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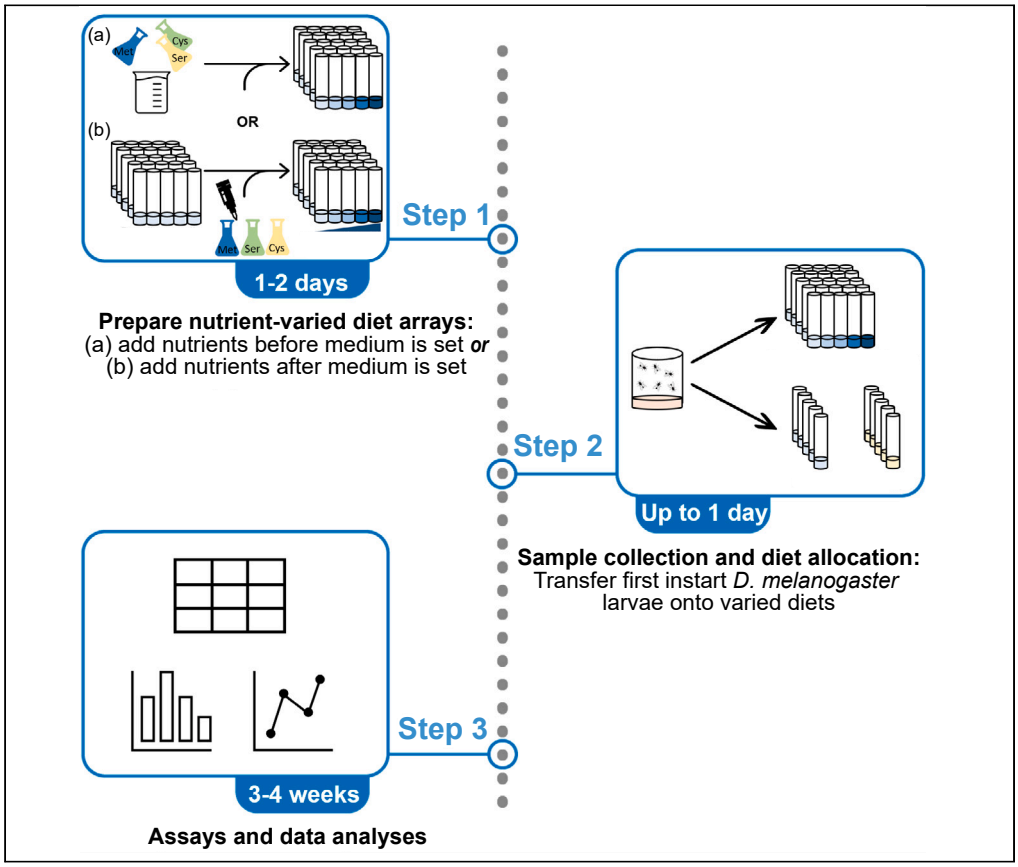
Protocol

A multi-nutrient array protocol to study disease-diet interactions in *Drosophila melanogaster*



The availability of a defined fully synthetic diet for the fruit fly *Drosophila melanogaster* allows for complete and precise manipulation of its nutritional environment. Here, we present a protocol for performing large-scale multivariate nutrient analysis via the traditional diet preparation approach, or by adding nutrient solutions to a baseline medium. We detail procedures from sample collection to data analysis. This protocol has applications for the study of nutrition-life trait interactions and nutrigenomics, to reveal interactions between genotype and diet composition.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for creating a fully synthetic diet for *Drosophila melanogaster*

Simplified multi-nutrient array by adding variable-nutrient solution to baseline medium

Pipeline to test diet effects on development and viability

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A multi-nutrient array protocol to study disease-diet interactions in *Drosophila melanogaster*

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SUMMARY

The availability of a defined fully synthetic diet for the fruit fly *Drosophila melanogaster* allows for complete and precise manipulation of its nutritional environment. Here, we present a protocol for performing large-scale multivariate nutrient analysis via the traditional diet preparation approach, or by adding nutrient solutions to a baseline medium. We detail procedures from sample collection to data analysis. This protocol has applications for the study of nutrition-life trait interactions and nutrigenomics, to reveal interactions between genotype and diet composition.

For complete details on the use and execution of this protocol, please refer to Martelli et al.[1] and Martelli et al.[2]

BEFORE YOU BEGIN

The protocol below describes the specific steps for creating a multivariate-nutrient array using a synthetic diet tailored for the model *Drosophila melanogaster*. It covers sample collection, diet preparation, and subsequent data collection and analysis. Here, we provide two diet preparation approaches for generating large-scale multi-nutrient arrays: (1) the traditional medium preparation technique where each different diet is prepared to completion separately, and in parallel, exemplified by the 3-dimensional methionine x serine x cysteine array, tested on a model of isolated sulfite oxidase deficiency (ISOD)[1]; and (2) a quicker technique where solutions containing the nutrients to be varied are added on top of a baseline medium, towards the end of the diet preparation protocol, exemplified by the 3-dimensional valine x leucine x isoleucine array, tested in a model of branched-chain amino acid transaminase (BCAT2) deficiency.[2] These protocols can be used as exemplars for the customization of any nutrient available in the fully synthetic medium, as well as to test the effects of supplements and drugs.

Before you begin, please make sure that all experiments involving *Drosophila* have been reviewed and approved according to the standards set by the institution. To perform the assay, you must first build healthy stock populations of the desired *Drosophila* strain(s). Before starting, expand the population size of the relevant stocks and maintain under controlled environmental conditions (25°C, 60% humidity and 12:12 light:dark cycle). To avoid overcrowding, standardize the total number of flies housed in each vial or bottle. Transfer stocks to fresh medium frequently to maintain healthy lines. We also suggest backcrossing the mutant strains to controls to standardize the genetic backgrounds.

Apple juice agar medium preparation

Timing: 3–3.5 h

1. Component preparation 0.5–1 h.
   a. Prepare a 5 L glass beaker and 10 Schott bottles each with 250 mL capacity.

   Note: This recipe yields ~10, 200 mL bottles of apple juice agar.

   b. Measure the following dry ingredients using a scale and place all in the 5 L beaker:
      i. Sucrose: 130.3 g.
      ii. Dextrose: 261 g.
   c. Measure wet ingredients as follows in separate measuring cylinders:
      i. Apple juice: 2024 mL.
      ii. Double-distilled water: 24 mL.
      iii. 10 M NaOH: 15 mL.
   d. To the 5 L beaker on a magnetic stirrer, add apple juice and double-distilled water.
   e. Add sucrose and dextrose, allow to dissolve.
   f. Add NaOH, allow to mix thoroughly.
2. Dispensing and autoclaving 2 h.
   a. Dispense ~200 mL solution into each of 10 Schott bottles each with 250 mL capacity.
   b. To each bottle, add 8 g of agar and autoclave 15 min at 120°C.
   c. Allow to cool to room temperature, secure lid and store at 4°C.

   Note: The amount of agar can vary as gelling properties of agar varies among suppliers.

   Note: After autoclaving, and cooling to room temperature, store bottles of apple juice agar at room temperature for up to 3 months until dispensing apple juice agar plates.

3. Preparation of apple juice agar plates 0.5 h.
   a. Melt 200 mL apple juice agar medium in bottle in a microwave.

   Note: Loosen lid before microwaving to allow pressure to escape and monitor the microwave closely to ensure it does not boil over.

   Note: For safety, handle the bottles with heat resistant gloves.

   b. Add 500 µL propionic acid and stir thoroughly by swirling the bottle.
   c. Pour the molten medium into Petri dishes and allow to cool and set at room temperature for up to 20 min. During cooling, cover the Petri dishes with cloth netting to prevent flies from contaminating the medium.
   d. Cover dishes with lids and store at 4°C until use.

   Note: 200 mL apple juice agar can make ~25 small plates (60 mm diameter petri dish) or ~10 large plates (90 mm diameter petri dish).

   Note: Plates can be stored at 4°C for up to 2 months.

Stock solutions preparation

Timing: up to 1 day

Table 1. Essential amino acids stock solution

Amino acids	Molar proportion of free amino acids required in baseline synthetic medium containing all amino acids	Molecular mass of amino acids (g/mol)	Ingredient	Molecular mass of ingredients (g/mol)	Mass of ingredients to be added to make a 200 mL stock solution (g)
L-Phenylalanine	0.03702	165.19	L-phenylalanine	165.19	3.33
L-Histidine	0.02555	155.15	L-histidine	155.15	2.16
L-Lysine	0.05660	146.19	L-lysine monohydrochloride	182.65	4.51
L-Arginine	0.05676	174.20	L-arginine monohydrochloride	210.66	5.38
L-Threonine	0.05635	119.12	L-threonine	119.12	3.65
L-Valine	0.06215	117.15	L-valine	117.15	3.96
L-Tryptophan	0.00954	204.23	L-tryptophan	204.23	1.06
L-Methionine	0.02452	149.22	L-methionine	149.21	1.99

4. Essential amino acids stock solution (33×) preparation 1 h.
   a. Measure the following essential amino acids using a scale and place in a 250 mL glass beaker (see Table 1):

   Note: The suppliers and product numbers of ingredients can be found in the key resources table. If other molecular forms of the amino acids, with different molecular masses, are used, re-calculation of the required mass of each amino acid will be needed to maintain the relative proportion of the free acids in the final diet.

   b. Add 200 mL milli-Q water and stir thoroughly with a magnetic stirrer.
   c. Adjust final pH of solutions to pH 4.5 using dropwise addition of HCl and monitoring with a pH meter. Mild heating may be required to solubilize.
   d. Filter sterilize into a sterile 250 mL Schott bottle.
   e. Store at 4°C.

   Note: Isoleucine and leucine are excluded from the essential amino acid stock solution due to their low solubility and thus will be added individually as powder when preparing the final medium during pre-autoclave preparation (see nutrient-varied diet arrays by adjusting the preparation of media step 1e). For diets requiring adjustment of their concentrations: (1) adjust the mass of powder added; or (2) prepare individual stock solutions, which are added afterwards as concentrates on the top of the gelled baseline synthetic medium and allowed to diffuse (see preparation of baseline synthetic diet).

   Note: Exclude essential amino acid(s) amenable to dietary manipulation when preparing the essential amino acid solution. To adjust concentrations of desired essential amino acids: (1) make individual stock solutions following the table above (e.g. methionine stock solution 1.99 g/200 mL) and add required volume of the stock during the post-autoclave medium preparation (see nutrient-varied diet arrays by adjusting the preparation of media step 3b); or (2) make individual stock solution(s) with desired essential amino acid(s), which are added afterwards as concentrates on the top of the gelled baseline synthetic medium and allowed to diffuse (see creating synthetic diets with varied levels of desired nutrients steps 8–9).

   Note: Essential amino acid stock solutions can be stored at 4°C for up to 1 year. If amino acids precipitate during storage, resolubilize with mild heating. Discard if the solution becomes cloudy or if any signs of microbial growth are detected.

5. Non-essential amino acids stock solution (33×) preparation 1 h.
   a. Measure the following non-essential amino acids using a scale and place in a 250 mL glass beaker (see Table 2):

Table 2. Non-essential amino acids stock solution

Amino acids	Molar proportion of free amino acids required in baseline synthetic medium containing all amino acids	Molecular mass of amino acids (g/mol)	Ingredient	Molecular mass of ingredients (g/mol)	Mass of ingredients to be added to make a 200 mL stock solution (g)
L-Alanine	0.07494	89.09	L-alanine	89.09	3.64
L-Aspartate	0.05338	133.10	L-aspartic acid sodium salt monohydrate	173.10	3.87
Glycine	0.06195	75.07	Glycine	75.07	2.53
L-Asparagine	0.04726	132.12	L-asparagine	132.12	3.40
L-Proline	0.05152	115.13	L-proline	115.13	3.23
L-Glutamine	0.04649	146.14	L-glutamine	146.14	3.70
L-Serine	0.07948	105.09	L-serine	105.09	4.55

Note: The suppliers and product numbers of ingredients can be found in the key resources table. If other molecular forms of the amino acids, with different molecular masses, are used, re-calculation of the required mass of each amino acid will be needed to maintain the relative proportion of the free acids in the final diet.

b. Add 200 mL milli-Q water and stir thoroughly with a magnetic stirrer.
c. Adjust final pH of solution to pH 4.5 using dropwise addition of 1 M HCl and monitoring with a pH meter. Mild heating may be required to solubilize.
d. Filter sterilize into a sterile 250 mL Schott bottle.
e. Store at 4°C.

Note: Tyrosine is excluded from the non-essential amino acid stock solution due to its low solubility and thus will be added in powdered form individually when preparing the final media during pre-autoclave preparation (see nutrient-varied arrays by adjusting the preparation of media step 1e). For diets requiring adjustment of tyrosine concentration: (1) adjust the mass of powder added; or (2) prepare individual tyrosine stock solutions, which are added afterwards as concentrates on the top of the gelled baseline synthetic medium and allowed to diffuse (see preparation of baseline synthetic diet).

Note: Cysteine is excluded from the non-essential amino acid stock solution and a separate cysteine stock is prepared (see step 6 below). This is because it precipitates over time and so keeping a separate stock allows prolonged storage of the non-essential amino acid mixture stock.

Note: Glutamate is excluded from the non-essential amino acid stock solution for flexibility (see step 6 below). For example, if an amino acid or a group of amino acids are omitted, glutamate can be adjusted to compensate for the resultant loss of total dietary nitrogen.

Note: Exclude non-essential amino acid(s) amenable to dietary manipulation when preparing the non-essential amino acid solution. To adjust concentrations of desired non-essential amino acids: (1) make individual stock solutions following the table above (e.g. serine stock solution 4.55 g/200 mL) and add required volume of the stock during the post-autoclave medium preparation (see nutrient-varied diet arrays by adjusting the preparation of media step 3b); or (2) make individual stock solution(s) with desired non-essential amino acid(s), which are added afterwards as concentrates on the top of the gelled baseline synthetic medium and allowed to diffuse (see creating synthetic diets with varied levels of desired nutrients steps 8–9).

Table 3. Cysteine and glutamate stock solutions

Amino acids	Molar ratios of free amino acids required in baseline synthetic medium containing all amino acids	Molecular mass of amino acids (g/mol)	Ingredient	Molecular mass of ingredients (g/mol)	Mass of ingredients to be added to make a 200 mL stock solution (g)
L-Cysteine	0.01707	121.16	L-cysteine hydrochloride	157.62	10
L-Glutamate	0.06258	147.13	L-glutamic acid monosodium salt hydrate	169.11	20

Note: Non-essential amino acid stock solution can be stored at 4°C for up to 1 year. If amino acids precipitate during storage, resolubilize with mild heating. Discard if the solution becomes cloudy or if any signs of microbial growth are detected.

6. Preparation of cysteine (10×) and glutamate (10×) stock solutions 1 h.
   a. Separately measure the following amino acids using a scale and place in separate labeled 250 mL glass beakers (see Table 3).

   Note: The suppliers and product numbers of ingredients can be found in the key resources table. If other molecular forms of the amino acids, with different molecular masses, are used, re-calculation of the required mass of each amino acid will be needed to maintain the relative proportion of the free acids in the final diet.

   b. To each beaker, add 200 mL milli-Q water and stir thoroughly with a magnetic stirrer.
   c. For each solution, filter sterilize into sterile 50 mL Falcon tubes.
   d. Store at 4°C.

   Note: For diets requiring adjustment of concentrations of cysteine and/or glutamate: (1) adjust the volume of stock solutions added during post-autoclave medium preparation (see nutrient-varied diet arrays by adjusting the preparation of media step 3b); or (2) add stock solutions afterwards as concentrates on the top of gelled baseline synthetic medium and allow to diffuse (see preparation of baseline synthetic diet).

   Note: Glutamate solution can be stored at 4°C for up to 1 year. Discard if the solution becomes cloudy or if any signs of microbial growth are detected.

   Note: Cysteine solution precipitates over time and can be stored at 4°C for up to 3 months. Discard if precipitate forms or becomes cloudy or if any signs of microbial growth are detected.

7. Nucleic acids and lipid head-group stock solution (125×) preparation 1–2 h.
   a. Measure the following ingredients using a scale and place them together in a labeled 1 L glass beaker.
      i. Choline chloride: 6.25 g.
      ii. Myo-inositol: 0.63 g.
      iii. Inosine: 8.13 g.
      iv. Uridine: 7.50 g.
   b. To the 1 L beaker add 1 L milli-Q water and stir thoroughly with a magnetic stirrer.
   c. Filter sterilize into sterile 50 mL Falcon tubes.
   d. Store at 4°C.

   Note: Nucleic acid/lipid solution can be stored at 4°C for up to 6 months.

8. Vitamin stock solution (47.6×) preparation 1–2 h.

a. Measure following ingredients using a scale and place together in a labeled 1 L glass beaker:
   i. Thiamine: 0.067 g.
   ii. Riboflavin: 0.033 g.
   iii. Nicotinic acid: 0.399 g.
   iv. Calcium pantothenate: 0.516 g.
   v. Pyridoxine: 0.083 g.
   vi. Biotin: 0.007 g.
b. Add 1 L milli-Q water to the beaker and stir thoroughly with a magnetic stirrer.
c. Filter sterilize into sterile 50 mL Falcon tubes.
d. Store at −20°C.

Note: Vitamin solution can be stored at −20°C for up to 1 year away from light.

Note: Once thawed, subsequently store vitamin solutions at 4°C for up to 3 months. (Do not subject to multiple freeze/thaw cycles).

9. Folic acid stock solution (1000×) preparation 1–2 h.
   a. Measure 0.5 g folic acid using a scale and place in a labeled 1 L glass beaker.
   b. Dissolve in 1 L milli-Q water by slow dropwise addition of 2 M NaOH and stir thoroughly with a magnetic stirrer.
   c. Filter sterilize the solution into sterile 50 mL Falcon tubes.
   d. Store at −20°C.

Note: Folic acid solution can be stored at −20°C for up to 1 year.

Note: Once thawed, subsequently store folic acid solutions at 4°C for up to 3 months. (Do not subject to multiple freeze/thaw cycles)

10. Acetate buffer stock (10×) preparation 2 h.
   a. Add 500 mL milli-Q water to a 1 L glass beaker.
   b. Add 30 mL acetic acid and 30 g KH_2PO_4, allow to dissolve.
   c. Slowly add 10 g $NaHCO_3$, allow to dissolve.
   d. Make up to 1 L with milli-Q water.
   e. Autoclave.
   f. Store at 4°C.

Note: Buffer can be stored at 4°C for up to 6 months. Discard if solution becomes cloudy.

11. Cholesterol stock solution (66.67×) preparation 0.5 h.
   a. Add 1 g of cholesterol to a sterile 50 mL Falcon tube.
   b. Make up to 50 mL with 100% ethanol.
   c. Store at −20°C.

Note: Cholesterol solution can be stored at −20°C for up to 1 year.

Note: Cholesterol will precipitate out of solution during storage, warm up the tube in a 37°C water bath to re-dissolve before each cook.

12. Trace element stock (1000×) solutions preparation 1 h.
   a. Measure the following ingredients using a scale and place in separate labeled 1 L glass beakers:
      i. $CaCl_2.2H_2O$: 250 g.
      ii. $MgSO_4$: 250 g.

iii. $CuSO_4.5H_2O$: 2.5 g.
iv. $FeSO_4.7H_2O$: 25 g.
v. $MnCl_2.4H_2O$: 1 g.
vi. $ZnSO_4.7H_2O$: 25 g.

b. To each beaker add 1 L milli-Q water and stir thoroughly with a magnetic stirrer.
c. For iron solution, filter sterilize into sterile 15 mL Falcon tubes.
d. For other metal solutions, filter sterilize into sterile 50 mL Falcon tubes.
e. Store calcium, magnesium, copper, manganese, and zinc solutions at room temperature. Store iron solution at −20°C.

Note: Iron solution can be stored at −20°C for up to 1 year. $FeSO_4$ will precipitate out of solution during storage, warm up the tube in a 37°C water bath to re-dissolve before each cook. Once thawed, subsequently store iron solutions at 4°C for up to 3 months. (Do not subject to multiple freeze/thaw cycles).

Note: Other trace element solutions can be stored at room temperature for up to 1 year (Discard if cloudy).

13. Nipagin stock (1×) solutions preparation 15 min.
    a. Measure 100 g nipagin (methyl 4-hydroxybenzoate) using a scale and place in a 1 L glass beaker.
    b. To the beaker add 1 L 100% ethanol and stir thoroughly with a magnetic stirrer.
    c. Store at room temperature.

Note: Nipagin stock solutions can be stored at room temperature for up to 6 months. Discard if solution becomes cloudy.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Organisms/strains		
D. melanogaster: w^{1118}	BDSC	RRID:BDSC_3605
D. melanogaster: $shop^{C15}$	Otto et al.[3]	N/A
Software and algorithms		
PRISM v9.0	GraphPad Software	N/A
R v4.2.2	www.r-project.org	N/A
RStudio	www.rstudio.com	N/A
Chemicals, peptides, and recombinant proteins		
L-arginine HCl	Sigma-Aldrich	A5131
L-alanine	Sigma-Aldrich	A7627
L-asparagine	Sigma-Aldrich	A0884
L-aspartic acid	Sigma-Aldrich	A6683
L-cysteine	Sigma-Aldrich	C1276
L-glutamic acid monosodium salt monohydrate	Sigma-Aldrich	G5889
L-glutamine	Sigma-Aldrich	G3126
Glycine	Sigma-Aldrich	G7126
L-histidine	Sigma-Aldrich	H8000
L-isoleucine	Sigma-Aldrich	I2752
L-leucine	Sigma-Aldrich	L8912
L-lysine HCl	Sigma-Aldrich	L5626
L-methionine	Sigma-Aldrich	M9625
L-phenylalanine	Sigma-Aldrich	P2126
L-proline	Sigma-Aldrich	P0380

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-serine	Sigma-Aldrich	S4500
L-threonine	Sigma-Aldrich	T8625
L-tryptophan	Sigma-Aldrich	T0254
L-tyrosine	Sigma-Aldrich	T3754
L-valine	Sigma-Aldrich	V0500
Agar	Sigma-Aldrich	A7002
Sucrose	Sigma-Aldrich	S1888
Cholesterol	Sigma-Aldrich	C8667
Choline chloride	Sigma-Aldrich	C1879
Myo-inositol	Sigma-Aldrich	I7508
Inosine	Sigma-Aldrich	I4125
Uridine	Sigma-Aldrich	U3750
Acetic acid	Thermo Fisher Scientific	AJA1
KH_2PO_4	Sigma-Aldrich	P9791
$NaHCO_3$	Sigma-Aldrich	S8875
$CaCl_2 \cdot 2H_2O$	Sigma-Aldrich	C7902
$CuSO_4 \cdot 5H_2O$	Sigma-Aldrich	C7631
$FeSO_4 \cdot 7H_2O$	Sigma-Aldrich	F7002
$MgSO_4$ (anhydrous)	Sigma-Aldrich	M7506
$MnCl_2 \cdot 4H_2O$	Sigma-Aldrich	M3634
$ZnSO_4 \cdot 7H_2O$	Sigma-Aldrich	Z0251
Thiamine (aneurin)	Sigma-Aldrich	T4625
Riboflavin	Sigma-Aldrich	R4500
Nicotinic acid	Sigma-Aldrich	N4126
Ca pantothenate	Sigma-Aldrich	21210
Pyridoxine-HCl	Sigma-Aldrich	P9755
Biotin	Sigma-Aldrich	B4501
Folic acid	Sigma-Aldrich	F7876
Propionic acid	Merck	8.00605.0500
Nipagin	Sigma-Aldrich	W271004-5KG-K
Absolute ethanol	Thermo Fisher Scientific	AJA214-2.5LPL
Others		
250 mL Schott bottle	Bio-Strategy	SCOT218013651
1 L Schott bottle	Bio-Strategy	SCOT218015455
5 L glass beaker	Bio-Strategy	SCOT211060701
1 L glass beaker	Bio-Strategy	SCOT211065408
250 mL glass beaker	Bio-Strategy	SCOT211063604
Measuring cylinder (1,000 mL)	Westlab	030316-1000
Autoclave	Tomy	SX-700E
60 mm Petri dishes	Techno Plas	*S6014S10*
90 mm Petri dishes	Techno Plas	TPG9014G
Filter (for filter sterilize solutions)	Neta Scientific	PALL-4612
Cloth netting	IKEA	ULLVIDE
Analytical balance	Mettler Toledo	ML204T
Heat-resistant gloves	Ansell	Crusader Flex
Magnetic stirrer	Thermo Scientific	Cimarec+
pH meter	Hanna	edge
Pipettes (P2–P1000)	Gilson	Pipetman
50 mL Falcon tube	In Vitro Technologies	FAL352070
15 mL Falcon tube	Bio-Strategy	BDAA352096
Drosophila culture vials	Pathtech	076-32-116
Trays for *Drosophila* culture vials	Pathtech	076-59-207BLU
Flugs to cap vials	Pathtech	076-59-200
Peristaltic pump	Cole-Palmer	Masterflex L/S 77200-60
Electric pipette controller	Thermo Scientific	9521

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Population cages for *Drosophila*	ProSciTech	LB700-050
Blunt metal probe	Livingstone	WND140SAH
Fine brush	Winc	25097633
Fluorescence microscope	Leica	M165 FC

STEP-BY-STEP METHOD DETAILS

Nutrient-varied diet arrays by adjusting the preparation of media

Timing: 1–2 days

This step describes procedures to generate a targeted diet array in which methionine, serine, and cysteine are co-varied by adjusting the preparation of media so that their concentrations are modified as part of the cooking procedure. This approach was used with *Drosophila shop* mutants in search of therapeutic diets.[1]

Note: This recipe yields ~150 vials, each with 6 mL of synthetic diet with one of the desired concentrations of methionine, serine and cysteine. This procedure needs to be repeated for each of the different concentration combinations.

1. Pre-autoclave preparation 0.5–1 h.
   a. Prepare 150 vials into trays.
   b. To a 1 L Schott bottle, add appropriate volume of milli-Q water (see "Total volume before autoclave (mL)" in Table S1).

   Note: Use measuring cylinder and micropipette to ensure accuracy.

   c. Add a stirrer rod to the bottle and mark the water level with a marker.
   d. Discard ~100 mL water.
   e. To the bottle, measure and add following ingredients:
      i. Agar: 7 g.
      ii. Isoleucine: 0.61 g.
      iii. Leucine: 1.11 g.
      iv. Tyrosine: 0.5 g.
      v. Sucrose: 17.13 g.
      vi. Cholesterol stock solution: 15 mL.
      vii. Trace element stock solutions: 1 mL each.
   f. Make up volume to the level of your marker with milli-Q water.
2. Autoclave 15 min at 120°C.
3. Post-autoclave medium preparation 1 h.
   a. Place bottle with molten medium on stirrer (set at 55°C), set mixing at a brisk pace.
   b. Measure and add the following ingredients:

   Note: Use electric pipette controller and micropipette to ensure accuracy.

      i. Acetate buffer stock: 100 mL.
      ii. Nucleic acid and lipid head-group stock solution: 8 mL.
      iii. Essential amino acid stock solution (without methionine): 32.6 mL.
      iv. Non-essential amino acid stock solution (without serine): 32.6 mL.
      v. Varying volumes of the Methionine stock solution: see "Methionine stock solution (mL)" in Table S1.

vi. Varying volumes of the Serine stock solution: see Table "Serine stock solution (mL)" in Table S1.
vii. Varying volumes of the Cysteine stock solution: see "Cysteine stock solution (mL)" in Table S1.
viii. Glutamate stock solution: 8.2 mL.
ix. Vitamin stock solution: 21 mL.
x. Folic acid stock solution: 1 mL.
xi. Propionic acid: 6 mL.
xii. Nipagin solution: 15 mL.

c. Dispense 6 mL into each vial with a peristaltic pump or repeat dispenser pipette.

4. Medium storage.
   a. Cover trays with cloth netting to prevent flies from contaminating the medium while cooling.
   b. Keep the food at room temperature overnight.
   c. Cap all vials and store at 4°C on the next day.

Note: Medium can be stored at 4°C for up to 3 months. Discard if there is any sign of drying (i.e., cracks in the medium or medium pulling away from the vial edges).

Nutrient-varied diet arrays by addition of solubilized nutrient solutions to baseline media

Timing: 1–2 days

This step describes procedures to generate a targeted diet array in which valine, leucine and isoleucine are co-varied via addition of solubilized nutrient solutions. This approach was used with *Drosophila BCAT2* mutants in search of therapeutic diets.[2] This technique involves the preparation of a gelled baseline synthetic medium to which nutrients to be varied are added as concentrated soluble solutions on top and allowed to mix by diffusion. This approach is faster and less laborious than the previous protocol in which each different diet is prepared individually. Before using this protocol for arrays of other dietary components, it is important to pilot the efficacy of the diffusion of the stock solutions into the gelled food. For essential amino acid dilutions, this can be done by assessing the effects of a small number of dilutions on development time or female egg production.

Preparation of baseline synthetic diet

Note: The baseline synthetic diet is one which contains all nutrients except the desired nutrient(s) to be varied in the experiment.

Note: This recipe yields ~150 vials with 6 mL of baseline synthetic diet.

5. Pre-autoclave preparation 0.5–1 h.
   a. Prepare 150 vials into trays.
   b. To a 1 L Schott bottle, add 706.5 mL milli-Q water.

Note: Use measuring cylinder and micropipette to ensure accuracy.

   c. Add a stirrer rod to the bottle and mark the water level with a marker.
   d. Discard ~100 mL water.
   e. To the bottle, measure and add following ingredients:
      i. Agar: 7 g.
      ii. Tyrosine: 0.5 g.
      iii. Sucrose: 17.13 g.
      iv. Cholesterol stock solution: 15 mL.
      v. Trace element stock solutions: 1 mL each.

f. Make up volume to the level of your marker with milli-Q water.

6. Autoclave 15 min at 120°C.
7. Post-autoclave medium preparation 1 h.
   a. Place bottle with molten medium on stirrer (set at 55°C), set mixing at a brisk pace.
   b. Measure and add the following ingredients:

   Note: Use electric pipette controller and micropipette to ensure accuracy.

      i. Acetate buffer stock: 100 mL.
      ii. Nucleic acid and lipid head-group stock solution: 8 mL.
      iii. Essential amino acid stock solution (without valine): 32.6 mL.
      iv. Non-essential amino acid stock solution: 32.6 mL.
      v. Glutamate stock solution: 8.2 mL.
      vi. Vitamin stock solution: 21 mL.
      vii. Folic acid stock solution: 1 mL.
      viii. Propionic acid: 6 mL.
      ix. Nipagin solution: 15 mL.

   c. Dispense 6 mL into each vial with a peristaltic pump or repeat dispenser pipette.

   Note: In this example, isoleucine, leucine and valine solutions are omitted from the recipe up to here as they will be added in varying amounts afterwards. Other nutrients may be manipulated as required.

8. Medium storage.
   a. Cover trays with cloth netting while medium cools to prevent flies from contaminating the medium.
   b. Keep the food at room temperature overnight.
   c. Cap all vials and store at 4°C on the next day.

   Note: Medium can be stored at 4°C for up to 3 months. Discard if there is any sign of drying (i.e., cracks in the medium or medium pulling away from the vial edges).

Creating synthetic diets with varied levels of desired nutrients

9. Preparing leucine, isoleucine, and valine stock solutions 1 h.
   a. Measure the following ingredients using a scale and place in separate labeled 250 mL glass beakers:
      i. Leucine: 4.4 g.
      ii. Isoleucine: 3.6 g.
      iii. Valine: 3.9 g.
   b. To each beaker add 100 mL of milli-Q water and stir thoroughly with a magnetic stirrer.
   c. Adjust final pH of solutions to pH 4.5 using dropwise addition of 1 M HCl and monitoring with a pH meter. Mild heating (50°C) may be required to solubilize.
   d. For each solution, filter sterilize into an autoclaved 250 mL Schott bottle.
   e. Store at 4°C.

   Note: Some amino acid solutions may precipitate at 4°C and must be warmed up at 50°C for solubilizing before usage. Discard if solution becomes cloudy during storage.

10. Pipetting amino acid solutions to create varied synthetic diets 1–3 h.
    a. Uncap vials containing baseline diet medium.
    b. Add the required volume of amino acid stock solutions (see Table S2) on top of the food surface in each vial (6 mL of baseline diet) using a micropipette.

Note: The additions of stock solutions should be sufficient to supply the nutrient to the level that would be found in 6 mL of final medium.

c. Cap vials and store them at 25°C, 60% humidity for 48 h to allow complete diffusion and absorption of amino acid solutions.

Note: Prepare at least 10 replicate vials of each different diet to be tested.

Note: To check for complete diffusion, gently swirl the vial and observe if any liquid is floating on the top of the medium.

Note: As a reference the volume of each individual amino acid solution to be added to a vial containing 6 mL of baseline diet in order to reach 100% concentration is shown in Table S3.

Note: Stock solutions of different amino acids may be further combined at different ratios using Table S3 as a reference to create diets containing different concentrations of desired nutrients.

Note: A maximum of 350 μL of nutrient solution should be pipetted on top of 6 mL of baseline diet to allow for evaporation of excess liquid as well as complete absorption and diffusion of the solutes.

Note: The concentration of amino acids stock solutions may be varied as required.

11. Check the medium daily for the absence of liquid and store at 4°C as soon as possible.

Note: Medium can be stored at 4°C for up to 3 months. Discard if there is any sign of drying (e.g. cracks or medium pulling away from the edges).

Sample collection and diet allocation

Timing: up to 1 day

This step describes the procedure to collect large numbers of developmentally synchronized *shop* (*shop/FM7-GFP*) mutant and w^{1118} control embryos or first-instar larvae.

Note: Since the *shop* gene is located on the X chromosome and hemizygous *shop* males are sterile, *shop* mutants are exclusively found in hemizygous males.

Note: FM7-GFP (Bloomington #9372) is a first chromosome balancer carrying a dominant mutation in the gene *Bar*, which can be identified by hearted-shape red eyes.

Note: All genotypes tested should be cultured and handled simultaneously under identical conditions.

12. Population cages setup 6.5–7 h.
    a. Prepare two population cages, each with an apple juice agar plate supplemented with a small ball of live yeast paste. Label with the tested genotype.
    b. From each of the *shop* and the w^{1118} stock, collect 200 females and 50 males, respectively.
    c. Transfer flies to the respective population cage.
    d. Allow flies to lay for 6 h.

Note: For the targeted multi-nutrient array with subsequent survival assay, only set up population cages for *shop*. Set up multiple cages (~5) to obtain sufficient numbers of embryos.

13. Collection of embryos and diet allocation 1.5–2 h.

Note: For the targeted multi-nutrient array with subsequent survival assay, only collect embryos using this method due to the large number of embryos required for testing on all diets. For a smaller-scale test (<10 diets in total), pick larvae following step 4 below. For developmental timing and larval viability assay, skip directly to 4, picking larvae.

a. Detach apple juice agar plates from population cages 6 h after the cage setup.
b. Add PBS to the surface of the plate and gently dislodge eggs with a paint brush. Transfer the egg suspension to a 15 mL Falcon tube. Allow embryos to settle for 1 min and remove excess PBS with a micropipette.
c. Set a micropipette to 5 μL with a micropipette tip cut to widen the bore or purchase wide mouth pipette tips so that eggs can freely pass through the end of the tip.
d. Collect 5 μL of densely packed eggs suspended in PBS. This is done by lowering the tip to the base of the egg mass and sharply releasing the plunger to suck up the eggs in PBS.
e. Dispense *shop* embryos with micropipette to the surface of the medium prepared in 2.

Note: Repeat for each of the vials containing modified diets with varied levels of focal nutrients (methionine, serine, and cysteine in this case).

14. Alternative collection method, pick first-instar larvae allocate to diets 1.5–2 h.
    a. Detach apple juice agar plates from population cages 6 h after the cage setup.
    b. Cover the plate with a lid and incubate the plate at 25°C for 24 h to hatch embryos.
    c. Use a blunt metal probe, collect first-instar larvae by touching the probe against them; they will stick. Carefully place the larvae in the vial containing appropriate diet:
        i. Defined complete synthetic diet (100% cysteine).
        ii. 0% cysteine diet.

Note: Select first-instar *shop* larvae against the florescent balancer FM7-GFP.

Note: For each diet, collect 5 replicate vials per genotype, with 20 larvae in each replicate vial.

Assays and data analyses

Timing: 3–4 weeks

This step describes procedures to collect and analyze the survivorship and developmental timing of *shop* animals relative to their control siblings raised on a multi-nutrient array of diets and statistical analyses.

15. Assaying survival from egg to adult of *shop* targeted multi-nutrient array.
    a. Check vials daily for 20 days and record following data for each vial:
        i. Total number of adult flies.
        ii. Total number of *shop* hemizygous males.

Note: The *shop* hemizygous males can be identified by white eyes.

Note: For this assay, the embryos were pipetted which resulted in variation in numbers of embryos per vial. As a result, the total number of adults per replicate vial ranges from 20 to 80 (see Table 4).

Table 4. Example of raw data collected for the survival assay of *shop* targeted multi-nutrient array

Ratio (met%: Ser%: Cys%)	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
	Total no.	*shop* no.	Total no.	*shop* no.	Total no.	*shop* no.	Total no.	*shop* no.	Total no.	*shop* no.
100:100:100	34	0	38	0	42	0	43	0	53	0
100:100:75	29	11	36	15	50	18	27	13	71	22
100:100:50	32	10	35	6	39	10	51	14	29	9
100:100:25	34	10	35	11	30	9	21	5	57	18
100:100:0	31	7	33	8	34	15	28	8	29	7

b. Calculate the *shop* survival proportion (total number of *shop* hemizygous males / total number of adult flies) for each vial.
c. Calculate the mean *shop* survival proportion across replicate vials for each diet.
d. Perform statistical analysis (see quantification and statistical analysis 1).

16. Developmental timing and larval viability assay.
    a. Check vials every 24 h for 20 days for the presence of pupae and adult. Record following data for each vial (see Table 5):
        i. Time of appearance of each pupa and cumulative number of pupae.
        ii. The final total number of pupae.
        iii. Total number of adult flies.

Note: When repeatedly checking the same vial for the appearance of new pupae, use a marker pen to mark the vial at the position of each newly recorded pupal case. This will identify each individual (for recording time to emergence as an adult) and help to avoid repeat counting or omissions.

b. Calculate larva-to-pupa and larva-to-adult viability for each vial as follows:
    i. larva-to-pupa viability: total number of pupae/total number of larvae.
    ii. larva-to-adult viability: total number of adult flies/total number of larvae.

Note: In this case, where larvae were picked for the developmental timing assay, there was a total number of 20 larvae for each diet and genotype tested.

c. Perform statistical analysis (see quantification and statistical analysis 2–3).

Table 5. Example of raw data collected for developmental timing and viability assays (only one replicate is shown for each condition)

	shop		w^{1118}	
Days after egg lay	Control diet	0% Cys	Control diet	0% Cys
9	0	0	0	0
10	0	0	0	0
11	0	0	0	0
12	0	0	0	0
13	0	1	0	0
14	0	4	0	1
15	0	7	4	5
16	0	13	8	13
17	0	15	14	17
18	0	18	17	20
19	0	20	17	20
20	0	20	17	20
Total pupae No.	0	20	17	20
Total adult No.	0	18	17	20

EXPECTED OUTCOMES

An example of data collected for the survival assay of *shop* targeted multi-nutrient array is shown in Table 4. Depleting methionine, serine, and cysteine individually or in combination rescues the lethality of *shop* to varying degrees and results in viable adults.[1] Moreover, cysteine-depleted diet restores the viability as well as developmental timing of *shop* to the control level (Table 5).

QUANTIFICATION AND STATISTICAL ANALYSIS

1. Survival assay of *shop* targeted multi-nutrient array.
   a. Using R studio or equivalent software, apply the linear mixed model (R package: lme4) to analyze the effects of methionine, serine, and cysteine concentrations and their interactions on *shop* survival proportion. Set replicates as a random effect.
2. Plotting the pupariation kinetics curves.
   a. Turn the raw data (cumulative number of pupae) at each time point into percentage as follows: cumulative pupariation (%) = (cumulative number of pupae/total number of larvae) × 100

   Note: Total number of larvae is 20 for each diet and genotype combination tested.

   b. Using GraphPad Prism or equivalent software, visualize timing of developmental progression by plotting cumulative pupariation (%) against time after egg laying (days).
3. Calculating dietary effects on time of development.
   a. Calculate the median time of development in days after egg laying from egg to pupa or egg to adult, for each individual replicate.
   b. Using R studio or equivalent software, apply a two-way ANOVA followed by Tukey's HSD multiple comparison test, accounting for genotype-diet interactions, comparing mutants versus controls for each diet individually, as well as the response of mutants (or controls) to the different diets.
4. Viability assay.
   a. Turn viability data into percentages as follows:
      i. Larva-to-pupa mean survivorship (%) = larva-to-pupa viability × 100.
      ii. Larva-to-adult mean survivorship (%) = larva-to-adult viability × 100.
   b. Using GraphPad Prism or equivalent software, plot larva-to-pupa viability and larva-to-adult viability as mean survivorship (%), respectively.
   c. Perform one-way ANOVA followed by Tukey's Honestly Significant Difference test to determine whether the means of different experimental groups are statistically different from one another.

LIMITATIONS

Flies reared on the complete synthetic medium are significantly delayed compared to flies reared on sugar-yeast diet. While this is helpful for determining the timing of development by allowing less frequent measurements, it remains unknown why it is the case. It is possible that nutrients required to promote growth are missing from this diet. This should be considered when interpreting nutrient-genotype interactions and caution should be taken when drawing conclusions.

Environmental factors play a significant role in experiments involving model organisms such as *Drosophila*. Therefore, maintaining consistent temperature, humidity and light:dark cycle, as well as proper food preparation and fly handling, are essential as these variables can influence development, behavior and responsiveness to dietary manipulations.

TROUBLESHOOTING

Problem 1

Contamination of fly cultures with other fly genotypes at sample collection and diet allocation step 12.

Potential solution

- Discard contaminated populations, inspect the stock for potential contaminations, and set up new population cages using the stock if no contamination is identified.
- Clearly label vials and bottles to minimize contamination.
- Ensure that different paintbrushes are used for different genotypes.

Problem 2

Agar does not solidify at medium storage step.

Potential solution

- Ensure that the buffer is added after autoclaving.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Matthew D.W. Piper (matthew.piper@monash.edu).

Technical contact

Questions about the technical specifics of performing the protocol should be directed to and will be fulfilled by the technical contact, Travis K. Johnson (T.Johnson@latrobe.edu.au).

Materials availability

This study did not generate new reagents.

Data and code availability

- This study did not generate original code.
- Any additional information required to reanalyze the data reported in this work is available from the lead contact upon request

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103225.

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AUTHOR CONTRIBUTIONS

Conceptualization, M.D.W.P. and T.K.J.; methodology, J.L., F.M., S.M., A.Q., T.K.J., and M.D.W.P.; investigation, J.L. and F.M.; visualization and analysis, J.L., F.M., M.D.W.P., and T.K.J.; funding acquisition, T.K.J. and M.D.W.P.; project administration, T.K.J. and M.D.W.P.; supervision, T.K.J. and M.D.W.P.; writing, J.L., F.M., T.K.J., and M.D.W.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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