20250219 MTHFS supplementation Folinic acid conversion

Status	Shipped
Date	@February 19, 2025 → February 20, 2025
Tags	💧 PURE ▶ Energy Mix
AI summary	Research on MTHFS supplementation and folinic acid conversion shows MTHFS is essential for converting folinic acid to 5,10-methenylTHF, with experiments indicating buffer contamination affects expression. Stability tests reveal minimal activity loss after freeze-thaw cycles.
Researchers	Yemo Ku
Keywords	PURE
Artifacts	Protein-Mix-03-02, tRNA-06-02, NEB Ribosome
Findings	Formylated Folinic Acid is fragile and will break PURE, unless used quickly or regenerated, SMS broke upon freeze thaw at MBC but not at AIC

Overview

*Summary of the research or experiment, particularly the **why** of the work.*

Background

Information and resources necessary for context; include links to other research notebooks as necessary.

DREAM

Track experimental progress here. Experiments are not done until written up. Collect results of sequences or series of experiments in Close-Out memos and link below.

- [ ] Sub-project: Folinic Acid
- [ ] Background: Folinic acid may be formylated by copurified enzymes

Contents

Notebook

ProSpec provided info

- **MW:** 25.4 kDa.
- Fused to a 20 AA His-tag at N-terminus
- **Protein concentration & buffer:** 0.5 ug/uL is formulated in 20mM Tris-HCl buffer (pH8.0), 200mM NaCl, 5mM DTT and 30% glycerol.
- **AA Sequence**

  MGSSHHHHHH SSGLVPRGSH MAAAAVSSAK RSLRGELKQR LRAMSAEERL RQSRVLSQKV IAHSEYQKSK RISIFLSMQD EIETEEIIKD IFQRGKICFI PRYRFQSNHM DMVRIESPEE ISLLPKTSWN IPQPGEGDVR EEALSTGGLD LIFMPGLGFD KHGNRLGRGK GYYDAYLKRC LQHQEVKPYT LALAFKEQIC LQVPVNENDM KVDEVLYEDS STA
- 25 ug ordered. If 0.5 mg/mL → 50 uL total volume

MTHFS & Folinic acid

- Literature specific activity: 0.07 to 1.83 pmoles of 5,10 methenylTHF/min/ ug protein
  - ~25x difference between low & high activity
- Folinic acid in PURE: 0.02 mM at 10 uL rxn volume → ~200 pmoles of folinic acid

💡**Thoughts on approaching experiment:**

- Will need to buffer exchange MTHFS into storage buffer without glycerol using a 3kDa Amicon filter. Currently in high salt (200 mM NaCl) buffer that may break PURE.
- Aim to add ~2 ug of protein, then in 1 hour, all the folinic acid should be converted to 5,10 methenylTHF
  - This is assuming the specific activity is ~2 pmoles folinic acid/ min/ ug

Goals

1. Identify if MTHFS is the discrepancy in how folinic acid is prepared between one-pot pure and Shimizu's PURE.
2. Produce reproducible SMS solution that can be freeze-thawed and lose only 10-15% functionality.

1. Identify if MTHFS is the discrepancy in how folinic acid is prepared between one-pot pure and shimizu's PURE.

Experimental set-up

- 3 small molecule solution with 7.5 mM Mg were created to test if MTHFS is the discrepancy how folinic acid is prepared between One-Pot PURE and Shimizu's PURE
  - SMS 03-06_1: no folinic acid
  - SMS 03-06_2: 5,10-methenyl-THF
    - Shimizu's method of converting folinic acid to 5,10-methenyl-THF in reducing and acidic conditions
  - SMS 03-06_3: folinic acid
    - 5 mM folinic acid (Leuvocornin, 511.50 g/mol): 2.9 mg of Leuvocornin was weighed in 1.5 mL microcentrifuge tube and dissolved in 1.134 mL filtered milliQ water.
- MTHFS
  - 40 of 50 uL was buffer exchanged into protein storage buffer (100 mM HEPES-KOH pH 7.6, 10 mM MgCl2, 100 mM KCl, 1 mM TCEP) using a 3 kDa amicon concentrator. MTHFS was diluted 320x from its original concentration.
    - Note: when diluting MTHFS, the rapid dilution caused some of the protein to precipitate.
  - Protein concentration determined with A280 measurement
    - MW: 25,400 kDa
    - Ext Coeff (M-1 cm-1): 16055

Protein	A280	A280/A230	Concentration (mg/mL)
MTHFS	1.4233	0.592	2.252
Buffer Exchanged MTHFS	0.4430	1.269	0.701

- Both MTHFS and buffer exchanged MTHFS will be tested in PURE due to the concentration difference and to determine the buffer effects.

PURE Timecourse

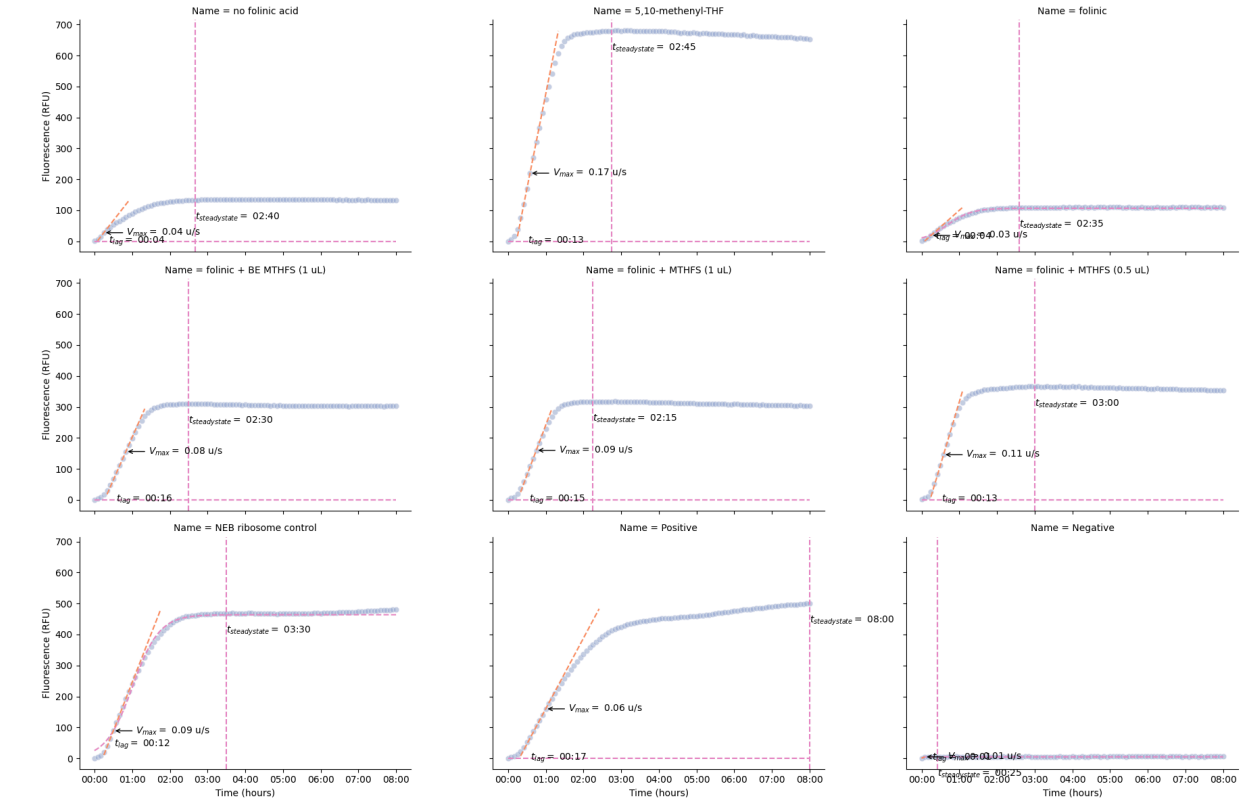
1. p-mix 03-02 + tRNA 06-02 + NEB ribosomes
    a. SMS-03-06_1 (7.5 mM) → no folinic acid
    b. SMS-03-06_2 (7.5 mM) → 5,10-methenyltetrahydrofolate
    c. SMS-03-06_3 (7.5 mM) → folinic acid in water
    d. SMS-03-06_3 (7.5 mM) → folinic acid in water + MTHFS (buffer exchanged 1 uL)
    e. SMS-03-06_3 (7.5 mM) → folinic acid in water + MTHFS (not buffer exchanged 1 uL)
    f. SMS-03-06_3 (7.5 mM) → folinic acid in water + MTHFS (not buffer exchanged 0.5 uL)
2. Ribosome + PURxpress control

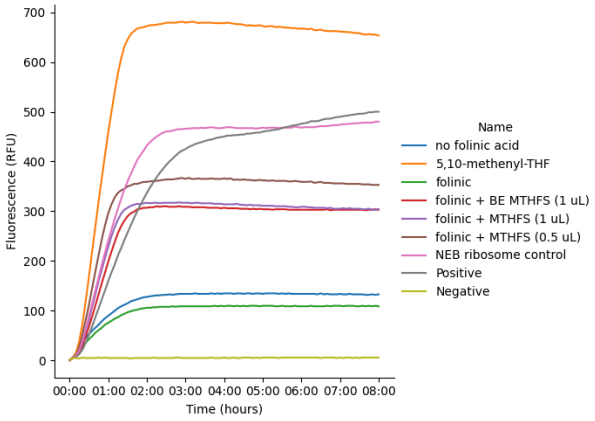
▼ Well layout

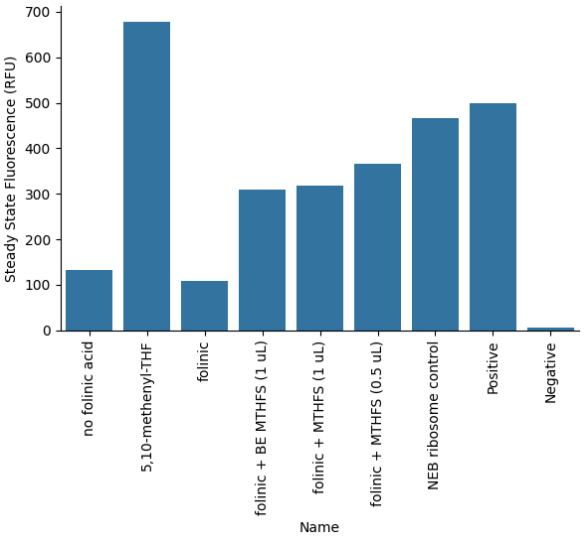
Well	B2	B4	B6	B8	B10	B12	B14
Sample #	1	2	3	4	5	6	7
Condition	no folinic acid	5,10-methenyl-THF	folinic	folinic + BE MTHFS (1 uL)	folinic + MTHFS (1 uL)	folinic + MTHFS (0.5 uL)	NEB ribo control
NEB ribosome	1.8	1.8	1.8	1.8	1.8	1.8	1.8
NEB factor mix****	-	-	-	-	-	-	1.2
bnext tRNA	1.00	1.00	1.00	1.00	1.00	1.00	-
SMS-03-06	4	4	4	4	4	4	-
bnext p-mix-03-03	1.3	1.3	1.3	1.3	1.3	1.3	-
NEB Solution A	-	-	-	-	-	-	4
NEB solution B	-	-	-	-	-	-	-
RNAse Inhibitor	0.4	0.4	0.4	0.4	0.4	0.4	0.4
DNA (deGFP)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H2O	1.00	1.00	1.00	0.00	0.00	0.50	2.10
MTHFS	0.00	0.00	0.00	1.00	1.00	0.50	
Total Volume	10.0	10.0	10.0	10.0	10.0	10.0	10

Results

▼ Kinetics







Conclusion:

1. MTHFS is required for folinic acid to be converted to the usable formyl donor (5,10-methenyl-THF → 10-FomyI-THF).
    a. Folinic acid alone (with no MTHFS) has little to no activity similar to when there is no folinic acid present.
2. When 5,10-methenyl-THF, it is quickly used to create f-met that is utilized in the the synthesis of deGFP.
    a. The high expression is unusual as it performs similar to the positive controls.

b. 5,10-methenyl-THF was added last to the SMS solution before quickly assembling the reaction and putting it on the plate reader.

3. Buffer contamination of MTHFS interferes with PURE reaction/ deGFP expression.
   a. 0.7 ug of BE MTHFS had similar activity as 2.25 ug of MTHFS added. Therefore, the reduction in deGFP expression is due to the salt carry over from MTHFS that interferes with PURE.
   b. 1.25 ug of MTHFS had more activity than 2.25 ug of MTHFS as only 0.5 uL of the sample was added to the PURE reaction.
      i. 20 mM vs 10 mM NaCl

2. Produce reproducible SMS solution that can be freeze-thawed and lose only 10-15% functionality.

Experimental Set-up

- SMS-03-06 variants (no folinic, 5,10-methenyl-THF, folinic) made @February 19, 2025 were stored at -20C and will be utilized again to determine the stability of SMS after a freeze-thaw.
- Since MTHFS has a buffer contamination interference with PURE, only 1.25 ug (0.5 uL of 2.25 ug/uL) will be tested. On the other hand, more buffer-exchanged (BE) MTHFS (1.25 ug, 1.61 uL of 0.7 ug/uL) will be added to determine if a higher concentration of MTHFS will result in higher deGFP expression.
- One last condition being tested will be

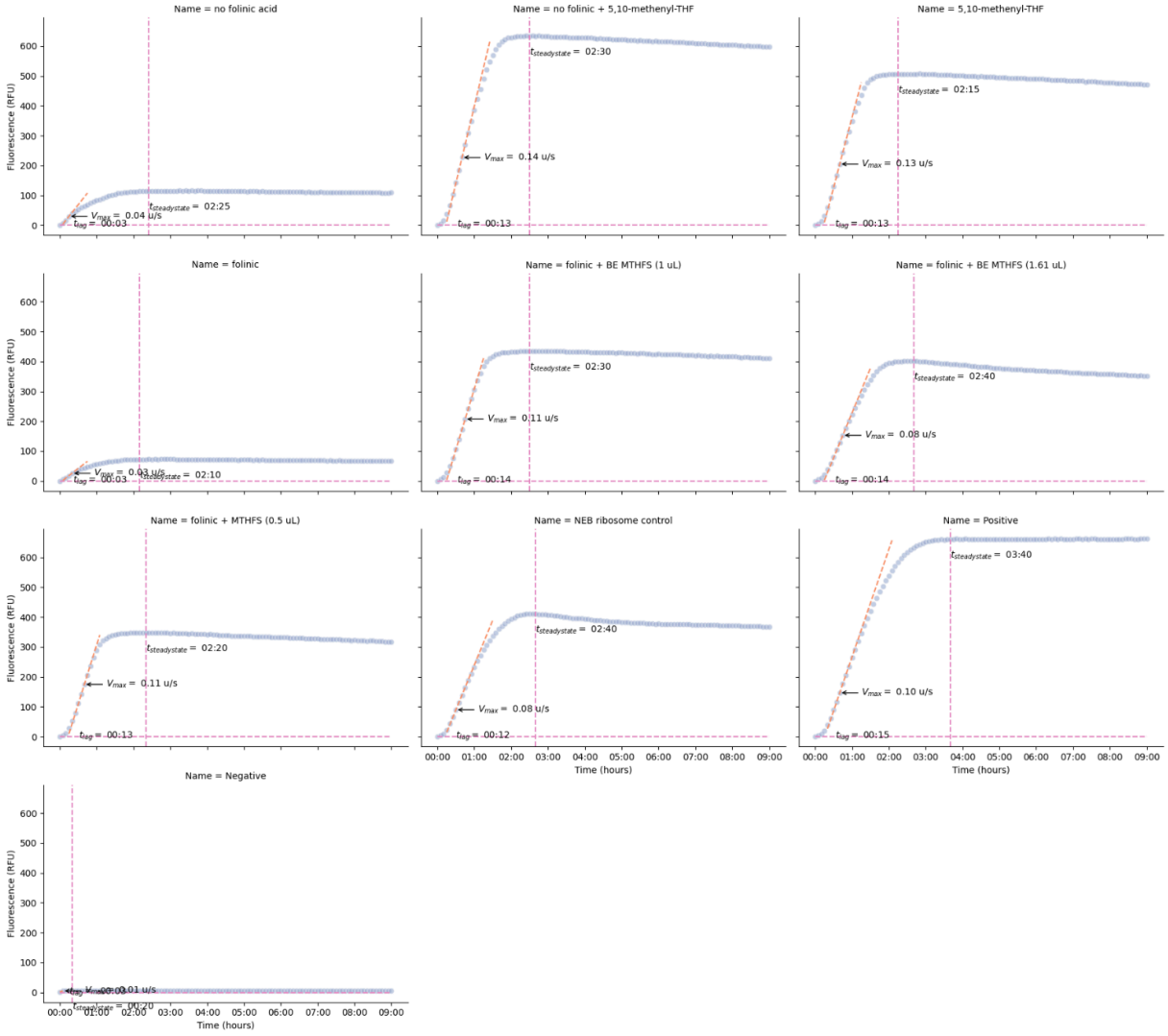
PURE Time-course

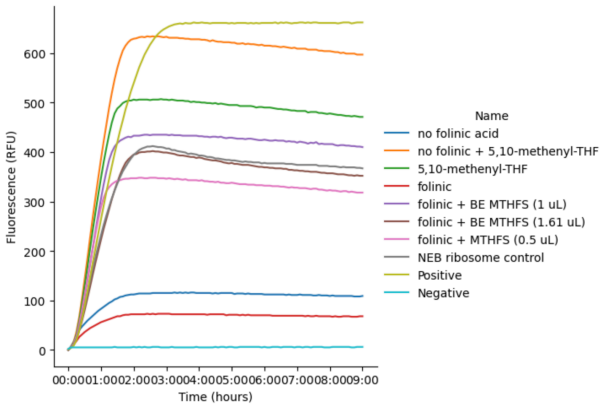
▼ Well layout

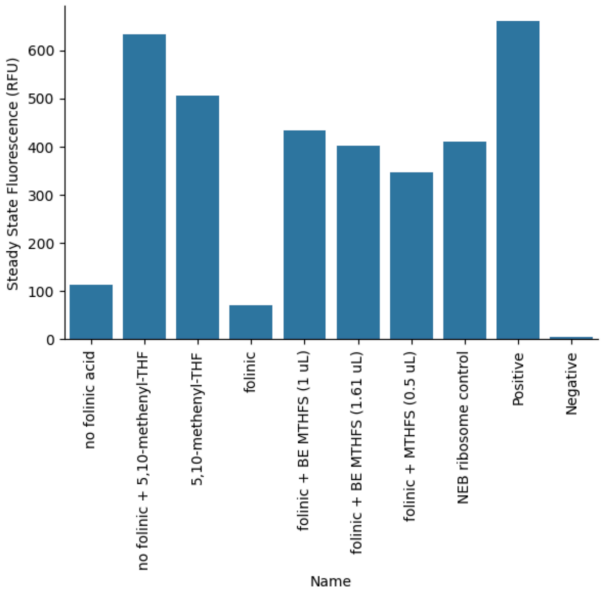
Well	D2	D4	D6	D8	D10	D12	D14
Sample #	1	2	3	4	5	6	7
Condition	**no folinic acid**	**no folinic + 5,10-methenyl-THF**	**5,10-methenyl-THF**	**folinic**	**folinic + BE MTHFS (1 uL)**	**folinic + BE MTHFS (1.61 uL)**	**folinic + MTHFS (uL)**
NEB ribosome	1.8	1.8	1.8	1.8	1.8	1.8	1.8
NEB factor mix	-	-	-	-	-	-	-
bnext tRNA	1.00	1.00	1.00	1.00	1.00	1.00	1.00
SMS-03-03	4	4	4	4	4	4	4
bnext p-mix-03-03	1.3	1.3	1.3	1.3	1.3	1.3	1.3
NEB Solution A	-	-	-	-	-	-	-
NEB solution B	-	-	-	-	-	-	-
RNAse Inhibitor	0.4	0.4	0.4	0.4	0.4	0.4	0.4
DNA (deGFP)	0.5	0.5	0.5	0.5	0.5	0.5	0.5
H2O	1.00	0.00	1.00	1.00	0.00	-	0.50
MTHFS	0.00	0.00	0.00	0.00	1.00	1.61	0.50
0.2 mM 5,10-methenyl-THF	-	1.00	-	-	-	-	-
Total Volume	10.0	10.0	10.0	10.0	10.0	10.61	10.0

Results

▼ Kinetics







Conclusion

- 5,10-methenyl-THF performed better than expected after a freeze thaw. Previously, we would observe a drastic decrease in performance after a freeze; However, in this experiment, we only observe ~25% decrease in activity.
- Other reagents in SMS is relatively stable and activity can be preserved if SMS is assembled without folinic acid and then supplemented right before the PURE time course. We tested this in this experiment by using no folinic acid freeze thawed & supplementing 1 uL of 0.2 mM folinic acid.
- Folinic acid is stable in SMS and we do not see a decrease

Meeting

1. Ribosomes (halted)
    a. Hydrophobic to do ribosome purification → blocked.
        i. Ribosome 03-02: Lavickova (waste of time)
        ii. QA + Ultracentrifugation → wait
    b. 1 mL sample column from sartorius (February 21st shipped)
    c. Test ultracentrifuge (To-do) → manage
2. Protein
    a. Blocking on Yen-Yu
        i. Cx- 43 build
        ii. Akshay needs help with 2 × 2 hours. W & F for shipping.
    b. Back-logged BL21 vs LysY/lac Iq 24-well plate most likely → back-logged DNA
        i. T7RNAP (March 10th).

    ii. LeuRS
    iii. IF2
    iv. ThrRS
c. Next week goals:
    i. Protein expression study
    ii. Protein induction study
d. MTHFS → ACJS deliver it
    i. Make him design the other proteins\