Point-by-point response to editor concerns (Revision #2).
We want to thank the editor for his thorough revision of the manuscript. Editor comments are italicized below. Our responses are in normal font. The red text indicates the additions in the revised manuscript.

Editor´s Comments

1) *For the protocol section please ensure you answer the "how" question, i.e., how is the step performed? Please include all specific details associated with the step to be performed.For the software steps please include a click by click Instructions of how to perform the steps for the software used in your experiment.Once done please highlight 2.75 pages of the protocol text including headings and spacings for filming purpose.*

   We have addressed these comments by revising the entire manuscript and we believed that all the "how" questions are now addressed accordingly with specific details for each case. The manuscript has been modified extensively and several paragraphs added (text in red) and two new supplementary files (button clicks) were added.

   For filming purpose, 2.75 pages of the protocol text has been highlighted.

   We have highlighted the text (yellow) as indicated by the editor for filming purposes.

2) *This part (library design) needs more detail for clarity.*

   This comment has been considered. The various points made have been corrected in the re-written protocol as follow:

   1. Oligonucleotide library design and preparation

   1.1. Library design

   1.1.1. Based on the following criteria design a library of oligonucleotide probes between 8 and 12-mer long to avoid secondary structure formation, with equal length for all probes:

   1.1.1.1. Include at least one DNA and one RNA random sequence containing a combination of adenine (A), guanine (G), cytosine (C) and thymine (T)/uracil (U) **(**DNA and RNA probes in **Table 1)**.

   1.1.1.2. Include polynucleotide sequences consisting of a single type of nucleotide to determine sequence dependence for a given nuclease activity (DNA-Poly A, DNA-Poly T, DNA-Poly C, DNA-Poly G, RNA-Poly A, RNA-Poly U, RNA-Poly C and RNA-Poly G in **Table 1**).

   1.1.1.3. Using the same sequence of nucleotide residues as the initial DNA and RNA sequences, include sequences that contain nucleoside analogues harboring chemical modifications at the 2'-position of the ribose sugar, such as 2'-Fluoro and 2'-O-Methyl, as a stringent step for increasing the selectivity of the nucleases.

1.1.1.3.1. Include fully modified sequences consisting entirely of the 2'-Fluoro nucleoside analogues or the 2'-O-Methyl nucleoside analogues (All 2'-F and All 2'-OMe, **Table 1**)

1.1.1.3.2. Include chimeric sequences consisting of unmodified natural purines and 2'-Fluoro pyrimidine nucleoside analogues (RNA Pur-2'F, **Table 1**).

1.1.1.3.3. Include chimeric sequences consisting of unmodified natural purines and 2'-O-Methyl pyrimidine nucleoside analogues (RNA Pur-2'OMe, **Table 1**).

1.1.1.3.4. Include chimeric sequences consisting of unmodified natural pyrimidines and 2'-Fluoro purine nucleoside analogues (RNA Pyr-2'F, T**able 1**).

1.1.1.3.5. Include chimeric sequences consisting of unmodified natural pyrimidines and 2'-O-Methyl purine nucleoside analogues (RNA Pyr-2'OMe, **Table 1**).

3) *Is there specific sequence to be taken into consideration? In which bacteria … how was this done? How far away are each probe? How many probe per gene or per strain? Please include all specific details.*

The probe sequences in this study (substrates for nucleases) are not based on the genome of a given bacteria. We apologize for the misunderstanding. The following sentence and NOTE were rephrased for clarity purposes:

1.1.1.4. Include at least one DNA and one RNA random sequence containing a combination of adenine (A), guanine (G), cytosine (C) and thymine (T)/uracil (U) (**DNA and RNA probes in Table 1**).

And

NOTE: The DNA and RNA sequences described in **Table 1** have been useful as the initial substrates for classifying an unknown type of nuclease activity, either DNase or RNase. These two sequences are recommended as the starting point for any screening, as they have shown a wide capability to detect nuclease activity profiles in bacteria and tissue samples. If nuclease activity is not observed with these DNA and RNA sequences, the design of additional oligonucleotides is required.

4) *How do you identify natural DNA in the bacterial species? Do you use any software for the library design?*

No particular software is used for library design. We are not looking for bacterial DNA sequences. This comment is also addressed by the answer at the previous point #3.

5) *How many sequences per kind are included for one test? Also how do you determine the length of the sequences?*

This comment has been considered. The various points made have been corrected in the re-written protocol.

The length of probes is now described in more detail in the protocol (see below):

1.1.2. Based on the following criteria design a library of oligonucleotide probes between 8 and 12-mer long to avoid secondary structure formation, with equal length for all probes:

The sequences of the library included in each test/screening round are now specified in section 5 of the protocol:

5.1. First Round of Screening:

5.1.1. Perform the nuclease activity assay (as described in section 4) using DNA, RNA and polynucleotide probes.

5.1.2. Evaluate the preference for DNA chemistry or RNA chemistry by comparing the number and performance (FD value) of DNA and RNA candidate probes.

5.1.3. Select type of nucleic acid chemistry rendering the greatest number of candidate probes (FD>3) and the best performing probes (FD value), as illustrated in Figure 1.

5.2. Second Round of Screening:

5.2.1. Perform the nuclease activity assay (as described in section 4) using fully modified probes and chimeric probes based on the nucleic acid chemistry selected in the first screening round.

5.2.2. Evaluate the preference towards sequences containing 2'-Fluoro and 2'-O-Methyl nucleoside analogues by comparing the results obtained for 2'-Fluoro and 2'-O-Methyl modified candidate probes.

5.2.3. Select the candidate type of nucleoside analog modification rendering the greatest number of candidate probes (FD>3) and the best performing probes (FD value), as illustrated in Figure 1.

6) *Are these against the bacterial genome? Which ones are chimeric in the table?*

An additional column has been added to Table 1 in order to clarify this point.

7) *What kind of bacteria?*
8) *Citation if any?*
9) *The abbreviation must be expanded during the first time use*
10) *Composition of the medium? Is the medium present on a Petri plate?*

These comments (7, 8, 9 and 10) have been addressed by adding a reference and

modifying point 2.1 in the protocol section:

2.1 To isolate individual bacterial colonies (*Salmonella* and *E.coli*), use the quadrant method[24] by streaking/rolling the bead directly onto a Petri dish containing Tryptic Soy Agar (TSA) medium supplemented with defibrinated sheep blood.

11) Of what? What volume of working dilution is prepared?

This comment has been addressed by editing the text in the protocol as follows:

4.1.1. Prepare a 20 µL working solution for each probe in 1.5 mL nuclease free microcentrifuge tubes by diluting (1:10 ratio) the stock solution (500 pmol/ µL) for a final working concentration of 50 pmol/ µL. For that purpose, mix 18 µL of Phosphate Buffer Saline (PBS) containing $MgCl_2$ and $CaCl_2$ with 2 µL of probe stock solution.

12) From step number?

13) How many bacterial supernatants are prepared? What is the difference? Are these different strains, species? Please bring out this clarity somewhere in the protocol

14) How many probes are prepared in this experiment? Do you add one probe per well? Please explain this part.

4.2.3. Carefully add 96 µL/tube of TSB sterile culture media, *Salmonella* supernatant or *E.coli* supernatant (from the step 3.4.). Subsequently, add 4 µL/tube of probe working solution accordingly. Perform this step at room temperature.

Note: 10 probes were used for the first round of the screening and 6 probes for the second round."

15) How? Using a pipette or plate mixer?

This information has been added by rephrasing the following sentence in the protocol

4.2.4. Mix thoroughly by pipetting up and down to obtain a homogenous solution. Avoid introducing air bubbles into the samples while mixing.

16) Do you mix all the probes or is it probe+ supernatant mixture?

This information has been clarified by modifying the following step in the protocol:

4.2.5. Load 95 µL of each solution (probe + supernatant or culture media) into a separate well of a black bottom, non-treated 96 well plate. Minimize the formation of bubbles in the wells upon loading by dispensing carefully with the tip close to the wall of the well.

17) Ensure how?

This comment has been addressed by rephrasing the protocol step as follows, to include the "how":

"Inspect visually the lid and check for pen markings or dust particle accumulation that may introduce measurement artifacts. Replace the lid for a new one if that is the case."

18) *Please include click by click instruction of how to perform this part. E.g., Click **open** to open the software. Then click **create** to create a new protocol. Manually enter 37 °C for the temperature… etc.*
19) *For all the sub steps please use complete sentences and show how it is done.*

We have addressed these comments by modified the supplementary figure 1, two supplementary figures with "button clicks" were added and we have rephrased the following paragraphs:

4.3. Measurement set up

4.3.3. Open the acquisition software (Gen5 3.05 or any other available compatible software) by clicking the software shortcut icon (Figure S1 A).

4.3.4. Select "Read Now" from the task manager window and choose "New…" to create the kinetic measurement protocol (Figure S1 B).

4.3.5. Click on "Set Temperature" in the dialog window titled "Procedure and select 37 °C. Confirm and save the settings by pressing "OK" (Figure S1C).

4.3.6. Click on "Start Kinetics" in the dialog window titled "Procedure". Then in the pop-up dialog window, titled "Kinetic Step", select 2 hours in the "Run Time" input box and 2 minutes in the "Interval" input box. Confirm and save the settings by pressing "OK" (Figure S1D).

4.3.7. Click on "Read" in the dialog window titled "Procedure". Then in the pop-up dialog window, titled "Read Method", select "Fluorescence Intensity" as a detection method, "Endpoint/Kinetic" as a read type and "Filters" as optics type. Confirm and save the settings by pressing "OK" (Figure S1E).

4.3.8. In the pop-up dialog window titled "Read Step", select "Green" from the "Filter Set". Confirm and save the settings by pressing "OK" (Figure S2A).

4.3.9. In the dialog window titled "Procedure", select "use lid" and click on "validate" to ensure that the created protocol is valid by receiving a pop-up dialog window (Figure S2B).

4.3.10. Select "protocol" in the menu bar and choose "procedure" (Figure S2C).

4.3.11. In the dialog window titled "Procedure", define the wells to be measured (Figure S2D).

4.3.12. Enter the name of the experiment in the file name input box (Figure S2E).

4.3.13. Load the plate with its lid into the plate reader. Ensure the plate is in the right orientation.

4.3.14. Start the acquisition by clicking "read new" button in the toolbar (Figure S2F).

4.4. Data Analysis

4.4.3. Click on one of the measured wells in the dialog window titled “Plate 1” (Figure S3A).

4.4.4. Click “select wells” and include all the measured wells in the “well selection dialog” window. Confirm and save the settings by pressing “OK”. (Figure S3B).

4.4.5. Select “data” in the dialog window titled “Plate 1” to visualize the tabulated results (Figure S3C).

4.4.6. Export the data to excel spread sheet by selecting “quick export” from the context menu (Figure S3C).

4.4.7. In the excel spread sheet label the data columns accordingly for each sample and probe.

4.4.8. Click “insert” in Excel menu bar. Select “line” from the toolbar and choose “line with markers” to generate kinetic graphs (x-axis: timeline of the reaction // y-axis: relative fluorescent units)

20) How is this done?

This comment has been addressed by including stepwise description of generating the graphs in the data analysis section.

21) Please include details of the screenings and how these were performed in your case. Third fourth and fifth screening set not described.

The third, fourth and fifth screening rounds are not described in the protocol as they were not performed experimentally, however, a mention to future rounds is made in the discussion and illustrated with the supplementary figures 4, 5 and 6.

22) How is this assessment performed?
23) How is this done?

These comments have been addressed by adding more details and steps to section 5 of the protocol (see below):

5.3. First Round of Screening:

5.3.1. Perform the nuclease activity assay (as described in section 4) using DNA, RNA and polynucleotide probes.

5.3.2. Evaluate the preference for DNA chemistry or RNA chemistry by comparing the number and performance (FD value) of DNA and RNA candidate probes.

5.3.3. Select type of nucleic acid chemistry rendering the most number of candidate probes (FD>3) and the best performing probes (FD value), as illustrated in Figure 1.

5.4. Second Round of Screening:

5.4.1. Perform the nuclease activity assay (as described in section 4) using fully modified probes and chimeric probes based on the nucleic acid chemistry selected in the first screening round.

5.4.2. Evaluate the preference towards sequences containing 2'-Fluoro and 2'-O-Methyl nucleoside analogues by comparing the results obtained for 2'-Fluoro and 2'-O-Methyl modified candidate probes.

5.4.3. Select the candidate type of nucleoside analog modification rendering the most number of candidate probes (FD>3) and the best performing probes (FD value), as illustrated in Figure 1.

24) *Please include this sentence somewhere in the introduction to bring out clarity in the protocol text.*

To address this comment, we have removed this sentence from the results section:

"We sought to identify the nuclease activity derived from Salmonella as a biomarker for the specific identification of this bacteria. Herein, we report on the methods for screening bacteria nuclease activity using kinetic analysis."

And substituted it for a similar sentence in the last paragraph of the introduction (line110), for clarity:

"As such, this approach was used to identify the nuclease activity derived from *Salmonella* for the specific identification of this bacteria. In the following protocol, we report on the methods for screening bacterial nuclease activity using kinetic analysis."

25) *How is this derived? What is Discretize time, TSB, E.coli and velocity and what do you infer from all these values. Need further description. Applicable to all the supplementary files.*

For clarity purposes, we have eliminated the term "Discretize" and more appropriate labels have been used to describe the supplementary files content. In addition, a more thorough legend description of the parameters included in supplementary files has been added as indicated below:

Supplementary file 1: Raw data from the first screening round. For each probe (labelled in red, on top), the acquisition time and the raw fluorescence values were reported for the three samples tested in this study (TSB, *E. coli* and *Salmonella*), along with the calculated rate value for each interval. The calculations were carried out as described in the methods section.

Supplementary file 2: Raw data from the second screening round. For each probe (labelled in red, on top), the acquisition time and the raw fluorescence values were reported for the

three samples tested in this study (TSB, *E. coli* and *Salmonella*), along with the calculated rate value for each interval. The calculations were carried out as described in the methods section

Supplementary file 3: Calculations based on the first screening round. For each probe (labelled in red, on top), the following calculations were performed for the three samples in this study (TSB, *E. coli* and *Salmonella*): maximum rate values, minimal and maximal interval time point, rate coefficient, fold difference values between *Salmonella* and *E.coli* over TSB and fold difference values between *Salmonella* and *E.coli (highlighted in yellow).* The calculations were carried out as described in the methods section and the calculation formulas and the step by step calculations are shown in the spreadsheet.

Supplementary file 4: Calculations based on the second screening round. For each probe (labelled in red, on top), the following calculations were performed for the three samples in this study (TSB, *E. coli* and *Salmonella*): maximum rate values, minimal and maximal interval time point, rate coefficient, fold difference values between *Salmonella* and *E.coli* over TSB and fold difference values between *Salmonella* and *E.coli (highlighted in yellow).* The calculations were carried out as described in the methods section and the calculation formulas and the step by step calculations are shown in the spreadsheet.