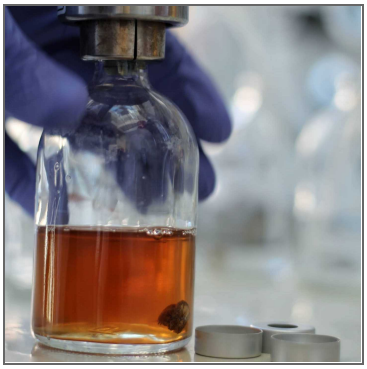


OCT 02, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.36wgq779kvk5/v1

Protocol Citation: Julia Vinzelj, Diana Young, Akshay Joshi, Sophia Strobl, Ljubica Begovic, Nico Peer, Magdalena Nagler, Rolf Warthmann, Veronika Flad, Urs Baier, Michael Lebuhn, Sabine Podmirseg 2023. Medium without CRF for Neocallimastigomycota. **protocols.io** https://dx.doi.org/10.17504/protocols.io.36wgq779kvk5/v1

License: This is an open access protocol distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Medium without CRF for Neocallimastigomycota

Julia Vinzelj[1], Diana Young[2], Akshay Joshi[3], Sophia Strobl[1], Ljubica Begovic[1], Nico Peer[1], Magdalena Nagler[1], Rolf Warthmann[4], Veronika Flad[2], Urs Baier[4], Michael Lebuhn[2], Sabine Podmirseg[1]

[1]University of Innsbruck, Austria;
[2]Landesanstalt für Landwirtschaft (LfL), Freising, Germany;
[3]Zurich University of Applied Sciences (ZHAW), Wädensil, Switzerland;
[4]Zurich University of Applied Sciences (ZHAW), Wädenswil, Switzerland

HiPoAF

Julia Vinzelj

DISCLAIMER

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

The protocol content here is for informational purposes only and does not constitute legal, medical, clinical, or safety advice, or otherwise; content added to protocols.io is not peer reviewed and may not have undergone a formal approval of any kind. Information presented in this protocol should not substitute for independent professional judgment, advice, diagnosis, or treatment. Any action you take or refrain from taking using or relying upon the information presented here is strictly at your own risk. You agree that neither the Company nor any of the authors, contributors, administrators, or anyone else associated with protocols.io, can be held responsible for your use of the information contained in or linked to this protocol or any of our Sites/Apps and Services.

Protocol status: Working
We use this protocol and it's working

Created: Feb 01, 2022

Last Modified: Oct 02, 2023

PROTOCOL integer ID: 57642

Keywords: anaerobic fungi, cultivation medium, anaerobic cultivation, neocallimastigomycota

ABSTRACT

This is the protocol for the standard, nutrient-rich, undefined cultivation medium *without* clarified rumen fluid (CRF) used within the HiPoAF project.

In previous publications this medium or slight variations of it have also been referred to as "defined medium" (since it does not contain CRF) - this, however, is not completely true, since it does contain undefined components (yeast extract, and tryptone).

If you need a completely defined medium, check out our protocol "Minimal Medium for AF".

IMAGE ATTRIBUTION

Image taken by Julia Vinzelj

MATERIALS

Here we list the ingredients of our standard, nutrient**-rich AF cultivation medium (*without* clarified rumen fluid), and the preparation of stock solutions needed for this medium:**

	Ingredient	Amount per litre
	Hemin Solution	2mL
	L-cysteine-HCl	1g
	MilliQ water	700mL
	Resazurin Solution	2mL
	Salt Soluion I	150mL
	Salt Solution II	150mL
	Sodium hydrogen carbonate (NaHCO3)	6g
	Tryptone	10g
	Xylan powder	2g
	Yeast Extract	3g
	Cellobiose	3g

Ingredients of 1 litre of standard cultivation medium with CRF. Xylan and cellobiose function as C-sources for AF and can be replaced by other C-sources (e.g. wheat straw, rice straw, avicel, glucose, etc.) as needed. For our culture collection we omit the cellobiose and just add xylan and wheat straw as C-

sources.

Hemin solution is prepared by dissolving [M] 1 mg/mL hemin powder in a 1:1 mixture of 96% ethanol and [M] 0.05 Molarity (M) NaOH. The solution is then filter sterilzed (0.22µm pore size) and stored at 4 °C until use.

Resazurin solution is prepared by dissolving [M] 1 mg/mL resazurin powder in MilliQ water and filter sterilizing it (0.22µm pore size). The solution is then stored at 4 °C until use.

Salt Solution I consists of [M] 3 g/L K_2HPO_4 dissolved in MilliQ water. It is autoclaved (121 °C , 00:20:00), and stored at 4 °C until use.

Salt Solution II consists per litre of 3 g KH_2PO_4, 6 g $(NH_4)_2SO_4$, 6 g NaCl, 0.6 g $MgSO_4.7H_2O$, and 0.6 g $CaCl_2.2H_2O$. The ingredients are dissolved in MilliQ water one after the other. The solution is then autoclaved (121 °C , 00:20:00), and stored at 4 °C until use.

Ingredient	g/L
CoCl2.6H2O	0.05
CuSO4	0.021
FeSO4.7H2O	0.20
H3BO3	0.25
MnCl2.4H2O	0.25
Na2MoO4.2H2O	0.28
Na2SeO3	0.04

	Ingredient	g/L
	NaVO3	0.03
	NiCl2.6H2O	0.25
	ZnSO4.7H2O	0.026

Trace Elements Solution composition. The chemicals are dissolved in 0.2M HCl solution. The solution is then aliquoted, autoclaved (121 °C , 00:20:00), and stored at 4°C.

PAS-212 antibiotic stock solution is prepared by slowly dissolving [M] 20 mg/mL Streptomycin sulfate, [M] 20 mg/mL Penicillin G sodium salt, and [M] 10 mg/mL Ampicillin sodium salt in distilled water. The mixture is then gassed with pure CO_2 for 00:10:00 , then closed with a rubber stopper and crimped to obtain and maintain anoxic conditions. With the help of needle and syringe the solution is filter sterilized into a sterile, anoxic, closed & crimped serum bottle and stored at 4 °C until use.

	Ingredient	g/L
	Biotin	0.2
	Calcium Pantothenate	0.6
	Folic acid	0.05
	Niacin	0.6
	Nicotinamide	1.0
	p-Aminobenzoic acid	0.05
	Pyridoxamine	0.1
	Riboflavin	0.2
	L-Thiamine hydrochloride	0.01
	Vitamin B12 (Cyanocobalamin)	0.02

Vitamin Solution composition. The chemicals are dissolved in MilliQ water and are then filter sterilized (0.22µm pore size) into aliquots. Niacin and Nicotinamide should be added under an extraction hood. Store at 4°C.

Serum bottles. For standard AF cultivation, we usually use glass serum bottles with an ND20 head and a total of volume of 120 mL. In those serum bottles we do not add more than 50 mL medium, to ensure an adequate amount of head space. Alternatively, smaller serum bottles with a total volume of 60 mL also work (filled with no more than 30 mL of medium). We recommend using the bulky serum bottles rather than the long, thin ones, because the longer, thinner ones tend to easily break and are less convenient when handling the bottles.

Rubber stoppers. The bottles are usually closed with 3-legged butyl rubber stoppers or with full-body black rubber stoppers, and crimped with a metal cap.

SAFETY WARNINGS

Safety information

Please make sure that you attend to all safety regulations regarding the handling of CO_2 gas.

Safety information

Be also aware of the risks of autoclaving closed serum bottles, and make sure your autoclave has an appropriate program for that.

BEFORE START INSTRUCTIONS

Before you start, make sure you have prepared all necessary stock solutions, and have all chemicals needed at hand (see Materials section for details).

You will need approx. 03:00:00 in total to prepare the medium (depending on the volume you prepare, it can take longer or might be faster).

Safety information

Please also ensure, that all necessary safety measures are in place (see also: Safety Warnings).

1 Add Salt Solution I, Salt Solution II, Yeast Extract, Tryptone, Hemin Solution, Resazurin Solution, Xylan powder (as an additional C-source for AF; can be omitted), and MilliQ water to a cooking pot.

2 Heat the mixture and simmer it until the colour changes to dark yellow. For us with 2 L of medium in a 5 L cooking pot, this takes about 00:20:00 to 00:30:00 . 30m

Note

Take care not to cook it too long. Otherwise you will lose a lot of water, making the medium more concentrated. You can also carefully heat the mixture in a microwave.

2.1 While the mixture is simmering, flush a large enough Erlmeyer flask (best chose one with two times the volume of the medium) with pure CO_2 for 00:02:00 . 2m

3 Carefully pour the mixture from the pot into the Erlmeyer flask. Cool it with ice-water while agitating it by magnetic stirrer and bubble it with pure CO_2.

4 Once the mixture has cooled to around 39 °C, add the trace elements solution, $NaHCO_3$, soluble C-source (like cellobiose, glucose, etc.; if necessary) and lastly the L-cysteine-HCl. Keep gassing

with CO_2, but adjust the gas flow in order to avoid bubbling/overflowing of the medium. Bubble with CO_2 until the medium turns yellow again.

4.1 If you want to have a simple, nutrient-rich cultivation medium for anaerobic fungi without clarified rumen fluid add milled wheat straw or pressed wheat straw pellets to your serum bottles. The combination of a complex C-source like wheat straw and a less complex one like xylan helps keeping the metabolism of AF active. Using less complex C-sources like glucose or cellobiose also works well, but it might inhibit parts of the carbohydrate metabolism of AF and can give rise to bacterial or yeast contamination if you are not handling your cultures carefully.

Note

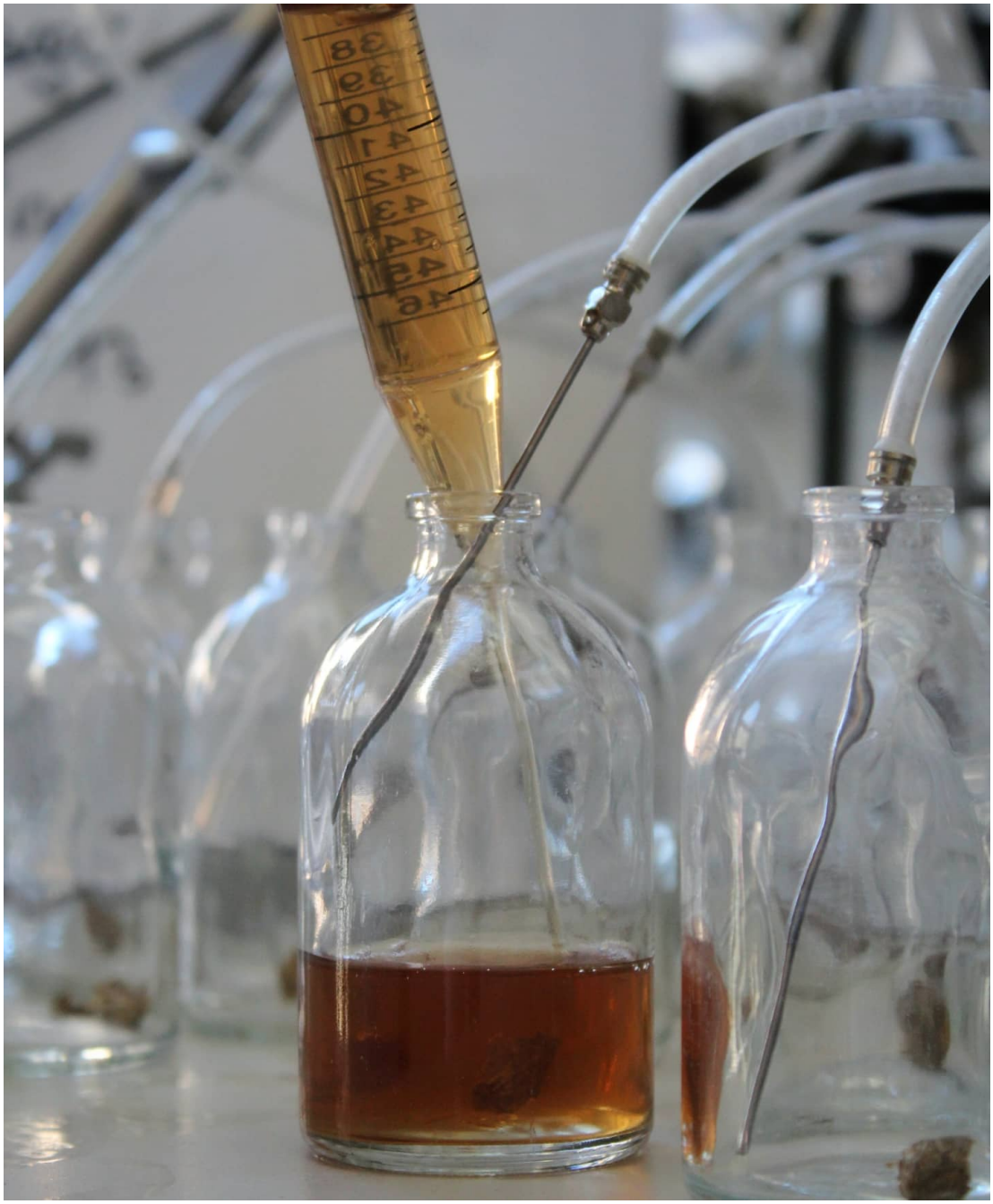
For example: for our standard culture collection medium, we use serum bottles with a total volume of 120 mL. We add 0.35 g wheat straw to those bottles, and add 45 mL medium (in step 6). We later add 0.5 mL vitamin solution only shortly before use and sub-culture 5 mL of an actively growing culture into this medium, which brings the total volume of liquids in this bottle up to 50.5 mL.

Pro-tip: For culture care (not for experiments), the pressed pure wheat straw pellets (e.g. horse feed as you might find in a pet store) are way more convenient to handle! You can simply eyeball the amount needed.

5 Once the medium turned yellowish, set the pH of the medium to pH 6.9 with the help of $NaHCO_3$ powder or 5M NaOH solution.

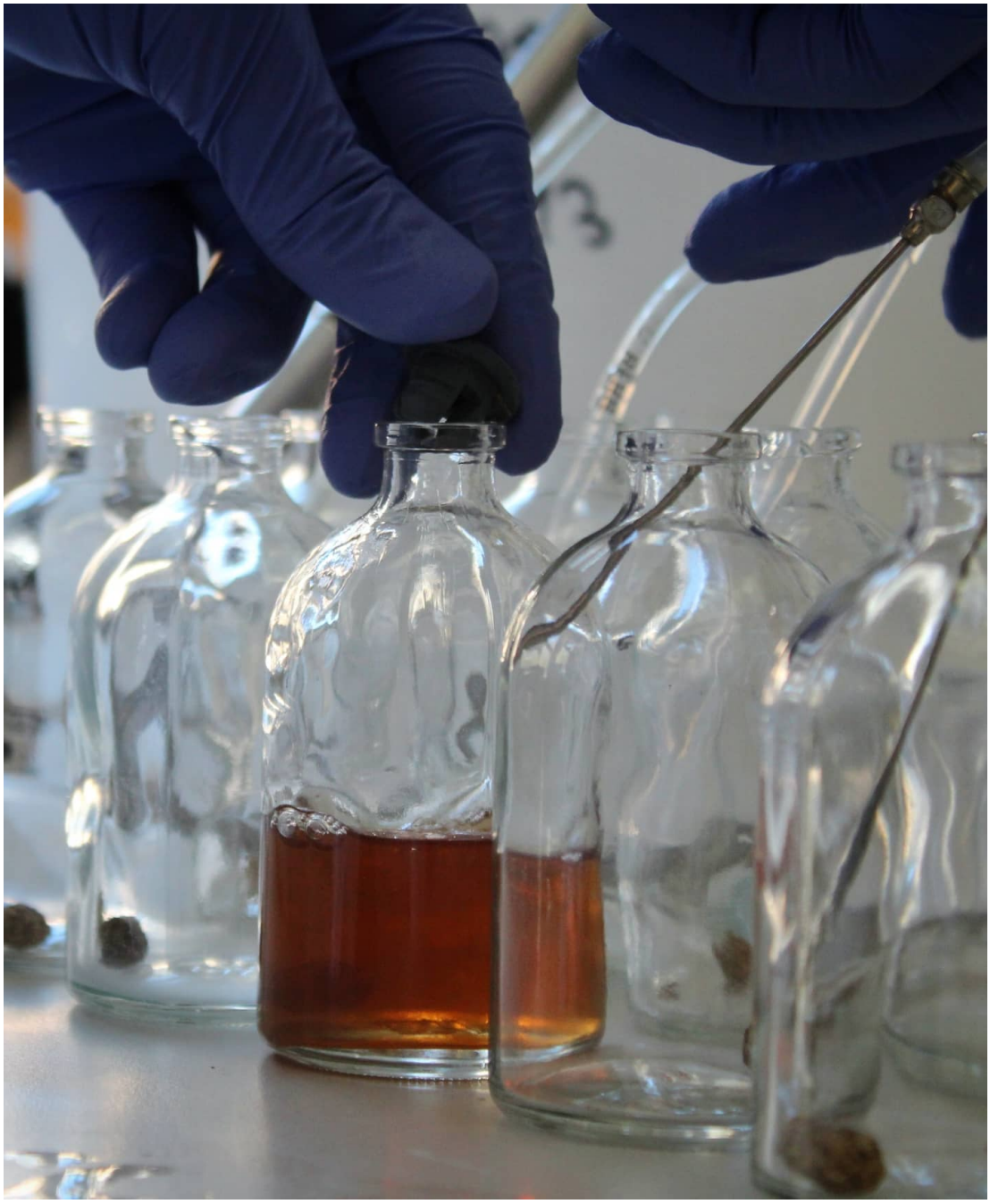
5.1 While setting the pH, flush your serum bottles with pure CO_2 for 1-2 minutes, to minimize oxygen contamination when aliquotting your medium in step 6.

6 Aliquot the medium into your serum bottles under continuous flow of CO_2 in the serum bottles and the Erlmeyer flask filled with medium. Depending on the amount of medium prepared this step takes around 00:30:00. 30m



Dispension of the medium into 120mL glass serum bottles (max. 50mL per bottle).
Picture copyright: Julia Vinzelj & Nico Peer

6.1 Take the gas hose out of the serum bottle and immediately close it with a rubber stopper, then crimp the bottle with an aluminium cap.

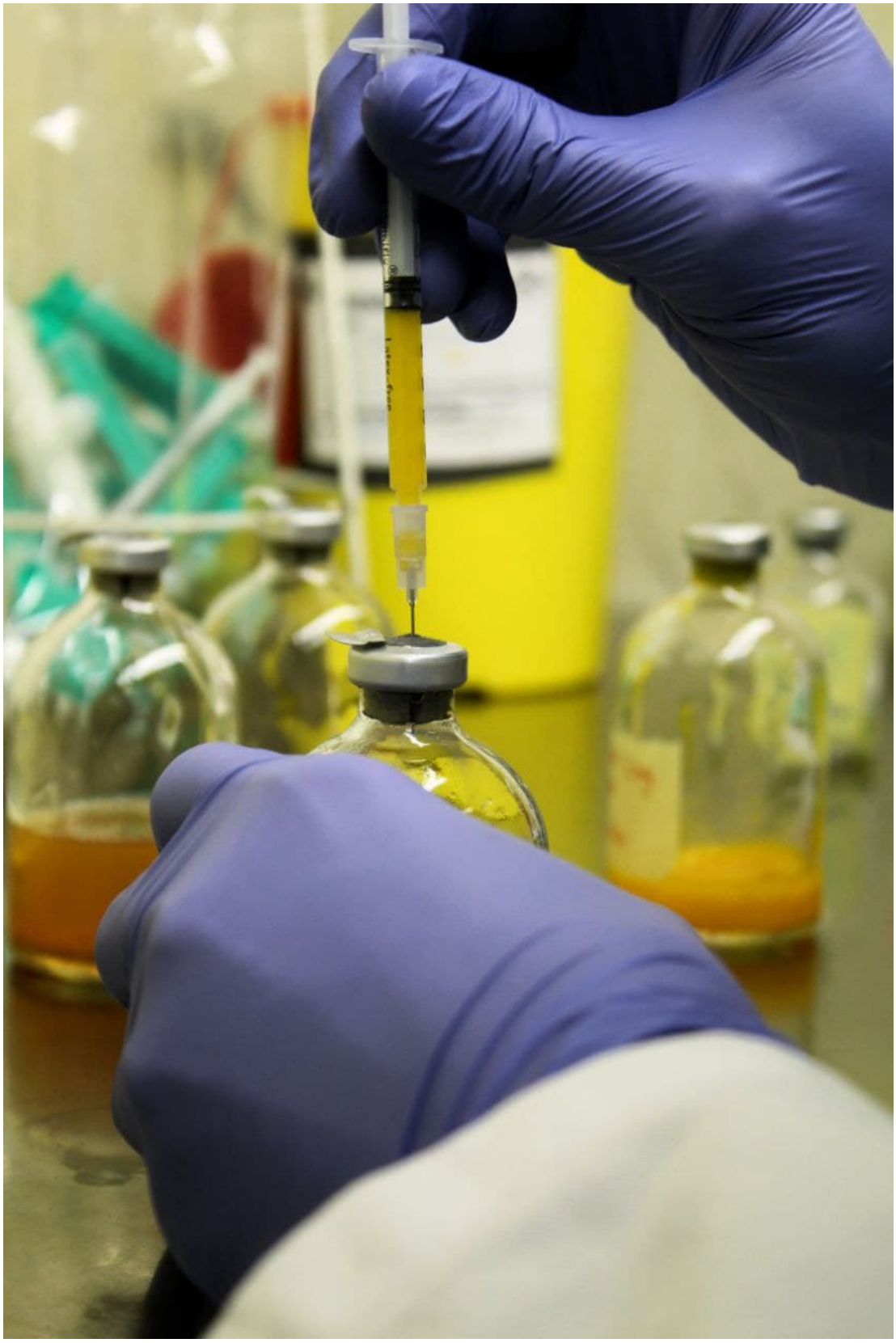


Closing of the serum bottles with 3-legged butyl-rubber stoppers after the medium has been dispensed.
Picture copyright: Julia Vinzelj & Nico Peer.

7 Autoclave the serum bottles at 121 °C for 00:20:00 . 20m

8 Store the bottles without regard to temperature for up to 2 months. 4 °C works as well as 37 °C for the duration of 2 months at least. Discard any bottles with medium that turned red.

8.1 Before use, they should be warmed to 39 °C. Also, 0.01mL vitamin solution must be added per mL of medium shortly before use by injection. If needed, antibiotics can be injected as well.



Injection of Vitamin Solution into sterile, pre-warmed serum bottles shortly before use.
Picture copyright: Julia Vinzelj & Nico Peer