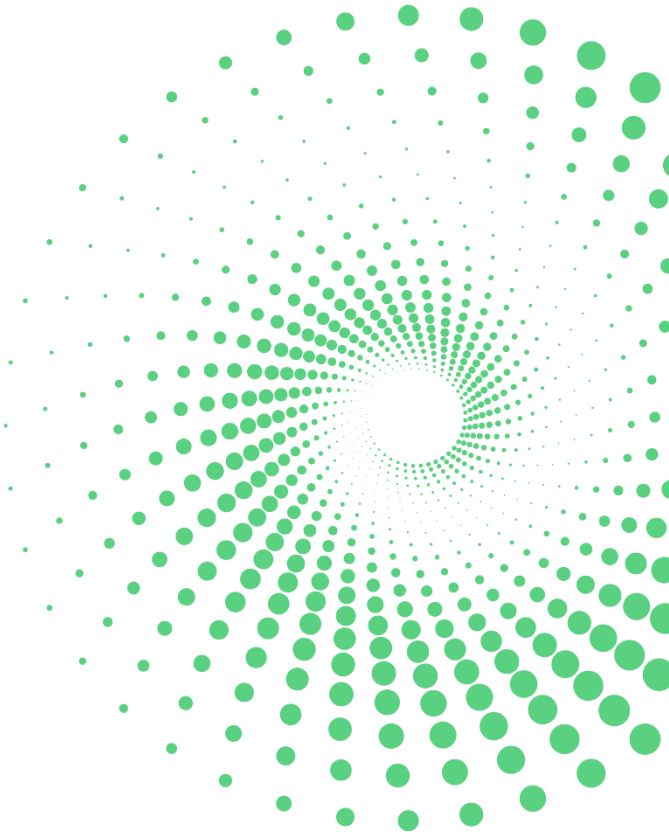


YCFA Medium. Full Recipe - Instructions

(Yeast Casitone Fatty Acids Broth)

Revision Date	July 2025
Revision Number	1.9

GMExpression
General Molecular Expression Service Pty Ltd

Index

1. Product information

2. Instructions for preparation

3. Instructions for YCFA anaerobic tubes

Appendix

FAQ

1. Product information

1.1. Summary

Product name	YCFA medium - Full Recipe
SKU	GMNB-YCFA03
Qty	Make up to 12.7 L of media

1.2. Application

- YCFA Medium is an enriched nonselective medium for cultivating most anaerobic bacteria and other fastidious microorganisms.
- Suitable for microbial culturomics & mimicking the gut environment to obtain gut microbiota isolates, specifically anaerobic strains.

1.3. Package contains

- Mixture A	279.5 g
- Mixture B	65 g
- Stock C | Volatile Fatty Acids	41.5 ml
- Stock D | Vitamin Solution 1 (0.22µm filter sterilised)	30 ml
- Stock E | Vitamin Solution 2 (0.22µm filter sterilised)	30 ml
- Stock F | 1M KOH Solution	50 ml
- Airtight PP Bags	10 pieces
- Heat-resistant rubbers	10 pieces

1.4. Components - Per 980mL of YCFA Medium - Full Recipe contains

Mixture A	
Tryptone	10.0 g
Yeast extract	2.5 g
Glucose	2.0 g
Maltose	2.0 g
Cellobiose	2.0 g
$MgSO_4 \cdot 7H_2O$	0.09 g
$CaCl_2 \cdot 2H_2O$	0.09 g
K_2HPO_4	0.46 g
KH_2PO_4	0.46 g
$(NH_4)_2SO_4$	0.92 g
NaCl	0.92 g
Resazurin	1 mg

Mixture B	
$NaHCO_3$	4 g
L-Cysteine-HCl	1 g
Hemin	10 mg

Stock C | Volatile fatty acids	
Acetic acid	2.025 ml
Propionic acid	0.715 ml
iso-Butyric acid	0.120 ml
n-Valeric acid	0.120 ml
iso-Valeric acid	0.120 ml

Stock D | Vitamin Solution 1	
Biotin	10 µg
Folic acid	50 µg
Pyridoxine-HCl	150 µg
Vitamin B12	10 µg
p-Aminobenzoic acid	30 µg

Stock E | Vitamin Solution 2	
Thiamine-HCl	50 µg
Riboflavin	50 µg

Stock F | KOH	
KOH solution, 1M	50 ml

1.5. Storage and Expiry Date

- This product can be shipped at ambient temperature. Upon receipt of the product, please store Stock D and Stock E at 4°C.
- When stored at room temperature (15~25°C), Mixture A, Mixture B, Stock C and Stock F can be used for three years without losing their performance; when stored at 2~6°C, Stock D and Stock E can be valid for one year.
- Prepared liquid media and agar plates are suggested to be vacuum sealed together with the oxygen absorber and stored at 4°C, and the shelf life will be one year.

1.6. Safety Notes

Mixture B: Mixture B is not vacuum-packed as the content may release a small quantity of carbon dioxide, which will not affect the use of the product.

Stock C: Stock C may emit an irritant vapour. The walls of the square bottle may fog over time. This is due to the oxidation of the PET by a small amount of anhydride in the carboxylic acid, which will not affect the normal use of the liquid.

The isobutyric and isovaleric acids contained in the liquid may emit an unpleasant odour; please operate in a fume hood. Keep the bottle sealed tightly after use.

The Valeric acid contained is harmful to aquatic organisms, and pouring the liquid directly into the sewer is strictly prohibited. Wear latex gloves and goggles to prevent skin/eye contamination and inhalation.

Stock F: Stock F is a corrosive liquid. Wear latex gloves and goggles to prevent skin/eye contamination when operating.

1.7. Cautions

- Always operate Stock C in a fume hood.
- Wear latex gloves and goggles to prevent skin/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.
- 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
- ☐ 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
- ☐ YCFA media
- ☐ Self-sealing plastic bags (several)
- ☐ Autoclave chamber

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

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.

Common-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 D and stock E in the AAE and loosen (half unscrew) the cap of the bottle for 30 minutes to allow efficient removal of the dissolved oxygen. Always tighten the cap of the stock D/E in the AAE after use.

Stock D/E (Vitamin solution) is prepared and bottled by filter sterilisation in air. Before first use, it is recommended to place it in the AAE to remove oxygen for strict anaerobic tests and culturing.

Step 1

Dissolve 21.44 g of mixture A and 3.1 ml of stock C in 900 ml double distilled or MQ water in a GL45-cap reagent bottle.

Operating in a fume hood when handling stock C (Volatile fatty acids) is strongly recommended.

From Step 2.0 to Step 2.4 is to heat the solution of mixture A and stock C, which can be skipped. However, heating the solution will enable sufficient reaction and solubility of the contents in mixture A and stock C, achieving better performance of the broth.

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 vacuum the air out of the bottle to remove the dissolved oxygen in the liquid and air in the bottle.

The vacuum method can best remove the oxygen dissolved within the liquid and the oxygen absorbed onto the solid surface.

Vacuum until bubbles emerge from the liquid. repeat at least twice with a 1-minute gap between each vacuum to allow the dissolved oxygen to rebuild its partial pressure at the top of the liquid, facilitating the best oxygen removal by the next round of vacuum.

Step 2.2 - (optional) - (if Step 2.1 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, and leave the apertured cap **slightly loosened**.

Open the rubber stopper for 20 minutes to best remove dissolved oxygen if Step 2.1 was skipped.

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.

The airtight PP bag is used to prevent the reagent bottle from inhaling oxygen-containing air. It also isolates any leaked internal liquid/vapour from escaping into the open air.

The bag is allowed to be 1/3 to half filled with AGs to best prevent enteral oxygen from entering the bottle during the next step of boiling. If AGs contain hydrogen, squeeze out as much AGs as possible before sealing the bag neck for safety.

Step 2.4 - (optional)

Heat the prepared 900 ml broth solution to about boiling in a microwave oven for 5~10 minutes, then leave the prepared 900 ml broth solution to rest and cool down to room temperature.

Observe if the rubber stopper is deformed by the pressure, and bubbles appear in the liquid. Switch off the microwave heating immediately when boiling starts, and switch on again when the rubber stopper has recovered its shape and the bubbles have disappeared (liquid has cooled down).

⚠ **Safety caution.** Please refer to *FAQ 15* for detailed suggested instructions.

Step 3

Add 5 g of mixture B and 2.05 ml of stock F to the 900 ml of prepared broth. Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle. Leave for a few minutes until the foam disappears, make up to 980 ml with double-distilled or MQ water and adjust the pH to 7.4 ~ 7.5 with 1M KOH (stock F) or 1M HCl.

It is better to operate this step and Step 4 in AAE. It can also be done in an air atmosphere to simplify the operation, as long as the YCFA broth is cool and not after long exposure to air (best within 10 minutes).

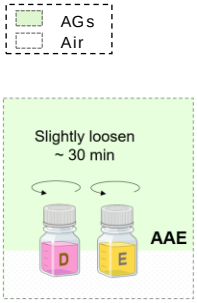
Mixture B contains $NaHCO_3$. When it meets acidic liquid, it will react and release carbon dioxide, and the foam will disappear after a few minutes of resting.

After Mixture B is dissolved, a tiny amount of insolubles may remain, which is hemin-mediated condensed protein/peptide (see *FAQ 14*). After adjusting the pH to basic, most of the insolubles will disappear. The remaining insolubles will not affect the bacterial culture.

- Tips: Freshly prepared YCFA broth will generally be acidic, with a pH range of 5 to 6. Using 5M KOH for rough adjustment and then 1M KOH for fine adjustment can save preparation time.

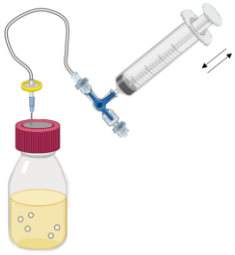
Step 4

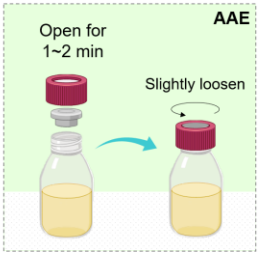
Rest the broth solution and let the insolubles precipitate, filtrate or directly pour the supernatant into two prepared clean reagent bottles.



Step 0 (Optional)

Step 1


Step 2.0 ~ 2.1 (optional)



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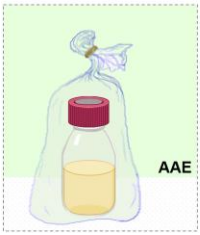

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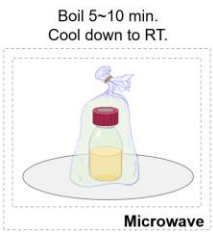
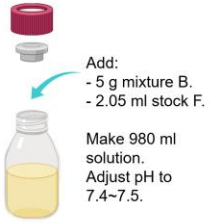


Step 2.4 (optional)



Step 3

Step 4

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

Step 5

In an anaerobic atmosphere environment, aliquot the broth solution into anaerobic tubes (e.g. Hungate Tubes), 10 ml per tube (1/3 ~1/2 of the tube volume, but no more than 1/2), and seal the tubes.

Tips: Check the colour before autoclave. If the colour of the broth is pale yellow or colourless, it is good and ready for autoclave. If the colour is light pink/red, it means there is oxygen in the solution. Check your AAE conditions or use the vacuum process (as in Step 2.1) to remove the oxygen.

Step 6

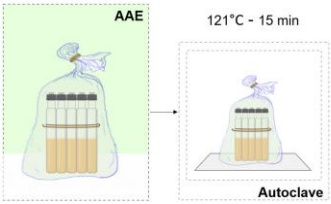
Use rubber bands to hold the anaerobic tubes together, place them in the airtight PP bag, and seal the neck of the bag with a rubber band. Then move out of the AAE and autoclave at 121°C for 15 minutes.

Optionally, before autoclave, the sealed anaerobic tubes can be vacuumed using the method of Step 2. The vacuum method can best remove the oxygen dissolved within the liquid, which is good for strict anaerobic tests.

Step 7

Before use, aseptically add 20 µl of stock D (vitamin solution 1) and 20 µl of stock E (vitamin solution 2) to each anaerobic tube (2 µl/mL broth solution) in an anaerobic atmosphere environment or using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*) to inject the vitamin solution through the rubber stopper.

Step 5



Step 6

Step 7

To prepare YCFA in bottles

Step 5

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

Tips: Check the colour before autoclave. If the colour of the broth is pale yellow or colourless, it is good and ready for autoclave. If the colour is light pink/red, it means there is oxygen in the solution. Check your AAE conditions or use the vacuum process (as Step 8.1) to remove the oxygen.

Step 7

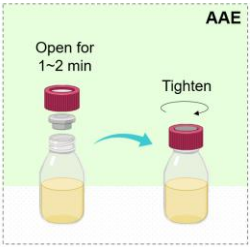
Put the bottle into an airtight PP bag and seal the neck of the bag with a rubber band, take it out of the AAE, then autoclave at 121°C for 15 minutes.

Step 8

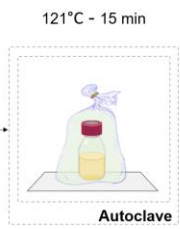
Before use, aseptically add 2 µl of stock D (vitamin solution 1) and 2 µl of stock E (vitamin solution 2) per mL of broth solution in an anaerobic atmosphere environment or using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*) to inject the vitamin solution through the rubber stopper.

Step 5

Step 5.1 (Optional)



Step 6



Step 7

Step 8

To prepare YCFA agar plates

Step 5

Dissolve 1~2 g of agar (1~2% w/w) in pH-adjusted broth, seal the bottle with a (butyl) rubber stopper and screw an apertured cap 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 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.

Tips: Check the colour before autoclave. If the colour of the broth is pale yellow or colourless, it is good and ready for autoclave. If the colour is light pink/red, it means there is oxygen in the solution, check your AAE conditions or use the vacuum process (as Step 8.1) to remove the oxygen.

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.

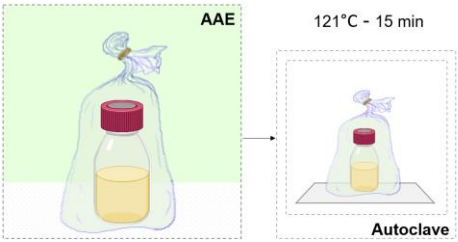
Step 8

In an anaerobic atmosphere environment, add 2 ml of stock D (vitamin solution1) and 2 ml of stock E (vitamin solution 2) to the broth solution (2 µl/ml broth solution) when the broth solution **cools down to 60°C** and mix well, quickly pour the YCFA agar media into sterile Petri dishes when hot and wait for the agar to cool and harden.

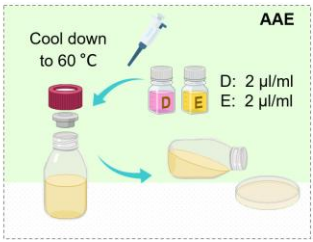
Step 5

Step 5.1 (Optional)

Step 6



Step 7



Step 8

Storage

It is recommended that unused prepared YCFA liquid media, stock D/E, anaerobic tubes and YCFA agar plates are packaged in an anaerobic atmosphere, vacuum sealed [3] together with the oxygen absorber [4][5] in plastic bags and stored at 4°C. The shelf life of prepared media is about half a year. The shelf life of stock D/E is about one year.

Storage Cautions:

When the liquid YCFA Medium is stored at low temperature and when the pH is lowered during the incubation process, a tiny amount of insolubles may precipitate slowly, which is hemin-mediated condensed protein/peptide (see *FAQ 14*) with low solubility, and only needs to be left to precipitate to the bottom of the bottle or the bottom of the tube, which is normal and will not affect the growth of bacteria.

.....

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

Common-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 D and stock E in the assembled self-sealing autoclave bag [4], seal the bag and loosen (half unscrew) the cap (twist from outside the bag), then use the assembled vacuum device [2] to vacuum the bag and rest for 5 minutes, then fill the AGs through the PTFE air valve into the autoclave bag and rest for 15 minutes to allow efficient remove of the dissolved oxygen. After use, always tighten the cap of stock D/E in the vacuumed self-sealing autoclave bag or the autoclave bag filled with AGs.

Stock D/E (Vitamin solution) is prepared and bottled by filter sterilisation in air. Before first use, it is recommended to place it in a vacuumed space to remove oxygen for strict anaerobic tests and culturing.

Before placing stock D/E in the self-sealing autoclave bag, the bottle surface of stock D/E and the inner surface of the self-sealing autoclave bag need to be sterilised with 70% alcohol.

Step 1

Dissolve 21.44 g of mixture A and 3.1 ml of stock C in 900 ml double distilled or MQ water in a GL45-cap reagent bottle.

Operating in a fume hood when handling Stock C (Volatile fatty acids) is strongly recommended.

From Step 2.0 to Step 2.2 is to heat the solution of mixture A and stock C, which can be skipped. However, heating the solution will enable sufficient reaction and solubility of the contents in mixture A and stock C, achieving better performance of the broth.

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 air out of the bottle to remove the dissolved oxygen in the liquid and air in the bottle.

The vacuum method can best remove the oxygen dissolved within the liquid and the oxygen absorbed to the solid surface.

Vacuum until bubbles emerge from the liquid, repeat at least twice with a 1-minute gap between each vacuum to allow the dissolved oxygen to rebuild its partial pressure at the top of the liquid, facilitating the best oxygen removal by the next round of vacuum.

Step 2.1 - (optional)

Put the bottle into an airtight PP bag [1], **slightly loosen** the apertured cap, squeeze out/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.

[Optional] Assembled self-sealing autoclave bag can also be used. Put the bottle into the autoclave bag, and seal the bag with an airtight seal stick. Vacuum the bag and then fill it with the AGs through the PTFE air valve into the autoclave bag.

The airtight PP bag is used to prevent the reagent bottle from inhaling oxygen-containing air. It also isolates any leaked internal liquid/vapour from escaping into the open air.

The bag is allowed to be 1/3 to half filled with AGs to best prevent enteral oxygen from entering the bottle during the next step of boiling. If AGs contain hydrogen, do not fill the bag with AGs for safety.

Step 2.2 - (optional)

Heat the prepared 900 ml broth solution to about boiling in a microwave oven for 5~10 minutes, then leave the prepared 900 ml broth solution to rest and cool down to room temperature.

Observe if the rubber stopper is deformed by the pressure and bubbles appear in the liquid, switch off the microwave heating immediately when boiling starts, and switch on again when the rubber stopper has recovered its shape and the bubbles have disappeared (liquid has cooled down).

⚠ **Safety caution.** Please refer to *FAQ 15* for detailed suggested instructions.

Step 3

Add 5 g of mixture B and 2.05 ml of stock F to the 900 ml of prepared broth. Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle. Leave for a few minutes until the foam disappears, make up to 980 ml with double-distilled or MQ water and adjust the pH to 7.4 ~ 7.5 with 1M KOH (stock F) or 1M HCl.

It is OK to operate in an air atmosphere to simplify the operation, as long as the YCFA broth is cool and not after long exposure to air (best within 10 minutes).

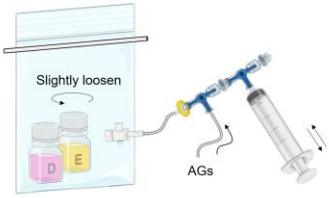
Mixture B contains $NaHCO_3$. When it meets acidic liquid, it will react and release carbon dioxide, and the foam will disappear after a few minutes of resting.

After Mixture B is dissolved, a tiny amount of insolubles may remain, which is hemin-mediated condensed protein/peptide (see *FAQ 14*). After adjusting the pH to basic, most of the insolubles will disappear. The remaining insolubles will not affect the bacterial culture.

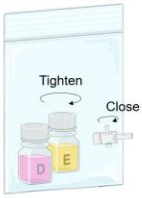
Tips: Freshly prepared YCFA broth will generally be acidic, with a pH range of 5 to 6. Using 5M KOH for rough adjustment and then 1M KOH for fine adjustment can save preparation time.

Step 4

Rest the broth solution and let the insolubles precipitate, filtrate or directly pour the supernatant into two prepared clean reagent bottles.



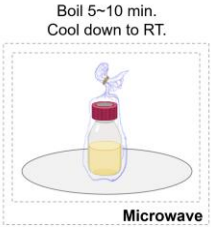
Step 0 (Optional)



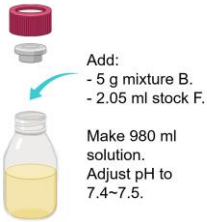
Step 1

Step 2.0 (optional)

Step 2.1 (optional)



Step 2.2 (optional)



Step 3

Step 4

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

Step 5

Aliquot the broth solution into anaerobic tubes (e.g. Hungate Tubes), 10 ml per tube (1/3 ~1/2 of the tube volume, but no more than 1/2), and seal the tubes. Then use the method of Step 2 to vacuum each sealed anaerobic tube.

Vacuum can remove dissolved oxygen and prevent oxidation of the broth during autoclaving.

Tips: Rest the vacuumed solution for about 10 minutes to check its colour, if turned to pale yellow or colourless, it means the oxygen has been totally removed and ready for autoclave. If the colour is still pink/red, repeat the vacuum process to remove the oxygen.

Step 5.1 - (Optional)

If working with an Anaerobic Preparation Kit Plus, after vacuuming the air out of the tubes, AGs can be injected into the tubes through the gas pump/exchange assembly.

Step 6

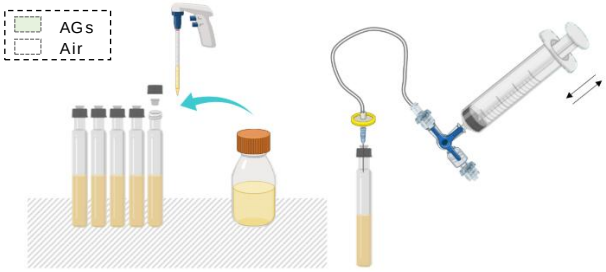
Use rubber bands to hold the anaerobic tubes together, place them in the airtight PP bag [1], squeeze/vacuum out as much air as possible and seal the neck of the bag with a rubber band [1]. Then place them together in the autoclave chamber. Autoclave at 121°C for 15 minutes.

The bag is allowed to be 1/3 to half filled with AGs to best prevent enteral oxygen from entering the bottle during autoclaving. If AGs contain hydrogen, do not fill the bag with AGs for safety.

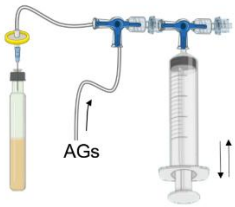
Step 7

Before use, aseptically add 20 µl of stock D (vitamin solution 1) and 20 µl of stock E (vitamin solution 2) to each anaerobic tube (2 µl/mL broth solution), using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*) to inject the vitamin solution through the rubber stopper, carefully avoid introducing air into the tube.

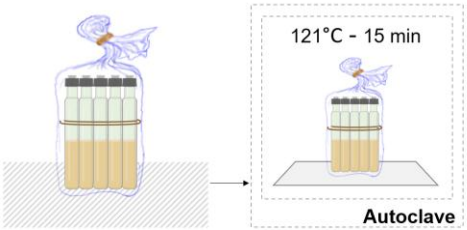
Squeeze the plunger until all air is expelled from the syringe needle before use.



Step 5



Step 5.1 (Optional)



Step 6

Step 7

To prepare YCFA in bottles

Step 5

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

Step 6

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.

Tips: Rest the vacuumed solution for about 10 minutes to check its colour; if it turns pale yellow or colourless, it means the oxygen has been totally removed and is ready for autoclave. If the colour is still pink/red, repeat the vacuum process to remove the oxygen.

Step 6.1 - (Optional)

If working with an Anaerobic Preparation Kit Plus, after vacuuming the air out of the bottle, AGs can be injected into the bottle through the gas pump/exchange assembly.

Step 7

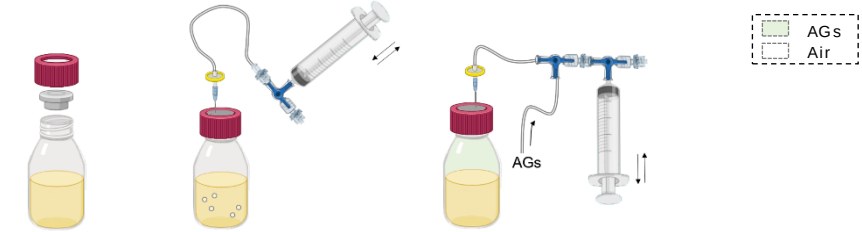
Tighten the apertured cap and put the bottle into an airtight PP bag, squeeze/vacuum out as much air as possible and seal the neck of the bag with a rubber band [1]. Then place them together in the autoclave chamber. Autoclave at 121°C for 15 minutes.

The bag is allowed to be 1/3 to half filled with AGs to best prevent enteral oxygen from entering the bottle during autoclaving. If AGs contain hydrogen, do not fill the bag with AGs for safety.

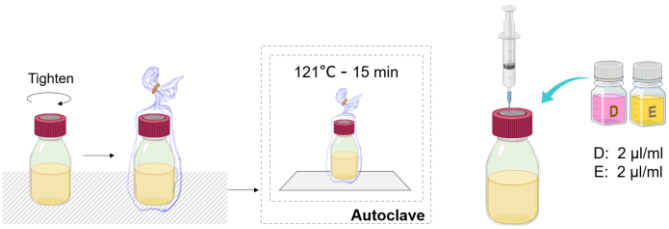
Step 8

Before use, aseptically add 2 µl of stock D (vitamin solution 1) and 2 µl of stock E (vitamin solution 2) per mL of broth solution into the bottle, suggested using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*) to inject the vitamin solution through the rubber stopper, carefully avoid introducing air into the bottle.

Squeeze the plunger until all air is expelled from the syringe needle before use.



Step 5 Step 6 Step 6.1 (Optional)



Step 7 Step 8

Storage

It is recommended that unused prepared YCFA liquid media and stock D/E should be packaged in an anaerobic atmosphere, vacuum sealed [3] together with the oxygen absorber [4][5] in plastic bags and stored at 4°C. The shelf life of prepared media is about half a year. The shelf life of stock D/E is about one year.

Storage Cautions:

When the liquid YCFA Medium is stored at low temperature and when the pH is lowered during the incubation process, a tiny amount of insolubles may precipitate slowly, which is hemin-mediated condensed protein/peptide (see *FAQ 14*) with low solubility, and only needs to be left to precipitate to the bottom of the bottle or the bottom of the tube, which is normal and will not affect the growth of bacteria.

.....

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

Common-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 according to the instructions.

Step 2

Dissolve 21.44 g of mixture A and 3.1 ml of stock C in 900 ml double distilled or MQ water in a GL45-cap reagent bottle.

Operating in a fume hood when handling stock C (Volatile fatty acids) is strongly recommended.

From Step 2.1 to Step 2.3 is to heat the solution of mixture A and stock C, which can be skipped. However, heating the solution will enable sufficient reaction and solubility of the contents in mixture A and stock C, achieving better performance of the broth.

Step 2.1 - (optional)

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

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 airtight PP bag is used to prevent the reagent bottle from inhaling oxygen-containing air. It also isolates any leaked internal liquid/vapour from escaping into the open air.

Step 2.3 - (optional)

Boil the prepared 900 ml broth solution in a microwave oven for 5~10 minutes, then leave the prepared 900 ml broth solution to rest and cool to room temperature.

Observe if the rubber stopper is deformed by the pressure and bubbles appear in the liquid, switch off the microwave heating immediately when boiling starts, and switch on again when the

rubber stopper has recovered its shape and the bubbles have disappeared (liquid has cooled down).

⚠ **Safety caution.** Please refer to *FAQ 15* for detailed suggested instructions.

Step 3

Add 5 g of mixture B and 2.05 ml of stock F to the 900 ml of prepared broth. Seal the bottle with a butyl rubber stopper [2] and screw an apertured PBT cap [2] onto the bottle. Leave for a few minutes until the foam disappears, make up to 980 ml with double-distilled or MQ water and adjust the pH to 7.4 ~ 7.5 with 1M KOH (stock F) or 1M HCl.

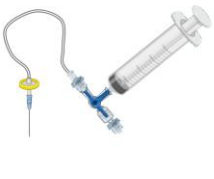
Mixture B contains $NaHCO_3$. When it meets acidic liquid, it will react and release carbon dioxide, and the foam will disappear after a few minutes of resting.

After Mixture B is dissolved, a tiny amount of insoluble material may remain, which is hemin-mediated condensed protein/peptide (see *FAQ 14*). After adjusting the pH to basic, most of the insolubles will disappear. The remaining insolubles will not affect the bacterial culture.

Freshly prepared YCFA broth will generally be acidic, with a pH range of 5 to 6. Using 5M KOH for rough adjustment and then 1M KOH for fine adjustment can save preparation time.

Step 4

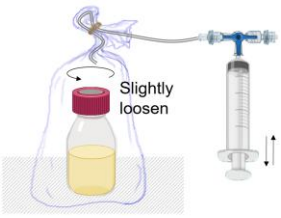
Rest the broth solution and let the insolubles precipitate, filtrate or directly pour the supernatant into two prepared clean reagent bottles.



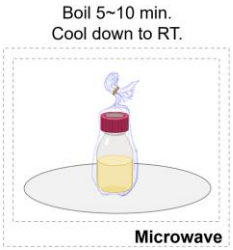
Step 1

Step 2

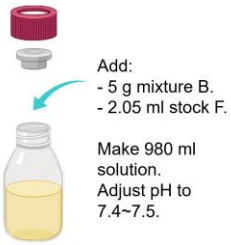
Step 2.1 (optional)



Step 2.2 (optional)



Step 2.3 (optional)



Step 3

Step 4

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

Step 5

Aliquot the broth solution into anaerobic tubes (e.g. Hungate Tubes), 10 ml per tube (1/3 ~1/2 of the tube volume, but no more than 1/2), and seal the tubes.

Step 6

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

Tips: Rest the vacuumed solution for about 10 minutes to check its colour, if turned to pale yellow or colourless, it means the oxygen has been totally removed and ready for autoclave. If the colour is still pink/red, repeat the vacuum process to remove the oxygen.

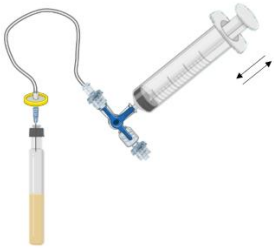
Step 7

Use rubber bands to hold the vacuumed anaerobic tubes together, place them in an airtight PP bag [2], 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.

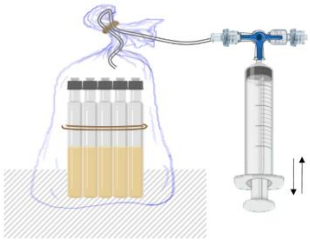
Step 8

Before use, puncture the rubber stopper and inject 20 µl of stock D (vitamin solution 1) and 20 µl of stock E (vitamin solution 2) aseptically (operate on a clean bench) into each Anaerobic tube (2 µl/mL broth solution) using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*).

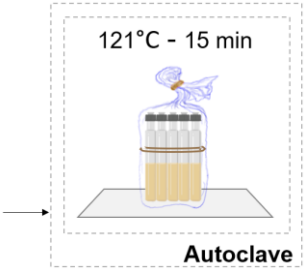
Step 5



Step 6



Step 7 (to be continued)



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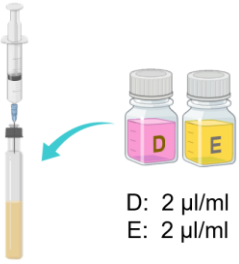


Step 8

To prepare YCFA 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.

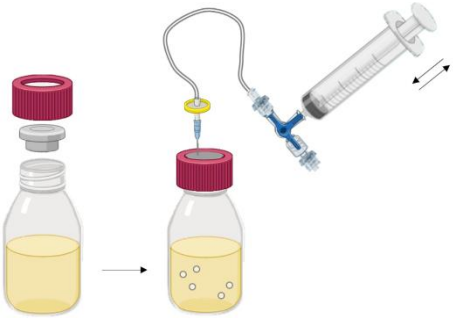
Tips: Rest the vacuumed solution for about 10 minutes to check its colour; if it turns pale yellow or colourless, it means the oxygen has been totally removed and is ready for autoclave. If the colour is still pink/red, repeat the vacuum process to remove the oxygen.

Step 6

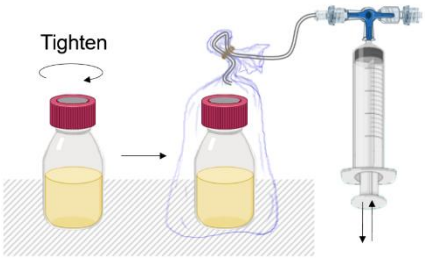
Tighten the apertured cap and put the bottle into the airtight PP bag [2], vacuum the bag with the assembled vacuum device [2] or oil-free vacuum pump and seal the bag, then autoclave at 121°C for 15 minutes.

Step 7

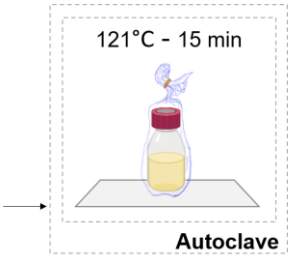
Before use, puncture the rubber stopper and inject 2 µl/ml of stock D and 2 µl/ml of stock E aseptically (operate on a clean bench) into the broth using a sterile syringe (preferred ultra-fine syringes shown in *section 2.1*).



Step 5



Step 6 (to be continued)



(Continued) Step 6

Step 7

Storage

It is recommended that unused prepared YCFA liquid media and stock D/E should be vacuum sealed [3] together with the oxygen absorber in plastic bags and stored at 4°C. The shelf life of prepared media is about half a year. The shelf life of stock D/E is about one year.

Storage Cautions:

When the liquid YCFA Medium is stored at low temperature and when the pH is lowered during the incubation process, a tiny amount of insolubles may precipitate slowly, which is hemin-mediated condensed protein/peptide (see *FAQ 14*) with low solubility, and only needs to be left to precipitate to the bottom of the bottle or the bottom of the tube, which is normal and will not affect the growth of bacteria.

.....

[1] Included in the YCFA 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] 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 YCFA anaerobic tubes

3.1. Summary

Product name	YCFA media broth in anaerobic tubes
SKU	GMNB-YCFA03-T020
Volume	10 ml broth in 20ml anaerobic tubes

3.2. Instructions for use

Stock D/E is prepared and bottled by filter sterilisation in air. Before first use, it is recommended to place it in an anaerobic atmosphere [1] (in an anaerobic bench or an anaerobic glove box filled with anaerobic incubation gas) and loosen (half unscrew) the cap of the bottle for 30 minutes to allow for an efficiently remove of the dissolved oxygen before proceeding with sampling and use. After use, the cap of the stock D/E bottle containing the remaining liquid should be tightened in an anaerobic atmosphere, and the stock D/E bottle should be placed in a sealed bag together with the oxygen absorber and stored at 4°C. If conditions allow, the sealing bag can be sealed with a vacuum sealer [2] to achieve a better oxygen-free condition.

The YCFA medium in the anaerobic tubes is prepared and filled under a pure nitrogen atmosphere. Before first use, it is recommended to sterilise the surface of the tubes by moistening with 70% alcohol, first with the cap and then unscrewing and wiping the threads and the rubber stopper. Unplug the rubber stopper in an anaerobic atmosphere (in an anaerobic bench or anaerobic glove box), or puncture the rubber stopper with a syringe needle to pump out the nitrogen gas and replace it with the anaerobic atmosphere, then add/inject stock D/E (Vitamin solution) and samples containing bacteria into the tubes.

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.

.....

[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

- Sterilise the anaerobic bench or anaerobic glove box with UV before use.
- It is recommended to sterilise reagent packages with 70% alcohol wiping on the surface before placing them into the anaerobic bench or in the anaerobic glove box.
- It is also recommended that anaerobic tubes are sterilised by wiping the opening at the top of the plastic cap and the corresponding exposed rubber stopper with cotton swabs containing 70% alcohol before puncturing with a syringe needle.
- It is recommended to use a pre-sterilised 23/25G (a minimum 20mm needle length) disposable syringe needle.
- The needle should be punctured vertically through the rubber stopper. Do not puncture the same site of the rubber stopper repeatedly to prevent loss of air-tightness, and it is recommended to puncture as far away as possible from the previous needle hole. Be aware of the

risk of cross-contamination and do not use the same needle to puncture different anaerobic tubes.

- For strictly anaerobic cultures, samples containing bacteria also need to be deoxygenated before injection (repeated vacuuming of the sample chamber followed by injection of AGs).
- After prolonged exposure to oxygen, YCFA medium containing Resazurin will slowly change its colour from pale yellow or colourless to light pink/red.

3.4. Cautions

When the liquid YCFA Medium is stored at low temperature and when the pH is lowered during the incubation process, a very small amount of insolubles may precipitate slowly, which is hemin-mediated condensed protein/peptide (see *FAQ 14*) with low solubility, and only needs to be left to precipitate to the bottom of the bottle or the bottom of the tube, which is normal and will not affect the growth of bacteria.

Appendix

I. Anaerobic Preparation Kit - Lite

Product name	Anaerobic Preparation Kit Plus
SKU	GMNB-APK01

Application

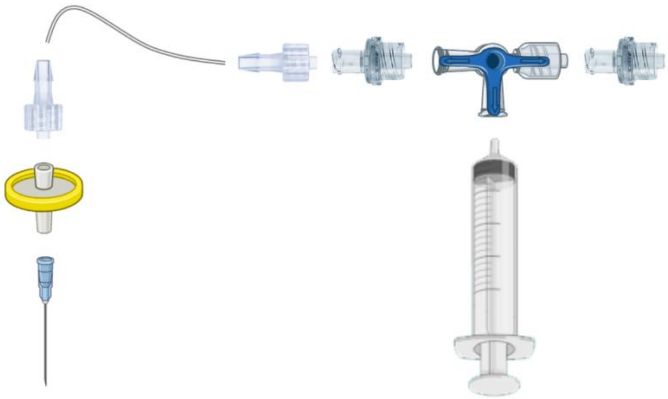
Used for vacuum-method anaerobic media preparation without AGs.

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 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

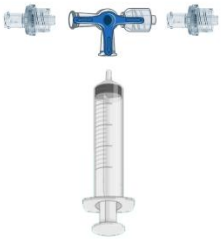
Module B: for preparing nitrogen from air (deoxygenate).

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.

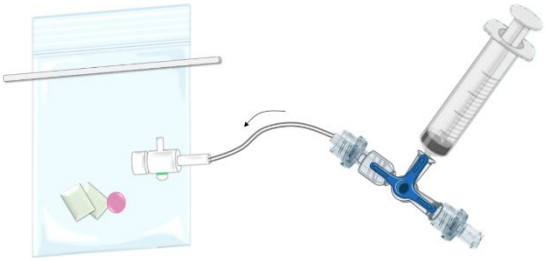
Note:

- 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.

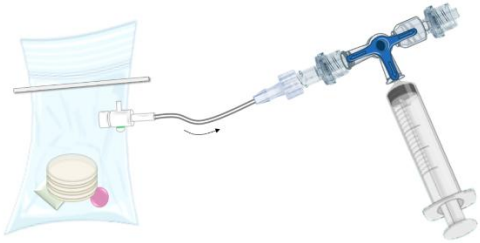


5. Switch off the PTFE valve. Allow the bag to rest (approximately 48 hours) and wait for the oxygen absorber to remove all oxygen from the bag until the colour of the oxygen indicator returns to red (prune red/red to watermelon red).
6. Connect the PTFE air valve to the 3-way valve using the PTFE tube (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.

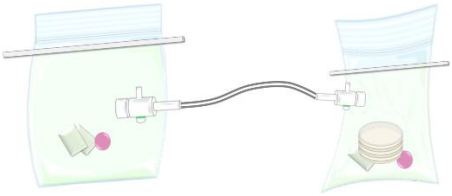
For anaerobic cultivation

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, following 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

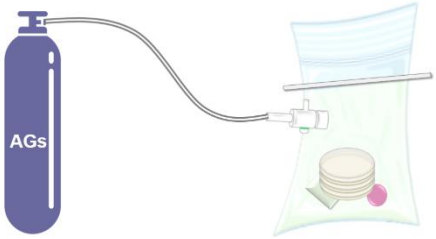
assembled air pump (Assembly of Module A: step 1.) and connect the PTFE air valve to the Luer male connector using a tube (φ6.0mm). Vacuum out all the air inside the bag using the air pump, and switch off the PTFE valve.



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

A1. Firstly, to prevent the foul-smelling fumes of the YCFA broth from escaping into the atmosphere when it is heated in a microwave oven or autoclave chamber.

Secondly, to prevent inhalation of the oxygen-containing air when the heated reagent bottle and anaerobic tubes cool down, because the container is then under negative pressure.

Q2. How to prepare YCFA media if there is no AAE, AGs, or Anaerobic Preparation Kit?

A2. Please follow the fast preparation instructions on our webpage. However, this cannot guarantee the effectiveness of the prepared media solution for strict anaerobic tests and culturing.

Q3. Is the material of the stopper important?

A3. Brombutyl rubber stoppers can provide better airtightness than silica rubber and keep airtight even after punctures with a needle.

Q4. Why should the reagent bottle should be tightened with a rubber stopper and aperture cap before autoclaving? Is there a risk of the bottle exploding during autoclaving?

A4. No. Once the rubber stopper has been punctured, the needle hole can be closed and kept airtight at normal pressure, whereas if the internal pressure is extreme, vapour can escape through the needle hole and the bottle will not explode.

Q5. Why the bottle cap should be loosened before being put into the microwave oven?

A5. This is to prevent the temperature of the liquid from getting too high in order to preserve the fatty acid content.

If the cap is tightened, the internal pressure may increase as the liquid heats up, raising the boiling point and causing the fatty acid to evaporate during boiling and be lost the next time the cap is opened.

Q6. Is the airtight PP bag plus the rubber stopper effective to be used for autoclaving the liquid in the bottle?

A6. External pressure in the autoclave chamber can pressurise the inert gas in the bag, and inert gas in the bag pressurises the gas in the bottle via the rubber stopper (a small amount of gas exchange is allowed between the bag and the bottle), finally, the balanced pressure in the bottle is equal to that in the autoclave chamber.

Another fact is that the majority proportion of the bottle's internal extreme pressure is caused by the liquid's evaporation (total partial pressures of each substance in the liquid phase) when hot, not transmitted from outside.

The sterilisation will be guaranteed as long as the pressure and temperature reach the designed level (121 °C / 205 kPa).

Q7. Can the airtight PP bag be used in an autoclave with the 'vacuum pulse' and 'support pressure' functions?

A7. The airtight PP bag included in the media kit is thin but durable; its airtightness is good even when sealed with a rubber band. Less internal inert gas/AGs have a chance to escape from the bag at low external pressure, especially in the case that the bag is sealed when half-filled. And even if the inert gas/AGs have been sucked out of the bag, it doesn't matter because its role is to prevent external oxygen from entering the bottle during autoclaving. Therefore, a vacuum pulse is not a problem.

The support pressure will also be OK as the rubber stopper and airtight PP bag plus internal inert gas/AGs form a flexible triple barrier that seals and prevents external gas (oxygen) from entering the bottle but allows pressure to be applied.

Q8. What colour indicates that the prepared broth is oxidised or contains oxygen?

A8. If the broth turns light pink/red rather than pale yellow or colourless, this means that there is oxygen in the broth and it is no longer suitable for strict anaerobic culture.

Q9. During preparation, the broth turns light pink/red, is this ok?

A9. Don't worry, it's normal for the broth to turn light pink/red, as long as the broth turns pale yellow just before (let the tubes/bottles rest for a while after vacuuming)/after autoclaving, the media is in good anaerobic condition.

Q10. Can the oxidised prepared YCFA broth be recovered for anaerobic culture?

A10. It is possible to recover the prepared YCFA broth after deoxygenation and prolonged exposure to anaerobic gas, but we don't recommend it because the media is easily contaminated during treatment, and the oxygen indicator loses its best colour change resolution (pink-colourless) after prolonged oxidation.

Q11. What are the best storage methods and conditions?

A11. Vacuum packaging with a bag of oxygen absorber and store in a refrigerator at 2~6 degrees centigrade.

Q12. Is a third-party anaerobic gas generating bag (e.g. Oxoid Anaerogen Sachet / Mitsubishi Anaerobic Gas Generating Bag) compatible?

A12. Third-party anaerobic gas-generating bags can be placed in the PP autoclave bag supplied with an Anaerobic Preparation Plus Kit to generate anaerobic gas and/or to anaerobically incubate tubes and plates.

Q13. Sterilisation notes for working in an anaerobic bench/glove box.

A13. As most commonly used anaerobic benches/glove boxes don't incorporate laminar or vertical HEPA-filtered airflow to best prevent microbial contamination, a minimum of 20 minutes of UV radiation and the use of 70% alcohol spray on the bench and wiping the surface of each bottle/container before operation is a must.

Don't forget to sterilise the rubber stopper and aperture cap, especially the area where the needle will be punctured.

Q14. What is the precipitated insoluble matter that occurs during the preparation of YCFA media and after culturing bacteria for a period of time?

A14. The precipitated insoluble matter is hemin-mediated condensed protein/peptide **[1]**. This phenomenon is common when bacteria are cultured with high concentrations of peptone plus blood components, and does not affect bacteria cultured normally.

Based on our experience, precipitation usually occurs during the preparation of YCFA media (before adjusting the pH to 6.5~7) and after prolonged culture, both at a relatively low pH. We deduced that the pI (isoelectric point) value of the flocculated protein is lower than 7. We have included some pictures of the precipitation during the preparation of the YCFA media for the customer's information.

[1] *Hemin is a key component of the coagulation process and can bind many proteins with specific affinity, altering their conformation and physicochemical properties. The core structure of Hemin, an iron porphyrin with iron ions prominently exposed from the porphyrin plane, binds to proteins with specific structures rich in amino acids such as histidine, altering the conformation of the protein (the imidazole side chain of the histidine residue commonly serves as a ligand in metalloproteins. An example is the axial base attached to Fe in*

myoglobin and haemoglobin), causing the proteins to cross-link together to form intermolecular network structures. Macroscopically, proteins can significantly change the pI value by cross-linking with each other, resulting in flocculation in solution.

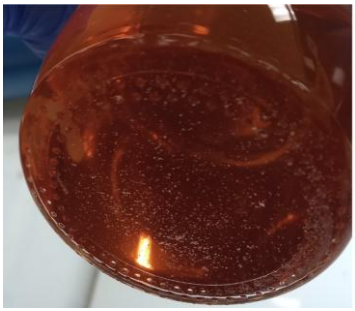


Figure 1 (Left)
White precipitate appears when Hemin/Cys-HCl is added to the broth, and the pH of the solution is around 4.7

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

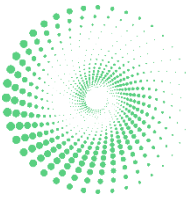
Q15. Why should the broth be boiled after adding volatile organic acid? What is the best and safest way to heat the broth in a sealed bottle in the microwave?

A15. This step aims to maximise the reaction between the volatile organic acid and the peptide and lipid content of the broth, producing organic esters or amides that mimic the chemical environment of the gut. At the same time, the pH of the broth is adjusted to avoid excessive acidity so that it cannot directly react with $NaHCO_3$ in the subsequent step, which releases carbon dioxide vigorously and forms alkali metal salts of the organic acid, facilitating the evaporation of the volatile organic acid during the subsequent autoclaving.

The boiling process should be conducted at 100°C for 5~10 minutes or at 85~95°C for 20~30 minutes. It is recommended to heat the broth using a water bath or microwave oven.

If using a water bath, the heating time can be doubled. Heat the broth at 85~95°C for 20~30 minutes. **DO NOT** open the bottle until cools down. Otherwise, there will be a risk of explosion when opening the cap, due to the steam pressure.

When using a microwave oven, to prevent explosions and to take into account the difficulty of visually observing a deformed rubber stopper, it is suggested to use a surface infrared thermometer to monitor the bottle's surface temperature and indirectly estimate the liquid's temperature (the surface temperature of the bottle is usually slightly lower than the liquid's temperature). Based on our experience, heating 500 ml of broth with a 1000-watt microwave in 30-second pulses, followed by the gentle elliptical swirling of the bottle (**DO WEAR** heat-resistant gloves) and checking with the thermometer, will take around 2~3 minutes to heat the liquid to 80~100°C. Maintain the temperature for the next 10-20 minutes by applying 10~15 second pulses every 2 minutes. **DO NOT** open the bottle until it has cooled down. Allow the broth to rest and cool to room temperature before proceeding to the next step.



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