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Detection and quantification of ricin-mediated 28S ribosomal depurination by digital droplet PCR

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ABSTRACT

Ricin acts to damage cells by producing a site of depurination in 28S ribosomal RNA. This depurination results in ribosome inactivation which inhibits protein synthesis and ultimately leads to cell death. We have developed a multiplexed digital droplet polymerase chain reaction assay that enables the objective measurement of toxin activity through quantitation of depurinated 28S rRNA molecules. This assay demonstrates the first use of digital PCR technology to measure ribotoxin-mediated damage. Depurination events were detected in ricin-treated lung cell cultures as early as 1 h, and within 9 h of exposure the maximum ribosomal damage of 70% was reached and was sustained for at least 24 h post-exposure.

1. Introduction

Ricin toxin is a 66-kDa ribosome-inactivating protein (RIP) derived from the seeds of the castor bean plant (*Ricinus communis*) and comprises an A- and B- polypeptide chain linked covalently by a single disulphide bond [1]. The B-chain facilitates toxin entry into mammalian cells by binding to β-1,4-linked terminal galactose residues which are present on cell surface glycoproteins and glycolipids. Ricin enters the cell by endocytosis, where a proportion of the ricin is transported via the Golgi apparatus to the endoplasmic reticulum [2,3]. Within the ER lumen, the A- and B-chains are separated by a resident protein disulphide isomerase and retrotranslocated to the cytosol via the Sec61 channel. The A-chain acts as an RNA *N*-glycosidase to inactivate ribosomes by cleaving an adenine at residue 4324 of the highly-conserved sarcin-ricin loop in eukaryotic 28S rRNA, an activity called depurination [4]. The depurinated site prevents the formation of an essential stem-loop configuration required for eukaryotic elongation factor 2 binding, thereby inhibiting protein synthesis, ultimately resulting in cell death [5].

The majority of ricin detection and diagnostic assays that have been developed are immunochemical and require the presence of ricin at a detectable concentration rather than measuring toxin function [6,7]. Assays for toxin activity have also been developed, where some detect the release of free adenine as a result of the ricin A-chain enzymatic activity while others detect the production of depurinated nucleic acids [1,8,9]. Depurinated nucleic acids have been detected through use of relative quantification polymerase chain reaction (qPCR) assays. For example, Melchior Jr and Tolleson [6] developed a real-time assay that directly measures the enzymatic activity of RIPs on 28S rRNA using SYBR green. A similar assay was used to assess the activity of ricin on 25S rRNA in yeast [10]. Falach et al. [11] developed an endpoint assay to measure truncated cDNA molecules reverse transcribed from depurinated 28S rRNA. Molecules (control and truncated cDNA) were labelled with fluorophores and were resolved using capillary electrophoresis. This assay was further refined and changed to a quantitative probe-based PCR assay but requires an additional step of ligating a synthetic oligo tag to the truncated cDNA molecule prior to real-time quantitative PCR (RT-qPCR) [7]. However, with the advent of digital PCR, there is now scope for PCR assays to yield accurate absolute counts of depurinated molecules for the first time, rather than counts generated from relative data.

There are different digital PCR systems available on the market based on either chip or droplet technology. The Bio-Rad QX200 system uses water-in-oil partitioning to generate around 20000 nanolitre-sized droplets for each sample. Template molecules are randomly allocated into the tiny droplets or partitions such that each of these partitions may contain no template or one or more copies of template. Each of these partitions undergoes PCR amplification, in parallel, through thermal cycling. Partitions are then analysed in order to determine the number of positive and negative partitions [12,13]. Poisson statistics

*Abbreviations:*SEM, standard error of the mean

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are applied to calculate the original target concentration based on the fraction of positive partitions and thus generate absolute counts for targets [13].

Digital droplet PCR (ddPCR) has advantages over conventional RT-qPCR. Unlike conventional RT-qPCR, it does not require the use of a standard curve to generate counts for unknown samples via absolute quantification [14]. It is also less sensitive to factors that can inhibit target amplification due to the partitioning of template molecules [15]. RT-qPCR requires sample fluorescence to cross a threshold level and the number of cycles needed to reach threshold is used to calculate original starting template quantities [16,17]. Threshold cycle can, however, be influenced by factors affecting PCR efficiency resulting in variable accuracy and precision of RT-qPCR [12]. The effects of PCR efficiency are not as significant for digital PCR as the reaction only needs to reach a threshold at which the product will be detected [18]. The precision and sensitivity of ddPCR increases as more partitions are analysed. Through partitioning, the limit of detection is high as each droplet is essentially a separate sample [19,20]. Furthermore, digital PCR requires low amounts of template and the results are shown to be more reproducible than conventional approaches [18]. Similar detection chemistries (SYBR green, Taqman probes) are also used in both digital PCR and RT-qPCR which facilitates assay conversion to digital format [21].

With the advent of ddPCR, there is now scope to improve the qPCR assay described by Melchior Jr and Tolleson [6] to enable absolute quantification of ricin-mediated damage. This work describes the first use of ddPCR technology to accurately measure time-dependent RIP-/ricin-mediated damage of rRNA. Given inhalation is a highly toxic exposure route for ricin [22], normal mouse lung cells exposed to ricin were used as an *in vitro* model to generate depurinated rRNA. This permits the absolute quantification of depurination events over time in physiologically-relevant cell populations. Furthermore, this method may be used to detect the activity of other traditional RIPs and toxins which possess the same enzymatic activity (such as abrin or Shiga Toxin) in any cell or biological sample that has the same 28S rRNA target sequence. In addition, this assay could also be utilised to evaluate novel type I and II RIPs that are predicted to have RIP activity due to their amino acid sequence homology with known RIPs.

2. Materials and methods

2.1. Cell culture

Cell culture consumables were purchased from Thermo Fisher (Scoresby, VIC, Australia) unless otherwise stated. Normal mouse lung cells (MM14.Lu) were sourced from the American Type Culture Collection (ATCC, Manassas, VA, USA). MM14.Lu cells were cultured in Dulbecco's Modified Eagle Medium with 1% L-glutamine and 5% foetal bovine serum, at 37 °C with 5% CO_2, in T75 flasks. Cells were cultured to 80% confluency prior to passaging, during which cells were washed twice with phosphate-buffered saline (Sigma, Castle Hill, NSW, Australia), detached with Trypsin-EDTA (0.25% w/v) for 2 min, harvested in cell culture medium and pelleted at 225 × *g* for 8 min. Cells were resuspended in culture medium in preparation for seeding.

2.2. Cell treatment

A total of 64 biological replicates were prepared in 16 × 4-well culture dishes (Sigma). Cells were seeded at a density of 2×10^5 cells/well. After overnight recovery at 37 °C with 5% CO_2, culture dishes were randomly and equally allocated to a treatment group or a time-matched control group. Each treatment or control time point had 4 biological replicates. Treatment consisted of exposure of cells to a final concentration of 1 nM ricin toxin which was a gift from Jane Holley (Defence Science and Technology Laboratories, Porton Down, UK) in culture medium. Time-matched control plates were incubated in cell culture medium only. All toxin manipulations were performed using personal protective equipment in a cytotoxic drug cabinet that was compliant with Australian Standards. Waste material was decontaminated in at least 0.125% (w/v) sodium hypochlorite in accordance with findings from Mackinnon and Alderton [23].

Time points for treatment were 1, 2, 4, 6, 9, 12, 18 and 24 h. At the end of each interval, cells were detached with trypsin (as previously described), counted, pelleted, snap frozen in liquid nitrogen and stored at −80 °C until required for RNA extraction.

2.3. RNA extraction and cDNA synthesis

Total RNA was isolated from cells using the RNAqueous kit (Thermo Fisher) with an additional DNase treatment, according to the manufacturer's instructions. The quantity and quality of isolated RNA were determined using a Qubit 2.0 fluorometer (Thermo Fisher) and Bioanalyzer 2100 with an RNA 6000 Nano kit (Agilent Technologies; Mulgrave, VIC, Australia). RNA purity was assessed using a NanoDrop 2000 (Thermo Fisher). RNA yield ranged from 25 to 93 ng/μL across all samples. RINs ranged from 8.8 to 10.

A 200 ng duplicate cDNA synthesis (20 μL total volume) was undertaken on each sample. Each cDNA synthesis reaction included two controls: one containing water only and another without reverse transcriptase. Synthesis of cDNA was performed using a Transcriptor First Strand cDNA Synthesis kit (Roche, North Ryde, NSW, Australia) following the manufacturer's instructions. Duplicate syntheses for each sample were then pooled upon completion to minimise variability in the synthesis step and cDNA was diluted 1/20000 for use in ddPCR.

2.4. Primer design

Primers were based on the dual amplicon format of Melchior Jr and Tolleson [6] with modifications. One set of control primers measures the total amount of 28S rRNA at a location close to the site of depurination. The second primer set detects the site of depurination. However, the small amplicon sizes produced by the Melchior Jr and Tolleson [6] primers were outside the specifications recommended for use as Taqman assays. Primers were therefore re-designed, with conservation of the depurination detection primer, to accommodate Taqman probes (Table 1). The new primers were designed using the human and mouse 28S rRNA sequence (reference code NR_003287.2 and NR_003279.1) available at https://www.ncbi.nlm.nih.gov/nuccore/1154491914/ (last accessed 16/11/2016). Primers and Taqman probes were designed using Primer 3 software freely available at http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi using the parameters as directed in the Bio-Rad QX200 user's manual. Probes contained 5′ HEX or FAM labels and 3′ BHQ-1 as the quencher (GeneWorks; Thebarton, SA, Australia).

2.5. Digital droplet PCR assay conditions

All primers were initially assessed via traditional endpoint PCR and analysed on a 1.5% (w/v) agarose gel stained with SYBR Safe dye (Thermo Fisher) to ensure specific product amplification (data not shown).

Table 1
Primer sequences and labels.

Primer name	Sequence 5′-3′	Amplicon Length (bp)
Ctrl_fwd	AATACAGACCGTGAAAGCGG	94
Ctrl_fwdP (HEX)	TTTGGGTTTTAAGCAGGAGG	
Ctrl_rev	AGCCAGTTATCCCTGTGGTA	
Depur_fwd	GGTTTAGACCGTCGTGAGAC	97
Depur_fwdP (FAM)	TGATGATGTGTTGTTGCCATG	
Depur_rev[a]	TCTGAACCTGCGGTTCCACA	

[a] Melchior Jr and Tolleson [6] depurination detection primer.