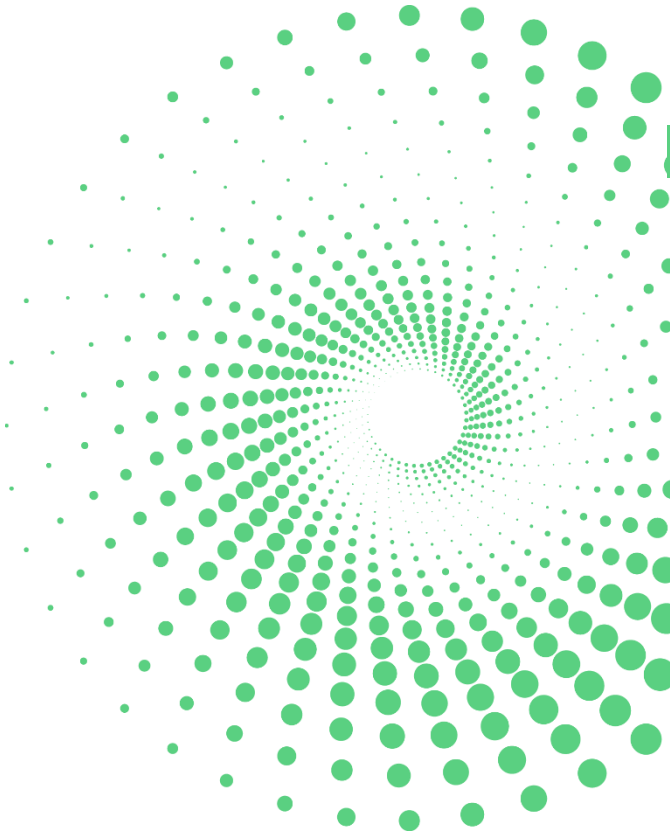

Instructions for Use

Gut Microbiota Medium

GMExpression® mucin-supplemented BHI formulation

Revision Date	August 2026
Revision Number	1.2

GMExpression
General Molecular Expression Service Pty Ltd

Anaerobic & Hypoxic culture media

GMExpression supplies a complete line of media formulated for the recovery, isolation and cultivation of oxygen-sensitive bacteria, for primary isolation, strain maintenance, method development and specialised laboratory applications. Twenty-eight products are listed, including enriched non-selective broths and agars, selective and differential plating sets, rumen-fluid and chopped-meat formulations, defined minimal media and the supplements that go with them. The whole line can be browsed and ordered online - Anaerobic & Hypoxic culture (https://shop.gmexpression.com/culture-media/Bacterial-culture-media/Anaerobic-Hypoxic).

Medium	Pack size
YCFA Medium, Full Recipe	For 12.7 L
YCFA Medium (Modified)	For 13 L
Modified Gifu Anaerobic Medium (mGAM)	For 5 L
Brain Heart Infusion (Supplemented), BHI-S	For 5 L
Modified M2 / M2GSC Medium - Rumen-Fluid Anaerobic Culturomics Broth	For 5 L
Chopped Meat Broth with Carbohydrates, GMExpression Modified	For 5 L
Peptone Yeast Extract Glucose Broth (PYG)	For 6 L
Wilkins-Chalgren Anaerobe Medium	For 5 L
TPY Medium for Bifidobacterium	For 5 L
Fastidious Anaerobe Agar (FAA, ATCC Medium 2895)	For 7 L
Columbia Blood Agar, with Hemin and Vitamin K_1	For 5 L
Mega + Minimal Medium Bundle	For 10 L
Bovine Rumen Fluid - cell-free sterile-filtered growth-factor supplement	50 ml
ATCC Trace Mineral & Vitamin Supplement	250 ml

Anaerobic & Hypoxic culture media

If you are not certain which medium suits a strain or an experiment, use our online selection tool: **Culturomics & Media Navigator** (https://shop.gmexpression.com/Culturomics-and-Media-Navigator.html). It holds 455 organism profiles across gut, oral, tissue, food, industrial, zoonotic, soil, extremophile and archaeal groups, and it searches in both directions:

- **Forward - from a microorganism to the recommended medium**. Select the target organism and the isolation objective, and the tool returns the recommended medium with its standard formulation per litre, the critical culture parameters, handling notes, detection methods and taxonomy.
- **Reverse - from a medium to the organisms it supports**. Start from a medium and see which organisms it is appropriate for, with their biosafety level, oxygen requirement, Gram reaction and growth environment.

Entries are drawn from authorised publications and databases, including NCBI/NIH, DSMZ, IJSEM, CLSI and ISO. For research use only.

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1. Product information

1.1. Summary

Product name	Gut Microbiota Medium (GMM)
SKU	GMNB-GMM01
Qty	Make up to 5 L of media
Reference identity	This product is the GMExpression® mucin-supplemented brain-heart infusion formulation, sold under the listing name *Gut Microbiota Medium (GMM)*. It is a different formulation from the defined medium published by Goodman and colleagues in 2011 under the same abbreviation. Cite this product by its formulation, not by the abbreviation alone.

1.2. Application

- GMM is an enriched, non-selective broth medium intended for use with anaerobic bacteria of the human gut, including fastidious and oxygen-sensitive taxa.
- A brain-heart infusion base is supplemented with yeast extract, a pancreatic digest of casein, four carbohydrates, porcine gastric mucin, hemin, Vitamin K_1, L-cysteine hydrochloride and five short-chain fatty acids.
- Suitable for microbial culturomics work requiring a mucin substrate and a short-chain-fatty-acid background.
- Research use only. Not for clinical or diagnostic use. Not for administration to humans or animals.
- **Establish performance for your strain, sample type, inoculum and gas atmosphere before relying on a result. Run the section 2 quality checks on every batch.**

1.3. Package contains

- Mixture A — 215 g
- Mixture B — 23 g
- Stock H | Hemin, 1.00 mg/ml in 0.05 M sodium hydroxide — 30 ml
- Stock K | Vitamin K_1, two formats, supplied on request
  - ❖ Format A | 0.50 mg/ml in 95 % ethanol, 0.22 µm filter-sterilised, 15 ml. Preparation instructions in section 2 are written for this format.
  - ❖ Format B | pure Vitamin K_1, 5 x 2 ml microtubes each holding about 2 mg, from which the user prepares the stock following the steps in FAQ 22.
- Stock S | Branched and C5 short-chain fatty acids — 30 ml
- Airtight PP Bags — 5 pieces
- Heat-resistant rubbers — 5 pieces

Note 1. Weigh 41.823 g of Mixture A and 4.500 g of Mixture B for every litre: the surplus in each container is a dispensing allowance, not an extra dose. **NEVER** tip a whole container into a 5 L batch. See FAQ 28.

Note 2. Mixture A can segregate in transit. Invert or tumble the bottle 10~15 times immediately before weighing each sub-portion, and weigh every litre individually rather than dividing the bottle by eye.

1.4. Components

Every quantity in this section is per 1.000 L of finished GMM. **Table A** lists what is in each container, with the dehydrated brain-heart infusion base as the single composite ingredient it is supplied as. **Table B** opens that base into its six published constituents and sets them against the quantities declared in Table A.

Table A - as formulated, per 1.000 L of finished GMM

Mixture A	**Per 1L**
Dehydrated Brain Heart Infusion (BHI) base	18.000 g
Yeast extract	5.000 g
Trypticase (pancreatic digest of casein)	5.000 g
D-Glucose (added separately)	2.000 g
Cellobiose	1.000 g
Maltose	1.000 g
Soluble starch	1.000 g
Sodium acetate	2.707 g
Sodium propionate	0.865 g
K_2HPO_4	2.000 g
KH_2PO_4	1.000 g
NaCl	2.000 g
$MgSO_4$ (hydration state per lot label)	0.200 g
$CaCl_2$ (hydration state per lot label)	0.050 g
Resazurin	0.001 g
Mixture A total	**41.823 g**

Mixture B	**Per 1L**
Porcine gastric mucin	4.000 g
L-Cysteine-HCl	0.500 g
Mixture B total	**4.500 g**

Stock H	**Per 1L**
Hemin	5.0 mg

Stock K	**Per 1L**
Vitamin K_1	1.0 mg

Stock S	**Per 1L**
iso-Butyric acid	88.1 mg
n-Valeric acid	102.1 mg
iso-Valeric acid	102.1 mg

Notes on the Table A entries

- **Porcine gastric mucin grade.** Sigma M2378 (Type II, crude) or M1778 (Type III, partially purified); the grade and catalogue number of the lot supplied are stated on the lot label and the certificate of analysis. The two grades are not interchangeable for sialidase-dependent work - see FAQ 10.
- **Hydration states.** The hydration state of each salt supplied is stated on the lot label. L-cysteine hydrochloride is the monohydrate, L-Cysteine-HCl-H_2O, M = 175.63 g/mol, so 0.500 g/L delivers 2.85 mmol/L.
- **L-cysteine is pre-blended with the mucin in Mixture B** and autoclaved in place; no separate sterile addition of reducing agent is required. See FAQ 10.
- **Stock H.** The 0.05 M sodium hydroxide is the solubilising agent, not an inert diluent: hemin, M = 651.9 g/mol, is practically insoluble in water. Use Stock H as supplied and do not dilute it. See FAQ 21.
- **Stock K - Format A carries ethanol into the medium.** The 2.00 ml dose of 95 % ethanol per litre leaves 0.19 % v/v ethanol in the finished medium. See FAQ 23.

Notes on Stock K Format B

Format B is a viscous yellow oil, not a solid: about 2 mg forms a thin film on the wall of the tube, so the tube is **NOT** empty. The preparation procedure is in FAQ 22.

- Reconstituted stock: **0.50 mg/ml** in 95 % or absolute ethanol - 2 mg made up to **4 ml** by four sequential 1 ml rinse-and-transfer steps, which is what recovers a 2 µL oil film quantitatively. The same concentration as Format A.
- Dose: **2.00 ml per litre**, the same as Format A, delivering 1.0 mg of Vitamin K_1 per litre and carrying the same 0.19 % v/v ethanol into the medium.
- Yield: 4 ml per tube, that is two 1 L doses; five tubes cover 10 L.
- Filter: **0.22 µm single-use syringe filter**, PTFE or nylon membrane in a polypropylene housing.

Never use water as the solvent: use 95 % or absolute ethanol. See FAQ 21.

Transfer accuracy, not solubility, sets the realised concentration of a Format B stock. Use Format A where a defined Vitamin K_1 concentration is required. See FAQ 22.

Notes on Stock S

- Stock S is non-sterile: never add it after autoclaving. See FAQ 5.
- Stock S is a 200-fold concentrate: dose 5.00 ml per litre to give 1.000 mmol/L of each acid. See FAQ 6.
- Stock S goes in with Mixture A at the dissolving step and is sterilised in the medium's own autoclave cycle. See FAQ 5.

Why the five fatty acids are separately packed

All five short-chain fatty acids (SCFAs) are added to the medium at the same stage before autoclaving. They are divided across two separate containers based on dry-blend compatibility.

1. **Acetate and propionate are supplied as their sodium salts inside Mixture A.** Sodium acetate and sodium propionate are stable, non-hygroscopic crystalline solids that blend and store well.
2. **The branched and longer-chain acids are supplied as the liquid Stock S.** iso-Butyric acid (melting point -47 °C), iso-valeric acid (-29 °C) and n-valeric acid (-34 °C) are liquids at room temperature, and their sodium salts are hygroscopic and waxy rather than free-flowing powders, so they cannot be dry-blended into Mixture A.

Table B - the brain-heart infusion base deconstructed

The 18.000 g of dehydrated brain-heart infusion base declared in Table A is itself a published six-component formula, and three of those components - glucose, sodium chloride and phosphate - are also declared separately in Mixture A. Table B gives the total of each of those three species per litre of finished medium, the identity and quantity of the animal-derived fractions (see 1.6), and the full component list needed to prepare this medium from individual raw materials rather than from a dehydrated base.

BHI component	per 1 L BHI	Contributed to 1 L GMM
Calf-brain infusion solids	7.7 g	3.746 g
Beef-heart infusion solids	9.8 g	4.768 g
Proteose peptone (pancreatic digest)	10.0 g	4.865 g
Dextrose (D-glucose)	2.0 g	0.973 g
Sodium chloride	5.0 g	2.432 g
Disodium phosphate	2.5 g	1.216 g
Sum	**37.0 g**	**18.000 g**

Integrated totals - Table B read against Table A

ingredient	Declared separately	From the BHI base	Total per 1 L GMM
D-Glucose	2.000 g	0.973 g	**2.973 g** (16.5 mmol/L)
NaCl	2.000 g	2.432 g	**4.432 g** (75.8 mmol/L)
Phosphate, total of all species	K_2HPO_4 2.000 g + KH_2PO_4 1.000 g	Na_2HPO_4 1.216 g	**27.40 mmol/L**
Peptide and protein nitrogen sources	Trypticase 5.000 g + Yeast extract 5.000 g	proteose peptone 4.865 g + brain 3.746 g + heart 4.768 g	**23.379 g**

Notes on the Table B

- Table B is the six-component brain-heart infusion formula cited to BD Difco 237500. See FAQ 3 for the sourcing and for how it varies between suppliers.
- **Confirm the hydration state of the brain-heart infusion base in use**. Table B and the phosphate totals assume anhydrous disodium phosphate; a hydrated base lowers the phosphate total. See FAQ 3.
- The integrated totals are supplier-independent; only the split between the three nitrogen rows varies by supplier. See FAQ 3.
- The base's declared reaction, pH 7.4 ± 0.2 at 25 °C, applies to the full-strength base at 37.0 g/L and is not a GMM specification. GMM's pre-autoclave target is 7.2 ± 0.2.
- Dextrose and glucose are the same substance. No brain-heart infusion supplier declares a hydration state for it, so the 2.0 g is read as anhydrous D-glucose.

Notes on pH values

The phosphate blend sets the pH of the fresh medium, not the acid load. GMM carries 3.0 mmol/L of free acid from Stock S against 27.40 mmol/L of total phosphate, a ratio of 0.11 : 1. Measure the pH first and adjust in whichever direction the reading requires; do not add alkali by default. FAQ 2 gives the worked arithmetic and the comparison with media that supply acetate and propionate as free acids.

Parameter	Specification
Pre-autoclave pH	7.2 ± 0.2 at 25 °C
Finished medium pH	7.0 ± 0.2 at 25 °C (6.8 to 7.2)
Sterilisation	121 °C, 15 min, slow exhaust
Reduction check	resazurin blue (dry) → pink (on boiling) → colourless (on reduction), before inoculation

- **The two pH figures are set at different points and are not expected to be identical.** See FAQ 2.
- **The resazurin check is qualitative.** The medium must be colourless before inoculation. Redox potential is not a specification of this product; measure it where your application requires a defined value. See FAQ 1.
- Record the pH immediately before and immediately after autoclaving, at 25 °C.
- **Volume reconciliation**. The batch is made up to **998 ml** before autoclaving; the 2.00 ml of Stock K added afterwards brings it to 1 L. The full ledger is in section 2.

1.5. Storage and Expiry Date

- This product can be shipped at ambient temperature. Upon receipt of the product, store **Mixture B**, **Stock H** and **Stock K** to a refrigerator at 2~8 °C. Stock S is stored at 15~25 °C by preference; 2~8 °C is acceptable.
- **Mixture A**. Store in the unopened bottle at room temperature (15~25 °C), dry and away from direct sunlight. After each use, tightly re-seal the bottle and return it to the same conditions. Weigh sub-portions promptly, and homogenise the container before each one; see Note 2 in 1.3. No post-opening shelf life is specified: use by the expiry date printed on the pack, and qualify any longer hold for your own.
- **Mixture B**. 2~8 °C, store with oxygen absorber and desiccant. After each use, tightly re-seal the bottle and return it to the same conditions. Use by the expiry date printed on the pack.
- **Stock H**. 2~8 °C, protected from light, in its aluminium-foil pouch. The pouch is the light protection; keep the bottle inside it between uses.
- **Stock K, Format A**. 2~8 °C, **strictly** protected from light, in its aluminium-foil pouch. Keep the cap tight. This is a flammable liquid: keep it away from ignition sources and **NEVER** autoclave the bottle.
- **Stock K, Format B**. 2~8 °C, **strictly** protected from light, and follow any storage condition printed on the tube label. The contents are **viscous oil**, not solid. Once reconstituted in ethanol, store the prepared stock as for Format A, in dark place at 2~8 °C, and treat it as a flammable liquid.
- **Stock S**, 15~25 °C preferred; 2~8 °C acceptable. **Do not freeze**. Whatever the storage temperature, bring the bottle to 15~25 °C, invert it 3~5 times and confirm a single clear phase before dispensing, inspecting it for turbidity or oily droplets first. Room temperature is preferred because the acids stay in solution more easily there. See FAQ 6.
- **Prepared medium, aerobic hold**. Hold broth and plates that have not been vacuum-sealed at 2~8 °C in the dark and use them promptly. They are not anaerobic: repeat the resazurin check in 1.4 before inoculation.

- **Prepared medium, anaerobic hold**. Vacuum-seal tubes, bottles or plates together with oxygen absorbers, and an oxygen indicator where available, and store at 2~8 °C. No shelf life is specified for prepared GMM; establish your own hold period before relying on stored medium. See FAQ 29.

1.6. Safety Notes

Mixture A: Dry blend of 15 components. Avoid generating dust when opening and weighing.

Mixture B: Contains fine animal-protein powder. Avoid generating dust. Open the bag and weigh inside a fume hood, a powder-weighing enclosure or a Class II cabinet; keep the bag low in the vessel and do not pour from height. Wear safety glasses with side shields, nitrile or latex gloves and a laboratory coat, and use a particulate respirator where dust cannot be contained or where an operator has a history of occupational asthma or protein-dust allergy. Wash hands after handling. Control dust as a precaution. Read the Safety Data Sheet supplied with your lot before handling. Mixture B also contains L-cysteine hydrochloride, an acidic solid. Mixture B is supplied non-sterile and is sterilised in the medium's own autoclave cycle; it must never be added after autoclaving.

Stock H: Hemin in 0.05 M sodium hydroxide. This is a dilute alkaline solution and is irritant to skin and eyes. Wear gloves and goggles. Do not pipette by mouth. The solution is deeply coloured and stains.

Stock K: Format A is a solution in 95 % ethanol and is a highly flammable liquid and vapour. Keep it away from heat, sparks, open flames and hot surfaces, keep the cap tight, and never autoclave the bottle. Format B tubes hold a neat oil; once reconstituted in ethanol, the prepared stock carries the same flammability. Ethanol vapour is also an eye and respiratory irritant.

Stock S: Operate Stock S in a fume hood and keep the bottle tightly sealed after use. It emits an irritant vapour, and the iso-butyric and iso-valeric acid it contains are detectable by odour at very low concentrations. Never pour Stock S, its residues or its rinsings into the sewer: n-valeric acid is harmful to aquatic organisms. Dispose of them as chemical waste through your institution's waste stream. Wear gloves and goggles to prevent skin and eye contamination and inhalation. Handle Stock S as a dilute organic acid: it carries 200 mmol/L of each of the three free acids.

Animal-derived components: this medium contains components of animal origin. The dehydrated brain-heart infusion base supplies bovine (calf) brain infusion solids, 3.746 g per litre of finished medium, and bovine heart infusion solids, 4.768 g per litre; Mixture B supplies porcine gastric mucin; the peptone and casein-digest fractions are of animal origin. No TSE, BSE, country-of-origin or animal-health certification accompanies this product. If your institution's biosafety, import or ethics approval requires an origin or TSE statement, request it for your specific lot before order. Handle the product as biological material of animal origin, observe your local biosafety rules and any national import or quarantine requirements for animal-derived laboratory materials, and do not use it in any application involving administration to humans or animals.

1.7. Cautions

- Always operate Stock S in a fume hood.
- Wear latex or nitrile gloves and goggles to prevent skin and eye contamination and inhalation.

- If skin or eyes are contacted, rinse immediately with plenty of water or saline solution and seek first aid if required.
- **Never pre-mix Stock H and Stock K, draw them into one pipette, or add Stock K into the Stock H bottle**. Where the optional route adds Stock H after autoclaving, add Stock H first, mix until the colour is uniform and no dark streaks remain, and only then add Stock K. See FAQ 21.
- **Protect Stock K and the supplemented medium from light at every stage**. Keep Stock K in its aluminium-foil pouch at 2~8 °C, return it to the pouch after each withdrawal, and never leave the bottle standing on an open bench. Switch the glove-box UV lamp off, and confirm it is off, before bringing Stock K or supplemented medium into the cabinet. See FAQ 24.
- When adjusting pH, add 1 M hydrochloric acid dropwise into the vortex of a vigorously stirred vessel. Never run acid onto a static surface and never add it as a bolus. See FAQ 10.
- Do not autoclave the medium twice.
- Do not clarify the bulk medium through a 0.22 µm filter**, and do not decant the dispersed mucin. The translucent opalescence is normal: carry it forward. See FAQ 10.
- Three deposits are handled differently: keep the translucent opalescence, leave only coarse flocculent material behind when decanting, and sterilise the made-up broth promptly rather than holding it in air. See FAQ 9.
- Invert or tumble the Mixture A bottle and the Mixture B bag 10~15 times before weighing any sub-portion. See FAQ 28.
- Keep Stock S at 15~25 °C by preference, or at 2~8 °C, never frozen, and inspect it for turbidity or oily droplets before every use. See FAQ 6.
- Stock K is a flammable liquid. Keep it away from ignition sources and never autoclave the bottle.
- Lab research use only. Not for clinical or diagnostic use, and not for administration to humans or animals.
- Emergency Phone: Company emergency call (24/7): +61 481 192 170

2. Instructions for preparation

2.1. Materials & equipment to prepare in advance:

- ☐ Personal protection: rubber gloves, goggles
- ☐ 70% alcohol & spray bottle
- ☐ Cotton swabs
- ☐ Oil-free vacuum pump
- ☐ Precision scale
- ☐ Magnetic stirrer and stirring follower
- ☐ Calibrated pH meter with temperature readout
- ☐ 3~4 clean 1000ml GL45 reagent bottles
- ☐ 25~50 anaerobic tubes with rubber stoppers and caps
- ☐ Tube racks
- ☐ 1ml pipette & pipette tips
- ☐ 200uL pipette & sterile pipette tips
- ☐ Pipette controller with 10ml serological pipette
- ☐ 1 Litre of MQ water or double-distilled water
- ☐ 1 Litre graduated cylinder
- ☐ Anaerobic preparation kit
- ☐ GMM media
- ☐ Self-sealing plastic bags (several)
- ☐ Autoclave chamber

Neither acid nor base is supplied. Have both 1 M HCl and 1 M NaOH on the bench before you begin; the adjustment may be needed in either direction. See FAQ 2. Record any substitution.

Optional:

- ☐ Anaerobic bench or anaerobic glove box
- ☐ 1.0 ml Ultra-fine syringes (see the image below-right for example)
- ☐ Vacuum sealer
- ☐ Vacuum plastic bags
- ☐ Oxygen absorber and oxygen indicator
- ☐ Surface Infrared Thermometer (see the image below-left for example)
- ☐ Heat-resistant gloves
- ☐ Fume hood, powder-weighing enclosure or Class II cabinet for weighing Mixture B [1]
- ☐ Particulate respirator, P2/N95 or better
- ☐ Agar, 15 g per litre, and sterile Petri dishes (agar plate route only; agar is not supplied with this kit - see FAQ 27)
- ☐ Water bath set to 50 °C (agar plate route only)
- ☐ Absolute or 95% ethanol, a 0.22 µm PTFE membrane filter in a polypropylene housing, and an inert gas (Stock K Format B only)

☐ Ethanol, the 0.22 µm filter and the inert gas are needed only if you are preparing a Format B Vitamin K_1 stock. See FAQ 22.

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[1]. Mixture B is a non-sterile fine animal-protein powder. See FAQ 12. Weigh it in a fume hood, powder-weighing enclosure or Class II cabinet; avoid dust, keep the bag low in the vessel and do not pour from height. Wear a P2/N95 respirator where dust cannot be contained. Read the Safety Data Sheet for your lot before handling.

2.2. If you have an anaerobic atmosphere working environment (AAE)

AAE means that the preparation can be processed in an anaerobic bench/chamber/glove box filled with anaerobic incubation gases, allowing strict anaerobic tests, culturing and YCFA agar plate preparation.

Commonly used anaerobic incubation gases (AGs):
- Triple gas mixture: 80% nitrogen, 10% hydrogen, 10% carbon dioxide.
- Double gas mixture: 80% nitrogen, 20% carbon dioxide.
- Pure nitrogen.

Text colours:
Blue - Explanation
Purple - Optional
Orange - Cautions

Step 0 - (optional)

Stock K in the AAE and half unscrew the cap for 30 minutes. Tighten the cap inside the AAE after every withdrawal.

Stock K is the only addition made after autoclaving. Stock H and Stock S go in at Step 1 and pass through the cycle, so neither needs separate deoxygenation. See FAQ 25.

Never pre-mix Stock H and Stock K, draw them into one pipette, or add Stock K into the Stock H bottle. Protect Stock K from light. See FAQ 21.

Step 1

Dissolve 41.823 g of Mixture A, 5.00 ml of Stock S and 5.00 ml of Stock H in 900 ml of double distilled or MQ water in a GL45-cap reagent bottle.

Handle Stock S in a fume hood; its C5 acids are detectable at parts-per-billion levels. See FAQ 5.

Bring the Stock S bottle to 15~25 °C, invert it 3~5 times and confirm a single clear phase before withdrawing a dose. This check applies whether the bottle was stored at room temperature or refrigerated. See FAQ 6.

Mixture A can segregate in transit; invert or tumble the bottle 10~15 times before weighing, and weigh each litre individually. See FAQ 28.

Steps 2.0 to 2.4 are optional. The heat step dissolves Mixture A and drives off dissolved oxygen; it has no other purpose. See FAQ 8.

Step 2.0 - (optional)

Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] or an apertured PP cap onto the bottle.

Step 2.1 - (optional)

Puncture the rubber stopper with the assembled vacuum device [2] and evacuate the bottle.

Vacuum until bubbles emerge from the liquid. Repeat at least twice, with a 1-minute gap between rounds. A pump substituted for the kit syringe must reach 3 kPa absolute or lower, or the liquid will not bubble and the step will not work; see FAQ 15.

See FAQ 14 for why evacuating the vessel removes oxygen more completely than purging.

Step 2.2 - (optional) - (if Step 2.1 processed, this step can be skipped)

Put the bottle into the anaerobic atmosphere environment (AAE). Open the rubber stopper for 1~2 minutes to remove oxygen from the bottle and allow the inert gas to fill. Then close the rubber stopper, and leave the apertured cap slightly loosened.

If Step 2.1 was skipped, leave the stopper open for 20 minutes instead of 1~2 minutes.

Step 2.3 - (optional)

Under the AAE, put the bottle into an airtight PP bag [1], seal the neck of the bag with a rubber band [1], and take it out of the AAE.

Fill the bag 1/3 to half with AG before sealing. If the AG contains hydrogen, squeeze out as much as possible before sealing the bag neck.

The bag keeps air off the bottle and contains odour. See FAQ 13.

Step 2.4 - (optional)

Heat the 910 ml of broth to 95~100 °C in a microwave oven or water bath for 10~12 minutes with frequent agitation, then leave it to cool.

Watch the stopper and the liquid. Switch the microwave off or take out of water bath as soon as boiling starts, and on again once the stopper has recovered its shape and the bubbles have gone.

The broth turns pink on heating. This is expected and is not a fault. See FAQ 1.

⚠ **Safety caution.** Loosen the cap before heating. Heat in 30-second pulses, swirling between pulses in heat-resistant gloves. Stop heating the moment boiling starts. Do not open the bottle until it has cooled: steam pressure in a hot sealed bottle ejects liquid when the cap is released. See FAQ 8 for the full heating procedure.

Step 3

If the broth was heated, let it cool to about 80 °C.

Add 4.500 g of Mixture B to the 910 ml of prepared broth with vigorous stirring, and continue stirring for 5~8 minutes.

Make up to 998 ml with double distilled or MQ water, and adjust the pH to 7.2 ± 0.2 at 25 °C with 1 M HCl or 1 M NaOH.

Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle. The aperture is the pressure-relief path for the autoclave; see FAQ 19.

Record the pH at 25 °C immediately before autoclaving.

Vitamin K_1 solution is added post-autoclave, once the medium has cooled to 50 °C or below. Stock S and Stock H went in at Step 1.

Hemin may alternatively be added after autoclaving; the default route charges it at Step 1. See FAQ 25.

Stock K may alternatively be added to the bulk medium at Step 3 before autoclaving, making up to 1000 ml instead of 998 ml. Never add it into the Stock H bottle or a shared diluent. See FAQ 25.

80 °C is a handling convention, not a mucin requirement. See FAQ 8.

Mucin disperses rather than dissolves; the translucent opalescence is normal and stays. See FAQ 10.

Mixture B does not effervesce; there is no waiting period. See FAQ 8.

A small quantity of coarse insolubles is expected at this stage and is not a failed batch. See FAQ 9.

Add 1 M HCl dropwise into the vortex of a vigorously stirred vessel, never onto a still surface and never as a bolus. See FAQ 10.

Measure the pH, then adjust in whichever direction the reading requires; do not reach for alkali by reflex. Record the direction and the volume of titrant used. See FAQ 2.

Sterilise the made-up broth promptly; do not hold it overnight. See FAQ 11.

Work Steps 3 and 4 in the AAE where possible. In air, keep the broth cool and complete both within 10 minutes.

Do not add Stock S after autoclaving; it is not a sterile supplement. See FAQ 5.

Running volume for a 1 L batch:

Stage	Operation	Running volume
1	Mixture A - 41.823 g, Stock S - 5.00 ml, Stock H - 5.00 ml into 900 ml of MQ water	910 ml
2	Mixture B 4.500 g added as a solid at about 80 °C	910 ml
3	pH adjusted, then made up with water	**998 ml**
4	Autoclave, 121 °C for 15 minutes	998 ml
5	Stock K 2.00 ml, after autoclaving, at 50 °C or below	**1000 ml**

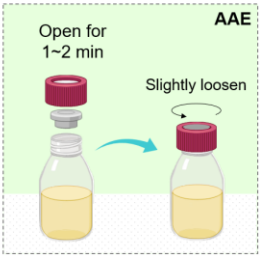
Step 4

If macroscopic particulates are observed, allow the broth to stand until the coarse insolubles settle. Carefully decant the broth into one or more clean reagent bottles, leaving only the heavy precipitate behind. Record the volume transferred to each bottle.

Do not attempt to remove the natural mucin opalescence; leave behind only the settled, coarse material. Do not clarify the bulk medium through a 0.22 µm filter. See FAQ 10.

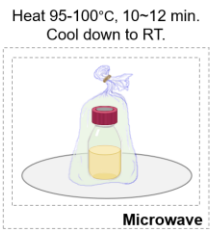
Step 0 (Optional)

Step 1 | Step 2.0 ~ Step 2.1 (Optional)



Step 2.2 (Optional)

Step 2.3 (Optional)



Step 2.4 (Optional)

Step 3

Step 4

To prepare GMM anaerobic tubes (e.g. Hungate Tubes)

Step 5

In the AAE, dispense 9.98 ml of broth into each 20 ml anaerobic tube (1/3~1/2 of the tube volume, never more than 1/2) and seal the tubes.

9.98 ml is the pre-autoclave fill, Stock S and Stock H included; 20 µL of Stock K after autoclaving gives 10.00 ml of finished medium.

Reduction check: a colourless to pale-straw broth is reduced and ready for the autoclave. Qualitative only. See FAQ 1.

If the broth stays pink, let the sealed medium stand and re-check, or evacuate it as in Step 2.1. See FAQ 1.

Step 6

Use rubber bands [1] to hold the anaerobic tubes together, place them in the airtight PP bag [1], and seal the neck of the bag with a rubber band. Then move out of the AAE and autoclave at 121 °C for 15 minutes on a slow-exhaust cycle.

Optionally, evacuate the sealed tubes before autoclaving using the method of Step 2.1. See FAQ 14.

Step 7

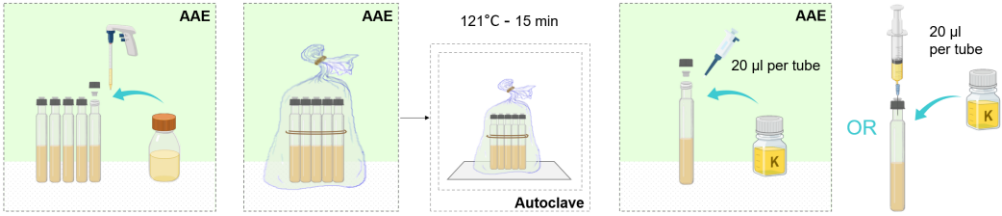
When the tubes have cooled to 50 °C or below, aseptically add 20 µL of Stock K (2.0 µL per ml) to each tube, working in the AAE or injecting through the rubber stopper with a sterile syringe. Invert each tube several times.

Step 8 - (Optional for quality control purposes)

If condition allows, record the pH of the dosed medium at 25 °C. The specification is **7.0 ± 0.2 at 25 °C (6.8 ~ 7.2).**

Measure the finished pH rather than predicting it from the pre-autoclave reading. See FAQ 2.

Measure on one sacrificial tube, or on a reference aliquot carried through the same cycle. The achievable window is no narrower than 7.0 ± 0.2.



Step 5 Step 6 Step 7

To prepare GMM in bottles

Step 5

Seal the bottle with a (butyl) rubber stopper [2] and screw an apertured cap [2] onto the bottle.

Step 5.1 - (Optional)

Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the air out of the bottle to remove the dissolved oxygen in the liquid.

Step 6 - (if Step 5.1 has been processed, this step can be skipped)

Put the bottle into the anaerobic atmosphere environment (AAE). Open the rubber stopper for 1~2 minutes to remove oxygen from the bottle and allow the inert gas to fill. Then close the rubber stopper, screw and **tighten** the apertured cap.

Reduction check: a colourless to pale-straw broth is reduced and ready; pink indicates oxygen. Qualitative only. See FAQ 1.

Step 7

Put the bottle into an airtight PP bag and seal the neck of the bag with a rubber band, take it out of the AAE, then autoclave at 121°C for 15 minutes on a slow-exhaust cycle.

Step 8

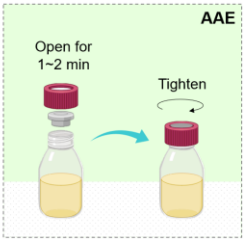
When the medium has cooled to 50 °C or below, aseptically add 2.00 ml of Stock K per litre, working in the AAE or injecting through the rubber stopper with a sterile syringe. Mix thoroughly.

Step 9 - (Optional for quality control purposes)

If condition allows, record the pH of the finished medium at 25 °C. The specification is **7.0 ± 0.2 at 25 °C (6.8 ~ 7.2).**

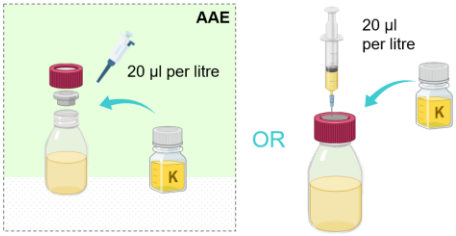
Step 5

Step 5.1 (Optional)



Step 6

Step 7



Step 8

To prepare GMM agar plates

Step 5

Dissolve 15 g of agar per litre in the pH-adjusted broth, seal the bottle with a (butyl) rubber stopper and screw an apertured cap onto the bottle.

Agar is not supplied with this kit. Record the loading used. See FAQ 27.

Step 5.1 - (Optional)

Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the air out of the bottle to best remove the dissolved oxygen in the liquid.

Step 6 - (if Step 5.1 has been processed, this step can be skipped)

Put the bottle into the anaerobic atmosphere environment (AAE). Open the rubber stopper for 1~2 minutes to remove oxygen from the bottle and allow the inert gas to fill. Then close the rubber stopper, screw and **tighten** the apertured cap.

Reduction check: a colourless to pale-straw broth is reduced and ready; pink indicates oxygen. Qualitative only. See FAQ 1.

Step 7

Put the bottle into an airtight PP bag and seal the neck of the bag with a rubber band, take it out of the AAE, then autoclave at 121°C for 15 minutes on a slow-exhaust cycle.

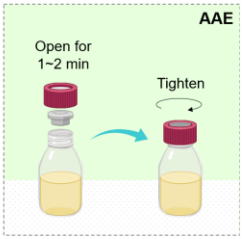
Hold the molten medium in a 50 °C water bath, then dose, mix and pour without delay. If it begins to set, re-melt gently and pour; never autoclave it a second time. See FAQ 27.

Step 9 - (Optional for quality control purposes)

Record the pH at 25 °C on a broth aliquot taken before the agar was added and then dosed with Stock K, or on the molten medium with a flat-surface electrode. The specification is 7.0 ± 0.2 at 25 °C (6.8 ~ 7.2).

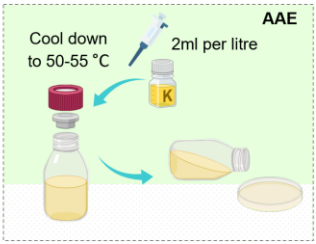
Step 5

Step 5.1 (Optional)



Step 6

Step 7



Step 8

Storage

Pack unused broth, tubes and plates in an anaerobic atmosphere, vacuum seal [3] them in plastic bags with an oxygen absorber [4][5], and store at 4 °C (2~8 °C band). For expiry dates and shelf life, see section 1.5.

The supplied mixtures and stocks are stored separately from the prepared media, as follows.

Item	Storage
Mixture A	RT, sealed.
Mixture B	2~8 °C, with oxygen absorber and desiccant.
Stock H - Hemin	2~8 °C, protected from light.
Stock K - Vitamin K_1 (both formats)	2~8 °C, strictly protected from light.
Stock S - SCFA mix	15~25 °C preferred. Do not freeze.

Storage Cautions:

- A small quantity of insolubles may precipitate slowly in medium held cold, or as the pH falls during incubation. Leave it to settle; it is the expected appearance of correctly prepared medium and not a failed batch. See FAQ 9.
- Never freeze Stock S. Keep the cap tight between uses. Store it at 15~25 °C by preference, or at 2~8 °C; either way bring it to 15~25 °C, invert it and confirm a single clear phase before dispensing. See FAQ 6.
- Set and record an in-use limit for Stock K after first opening; one month is standard practice. See FAQ 22.

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[1] Included in the GMM medium package.
[2] Included in the Anaerobic Preparation Kit Lite/Plus. Follow the kit instructions and assemble in advance before use.
[3] It is OK to use a domestic vacuum sealer, which is usually used for food packaging.
[4] Only available in/using the Anaerobic Preparation Kit Plus. Follow the kit instructions and assemble in advance before use.
[5] It is OK to use a food-grade oxygen absorber.

2.3. If you DO NOT have an anaerobic atmosphere working environment (AAE), but HAVE anaerobic gases (AGs) and an anaerobic preparation kit

AAE means that the preparation can be processed in an anaerobic bench/chamber/glove box filled with anaerobic incubation gases, allowing strict anaerobic tests, culturing and YCFA agar plate preparation.

Without AAE, GMM agar plates cannot be prepared, whereas GMM anaerobic tubes (e.g. Hungate tubes) can be prepared, sample-loaded and cultured [4] using an anaerobic preparation kit with or without AGs.

Commonly used anaerobic incubation gases (AGs):
- Triple gas mixture: 80% nitrogen, 10% hydrogen, 10% carbon dioxide.
- Double gas mixture: 80% nitrogen, 20% carbon dioxide.
- Pure nitrogen.

Text colours:
Blue - Explanation
Purple - Optional
Orange - Cautions

Step 0 - (optional)

Place Stock K in the assembled self-sealing autoclave bag [4] and seal the bag. Half unscrew the cap, twisting from outside the bag. Vacuum the bag with the assembled vacuum device [2] and leave it 5 minutes. Fill the bag with AGs through the PTFE air valve and leave it 15 minutes. After each withdrawal, tighten the Stock K cap inside the vacuumed or AG-filled bag.

Before bagging, wipe the Stock K bottle and the inner surface of the self-sealing autoclave bag with 70 % alcohol.

Stock K is the only stock added after autoclaving, so it is the only one needing separate deoxygenation; Stock S and Stock H pass through the autoclave with the medium. See FAQ 25.

Step 1

Dissolve 41.823 g of Mixture A, 5.00 ml of Stock S and 5.00 ml of Stock H in 900 ml double distilled or MQ water in a GL45-cap reagent bottle.

Handle Stock S in a fume hood: the C5 acids are detectable at parts-per-billion levels. See FAQ 5.

Hold Stock S at 15 to 25 °C by preference, or at 2 to 8 °C. Do not freeze or filter it. Bring it to 15 to 25 °C and mix to a single clear phase before dispensing. See FAQ 6.

5.00 ml of Stock S per litre delivers 1.000 mmol/L each of iso-butyric, iso-valeric and n-valeric acid. See FAQ 7.

Mixture A can segregate in transit: invert or tumble the bottle 10~15 times before weighing, and weigh each litre individually. See FAQ 28.

Steps 2.0 to 2.2 are optional. Heating only dissolves the blend and drives off dissolved oxygen. See FAQ 8.

Step 2.0 - (optional)

Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] or an apertured PP cap onto the bottle.

Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the bottle.

Vacuuming removes oxygen from the liquid itself, not only from the headspace. See FAQ 13.

Vacuum until bubbles emerge, then repeat at least twice with a 1-minute gap between cycles. See FAQ 13. A pump substituted for the kit syringe must reach 3 kPa absolute or lower; see FAQ 15.

Step 2.1 - (optional)

Put the bottle into an airtight PP bag [1], **slightly loosen** the apertured cap, squeeze out or vacuum out as much air as possible and seal the neck of the bag with a rubber band [1]. Then put them together in a microwave oven or water bath.

Alternatively, seal the bottle into an assembled self-sealing autoclave bag with the seal stick, then vacuum the bag and fill it with AGs through the PTFE air valve.

The bag keeps air out of the bottle and contains any leakage. See FAQ 13.

Fill the bag 1/3 to half with AGs to keep external oxygen out of the bottle. Never fill the bag with AGs that contain hydrogen before heating.

Step 2.2 - (optional)

Heat the prepared 910 ml broth solution to 95~100 °C in a microwave oven or water bath for 10~12 minutes, with frequent agitation. Then leave the solution to rest and cool down.

Agitate frequently: unmixed starch and infusion solids settle and scorch on the base of the bottle.

Switch the microwave off or take out of water bath as soon as boiling starts, and on again only once the stopper has recovered its shape and the bubbles have gone. See FAQ 8.

⚠ **Safety caution.** Loosen the cap before heating. Heat in 30-second pulses, swirling between pulses in heat-resistant gloves. Stop heating the moment boiling starts. Do not open the bottle until it has cooled: steam pressure in a hot sealed bottle ejects liquid when the cap is released. See FAQ 8 for the full heating procedure.

Step 3

If the broth was heated, let it cool to about 80 °C. Add 4.500 g of Mixture B to the 910 ml of prepared broth with vigorous stirring and continue stirring for 5~8 minutes. Stock K, vitamin K_1, is added post-autoclave, once the broth has cooled to 50 °C or below. Stock S and Stock H are already present from Step 1. Make up to 998 ml with double-distilled or MQ water and adjust the pH to 7.2 ± 0.2 at 25 °C. Record the pH at 25 °C immediately before autoclaving. Then seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle.

Optional: add hemin after autoclaving. Hold the 5.00 ml of Stock H back at Step 1, make up to 993 ml instead of 998 ml, and add it with Stock K once the medium is at 50 °C or below, hemin first and mixed in before the vitamin K_1. The pre-autoclave route is the default. See FAQ 5.

Optional: add Stock K before autoclaving. Add the 2.00 ml of Stock K to the bulk medium at this step and make up to 1000 ml instead of 998 ml. Add it to the bulk medium only, never into the Stock H bottle. Keep the batch dark, and treat the 1.0 mg/L as a nominal dose. See FAQ 24.

Mucin disperses rather than dissolves and there is no effervescence; the translucent opalescence is normal and is carried forward. See FAQ 10.

Add Stock H and Stock K separately to the bulk medium. Never pre-mix or co-dilute them, and never add Stock K into the Stock H bottle. See FAQ 21.

Stock H is dosed as supplied: its dilute alkali is the solubilising agent, not an inert diluent. See FAQ 21.

Stock K is flammable: keep it away from ignition sources and never autoclave the bottle. See FAQ 23 and section 1.7.

Add 1 M HCl slowly and dropwise into the vortex of a vigorously stirred vessel, never onto a still surface and never as a bolus. See FAQ 10.

Measure the pH first, then adjust to 7.2 ± 0.2 at 25 °C with 1 M HCl or 1 M NaOH as the reading requires; do not add alkali by default. See FAQ 2.

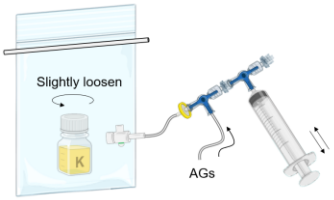
A small amount of coarse insoluble material at this stage is the expected appearance. See FAQ 9.

Sterilise promptly. Do not hold the made-up broth open to air or overnight. See FAQ 11.

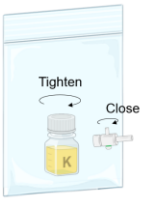
Step 4

If macroscopic particulates are observed, allow the broth to stand until the coarse insolubles settle. Carefully decant the broth into one or more clean reagent bottles, leaving only the heavy precipitate behind. Record the volume transferred to each bottle.

Do not attempt to remove the natural mucin opalescence; leave behind only the settled, coarse material. Do not clarify the bulk medium through a 0.22 µm filter. See FAQ 10.



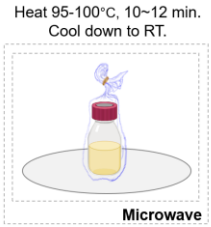
Step 0 (Optional)



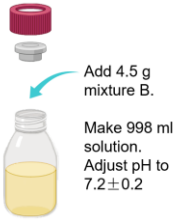
Step 1

Step 2.0 (optional)

Step 2.1 (optional)



Step 2.2 (optional)



Step 3

Step 4

To prepare GMM anaerobic tubes (e.g. Hungate Tubes)

Step 5

Aliquot 9.98 ml of broth into each 20 ml anaerobic tube (e.g. Hungate tube), which is 1/3~1/2 of the tube volume and never more than 1/2, and seal the tubes. Vacuum each sealed tube as in Step 2.0.

Each tube takes 9.98 ml. The 20 µL of Stock K added after autoclaving brings it to 10 ml.

Rest the vacuumed tubes for about 10 minutes and check the colour. Colourless means the oxygen has gone and the tubes are ready for autoclaving; if they are still pink or red, repeat the vacuum. See FAQ 1.

Step 5.1 - (Optional)

If working with an Anaerobic Preparation Kit Plus, after vacuuming tubes, inject AGs into the tubes through the gas pump/exchange assembly.

Step 6

Use rubber bands to hold the anaerobic tubes together, place them in the airtight PP bag [1], squeeze/vacuum out as much air as possible and seal the neck of the bag with a rubber band [1]. Then place them together in the autoclave chamber. Autoclave at 121°C for 15 minutes on slow exhaust.

Fill the bag 1/3 to half with AGs to keep external oxygen out of the tubes. Never fill the bag with AGs that contain hydrogen before autoclaving.

Step 7

Let the autoclaved tubes cool to 50 °C or below. Then aseptically add 20 µL of Stock K to each tube, using a sterile syringe (preferred ultra-fine syringes shown in section 2.1) to inject it through the rubber stopper, carefully avoiding the introduction of air into the tube. Mix by inverting the tube gently two or three times after the injection.

☐ The syringe and needle are sterile and have not touched any surface.

☐ A sterile syringe is used for Stock K, and a separate one for the sample.

☐ 20 µL of Stock K has been drawn for a 9.98 ml fill, or 2.0 µL per ml for any other fill volume.

☐ The needle points up and the plunger has been squeezed until all air is expelled and liquid stands at the needle tip.

☐ The tube has cooled to 50 °C or below.

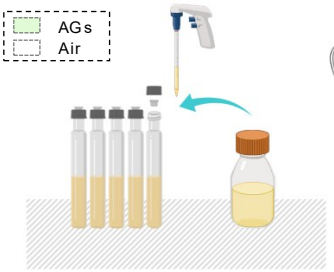
Dose by volume, not by tube: any other fill volume takes 2.0 µL of Stock K per ml.

This is an open-bench transfer into a sealed, reduced tube. Work on a clean bench, and wipe the stopper with 70 % alcohol and let it dry before puncturing. Before each injection, inject slowly and withdraw the needle. Do not inject air, vent the tube or loosen the stopper; 20 µL needs no pressure equalisation.

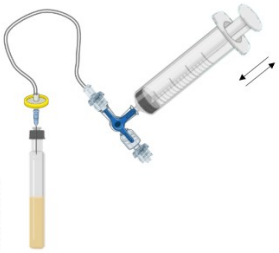
Step 8 - (Optional for quality control purposes)

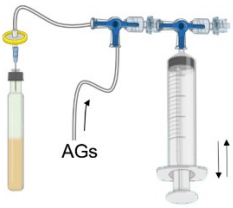
Record the pH of one sacrificial tube at 25 °C after Stock K has been added and mixed.

The specification for the finished medium is pH 7.0 ± 0.2 at 25 °C (6.8 to 7.2). See FAQ 2.

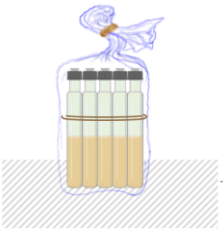


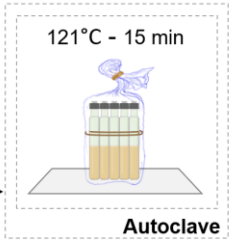
Step 5



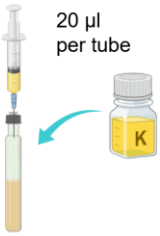


Step 5.1 (Optional)





Step 6



Step 7

To prepare GMM in bottles

Step 5

Seal the bottle with a butyl rubber stopper [2] and screw an apertured cap [2] onto the bottle.

Step 6

Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the air out of the bottle to remove the dissolved oxygen in the liquid.

Rest the vacuumed bottle for about 10 minutes and check the colour. Colourless means the oxygen has gone and the bottle is ready for autoclaving; if it is still pink or red, repeat the vacuum. See FAQ 1.

Step 6.1 - (Optional)

If working with an Anaerobic Preparation Kit Plus, after vacuuming the bottle, inject AGs into the vacuumed bottle through the gas pump/exchange assembly.

Step 7

Tighten the apertured cap and put the bottle into an airtight PP bag, squeeze or vacuum out as much air as possible and seal the neck of the bag with a rubber band [1]. Then place them together in the autoclave chamber. Autoclave at 121°C for 15 minutes on slow exhaust.

Fill the bag 1/3 to half with AGs to keep external oxygen out of the bottle. Never fill the bag with AGs that contain hydrogen before autoclaving.

Step 8

Let the autoclaved bottle cool to 50 °C or below. Then aseptically add Stock K at 2 ml per litre of broth solution (2 µL per ml), using a sterile syringe (preferred ultra-fine syringes shown in section 2.1) to inject it through the rubber stopper, carefully avoiding the introduction of air into the bottle. Mix by inverting or swirling the bottle until the medium is uniform.

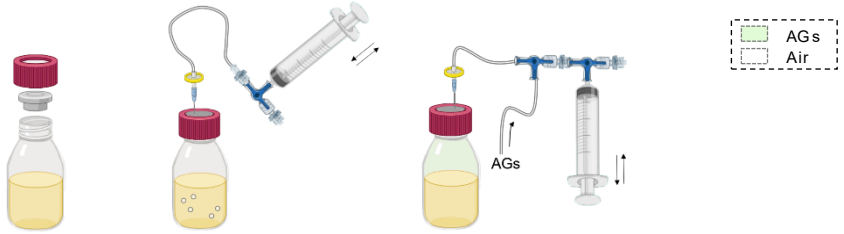
Dose from the volume recorded at Step 4, not from the nominal batch size: each bottle takes 2 µL of Stock K per ml it holds.

Work on a clean bench. Wipe the stopper with 70 % alcohol and let it dry, expel all air from the syringe, then inject slowly and do not vent the bottle.

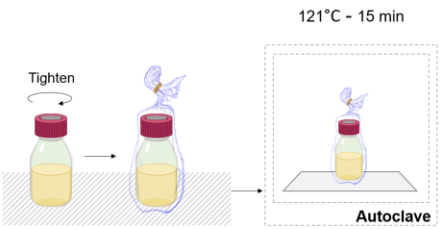
Step 9 - (Optional for quality control purposes)

Record the pH after Stock K has been added and mixed, at 25 °C.

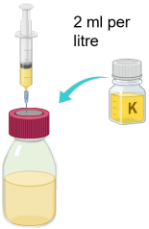
The specification for the finished medium is pH 7.0 ± 0.2 at 25 °C (6.8 to 7.2). See FAQ 2.



Step 5 Step 6 Step 6.1 (Optional)



Step 7



Step 8

Storage

Pack unused prepared GMM broth in an anaerobic atmosphere, vacuum seal [3] them in plastic bags with an oxygen absorber [4][5], and store at 4 °C (2~8 °C band). For expiry dates and shelf life, see section 1.5.

The supplied mixtures and stocks are stored separately from the prepared media, as follows.

Item	Storage
Mixture A	RT, sealed.
Mixture B	2~8 °C, with oxygen absorber and desiccant.
Stock H - Hemin	2~8 °C, protected from light.
Stock K - Vitamin K_1 (both formats)	2~8 °C, strictly protected from light.
Stock S - SCFA mix	15~25 °C preferred. Do not freeze.

Storage Cautions:

- A small quantity of insolubles may precipitate slowly in medium held cold, or as the pH falls during incubation. Leave it to settle; it is the expected appearance of correctly prepared medium and not a failed batch. See FAQ 9.
- Never freeze Stock S. Keep the cap tight between uses. Store it at 15~25 °C by preference, or at 2~8 °C; either way bring it to 15~25 °C, invert it and confirm a single clear phase before dispensing. See FAQ 6.
- Set and record an in-use limit for Stock K after first opening; one month is standard practice. See FAQ 22.

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[1] Included in the GMM medium package.

[2] Included in the Anaerobic Preparation Kit Lite/Plus. Follow the kit instructions and assemble in advance before use.

[3] It is OK to use a domestic vacuum sealer, which is usually used for food packaging.

[4] Only available in/using the Anaerobic Preparation Kit Plus. Follow the kit instructions and assemble in advance before use.

[5] It is OK to use a food-grade oxygen absorber.

2.4. If you DO NOT have an AAE or AGs - work with an Anaerobic Preparation Kit

AAE means that the preparation can be processed in an anaerobic bench/chamber/glove box filled with anaerobic incubation gases, allowing strict anaerobic tests, culturing and YCFA agar plate preparation.

Without AAE, GMM agar plates cannot be prepared, GMM anaerobic tubes (e.g. Hungate tubes) and GMM in bottles can be prepared, sample-loaded and cultured [4] using an anaerobic preparation kit with or without AGs.

Commonly used anaerobic incubation gases (AGs):
- Triple gas mixture: 80% nitrogen, 10% hydrogen, 10% carbon dioxide.
- Double gas mixture: 80% nitrogen, 20% carbon dioxide.
- Pure nitrogen.

Text colours:
Blue - Explanation
Purple - Optional
Orange - Cautions

Step 1

Open the anaerobic preparation kit and assemble the vacuum device and the gas preparation bag in advance, following the instructions.

Stock K is added after autoclaving and cannot be deoxygenated without AGs. Keep the cap tight, withdraw each dose promptly and return the bottle to 2~8 °C, protected from light. See FAQ 24.

Step 2

Dissolve 41.823 g of Mixture A, 5.00 ml of Stock S and 5.00 ml of Stock H in 900 ml double distilled or MQ water in a GL45-cap reagent bottle.

Handle Stock S in a fume hood. See FAQ 5. Store Stock S at RT. Never freeze or filter it. Bring it to 15 to 25 °C and mix until a single clear phase is visible before dispensing. See FAQ 6.

Stock S is a 200 mmol/L solution of each of the three acids, a 200-fold concentrate; 5.00 ml/L delivers 1.000 mmol/L of each. See FAQ 7.

Acetate and propionate are inside Mixture A; the three branched and longer-chain acids go in here as Stock S. See FAQ 4.

Mixture A can segregate in transit: invert or tumble the bottle 10~15 times before weighing, and weigh each litre individually. See FAQ 28.

Steps 2.1 to 2.3 are optional. Heating only dissolves the blend and drives out dissolved oxygen. See FAQ 8.

Step 2.1 - (optional)

Seal the bottle with a butyl rubber stopper [2] and screw an apertured cap [2] onto the bottle. Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the air out of the bottle to remove the dissolved oxygen in the liquid and the air in the bottle.

Vacuum until bubbles emerge from the liquid, then repeat at least twice with a 1-minute gap between rounds. See FAQ 14.

Step 2.2 - (optional)

Put the bottle into an airtight PP bag [1], **slightly loosen** the apertured cap, vacuum the bag with the assembled vacuum device [2] or using an oil-free vacuum pump, seal the neck of the bag with a rubber band [1].

The bag keeps oxygen-free gas against the bottle and contains any leaked liquid or vapour. See FAQ 13.

Step 2.3 - (optional)

Heat the prepared 910 ml broth solution to 95~100 °C in a microwave oven or water bath for 10~12 minutes, with frequent agitation. Then leave the solution to rest and cool down.

Agitate frequently: unmixed starch and infusion solids scorch on the base of the bottle.

Watch the stopper and the liquid: switch the microwave off or take out of water bath as soon as boiling starts, and switch it on again only when the stopper has recovered its shape and the bubbles have gone. See FAQ 8.

⚠ **Safety caution.** Loosen the cap before heating. Heat in 30-second pulses, swirling between pulses in heat-resistant gloves. Stop heating the moment boiling starts. Do not open the bottle until it has cooled: steam pressure in a hot sealed bottle ejects liquid when the cap is released. See FAQ 8 for the full heating procedure.

Step 3

If the broth was heated, let it cool to about 80 °C. Add 4.500 g of Mixture B to the 910 ml of prepared broth with vigorous stirring and continue stirring for 5~8 minutes. Stock K, vitamin K_1, is added post-autoclave, once the broth has cooled to 50 °C or below. Stock S and Stock H went in at the Mixture A dissolving step. Make up to 998 ml with double-distilled or MQ water and adjust the pH to 7.2 ± 0.2 at 25 °C. Record the pH at 25 °C immediately before autoclaving. Then seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle.

Optional: hemin may instead be added after autoclaving; the default route puts Stock H in at Step 2, before the cycle. See FAQ 26.

Optional: Stock K may instead be added to the bulk medium before autoclaving, making up to 1000 ml rather than 998 ml. See FAQ 26.

Mixture B hydrates slowly rather than dissolving; the translucent opalescence that results is normal and must be carried forward. See FAQ 10.

Never pre-mix or co-dilute Stock H and Stock K, and never add Stock K into the Stock H bottle or a shared diluent. Add each stock separately to the bulk medium. See FAQ 21.

Stock K is flammable: keep the bottle away from ignition sources and never autoclave it. Format A carries 0.19 % v/v ethanol into the medium. See FAQ 23.

Add 1 M HCl dropwise into the vortex of a vigorously stirred vessel, never onto a still surface and never as a bolus. See FAQ 10.

Measure the pH first, then adjust in whichever direction the reading requires with 1 M HCl or 1 M NaOH. Do not add alkali by default. See FAQ 2.

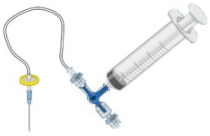
A small amount of coarse insoluble material after Mixture B is expected. See FAQ 9.

Sterilise promptly. Do not hold the made-up broth open to air or overnight. See FAQ 11.

Step 4

If macroscopic particulates are observed, allow the broth to stand until the coarse insolubles settle. Carefully decant the broth into one or more clean reagent bottles, leaving only the heavy precipitate behind. Record the volume transferred to each bottle.

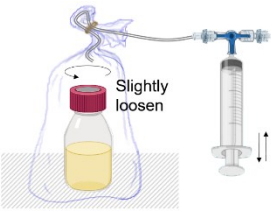
Do not attempt to remove the natural mucin opalescence; leave behind only the settled, coarse material. Do not clarify the bulk medium through a 0.22 µm filter. See FAQ 10.



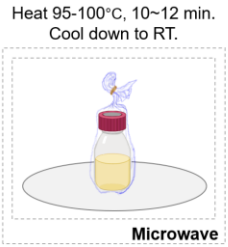
Step 1

Step 2

Step 2.1 (optional)



Step 2.2 (optional)



Step 2.3 (optional)

Step 3

Step 4

To prepare GMM anaerobic tubes (e.g. Hungate Tubes)

Step 5

Aliquot the broth solution into 20 ml anaerobic tubes (e.g. Hungate Tubes), 9.98 ml per tube (1/3~1/2 of the tube volume, never more than 1/2), and seal the tubes.

9.98 ml plus the 20 µL of Stock K added after autoclaving gives 10.00 ml of finished medium. See FAQ 28.

Step 6

Puncture the rubber stoppers of the anaerobic tubes with the assembled vacuum device [2] and vacuum the air out of each tube.

Rest the vacuumed tubes about 10 minutes and check the colour; if it is still pink or red, repeat the vacuum. See FAQ 1.

Step 7

Use rubber bands to hold the vacuumed anaerobic tubes together, place them in an airtight PP bag [1], vacuum the bag with the assembled vacuum device [2] or oil-free vacuum pump and seal the bag. Then autoclave at 121°C for 15 minutes on slow exhaust.

Step 8

Let the autoclaved tubes cool to 50 °C or below. Then puncture the rubber stopper and inject 20 µL of Stock K aseptically (operate on a clean bench) into each tube (2.0 µL per ml of broth solution), using a sterile syringe (see section 2.1 for suitable ultra-fine syringes). Mix by inverting the tube gently two or three times after the injection.

☐ The syringe and needle are sterile and have not touched any surface.

☐ A sterile syringe is used for Stock K, and a separate one for the sample.

☐ 20 µL of Stock K has been drawn for a 9.98 ml fill, or 2.0 µL per ml for any other fill volume.

☐ The needle points up and the plunger has been squeezed until all air is expelled and liquid stands at the needle tip.

☐ The tube has cooled to 50 °C or below.

Dose by volume, not by tube: a tube holding a different volume takes 2.0 µL/ml of Stock K.

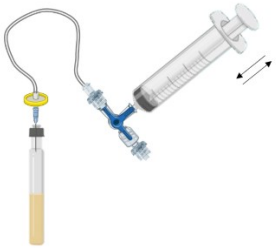
Wipe the rubber stopper with 70 % alcohol and let it dry before puncturing. Before each injection: inject slowly and withdraw the needle; do not inject air, vent the tube, or loosen or lift the stopper. A 20 µL volume needs no pressure equalisation

Step 9 - (Optional for quality control purposes)

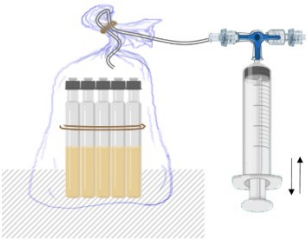
Record the pH after Stock K has been added and mixed, at 25 °C, on one sacrificial tube.

The specification for the finished medium is pH 7.0 ± 0.2 at 25 °C (6.8 to 7.2). See FAQ 2.

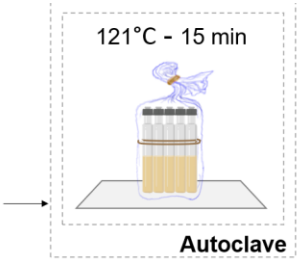
Step 5



Step 6



Step 7 (to be continued)



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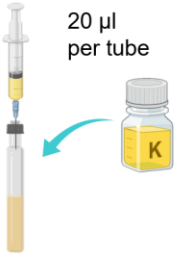


Step 8

To prepare GMM in bottles

Step 5

Seal the bottle with a butyl rubber stopper [2] and screw an apertured cap [2] onto the bottle. Puncture the rubber stopper with the assembled vacuum device [2] and vacuum the air out of the bottle.

Rest the vacuumed bottle about 10 minutes and check the colour; if it is still pink or red, repeat the vacuum. See FAQ 1.

Step 6

Tighten the apertured cap and put the bottle into the airtight PP bag [1], vacuum the bag with the assembled vacuum device [2] or oil-free vacuum pump and seal the bag, then autoclave at 121°C for 15 minutes on slow exhaust.

Step 7

Let the autoclaved bottle cool to 50 °C or below. Then puncture the rubber stopper and inject Stock K aseptically (operate on a clean bench) at 2.0 µL per ml of broth, that is 2.00 ml per litre, using a sterile syringe (see section 2.1 for suitable ultra-fine syringes). Mix by inverting or swirling the bottle until the medium is uniform.

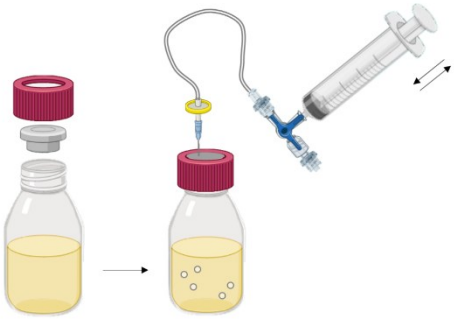
Dose from the volume recorded at Step 4, not from the nominal batch size: each bottle takes 2 µL of Stock K per ml it actually holds.

Wipe the rubber stopper with 70 % alcohol and let it dry. Expel all air from the needle before use, inject slowly and do not vent the bottle.

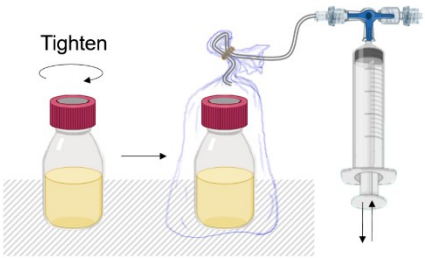
Step 8 - (Optional for quality control purposes)

Record the pH after Stock K has been added and mixed, at 25 °C.

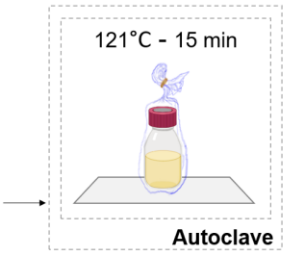
The specification for the finished medium is pH 7.0 ± 0.2 at 25 °C (6.8 to 7.2). See FAQ 2.



Step 5



Step 6 (to be continued)



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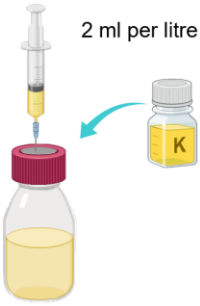

Step 7

Storage

Pack unused prepared GMM broth in an anaerobic atmosphere, vacuum seal [3] them in plastic bags with an oxygen absorber [4][5], and store at 4 °C (2~8 °C band). For expiry dates and shelf life, see section 1.5.

The supplied mixtures and stocks are stored separately from the prepared media, as follows.

Item	Storage
Mixture A	RT, sealed.
Mixture B	2~8 °C, with oxygen absorber and desiccant.
Stock H - Hemin	2~8 °C, protected from light.
Stock K - Vitamin K_1 (both formats)	2~8 °C, strictly protected from light.
Stock S - SCFA mix	15~25 °C preferred. Do not freeze.

Storage Cautions:

- A small quantity of insolubles may precipitate slowly in medium held cold, or as the pH falls during incubation. Leave it to settle; it is the expected appearance of correctly prepared medium and not a failed batch. See FAQ 9.
- Never freeze Stock S. Keep the cap tight between uses. Store it at 15~25 °C by preference, or at 2~8 °C; either way bring it to 15~25 °C, invert it and confirm a single clear phase before dispensing. See FAQ 6.
- Set and record an in-use limit for Stock K after first opening; one month is standard practice. See FAQ 22.

[1] Included in the GMM medium package.
[2] Included in the Anaerobic Preparation Kit Lite/Plus. Follow the kit instructions and assemble in advance before use.
[3] It is OK to use a domestic vacuum sealer, which is usually used for food packaging.
[4] Only available in/using the Anaerobic Preparation Kit Plus. Follow the kit instructions and assemble in advance before use.
[5] It is OK to use a food-grade oxygen absorber.

3. Instructions for GMM anaerobic tubes

3.1. Summary

Product name	YCFA modified media broth in anaerobic tubes
SKU	GMNB-GMM01-T020
Volume	10 ml of broth in 20 ml anaerobic tubes

3.2. Instructions for use

Each tube is filled with 9.98 ml of sterilised GMM broth under a pure nitrogen atmosphere. Mixture A, Mixture B, Stock S and Stock H are already present in the medium. Stock K is the only addition the user makes: 20 µL per tube, which brings the tube to 10.00 ml of finished medium. Research use only.

Stock K is supplied sterile and bottled in air.

- Before first use, place the bottle in an anaerobic atmosphere [1] (anaerobic bench or anaerobic glove box) and loosen the cap half a turn for 30 minutes.
- Withdraw the dose inside the enclosure, then tighten the cap while the bottle is still in the anaerobic atmosphere.
- Return the bottle to a sealed bag with an oxygen absorber, and vacuum-seal the bag [2] where a vacuum sealer is available.

Preparation:

1. Wipe the tube with 70 % alcohol: the cap first, then, after unscrewing it, the threads and the rubber stopper.
2. In an anaerobic bench or anaerobic glove box, either unplug the rubber stopper, or leave it seated and puncture it with a syringe needle to pump out the nitrogen and replace it with the anaerobic atmosphere.
3. Add 20 µL of Stock K per tube and invert the tube gently two or three times to mix.

Check that the medium is colourless to pale straw, then inoculate. Do not inoculate a tube that reads pink. The resazurin check is qualitative; FAQ 1 gives what the colour does and does not report, and what to do with a tube that stays pink.

In anaerobic environments, the stopper can be removed, and a solution or sample can be added through a pipette head. Alternatively, a syringe needle can be used to puncture the stopper, which is held in place by a tightly screwed cap.

In an aerobic atmosphere, ONLY a syringe needle puncture can be used to add solution or sample.

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[1] Triple gas mixture: 80% nitrogen, 10% hydrogen, 10% carbon dioxide or double gas mixture: 80% nitrogen, 20% carbon dioxide.

[2] It is OK to use a domestic vacuum sealer, which is usually used for food packaging.

3.3. Operating Notes

- Store Stock K as specified in 1.5 - cold, dark, away from ignition sources - and never autoclave it. See FAQ 29 and FAQ 24.

- Stock S and Stock H are already in the broth; Stock K is the only addition made at the tube. See FAQ 25.
- Use a separate sterile syringe and needle for Stock K and for the sample. Never draw both into one syringe.
- UV-irradiate the anaerobic bench or glove box before use, and wipe every item taken into it with 70 % alcohol. See FAQ 25.
- Wipe the exposed top of the plastic cap and the rubber stopper with a 70 % alcohol swab before puncturing.
- Use a pre-sterilised 23/25G disposable syringe needle, minimum 20 mm needle length.
- 20 µL is a small transfer. Measure it with a 200 µL pipette and a sterile tip when the stopper has been removed inside the enclosure, or with a 1.0 ml ultra-fine syringe when injecting through the stopper.
- For strictly anaerobic cultures, deoxygenate the sample before injection: vacuum the sample chamber repeatedly and refill it with anaerobic gas.
- Do not autoclave the tubes a second time. The medium has already been sterilised at 121 °C for 15 min.

3.4. Cautions

- A very small amount of coarse insoluble matter may settle slowly in cold storage and as the pH falls during incubation. Leave it at the bottom of the tube. See FAQ 9.
- The medium contains 4.000 g of porcine gastric mucin per litre, which disperses rather than dissolves. A translucent opalescence is normal. Do not clarify or filter the broth. See FAQ 10.
- A fine white haze or flocculent deposit after prolonged exposure to air is cystine, and is distinct from the mucin opalescence and the coarse settling deposit. See FAQ 9.
- A pink or red broth indicates that the medium has been oxidised. See 3.2 and 3.3.
- The medium contains components of animal origin, including bovine calf-brain infusion solids and porcine gastric mucin. Handle it as biological material of animal origin and observe local biosafety rules. Country of origin, species, tissue and any transmissible-spongiform-encephalopathy statement for a given lot are given in the Safety Data Sheet and the lot certificate of analysis. See Section 1.6.
- Wear gloves and goggles. If skin or eyes are contacted, rinse immediately with plenty of water or saline solution and seek first aid if required.
- Research use only. Not for clinical or diagnostic use.

Appendix

I. Anaerobic Preparation Kit - Lite

Product name	Anaerobic Preparation Kit Plus
SKU	GMNB-APK01

Application

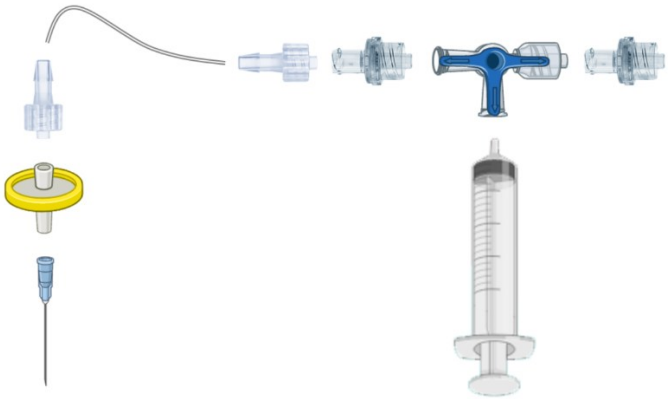
Used for vacuum-method anaerobic media preparation without AGs.

Contains

-	25cm PTFE tube [1] (φ4.0mm)	1 piece
-	Disposable filter (φ25mm 0.22μm)	1 piece
-	Luer male connector [1] (φ4.0mm)	2 pieces
-	Three-way Luer connector [1]	1 piece
-	Check valve (prevents backflow) [1]	2 pieces
-	100ml syringe for fast vacuum [1]	1 piece
-	23G syringe needle	3 pieces
-	GL45 PBT cap [1][2] with 1x Butyl Rubber stopper [2][3]	1 piece

Assembling (video clips can be found on YouTube)

1. Air pump assembling: Connect one Luer male connector to a check valve, then connect the check valve to a three-way Luer connector, then connect another check valve to the other end of the three-way Luer connector, and finally connect the 100ml syringe to the middle outlet of the three-way Luer connector.
2. Connect two Luer male connectors with the PTFE tube.
3. Connect the syringe needle to the disposable filter, then connect the filter to the free Luer male connector.
4. Try to test the airtightness of the assembly, push and pull the syringe piston and test if such an assembly can pump air from the syringe needle to the back-end check valve.



Note:

- Connect the air pump assembly to the needle and the unit can be used to vacuum the rubber-stoppered GL45 reagent bottle.
- Connect the air pump assembly to the PTFE tube only and the unit can also be used to vacuum the airtight bag.

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[1] Reusable
[2] Can be purchased separately
[3] Max 7 times of needle puncture

II. Anaerobic Preparation Kit - Plus

Product name	Anaerobic Preparation Kit Plus
SKU	GMNB-APK02

Application

Used for vacuum-method anaerobic media preparation without AGs, including a nitrogen generation module.

Contains:

Module A: Basic tools for vacuum-style preparation without AGs.

- 25cm PTFE tube [1] (φ4.0mm)	1 piece
- Disposable filter (φ25mm 0.22μm)	1 piece
- Luer male connector [1] (φ4.0mm)	2 pieces
- Three-way Luer connector [1]	2 pieces
- Check valve (prevents backflow) [1]	2 pieces
- 100ml syringe for fast vacuum [1]	1 piece
- 23G syringe needle	5 pieces
- GL45 PBT cap [1][2]	1 piece
- GL45 Butyl Rubber stopper [2][3]	2 pieces

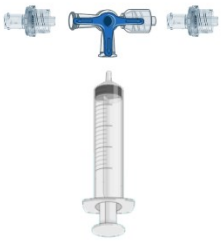
Module B: for preparing nitrogen from air (deoxygenate).

Nitrogen (deoxygenated air) can be further introduced into the GL45 bottle through Module A after vacuuming to best protect the media from oxygen.

- Autoclave bag airtight seal stick [1][5]	1 piece
- PTFE air valve can attach to autoclave bag [1]	2 pieces
- Self-sealing autoclave bag [1][2][4]	10 pieces
- 500cc oxygen absorber [1][2]	10 pieces
- Oxygen indicator paper [1][2]	5 pieces
- 25cm PTFE tube [1] (φ6.0mm)	1 piece
- Luer male connector [1] (φ6.0mm)	1 piece

Assembly of Module A (video clips can be found on YouTube)

1. Connect the check valve to a three-way Luer connector, then connect another check valve to the opposite end of the three-way Luer connector, and finally connect the 100ml syringe to the middle outlet of the three-way Luer connector. -- An air pump assembly is prepared.



2. Connect a three-way Luer connector to the inlet side of the above assembly's check valve. Connect one Luer male (φ4.0mm) connector to the far end of the second three-way Luer connector and then connect the second three-way Luer connector's vertical inlet with the AGs' pipeline. -- An air exchange assembly is prepared.

   The AGs' pipeline and connected Luer male connector need to be selected based on different types of AGs' regulators. A φ6.0mm pipe and a φ6.0mm Luer male connector can be found in the module B pack.

3. Connect two Luer male connectors (φ4.0mm) with the PTFE tube (φ4.0mm).
4. Connect the syringe needle to the disposable filter, then connect the filter to the free Luer male connector.

5. Try to test the airtightness of the assembly, push and pull the syringe piston and test if such an assembly can pump air from the syringe needle to the back-end check valve.

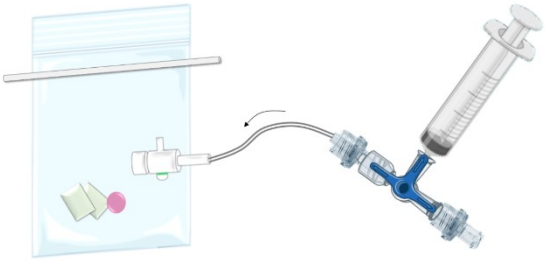
Note:

- Connect the air exchange assembly to the needle and the unit can be used to vacuum the rubber-stoppered GL45 reagent bottle and exchange air with AGs.
- Connect the air pump assembly to the PTFE tube only and the unit can also be used to vacuum the airtight bag/ self-sealing autoclave bag.

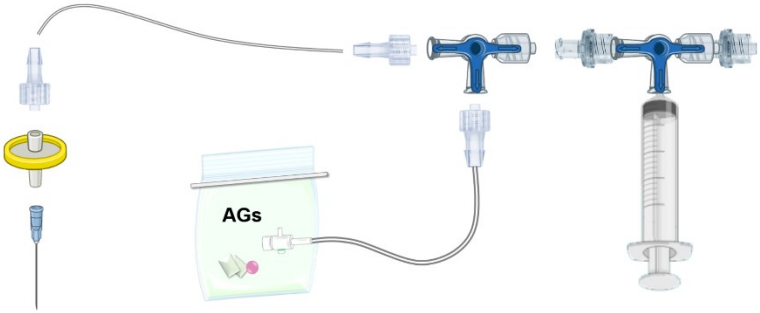
Assembly of Module B

To generate inert gas

1. Punch a hole in the body of the self-sealing autoclave bag, insert the PTFE air valve into the hole and tighten the washer.
2. Insert an oxygen absorber and a piece of oxygen indicator paper into the bag.
3. Double-seal the bag by compressing the complementary grooves and attach an airtight seal stick near the bag's mouth.
4. Connect an assembled air pump (Assembly of Module A: step 1) and the PTFE air valve via a tube (φ6.0mm). Inject air into the bag through the PTFE air valve and squeeze the bag body to check the airtightness.



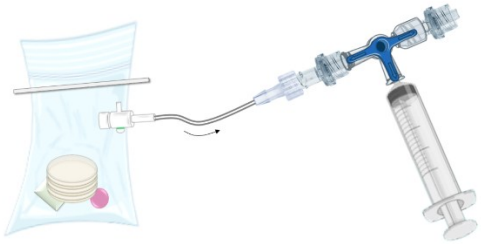
5. Switch off the PTFE valve. Allow the bag to rest (approximately 48 hours) and wait for the oxygen absorber to remove all oxygen from the bag until the colour of the oxygen indicator returns to red (prune red/red to watermelon red).
6. Connect the PTFE air valve to the 3-way valve using the PTFE tube (φ6.0mm) and a Luer male connector (φ6.0mm), and the oxygen-free gas is ready to fill the reagent bottle and airtight bag, acting as an inert gas. Refer to FAQ 16 if it is difficult to connect the PTFE tube to the Luer male connector.



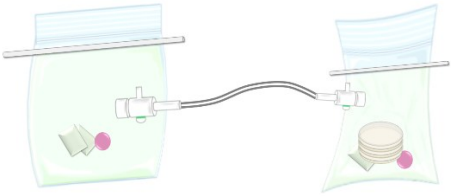
For anaerobic cultivation

1. Take two self-sealing autoclave bags, punch a hole in the body and attach the PTFE air valve to each bag.
2. Use one to prepare the inert gas, follow the protocol above.
3. Place the anaerobic tubes/plates to be cultured together with an oxygen scavenger and a piece of oxygen indicator paper in another bag (cultivation bag).

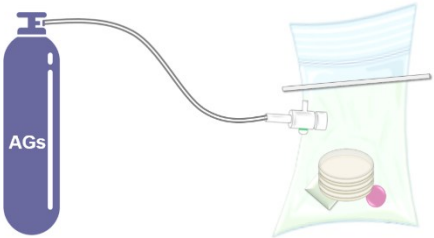
4. Double-seal the second bag by compressing the complementary grooves and placing an airtight seal stick near the bag's mouth. Connect a Luer male connector (φ6.0mm) to an assembled air pump (Assembly of Module A: step 1.) and connect the PTFE air valve to the Luer male connector using a tube (φ6.0mm). Vacuum out all the air inside the bag using the air pump, and switch off the PTFE valve.



5. Connect the two PTFE air valves using the PTFE tube (φ6.0mm), switch on the two valves and push the inert gas from the first bag into the second by gently compressing the bag body.



Or if you have AGs, the AGs can be directly used to fill the cultivation bag, instead of generating inter gas.



6. Switch off the valve, and put the cultivation bag into the incubator.

Note:

- Connect the air pump assembly to the needle and the unit can be used to vacuum the rubber-stoppered GL45 reagent bottle.
- Connect the air pump assembly to the PTFE tube only and the unit can also be used to vacuum the airtight bag.

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[1] Reusable.
[2] Can be purchased separately.
[3] Max 7 times of needle puncture.
[4] Not the airtight PP bags provided along with the YCFA media.
[5] The Seal Stick is made of PE, not heat-resistant. DO NOT autoclave.

FAQ

Q1. What colour shows that the broth is oxidised or contains oxygen, and is it normal for the broth to turn pink during preparation?

A1. Pink is the colour that reports oxygen, and a pink stage during preparation is normal. The two readings are separate and both are needed: pink while the broth is being heated belongs to the indicator's own sequence, while pink in a medium that should already be colourless means the medium has taken up oxygen.

The pink stage during preparation. Resazurin in GMM passes through three states. The dry blend is blue. The broth turns pink when it is heated, because resazurin is converted to resorufin, and that step is irreversible. The broth then turns colourless as it is reduced. The change to pink on heating is expected and is not a fault. The colour that has to be checked is the one at the end.

Pink in a finished medium. A reduced GMM broth is a translucent, colourless to pale straw liquid, made slightly opalescent by the mucin (FAQ 10). The observation that matters is the absence of pink. If the medium is pink at the point of inoculation it has taken up oxygen. Do not inoculate a vessel that reads pink, and read a deeper pink to red the same way.

If the medium is still pink, close the vessel, leave the reduced medium to stand and check again. Standing at 37 °C accelerates it. If it stays pink, suspect oxygen ingress at the septum or the closure rather than a failed medium. Check that the stopper is seated, that the puncture sites have resealed, and that the vessel was held under the oxygen-free cover specified by the preparation route you are following while it cooled. For a batch that has clearly oxidised, see FAQ 11.

Read the colour as a check on the batch in hand, and do not read a redox potential off it. The indicator reports a threshold and not a number: colourless indicates roughly -110 mV or lower, and nothing more precise than that. Measure with a redox electrode where a numerical potential is required. L-cysteine at about 3 mmol/L has been reported to poise a medium near -79 to -95 mV, so decolourisation is not guaranteed by the cysteine dose alone. Organisms that require below -300 mV may need a further reducing agent, which this kit does not supply.

Q2. Does the fresh broth have to be raised with 1 M alkali, and how does the phosphate buffer set the pH?

A2. No. Measure the pH first, then adjust in whichever direction the reading requires. The two kinds of medium present their buffer with completely different acid loads, and the worked note at the end of this item is the whole of the arithmetic.

Media that supply their acetate and propionate as free acetic and propionic acid - the YCFA family among them - carry about 49.43 mmol/L of free acid against about 6.02 mmol/L of phosphate, an acid-to-buffer ratio of 8.2 : 1. The phosphate is swamped, the pH is set by the acetic acid and acetate pair itself, the freshly made broth reads pH 5 to 6, and it is raised with 1 M potassium hydroxide.

GMM is built the other way round. Acetate and propionate are supplied as their anhydrous sodium salts in Mixture A, at 33.00 mmol/L and 9.00 mmol/L respectively, so the only free acid in the medium is the 3.0 mmol/L contributed by Stock S, against 27.40 mmol/L of total phosphate - a ratio of 0.11 : 1, seventy-five times lower.

That is not a direction of travel. The absence of a large acid load means GMM does not read low *for that reason*; it does not make the medium alkaline. The sodium-salt format's own net contribution is about -0.012 pH units, and the brain-heart infusion base contributes organic acidity that the deconstruction in Table B does not model. Do not expect the pH 5 to 6 reading of a free-acid formulation, and do not reach for alkali by reflex. Measure the pH at 25 °C and adjust to 7.2 ± 0.2 in whichever direction the reading requires, with 1 M hydrochloric acid or 1 M sodium hydroxide.

Neither titrant is supplied with this kit, and the direction of the adjustment is not predictable from the formulation, so prepare or purchase both 1 M HCl and 1 M NaOH and have them on the bench before you begin; see section 2.1. Record the direction and the volume of titrant used: it is lot-dependent, and knowing it for your lot makes subsequent batches faster.

No potassium hydroxide solution is supplied, and there is no KOH anywhere in this kit. Do not substitute KOH for NaOH without recording the change. Where acid is needed, add the 1 M HCl dropwise into the vortex of a vigorously stirred vessel, never onto a still surface and never as a bolus (FAQ 10).

The two pH specifications. The pre-autoclave figure is 7.2 ± 0.2 at 25 °C; the finished medium is 7.0 ± 0.2 at 25 °C, that is 6.8 to 7.2.

The two figures are set at different points and are not expected to be identical. Stock S and Stock H are both in the vessel when the pH is adjusted, so that single adjustment accounts for the acid load and for hemin's slight alkalinity alike. The one addition made after autoclaving on the default route, 2.00 ml of Stock K per litre, is a neutral ethanolic solution and does not move the pH appreciably. The difference between the two readings is therefore the autoclave cycle's own effect - 121 °C for 15 min - which varies with load, fill volume and vessel, and which is why the finished-medium window is wider than the pre-autoclave tolerance. Measure both and record them against the batch: the pH immediately before and immediately after autoclaving, at 25 °C.

Worked note - the phosphate buffer and the pH arithmetic

The buffer is the sum of three salts, two declared in Mixture A and one carried in by the brain-heart infusion base:

- K_2HPO_4, 2.000 g/L divided by 174.18 = **11.482 mmol/L** as HPO_4^{2-}
- KH_2PO_4, 1.000 g/L divided by 136.09 = **7.348 mmol/L** as $H_2PO_4^-$
- Na_2HPO_4, 1.216 g/L divided by 141.96 = **8.566 mmol/L** as HPO_4^{2-}, from the base

Total phosphate is therefore **27.40 mmol/L**, split HPO_4^{2-} **20.05** and $H_2PO_4^{2-}$ **7.35**. The buffer capacity at pH 7.2 is about **15.8 mmol/L per pH unit**. That capacity is what makes the Stock S acid load tractable.

The 27.40 mmol/L figure assumes anhydrous disodium phosphate in the brain-heart infusion base; see the FAQ item on the composition basis of the brain-heart infusion base for the full condition.

Taking the phosphate pair alone at zero ionic strength: pH = 7.20 + log(20.05/7.35) = **7.64**.

Precision. This medium has an ionic strength of about 0.18 mol/L. By the Davies equation the apparent second dissociation constant falls from 7.20 to about 6.83, which moves the phosphate-pair figure from 7.64 to about **7.26**. Calculated absolute pH values therefore carry a systematic error of roughly 0.3 to 0.4 units before the unmodelled organic matrix is considered. **They are indications, not predictions**. pH *shifts*, by contrast, are robust, because the activity term cancels to first order. Dosed into the finished medium instead, the three acids of Stock S would give a computed fall of -0.192 at pKa2 7.20 and of -0.208 at 6.83.

The Stock S acid load - the number to use is about -0.2 pH units. Stock S contributes 3.0 mmol/L of free monocarboxylic acid, pKa about 4.8 and so more than 99 % ionised at pH 7.2, against a medium holding 13.70 mmol/L of each phosphate species at pH 7.20: pH = 7.20 + log((13.70 - 3.00)/(13.70 + 3.00)) = **7.007**, a fall of about **0.19** units. Stock S goes in with Mixture A at the dissolving step, before the pH is set, so this is the load the adjustment absorbs rather than a later fall.

Stock H. 5 ml per litre of 0.05 M sodium hydroxide brings in **0.25 mmol/L** of sodium hydroxide and shifts the pH by **+0.016** units. Stock H goes in at the dissolving step as well, so the same adjustment absorbs it; it is negligible against 27.40 mmol/L of phosphate either way.

Q3. Where do the brain-heart infusion figures in section 1.4 come from, and do they change with the supplier of the base?

A3. From the GMExpression brain-heart infusion base, GMNB-BHIS01. That base is what this formulation was specified against, and section 1.4 scales its published six-component formula to the 18.000 g that Table A declares.

If you source the base elsewhere, check it against the rows below first. The kit does not require the GMExpression base, and the suppliers named under *Where each row is cited* are suitable alternatives. Their formulations are close but not identical, and three quantities in section 1.4 are derived from the base rather than weighed into the kit: the integrated D-glucose total, the integrated sodium chloride total, and the total phosphate, which in turn sets the buffer arithmetic and the pH behaviour given in FAQ 2. The notes below record which rows hold across the bases examined and which do not. Where the base you intend to use departs from them, re-derive the affected total for that base before relying on the concentration declared in section 1.4, and record the base and its lot on the batch sheet.

A dehydrated brain-heart infusion broth is 37.0 g per litre at full strength. GMM takes 18.000 g of it per litre, so every row of the base formula is scaled by one factor, f = 18.000/37.000 = 0.486486.

BHI component	per 1 L base	Contributed to 1 L GMM
Calf-brain infusion solids (from 200 g brain)	7.7 g	3.746 g
Beef-heart infusion solids (from 250 g heart)	9.8 g	4.768 g
Proteose peptone (pancreatic digest)	10.0 g	4.865 g
Dextrose (D-glucose)	2.0 g	0.973 g
Sodium chloride	5.0 g	2.432 g
Disodium phosphate (Na_2HPO_4)	2.5 g	1.216 g
Sum	**37.0 g**	**18.000 g**

Where each row is cited. The six rows are those published for the GMExpression Brain-Heart-Infusion listing, GMNB-BHIS01. BD Difco 237500 publishes the same two infusion-solids figures. ATCC Medium 44 corroborates the peptone, dextrose, sodium chloride, phosphate and pH rows, but it states tissue masses rather than solids masses, so it cannot carry the 7.7 g and 9.8 g figures. BBL Brain Heart Infusion is a different composition at the same 37.0 g total, with dextrose at 3.0 g, and is not the base deconstructed in Table B.

The integrated totals are supplier-independent. Across every six-component 37.0 g brain-heart infusion broth examined - BD Difco, Oxoid CM1135, Merck 110493, Liofilchem 610008, HiMedia M210I and Condalab 1400 - dextrose or glucose 2.0 g, sodium chloride 5.0 g and disodium phosphate 2.5 g are invariant, and the three nitrogen lines always total 27.5 g. Only the split between the individual nitrogen rows is supplier-specific: an Oxoid-type base gives brain 6.081 g, heart 2.432 g and peptone 4.865 g, the same nitrogen subtotal, since the three rows always total 27.5 g in the base. The single exception is BBL Brain Heart Infusion, whose dextrose is 3.0 g. So the row-by-row nitrogen figures follow the base, while the integrated glucose, sodium chloride and phosphate totals in section 1.4 stand across all six of the bases above.

Disodium phosphate is taken as anhydrous, M = 141.96 g/mol, as specified by Merck GranuCult 110493 and by Liofilchem; BD, ATCC, Oxoid, HiMedia and Condalab state no hydration state. Confirm the hydration state declared for the base in use: on the dodecahydrate the same 1.216 g would contribute 3.395 instead of 8.566 mmol/L and the total phosphate would fall from 27.40 to 22.23 mmol/L, which would invalidate the phosphate totals in section 1.4 and the buffer arithmetic in FAQ 2.

The declared reaction of the dehydrated base, **pH 7.4 ± 0.2 at 25 °C**, is the pH of the full-strength base at 37.0 g per litre. It is **not** a GMM specification. GMM's pre-autoclave target is 7.2 ± 0.2.

Dextrose and glucose are the same substance; the naming splits by supplier house style. No brain-heart infusion supplier specifies a hydration state for it, so the 2.0 g is read as anhydrous D-glucose.

The brain and heart rows do double duty. They are also the animal-derived fractions declared in section 1.6, at 3.746 g and 4.768 g per litre of finished medium, and those two figures follow the BD Difco split rather than an Oxoid-type one.

Q4. In what form are the five short-chain fatty acids supplied, and why do they arrive in two packages?

A4. Acetate and propionate are supplied as anhydrous sodium salts inside the Mixture A dry blend. The three branched and C5 acids are supplied as free acids in the liquid concentrate Stock S. All five reach the finished medium at the concentrations the formulation declares.

Acid	Package	Supplied as	Mass per 1L of medium	In the finished medium
Acetate	Mixture A	Sodium acetate, anhydrous, powder	2.707 g	33.00 mmol/L
Propionate	Mixture A	Sodium propionate, anhydrous, powder	0.865 g	9.00 mmol/L
iso-Butyric acid	Stock S	Free acid, liquid concentrate	88.1 mg	1.000 mmol/L
iso-Valeric acid	Stock S	Free acid, liquid concentrate	102.1 mg	1.000 mmol/L
n-Valeric acid	Stock S	Free acid, liquid concentrate	102.1 mg	1.000 mmol/L

The 5 L kit carries 13.536 g of sodium acetate and 4.323 g of sodium propionate inside Mixture A. Stock S is a 200 mmol/L concentrate of each of the three acids, so the 5.00 ml/L dose delivers the 1.000 mmol/L figures in the table.

The division between the two packages is a packaging decision, not a chemical one. Sodium acetate and sodium propionate are stable, non-hygroscopic crystalline solids: free-flowing powders that dry-blend and store well. iso-Butyric acid (melting point -47°C), iso-valeric acid (-29 °C) and n-valeric acid (-34 °C) are liquids at room temperature, and their sodium salts are hygroscopic and waxy rather than free-flowing, so they cannot be dry-blended into Mixture A. Supplied instead as a liquid concentrate, the three acids are dosed at the Mixture A dissolving step; FAQ 5 gives the reason that is before autoclaving and not after.

Two points follow.

The two salts add 3.572 g per litre, and the weighing figure for Mixture A is 41.823 g per litre. Five litres therefore need 209.113 g, and the kit supplies 215 g. Weigh 41.823 g per litre.

The medium carries only the 3.0 mmol/L of free acid contributed by Stock S. What sets the pH of the fresh medium is the phosphate blend, and the pH consequence of supplying acetate and propionate as sodium salts rather than as free acids is given in FAQ 2.

Q5. Why is Stock S added before autoclaving, when it contains volatile acids?

A5. Because the acids are not meaningfully volatile at this pH, and because Stock S is not a sterile supplement.

At the working pH only about 0.4 % of each acid is present in the volatile, un-ionised form; the rest is the non-volatile carboxylate anion. For a monoprotic acid of pKa 4.8 at pH 7.2 the undissociated fraction is 1 / (1 + 10^(7.2 - 4.8)) = 0.004. The boiling points of the three acids, 154 °C, 176 °C and 187 °C, are all above that of water, so they are not the first thing to leave a heated vessel. Only the un-ionised fraction can partition into the headspace at all, and in a bottle closed with a butyl rubber stopper it cannot escape the vessel. Steam distillation of these acids requires deliberate acidification below pH 4, which this medium never approaches.

Dosing Stock S at the Mixture A dissolving step also puts it through the autoclave cycle with the medium, so the three acids reach the finished medium without an aseptic transfer; FAQ 4 gives the form in which each of the five acids is supplied. Published anaerobe media do the same thing. DSMZ Medium 1611 and JCM Medium 1130 both carry a volatile fatty-acid blend through the sterilisation step at comparable concentrations. Adding the acids before autoclaving is the standard arrangement.

Stock S is not supplied as a sterile supplement. It is sterilised in the medium's own autoclave cycle. Adding it after autoclaving would introduce an unsterilised liquid into a sterile medium, and that is the decisive reason not to do it.

Handle Stock S in a fume hood at the Mixture A dissolving step of the preparation route you are following. iso-Valeric and n-valeric acid have odour thresholds in the parts-per-billion range, and the odour is persistent. See 1.6.

Storage of Stock S, and the single-clear-phase check before a dose is withdrawn, are in FAQ 6.

Q6. How should Stock S be stored, and can it be refrigerated?

A6. Hold it at 15~25 °C by preference. Refrigeration at 2~8 °C is permitted and does not harm the acids, but the margin to saturation is narrower cold, so the check before each dose is what makes either temperature safe.

Store Stock S at 15~25 °C by preference, or at 2~8 °C. Do not freeze it. Whatever the storage temperature, bring the bottle to 15~25 °C, invert it 3~5 times and confirm a single clear phase before each dose is withdrawn.

Where the margin actually is. Stock S is a 200-fold concentrate: 1.8708 ml of neat acid in 30 ml, 6.24 % v/v, 58.5 g/L of total free acid. At 0.600 mol/L with a mean pKa of 4.80 the solution self-buffers to about pH 2.5, so 99.5 % of it is the undissociated free acid, which is the sparingly soluble form. Against the compiled aqueous solubilities at 20~25 °C:

Acid	In Stock S	Aqueous solubility	Of saturation
iso-Butyric	17.62 g/L	167 g/L at 20 °C	11 %
iso-Valeric	20.43 g/L	40.7 g/L at 20 °C	50 %
n-Valeric	20.43 g/L	24 g/L at 25 °C	85 %

n-Valeric acid is the limiting component, and its 24 g/L is the best-supported figure in the set: the compiled value and an independent ratio statement, one volume of acid in forty of water, agree to within 2 %. Published values for this acid do range from 24 to 40 g/L, so 85 % is the conservative end of a 51~85 % spread rather than a settled number. iso-Butyric acid is the only one of the three with measured cold data, and it is the safest: the water-rich phase boundary of that binary is about 16.4 % w/w at 4 °C against the product's 1.78 % w/w, a margin of roughly nine-fold.

What is not established. No aqueous solubility has been measured for either C5 acid at or below 16 °C, and no phase boundary has ever been determined for water with more than one of these three acids. Short-chain carboxylic acid dissolution is endothermic at 25 °C, so solubility falls as the temperature falls; the direction is adverse but the magnitude over 25 °C

to 4 °C is unmeasured for these acids. That gap is why refrigeration is permitted rather than recommended, and why the single-phase check is mandatory at either temperature.

If the bottle has been chilled, in transit or in store, stand it at 15~25 °C for at least an hour, then look at the meniscus and the inside of the neck for an oily film or droplets as well as at the bulk liquid for haze. Invert it 3~5 times, confirm a single clear phase, and only then withdraw the dose. If a haze or a second phase is still visible after a further hour at room temperature and remixing, do not use that bottle and do not try to clarify it.

What a cloudy Stock S means. It is not frozen and it has not crystallised: all three acids melt below -29 °C. A haze or fine droplets are a separate, oil-like acid-rich phase that has come out of the water. A 5.00 ml aliquot drawn from a two-phase stock is no longer representative, and it will under-deliver all three acids.

Never clarify Stock S by filtration. Filtering a two-phase stock removes acid, not contaminant, and silently lowers the delivered dose.

Keep the cap tight between uses. The three acids are volatile, and evaporative loss concentrates what is left and reduces the margin further.

The acids themselves are stable at either temperature. None of the three carries a hydrolysable or autoxidisable group, so there is no degradation pathway for cold storage to slow. Microbial growth is excluded as well, and not by the pH: the formulation contains no nitrogen and no phosphorus, so biomass synthesis is stoichiometrically impossible in it.

Q7. Can Stock S be omitted?

A7. Not without changing the medium.

Stock S delivers 1 mmol/L each of iso-butyric, iso-valeric and n-valeric acid to the finished medium. Those three acids are part of the declared formulation. A preparation made without them is a different medium, and it should not be reported as this one.

Two points if it is left out.

The volume ledger does not change. Stock S goes in at Step 1, before the broth is made up, so the make-up to 998 ml per litre absorbs the missing 5.00 ml as water, and every other declared concentration is unaffected. Stock K still supplies the last 2.00 ml. Make up to 998 ml as usual, and record that Stock S was left out.

Growth and recovery performance for this medium, with or without Stock S, is not established. Whether a particular organism requires these three acids is likewise not established. Establish performance for your strain, sample type, inoculum and gas atmosphere before relying on a result.

If the acids have to be left out for an experimental reason, for instance where a short-chain fatty acid is itself the variable under study, treat the result as obtained in a modified medium and state the modification. Research use only

Q8. What is the best and safest way to heat the broth in a sealed bottle in a microwave oven, and why must the cap be loosened first?

A8. GMM's heat step is for dissolution and deoxygenation only: it dissolves Mixture A and drives dissolved oxygen out of the water. Hold the broth at 95~100 °C with frequent agitation for 10~12 min. A water bath or a microwave oven is suitable. In a water bath, allow a longer time to reach temperature and then hold for the same 10~12 min.

Loosen the cap before heating. Do not remove it. Loosening the cap is a pressure-safety measure. A closed bottle heated in a microwave oven builds internal pressure; the pressure raises the boiling point, so the liquid overshoots the intended temperature and the stopper deforms, and opening a hot, pressurised bottle then releases steam and hot broth. A loose cap lets that pressure escape as it builds and keeps the broth at the temperature you are aiming for.

The three C4-C5 acids of Stock S are in the bottle at this stage. At the pH of the broth they are effectively non-volatile, so they place no requirement on the closure and play no part in how the heat step is run. The cap is loosened for pressure safety alone. FAQ 5 gives the quantitative basis for the acids being non-volatile at this pH.

Deformation of a rubber stopper is hard to judge by eye, so monitor the bottle's surface temperature with a surface infrared thermometer and read the liquid temperature from it. The bottle surface is usually a little cooler than the liquid.

Heat in 30-second pulses. Between pulses, swirl the bottle gently in an ellipse, wearing heat-resistant gloves, and check the temperature. 500 ml of broth in a 1000-watt oven reaches 95~100 °C in about 2-3 minutes. Bring it to 95~100 °C and hold it there for the next 10~12 min with 10-15 second pulses every 2 minutes.

⚠ **Safety caution.** Do not open the bottle until it has cooled. Steam pressure in a hot sealed bottle will eject liquid when the cap is released. Wear heat-resistant gloves whenever the bottle is handled hot.

Let the broth cool to about 80 °C before Mixture B is charged. 80 °C is a process convention and is not a demonstrated requirement. The reason for it is operator safety and handling: charging 4.500 g of powder into near-boiling liquid causes bumping and boil-over, and a lower temperature reduces the surface foam and clumping that form while the powder wets out. It is not there to protect the mucin.

Q9. What is the insoluble matter that appears during preparation, and after a period of culture?

A9. GMM can show three different deposits. They are not the same thing and they are not handled the same way.

Deposit	When, and what it looks like	What to do
Hemin-mediated condensed protein and peptide	Coarse flocculent material, pale when it first appears and darkening as the pH is raised. Forms during preparation, and again after prolonged culture.	Let it settle, then transfer the medium off the coarse material only. See section 2.2, Step 4.
Mucin dispersion	A fine, even, translucent opalescence through the whole liquid. Present from the moment Mixture B is added.	Carry it forward. Do not decant it and do not filter it out. The mucin is a declared substrate of this medium. See FAQ 10.
Cystine	A fine white haze or flocculent deposit, appearing after the made-up broth has stood exposed to air.	Avoid it rather than treat it: sterilise promptly instead of holding the broth made up. See FAQ 11.

The coarse deposit is hemin-mediated condensed protein and peptide, and it is common in media that combine a high peptone load with blood-derived components. It is the expected appearance of such a medium and does not indicate a failed batch. GMM supplies 23.379 g of peptide and protein nitrogen sources per litre and 5.0 mg of hemin per litre, so the same chemistry applies here.

The mechanism. Hemin is an iron porphyrin whose iron is prominently exposed from the porphyrin plane, and it binds proteins rich in residues such as histidine with specific affinity, the imidazole side chain acting as an axial ligand in the way it does in myoglobin and haemoglobin. Binding alters the conformation of the protein and cross-links protein molecules into intermolecular networks. Cross-linking shifts the isoelectric point, and the result is flocculation in solution. The deposit appears at a relatively low pH, which indicates that the isoelectric point of the flocculated protein lies below 7.

The two photographs below show the appearance and the colour change in the YCFA reference medium, which passes through a markedly lower pH than GMM. GMM is held at 7.2 ± 0.2 and does not reach the pH values given in the captions.

Figure 1 (Left)
White precipitate appears when Hemin/Cys-HCl is added to the broth, and the pH of the solution is around 4.7

Figure 2 (Right)
The amount of white precipitate decreases, and the colour becomes black after adjusting the pH to 6.7

Q10. Why does the mucin not dissolve completely, why must the medium not be filtered clear, and why is the L-cysteine blended in with it?

A10. Mucin disperses. It does not dissolve.

Porcine gastric mucin is supplied at 4.000 g per litre inside Mixture B. The supplier declares a solubility in dilute alkali only, and declares no aqueous solubility. In water the material forms a dispersion, not a solution, and a translucent opalescence through the whole liquid is the normal and expected appearance of correctly prepared GMM. It is not turbidity from growth, and it is not a failure to dissolve something that ought to have dissolved.

The medium must not be filtered clear, for one reason: the mucin is a declared substrate of this medium, and removing it removes the component the medium is formulated around.

- Do not pass the bulk medium through a 0.22 µm filter.
- Do not decant the mucin. At the settling and transfer step, leave behind coarse precipitate only. See FAQ 9 for the three deposits and how they differ.
- Mixture B is supplied non-sterile and is sterilised in the pack's own autoclave cycle. It must therefore be added before autoclaving and never after, and the medium must not be autoclaved twice.

Handling. Cool the broth to about 80 °C before Mixture B is charged, add it with vigorous stirring, and continue stirring for 5-8 min. 80 °C is a process convention and is not a demonstrated requirement; the reason for it is given in FAQ 8. There is no gas-generating salt anywhere in this formulation, so nothing effervesces when Mixture B is charged and there is no waiting period. The behaviour to expect is slow hydration, not gas.

⚠ **Safety caution.** Add 1 M HCl dropwise, into the vortex of a vigorously stirred medium. Never run acid onto a static broth and never add it as a bolus. Mucin precipitates in weak acid, and native porcine gastric mucin forms a gel near pH 4. A clot formed by a local fall in pH will not redisperse.

Grade and loading. Sigma M2378 (Type II, crude) and M1778 (Type III, partially purified) are both acceptable for this medium, but they are not interchangeable for sialidase-dependent

work: Type II specifies bound sialic acid at 1.2 % or less with no lower limit, and Type III at 0.5-1.5 %. The grade and catalogue number of the lot supplied are stated on the lot label and the certificate of analysis. See section 1.4. The 4.0 g per litre loading sits at the upper end of published practice for mucin-supplemented anaerobe media, and inside it.

The quantitative extent of any change to the mucin glycans across a 121 °C, 15 min cycle is not established. Autoclaving mucin-containing media is the established route in published mucin-utilisation methods and at reference culture collections. Qualify glycan-dependent work against the medium you prepare.

The L-cysteine in Mixture B. The L-cysteine hydrochloride monohydrate shares the pouch with the mucin and is autoclaved in place with it, so no separate sterile addition of reducing agent is required. The salt supplied is the monohydrate, L-Cysteine-HCl-H_2O, M = 175.63 g/mol, so 0.500 g per litre delivers 2.85 mmol/L.

Autoclaving the reducing agent in place is a convention of this formulation rather than a demonstrated requirement of it. General anaerobe guidance adds reducing agents to the already-autoclaved medium from a nitrogen-sparged stock; published reference media autoclave L-cysteine hydrochloride in place at 0.40-0.50 g/L, which is the arrangement used here. Pre-blending it with the mucin keeps the two components in one pouch and takes a sterile addition off the bench.

Q11. Can oxidised GMM broth be recovered for anaerobic culture?

A11. It can be deoxygenated again and held under anaerobic gas, but recovery is not recommended. The medium is easily contaminated during the treatment, and the indicator loses its colour-change resolution between pink and colourless after prolonged oxidation, so the check you rely on becomes less informative.

Two points specific to GMM.

Do not autoclave the medium a second time. One cycle is what the formulation assumes, and repeat cycles are not supported.

A fine white haze or a flocculent deposit that appears after the made-up broth has stood exposed to air is cystine, formed by oxidation of the L-cysteine in Mixture B. Complete oxidation of the 2.85 mmol/L of L-cysteine would give 0.342 g/L of cystine against a solubility limit of 0.112 g/L, about three times over. Sterilise the broth promptly rather than holding it made up. This deposit is not the same as either of the two described in FAQ 9.

Q12. What precautions does the Mixture B powder need when it is weighed, and why is there no exposure limit to work to?

A12. Because there is no limit to work to. The dust is controlled by containing it, not by measuring it.

Mixture B is a fine animal-protein powder: a two-component dry blend of porcine gastric mucin and L-cysteine hydrochloride monohydrate, from which 4.500 g is weighed for every litre of finished medium. The powder is light and lifts readily when the bag is opened, tipped or set down hard, so the whole of the handling practice is aimed at keeping it out of the breathing zone and inside the vessel.

Section 1.6 sets out the controls: an enclosure for the weighing, the bag kept low in the receiving vessel, the personal protective equipment, and a particulate respirator where the dust cannot be contained. Work to 1.6, not to this item.

Containment also keeps the weighed mass right. Mixture B carries only a small dispensing margin, and powder lost to dust or to a spill cannot be made up from elsewhere in the kit. See FAQ 28.

Mixture B is supplied non-sterile and is charged into the broth as a solid before sterilisation, never into sterile medium. FAQ 10 sets out that rule.

Porcine gastric mucin has no harmonised hazard classification, and suppliers classify it differently from one another. No occupational exposure limit has been set for mucin, so

there is no airborne figure to work to and nothing to measure a weighing enclosure against. The practice in 1.6 rests on the general handling of a fine protein powder of animal origin, not on a classification or a limit. Section 1.6 carries the occupational hazard boundary for this product and the animal-derived-components disclosure.

Q13. What is the purpose of the airtight bag that encloses the reagent bottle and the anaerobic tubes?

A13. Two reasons.

First, to keep oxygen-containing air out of the vessel as it cools. A heated, closed container goes under negative pressure while it cools, and whatever surrounds it is drawn in through the septum and the closure. The bag holds an oxygen-free gas against the outside of the bottle, so what is drawn in is inert gas and not air.

Second, to contain odour. Stock S goes in at Step 1, so the medium carries iso-butyric, iso-valeric and n-valeric acid from the dissolving step onwards, and iso-valeric and n-valeric acid have odour thresholds in the parts-per-billion range. Handle Stock S in a fume hood at Step 1 (FAQ 5). Keep finished tubes and bottles bagged.

Q14. Why is the sealed bottle evacuated rather than simply having its headspace swept with an oxygen-free gas, and why are the vacuum rounds repeated?

A14. Because purging and evacuation do different things to the same vessel, and because each round of evacuation can only take a share of what the round before it left behind.

Purging replaces the gas in the headspace. It sweeps the air out from above the liquid and leaves an oxygen-free gas in its place, but it does not reach into the broth. The oxygen already dissolved in the water stays dissolved, and it leaves only slowly, as the liquid drifts into equilibrium with whatever now sits above it.

Evacuation acts on the liquid. Reducing the pressure in a closed vessel reduces the partial pressure of every gas in contact with the broth, and the water is then holding more dissolved oxygen than that lower pressure will support. The excess comes out of solution, rises as bubbles, and is carried away with the headspace gas as the vessel is pumped. That is why the end-point is bubbles emerging from the liquid rather than a figure on a gauge: the bubbles are dissolved gas leaving the water, which is the fraction purging cannot take. It also follows that the vessel has to be closed for any of this to happen. The stopper is what allows the pressure above the liquid to be lowered at all; the apertured cap holds it in place, and the septum, punctured for each round, has to reseal between them (FAQ 18).

Repeated rounds are not the same as one long pull. A single evacuation removes the gas that has reached the headspace while the pump is running, and the liquid cannot give up all of its dissolved oxygen in that time, because diffusion out of the bulk of the liquid is slower than the removal of gas from above it. Releasing the vacuum lets broth and headspace re-equilibrate: oxygen still held in the liquid redistributes into the gas above it, where the next round can remove it. Each round therefore takes a share of what remains, and repeated short rounds remove more oxygen than one pull of the same total length. The 1-minute gap is there for that re-equilibration; it is the interval, not the pumping, that brings the next fraction within reach. Drawing a single round out beyond its end-point mostly removes water vapour rather than oxygen.

Whether the vacuum comes from an assembled vacuum device or from an oil-free vacuum pump, and whichever preparation route you are following, the reason for the cycling is the same.

How much oxygen the rounds remove is not quantified for this medium, and no dissolved-oxygen figure is attached to any number of rounds. At least twice with a 1-minute gap is a practical minimum rather than a measured end-point, which is why the check that follows is the colour of the medium. What that colour does and does not tell you is in FAQ 1.

Q15. Why does vacuum deoxygenation fail with some laboratory vacuum pumps?

A15. Because most general-purpose laboratory pumps cannot reach a low enough absolute pressure. Removing dissolved oxygen is a pressure problem, not a running-time problem: a pump that cannot reach the threshold will not get there by being left running for longer.

There are two thresholds, and the second is the one that matters.

Absolute pressure in the vessel	What happens
Below about **21 kPa** (210 mbar)	Net removal begins. Oxygen is 20.95 % of air, so it exerts 21.2 kPa at one atmosphere; below that the headspace holds less oxygen than the liquid is equilibrated with, and dissolved oxygen starts to leave.
At or below the **water vapour pressure** - **2.3 kPa** (23 mbar) at 20 °C, **3.2 kPa** (32 mbar) at 25 °C	The liquid boils at room temperature. The rising vapour sweeps dissolved gas out with it, and removal becomes rapid and substantially complete.

Between those two figures oxygen leaves slowly and incompletely. Reaching the second is what makes the step work. Water at 25 °C holds about 8.3 mg/L of dissolved oxygen at air saturation, roughly 6 ml/L as gas, and that is what has to come out.

Judge a pump by its ultimate absolute pressure, not by its type.

Pump	Typical ultimate pressure	Suitable
Single-stage diaphragm	16~21 kPa (160~210 mbar)	**No.** It sits above the water vapour pressure, so the liquid never boils
Multi-stage oil-free chemical diaphragm	0.7~3 kPa (7~30 mbar)	Yes
Two-stage oil-sealed rotary vane	below 0.1 kPa (1 mbar)	Yes, with the reservations below
Water aspirator	set by the water temperature, about 2.3~4 kPa	Marginal, and it drifts with the supply temperature
The vacuum syringe in the Anaerobic Preparation Kit, Lite or Plus	close to 3 kPa (30 mbar)	Yes

Read the sign convention carefully. A pump advertised as "-85 kPa" is quoting gauge pressure against one atmosphere, which is **16 kPa absolute**, not 85 kPa of vacuum. The figure that governs this step is the absolute one. A single-stage diaphragm pump quoted at -80 to -85 kPa reaches 21 to 16 kPa absolute, which is five to nine times the pressure at which water boils at room temperature.

How to tell it is working: watch the liquid, not the gauge. Successful deoxygenation shows as continuous fine bubbling from within the body of the liquid - vacuum boiling - sustained for as long as the vacuum is held. A few bubbles clinging to the glass, or breaking only at the surface, are dissolved air coming out of the near-surface layer and are not the same thing. If no bubble stream develops, the pump has not reached the threshold and the step has not worked, however long it is left running.

This is why the preparation routes call for the vacuum to be drawn until bubbles emerge and then released and repeated rather than held once and for long. Each cycle lets the dissolved gas remaining in the bulk re-equilibrate toward the headspace, and the next cycle takes that fraction too. Prolonged boiling under a single deep vacuum removes water as well as gas and concentrates the medium.

If an oil-sealed rotary vane pump is used, protect it and the room. Water vapour drawn into the oil degrades it, so run the gas ballast or fit a cold trap between the vessel and the pump; the oil mist in the exhaust makes these pumps unsuitable for a clean bench without filtration, and they are noisy. These are the practical reasons a laboratory often has only a diaphragm pump, and they do not change the pressure arithmetic above.

This item applies to any anaerobic medium prepared by vacuum deoxygenation, not only to this product.

Q16. Is a third-party anaerobic gas-generating sachet compatible?

A16. Yes. A third-party anaerobic gas-generating bag or sachet, for example an Oxoid AnaeroGen sachet or a Mitsubishi anaerobic gas-generating bag, can be placed inside the PP autoclave bag supplied with the Anaerobic Preparation Kit Plus (GMNB-APK02, Appendix II), to generate anaerobic gas and to incubate tubes and plates anaerobically.

Q17. How is GMM prepared if there is no AAE, no anaerobic gases and no Anaerobic Preparation Kit?

A17. Follow the fast preparation notes for this medium on its product page in the GMExpression online shop. That route cannot be relied on for strict anaerobic work. Without a means of excluding oxygen while the medium is aliquoted and while it cools, the medium takes up oxygen and the resazurin will not stay colourless.

Q18. Is the material of the stopper important?

A18. Yes. Bromobutyl rubber stoppers give better airtightness than silicone rubber, and they stay airtight after being punctured with a needle.

For GMM this matters at one step in particular. Stock K is injected through the septum after the medium has been sterilised, so the puncture has to reseal. A stopper that does not reseal admits air at the moment the medium is finished and ready to inoculate.

Q19. How should the reagent bottle be closed for autoclaving, can the bottle burst, and is an airtight PP bag plus a rubber stopper enough?

A19. Close the bottle with a butyl rubber stopper and an apertured cap over it, then seal it inside the airtight PP bag. That closure is enough for autoclaving the liquid, and the bottle does not burst.

Both parts of the answer rest on one pressure chain. Chamber pressure pressurises the gas in the bag, and the gas in the bag pressurises the gas in the bottle through the stopper, because a small amount of gas exchange between bag and bottle is allowed. The pressure in the bottle therefore equalises with the pressure in the chamber, and the closure is never asked to hold the chamber's pressure as a difference across it. Most of the bottle's internal pressure when hot comes from the liquid's own evaporation, not from pressure transmitted from outside.

Why the stopper and the apertured cap. Once the stopper has been punctured, the needle hole closes and stays airtight at normal pressure. If the internal pressure becomes extreme, vapour escapes through the needle hole and the bottle does not burst. The apertured cap holds the stopper in place and leaves that relief path open.

Use an apertured cap, not a solid one. The aperture is what keeps the relief path through the stopper open.

Why the bag and the stopper are enough. Because the two gas volumes equalise with the chamber, the bag is not called on to resist the cycle. What it has to do is keep air off the outside of the bottle, whether it is gas-filled or evacuated as the preparation route you are following directs, and that is what it does; see FAQ 13 for the bag itself. Sterilisation is achieved provided the cycle reaches 121 °C and is held for 15 min on a slow-exhaust liquid cycle.

Close and bag the bottle at the point the preparation route you are following sets out. For autoclaves with vacuum-pulse and support-pressure functions, see FAQ 20.

Q20. Can the airtight PP bag be used in an autoclave with vacuum-pulse and support-pressure functions?

A20. Both are acceptable.

The bag supplied with the kit is thin but durable, and its airtightness is good even when it is closed with a rubber band. Little of the gas inside escapes at low external pressure, particularly if the bag was sealed only half filled. Even if the gas is drawn out of the bag completely it does not matter, because the gas is there to keep external oxygen out of the bottle during autoclaving, not to pressurise it. A vacuum pulse is therefore not a problem.

Support pressure is also acceptable. The rubber stopper, the airtight PP bag and the gas inside it form a flexible triple barrier: it seals against external gas while still allowing pressure to be applied.

Q21. Can vitamin K_1 be dissolved in water?

A21. No. Pure water must never be used as the solvent for vitamin K_1.

Vitamin K_1 (phylloquinone) is practically insoluble in water. It is soluble in ethanol to about 25 mg/ml (Merck Index; Cayman). Stock K Format A is supplied as 0.50 mg/ml in 95 % ethanol, and the Format B stock is reconstituted to the same 0.50 mg/ml. Both are far below that solubility.

Acceptable solvents for a Format B stock are absolute ethanol, 95 % ethanol, or another suitable organic solvent. Purge the solvent with an inert gas before dissolving.

What goes wrong if water is used. The vitamin K_1 does not dissolve. It stays as oil on the wall of the tube and on the pipette tip, and what you draw up is water carrying a variable, unknown amount of suspended oil. The dose delivered to the medium is then unknown, and it will be far below 1.0 mg/L. The error is easy to miss, because about 2 mg of vitamin K_1 is only about 2.1 µL of oil, a thin film rather than a visible pellet, so there is nothing obvious left behind to warn you.

Two further rules follow from the same chemistry.

Never dilute Stock K Format A with water. It is already a 95 % ethanol solution, and adding water drops the vitamin K_1 out of solution.

Never pre-mix or co-dilute Stock H and Stock K. Do not add Stock K into the Stock H bottle, and do not put the two into a shared diluent. Vitamin K_1 is degraded by alkali hydroxides, and Stock H is made up in 0.05 M NaOH. Add each stock separately to the near-neutral bulk medium. That is what keeps the vitamin K_1 intact.

In the finished medium the vitamin K_1 is dispersed in the peptide and lipid matrix rather than dissolved in the water. Dispersed is the right word. The medium is not a solution of vitamin K_1 and does not need to be.

The other stock's solvent. The 0.05 M sodium hydroxide in Stock H is the solubilising agent, not an inert diluent, which is why Stock H is dosed as supplied and never diluted.

Hemin, M = 651.9 g/mol, is practically insoluble in water. Dilute base deprotonates its two propionate side chains and displaces the axial chloride to form haematin, so the minimum stoichiometric demand is 3 mol of hydroxide per mol of hemin. At 1.00 mg/ml hemin, 0.05 M sodium hydroxide is a 32.6 : 1 molar excess, about twelve times the tightest documented working ratio of 2.61 : 1 with sonication. The excess is what makes the stock dissolve reproducibly and stay in solution in the bottle.

Use Stock H as supplied and do not dilute it. Dose it at 5.00 ml per litre, which delivers 5.0 mg of hemin per litre.

The alkali that reaches the medium is small, and FAQ 2 gives the shift it produces.

Q22. Should Format A or Format B of Stock K be used, and how is Format B prepared?

A22. Use Format A for routine preparation. It is supplied ready to use and filter-sterilised, its concentration is defined, and the delivered dose of vitamin K_1 is the more reproducible of the two. Dose it at 2.00 ml per litre, which delivers 1.0 mg of vitamin K_1 per litre.

Use Format B when the ethanol carried in by Format A matters, because the recommended Format B stock carries one tenth as much (FAQ 23), or when a solid with a longer shelf life is preferred to a made-up ethanolic solution.

Format B is supplied as five 2 ml microtubes, each holding about 2 mg of vitamin K_1, about 10 mg per kit. Phylloquinone is a viscous yellow oil at room temperature, with a melting point near -20 °C. It is not a solid. About 2 mg at a density of 0.964 g/ml is about 2.1 µL, which is a thin film on the wall of the tube and not a visible pellet. The tube is not empty.

Format B's limitation is transfer-accuracy, not solubility. Recovering about 2 µL of a viscous oil quantitatively is the real difficulty, so a stock prepared from Format B carries a larger concentration uncertainty than Format A. That is acceptable for a growth factor dosed at 1.0 mg/L. If you need a defined vitamin K_1 concentration, use Format A.

The purity of Format B is supplier- and lot-specific. Vitamin K_1 is sold at purities from 80 % upward, because of E/Z isomerism at the C2' double bond. Take the purity figure from the lot certificate of analysis.

To prepare a vitamin K_1 stock from Format B

Vitamin K_1 dissolves slowly, even in 95 % or absolute ethanol, and about 2 mg is a film of roughly 2.1 µL on the wall of the tube. The stock is therefore built by sequential rinsing, which is what recovers the oil quantitatively. A single addition of solvent does not.

Three concentrations are possible, and they differ only in how much ethanol they carry into the medium. All three deliver the same 1.0 mg of vitamin K_1 per litre.

Stock	2 mg made up to	Dose per litre	Ethanol in the medium	Doses per tube
0.5 mg/ml - default, matching Format A	4.00 ml	2.00 ml	0.190 % v/v	2
2.0 mg/ml	1.00 ml	0.50 ml	0.048 % v/v	2
5.0 mg/ml	0.40 ml	0.20 ml	0.019 % v/v	2

Use the 0.5 mg/ml preparation unless you have a reason not to: it is the one the four-rinse procedure below is written for, and it is the concentration supplied as Format A. Choose 2.0 or 5.0 mg/ml if you want the lowest possible ethanol load - for example when working with a strain whose ethanol tolerance you do not know.

Prepare a concentrated stock in the microcentrifuge tube itself and use it within a short period. Do not make a concentrated vitamin K_1 stock in a larger storage bottle and keep it: concentrated ethanolic vitamin K_1 deposits an oily condensate on the vessel wall over prolonged storage, even when it dissolved completely to begin with, and the dose then becomes inaccurate. The same applies to any lipophilic quinone stock you prepare yourself, menadione included.

Menadione, vitamin K_3, is a different compound from the phylloquinone this formulation specifies. Substituting it is a formulation change and is yours to validate.

At 2.0 and 5.0 mg/ml the four-rinse transfer below cannot be used, because the whole stock is smaller than one rinse. Add the ethanol directly to the microtube, cap it, and vortex - with brief sonication at 30 °C or below if the oil film persists - until no film remains on the wall when the tube is held to the light. Dose directly from the tube. The loss is then the tube's dead volume, about 20 µL, rather than a transfer loss.

Step 1

Read the mass of vitamin K_1 printed on the tube label. Use that figure, not the nominal 2 mg. Let the tube reach room temperature before opening it, and purge the ethanol with an inert gas before use.

Step 2

Add **1 ml** of solvent - 95 % ethanol, absolute ethanol or another suitable organic solvent - to the 2 ml tube. Cap it and vortex thoroughly to dissolve as much of the oil as possible.

Step 3

Transfer the solution into a larger tube, for example a 10 ml centrifuge tube. This tube also serves to bring the stock to its final volume.

Step 4

Add a further **1 ml** of solvent to the original 2 ml tube, vortex again to take up the residue, and pour the solution into the same 10 ml tube.

Step 5

Repeat Step 4 until **four 1 ml rinses in total** have been transferred, giving **4 ml** of combined solution. Inspect the 2 ml tube against the light: no oil film should remain on the wall.

Step 6

The stock is **2 mg / 4 ml = 0.50 mg/ml**, the same concentration as Format A. For a tube labelled m mg rather than 2.0 mg, make up to m / 0.50 ml so the stock is still 0.50 mg/ml.

Step 7

Sterilise through a **0.22 µm single-use syringe filter** into a sterile amber vial.

Do not use a filter with an acrylic or MBS housing, and do not use a cellulose-nitrate membrane: manufacturer compatibility tables rate these unsatisfactory for high-concentration ethanol. A PTFE or nylon membrane in a polypropylene housing is correct. Polyethersulfone and cellulose-acetate membranes are rated ethanol-compatible by their manufacturers and are not contraindicated. The housing, not the membrane, is the usual point of failure, so check the housing material of the filter you are holding.

No filtrate is discarded. Filtration removes volume, not concentration: the filter holds back roughly 0.1 ml on a 13 mm unit, which reduces the recovered volume slightly and leaves the 0.50 mg/ml unchanged. Use a small-diameter filter to keep that loss down. Discard the first drops only if the filter's own instructions call for priming, and note that doing so costs usable volume.

Step 8

Dose at **2 ml per litre** of medium, exactly as for Format A. That delivers 1.0 mg of vitamin K_1 per litre and carries the same 0.19 % v/v of ethanol.
Each tube yields 4 ml, which is two 1 L doses. Five tubes therefore cover 10 L, twice the nominal pack size.

Store both formats at 2~8 °C, protected from light. Standard practice for ethanolic vitamin K_1 stocks is to discard them about one month after first opening.

Format A supplies 15 ml, which is 7.5 doses, covering 5 L plus 50 %: a larger margin than the 20 % used elsewhere in this kit, allowed for ethanol evaporation on repeated opening and for bottle dead volume. That margin assumes use spread over time, and a one-month in-use limit makes 7.5 doses unusable in a laboratory that prepares one litre at a time. A laboratory preparing small batches infrequently should use Format B.

Q23. Stock K Format A brings ethanol into the medium. How much, and does it matter?

A23. 2 ml of 95 % ethanol per litre is 1.90 ml of absolute ethanol per litre: 0.19 % v/v, about 32.5 mmol/L.

That is the whole of it. Stock K is added after autoclaving, so no ethanol is lost to the vapour phase: unlike a pre-autoclave addition, the calculated figure and the figure present at inoculation are the same.

Is it a usual amount? Not quite. Across nine standard anaerobe formulations the documented ethanolic carry-over range is 0.019-0.100 % v/v, and Format A is about twice the top of that range.

Format A is supplied at 0.50 mg/ml for reasons that only show up over a product's shelf life. More concentrated ethanolic vitamin K_1 stocks tend to deposit an oily condensate on the inner wall of the bottle after prolonged storage, even when the vitamin was fully dissolved to begin with; the active material is then neither in solution nor reliably delivered, and the dose becomes inaccurate. At 0.50 mg/ml the stock prepares reproducibly, loses little during 0.22 µm filtration, stores without oily deposition, and doses accurately with an ordinary pipette.

If you want less ethanol in the medium, raise the concentration of a Format B stock rather than of Format A. A Format B stock is made in a microcentrifuge tube and used within a short period, so the long-term deposition that rules out a concentrated Format A does not arise. See FAQ 22 for the three preparations and the ethanol each one carries.

Is it likely to matter? The evidence is partial:

- Gut isolates including Bacteroides species and Clostridium cluster XIVa organisms have been reported growing in broth containing 5 % v/v ethanol.
- For mixed faecal anaerobes, 50 % inhibition of growth rate has been reported near 2.8 % v/v, and no growth near 8 % v/v.
- Against 0.19 % v/v, those figures are margins of roughly 15 to 40 times.
- Ethanol dose-response is not established for *Akkermansia muciniphila*, *Faecalibacterium prausnitzii* or *Roseburia* species. Qualify the ethanol load for these three genera in your own application.
- Ethanol-related effects in this medium are not established for any organism. Establish them for your strain before relying on a result.

For ethanol-sensitive or uncharacterised isolates, and for any study in which ethanol is itself a variable, note that Format B is reconstituted to the same 0.50 mg/ml and dosed at the same 2.00 ml per litre, so it carries the same 0.19 % v/v and is not an ethanol-sparing alternative. Include a solvent-only control wherever ethanol sensitivity is in question.

For context on the vitamin K_1 dose itself: 1.0 mg per litre is the CLSI-recommended final concentration, and it sits inside the 0.5-10 mg/L range used across standard anaerobe media.

Stock K is a flammable liquid. Keep it away from ignition sources. Never autoclave the Stock K bottle.

Q24. How should vitamin K_1 be protected from light?

A24. Treat light as the main threat to vitamin K_1, not heat.

Protect Stock K from light at every stage, not only in storage: keep it in its aluminium-foil pouch at 2~8 °C, return it to the pouch immediately after each withdrawal, and never leave the bottle or a working dilution standing on an open bench. In edible oils, phylloquinone in clear glass lost 46 to 59 % after two days under fluorescent light and 87 to 94 % after two days of daylight through a window, against 11 % after 40 minutes at 185 to 190 °C; amber glass gave no significant loss (Ferland and Sadowski, J Agric Food Chem 1992;40:1869-1873).

In practice:

- Work under subdued light when dispensing Stock K, and keep the bottle in its pouch between withdrawals. Amber glass is an acceptable substitute for the pouch.
- **Switch the glove-box UV lamp off, and confirm it is off, before bringing Stock K or supplemented medium into the cabinet.** A routine 20-minute UV decontamination is a far larger dose than anything the medium meets on the bench. If your workflow runs UV before each session, stage the stock outside the cabinet and bring it in after the lamp has finished.
- Store supplemented medium in the dark. Foil-wrap bottles of prepared broth, or keep them in a closed box in the refrigerator. Tubes and plates go back into their light-protective packaging.
- The concentrated stock is the more valuable target: 0.50 mg/ml in a 15 ml bottle is several litres' worth of vitamin K_1 in one place, where the finished medium holds 1.0 mg/L. Protect both, but protect the stock first.

Photodegradation in this medium and in this stock is not established. The edible-oil figures bound the hazard rather than quantify it for this product. Minimise light exposure at every step.

Q25. How is an anaerobic bench or glove box prepared before work?

A25. Most anaerobic benches and glove boxes have no laminar or vertical HEPA-filtered airflow, so they do not control microbial contamination on their own. Before work, give the enclosure a minimum of 20 minutes of UV irradiation, then spray the bench and the outside of every bottle and container with 70 % alcohol and wipe them down.

Sterilise the rubber stopper and the apertured cap as well, particularly the area the needle will puncture.

For GMM this matters at one specific point. On the default route Stock K is the only addition made inside the enclosure after the medium has been sterilised, so that bottle and the septum it is injected through are the last opportunity to introduce a contaminant into a finished medium.

Stock K also has to be de-oxygenated before first use. It is supplied sterile, but it is bottled in air. Because it is dosed into the medium after sterilisation, any oxygen it carries goes straight into the finished medium instead of being driven off in the cycle. De-oxygenate it by the method the preparation route you are following sets out: where the bottle can be opened directly into an oxygen-free atmosphere, loosen the cap half a turn and leave it for 30 minutes; where it cannot, the route substitutes a vacuum-and-gas-fill cycle in a sealed bag. Withdraw each dose inside that atmosphere, and tighten the cap before the bottle leaves it.

Finish the UV decontamination and confirm the lamp is off before Stock K goes into the enclosure. See FAQ 24.

Stock S and Stock H need no separate de-oxygenation on the default route. Both go in at the Mixture A dissolving step and pass through the autoclave with the medium, so the cycle deals with the oxygen they carry. Where Stock H is held back and added after the cycle instead, treat it as this item treats Stock K.

Where the preparation route you are following gives you no anaerobic atmosphere to open the Stock K bottle into, keep the cap tight, withdraw each dose promptly, and return the bottle to the storage conditions in section 1.5, protected from light. See FAQ 24.

Q26. Can Hemin be added after autoclaving, or vitamin K_1 before it?

A26. Both variants are permissible. Neither is the default.

On the default route Stock S and Stock H both go in at the Mixture A dissolving step and are sterilised in the medium's own cycle, and Stock K is the only addition made after autoclaving. Only Stock H and Stock K are in question here. The placement of Stock S is not optional, because it is not supplied as a sterile supplement (FAQ 5).

Keep the default arrangement unless you have a reason not to. The volume ledger and the pH adjustment printed with the preparation route you are following are written for it: make up to 998 ml, adjust to 7.2 ± 0.2 at 25 °C, autoclave at 121 °C for 15 min, then add 2.00 ml of Stock K to reach 1000 ml, the finished medium being 7.0 ± 0.2 at 25 °C. Per vessel, 9.98 ml of pre-autoclave broth plus 20 µL of Stock K gives 10.00 ml. A user taking either variant below adjusts the make-up volume as given for that variant and changes nothing else in the ledger. Each variant below is an alternative to the default, not to the other: the make-up volumes given assume one change only, and a batch that takes both is outside the ledger printed with the preparation route you are following.

Hemin after autoclaving.

Stock H is supplied sterile at 1 mg/ml, so the 5 ml per litre can be held back. Make up to 993 ml instead of 998 ml, and add the 5.00 ml of Stock H together with the 2.00 ml of Stock K once the medium is at 50 °C or below, hemin first and mixed thoroughly before the vitamin K_1 goes in. The delivered dose is unchanged at 5.0 mg/L of hemin.

The reason the default charges Hemin before the cycle is contamination control. Every additional sterile transfer into a sterilised medium is another opportunity for contamination, and this variant adds one. Hemin also tolerates autoclaving: the FDA Bacteriological Analytical Manual and DSMZ both sterilise hemin stocks at 121 °C for 15 min, and BBL Schaedler Agar carries hemin through the autoclave in its dehydrated base. Performance equivalence of the two arrangements has not been established.

Add the two stocks separately and in that order. Mixing the hemin in thoroughly first disperses its dilute alkali before the vitamin K_1 arrives. Never pre-mix or co-dilute them, never add Stock K into the Stock H bottle, and never draw both from one pipette or syringe (FAQ 21).

On this variant the pre-autoclave pH adjustment is made without the dilute alkali of Stock H in the vessel, so that contribution is no longer absorbed by the adjustment and falls instead on the finished medium. FAQ 2 gives the size of the shift, which is negligible.

Vitamin K_1 before autoclaving.

Charge the 2.00 ml of Stock K into the bulk medium before autoclaving, at the Mixture A dissolving step, and make up to 1000 ml instead of 998 ml. This removes the aseptic post-autoclave transfer altogether, which on the default route is the last opportunity to introduce a contaminant into a finished medium (FAQ 25).

Add it to the bulk medium only. Never into the Stock H bottle and never into a shared diluent: vitamin K_1 is degraded by alkali hydroxides, and Stock H is made up in 0.05 M sodium hydroxide (FAQ 21).

Phylloquinone is reported to be relatively stable to moist heat, and several standard anaerobe media carry vitamin K_1 through 121 °C for 15 min in the dehydrated base. How much of the 0.50 mg/ml stock's vitamin K_1 is still present at the point of inoculation after a cycle has not been established, so treat the 1.0 mg/L as a nominal dose on this variant.

Charging Stock K into the medium is not the same thing as autoclaving the stock. The Stock K bottle itself is never autoclaved (FAQ 23). On this variant the ethanol Format A carries in is no longer bounded by the figure FAQ 23 gives, because that figure assumes an addition made after the cycle; a pre-autoclave addition loses some of the ethanol to the vapour phase, and how much is not established.

Light applies to both arrangements. Light, not heat, is the documented mode of loss for vitamin K_1, so keep the batch dark whichever arrangement you take (FAQ 24).

Record which arrangement was used with the batch, and qualify whichever one you adopt for your own application.

Q27. Can GMM agar plates be made?

A27. Yes. Follow the plate route in section 2.2. Three points govern it.

Agar is not supplied with this kit. The loading for GMM plates is 15 g per litre. Dissolve it in the pH-adjusted, made-up broth, as section 2.2 does, and record the figure used.

Stock S and Stock H are already in the medium: both go in with Mixture A at Step 1 and pass through the autoclave. Stock K is the only addition left, 2.00 ml per litre at 50~55 °C. Molten agar is close to its gelling range at that temperature, so work in this order: autoclave, cool to 50~55 °C, add Stock K, mix, then pour immediately without creating foam. If the agar sets before Stock K has been added, re-melt it gently, cool again to 50~55 °C, and then add it. Do not autoclave it again.

No pouring temperature is specified within the 50~55 °C window the agar route uses. A laboratory making plates routinely should establish its own working temperature and record it.

The plates will be opalescent, because the mucin is carried through (FAQ 10). That is the expected appearance.

Q28. Can a batch smaller than 1 L be made?

A28. Yes, but homogenise the dry blends first. That is the single most likely source of error in a sub-litre batch.

Mixture A is a 15-component dry blend and can segregate in transit. Resazurin is 24 ppm of the blend and calcium chloride is 0.12 %. Invert or tumble the Mixture A bottle 10-15 times before weighing out any sub-portion. Mixture B is a two-component blend of mucin and L-cysteine and must be homogenised the same way.

Weigh each litre out individually rather than dividing a container by eye. Mixture A and Mixture B carry only a small dispensing margin: the kit supplies 215 g and 23 g against the 209.113 g and 22.500 g that five litres need, so the margin absorbs ordinary weighing error but not a spill.

To scale, multiply every line of the volume ledger in section 2.2 by the same factor, and keep the same number of decimal places as the per-litre figures. The ratios that do not change with batch size are:

- Mixture A 41.823 g per litre, and Mixture B 4.500 g per litre
- Stock H 5.00 ml per litre
- Stock K 2.00 ml per litre, Format A or Format B (both are 0.50 mg/ml)
- Stock S 5.00 ml per litre, added with Mixture A at the dissolving step
- Make up with water, before autoclaving, to the final volume less the volume of Stock K, that is 998 ml per litre. Stock S and Stock H are already in the vessel from the Mixture A dissolving step

Per vessel: a 20 ml anaerobic tube filled with 9.98 ml of pre-autoclave broth, Stock S and Stock H already included, receives 20 µL of Stock K.

The practical limit is your balance, not the formulation. Calcium chloride at 0.050 g per litre and resazurin at 0.001 g per litre are the components that a poorly homogenised small sub-portion will get wrong, and resazurin is the one you will notice, because the colour check stops working

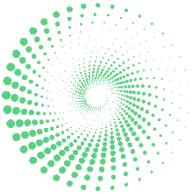
Q29. What are the best storage methods and conditions?

A29. For prepared medium there are two holds, as section 1.5 defines them. The anaerobic hold: vacuum-pack the tubes, bottles or plates together with a bag of oxygen absorber, and store refrigerated at 4 °C (2~8 °C band). The aerobic hold: broth, bottles and plates that have not been vacuum-sealed, held at the same temperature in the dark. They are not anaerobic: repeat the resazurin check in section 1.4 before inoculation.

No shelf life is specified for prepared GMM, in either the aerobic or the anaerobic hold. Establish a hold period for your own storage arrangement, and verify the medium before each use rather than relying on an elapsed time.

For the kit components, see section 1.5, which gives the temperature, light and flammability conditions for each stock and for both dry blends: Stock H and Stock K at 2~8 °C protected from light, Stock K kept away from ignition sources and never autoclaved, and Stock S at 15~25 °C by preference or at 2~8 °C, never frozen (FAQ 6).

Keep the Mixture A bottle and the Mixture B bag closed. Each carries only a small dispensing margin over the 5 L requirement, so the margin absorbs ordinary weighing error but not a spill, and a spill cannot be made up from elsewhere in the kit. See FAQ 28.



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