INTRODUCTION

In times of global population growth and an increased lifespan, the demand for organs and tissue-based regenerative strategies is ever increasing.1 Due to a shortage of available organ donors, usage of animal tissues (xenografts) has emerged as an alternative for transplantation and scaffold-based tissue engineering. 2,3 However, xenografts need to be processed to prevent graft rejection and inflammatory reactions.3 For this, removal of surface antigens as well as animal DNA from the source tissue is required, a process called decellularization. While surface antigens may lead to hyperacute graft rejection,4 DNA from animals harbors endogenous retroviruses that may be transmitted to patients. 5 Additionally, extracellular DNA is known to cause inflammatory reactions via several signaling pathways, necessitating DNA-removal from the tissue.6,7 Consequently, accurate measurements are needed to detect short fragmented DNA (sfDNA)

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remaining in decellularized tissues. However, processing of decel-
lularized samples for DNA quantification may affect the results of
such quantification, depending on the method. In the following para-
graphs, common methods for DNA extraction are explained in more
detail.

Before the amount of residual DNA in decellularized tissues can
be assessed, the tissue is digested, the cells are lysed (if any remain),
and the DNA is dissolved in the buffer solution. Thereafter, the
amount of DNA can be directly assessed by adding a fluorescent
probe to the digested sample. Alternatively, the DNA can be further
purified from the sample using an extraction procedure (Figure 1),
exploring its physicochemical properties. Phenol/chloroform-ex-
traction of DNA exploits differences in solubility of DNA vs. pro-
teins and lipids in water-/oil-based solvents, respectively. A mixture
of 25:24:1 phenol/chloroform/isoamyl alcohol is added to the sam-
ple, which is then vortexed for emulsification before centrifugation
to ensure phase separation. While lipids are dissolved in the organic
phase, proteins remain at the interphase and DNA in the aqueous
supernatant, which can be transferred into a new vessel for quanti-
fication and further analysis.8,9

Alternatively, high concentrations of salt can be used to precip-
itate proteins and cellular debris, due to their hydrophobicity, while
the DNA remains in the supernatant. This method is often preferred
over the phenol/chloroform-based extraction, as it does not rely on
hazardous chemicals.8,10

Solid-phase extraction exploits interactions of DNA with a solid
substrate, such as silica resin/beads in the presence of chaotropic
salts, allowing for rapid purification of DNA from digested samples.
Immobilization of DNA to the silica-surface is based on electrostatic
interactions, only allowing for release in the presence of hypotonic
buffers. Especially for sfDNA, however, this does not recover the
total amount. Investigations into the recovery of sfDNA from sol-
id-phase extraction kits have shown that for DNA fragments < 50 bp
and < 100 bp, only about 16.5% and 27.7% (median across various
extraction kits) are recovered, respectively.11-13

In the context of decellularized tissues, the method chosen for
sfDNA extraction therefore biases the interpretation of results. This
has subsequent consequences for suitability for in-patient applica-
tions due to possible immunological side-effects. Here, we review
DNA extraction methods used in decellularization studies, discuss
their effect on clinically safe use and identify suitable methods for
DNA quantification in decellularized tissues.

2 | RESULTS AND DISCUSSION

For this study, PubMed was searched for papers on decellulariza-
tion methods for tissues by searching for “decellulariz*” OR “decel-
lularis*” in title/abstract AND “DNA” as text word (see Appendix
S1). 387 publications were reviewed for their DNA quantification
approach for decellularized tissues. Over the past 20 years, a clear
trend can be seen with an ever-increasing number of publications
describing protocols for decellularization of various tissues and uses
thereof in regenerative medicine (Figure 2). Reducing the amount of
residual DNA in these decellularized tissues is crucial for their fur-
ther clinical application.

A large portion of research groups quantify residual DNA lev-
eels after extracting the DNA from the tissue lysate (ca. 70%), with a
steady popularity of spin-column silica-based solid-phase extraction
(Figure 3). All in all, ~50% of the conducted decellularization stud-
ies extract DNA via solid-phase adsorption prior to quantification,
while 15% use an organic extraction protocol and 5% extract DNA
via salting-out protocols. A consistent majority of research groups
rely on silica-based DNA extraction for quantification of residual
DNA, while a minority relies on specific interactions between DNA-
binding dye facilitating direct detection in crude tissue lysates.

Recently, emerging evidence suggests that commercially avail-
able solid silica phase DNA extraction kits do not recover small
dsDNA-fragments from solution, as typically found in decellular-
ized tissue.11 This underestimates the amount of measured residual
DNA, which can thus impact the clinical usability. DNA interacts
with silica electrostatically and hydrophobically via the negatively
charged backbone (phosphate-ions) and positively charged silica.14
This means that there are more DNA-resin-bonds found for large

FIGURE 1 Overview of common DNA
extraction protocols used on tissue lysate
DNA-molecules than small ones. Small fragments can consequently get lost in subsequent washing steps due to the applied shear forces from centrifugation/pipetting that rupture the bonds. The DNA fragment size distribution in decellularized samples is dependent on the chosen decellularization protocol, enzyme concentration, and incubation time. Assuming optimization of DNA digestive conditions, the problem of DNA underestimation just becomes exacerbated, as fragments will become increasingly smaller, making them harder to accurately quantify after silica-based solid-phase extraction methods. Based on the median relative recovery of fragments < 200 bp estimated from Cook et al., this would lead to a gross underestimation of DNA content. Results from Tsai et al. and our group (Figure 4) suggest that obtained values for sfDNA may differ by as much as 10-fold. This effect may even be increased in the presence of ceramics, but may be less pronounced for perfusion-based decellularization processes flushing out small DNA fragments, thus reducing the potential underestimation by DNA extraction. Modifying the surface structure of the solid phase used, may improve sfDNA recovery, however, the investigated fragment sizes are often still > 100 bp and the studies’ results might not necessarily apply to even smaller fragments of DNA as found in decellularized tissues. One study investigated sfDNA adsorption onto silica-coated magnetite particles, achieving >54% adsorption of available sfDNA (80-160 bp). To the best of our knowledge, none of these modified substrates are readily commercially available. Careful adjustment of buffer-conditions may increase yield of fragments ≥ 20 bp, but are not discussed in commercially available kits and therefore not routinely used. Complicating the situation is the lack of available information on the smallest extractable sfDNA using commercially available extraction kits (Table 1). Most groups use silica-membrane-based extraction kits, optimized for genomic DNA extraction from tissues, that is, optimized for large DNA fragment recovery. Moreover, these kits often do not state a lower limit of extraction concerning DNA fragment sizes. This highlights the need for either determining and correcting for the relative loss of sfDNA prior to using commercially available kits, or employing extraction methods that are more suitable for small fragment recovery.

Our search did not result in studies examining potential bias toward certain DNA fragment sizes based on their solubility in presence of high ion-concentrations. The suitability of salting-out proteins for purification of DNA is therefore difficult to judge. From a usability standpoint, while organic extraction protocols do seem to enable sfDNA recovery, they employ hazardous chemicals unfit for...
| Used kit (Supplier) | Studies | Lower DNA fragment size extraction limit | Usual size range of extracted DNA fragments |
|---------------------|---------|-----------------------------------------|------------------------------------------|
| **AccuPrep®, Genomic DNA Extraction Kit (Bioneer)** | 1 0.54 | Not stated | Not stated |
| **AllPrep DNA/ RNA Mini Kit (Qiagen)** | 1 0.54 | Not stated | 15-30 kbp |
| **Favorprep™ Tissue Genomic DNA Extraction Mini Kit (Favorgen)** | 1 0.54 | Not stated | Not stated |
| **Genomic-tip 500/G (Qiagen)** | 1 0.54 | Not stated | 20-150 kbp |
| **Genomic DNA isolation kit (DENAzist)** | 1 0.54 | Not stated | Not stated |
| **Illustra™ Tissue and Cells Genomic Prep Mini Spin Kit (GE Healthcare)** | 1 0.54 | >20 kbp | Not stated |
| **Invisorb Spin Tissue Midi Kit (Invitek)** | 1 0.54 | >180 bp | Not stated |
| **LaboPass Tissue DNA Purification Kit (Hokkaido System Science Co. Ltd.)** | 1 0.54 | Not stated | Not stated |
| **peqGOLD Tissue DNA Mini Kit (peqlab)** | 1 0.54 | Not stated | Not stated |
| **PrimePrep Genomic DNA Isolation Kit (Genet Bio)** | 1 0.54 | Not stated | Not stated |
| **G-spin™ Total DNA Extraction Kit (iNtRON Biotechnology)** | 1 0.54 | Not stated | 20-30 kbp |
| **UltraClean tissue and cell DNA isolation kit (Mo Bio Laboratories)** | 1 0.54 | Not stated | Not stated |
| **Isolate II Genomic DNA Kit (Bioline GmbH)** | 2 1.08 | Not stated | Not stated |
| **NucleoSpin kit (Macherey-Nagel)** | 2 1.08 | Not stated | Not stated |
| **QIAamp DNA FFPE tissue kit (Qiagen)** | 2 1.08 | Not stated | Not stated |
| **ReliaPrep™ gDNA Tissue Miniprep System (Promega)** | 2 1.08 | Not stated | Not stated |
| **GeneJet DNA purification kit (Thermo Scientific)** | 4 2.15 | >30 kbp | Not stated |
| **GenElute mammalian genomic DNA miniprep kit (Sigma-Aldrich)** | 8 4.30 | Not stated | Not stated |
| **TIANamp Genomic DNA assay kit (Tiangen Biotech)** | 9 4.84 | Not stated | Not stated |
large-scale and routine use, while salt precipitation can be accomplished with non-hazardous chemicals like NaCl.

Typically, DNA concentration after extraction and purification is measured spectrophotometrically, assessing the absorption value at 260 nm. Alternatively, colorimetric quantitation is used, and more sensitive in the sub-µg range. A different approach utilizes quantitative real-time PCR for DNA quantification, of which the reproducibility is however dependent on the initial DNA extraction method chosen, as well as potential interference from non-DNA components in the sample itself.

Probably easiest is the direct quantification of DNA in digested tissue samples. This has obvious consequences for the detection method, as other tissue components and the homogeneity of the lysate will affect spectrophotometric approaches. Addition of a fluorophore, however, has been demonstrated to be a highly sensitive and reproducible approach for DNA detection in whole blood, serum, urine, and in the presence of proteins and glycosaminoglycans. Especially sensitive for detection of DNA in low amounts are PicoGreen and SYBR Green, contrary to ethidium bromide and Hoechst-based dyes. The binding site sizes of all probes are smaller than the DNA fragments produced by commonly used DNases used in decellularization protocols (e.g., Benzonase cleaves DNA to fragments of ca. 5 bp in size, while DNase I leaves fragments of ≥ 10 bp size), enabling them to detect even small fragments to varying degrees. Although these fluorescent dyes exhibit sequence-dependent specificity, with Hoechst and SYBR Green preferentially binding to AT-rich sequences whereas PicoGreen binds more often to GC-rich regions, this effect is most likely negligible in the context of apoptotic cells allowing for an estimate of permissible DNA levels in xenografts at the site in question.

Currently, there are no published systematic studies investigating the relation between residual DNA amount, fragment length, and immunological reaction in vivo, following xenotransplantation. A key player in extracellular dsDNA-recognition and downstream signaling is interleukin 26 (IL-26; Figure 5). The binding site size for DNA on IL-26 is not defined yet; however, a minimal fragment size of >6 bp for DNA to be bound is expected, based on the predicted recognition site and the biophysical structure of amino acid α-helices as well as DNA. Recognition of non-self DNA via IL-26 and subsequent transfer into the cell has been tied to cyclic GMP-AMP synthase and Stimulator of Interferon Genes (cGAS-STING)-mediated inflammation in myeloid cells, which ultimately leads to production of TNFα, and IL-1β and IL-6 activation. Once in the cytosol,
another signaling pathway including the Absent In Melanoma 2 (AIM2) protein recognizing the DNA ultimately leads to activation of the inflammasome and downstream maturation of IL-1β and IL-18. cGAS-STING and AIM2 bind to DNA of >50 and >80 bp, respectively, reflecting the need for accurate determination of sfDNA content in decellularized tissues.

3 | CONCLUSION

Immunological sensing of DNA is one possible adverse reaction to xenotransplants in vivo. Accurate determination of DNA amount and fragment size distribution is therefore paramount in assessing the clinical suitability of decellularized tissues. From the currently available facts, DNA extraction from decellularized tissues via silica-based approaches is not advisable due to depletion of sfDNA, leading to an underestimation of total DNA content. More suitable are solvent-based extraction methods utilizing, for example, phenol/chloroform, or methods selectively precipitating proteins and cell debris for DNA isolation. Alternatively, direct assessment of DNA in tissue lysate can be performed. As no extraction procedure is performed, no bias in DNA detection is given, and the obtained value is expected to more accurately reflect residual DNA in the sample.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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