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RESEARCH ARTICLE

PROFICIENCY TESTING AND PRE-DIAGNOSTIC CHARACTERIZATION OF EXTRACTED DNA FOR EXTERNAL QUALITY ASSURANCE

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ABSTRACT

Background: Current external quality assurance (EQA) programs for DNA extraction require participating laboratories to perform DNA extraction on an EQA provided fresh blood sample or specific tissue type. However, this strategy is too restrictive for laboratories who regularly perform DNA extraction on other tissue types or for biobanks who have vast stores of archived specimens that are unrelated to the EQA material. To address this, a pilot EQA program was developed by the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) where laboratories were requested to directly submit to the RCPAQAP five DNA extracts that were of specific interest to them.

Methods: Three complementary measuring strategies were used for quality assessing all received DNA extracts. Total DNA integrity was analysed using a DNA TapeStation 4200 (Agilent Technologies) and pre-diagnostic validation was performed using multiplex-PCR and real-time PCR.

Results: A total of 89 DNA extracts isolated from 16 different tissue types were submitted for quality assessment between 2016 and 2017. 97% (86/89) of DNA extracts were concordant for DNA integrity with three samples (3%) being discordant. Two of the three discordant samples were however concordant for multiplex and real-time PCR. Only one DNA extract failed all three measuring platforms.

Conclusion: The data from this study indicate that DNA extracted from multiple tissue types using an array of extraction platforms can be quality assessed and diagnostically pre-validated at the same time. The RCPAQAP are the first EQA provider to offer this type of proficiency testing program and are developing similar strategies for future fixed-formalin paraffin-embedded and circulating free DNA testing.

INTRODUCTION

Analyses of DNA and RNA is fundamental for most human genetic disease diagnostics. The reproducibility of DNA amplification sequence information that accurately reflects the original clinical sample is essential for all downstream clinical diagnostic interpretations. For many molecular genetics tests, it is critical that the quality of the DNA extract used for clinical testing be of the highest standard possible. In addition, there is a key requirement for diagnostic and research laboratories to demonstrate acceptable levels of proficiency performance to maintain or apply for diagnostic accreditation respectfully.

The emergence of high throughput and high sensitive DNA analyzing technology has improved routine clinical diagnostics, and as such, there are now over 54,000 genetic tests being offered and performed on over 10,000 genetic disorders globally (https://www.ncbi.nlm.nih.gov/gtr). Given the plethora of molecular genetic tests, it is critical that the quality of the initial DNA extract used for diagnostic analyses be at a resolution that is fit for purpose for the specific test being performed. For example, multiple DNA extraction platforms exist with the quality of the DNA isolate being reflective of the specific platform used. However, downstream analyses can nonetheless still be performed on such DNA extracts (Schuurman, 2007; Oldham, 2012 and Perry, 2014). These data indicate that DNA quality can greatly vary but still be applicable for genetic testing.

Current key technologies used for DNA analyses include next generation sequencing, Sanger sequencing, microarray, allele-specific PCR, multiplex ligated probe amplification, real-time PCR and digital PCR (Sherwood, 2017; Khubaib, 2017; Alhourani, 2014; van Ginkel, 2017 and Patel, 2016). The development of sensitive and high throughput DNA testing technologies has allowed laboratories to improve upon their clinical report turnaround times as well as increasing confidence in the reported diagnostic DNA data (Sherwood, 2017; Bourchany, 2017 and Lemon, 2017). However, the accuracy and quality of sensitive genetic test data are directly dependent on the quality of the clinical DNA extract (Melton-Kreft, 2016). Most DNA diagnosing technologies using high sensitive tests therefore require the clinical DNA test sample to be of high quality for reliable and accurate data production. Thus, the efficient extraction of DNA from human tissue is paramount for downstream sensitive genetic diagnostic

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applications (Permenter, 2015; Nechifor-Boilă, 2017). Recovery of DNA extracts that are of low quality can fail to amplify, or for formalin-fixed paraffin-embedded (FFPE) DNA, lead to false positive or negative DNA variants being recorded which may impacton the clinical management of the patient (Do, 2015 and Einaga, 2017). It is therefore key for laboratories to know the quality of their extracted DNA prior to genetic analyses. This is particularly important for laboratories who refer extracted DNA to other facilities who require high quality DNA for sensitive next generation sequencing type assays. The availability of EQA thus allows laboratory DNA to be quality tested to ensure that DNA extracts are at acceptable resolution levels required for diagnostic analyses (Kalman, 2013 and Cree, 2014).

EQA programs exist to monitor the proficiency of diagnostic testing laboratories so that consistency and accuracy of the tests being performed can be maintained and improved where required. Current EQA for DNA extraction involves the EQA provider supplying a specific tissue sample (primarily a blood sample) to the laboratory so that proficiency testing can be performed (Raggi, 2003; Orlando, 2007; Malentacchi, 2016; Meini,, 2016 and Patton, 2014). EQA providers have focused on assessing DNA purity and yield, and in the generation oflaboratory produced PCR products (derived from the EQA provided material) as a strategy for proficiency testing the quality of the initial DNA (or RNA) extraction process (Permenter, 2015; Orlando, 2007; Ramsden, 2006). RCPAQAP developed However, EQA assessments of PCR products generated from different laboratories can be unreliable given that different PCR amplification platforms and PCR reagents may have different DNA amplification efficiencies, and this will be reflected in the quality and quantity of the PCR products produced. In addition, laboratories may also characterize DNA from multiple archived tissue types or from specific tissues supplied by biobanks and these are unlikely to be related to the EQA material (Ahmad-Nejad, 2015). To address this, the RCPAQAP developed a novel EQA program for quality assessment of DNA extracts isolated from multiple tissues. Essentially, laboratories extracted their own DNA from any tissue type that was of specific interest to them and directly submitted these to the RCPAQAP for analyses. The RCPAQAP proficiency test incorporated different measuring strategies designed to measure whole genome DNA integrity and to validate each DNA extract in terms of PCR amplification across 16 different gene loci. A DNA TapeStation 4200 (Agilent Technologies) was used for DNA integrity analyses and multiplex PCR and real-time PCR were used for validation. For the PCR validation step, any inter-platform variability were removed since the same platform and PCR reagents were used for all DNA extracts. The first initial trial of the EQA was performed in 2016 and involved six laboratories submitting a total of 34 different DNA extracted samples. A second pilot survey was performed in 2017 with 11 laboratories submitting a total of 55 extracted DNA samples for analysis. Here we report the results of the two surveys.

MATERIALS AND METHODS

Laboratories: A total of 17 laboratories (15 from Australia, 1 from New Zealand and 1 from South Africa) participated in this two-year DNA extraction pilot program (6 laboratories in 2016 and 11 laboratories in 2017). All laboratories perform multiple DNA diagnostic testing in addition to storing and isolating DNA from different tissue types.

Procedure: Participating laboratories were instructed to extract total DNA from any of five recently received (or archived) tissue and to forward each DNA extract to the RCPAQAP for quality assessment. Information relating to the DNA extraction process including equipment and kits used, measured DNA concentration (ng/µl) and 260/280 and 260/230 ratios were also requested. RCPAQAP stored fixed-formalin paraffin-embedded (FFPE) tissue DNA were extracted using the Gene Read DNA FFPE Kit (Qiagen, Hilden, Germany) and were used as fragmented controls for DNA integrity testing.

Quality measurement of DNA: The RCPAQAP evaluated each DNA extract using three different measuring strategies; (i) total DNA integrity analysis using a DNA TapeStation 4200 (Agilent Technologies, Santa Clara, USA); (ii) analysisof DNA using multiplex-PCR; and (iii) analysis of DNA by real-time PCR. For DNA integrity analysis, quality assessment was performed to determine the level of DNA recovery and degradation in relation to the extraction process. For multiplex PCR and real-time PCR analyses, the amplification of 16 gene loci (Table 1) were used to determine the applicability of each DNA extract for downstream applications. For the 2016 DNA extracted samples, only total DNA integrity were performed for the initial quality assessment trial as limited DNA extract material were supplied. For the 2017 samples, more DNA extractes were requested so that multiplex-PCR and real-time PCR strategies could be incorporated for a more comprehensive quality assessment.

DNA Integrity (DIN): The integrity of each DNA extract was initially measured on the DNA Tape Station 4200. The Tape Station is a micro fluidic platform which assesses the quantity and integrity of genomic DNA in the sizing range of 200 to >60000 bp. A software algorithm produces a virtual gel image and a DNA Integrity Number (DIN) that are representative of whole genomic DNA integrity. The DIN ranges from 0 (highly degraded DNA) to 10 (highly intact DNA). DNA extracts were diluted to a working concentration of 50ng/µl. Extracts that measured less than 50ng/µl were analysed undiluted.

DIN assessment criteria: A Z-score calculation of each DIN value was used to determine population concordance of each DNA extract. Z-scores within +/- 2 standard deviations from the mean (i.e., agree with 95% of all participant data) were regarded as good quality DNA extracts and were considered to be concordant. In contrast, Z-scores greater than 2 standard deviations below the mean (i.e., disagree with 95% of all participant data) were considered discordant to the population data and were categorised as highly fragmented DNA.

The Z-score calculation is: Z = (DIN score – population mean DIN score) / standard deviation of the population DIN data
Example: Z = (2.9 – 7.86) / 1.18 = -4.2 (i.e., 4.2 standard deviations below the mean and discordant)

Multiplex PCR Analysis (M-PCR): M-PCR was performed across five gene loci (*AFF1* [600bp and 400bp], *ZBTB16* [300bp], *RAG1* [200bp], *TBXAS1* [100bp]) as previously reported (van Dongen, 2003).

M-PCR assessment criteria: Five amplification products were expected to be amplified from each single PCR reaction. For this endpoint PCR assay, amplification of one to five PCR products were considered concordant for each tissue DNA extract. In contrast, tissue DNA extracts that failed to amplify across all five gene loci were considered discordant.

Table 1. Gene loci used for RT-PCR (RT) and M-PCR (M) assessment of extracted DNA. The catalogue number refers to Thermo Fisher Scientific TaqMan assays

Gene	Chromosome	RefSeq	Description	Dye	Size (bp)	Assay*	Catalogue No./Reference
LRP1B	2	NG_051023.1	LDL receptor related protein 1B	FAM	114	RT	Hs02501162_cn
ROBO2	3	NG_027734.1	roundabout guidance receptor 2	FAM	77	RT	Hs03227040_cn
TERT	5	NG_009265.1	telomerase reverse transcriptase	VIC	88	RT	4403315
PDE4D	5	NG_027957.1	phosphodiesterase 4D	FAM	110	RT	Hs04290984_cn
EYS	6	NG_023443.2	eyes shut homolog (Drosophila)	FAM	110	RT	Hs04321318_cn
CNTNAP2	7	NG_007092.2	contactin associated protein like 2	FAM	107	RT	Hs05018255_cn
ASTN2	9	NG_021409.1	astrotactin 2	FAM	102	RT	Hs06843013_cn
PRKG1	10	NG_029982.1	protein kinase, cGMP-dependent, type I	FAM	82	RT	Hs03731145_cn
CNTN5	11	NG_047156.1	contactin 5	FAM	105	RT	Hs05228401_cn
RBFOX1	16	NG_011881.1	RNA binding fox-1 homolog 1	FAM	110	RT	Hs03953793_cn
DMD	X	NG_012232.1	dystrophin	FAM	105	RT	Hs00129944_cn
AFF1 (AF4)	4	NC_018915.2	AF4/FMR2 family member 1	None	600	M	van Dongen et al., (2003)
AFF1 (AF4)	4	NC_018915.2	AF4/FMR2 family member 1	None	400	M	van Dongen et al., (2003)
ZBTB16 (PLZF)	11	NG_012140.2	zinc finger and BTB domain containing 16	None	300	M	van Dongen et al., (2003)
RAG1	11	NG_007528.1	Recombination activating gene 1	None	200	M	van Dongen et al., (2003)
TBXAS1	7	NG_008422.2	Thromboxane A synthase 1	None	100	M	van Dongen et al., (2003)

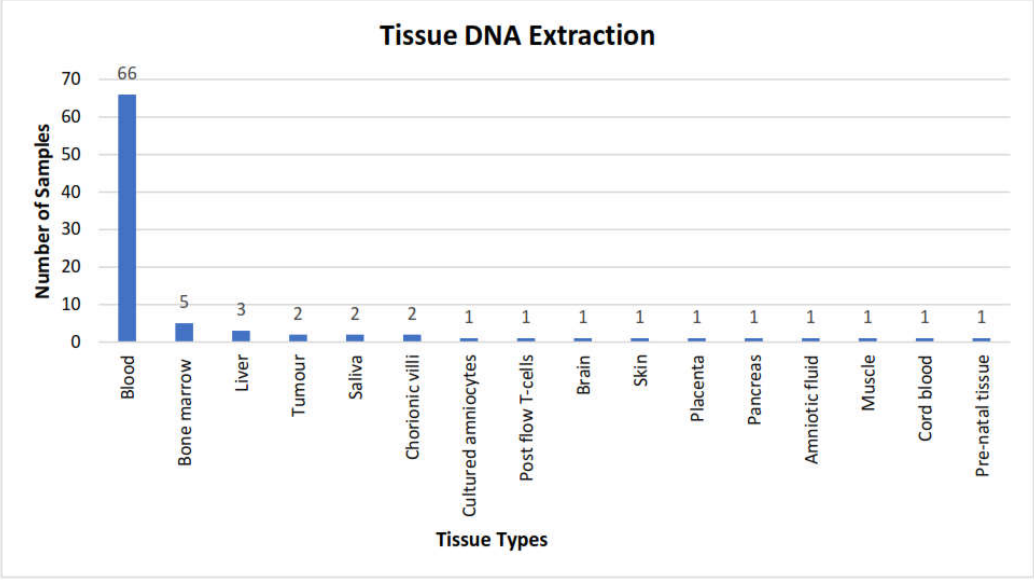

Figure 1. DNA received from multiple tissues in the RCPAQAP 2016 and 2017 DNA Extraction EQA program

Real-time PCR (RT-PCR): RT-PCR was performed using the Quant Studio 3 System (Thermo Fisher Scientific, Massachusetts, USA). TaqMan assays (Thermo Fisher Scientific, Table 1) representing 11 gene loci were used to amplify DNA extracted from each tissue type. TaqMan assays were performed in duplicate using 96-well plates. Briefly, a total volume of 17.4µl volumes consisting of a final concentration of 1X PCR master mix (Thermo Fisher Scientific), 1X TaqMan primer/probe mix (Thermo Fisher Scientific) and 10ng of DNA were used in accordance with the manufacturers protocols (Thermo Fisher Scientific). Cycling parameters were 96°C for 10 min, followed by 39 cycles of 98°C for 30s and 60°C for 2min, followed by 60°C for 2min, and holding at 10°C. Analyses of gene loci cycle threshold (Ct) values for all plates were determined using the Data Assist program (Thermo Fisher Scientific).

RQ-PCR assessment criteria: The RT-PCR assays were expected to generate a Ct value for each of the 11 genes (*TERT, LRP1B, RBFOX1, ROBO2, DMD, PDE4D, EYS, CNTNAP2, ASTN2, PRKG1, CNTN5*) to confirm DNA amplification. The Ct values for each gene and tissue type were then averaged to identify the mean and standard deviation to derive a Z-score for each individual gene for each tissue type. The mean gene Z-score for the genes in each tissue type were then derived to identify a final tissue-specific Z-score. Z-scores within +/- 2 standard deviations from the mean were considered concordant. In contrast, Z-scores deviating +/- 2 standard deviations from the mean were considered discordant.

RESULTS

DNA tissue extracts

A total of 89 DNA extracts from 16 different tissues were submitted for DNA quality assessment between 2016 and 2017.Blood DNA represented 74% (66/89) of all samples received with DNA from bone marrow (6%), liver (3%), tumour (2%), saliva (2%), chorionic villi (2%), cultured amniocytes (1%), post flow T-cells (1%), brain (1%), skin (1%), placenta (1%), pancreas (1%), amniotic fluid (1%), muscle (1%), cord blood (1%), and pre-natal tissue (1%) also being submitted for quality testing (Figure 1). Laboratory submitted DNA were extracted using 26 different strategies ranging from commercially available kits to optimized in-house methods (Supplementary Table 1).

Quality measurement of DNA

DNA integrity assessment

The generated data from the DNA Tape Station 4200 identified that 93% (83/89) of all submitted 2016 and 2017 DNA extracts had DIN values >6 and reflect a high density migrating genomic band at ≥48500 bp (Figure 2). In contrast, the control FFPE DNA extracts produced low DIN values (<6) and demonstrated an absence of a high density migrating genomic band with significant DNA fragmentation and smearing (Figure 2). Z-score analysis of the DIN values identified that 97% (86/89) of the DNA extracts were considered concordant to the population dataset. In contrast, three samples (T20 [mouth wash], T31 [blood] and T53 [muscle]) received a Z-score greater than 2 standard deviations below the mean and were considered discordant (Figure 3).

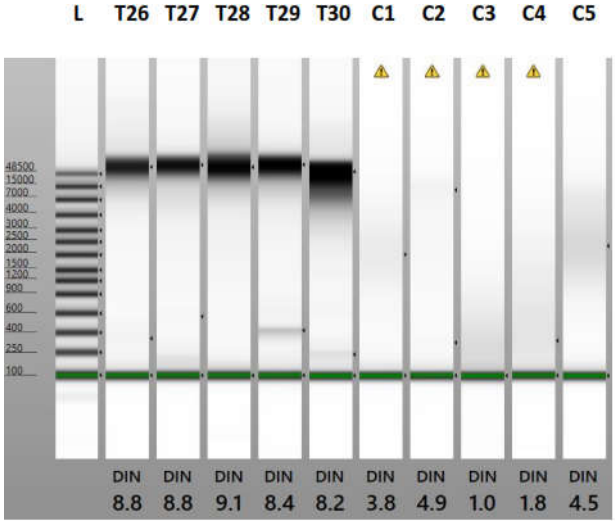

Figure 2. Representative DNA TapeStation 4200 virtual gel images of submitted DNA extracts. The lower band represents an internal 100bp DNA standard of known concentration. The triangles at the top of the gel indicate that sample concentration is outside the functional range for an optimal DIN calculation. The small arrow heads represent regions of fragmented DNA. Ladder (L) size range from 100bp to 48500bp. Samples T26 - T30 represent DNA isolated from blood; samples C1 – C5 represent DNA isolated from FFPE tissue and serve as fragmentation DNA controls. Refer to Supplementary Table 1 for tissue and extraction details.

M-PCR assessment: M-PCR was only performed on the 2017 DNA extracts. Of the 55 DNA extracts submitted, 5% (3/55) were depleted of DNA and could not be assessed. Of the remaining 52 DNA extracts, 98% (51/52) samples could be amplified across all five gene loci and were considered concordant and 2% (1/55) failed to amplify. In addition, two of the three DNA extracts discordant for DNA integrity (T20 and T53) were successfully amplified and considered concordant. The DNA extract that failed to amplify was the DNA integrity discordant T31 tissue sample (Figure 4).

RT-PCR assessment: RT-PCR was only performed on the 2017 DNA extracts. Z-score analyses of all genes and tissues Ct data indicated that 98% (51/52) samples were concordant including two of the three DNA extracts discordant for DNA integrity (T20 and T53) (Figure 5). Three samples were depleted of DNA and were not assessed. The remaining DNA extract that failed to amplify was the DNA integrity discordant T31 tissue sample.

DISCUSSION

Multiple commercially available tissue-specific DNA extraction platforms/kits are offered globally with the levels of DNA extraction efficiency being largely dependent on the type of platforms/kits used (Orlando et al., 2000 and Malentacchi, 2016). This EQA was therefore designed to evaluate and validate DNA extracted from multiple platforms representative of commercially available kits to in-house developed assays for downstream usage. Three separate testing strategies were incorporated to quality assess each DNA isolate. This combined testing approach allowed for a more comprehensive assessment of each DNA extract. For example, the generation of a DNA TapeStation 4200 virtual gel image in combination with a DIN increases confidence in the quality of the DNA extract.

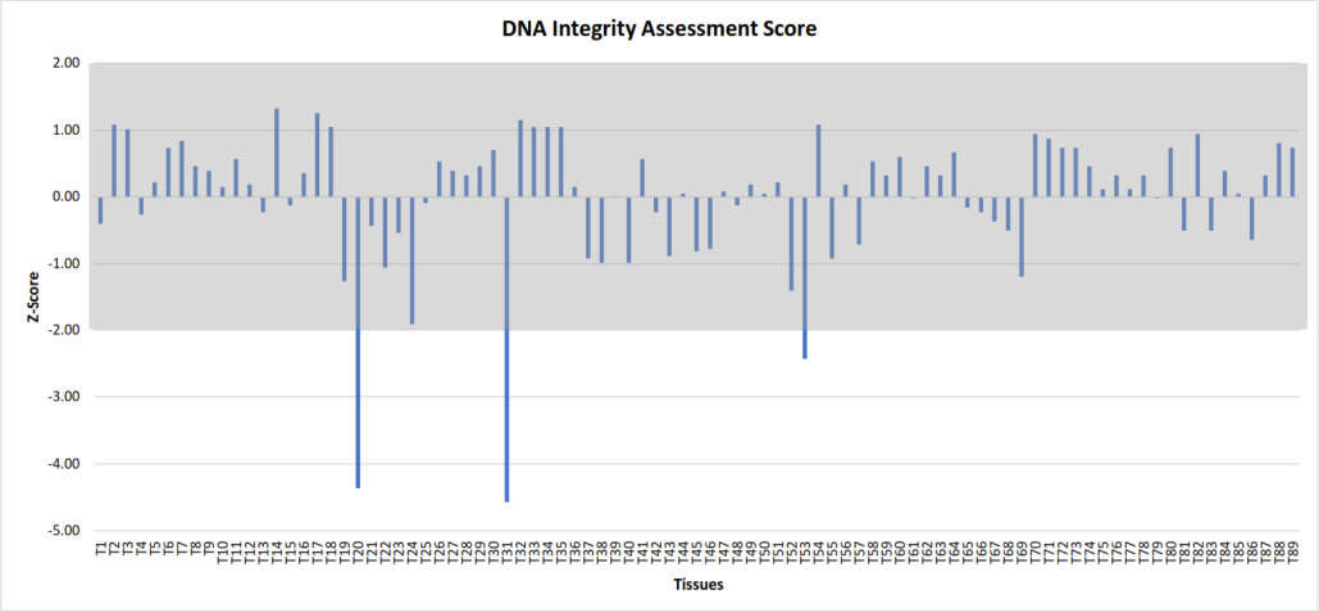

Figure 3. Tissue DNA integrity Z-scores in the 2016 and 2017 DNA extraction pilot EQA program. DNA integrity Z-scores within two standard deviations of the data population mean (grey shaded area) are considered concordant. DNA integrity Z-scores below two standard deviations of the data population mean (outside shaded area) are discordant to the population dataset. Refer to Supplementary Table 1 for tissue and DNA extraction details

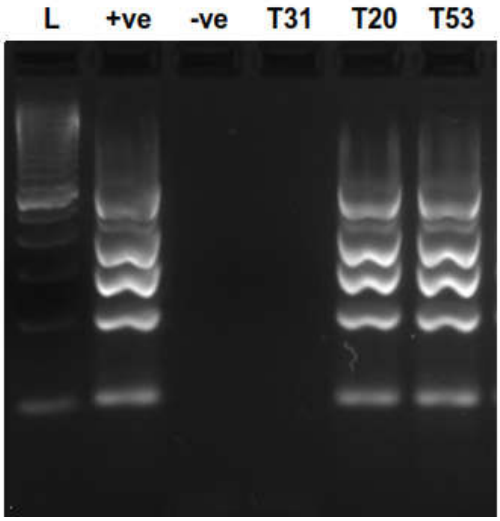

Figure 4. M-PCR gel electrophoresis of samples that were discordant for DNA integrity. L (600bp DNA ladder); +ve (positive DNA control); -ve (negative DNA control); T31 (blood DNA); T20 (mouth wash DNA); T53 (muscle DNA)

In addition, gel images and the DIN are generated regardless of the presence of contaminants in the DNA isolate. In particular, the DIN represents an objective measure for total DNA integrity (Gassmann, 2014 and Kong, 2017). However, analyses of DNA integrity using the DNA 4200 TapeStation in isolation was not considered fully adequate for EQA testing, given that DNA extracts with high DIN values may contain contaminants that impede downstream DNA amplification, and DNA extracts with low DIN values may still be amenable for DNA diagnostics. To address this, the RCPAQAP included the techniques of M-PCR and RT-PCR as part of the 2017 pilot EQA scheme to validate the amplifiable capacity of all DNA isolates for downstream genetic analyses. However, deamination of cytosine to uracil remains an issue with archived tissue and can be problematic for PCR amplification (Bourgon, 2014 and Serizawa et al., 2015). Failure to amplify archived DNA may indicate an underlying process of deamination and can represent an incidental finding as part of this EQA.

No incidental findings were found in this study, but any such discoveries would nonetheless be reported to the participating laboratory since it may or may not be clinically relevant.

For this study, laboratory DNA were extracted from 16 different tissue types using a total of 26 different DNA extracting platforms (Figure 1 and Supplementary Table 1). EQA quality assessment of DNA integrity supported previous reports (Gassmann, 2014 and Kong, 2017), that high DIN values were representative of good quality DNA integrity with low DIN values being associated with increased levels of DNA fragmentation and poor DNA integrity (Figure 2). Of the 89 DNA samples extracted from the 16 different tissue types, 97% (86/89) were deemed to be concordant for good DNA quality and three samples (T20, T31 and T53) were discordant (Figure 3). However, two of the three discordant samples (T20 and T53) were nonetheless concordant for both M-PCR and RT-PCR assessment (Figure 5) and were considered applicable for downstream diagnostic analyses.

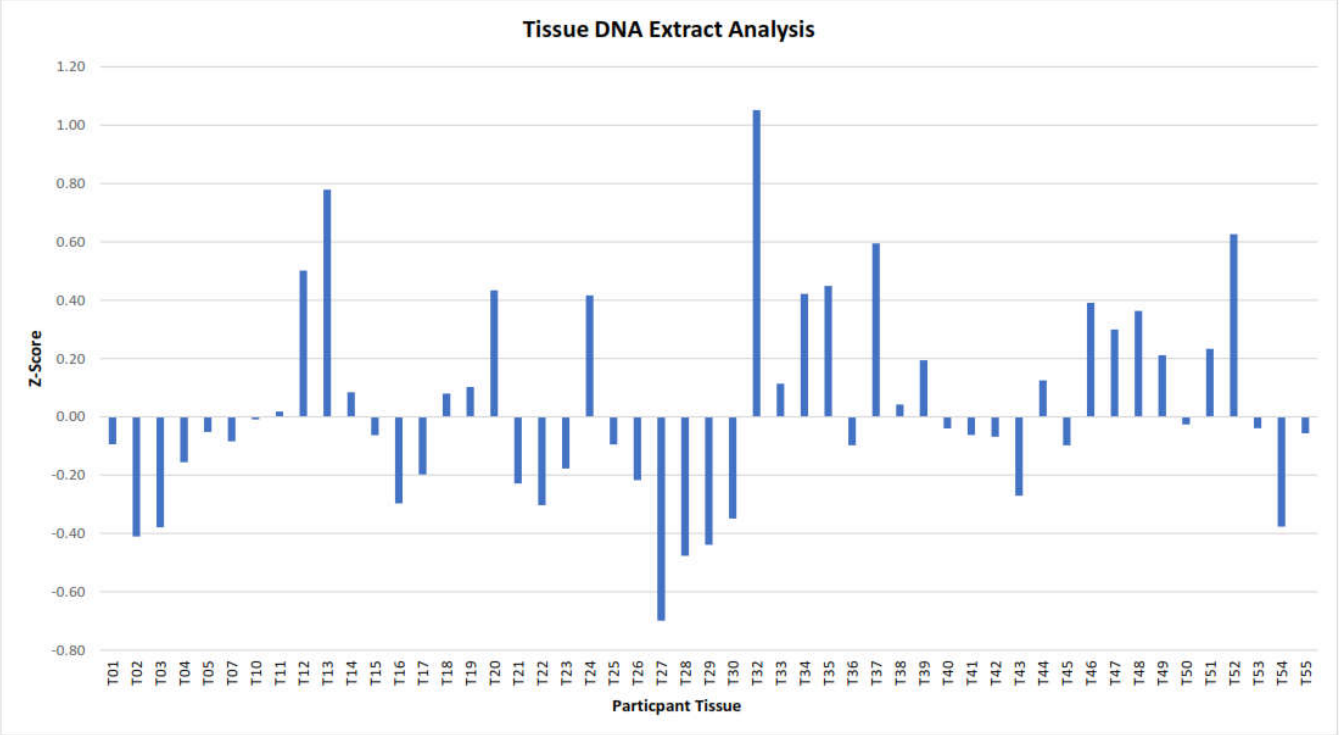

Figure 5. Concordance testing using real-time PCR. The calculated Z-scores from the Ct values are within +/- 2 standard deviations from the mean (0) and are considered concordant. Refer to Supplementary Table 1 for tissue and DNA extraction details

The T31 sample (blood DNA extract) could not be amplified by either M-PCR or RT-PCR and is indicative of a poor DNA extract that is unsuitable for DNA diagnostic characterization. These data support similar findings reported recently for endpoint PCR and RT-PCR assays that were used to determine the efficiency of different DNA extraction methods (Koshy, 2017). Importantly, the key finding from this study is that none of the 26 DNA extraction strategies used were identified as underperforming (see Supplementary Table 1 for tissues and extraction methods used).

EQA proficiency testing is rapidly playing a more fundamental role for laboratory diagnostics. It is therefore critical that the initial clinical DNA material to be tested be of the highest pre-diagnostic standard for high sensitive technologies or be at a resolution that is fit for diagnostic purpose using the less sensitive techniques. Laboratories therefore need to know when high quality DNA is required and be able to produce it when necessary, particularly if the laboratory needs to refer the DNA to facilities using high sensitive applications. This study demonstrates that participation in an EQA pre-validation testing program can be beneficial since quality assessments are performed directly on the clinical materials to be genetically characterized. This is important since the availability and supply of EQA DNA extraction reference testing material would be an unrealistic goal since vast amounts of different tissue types would be required to meet the demand from laboratories.

Conclusion

This combined methodologic EQA offers an alternative approach that additionally quality assesses and pre-validates each laboratory supplied DNA extract at the same time. This EQA should therefore help raise confidence that the DNA are applicable for downstream diagnostic genetic testing and are suitable for low or high sensitive testing assays. An additional benefit of this RCPAQAP developed scheme, is that all submitted DNA extracts can be quality validated from any organ tissue type and is particularly useful for laboratories and tissue biobanks performing DNA extraction procedures on tissues other than blood. Based on the findings from this study, similar strategies are currently being devised for DNA extracted from FFPE tissue and for circulating free DNA (cfDNA) isolated from blood sera. Importantly, cfDNA testing is rapidly growing in cancer diagnostics and an EQA is currently unavailable to proficiency test the cfDNA extraction process.

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