A comprehensive model of DNA fragmentation for the preservation of High Molecular Weight DNA

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ABSTRACT

During DNA extraction the DNA molecule undergoes physical and chemical shearing, causing the DNA to fragment into shorter and shorter pieces. Under common laboratory conditions this fragmentation yields DNA fragments of 5-35 kilobases (kb) in length. This fragment length is more than sufficient for DNA sequencing using short-read technologies which generate reads 50-600 bp in length, but insufficient for long-read sequencing and linked reads where fragment lengths of more than 40 kb may be desirable.

This study provides a theoretical framework for quality management to ensure access to high molecular weight DNA in samples. Shearing can be divided into physical and chemical shearing which generate different patterns of fragmentation. Exposure to physical shearing creates a characteristic fragment length where DNA fragments are cut in half by shear stress. This characteristic length can be measured using gel electrophoresis or instruments for DNA fragment analysis. Chemical shearing generates randomly distributed fragment lengths visible as a smear of DNA below the peak fragment length. By measuring the peak of DNA fragment length and the proportion of very short DNA fragments both sources of shearing can be measured using commonly used laboratory techniques, providing a suitable quantification of DNA integrity of DNA for sequencing with long-read technologies.

INTRODUCTION

The structure of the DNA molecule has been known for more than 60 years and it is without a doubt the most intensively studied molecule in our bodies. However, there has been little need to evaluate the mechanisms of DNA fragmentation at length scales beyond a few thousand base pairs (Mulcahy et al., 2016). With the rising importance of long-read sequencing technologies and mapping technologies utilising linked reads (Schadt et al., 2010; Goodwin et al., 2016) it is however evident that a greater understanding of DNA structural integrity and fragmentation must be achieved to support better quality management practices for high-molecular-weight DNA (HMW-DNA) (English et al., 2012; Malentacchi et al., 2015; Goodwin et al., 2016). DNA is generally considered a relatively stable analyte as it is highly resistant to physical shear at the lengths required for PCR and short-read next generation sequencing (NGS) (Morente et al., 2006; Lou et al., 2014; Robb et al., 2014; Ellervik and Vaught, 2015). Prevention of chemical degradation over short periods of time is achieved using routine procedures. Due to this perceived stability, the research on the preservation of HMW-DNA has long been neglected (Anchordoquy and Molina, 2007). Purity, yield and amplification success are seen as sufficient quality parameters when evaluating DNA sample quality making it hard to estimate if DNA in samples is suitable for long-read sequencing (Bustin et al., 2009; Le Page et al., 2013; Mathay et al., 2016; Mulcahy et al., 2016; Office of Biorepositories and Biospecimen Research et al., n.d.).

In this study we create a comprehensive framework of the significant factors defining the fragment length distribution of DNA provided for sequencing. Biobank research and published method papers
provide valuable empirical data on fragmentation observed under common laboratory conditions. Yet, data is too sparse to untangle the potential pre-analytical variables and complex interdependencies necessary to identify the key molecular mechanisms contributing to fragmentation (Robb et al., 2014; Malentacchi et al., 2016a). Therefore, this work takes an approach where experimental data is assessed and theories are generated by a wide variety of fields affected by DNA fragmentation. By merging data from the following fields, we are thereby able to present a model explaining the key contributing factors to DNA fragmentation when preparing biospecimen for high throughput genomics research:

- Early research on the physical properties of DNA conducted in the years after identifying the structure of the DNA helix (Bowman and Davidson, 1972; Adam and Zimm, 1977; Dancis, 1978).

- Investigations into the chemical properties and degradation of DNA in vitro/in vivo (Lindahl, 1993) that ultimately led to the discovery of cellular repair mechanisms of DNA and the Nobel prize for these discoveries.

- Archaeological research investigating the decay of DNA over long periods of time and the decay products generated during the process. Indicating that the initial rate of decay is substantially higher than can be explained by depurination (Sawyer et al., 2012) but that long term fragmentation follow a 1st order reaction where depurination is a key driving factor (Overballe-Petersen et al., 2012) which for genomic DNA is close to what we can expect from depurination at pH 7.5 (Allentoft et al., 2012).

- Forensic research provides a baseline for DNA fragmentation patterns in different tissues under conditions where no stabilization of samples is provided (Bär et al., 1988; Johnson and Ferris, 2002).

- The stability of plasmids, linearized plasmids and genomic DNA for pharmaceutical applications (Evans et al., 2000). In particular, it should be noted that a similar survey to determine pharmacologically relevant factors of DNA degradation has been published (Rossmanith et al., 2011). Under these conditions DNA degradation due to depurination was estimated to be the dominant factor for degradation of samples.

- Polymer science and physical chemistry studying the behaviour of polymers (Vanapalli et al., 2006), and simulating electrostatic interactions in silico explaining the influence of folding and protection of DNA under high ionic-strength conditions (Cinque et al., 2010; Jiang et al., 2015).

A large number of potentially relevant chemical mechanisms for degradation have been identified in pharmacological research studying the degradation of DNA-based pharmaceuticals. For the purposes of DNA extraction, using aqueous solutions oxidation and depurination are estimated to be the relevant factors (Pogocki and Schöneich, 2000; Rossmanith et al., 2011). DNA sequencing of archaeological findings support these conclusions as they indicate that even DNA retrieved in conditions far from optimal show a degradation pattern consistent with depurination being the main source of fragmentation (Allentoft et al., 2012). By adjusting for this baseline of degradation other, more situational, factors for degradation such as the influence of specific DNA extraction can be identified and used to generate a minimal viable explanation for DNA fragmentation under laboratory conditions.

**A MINIMAL EXPLANATION OF DNA FRAGMENTATION**

When tracking DNA fragmentation in a laboratory environment the degradation process of DNA is initiated at the start of the warm ischemia time for tissue collection or blood draw (or equivalent) when collecting fluids (Johnson and Ferris, 2002; Betsou et al., 2010).
Chemical factors

The disruption of the cellular environment triggers a number of processes leading to the degradation of DNA by oxidizing attacks and degradation by nucleases, unless preservation measures are taken (Elmore, 2007; Rossmanith et al., 2011). This process can be halted through freezing or drying of samples. It can also be prevented by the addition of preservatives, in the form of chelating agents which bind metal ions that catalyse the production of hydroxyl radicals as well as Mg\(^{2+}\) ions in Mg\(^{2+}\)-dependent endonucleases (Lahiri and Schnabel, 1993; Rossmanith et al., 2011). Chemical degradation by these pathways is the immediate threat to DNA integrity and may rapidly produce a smear of short fragments. At the same time, these factors make DNA vulnerable to physical stress by "nicking" (the introduction of single strand breaks in double stranded DNA), but this issue is easily dealt with by the addition of buffers containing chelators, or a combination of EDTA + ethanol, which are highly effective in preventing degradation by oxidizing attacks (Evans et al., 2000). It should, however, be noted that EDTA on its own actually catalyses the formation of hydroxyl radicals and therefore increases degradation rates unless combined with a radical scavenger (Evans et al., 2000).

The other major cause of chemical shearing of DNA is depurination followed by β-elimination which is an acid-catalysed first order reaction (An et al., 2014). As the reaction is driven by protonation it is impossible to completely prevent it in aqueous solutions, and the complete dehydration of DNA requires encapsulation to protect the sample from atmospheric water as the DNA molecule binds water in its minor and major groove (Bonnet et al., 2010). Storing samples at pH 8 (Brunstein, 2015), reducing molecular mobility and temperature, along with the avoidance of primary amines, can reduce the reaction rate to levels sufficiently low to ensure that DNA maintain integrity for most long-read purposes for centuries (An et al., 2014).

Physical stress

Fragmentation due to physical stress has an immediate impact on HMW-DNA. Susceptibility to physical stress is directly dependent on the length and folding of DNA molecules (Anchordoquy and Molina, 2007; Cinque et al., 2010; Lengsfeld et al., 2011), and during extraction DNA is subjected to physical forces generating a peak abundance of 5-35 kb under common laboratory conditions (Malentacchi et al., 2013, 2016b). Laminar flows have for a long time been seen as the main culprit behind DNA fragmentation (Dancis, 1978), but chemists specialising in the study of polymers in turbulent flow have provided convincing evidence indicating that turbulence dominates the influence of laminar flows under most conditions (Vanapalli et al., 2006). These conclusions are further supported by the teams of Anchordoquy (Lengsfeld et al., 2011) and Baigl (Cinque et al., 2010) who have investigated the factors influencing the degradation of DNA for pharmaceutical applications (Anchordoquy and Molina, 2007).

These conclusions on physical shear stress significantly simplify the work to minimise the influence of physical shear in DNA processing, as it means that most DNA fragmentation occurs when the contour length of DNA exceeds the Kolmogorov length of a turbulent flow. The Kolmogorov length scale defines the smallest possible eddies occurring in a system before they dissipate into energy. To understand this connection between quantum mechanics and biology it is necessary to know that all turbulence can be modelled as consisting of small vortices that in turn consist of smaller vortices. At a certain threshold, it is no longer possible to divide vortices into smaller ones, and at the Kolmogorov length scale these distances are so small that the spatial movement of molecules turns into vibrations (i.e. heat). Calculations by Vanapalli et al. have proven that the pull of a DNA molecule stuck in two vortices at the same time is sufficient to break the strand in half and that this is the primary cause of fragmentation under most hydrodynamic conditions (Vanapalli et al., 2006). During sample processing fragmentation occurs over time until all DNA molecules have been degraded to a length where all fragments are short enough to remain within a single eddy, which is consistent with experimental results with commonly used laboratory equipment, such as vortex mixers and pipettes (Yoo et al.,
2011), and results suggest that the breakage rate is a function of the shear rate rather than the shear stress (Bowman and Davidson, 1972; Meacle et al., 2007).

Freezing and long-term storage

Finally, DNA can be fragmented by freezing, thawing, and long-term storage. Repeated freeze-thaw cycles are the most significant source of extensive damage but continued chemical reactions while the samples are frozen also play a role. Researchers frequently assume that nothing happens to DNA inside a frozen sample (Hubel et al., 2014), but in reality both chemical processes and physical movement occur even at low temperatures. Between -39 and -50 °C, the DNA molecule becomes rigid and brittle as it enters a glassy state (Norberg and Nilsson, 1996) while protons in the water remain mobile even at temperatures close to 268 °C (Benton et al., 2016). There is a lack of long term studies at -80 °C or -196 °C, and the complex behaviour of water at low temperatures makes it impossible to estimate at what temperatures chemical activity generated by protons ceases to occur. Using the Arrhenius equation to estimate an upper bound of activity we can, however, estimate that a strand break occur at a rate of approximately once every 5 seconds in a 3.2 billion base pair genome at 25 °C and pH 8. Storage in a -80 °C freezer reduces the rate to less than once per 10,000 years (An et al., 2014).

During freezing and thawing two other factors may contribute to the fragmentation of DNA (Anchordoquy et al., 2001; Shao et al., 2012). As a sample cools beneath the freezing temperature of fluids, it will undergo nucleation where water molecules (or other fluids) start assembling into crystals. Solutes are not incorporated into the solid ice crystals, resulting in the sample separating into solid components and unfrozen liquid components with high concentration of solutes. Creating an environment where two sources of DNA shearing has been proposed (Anchordoquy et al., 2001; Brunstein, 2015):

- As ice crystals form, solutes are crowded into gaps with increasingly high concentrations of solutes, thereby drastically increasing the potential for oxidising attacks and protonation of the DNA chain, leading to chemical shearing of the DNA polymer.

- As ice crystals form, DNA polymers are partially embedded within the ice crystals. The mechanical stress could thereby lead to the breakage of DNA molecules with a higher likelihood of breakage occurring in longer polymers.

Based on laboratory experiments, it is evident that chemical shearing is present under certain circumstances (Davis et al., 2000) but can be completely avoided as highly purified samples of short DNA fragments (<2 kb) have remained intact during repeated freeze-thawing in several studies (Ross et al., 1990; Thornton et al., 2005; Integrated DNA Technologies, 2014; Rossmanith et al., 2011). Rossmanith et al. could even show that short amplicons (274 bp) remained intact while the same amplicons when located within longer genomic sequences from Salmonella typhimurium (4857 kb) and Listeria monocytogenes (1160 kb) were sheared during their freeze-thaw experiments (Rossmanith et al., 2011).

Research in cryopreservation of oocytes, embryos and sperm (Amidi et al., 2016; Kopeika et al., 2015) suggest that shearing of DNA during freeze-thaw can be reduced by the addition of antioxidants, indicating that oxidation of DNA by radicals may, at least in vivo, be a significant cause of DNA fragmentation. Such conclusions must however be considered highly tentative as there is a substantial differences in the chemical environment inside the cell compared to the in vitro conditions covered by this study. The safeguards necessary to avoid physical shearing by ice crystal formation are also poorly understood. The addition of glycerol (Calcott and Gargett, 1981; Schaudien et al., 2007) is known to protect DNA during freeze-thaw cycles but we have been unable to determine why samples seemingly subjected to physical shearing may converge to lengths anywhere between 2000 bp and
25 000 bp in different studies (Röder et al., 2010; Ross et al., 1990; Schaudien et al., 2007; Shao et al., 2012).

**DISCUSSION**

Long-read sequencing enables studies of complex genomic events, e.g. structural re-arrangements, copy number variations, repeat expansions, etc. Hence, it is obvious that the long-read technologies, such as PacBio, 10x Genomics and Oxford Nanopore will be increasingly popular. This constitutes a challenge for laboratories and biobanks, as best practice routines for storage of biological samples and DNA extractions need to be adjusted in order to allow delivery of HMW-DNA suitable for long-read sequencing (Malentacchi et al., 2016a). Currently, the Oxford Nanopore MinION, GridION and PromethION are able to sequence reads up to 1 Mbp in length (Oxford Nanopore Technologies, 2017). The optimal results obtained by 10x Chromium systems are obtained if the sample contains a high number of molecules above 50 kbp, and up to a few hundred kilobases, and the PacBio systems can produce reads up to 60-70 kbp (Bickhart et al., 2017; Lok et al., 2017; Paajanen et al., 2017). These numbers will most likely rise due to technology development, and biobanks should be ready to be able to provide researchers with DNA samples of adequate quality and quantity.

Current biobanking practices provide DNA with a high yield and purity when tested, but suffer from a high level of variability in regards to DNA integrity (Malentacchi et al., 2013, 2015). Improved protocols and subtle alterations of protocols can, however, provide significant improvements. The SPIDIA-ring trials show that biobanks are able to rapidly improve their operations when provided with sufficient guidance (Malentacchi et al., 2016a). It is therefore important that an acceptable definition of DNA fragmentation and robust units of measurement are made available to the research community to improve the availability of HMW-DNA for researchers. In this regard the SPIDIA project (Malentacchi et al., 2013) and the Global Genome Biodiversity Network (GGBN) (Mulcahy et al., 2016) have provided pioneering work on the subject on how to objectively evaluate and compare DNA fragmentation in samples using gel-electrophoresis and a HindIII ladder. Both projects propose a “greater than X kb” approach where the percentage of DNA above a certain “DNA threshold” is measured and used as an indicator of DNA integrity. The SPIDIA project used the 4.36 kb size marker as its X-value and supplemented it with the measure peak intensity to provide an assessment of DNA integrity (Ciniselli et al., 2015). The GGBN on the other hand decided to forego the usage of a peak intensity and instead proposed the X value to be dependent on the intended utilization of samples but with the 9.416 bp size marker as a default as it is substantially longer than standard HTS reads and would be an appropriate minimum for long-read sequencing (Mulcahy et al., 2016).

The influence of physical shear stress and chemical degradation are, however, substantially different from each other. We therefore believe that two separate measures of DNA fragmentation are necessary to help researchers to evaluate the integrity of their DNA in an adequate manner.

**The characteristic fragment length (CFL):** The most abundant fragment length of the sample. In gel-electrophoresis (both conventional and capillary), this value corresponds to the peak intensity of the signal.

**The smear ratio (SR):** The proportion of DNA with a fragment length of less than 500 bp. This value can be easily calculated using the imageJ open source software to integrate over the signal intensity curve as described by Mulcahy et al. (Mulcahy et al., 2016).

The characteristic fragment length is routinely reported when DNA integrity is assessed using gel-electrophoresis. Based on the conclusions drawn in this paper it can be deemed suitable as a direct but conservative estimate of how physical shear stress limits the DNA fragment length in a sample.

The smear ratio provides an estimate of the proportion of DNA that has been shredded to short fragment lengths due to freeze-thaw shearing or chemical degradation. From a technical perspective the work to produce a “less than X kb” measurement is identical to the “greater than X kb” proposed by
GGBN, but it provides researchers with a more informative measurement. Samples with highly preserved content will have a lopsided distribution around the characteristic fragment length with a long tail of shorter DNA fragments. Changes in the maximum fragment length by physical shear stress will easily be detected by a shift in peak fragment length and a “less than X kb” measurement provides a corresponding estimate of the amount of DNA being sheared to very short fragments due to chemical degradation and/or freeze-thaw.

These two measurements therefore provide laboratories with the means to monitor changes in processing and allow end users to assess the potential of obtaining sufficiently HMW DNA from a resource. For advanced users and quality management it is, however, still attractive to also make the entire fragment distribution plot available (Permenter et al., 2015). Based on the experience of National Genomics Infrastructure - Sweden, the following instrumentation can provide researchers with this type of information: Agilent Bioanalyser (Agilent Technologies), Fragment Analyzer (Advanced Analytical) and Femto Pulse (Advanced Analytical), where two latter systems can process at least 8 samples in parallel providing both the characteristic fragment length and smear ratio in a single analysis (figure 1).

The first megabase plus read was recently announced using the MinION technology from Oxford Nanopore (Oxford Nanopore Technologies, 2017). At such long-read lengths, currently standard methods require modifications and gentle DNA extraction methods such as phenol–chloroform extraction (for reference the Rad003 protocol used by Loman lab to extract DNA for generating the megabase-length read is available at http://lab.loman.net/protocols/). The shearing of DNA caused by the extraction step itself is, at least in high-throughput laboratories such as biobanks, likely to generate fragments with a characteristic length of more than 50 kb, while turbulent forces during other parts of the process restrict the fragment length to 5-35 kb (Shao et al., 2012; Malentacchi et al., 2015). Among commonly used extraction methods, precipitation based DNA extraction generate the highest molecular weight of DNA (Malentacchi et al., 2015) but variations in fragment length (Shao et al., 2012) and other quality parameters mean that kit-by-kit comparisons will be necessary for the selection of suitable best practice procedures for DNA extraction depending on the intended usage of the samples.

CONCLUSIONS

Based on the model presented above, laboratories are advised to focus on three areas if they wish to increase the integrity of extracted DNA samples (table 1). During the retrieval of samples it is important to quickly stabilise the sample by preventing enzymatic degradation and generation of free radicals within the sample. After the cells have been lysed it is important to handle the sample with outmost care to minimise physical shearing (gentle mixing, slow pipetting, using wide-bore pipette tips, etc) and then store DNA at low temperature or in a completely dehydrated state to avoid depurination.

Physical degradation by shear stress is mainly caused by microscale turbulence. The turbulence fragments DNA until fragments reach a characteristic peak length. Physical forces are strongest in the centre of the DNA polymer but nicks and random events may cause strand breaks elsewhere. This generates a characteristic peak length roughly corresponding to half the length of the smallest fragment length prone to degradation in the system. When measured by gel electrophoresis this generates a characteristic pattern with a peak intensity of fluorescence followed by a long tail smear below the peak and a shorter upstream section of fragments that, due to random/chemical effects, reached a length protected from turbulence but still longer than half the critical length.

Freeze-thaw events may contribute significant damage to DNA but is highly dependent on parameters such as polymer size, ion strength, rate of temperature drops, presence of nicks etc. Currently, there is no generalized theory to predict fragmentation but preservation with means such as storage in 50 % glycerol has been shown to prevent shearing. Furthermore, it seems that variations in upstream
protocols have a profound impact on the vulnerability to freeze-thaw cycles as evidenced by in-house testing not identifying any fragmentation of samples exposed to repeated freeze-thaw cycles.

To support further work on maintaining DNA integrity it is important that researchers report relevant data on the integrity of DNA. DNA integrity data is often omitted or reported using inadequate measurements such as electrophoresis with *Hin*<sub>pol</sub>II digested λ DNA, which does not provide relevant information above 23 kb. Current literature on maintaining longer DNA for high throughput applications is therefore limited and this study provides a foundation for further work in quality management and process optimisation for the production of HMW-DNA for sequencing.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Example of high quality DNA with minimal smear (section B) and a characteristic fragment length of 129 292 bp as measured by the Femtopulse (Advanced Analytical). High quality samples often contain a plateau on the right hand side of the characteristic fragment length, meaning that the CFL should not be interpreted as a measure of the longest fragment length available for sequencing. But rather as a measurement of the turbulent forces restricting the fragment length of the sample. To provide longer reads suitable for long-read sequencing or linked-read technology, the DNA extraction process can be altered to reduce physical shearing. With continuous monitoring it is also possible to quickly any inadvertent changes influencing DNA integrity as chemical shearing an increased smear ratio or a drop in characteristic fragment length is immediately measured using the above described technique.
Table 1, key parameters for identifying causes of DNA fragmentation and how to reduce it.

<table>
<thead>
<tr>
<th>Source</th>
<th>Time scale</th>
<th>Mechanism</th>
<th>Threat profile</th>
<th>Preventive measure</th>
<th>Damage pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample collection</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Enzymatic degradation</td>
<td>Immediate</td>
<td>Naturally occurring DNAs attack the DNA polymer</td>
<td>An immediate threat to the integrity of DNA unless enzymes are inhibited or destroyed.</td>
<td>Addition of Proteinase degrading the protein or chelators binding metal ions functioning as co-factors.</td>
<td>DNA fragmentation with specific cleavage sites</td>
</tr>
<tr>
<td>Oxidation</td>
<td>Seconds to years</td>
<td>Metal ions catalysing the production of hydroxyl radicals</td>
<td>An immediate threat to the integrity of DNA unless chelating agents and/or a radical scavenger is added.</td>
<td>Addition of chelators or EDTA + Ethanol</td>
<td>Smearing</td>
</tr>
<tr>
<td>Turbulence</td>
<td>Immediate</td>
<td>Physical shearing</td>
<td>Poorly optimised machinery and careless handling of sample will cause physical shearing of the DNA.</td>
<td>Freezing, dehydration or maintaining a high P/E-value</td>
<td>Characteristic fragment length</td>
</tr>
<tr>
<td>Depurination</td>
<td>Weeks to years</td>
<td>Protonation of purines lead to depurination followed by beta elimination</td>
<td>A slow reaction that may cause issues under acidic conditions or long term storage at room temperature.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>Immediate</td>
<td>Physical shearing</td>
<td>The damage mechanisms of freeze-thawing DNA are still poorly understood in vitro but damage is proportional to the number of freeze-thaw cycles the sample has undergone. Flash freezing or the addition of a compound allowing samples to vitrify rather than form ice crystals seem effective.</td>
<td>Addition of a protective agent such as glycerol or flash freezing to low temperatures in order to prevent large ice crystals from forming.</td>
<td>Characteristic fragment length</td>
</tr>
<tr>
<td>Freeze-thaw</td>
<td>Immediate</td>
<td>Crowding of solutes leading to hydrolysis and/or oxidation</td>
<td>The damage mechanisms of freeze-thawing DNA are still poorly understood in vitro but damage is proportional to the number of freeze-thaw cycles the sample has undergone. Flash freezing or the addition of a compound allowing samples to vitrify rather than form ice crystals seem effective as solutes are not crowded into the vicinity of the DNA polymer. Minimising the number of freeze-thaw cycles is also an obvious solution.</td>
<td>Maintaining a high purity of extracted DNA and possibly addition of protective agents.</td>
<td></td>
</tr>
<tr>
<td>Depurination</td>
<td>Weeks to years</td>
<td>Protonation of purines leading to depurination followed by beta elimination</td>
<td>A slow reaction that may cause issues under acidic conditions or long term storage at high temperature or with insufficient removal of water</td>
<td>Low temperature storage or complete dehydration.</td>
<td></td>
</tr>
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</table>

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