

10-EXPERIMENT PORTFOLIO: MAXIMIZE CDK STEADY STATE

Simplified Technical English (STE) Engineering Summary | Bayesian Optimization (GES-1..8) + Literature & Surrogate Ridge Synthesis (EXP-5..10)

PRIMARY OBJECTIVE
CDK ≥ 4.0 μM

FIXED BASELINE REAGENTS & MASTER-MIX PARAMETERS (APPLIED TO ALL 10 RUNS)

DNA Template: pET21a-2xBroccoli-mScarlet at 1.0 nM

SMix / PMix IDs: AR-1449 (1.0 X) / AR-1430

PMix Dispense: 1.80 mg/mL (Nom) | 1.89 mg/mL (Actual R3)

Ribosome / tRNA: AR-907 / AR-1179

Cofactors: [FAD] = 0.20 mM | [TPP] = 2.0 mM

Phosphate Stock: pH 7.0 (61.5:38.5 K₂HPO₄:KH₂PO₄)

MASTER PARAMETER & SURROGATE MODEL PREDICTION MATRIX

Exp ID	Source / Design Strategy	[CP] (mM)	[K-Phos] (mM)	[Mg-Glu] (mM)	[Na-Pyr] (mM)	[Pox5] / [AckA] / [Kate] (μM)	Pred CDK Steady State (μM) (Nominal / Actual 1.89 mM)	Pred t _{final} Norm. (μM) (Nominal / Actual 1.89 mM)	Pred Time (h)
1. GES-3	Bayesian Opt (GES-2/3 Twin)	17.64	43.16	14.65	19.76	3.03 / 13.89 / 2.38	3.48 ± 0.40 / 3.75 ± 0.20	3.60 ± 0.40 / 3.85 ± 0.20	4.31 ± 0.20
2. GES-1	Bayesian Opt (GES-1 Upper P _i)	18.44	44.90	14.83	19.34	3.03 / 13.89 / 2.38	3.46 ± 0.40 / 3.75 ± 0.20	3.58 ± 0.40 / 3.85 ± 0.20	4.34 ± 0.20
3. GES-4	Bayesian Opt (High-CP/P _i Extrap)	96.13	100.00	37.11	5.00	3.03 / 13.89 / 2.38	3.58 ± 0.90 (extrap)	3.58 ± 0.90	5.52 ± 0.50
4. GES-8	Bayesian Opt (Dual Saturation)	100.00	100.00	38.38	48.73	3.03 / 13.89 / 2.38	3.38 ± 1.00 (extrap)	3.35 ± 0.90	6.13 ± 0.50
5. EXP-5	Model Ridge Peak (r1020_F13)	19.00	35.00	16.50	18.00	3.03 / 13.89 / 2.38	3.30 ± 0.30 / 3.80 ± 0.20	3.40 ± 0.30 / 3.90 ± 0.20	3.93 ± 0.08
6. EXP-6	Lit. Mg ²⁺ × P _i Ridge (r1018_E18)	20.00	46.00	20.00	18.00	3.03 / 13.89 / 2.38	3.20 ± 0.30 / 3.70 ± 0.20	3.30 ± 0.40 / 3.80 ± 0.20	4.07 ± 0.09
7. EXP-7	Low-Pyr / Reduced-CP Synergy	15.00	40.00	18.00	22.00	3.03 / 13.89 / 2.38	3.40 ± 0.30 / 3.70 ± 0.20	3.50 ± 0.40 / 3.80 ± 0.20	3.92 ± 0.08
8. EXP-8	Low-CP / High-PAP Flux (r1018_F9)	10.00	37.00	15.50	36.00	3.03 / 13.89 / 2.38	3.30 ± 0.30 / 3.60 ± 0.20	3.40 ± 0.30 / 3.70 ± 0.20	4.22 ± 0.08
9. EXP-9	Controlled High-P _i Step-Out	25.00	55.00	22.00	20.00	3.03 / 13.89 / 2.38	3.20 ± 0.40 / 3.70 ± 0.30	3.30 ± 0.40 / 3.80 ± 0.30	4.39 ± 0.13
10. EXP-10	Enzyme Boost + Optimal Substrate	19.00	35.00	16.50	25.00	5.00 / 20.00 / 6.00	2.70 ± 0.50 (unexplored)	2.70 ± 0.60	4.20 ± 0.30

EXPERIMENT-BY-EXPERIMENT ENGINEERING JUSTIFICATIONS (RUNS 1 TO 10)

1. GES-3 BAYESIAN OPT RIDGE PICK

[CP]: 17.64 mM | [K-Phos]: 43.16 mM | [Mg-Glu]: 14.65 mM | [Na-Pyr]: 19.76 mM | Enz: 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Ranked #3 in batch GES-1..4 (GES-2 is an identical twin). Nearest neighbors are r1020_G6 (3.58 μ M, similarity 0.83), top workspace hit r1020_F13 (3.99 μ M steady state / 4.08 μ M t_{final} , similarity 0.51), and r1020_L15 (3.72 μ M, similarity 0.51). Model predicts 3.48 ± 0.40 μ M (nominal 1.80 PMix) and 3.75 ± 0.20 μ M (3.85 ± 0.20 μ M t_{final}) at actual Round 3 1.89 mg/mL PMix.

LITERATURE & CORPUS EVIDENCE:

Uses pH 7.0 potassium phosphate buffer (61.5:38.5 dibasic:monobasic ratio; Notion_Discovery_Plate_PAP_Energy_Module_261006_(1).pdf, p. 5) shown in Yadav et al. (2025, 10.1021/acssynbio.4c00697, PAP_SY_(2).pdf Fig. 2a & S19) to activate Pox5 without acidifying the reaction.

MECHANISTIC / BIO-REASONING:

Elevating P_i to 43.16 mM (18 $\times$ above Pox5 $K_m = 2.3$ mM; PAP_SY_SupportingInfo_(1).pdf Table S2) drives pyruvate + $P_i \rightarrow$ acetyl-P condensation while buffering acetic acid accumulation. Stepping Mg glutamate up from 12.5 mM (SOP) to 14.65 mM compensates for weak HPO_4^{2-} chelation ($K_d \approx 10$ –60 mM), preserving free Mg^{2+} for 70S ribosome stability and tRNA synthetases.

2. GES-1 BAYESIAN OPT UPPER- P_i PICK

[CP]: 18.44 mM | [K-Phos]: 44.90 mM | [Mg-Glu]: 14.83 mM | [Na-Pyr]: 19.34 mM | Enz: 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Ranked #2 in batch GES-1..4. Steps [K-phosphate] (44.90 mM) and [CP] (18.44 mM) up to the upper edge of explored space (46.29 mM max observed). Nearest runs are r1020_G6 (3.58 μ M, similarity 0.84), r1020_F13 (3.99 μ M), and r1020_L15 (3.72 μ M). Model predicts 3.46 ± 0.40 μ M (nominal MM) and 3.75 ± 0.20 μ M (actual R3 MM).

LITERATURE & CORPUS EVIDENCE:

Directly mirrors Condition 8/14 of the cCCD in PAP_SY_SupportingInfo_(1).pdf (Table S3: 45.5–50 mM phosphate), where high phosphate maintained high yield only when paired with elevated Mg^{2+} .

MECHANISTIC / BIO-REASONING:

Tests the upper edge of the ~45 mM phosphate buffer regime while keeping [Na pyruvate] below 20 mM. In Yadav et al. (PAP_SY_(2).pdf Section 2.5, Fig. S15), keeping initial pyruvate near 18–20 mM minimizes early O_2 consumption by Pox5, preventing anaerobic delay of both translation initiation and O_2 -dependent mScarlet chromophore maturation.

3. GES-4 HIGH-VARIANCE BOUNDARY EXTRAPOLATION

[CP]: 96.13 mM | [K-Phos]: 100.00 mM | [Mg-Glu]: 37.11 mM | [Na-Pyr]: 5.00 mM | Enz: 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Ranked #1 in GES-1..4 by hit probability ($1.1\text{e-}12$) when CDK completion time had a goal of ≥ 9.0 h. Extrapolates outside observed data in 3 dimensions: CP = 96.1 mM (max obs 42.4 mM), K-Phos = 100 mM (max obs 46.3 mM), and Mg-Glu = 37.1 mM (max obs 24.8 mM), while pinning Na-Pyr at 5.0 mM. Model predicts 3.58 ± 0.90 μ M and 5.52 ± 0.50 h; large epistemic uncertainty (± 0.90 μ M) drives upper-tail hit probability.

LITERATURE & CORPUS EVIDENCE:

Tests the upper [5, 100] mM bounds for CP, K-phosphate, and pyruvate specified in Notion_Discovery_Plate_PAP_Energy_Module_261006_(1).pdf (Page 2).

MECHANISTIC / BIO-REASONING:

Extreme CP-dominated / phosphate-buffered boundary test: 96 mM CP floods the CK reaction for rapid ATP regeneration while 100 mM pH 7 K-phosphate + 37.1 mM Mg-Glu buffers pH and maintains free Mg^{2+} ($\text{Mg:P}_i \approx 0.37$). Pinning pyruvate at 5 mM avoids Pox5-driven O_2 depletion (PAP_SY Fig. S15), though 196 mM total CP + P_i carries high ionic/osmotic strength risk.

4. GES-8 DUAL-ENERGY SATURATION EXTRAPOLATION

[CP]: 100.00 mM | [K-Phos]: 100.00 mM | [Mg-Glu]: 38.38 mM | [Na-Pyr]: 48.73 mM | **Enz:** 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Ranked #2 in GES-5.9 (hit_probability = 0.0032 for goals time $\geq$ 7 h and yield $\geq$ 4 μ M). Nearest runs are high-CP R2 wells r1018_H19/L15/I8 (42.4 mM CP , 36.7 mM P_i , 15.4 mM Mg-Glu , 36.0 mM Pyr $\rightarrow$ 1.96–2.41 μ M) vs r1018_F19 (where raising Mg-Glu to **24.5 mM** jumped yield to **3.69 μ M** and 5.25 h). Model predicts 3.38 ± 1.00 μ M and 6.13 ± 0.50 h .

LITERATURE & CORPUS EVIDENCE:

Tests the upper corner of Notion_Discovery_Plate_PAP_Energy_Module_261006_(1).pdf (Page 2) small-molecule ranges (100 mM CP , 100 mM K-Phos) with moderate-high pyruvate (48.7 mM , matching the 50 mM axial point in PAP_SY_SupportingInfo_(1).pdf Table S3 Condition 13).

MECHANISTIC / BIO-REASONING:

In Round 2, 42 mM CP + 36 mM P_i + 36 mM Pyr failed at 15.4 mM Mg-Glu (r1018_I8 : 2.41 μ M) due to Mg²⁺ sequestration by CP and P_i, but succeeded when Mg-Glu was raised to 24.5 mM (r1018_F19 : 3.69 μ M). GES-8 scales that trend to 100 mM CP + 100 mM P_i by raising Mg-Glu to 38.38 mM to test if massive dual-substrate loading sustains synthesis past 6 hours .

5. EXP-5 MODEL RIDGE PEAK & R3 TOP HIT NEIGHBOR

[CP]: 19.00 mM | [K-Phos]: 35.00 mM | [Mg-Glu]: 16.50 mM | [Na-Pyr]: 18.00 mM | **Enz:** 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Directly targets the neighborhood of the #1 highest-yielding PAP well in the workspace, r1020_F13 (19.12 mM CP , 34.87 mM P_i , 16.26 mM Mg-Glu , 18.04 mM Pyr $\rightarrow$ **3.993 μ M steady state** / **4.082 μ M t_{final}**) and replicate r1020_L15 (3.724 μ M). Achieves the **highest model-predicted mean in the entire design space:** **3.80 $\pm$ 0.20 μ M** steady state and **3.90 $\pm$ 0.20 μ M t_{final}** (3.93 $\pm$ 0.08 h) at R3 master-mix levels.

LITERATURE & CORPUS EVIDENCE:

Shifts the Yadav et al. (2025) center point (20 mM CP , 27.5 mM P_i , 12.5 mM Mg-Glu , 27.5 mM Pyr ; PAP_SY_(2).pdf Table S5 & 261008_PAP-LC_expt-setup.xlsx) along the positive Mg²⁺ $\times$ P_i interaction vector ($\beta_4 = +0.333$, Table S4) while reducing pyruvate to 18 mM (matching Validation Condition 18 in Table S3: 16.88 mM Pyr , 34.88 mM P_i).

MECHANISTIC / BIO-REASONING:

Balances all four competing biochemical constraints simultaneously: (1) 19 mM CP fuels hour 0–1 ATP demand before PAP ramps up; (2) 18 mM pyruvate avoids early O₂ exhaustion and keeps H₂O₂ below its 5 mM IC₅₀ (Fig. S10); (3) 35 mM K-phosphate (pH 7) buffers late-stage acetic acid and recycles P_i; and (4) 16.5 mM Mg-Glu (+4 mM above SOP) offsets MgHPO₄ complexation so ribosomes stay intact through ~4 hours .

6. EXP-6 LITERATURE MG²⁺ $\times$ P_i CHELATION RIDGE

[CP]: 20.00 mM | [K-Phos]: 46.00 mM | [Mg-Glu]: 20.00 mM | [Na-Pyr]: 18.00 mM | **Enz:** 3.03 / 13.89 / 2.38 μ M

WORKSPACE & MODEL EVIDENCE:

Anchored on the #1 Round 2 small-molecule sweep condition r1018_E18 (19.01 mM CP , 46.29 mM P_i , 20.24 mM Mg-Glu , 18.19 mM Pyr), which achieved **3.850 μ M steady state** and **3.989 μ M t_{final}** (replicates r1018_J13 at 3.28 μ M , r1018_I6 at 3.02 μ M). Model predicts **3.70 $\pm$ 0.20 μ M** steady state and **3.80 $\pm$ 0.20 μ M t_{final}** (4.07 $\pm$ 0.09 h).

LITERATURE & CORPUS EVIDENCE:

Directly tests the high-phosphate / high-magnesium corner (45.5 mM P_i , 18.5 mM Mg-Glu) from PAP_SY_SupportingInfo_(1).pdf (Fig. S15 bottom panel & Table S3 Condition 7/8), where Yadav et al. showed that at 18.5 mM Mg²⁺ + 45.5 mM P_i , lowering pyruvate reduces lag time while sustaining high expression.

MECHANISTIC / BIO-REASONING:

In R2 data, 46.2 mM K-phosphate with standard 12.0 mM Mg-Glu (r1018_E6 , D7 , K14) crashed yield to **0.50–0.67 μ M** because 46 mM phosphate chelates ~6–8 mM Mg²⁺, starving ribosomes of free Mg²⁺. Raising Mg glutamate to **20.0 mM** restores free Mg²⁺ to ~12 mM while providing 46 mM pH 7 buffer capacity.

7. EXP-7 LOW-PYRUVATE / REDUCED-CP SYNERGY[CP]: 15.00 mM | [K-Phos]: 40.00 mM | [Mg-Glu]: 18.00 mM | [Na-Pyr]: 22.00 mM | Enz: 3.03 / 13.89 / 2.38 μ M**WORKSPACE & MODEL EVIDENCE:**

Interpolates the unmeasured midpoint between top clusters r1020_F13 (34.9 mM P_i , 16.3 mM Mg-Glu $\rightarrow$ 3.99 μ M) and r1018_E18 (46.3 mM P_i , 20.2 mM Mg-Glu $\rightarrow$ 3.85 μ M), while stepping CP down to 15.0 mM (as in r1020_G18 at 14.4 mM CP $\rightarrow$ 3.40 μ M steady state / 3.49 μ M t_{final}). Model predicts **3.70 $\pm$ 0.20 μ M** steady state and **3.80 $\pm$ 0.20 μ M** t_{final} (3.92 $\pm$ 0.08 h).

LITERATURE & CORPUS EVIDENCE:

Follows the contour ridge of Figure 3d in PAP_SY_(2).pdf (40 mM P_i paired with 18 mM Mg-Glu) and tests whether slightly lower CP (15 mM vs 20 mM) reduces free P_i release from CP hydrolysis during the first 2 hours.

MECHANISTIC / BIO-REASONING:

Every 1 mM of CP hydrolyzed by CK releases 1 mM of unbuffered inorganic phosphate. Starting at 15 mM CP instead of 20 mM lowers the acute P_i surge during peak translation (1.5–2.5 h), while 22 mM pyruvate + 40 mM K-phosphate + 18 mM Mg-Glu sustains ATP regeneration via Pox5/AckA without crossing the > 27 mM pyruvate lag threshold.

8. EXP-8 LOW-CP / HIGH-PAP FLUX SHIFT[CP]: 10.00 mM | [K-Phos]: 37.00 mM | [Mg-Glu]: 15.50 mM | [Na-Pyr]: 36.00 mM | Enz: 3.03 / 13.89 / 2.38 μ M**WORKSPACE & MODEL EVIDENCE:**

Grounded in Round 2 well r1018_F9 (9.44 mM CP , 36.78 mM P_i , 15.47 mM Mg-Glu , 36.14 mM Pyr), which achieved **3.525 μ M steady state** (3.604 μ M t_{final}), and replicates r1018_J15 (3.31 μ M) and K16 (3.22 μ M). Model predicts **3.60 $\pm$ 0.20 μ M** steady state and **3.70 $\pm$ 0.20 μ M** t_{final} (4.22 $\pm$ 0.08 h).

LITERATURE & CORPUS EVIDENCE:

Directly tests the Validation Condition 16 regime from PAP_SY_SupportingInfo_(1).pdf (Table S3: 39.38 mM pyruvate , 44.96 mM phosphate) combined with a halved CP concentration (10 mM vs 20 mM SOP in 261008_PAP-LC_expt-setup.xlsx).

MECHANISTIC / BIO-REASONING:

Tests a PAP-dominant, low-CP energy regime: cutting CP in half (10 mM) reduces initial osmotic load and creatine accumulation, while 36 mM pyruvate + 37 mM K-phosphate supplies ~2 $\times$ carbon/phosphate flux through Pox5 and AckA to extend reaction duration (4.22 h). Because CP is only 10 mM (less Mg²⁺ chelation and lower P_i dump), 15.5 mM Mg-Glu maintains free Mg²⁺.

9. EXP-9 CONTROLLED HIGH-P_i STEP-OUT (55 mM)[CP]: 25.00 mM | [K-Phos]: 55.00 mM | [Mg-Glu]: 22.00 mM | [Na-Pyr]: 20.00 mM | Enz: 3.03 / 13.89 / 2.38 μ M**WORKSPACE & MODEL EVIDENCE:**

Controlled step-out just beyond the highest observed K-phosphate (46.29 mM in r1018_E18), rather than jumping straight to 100 mM like GES-4/8 . Model predicts **3.70 $\pm$ 0.30 μ M** steady state and **3.80 $\pm$ 0.30 μ M** t_{final} (4.39 $\pm$ 0.13 h)—combining high predicted mean with moderate uncertainty ($\pm$ 0.30 μ M) so the upper confidence bound exceeds **> 4.0 μ M** .

LITERATURE & CORPUS EVIDENCE:

In PAP_SY_(2).pdf (Page 6, citing Calhoun & Swartz 2005 and Smith & Alberty 1956), Yadav et al. note that dibasic phosphate inhibition in CFPS occurs above ~50 mM unless Mg²⁺ sequestration (K_d $\approx$ 10–60 mM) is stoichiometrically compensated.

MECHANISTIC / BIO-REASONING:

In R2/R3, yield had not yet peaked at 46.3 mM K-phosphate (r1018_E18 gave 3.85 μ M steady state / 3.99 μ M t_{final} when paired with 20.2 mM Mg-Glu). Stepping K-phosphate to 55 mM with proportionally scaled Mg glutamate (22.0 mM) and 25 mM CP tests whether stronger pH 7 buffering and higher P_i driving force push yield past 4.1 μ M without 100 mM ionic shock.

10. EXP-10 **CORPUS ENZYME BOOST + OPTIMAL SUBSTRATE**

[CP]: 19.00 mM | [K-Phos]: 35.00 mM | [Mg-Glu]: 16.50 mM | [Na-Pyr]: 25.00 mM | Enz: 5.00 / 20.00 / 6.00 μ M

WORKSPACE & MODEL EVIDENCE:

All R1–R3 runs held enzymes fixed near 3.03 / 13.89 / 2.38 μ M ; consequently, the GP model reverts toward the prior mean (2.70 $\pm$ 0.50 μ M) when enzyme concentrations change. However, `get_ges_justification_context` shows the model's largest positive yield sensitivity (+4 to +8 uM/unit) is on Expected [PMix] , which covaries 1:1 with Pox5 , AckA , and KatE across R1–R3.

LITERATURE & CORPUS EVIDENCE:

Notion_Discovery_Plate_PAP_Energy_Module_261006_(1).pdf (p. 2 & 5) specifies testing Pox5 (1–20 μ M) , AckA (2–30 μ M) , and KatE (1–20 μ M) , and 261008_PAP-LC_expt-setup.xlsx ("cytosol ref") shows stock Pox5 (AR-1393) is only **34.8–40.8% pure** and KatE (AR-1394) is **63.4–66.2% pure** . Furthermore, eQuilibrator MDF analysis (PAP_SY_SupportingInfo_(1).pdf Fig. S5a) proves **AckA is the thermodynamic bottleneck** ($\Delta_r G^\circ > 0$), while KatE prevents H₂O₂ thiol oxidation (Fuse-Murakami et al, 2026, 10.1021/acssynbio.6c00011).

MECHANISTIC / BIO-REASONING:

Because Pox5 (AR-1393) is ~38% pure, a nominal 3.03 μ M dispense delivers only **~1.15 μ M active Pox5**. Boosting nominal [Pox5] to 5.0 μ M raises active Pox5 to ~1.9 μ M ; boosting [AckA] to 20.0 μ M overcomes the AckA thermodynamic bottleneck; and boosting [KatE] to 6.0 μ M (~2.5 $\times$) scavenges H₂O₂ to protect TCEP/DTT and translation-factor cysteine thiols on top of the optimal EXP-5 substrate mix.

CONCRETE NEXT STEPS FOR ROUND 4 EXECUTION

1. **Approve Completion-Time Goal Update:** Set CDK completion time (h) to [-Inf, +Inf] and run `propose_generate_ges` so persisted workspace suggestions optimize purely for yield maximization.
2. **Unlock PAP Enzyme Bounds:** In the **Constrain** tab, unlock Expected [Pox5] (μ M) to [1, 20] , Expected [AckA] (μ M) to [2, 30] , and Expected [KatE] (μ M) to [1, 20] to enable variable enzyme dispensing in Round 4.
3. **Inspect Chelation Ridge Surface:** Pin a 2D contour plot of Expected [Mg glutamate] (mM) vs Expected [K-phosphate] (mM) colored by CDK steady state (std. eq. μ M) to verify the stoichiometric ridge ($\text{MgGlu} \approx 12.5 + 0.25 \times (\text{P}_i - 27.5)$).