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## A SARS-CoV-2 Synthetic Control Mixture Preparation Protocol

Jessica A

Jannatul Ferdous<sup>1</sup>, April N Harris<sup>1</sup>, wtaylo<sup>1</sup>, Schlueter<sup>1</sup>,  
Cynthia  
Gibas<sup>1</sup>

<sup>1</sup>UNC Charlotte

UNC Charlotte COVID WBE

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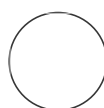
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**Protocol status:** Working  
We use this protocol and it's working

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Jannatul Ferdous

### ABSTRACT

Wastewater-based sequencing surveillance has been a powerful tool for monitoring SARS-CoV-2 and making public health decisions. Along with sequencing wastewater as a medium, this approach has additional complexity in the deconvolution process. The evaluation of existing bioinformatic deconvolution tools with a dataset with known composition and concentration is expected to aid in mitigating the complexity of deconvolution. This is a protocol for generating a golden standard dataset with synthetic RNA control mixtures that is reproducible , easy to adapt to any other synthetic controls and sequencing platform and can be applied to performance evaluation studies.

### GUIDELINES

- Always keep the RNA controls in -80 freezers if not using immediately.
- Plan ahead to work with these controls to minimize the freeze and thaw cycle.

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**Funders**

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## MATERIALS

- Control 15 (Alpha-103909)
- Control 17 (Gamma-104044)
- Control 23 (Delta-104533)
- Control 48 (Omicron - BA.1 lineage-105204)
- Control 51 (B.1.1.529+BA.2-England-105346)
- Control 2 (Wuhan hu-1 from china-102024)
- Control 6 (Wuhan hu-1 from California-102918)
- Control 50 (B.1.1.529+BA.2-Australia-105345)
- Control 64 (BA.5-England-106196)
- Control 62 (B.2.12.1-Denmark-105865)
- Control 66 (BA.4-Texas-106198)
- Control 67 (BA.4-California-106199)
- Control 63 (B.2.12.1-USA-105857)
- Control 65 (BA.5-USA-106197)
- Control 19 (Iota-104529)

## Selection of Synthetic Controls

- 1 Synthetic RNA controls were selected from Twist Biosciences ([TwistBioscienceSyntheticViralControl](#)). For this project, 15 different synthetic controls were chosen, however, any combination of controls can be ordered and combined for a similar analysis.
- 2 Synthetic controls chosen for Ferdous et al. 2023 - Control 15 (Alpha-103909), Control 17 (Gamma-104044), Control 23 (Delta-104533), Control 48 (Omicron - BA.1 lineage-105204), Control 51 (B.1.1.529+BA.2-England-105346), Control 2 (Wuhan hu-1 from china-102024), Control 6 (Wuhan hu-1 from California-102918), Control 50 (B.1.1.529+BA.2-Australia-105345), Control 64 (BA.5-England-106196), Control 62 (B.2.12.1-Denmark-105865), Control 66 (BA.4-Texas-106198), Control 67 (BA.4-California-106199), Control 63 (B.2.12.1-USA-105857), Control 65 (BA.5-USA-106197) and Control 19 (Iota-104529).
- 3 Store the controls in -80 unless used immediately. It is crucial to minimize the number of freeze and thaw cycles with these RNA controls. So plan accordingly.

## Dilution of Synthetic Controls

- 4 Dilution can be modified based on the specific concentration requirement of the research design. For this dataset, dilute the controls in a 1:100 ratio as each control is provided in a standard format, which consists of 100ul at a concentration of approximately one million copies per microliter.

- 5 Take 15 1.5ml eppendorf tubes and sterilize them with UV radiation.
- 6 Label the tubes as "Diluted Control x-date" with respective control name and date.
- 7 Add 99ul Nuclease free water to the tubes.  
Add 1ul of each control in respective tubes .
- 8 Mix properly with gentle vortexing.
- 9 This diluted controls will be used as our stock to make control mixtures. Store the controls in -80 unless used immediately.

## Creating Proper Documentation

- 10 It is crucial to maintain proper documentation while making the control mixture to make sure that the ground truth is completely error-free.
- 11 We created a spreadsheet with columns "Mixture name"(Name of the mixture), "Controls"(controls that are added in this mixture), and "Amount"(Amount of each control added in this mixture).
- 12 Fill in the information accordingly.

- 13 Use this spreadsheet as a guideline while preparing the controls in the lab-[ControlMixtureSpreadsheet](#)

## Preparation of Synthetic Control Mixtures

- 14 Take out all the diluted controls from the freezer and thaw them in room temperature.
- 15 Take 45 eppendorf tubes and decontaminate them with UV.  
Label them as the Mixtures names and organize them in eppendorf tube holder.
- 16 As per the spreadsheet above, take the tube of Mixture "All" and add all the control in 0.5ul amount.  
Add 0.5 ul of NFW to that solution as well.  
Remember to change tips in between adding controls.
- 17 Close the lid and mix by gentle vortexing.  
Mixtue "All" is now completely prepared.
- 18 Prepare rest of the mixtures as per the spreadsheet.
- 19 Store all the control mixtures in -80 unless used immediately.

## Sequencing

- 20 Follow the standard procedure of Oxford Nanopore sequencing library prep and loading ([Protocol](#)) for sequencing the synthetic control mixtures.

**21** This can be modified according to the preferred choice of primers and sequencing platform.