

Short communication

Quantitative analysis of WRN exonuclease activity by isotope dilution mass spectrometry

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A B S T R A C T

Werner syndrome is a disorder characterized by a premature aging phenotype. The disease is caused by mutations in the *WRN* gene which encodes a DNA helicase/exonuclease which is involved in multiple aspects of DNA metabolism. Current methods mostly rely on radiometric techniques to assess WRN exonuclease activity. Here we present an alternative, quantitative approach based on non-radioactive isotope dilution mass spectrometry (LC-MS/MS). A oligoduplex substrate mimicking the telomeric sequence was used for method development. Released nucleotides, which correlate with the degree of oligoduplex degradation, were dephosphorylated, purified, and quantified by LC-MS/MS. Heavy-isotope-labeled internal standards were used to account for technical variability. The method was validated in terms of reproducibility, time-course and concentration-dependency of the reaction. As shown in this study, the LC-MS/MS method can assess exonuclease activity of WRN mutants, WRN's substrate and strand specificity, and modulatory effects of WRN interaction partners and posttranslational modifications. Moreover, it can be used to analyze the selectivity and processivity of WRN exonuclease and allows the screening of small molecules for WRN exonuclease inhibitors. Importantly, this approach can easily be adapted to study nucleases other than WRN. This is of general interest, because exonucleases are key players in DNA metabolism and aging mechanisms.

Keywords:

Werner syndrome
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Werner syndrome (WS) is a rare autosomal recessive disorder characterized by a segmental premature aging phenotype, including early onset of atherosclerosis, osteoporosis, and a high cancer incidence. The disease is caused by loss-of-function mutations in the *WRN* gene which encodes the WRN protein, a member of the RecQ helicase family. On the cellular level, fibroblasts derived from WS patients display genomic instability and a reduced replicative life span (Kudlow et al., 2007). This phenotype is in accordance with experimental data demonstrating that WRN is involved in multiple aspects of DNA metabolism, such as DNA replication, genomic maintenance, and telomere regulation (Bohr, 2008; Reddy et al., 2010; Rossi et al., 2010). In contrast to the other five members of the human RecQ helicase family, WRN also possesses a unique 3' → 5' exonuclease activity (Huang et al., 1998).

The WRN exonuclease cleaves the DNA phosphodiester bond and releases free 5'-dNMPs from the DNA strand (Kamath-Loeb et al., 1998). To elicit its exonuclease activity, WRN requires a 3' recessed end (5'-overhang) substrate. WRN does not degrade duplex DNA with blunt ends, unless the substrate also contains a junction or alternate DNA structures such as a fork (Brosh et al., 2006; Shen and Loeb, 2000). It is largely inactive on short single-stranded DNA substrates (Kamath-Loeb et al., 1998), but longer ssDNA substrates are efficiently degraded (Machwe et al., 2006). Its activity is regulated by posttranslational modifications and protein interactions. For instance, phosphorylation of WRN by DNA-PK inhibits its exonuclease activity (Karmakar et al., 2002; Yannone et al., 2001). In addition, p53, BLM, and PARP1 cause inhibitory effects (Brosh et al., 2001; Sommers et al., 2005; von Kobbe et al., 2002, 2004), whereas the Ku70/80 complex stimulates exonuclease activity (Cooper et al., 2000; Kudlow et al., 2007; Li and Comai, 2000, 2001).

Standard methods to assess WRN exonuclease activity utilize radioactively or fluorescently 5' end-labeled DNA substrates to detect the degradation of the full-length DNA molecules (Boubriak et al., 2009; Brosh et al., 2006). Here we present an alternative approach to assess WRN exonuclease activity based on isotope dilution mass spectrometry (LC-MS/MS). This method may be

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particularly useful in two situations: Firstly, for laboratories that wish to replace the common radioactive assays with a non-radioactive one and, secondly, the method can be incorporated into high throughput screening approaches for small molecules that affect exonuclease activity.

We have validated our newly developed method and compared it to a modified version of an established protocol that uses a 5'-biotin-end-labeled DNA substrate to detect activity of recombinant WRN exonuclease (Brosh et al., 2006) (Suppl. Fig. 1). Importantly, using a telomeric substrate mimics one of the key functions of WRN which is to operate at the telomere (Bohr, 2008). To assess if this oligoduplex indeed serves as a suitable substrate for WRN in our hands, an exonuclease reaction was carried out as published previously (Brosh et al., 2006). The reaction mixture contained 75 fmol of the oligoduplex and 0.1–1 pmol of recombinant WRN. Subsequently, digestion products were resolved by denaturing polyacrylamide gel electrophoresis (PAGE) and biotin was detected by streptavidin-POD (Suppl. Fig. 2). In this method, loss of signal intensity of the full-length end-labeled DNA substrate was used as readout to assess exonuclease activity. As is evident from Suppl. Fig. 2, WRN efficiently catalyzes the degradation of this oligoduplex to truncated DNA molecules of various lengths in a concentration dependent manner. Initial degradation of the substrate is visible at an enzyme to substrate ratio (E/S) of ~ 1 (10 nM WRN) and reached saturation at an E/S of ~ 8 (60 nM WRN) with a maximum efficiency of $\sim 80\%$.

Instead of detecting the shortened DNA substrate, the rationale of the LC-MS/MS-based method is to detect degradation end-products, *i.e.*, free nucleosides, to assess WRN exonuclease activity. To allow comparability to the biotin-end-labeling technique, the same substrate and identical reaction conditions were chosen to develop the LC-MS/MS-based method. Since the oligoduplex contains a repetitive telomeric sequence (TTAGGG)₄, LC-MS/MS quantification of free 2'-deoxyguanosine (dG) was expected to be a suitable readout to assess WRN exonuclease activity. Fig. 1 shows a flow chart of the experimental procedure; a detailed protocol is available in the supplementary information section. Briefly, after the exonuclease reaction was carried out, samples were placed on ice and ¹⁵N-labeled dG was added as an internal standard to the

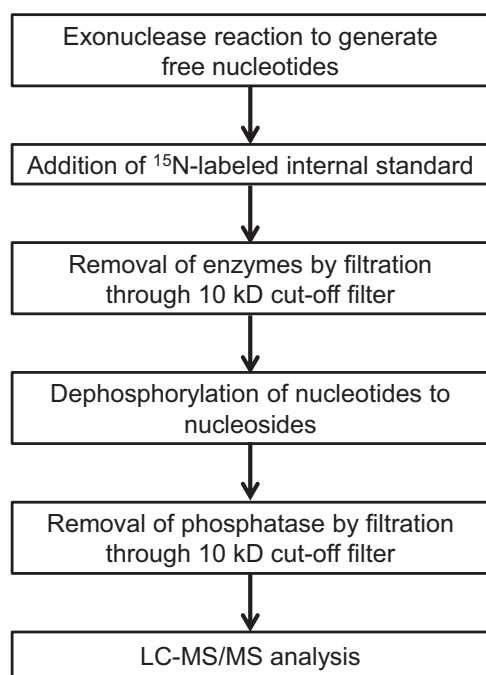


Fig. 1. Flow chart for the LC-MS/MS quantification of WRN exonuclease activity.

reaction mixture to account for technical variability during sample work-up and mass spectrometric measurement. Thereafter, recombinant WRN was removed by spin column filtration, followed by dephosphorylation of the nucleotides to nucleosides using alkaline phosphatase (AP), removal of the phosphatase by spin column filtration, and subsequent LC-MS/MS analysis. The recovery rate of the internal standard was usually $\sim 70\%$. Typical LC-MS/MS chromatograms of unlabeled and ¹⁵N-labeled dG as well as a calibration curve are shown in Suppl. Fig. 3.

The method shows adequate assay-to-assay variability (Suppl. Fig. 4A) and can be performed in 1–2 days dependent on the time chosen for AP digestion (Suppl. Fig. 4B; NB: no significant differences in the quantities of dG were observed between 4-h and overnight AP digestion, indicating that an AP digestion time of 4 h is sufficient for complete dephosphorylation of dGMP).

As shown in Fig. 2, free dG was detected in a time and concentration dependent manner. Since the reaction is still in its dynamic range after 15 min in terms of release of free dGMP (Fig. 2A), a reaction time of 45 min was chosen for the following experiments to achieve maximum oligoduplex degradation. In agreement with results obtained from biotin-end-labeling technique, WRN activity was already detected at an E/S of ~ 1 (5–10 nM WRN) and reached saturation at an E/S of ~ 8 (60 nM WRN) (Fig. 2B). A maximum of 32% of the expected total amount of dG was detected in WRN-digested samples. The lower efficiency of WRN compared to phosphodiesterase (PDE) may be related to incomplete annealing of the DNA strands and therefore to incomplete digestion by WRN. A WRN mutant with a mutation in the exonuclease domain (WRN-E84A, X-WRN) (Huang et al., 1998; Machwe et al., 2000) showed strongly diminished exonuclease activity compared to WT-WRN (Fig. 2C). The residual exonuclease activity observed in the X-WRN sample may be related to the sensitivity of the LC-MS/MS method, to some contamination with Zn²⁺ ions, which can trigger minimal exonuclease activity of X-WRN (Choudhary et al., 2004), or to contamination of the X-WRN protein with an unknown nuclease.

Conceivable applications of the LC-MS/MS-based method include studying effects of factors that modulate WRN exonuclease activity, such as posttranslational modifications and protein interaction partners. For example, PARP1, which catalyzes the synthesis of the biopolymer poly(ADP-ribose) upon genotoxic stress, is an established interaction partner of WRN (Rossi et al., 2010; Rouleau et al., 2010). Previously, it was shown that PARP1 inhibits both WRN's helicase and exonuclease activities (von Kobbe et al., 2004). In agreement with these results, PARP1 led to an inhibition of WRN exonuclease activity by up to 80% as detected by LC-MS/MS analysis (Fig. 2D).

Furthermore, the method can be applied to study WRN's substrate and strand specificity. To this end, we tested several newly designed WRN oligoduplex substrates which are compatible with our assay, *i.e.* an optimized forked oligoduplex, a blunt-ended oligoduplex, and oligoduplexes containing a 4-way junction and a 5'-overhang (Suppl. Table 1 for full sequence details). All substrates comprised a strand that contains only dG, dA, dT ('dG' strand) within a repetitive sequence element (XXXGGG)₆ (X = A,T in alternating sequence to assure annealing specificity) and a complementary strand that only contains dC, dA, dT ('dC' strand). Except for the blunt-ended substrate, the dG-strand was expected to be accessible for WRN exonuclease activity. In agreement with previous reports (Brosh et al., 2006; Kamath-Loeb et al., 1998), oligoduplexes which contained the fork and 4-way junction served as efficient substrates for WRN exonuclease, whereas the blunt ended oligoduplex showed almost no release of free dG (Fig. 3A). Moreover, significant WRN exonuclease activity was detected with the 5'-overhang substrate, however to a lesser extent than with the fork and 4-way junction oligoduplexes. As expected, WRN was not

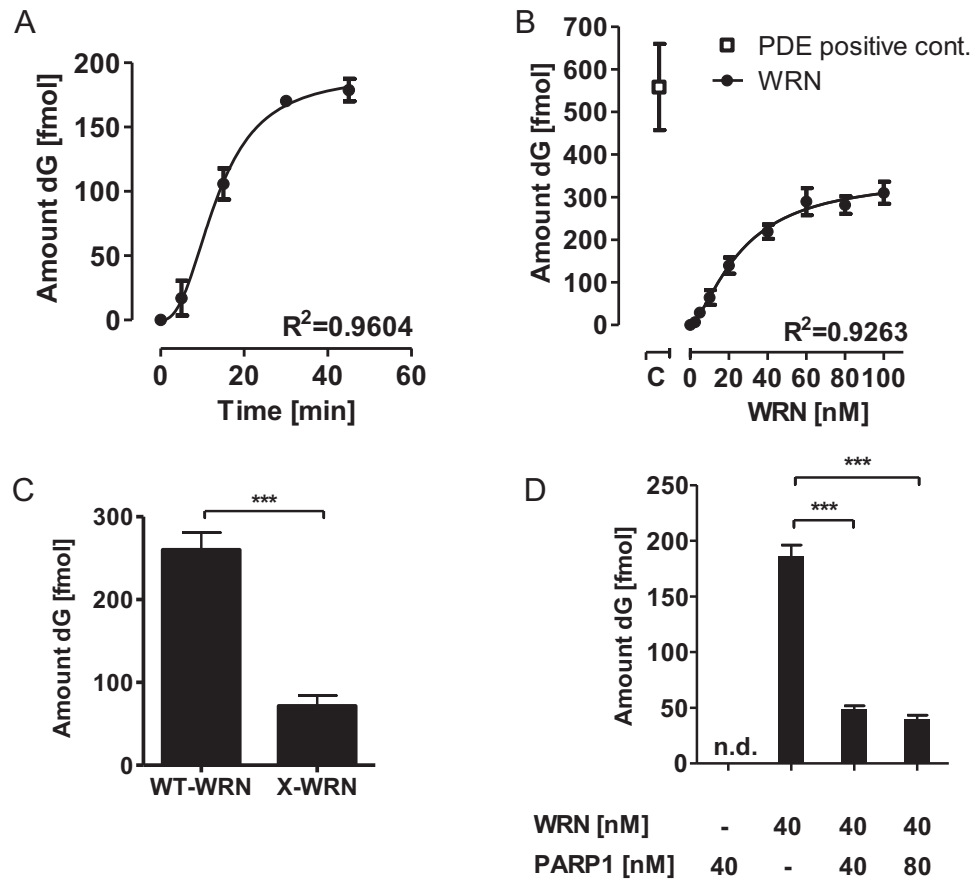


Fig. 2. Analysis of WRN exonuclease activity by isotope dilution mass spectrometry. WRN degrades an oligoduplex substrate mimicking the telomeric repeat sequence (TTAGGG)₄ to mononucleotides. Due to the repetitive sequence of the oligoduplex the amount of free 2'-deoxyguanosine (dG) correlates with the extent of oligoduplex degradation. Prior to LC-MS/MS analysis, nucleotides were dephosphorylated using alkaline phosphatase. Samples were subjected to LC-MS/MS analysis monitoring the transition m/z 268 \rightarrow 152 (dG) in the multiple reaction monitoring (MRM) mode. ¹⁵N-labeled dG was added as an internal standard directly after the reaction to account for technical variability during sample work-up and LC-MS/MS analysis; the ion transition m/z 273 \rightarrow 157 was monitored in MRM mode. (A) Time course analysis of WRN exonuclease activity using 40 nM WRN shows that the reaction reached a plateau after 30 min. (B) WRN degrades the oligoduplex in a concentration dependent manner. After incubation with 60–100 nM WRN for 45 min, the reaction reached a plateau. Incubation of the oligoduplex with snake venom phosphodiesterase (PDE) served as a positive control. (C) A mutation in the WRN exonuclease domain (WRN-E84A, X-WRN) led to a strongly diminished exonuclease activity (40 nM of WT-WRN and X-WRN, respectively; reactions were run for 45 min) (D) Recombinant PARP1 inhibits WRN exonuclease activity. Concentrations of WRN and PARP1 were used as indicated; reactions were run for 45 min. Oligoduplex substrate was used in a concentration of 75 fmol (A, B, D) or 100 fmol (C) per reaction. Statistical analysis was performed using one-way ANOVA following Bonferroni's multiple comparison test. *** p < 0.0001. Data represent means \pm SEM ($N \geq 3$); R represents the non-linear regression coefficient. n.d., not detectable.

able to degrade the complementary strand as efficiently as evaluated by analyzing the release of free dC (Fig. 3B). In summary, the method described herein opens up new possibilities to study substrate and strand specificity of WRN in particular and exonucleases in general.

In conclusion, we have developed a novel, non-radioactive LC-MS/MS-based quantitative method to assess WRN exonuclease function and activity with high sensitivity, accuracy, and precision. As mass spectrometry is a common tool in molecular bioscience, this method represents a reliable and easy-to-use alternative to existing techniques with 5' end-labeled oligonucleotides. Our method is comparable to existing radiometric techniques in terms of workload and economic aspects, but can be performed in higher throughput and enables mass spectrometric structural characterization and full quantification of the exonuclease digestion products. For this reason this new method should be instrumental for several applications. As demonstrated in this study, the LC-MS/MS method can be used to study exonuclease activity of WRN mutants (Fig. 2C), modulatory effects of WRN interaction partners and posttranslational modifications on its exonuclease activity (Fig. 2D), and WRN's substrate and strand specificity (Fig. 3). Moreover, the LC-MS/MS method can be

employed to analyze the selectivity and processivity of WRN exonuclease with high specificity. For example, as shown by classical radiometric techniques, WRN exonuclease is selectively blocked by specific base modifications such as 8-oxo-guanine, 8-oxo-adenine, and cholesterol adducts, but is active on other lesions such as uracil or hypoxanthine (Bukowy et al., 2008; Machwe et al., 2000). Since released base adducts can be unequivocally identified, characterized, and quantified by mass spectrometry, the current method represents a promising approach to extend such studies. In addition, this method allows the screening of small molecule libraries for potential WRN exonuclease inhibitors. In analogy to a recently identified WRN helicase inhibitor (Aggarwal et al., 2011), such potentially existing exonuclease inhibitors could be useful tools to study the function of WRN in a cellular context and may also have therapeutic potential in cancer treatment. We are currently planning such screening approaches for finding inhibitors or stimulators of the WRN exonuclease activity at nanomolar levels. Finally, the LC-MS/MS-based method can be extended and adapted to study the activity, specificity, and processivity of nucleases other than WRN. Experiments with *Escherichia coli* ExoI show the feasibility of such extended applications (Fig. 4).

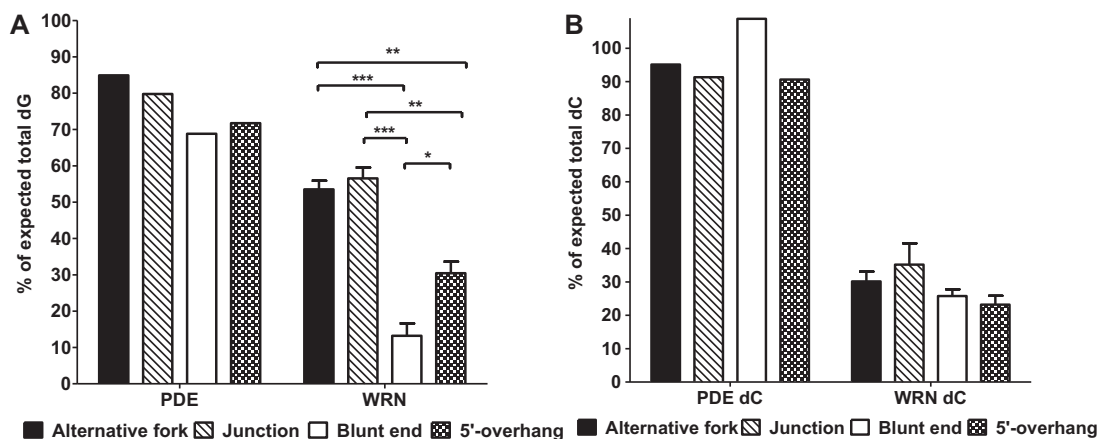


Fig. 3. Analysis of WRN substrate and strand specificity. WRN substrate specificity was tested with several oligoduplex substrates containing the repetitive sequence element (XXXGGG)₆ (X = A,T). Reaction mixtures contained 100 fmol of each oligoduplex, i.e., an optimized fork ("alternative fork"), a 4-way junction containing oligoduplex, a blunt ended oligoduplex, and an oligoduplex with a 5' overhang (for sequence information see SI section). ¹⁵N-dG and ¹⁵N/¹³C-dC were added as internal standards directly after the reaction to account for technical variability during sample work-up and LC-MS/MS analysis. Samples were analyzed in multiple reaction monitoring (MRM) mode with the following transitions: ¹⁴N-dG (*m/z* 268 → 152), ¹⁵N-dG (*m/z* 273 → 157), ¹⁴N/¹²C-dC (*m/z* 228 → 112) and ¹⁵N/¹³C-dC (*m/z* 240 → 119). PDE digestion served as a positive control and led to the release of 70–100% of the expected total amount of dG (A) and dC (B) of each oligonucleotide. WRN exonuclease reaction was performed in the presence of 40 nM WRN for 45 min. (A) Evaluation of the release of free dG of substrates as indicated. Whereas the blunt-ended oligoduplex served as a poor substrate for WRN exonuclease, the 5'-overhang led to the release of 30% of total dG, and the forked and 4-way-junction-containing substrates served as efficient substrates (>50% release of expected total dG). (B) WRN activity on the dG-complementary strand of the different substrates was tested by detecting the release of free dC. In contrast to the WRN-dependent degradation of the dG-strands, as expected the dC-strands served as poor substrates for WRN exonuclease showing a release of <35% of the expected total amount of dC. WRN data represent mean ± SEM (*N* = 3). PDE data represent means from two independent experiments. WRN data was evaluated by Student's *t*-test for statistical significant differences. **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

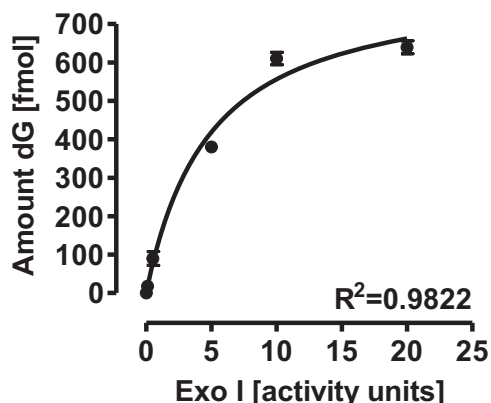


Fig. 4. Analysis of Exo I activity. LC-MS/MS quantification of Exo I activity. *E. coli* Exo I degrades a single-stranded oligonucleotide substrate (75 fmol), containing a repetitive element (TTAGGG)₄, to mononucleotides in a concentration dependent manner. Data are means ± SEM (*N* = 3). *R* represents the non-linear regression coefficient.

A limitation of the assay includes that access to an LC-ESI-MS/MS system is a prerequisite for its use. Moreover, it is limited to study exonuclease activity in non-cellular systems and so far it is not applicable to study the exonuclease directionality.

In summary, the LC-MS/MS-based exonuclease activity assay adds to the armamentarium of methods to study DNA metabolism in general and, as demonstrated in this study, in particular in the field of molecular aging research.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.mad.2012.06.005>.

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