Strain Requirement Checklist

The following checklist can be used as a quick reference to ensure your strains meet our submission criteria.

Samples that do not meet one or more of the criteria below can be refused, or fail to sequence entirely, so please ensure you read the entire document to avoid any issues or delays.

Tubes:	Receive tubes from us	☐
Sample ID Labels:	Affix sample ID labels lengthwise	☐
Sample Info:	Upload to project portal	☐
Culture:	Grow your bacteria	☐
Transfer:	4×10^9 - 6×10^9 cells or 30 - 50 mg	☐
Ship:	Package and label correctly	☐

Overview

Preparing bacteria for submission to MicrobesNG is easy - we provide tubes containing a buffer, which inactivates the cells and stabilises the DNA. The buffer also effectively inactivates pathogens so you can send bacterial samples from around the world, hassle free.

- GMO's and CL/BSL-3 strains accepted
- Ship at ambient temperature, through regular post

We do not grow your bacteria - instead, we extract DNA directly from the material that you send. For this reason, you need to send between **4 x 10^9 to 6 x 10^9** cells, or a pellet that weighs between **30 and 50 mg** when wet.

This protocol will give you some pointers on how to:

- Grow a pure culture
- Quantify the number of cells
- Harvest the cells
- Ensure that the ratio of cells to buffer is correct so that all the cells are properly inactivated, and the DNA is stabilised

You can see a visual summary of the process in Appendix A.

Inactivation Buffer

The tubes that we send you contain 500 µL of DNA/RNA Shield from Zymo Research. This buffer effectively inactivates pathogens, including tough-to-lyse microbes, without the need for any additional steps. Samples are stable at 4-25˚C for >2 years, and indefinitely at -20˚C. When exposed to low temperatures, the buffer may crystalise. This can be resolved by warming the buffer to room temperature and mixing.

We ask you to read the Safety Data Sheet for DNA/RNA Shield, which can be accessed via the manufacturer's website.

If you prefer to provide your own tubes, we require 2 mL screw cap tubes with an outer diameter of 11 mm, which is the standard diameter for microcentrifuge rotors. Tubes must contain 500 µL of DNA/RNA Shield, which can be purchased from Zymo, or one of their regional distributors. You then can print your sample ID labels from your project portal; note that we do not accept handwritten labels.

Sample Information

Before sending your samples, it's vital that you submit your sample information in your online project portal. We need to know the species of the organisms you are sending to ensure we can meet our guarantees on coverage, and provide the best QC possible.

If you do not know the species, we can accept identification to genus level, however this may impact the QC of your sequencing data. If you don't know the identity of your organism, then please contact us at info@microbesng.com.

We will not be able to accept your samples if you have not entered the sample information in your project portal.

Growing your cells

We all know how to grow bacteria, but here are some steps to ensure you send a pure, clonal bacterial strain. Purity is important for obtaining the highest quality assembly.

1. Prepare an agar plate of the strain you wish to sequence.
2. Visually inspect the plate to ensure there is a single phenotype.
3. Pick a single colony and subculture either in liquid broth, or on an agar plate.

If you grow your cells in liquid broth, we recommend that you resuspend a single colony in 200 µL of a suitable sterile buffer (e.g. PBS), and use 100 µL as an inoculum. Following culture, we would recommend plating an aliquot of this fresh growth to ensure no contamination occurred during the process.

If you grow your cells on agar plates, aim to have a lawn covering between ½ to ⅔ of the plate, and streak the rest of the plate to obtain single colonies to check the culture is pure. Depending on the species you may need to prepare more than one plate.

Quantifying your cells

There are several ways of quantifying bacterial growth. This protocol uses **O**ptical **D**ensity at **600** nm (**OD600**), which is suitable for many organisms - but not all. This method requires access to a spectrophotometer, and we recommend that you follow the relevant instruction manual for your specific instrument to ensure you obtain accurate readings. If you're new to OD, check out Appendix C.

If OD is not a good fit for your organism, you can also centrifuge your bacteria and send us a pellet that weighs between **30 and 50 mg** when wet. Alternatively, you might consider plate counts of serial dilutions (e.g. Miles & Misra) or flow cytometry.

If you subcultured your pure single colony into a **liquid broth**, you should aim to harvest cells during the mid log/exponential growth phase. This is typically between an OD600 of 0.5 - 1, depending on the organism you are culturing. Mix the culture thoroughly before measuring the optical density and blank using sterile broth.

If you grew your bacteria on an **agar plate**, create a bacterial suspension by harvesting ½ to ⅔ of total fresh growth from the plate with a sterile loop and resuspend it in 5 - 10 mL of a suitable sterile buffer (e.g. PBS). You can then blank the spectrophotometer with an aliquot of the sterile buffer, and measure the optical density of an aliquot of your bacterial suspension.

OD measures are only valid when they are below 1.0, so you may need to prepare a serial dilution of your bacterial suspension, with the series continuing until one of the dilutions has an OD below 1.0. You can then calculate the OD of the stock culture by multiplying the measured value by the dilution factor (Appendix C).

Harvesting cells

We require 4 x 10^9 - 6 x 10^9 cells - this ensures that the cells are inactivated and safe for transport and handling. A quick way to determine the number of cells is to use an equivalent OD[1] of 8 - 12.

For example, if you're harvesting a liquid culture at an OD600 of 0.8, you would need to process 10 - 15 mL. If the stock bacterial suspension you prepared from an agar plate has an OD600 of 80, you would need 0.10 - 0.15 mL. A worked example is provided in Appendix B.

[1] The equivalent OD is the OD value that a 1 mL cell suspension would have if it contained the desired number of cells

Take an appropriate volume and centrifuge to pellet the bacterial cells. Discard the supernatant, and wash the pellet with 1 mL of sterile buffer (e.g. PBS), before centrifuging a second time.

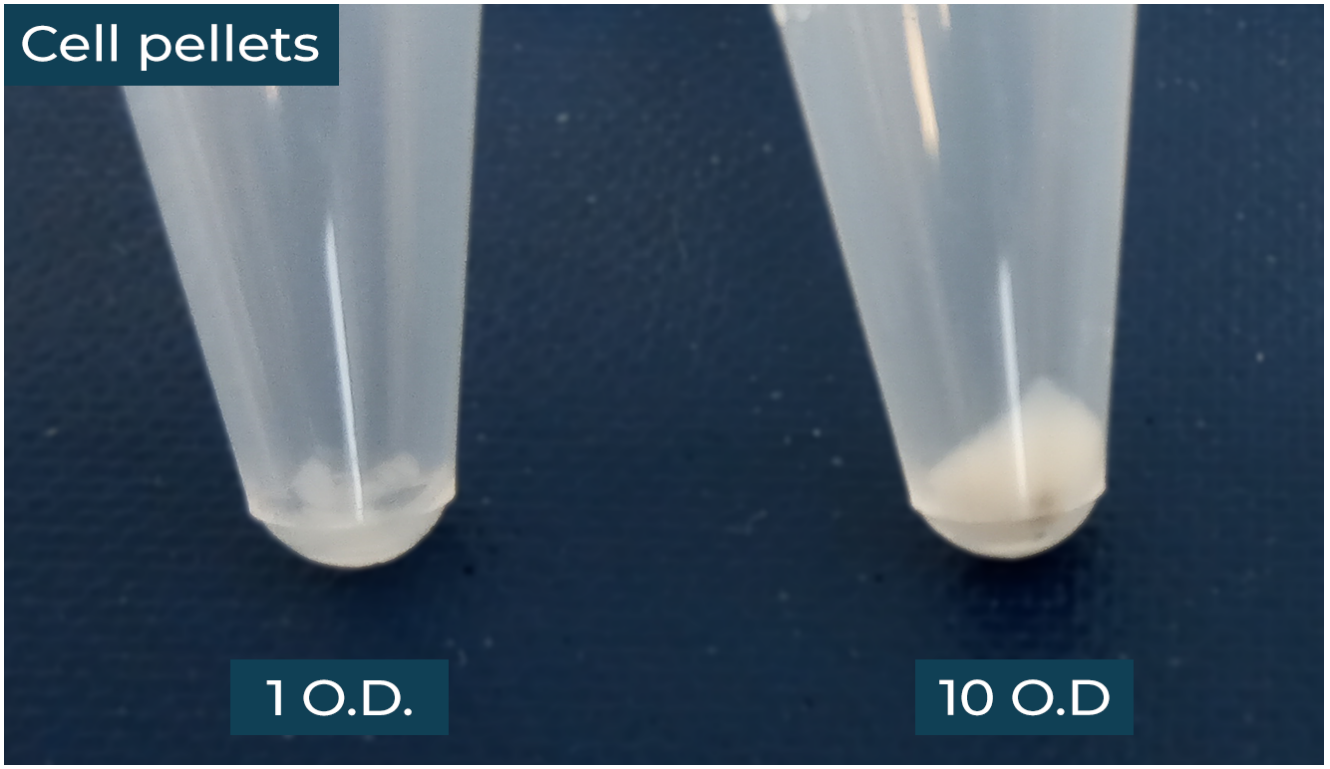


Figure 1. Pelleted *S. aureus* cells; the right tube contains cells at an equivalent OD600 of 10, or ~40 mg in wet weight. The pellet on the left shows an example of equivalent OD600 of 1, which is insufficient for submission.

Take 0.5 mL of the inactivation buffer from the sample tubes provided to you, and resuspend the bacterial pellet.

Transfer this resuspension back into your sample tube, ensure the lid is tightly fastened, and affix the MicrobesNG Sample ID label we sent with your tubes.

Please attach labels to your sample tubes lengthwise, **not around the tube** (Figure 2).

Figure 2. How to correctly apply labels to your tubes. Please use the tubes we provide, or if supplying your own, ensure they are 2 mL thread capped tubes containing 500 µL of DNA/RNA Shield Inactivation Buffer.

We will not be able to accept your samples if you use incorrect tubes or labelling, or if you have not entered the sample information in your project portal.

Shipping

If you expect your shipment to arrive within 5-7 days, the samples can be sent at ambient temperature. If your package will be in transit for longer than this, we recommend including ice packs.

To prevent your samples from leaking during shipment, place the tubes in a rigid tube box, with individual spacers. Placing your tubes in the box in order of the sample ID number greatly reduces handling, and will give you the best possible turnaround time.

If we sent your buffer tubes in a plastic box, please use this to ship your samples to us.

If you are sending more than one project in the same shipment, please **use a separate box for each project** and clearly label each box with the project number. If you are not able to use a box, please **separate tubes from different projects** in another way, e.g. inside a falcon tube or zip lock bag.

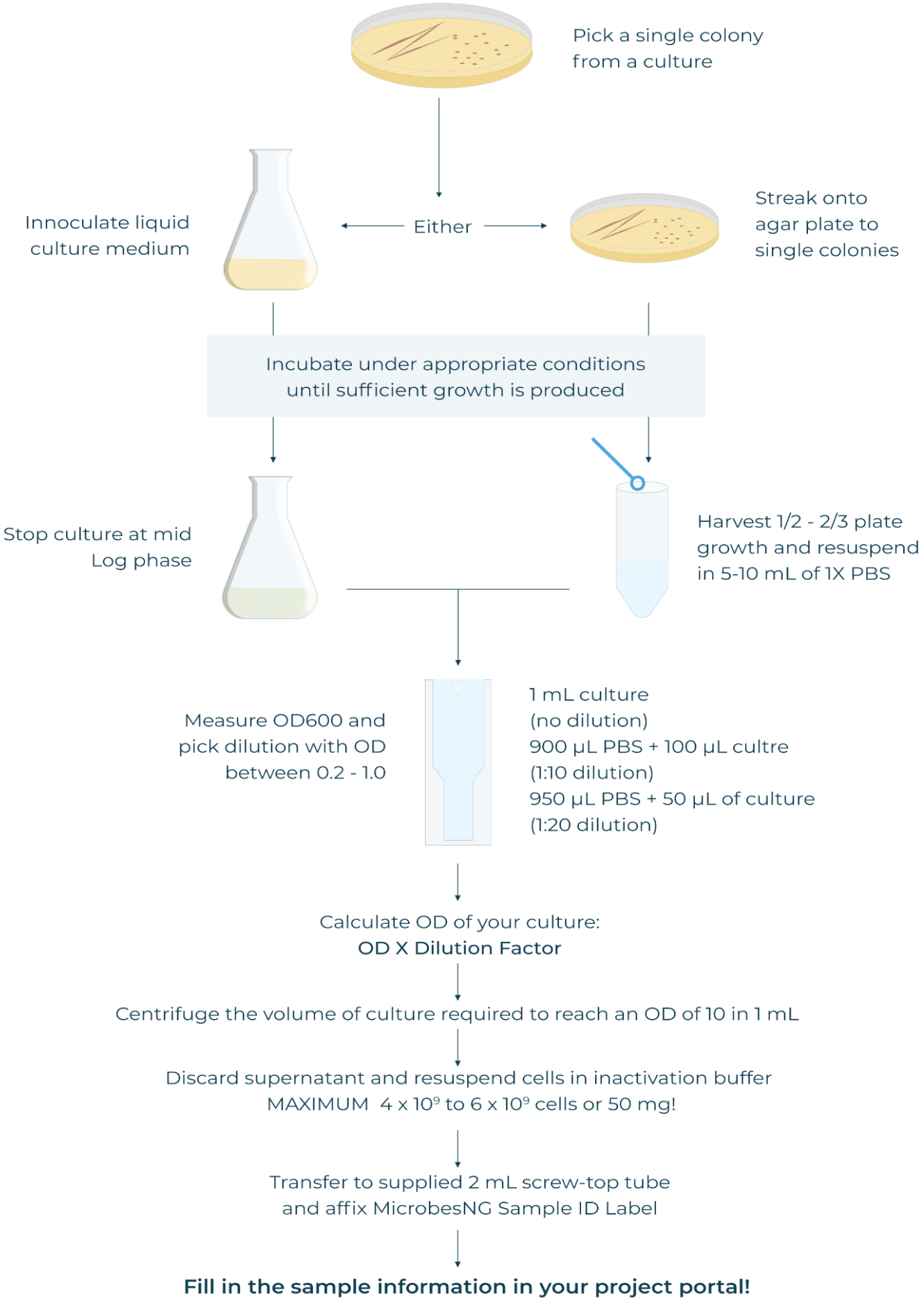
Place the rigid tube box inside an outer package (e.g. polystyrene or cardboard box) along with an absorbent material to catch any leaks, and protect it with suitable packaging material.

Log onto your project portal to print the return label and attach it to the outside of your package. Using this label will help our Laboratory Team to identify your project and ensure your samples enter the correct pipeline as quickly as possible. We recommend that you send your parcel using a tracked service.

Make sure that you have entered the sample information in your project portal before shipping your samples! We use this information to optimise the extraction process.

We will NOT be able to accept your samples if you have not entered the sample information in your project portal.

Appendix A - Visual summary of workflow



Appendix B - Worked example of optical density calculation

- You start with 20 mL of a bacterial suspension.
- You remove 100 µL and place it into a 1 cm cuvette with 900 µL of sterile broth, creating a 1:10 dilution (i.e. dilution factor of 10).
- You place the cuvette in a spectrophotometer (blanked with sterile broth) and measure the absorbance at 600 nm.
- The result is 0.25. This is the observed OD. The target OD is 10.
- Calculate the OD of your culture by multiplying the observed OD by the dilution factor: 0.25 x 10 = 2.5
- Now you can use this formula to obtain a target or calculated OD:

  (target OD)/(observed OD) = mL of culture

  10 ÷ 2.5 = 4 mL of culture
- Transfer the appropriate volume of culture to a suitable tube and centrifuge to pellet the cells. In the example above you would transfer 4 mL of culture.
- Discard the supernatant, and wash the pellet with 1 mL of sterile buffer (e.g. PBS), before centrifuging a second time.
- Resuspend the pellet in 0.5 mL of inactivation buffer.
- Transfer this cell suspension back into the provided 2 ml screw top tubes.

Appendix C - Guide to **O**ptical **D**ensity (OD)

Optical **D**ensity (OD) measures light scattering caused by the bacteria in your culture; the higher the number of bacterial cells, the more the light will be scattered.

The relationship between OD and the number of cells depends on

- The spectrophotometer being used.
- The length of the light path through the culture (i.e. Beer-Lambert Law).
- The size and shape of the bacterial cells.
- The cell culture density.

Tips for OD success

Spectrophotometer Blanking

Your culture media has an inherent absorption, so make sure you're using a sterile aliquot of your culture media to account for the basal absorption of the liquid.

Beer-Lambert made easy

As noted above, the relationship between OD and number of cells is dependent on the length of the light path. To make things easy, it's best to use a 1 cm cuvette.

Culture Homogeneity

Mix the culture well before sampling, and take the measurement straight away! The cells start to settle in less than a minute, which leads to inaccurate results. You can always put some parafilm on top of your cuvette and invert it a few times before you put it in the spec.

An OD reading above 1 is not accurate!

This is beyond the dynamic range of most spectrophotometers, which means that the readings do not increase linearly as cell concentration increases. If you get an OD reading above 1, dilute your sample and measure it again. You can then use the dilution factor to calculate the OD of the undiluted culture.

Appendix C - Guide to **O**ptical **D**ensity (OD)

Weird bacteria? Calibrate!

The OD values we provide in our protocol are generic, and may not suit your weird and wacky bacteria. If you suspect this might be the case, it's best to calibrate!

Calibrating Your OD600 Measurements

Begin with a culture of the cells you are working on, then perform dilutions so that you end up with a series of samples with ODs of =2, 1, 0.8, 0.6, 0.4, 0.2, and 0.1 on your spec. For each of your measured OD's, carry out plate counts of serial dilutions (e.g. Miles & Misra).

The number of colonies formed multiplied by the dilution factor will give you the number of colony forming units/mL in the original sample (CFU/mL). These values can then be used to construct a calibration curve of OD vs. CFU/mL.

The conversion factor for your spectrophotometer (CFU/mL represented by 1 OD unit) will be equal to the gradient of the linear portion of the curve (normally up to about OD=1). You can now use this factor to convert your OD reading to cells/mL. This number will be absolute and comparable between experiments, as long as you remember to repeat the calibration if you change to a different spectrophotometer.