

PAP - IGOR round review

Here is the round-by-round breakdown of the **landscapes, hypotheses, and key findings** in Simplified Technical English (STE).

R0 (10/07, 10/08, 10/15, 10/16 — 4 Runs, 196 Wells)

- **Landscape Explored:**
 - Energy pathways: CP/CK alone vs. PAP alone vs. PAP + CP/CK combined.
 - Dispense workflow: proteins inside the Master Mix (MM) vs. separated (early / late), and RNAsin (0 vs. 1000 U/mL).
 - Template and salt: pOpen vs. pET21a plasmid (0.5–4 nM) and initial [MgGlu] (8–16 mM).
 - **Hypotheses Tested:**
 - **H1:** Integrating PAP with CP/CK recycles P_i and extends protein synthesis beyond the CP/CK limit ($\sim 1.1\text{--}2.3\text{ }\mu\text{M}$).
 - **H2:** Pre-mixing PAP enzymes (Pox5/AckA/KatE) with substrates in the Master Mix causes premature turnover, gas bubbles, and flatlined wells.
 - **Key Findings:**
 - Removing RNAsin alone (r1008) did not fix flatlined PAP wells ($\sim 0.55\text{ }\mu\text{M}$).
 - Separating PAP-PMix (PMix + Pox5 + AckA + KatE) from PAP-MM (r1016) eliminated flatlines and boosted yield 5–7 $\times$ to **3.0–3.99 μM** (r1016_E17).
 - pET21a at 1 nM outperformed pOpen and higher [DNA] (2–4 nM) at baseline Mg^{2+} .
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R1 (10/18 — 1 Run, 84 Wells)

- **Landscape Explored:**
 - 4D small-molecule central composite DOE across [MgGlu] (3.8–24.8 mM), [CP] (0–42.4 mM), [Na-Pyr] (0–54.2 mM), and [K-phos] (0–46.3 mM).
- **Hypotheses Tested:**
 - **H1 ($\text{Mg}^{2+} \times P_i$ /CP Chelation Ridge):** High [K-phos] and [CP] chelate free Mg^{2+} and starve ribosomes unless [MgGlu] is increased proportionally.
 - **H2 (Pyruvate Lag vs. Fuel):** High [Na-Pyr] ($>45\text{ mM}$) increases early O_2 consumption and lag time, while moderate [Na-Pyr] (18–36 mM) balances rate and lifetime.

- **Key Findings:**

- Low [MgGlu] (3.8–9.6 mM) with moderate/high [K-phos] or [CP] crashed yield to 0.00–0.97 uM .
 - High [K-phos] (46.3 mM) failed at 12 mM [MgGlu] (0.50–0.67 uM in r1018_E6), but jumped to 3.85 uM when [MgGlu] was raised to 20.2 mM (r1018_E18 at 18.2 mM Pyr).
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R2 (10/20 — 1 Run, 56 Wells)

- **Landscape Explored:**

- Low- P_i (5–18 mM [K-phos]) vs. High- P_i (35–45 mM [K-phos]) regimes at compensated [MgGlu] (13.5–20.5 mM).
- Untested literature optima from Yadav et al. Table S3 (SY-V16-c20 , SY-V18-c0 , SY-V18-c20) and space-filling anchors (anchor-A , anchor-B).

- **Hypotheses Tested:**

- **H1 (Low- P_i Chelation Relief):** Starting with low [K-phos] (5–15 mM) and relying on P_i released from CP hydrolysis could reduce Mg^{2+} chelation (GES-10 , Exp-1-BL , Exp-3-BL , HI-CP-lowPi).
- **H2 (High- P_i Buffering & Mass Action):** Alternatively, 35–45 mM pH 7 [K-phos] is required to buffer acetic acid and drive Pox5 flux when paired with 16 mM [MgGlu] (SY-V18-c20 , SY-V16-c20).

- **Key Findings:**

- **H1 was refuted and H2 won:** Low [K-phos] (5–15 mM) capped out at 2.35–2.89 uM , whereas high [K-phos] (34.9–44.0 mM) with 16 mM [MgGlu] hit 3.99 uM (r1020_F13 , SY-V18-c20) and 3.81 uM (r1020_K12 , SY-V16-c20).
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R3 (10/21 — 1 Run, 56 Wells)

- **Landscape Explored:**

- High- P_i ridge refinement ([K-phos] = 35–50 mM , [CP] = 14–26 mM , [MgGlu] = 13–18.8 mM , [Na-Pyr] = 18–35 mM).
- Template [DNA] concentration (1 nM vs. 3 nM) across all pathway controls and top PAP+CP recipes.

- **Hypotheses Tested:**

- **H1:** Stepping [K-phos] up to 48–50 mM with 20–26 mM [CP] and 16.5–18.8 mM [MgGlu] (GES-5 , HiCP25_HiPhos50) maximizes dual-pathway ATP supply.

- **H2:** Tripling [DNA] (1 nM → 3 nM) with high-capacity PAP+CP energy regeneration will increase total mRNA and protein yield.
 - **Key Findings:**
 - **H1 confirmed:** HiCP25_HiPhos50 (50 mM K-phos, 26 mM CP, 18.8 mM Mg) and GES-5 (46 mM K-phos, 18 mM CP, 16.9 mM Mg) were the **#1 and #2 highest conditions on the plate**.
 - **H2 refuted:** 3 nM [DNA] reduced yield by **37–48%** and shortened completion time by ~1.0 h across every pathway due to excess transcription draining rNTPs and chelating Mg²⁺.
 - Plate-wide PAP yield ceiling dropped vs. R2 while CKonly held steady (1.99 uM vs. 2.23 uM), implicating [total protein] / PAP-PMix enzyme capacity as the next limiting factor.
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R4 (10/22 — 1 Run, 60 Wells)

- **Landscape Explored:**
 - Focused high-yield optimum box: [K-phos] (34–55 mM), [CP] (20–32 mM), [MgGlu] (14–20 mM), [Na-Pyr] (17–35.4 mM), locked at [DNA] = 1 nM .
 - Coupled [total protein] titration (0.8x , 1.0x , 1.2x PAP-PMix = 1.44 , 1.80 , 2.16 mg/mL PMix + proportional Pox5/AckA/KatE).
- **Hypotheses Tested:**
 - **H1 (Macromolecular Bottleneck Relief):** Once small-molecule fuel (35–55 mM K-phos, 20–32 mM CP, 16–20 mM Mg) is saturated, increasing [total protein] (1.2x PAP-PMix) relieves translation-factor (EF-Tu) and Pox5/AckA/KatE enzyme bottlenecks.
 - **H2 (Pathway-Specific Protein Sensitivity):** Comparing 0.8x / 1.0x / 1.2x PAP-PMix on CKonly vs. ctr and top PAP+CP hits (SY-V18-c20 , SY-V16-c20 , GES-5 , HiCP25_HiPhos50 , GES-13 , GES-17) separates translation-factor scaling from PAP enzyme scaling.