Evaluation of Alcalase pretreatment for *Chlamydomonas reinhardtii* CRISPR knock-in

To edit cell-walled *C. reinhardtii*, you must first digest the wall to deliver Cas9 RNPs and donor DNA. We tried the off-the-shelf enzyme Alcalase and found that it digested walls, but sharply reduced viability, and we recovered no CRISPR knock-ins. A one-time cautionary result.

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Purpose

We wanted to use CRISPR-Cas9-mediated homology-directed repair (HDR) in *C. reinhardtii* to insert genomic landing pads as part of a larger project. In cell-walled algae, delivering Cas9 ribonucleoproteins (RNPs) and donor DNA requires first digesting that wall. The standard approach uses autolysin, a gamete-derived protease that must be prepared in-house over several days and varies in activity between batches. We tested whether Alcalase, a commercially available serine endopeptidase, could serve as an off-the-shelf alternative.

We tested Alcalase pretreatment and compared it to GeneArt MAX Efficiency Transformation Reagent for Algae, which facilitates transformation and random integration of donor DNA. We electroporated Cas9 RNPs carrying one of four guide RNAs plus an antibiotic-resistance donor gene into our cells and found that Alcalase digested cell walls, but reduced viability independent of electroporation. Further, surviving cells were less likely to produce nourseothricin (NAT)-resistant colonies. Amplicon sequencing of NAT-resistant colonies detected no knock-in events or target-site indels with either method, indicating that on-target editing efficiency was low and NAT resistance arose primarily from off-target genomic integration of donor DNA.

We stopped pursuing this work after bigger-picture priorities shifted, but are sharing our initial findings in case this is useful to others considering this approach. One attempt under one set of conditions isn't enough to rule out Alcalase pretreatment for targeted gene integration in *Chlamydomonas*, and we think this could be worth pursuing using gentler conditions.

We've put this effort on ice!

#TechnicalGap #StrategicMisalignment

We've paused targeted integration work in *C. reinhardtii* after this attempt because optimizing this approach would likely require additional time and resources, and our research priorities have shifted. The "Next steps" we outline provide a reasonable path forward should this work be revisited. The underlying landing pad concept remains on the table if and when that changes.

Learn more about the Icebox and the different reasons we ice projects.

Caveats: What this pub does and doesn't claim

We're sharing the conditions we tested, what we observed, and our interpretation of the results. This was a single, unreplicated experiment performed by one experimenter using one batch of each key reagent, so our conclusions are limited to the conditions described here and shouldn't be read as an evaluation of any individual component of the workflow. These findings may help others considering Alcalase pretreatment for CRISPR knock-in in *Chlamydomonas* to avoid

unproductive approaches, refine experimental designs, or identify conditions worth exploring further.

We aren't ruling out that Alcalase could work under different conditions — we encourage others to learn from our efforts and let us know if you're able to make it work.

Background and goals

In a past publication [1], we outlined a proposed framework for building modular CRISPR landing pads in *Chlamydomonas reinhardtii*, a strategy for installing repeatable targeted knock-ins at a fixed safe-harbor locus that could enable a scalable platform for deep mutational scanning, engineered protein variant screening, optogenetic discovery, or multi-gene pathway refactoring. As a prerequisite for this platform, we attempted to establish a robust, high-throughput-compatible CRISPR-Cas9 knock-in system for *C. reinhardtii*.

Moving from random, non-homologous end joining (NHEJ)-mediated genome integration to targeted insertion via CRISPR-Cas9-mediated homology-directed repair (HDR) places stricter demands on delivery. A selection cassette only has to enter a cell and integrate somewhere, so even inefficient delivery yields colonies. Targeted insertion requires that the Cas9 ribonucleoprotein (RNP) and donor reach the same cell and that repair proceeds by HDR rather than NHEJ, making the events far rarer [2]. Getting both cargoes across an intact cell wall at sufficient frequency remains a technical challenge, and much of the *Chlamydomonas* CRISPR literature sidesteps it by working in cell-wall-less or cell-wall-deficient strains like cw15 [3]. However, several of our phenotyping efforts, including swimming, growth in marine broth, and overall cellular fitness, depend on an intact cell wall [4]. We therefore aimed to develop a high-throughput-compatible method for transforming cell-walled strains. Autolysin, an enzyme derived from *C. reinhardtii* gametes, is an established route [5] [6], but its preparation is a multi-day process that requires mating specific mt^+ and mt^- strains to harvest the enzyme, the enzyme should be used soon after preparation to avoid loss of activity, and batch-to-batch yields can be inconsistent [7].

As an alternative to autolysin or wall-less mutants, we tested whether commercial enzymes and chemical methods could substitute for robust wall permeabilization. Alcalase, a commercially available serine endopeptidase, is an attractive option: It's available off the shelf, has been applied to CRISPR gene editing of *Chlorella sorokiniana*, another green microalga [8], and has been shown to digest *C. reinhardtii* cell walls [9]. We wanted to test whether Alcalase enzymatic pretreatment could support CRISPR-Cas9-mediated HDR in *C. reinhardtii*. We tested it alongside GeneArt MAX Efficiency Transformation Reagent for Algae (hereafter, "GeneArt"), a non-enzymatic chemical reagent commonly used for *C. reinhardtii* transformation [10] that has also been applied to CRISPR editing [11]. GeneArt is our standard approach for random, NHEJ-based integration of linear DNA, but we haven't tested it for CRISPR applications; we included it as a comparator for permeabilization, viability, and NAT resistance. As a secondary variable, we also tested whether plate- vs. flask-grown cells differ in susceptibility to cell-wall permeabilization and subsequent transformation, since different protocols have successfully used one or the other [6] [12] without a direct comparison.

To compare these two permeabilization strategies, we needed a fast, easily scored readout of transformation and knock-in efficiency. The pale-green, truncated light-harvesting antenna (TLA) phenotype produced by disrupting *CpSRP43* (also called *TLA3*) [13] or *CpSRP54* (also called *TLA4*) [14] fit this need, letting us assess editing efficiency by eye alongside genotyping. To support future landing pad integrations, we also targeted *SRTA*, a Sir2-type histone deacetylase, which has been identified as a major driver of transgene silencing in *C. reinhardtii* [15].

The approach

We evaluated different cell wall permeabilization and culture strategies for CRISPR-Cas9-mediated knock-in in *C. reinhardtii* (Figure 1).

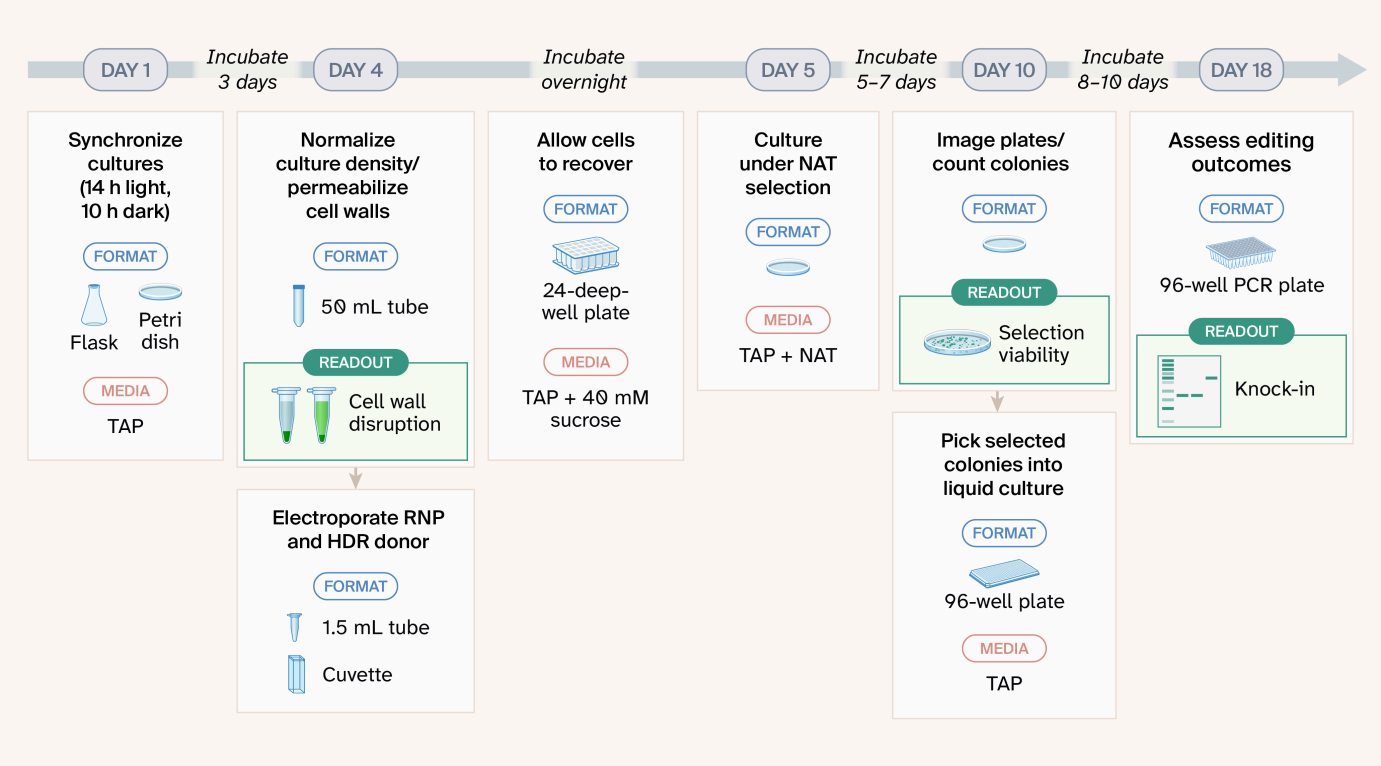

Figure 1. **Our CRISPR knock-in workflow spans ~18 days from culture synchronization to genotyping.**

Guide RNA and linear HDR donor preparation

Dual-guide RNA preparation

We used Geneious Prime (v2026.0.2) to design four dual-guide RNAs targeting exon 1 of three genomic loci: *CpSRP43*, *CpSRP54*, and *SRTA* (two guides: SRTA_KO_g01 and SRTA_KO_g02) (Table 1). We ordered Alt-R crRNAs and a universal Alt-R tracrRNA from Integrated DNA Technologies (IDT, #1072533). We reconstituted each crRNA to 100 µM in 20 µL of nuclease-free duplex buffer (IDT; 30 mM HEPES pH 7.5, 100 mM potassium acetate) and the tracrRNA to 100 µM in 200 µL of the same buffer. To form each crRNA duplex, we combined 5 µL of 100 µM crRNA with 5 µL of 100 µM tracrRNA in a 10 µL reaction, heated to 95 °C for 5 min, then cooled to room temperature. We prepared dual-guide RNAs for *CpSRP43* and *CpSRP54* in advance and stored them at −20 °C until the day of transformation; we prepared *SRTA* dual-guide RNAs fresh on the day of transformation.

HDR donor template

We adapted HDR donor architecture from Nievergelt et al. [6]. Donors consisted of a nourseothricin (NAT) resistance cassette, consisting of the HSP70A–RBCS2 fusion

promoter, RBCS2 intron 1, NAT coding sequence, and RBCS2 terminator (HSP70A–RBCS2::RBCS2i1::NAT::RBCS2-T) flanked by 50 bp locus-specific homology arms and a SapI restriction enzyme site to facilitate excision (Figure 2). Twist Bioscience synthesized donors in a high-copy cloning vector (pTwist Amp High Copy). We linearized plasmids by SapI digestion (NEB, #R0569S) and purified the resulting 1,589 bp donor fragments by gel extraction using a Zymoclean Gel DNA Recovery Kit (Zymo, #D4001). We used a total of 1 µg of linearized HDR donor template per transformation.

▶ Expand to see a supplemental diagram of our HDR donor architecture and a table of guide RNA design details.

Cas9 RNP assembly

We assembled RNP complexes following Nievergelt et al. [6], mixing our annealed dual-guide RNA and Alt-R *Streptococcus pyogenes* Cas9 V3 (IDT, #1081058) in equimolar amounts at room temperature for approximately 5–10 min, then transferred to ice. For each 20 µL reaction, we combined 2 µL of 50 µM guide RNA with 1.8 µL of Cas9 stock and 16.2 µL of nuclease-free duplex buffer, reaching a final concentration of 5 µM each. Each 20 µL RNP preparation was sufficient for four transformation reactions (4 µL per reaction).

Growth media formulations

We prepared growth media using the following recipes.

TAP (Tris-acetate-phosphate) medium

7.5 mM NH_4Cl, 340 µM $CaCl_2{\cdot}2H_2O$, 406 µM $MgSO_4{\cdot}7H_2O$, 620 µM K_2HPO_4, 411 µM KH_2PO_4, 20 mM Trizma base, 17.5 mM glacial acetic acid, with Hutner's trace elements solution at final concentrations of 134 µM $Na_2EDTA{\cdot}2H_2O$, 76.5 µM $ZnSO_4{\cdot}7H_2O$, 184 µM H_3BO_3, 25.6 µM $MnCl_2{\cdot}4H_2O$, 17.9 µM $FeSO_4{\cdot}7H_2O$, 6.8 µM $CoCl_2{\cdot}6H_2O$, 6.3 µM $CuSO_4{\cdot}5H_2O$, and 0.9 µM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$. We dissolved the components in ultrapure water.

M−N (nitrogen-free minimal medium)

1.7 mM $Na_3C_6H_5O_7{\cdot}2H_2O$, 37 µM $FeCl_3{\cdot}6H_2O$, 360 µM $CaCl_2{\cdot}2H_2O$, 1.22 mM $MgSO_4{\cdot}7H_2O$, 657 µM $K_2HPO_4{\cdot}3H_2O$, with trace elements (16.2 µM H_3BO_3, 3.5 µM $ZnSO_4{\cdot}7H_2O$, 1.8 µM $MnSO_4{\cdot}H_2O$, 0.84 µM $CoCl_2{\cdot}6H_2O$, 0.83 µM $Na_2MoO_4{\cdot}2H_2O$, 0.28 µM $CuSO_4{\cdot}5H_2O$). We adjusted pH to 6.8 and dissolved in ultrapure water.

Growing *C. reinhardtii*

We used the *C. reinhardtii* wild-type (WT) strain CC-124 (Chlamydomonas Resource Center) for all transformations. We maintained cultures on TAP + 1.5% agar plates and in TAP liquid medium. We synchronized CC-124 cultures under a 14 h light/10 h dark cycle starting four days before transformation, in custom-built light boxes fitted with Plant 4.0 LED Nano lamps (Fluval, #16875) set to 100% red, 100% blue, 25% all other wavelengths, giving 11,000 lux measured at the culture surface. For plate-derived cells, we transferred approximately half an inoculating loop of cells from a solid TAP maintenance plate to a fresh TAP agar plate, spreading with 100 µL of TAP medium, and grew them at ambient laboratory temperature without shaking. For liquid cultures, we inoculated 50 mL of TAP in a 250 mL flask at a 1 ,000 dilution from a starter liquid culture derived from the same maintenance line and grew cells at 25 °C with shaking at 250 rpm.

The afternoon before transformation, we measured the optical density (OD) of the liquid culture at 750 nm (OD_{750} = 0.49) using a Thermo Scientific NanoDrop OneC spectrophotometer, and set up an overnight dilution (1) in fresh TAP. On the morning of transformation day, we allowed the culture to reach mid-log phase (OD_{750} = 0.3), which we obtained at 12 pm, before harvesting the cells. To harvest the plate culture, we used a 25 cm cell scraper (VWR, #734-2602) to remove cells from the plate and resuspended them in 10 mL of TAP, then measured OD (OD_{750} = 3.3), which we diluted 10× to OD_{750} ≈ 0.33. We split 50 mL of each culture evenly into two 50 mL tubes (25 mL per tube), giving us two flask cultures and two plate cultures, which we centrifuged at 3,000 × g for 5 min at room temperature to pellet the cells.

Cell wall removal

We compared Alcalase (Sigma-Aldrich, #126741) cell wall removal treatment to washing with GeneArt MAX Efficiency Transformation Reagent for Algae (Thermo Fisher Scientific, #A24229), benchmarked against a no-enzyme M−N control, following Hwang et al. [9] with minor modifications. For Alcalase treatment, we resuspended pelleted cells in 10 mL of 1 dilution of Alcalase in TAP and incubated for 1 h in an LED-illuminated grow tent (approximately 7,000 lux at the shelf surface, broad-spectrum white LEDs; hereafter, "grow tent") at ambient laboratory temperature without shaking, then pelleted and washed twice with 10 mL TAP + 40 mM sucrose (TAP + Suc). The no-enzyme control followed the same procedure with M−N in place of Alcalase/TAP. For the GeneArt treatment, we washed

cells twice in 10 mL of undiluted GeneArt MAX Efficiency reagent. After washing, we resuspended all pellets in 2.5 mL of their respective buffer (TAP + Suc or GeneArt reagent) and dispensed 250 µL aliquots in 1.5 mL microcentrifuge tubes on ice.

Cell wall leakage assay

To assess the efficiency of each cell wall degradation method, we performed an OD-based leakage assay. In this assay, cells with weakened cell walls are more susceptible to detergent-induced damage, so pigment leakage detected by OD reads out the degree of cell wall degradation [9]. We transferred 1 mL from each condition (plate or flask, treated with Alcalase, GeneArt, or M−N) to fresh 1.5 mL tubes and measured the OD values at 662 nm (OD_1) using a Thermo Scientific NanoDrop OneC spectrophotometer. We pelleted cells at 5,000 × g for 5 min and discarded the supernatant. We then resuspended the pellet in either 1 mL of 100% TAP or a 50% TAP/50% 0.1% Triton X-100 mixture, vortexed for 30 s, pelleted again at 5,000 × g for 5 min, and measured the OD value of the resulting supernatant (OD_2). We calculated leakage as $(\mathrm{OD}_2/\mathrm{OD}_1) \times 100\%$.

Electroporation and recovery

We pre-chilled 4 mm electroporation cuvettes (VWR, #89047-210) at −20 °C for at least 5 min before use. For each transformation reaction, we added 1 µg of linearized HDR donor template and 4 µL of assembled RNP complex to 250 µL of prepared cells, incubated the mixture on ice for 5 min, and then transferred it to a cuvette. We electroporated using a Gene Pulser Xcell electroporator (Bio-Rad) at 500 V, 50 µF, and 800 Ω. After electroporation, we added 1 mL of TAP + 40 mM sucrose directly to the cuvette and transferred the cells (~1.2 mL total) to a well of a 24-well plate. For non-electroporated controls, we omitted the electroporation pulse, then samples were processed alongside the transformed samples through all subsequent steps. We rested cells at room temperature for up to 15 min, then covered plates with aluminum foil and incubated overnight at room temperature in the dark at 150 rpm.

The following day, we centrifuged the cultures at 1,500 × g for 5 min, discarded the supernatant, and resuspended each pellet in 100 µL of TAP medium. We plated the entire 100 µL onto 100 mm petri dishes containing TAP agar supplemented with 7.5 µg/mL NAT for selection. For control samples (no RNP, no donor), we diluted cells an additional 100× before plating 100 µL onto 100 mm petri dishes

containing TAP agar without antibiotics. We placed all plates in our grow tent and incubated at ambient temperature for approximately 5–7 days until colonies were visible.

Colony imaging and counting

We imaged colonies at 600 dpi using an Epson V700 flatbed scanner. We scanned 100 mm TAP agar plates seven days after plating to count colonies, guide colony picking, and classify colony size (small, medium, or large). We scanned plates again 12 days after plating to count colonies and monitor colony growth and phenotype progression, immediately before transferring colonies to 1 mL cultures in 96-deep-well plates. We counted colonies using the multi-point tool in Fiji (v2.16.0/1.54p) **[18]**.

Colony PCR and amplicon sequencing

We screened putative transformants by colony PCR using locus-specific primers flanking the target insertion site (expected product ~0.5 kb without insert, ~2 kb with HDR insert) (Table 2).

▶ Expand to see primer sequences.

We used 1 µL of cell culture from our 96-deep-well plates (after ~8–10 days) directly as the template for all PCR reactions. To attempt amplification of full-length donor integrations (~2 kb), we used PrimeSTAR GXL Premix Fast, Dye Plus (Takara Bio, #R052A) in 20 µL reactions (10 µL GXL 2× mix, 0.4 µL of each 10 µM primer, 1 µL template, 8.2 µL water) with the following program: 98 °C for 1 min; 30 cycles of 98 °C for 10 s, 60 °C for 5 s, 68 °C for 10 s; then hold at 10 °C.

We ran colony PCR reactions on either a 2% agarose pre-cast E-Gel (Invitrogen, #G700802) or a self-cast 1% agarose gel. We were distinguishing between products of roughly 500 bp and 2 kb, which both formats separate easily, so the choice of gel doesn't affect the results. We then submitted unpurified PCR products from 40 samples to Angstrom Innovation for amplicon sequencing (Oxford Nanopore Technologies). We compared the resulting sequences to the *C. reinhardtii* v5.6 reference genome using Geneious Prime (v2026.0.2).

Our plasmid maps, colony plate images, PCR gel images, and

amplicon sequencing **data** are available on Zenodo.

Pub preparation

We used Claude (Opus 4.8, Opus 5) to suggest wording ideas, copy-edit draft text to match Arcadia's style, clarify and streamline text, and generate figure mockups. We used ChatGPT (GPT-5.6 Luna) to suggest wording ideas and to clarify and streamline text. We also used ChatGPT (GPT-5.6 Sol) to identify reproducibility/clarity issues in our draft, and we corrected the issues it found. Additionally, Gemini (Gemini 3.6 Flash) suggested papers on relevant science, we did further reading, and we cited some of this literature. We reviewed all AI-assisted content and take responsibility for its accuracy and integrity.

We used arcadia-pycolor (v0.8.0) [19] to generate figures before manual adjustment.

The results

The primary objective of this study was to determine whether Alcalase could serve as an effective cell wall permeabilization method prior to electroporation for CRISPR knock-in. To assess its performance, we compared Alcalase pretreatment with GeneArt, a commonly used commercial reagent that increases transformation efficiency in walled *Chlamydomonas* strains without requiring wall digestion [10]. We tested two starting material formats for the synchronized cultures: cells grown to a lawn on TAP agar plates versus cells grown to mid-log phase in liquid TAP medium. We assessed whether growth format during synchronization affected cell-wall digestion and subsequent transformation outcomes. These two conditions, combined with four guide RNAs targeting three loci (*CpSRP43*, *CpSRP54*, *SRTA*), gave 16 transformation conditions, as summarized in the table below (Table 3). For each treatment, we also included a control with no RNP or donor, with and without electroporation, to assess baseline viability and background NAT resistance independent of editing (Table 3).

Source	Treatment	Target	Guide RNA	Condition
Flask	Alcalase	*TLA3*	CpSRP43_KO_g01	Electroporated, plated on TAP + NAT
		TLA4	CpSRP54_KO_g01	
		SRTA	SRTA_KO_g01	
		SRTA	SRTA_KO_g02	
Plate		*TLA3*	CpSRP43_KO_g01	
		TLA4	CpSRP54_KO_g01	
		SRTA	SRTA_KO_g01	
		SRTA	SRTA_KO_g02	
Flask	GeneArt	*TLA3*	CpSRP43_KO_g01	
		TLA4	CpSRP54_KO_g01	
		SRTA	SRTA_KO_g01	
		SRTA	SRTA_KO_g02	
Plate		*TLA3*	CpSRP43_KO_g01	
		TLA4	CpSRP54_KO_g01	
		SRTA	SRTA_KO_g01	
		SRTA	SRTA_KO_g02	
Flask	Alcalase	–	–	Electroporated, plated on TAP + NAT and TAP (100× dilution)
Plate				
Flask	GeneArt			
Plate				
Flask	Alcalase			Non-electroporated, plated on TAP (100×

Plate				dilution)
Flask	GeneArt			
Plate				

Table 3. **Experimental conditions.**

"Flask" refers to cells in liquid culture; "plate" refers to cells picked from plate and resuspended in TAP medium prior to Alcalase or GeneArt treatment. For conditions without a guide RNA listed, we omitted both the RNP and the donor DNA.

Alcalase reduced viability and NAT resistance

Efficient delivery of Cas9 RNP and exogenous DNA depends on cell wall permeabilization, so we first confirmed that Alcalase treatment had disrupted the algal cell wall. We probed cell wall integrity by checking for detergent-induced pigment release — the fraction of each culture's OD_{662} released into the supernatant after a Triton X-100 vortex — indexed against GeneArt washing and a no-digestion control (M−N). Pigmentation release was approximately 22% for Alcalase, 11% for GeneArt washing, and 6% for the M−N control (Figure 3), confirming that Alcalase treatment increased cell wall permeability. As these counts came from the same cultures that we used for downstream transformations, each measurement represents $n = 1$, rather than replicated conditions. The leakage assay confirms that Alcalase treatment compromises the cell wall, but pigment release doesn't tell us whether those cells were still viable, so we turned to colony counts.

Because only surviving cells form colonies, we plated an aliquot of each culture used for the leakage assay onto TAP plates and counted the resulting colonies. We found that cell wall permeabilization using Alcalase came at a cost: Under the conditions we used, Alcalase was substantially more cytotoxic than GeneArt. We started with equal cell numbers across conditions (normalized by OD_{750}) prior to cell wall treatment. We didn't renormalize cell density after treatment, so colony counts from a fixed plated volume can estimate viability. Starting from the same initial cell density, Alcalase-treated cultures yielded 35 and 68 colonies (flask and plate, respectively), whereas GeneArt-treated cultures exceeded our reliable counting ceiling of ~1,200 colonies, representing at least a 10-fold difference (Table 4 and Figure 4). By this measure, Alcalase caused substantial cell death independent of electroporation.

Guide RNA	**Ele**
–	No
–	Yes
–	Yes
CpSRP43_KO_g01	Yes
CpSRP54_KO_g01	Yes
SRTA_KO_g01	Yes
SRTA_KO_g02	Yes

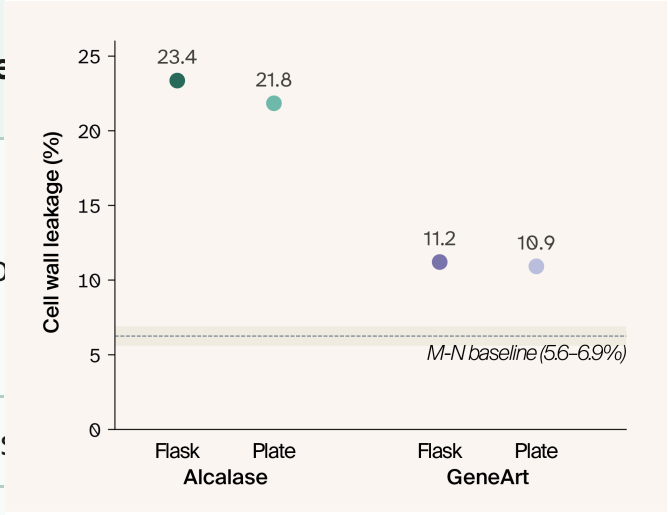

Figure 3. **Alcalase increased *Chlamydomonas* cell wall permeability.**

Table 4. **Colony counts separated by treatment and culture format.**
For conditions without a guide RNA listed, we omitted both the RNP and the donor DNA.

Cytotoxicity before electroporation would matter less if the survivors transformed well, so we next asked how they tolerated the pulse. To isolate the contribution of electroporation to cell death, we plated electroporated cultures at the same volume as the corresponding non-electroporated cultures, allowing direct comparison. We estimated apparent survival as the ratio of electroporated to non-electroporated colony counts from the same culture. Among Alcalase survivors, flask-grown cells tolerated electroporation approximately two-fold better than plate-grown cells, 69% vs. 38%, respectively (Table 4 and Figure 4). We couldn't

make this comparison for GeneArt, since both of its non-electroporated densities exceeded our reliable counting limit.

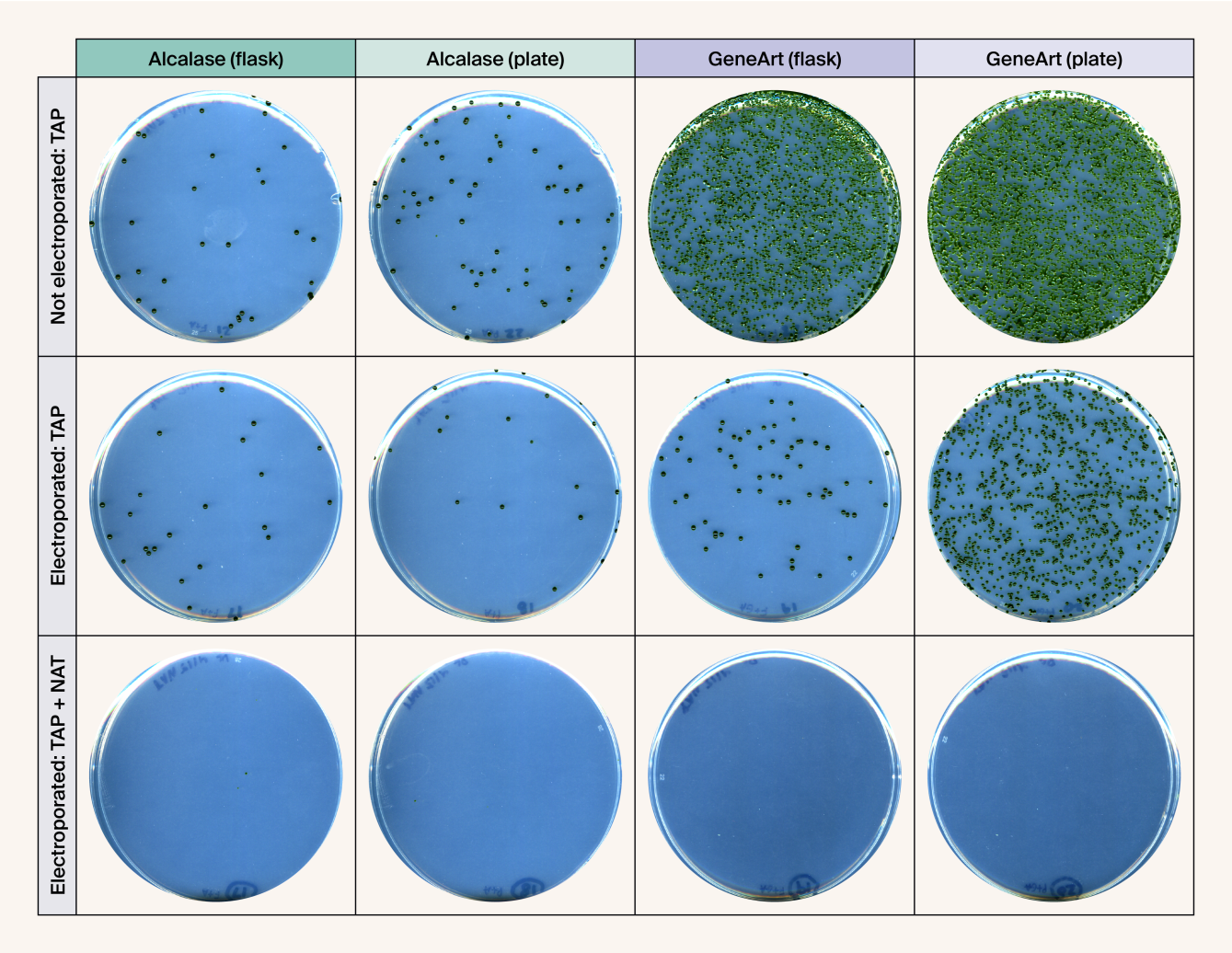

Figure 4. **Control plates lacking both RNP and donor quantified in Table 4, illustrating colony density differences between Alcalase and GeneArt treatments.**

Low NAT-resistant colony counts would be uninformative if selection itself were leaky, so we next assessed the frequency of spontaneous NAT resistance (selection escape) in the absence of genome editing. NAT-resistant colonies in control transformations lacking both RNP and donor were rare under both treatments. Despite plating at 100-fold greater density than the corresponding TAP-only plates, Alcalase-treated samples yielded only a few colonies on TAP + NAT, while the GeneArt-treated samples yielded none (Table 4). These results indicate that NAT selection was effective, and selection escape occurred at a very low frequency.

Given this low background, we next evaluated whether RNP delivery and HDR donor introduction resulted in an increased number of NAT-resistant colonies. Across all four guides and both starting culture formats, Alcalase-treated cells yielded zero to 16 colony-forming units (CFU) per plate on TAP + NAT after electroporation, with six of eight treatments indistinguishable from the matched no-donor control. The exceptions were the two *SRTA* guides in flask-grown cultures, which yielded 15 and 16 colonies (Table 4). In contrast, GeneArt-treated cultures yielded far more NAT-resistant colonies (Table 4). After normalizing to the viable cells recovered from their matched no-donor controls, GeneArt-treated cultures still yielded more NAT-resistant colonies under both starting culture conditions (Figure 5), indicating that differences in survival alone don't explain the disparity.

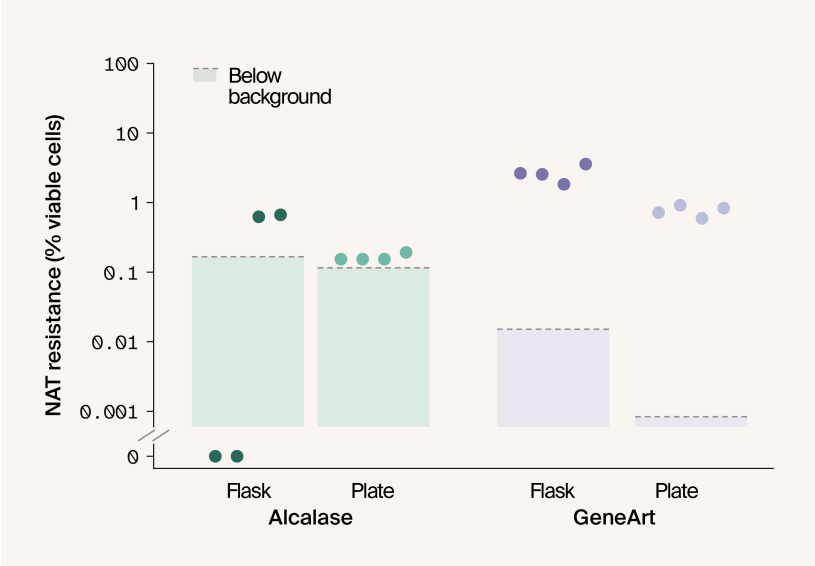

Figure 5. **Alcalase resulted in lower NAT resistance than GeneArt in both culture conditions.** Each point is one guide RNA. We plotted NAT-resistant colonies on TAP + NAT as a percentage of the viable cells in the matched electroporated no-RNP, no-donor control, plated on TAP without NAT. Alcalase controls yielded four and three colonies, respectively, while GeneArt controls yielded no colonies and were therefore plotted at the single-colony detection limit ("below background").

Since GeneArt-treated cultures retained many more viable cells before electroporation than Alcalase-treated cultures, we can't attribute the higher frequency of NAT resistance (which likely reflects random donor integration) solely to the transformation reagent. That said, the marked increase in NAT-resistant colonies following GeneArt treatment, compared with the minimal increase

observed after Alcalase treatment (Figure 5), is consistent with less efficient donor DNA delivery or integration under the Alcalase condition.

Taken together, the data suggest that Alcalase was cytotoxic and killed many cells prior to electroporation. Although many of the surviving cells appeared to tolerate electroporation, they produced far fewer NAT-resistant colonies than GeneArt-treated cells. Most Alcalase conditions remained at background levels of spontaneous NAT resistance, with only the two *SRTA* guides showing a modest increase above background (Figure 5).

▶ **Why might cells that survive Alcalase treatment be less competent?**

Colony phenotype was too variable to screen for *TLA* knockouts

▶ Expand to see why colony color didn't work as a proxy for editing efficiency.

We didn’t observe any donor integration or other CRISPR edits at target loci

To measure editing efficiency directly, we picked 201 NAT-resistant colonies spanning a range of size and color phenotypes and transferred them into 96-deep-well plates containing 1 mL TAP per well. After 8–10 days of growth, we used 1 µL of culture directly as PCR template. We used locus-specific primers and PrimeSTAR GXL: We expected the wild-type allele to appear as a ~0.5 kb band and a successful HDR event to yield a ~2 kb band (Table 2). We excluded 10 samples due to evaporation. Of the remaining 191 colonies, 172 yielded a ~0.5 kb amplicon, and the rest showed no band (Table 5). This indicates that on-target HDR integration was rare or absent.

To look for small polymorphisms that wouldn't be distinguishable from WT on a gel, we performed amplicon sequencing on unpurified colony PCR products from 40 NAT-resistant colonies (10 per guide RNA), spanning both treatments, both starting culture formats, and available colony sizes (Table 5). All 40 were wild type at the target locus, with no indels and no HDR integration detected.

Colony morphology	Colony PCR with locus-specific primers	# submitted for amplicon sequencing
Small	36/45 (80%)	17
Medium	61/67 (91%)	14
Large	75/79 (95%)	9
Total (combined)	172/191 (90%)	40
Outcome	No insert detected	No editing events detected

Table 5. **We detected zero CRISPR integration or editing events.**
Colony PCR values show amplicon-positive colonies/total colonies screened (%). All amplicons were wild-type-sized, with no shifts, indicating no detectable on-target donor integration.

Together, these results suggest that the donor DNA was delivered and integrated in cells that gained antibiotic resistance, but most likely off-target by NHEJ rather than on-target by HDR. Because our end goal is applying this method to high-throughput CRISPR screens, which demands a high rate of on-target editing, and because CRISPR editing was undetectable, we chose not to pursue further characterization of the exact editing rate.

Key takeaways

We tested whether Alcalase, an off-the-shelf enzyme, could digest *C. reinhardtii* cell walls to facilitate CRISPR-targeted DNA integration. Under the single set of conditions we tried, Alcalase killed most cells independent of electroporation, produced far fewer transformants than GeneArt (a standard chemical transformation reagent), and both reagent treatments resulted only in random donor insertion rather than integration at our target sites. We also compared flask- and plate-grown starting cultures and found that both formats yielded NAT-resistant colonies, but with no on-target events under either, we can't say whether one format is better for knock-in specifically.

We can't rule out that Alcalase would work under gentler conditions: This is one attempt with one reagent batch by one experimenter that we're sharing as a practical caution.

Next steps

Here's what we'd do next if we picked this project back up:

1. **Develop a robust, CRISPR-compatible cell wall digestion method.** Autolysin is the wall-digestion enzyme used in most published *Chlamydomonas* knock-in protocols, and recent work points to how to improve it: Shetty et al. [20] raised mating efficiency by growing cultures at 28 °C rather than 18 °C. For Alcalase, a recent paper in a different alga [21] points to two levers that might address the harshness we saw: osmotic support during and after digestion, and titrating down the enzyme dose to avoid over-digestion. Their organism is marine, so the specific conditions won't transfer directly to freshwater *C. reinhardtii*, but both principles are worth testing here.

With this cell wall digestion method, we would:

2. **Separate delivery failures from editing failures.** In this experiment, we couldn't separate RNP delivery failure from editing or repair failure. We'd restore a visual screen to confirm the guides, donor, and selection work, and also include a donor-only, no-RNP control for estimating off-target NHEJ.
3. **Optimize on-target editing.** One common strategy for enriching edited cells is co-targeting an endogenous selectable marker alongside the desired edit. For example, a drug-resistance-conferring point mutation can be introduced at a second locus, allowing survival to serve as a proxy for editing activity. This can enrich for desired edits because cells that successfully edit the co-target are more likely to have also acquired the desired edit [22] [23]. However, this approach does not directly report editing at the intended locus, and its effectiveness depends on the relationship between editing efficiencies at the two sites. In parallel, we'd also tune reaction conditions to improve knock-in efficiency, including the RNP and donor concentrations and their ratio, which improved knock-in efficiency to ~37% in one study [24].

Weigh in!

- Alcalase pretreatment under our conditions was too rough and cut viability sharply. Has anyone gotten Alcalase pretreatment to work in *C. reinhardtii*? If so, what digestion time, concentration, and recovery conditions did you use?
- If you've gotten autolysin preparations working consistently, which factors mattered most?

- If we try making targeted knock-ins again, what "need-to-haves" do you think we missed?

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Contributors (A–Z)

- **Audrey Bell**: Visualization
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- **Megan L. Hochstrasser**: Editing
- **James Kraemer**: Editing, Supervision
- **Cameron Dale MacQuarrie**: Critical feedback, Resources

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