs (p154, p155) all passed restriction digest screening and sequence verification. STEM Objective #1 was met.

The m971 scFv gBlock was synthesized by IDT on the first attempt. The m972 scFv required in-house recovery by staircase PCR assembly after two failed IDT synthesis attempts; the recovery, root-cause analysis, and resulting schedule impact are described in §3.1.1. Figure 2 shows gel electrophoresis confirmation of the 720-bp m972 full-length product; a clean single band at the expected size confirmed successful de novo synthesis.

4.2 Lentiviral Production and Titers

All six constructs produced functional lentivirus exceeding the >=10^6 TU/mL threshold specified in STEM Objective #2 (Table 2, Figure 4). Functional titers were derived from p24 ELISA and flow cytometry data via the Poisson single-hit approximation (§3.2).

Figure 4. Lentiviral functional titers. All constructs exceeded the 10^6 TU/mL STEM Objective #2 threshold (dashed line). BBz CAR constructs (p156, p157) produced titers approximately 3- to 5-fold higher than SynNotch-BFP constructs (p158, p159), consistent with the larger transgene size of the single-vector SynNotch architecture.

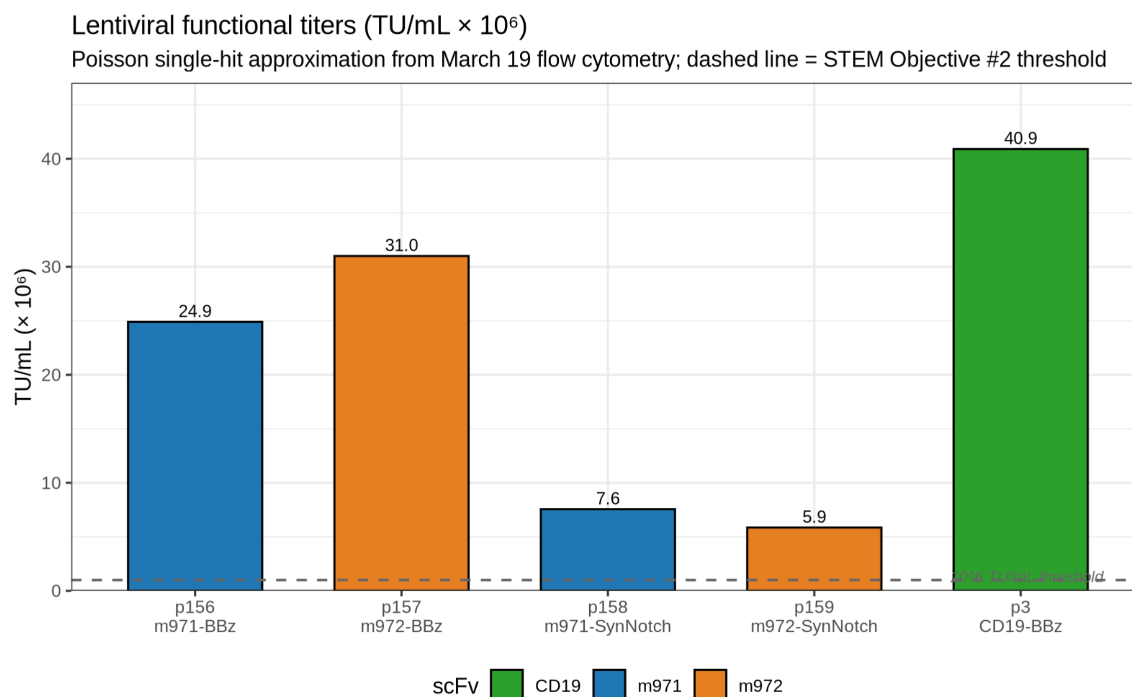


Table 2. Lentiviral titers for all constructs.

Construct	Plasmid	scFv	TU/mL	Source
pELPS-CD22-m971-BBz	p156	m971	2.49 x 10^7	Direct (flow)
pELPS-CD22-m972-BBz	p157	m972	3.10 x 10^7	Direct (flow)
UAS-CD22-m971-SynNotch-BFP	p158	m971	7.56 x 10^6	Direct (flow)
UAS-CD22-m972-SynNotch-BFP	p159	m972	5.86 x 10^6	Direct (flow)
CD19-BBz (positive control)	p3	CD19	4.09 x 10^7	Direct (flow)

4.3 Transduction Efficiency and CAR Expression

Transduction efficiency was assessed by anti-G4S Pacific Blue flow cytometry approximately 7 days post-transduction (§3.5). Post-enrichment FL12-positive percentages confirmed maintained CAR surface expression in the three BBz construct populations (Table 3, Figure 5).

SynNotch constructs (p158, p159) are excluded from this analysis because the FL12 channel cannot distinguish anti-G4S Pacific Blue staining from BFP reporter signal

(§3.4). SynNotch Jurkat populations were magnetically enriched to high positivity via the anti-G4S/Dynabead protocol (§3.5); the absence of a constitutive transduction marker is a limitation discussed in §5.3.

Figure 5. All BBz constructs maintained detectable CAR surface expression post-enrichment. Anti-G4S Pacific Blue staining approximately 7 days post-transduction confirms sustained expression in enriched populations. SynNotch constructs are excluded due to FL12/BFP spectral overlap (§3.4). Error bars: Wilson 95% CIs.

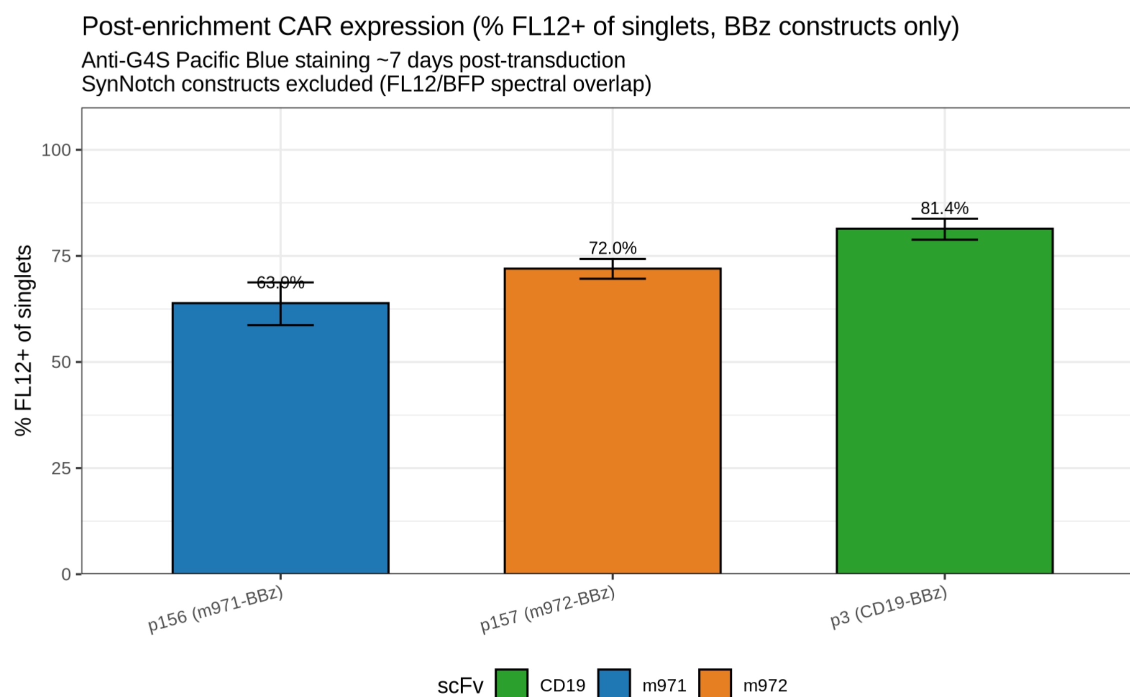


Table 3. Post-enrichment CAR expression (BBz constructs).

Construct	scFv	Arm	Post-Enrichment FL12+ (%)
p156	m971	BBz CAR	63.9
p157	m972	BBz CAR	72.0
p3	CD19	BBz CAR (control)	81.4

Pre-enrichment raw transduction efficiencies were below the proposal's 30% threshold for the CD22 constructs (m972-BBz = 21.8%, m971-BBz = 27.0% pre-enrichment, by event-level gating). However, all functional assays used populations that had been magnetically enriched to approximately 100% CAR-positive cells (§3.5), and the post-enrichment values in Table 3 represent confirmation of maintained expression after enrichment and expansion, not raw transduction efficiency. STEM Objective #3 was therefore met through the combined transduction and magnetic enrichment pipeline.

4.4 BBz CAR Cytotoxicity Timecourse (Primary Endpoint)

The cytotoxicity timecourse was the primary functional endpoint specified in the project proposal. Magnetically enriched CAR-T populations were co-cultured with NALM-6-GFP target cells at three E:T ratios (1:1, 5:1, 10:1) and harvested at 24, 48, and 72 hours (§3.6.1). Target cell survival was quantified as %GFP-positive of singlets; lower values indicate more effective killing. Representative gating is shown in Figure 6; the full timecourse killing data are presented in Figure 7; full per-condition statistics are provided in Appendix D.

Figure 6. Representative gating walkthrough (timecourse, m971-BBz, 10:1 / 72h). Four-panel gating hierarchy: (1) scatter gate, (2) singlets (FSC-H vs. FSC-A), (3) GFP-positive target identification (FL1-A), (4) CAR-positive T cells within the GFP-negative fraction (FL12-A). Red dashed lines indicate approximate gate positions for visualization; quantitative analysis used original interactive gates drawn in Floreada and exported as Gating-ML (§3.7).

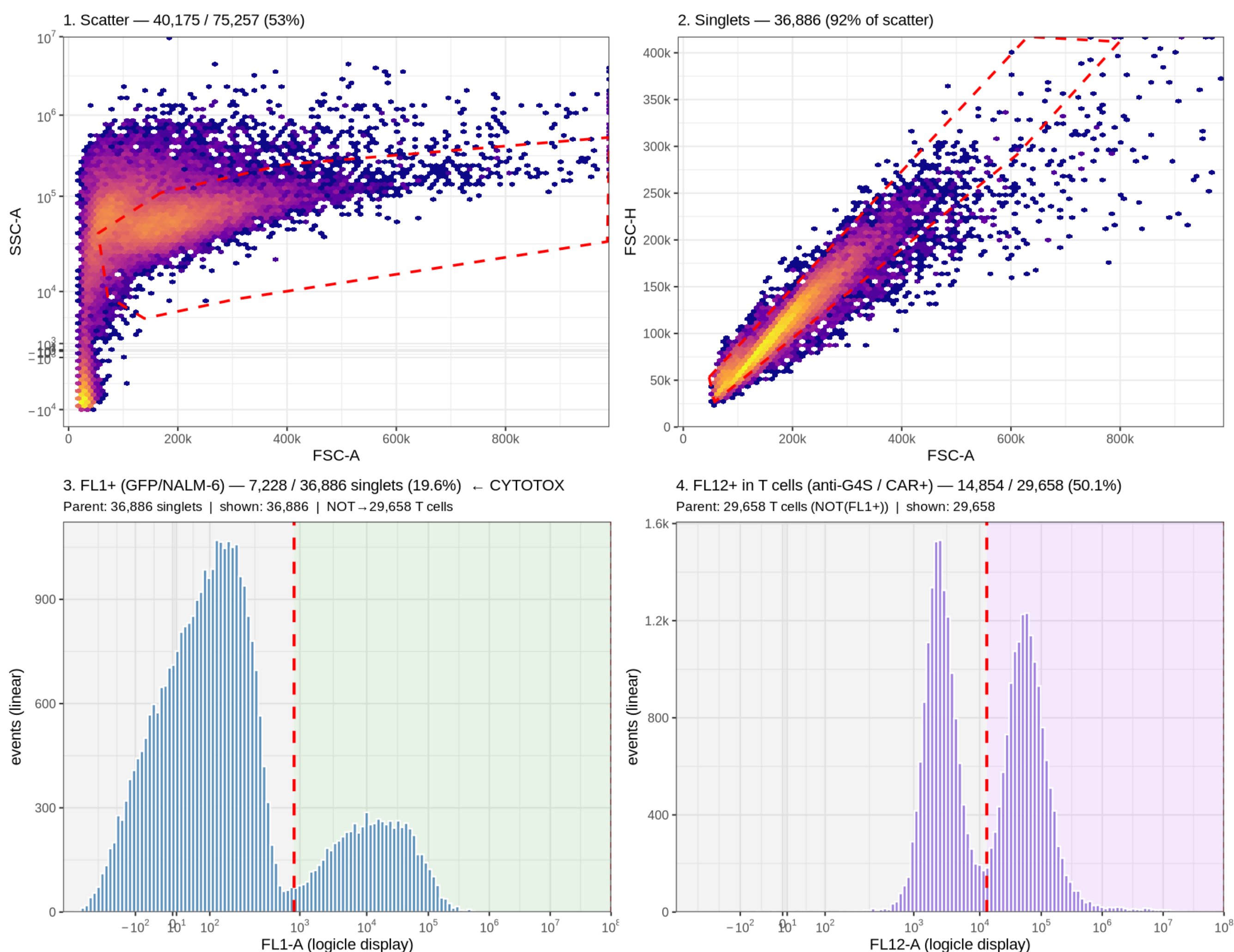
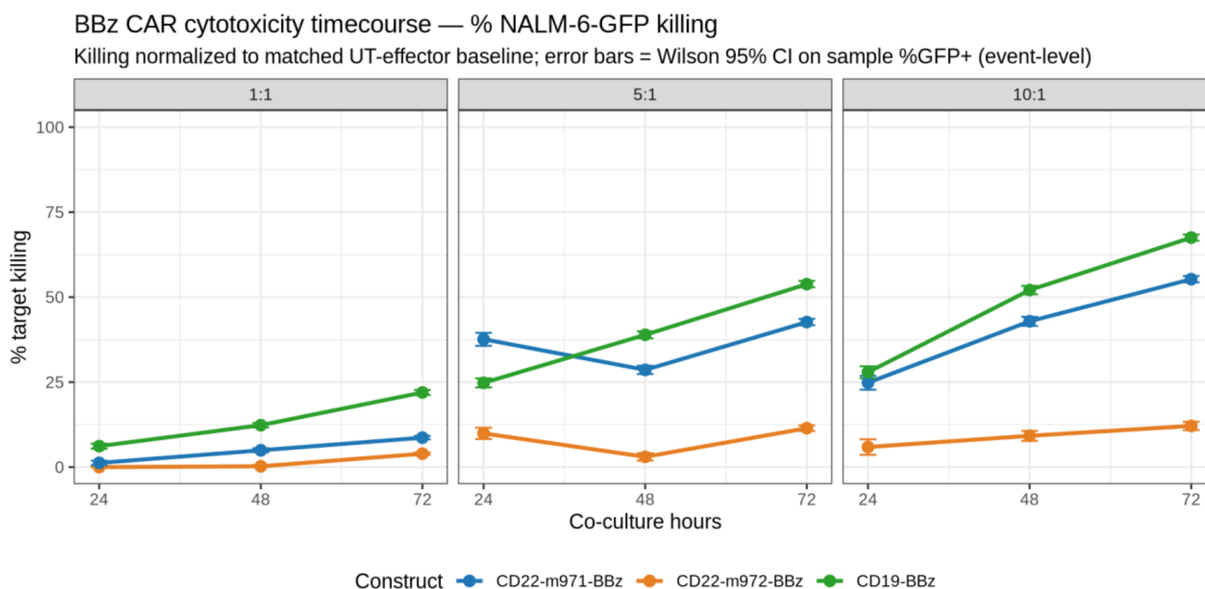


Figure 7. BBz CAR cytotoxicity timecourse. Percent target killing normalized to matched untransduced (UT) baseline at each E:T ratio and timepoint. m971-BBz (blue) outperforms m972-BBz (orange) at 5:1 and 10:1, with the gap widening over time. Error bars: Wilson 95% CIs.



High E:T ratios (5:1 and 10:1). At high effector-to-target ratios, m971-BBz consistently outperformed m972-BBz in target cell elimination, and the magnitude of this difference increased over time. At 10:1 / 72 hours, m972-BBz target survival (38.5%) was nearly double that of m971-BBz (19.6%) and almost three-fold higher than the CD19-BBz positive control (14.2%). The same pattern held at 5:1 / 72 hours, where m972-BBz showed 55.4% target survival versus 35.9% for m971-BBz and 28.9% for CD19-BBz. All pairwise comparisons at both E:T ratios were significant (Fisher's exact $p < 0.0001$; full statistics in Appendix D).

Figure 7 shows that m971-BBz reaches its killing plateau by the 48-hour timepoint at high E:T ratios, while m972-BBz killing continues to progress more slowly through the full 72-hour window; the gap between the two constructs widens over time rather than converging. At 10:1, the m972-vs-m971 difference in target survival grew from 2.9 percentage points at 24 hours to 18.9 percentage points at 72 hours, indicating a kinetic divergence in killing efficiency that anticipates the rechallenge results in §4.5.

Low E:T ratio (1:1). At the 1:1 ratio, killing was limited for all constructs. At 72 hours, target survival was 89.8% for m972-BBz, 85.4% for m971-BBz, and 73.0% for CD19-BBz, compared to 93.5% for the untransduced control. Pairwise differences were statistically significant (all $p < 0.0001$) but modest in absolute terms. The m971 advantage over m972 was 4.4 percentage points at 1:1 versus 18.9 percentage points at

10:1, indicating that the same directional advantage was present at both E:T ratios but scaled with effector dose.

Untransduced controls. The UT negative control showed expected dose-dependent target cell survival across all conditions, confirming that the observed killing in CAR-T conditions was antigen-specific rather than attributable to allogeneic T-cell reactivity. At 10:1 / 72 hours, UT co-cultures retained 43.8% GFP-positive targets (reflecting T-cell overgrowth diluting the GFP fraction), compared to 14.2-38.5% in the CAR conditions.

4.5 BBz CAR Rechallenge Series (RC-1 through RC-5)

A five-round serial rechallenge assay was added to the original experimental design to assess sustained killing capacity beyond the single-timepoint co-culture specified in the proposal (§3.6.2). This assay tested sustained killing under repeated antigen exposure at 1:1 E:T. Two readouts were quantified per round: %GFP-positive of singlets (target cell survival) and %CAR-positive of GFP-negative T cells (CAR surface retention). Figure 8 presents the rechallenge killing and CAR retention data across all five rounds.

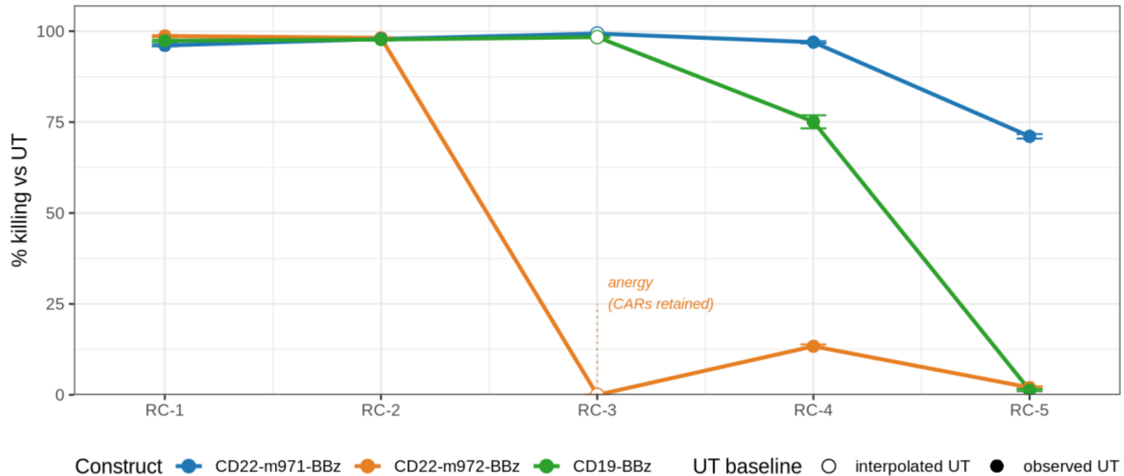
Figure 8. Rechallenge series. (A) Target killing normalized to per-round UT baseline. m972-BBz (orange) loses killing at RC-3; m971-BBz (blue) sustains >70% killing through RC-5. Open circle: interpolated UT. (B) CAR-positive retention (%FL12+ of GFP-negative T cells).

Rechallenge series — two-step exhaustion mechanism

m972 loses killing FIRST (RC-3 anergy, CARs still retained at 43%), then the anergic CAR+ cells die (RC-5 attrition, 0.7% CAR+).

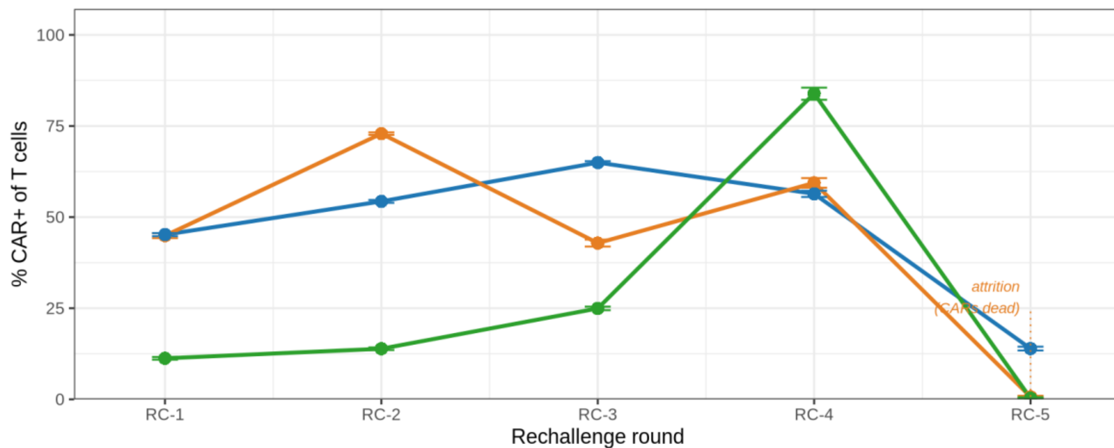
A. Target killing — % vs per-round UT baseline

% killing = 1 − (sample %GFP+ / per-round UT %GFP+); higher = more killing



B. CAR+ retention — % of NOT(FL1+) T cells

anti-G4S FL12+ in the NOT(FL1+) parent; higher = more CARs still on effectors



Rechallenge T:E ratio higher than timecourse → masks early m972 weakness in RC-1/RC-2 (target-limited regime).
RC-3 had no UT co-culture; UT baseline interpolated linearly from RC-2 (47.8%) and RC-4 (93.3%).

Early rounds (RC-1, RC-2): equivalent killing. In the first two rechallenge rounds, both m971-BBz and m972-BBz demonstrated robust target elimination, each clearing >98% of target cells. At RC-1, target survival was 0.5% for m972-BBz and 1.5% for m971-BBz; at RC-2, both remained below 1.1%. The UT control at RC-1 showed 38.7% target survival, confirming that CAR-mediated killing was the dominant mechanism. CAR surface retention was equivalent between m971-BBz and m972-BBz at RC-1 (45.2% vs. 44.9%; $p = 0.47$, not significant).

RC-3: m972 functional anergy. At the third rechallenge round, m972-BBz showed a sudden and complete loss of killing capacity. Target survival rose to 74.9%, while m971-BBz maintained near-complete killing at 0.5% survival and CD19-BBz at 1.1% (all pairwise $p < 0.0001$). Critically, CAR surface expression on m972-BBz T cells remained at 42.9% at RC-3; the CARs were still on the cell surface, but the cells had stopped killing. This dissociation between CAR retention and functional output is the hallmark of functional anergy rather than CAR downregulation or T-cell death. No untransduced co-culture was acquired at RC-3; the UT baseline for this round was linearly interpolated between RC-2 (47.8%) and RC-4 (93.3%) values, as described in §3.8.

RC-4 and RC-5: m972 population attrition. By RC-4, m972-BBz target survival remained elevated at 80.8%, while m971-BBz continued effective killing at 2.8% survival. CD19-BBz showed partial loss of function at RC-4, with target survival rising to 23.2%, suggesting the positive control was also experiencing antigen-driven stress by round 4.

At RC-5, the m972-BBz CAR-positive T-cell population had collapsed: only 0.7% of T cells retained CAR surface expression (approximately 31 of 4,533 T cells), compared to 13.9% for m971-BBz. Target survival in the m972-BBz condition was 90.1%, essentially equivalent to the UT control at 91.9%. CD19-BBz showed a parallel collapse: 0.3% CAR-positive and 90.7% target survival. Only m971-BBz retained meaningful CAR expression and killing capacity at RC-5, with 26.6% target survival ($p < 0.0001$ vs. both m972 and CD19).

Two-step exhaustion pattern. Figure 8 shows that m972-BBz loss of function proceeds through two distinct phases: (1) killing collapse at RC-3 with CARs still on the surface (~43% CAR-positive), followed by (2) loss of the CAR-positive population itself by RC-5 (0.7% CAR-positive). The mechanistic interpretation of this two-phase trajectory is discussed in Section 5.1.

4.6 SynNotch BFP Reporter Activation (Jurkats)

The SynNotch BFP assay measured antigen-dependent transcriptional activation in magnetically enriched Jurkat populations co-cultured with NALM-6-GFP targets at two T:E ratios (§3.6.3). BFP induction was quantified as %BFP-positive of singlets on the FL12 channel, with the BFP-positive threshold set from the UT negative sample (0.6%

BFP-positive background). Representative gating is shown in Figure 9; quantitative results are presented in Figure 10.

Figure 9. Representative SynNotch BFP gating walkthrough (m971-SynNotch, T:E 1.0). Six-panel layout: (1) scatter gate, (2) singlets, (3) FL12 x FL1 with ellipsoid BFP+ gate (40.2% BFP+), (4) FL12-A histogram. Panels 5-6 show the same gates applied to the UT negative control (0.6% BFP+), demonstrating that the gate excludes background events. An ellipsoid gate on FL12 x FL1 was used rather than a simple FL12-A threshold to account for spectral compensation effects between the Pacific Blue and FITC channels on the CytoFLEX LX. Red dashed ellipse is the original Floreada gate exported via Gating-ML (§3.7).

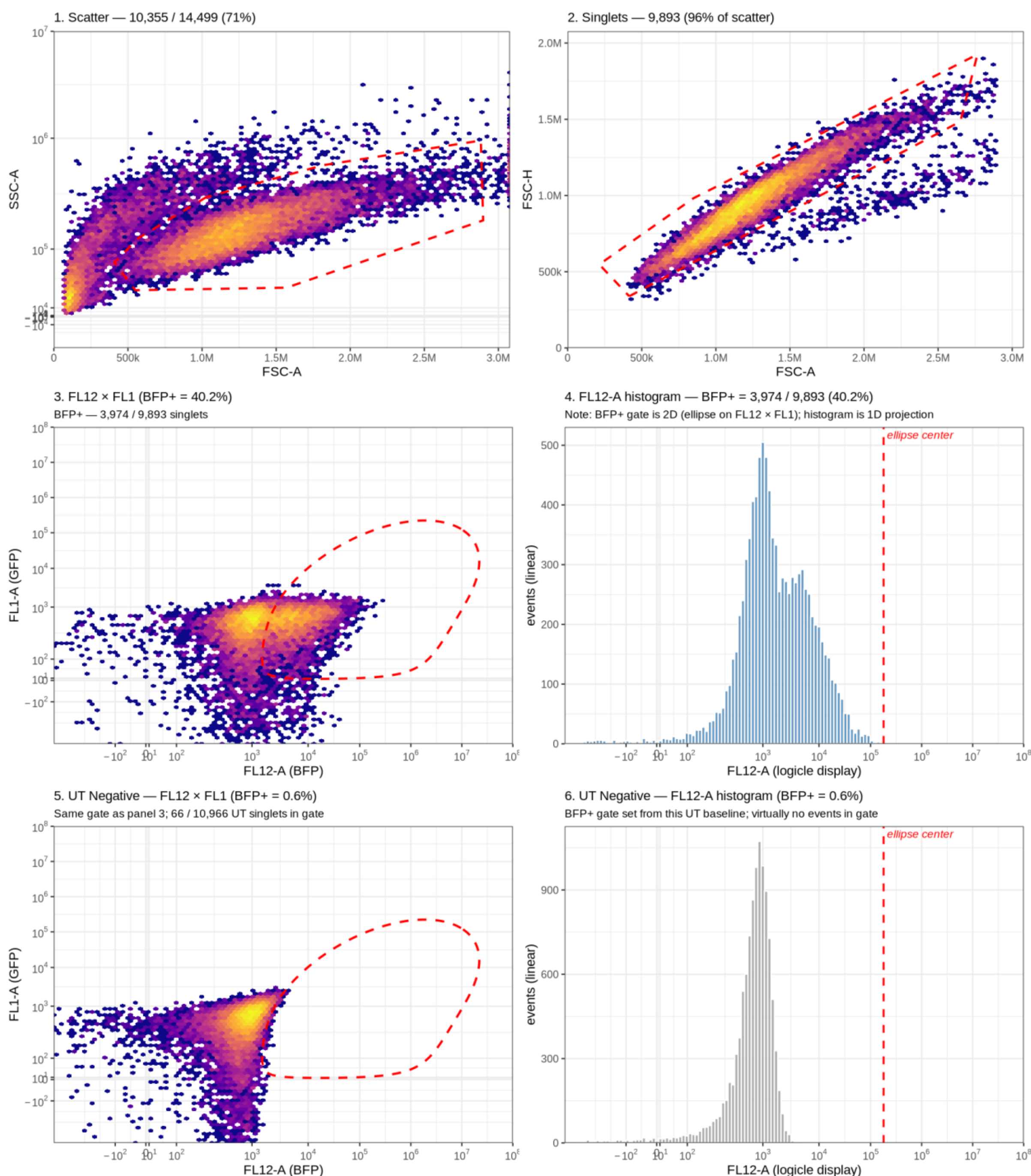
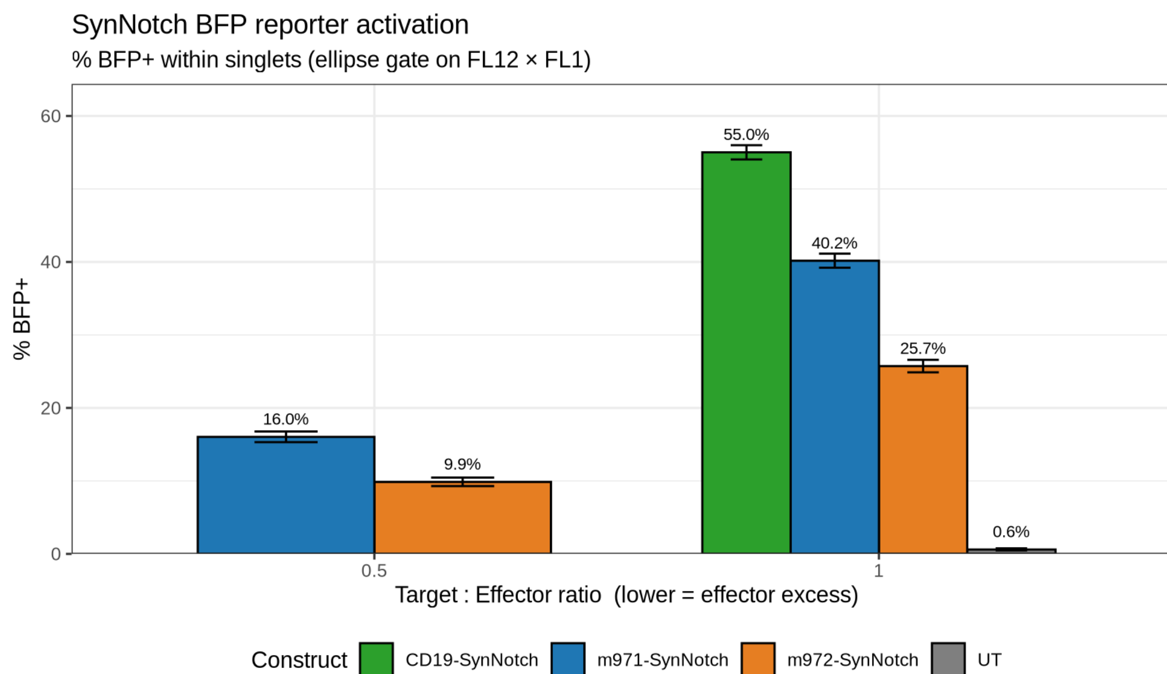


Figure 10. SynNotch BFP reporter activation. Percent BFP-positive within singlets for each SynNotch construct at T:E 0.5 and T:E 1.0. CD19-SynNotch positive control (p2 + p17, green) tested at T:E 1.0 only. m971-SynNotch (blue) produces higher activation than m972-SynNotch (orange) at both ratios. UT (grey) shows the BFP-negative baseline. Gating as described in Figure 9.



CD19-SynNotch control = p2 + p17 two-component (T:E = 1.0 only). m971/m972-SynNotch = single vectors, tested at T:E 0.5 and 1.0.
UT = untransduced Jurkats + NALM-6 (BFP-negative baseline). Ellipse gate on FL12 × FL1 accounts for Pacific Blue / BFP spectral overlap.

T:E 1.0 (equal target and effector numbers). At the 1:1 target-to-effector ratio, the CD19-SynNotch positive control (p2 + p17) produced the highest activation at 55.0% BFP-positive. m971-SynNotch (p158) showed 40.2% activation and m972-SynNotch (p159) showed 25.7% (all pairwise $p < 0.0001$). m971 produced approximately 1.6-fold higher BFP activation than m972 in the SynNotch context, a directionally consistent advantage with the BBz cytotoxicity results in §4.4.

T:E 0.5 (effector excess). At the 0.5:1 T:E ratio (fewer targets per effector), activation levels were lower across all constructs as expected: m971-SynNotch = 16.0%, m972-SynNotch = 9.9% ($p < 0.0001$). The CD19 positive control was not tested at T:E 0.5.

Interpretation context. These results are exploratory due to the single-replicate, single-timepoint design (§3.6.3) and the absence of a constitutive transduction marker on the p158/p159 constructs (§3.4). Because the Jurkat populations were magnetically enriched to approximately 100% SynNotch-positive before the assay, the %BFP-positive

readout primarily reflects SynNotch activation rate rather than the joint probability of transduction and activation. The consistent m971 > m972 activation pattern across both T:E ratios supports the conclusion that this difference is scFv-dependent rather than an artifact of differential transduction.

4.7 Cross-Readout Comparison

Figure 11. Cross-context scFv comparison. Top row: raw effectiveness (% killing at 10:1 / 72h for BBz CAR; % BFP-positive at T:E 1.0 for SynNotch), with UT (untransduced negative control) baseline included for reference. Bottom row: same data normalized to CD19 positive control = 100%. m972 retains 47% of CD19 effectiveness in SynNotch activation but only 18% in BBz killing, indicating a larger m971 advantage in the cytotoxic context.

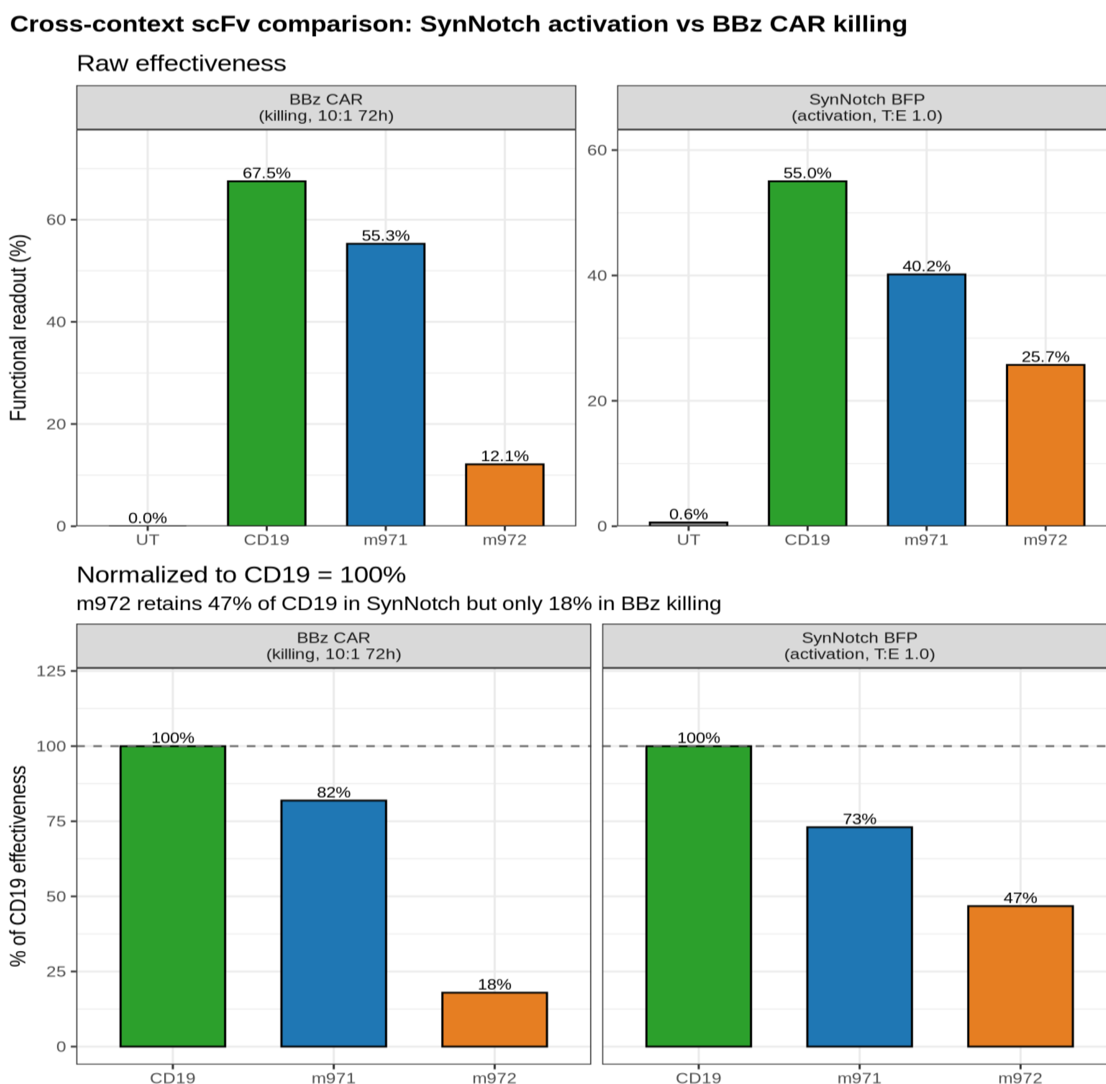


Figure 11 summarizes the m971-vs-m972 comparison across all three functional readouts. In every assay and condition tested, m971 outperformed m972:

- **BBz cytotoxicity (§4.4):** m971-BBz produced significantly lower target survival than m972-BBz at 5:1 and 10:1 E:T ratios across all timepoints (all $p < 0.0001$). The difference was largest at 10:1 / 72 hours (19.6% vs. 38.5% target survival).
- **Rechallenge durability (§4.5):** m971-BBz maintained killing capacity through all five rechallenge rounds, while m972-BBz underwent complete functional collapse by RC-3 (74.9% target survival) followed by CAR-positive population loss by RC-5 (0.7% CAR-positive).
- **SynNotch BFP activation (§4.6):** m971-SynNotch produced 40.2% BFP-positive activation versus 25.7% for m972-SynNotch at T:E 1.0 ($p < 0.0001$).

Across all three functional readouts, m971 outperformed m972 in every assay and condition tested. The interpretation of this consistent, unidirectional finding and its relationship to the project proposal's original hypothesis is discussed in Section 5.

The Incucyte S3 live-cell kinetic killing assay, which was proposed as a stretch objective to generate real-time EC50 values, was not performed due to the timeline compression caused by the m972 cloning delay (§3.1.1, §3.6.4). Its potential value is discussed in Section 5.4 (Future Work).

4.8 Business Deliverables

Two business deliverables were produced alongside the STEM experimental work, addressing Business Objectives #5 and #6 from the project proposal.

Standard Operating Procedure (Business Objective #5). A complete SOP (SOP-PL-001) for the scFv A/B testing workflow was developed, codifying the end-to-end pipeline validated during this project into a reusable protocol for future scFv screening campaigns at the Pulsipher Laboratory. The SOP defines six stages with QC checkpoints and go/no-go decision criteria at each transition:

Stage	Activity	QC Checkpoint
1	Construct design	Sequence sourced and codon-optimized
2	Cloning and verification	Plasmidsaurus nanopore sequencing PASS
3	Lentiviral production	p24 ELISA titer >=10^6 TU/mL
4	Transduction and enrichment	Anti-G4S+ expression confirmed by flow
5	Functional screening	Co-culture assays completed per design
6	Analysis and decision	Lead scFv recommendation memo to PI

The SOP incorporates lessons learned from this project, including codon diversification for GC-rich linker regions to mitigate gBlock synthesis risk (§3.1.1), the magnetic enrichment protocol as a standard step rather than an optional add-on, and the recommendation to include a constitutive transduction marker in all future constructs. Total estimated timeline is 7 to 9 weeks for a clean run, with 2 to 3 weeks of additional float budgeted for synthesis or cloning failures. The full SOP is provided in Appendix E.

Cost-per-construct analysis (Business Objective #6). A make-vs-buy analysis was performed comparing in-house scFv construct production and testing against outsourced alternatives at two scopes: plasmid construction alone, and the full A/B screening workflow. Table 4 summarizes the key cost findings.

Table 4. In-house vs. outsourced cost comparison.

Scope	In-House	Outsourced	Ratio
Plasmid construction (per construct)	~$844	~$400-1,100 (VectorBuilder/GenScript)	Comparable
Full A/B workflow (per final construct)	~$2,163	~$3,150-4,400 (CRO estimate)	In-house ~2x cheaper
Total project cost (4 final constructs)	~$8,651	~$12,600-17,600	In-house saves ~$4,000-9,000
Total personnel hours	~180 h	~80-90 h (in-house portion only)	Outsourcing saves ~50% of labor

In-house production is the recommended default for routine scFv screening at the Pulsipher Laboratory because it is approximately half the cost of outsourcing, provides faster failure recovery, and maintains full IP control. Outsourcing is a reasonable alternative for plasmid construction specifically when internal capacity is limited, as per-plasmid costs are comparable. The full cost analysis with line-item reagent accounting, labor rate documentation, and CRO quote specifications is provided in Appendix F.

5. Discussion and Recommendations

5.1 Interpretation: m971 vs. m972 Across Functional Contexts

Across all three functional readouts evaluated in this study, m971 consistently outperformed m972: lower target survival in the BBz CAR cytotoxicity timecourse, sustained killing through five serial rechallenge rounds where m972 collapsed, and higher BFP reporter activation in the SynNotch transcriptional context (§4.7, Figure 11). All primary pairwise comparisons favored m971 ($p < 0.0001$). These results favor m971 as the lead candidate for both the CAR effector and SynNotch gating roles in the laboratory's prime-and-kill program.

Before interpreting the mechanistic basis for this finding, I want to note what the data did not show. The project was designed to test whether m972's higher CD22 binding affinity might confer an advantage in the SynNotch gating context, even if m971 remained superior for direct cytotoxic killing. The data do not support that hypothesis; m971 outperformed m972 in both functional roles. While this simplifies the construct selection decision for the laboratory, it also means that the higher-affinity variant does not fill the niche that motivated the dual-context experimental design.

The m971/m972 Paradox

The project proposal framed the central scientific question around a paradox in the CD22 scFv literature: m972 binds CD22 with approximately 10-fold higher affinity than m971, yet m971-based clinical constructs have demonstrated more robust anti-leukemic activity (Haso et al., 2013). Three hypotheses were on the table at the outset: (1) epitope accessibility, where the m972 epitope may be sterically hindered on B-ALL cell surfaces; (2) binding kinetics affecting serial killing, where high-affinity m972 may bind so tightly that it cannot disengage and re-engage successive targets; and (3) tonic signaling differences, where m972 may signal constitutively without antigen, leading to premature exhaustion (Long et al., 2015).

I interpret the data from this project as most consistent with a combination of hypotheses #2 and #3, and the rechallenge series provides the clearest mechanistic window into the failure mode. The two-step exhaustion pattern observed in m972-BBz (Figure 8) is particularly informative:

Phase 1: Functional anergy (RC-3). At RC-3, m972-BBz T cells retained 42.9% CAR surface expression but had lost the ability to kill targets (74.9% target survival vs. 0.5% for m971). This dissociation between surface expression and function is the hallmark of T-cell anergy rather than CAR downregulation or cell death. High-affinity m972 CARs may form such stable immunological synapses that the T cells cannot efficiently disengage from killed targets and re-engage new ones; the cumulative cost of prolonged synapse formation across three antigen exposures manifests as functional exhaustion. The m971 CARs, with lower binding affinity, may form shorter-duration synapses that allow more efficient serial target engagement.

Phase 2: Population attrition (RC-5). By RC-5, the m972-BBz CAR-positive population collapsed to 0.68% (~31 of 4,533 T cells), with target survival essentially equivalent to the untransduced control. This second phase is consistent with exhaustion-driven cell death mediated by sustained tonic signaling through the high-affinity scFv. Tonic signaling is a recognized driver of CAR-T exhaustion (Long et al., 2015), and m972's higher affinity may lower the activation threshold sufficiently that incidental receptor clustering triggers signaling even between rechallenge rounds, driving the exhausted cells past a point of no return.

Disposition of hypothesis #1 (epitope accessibility). Epitope accessibility would predict a uniform deficit in m972 function across all timepoints and assay contexts; it would not explain the kinetic divergence pattern in which m972 performs equivalently to m971 in early rechallenge rounds (RC-1 and RC-2: both constructs cleared >98% of targets) but diverges sharply at RC-3. The data therefore do not support epitope accessibility as the primary mechanism for the m971/m972 performance difference. However, it was well beyond the scope of this project to measure binding kinetics or tonic signaling, so the mechanistic interpretation remains correlative.

The Role-Specificity Question

The project proposal hypothesized that scFv requirements might differ between SynNotch gating and CAR-mediated killing, providing the scientific rationale for testing both constructs in both functional contexts. Specifically, it was plausible that a higher-affinity scFv (m972) could be advantageous for SynNotch gating, where sustained antigen engagement drives Notch1 cleavage and Gal4-VP64 release, while a lower-affinity scFv (m971) might perform better for cytotoxic killing, where rapid target disengagement enables serial killing.

The data do not support this hypothesis. m971 outperformed m972 in both contexts: BBz cytotoxicity (19.6% vs. 38.5% target survival at 10:1 / 72 h), sustained killing (maintained through RC-5 vs. collapsed at RC-3), and SynNotch BFP activation (40.2% vs. 25.7% at T:E 1.0). The lack of role-specificity is itself a mechanistic finding worth noting. It suggests that the binding-kinetics property that impairs m972 in serial killing also limits its SynNotch transcriptional output. One plausible explanation is that SynNotch activation, like serial killing, benefits from productive engagement followed by release. The Notch1 cleavage event that initiates the SynNotch signaling cascade requires mechanical force generated during receptor-ligand interaction (Roybal et al., 2016). If m972's high affinity results in overly stable receptor-ligand complexes, the conformational strain required for efficient Notch1 S2 cleavage may be suboptimal, reducing per-encounter activation probability. The approximately 1.6-fold difference in %BFP-positive (m971 vs. m972) compared to the modest difference in per-cell BFP median fluorescence intensity (§4.6) supports this interpretation: m971's advantage is primarily in the fraction of cells that activate, not in the intensity of activation per cell, consistent with a model in which m971 enables a higher probability of productive cleavage events per encounter.

5.2 Implications for the Pulsipher Laboratory

These findings inform the Pulsipher Laboratory's ongoing development of a prime-and-kill CAR-T architecture targeting B-ALL. The prime-and-kill design requires selecting an scFv for two distinct functional roles: a SynNotch gate scFv that detects antigen A and drives transcription, and a CAR effector scFv that kills cells expressing antigen B. The project proposal specified that this comparative study would generate "evidence-based recommendations for lead scFv selection for each functional role" as a key sponsor deliverable.

The original design question was whether these two roles might require different scFvs optimized for different binding properties. The data simplifies this decision substantially: m971 is the stronger performer in both roles. This means the laboratory does not need to pursue a split-scFv design in the next phase of construct development. For the CAR effector role, m971-BBz (p156) demonstrated superior acute cytotoxicity and, critically, sustained killing capacity under serial antigen challenge. For the SynNotch gating role, m971-SynNotch (p158) produced higher BFP activation at both

T:E ratios tested. I therefore recommend advancing m971 as the lead CD22-targeting scFv for both arms of the prime-and-kill program.

This recommendation carries a practical benefit beyond the biology: using the same scFv for both roles reduces the number of construct variants that the laboratory must carry forward into confirmatory studies, simplifying the cloning, viral production, and QC pipeline for the next development phase.

The m972 variant is not without value. Its demonstrated high-affinity binding to CD22 and its successful in-house synthesis (§3.1.1) mean it remains available as a backup candidate or as a reference construct for future studies examining affinity-dependent CAR-T phenomena. The two-step exhaustion phenotype observed in the rechallenge assay (§4.5) is itself a useful tool for the laboratory: m972 could serve as a positive control for exhaustion in future assays designed to evaluate anti-exhaustion interventions (e.g., checkpoint blockade, epigenetic modifiers, or armored CAR designs).

Implications for the Laboratory's Screening Pipeline

Beyond the STEM findings, the two business deliverables (§4.8) have practical implications for the laboratory's screening pipeline. Before this project, the laboratory had no standardized process for comparative scFv evaluation; each campaign was designed ad hoc. The SOP (SOP-PL-001, Appendix E) changes that by providing a repeatable six-stage pipeline that any trained lab member can execute, with lessons from this project's failures embedded as standing practice.

The cost-per-construct analysis (Appendix F, Table 4) demonstrates that the in-house workflow is approximately half the cost of outsourcing (~$2,163 vs. ~$3,150-4,400 per final construct), and this advantage increases with screening volume as fixed costs amortize across more constructs. The IDT synthesis failure also provided an unplanned data point on the hidden costs of vendor dependencies: the in-house staircase PCR recovery took approximately one week at ~$150-300, while a vendor re-order would have required 2-3 additional weeks. For a laboratory on a fixed academic timeline, that recovery capability is a strategic asset.

Objective Scorecard

All six project objectives were addressed, though several were modified from their original specification due to the schedule compression caused by the m972 cloning delay. STEM Objectives #1 (construct generation) and #2 (lentiviral titers exceeding

10^6 TU/mL) were fully met. STEM Objective #3 (transduction efficiency >30%) was met through the combined transduction and magnetic enrichment pipeline rather than by raw transduction efficiency alone (§4.3). STEM Objective #4 (functional comparison of m971 vs. m972) was met and exceeded, with three complementary readouts including the rechallenge series that was added beyond the proposal scope. Business Objectives #5 (SOP) and #6 (cost analysis) were both completed as specified. The modifications to how objectives were achieved are detailed in §5.5 (Table 5).

5.3 Limitations

Several limitations should be considered when interpreting the results of this study.

Single biological replicate (N(biological) = 1). All functional assays used a single PBMC donor preparation (BBz arm) and a single Jurkat transduction batch (SynNotch arm), a direct consequence of the schedule compression from the m972 cloning delay (§3.1.1). The statistical framework and its interpretive boundaries are described in §3.8; in brief, the reported p-values confirm that proportions differ within this experiment but do not address biological reproducibility across independent donors. This is the single most important limitation of the study. Replication is the highest-priority future work item (§5.4).

No CD22-Fc functional QC for antigen binding. CD22-Fc/APC staining was dropped due to reagent cost (§3.5). The anti-G4S antibody confirms CAR surface expression but does not directly verify antigen-binding competence. This is a minor limitation given that m971 and m972 are literature-validated scFvs (Haso et al., 2013), and the functional assays themselves confirm target engagement.

Incucyte kinetic assay not performed. This stretch objective was dropped due to the cloning delay (§3.6.4). Its absence means the serial-killing hypothesis (§5.1) is supported by correlative endpoint evidence rather than direct kinetic measurements.

SynNotch assay in Jurkats only. The SynNotch assay was limited to Jurkat T cells rather than run in parallel with primary T cells, as a triage decision following the cloning delay (§3.4). Jurkats provide a controlled baseline for scFv-dependent activation comparisons but do not fully recapitulate primary T-cell biology; these results should be considered exploratory.

Absence of constitutive transduction marker on SynNotch constructs. The p158/p159 constructs lacked a constitutive mCherry marker (§3.4), meaning the %BFP-

positive readout reflects the joint probability of transduction and activation. Pre-assay magnetic enrichment to high positivity (§3.5) partially mitigates this, and the consistent m971 > m972 pattern at both T:E ratios argues against a transduction artifact.

Additional methodological notes. Three further points are worth noting, though none biases the between-construct comparisons. First, all flow cytometry was performed on formalin-fixed cells, precluding live/dead discrimination; future experiments should consider live-cell acquisition with a viability dye. Second, no untransduced co-culture was acquired at rechallenge round 3; the UT baseline was linearly interpolated between RC-2 and RC-4 (§3.8). Third, the shift from the proposal's GraphPad Prism / two-way ANOVA approach to R / Fisher's exact tests was driven by the N(biological) = 1 constraint and is described in §3.8.

5.4 Possible Future Work

The following future work items are organized to address the limitations identified in §5.3, with each limitation paired to the specific experiment or modification that would resolve it.

Biological replication across additional donors. As recommended in §5.3, replication in 2-3 additional PBMC donors is the highest-priority next step. Much of the cloning and viral production work has been completed; the existing validated stocks enable efficient re-execution. Replication would enable cross-replicate statistical inference (the two-way ANOVA originally specified in the proposal) and confirm that the m971 advantage is scFv-dependent rather than donor-specific. Estimated cost: approximately $2,000 in reagents per donor; timeline: 4-6 weeks per round.

Addition of constitutive mCherry transduction marker. Next-generation SynNotch-BFP constructs (p158 v2, p159 v2) should incorporate a PGK-mCherry cassette, matching the two-component p2 + p17 positive control architecture, to enable clean separation of transduction efficiency from SynNotch activation rate. This is a single cloning step using the same inverse PCR and NEB HiFi assembly workflow validated in this project. Estimated timeline: 4 weeks for cloning, sequence verification, and viral packaging. This addition would strengthen any future A/B screening campaign that uses a SynNotch reporter readout and would provide independent confirmation of the activation differences observed here.

Primary T-cell SynNotch assay. The SynNotch BFP comparison should be repeated in primary human T cells to confirm that the Jurkat results translate to the cell type relevant for clinical application. This would also enable assessment of whether primary T-cell-specific factors (e.g., endogenous Notch signaling, activation state variability) modify the scFv-dependent activation differences observed in Jurkats.

Incucyte S3 kinetic killing assay. This assay, originally proposed as a stretch objective, would generate real-time killing curves over 72 hours and enable EC50 calculations for each scFv variant. It would provide the most direct test of the serial-killing / binding-kinetics hypothesis proposed in §5.1. If m971 shows faster initial killing kinetics and a more sustained killing plateau compared to m972, this would directly support the hypothesis that lower-affinity CARs enable more efficient serial target engagement. The Incucyte instrument is available at the Huntsman Cancer Institute, and the assay can be performed using existing viral stocks.

CD22-Fc/APC antigen-binding QC. Including CD22-Fc/APC staining in the next round of experiments would provide direct confirmation of antigen-binding competence alongside the anti-G4S expression QC. This addresses a minor limitation but would strengthen the interpretive framework, particularly if the study is expanded to include additional scFv variants.

In vivo validation. The ultimate test of the m971 lead-construct recommendation is in vivo efficacy. An NSG mouse B-ALL xenograft model, using NALM-6 or a patient-derived xenograft, would evaluate the m971-based prime-and-kill construct under conditions that include tumor microenvironment immunosuppression, T-cell trafficking and persistence, and sustained antigen pressure over weeks rather than days. This is a longer-term objective that should follow successful biological replication and primary-T-cell SynNotch confirmation in vitro.

Full prime-and-kill construct assembly. The modular comparison performed in this project (testing each scFv in isolated CAR and SynNotch contexts) was a necessary first step. The next phase should assemble the complete prime-and-kill circuit using m971 for both the SynNotch gate (detecting one antigen) and the inducible CAR (killing via a second antigen), then test the integrated system for AND-gate logic fidelity, on-target specificity, and off-target safety.

5.5 Proposal vs. Actual Summary

Table 5 summarizes all deviations between the project as proposed and as executed. All scope reductions trace to the m972 gBlock synthesis failure (§3.1.1), which consumed all schedule float and forced the triage decisions described in §3.1.1.

In retrospect, this project was over-scoped for the available timeline. The proposal committed to four constructs, three functional assays, two business deliverables, and biological replicates within a nine-week bench window that assumed a two-week cloning phase. A more conservative scope might have designated the SynNotch arm as a stretch objective or ordered backup synthesis from a second vendor at the outset. The lesson is that projects dependent on a critical-path cloning step with vendor risk should budget at least two weeks of dedicated float for that step. This observation is developed further in the professional reflection (§6).

Table 5. Proposal vs. Actual Execution Summary.

Planned (Proposal)	Executed (Actual)	Reason for Change
4 lentiviral constructs by Week 2	6 constructs completed (4 final + 2 precursor); approximately 1 month delayed	IDT m972 gBlock synthesis failure; recovered by in-house staircase PCR assembly
Co-culture cytotoxicity timecourse (E:T 1:1 / 5:1 / 10:1 at 24 / 48 / 72 h)	Cytotoxicity timecourse completed at proposed E:T and timepoint grid, plus 5-round serial rechallenge series (added)	Rechallenge added as orthogonal readout for sustained killing capacity
SynNotch BFP in primary T cells and Jurkats in parallel	SynNotch BFP in Jurkats only	Time pressure after cloning slip; Jurkats provide a more controlled reporter baseline
CD22-Fc/APC + anti-G4S linker for transduction QC	Anti-G4S linker on Pacific Blue only	Budget constraint; CD22-Fc reagent cost
GraphPad Prism with two-way ANOVA and Bonferroni correction	R with event-level Fisher's exact test and Wilson CIs	Tooling preference for reproducibility; ANOVA not appropriate at N(biological) = 1
Incucyte S3 kinetic killing assay (stretch objective)	Not performed	Stretch goal explicitly contingent on schedule float; float consumed by cloning delay
9-week timeline (Jan 26 to Apr 1)	Bench work completed Apr 3; report drafting begun Apr 6	Cloning delay absorbed all schedule contingency

6. Professional Reflection

The gap between knowing and doing. This project continuously showed me the distance between having knowledge and applying it. One of my favorite classes in the PSM program was project management; I love thinking about these sorts of big-picture, strategic decision making and planning problems. Yet, my love for phrases like "The first 90% of the project takes 90% of the time. The last 10% takes the other 90%." did not protect me from the planning fallacy. I *definitely* planned this project along optimistic timelines, and I knew it.

Scope, rigor, and what I'm proud of. I am disappointed that, because of the (inevitable) unexpected delays and failures, this project does not reflect the scientific rigor I would have preferred for my PSM final project; to be precise, I really would have liked to complete the Incucyte analysis, and given more time all experiments could have been done in triplicate rather than n=1. If I had another month, I probably would have redone the viral production and transduction, to have a higher starting titer, but this was completely infeasible in the time frame I set. Still, I am proud that I was able to pull it together to the extent I did given the limited timeline and the over-scoped goals. In larger part, this project went as well as it did because of the knowledge I gained in the PSM Biotechnology program, and therefore as a reflection of what I learned during this course I think it was a success.

Connections to PSM coursework. There is no doubt that my project management and planning skills have vastly improved since I started this program. So many of the planned contingencies that allowed me to bring this project to its current state would never have occurred to me before I started the project. The technical aspects of the project also directly reflected my coursework. Applied Statistics was my first exposure to R, which formed the backbone of my analysis work here and much of my work in my current employment. Molecular Biology provided the cloning and PCR foundations that made the staircase-PCR recovery possible. Cell Structure and Signalling informed my understanding of the GAL4/UAS transcriptional activation system underlying the SynNotch design. Molecular Mechanisms of Oncology gave me the context to identify m971 and m972 as CD22-targeting variants worth evaluating against the parental FMC63 construct. Bioanalytical Chemistry grounded the flow cytometry and spectral analysis work that ran throughout the project.

Technical skills developed through failure. The technical skills I developed during this project were, in many ways, the product of things going wrong. When IDT was unable to synthesize the m972 gBlock after two attempts, I designed and executed a staircase PCR assembly from 24 overlapping oligonucleotides to synthesize the 720 bp scFv de novo. That recovery would not have been possible without the molecular cloning foundations I built in Molecular Biology; nor would I have thought to attempt it before this program. I probably could not have accomplished it without the teaching and support of my lab colleague Payton Utzman, who helped me design and complete the synthesis in a timely and correct manner.

What I learned about working in this sector. Working in the Pulsipher Lab taught me lessons about how science operates in an academic translational setting that I would not have learned in a classroom. The IDT synthesis failure was the clearest example: outsourcing gene synthesis looked like the cheaper and faster option in my proposal cost analysis, but the failure mode was not priced into the quote. That single vendor failure cascaded into a one-month schedule slip, which forced me to drop the Incucyte kinetic killing assay entirely and limit SynNotch testing to Jurkats only instead of running it in parallel with primary T cells. The lesson I took from this is that make-vs-buy decisions in a research setting need to account for recovery time when the "buy" option fails, not just the sticker price.

What I would do differently. If I were to do this project again, there are several things I would change. First, I would order backup gBlocks from a second vendor in parallel for any high-risk synthetic fragments; the IDT failure could have been absorbed without timeline impact if a redundant order had been placed at the start. Second, I would triage scope earlier. Yes, I knew we wouldn't make it to Incucyte by the time the second IDT synthesis failed, but I *should* have triaged that item before the project even began; fortunately, listing that item as a "stretch goal" gave me a smooth exit ramp.

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7. REFERENCES

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Appendix A: Plasmid Maps and Sequence Verification

Annotated plasmid maps for all constructs. Final constructs (p156-p159) were used in functional assays; precursor constructs (p154-p155) were cloned as intermediates. All verified by whole-plasmid nanopore sequencing (Plasmidsaurus).

A.1 Final BBz CAR Constructs

p156 — pELPS-CD22-m971-BBz
9,186 bp

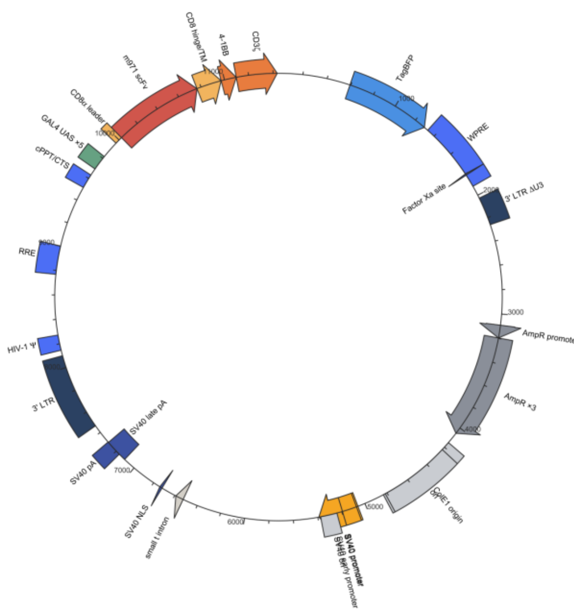
p157 — pELPS-CD22-m972-BBz
9,168 bp

A.2 Final SynNotch-BFP Constructs

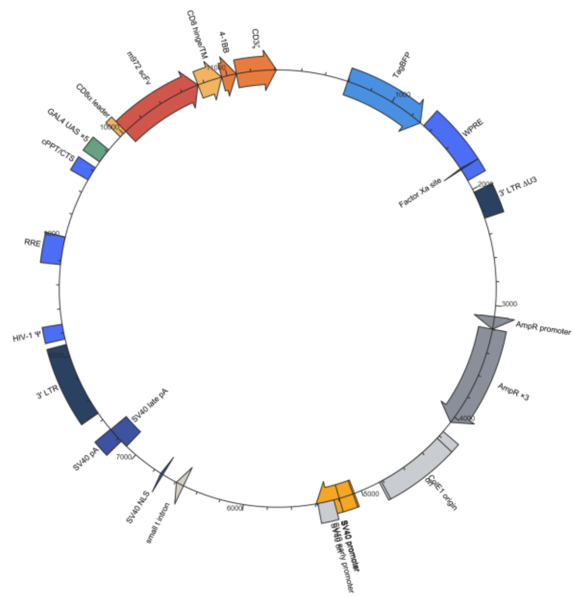
p158 — UAS-CD22-m971-CAR-BFP
12,759 bp

p159 — UAS-CD22-m972-CAR-BFP
12,741 bp

p158B — UAS-CD22-m971-CAR-BFP
11,443 bp

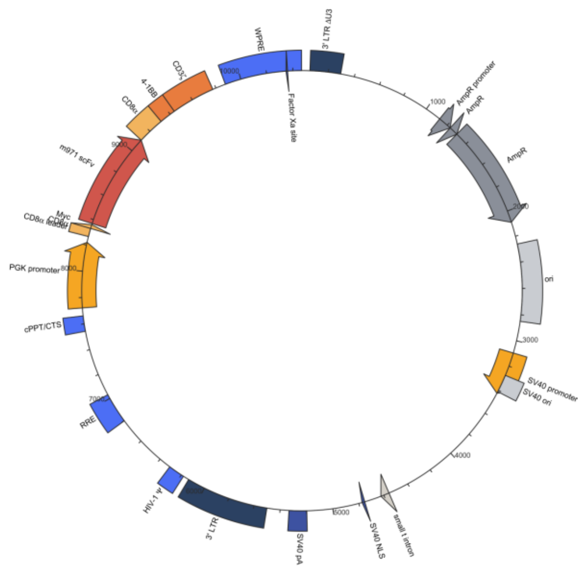


p159B — UAS-CD22-m972-CAR-BFP
11,425 bp

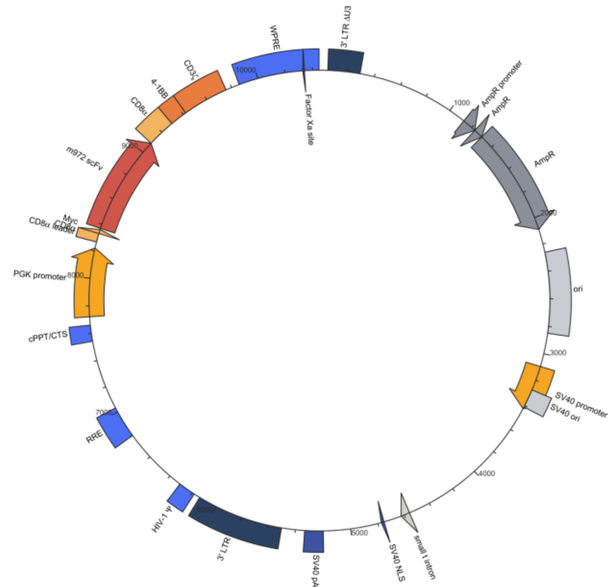


A.3 Precursor Constructs (Not Used in Functional Assays)

p154 — CD22-m971-41BBz
10,468 bp



p155 — CD22-m972-41BBz
10,450 bp



A.4 Sequence Verification

Plasmid	Construct	Expected (bp)	Observed (bp)	Status
p154	CD22-m971-41BBz	10,468	10,468	Verified
p155	CD22-m972-41BBz	10,450	10,450	Verified
p156	pELPS-CD22-m971-BBz	9,186	9,186	Verified
p157	pELPS-CD22-m972-BBz	9,168	9,168	Verified
p158	UAS-CD22-m971-CAR-BFP	12,759	12,759	Verified
p159	UAS-CD22-m972-CAR-BFP	12,741	12,741	Verified

The 18 bp systematic size difference between each m971/m972 pair reflects the intrinsic length difference between the two scFv coding sequences.

Appendix B: Spectral Panel Reference

This appendix provides the full cytometer channel configuration and staining panel for all flow cytometry experiments in this project, referenced from Section 3.7 (Flow Cytometry Acquisition and Analysis). All acquisition was performed on a Beckman Coulter CytoFLEX LX (serial BB09006) using CytExpert software.

B.1 Instrument Configuration

Parameter	Value
Instrument	Beckman Coulter CytoFLEX LX
Serial number	BB09006
Operator	Pulsipher
Software	CytExpert
Compensation	Embedded in FCS metadata ($SPILLOVER key); applied automatically during analysis
Pulse parameters	Both height (-H) and area (-A) recorded; gating uses area

B.2 Active Channels

Only two fluorescence channels carried biology-relevant signal across all experiments in this project. All other channels were present in the acquisition template but unused.

Channel	Filter	Fluorophore	Marker	Detection role	Assays
FL1	525/40 (blue laser)	FITC / GFP	NALM-6-GFP target cells	Target identification	All (timecourse, rechallenge, SynNotch)
FL12	450/45 (violet laser)	Pacific Blue	Anti-G4S linker antibody (CAR detection)	CAR expression	BBz timecourse, rechallenge, TD-efficiency
FL12	450/45 (violet laser)	BFP	SynNotch-induced BFP reporter	SynNotch activation	SynNotch BFP co-culture

Note: FL12 serves dual roles across experimental arms. In BBz CAR experiments, Pacific Blue signal from the anti-G4S linker antibody identifies CAR-positive T cells. In the SynNotch arm, the same channel reads construct-encoded BFP induction. This spectral overlap between the anti-G4S detection antibody and the BFP reporter is the reason SynNotch constructs (p158/p159) cannot be assayed for transduction efficiency via the standard FL12 staining protocol (Section 5.3).

B.3 Rechallenge / Timecourse / TD-Efficiency Template (28 Parameters)

Used by: cytotoxicity timecourse (2026-03-28), rechallenge rounds RC-1 through RC-5, and initial transduction efficiency measurements.

Channel	$PnN	$PnS (filter)	Laser / filter	PMT Gain	Role in this project
—	FSC-H	FSC-H	Forward scatter, height	—	Doublet exclusion (FSC-H vs FSC-A)
—	FSC-A	FSC-A	Forward scatter, area	—	Scatter gating, singlet gate
—	SSC-H	SSC-H	Side scatter, height	—	—
—	SSC-A	SSC-A	Side scatter, area	—	Scatter gating (FSC-A vs SSC-A)

Channel	$PnN	$PnS (filter)	Laser / filter	PMT Gain	Role in this project
FL1	FL1-A	FITC-A	Blue 525/40	10	**GFP: NALM-6 target identification**
FL2	FL2-A	B610-20-A	Blue 610/20	182	Unused
FL4	FL4-A	PE-A	Yellow-green 585/42	50	Unused
FL5	FL5-A	mCherry-A	Yellow-green 610/20	176	Unused
FL8	FL8-A	PE-Cy7-A	Yellow-green 780/60	600	Unused
FL9	FL9-A	APC-A	Red 660/20	382	Unused
FL11	FL11-A	APC-Cy7-A	Red 780/60	700	Unused
FL12	FL12-A	V450-45-A	Violet 450/45	200	**Pacific Blue: CAR detection (anti-G4S)**
FL13	FL13-A	BV510-A	Violet 525/40	300	Unused
FL14	FL14-A	BV605-A	Violet 610/20	600	Unused
FL15	FL15-A	BV650-A	Violet 660/20	800	Unused
—	FSC-Width	FSC-Width	Pulse width	—	—
—	Time	Time	Acquisition time	—	—

B.4 SynNotch BFP Template (14 Parameters)

Used by: SynNotch BFP co-culture experiment (2026-03-26). A stripped-down acquisition template with four fluorescence channels.

Channel	$PnN	$PnS (filter)	Laser / filter	Role in this project
—	FSC-H	FSC-H	Forward scatter, height	Doublet exclusion
—	FSC-A	FSC-A	Forward scatter, area	Scatter gating
—	SSC-H	SSC-H	Side scatter, height	—
—	SSC-A	SSC-A	Side scatter, area	Scatter gating
FL1	FL1-A	FITC-A	Blue 525/40	**GFP: NALM-6-GFP target cells**
FL4	FL4-A	PE-A	Yellow-green 585/42	Unused
FL5	FL5-A	mCherry-A	Yellow-green 610/20	Unused
FL12	FL12-A	V450-45-A	Violet 450/45	**BFP: SynNotch induced reporter**
—	FSC-Width	FSC-Width	Pulse width	—
—	Time	Time	Acquisition time	—

FL5 (mCherry) was included in the acquisition template as a potential transduction marker channel. However, the CD22 SynNotch-BFP constructs (p158, p159) do not encode a constitutive mCherry marker; no FL5 signal above autofluorescence was observed in any sample including the CD19 positive control. This is a methods limitation discussed in Section 5.3.

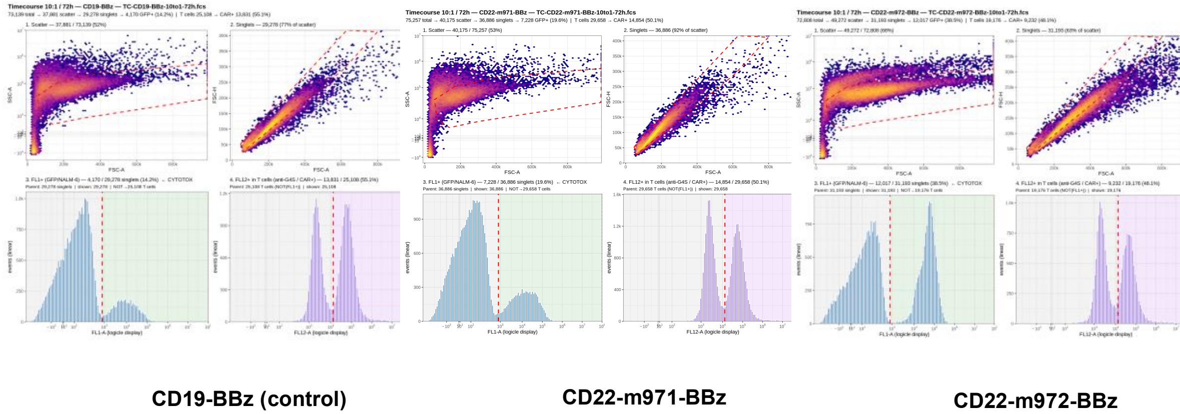
B.5 Control Samples

Sample type	Abbreviation	Purpose	Used for
Untransduced, unstained	UT / UTUS	Pure autofluorescence baseline	Positivity threshold derivation
Untransduced, stained	UTs	Antibody background (non-specific binding)	Background subtraction from raw %CAR-positive
NALM-6-GFP alone	GFP Pos	GFP channel reference; confirms no FL1 bleed into FL12	Channel crosstalk verification
CD19-BBz (p3)	CD19 positive control	Known-functional CAR; internal benchmark	Performance ranking across all BBz assays
p2 + p17 two-component	CD19-SynNotch positive control	Known-functional SynNotch circuit	BFP induction benchmark

Appendix C: Detailed Gating Strategies

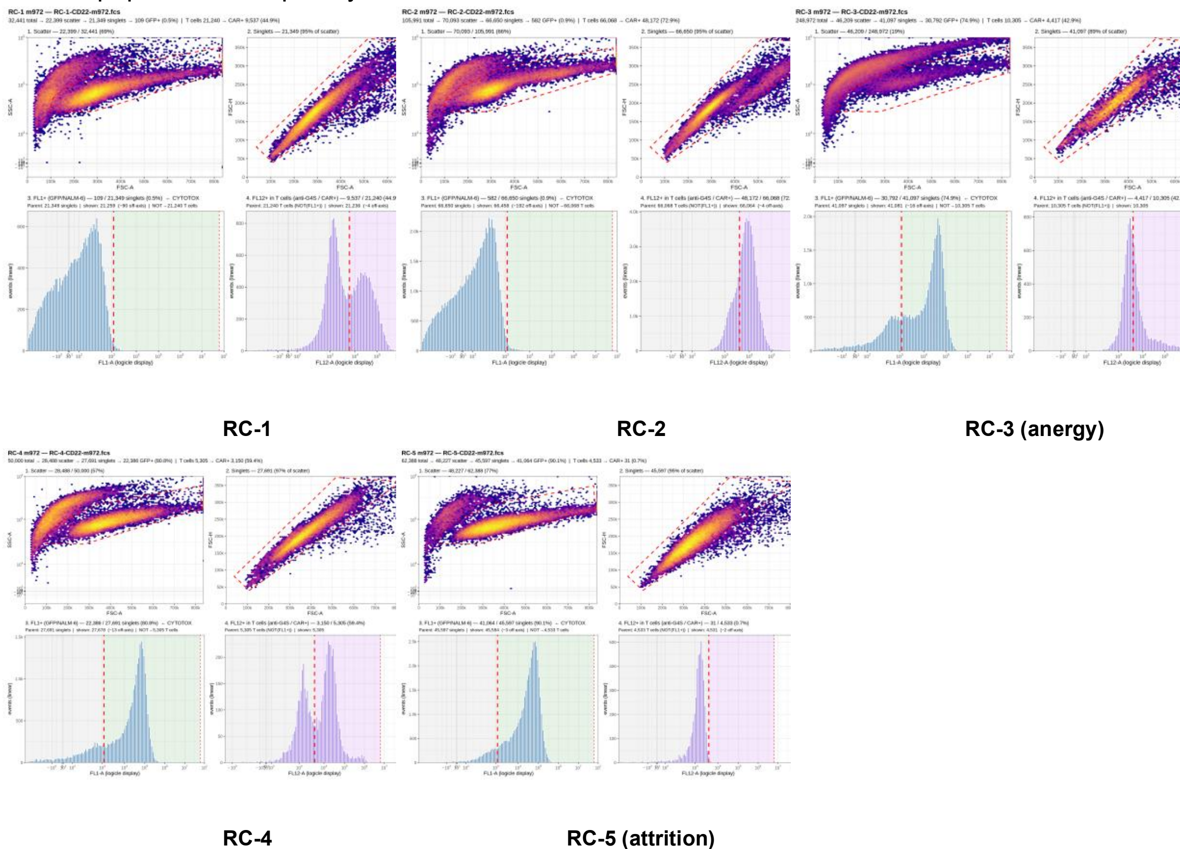
All gates were drawn manually in Floreada and exported via Gating-ML. BBz CAR hierarchy: scatter gate, singlets (FSC-H vs FSC-A), GFP+ targets (FL1-A), Boolean NOT for GFP-negative T cells, then FL12+ for CAR detection. SynNotch: ellipsoid gate on FL12 x FL1.

C.1 Cytotoxicity Timecourse Gating (10:1, 72h)

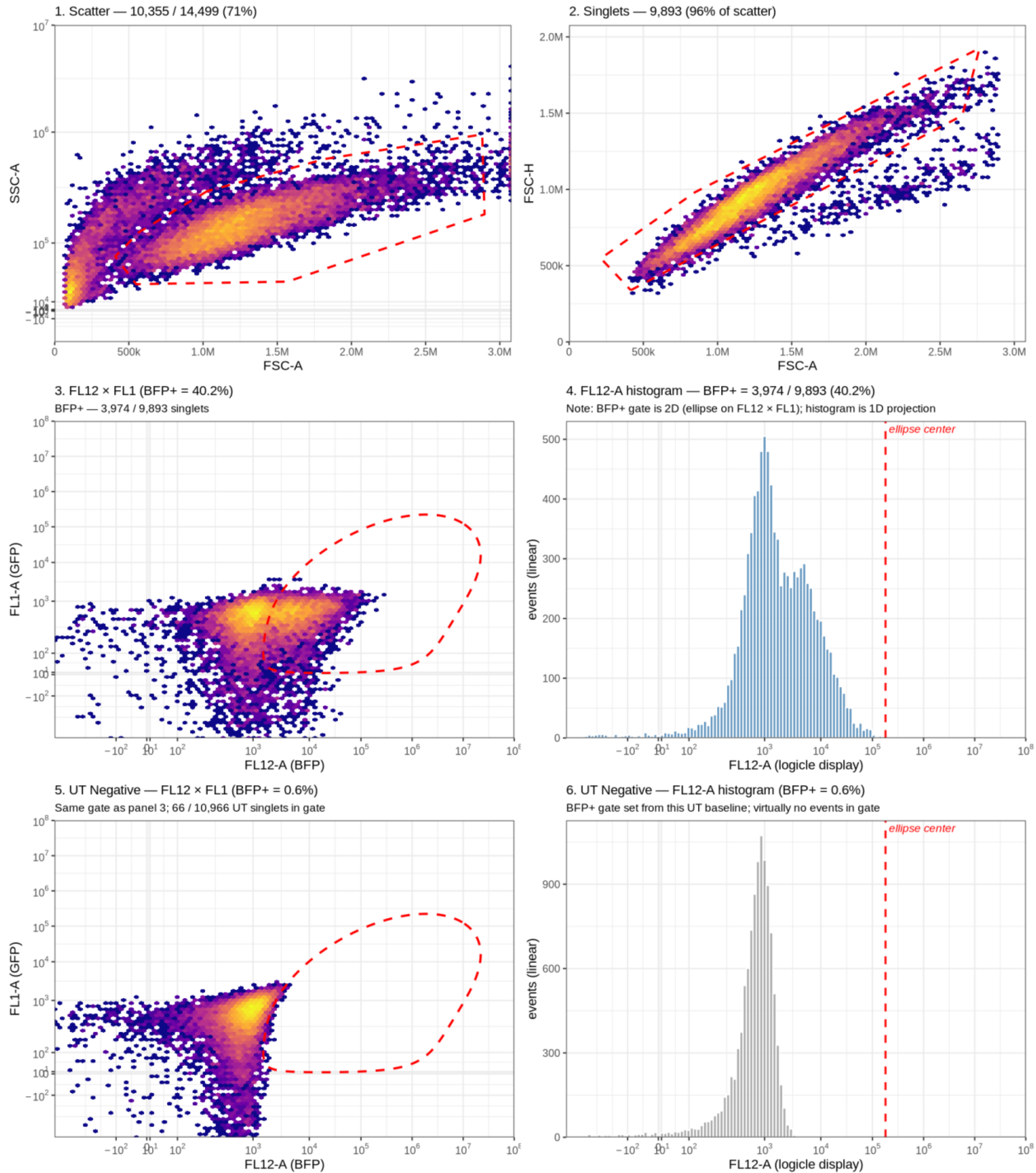


C.2 m972-BBz Rechallenge Progression (RC-1 through RC-5)

Two-step exhaustion trajectory: functional anergy at RC-3 (CARs present, killing lost), then CAR+ population collapse by RC-5.



C.3 SynNotch BFP Gating (m971, T:E 1.0)



Appendix D: Full Statistical Output

This appendix contains the complete pairwise statistical comparisons for all experiments, referenced from Section 4 (Results). The summary tables in Section 4 report headline comparisons; the full output is provided here for completeness.

D.1 Statistical Methods

All proportions are reported with Wilson 95% confidence intervals computed from event counts within each sample. These reflect **measurement precision** given the number of gated events, not biological reproducibility across independent experimental runs (N_bio = 1 per condition due to the IDT m972 gBlock synthesis failure that compressed the assay schedule; Section 3.1.1).

Pairwise construct comparisons use Fisher's exact test on 2x2 contingency tables of positive/negative event counts within the same experimental condition. Significance codes: `****` $p < 0.0001$, `***` $p < 0.001$, `**` $p < 0.01$, `*` $p < 0.05$, `ns` not significant.

D.2 Cytotoxicity Timecourse

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
10:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	48.00	47.47–48.54	50.51	49.99–51.02	+2.51	4.33e-11	****
10:1 / 24h	CD22-m972-BBz vs CD19-BBz	48.00	47.47–48.54	55.94	55.48–56.39	+7.94	< 2.2e-16	****
10:1 / 24h	CD22-m971-BBz vs CD19-BBz	50.51	49.99–51.02	55.94	55.48–56.39	+5.43	< 2.2e-16	****
10:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	49.68	49.12–50.24	51.13	50.56–51.70	+1.45	0.000402	***
10:1 / 48h	CD22-m972-BBz vs CD19-BBz	49.68	49.12–50.24	56.19	55.63–56.75	+6.51	< 2.2e-16	****
10:1 / 48h	CD22-m971-BBz vs CD19-BBz	51.13	50.56–51.70	56.19	55.63–56.75	+5.06	< 2.2e-16	****
10:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	48.14	47.44–48.85	50.08	49.52–50.65	+1.94	2.80e-05	****
10:1 / 72h	CD22-m972-BBz vs CD19-BBz	48.14	47.44–48.85	55.09	54.47–55.70	+6.95	< 2.2e-16	****
10:1 / 72h	CD22-m971-BBz vs CD19-BBz	50.08	49.52–50.65	55.09	54.47–55.70	+5.01	< 2.2e-16	****
5:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	48.95	48.31–49.58	52.56	51.77–53.35	+3.61	3.40e-12	****
5:1 / 24h	CD22-m972-BBz vs CD19-BBz	48.95	48.31–49.58	55.83	55.30–56.35	+6.88	< 2.2e-16	****
5:1 / 24h	CD22-m971-BBz vs CD19-BBz	52.56	51.77–53.35	55.83	55.30–56.35	+3.27	1.66e-11	****
5:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	48.31	47.68–48.94	50.21	49.52–50.91	+1.90	7.48e-05	****
5:1 / 48h	CD22-m972-BBz vs CD19-BBz	48.31	47.68–48.94	55.27	54.68–55.86	+6.96	< 2.2e-16	****
5:1 / 48h	CD22-m971-BBz vs CD19-BBz	50.21	49.52–50.91	55.27	54.68–55.86	+5.06	< 2.2e-16	****
5:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	47.31	46.51–48.11	49.97	49.21–50.73	+2.66	2.36e-06	****
5:1 / 72h	CD22-m972-BBz vs CD19-BBz	47.31	46.51–48.11	53.47	52.70–54.24	+6.16	< 2.2e-16	****
5:1 / 72h	CD22-m971-BBz vs CD19-BBz	49.97	49.21–50.73	53.47	52.70–54.24	+3.50	2.32e-10	****
1:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	49.01	48.19–49.84	50.35	49.56–51.14	+1.34	0.021795	*
1:1 / 24h	CD22-m972-BBz vs CD19-BBz	49.01	48.19–49.84	55.72	54.87–56.57	+6.71	< 2.2e-16	****
1:1 / 24h	CD22-m971-BBz vs CD19-BBz	50.35	49.56–51.14	55.72	54.87–56.57	+5.37	< 2.2e-16	****
1:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	49.07	48.03–50.11	50.64	49.39–51.89	+1.57	0.058926	ns
1:1 / 48h	CD22-m972-BBz vs CD19-BBz	49.07	48.03–50.11	51.64	50.53–52.75	+2.57	0.000961	***
1:1 / 48h	CD22-m971-BBz vs CD19-BBz	50.64	49.39–51.89	51.64	50.53–52.75	+1.00	0.246515	ns
1:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	47.25	45.47–49.04	45.65	43.95–47.36	-1.60	0.205567	ns
1:1 / 72h	CD22-m972-BBz vs CD19-BBz	47.25	45.47–49.04	46.23	44.76–47.71	-1.02	0.392647	ns
1:1 / 72h	CD22-m971-BBz vs CD19-BBz	45.65	43.95–47.36	46.23	44.76–47.71	+0.58	0.626579	ns

D.2.2 %GFP-Positive of Singlets (Target Survival)

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
10:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	14.32	13.97–14.67	11.44	11.14–11.75	-2.88	< 2.2e-16	****
10:1 / 24h	CD22-m972-BBz vs CD19-BBz	14.32	13.97–14.67	10.97	10.71–11.24	-3.35	< 2.2e-16	****
10:1 / 24h	CD22-m971-BBz vs CD19-BBz	11.44	11.14–11.75	10.97	10.71–11.24	-0.47	0.024787	*
10:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	26.69	26.27–27.12	16.79	16.41–17.19	-9.90	< 2.2e-16	****
10:1 / 48h	CD22-m972-BBz vs CD19-BBz	26.69	26.27–27.12	14.09	13.73–14.45	-12.60	< 2.2e-16	****
10:1 / 48h	CD22-m971-BBz vs CD19-BBz	16.79	16.41–17.19	14.09	13.73–14.45	-2.70	< 2.2e-16	****
10:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	38.52	37.99–39.07	19.60	19.19–20.00	-18.92	< 2.2e-16	****
10:1 / 72h	CD22-m972-BBz vs CD19-BBz	38.52	37.99–39.07	14.24	13.85–14.65	-24.28	< 2.2e-16	****
10:1 / 72h	CD22-m971-BBz vs CD19-BBz	19.60	19.19–20.00	14.24	13.85–14.65	-5.36	< 2.2e-16	****
5:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	26.05	25.57–26.53	18.04	17.50–18.61	-8.01	< 2.2e-16	****
5:1 / 24h	CD22-m972-BBz vs CD19-BBz	26.05	25.57–26.53	21.75	21.37–22.14	-4.30	< 2.2e-16	****
5:1 / 24h	CD22-m971-BBz vs CD19-BBz	18.04	17.50–18.61	21.75	21.37–22.14	+3.71	< 2.2e-16	****
5:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	42.58	42.11–43.06	31.35	30.82–31.89	-11.23	< 2.2e-16	****
5:1 / 48h	CD22-m972-BBz vs CD19-BBz	42.58	42.11–43.06	26.82	26.37–27.27	-15.76	< 2.2e-16	****
5:1 / 48h	CD22-m971-BBz vs CD19-BBz	31.35	30.82–31.89	26.82	26.37–27.27	-4.53	< 2.2e-16	****
5:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	55.37	54.83–55.90	35.85	35.27–36.44	-19.52	< 2.2e-16	****
5:1 / 72h	CD22-m972-BBz vs CD19-BBz	55.37	54.83–55.90	28.87	28.28–29.46	-26.50	< 2.2e-16	****
5:1 / 72h	CD22-m971-BBz vs CD19-BBz	35.85	35.27–36.44	28.87	28.28–29.46	-6.98	< 2.2e-16	****
1:1 / 24h	CD22-m972-BBz vs CD22-m971-BBz	67.51	67.07–67.95	65.99	65.55–66.42	-1.52	1.58e-06	****
1:1 / 24h	CD22-m972-BBz vs CD19-BBz	67.51	67.07–67.95	62.70	62.19–63.20	-4.81	< 2.2e-16	****
1:1 / 24h	CD22-m971-BBz vs CD19-BBz	65.99	65.55–66.42	62.70	62.19–63.20	-3.29	< 2.2e-16	****
1:1 / 48h	CD22-m972-BBz vs CD22-m971-BBz	81.63	81.29–81.97	77.78	77.28–78.26	-3.85	< 2.2e-16	****
1:1 / 48h	CD22-m972-BBz vs CD19-BBz	81.63	81.29–81.97	71.74	71.21–72.27	-9.89	< 2.2e-16	****
1:1 / 48h	CD22-m971-BBz vs CD19-BBz	77.78	77.28–78.26	71.74	71.21–72.27	-6.04	< 2.2e-16	****
1:1 / 72h	CD22-m972-BBz vs CD22-m971-BBz	89.83	89.48–90.17	85.42	84.95–85.88	-4.41	< 2.2e-16	****
1:1 / 72h	CD22-m972-BBz vs CD19-BBz	89.83	89.48–90.17	72.99	72.30–73.67	-16.84	< 2.2e-16	****
1:1 / 72h	CD22-m971-BBz vs CD19-BBz	85.42	84.95–85.88	72.99	72.30–73.67	-12.43	< 2.2e-16	****

D.3 Rechallenge Series

D.3.1 %CAR-Positive of T Cells

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
RC-1	CD22-m972-BBz vs CD22-m971-BBz	44.90	44.23–45.57	45.19	44.77–45.62	+0.29	0.472556	ns
RC-1	CD22-m972-BBz vs CD19-BBz	44.90	44.23–45.57	11.25	10.89–11.62	-33.65	< 2.2e-16	****
RC-1	CD22-m971-BBz vs CD19-BBz	45.19	44.77–45.62	11.25	10.89–11.62	-33.94	< 2.2e-16	****
RC-2	CD22-m972-BBz vs CD22-m971-BBz	72.91	72.57–73.25	54.32	53.91–54.72	-18.59	< 2.2e-16	****
RC-2	CD22-m972-BBz vs CD19-BBz	72.91	72.57–73.25	13.88	13.52–14.24	-59.03	< 2.2e-16	****
RC-2	CD22-m971-BBz vs CD19-BBz	54.32	53.91–54.72	13.88	13.52–14.24	-40.44	< 2.2e-16	****
RC-3	CD22-m972-BBz vs CD22-m971-BBz	42.87	41.92–43.83	64.96	64.56–65.35	+22.09	< 2.2e-16	****
RC-3	CD22-m972-BBz vs CD19-BBz	42.87	41.92–43.83	24.94	24.46–25.43	-17.93	< 2.2e-16	****
RC-3	CD22-m971-BBz vs CD19-BBz	64.96	64.56–65.35	24.94	24.46–25.43	-40.02	< 2.2e-16	****
RC-4	CD22-m972-BBz vs CD22-m971-BBz	59.40	58.07–60.71	56.39	55.52–57.26	-3.01	0.000220	***
RC-4	CD22-m972-BBz vs CD19-BBz	59.40	58.07–60.71	83.92	82.19–85.52	+24.52	< 2.2e-16	****
RC-4	CD22-m971-BBz vs CD19-BBz	56.39	55.52–57.26	83.92	82.19–85.52	+27.53	< 2.2e-16	****
RC-5	CD22-m972-BBz vs CD22-m971-BBz	0.68	0.48–0.97	13.91	13.40–14.44	+13.23	< 2.2e-16	****
RC-5	CD22-m972-BBz vs CD19-BBz	0.68	0.48–0.97	0.32	0.18–0.56	-0.36	0.021284	*
RC-5	CD22-m971-BBz vs CD19-BBz	13.91	13.40–14.44	0.32	0.18–0.56	-13.59	< 2.2e-16	****

D.3.2 %GFP-Positive of Singlets (Target Survival)

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
RC-1	CD22-m972-BBz vs CD22-m971-BBz	0.51	0.42–0.61	1.51	1.41–1.61	+1.00	< 2.2e-16	****
RC-1	CD22-m972-BBz vs CD19-BBz	0.51	0.42–0.61	1.02	0.91–1.14	+0.51	1.04e-10	****
RC-1	CD22-m971-BBz vs CD19-BBz	1.51	1.41–1.61	1.02	0.91–1.14	-0.49	3.27e-09	****
RC-2	CD22-m972-BBz vs CD22-m971-BBz	0.87	0.81–0.95	1.02	0.94–1.10	+0.15	0.007944	**
RC-2	CD22-m972-BBz vs CD19-BBz	0.87	0.81–0.95	1.09	0.99–1.21	+0.22	0.000765	***
RC-2	CD22-m971-BBz vs CD19-BBz	1.02	0.94–1.10	1.09	0.99–1.21	+0.07	0.290795	ns
RC-3	CD22-m972-BBz vs CD22-m971-BBz	74.93	74.50–75.34	0.46	0.41–0.52	-74.47	< 2.2e-16	****
RC-3	CD22-m972-BBz vs CD19-BBz	74.93	74.50–75.34	1.14	1.03–1.27	-73.79	< 2.2e-16	****
RC-3	CD22-m971-BBz vs CD19-BBz	0.46	0.41–0.52	1.14	1.03–1.27	+0.68	< 2.2e-16	****
RC-4	CD22-m972-BBz vs CD22-m971-BBz	80.84	80.37–81.30	2.82	2.55–3.12	-78.02	< 2.2e-16	****
RC-4	CD22-m972-BBz vs CD19-BBz	80.84	80.37–81.30	23.20	21.57–24.93	-57.64	< 2.2e-16	****

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
RC-4	CD22-m971-BBz vs CD19-BBz	2.82	2.55–3.12	23.20	21.57–24.93	+20.38	< 2.2e-16	****
RC-5	CD22-m972-BBz vs CD22-m971-BBz	90.06	89.78–90.33	26.57	26.00–27.14	-63.49	< 2.2e-16	****
RC-5	CD22-m972-BBz vs CD19-BBz	90.06	89.78–90.33	90.72	90.44–91.00	+0.66	0.000986	***
RC-5	CD22-m971-BBz vs CD19-BBz	26.57	26.00–27.14	90.72	90.44–91.00	+64.15	< 2.2e-16	****

D.4 SynNotch BFP Reporter

D.4.1 %BFP-Positive of Singlets

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
T:E 0.5	m972-SynNotch vs m971-SynNotch	9.86	9.29–10.46	16.03	15.31–16.77	+6.17	< 2.2e-16	****
T:E 1.0	m972-SynNotch vs m971-SynNotch	25.73	24.88–26.59	40.17	39.21–41.14	+14.44	< 2.2e-16	****
T:E 1.0	m972-SynNotch vs CD19-SynNotch	25.73	24.88–26.59	55.02	54.05–56.00	+29.29	< 2.2e-16	****
T:E 1.0	m971-SynNotch vs CD19-SynNotch	40.17	39.21–41.14	55.02	54.05–56.00	+14.85	< 2.2e-16	****

D.5 Transduction Efficiency (Pre-Enrichment)

D.5.1 %FL12-Positive of Singlets

Condition	Comparison	%A	95% CI	%B	95% CI	Delta	Fisher p	sig
pre-enrichment	m972-BBz vs m971-BBz	21.84	21.39–22.30	27.04	26.53–27.55	+5.20	< 2.2e-16	****
pre-enrichment	m972-BBz vs CD19-BBz	21.84	21.39–22.30	81.41	78.81–83.76	+59.57	< 2.2e-16	****
pre-enrichment	m971-BBz vs CD19-BBz	27.04	26.53–27.55	81.41	78.81–83.76	+54.37	< 2.2e-16	****

D.6 Summary Statistics

Experiment	Total comparisons	Significant (p < 0.05)	Not significant
Cytotoxicity timecourse	54	48	6
Rechallenge (RC-1 to RC-5)	30	29	1
SynNotch BFP	4	4	0
Transduction efficiency	3	3	0
Total	**91**	**84**	**7**

Non-significant comparisons cluster at the 1:1 E:T ratio at 48h and 72h in the cytotoxicity timecourse, consistent with target cell excess overwhelming all three constructs at the lowest effector dose (Section 4.2). The single non-significant rechallenge comparison is RC-2 %GFP m971 vs CD19 ($p = 0.29$), where both constructs maintain near-complete killing.

Appendix E: SOP - scFv A/B Testing Workflow for CAR-T Construct Development

Field	Value
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Approval

Role	Name	Signature	Date
Author	Tyler Henderson		
PI / Reviewer	Dr. Michael Pulsipher		

This SOP codifies the end-to-end workflow for comparing candidate scFvs in CAR-T or CAR-adjacent architectures, as developed and validated during the m971/m972 CD22 project at the Pulsipher Laboratory. It is intended as a reusable protocol for future scFv screening campaigns, with project-specific parameters left as guided fill-ins and lessons from the first run incorporated as standing practice.

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1. Purpose and Scope

Purpose: This SOP defines a reproducible workflow for comparing two or more candidate scFvs in a CAR-T or CAR-adjacent context (e.g., SynNotch, prime-and-kill architectures) to inform lead scFv selection for downstream therapeutic development.

Scope: Covers the full workflow from **construct design** to a **data-driven lead scFv recommendation**. Assumes the Pulsipher Laboratory's standing environment: BSL-2 tissue culture, NEB HiFi assembly cloning, third-generation lentiviral packaging, primary human T-cell work with PBMC-derived effectors, Beckman CytoFLEX LX flow cytometry, and R-based downstream analysis.

Out of scope:

- De novo scFv discovery (phage display, immunization, computational modeling)
- Full preclinical efficacy studies (in-vivo NSG mouse work, EC50 refinement at multiple doses in multiple donors)
- GMP / clinical manufacturing
- Regulatory filing documentation

Intended users: Pulsipher Lab researchers (graduate students, postdocs, research associates) running a comparative scFv screen on a new target or a new scFv candidate set.

2. When to use this SOP

Use this workflow when **any of the following** are true:

- Two or more candidate scFvs for the same antigen need direct head-to-head comparison
- A literature-reported scFv is being adapted to a new architecture (e.g., moving an scFv from a published BBz CAR to a SynNotch context)
- A new scFv needs to be validated against an established lab benchmark before committing to further engineering
- The lab is evaluating a make-vs-buy decision for a specific scFv (e.g., synthesize in-house vs license from a commercial supplier)

Do **not** use this SOP as the primary protocol for:

- Time-sensitive clinical translation (use GMP-compatible workflows)
- Comparisons that require in-vivo readouts (use the animal facility protocols and NSG mouse SOPs)

3. Prerequisites and Inputs

Information you need before starting

- [ ] **Target antigen sequence** (full-length; for NALM-6-based B-ALL work, CD19 and CD22 sequences are standing references in the lab)
- [ ] **Candidate scFv sequences** (VH-linker-VL) for each variant being compared
- [ ] **Architectural context(s)** - BBz CAR, SynNotch, prime-and-kill combinations, other
- [ ] **Intended downstream application** - in-vitro screen only, or feeds into a preclinical program
- [ ] **Cell line source** - clonal cell lines (e.g., Jurkat, NALM-6, Raji) available in the lab, or primary cells (PBMCs + donor source)
- [ ] **Target screening depth** - how many E:T ratios x timepoints x replicates is "enough"
- [ ] **Decision criteria** - what would need to be true for an scFv to be declared the lead, and what would be a disqualifying failure

Reagents and consumables

Lab-standing reagents (assumed available):

- Lentiviral packaging plasmids: psPAX2 (p6) and pMD2.G/VSV-G (p5), maintained as in-house glycerol stocks
- NEB HiFi DNA Assembly Master Mix (E2621)
- Q5 High-Fidelity 2x Master Mix (NEB M0492)
- NEB Stable Competent Cells (C3040)
- Restriction/cloning enzymes: PaqCI, DpnI, rSAP (NEB)
- RPMI 1640 + DMEM (high glucose, GlutaMAX) + FBS (heat-inactivated, premium) + penicillin-streptomycin (100x)
- IL-2 (recombinant human, GMP grade)
- Dynabeads Human T-Activator CD3/CD28 (Thermo Fisher)
- Dynabeads M-280 Streptavidin (Thermo Fisher) + DynaMag magnetic stand
- RetroNectin (Takara Bio, recombinant human fibronectin fragment)
- Lenti-X Concentrator (Takara Bio)
- PEI 25K transfection grade (1 mg/mL stock, Polysciences)
- Opti-MEM I Reduced Serum Medium (Gibco)
- Anti-G4S linker biotinylated mAb (CST #17621) for magnetic enrichment
- Anti-G4S linker Pacific Blue conjugate (CST #44962) for flow cytometry
- Polybrene transfection reagent
- Trypsin-EDTA (0.25%)
- Agarose (molecular biology grade), SYBR Safe gel stain, 1 kb Plus DNA Ladder

- PBS, DPBS, FACS buffer (PBS + 2% FBS), nuclease-free water
- LB broth (powder), ampicillin (100 mg/mL stock), LB-Amp agar plates, glycerol (50% stock)
- PureLink Quick Plasmid Miniprep Kit + PureLink HiPure Plasmid Maxiprep Kit + PureLink Gel Extraction Kit (Invitrogen)
- Tissue culture plasticware: 10 cm dishes, 6-well/24-well/48-well/96-well plates, T-25/T-75 flasks, cryovials

Project-specific reagents (order fresh for each A/B run):

- Custom gBlocks or staircase PCR oligos (IDT) encoding each candidate scFv with flanking homology arms
- Custom cloning primers (Genewiz/IDT plate order - iPCR backbone primers + insert-specific primers)
- Cryopreserved PBMCs (STEMCELL Technologies, 1 vial per donor)
- Lenti-X p24 Rapid Titer ELISA Kit (Takara Bio) - 1 kit covers multiple constructs
- Plasmidsaurus whole-plasmid nanopore sequencing submissions (~$15-20 per sample)
- Target cell line (e.g., NALM-6 GFP for CD19/CD22 work) - thaw from lab LN2 stock ~1 week before co-culture
- Jurkat T cells (ATCC E6-1) - thaw from lab LN2 stock if SynNotch arm is included

Equipment and software

- BSL-2 tissue culture hood, incubator (37 degrees C, 5% CO_2), centrifuges
- NEB HiFi assembly kit and E. coli transformation workflow
- Thermocycler (Q5 or similar high-fidelity polymerase)
- Gel electrophoresis + image capture (BioRad GelDoc or equivalent)
- Plasmidsaurus account for whole-plasmid nanopore sequencing
- HEK293T cells for lentiviral packaging; Lenti-X concentrator
- p24 ELISA kit (or equivalent titer quantification)
- RetroNectin-coated plates for T-cell transduction
- Primary anti-G4S linker antibody (biotinylated, for magnetic enrichment) + streptavidin Dynabeads + DynaMag
- Pacific Blue-conjugated anti-G4S linker antibody (CST #44962) for flow cytometry CAR detection
- Beckman Coulter CytoFLEX LX (10-laser) + CytExpert software
- R + RStudio + Bioconductor (flowCore, flowWorkspace, ggcyto, rstatix, ggplot2) for analysis
- SnapGene Molecular Biology Software
- Floreada (Linux-based, free) flow cytometry analysis software

4. Workflow Overview

```
Stage 1: Construct design         (~1 week)
    |
Stage 2: Cloning + verification   (~2-3 weeks; budget extra for gBlock/synthesis risk)
    |  - Plasmidsaurus PASS / FAIL checkpoint
Stage 3: Lentiviral production    (~1 week)
    |  - p24 ELISA titer PASS / FAIL checkpoint
Stage 4: Transduction + enrichment (~1 week)
    |  - anti-G4S+ TD efficiency checkpoint
Stage 5: Functional screening      (~1-2 weeks)
    |
Stage 6: Analysis + decision       (~1 week)
    |
Lead scFv recommendation memo to PI + sponsor
```

Total elapsed time: ~7-9 weeks for a clean run. **Budget an extra 2-3 weeks of float** for synthesis / cloning risk; the single biggest schedule failure mode is an unexpected gBlock or PCR failure in Stage 2 (see Section 14 for the m972 lessons).

5. Stage 1 - Construct Design

5.1 Sequence sourcing

- Pull the VH and VL sequences from the published literature or patent filings for each candidate scFv. Document the source in the construct record.
- Codon-optimize for human expression if the source is a non-human clone. The lab uses IDT's integrated codon-optimization service when ordering gBlocks; for in-house staircase PCR assembly, apply manual codon diversification if the sequence has repetitive or high-GC regions (see Section 14, lesson 1).
- Add the (G4S)3 interdomain linker between VH and VL. Confirm the linker choice matches the CAR architecture being used.

5.2 Backbone selection

- For **BBz CAR** comparisons: use the lab's standing pELPS-CD8a-4-1BB-CD3z backbone (p3 in the lab nomenclature is the CD19-BBz positive control and is the inverse-PCR template for new scFv swaps)
- For **SynNotch-BFP** comparisons: use the lab's standing UAS-inducible SynNotch-BFP backbone (p1 in the lab nomenclature). **If constitutive transduction marker separability is needed, verify the backbone includes constitutive mCherry or equivalent; the m971/m972 project backbones did not, and this was a flagged methods limitation.**
- Backbone naming confirmed as of the m971/m972 project (2026): p3 = pELPS CD19-BBz (BBz template), p1 = pHR-UAS-CAR-BFP (SynNotch template). Verify naming is current before each new project; the lab's plasmid numbering is sequential and these designations could shift if new backbones are adopted.

5.3 Synthesis strategy decision tree

For each scFv to be built:

Estimate synthesizability. Submit the sequence to the IDT gBlock complexity scorer (or equivalent at another vendor). If the complexity flag is below the "high risk" threshold, proceed to gBlock order.

If high-risk: place the gBlock order first and allow it to proceed through the vendor's synthesis pipeline. **Only if the gBlock fails** (vendor cancellation or repeated QC failure), fall back to designing a staircase PCR assembly from tiled oligos in-house. **Exception:** if the synthesis step is on the critical path of a time-critical project, it may be worth ordering both the gBlock and staircase PCR oligos in parallel to avoid schedule risk, but this is the exception, not the default, since ordering both when not time-critical wastes money on unnecessary oligos. See Section 14 for the m972 gBlock failure that motivated this guidance.

If very high-risk or a history of failure exists: default directly to in-house staircase PCR synthesis from tiled oligos. This is slower (~1 extra week for oligo design and assembly) but has no vendor-dependent failure mode.

Document the decision. Capture the risk score and the chosen strategy in the lab notebook entry for the construct.

5.4 Primer / oligo design

- Use SnapGene to design HiFi assembly primers with >=20 bp homology arms at both ends of the insert.
- For staircase PCR assembly (backup path), design tiled 60 bp oligos with 30 bp overlap. The m971/m972 project's staircase PCR recovery of the m972 scFv (24 tiled oligos, documented in the 2026-02-09 lab notebook entry) serves as a reference implementation.
- Maintain all primer / oligo records in Benchling and BenchBuddy.

5.5 QC Checkpoint 1 - Design review

Before placing any synthesis order, confirm with a second set of eyes (PI, supervisor, or experienced coworker):

- [ ] Sequences are the correct variant of each scFv (no VH/VL swap errors)
- [ ] Linker length and composition is intended
- [ ] HiFi homology arms align with the chosen backbone's entry points
- [ ] Order is placed under the correct project budget code

6. Stage 2 - Cloning and Sequence Verification

6.1 Standard NEB HiFi assembly

- Follow NEB HiFi protocol per manufacturer's instructions
- Transform into NEB Stable Competent Cells (C3040, high efficiency); the lab standard for lentiviral backbone work due to low recombination at LTR repeats
- Plate on LB + ampicillin (100 ug/mL), incubate overnight at 30 degrees C for lentiviral constructs (reduces recombination at repetitive elements; 20-22 h at 30 degrees C produces smaller colonies than 37 degrees C overnight)
- Pick 4-6 colonies per construct for screening

6.2 Pre-sequencing colony screen (restriction digest)

- Miniprep colonies using PureLink Quick Plasmid Miniprep Kit (Invitrogen) per manufacturer's protocol
- Run a diagnostic restriction digest with enzymes chosen to distinguish correct insert from empty backbone or mis-ligations. **Enzyme selection is project-specific**; choose a pair that cuts once inside the insert and once in the backbone (or flanking the insert) to produce a distinctive banding pattern. Use SnapGene's restriction map view to simulate the digest and predict expected bands before cutting.
- Run digests on a 1% agarose gel with a 1 kb Plus DNA Ladder (NEB/Invitrogen)
- **Before each new project**, generate and archive the expected band patterns for your specific constructs from SnapGene and save to the project folder in BenchBuddy
- **Advance the colony with the cleanest expected pattern to Plasmidsaurus sequencing**

6.3 Plasmidsaurus whole-plasmid nanopore verification

- Submit each passing colony to Plasmidsaurus for whole-plasmid nanopore sequencing (standard turnaround ~2 business days)
- Review the Plasmidsaurus coverage plot and SNV call summary on receipt
- **Pass criteria:** zero SNVs in the coding sequence of the scFv + signaling domains, >=30x median coverage across the construct, no deletions/insertions in the scFv region

6.4 QC Checkpoint 2 - Plasmidsaurus PASS / FAIL

PASS: sequence matches design in all critical regions (scFv, linker, CAR domains, reporter ORF if present); advance to Stage 3

FAIL:

- Silent mutations outside coding regions: **advance with a footnote in the construct record**, but document
- SNVs in coding regions: **re-screen additional colonies** from the same transformation, or re-transform and pick fresh colonies
- Structural errors (deletions/insertions in the insert): **re-do the HiFi assembly** from the original PCR-amplified insert
- If the insert itself is suspect: **re-amplify or re-order** the insert fragment

Archive: on a pass, bank a glycerol stock (500 uL overnight culture + 500 uL 50% glycerol; 25% final, flash-frozen in LN2 or dry ice/EtOH, stored at -80 degrees C; label: plasmid ID + date + initials). Log the stock location in BenchBuddy. Record the Plasmidsaurus FASTA and QC PDF in the project folder in BenchBuddy.

7. Stage 3 - Lentiviral Production and Titering

7.1 Production

- HEK293T (Lenti-X 293T) transient transfection with the lentiviral transfer vector + 3rd-generation packaging plasmids: psPAX2 (p6, packaging) + pMD2.G/VSV-G (p5, envelope). **DNA ratio: 3:2:1 (transfer : psPAX2 : VSV-G).**
- Transfection reagent: PEI 25K (1 mg/mL stock), **3:1 w/w PEI:DNA ratio**. Mix Tube A (DNA + Opti-MEM) and Tube B (PEI + Opti-MEM), incubate 15-20 min at RT, add dropwise to cells at ~70-80% confluency on 10 cm dishes (2 plates per construct).
- 48 h and 72 h supernatant harvests, pooled, 0.45 um PVDF syringe filtered
- Concentration: Takara Lenti-X Concentrator per manufacturer's protocol (1:3 concentrator:supernatant), resuspend in **160 uL per construct in R10 + 10% sucrose** (cryoprotectant for -80 degrees C storage)
- Aliquot into cryovials (avoid freeze/thaw; small aliquots at -80 degrees C)

7.2 p24 ELISA titer quantification

- Run p24 ELISA on each lentiviral prep per the kit's protocol
- Convert p24 to TU/mL using the lab's empirical conversion factor. The m971/m972 project derived **~5,917 TU/pg p24** (mean of p156 = 7,866 and p157 = 3,968 TU/pg) from BBz constructs with known functional titers. This factor is construct-dependent; recalibrate for each new backbone class by comparing p24 ELISA to a functional titer assay (e.g., serial dilution + flow cytometry for CAR+ on Jurkats).
- Report titer in TU/mL and in total TU per harvest

7.3 QC Checkpoint 3 - Titer PASS / FAIL

- **PASS criterion:** >=10^6 TU/mL for each construct. This threshold was used in the m971/m972 project (all constructs passed: BBz at ~2.5-3.1 x 10^7 TU/mL, SynNotch at ~6-8 x 10^6 TU/mL) and is aligned with the lab's standing minimum for primary T-cell transduction at practical MOIs.
- **FAIL response:** re-do the HEK293T transfection (most common failure mode is transfection efficiency); if repeated failure, troubleshoot packaging plasmid quality or HEK293T passage number

8. Stage 4 - Transduction and CAR+ Enrichment

8.1 Cell source prep

For primary T-cell (BBz CAR) arm:

- PBMC isolation from **STEMCELL Technologies** (cryopreserved, cat. #70025), donor lot recorded in lab notebook
- CD3+ T-cell isolation: BioLegend MojoSort Human CD3 negative selection per manufacturer protocol
- Post-MojoSort purity check: **not routinely performed at the Pulsipher Lab** (lab convention; does not bias between-construct comparisons in a shared PBMC prep). Document this choice in the construct record.
- Activation: Dynabeads Human T-Activator CD3/CD28 at 1:1 bead:cell ratio in complete RPMI (R10) + IL-2 at **100 IU/mL** during activation, overnight (~24 h pre-transduction). Post-bead removal (DynaMag, day 3-4): reduce IL-2 to **50 IU/mL** for expansion and post-transduction maintenance. Rechallenge co-cultures: return to **100 IU/mL**.

For Jurkat (SynNotch) arm:

- Jurkat T-cell line (ATCC E6-1 clone, lab LN2 stock), maintained in **R10** (RPMI 1640 + 10% FBS + 1% pen/strep), passaged every 2-3 days
- No activation step required for Jurkats; transduce at log-phase growth

8.2 Transduction

- RetroNectin-coated 48-well plates per Takara Bio manufacturer protocol (the m971/m972 project used 4 wells per construct x 6 constructs = 24 wells; specific coating concentration followed manufacturer instructions)
- MOI 5-10 for primary T cells; MOI 5-10 for Jurkats (the m971/m972 project used 20 uL concentrated virus per well from ~100x stock onto 500K cells/well; calculate effective MOI from your p24 titer)
- Media changes every 48 h post-transduction
- Expand primary T cells for 5 days post-transduction in IL-2-supplemented media before the enrichment step

8.3 CAR+ magnetic enrichment (primary T cells)

> **Critical workflow step** - standard Pulsipher Lab practice for isolating a high-purity CAR+ effector population before functional assays.

- Stain expanded T cells with **primary anti-G4S linker biotinylated antibody** (CST #17621) at **1 uL per million cells** for **15 minutes on ice**
- Wash, bind **streptavidin-conjugated Dynabeads** per manufacturer's protocol
- Magnetic separation on DynaMag; collect the positive fraction
- Expected post-enrichment CAR+ purity: >=95%, typically approaching 100%
- **Jurkat SynNotch arm does NOT use magnetic enrichment**; the SynNotch readout is gated on the transduced population by flow cytometry in the final assay

8.4 QC Checkpoint 4 - Transduction efficiency and enrichment success

- Run a small aliquot on the CytoFLEX, stained with Pacific Blue anti-G4S linker (CST #44962), before and after magnetic enrichment
- **Pre-enrichment TD%:** document for process improvement (the raw transduction efficiency is a useful data point even if enrichment brings the functional population to ~100%)
- **Post-enrichment CAR+ purity:** >=95% required to proceed to Stage 5. If below 95%, investigate bead-binding efficiency and consider a second magnetic round.

9. Stage 5 - Functional Screening

9.1 Screen design matrix

Every scFv A/B run should generate **both** an acute readout and a durability readout.

Ratio convention note: BBz CAR cytotoxicity and rechallenge assays use **E:T** (effector:target) ratios, since the effector cell number is the independent variable. SynNotch BFP induction assays use **T:E** (target:effector) ratios, since the target (antigen source) is titrated against a fixed Jurkat effector population. Maintain this convention consistently in filenames, figures, and statistical models.

Readout	Question answered	Assay
Acute co-culture cytotoxicity timecourse	Which scFv kills faster / deeper at standard E:T and timepoints?	BBz CAR-T vs target (e.g., NALM-6-GFP), E:T 1:1/5:1/10:1, 24/48/72 h
Rechallenge series	Which scFv sustains killing across repeated antigen exposures? (Proxy for in-vivo durability.)	Same effectors re-challenged with fresh targets over 5 rounds
SynNotch BFP induction (if the scFv is also being tested in SynNotch context)	Which scFv activates the reporter more strongly at each T:E ratio?	Jurkat-SynNotch + target, T:E 0.5 and 1.0, single timepoint

9.2 Acute cytotoxicity timecourse (BBz CAR)

- Target cells: **NALM-6 GFP** (human B-ALL, stably expressing GFP; cultured in R10 without selection antibiotic; GFP fluorescence serves as viability proxy; surviving targets remain GFP+, killed targets lose signal). **Seeding density:** 1 x 10^5 targets per well in 96-well flat-bottom plates (200 uL/well) for timecourse; 1 x 10^6 targets per well in 24-well plates (2 mL/well) for rechallenge. Thaw NALM-6 GFP from LN2 stock ~1 week before first co-culture.
- Effector cells: post-enrichment CAR+ T cells at E:T 1:1, 5:1, 10:1

- Controls per session: **UT effectors** (untransduced negative), **CD19-BBz effectors** (lab positive control, uses p3), **target-only** (no effectors, tumor baseline), **UTs/UTUS** controls for antibody background and autofluorescence
- Acquisition: flow cytometry at 24, 48, 72 h, gated on the target-specific marker (GFP) in the live singlet population
- **Session controls are non-negotiable**; the m971/m972 analysis relies on per-session autofluorescence baselines (UTUS controls) to set the GFP+ gating threshold, and session drift in the cytometer is otherwise a confound

9.3 Rechallenge series

- Run 3-5 rechallenge rounds (the m971/m972 project ran 5 rounds from 2026-03-26 to 2026-04-03)
- Each round: re-seed the same effector population against a fresh 1 x 10^6 target population (24-well, 2 mL), acquire flow cytometry at **~48 hours post-seeding** (the m971/m972 project ran ~2-day intervals between rounds)
- Maintain the same per-session control panel (UT, CD19-BBz, target-only, UTs, UTUS) in every round
- The killing-vs-round curve is the primary durability readout

9.4 SynNotch BFP induction (if applicable)

- Jurkat-SynNotch effectors + target at T:E 0.5 and 1.0
- Single timepoint at **~48 hours** post-co-culture setup (the m971/m972 project set up ~2026-03-24, acquired ~2026-03-26)
- Controls: UT Jurkats (untransduced negative), CD19-SynNotch (positive activation control for any CD22-independent SynNotch activation)
- Readout: %BFP+ within the gated lymphocyte population; if a constitutive transduction marker is present in the construct (e.g., mCherry), gate on that first and report %BFP+ within mCherry+. **If no transduction marker is present, fall back to %BFP+ within all lymphocytes and document the limitation in the final report; this was the case for the m971/m972 SynNotch constructs.**

9.5 QC Checkpoint 5 - Session controls on every acquisition

Before recording any sample data in a session, confirm:

- [ ] All per-session controls are acquired (UT, UTs, UTUS, target-only baseline)
- [ ] Compensation matrix loaded (auto-compensated by CytExpert or imported from the lab's standing compensation file)
- [ ] Event count >=10,000 singlets per sample (the m971/m972 project flagged samples below this threshold as low-confidence; e.g., p154 TD check had only 346 singlets and was flagged)
- [ ] All files saved with the standing filename convention: **RC-{#}-{construct}** for rechallenge, **TC-{construct}-{E:T}-{timepoint}h** for timecourse, **BFP-{construct}-{T:E}** for SynNotch, **{date}-{construct}-Transduction** for TD checks, controls as **UT_control_session{#}** / **UTUS_control_session{#}** / **NALM6-GFP-alone-session{#}**

10. Stage 6 - Data Analysis and Go/No-Go Decision

10.1 Analysis pipeline

- Use the lab's R/flowCore-based analysis pipeline as the template (scripts 01-06 covering inspection, gating, statistics, and figures)
- Fill in the panel configuration file at the start of each project; see the Pulsipher Lab's standing CytoFLEX channel layout and marker assignment reference
- Run inspection, gating, stats, and figures sequentially through the pipeline
- Export per-sample stats to a long-format CSV indexed by construct x condition x timepoint x replicate
- Archive the full R session info alongside the stats CSV for reproducibility

10.2 Statistical test

- **Two-way ANOVA** for each functional readout (construct x {round | E:T | timepoint}) with multiple-comparisons correction (Tukey HSD recommended; Bonferroni acceptable)
- Report effect sizes alongside p-values
- Significance threshold: $p < 0.05$ (adjusted)

10.3 Decision criteria

A **lead scFv recommendation** requires **all** of the following to be true:

- [ ] **Acute cytotoxicity:** the candidate scFv's killing curve is statistically indistinguishable from or better than the CD19-BBz positive control in at least one E:T condition at 72 h (Tukey HSD, adjusted $p > 0.05$ for "indistinguishable from positive control" or adjusted $p < 0.05$ for "better than comparator scFv")
- [ ] **Rechallenge durability:** the candidate scFv maintains >=50% of its round-1 killing capacity through round 3 of the rechallenge series, and is not statistically worse than the comparator scFv
- [ ] **SynNotch activation** *(if applicable)*: the candidate scFv produces BFP induction above the UT Negative baseline at both T:E ratios, and (where relevant) performs at least as well as the comparator
- [ ] **Expression QC:** anti-G4S linker surface expression on the post-enrichment population is >=95% CAR+
- [ ] **Cloning integrity:** Plasmidsaurus PASS with zero SNVs in the scFv + signaling domains
- [ ] **Reproducibility:** at least **2 independent PBMC donors** (the m971/m972 project ran a single donor, which is a documented limitation; single-donor data cannot distinguish construct-intrinsic effects from donor-specific T-cell variability. For future A/B runs, budget for >=2 donors to establish reproducibility.)

A candidate scFv can be disqualified for any of the following:

- Cloning failure (unrecoverable SNV or frame-shift in the insert)
- Titer $<10^6$ TU/mL after two production attempts
- Post-enrichment CAR+ purity <90%
- Statistically worse than the UT negative control in acute cytotoxicity (i.e., no measurable killing above baseline)
- Rechallenge durability drops below 25% of round-1 capacity by round 3

10.4 Lead scFv recommendation memo

When decision criteria are met, produce a **one-page recommendation memo** for the PI containing:

- Construct identity and plasmid archive location
- Summary of cytotoxicity, rechallenge, and (if applicable) SynNotch results with one headline figure each
- Explicit recommendation statement ("We recommend [scFv X] for the BBz CAR effector role / SynNotch sensor role")
- Caveats and limitations
- Recommended next steps (additional donors, dose-response refinement, in-vivo validation)

A template for this memo is provided in Sub-appendix D below.

11. QC Checkpoints Summary

#	Stage	Checkpoint	Pass criterion	Fail response
1	Design	Design review	Sequence correct, HiFi arms aligned, budget confirmed	Fix and re-review before ordering
2	Cloning	Plasmidsaurus	0 SNVs in scFv + CAR domains, >=30x coverage	Re-screen colonies or re-assemble
3	Production	p24 ELISA	>=10^6 TU/mL	Re-transfect; troubleshoot HEK293T / plasmids
4	Transduction	CAR+ enrichment	>=95% CAR+ post-enrichment	Second magnetic round; troubleshoot antibody binding
5	Acquisition	Session controls	All UT/UTs/UTUS controls acquired, event count >=minimum	Re-acquire session
6	Analysis	Go/No-Go criteria (Section 10.3)	All items checked	Disqualify candidate or collect more data

12. Decision Criteria Summary

See Section 10.3 for the full decision framework. The short version:

- **Every final construct must pass Plasmidsaurus** (zero coding-region SNVs).
- **Every construct must hit >=10^6 TU/mL** at titer.
- **Every functional readout must have per-session controls** (UT, UTs, UTUS).
- **A lead scFv recommendation requires** acute killing at-or-above CD19-BBz positive control, sustained killing through rechallenge round 3, and >=2 biological replicates (or a documented limitation if fewer).

13. Timeline and Resource Estimates

Stage	Duration (clean run)	Duration (with cloning risk)
1. Design	1 week	1 week
2. Cloning + verification	2 weeks	3-5 weeks
3. Production + titering	1 week	1 week
4. Transduction + enrichment	1 week	1 week
5. Functional screening	1-2 weeks	1-2 weeks
6. Analysis + decision	1 week	1 week
Total	**~7 weeks**	**~9-11 weeks**

Always budget for Stage 2 risk. The m971/m972 project assumed a clean 2-week cloning window and slipped ~4 weeks due to the IDT m972 gBlock failure, which consumed all downstream schedule float (see Section 14).

Approximate per-construct cost (m971/m972 project data point, full scope): ~$2,163 per final construct (reagents ~$902 + labor ~$1,261 at mixed PI-provided rates). Amortized across all 6 constructs produced (p154-p159, including precursors): ~**$1,442** per construct. For a repeat project reusing iPCR-ready backbones with no staircase-PCR recovery: ~**$1,940** per final construct. See Appendix F, Section 1.4 for the full cost breakdown.

14. Lessons Incorporated from the m971/m972 Project

The m971/m972 PSM project was the **first end-to-end run of this workflow**. Several process improvements baked into this SOP come directly from that project's experience:

- **Synthesis risk is not negligible.** IDT was unable to synthesize the m972 gBlock after **two attempts**, which cost ~4 weeks and forced scope cuts (Incucyte dropped, SynNotch arm collapsed to Jurkats only). **Mitigation:** for high-complexity-score sequences, order the gBlock first but be prepared to pivot quickly to an in-house staircase PCR assembly if the vendor fails. Have oligo designs roughed out so the fallback path can be initiated immediately on a vendor cancellation notice rather than starting from scratch. If the synthesis step sits on a time-critical path, ordering both in parallel is justified; otherwise, default to the sequential approach to avoid paying for oligos that may never be used.
- **Staircase PCR is a viable rescue.** The m972 scFv was successfully assembled in-house from 24 tiled 60 bp oligos (documented in the 2026-02-09 lab notebook entry). This demonstrated that the lab can execute gene synthesis in-house when vendor synthesis fails. Adopt this as the standing fallback path.
- **Transduction marker separability matters.** The m971/m972 SynNotch constructs lacked a constitutive mCherry transduction marker, which prevented separating (transduction rate) from (SynNotch activation rate) in the final readout. **Mitigation:** add a constitutive fluorescent marker (mCherry or equivalent) to all future SynNotch constructs, and check the backbone at Stage 1.
- **Panel documentation must be captured at acquisition time.** The m971/m972 project deferred panel configuration completion to post-acquisition, creating ambiguity during downstream gating. **Mitigation:** capture and archive the channel-to-fluorophore-to-marker mapping **at the same time the acquisition template is set up**, not afterward.
- **Pre-lock the analysis pipeline before bench work begins.** The m971/m972 analysis was scaffolded after data acquisition, delaying results by 1-2 weeks. **Mitigation:** run Stage 6 scripts against synthetic or representative FCS files before real data exists, so the pipeline is debugged and ready to consume data the moment it arrives.
- **CD22-Fc / APC antigen staining is a nice-to-have, not a blocker.** The m971/m972 project dropped CD22-Fc/APC for budget reasons and relied on anti-G4S linker for expression verification. This is defensible; it measures CAR surface expression cleanly but does not confirm antigen-binding competence at the QC step. For scFvs where antigen-binding competence is already established in the literature, anti-G4S alone is sufficient; for novel scFvs, budget for CD22-Fc (or equivalent antigen-conjugate) staining.
- **Rechallenge is worth the extra bench time.** The rechallenge series was added beyond the original m971/m972 proposal and became the single most clinically relevant readout. Include it as a default readout in every future A/B run.
- **R/flowCore is the analysis tool of record.** R has superseded GraphPad Prism for this workflow because the full pipeline (loading, gating, stats, figures) is archivable code. Every future A/B run should start from the m971/m972 project's R pipeline as the template.

15. Sub-appendices

Sub-appendix A - Reference to the m971/m972 project materials

The following project materials are archived in the m971/m972 project repository and BenchBuddy:

- **Staircase PCR reference protocol:** documented in the 2026-02-09 lab notebook entry
- **R analysis pipeline template:** six-script pipeline (inspect, gate, stats, figures) in the project analysis directory
- **Panel configuration template:** CytoFLEX channel-to-fluorophore-to-marker mapping file
- **Project state handoff document** and **Proposal-vs-actual delta tracker**

Sub-appendix B - Standing reagent/catalog list

The complete 76-item reagent inventory with IPN, vendor, catalog number, and unit pricing is maintained in the Pulsipher Lab's BenchBuddy system. The "Lab-standing" reagent list in Section 3 of this SOP is the distilled version for bench use; the BenchBuddy inventory is the procurement-level reference.

Sub-appendix C - CytoFLEX LX channel layout reference

The two acquisition templates used by the m971/m972 project (28-parameter rechallenge/timecourse and 14-parameter SynNotch) plus the channel-to-fluorophore-to-marker mapping are maintained in the project's panel configuration file. Consult these before setting up a new acquisition template for a future project.

Sub-appendix D - Lead recommendation memo template

Lead scFv Recommendation Memo
Project: [project name]
Date: [date]
Author: [name]
PI: [PI name]
Recommendation
Lead scFv for [BBz CAR / SynNotch / both]: [scFv name]
One-sentence rationale: [e.g., "m971 is recommended as the lead BBz CAR scFv based on superior rechallenge durability and equivalent acute cytotoxicity relative to m972."]
Constructs tested

Construct	scFv	Architecture	Plasmid ID	Backbone

Positive control: [e.g., CD19-BBz (p3)]
Negative control: [e.g., untransduced T cells]
Key results

Acute cytotoxicity (timecourse): [1-2 sentences + one summary figure or table. State which scFv killed more effectively at which E:T ratios and timepoints, and whether the difference was statistically significant.]

Rechallenge durability: [1-2 sentences + one summary figure or table. State which scFv maintained killing capacity across rounds, and at which round the comparator dropped off (if applicable).]

SynNotch activation (if applicable): [1-2 sentences. State BFP induction levels at each T:E ratio and whether the lead scFv activated the reporter above baseline.]

Expression QC: [1-2 sentences. Post-enrichment CAR+ %, titer, Plasmidsaurus verification status.]

Limitations

- [ ] Single PBMC donor / number of biological replicates: [state N]
- [ ] [any scope cuts, assays not run, or known confounds]
- [ ] [any construct-level caveats, e.g., no constitutive transduction marker on SynNotch constructs]

Recommended next steps

[e.g., "Repeat with 1-2 additional PBMC donors to confirm donor-independence."]
[e.g., "Advance lead scFv into prime-and-kill architecture with SynNotch gating."]
[e.g., "Evaluate in NSG mouse model if preclinical path is warranted."]

This memo is the terminal deliverable of the scFv A/B testing workflow (Section 10.4).

Appendix F: Cost-per-Construct Analysis - In-House vs. Outsourced

Document: PSM Professional Project Business Objective #6 / Appendix F of the final report
Author: Tyler Henderson
Version: 1.1 (2026-04-11)

This appendix presents a make-vs-buy analysis for scFv A/B testing construct production, using the m971/m972 CD22 CAR project as the primary cost data point. It compares in-house costs against commercial plasmid synthesis services and contract research organizations at two scopes: plasmid construction alone, and the full screening workflow from design through functional data.

Executive summary

This analysis addresses two make-vs-buy questions at different scopes:

Question 1 - Plasmid construction: What does it cost to produce a sequence-verified, maxiprepped lentiviral transfer plasmid in-house versus ordering from a commercial plasmid synthesis service?

Answer: In-house plasmid construction cost **~$844 per construct** in the m971/m972 project (or ~$624 for a clean run without the m972 gBlock recovery). Commercial plasmid synthesis services (VectorBuilder, GenScript, Twist) range from **~$400-$1,100 per construct** for a turnkey "send sequence, receive transfection-ready plasmid" deliverable, with VectorBuilder at **~$400-700** being the most competitive all-in option. In-house and outsourced plasmid costs are comparable at current rates. The in-house advantage is iteration speed (re-attempt a failed cloning the next day vs. a new vendor cycle) and the ability to recover from synthesis failures in-house (as demonstrated by the m972 staircase PCR recovery). The outsourced advantage is reduced bench time (~4 h/plasmid management vs. ~16 h/plasmid bench work).

Question 2 - Full A/B testing workflow: What does the complete scFv screening pipeline cost, from construct design through functional data and analysis, in-house versus outsourcing the entire workflow to a CRO?

Answer: The full in-house workflow cost **~$2,163 per final construct** (~$8,651 total for 4 constructs). Outsourcing the equivalent scope to a CRO would cost an estimated **~$3,150-$4,400 per construct** (from published CRO pricing), making in-house **~2x cheaper**. Outsourcing saves ~50-55 person-hours (~30%), but does not reduce calendar time because the functional assay and analysis stages (~93 h, ~4 weeks) must be done in-house regardless. The in-house path also provides faster failure recovery, full IP control, and trainee skill development.

Overall recommendation: In-house production is the default for routine scFv A/B screening at the Pulsipher Laboratory. For plasmid construction specifically, outsourcing to a service like VectorBuilder is a reasonable alternative when internal capacity is limited, since the per-plasmid cost is comparable. Outsourcing the full workflow is cost-effective only at higher institutional labor rates or for GMP/clinical-grade work.

Analytical approach

- **Cost the actual m971/m972 project** (in-house) as a concrete data point, broken out by stage so that plasmid construction and downstream functional work can be compared independently.
- **Collect outsourced pricing at two scopes:** (a) plasmid construction only, from commercial gene synthesis and cloning services, and (b) the full A/B workflow, from CROs and academic vector cores.
- **Normalize** to a per-construct cost at each scope level so the paths are comparable.
- **Compare hours and calendar time**, not just dollars; outsourcing shifts labor rather than eliminating it.
- **Account for non-cash factors** the dollar numbers don't capture: timeline control, IP, iteration speed, failure recovery, capability building.
- **Include the IDT failure as a concrete risk**; outsourcing has failure modes that aren't priced into the quote, and this project has direct evidence of one.

Part 1 - In-house cost accounting (the m971/m972 project)

1.1 Reagent costs by stage

The complete line-item inventory (76 items with vendor, catalog number, quantity, unit price, price source date, and allocation rule) is maintained in the Pulsipher Lab's BenchBuddy system. Every catalogued line is priced against the lab's internal catalog (IPN-indexed), with allocation rules reflecting fractional usage, fixed charges, whole-unit consumption, lab stock (no direct charge), and vendor-direct items not in the catalog system.

The table below summarizes the **catalogued** costs by stage. Items not in the Pulsipher catalog (lab stock, vendor-direct) are called out separately in Section 1.1.1.

Stage	Catalogued cost	Dominant line items
Design / Synthesis (primers)	**$500.00**	Genewiz blanket PO allocation for the 43-primer cloning plate (iPCR backbone primers + insert-specific primers for all 6 constructs, 1/2 of one plate budget)
Cloning	**$569.60**	Plasmidsaurus sequencing ($210; 6 culture + 6 plasmid submissions); PureLink HiPure maxiprep ($106.60; 6 preps); NEB Stable competent cells ($76.20; 6 transformations); NEB HiFi Assembly ($68.16; 6 rxns); Q5 HiFi MM ($34.88); gel supplies + extraction kit (~$42); restriction/cloning enzymes PaqCI+DpnI+GC enhancer (~$14); PureLink miniprep ($18.05)
Lentivirus production	**$702.24**	Lenti-X 293T cells ($422.00; 1 vial, the single largest reagent line); Lenti-X Concentrator ($139.54; ~80 mL); complete DMEM ($59.12); Lenti-X p24 Rapid Titer ELISA ($40.94; 6 wells); TC plates + filters + Opti-MEM + PEI + trypsin + sucrose (~$41)
T-cell prep	**$153.74**	EasySep Human T cell isolation kit ($113.62; single isolation); rhIL-2 GMP ($32.40); Dynabeads CD3/CD28 activator ($7.72)
Transduction	**$409.15**	Premium FBS for R10 ($234.00; ~300 mL across 6 weeks of T-cell culture); complete RPMI ($81.24; ~3 L); RetroNectin ($85.00; 24 wells coated); Pen-Strep + 48-well plates + polybrene (~$9)
CAR enrichment	**$49.73**	Biotinylated anti-G4S mAb CST #17621 ($43.98); Streptavidin Dynabeads M-280 ($5.75)
Flow cytometry	**$120.85**	Anti-G4S Pacific Blue CST #44962 ($102.60; ~20 uL across TD check + rechallenge + timecourse); 96-well round-bottom plates for acquisition ($18.25)
Functional assays (plasticware only)	**$20.18**	Three TC plates: one 24-well for rechallenge, one 96-well for timecourse, one 24-well for SynNotch
General consumables	**$412.59**	VWR filter pipette tips ($334.65; dominant line, ~2,000 tips across 6 weeks); serological pipettes ($22.43); HEK293T maintenance DMEM ($29.56); conicals + microcentrifuge + cryovials + nuclease-free water (~$25)
Subtotal - catalogued reagents	**$2,938.08**	
+ IDT direct orders (Section 1.1.1a)	~$427	gBlocks (2 x $75) + staircase PCR oligos (~$267) + IDTE (~$10)
+ Vendor-direct (Section 1.1.1b)	~$241	STEMCELL PBMCs (~$203) + NEB rSAP + Corning flasks
Total reagents and consumables	**~$3,606**	Lab-stock items used but not costed (Section 1.1.1c)

Per-construct cost (reagents only): ~$3,606 / 4 final constructs = **~$902 per final construct**. Amortized across all 6 constructs made (p154/p155 precursors + p156-p159 final): **~$601 per construct**. For the fully-loaded per-construct cost including labor, see Section 1.4.

1.1.1 Gaps in the catalogued total

The $2,938.08 subtotal **does not include** four categories of spend:

(a) IDT direct orders - These were ordered directly from IDT and do not route through the Pulsipher catalog system. gBlock costs confirmed at institutional pricing; oligo costs estimated from university core facility IDT pricing ($0.18/base at 25 nmol standard desalt).

Item	Quantity	Cost	Source
m971 scFv gBlock (successful)	1 x ~750 bp	**$75.00**	Confirmed institutional rate
m972 gBlock attempt 1 (FAILED)	1	**$0.00**	IDT did not bill for failed synthesis
m972 gBlock attempt 2 (FAILED)	1	**$0.00**	IDT did not bill for failed synthesis
m972 codon-diversified reorder	1 x ~750 bp	**$75.00**	Same IDT gBlock pricing
m972 staircase PCR oligos	24 x 60-mer, std desalt	**~$259**	Est. 24 x 60 bases x $0.18/base
m972 outer flanking primers	2 x 22-mer, std desalt	**~$8**	Est. 2 x 22 bases x $0.18/base
IDTE buffer pH 8.0	1 tube (~1 mL)	**~$10**	Est. IDT catalog
IDT direct subtotal		**~$427**	

(b) Vendor-direct (not in Pulsipher catalog) - estimated from distributor list prices:

Item	Quantity	Cost	Source
Cryopreserved PBMCs (frozen)	1 vial	**~$203**	STEMCELL Technologies cat. #70025, Fisher Scientific list price $203.35
rSAP (NEB M0371S, 500 U)	1 uL (1 U of 500 U pack)	**~$0.12**	NEB list $58/500 U, fractional allocation
T-25 / T-75 culture flasks	~10 flasks	**~$38**	Est. ~$3.84/flask from Corning 430641U ($384/100-case)
Vendor-direct subtotal		**~$241**	

(c) Lab stock - these items were used in the project but are difficult to quantify as direct project costs. They represent standing lab infrastructure whose costs are absorbed across many projects:

- psPAX2 and pMD2.G packaging plasmids (produced in-house from Addgene deposits)
- NALM-6 GFP target cell line, Jurkat E6-1 cell line (maintained as standing lab stocks)
- FACS buffer (PBS + 2% FBS, made in-lab), SOC, LB broth, ampicillin, LB-Amp agar plates, PBS, DPBS (all prepared from bulk powder stocks)
- Glycerol, PFA, streptavidin-Pacific Blue conjugate (lab stock aliquots)

A CRO comparison should account for the fact that these represent real spend; a CRO would charge for equivalent materials. For the in-house accounting, these costs are captured implicitly in the combined labor + overhead rate provided by the PI (see Sections 1.2-1.3).

Updated reagent subtotal (all sources):

Source	Cost
Catalogued (Pulsipher Lab catalog)	$2,938.08
IDT direct orders	~$427
Vendor-direct (STEMCELL, NEB, Corning)	~$241
Lab stock (used, difficult to quantify)	not costed
Total reagents and consumables	**~$3,606**

1.2 Personnel time by stage

The biggest hidden cost. Use a reasonable hourly rate for a graduate researcher (or the lab's standing fully-loaded rate).

Estimated hours from notebook timeline reconstruction (dates from lab entries):

Stage	Dates	Calendar days	My hours (estimate)	Supervisor hours	Other	Notes
1. Design + oligo ordering	Feb 6 - Feb 20	14	~15 h	~2 h	~4 h (Payton)	Computational validation, primer design, staircase PCR oligo design, IDT ordering
2. Cloning (iPCR + HiFi + screening + staircase PCR)	Feb 21 - Feb 28	7	~30 h	~2 h	~8 h (Payton)	Full bench days: iPCR, insert PCRs, HiFi assembly, transformation, colony picking, miniprep, Plasmidsaurus submission, glycerol stocks
2b. IDT gBlock failure wait time (not active bench)	Jan 26 - Feb 20	~25	~3 h	0	0	Time spent communicating with IDT, monitoring order status; mostly idle wait time that consumed calendar schedule
3. Maxiprep + lentivirus production + titering	Mar 1 - Mar 9	8	~25 h	~1 h	0	Maxiprep, HEK293T thaw/passage/seeding, PEI transfection, 48h+72h harvests, Lenti-X concentration, p24 ELISA
4. T-cell prep + transduction + enrichment	Mar 1 - Mar 12	11	~20 h	~1 h	0	PBMC thaw, T-cell isolation, Dynabead activation/removal, Jurkat thaw/culture, RetroNectin transduction (overlaps with stage 3)
4b. CAR+ magnetic selection	Feb 28	1	~4 h	0	0	Anti-G4S/Dynabead enrichment of all 4 constructs
5. Functional assays	Mar 17 - Apr 3	17	~35 h	~2 h	0	NALM-6 thaw/expansion, rechallenge setup + 5 round readouts, cytotoxicity timecourse setup + 3 acquisitions, SynNotch BFP co-culture, TD flow check
6. Data analysis + report writing	Apr 6 - present	ongoing	~30 h	~2 h	0	R pipeline development, data organization, report scaffolding, AI-assisted analysis
TOTAL	**Jan 26 - Apr 10**	**~74 days**	**~162 h**	**~10 h**	**~8 h**	

Hourly rates - provided by the PI for this project. Rates differ by role to reflect different institutional cost structures.

Personnel	Hours	Rate	Cost
Graduate researcher (T. Henderson)	162 h	$22.50	**$3,645.00**
Supervisor (Dr. Candi Deimundo Roura)	10 h	$100.00	**$1,000.00**
Payton Utzman (lab colleague - staircase PCR)	8 h	$50.00	**$400.00**
Total labor + overhead	**180 h**		**$5,045.00**

1.3 Equipment utilization and overhead allocation

The project proposal listed equipment utilization as a cost component. In practice, equipment costs are **not separately itemized** here because they are already captured in the PI-provided labor rates. The Pulsipher Lab uses a blended rate structure where each hourly rate reflects the institutional cost of a researcher using existing lab infrastructure; there is no per-instrument access fee or hourly equipment charge in this lab's cost model. The major equipment used (CytoFLEX LX flow cytometer, biosafety cabinet, CO2 incubator, thermocycler, centrifuges, gel imaging system) is shared infrastructure whose acquisition and maintenance costs are amortized across the lab's full project portfolio and funded through the department, not charged to individual projects.

This is a common structure in academic core-funded labs and differs from fee-for-service core facilities (which do charge per-instrument-hour). If the Pulsipher Lab were to adopt per-instrument pricing in the future, the CytoFLEX LX (~12 h of acquisition time across all sessions) would be the largest equipment line item, typically $25-75/h at academic core rates, adding ~$300-900 to the project total.

For the CRO comparison in Part 3, note that a CRO quote includes its own equipment amortization, facility overhead, and profit margin in the per-construct price, so the comparison is apples-to-apples at the total-cost level even though the cost structures differ.

1.4 Total in-house project cost

Category	Cost	Notes
Reagents and consumables - catalogued	$2,938.08	76 items from Pulsipher Lab catalog (Section 1.1)
Reagents and consumables - IDT direct	~$427	gBlocks ($150) + staircase PCR oligos (~$267) + IDTE (~$10) (Section 1.1.1a)
Reagents and consumables - vendor-direct	~$241	STEMCELL PBMCs (~$203) + NEB rSAP + Corning flasks (Section 1.1.1b)
Lab stock (used, not costed)	N/A	Packaging plasmids, cell lines, bulk buffers/media powders (Section 1.1.1c)
Labor + overhead (180 h, mixed rates)	$5,045.00	162 h grad researcher @ $22.50 + 10 h supervisor @ $100 + 8 h colleague @ $50 (Section 1.2)
TOTAL	**~$8,651**	

Constructs produced: 4 final lentiviral constructs (p156, p157, p158, p159) + 2 precursor constructs (p154, p155) = **4 functionally final + 2 precursor**.

Cost per final construct (in-house, m971/m972 project):

~$8,651 / 4 final constructs = **~$2,163 per final construct**

Amortized across all 6 constructs actually produced: ~$8,651 / 6 = **~$1,442 per construct**.

Cost breakdown insight: Reagents (~$3,606, ~42%) and labor (~$5,045, ~58%) are the two dominant cost categories, with labor slightly larger due to the supervisor rate ($100/h). Within reagents, the top 5 line items (Lenti-X 293T cells $422, pipette tips $335, FBS $234, staircase PCR oligos ~$259, Plasmidsaurus sequencing $210) account for ~$1,460, about 40% of the reagent total.

Part 2 - Outsourced quote collection

2.1 Outsourced plasmid synthesis services (published pricing)

Formal quotes were not collected for this project due to timeline constraints. The estimates below are drawn from published pricing on vendor websites, academic contract pricing sheets, and vector core fee schedules (accessed April 2026).

Scope: turnkey plasmid construction (gene synthesis + cloning into lentiviral backbone + sequence verification + EndoFree maxiprep, >=500 ug delivered)

Vendor	Per-plasmid estimate	Turnaround	Includes	Notes
VectorBuilder (all-in quote)	**$400-700**	7-14 BD	Synthesis, cloning, sequencing, EndoFree maxiprep	Best turnkey option; instant online quote via CAR vector design tool; 10-15% batch discount for 4+ constructs
GenScript Economy ($0.19-0.29/bp + subcloning + maxiprep)	**$650-850**	15-25 + 6 BD prep	Synthesis, subcloning, Sanger verification, EndoFree maxiprep	Slowest but reliable; academic contracts 10-20% off list
GenScript Standard ($0.35-0.45/bp)	**$850-1,100**	8-15 + 6 BD prep	Same as economy	No complexity surcharge
Twist Bioscience ($0.09/bp + custom vector onboarding)	**$350-600** (Twist vector) or **$850+** (custom backbone)	8-13 BD	Synthesis, NGS verification, 5 ug miniprep default	Cheapest per-bp; $500 one-time custom vector onboarding fee amortizes to ~$85-125/plasmid over 4-6 constructs; EndoFree maxiprep by separate arrangement
Azenta/GENEWIZ (quote-based)	**$500-900** (estimated)	5-15 BD	Synthesis, cloning, verification	Must request quote; includes vector onboarding
Bio Basic ($0.09/bp + subcloning)	**$400-550** + DIY maxiprep	18-24 BD	Synthesis, subcloning, Sanger verification	Budget option; maxiprep not offered as service
Synbio Technologies (~$0.15/bp clonal)	**$300-500** + DIY prep	5-15 BD	Synthesis, verification	Competitive on short genes; no maxiprep service
WashU Hope Center Vector Core (academic)	**$750-1,320**	Variable	Subcloning, QC	Requires customer-supplied insert; higher end is for complex assemblies

Estimated range: $400 - $1,100 per plasmid (turnkey with maxiprep). Midpoint: ~$650.
Sources: VectorBuilder Vector Cloning service page, GenScript Gene Synthesis service page, GenScript-PSU Academic Contract 2024-2025, GenScript Plasmid Prep service page, Twist Bioscience Clonal Genes FAQ, Bio Basic Genes Pricing page, Synbio Technologies Whole Gene Synthesis page, WashU Hope Center Viral Vectors Core charges page. Accessed April 2026.

2.2 Outsourced full-workflow estimates (CRO + vector core pricing)

For comparison at the full-workflow scope (cloning + lentivirus production + titering + optional functional validation), pricing was estimated from published CRO and vector core fee schedules.

Scope (b): Plasmid construction + lentivirus production + p24 titering

Vendor / Source	Per-construct estimate	Notes
VectorBuilder Medium scale (1 mL, >3x10^8 TU/mL)	$649 (virus only)	Add $350-$860 for cloning = $1,000-$1,500 total
VectorBuilder Large scale (1 mL, >2x10^9 TU/mL)	$1,099 (virus only)	Add cloning = $1,450-$1,960 total
VectorBuilder Ultra-purified Large	$1,699 (virus only)	Add cloning = $2,050-$2,560 total
University of Iowa Vector Core (500 uL concentrated HIV lenti)	$2,377 (virus only)	Requires customer-supplied transfer plasmid
WashU Hope Center (large-scale lenti + FACS titer)	$2,160 (virus + titer)	Add $750-$1,320 for cloning = $2,910-$3,480 total

Estimated range (Scope b): $1,800 - $4,500 per construct

Scope (c): Full service including functional CAR expression validation

No vendor publishes fixed pricing for functional CAR validation (T-cell transduction + flow cytometry). Creative Biolabs and Charles River offer this as custom-quoted work. Based on academic core fees and labor intensity, an additional $500 - $2,000 per construct is expected for functional validation.

Estimated range (Scope c): $2,300 - $6,500 per construct

Sources: VectorBuilder Lentivirus Packaging service page, VectorBuilder Plasmid DNA Prep service page, Twist Bioscience Clonal Genes FAQ, GenScript FLASH Gene service page, WashU Hope Center Vector Core charges page, University of Iowa Vector Core lentivirus products page. All accessed April 2026.

2.3 Normalized per-construct outsourced cost

Using the midpoint of each range as the representative estimate:

- **Plasmid construction only (Section 2.2):** ~$650 (range $400 - $1,100)
- **Plasmid + virus + titer (Section 2.3 scope b):** ~$3,150 (range $1,800 - $4,500)
- **Full A/B workflow (Section 2.3 scope c):** ~$4,400 (range $2,300 - $6,500)

These are the comparison points for Parts 3 and 4.

Part 2.5 - gBlock ordering vs in-house staircase PCR: cost and time comparison

This is a micro-level make-vs-buy analysis within the cloning stage, directly informed by the m972 experience. It feeds into the broader project-level analysis in Part 3 and provides a concrete data point for the report's Reflection narrative.

The m972 scenario (what actually happened)

Path	Money cost	Time cost	Outcome
IDT gBlock attempt 1	$0 (IDT did not charge for failure)	~2 weeks turnaround (wasted)	Failed
IDT gBlock attempt 2	$0 (IDT did not charge for failure)	~2 weeks turnaround (wasted)	Failed
In-house staircase PCR	Oligo cost + enzyme cost (see below)	~1 week (design + execution)	Succeeded
Total actual	Oligo cost only	~5 weeks elapsed (2 failed IDT rounds + 1 week staircase PCR)	Complete

Cost comparison: gBlock vs staircase PCR for a single 720 bp scFv

Item	IDT gBlock (when it works)	In-house staircase PCR
Gene fragment / oligos	**$75** (1 x IDT gBlock, ~750 bp; confirmed institutional pricing)	**~$267** (24 x 60-mer oligos at $0.18/base = ~$259 + 2 x 22-mer primers at $0.18/base = ~$8; IDT 25 nmol standard desalt, per university core pricing)
Enzyme / reagents	N/A (gBlock arrives ready to use)	**~$18** (Q5 HiFi MM ~3 rxns, PureLink gel extraction ~1 rxn, agarose/ladder/stain allocation, buffers/consumables; all allocated fractionally from Section 1.1 Cloning)
Sequencing	N/A	N/A (not performed; verified post-cloning via Plasmidsaurus)
Reagent subtotal	**$75**	**~$285**

Key observation: At institutional gBlock pricing ($75), the gBlock path is **~3.8x cheaper** in reagent cost than staircase PCR (~$285). The default strategy should be to **order the gBlock first** and only order staircase PCR oligos if the gBlock fails. The ~$285 in oligos is a recovery cost, not a standing insurance premium. However, if the synthesis step sits on a critical path in a time-constrained project (as it did here), the ~$285 redundancy premium may be worth paying up front to avoid a ~4-week schedule catastrophe, but this is the exception, not the default.

At standard (non-institutional) IDT gBlock pricing ($150-250 for a ~750 bp fragment), the cost gap narrows and staircase PCR becomes roughly comparable. The lesson is institution-dependent: labs with favorable gBlock pricing should default to gBlock-first with staircase PCR as a contingency; labs paying full list may want to consider staircase PCR as a primary path for sequences with known synthesis risk factors.

Time comparison

Activity	IDT gBlock (clean run)	IDT gBlock (with failure)	In-house staircase PCR
Design time	~1 h (upload sequence to IDT)	Same	~4 h (tile oligos, check overlaps, order)
Vendor turnaround	5-10 business days	5-10 days x N attempts	N/A
Bench execution	N/A (arrives ready)	N/A	~6 h (resuspension + assembly + amplification + gel verification + cleanup)
Total hands-on time	~1 h	~1 h (x N orders)	~10 h
Total elapsed time	~2 weeks	~2 weeks x N attempts	~1 week (from oligo arrival to verified product)
Oligo delivery	N/A	N/A	~3-5 business days (standard shipping from IDT)

Key insight for future projects

Scenario	Recommended path	Rationale
Low-complexity scFv, no time pressure	**IDT gBlock**	Cheaper, less hands-on time, reliable for most sequences. Order staircase PCR oligos only if the gBlock fails.
High-complexity scFv (GC-rich, repetitive linkers), no critical-path time pressure	**IDT gBlock first; design staircase PCR oligos as contingency**	Order the gBlock. Have the staircase PCR oligo design ready but do not order oligos unless the gBlock fails. The ~$285 in oligos is a recovery cost, not a default spend.
High-complexity scFv on a critical path in a time-critical project	**Parallel: order gBlock + staircase PCR oligos simultaneously**	When a synthesis failure would cause an unrecoverable schedule collapse, ~$285 upfront in oligos is worth the redundancy premium. Total parallel cost ~$360 ($75 gBlock + $285 staircase PCR). If the gBlock arrives clean, discard the oligos. This is the exception for high-stakes timelines, not the default.
Any sequence after a first IDT failure	**Switch immediately to staircase PCR**	The m972 project waited for a second IDT attempt before switching, costing an additional ~2 weeks. After one failure, the probability of a second failure on the same sequence is high.
GMP/regulatory context	**IDT gBlock (with certificate of analysis)**	Vendor-provided CoA is valuable for regulatory documentation; in-house staircase PCR would need additional QC documentation

Time-cost framing for the report

The m972 IDT failure cost **zero dollars** (IDT didn't charge for two failed attempts) but cost **~4 weeks of project timeline**, which is the most expensive resource in a 9-week project. The staircase PCR recovery cost **~$285** in oligos/enzymes and **~10 hours of bench time** (~$225 at the PI's $22.50/h rate = **~$510 fully loaded**), but saved the project from a potentially unrecoverable schedule collapse. The lesson: **the default should be to order the gBlock and have a staircase PCR design ready as a contingency. If the gBlock fails, switch immediately; do not wait for a vendor re-attempt on the same sequence.** For projects where the synthesis step sits on a critical path with no schedule float, pre-ordering staircase PCR oligos in parallel (~$285 redundancy premium) may be justified, but this is an exception for high-stakes timelines, not standard practice. Could you thoroughly review this report

Part 3 - Break-even analysis

3.1 Side-by-side comparison: plasmid construction

This is the tighter comparison: the cost of producing a sequence-verified, maxiprepped lentiviral transfer plasmid, in-house versus ordering from a commercial service.

Category	In-house (actual)	In-house (clean run)	Outsourced (service midpoint)
Per-plasmid cost	**~$844**	**~$624**	**~$650** (range $400-1,100)
Reagent component	~$374	~$289	included in service price
Labor component	~$470 (mixed rates, ~16 h/plasmid)	~$335 (~12 h/plasmid)	~$90 (~4 h/plasmid management at $22.50)
Bench time per plasmid	~16 h	~12 h	~0 h (vendor does the work)
Management time per plasmid	0 h	0 h	~4 h (specs, review, QC)
Calendar time (4 plasmids)	~7-8 weeks (with slip)	~3-4 weeks	~2-4 weeks (vendor turnaround)

In-house "actual" includes the m972 gBlock failure and staircase PCR recovery costs. "Clean run" removes ~$342 in recovery reagents and ~$538 of recovery labor (Tyler ~15 h + Payton ~4 h), representing the expected cost for a future project using the same backbones.

In-house per-plasmid cost: (Design/Synthesis $500 + IDT direct ~$427 + Cloning $570 reagents) + (Tyler 48 h x $22.50 + Supervisor 4 h x $100 + Payton 8 h x $50 = $1,880 labor) = ~$3,377 / 4 = ~$844. Clean run: (~$3,377 - $342 reagents - $538 labor) / 4 = ~$624.

Key finding: For a clean run, in-house plasmid construction (~$624) is competitive with the cheapest commercial services (VectorBuilder ~$400-700). With recovery costs included (~$844), outsourcing is slightly cheaper at the midpoint (~$650). The decision between paths rests on capacity, speed, and failure-recovery considerations rather than pure cost.

3.1b Side-by-side comparison: full A/B testing workflow

This is the broader comparison: the full scFv screening pipeline from design through functional data.

Category	In-house (actual)	Outsourced (CRO midpoint)	Delta
Per-construct cost (plasmid + virus + titer)	**~$1,270**	**~$3,150**	outsourced 2.5x
Per-construct cost (full scope)	**~$2,163**	**~$4,400**	outsourced 2.0x
Timeline (clean run)	~7 weeks	~8-12 weeks	in-house faster
Timeline (with cloning slip)	~11 weeks	~10-14 weeks	comparable

In-house per-construct costs:

- *Plasmid + virus: add Lentivirus reagents $702 + lentivirus labor (Tyler 25 h x $22.50 + Supervisor 1 h x $100) = ~$5,082 / 4 = ~$1,270*
- *Full scope: total project ~$8,651 / 4 = ~$2,163*

Key finding: At full-workflow scope, in-house is **~2x cheaper** than outsourcing. The cost gap widens beyond the plasmid stage because lentivirus production and functional assays carry high CRO markups.

3.1.3 Personnel hours comparison

Outsourcing does not eliminate labor; it shifts it. The table below compares hours for two outsourcing scenarios: plasmid-only (outsource stages 1-2, do everything else in-house) and full-workflow (outsource stages 1-3).

Stage	In-house (actual)	Outsource plasmid only	Outsource stages 1-3	Notes
1. Design + ordering	~21 h	~8 h	~8 h	Outsourced: write specs, review vendor proposals, approve.
2. Cloning + sequencing	~44 h	~5 h	~5 h	Outsourced: review deliverables + QC only.
2b. Vendor failure management	~3 h	~3 h	~3 h	Calendar slip occurs either way.
3. Lentivirus production + titering	~26 h	~26 h	~3 h	In-house if only plasmid is outsourced.
4. T-cell prep + transduction + enrichment	~24 h	~24 h	~24 h	In-house regardless.
5. Functional assays	~37 h	~37 h	~37 h	In-house regardless.
6. Data analysis + reporting	~32 h	~32 h	~32 h	In-house regardless.
Project management overhead	0 h	~8 h	~10-15 h	Vendor communication, shipping, QC review.
Total	**~180 h**	**~143 h**	**~125-130 h**	
Bench hours saved	-	**~37 h (21%)**	**~50-55 h (30%)**	

Conclusion: Outsourcing plasmid construction alone saves ~37 bench hours (~21%), mostly the cloning stage. Outsourcing through virus production saves ~50-55 h (~30%), but the functional assay and analysis stages (~93 h) are in-house regardless. Both outsourcing paths add project management overhead that does not exist in the fully in-house workflow.

3.1.4 Timeline comparison

Scenario	In-house	Outsource plasmid only	Outsource stages 1-3
Plasmid construction	~3-4 weeks (clean)	~2-4 weeks (vendor)	~2-4 weeks (vendor)
Lentivirus production	~1-2 weeks	~1-2 weeks (in-house)	~2-3 weeks (vendor, add to plasmid turnaround)
Functional + analysis	~4 weeks	~4 weeks	~4 weeks
Total (clean run)	**~7 weeks**	**~7-10 weeks**	**~8-12 weeks**
Total (with cloning slip)	**~11 weeks**	**~9-12 weeks**	**~10-14 weeks**

Conclusion: Outsourcing plasmid construction may save 1-2 weeks on a clean run (vendor turnaround can be faster than in-house bench work), but does not help with the downstream stages. In the event of a synthesis failure, the in-house path can recover faster (start staircase PCR the next day) while an outsourced path requires a new vendor cycle.

3.2 Sensitivity analysis: at what labor rate does outsourcing win?

A traditional fixed-vs-marginal break-even on volume is not the right frame here. The Pulsipher Lab already has all the infrastructure in place (no incremental fixed cost), and the in-house marginal cost (~$2,163/construct) is below the outsourced price (~$4,400) at every volume. Volume break-even only matters when a lab is deciding whether to *build* infrastructure; this lab already has it.

The more useful question is: **at what hourly labor rate does outsourcing become the cheaper path?** Labor is 58% of the in-house cost, and the grad student rate ($22.50/h for 162 of 180 total hours) is the dominant variable. Holding reagent costs and supervisor/colleague hours constant, I can solve for the grad student rate *r* at which in-house cost equals the outsourced midpoint.

Full-workflow crossover (in-house vs. CRO at ~$4,400/construct):

```
Total in-house = Reagents + Tyler labor + Supervisor labor + Payton labor
               = $3,606 + (162 x r) + $1,000 + $400

Set equal to outsourced total for 4 constructs:
$5,006 + 162r = 4 x $4,400 = $17,600
162r = $12,594
r = ~$77.74/h
```

At the current $22.50/h, in-house is ~2x cheaper. Outsourcing the full workflow would not become competitive until the grad student rate exceeds **~$78/h**, which is well above typical academic rates even at R1 institutions with full fringe-loaded costs ($45-65/h). The in-house advantage at full-workflow scope is robust.

Plasmid-only crossover (in-house clean run vs. synthesis service at ~$650/plasmid):

```
Plasmid stages in-house (clean run) = $1,155 reagents + (33 x r) Tyler + $400 supervisor
Set equal to outsourced total for 4 plasmids:
$1,555 + 33r = 4 x $650 = $2,600
33r = $1,045
r = ~$31.67/h
```

At the current $22.50/h, a clean-run plasmid costs ~$624 in-house vs. ~$650 outsourced; nearly equal. The crossover is **~$32/h**, which is within range of many academic institutions' fully-loaded rates. This confirms that plasmid-only outsourcing is a genuinely competitive option at current market pricing, and the decision should rest on non-cash factors (Section 3.3) rather than cost alone.

Summary of crossover rates:

Scope	Outsourced midpoint	Crossover grad student rate	Current rate	Margin
Plasmid only (clean run)	~$650/plasmid	**~$32/h**	$22.50/h	Narrow; outsourcing competitive
Full workflow (scope c)	~$4,400/construct	**~$78/h**	$22.50/h	Wide; in-house strongly favored

Interpretation: The in-house cost advantage is driven almost entirely by the low grad student rate. For plasmid construction alone, outsourcing is already cost-competitive and should be decided on capacity and speed. For the full workflow, in-house remains the clear winner unless institutional labor rates rise substantially above current levels.

3.3 Non-cash factors (the decision matrix)

A pure dollar comparison misses most of the story. Weigh these explicitly:

Factor	In-house	Outsourced	Notes
Timeline control	Full control, but vulnerable to internal bottlenecks	External dependency, vulnerable to CRO queue and failure modes	The IDT failure is a concrete data point; outsourcing has real failure risk
Timeline predictability	Variable (this project had a 4-week slip)	Variable (CRO capacity fluctuates)	Neither is a clear winner
IP control	Full lab ownership	Depends on contract terms; some CROs retain rights	Worth reading the fine print on each quote
Iteration speed	Fast; can re-do a construct in days if a problem is found	Slow; every re-do is a new contract cycle	**In-house wins decisively here**; critical for screening workflows
Capability building	Builds lab expertise and institutional knowledge	No internal skill development	**In-house wins for lab strategy**
Failure handling	Lab absorbs the cost of failures but also learns from them	Pay for successes only (usually); failures are on the vendor	Mixed; m971/m972 project shows vendor failures can still cost the project its timeline
Volume flexibility	Linear in marginal cost	Fixed per-unit, hard to get volume discounts for small orders	In-house wins at higher volumes; outsourced wins at very low volumes
Quality control	Visible and debuggable	Black box	**In-house wins for protocol-sensitive work**

Factor	In-house	Outsourced	Notes
Budget timing	Distributed across grants	Single lump-sum commitment	Outsourced is easier to budget in discrete chunks
Hands-on training for trainees	Direct	None	**In-house wins for an academic training environment**

3.4 The IDT failure as a data point on outsourcing risk

This is the single most concrete lesson from the m971/m972 project.

What happened:

- The m972 scFv gBlock was ordered from IDT, a reputable, industry-standard commercial DNA synthesis vendor.
- IDT was unable to synthesize the fragment after **two separate attempts**.
- The failure was absorbed by in-house staircase PCR assembly of the scFv from 24 tiled oligos, a workflow the lab had the capability to execute.
- Timeline impact: **~4 weeks of schedule slip**, which consumed all downstream float and forced scope cuts (Incucyte dropped, SynNotch arm collapsed to Jurkats only).

What this tells the make-vs-buy analysis:

Commercial synthesis has a non-zero failure rate, and that failure rate is not priced into the quote. For a clean sequence the failure rate is vanishingly small, but for sequences with complex secondary structure or repetitive elements (like some scFvs), the failure rate can be meaningful.

The cost of a synthesis failure is not just the synthesis fee; it's the downstream project timeline, which can be substantially larger.

In-house capability serves as a risk backstop even if it's not the primary execution path. If the lab can execute staircase PCR when a vendor fails, the worst-case scenario is a longer in-house path, not a dead project. A lab that had fully outsourced would have had no recovery.

The default strategy is to order the gBlock first and have a staircase PCR design ready as a contingency. If the gBlock fails, switch immediately to staircase PCR without waiting for vendor re-attempts. For projects where the synthesis step sits on a critical path with no schedule float, pre-ordering staircase PCR oligos in parallel may justify the small redundancy premium to avoid a multi-week schedule catastrophe.

This failure mode is **not visible in a standard cost-per-construct comparison** but should be a named line item in the risk-adjusted analysis.

Part 4 - Recommendation framework

4.1 Recommendation

Plasmid construction (in-house vs. synthesis service):

In-house plasmid construction and commercial synthesis services are **cost-competitive** at the current rate structure. The in-house cost is ~$624-844 per plasmid (depending on whether recovery from a synthesis failure is needed) versus ~$400-1,100 from a commercial service (midpoint ~$650). The cost difference is not large enough to drive the decision on its own. The deciding factors are:

- **In-house advantages:** Faster failure recovery (staircase PCR can start the next day vs. a new vendor cycle), no vendor dependency for timeline-critical steps, hands-on training value, full QC visibility. In-house requires ~16 h/plasmid of bench time versus ~4 h/plasmid of management time for outsourced, but the bench time builds institutional cloning capability.
- **Outsourced advantages:** Frees ~12 h/plasmid of bench time for other work, may be 1-2 weeks faster on a clean run (vendor turnaround 2-4 weeks vs. in-house 3-4 weeks), no consumable inventory management.

- **Recommendation:** Default to in-house for routine work. Outsource plasmid construction (e.g., VectorBuilder at ~$400-700/plasmid) when internal capacity is limited, when the researcher's time is better spent on other tasks, or when speed on a clean run is the priority.

Full A/B testing workflow (in-house vs. CRO):

In-house is the clear winner at full-workflow scope. The in-house cost of ~$2,163 per construct is **~2x cheaper** than the estimated ~$3,150-$4,400 outsourced. Outsourcing saves ~50-55 person-hours (~30%) by offloading stages 1-3, but the functional assay, analysis, and reporting stages (~93 h, ~4 weeks) must be done in-house regardless, so outsourcing does not meaningfully reduce total project duration (~7 weeks in-house vs. ~8-12 weeks outsourced). The in-house path also provides faster failure iteration, full IP control, and trainee skill development.

Outsourcing the full workflow is recommended only for GMP/clinical-grade work requiring vendor certification, or when internal capacity is completely unavailable for an extended period.

4.2 Scenario-specific guidance

Scenario	Recommendation
Routine scFv A/B screens (>=4 constructs/year)	In-house; amortizes fixed costs over enough projects
One-off urgent timeline with no internal capacity	Outsourced; pay the premium for turnaround
Highly complex sequences with known synthesis failure risk	**In-house with contingency**; order gBlock first; have staircase PCR design ready as fallback. Only pre-order oligos in parallel if the step is on a critical path with no schedule float.
Novel scFv validation (unknown antigen-binding behavior)	In-house; iteration speed matters more than per-unit cost
Late-stage optimization of a committed lead	Either; decide on timeline pressure and IP considerations
GMP / clinical-grade work	Outsourced (specialized GMP vendor); NOT this workflow

4.3 Process improvements for future in-house runs

The m971/m972 project provides several concrete process improvements that would reduce future in-house cost:

Staircase PCR contingency design. For high-risk gBlock orders (GC-rich, repetitive linkers), design the staircase PCR oligo set in advance but do not order until a gBlock failure is confirmed. This costs only design time (~4 h) and eliminates the oligo-design delay if a switch is needed. Exception: if the synthesis step is on a critical path with no schedule float, pre-ordering oligos in parallel (~$285 per construct) may be worth the redundancy premium to avoid a multi-week schedule slip.

Pre-locked analysis pipeline. The m971/m972 analysis was built after data acquisition, adding 1-2 weeks to the Stage 6 timeline. Building the R analysis pipeline before bench work starts (as a dry-run against synthetic FCS data) saves time and personnel cost.

Standing reagent inventory. Many reagents in this project had to be ordered mid-project, adding procurement delays. Maintaining a standing inventory of high-usage reagents (HiFi mix, Q5, RetroNectin, anti-G4S-biotin) reduces per-project reagent ordering overhead.

Reusable infrastructure. The pELPS backbone (p3) and SynNotch backbone (p1) are now inverse-PCR-ready starting points for any future scFv swap. Future A/B runs don't need to re-build these backbones.

Part 5 - Executive Summary Memo (one-page format for the Pulsipher Lab)

TO: Dr. Michael Pulsipher, Pulsipher Laboratory
FROM: Tyler Henderson
RE: Make-vs-buy recommendation for scFv A/B testing construct production
DATE: 2026-04-11
SOURCE DATA: m971/m972 CD22 CAR project (PSM Professional Project, Jan-Apr 2026)

Bottom line

In-house scFv A/B testing is **~2x cheaper** than outsourcing the full workflow and provides faster failure recovery, full IP control, and trainee development. For plasmid construction alone, outsourcing is cost-competitive and reasonable when internal capacity is limited.

Key numbers

Metric	In-house	Outsourced	Ratio
Per-construct cost (full scope)	~$2,163	~$4,400	In-house 2.0x cheaper
Per-plasmid cost (clean run)	~$624	~$650	Near-parity
Total project (4 constructs)	~$8,651	~$17,600	In-house 2.0x cheaper
Personnel hours	180 h	125-130 h	Outsource saves ~30%
Calendar time (clean run)	~7 weeks	~8-12 weeks	In-house faster

When to outsource

Scenario	Path
Routine scFv screens, capacity available	**In-house**
Plasmid cloning, capacity limited	**Outsource plasmid** (VectorBuilder, ~$400-700)
Urgent one-off, no bench availability	**Outsource through virus**
GMP / clinical-grade constructs	**Outsource to GMP vendor**

In-house loses its cost advantage when the grad student rate exceeds **~$32/h** for plasmid-only scope or **~$78/h** for the full workflow. At the current PI-provided rate of $22.50/h, in-house is favored at full scope. For plasmid construction alone, the cost decision is essentially neutral; decide on capacity and speed.

Risk note

The m972 IDT gBlock failure (two failed synthesis attempts, ~4 weeks of schedule slip) demonstrates that outsourced synthesis has real failure modes not priced into vendor quotes. The in-house staircase PCR capability served as a critical risk backstop. Maintain this capability regardless of the primary production path.

Full analysis: Parts 1-4 of this appendix.

Appendix G: Detailed Protocols

This appendix contains the step-level protocol parameters referenced from Section 3 (Methods). These protocols were performed as described below unless otherwise noted in the corresponding body section.

G.1 Lentiviral Transfection Protocol (referenced from §3.2)

Three-plasmid co-transfection was performed in 10-cm tissue culture plates using polyethylenimine (PEI, 1 mg/mL stock) at a 3:1 PEI:DNA weight ratio. Each plate received 9 µg total DNA at a 3:2:1 molar ratio of transfer vector to psPAX2 packaging plasmid (Gag-Pol) to pMD2.G envelope plasmid (VSV-G). DNA and PEI were complexed separately in Opti-MEM (Thermo Fisher), combined, and incubated at room temperature for 15 minutes before dropwise addition to plates. Two plates were transfected per construct (12 plates total for six constructs). Media was changed approximately 6 hours post-transfection with fresh complete DMEM (10% FBS, 1% penicillin-streptomycin).

G.2 Viral Supernatant Harvest and Concentration (referenced from §3.2)

Viral supernatants were collected at 48 hours and 72 hours post-transfection, filtered through 0.45-µm PVDF syringe filters to remove cell debris, and pooled (approximately 40 mL per construct from two plates and two harvests). Pooled supernatants were concentrated using Takara Lenti-X Concentrator at a 1:3 ratio (concentrator to supernatant, v/v), incubated overnight at 4°C, and centrifuged at 1,500 x g for 45 minutes at 4°C. The supernatant was carefully aspirated and the resulting pellet was resuspended in 160 µL R10 medium (RPMI 1640 + 10% FBS) supplemented with 10% sucrose per construct, yielding approximately 100-fold concentration. Resuspended virus was aliquoted (20 µL per tube) to avoid repeated freeze-thaw cycles, flash-frozen on dry ice, and stored at -80°C.

G.3 RetroNectin/Spinoculation Transduction Protocol (referenced from §3.3)

Plate coating. 48-well tissue culture plates were coated with RetroNectin (Takara Bio) at 20 µg/mL in PBS, incubated at 4°C overnight, and blocked with 2% BSA in PBS for 30 minutes at room temperature. Wells were washed once with PBS before use.

Cell plating and virus addition. Each well received 500,000 cells in 500 µL R10 medium. Concentrated lentivirus (20 µL per well) was added directly to the cell suspension. Polybrene (hexadimethrine bromide) was added at a final concentration of 2 µg/mL to enhance transduction efficiency.

Spinoculation. Plates were centrifuged at 800 to 1,000 x g for 30 to 60 minutes at 32°C in a swinging-bucket centrifuge.

Post-spinoculation. Plates were returned to the incubator (37°C, 5% CO2). Media was changed the following day to remove residual polybrene and unincorporated virus.

G.4 Anti-G4S/Dynabead Magnetic Enrichment Protocol (referenced from §3.5)

Antibody labeling. Cells were resuspended in FACS buffer (PBS + 2% FBS) on ice. Biotinylated anti-G4S linker antibody (E7O2V, Cell Signaling Technology) was added at 1 µL per million cells. Cells were incubated for 15 minutes on ice.

Wash. Cells were washed twice with 10 volumes cold FACS buffer (300 x g, 5 minutes, 4°C) to remove unbound antibody.

Bead labeling. Thermo Fisher Streptavidin Dynabeads M-280 were added at 25 µL per 10^7 cells. Cells were incubated for 15 minutes on ice with gentle mixing every 5 minutes.

Magnetic separation. Tubes were placed in a DynaMag-2 magnet for 5 minutes. The supernatant (negative fraction, containing untransduced cells) was carefully removed and discarded.

Wash and resuspension. The bead-bound positive fraction was washed twice with cold FACS buffer (2 minutes per wash in magnet). After the final wash, cells were resuspended in the appropriate culture medium:

- BBz CAR constructs (primary T cells): R10 + 100 IU/mL IL-2
- SynNotch-BFP constructs (Jurkats): R10 without IL-2

G.5 Staircase PCR Assembly Protocol (referenced from §3.1.1)

The m972 scFv (720 bp) was assembled from 24 tiled 60-bp oligonucleotides alternating between top and bottom strands, with 30-bp overlap between adjacent oligos. The protocol consisted of two steps:

Primerless assembly. All 24 oligos were pooled at equimolar concentration (25 nM each) with Q5 High-Fidelity DNA Polymerase (NEB) in a 50-µL reaction. Cycling conditions: 98°C initial denaturation for 30 seconds, then 30 cycles of 98°C for 10 seconds, 55°C annealing for 30 seconds, and 72°C extension for 30 seconds, followed by a 2-minute final extension at 72°C. No external primers were added; extension occurs by overlap hybridization between adjacent oligos.

Outer-primer amplification. The primerless assembly product (1 µL) was used as template in a fresh 50-µL Q5 reaction with 22-bp flanking primers at 0.5 µM each. Cycling conditions: 98°C for 30 seconds, then 30 cycles of 98°C for 10 seconds, 65°C annealing for 30 seconds, and 72°C extension for 30 seconds, followed by a 2-minute final extension.

The full-length 720-bp product was verified by 1% agarose gel electrophoresis (150 V, 20 minutes) as a clean single band with no detectable off-target products (Figure 2, §3.1.1).

G.6 Co-Culture Standard Conditions (referenced from §3.6.1, §3.6.2)

All co-culture assays used complete T-cell medium: RPMI 1640 + 10% FBS + 1% penicillin-streptomycin + 100 IU/mL IL-2 (BBz CAR primary T-cell assays) or RPMI 1640 + 10% FBS + 1% penicillin-streptomycin without IL-2 (SynNotch Jurkat assays). All co-cultures were maintained at 37°C / 5% CO_2.

Cytotoxicity timecourse (§3.6.1). 96-well flat-bottom plates, 200 µL per well. E:T ratios of 1:1, 5:1, and 10:1 corresponding to 1×10^5, 5×10^5, and 1×10^6 effector cells per 1×10^5 NALM-6-GFP target cells. Session controls included NALM-6-GFP alone, stained UT, and unstained UT acquired at each harvest session.

Serial rechallenge (§3.6.2). 24-well plates, 2 mL per well. 1:1 ratio (1×10^6 effectors : 1×10^6 targets). All five rounds used the same effector population maintained in the original well.