Determining the Vitamin Supplementation Requirements of the Methanogenic Archaeon *Methanosarcina acetivorans*

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Abstract

Obligate anaerobic archaea known as methanogens produce methane by reducing carbon-containing substrates in a process known as methanogenesis. *Methanosarcina acetivorans* are of particular interest due to their adaptable genome and use of several methanogenesis pathways, resulting in high bioengineering potential for biofuels and industrial products. Laboratory cultures of methanogens are grown in Balch tubes with a high-salt growth media to provide the environment and substrates for metabolism. This generalized HS media includes the exogenous addition of ten distinct vitamins to assist growth. However, the exact function of these vitamins during methanogenesis has yet to be fully explained.

As such, this project aims to identify the most metabolically stimulating vitamins in the growth media to streamline resources and optimize the growth of the utilized *Methanosarcina acetivorans*. This would be beneficial in lowering materials costs and increasing efficiency for industrial-scale production. Furthermore, this project aims to contribute to closing knowledge gaps about methanogenesis biosynthetic pathways by explicating the relevant genes used in the pathways. This would serve to promote future research questions and increase understanding of methanogen biochemistry.

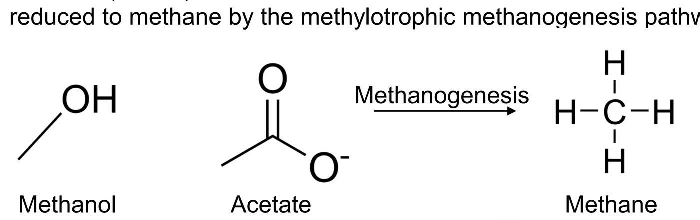
Research Questions

- Which vitamins are required, stimulatory, or neutral to the growth of *Methansarcina acetivorans*?
- Can the genes required for vitamin uptake, biosynthesis, and metabolism be identified by comparing gene expression patterns between growth conditions?

Methods

- Four starting cultures inoculated from the parental strain NB34 under different substrate and vitamin conditions are grown: methanol no-vitamin, methanol full-vitamin, acetate full-vitamin, and acetate no-vitamin.
- *M. acetivorans* is capable of surviving with no vitamins as a prototroph.

Figure 1. Chemical structures of the two substrates, methanol(MeOH) and acetate, and methane. Both substrates are reduced to methane by the methylotrophic methanogenesis pathway.



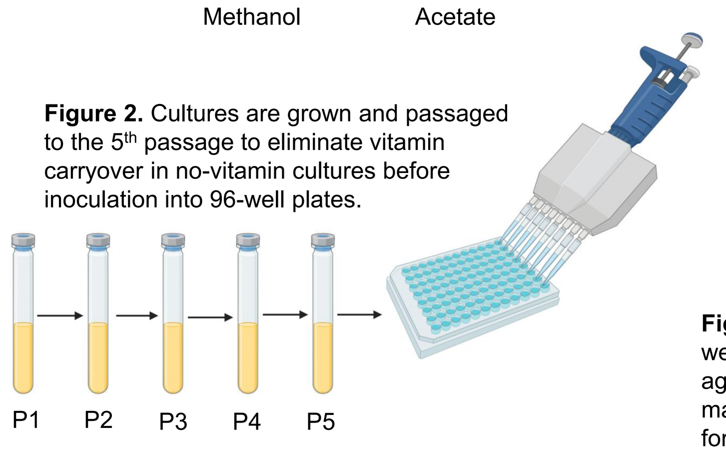


Figure 2. Cultures are grown and passaged to the 5th passage to eliminate vitamin carryover in no-vitamin cultures before inoculation into 96-well plates.

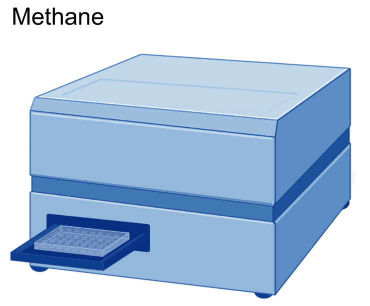


Figure 3. The absorbance of the plate wells is read every 30 minutes, with agitation and temperature control maintained throughout. Total time spent for readings varies by substrate.

Figure 4. Plate layout of the 10 listed vitamin conditions and controls. Water in the surrounding outer wells to prevent samples from drying out. Replicate data blanked using the no cell, no vitamin blank wells.

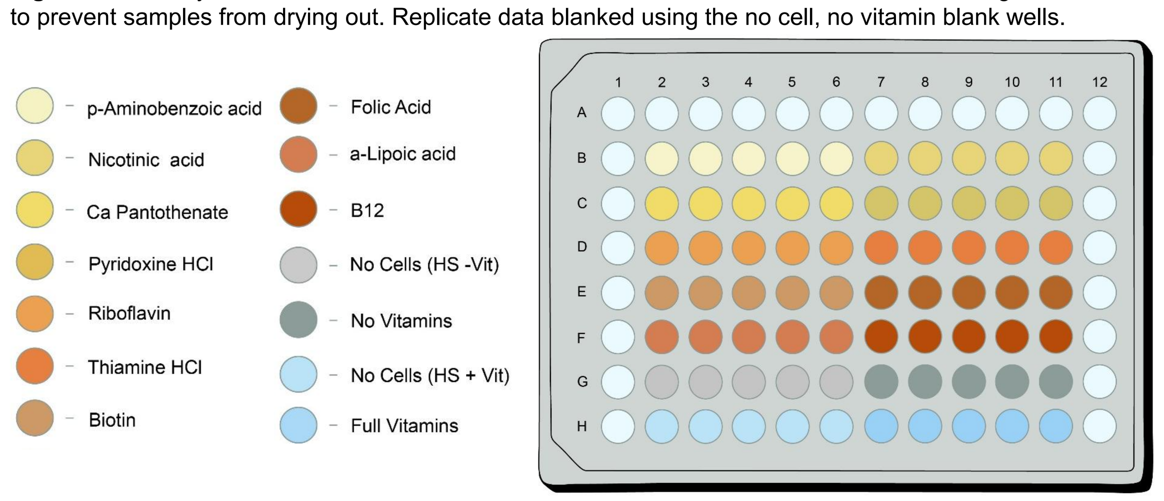
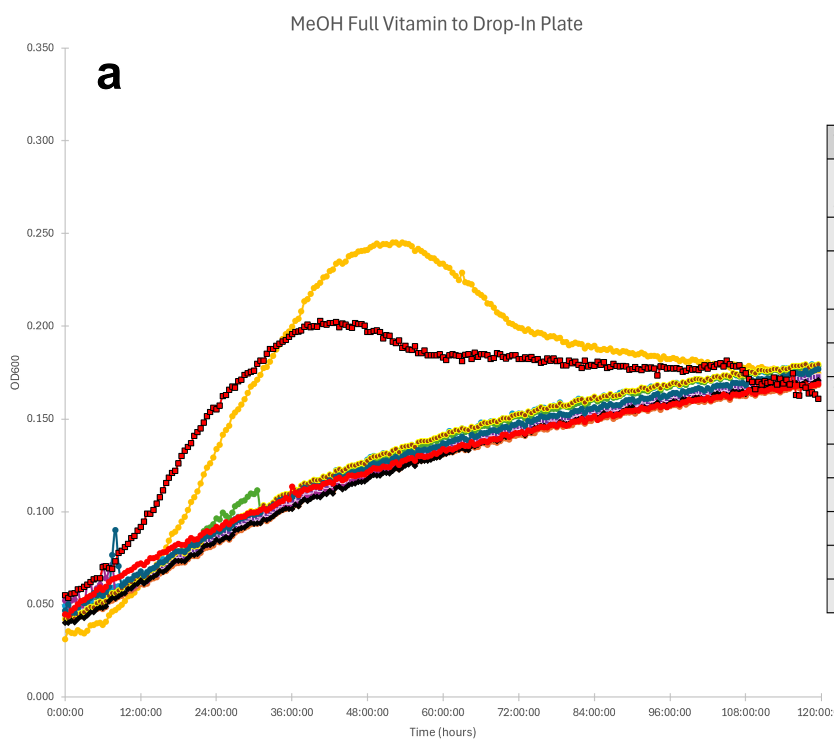
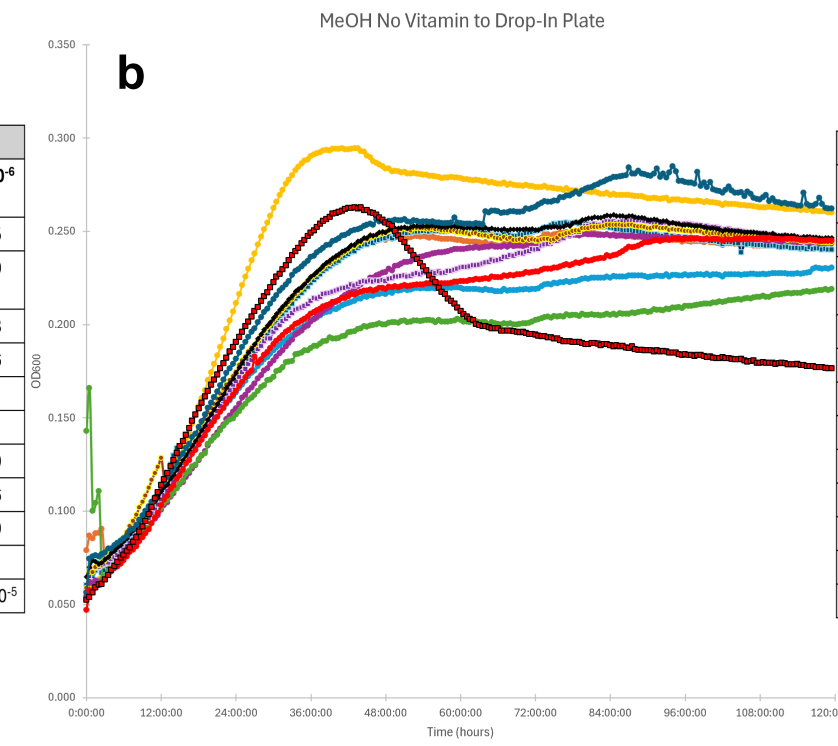


Plate Growth Curves

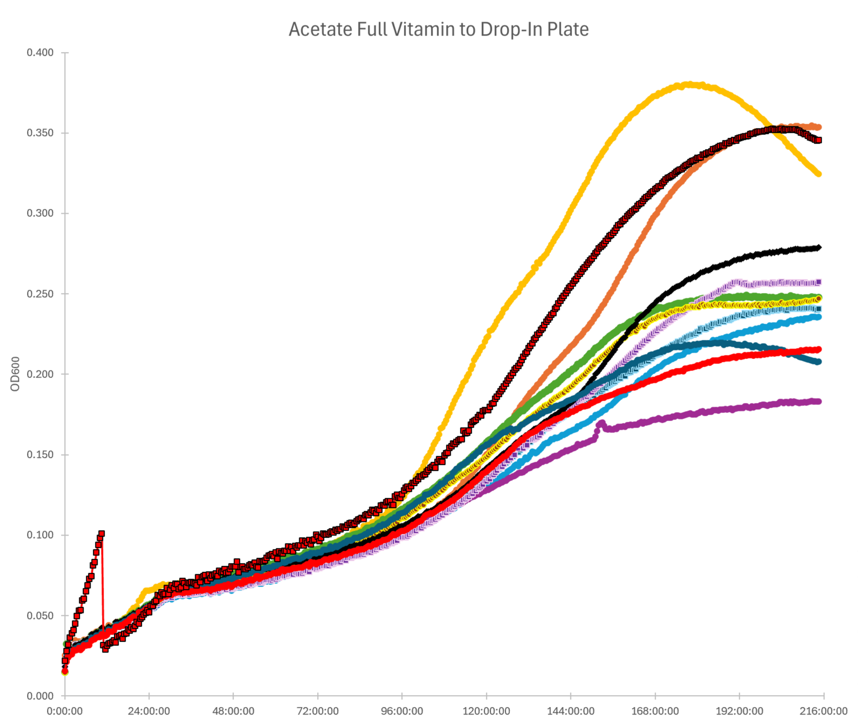


Vitamin	X̄ DT	vs. FV	vs. NV
p-Aminobenzoic Acid	19.772	**0.3348**	**5.6 x10^-6**
Nicotinic Acid	61.609	2.9 x 10^-7	0.0035
Ca Pantothenate	63.324	5.7 x 10^-7	0.0079
Pyridoxine HCL	62.626	5.5 x 10^-7	0.0068
Riboflavin	69.306	8.0 x 10^-9	0.0296
Thiamine HCL	58.666	4.7 x 10^-4	0.0031
Biotin	62.226	2.5 x 10^-7	0.0041
Folic Acid	67.547	1.1 x 10^-7	0.0170
a-Lipoic Acid	63.797	2.7 x 10^-8	0.0076
B12	69.966	4.3 x 10^-5	0.0550
No Vitamin	81.253	2.9 x 10^-5	1
Full Vitamin	24.455	1	2.9 x 10^-5



Vitamin	X̄ DT	vs. FV	vs. NV
p-Aminobenzoic Acid	18.628	**0.0891**	**0.0041**
Nicotinic Acid	27.705	0.0006	0.8487
Ca Pantothenate	27.886	0.0012	0.9412
Pyridoxine HCL	31.800	3.2 x 10^-5	0.0873
Riboflavin	28.551	0.0012	0.8078
Thiamine HCL	31.900	0.0027	0.1717
Biotin	28.045	0.0033	0.9971
Folic Acid	28.509	0.0089	0.8512
a-Lipoic Acid	28.059	0.0002	0.9900
B12	29.000	0.0001	0.6114
No Vitamin	28.037	0.0062	1
Full Vitamin	20.626	1	0.0062

Figure 5. Averaged growth curves (n=4-5) under different vitamin conditions over 120 hours and respective T-test results. Plates were inoculated from a MeOH culture grown with a) full-vitamin additions or b) no vitamin additions. Mean doubling times were calculated from the slope of the ln growth and compared to the full-vitamin and no-vitamin controls via a T-test of significance. Both plates indicate p-aminobenzoic acid(PABA) as an important vitamin for growth with MeOH as a substrate. Starting from a full-vitamin culture causes vitamin carryover in the plates, which can affect the results of a).



Vitamin	X̄ DT	vs. FV	vs. NV
p-Aminobenzoic Acid	41.720	**0.0556**	**0.0032**
Nicotinic Acid	67.210	**0.5584**	**0.0329**
Ca Pantothenate	86.334	0.1103	0.3674
Pyridoxine HCL	81.379	0.2258	0.2663
Riboflavin	123.93	0.0046	0.1997
Thiamine HCL	84.376	0.1099	0.2783
Biotin	79.433	0.3263	0.2704
Folic Acid	84.131	0.1599	0.3219
a-Lipoic Acid	74.140	0.4459	0.1506
B12	89.049	0.0741	0.4504
No Vitamin	100.79	0.0217	1
Full Vitamin	61.751	1	0.0217

Figure 6. Averaged growth curves (n=3-5) under different vitamin conditions over 216 hours and respective T-test results. The plate was inoculated from an acetate culture grown with full-vitamin additions. Most mean doubling times fall between the full-vitamin and no-vitamin rates, resulting in no significant difference compared to either control. PABA and nicotinic acid are of interest for having a p<0.05 when compared to the no-vitamin growth. p<0.05 for PABA versus full-vitamin growth because PABA has a faster doubling time.

Acetate Drop-In Plate From a No-Vitamin Culture?

- It is possible that acetate-grown cultures can not adapt to survive without added vitamins, but MeOH-grown cultures can
- Plate from P1 is possible for validating this hypothesis

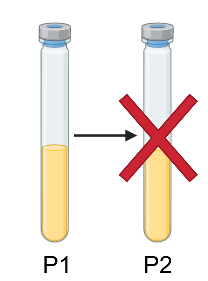
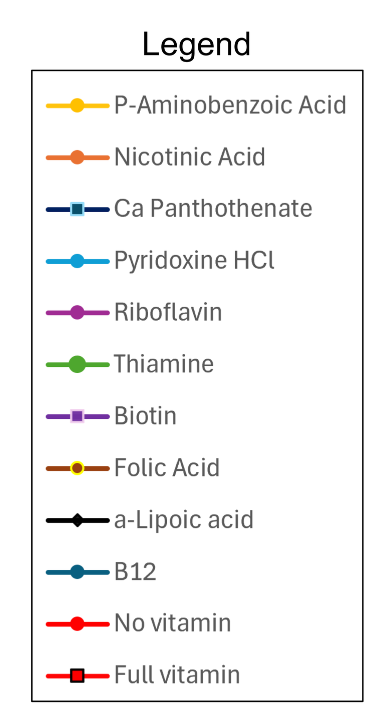


Figure 7. Acetate no-vitamin cultures will not grow into a second passage. Growth in P1 might be from vitamin carryover.



Balch Tube Growth Curves

- Based on the plate results, the growth of p-aminobenzoic acid (PABA) in MeOH was scaled up for observation in Balch tubes.
- PABA only, full-vitamin, and no-vitamin replicates inoculated from the same tube.
- Essentially, a scaled-up version of MeOH plate b) focusing on PABA only

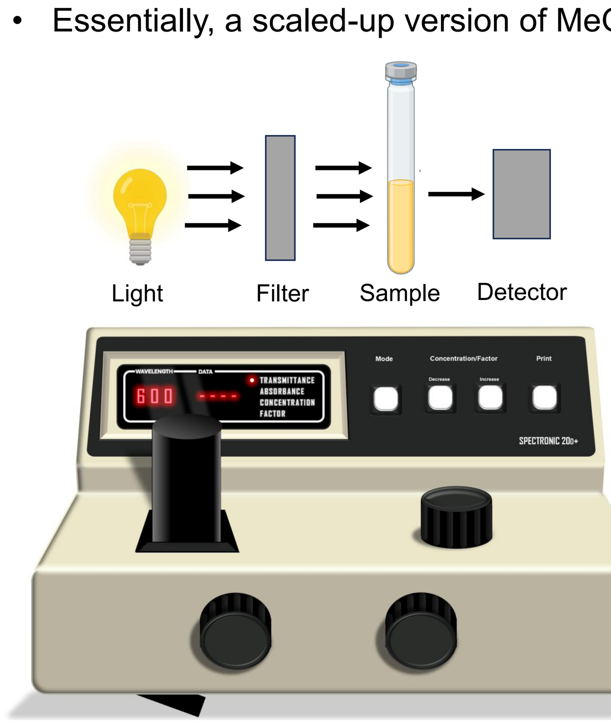
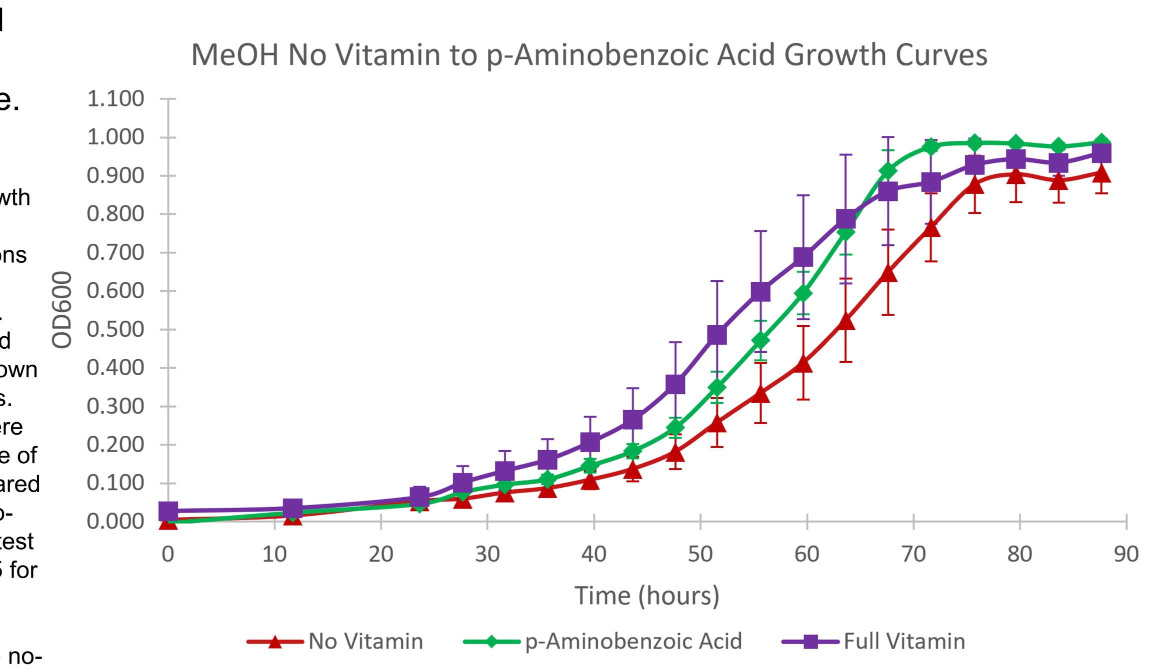


Figure 8. Optical density (OD) read by absorbance at 600nm on a Spectronic 20. Readings occur every 12 hours before samples hit the exponential growth phase, after which they are read every 4 hours.

Figure 9. Averaged growth curves (n=5-10) under different vitamin conditions over 90 hours and respective T-test results. Samples were inoculated from a MeOH culture grown with no-vitamin additions. Mean doubling times were calculated from the slope of the ln growth and compared to the full-vitamin and no-vitamin controls via a T-test of significance. A p>0.05 for full-vitamin versus no-vitamin growth indicates culture adaptation to the no-vitamin environment. p<0.05 for PABA versus no-vitamin, and the doubling time is shorter than that of the full-vitamin control.



Vitamin	X̄ DT	vs. FV	vs. NV
p-Aminobenzoic Acid	9.7775	0.1650	0.0247
No Vitamin	10.4823	0.7699	1
Full Vitamin	10.3559	1	0.7699

Future Work

- Investigate the theoretical basis behind why p-aminobenzoic acid(PABA) and nicotinic acid might be significant in methanogenesis.

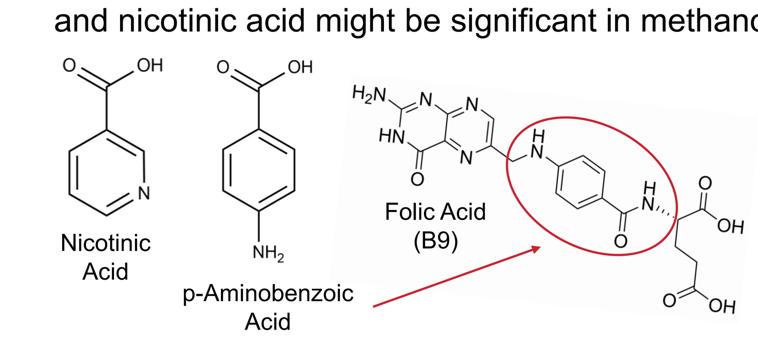


Figure 10. Chemical structures of nicotinic acid, PABA, and folic acid. Nicotinic acid and PABA have very similar structures. PABA is a constituent unit of another vitamin addition, folic acid,

- Complete an acetate no vitamin drop-in plate from P1. Do further acetate full vitamin drop-out plates with vitamin combinations.

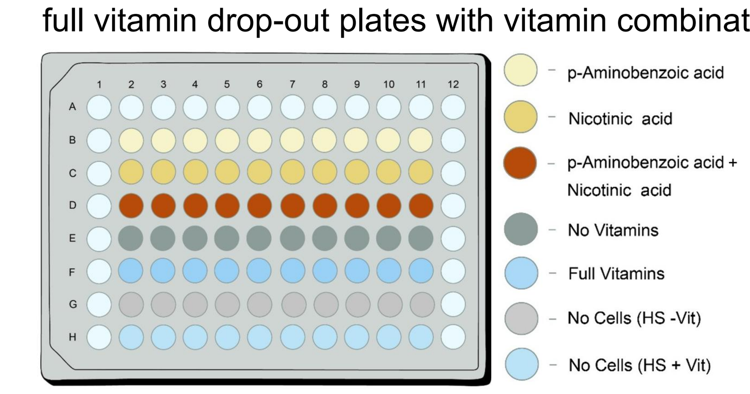


Figure 11. Tentative example of a plate set-up for vitamin combinations. It will also help to support initial plate results with more replicates under each growth condition.

- Compare gene expression under different vitamin conditions

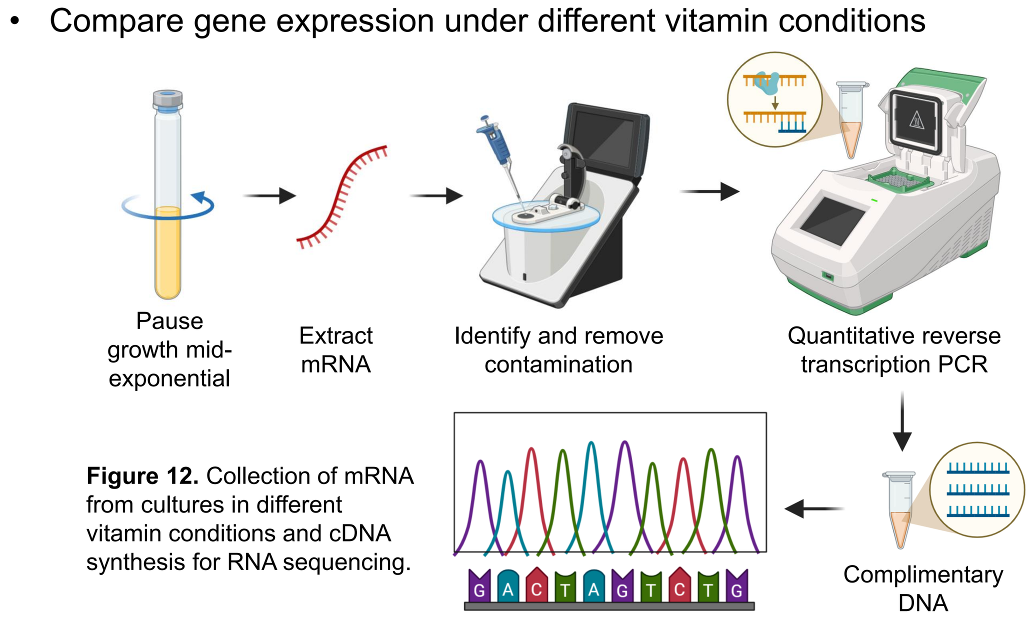


Figure 12. Collection of mRNA from cultures in different vitamin conditions and cDNA synthesis for RNA sequencing.

Acknowledgements

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