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SHORT COMMUNICATION

Development of a multiplex assay to determine the expression of mitochondrial genes in human skeletal muscle

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Abstract

Skeletal muscle is an important endocrine tissue demonstrating plasticity in response to external stimuli, including exercise and nutrition. Mitochondrial biogenesis is a common hallmark of adaptations to aerobic exercise training. Furthermore, altered expression of several genes implicated in the regulation of mitochondrial biogenesis, substrate oxidation and nicotinamide adenine dinucleotide (NAD^+) biosynthesis following acute exercise underpins longer-term muscle metabolic adaptations. Gene expression is typically measured using real-time quantitative PCR platforms. However, interest has developed in the design of multiplex gene expression assays (GeXP) using the GenomeLab GeXP™ genetic analysis system, which can simultaneously quantify gene expression of multiple targets, holding distinct advantages in terms of throughput, limiting technical error, cost effectiveness, and quantifying gene co-expression. This study describes the development of a custom-designed GeXP assay incorporating the measurement of proposed regulators of mitochondrial biogenesis, substrate oxidation, and NAD^+ biosynthetic capacity in human skeletal muscle and characterises the resting gene expression (overnight fasted and non-exercised) signature within a group of young, healthy, recreationally active males. The design of GeXP-based assays provides the capacity to more accurately characterise the regulation of a targeted group of genes with specific regulatory functions, a potentially advantageous development for future investigations of the regulation of muscle metabolism by exercise and/or nutrition.

KEYWORDS
gene expression, mitochondria, skeletal muscle biopsy

1 | INTRODUCTION

A central aim of human skeletal muscle physiological research is to evaluate changes in gene expression in response to external stimuli such as exercise and nutrition (Craig et al., 2015). The molecular signalling pathways which are responsive to such stimuli and underpin long-term skeletal muscle adaptations are inherently complex and are not fully characterised (Egan & Zierath, 2013). Mitigating technical challenges arising from different methods of gene expression quantification in skeletal muscle is critical for ensuring accuracy in data synthesis.

A classic method of evaluating the acute adaptive responses in muscle is through quantitative PCR (qPCR). This technique is commonly used in muscle physiology research (Kuang et al., 2018), although it is not without limitations. Namely, qPCR allows for only a single gene to be analysed per reaction plate. If one wishes to determine expression changes in a large quantity of genes, the resultant number of assays required gives rise to a greater potential

for inter-assay variation in terms of reaction efficiencies and technical error (Edwards & Gibbs, 1994). Moreover, in relative quantification, the traditionally used delta cycle threshold (C_T) method (Livak & Schmittgen, 2001) relies on running a PCR reaction in a separate reaction/plate(s) with a selected reference gene(s), though increasingly these are now run in duplex (Ishii et al., 2007). This again introduces further technical error compared to multiplex gene expression assays GeXP, which detects expression of experimental and reference genes in a single sample. The GeXP genetic analysis system (GenomeLab GeXP™, AB Sciex Pte Ltd., Framingham, MA, USA) is a platform providing a medium-throughput alternative to other quantification methods, using multiplexed gene expression analyses (Drew et al., 2011).

Advantages of GeXP also include the incorporation of multiple reference genes to increase reliability of results and the conservation of extracted RNA samples collected during experimental trials (Drew et al., 2011; Edwards & Gibbs, 1994), important considerations given the technical challenges involved with human skeletal muscle sampling (Hayot et al., 2005). GeXP assays have been previously validated with macroarray and qPCR experiments, showing comparable gene expression profiles to these methods (Drew et al., 2011). Advantages of PCR-based methods in comparison to hybridisation-based methods such as macroarrays include more reliable quantification of genes with very low or high abundance in a biological sample. Hybridisation-based methods depend upon the hybridisation of labelled cDNA to probe regions on a nylon filter, while qPCR and GeXP analyses both incorporate PCR amplification using primer assays. Consequently, the reverse transcription of cDNA for subsequent use in PCR-based experiments differs markedly from the preparation of labelled cDNA for hybridisation-based approaches, which can affect downstream results (Drew et al., 2011). Global gene expression analysis platforms such as RNA-Seq are demonstrated as robust quantification methods, but the associated costs and expertise required for these techniques are notable limitations (Wang et al., 2009; Whitley et al., 2016). Often genes identified in RNA-seq need further validation by qPCR. GeXP assays are also established as high throughput methods to rapidly detect multiple pathogens in clinical settings (Huang et al., 2020; Wang et al., 2016), and distinguish gene expression signatures which reflect pathological changes in different tissues (Drew et al., 2014b, Farquharson et al., 2012), demonstrating their utility for both research and diagnostic purposes.

Studies which have used array-based quantification of gene expression typically require greater quantities of RNA for cDNA synthesis when compared with GeXP (Rundqvist et al., 2019). Similarly, recommendations for qPCR-based assays suggest using greater quantities of total RNA compared with GeXP (Bhatnagar et al., 2012). GeXP enables gene expression quantification using comparatively small amounts of total RNA, an important consideration given the technical and practical challenges which are involved in obtaining large quantities of human skeletal muscle biopsy samples. This is especially evident for researchers interested in using the minimally invasive skeletal muscle microbiopsy technique (Hayot et al., 2005). This technique obtains a lower overall yield of skeletal muscle biopsy sample (~20 mg) compared to the more commonly used Bergstrom and Weil–Blakesley conchotome techniques, which typically obtain yields in the range of 100–300 mg of muscle tissue (Baczynska et al., 2016; Kuang et al., 2018). Research studies are increasingly implementing the microbiopsy technique given the practical advantages compared with Bergstrom and conchotome methods. However, since the microbiopsy technique obtains a lower overall sample yield, identifying high throughput gene quantification methods which can accurately evaluate the coregulation of genes while conserving RNA samples is warranted.

Given the technical challenges involved in human muscle biopsy sampling and subsequent gene expression analyses, the development of a GeXP assay to assess mitochondrial gene expression in human skeletal muscle may be advantageous. The aim of this study was to describe the development of a custom-designed GeXP assay, termed here the hMitoplex, evaluating mRNA expression levels of a targeted group of mitochondrial genes in human skeletal muscle which are proposedly regulated by exercise and nutritional factors. This report describes the methodology employed for developing the hMitoplex or other multiplex gene expression assays which may be developed in future research. In addition, to demonstrate the use of this assay, this study characterised the resting gene expression (overnight fasted and non-exercised) signature within a group of young, healthy, recreationally active males.

New Findings

- **What is the central question of this study?**
  Can a custom-designed multiplex gene expression assay be used to quantify expression levels of a targeted group of mitochondrial genes in human skeletal muscle?
- **What is the main finding and its importance?**
  A custom-designed GeXP multiplex assay was developed, and the ability to accurately quantify expression of a targeted set of mitochondrial genes in human skeletal muscle was demonstrated. It holds distinct methodological and practical advantages over other commonly used quantification methods.

2 | METHODS

2.1 | Skeletal muscle biopsies and experimental procedures

All experimental procedures were approved by the University of Limerick Faculty of Education and Health Sciences Research Ethics

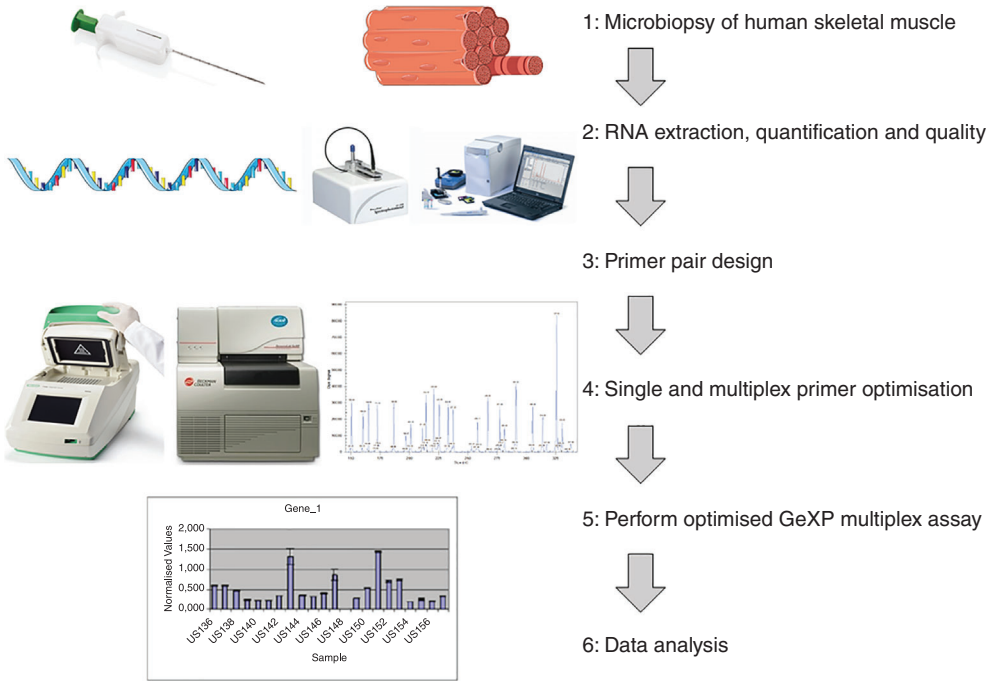

FIGURE 1 Overview of key steps in GeXP hMitoplex workflow. Skeletal muscle microbiopsy samples are snap-frozen in liquid nitrogen and stored at −80°C. RNA is extracted and assessed for yield and quality. Primer testing in singleplex and multiplex is performed to compare the level of agreement between quantification methods, and subsequent optimisation of multiplexed primer pairs is conducted on primer products obtained in multiplex reactions. Once optimised, final GeXP experiments can be implemented and data analyses performed using the appropriate methods

Committee (2016_18_11_EHS), in accordance with the *Declaration of Helsinki* except for registration in a database. All participants provided written informed consent prior to participation. An overview of the workflow involved in the development of the hMitoplex is presented in Figure 1. Skeletal muscle biopsies (mean ± SD 19.0 ± 3.3 mg) were obtained from m. vastus lateralis under local anaesthetic (1% lidocaine) by a medical professional using the microbiopsy technique (Medax Biofeather; San Possidonio, MO, Italy). Participants were recreationally active ($\dot{V}_{O_2max}$ < 50 (42.3 ± 4.8) ml kg^{-1} min^{-1}) healthy males (n = 37), aged 18–35 (25.0 ± 4.1) years, and non-obese (BMI < 30 (25.5 ± 2.2) kg m^{-2}). Biopsies were collected under resting conditions, with participants having attended the lab following an overnight fast of ≥10 h, having refrained from caffeine and alcohol intake for 12 and 24 h, respectively. Participants also refrained from vigorous exercise for the previous 48 h.

2.2 | Selection of target genes

The first stage of assay development comprised the selection of target genes with proposed roles in skeletal muscle metabolism, specifically in terms of regulation of mitochondrial adaptation, substrate oxidation and NAD^+ biosynthetic capacity. A summary list of each target gene and their purported role(s) in the regulation of muscle metabolism is provided in Table 1. The hMitoplex incorporates 25 gene targets, including seven mediators of mitochondrial adaptation, five targets with putative roles in regulating substrate oxidation preferences, four of the sirtuin genes, six proposed regulators of the NAD^+ biosynthesis and salvage pathways, together with three potential reference genes (*PPIA*, *PSMB6* and *UBE2D2*) and a synthetic reference messenger RNA transcript (Kan^r) for measuring relative quantification of gene expression and reaction efficiencies, respectively. The targets incorporated in the hMitoplex are shown to be altered in response to exercise and/or nutritional factors (Granata et al., 2018; Pillon et al., 2020; Rundqvist et al., 2019). Sequences used for primer assay design were downloaded from the National Centre for Biotechnology Information (NCBI) consensus coding sequences (CCDS) project. Functional enrichment analysis was performed on hMitoplex target genes using the Gene Ontology database (Ashburner et al., 2000).

Gene ontology (GO) analysis of hMitoplex genes indicated that biological processes with the greatest predicted fold enrichment included regulation of fatty acid oxidation, regulation of cellular ketone metabolic processes, NAD^+ biosynthetic processes, mitochondrion organisation, and peptidyl-lysine deacetylation, among others. Molecular functions with predicted enrichment included NAD^+ binding, NAD^+-dependent protein deacetylase activity, NAD^+ ADP-ribosyltransferase activity, small molecule binding and transcription factor binding. Enriched cellular components included the mitochondrion, mitochondrial matrix, organelle inner membrane and mitochondrial respiratory chain complex IV, among others.

2.3 | Primer design

The DNASTAR Lasergene and EditSeq software packages (DNASTAR; Madison, Wisconsin, USA) were used to identify suitable gene-

TABLE 1 Accession numbers, putative roles in skeletal muscle metabolism, product sizes and primer pair sequences for hMitoplex target genes

Gene	Accession number	Putative role in skeletal muscle metabolism	Product size	Forward primer sequence (5′−3′)	Reverse primer sequence (5′−3′)	Reverse primer dilution (nM)	Forward primer dilution (nM)
Tfam	NM_003201.3	Transcription factor regulating mtDNA expression and transcription of mitochondrial DNA-encoded subunits of the ETS (Granata et al., 2018; Pillon et al., 2020)	137	AGGTGACACTATAGAATAGT TGTCCAAAGAAACCTGTA	GTACGACTCACTATAGGGAAT TAGTTCTGTAGTTTTTGCAT	7.8	200
COX IV	NM_001861.5	Subunit of the ETC in the mitochondrial membrane. Regulation site for oxidative phosphorylation (Pillon et al., 2020)	155	AGGTGACACTATAGAATAAGAG CTTTGCTGAGATGA	GTACGACTCACTATAGGGACCG TACACATAGTGCTTCT	7.8	200
PGC-1α	NM_001330751.1	Regulates transcription of mitochondrial genes and transcription factors involved in various metabolic processes (Granata et al., 2018; Pillon et al., 2020; Rundqvist et al., 2019)	338	AGGTGACACTATAGAATACA GTCCTCACAGAGACACTA	GTACGACTCACTATAGGGAC TCCATGAATTCTCAGTCTT	31.25	200
PPAR-δ	NM_006238.5	Transcription factor which regulates fatty acid oxidation (Pillon et al., 2020; Rundqvist et al., 2019)	361	AGGTGACACTATAGAATATTC CACTACGGTGTTCAT	GTACGACTCACTATAGGGAGTT GAAGTTTTTCAGGTAGG	62.5	200
NRF1	NM_001040110.1	Regulates mitochondrial gene transcription, encoding a number ETC proteins (Granata et al., 2018; Pillon et al., 2020)	229	AGGTGACACTATAGAATATTGAG TCTAATCCATCTATCC	GTACGACTCACTATAGGGATCT TGTACTTACGCACCAC	62.5	200
GABPA/ NRF2	NM_002040.4	Mediates antioxidant defences, controls expression of mitochondrial genes (Granata et al., 2018; Pillon et al., 2020)	240	AGGTGACACTATAGAATATTTG ACCAAGGAGTAAAAAC	GTACGACTCACTATAGGGAGTG CCATCAAGAGTTATCA	62.5	200
UCP3	NM_003356.4	Uncoupling protein which regulates energy expenditure by uncoupling oxidative phosphorylation, producing heat without ATP synthesis (Pillon et al., 2020; Rundqvist *et al.*, 2019)	269	AGGTGACACTATAGAATACA TGAGGAATGCTATCGT	GTACGACTCACTATAGGGAC ATCTTTATCATACAGTCGAG	7.8	200

(Continues)

TABLE 1 (Continued)

Gene	Accession number	Putative role in skeletal muscle metabolism	Product size	Forward primer sequence (5′–3′)	Reverse primer sequence (5′–3′)	Reverse primer dilution (nM)	Forward primer dilution (nM)
SIRT1	NM_001142498.1	Deacetylase with downstream effects on other metabolic proteins. Modulates substrate oxidation and mitochondrial adaptation (Pillon et al., 2020)	245	AGGTGACACTATAGAATAGGAT TTGGGACTGATGGAGA	GTACGACTCACTATAGGGACAC CTTTCTGGTTTCCTTGC	15.6	200
SIRT3	NM_001017524.2	Deacetylase which upregulates activity of mitochondrial fatty acid oxidation enzymes (Pillon et al., 2020)	281	AGGTGACACTATAGAATAAGA ACATCGATGGGCTTGAG	GTACGACTCACTATAGGGAAA ATCAACCACATGCAGCAA	62.5	200
SIRT4	NM_012240.2	Deacetylase which inhibits activity of mitochondrial fatty acid oxidation enzymes (Pillon et al., 2020)	149	AGGTGACACTATAGAATAAGGT CAGAAAAAGTGGGGCT	GTACGACTCACTATAGGGACTA CGAAGTTTCTCGCCCAG	500	200
SIRT5	NM_001193267.2	Deacetylates cytochrome *c*, and exerts regulatory effects on ketogenic pathways (Pillon et al., 2020)	202	AGGTGACACTATAGAATACA GGAAAAGGTGCTCCAGAA	GTACGACTCACTATAGGGAC GAGCTCTCTGTCAACCTCC	62.5	200
GLUT4	NM_001042.3	Skeletal muscle glucose transporter. Regulates insulin sensitivity and glycogen synthesis (Pillon et al., 2020)	275	AGGTGACACTATAGAATATC TATTATTCGACCAGCATC	GTACGACTCACTATAGGGAA CAATGGAGACGTAGCTC	62.5	200
CD36	NM_001001547.3	Sarcolemmal FFA transporter. Rate-limiting enzyme in fatty acid oxidation (Pillon et al., 2020)	143	AGGTGACACTATAGAATAGA AGTTTACAGACAGTTTTGG	GTACGACTCACTATAGGGAC TCTGTACGTATAAGGACCTC	7.8	200
CPT1A	NM_001876.4	Mitochondrial FFA transporter. Rate-limiting enzyme in fatty acid oxidation (Pillon et al., 2020; Rundqvist et al., 2019)	167	AGGTGACACTATAGAATAGT TCATCAGATTCAAGAACG	GTACGACTCACTATAGGGAT GCAATTATTCCTAACGAG	31.25	200
PDK4	NM_002612.4	Mitochondrial protein which regulates substrate oxidation preferences (Pillon et al., 2020; Rundqvist et al., 2019)	192	AGGTGACACTATAGAATAGT GTATGTTCCTTCTCACCT	GTACGACTCACTATAGGGAT CTGAAATCTTAATGGTAAGG	1.95	200
ENHO	NM_198573.3	Encodes adropin, a putative modulator of substrate oxidation secreted from peripheral tissues (Pillon et al., 2020)	214	AGGTGACACTATAGAATAGC CCTCATCGCCATCGTCTG	GTACGACTCACTATAGGGAG CTGGGCTTCTGGGGTGGTG	1500	200

(Continues)

TABLE 1 (Continued)

Gene	Accession number	Putative role in skeletal muscle metabolism	Product size	Forward primer sequence (5′−3′)	Reverse primer sequence (5′−3′)	Reverse primer dilution (nM)	Forward primer dilution (nM)
NMNAT1	NM_001297778.1	Intermediary of the NAD^+ biosynthetic pathway (Pillon et al., 2020)	221	AGGTGACACTATAGAATAG GGTCATCATGGCAGAACTT	GTACGACTCACTATAGGGA CTCTTCCTTCCAGGCCTTTC	15.6	200
NMNAT3	NM_178177.4	Intermediary of the NAD^+ biosynthetic pathway (Pillon et al., 2020)	184	AGGTGACACTATAGAATAA GAAGTTTGGCTTGGTGTGC	GTACGACTCACTATAGGGA GCCTGATGTATGTGGCACTG	62.5	200
NMRK2	NM_170678.2	Key rate-limiting enzyme in the exogenous NAD^+ biosynthetic pathway (Pillon et al., 2020; Rundqvist et al., 2019)	161	AGGTGACACTATAGAATAC GCAACTACACAGTCCCTGA	GTACGACTCACTATAGGGA ACTTCATGCCGTCCAGGTAG	62.5	200
NAMPT	NM_005746.2	Key rate-limiting enzyme in the endogenous NAD^+ biosynthetic pathway (Pillon et al., 2020)	287	AGGTGACACTATAGAATAGG CCTTGGGATTAACGTCTT	GTACGACTCACTATAGGGAG ATGTGCTGCTTCCAGTTCA	15.6	200
NNMT	NM_006169.2	Modulates nicotinamide precursor levels required for NAD^+ biosynthesis, in turn affecting NAD^+ salvage pathway. Putative role in fatty acid oxidation (Pillon et al., 2020; Rundqvist et al., 2019)	311	AGGTGACACTATAGAATAG AATCAGGCTTCACCTCCAA	GTACGACTCACTATAGGGA CCTGCAGGTTCTGGTCTGAG	62.5	200
TDO2	NM_005651.4	Enzyme that plays a critical role in tryptophan metabolism by catalysing the rate-limiting step of the kynurenine pathway. Potential regulatory role in NAD^+ biosynthesis from exogenous sources (Pillon et al., 2020)	251	AGGTGACACTATAGAATAGG CAGCGAAGAAGACAAATC	GTACGACTCACTATAGGGAC AGAATCCAACTCCCAGAGG	1500	200
UBE2D2	NM_181838.1	Reference gene	172	AGGTGACACTATAGAATACA GCACAGTGTTCAGCAGGT	GTACGACTCACTATAGGGAT GAAGGGGTAATCTGTTGGG	0.48	200
PSMB6	NM_001270481.1	Reference gene	209	AGGTGACACTATAGAATAGAACA ACCACTGGGTCCTAC	GTACGACTCACTATAGGGAACCA GTGGAGGCTCATTCAG	31.25	200
PPIA	NM_021130.5	Reference gene	235	AGGTGACACTATAGAATAGGGT TTATGTGTCAGGGTGG	GTACGACTCACTATAGGGAGCC ATCCAACCACTCAGTCT	31.25	200

specific primers for reverse transcription and PCR amplification in accordance with user guidelines for the Genome Lab GeXP system, as published previously (Drew et al., 2011, 2104a, 2016). Reverse PCR primers were designed with a 3′ gene-specific sequence and a 5′ end consisting of a 19-base universal priming sequence. The forward PCR primers were designed with a 3′ gene-specific sequence and a 5′end consisting of a different 18-nucleotide universal priming sequence. Primer sequences were designed using NCBI Primer-BLAST to ensure specific amplification of the designed PCR fragments. NCBI Nucleotide BLAST identified if primer sequences had high homology to other genes detectable in human skeletal muscle. Primers with universal sequences were purchased from Sigma-Genosys (Haverhill, UK).

2.4 | RNA extractions

Total RNA was extracted from human skeletal muscle samples (19.0 ± 3.3 mg) using an RNeasy Plus Universal Mini Kit following homogenisation in QIAzol lysis reagent and a precellys 24 bead-mill homogeniser (Bertin Technologies; Montigny-le-Bretonneux, France). To increase RNA yield, kit instructions were modified by replacing ethanol with isopropanol to precipitate the RNA (Kuang et al., 2018). A genomic DNA elimination step was included in the kit to remove genomic DNA from total RNA. RNA purity was quantified using a NanoDrop ND-1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific; Waltham, MA, USA) and the 260: 280 nm absorbance ratio. The 260: 230 nm absorbance ratio was used to evaluate the degree of chemical contamination in each RNA sample. RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA, USA). According to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al., 2009), information about RNA quality and integrity should be reported for qPCR experiments. Based on these recommendations, results for RNA integrity number (8.1 ± 0.3) from tapestation analysis, as well as the A260: 280 (2.0 ± 0.3) and A260: 230 ratios (1.0 ± 0.4) obtained from Nanodrop analysis indicated that RNA samples extracted for the purposes of this study were of sufficient quality and integrity.

2.5 | Primer testing, singleplex and multiplex optimisation

The hMitoplex was optimised using total RNA (50 ng per reaction) extracted from human skeletal muscle samples which were mixed to make a template pool, and the Genome Lab GeXP start Kit (Beckman Coulter; Brea, CA, USA), according to the manufacturer's instructions and as published previously (Drew et al., 2011, 2014a, 2016). Individual primer pairs were initially tested using a reverse primer mix (500 nM) incorporating the entire set of primers in conjunction with each forward primer (200 nM) individually to ensure a single amplicon of the correct size was generated for each of the designed primer pairs. In total, one primer pair redesign was required for a single hMitoplex target (*ENHO*), while the remaining targets were detectable at the correct amplicon size. Upon redesign, an amplicon was detected at the correct nucleotide size using the second *ENHO* primer pair.

Following generation of singleplex reaction products at the predesigned product size for each target, reverse and forward primers were tested at the same concentration in multiplex to compare the relative expression levels of each hMitoplex target. A Bland–Altman plot was made to compare the level of agreement between the two methods of gene quantification (normalised to the reference gene *UBE2D2*) for each individual target (Bland & Altman, 1999). Normalisation to other reference genes incorporated in the hMitoplex, *PSMB6* and *PPIA*, was also performed. The NormFinder statistical program (Andersen et al., 2004) was used to determine the stability of the reference genes. All three reference genes were deemed stable; however, NormFinder indicated that *UBE2D2* was most stable and it was therefore selected for normalisation of all data going forward. Additionally, *UBE2D2* has previously been used in multiplex gene expression assays and demonstrated as stable in human and animal tissues (Drew et al., 2015, 2016).

Subsequently, optimisation of the hMitoplex incorporating multiplexed primer pairs was conducted on primer products obtained in multiplex reactions. Attenuation was then conducted on reverse primer concentrations according to manufacturer's instructions to determine the optimal dilution factor for each gene target to generate an appropriate dynamic range of signals within a measurable linear range. Where amplicons of a specific target tested with a 500 nM reverse primer concentration were detected above the appropriate dynamic range, a new reverse primer mix was prepared with its concentration initially reduced to 125 nM and retested. If still above the appropriate dynamic range, this concentration was decreased by half upon each retest until this range was reached. This process was performed for all but three hMitoplex targets. Where amplicons of a specific target with a 500 nM concentration were not detected in the appropriate dynamic range, reverse primer concentrations were doubled to 1000 nM, and if still undetectable tripled to 1500 nM to detect these targets within the appropriate dynamic range. Final primer concentrations for hMitoplex profiling are provided in Table 1.

2.6 | Experimental procedures for GeXP hMitoplex

Detailed procedures for GeXP experiments are described here and in further detail in previous publications (Drew et al., 2011, 2014a, 2016). A master mix of reagents (3 μl DNAse/RNAse-free H_2O, 4 μl reverse transcription buffer 5, 1 μl reverse transcriptase, 5 μl pre-diluted Kan^r RNA) was prepared for reverse-transcription reactions conducted in 96-well plate format as detailed in the Genome Lab GeXP Start Kit (Beckman Coulter) instructions, coupled with the attenuated reverse primer multiplex mix and using 50 ng total RNA per well. An aliquot (9.3 μl) of each reverse-transcription reaction was transferred to a new 96-well PCR plate (Abgene, Epsom, UK) and PCR amplified with the addition of the GeXP Start Kit PCR reaction mix prepared according to the manufacturer's instructions (4 μl PCR buffer 5×, 4 μl

25 mM $MgCl_2$ (Thermo-start), 0.7 μl Thermo-start DNA polymerase (A85022)), coupled with a 200 nM forward primer multiplex mix. Reverse transcription and PCR amplification steps were performed using a Bio-Rad T100 Thermal Cycler (Bio-Rad Laboratories; Hercules, CA, USA) and the respective reaction programme protocols as detailed in the GeXP Start Kit instructions. The PCR products were prepared for fragment analysis using the Beckman Coulter CEQ 8000 GeXP Genetic Analysis system. An aliquot of this PCR reaction (2 μl) was diluted with DNase/RNase-free water. This mixture was subsequently added to a sample loading solution and CEQ DNA Size Standard 400 (Beckman Coulter) according to the manufacturer's instructions in a new 96-well CEQ electrophoresis plate, giving a final dilution of 1:300. Capillary electrophoresis and fragment separation of amplicons were performed on the GeXP Genetic Analysis System as previously described (Drew et al., 2011).

2.7 | Electrophoresis analysis

Following capillary electrophoresis and fragment separation of GeXP amplicons, a size fragment analysis was performed using the fragment analysis module of the GenomeLab GeXP system software to generate electropherograms representing the electrophoresed and separated fragments generated by GeXP eXpress profiling, as previously described (Drew et al., 2011). Normalised peak area values against the incorporated reference genes (*UBE2D2*, *PPIA* and *PSMB6*) were calculated using the GeXP eXpress Analysis software.

2.8 | Statistical analysis

An intraclass correlation coefficient was calculated to determine test–retest reliability of quantified gene expression between singleplex and multiplex detection methods. Data for the overall study cohort are presented as mean ± 95% confidence intervals (CI) unless otherwise stated.

3 | RESULTS

3.1 | Primer testing

Gene expression of hMitoplex targets was quantified in singleplex and multiplex analysis, normalised to the reference gene *UBE2D2*. For completeness, relative quantification was also performed using the other two reference genes, *PSMB6* and *PPIA*, in both instances showing similar results in terms of the overall gene expression profiles and variance compared with *UBE2D2* normalisation. Two targets, *ENHO* and *TDO2*, were detected when measured in singleplex, but not in multiplex analysis and were excluded from the analysis. Bland–Altman analyses comparing the level of agreement between each method of gene expression quantification for individual targets are presented in Figure 2.

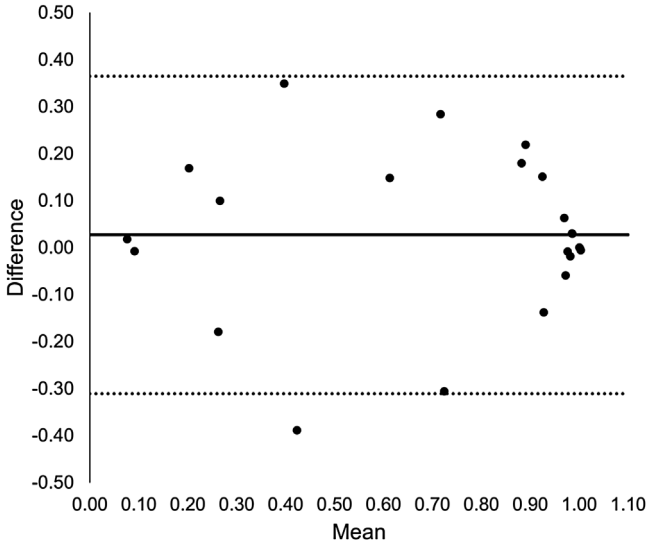

FIGURE 2 Bland–Altman plot showing 95% limits of agreement between hMitoplex primers tested in singleplex and multiplex, normalised to *UBE2D2*, in human skeletal muscle. Upper and lower levels of agreement are denoted by the dotted lines in the figure, while the level of bias is characterised by the continuous line. *ENHO* and *TDO2* were detected in singleplex near the acceptable lower level of detection but were not within this range when tested in multiplex analysis, suggesting that the abundance of these targets is very low in human skeletal muscle or poor primer performance/interactions. Since these targets were not within the reliable range of detection in multiplex analysis, they are not included in the Bland–Altman plot. The Bland–Altman plot indicated that *NRF1* was the only target which was outside of the 95% limits of agreement (upper limit 0.37; lower limit −0.31; bias 0.03) when measured in multiplex compared with singleplex quantification. Test–retest reliability expressed by an intraclass correlation coefficient for the remaining 23 genes was 0.88 (95% CI: 0.74–0.95)

3.2 | hMitoplex gene expression signatures

The hMitoplex gene expression signature in the sample cohort normalised to each reference gene is presented in Figure 3a–c. The expression profiles appeared broadly similar when target genes were normalised to each reference gene. In each instance all genes were reliably detected except for *TDO2* and *ENHO*, which may indicate that these genes have comparatively low expression in contrast with the other genes in this multiplex or that the primer efficiency was affected in the multiplex compared with singleplex quantification method for these targets. Since *TDO2* and *ENHO* were not reliably detected in multiplex analysis the data for these targets are not presented. Inter-individual variance in gene expression was largest in targets such as *PDK4* (standard deviation 0.21; individual range 0.03–0.87), *UCP3* (0.13; 0.17–0.69), *NAMPT* (0.13; 0.18–0.73) and *Tfam* (0.12; 0.25–0.90), while variance was smallest in targets including *NRF1* (0.02; 0.07–0.15), *SIRT3* (0.04; 0.13–0.28), *SIRT4* (0.03; 0.17-0.31), *SIRT5* (0.02; 0.07–0.16) and *NMNAT3* (0.03; 0.24–0.38).

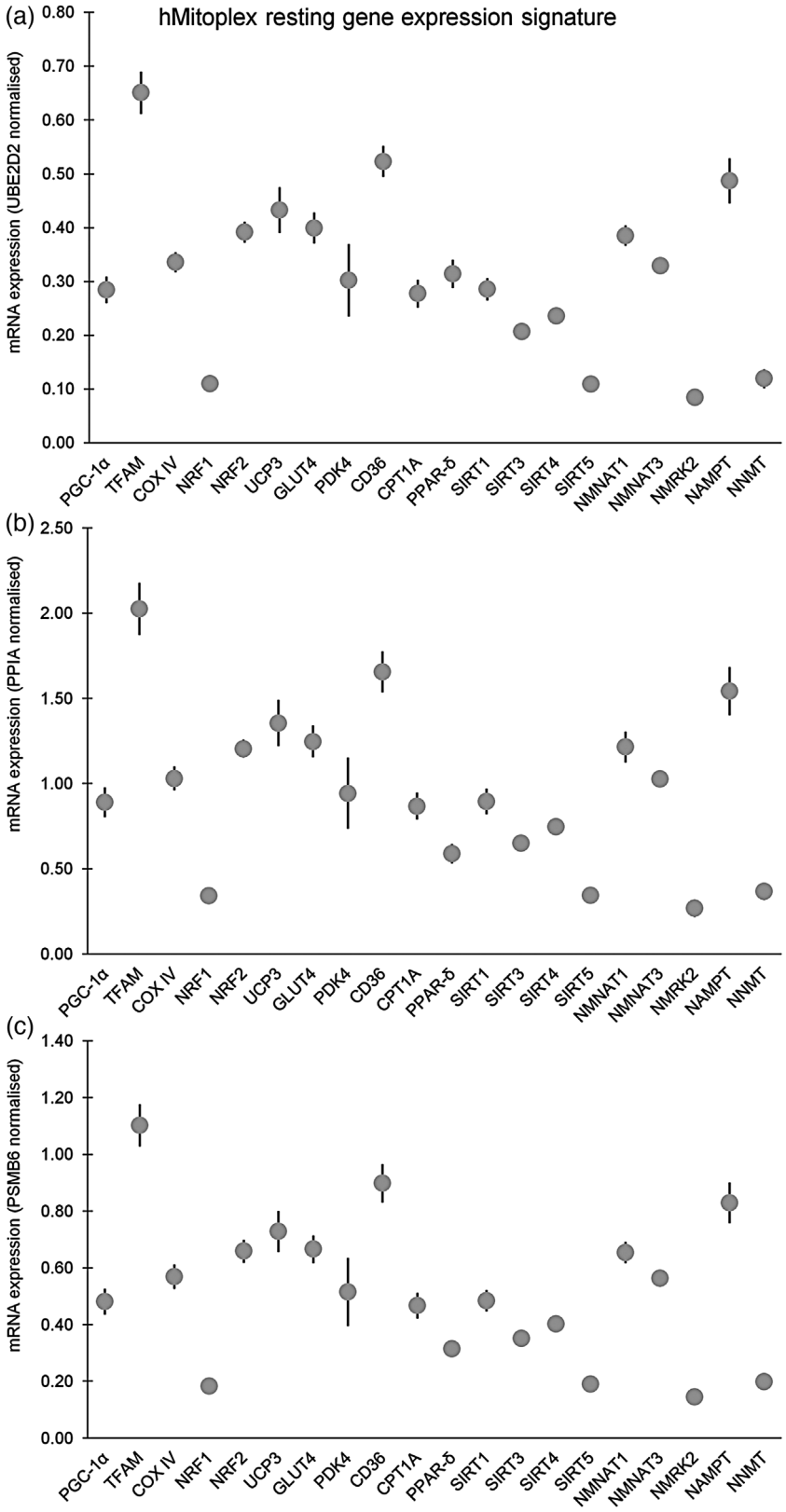

FIGURE 3 hMitoplex resting gene expression signatures in human skeletal muscle normalised to *UBE2D2* (a), *PPIA* (b) and *PSMB6* (c). Data are presented as means ± 95% confidence intervals

4 | DISCUSSION

Previous studies have reported on the development of custom-designed multiplex gene expression assays to determine expression of targeted groups of genes regulating various metabolic processes, including inflammation, NAD^+-dependent deacetylase activity, and oxidative stress, among others (Drew et al., 2011, 2014a, 2015, 2016; Gray et al., 2018). Additionally, studies have also validated GeXP assays for use in pathogen detection in clinical settings (Huang *et al*., 2020; Wang *et al*., 2016), and for characterisation of pathologies in various tissues (Drew et al., 2014b; Farquharson et al., 2012), highlighting their potential for use in multiple research settings. To our knowledge, this is the first study to design a GeXP assay to determine expression of a targeted set of genes implicated in regulating mitochondrial biogenesis, substrate metabolism and NAD^+ biosynthetic capacity in human skeletal muscle.

Fragment sizes of hMitoplex targets are calculated and normalisation to a selected reference gene is then completed. The ratio of target gene to reference gene peak area is used to quantify relative gene expression. GeXP assays hold the advantage of incorporating multiple reference genes, a recommended practice to ensure accurate quantification of gene expression (Bustin, 2010; Vandesompele et al., 2002). The overall gene expression profiles observed here were similar when normalised to each selected reference gene, supporting the reliability of these results and the rationale for using multiple reference genes. Additional advantages of GeXP include the reduced technical error due to quantifying expression levels of target and reference genes simultaneously in a single experimental sample, more accurate assessment of gene co-expression, conservation of extracted RNA samples, as well as savings on time commitments and overall costs if planning to analyse several target genes (Edwards & Gibbs, 1994). For example, quantifying the expression of the entire set of genes in the hMitoplex using qPCR would require a 25-fold greater quantity of template RNA, along with substantially increased time commitments and potential for technical error. GeXP assays also hold advantages over hybridisation-based methods, including the high background levels in macro/microarrays due to cross-hybridisation (Wang *et al*., 2009), and the more limited range of detection due to background and signal saturation compared to PCR-based techniques, which affects accuracy of gene quantification of targets with very low or high abundance (Drew et al., 2011; Wang *et al*., 2009). While next generation sequencing methods such as RNA-Seq are shown to provide superior sensitivity for quantification of global gene expression profiles, these approaches are not without their limitations in coverage of the whole transcriptome and remain costly and time consuming, which are significant limiting factors (Lahens *et al*., 2014; Sena *et al*., 2018; Wang *et al*., 2009). Moreover the lack of standardisation between sequencing platforms can affect the reproducibility of experimental results (Whitley *et al*., 2016), potentially indicative that in-depth specialist knowledge is required for these platforms. As such, GeXP-based assays have use as alternative strategies to quantify gene expression, holding specific advantages over both hybridisation and sequencing-based approaches. GeXP may be an especially useful tool for practitioners using the microbiopsy technique, given the lower overall biopsy sample yield obtained with this approach.

Bland–Altman analysis which was performed comparing quantified gene expression profiles in singleplex vs. multiplex indicated that *NRF1* was the only target which was outside the 95% limits of agreement. These data, coupled with findings from intraclass correlation analysis indicated adequate test–retest reliability between quantification methods with the exception of *NRF1*. These overall findings support the assertion that quantification using GeXP assays is reliable for detecting gene expression in human skeletal muscle when compared with standard singleplex quantification. These findings also support the determination of gene expression in human skeletal muscle (using as little as 50 ng of extracted RNA per PCR reaction) for 20 mitochondrial genes using the hMitoplex GeXP assay described here. Given the findings showing that *NRF1* expression was outside of the limits of agreement between multiplex and singleplex detection methods, careful considerations are necessary when interpreting the quantification of this gene in the hMitoplex. This may be due to interaction with other primers and can be assessed further when a set of treatments are applied across samples. Another important consideration when contextualising the level of agreement between these methods is that variation in some of the more lowly expressed targets may have a greater impact on accuracy of quantification compared to a similar amount of variation in more highly expressed targets. However, it is important to note the overall high level of agreement between targets detected in singleplex and multiplex analysis. Previous research has validated GeXP multiplex assays in comparison with qPCR showing good agreement (Drew et al., 2011), providing further support for the efficacy of this technique.

ENHO and *TDO2* were not within the lower limits of detection when tested in multiplex, but were detected near the lower end of the linear detection range in singleplex. The reasons for these discrepancies in the quantification of *ENHO* and *TDO2* compared with the remaining hMitoplex targets are not fully clear. One possible explanation may be that both targets have low abundance in human skeletal muscle and are thus not within the lower limits of detection for multiplex testing. This reasoning is supported by the fact that to our knowledge, though these genes are implicated in metabolism, no previous studies have reported on the gene expression of either of these targets in human skeletal muscle. Research studies have previously reported on the gene expression quantification of many other hMitoplex targets in human skeletal muscle *in vitro* or *in vivo* (Granata *et al*., 2018; Pillon *et al*., 2020; Rundqvist *et al*., 2019). This also outlines a potential limitation of GeXP-based assays for quantifying expression of very lowly expressed targets in a specific tissue. In this case the inability to accurately quantify *TDO2* and *ENHO* in skeletal muscle may point to a narrower range of detection in multiplex compared with singleplex quantification.

In conclusion, we have described the development of a custom-designed GeXP multiplex assay and demonstrated the ability to accurately quantify expression of a targeted set of multiple mitochondrial genes in a small quantity (5–10 mg) of human skeletal muscle. This GeXP multiplex is valuable as a tool to generate a

profile/signature of multiple targets in skeletal muscle tissue, as demonstrated by the profile of resting mitochondrial gene expression in a population of young, healthy recreationally active males reported here. An application of the assay in exercised/nutrient treated samples is not included here and is a limitation of this study. Other proposed advantages of GeXP multiplex assays have been outlined in comparison with other techniques, including reduced technical error due to quantifying gene expression levels of multiple targets simultaneously, incorporation of multiple reference genes, conservation of extracted RNA samples, time efficiency and cost savings. The methodological challenges encountered in the development of GeXP assays and how these can be addressed have been outlined in this report. The hMitoplex described here, may be useful in studies involving perturbations to human skeletal muscle by establishing a gene expression signature in response to treatment.

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COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

T.P.A., A.J.F., J.E.D. and B.P.C. were responsible for study conceptualisation and experimental design. T.P.A. and A.J.F. completed analysis on skeletal muscle biopsy samples. T.P.A., A.J.F., J.E.D. and B.P.C. researched, interpreted and analysed data. T.P.A., A.J.F., J.E.D. and B.P.C. wrote the manuscript. All authors critically revised the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in Table 1 and Figures 2 and 3.

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