Research article

Implementing routine monitoring for nuclease contamination of equipment and consumables into the quality Management system of a laboratory

Estelle Henry , Eleftheria Charalambous , Fay Betsou , William Mathieson [*]

Integrated Biobank of Luxembourg, 1 rue Louis Rech, Dudelange, L-3555, Luxembourg

ABSTRACT

Nucleases are ubiquitous in the environment, present in biospecimens and widely used in many laboratory processes. However, in the wrong context, as contaminants, they have catastrophic potential because of their ability to rapidly degrade nucleic acids whilst retaining high resilience to inactivation. Although laboratories undertake rigorous precautions to prevent nuclease contamination, such measures are not infallible. In 2015, we devised and integrated a novel routine nuclease testing regimen into our Quality Management System that uses cleavable, fluorescent DNA and RNA substrates to detect, monitor and control for nuclease contamination in our laboratory processes, equipment and consumables. The testing regimen enables us to identify higher-risk activities, design our laboratory workflows such that risk is minimized and help fulfil our obligations in respect of ISO 20387:2018 General Requirements for Biobanking and ISO 17025 Testing and Calibrations Laboratory standards, both of which stipulate that environmental conditions in our laboratory must be monitored with defined quality control criteria. In seventeen rounds of testing (30 Test Items per round), 1.1 % of RNase tests and 0.2 % of DNase tests returned elevated nuclease levels ($\geq 2.90 \times 10^{-9}$ U RNase or 1.67×10^{-3} U DNase) and we were able to take remedial action. In no instance was an elevated nuclease level consequential in terms of an impact on sample quality. We present our protocols, results and observations.

1. Introduction

Deoxyribonucleases (DNases) and ribonucleases (RNases) form very diverse groups of enzymes found in both eukaryotes and prokaryotes. In humans, certain DNases and RNases are organ-specific in their expression, but overall, they are present in all tissue types including on the surface of skin, and are secreted into blood, tears, saliva and perspiration [1–4]. In addition to performing a multitude of critical biological functions in vivo, purified nucleases are also widely used in the laboratory: RNase is used in DNA extractions to eliminate RNA and prevent it coeluting with DNA, DNase likewise in RNA extractions and both can be used in protein purification. DNases are also used in RT-PCR preparation, in cell culture (to prevent clumping) and as restriction enzymes in gene cloning, SNP analysis, library preparation and Southern blotting. Although hugely beneficial when used in the correct context, nucleases as contaminants have the potential to be catastrophic because they can very readily degrade nucleic acid analytes. Nucleases have optimal activity at a pH range of 6–8, which is comparable with the buffers used to elute, store and test nucleic acids [2,4]. In addition, some nucleases are highly resilient to degradation and inactivation, on account of their disulphide bonds, with for example RNase A (commonly used in DNA extractions) retaining its activity despite being autoclaved at 121 °C for 20 min and being resilient to extremes of pH and the protein denaturant urea [5,6].

Laboratories instigate precautionary measures to mitigate the likelihood of nucleases contaminating reagents, consumables and equipment. Contamination could originate from microorganisms and fungi present in the environment, or via hair, skin flakes or

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perspiration from laboratory staff. Standard precautions include the regular changing of gloves and laboratory coats, using consumables and reagents that are certified nuclease-free, maintaining a set of dedicated pipettes and consumables for work with nucleases, spatially separating DNA extractions that use RNases from RNA extractions using DNases and designating "nuclease-free zones" where downstream laboratory work involving nucleic acids is undertaken [7]. Mathay et al. present an example of what happens when an RNA sample is contaminated with RNases present on human skin when they deliberately exposed purified RNA to a finger for 3 s, after which its RNA Integrity Number decreased from 7.5 to 4.1 [8].

We, like many other laboratories, perform large numbers of nucleic acid extractions from a range of biospecimen types for multiple research projects and organisations. We therefore consider it critical to have demonstrable procedures in place to control and monitor for nuclease contamination. In 2015, as part of our Quality Management System, we implemented a (now) biannual nuclease-testing regime that we apply to our laboratory equipment, reagents and consumables. Although its primary function is to provide a documented record of current and historical nuclease levels in our laboratories, we have found that the regime has additional benefits: reinforcing the message that all staff and visitors must maintain vigilance against nuclease contamination, identifying potential weaknesses in our routine cleaning protocols before they become critical and enabling us to have a Standard Operating Procedure in place that can readily be applied to test any supplemental items on an *ad hoc* basis. For the benefit of other laboratories considering implementing a similar regime, we present our protocols, results and observations.

2. Materials and methods

2.1. Selection and preparation of test items

Any equipment, consumable or reagent used to extract, manipulate or analyse nucleic acid is eligible for inclusion in the nuclease testing regime. However, a balance is sought, so the regime is sufficiently thorough to be meaningful whilst not being of undue burden in its magnitude. We currently have 30 "Test Items", assigned to five broad categories, described below and listed in Table 1. As each Test Item is prepared for testing, the salient information pertinent to it (laboratory room number, run ID, date of collection, lot number etc.) is recorded on a dedicated "Assay Sheet and Report" form. A summary of the process of preparing items for testing is shown in Fig. 1.

Table 1
List of items that are routinely tested for RNase and DNase contamination.

Test Item no.	Description	Test item category	Function in our laboratory
1	Blank from Tecan liquid handler	Blank extraction	Liquid handler/PBMC isolation from plasma
2	Blank from Chemagen robot (24 rod head)	Blank extraction	DNA extraction from buffy coat/whole blood (24 samples simultaneously)
3	Blank from Chemagen robot (96 rod head)	Blank extraction	DNA extraction from stool/FFPE tissue (96 samples simultaneously)
4	Blank from QIACube robot	Blank extraction	RNA extractions from various biospecimen types
5	Blank from BioRobot	Blank extraction	RNA extractions from PAXgene blood tubes
6	Elution buffer: Chemagen 24 extractions kit	Buffer	Elution buffer used in Chemagen extractions from blood
7	Elution buffer: Chemagen 96 extractions kit	Buffer	Elution buffer used in Chemagen extractions from stool
8	Magnetic bead reservoir: Chemagen	Equipment	Trough from which Chemagen robot collects magnetic beads
9	Tips (900 μl): Chemagen	Consumables	Tips used by Chemagen robot
10	Tips (175 μl): Chemagen	Consumables	Tips used by Chemagen robot
11	Sleeve for Chemagen 96 rod head	Consumables	Sleeve that covers 96-rod head during extractions used by Chemagen robot
12	Sleeve for Chemagen 24 rod head	Consumables	As above, but for 24-rod head
13	Tips (1100 μl): BioRobot	Consumables	Tips used by BioRobot robot
14	Elution buffer reservoir: BioRobot	Consumables	Trough that contains elution buffer during BioRobot extraction
15	Tips (1000 μl): QiaCube robot	Consumables	Tips used by QiaCube robot
16	Tips (200 μl): QiaCube robot	Consumables	Tips used by QiaCube robot
17	Tips (1000 μl): Tecan liquid handler	Consumables	Tips used by Tecan liquid handler
18	Tecan buffer reservoir	Consumables	Trough that contains buffer during Tecan PBMC isolation
19	Take 3 plate (Synergy spectrophotometer)	Equipment	Microvolume plate used to load nucleic acid for quantification (16 samples)
20	Take 3 plate trio (Synergy spectrophotometer)	Equipment	As above but for 48 samples
21	Milli-Q water	Buffer	Water from ultrapurification system (numerous uses)
22	Ice from ice machine (melted)	Equipment	Crushed ice from ice machine (maintain cold chain for samples/reagents in tubes)
23	Matrix sample storage tubes	Consumables	500 μl tube used for storing aliquotted nucleic acids
24	Tips for manual pipettes	Consumables	Random selection of tips from pipette boxes open on lab benches (various sizes)
25	CryoXtract probes	Consumables	Probes used to core frozen tissue for extractions using CryoXtract frozen sample aliquotter
26	TissueLyzer beads	Consumables	Stainless steel tissue homogenization beads for TissueLyzer bead mill
27	Centrifuge tubes (0.5, 1.5 and 2 ml)	Consumables	Centrifuge tubes with multiple lab uses
28	50 ml centrifuge tubes	Consumables	Centrifuge tubes with multiple lab uses
29	15 ml centrifuge tubes	Consumables	Centrifuge tubes with multiple lab uses
30	25 ml Serological pipette	Consumables	Serological pipette with multiple lab uses

The assay requires test samples to be in the liquid phase at 80 μl per well, so we collect 350 μl of each Test Item to enable us to perform both the RNase and DNase assay in duplicate.

Category 1: Blanks from nucleic acid extractions and liquid sample aliquotting runs (Test Items 1–5)

Blanks are generated in the weeks prior to nuclease testing by including an additional extraction in a routine extraction/aliquotting run, but with a buffer rather than a biospecimen as the starting material (e.g. lysis buffer in place of homogenized tissue or PBS in place of a liquid biospecimen). Although the blanks ultimately contain only elution buffer, they have undergone exactly the same processing steps as the biospecimens processed in the same run. Blanks are stored at −80 °C pending their testing. In the absence of a blank, a nucleic acid extract or a liquid biospecimen aliquot could be used, provided it is surplus to other requirements and the appropriate patient consent and ethical approvals are in place.

Our nucleic acid extraction blanks are generated by the QIAcube and BioRobot robots (both Qiagen) we use for RNA extractions and a Chemagen MSM1 robot (PerkinElmer) that we use for DNA extractions. The blanks consist of elution buffer at the volumes
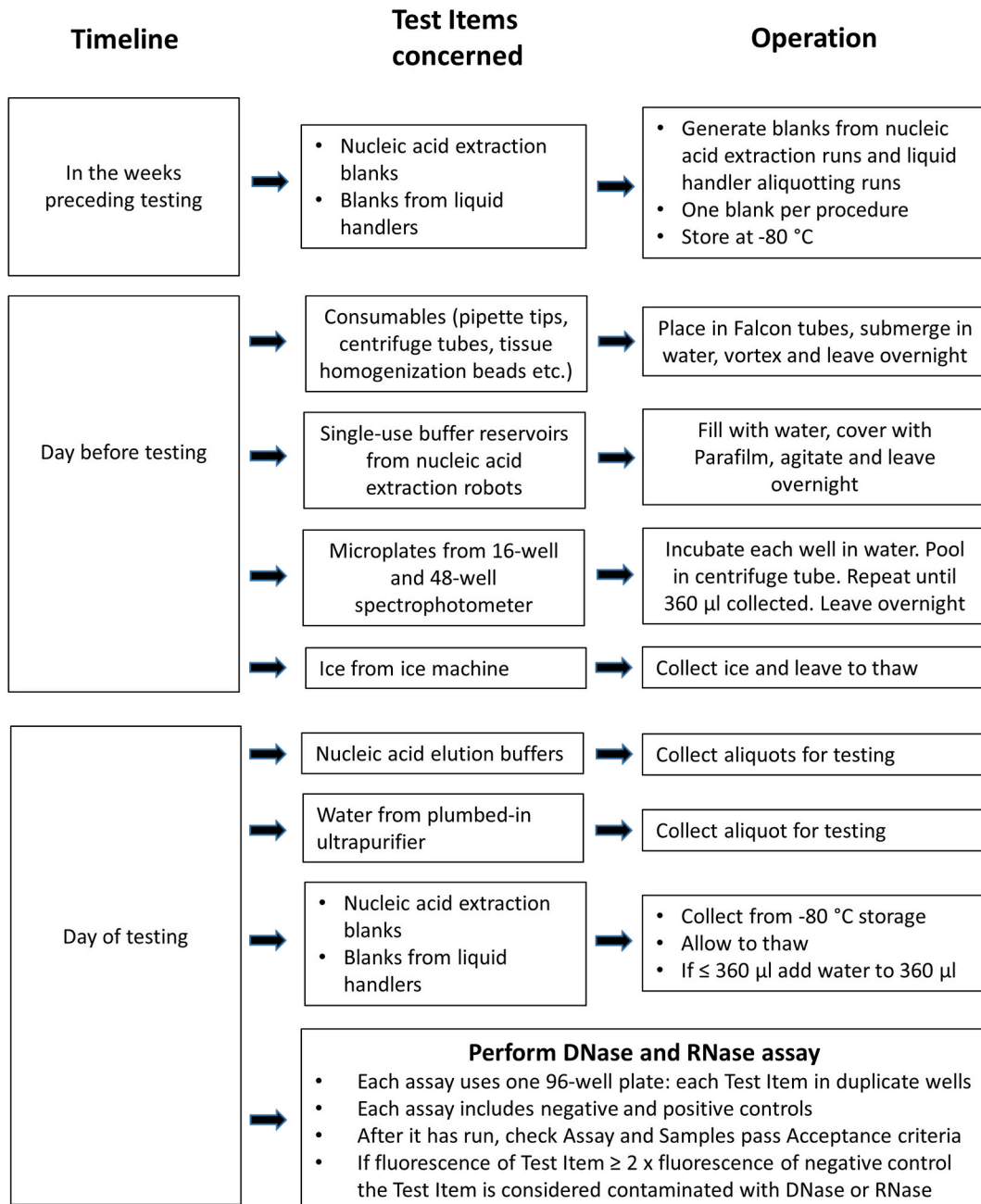

Fig. 1. Schematic of how Test Items are prepared, including the time line.

specified and programmed in the extraction protocols used: 50 or 80 μl for RNA (depending on whether the extraction protocol selected was for blood or tissue) and 80, 250 or 300 μl for DNA (depending on whether the protocol was for tissue, stool or blood). These volumes are less than the 350 μl we require in total, so we either run multiple blanks or add water to obtain the required volume. We also test blanks from our Tecan liquid handling robot (Tecan Group Ltd), which is used to automatically process blood in K_2EDTA tubes to deliver one aliquot of peripheral blood mononuclear cells (PBMCs) in 1 ml CryoStor CS10 cell freezing medium (Stemcell Technologies) plus 12 aliquots of plasma, each of 220 μl, per blood tube. The resultant blanks are either 1 ml of CryoStor CS10 or 220 μl water (in place of a plasma aliquot). Blanks are stored at −80 °C pending testing.

2.2. Category 2: buffers (test items 6, 7 and 21)

Aliquots of DNA elution buffers used by the Chemagen MSM1 robot are directly tested from their storage bottles because these bottles contain relatively large volumes (200 ml) and are opened multiple times over several months. Although the same elution buffers are tested as blanks, we additionally test the buffers direct from their bottles because sometimes several weeks have elapsed between the generation of the blank and the test. Elution buffers from other nucleic acid extraction kits can be tested as "*ad hoc*" items if considered necessary. Lysis and wash buffers cannot be tested on account of their chemical incompatibility with the nuclease assays. An aliquot of ultrapure water from our Milli-Q water purifier (Merck Millipore), which is plumbed into the building's centralised deionized water system is also directly tested. All test Items in this category are collected on the day that the assays are run.

2.3. Category 3: consumables (test items 9–18 and 23–30)

On the day before the assays are to be run, consumables (pipette tips, centrifuge tubes, cryotubes, beads from our TissueLyzer bead mill etc.) are randomly taken from boxes currently in use on lab benches, then, depending on their size, transferred to either a sterile 15 ml or 50 ml Falcon tube (one Test Item-type per Falcon tube). Sterile water, certified nuclease-free and not treated with (the RNase inhibitor) diethyl pyrocarbonate (Ambion AM9932) is then added until the Test Items are submerged. Consumable lot numbers and the laboratory room IDs are recorded on the "Assay Sheet and Report" form. The Falcon tubes are vortexed thoroughly every 10 min for 1 h then left to soak overnight. External and internal surfaces of centrifuge and cryotubes are both tested by additionally sealing water inside each tube, then pooling it after the overnight incubation. The single-use buffer reservoirs used to load wash and elution buffers into our Chemagen MSM1 DNA extraction robot are tested by taking a reservoir from the package currently in use, partially filling it with 10 ml sterile water, covering it with Parafilm (Bemis), tilting/rocking it every 10 min for 1 h then leaving it to soak overnight.

2.4. Equipment (test items 8, 19, 20 and 22)

Items in this category are prepared for testing the day before the assays are run. The reusable trough used to hold the DNA-binding magnetic beads used by our Chemagen MSM1 DNA extraction robot is tested in the same way as the buffer reservoirs (see above).The 16-sample and 48-sample microvolume plates that hold 3 μl aliquots of purified nucleic acid for quantification by spectrophotometry in our Synergy Mx microplate reader (BioTek Instruments) are separately tested by aliquotting 10 μl sterile water in each well, incubating it for 30 min, then pooling it into 1.5 ml Centrifuge tubes (one tube per plate). This process is repeated then an additional 30 μl water added for the 16-sample plate to generate the 350 μl required for testing. A 50 ml Falcon Tube is filled with ice from our ice machine (which is plumbed into the building's centralised deionized water system) then left to thaw.

2.5. Internal Processing Negative Controls

Internal Processing Negative Controls (IPNC) consist of Serological pipettes and Falcon Tubes taken from the same packages as those used to prepare Test Items for analyses. In the event that a Test Item prepared using a serological pipette or Falcon Tube returns a positive result we require the IPNCs to be negative before assigning the contamination to the Test Item rather than the consumables used to prepare it for testing. We prepare the 15 ml and 50 ml Falcon Tube IPNCs at the same time as Test Items in the consumables category by filling them with 15 ml sterile water, vortexing them with the same regularity as Test Items then leaving them overnight. The IPNC serological pipette is also prepared for testing the day before the assays are performed by filling and aspirating a 15 ml aliquot of sterile water 20 times in a 50 ml Falcon tube then leaving it overnight.

2.6. Nuclease assay

Nuclease levels are quantified using the RNaseAlert and DNaseAlert QC systems (ThermoFisher Scientific 4479769 and AM1970 respectively). Both return quantitative values for nuclease activity using a substrate consisting of a short length of RNA or DNA, with a fluorophore attached at one end and a quencher at the other. When the nucleic acid is cleaved by a nuclease, the fluorophore is liberated from its quencher and fluoresces when excited at the appropriate wavelength, with the magnitude of fluorescence being proportional to the quantity of nuclease present in the test sample.

RNaseAlert and DNaseAlert assays are performed separately in black 96 well plates, according to the manufacturer's instructions. Tests are performed in duplicate, each consisting of 80 μl test sample plus 20 μl of the RNaseAlert or DNaseAlert substrate solubilized in their buffers (provided in the kits). The capacity of each plate is 44 Test Items plus a duplicated positive control (RNase A or DNase 1 in place of a Test Item) and a quadruplicated Assay Negative Control (ANC) consisting of nuclease-free water in place of a Test Item.

Duplicated blanks consist of 100 μl water only. The plates are incubated for 1 h at 37 °C in the dark (the assay kit handbooks state 30 min–1 h incubation) then read on a Synergy Mx spectrofluorometer set at the fluorophores' excitation/emission maxima of 490/520 nm and 535/556 nm for RNaseAlert and DNaseAlert respectively and its default and intermediate gain setting of 100. Results are returned as blank-deducted Relative Fluorescence Units (RFU).

2.7. Interpretation and reporting of results

Each assay has four "Assay Acceptance Criteria" that relate to the controls and two "Sample Acceptance Criteria" that relate to the Test Items (specified in Table 2). If the assay fails an Assay Acceptance Criterion it is repeated in its entirety, including all Test Items. The Assay Acceptance Criteria consist of maximum RFU values for the ANCs, minimum RFU values for the Positive Controls (the ANC x 15) and a maximum coefficient of variation (CV) between the replicate wells of the positive and negative controls. Together, these ensure that each assay meets predefined criteria for minimum dynamic range and sensitivity, which are critical if we are to be confident that low-level nuclease concentrations would be detectable, even when all Test Items return a negative result. Provided the assay passes all its Acceptance Criteria, each Test Item's result is also accepted if it passes the Sample Acceptance Criteria (its RFU cannot be less than half that of the negative control and the duplicate wells in the assay must have a minimum CV). Test Items that fail a Sample Acceptance Criterion are re-tested, with the repeated test incorporating a fresh, replicated positive control and ANC. When a Test Item passes its Sample Acceptance Criteria it means that its RFU can be accepted, not that it is uncontaminated with a nuclease. Our protocol includes provision for "Conditional Acceptance", where a failure in either Assay or Sample Acceptance Criteria can be overridden when it is evident that the failure is inconsequential.

Acceptance criteria values were experimentally determined initially but have since been updated on three occasions to reflect the performance of the assays and their variation over time. The assay kits' handbooks point out that the performance of the assays depend on the sensitivity of the spectrofluorometer used and its gain settings, so the handbooks do not specify acceptance criteria, other than stating that the positive control RFU should be a minimum of 20 times that of the negative control [9,10]. The changes we have made to the acceptance criteria over time consist of reducing the coefficient of variation between duplicates, defining the extent to which Test Items can return an RFU below that of the ANC and reducing the magnitude of the allowed difference in RFU between the positive control and the ANC from 20 to 15 times.

For each Test Item, a "Contamination Ratio" (CR) is calculated: CR = mean Test Item RFU divided by mean ANC RFU. The assays' manufacturer states a CR threshold of 2–3 should be applied in both RNaseAlert and DNaseAlert assays, with Test Items retuning higher CRs considered contaminated [9,10]. We use the lower threshold, and take preventative action when a Test Item returns a CR $\geq$ 2.0. The precise nature of the action takes into account the magnitude of the CR of the Test Item, the CR of any related Test Items from the same laboratory room or process and historical data.

All results are reported on the "Assay Sheet and Report" previously used to record the Test Items' ID, lot numbers, laboratory room numbers etc. and the lot numbers of the nuclease assay kits used. All results are also manually added to an Excel database that contains all historic Test Item CRs and control RFUs, then reviewed for inconsistencies compared to historic data.

2.8. Assay sensitivity

Both nuclease assays are quantitative in that RFUs are proportional to nuclease activity, but the CR threshold is driven by the ANC. The quantity of nuclease required to generate CR = 2.0 were therefore validated experimentally. It was not possible to use the nucleases supplied in the DNaseAlert and RNaseAlert kits as positive controls for these sensitivity experiments because the manufacturer refused to provide data pertaining to their concentration or activity, citing commercial secrecy.

For the RNaseAlert assay, RNase A supplied at 100 U/ml and 4 mg/ml (Qiagen 158922) was diluted in water to create a series of 20 doubling dilutions from 2.50×10^{-7} to 4.77×10^{-13} U/μl ($10–1.91 \times 10^{-5}$ pg/μl), then assayed in triplicate wells at 80 μl RNase in a total volume of 100 μl/well. Qiagen defines 1 Unit of RNase to be the quantity that degrades RNA such that its velocity constant *k* is equal to 1 Kunitz unit at 25 °C and pH 5.0 (Qiagen Technical Service pers. comm.) [11].

For the DNaseAlert assay, DNase I supplied at 1000 U/ml (ThermoFisher Scientific EN0521) was used to create 20 doubling dilutions from 5.0×10^{-2} to 9.5×10^{-8} U/μl. ThermoFisher Scientific define 1 Unit of DNase to be the quantity that completely degrades 1 μg plasmid DNA in 10 min at 37 °C, with *k* being equal to 0.3 Kunitz units [11]. The manufacturer does not provide the concentration in mg/ml. As in the RNaseAlert assay, 80 μl each dilution was run in triplicate in a total volume of 100 μl/well.

Each assay was repeated three times, then mean RFUs plotted against nuclease concentrations using SigmaPlot *v*.13.5 (Systat

Table 2
Assay and Sample Acceptance Criteria. If the Assay Acceptance criteria are not met the entire assay is repeated, including all Test Item samples. If Assay Acceptance Criteria are met, individual Test Items are re-assayed with Positive and Assay Negative Controls if they fail Sample Acceptance Criteria. ANC: Assay Negative Control, RFU: Relative Fluorescence Units, CV: Coefficient of Variation.

|	Assay Acceptance Criteria				Sample Acceptance Criteria	
	Maximum ANC RFU	CV of ANC technical replicates	Minimum Positive Control RFU	CV of Positive Control technical replicates	Test Item Minimum RFU	CV of Test Item technical replicates
RNaseAlert	5000	<40 %	ANC RFU x 15	<30 %	ANC RFU/2	<30 %
DNaseAlert	1000	<30 %				
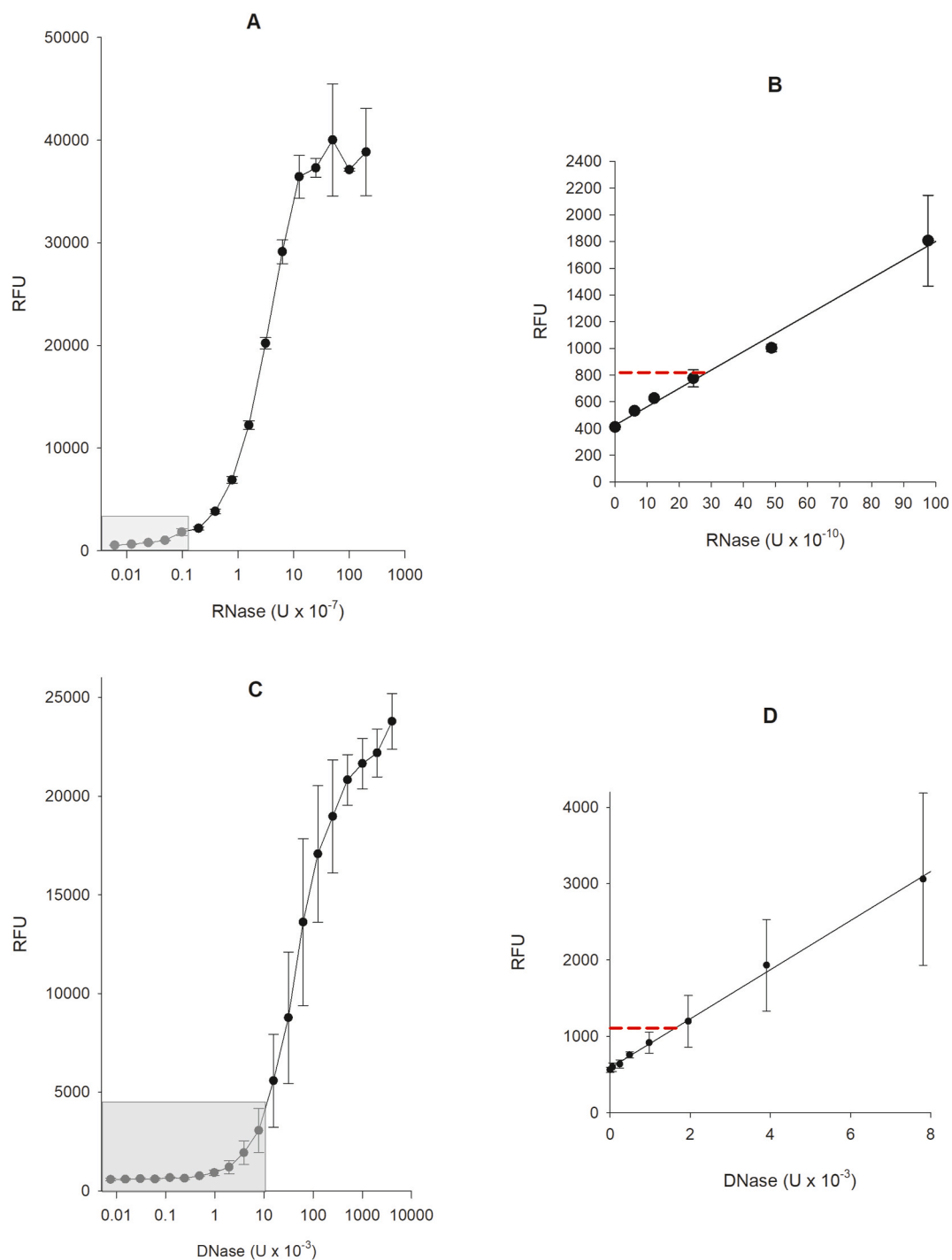
Fig. 2. Determination of the Contamination Ratio (CR) = 2 threshold for RNaseAlert (A & B) and DNaseAlert (C & D) assays. *X*-axes are Units of nuclease per well (divide by 80 for Units/μl). Data are the mean of triplicate assays and error bars are Standard Deviation. A & C: the entire range of doubling dilutions. Zero data points on *x*-axis not shown because scale is logarithmic. B: The lower concentrations from A (denoted by shaded box) but with linear-scaled *x*-axis and zero data point included. The red dotted line denotes the CR = 2 threshold and the solid line is the regression line used to calculate the quantity of RNase that equates to CR = 2 (2.90×10^{-9} U RNase). D: same as B, but for the DNaseAlert assay; the CR = 2 threshold equates to 1.67×10^{-3} U DNase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Software). The linear regression equation from the lower concentrations of nuclease (where RFU and nuclease concentration had a linear relationship) was used to establish the CR = 2 thresholds in terms of nuclease concentration. The CR = 2 threshold is 2 x ANC.

3. Results

3.1. Assigning nuclease activity values to Contamination Ratios

The RNaseAlert assay returned the expected sigmoid curve over the entire set of RNase dilutions (Fig. 2A). The ANC returned a mean RFU of 411.7, standard deviation (SD) = 15.4, so the CR = 2 threshold was 823.3 RFU. Plotting the data from RNase concentrations of $0–9.77 \times 10^{-9}$ U RNase (411.7–1806.0 RFU) returned the equation $y = 3433.8\,x + 426.27$ ($R^2 = 0.98$), so we assess the quantity of RNase required to generate a CR threshold of 2.0 to be 2.90×10^{-9} U (or 0.116 pg) (Fig. 2B). For liquid Test Items, the CR = 2 threshold would be generated by 3.63×10^{-11} U (or 1.45×10^{-3} pg) RNase/µl.
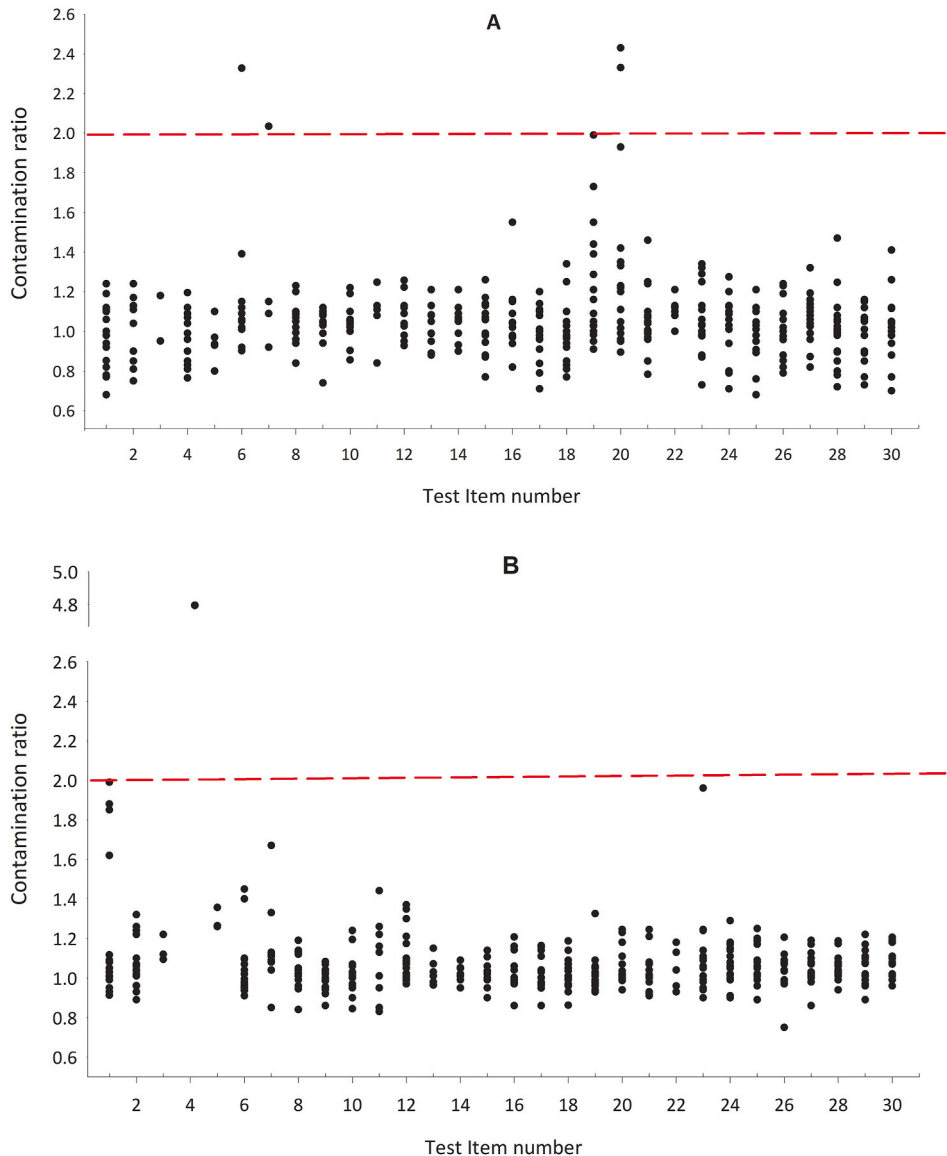
Fig. 3. Results from the 17 rounds of RNase (A) and DNase (B) testing performed between November 2015 and May 2023. The Test Item numbers are as per Table 1 and the red dotted line denotes the Contamination Ratio (CR) = 2 threshold denoting "contamination". Each data point is an individual test result. The number of tests performed per Test Item reflects the age and usage of equipment and the dates that a decision was made to include the Test Item into the regimen. For example, Test Item 21 (water from our water purification system) has been tested on every occasion but Test Item 22 (ice from our ice machine) has only been tested five times due to its later inclusion into the regime. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The DNaseAlert assay also returned a sigmoid curve over the entire set of dilutions (Fig. 2C). The mean RFU for the ANC was 561.5 (SD 35.3) so the CR = 2 threshold was 1123.1 RFU. The 0 to 7.81 x 10^{-3} U DNase concentrations (561.5–3057.7 RFU) returned the equation $y = 321826\ x + 585.33$ ($R^2 = 0.998$), so we assess the quantity of DNase required to generate a CR = 2 threshold to be 1.67 x 10^{-3} U DNase (Fig. 2D). For liquid test items, the CR = 2 threshold would be generated by 2.09 x 10^{-5} U DNase/µl.

3.2. Nuclease test Item results

We have performed 17 rounds of nuclease testing since we implemented the regimen in November 2015. We initially tested every four months, then reduced that to every 6 months after 3 years because of the consistent negative results. There was one occasion when testing could not be performed for operational reasons. During this time, RNaseAlert assays have failed Assay Acceptance Criteria twice and DNaseAlert assays once (always because the difference in RFU between the positive control and ANC was insufficient). Assays have needed to be repeated because individual Test Items failed Sample Acceptance Criteria on 8 and 4 occasions for RNaseAlert and DNaseAlert assays respectively (always because the CV between the replicates was too high).

Fig. 3 presents the variability in CR over time for each Test Item in respect of RNaseAlert (Fig. 3A) and DNaseAlert (Fig. 3B). A CR of 1.0 means that the RFU of the Test Item equals that of the ANC (i.e. background fluorescence), and for all Test Samples, median CRs are close to these values: 1.0–1.2 for RNaseAlert and 1.0–1.3 for the DNaseAlert. Sometimes Test Items had lower RFU than the ANC (30.9 % and 37.6 % for RNase and DNase assays respectively) returning a CR < 1.0 (minimum Test sample CR = 0.7). The mean RFU of the ANCs and Positive Controls are 1673 (CV 10.8 %) and 54,452 (CV 8.2 %) in the RNaseAlert assays and 449 (CV = 3.9 %) and 15,973 (CV 5.0 %)in the DNaseAlert assays.

3.3. RNase assay

For RNaseAlert (Fig. 2A), a total of 358 tests have been performed on the 30 Test Items, of which 4 tests (1.1 % of the total) returned a CR ≥ 2.0, denoting RNase contamination. Test Items 6 and 7 are different bottles of DNA elution buffer used by our Chemagen MSM1 DNA extraction robot (see Table 1), and both returned elevated RNase CRs of 2.33 and 2.03 respectively in the same test (May 2017). Excluding these elevated CRs, their mean CRs are 1.04 (SD 0.04) and 1.05 (SD 0.12) respectively. The bottles are located in our dedicated DNA extraction lab, where RNase is used to prevent RNA coeluting with extracted DNA: i.e. in an area with an inherently elevated risk of RNase contamination. The bottles were sealed in a plastic bag and discarded. The blank extractions returned by the Chemagen MSM1 robot (i.e. Test items 2 and 3) were from the previous run that used these two bottles of buffer and as both were negative for RNase in the same test (CRs of 0.75 and 0.95 respectively), so we concluded that the contamination was inconsequential. The preventative action consisted of replacing the bottles of buffers, after which the CR returned to normal.

Test Item 20 (the 16-well microplate used to quantify nucleic acid in our spectrophotometer) returned RNase CRs of 2.43 in April 2018 and 2.33 in January 2020. Its mean CR (excluding these elevated values) is 1.14 (SD 0.18). After thorough cleaning and decontamination with RNaseZap (Ambion), 2 % Tween 20 (Sigma Aldrich) and 70 % ethanol (Merck) the microplate's CR returned to normal levels in both instances. As nucleic acid applied to the microplate is cleaned off and disposed of after analysis, no downstream contamination of purified nucleic acid can have occurred, so the contamination event was deemed inconsequential and no remedial action in respect of previously-quantified extracts was deemed necessary.

3.4. DNase assay

For the DNase assay (Fig. 2B), 1 of the 408 tests performed (0.2 % of the total) returned a CR ≥ 2. Test Item 4 (the blank from our QiaCube RNA extraction robot) returned a DNase CR of 4.79 in December 2018. Excluding this elevated value, the mean CR for this Test Item is 1.14 (SD 0.21). We perform RNA extractions in a dedicated laboratory with dedicated equipment because we use DNase to prevent the coelution of DNA with extracted RNA [12]. The QiaCube robot was decontaminated with DNaseZap (Ambion) then 70 % ethanol (Merck), then twelve RNA extractions performed and tested in conjunction with an RNA extract from the QIAcube run that preceded that which generated the positive blank: all were DNase-negative (mean CR 1.01 (SD 0.12)), so no further remedial action was taken.

The CR from Test Item 1 (blanks from our Tecan PBMC isolation/urine aliquotting robot) has never exceeded the CR = 2 threshold, but the CR became intermittently elevated compared to historic values in four rounds of testing from August 2019. Excluding these elevated values, this Test Item returned CRs of 0.91–1.11 (mean 1.02, SD 0.06, $n = 12$), but the elevated CRs were 1.62–1.99 (mean 1.84, SD 0.16, $n = 4$). The elevated CRs were a consequence of differences in the blanks that were assayed, which in turn related to different applications of the Tecan robot. In the elevated tests the blanks were CryoStor CS10 Cell Freezing Medium (reflecting the PBMC isolation protocol that the Tecan is used for) but in the remaining 12 tests the blanks were water (representing the plasma that the Tecan also aliquots). A kinetic test with measurements at time points 0, 30 min and 60 min established that CryoStor CS10, taken fresh from a sealed bottle, had a stable RFU approximately double that of the (water) ANC (and therefore similar to the CR = 2 threshold), which, unlike DNase controls, did not increase with incubation time. The elevated CR for the Tecan robot was therefore assigned to a chemically-induced increase in background fluorescence in the DNase assay in respect of CryoStor CS10, rather than DNase contamination.

4. Discussion

It is commonplace for clinical biospecimens to yield partially degraded nucleic acids, because in vivo nucleases remain active whilst biospecimens undergo transport and processing, until they are stabilized (typically by fixation in formalin, stabilization in a nucleic acid stabilizer or by being frozen). The extent of the degradation depends on how stringent the collection/transport/processing protocols are in terms of controlling critical preanalytical variables such as ischemic time. In the absence of routine nuclease testing, a nuclease contamination event in a laboratory could easily go undetected simply because it is normal to observe partially degraded nucleic acid following extraction, so it would be logical for a laboratory to assume such degradation is a consequence of upstream preanalytical events rather than contamination in their laboratory.

Other than by direct nuclease testing, we see few methods by which laboratories could confidently identify nuclease contamination when at a low or intermediate level. The adoption of standardized collection and processing protocols prevents the uncontrolled degradation of biospecimens, but it is difficult to harmonize these across different institutions or achieve universal adherence to a given protocol. Also, what is considered to be a "high quality" biospecimen depends on the analyte being studied and the downstream analytical platform(s) being applied. The Standard Preanalytical Code (SPREC) is a numerical/alphabetical code that assigns the specific preanalytical details to each biospecimen, thereby enabling those that received suboptimal handling and therefore likely to return degraded nucleic acids to be identified [13,14]. However, in our experience, most biospecimens in laboratories other than professional biobanks are not annotated with SPREC codes, so their preanalytic parameters are unknown.

Participation in a Proficiency Testing scheme would be beneficial, because these enable a laboratory to compare their processes with their peers, using the same starting material [15–17]. Consequently, a report advising a laboratory that their processes yield nucleic acids that are unusually degraded suggests there could be a nuclease contamination issue. However, such schemes only cover certain laboratory processes, usually on an annual basis only, and there could be other factors accounting for the results (e.g. excessive homogenization of tissue). We believe that implementing routine nuclease testing on a biannual or triannual basis, with the option of performing additional *ad hoc* tests when necessary is the most effective way to monitor for nuclease contamination. In our hands, using the RNaseAlert and DNaseAlert assays, the quantity of RNase required to trigger a "contamination" call is 2.90×10^{-9} U (or 0.116 pg) for solid Test Items and 3.63×10^{-11} U (or 1.45×10^{-3} pg) RNase/µl for liquids. For DNase, we calculate the "contaminated" call to occur at 1.67×10^{-3} U DNase for solid Test Items and 2.09×10^{-5} U DNase/µl for liquid Test Items. When comparing results between different laboratories and selecting nucleases to use as controls it is important to note that the definition of a nuclease activity "unit" can differ, so it is important to establish how a manufacturer's unit equates to a standardized unit (e.g. a Kunitz Unit) when comparing nuclease activity data [11].

The sensitivity of the assays depends on the type of spectrofluorometer used and its gain settings, and so it cannot be assumed that other laboratories would have comparable results to ours. Assay sensitivity could be improved by increasing gain settings, but the consequence of this might be that the RFU of the positive control exceeds the detection range of the spectrofluorometer and the difference in RFU between the negative and positive controls decreases (we use the default gain settings of our spectrofluorometer). Assay sensitivity is also dependent to some extent on the incubation time. The assay kits' handbooks recommend 30–60 min incubation and we selected the longer incubation to maximise sensitivity.

Although every item in our laboratory is eligible for testing (with the exception of chemicals that are incompatible with the assays because they have dark colour, high ionic strength, extremes of pH or are chaotropic), it is impractical to test everything. We focus on the consumables, elution buffers and extraction robots located in areas where the risk of contamination is highest: nucleic acid extraction laboratories where nucleases are actively used. We also test ice from our plumbed in ice machine because tubes containing nucleic acid are placed in ice to their necks and the purity of the incoming water supply is outside of our control. Likewise, we test the water delivered by our water purifier (also connected to an external supply) because we use it to make up buffers for processes such as PCR or gel electrophoresis that directly manipulate nucleic acids.

In the absence of nuclease testing, laboratories are solely relying on preventative measures such as the use of laboratory coats/gloves and designating areas of laboratory space to be nuclease-free to prevent nuclease contamination. Despite our adopting such measures, we found 1.1 % ($n = 4$) of our RNase tests and 0.2 % ($n = 1$) of our DNase tests to be contaminated. Two of the four RNase-positive tests were from Test items located in our dedicated DNA extraction laboratory, where RNase is actively used, and the only DNase-positive test originated in our dedicated RNA extraction laboratory, where DNase is actively used. It could be considered debatable how consequential it is for an RNA extract to contain some DNase, or for a DNA extract to contain some RNase when these enzymes are used in the respective extraction protocols anyway. However, given that downstream equipment such as nano-electrophoresis platforms and PCR machines are generally used for both DNA and RNA, we consider that all nucleic acid extracts must be nuclease-free. Our findings demonstrate the importance of spatially separating DNA extractions from RNA extractions, using dedicated equipment and consumables for each and ideally performing them in separate laboratories. We also recommend that laboratory technicians change gloves immediately after using any nuclease-containing reagent.

We recorded elevated levels of DNase in the blanks from our Tecan robot when blanks consisted of CryoStor PBMC freezing medium, but not when they were water. Whilst still below the threshold denoting contamination, the DNase levels were clearly higher than background. Our kinetic tests concluded that these results are most likely caused by a chemical incompatibility between the CryoStor CS10 PBMC preservation medium and the DNaseAlert substrate/assay, but nevertheless, we cannot totally exclude the possibility that CryoStor CS10 has low levels of nuclease activity. However, studies using this medium have consistently shown that PBMC viability is not impaired with CryoStor CS10 compared to alternative cryopreservation media [18,19]. In addition, extracellular DNases are present in the plasma of both healthy and diseased individuals, so PBMCs likely have an inherent resilience to low levels of extracellular DNase [20–23]. We are therefore confident that no changes are required to our PBMC isolation protocol.

In their review of Crisis Management for Biobanks, Parry-Jones et al. describe how risk can be calculated by assigning numerical values on a scale of 1–5 for both the probability of a damaging event occurring and the magnitude of the impact should that event occur, then multiplying these two numbers together and noting where the product falls on a matrix of risk categories, from very low to very high risk [24]. If we apply this model to the risk of nuclease contamination in our biobank, where we perform nucleic acid extractions for researchers, were we not to perform the nuclease testing, we would categorize the risk as "high" because we assign the probability of some kind of nuclease contamination to be 4/5 and the impact should such an event occur as 3/5. Performing the nuclease testing enables us to more reliably assign a probability value to the likelihood of an event occurring because we now have data. We can also lower the risk category to "intermediate", because by monitoring we lower both the likelihood of a contaminating event occurring and its impact. By comparing ongoing testing data with historical data and having a protocol in place that can readily be applied on an *ad hoc* basis, we are able to identify equipment and processes that are vulnerable to contamination and implement preventative or remedial action in an expeditious and targeted way.

The requirements of ISO 20387:2018 General Requirements for Biobanking and ISO 17025 Testing and Calibrations Laboratory standards stipulate that laboratories must monitor and control their environmental conditions with defined quality control criteria to ensure their fitness for purpose, and to avoid adversely impacting their biological material or test items. We use our nuclease testing regimen as a quality indicator that demonstrably fulfils these criteria, providing us with documented, systematic feedback that our laboratory processes have resilience to nuclease contamination. Regardless of whether a laboratory is accredited or not, we encourage them to implement a similar regime.

5. Conclusion

In 8 years and 17 rounds of nuclease testing we demonstrate that the precautions we take to prevent nuclease contamination (which are standard laboratory practice) are generally effective. However, we also show that despite said precautions, nuclease contamination can still occur where processes that actively use nucleases are performed. Equipment used immediately downstream of these processes is also at risk. We found 0.2 and 1.1 % of tests to be positive for RNase and DNase respectively. Given that the consequences of nuclease contamination of nucleic acid samples are potentially catastrophic, we recommend other laboratories implement a similar testing regime. When performed regularly, laboratories can use the results to identify specific high-risk activities, then focus the testing in these areas or undertake more stringent preventative measures such as reorganising a lab to better quarantine DNA extractions from RNA extractions.

Additional information

No additional information is available for this paper.

Data availability statement

All raw data is available from the corresponding author.

CRediT authorship contribution statement

Estelle Henry: Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eleftheria Charalambous:** Investigation, Formal analysis. **Fay Betsou:** Funding acquisition, Conceptualization. **William Mathieson:** Writing - review & editing, Writing - original draft, Validation, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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