Biodistribution and Retention of Locally Administered Human Mesenchymal Stromal Cells: Quantitative PCR-Based Detection of Human DNA in Murine Organs

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Abstract

Background. Determining the distributive fate and retention of a cell therapy product after administration is an essential part of characterizing its biosafety profile. Therefore, regulatory guidelines stipulate that biodistribution assays are a requirement prior to advancing a cell therapy to the clinic. Here the development of a highly sensitive quantitative polymerase chain reaction (qPCR) based method of tracking the biodistribution and retention of human mesenchymal stromal cells (hMSCs) in mice, rats or rabbits is described. *Methods.* A primer-probe based qPCR assay was developed to detect and quantify human Alu sequences in a heterogeneous sample of human DNA (hDNA) and murine DNA from whole organ genomic DNA extracts. The assay measures the amount of human genomic DNA by amplifying a 31-base pair sequence of the human Alu (hAlu) repeat sequence, thus enabling the detection of 0.1 human cell in 1.5 x 10^6 heterogeneous cells. *Results.* Using this assay we investigated the biodistribution of 3 x 10^5 intramuscularly injected hMSCs in Balb/c nude mice. Genomic DNA was extracted from murine organs and hAlu sequences were quantified using qPCR analysis. After 3 months, hDNA ranging from 0.07-0.58 % was detected only at the injection sites and not in the distal tissues of the mice. *Discussion.* This assay represents a reproducible, sensitive method of detecting hDNA in rodent and lapine models. This manuscript describes the method employed to generate preclinical biodistribution data that was accepted by regulatory bodies in support of a clinical trial application.

Keywords: *biodistribution, cell therapy; genomic DNA, human Alu sequence; mesenchymal stromal cell, polymerase chain reaction, translational stem cell research.*

Introduction

Determining the distributive fate and retention of cell therapy (CT) products after administration are an essential part of characterizing the product's mechanism of action (MOA) and biosafety profile. The therapeutic cell's phenotype, efficacy and migratory potential are influenced by the formulation of the CT product as well as by the route of administration (ROA) and the micro-environment in which the cells reside in the host. Concerns surrounding the *in vivo* acquisition of cellular autonomy resulting in ectopic tissue formation prompt regulatory authorities to require stringent pre-clinical investigations into the biodistribution of the administered CT[1].

For CT products it is vital that reproducible, sensitive, quantitative assays are developed and applied to evaluate the persistence and distribution of cells after administration. Regulatory guidelines stipulate that CT product safety is determined using risk-based approaches such that the assays developed to determine biosafety for the intended host consider and directly address any risks posed to the intended host[1-4]. Unlike small molecule pharmaceuticals, the biological complexity of living cells does not make them suitable for routine absorption, distribution metabolism and excretion and pharmacokinetic testing[1]. As a result, biodistribution assays are a regulatory requirement for advancing a CT to the clinic. Biodistribution studies can provide data on CT product localization or migration over time as well as *in vivo* survival and differentiation in the case of progenitor cell-based CT[4].

The biodistributive profile of a CT product has safety and efficacy implications, addressing questions such as: Are the cells reaching the reparative site of interest in the host? Are they engrafting in numbers sufficient to elicit the desired response? How long do they persist in the host? Laboratories worldwide have used a wide range of techniques in attempts to determine the distribution of transplanted cells[5, 6]. Microscopic visualization of histological samples has been widely used to detect the presence of the transplanted cells in pre-clinical models using a variety of cell labeling techniques, such as membrane dyes (PKH26, DII [1,1'Dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate]) or nuclear dyes (Hoechst 33342, bromodeoxyuridine, DAPI [4'6-diamidino-2-phenylindole])[6]. However, cellular labeling methodologies are susceptible to dilution with cellular division reducing the label below the limits of detection[6]. Furthermore, the labor-intensive histological techniques required to locate the CT *in vivo* can be subject to sampling error, leading to variability and thus reduced sensitivity and reliability of the results[6].

Genetic modification of the intended CT can allow for the identification of the administered cells without concern about dilution of the label. However, the required use of cellular labeling molecules can have consequences on CT product functions and may potentially alter the biodistributive fate of the cells[4, 7]. Modification of the pre-clinical CT product with genes encoding proteins which can be visualized via microscopy (green fluorescent protein, enhanced green fluorescent protein, red fluorescent protein, yellow fluorescent protein, β-galactosidase and mCherry) can provide quantitative information about cellular location and survival, however gene silencing in long-term studies may result in a decrease in signal with time[6]. Furthermore autofluorescence within the tissues of interest and uptake of the fluorescent protein by adjacent cells, such as macrophages, can result in false positives thus compromising the accuracy of the results[6]. Newer technologies are emerging in which non-invasive imaging can provide real time *in vivo* tracking of the transplanted CT[5, 7-9]. Such imaging modalities are exciting as they enable the investigators to obtain dynamic measurements of cellular viability and location after administration. However their application relies upon suboptimal extensive cell labeling. This strategy may not be ideal as the regulatory authority requires that the pre-clinical studies to support the first in human application must be completed using the final cell product intended for human use[1, 2].

Real-time, quantitative PCR (qPCR) is a relatively inexpensive technique that bypasses the disadvantages associated with other cellular detection methods[6]. qPCR enables the accurate and sensitive detection of transplanted cells via their cell-specific DNA sequences within the whole host organ, minimalizing sampling errors[6, 10]. The Alu sequence remains the marker of choice when assessing the biodistrubution of transplanted cells in xenogenic models, due to genomic repetition and species specificity. The human Alu (hAlu) sequence can be amplified and quantified by qPCR from genomic DNA (gDNA) with a high degree of accuracy[6, 10].

Here we describe the development of an accurate, reproducible, quantitative and inexpensive qPCR-based method of tracking the biodistributive fate of human cells in xenogenic models. The assay is a primer-probe based PCR assay using custom-made primers to detect and quantify the hAlu sequences in a heterogeneous sample of human DNA (hDNA) and murine DNA (mDNA) from whole organ gDNA extracts. The assay enables the quantification of human gDNA by amplifying the human-specific hAlu repeat sequence[11] with a sensitivity to detect the DNA equivalent of 0.1 human cell in 1.5 x 10^6 heterogeneous cells.

Materials & Methods

Human Bone Marrow Mesenchymal Stromal Cell Isolation and Culture

Human mesenchymal stromal cells (hMSCS) were isolated from adult bone marrow and cultured expanded in accordance with local ethical approval and regulatory body-approved good manufacturing practice (GMP) protocols. Upon receipt, the bone marrow aspirate was washed with Dulbecco's Phosphate Buffered Saline (DPBS) and centrifuged at 900*g*. A 4% acetic acid wash was performed on a sample of the marrow to lyse the red blood cells and enable an accurate mononuclear cell (MNC) count. MNCs, plated at 40-50 million per 175cm^2 were cultured expanded in monolayer with complete medium (α-mimimal essential media supplemented with 10% selected fetal bovine serum [FBS]) in 5% CO_2 at 37°C. On day 3, fresh medium was added to the culture. On day 5, the cultures were washed with DPBS to remove non-adherent cells and fresh complete medium was added to each flask. When the monolayer reached 80-90% confluence, the adherent cells were washed with DPBS and detached from the culture plastic with 0.25% tryspin/ethylenediaminetetraacetic acid (EDTA). The dissociated cells were centrifuged at 400*g* for 5 minutes. The resultant pellet was resuspended in complete fresh medium and the cellular yield determined. hMSCs were further sub-cultured by seeding 3 x 10^6 cells in a triple flask through two passages. hMSCs were cryopreserved at of a dose of 2 x 10^6 per ml in FBS combined with 10% dimethyl sulfoxide.

Animal Husbandry

Animal care and administration of the hMSCs were conducted in Charles River, a good laboratory practices (GLP) certified site. Approval was obtained under the Animal Scientific Procedures Act (ASPA) 1986 by the Home Office in Scotland before initiation of the study. Male and female BALB/c Nude mice (Hsd-*Foxn1*^{nu}) were obtained from Harlan UK Ltd, (Oxon, UK) and maintained on a Teklad Rodent Diet 2919. The diet and water were provided *ad libitium* except during designated procedures. During the acclimation period and study duration, animals were housed in a limited access rodent facility and kept in groups of two or three per cage in appropriately sized polycarbronate/polypropylene cages with stainless steel grid tops and solid bottoms. Each cage was fitted with a filter top and had sterilized white wood shavings. The mice were allowed a *ca* 2 week acclimation period to the Charles River facility conditions (19°C -23°C, 40%-85% relative humidity and a twelve-hour light/dark cycle) prior to inclusion in the study.

hMSC Preparation

Bone marrow derived hMSCs were thawed and prepared immediately before injection. The cells were removed from liquid nitrogen, thawed in a 37°C water bath and transferred directly into tubes containing 4 mL of saline vehicle. The cryovial was washed once to ensure that all cells were removed. A cell count was performed using the trypan blue exclusion method. A cell suspension containing 300,000 cells was transferred into 15 mL conical tubes and centrifuged at 400*g* for 5 minutes at room temperature. The supernatant from the centrifuged cells was discarded and the pellet of hMSCs was resuspended in 150 µL of saline vehicle. The cell suspension was mixed well and transferred to a sterile cryovial, then to three insulin syringes containing 50 µL each. The hMSCs were administered to the animals within 2 hours of resuspension in saline.

hMSC Transplantation

hMSCs were administered at a dose of 3 x 10^5 cells in 150 µL per animal. The total volume was divided between 3 injection sites (50 µL per site), two in the thigh and one in the calf on the right leg. Each injection was administered over *ca* 1 to 3 seconds. The control animals received 3 injections containing a total of 150 µL of saline in a similar manner. Animals in each group were subjected to termination at 3 months after the hMSCs administration.

Necropsy and Tissue Collection

The following tissues, in the following order, were harvested from all animals at necropsy: liver, kidneys, heart, lungs, brain and spleen. To harvest the injection site, the complete right leg was collected, without the foot attached, including the lateral head of gastrocnemius muscle, semitendinosus muscle, semimembranosus muscle, adductor muscle and the calf muscle. Tissues were collected into RNAse-free 1.5 mL Eppendorfs, frozen in liquid nitrogen and stored at -80ºC. When required, the tissues were removed from -80°C and placed on ice to thaw. Using a sterile pipette tip, the tissues were removed from the tube and weighed on a small sterile tissue culture dish. The right thigh and calf regions (injection sites) were dissected and similarly weighed.

gDNA Extraction and Quantification

For a detailed protocol of DNA isolation and qPCR amplification of hAlu, please refer to the supplementary data.

Due to the sensitivity of this assay to detect hDNA, heightened measures to avoid hDNA contamination were required, such as a dedicated set of pipettes, frequent changing of gloves, the donning of protective eyewear, utilization of sterile disposables and filtered pipette tips. To gain an accurate, representative profile of hDNA content in each organ, the full organ was homogenized. Briefly, the organs were placed on a 100 μm cell filter strainer and mechanically dissociated using a sterile pestle before gDNA isolation. Using a Bioline DNA extraction kit, manufacturer's protocol was then scaled based on the weight of each organ, adding a proportional volume of lysis buffer and proteinase k (20 mg/mL) to each organ. The tissue suspension was incubated in a shaker at 55°C for 12-20 hours. Lysis buffer was then added to each tissue digest, followed by further incubation for 10 minutes at 70°C. A volume of the tissue lysate corresponding to 25 mg tissue was placed in the DNA spin column. The silica membrane with bound DNA was washed and the DNA eluted by adding 50 μL of elution buffer preheated to 70°C in a 3-minute incubation. The elution step was repeated to generate 100 μL of pooled eluted DNA.

Human-Specific Alu qPCR Primers

In an effort to determine the most efficient and sensitive qPCR methodology to detect hDNA, qPCR primers were designed for both SYBR-based and primer-probe based qPCR assays targeting the unique human-specific sequence of the Alu repeat[11] (Figure 1). The forward primer for the SYBR qPCR assay annealed upstream of the human specific hAlu sequence (5'-CGC CTG TAA TCC AGC TAC TC-3') while the reverse primer annealed primarily within the hAlu-specific sequence (5'-ATC TCG GCT CAC TGC AAC-3') ensuring amplification of only hAlu sequences and not Alu of the murine host (Figure 1A). For the primer-probe based assay the forward primer was designed to anneal upstream of the human specific Alu sequence (5'-TGG TGG CTC TCT CCT GTA AT-3') and the reverse primer designed to primarily anneal within the human-specific Alu sequence (5'-GAT CTC GGC TCA CTG CAA C-3'), resulting in a 96 (bp) amplicon. The probe was designed to bind between the two primers (5'-

TGA GGC AGG AGA ATC GCT TGA ACC-3') upstream of the hAlu specific sequence (Figure 1B). In the state-of-the-art McBride *et al* publication [12], the forward primer used was 5'- CAT GGT GAA ACC CCG TCT CTA – 3' along with the reverse primer 5'-GCC TCA GCC TCC CGA GTA G-3' and probe 5'- FAM- ATT AGC CGG GCG TGG TGG CG-TAMRA-3' (Figure 1C).

DNA Quantification and qPCR

The concentration of gDNA isolated from each murine organ was quantified using a Quant-iT PicoGreen dsDNA assay according to the manufacturer's instructions. For the SYBR Green technique, qPCR was performed in a volume of 25 µL that contained 12.5 µL of qPCR Sensimix , 0.4 µmol/L of forward and reverse primer and 200 ng of target template gDNA diluted in water. For the primer-probe based technique, qPCR was performed in a volume of 20 µl that contained 10 µl of qPCR master mix, 1 µl of primer-probe solution (FAM-MGB) and 100 ng of target template gDNA diluted to the final volume in water.

The SYBR Green PCR reactions were incubated at 95°C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds followed by 60°C for 1 minute. For the primer-probe reaction, the PCR samples were incubated at 50°C for 2 minutes and 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. All qPCR assays were performed in duplicate and the average values presented. qPCR assays were performed using StepOne Plus real time PCR machines. StepOne software was used to calculate crossing threshold (Ct) values for standards and samples using the automatic setting of baseline and threshold. Standard curves were generated by adding 10-fold serial dilutions (200 ng-0 ng) of hDNA on each PCR plate where a difference of 1.5 Cts less than that of the negative (0 ng) control was required to determine the lowest end of the assay's dynamic range.

Calculating hMSC Equivalents from hDNA Weight

By scatter plotting the log 10 of the standard concentrations versus the (Ct) values and determining the equation of the best fit line, the number of human cell equivalents in each qPCR well was calculated. After mathematically correcting for sampling and dilutions, the human cell equivalent per 25 mg tissue was scaled according to the organ weight to give the

total number of human cell equivalents contained within the murine organ. The human and mouse haploid genomes each contain approximately 3.3 x 10^9 bp DNA [13]. This value assumes all cells are diploid, although the authors acknowledge a percentage of cells will be undergoing DNA synthesis, mitosis or cytokinesis. To calculate the mass of the diploid genome, 3.3 x 10^9 bp was multiplied by 1.096^{-21}g/bp and multiplied by 2 (representing the conversion to a diploid genome), thus the total mass of the human or murine diploid genome is 6.67 pg. Therefore, a qPCR well containing 100 ng of gDNA was considered the equivalent of profiling 15,151.5 cells.

As 300,000 human cells were delivered intramuscularly, an equivalent of 2 µg of hDNA was administered (or 300,000 multiplied by 6.67 pg). Therefore, to calculate the percentage of administered hDNA retained within the organ, the amount of hDNA identified per organ was divided by 2 µg, then multiplied by 100.

Results

Specificity of the hAlu Primers: SYBR Green Vs Primer-Probe

To confirm primer specificity for hDNA in a heterogeneous solution with mDNA and determine the most sensitive qPCR methodology, both SYBR and primer-probe qPCRs were conducted (Figure 2). Positive control samples contained 100 ng hDNA and 100 ng mDNA, whereas negative control samples contained 0 ng hDNA and 200 ng mDNA. In the SYBR Green-based assay, the presence of hDNA (red amplification line, Figure 2A) resulted in a Ct value of 7.89 whereas the negative sample (yellow line, Figure 2A) generated a Ct value of 28.62. When using a primer-probe based assay, the presence of hDNA (purple amplification line, Figure 2B) resulted in a Ct value of 16.80 whereas the absence of hDNA (pink amplification line Figure 2B) resulted in a Ct of 36.60. In both the SYBR and primer-probe assays, a distinct difference was observed between the positive and negative samples, demonstrating amplification of the hAlu sequence. However, the SYBR based assay had notably higher Ct values in the negative sample, indicating increased background as compared to the primer-probe based assay. It was, therefore determined that the primer-probe based assay would be utilized in biodistributive analysis as the low background signal enables greater qPCR sensitivity.

To ensure that the mouse organ gDNA isolation methodology did not result in the retention of residual chemicals with potential to inhibit the qPCR reaction, serial dilutions of commercially obtained hDNA were analyzed by qPCR in the presence or absence of 200ng of isolated organ mDNA (Table I). It was observed that the highest point on the standard curve, containing 200 ng of hDNA, was saturated rendering it indistinguishable from the adjacent point on the standard curve containing 10-fold less DNA. The lowest point on the standard curve (containing 0.0002 ng hDNA) was distinguishable from the negative control (0 ng hDNA) by 1.5 Cts. The samples containing 20 – 0.0002 ng hDNA, as anticipated, confirmed the absence of non-specific background amplification of murine Alu sequences as well as the absence of residual inhibitory chemicals from the gDNA extraction protocol as evidenced by the maintained Ct values in the presence or absence of mDNA. Additional analysis of serially diluted hDNA assayed in the presence of 200 ng of rat or rabbit gDNA confirmed primer specificity for hDNA sequences as there is no increase in Ct values in the presence of xenogeneic gDNA (Table II).

Efficiency and Sensitivity of the hAlu Primer-Probe Combination

To compare the efficiency of the newly developed primer-probe combination with the current state-of-the-art in identifying hDNA in heterogeneous gDNA samples, a standard curve was created (Figure 3). qPCR was performed on 10-fold dilutions of human DNA from 200 ng to 0.0002 ng of hDNA using either the primer-probe sequence described in Figure 1B (Figure 3A) or the primer-probe set from McBride *et al*[12] (Figure 3B). The resultant Ct values were logarithmically graphed to calculate primer efficiency (Figure 3C). Both the McBride *et al.* [12]. and currently presented primer-probe combinations exhibited comparable efficiency at 98.0 and 99.4%, respectively.

The primer-probe combination presented in this manuscript produced less background signal compared to the McBride *et al.* [12] primer-probe combination, as demonstrated by the increased negative control Ct values (purple amplification curves in Figures 3A and B). This reduction in background signal, by 10.1 Cts, enabled a distinction between the negative controls and samples containing 0.0002 ng hDNA, samples that were indistinguishable from the negative control when using the McBride *et al.* [12] primers. Therefore, the currently described primer-probe combination is 100 times more sensitive than the current state of the art (Figure 3C). By converting hDNA weight to a cellular equivalent, the McBride primer-

probe combinations detected the DNA equivalent of 3 cells (Figure 3D) while the in house primer-probe combination allowed for the detection of the hDNA equivalent of 0.03 cells (Figure 3D).

Quantifying the qPCR assay's sensitivity was accomplished by conducting qPCR on hDNA mixed with increasing quantities of mDNA to a maximum of 3 μg of mDNA (or a cellular equivalent of 454,545 cells). In the presence of 3 μg of mDNA, a Ct value of 35.04 was observed in the 0.0002 ng standard whereas a Ct value of 37.46 was observed in the 0 ng standard (Table III). The 2.02 Ct difference observed between the 0.0002ng and 0 ng standard indicates that hDNA can still be reliably detected in the presence of 3 μg of mDNA. Although the upper limit of assay sensitivity is yet to be determined, these data demonstrate that the hDNA equivalent of 0.03 human cells can be detected in a heterogenous mixture of DNA from 454,545 cells, or 0.1 human cells in 1.5 x 10^6 heterogeneous cells..

Biodistribution of hMSCs after Intramuscular Administration to Nude Mice

In support of hMSC-based CT translation to the clinic, the pre-clinical biodistribution and retention of hMSCs was evaluated 3 months after intramuscular administration to Balb/c Nude mice. The 3 month time-point was chosen to coincide with parallel acute exposure toxicology studies. Control animals (n=5 males; n=5 females) received intramuscular injections of saline vehicle alone whereas hMSC treated animals (n=5 male; n=5 female) received intramuscular injections of saline with 300,000 hMSCs. Three months after local administration, eight critical organs were harvested, their gDNA extracted and qPCR analysis executed (Table IV). The resultant Ct values ranged from 34.42 to 35.36 in control males 34.35 to 36.30 in control females. Ct values higher than the lowest valid point on the standard curve were interpreted as background and were deemed non-detectable (ND). There was no hDNA detected in the organs from saline treated control animals.

In cell-treated animals, Ct values in male and female animals ranged from 26.83 to 35.94 and 28.67 to 36.23, respectively. Samples from the heart, lung, brain, liver, kidney and spleen were negative for hDNA in treated animals of both sexes. However, hDNA was detected in the thigh and calf samples, the sites of cell injection, in hMSC administered groups. Male thigh and calf samples retained the DNA equivalent of 0.20% and 0.58% of administered

human cells respectively while female thigh and calf samples retained the DNA equivalent of 0.07% and 0.13% of administered human cells, respectively.

Discussion

Patient safety is a paramount consideration when developing CT products. Early investment in the biological characterization of the therapeutic cell's phenotype, activity and migration upon administration is essential to ensure that the CT product is of a high quality, safe and efficacious when applied clinically. More specifically, it is critical to know where the CT product resides upon administration to ensure complementary toxicity assessments are conducted. Although the biologic safety of MSCs has been confirmed in clinical trials [14, 15], the lack of sufficient techniques to track cells after administration in humans means that the biodistribution of the transplanted cells remains largely unknown. As a result, regulatory agencies are now requiring preclinical evaluation of CT biodistribution as a prerequisite to first in human (FIH) trial initiation[1, 2, 16] .

Various methods such as imaging modalities, immunohistochemistry and flow cytometry have been used to assess the *in vivo* distribution of transplanted cells in multiple pre-clinical models[6-10]. However, the sensitivity and qualitative nature of such assays to detect a CT *in vivo* remains a concern. qPCR quantification of hAlu sequences represents one of the most efficient and sensitive techniques currently available. Here we describe the development of a versatile qPCR assay capable of amplifying the highly repetitive human-specific Alu DNA sequence[11] in the presence of mouse, rat or rabbit gDNA.

In this investigation, a non-pathological murine *in vivo* model was used for two reasons. First, the objective of the *in vivo* study was to confirm the *in vitro* assay development in a live model by isolating and identifying hDNA from viable tissue as an alternative to combining isolated gDNA from different organisms in a PCR assay. Second, the aim of this investigation was to develop and disseminate a methodology that was applicable to all pre-clinical disease models and not restricted to one clinical application. Therefore, to achieve these objectives, a non-pathological *in vivo* model is sufficient.

The versatility of this assay at detecting hDNA in a variety of pre-clinical models extends its potential utility beyond laboratory scale small rodents to the large animal models

(goat, horse, dog) required to translate a CT from the laboratory to the clinic. Due to its highly repetitive nature, targeting the Alu sequence will enable the detection of fragments of one cell in a xenogenic tissue sample. Although many groups have described PCR-based techniques for the detection of human cells in xenotransplantation systems, each qPCR protocol differs in its degree of sensitivity. Using the primer targeting strategy described herein with SYBR Green qPCR detection, we were able to detect 1 hMSC in 100 murine cells, superior to the detection limits reported by Song *et al*. [17] of 1 human cells in 20 murine cells, but inferior to those described by Toupet *et* al. [18] and Prigent *et al.* [10], detecting 1 adipose-derived hMSC in 41,000 murine cells or one hMSC in 200,000 murine cells, respectively. Although used to detect circulating human tumor cells, the SYBR Green-based protocol described by Schneider *et al.* [19] was far superior to our initial experimentation, detecting 1 human cell in 1 x 10^6 murine cells.

By developing this human specific Alu-targeting strategy into a more sensitive primer-probe based qPCR protocol, we were able to reduce the assay background fluorescence and thereby enable the detection of 0.1 human cell equivalents in 1.5 x 10^6 murine cells. With comparable methodology, Alcoser *et al.* [20] and Ramot *et al.* [21] described the detection of 1 human tumor cell in 149 murine cells or the identification of 1 placental-derived human stromal cell in 99,950 murine cells, respectively. Most recently, Priest *et al.* published the quantification of 1.4 human embryonic cell-derived oligodendrocytes in a heterogeneous mixture of 1.5 x 10^6 cells following their direct administration to the rat spinal cord [22]. However, the publication by McBride *et al.* [12] was of primary interest as it most closely reflected our intended application of quantifying the biodistribution of hMSCs in a murine model. We, therefore, compared the primer-probe combination developed herein with the sensitivity and efficiency of the state-of-the-art sequences described by McBride *et al* [12]. Although both assays retained comparable, high levels of efficiency, they differed largely in sensitivity. The protocol described herein surpassed the previously described detection of one hMSC in 20,000 cells by detecting equivalent of 0.1 hMSC in 1.5 x 10^6 murine cells (Table III), advancing the state-of-the-art of qPCR-based biodistribution assays beyond all previously published protocols [12, 18, 19, 21, 23, 24] . It is hypothesized this increase in sensitivity is a result of the differences in primer alignment. The primers described in this manuscript specifically target the human specific sequence in the Alu repeat, while the McBride primers do not (Figure I).

In support of a regulatory submission for FIH testing, it is advised to evaluate the intended human product in genetically immunodeficient models as this creates an immunotolerant environment for the human cellular component [1, 2, 16]. In this study, immunodeficient Balb/c Nude mice received an intramuscular administration of clinical-grade, GMP produced hMSCs via the route of administration intended for a proposed FIH study. Three months subsequent to hMSC administration, the critical organs were harvested and qPCR analysis conducted to localize and quantify the persisting hDNA. Within the limits of detection of this assay, no hDNA was detected in the brain, heart, lungs, kidneys, spleen or liver of animals that received hMSCs, indicating the CT product does not migrate and reside in these satellite sites. Moreover, the data demonstrate that after 3 months, small quantities of DNA derived from the hMSCs were retained within the muscle, at the site of administration. 0.07-0.20% of DNA from the administered hMSCs was retained in the thigh, while 0.13-0.58% was detected in the calf, similar to the cellular retention profiles previously reported in similar studies [18, 21]. It should be noted that these results were obtained in a health animal. A disease model may affect persistence and migration of the cells.

Upon identifying persisting CT DNA, the critical concern is the viability, safety and function of the residual cells. Are they alive and active, residual and senescent, or is the assay detecting CT that has been engulfed by local macrophages? The reliable quantification of hDNA isolated from dead cells is unlikely as gDNA degradation by caspases and DNAses occurs nearly immediately upon phagocytosis [25-27]. Further, data from Schneider *et al.* [19] support the theory that qPCR for hAlu amplifies DNA from live cells by showing a correlation with hAlu intensity and human cell proliferation marker Ki-67 from murine gDNA extracts prepared following the injection of H460M2 tumor cells into immunodeficient mice. Similarly, Prigent *et al.* [10] demonstrate that targeting hAlu sequences by qPCR provides quantification of live cells by showing a positive correlation between their qPCR data and histological localization of human cells actively transcribing a transgene. Therefore it is here hypothesized that hDNA identified in a murine organ sample was isolated from a viable hMSC

Herein we have developed an inexpensive, sensitive and regulatory body-accepted qPCR methodology to track unmodified bone marrow-derived hMSCs in mouse, rat and rabbit models. This assay is advantageous as it can provide accurate and precise quantification of small amounts of hDNA with a high degree of sensitivity. The qPCR assay described in this methods article can, therefore, be used a universally standardized method of quantitatively evaluating human CT engraftment, persistence and proliferation in support of FIH CT products.

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Table Legends

Table I. Values are mean of two replicates (standard deviation). hAlu primers amplify specifically hDNA in the presence of mDNA. Serial dilutions of genomic hDNA created in the presence of 0 ng or 200 ng of mDNA demonstrate the species-specific amplification of hAlu and not mAlu. Comparable Ct values with and without the addition of mDNA to the sample indicates that the hAlu sequence supports the qPCR reaction while the mDNA is not amplified.

Table II. Values are mean of two replicates (standard deviation). hAlu primers amplify hDNA and not rat or rabbit gDNA. Serial dilutions of genomic hDNA alone or in combination with 200 ng of rat or rabbit DNA resulted in comparable Ct values, demonstrating the specific qPCR amplification of hAlu and not rat or rabbit Alu sequences.

Table III. Values are mean of two replicates (standard deviation). Sensitivity of the qPCR assay. To establish the sensitivity of this newly developed biodistribution assay, 0.0002 ng of hDNA was combined with 3 μg mDNA and assayed by qPCR. A distinct >1.5 Ct difference was observed between the no template control and samples containing hDNA, demonstrating the potential to identify 0.1 human cell equivalents in a heterogeneous mixture of 1.5×10^6 cell equivalents.

Table IV. Any Ct value less than the limits of detection was deemed as ND. Biodistribution of bone marrow-derived hMSCs 3 months after intramuscular injection into Balb/c nude mice. The presence or absence of hDNA in murine organs was assayed by primer-probe qPCR analysis of gDNA extracted from male or female Balb/c nude thigh muscle, calf muscle, heart, lungs, brain, liver, kidneys and spleen. With n = 5 mice in each group, the Ct values were combined and the mean ± standard deviation (SD) calculated. No hDNA was observed in the untreated male or female animals. In animals receiving hMSCs, 0.07-0.58% of the administered hDNA was detected in the thigh or calf muscle injection sites of the cell-treated mice. No hDNA was detected in any distal organs.

Click here to view linked References

Table 1

Mixed Human and Mouse gDNA Templates: Average Ct Values

Standard hDNA (ng)	- mDNA	+ mDNA
200	16.44 (0.05)	16.52 (0.03)
20	18.69 (0.00)	18.67 (0.05)
2	21.94 (0.20)	22.16 (0.10)
0.2	25.49 (0.03)	26.30 (0.76)
0.02	29.22 (0.05)	29.61 (0.03)
0.002	32.86 (0.03)	32.87 (0.06)
0.00002	35.23 (0.10)	35.26 (0.17)
0	36.17 (0.32)	36.87 (0.09)

Note: Values are mean of two replicates (standard deviation).

Click here to view linked References

Table 2

Mixed Human, Rat and Rabbit gDNA Templates: Average Ct Values

Standard (ng)	Standard hDNA	200ng Rat DNA	200ng Rabbit DNA
200	16.62 (0.09)	16. 41 (0.01)	16.54 (0.45)
20	17.05 (0.53)	17.35 (0.01)	17.32 (0.01)
2	20.33 (0.42)	20.77 (0.32)	20.68 (0.01)
0.2	23.53 (0.08)	23.99 (0.09)	24.01 (0.08)
0.02	27.27 (0.15)	27.69 (0.09)	27.67 (0.20)
0.002	31.10 (0.39)	31.15 (0.03)	31.37 (0.04)
0.0002	33.93 (0.07)	34.25 (0.32)	34.02 (0.08)
0	35.73 (0.99)	35.93 (0.03)	35.89 (0.05)

Note: Values are mean of two replicates (standard deviation).

Table 3

Sensitivity of the qPCR Assay

Cellular Equivalent	Standard hDNA (ng)	3µg mDNA
0.03	0.0002 ng	35.44 (0.02)
0	0 ng	37.46 (0.67)

Note: Values are mean Ct values of two replicates (standard deviation).

Table 4

Organ Ct Values: Male and Female Group Mean Values

Group/sex		Thigh	Calf	Heart	Lung	Brain	Liver	Kidney	Spleen
Control Male	Mean	34.74	34.53	34.99	35.20	34.42	35.04	35.03	35.36
	SD	1.14	1.44	1.43	1.19	1.48	0.76	0.63	1.21
	% DNA	ND	ND	ND	ND	ND	ND	ND	ND
hMSC Male	Mean	28.74	26.83	35.85	35.65	35.21	35.77	35.94	35.87
	SD	1.47	1.91	1.19	0.69	0.16	0.73	0.99	1.22
	% DNA	0.20	0.58	ND	ND	ND	ND	ND	ND
Control Female	Mean	34.35	34.45	34.42	35.78	35.21	35.23	35.81	36.30
	SD	0.56	0.78	1.02	0.49	1.14	0.97	0.52	0.77
	% DNA	ND	ND	ND	ND	ND	ND	ND	ND
hMSC Female	Mean	30.27	28.67	35.75	36.23	35.90	36.05	36.14	36.19
	SD	1.32	1.78	0.80	1.41	0.70	0.66	0.57	1.61
	% DNA	0.07	0.13	ND	ND	ND	ND	ND	ND

Note: Any Ct value below the limits of detection was deemed as non detectable (ND).

Figure Legends

Figure 1. Primer sequence alignment with the hAlu genomic repeat. Pictorial representation of hAlu repeats with the SYBR Green primer (A) and primer-probe (B) alignments described herein, compared to the previously published primer-probe sequences [12] (C), illustrates a primer design (A, B) targeting the 31 bp of human-specific sequence [11] within the Alu DNA repeat (bold). In all assays, the forward primer (dashed underscore) binds in a generic region of the Alu DNA sequence. The probe (underscored) binds between the forward and reverse primers (B, C) to a xeno-conserved Alu sequence. The reverse primer, however, is targeted (A, B) to the 31-bp conserved sequence for specificity (double underscore) while in previously published reports it binds in a generic region of the hAlu repeat, resulting in reduced specificity (C).

Figure 2. Representative examples of real-time qPCR amplification curves demonstrating the specificity of hAlu primers used in SYBR Green and primer-probe assays. PCR reactions containing templates of 100 ng hDNA with 100 ng mDNA (positive control) or 200 ng mDNA (negative control) were assayed in SYBR Green (A) or primer-probe qPCR assays (B). DNA amplification was clearly detected in samples containing hDNA (red amplification line in A; purple amplification line in B) with the no template control background amplification observed in PCR samples containing only mDNA (yellow amplification line in A; pink amplification line in B). A notable reduction in background signal, visualized as a shift to the right of the negative control amplification curves, was observed when using primer-probe assays as compared to SYBR green assays.

Figure 3: The linear range and comparative efficiency of primer-probe based qPCR reactions. The efficiency of two primer-probe combinations were compared by amplifying hDNA standard curves ranging from 200 ng - 0.0002 ng of hDNA per qPCR well. The amplification plots of the in-house custom primer-probe (A) and the state-of-the-art McBride *et al.* primer-probe combination (B) indicate a positive signal in both assays in the presence of hDNA with an approximate 3.3 Ct reduction with a 10-fold decrease in DNA concentration. Standard curves derived from the mean Ct values in (A) and (B) were plotted against the $\log_{10}$ hDNA concentration (C), presented in tabular format in D, to calculate a comparable 98% efficacy

of the state- of-the-art *McBride et al*. primer-probe combination (red dot plot) or 99% with the in house primer-probe (blue dot plot) combination.

Figure 1

A

GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGTGGATCACCTGAGGTC
AGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCCGGGCGT
GGTGGCGCGCGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTGG
AGG**TTGCAGTGAGCCGAGATCGCGCCACTGCACT**CCAGCCTGGGCGACAGAGCGAGACTCCGTCTCA

B

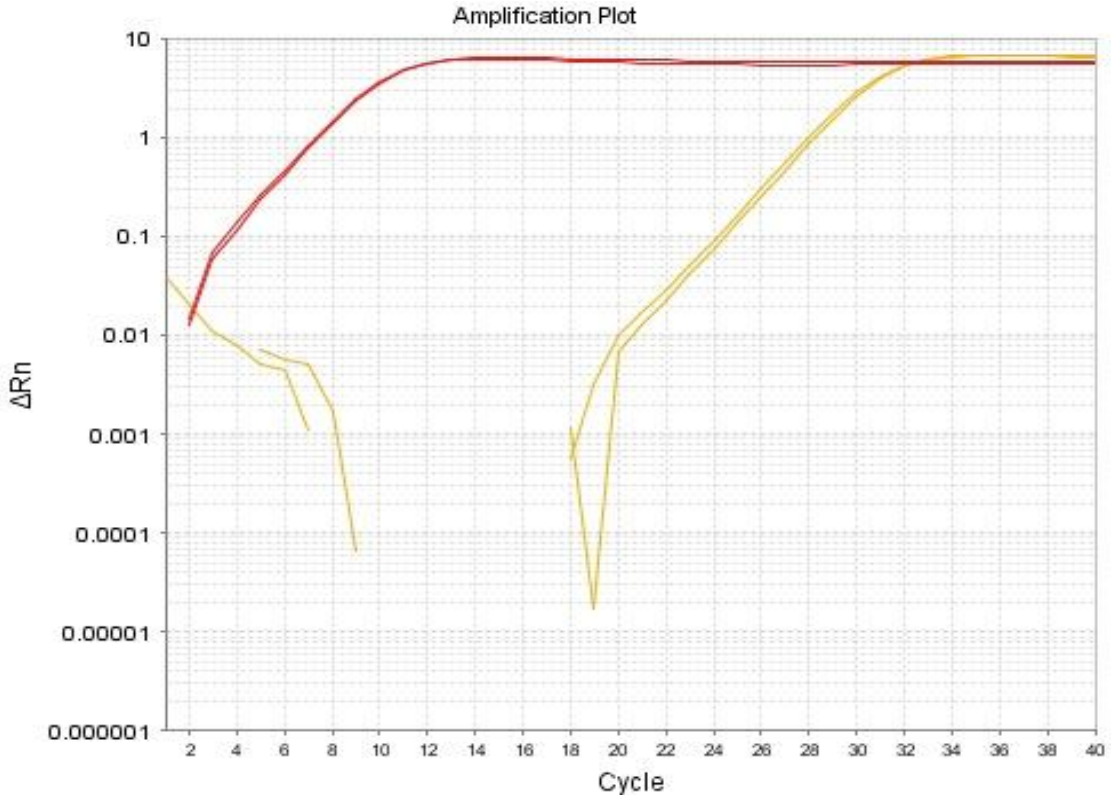
GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGTGGATCACCTGAGGTC
AGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCCGGGCG
TGGTGGCGCGCGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTG
GAGG**TTGCAGTGAGCCGAGATCGCGCCACTGCACT**CCAGCCTGGGCGACAGAGCGAGACTCCGTCTCA

C

GGCTGGGCGTGGTGGCTCACGCCTGTAATCCCAGCACTTTGGGAGGCCGAGGTGGGTGGATCACCTGAGGTC
AGGAGTTCAAGACCAGCCTGGCCAACATGGTGAAACCCCGTCTCTACTAAAAATACAAAAATTAGCCGGGCGT
GGTGGCGCGCGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATCGCTTGAACCCGGGAGGTGG
AGG**TTGCAGTGAGCCGAGATCGCGCCACTGCACT**CCAGCCTGGGCGACAGAGCGAGACTCCGTCTCA

Figure 2

A


Amplification Plot
ΔRn
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

B

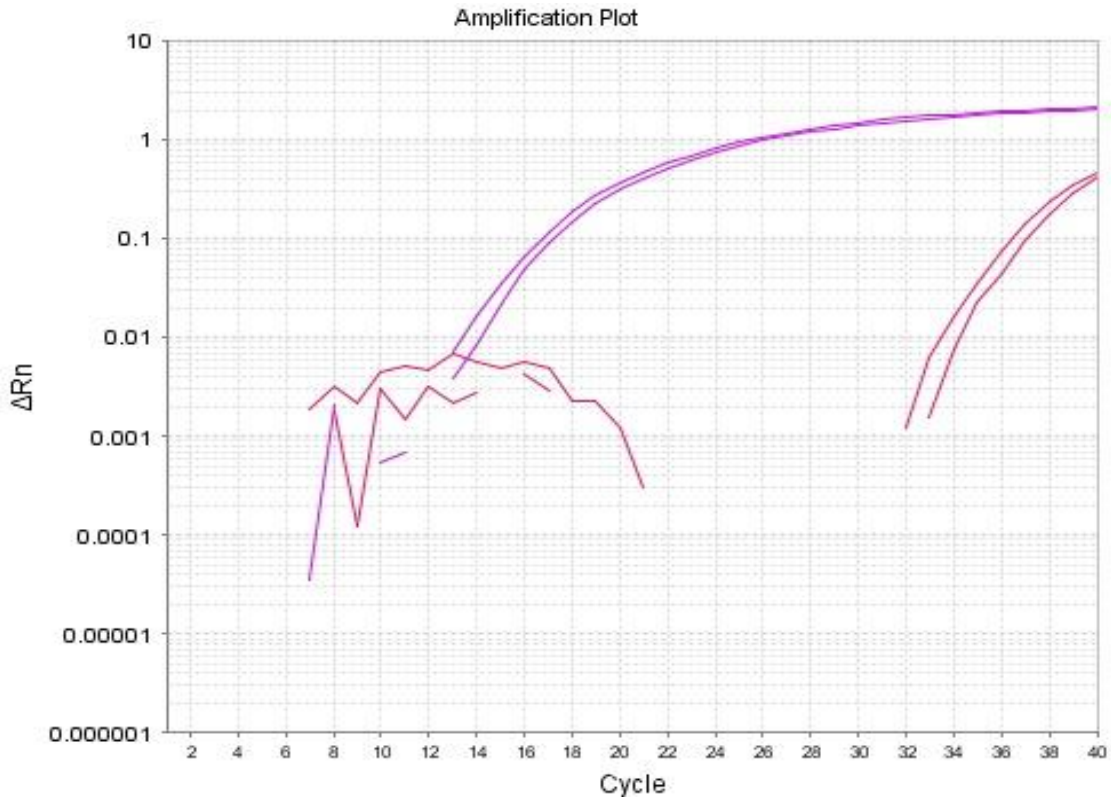
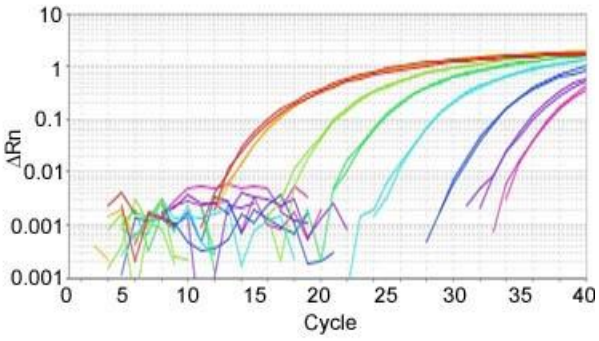
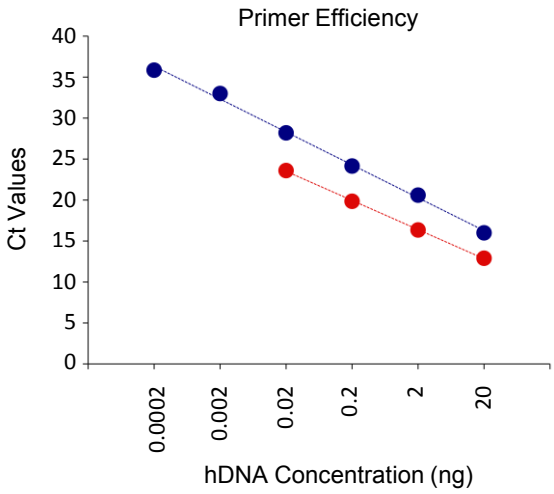

Amplification Plot
ΔRn
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Cycle

Figure 3

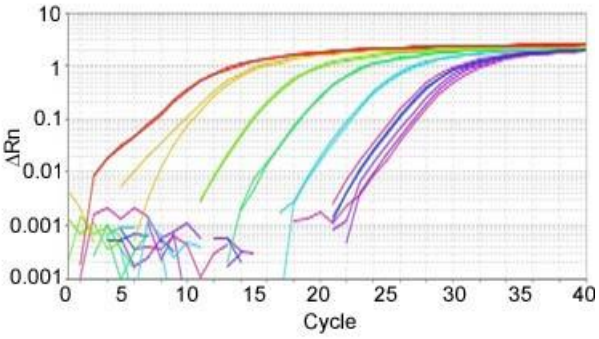
A



C



B



D

qPCR Standard Curve Ct Values

hDNA (ng)	Cellular Equivalent	Ct	McBride Ct
200	30303	15.40	12.45
20	3030	16.00	12.90
2	303	20.60	16.35
0.2	30	24.10	19.85
0.02	3	28.20	23.60
0.002	0.3	33.00	27.20
0.0002	0.03	35.80	28.10
0	0	37.60	27.70

1. Purpose

1.1. Preclinical biodistribution studies tracking the distribution of human mesenchymal stromal cells (hMSCs) after administration are a Health Products Regulatory Authority (HPRA) regulatory requirement prior to a clinical trial initiation. This SOP describes a method to detect the presence/absence of human genomic DNA (hDNA) and to quantify the level of hDNA in tissues of mice that have received intramuscular injections of hMSCs. The identification of hDNA is not intended to indicate the viability of the cell, but only the presence or absence of hDNA and the original cellular equivalent containing that quantity of hDNA.

Quantitative polymerase chain reaction (qPCR) is a method that uses fluorescent indicators to monitor the production of amplification products during each cycle of the PCR reaction. The accumulation of the fluorescent signal is measured at the exponential phase of the reaction to enable rapid and precise quantitation of the PCR product of interest. Here the presence of hDNA within murine tissues will be detected using qPCR, which is specific for the human Alu sequence.

This procedure uses qPCR to detect hDNA by amplifying the human Alu sequence in whole organ DNA extracts.

2. Scope

2.1. This procedure details how to isolate DNA from rodent or lapine tissues, quantify the amount of DNA and perform qPCR to detect the presence/absence of hDNA.

3. Nomenclature

µl	Microliters
Alu	Dimeric sequences derived from the 7SL RNA gene that are approximately 300 base pairs long. Alu insertional elements are one of the most abundant SINES (short interspersed elements) in the human genome.
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
g	Grams
hDNA	Human DNA
hMSCs	Human mesenchymal stem cells
HPRA	Health Products Regulatory Authority

mg	Milligrams
ml	Milliliters
ng	Nanograms
qPCR	Quantitative polymerase chain reaction
QC	Quality control
SOP	Standard operating procedure
Standard Curve	A graph that is created using known dilutions of human genomic DNA and is used for quantification.

4. Materials and Equipment

Materials	Manufacturer	Catalogue Number
Serological pipettes: 5 ml 10ml 25ml	 Sarstedt Sarstedt Sarstedt	 86.1253.001 86.1254.001 86.1685.001
50ml Centrifuge tubes	Sarstedt	62.547.254
Micropipette tips 10 µl	TipOne	S1120-3810
Micropipette tips 20 µl	TipOne	S112-1810
Micropipette tips 200 µl	TipOne	S1120-8810
Micropipette tips 1000 µl	TipOne	S1122-1830
Cell strainer, 100 µm yellow	Fisherbrand	22363549
10 ml syringe	BD Emerald	307736
Forward primer TGGTGGCTCTCTCCTGTAAT	Biosciences	Custom manufactured
Probe TGAGGCAGGAGAATCGCTTGAACC FAM-MGB	Biosciences	Custom manufactured
Reverse primer GATCTCGGCTCACTGCAAC	Biosciences	Custom manufactured
PCR Water	Bioline	BIO-37080
Ethanol 200 Proof	Sigma Aldrich	E7023-500ml
Microtube 1.5ml	Sarstedt	72.960.001
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml)	Applied Biosystems	4346906
ISOLATE II Genomic DNA Kit	Bioline	BIO-52067
MicroAMP Optical Adhesive Film	Applied Biosystems	4311971
Human DNA (200ng/µl)	Bioline	BIO-35025
Proteinase K 20mg/ml	Bioline	BIO37084
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen	P7589
96F Non Treated Black Microwell SI	Thermo Scientific/NUNC	37105
PCR Water	Bioline	BIO-37080
FG TaqMan Gene Expression Mastermix	Applied Biosystems	4369016
Tissue Culture Dish VENTS NUNCLON D SI	Thermo Scientific/NUNC	50288

Procedure

4.1. Record all equipment/materials used throughout this protocol in Forms 5-8.

4.2. Anytime a disposable plastic filter tip or syringe plunger comes in contact with a tissue sample, use it with that one and only tissue sample and dispose of it immediately upon use to avoid cross contamination between samples.

4.3. Wear gloves, goggles, hairnet, a lab coat and any other required personal protective equipment for all procedures to avoid contamination of samples with hDNA.

4.4. Complete tissue digestion

4.4.1. Remove buffer GL from the Isolate II kit and leave at room temperature.

4.4.2. Remove the proteinase K (20mg/ml) from the -20 degree Celsius (°C) freezer and store it in a 4 °C refrigerator until required for addition to the tissue lysate.

4.4.3. Pre-heat the oven/incubator to 55 +/- 5 °C and insert the temperature monitor.

4.4.4. Remove tissues from -80 °C freezer and place in bucket of ice such that the lid/cap is above the ice level to avoid contamination.

4.4.5. Remove the 25mm Nunclon tissue culture dish from the sterile packaging.

4.4.6. Label the Nunclon dish base with the organ name and animal number.

4.4.7. Transfer labeled 25mm dishes to the bench adjacent to the microbalance. Place the base or lid of one dish in the center of the balance.

4.4.8. Close microbalance doors and tare to 0.0000 g.

4.4.9. Remove labeled dish from the balance and place on the bench top. With a P1000 pipette and clean tip, remove the whole organ sample sample from the cryovial and place it in the 25mm dish.

4.4.10. Place the dish containing the tissue in the balance and record the weight of the tissue in g on Form 1.

4.4.11. Remove the 25mm dish from the balance, replace the lid and store the organ on ice.

4.4.12. Repeat steps 4.4.8 through 4.4.11 for each individual tissue.

4.4.13. On Form 1, with the use of Annex 1, convert the weight of the tissue from g to mg by multiplying by 1,000.

4.4.14. Using the chart in Annex 1, determine the amount of buffer GL and proteinase K that is required to digest each tissue. Record these volumes adjacent to the respective tissue in Form 1.

4.4.15. Label the lid and side of a sterile 50ml Sarstedt tube with the tissue name and animal ID number, creating a tube for each individual tissue.

4.4.16. Unwrap a sterile 100 µm Fisherbrand cell filter and place it directly in the open 50ml tube (in a rack), using a fresh filter for each tissue. Place one organ on the filter.

4.4.17. Using the plunger from a sterile BD Emerald 10ml syringe press the tissue onto the sterile cell filter.

4.4.18. Add the appropriate amount of buffer GL (according to Annex 1) to the top of the cell filter using a P1000 and/or P200 pipette and P1000/P200 tip, moistening the entire cell filter surface. Continue to press the moistened tissue through the filter.

4.4.19. Scrape the underside of the cell filter with the plunger to remove homogenized tissue. Rinse any adherent tissue from the plunger with the buffer GL flow-through using a P1000 pipette and tip.

4.4.20. Any small tissue clumps remaining in the top of the cell filter are scraped into the GL buffer flow through with a P1000 pipette and P1000 pipette tip.

4.4.21. Add the appropriate volume of proteinase K (according to Annex 1) to the homogenized tissue in buffer GL using a P200 pipette and P200 pipette tip.

4.4.22. Scrape any adherent tissue from the side of the 50ml tube into the GL buffer with a clean P1000 tip.

4.4.23. Place the homogenized tissue in a 50ml tube and leave it on the bench top on ice.

4.4.24. Repeat steps 4.4.16 through 4.4.23 for each remaining tissue.

4.4.25. Vortex the 50ml tubes vigorously at maximum speed for 15 seconds.

4.4.26. Place the 50ml tubes in a heat resistant rack and transport them to the pre-heated shaker/incubator.

4.4.27. Place the samples inside the incubator and rock at a frequency of 70 strokes per minute for 12-20 hours. Record the start time of the incubation on Form 1.

4.5. Genomic DNA Isolation from Digested Tissues

4.5.1. Pre-heat a small incubator to 70 °C.

4.5.2. Pre-heat the elution buffer to 70 °C within the small incubator in 4.5.1.

4.5.3. Remove the digested tissue from the shaker/incubator from step 4.4.27. Vortex the sample and ensure all tissue is fully digested. Record incubation end time on Form 2.

4.5.4. To each tissue digest, directly add the appropriate volume of G3 lysis buffer as determined in Annex 1, recording this volume in Form 2.

4.5.4.1. When using a new kit, G3 is created by combining G1 and G2 according to the manufacturer's instructions.

4.5.5. Vortex vigorously at maximum speed for 15 seconds.

4.5.6. Incubate the tissue digest at 70 °C for 10 minutes.

4.5.7. Remove the 50ml tube from the incubator.

4.5.8. Add the appropriate volume of ethanol as dictated in Annex 1, recording the volume added in document 2.

4.5.9. Vortex vigorously at maximum speed for 15 seconds.

4.5.10. Using a 5, 10 or 25 ml serological pipette as appropriate, measure the total volume of the contents of the tube and record in Form 2.

4.5.11. Calculate the volume of lysate containing 25mg of tissue.

4.5.11.1. Divide the total volume of tissue lysate (in ml) by tissue weight (in mg).

4.5.11.2. Multiply the product of 4.5.11.1 by 25 (mg) to determine the lysate volume (ml) equivalent to 25 mg of tissue.

4.5.11.3. Record this value in Form 2.

4.5.11.4. At this point the tissue lysates can be stored at -20 °C until the DNA isolation step. Record the freezer number and shelf number where the samples are stored in Form 2.

4.6. Isolation of Genomic DNA

4.6.1. If required, remove the tissue lysate tubes from the -20 °C and let them thaw over ice.

4.6.2. Label ISOLATE II spin column lid with sample name and animal number. Place it in a provided collection tube. Create one column/tube combination for each tissue sample.

4.6.3. Add the calculated lysate volume equivalent to 25mg of tissue (from Form 2) to each spin column and centrifuge in the Eppendorf microcentrifuge 54159 at 13000 rpm for 1 min.

4.6.4. Freeze the remaining tissue digest from step 4.6.1 at -20 °C. Record the freezer number and shelf number where samples are stored in Form 2.

4.6.5. Discard the flow through and recombine the spin column and collection tube.

4.6.6. Add 500 µl of GW1 wash buffer to each collection tube and spin at 13000 rpm for 1 min.

4.6.7. Discard the flow through and replace the spin column into the collection tube.

4.6.8. Add 600 µl of GW2 wash buffer and spin at 13000 rpm for 1 min.

4.6.8.1. When using a new kit, ethanol needs to be added to GW2 before use according to the manufacturer's instructions.

4.6.9. Discard the flow through and spin for 1 minute at 13000 rpm to remove excess ethanol.

4.6.10. Discard flow tube and place the spin column in a labeled 1.5ml Eppendorf tube.

4.6.11. Elute sample DNA by adding 50 µl of preheated elution buffer G (4.5.2) to the membrane followed by 3 min incubation at room temperature.

4.6.12. Centrifuge the spin column at 13000rpm for 1 min. Repeat step 4.6.11 on the same spin column with a fresh 50 µl elution buffer, combining this flow through with the flow through of step 4.6.11 to make 100 µl of eluted DNA total.

4.6.13. Freeze DNA at -20 °C until use or store on ice if proceeding to pico green analysis.

4.7. Pico Green Analysis of DNA Concentration

4.7.1. Remove the Quant-iT Pico green dsDNA assay kit from the -20 °C freezer and allow reagents to thaw on the bench top.

4.7.2. If the DNA samples from 4.6.13 are frozen, place them in an ice bucket and allow them to thaw. Lightly vortex the samples briefly before proceeding.

4.7.3. Dilute the 20x TE stock by removing 1ml of stock (with a P1000 pipette and pipette tip) and placing it in a 50ml Sarstedt tube. Add 19ml of deionized water, replace the cap, vortex and invert the solution several times to mix.

4.7.4. Dilute the Pico Green solution 200x in 1xTE prepared in step 4.7.3 to generate enough dye for 100 µl per well for all samples and standards. Once made, vortex it, then shield it from light. This solution must be made up fresh for each assay.

4.7.5. Dilute the dsDNA standard stock (contained in the Quant-iT PicoGreen dsDNA assay kit) 50 fold (example: 20 µl DNA stock to 980 µl 1x TE from 4.7.3) in a 1.5 ml Eppendorf tube. Vortex the closed tube briefly to mix the solution and store it on ice.

4.7.6. From the DNA standard solution prepare the 8 dsDNA standards according to table 1 below. Use fresh P20, P200 and P1000 pipettes and pipette tips as appropriate, creating each solution in a clean 1.5 ml Eppendorf tube. Vortex the closed tubes, then store them on ice until use.

4.7.6.1. Use the dsDNA stock created in 4.7.6 combined with the TE diluted in4.7.3 to create each standard.

DNA Stock (µl)	1x TE (µl)	Final DNA Concentration (ng/µl)
400	0	2
200	200	1
100	300	0.5
40	360	0.2
20	380	0.1
10	390	0.05
4	396	0.02
0	400	0

Table 1: Preparation of hDNA standards for the Pico Green assay

4.7.7. Label a fresh 1.5 ml Eppendorf tube for each digested tissue sample with the organ name and animal ID number.

4.7.7.1. Dilute each muscle, brain, heart or spleen tissue DNA sample (1:200) individually in the tube from 4.7.7 by combining 2 µl of sample with 398 µl of 1xTE from step 4.7.3. Vortex.

4.7.7.2. Dilute each lung, liver, kidney DNA sample (1:500) individually in the tube from 4.7.7 by combining 2 µl of sample with 998 µl of 1xTE from step 4.7.3. Vortex.

4.7.8. Place 100 µl of diluted standard (4.7.6) or sample (4.7.7.1 or 4.7.7.2) into each of two wells of a non-treated black 96 microwell plate according to the plate diagram in Annex 2.

4.7.9. Add 100 µl of Pico Green solution (4.7.4) to each standard and sample.

4.7.10. Incubate the plate at room temperature sheltered from light for 3 minutes.

4.7.11. Place the 96 well plate into the plate reader.

4.7.12. Select the protocol Fluorescein 485/535nm, 0.1 seconds ensuring the plate reader reads from the top of the well. Highlight the appropriate wells to be measured (labeled in Annex 2) and click save.

4.7.13. Initiate the plate reader to take measurements.

4.7.14. Export the resultant data in a Microsoft Excel sheet.

4.7.15. Using Excel, calculate the concentration of the DNA sample.

4.7.15.1. Average the duplicate values for each standard in the standard curve.

4.7.15.2. Plot the standard curve in an XY Scatter graph such that the ng/µl of DNA are along the X-axis and the fluorescence emission at 535nm is on the Y-axis.

4.7.15.3. Draw a line of best fit and determine the line equation. Ensure the R^2 value is greater than 0.98.

4.7.15.4. Average the duplicate values for each digested tissue DNA sample. Ensure the sample values fit within the standard curve. If not, repeat the entire assay, adjusting sample dilutions accordingly.

4.7.15.5. Using the line equation, calculate the DNA content (ng/µl) within each digested tissue sample well.

4.7.15.6. Multiply the value in 4.7.15.5 by the dilution factor (200 or 500 as appropriate in sections 4.7.7.1 and 4.7.7.2) to determine DNA concentration in the genomic DNA sample.

4.7.15.7. To determine the volume (µl) containing 100 ng of DNA, divide 100 by the DNA concentration from 4.7.15.5

4.8. qPCR Analysis

4.8.1. Remove from the freezer all genomic DNA samples, hDNA standards and the primer/probe mix. Allow them to thaw in an ice bucket. Remove from the refrigerator the Taqman master mix and store it on ice on the bench top.

4.8.2. Immediately before the qPCR assay, create a solution of primers/probe suspended in Taqman mastermix. Store the solution on ice for no longer than 2 hours.

4.8.2.1. For the number of assay well plus 2 additional wells, pipette 10 µl of Taqman mastermix into a clean Eppendorf tube (ex: for 10 samples, prepare a mix for 12 wells by pipetting 120 µl of mastermix) followed by 1 µl of primer/probe solution (ex: for 12 wells, add 12 µl of primer/probe mix to the 120 µl of mastermix).

4.8.2.2. Record the volumes used in Form 3.

4.8.3. Pipette 11 µl of the solution from step 4.8.2 into each standard or sample well of a 96 well qPCR plate

4.8.4. Into each standard well (columns 1 and 2, rows A-G), pipette 8 µl of PCR water and 1 µl of the appropriate standard.

4.8.4.1. To create qPCR standards of hDNA, combine the following as illustrated in table 2 in a fresh 1.5 ml Eppendorf tube. Store the standards at -20 °C thawing in an ice bucket before use.

Standard (ng/well)	hDNA	PCR-quality water
200	1 µl of Bioline stock DNA	0 µl
20	1 µl of Bioline stock DNA	9 µl
2	1 µl of Bioline stock DNA	99 µl
0.2	1 µl of Bioline stock DNA	999 µl
0.02	1 µl of the 0.2 ng/well stock	9 µl
0.002	1 µl of the 0.2ng/well stock	99 µl
0.0002	1 µl of the 0.2ng/well stock	999 µl
0	0 µl	1000 µl

Table 2: Preparation of hDNA standards for the qPCR reaction

4.8.5. Into each sample well, pipette 100 ng of organ DNA according to the calculations in step 4.7.15.7 as per Form 3.

4.8.6. Into each sample well, pipette the appropriate volume of PCR water according to the volume determined in Form 3.

4.8.7. Remove the adhesive backing from one piece of Microamp optical adhesive film and adhere the film to the top of the plate, avoiding fingerprints.

4.8.8. Conduct the qPCR assay according to the equipment manufacturer's instructions such that samples are incubated for 2 minutes at 50°C followed by 10 minutes at 95°C, then 40 cycling steps from 95°C for 15 seconds to 60°C for 1 minute.

4.9. **Data Analysis**

4.9.1. Using Excel, average the replicate data for each standard curve and sample value.

4.9.2. Plot the standard curve values on a XY Scatter graph such that the Predicted Number of human genomes (per well) is on the logarithmic X-axis and the average Ct value on the Y-axis.

4.9.2.1. Regularly when plotting the Standard Curve, the upper standard of 200ng is saturated and should be eliminated from the assay. Eliminate this point if there is less than a 3.33 Ct difference between the 200 ng standard and 20 ng standard.

4.9.2.2. If the lowest standard of 0.0002 ng is indistinguishable from the 0ng standard and should be eliminated. Eliminate this point if there are less than 1.5 Cts between the standard and the negative control well.

4.9.2.3. To determine the predicted number of human genomes per well based on DNA weight for each point on the standard curve, consult the table below.

hDNA Standard (ng)	Equivalent Number of Human Genomes Based on DNA
200	30,303
20	3,030
2	303
0.2	30
0.02	3
0.002	0.3
0.0002	0.03
0	0

Table 3: **QPCR standard curve values for ex vivo quantification of human genomes**

4.9.3. Draw the best fit line and determine the line equation.

4.9.4. Using the line equation, determine the number of human genomes detected in each PCR well (i.e.in each 100 ng DNA sample analyzed).

4.9.5. Calculate the total DNA (ng) isolated from each 25 mg tissue sample by multiplying the volume of elution buffer (4.6.12) used to extract the DNA from the column (usually 100 µl) by the DNA concentration of the extracted DNA (ng/µl) determined in 4.7.15.5.

4.9.6. Calculate the ng DNA isolated per organ by multiplying the total DNA in 25 mg of tissue (4.9.5), divided by 25, by the weight of the whole organ (4.4.13) in mg.

4.9.7. Determine the number of human genomes in a ng of genomic organ DNA by dividing 4.9.4 by 100.

4.9.8. Determine the number of human genomes in an organ by multiplying 4.9.7 by 4.9.6.

Form 1: Tissue Digestion Buffer Composition

Date: _____

Animal ID Number: _____

Tissue	Volume G3 (ml)	Volume Ethanol (ml)	Total Volume Digested Tissue (ml)	Volume of 25mg of Tissue (ml)
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

Start time of incubation _____

Recorded By: _____

Approved By: _____

Form 2: Tissue Digestion Buffer Composition

Date: _____

Animal ID Number: _____

Tissue	Volume G3 (ml)	Volume Ethanol (ml)	Total Volume Digested Tissue (ml)	Volume of 25mg of Tissue (ml)
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

End time of incubation _____

-20 °C Freezer number _____

-20 °C Freezer shelf number _____

Recorded By: _____

Approved By: _____

Form 3: Dilution of DNA sample for qPCR

Date: _____

Animal ID Number: _____

Tissue	Stock DNA Concentration (ng/µl)	Volume Containing 100 ng DNA (µl)	Volume of Water to Add to Reach 9 µl total	Initial Upon Addition To Well
Brain				
Lung				
Liver				
Heart				
Kidney				
Spleen				
Right Thigh				
Right Calf				

Recorded By: _____

Approved By: _____

Form 4 : Verification of the qPCR setup

Date: _____

Animal ID Number (s): _____

Experimental Name : _____

Notebook number/page of resultant data_____

Setup	Verification of qPCR Settings (Yes/No)
Experimental properties	
Plate Setup	
Run Method	
Reaction Setup	
Save	
Start Run	

Recorded By: _____

Approved By: _____

Form 5: Record of Lot Numbers for Assay Materials: Tissue Digest

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Cell strainer, 100 µm yellow	Fisherbrand		
10 ml syringe	BD Emerald		
50ml Centrifuge tubes	Sarstedt		
Ethanol 200 proof	Sigma Aldrich		
Proteinase K 20mg/ml	Bioline		
Isolate II genomic DNA kit	Bioline		
Tissue Culture Dish VENTS NUNCLON D SI	Thermo Scientific/NUNC		

Form 6: Record of Lot Numbers for Assay Materials: DNA Purification

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Ethanol 200 Proof	Sigma Aldrich		
Microtube 1.5ml	Sarstedt		
ISOLATE II Genomic DNA Kit	Bioline		

Form 7: Record of Lot Numbers for Assay Materials: Pico Green

Date: _____ Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Serological pipettes: 5 ml 10ml 25ml	Sarstedt		
50ml Centrifuge tubes	Sarstedt		
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Microtube 1.5ml	Sarstedt		
Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen		
96F Non Treated Black microwell SI	Thermo Scientific/NUNC		

Form 8: Record of Lot Numbers for Assay Materials: qPCR

Date: _____

Animal ID Number (s): _____

Materials	Manufacturer	Lot number	Initials
Micropipette tips 10 µl	TipOne		
Micropipette tips 20 µl	TipOne		
Micropipette tips 200 µl	TipOne		
Micropipette tips 1000 µl	TipOne		
Taqman primer/probe solution	Bioline		
PCR Water	Bioline		
Microtube 1.5ml	Sarstedt		
MicroAmp Fast Optical 96-Well Reaction Plate with Barcode (0.1ml)	Applied Biosystems		
MicroAMP Optical Adhesive Film	Applied Biosystems		
Human DNA standard	Bioline		
PCR Water	Bioline		
Taqman mastermix	Applied Biosystems		

Annex 1: DNA Isolation Calculations

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
0.10	100.00	0.72	100.00	0.80	0.84
0.11	110.00	0.79	110.00	0.88	0.92
0.12	120.00	0.86	120.00	0.96	1.01
0.13	130.00	0.94	130.00	1.04	1.09
0.14	140.00	1.01	140.00	1.12	1.18
0.15	150.00	1.08	150.00	1.20	1.26
0.16	160.00	1.15	160.00	1.28	1.34
0.17	170.00	1.22	170.00	1.36	1.43
0.18	180.00	1.30	180.00	1.44	1.51
0.19	190.00	1.37	190.00	1.52	1.60
0.20	200.00	1.44	200.00	1.60	1.68
0.21	210.00	1.51	210.00	1.68	1.76
0.22	220.00	1.58	220.00	1.76	1.85
0.23	230.00	1.66	230.00	1.84	1.93
0.24	240.00	1.73	240.00	1.92	2.02
0.25	250.00	1.80	250.00	2.00	2.10
0.26	260.00	1.87	260.00	2.08	2.18
0.27	270.00	1.94	270.00	2.16	2.27
0.28	280.00	2.02	280.00	2.24	2.35
0.29	290.00	2.09	290.00	2.32	2.44
0.30	300.00	2.16	300.00	2.40	2.52
0.31	310.00	2.23	310.00	2.48	2.60
0.32	320.00	2.30	320.00	2.56	2.69
0.33	330.00	2.38	330.00	2.64	2.77
0.34	340.00	2.45	340.00	2.72	2.86
0.35	350.00	2.52	350.00	2.80	2.94
0.36	360.00	2.59	360.00	2.88	3.02
0.37	370.00	2.66	370.00	2.96	3.11
0.38	380.00	2.74	380.00	3.04	3.19

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
0.39	390.00	2.81	390.00	3.12	3.28
0.40	400.00	2.88	400.00	3.20	3.36
0.41	410.00	2.95	410.00	3.28	3.44
0.42	420.00	3.02	420.00	3.36	3.53
0.43	430.00	3.10	430.00	3.44	3.61
0.44	440.00	3.17	440.00	3.52	3.70
0.45	450.00	3.24	450.00	3.60	3.78
0.46	460.00	3.31	460.00	3.68	3.86
0.47	470.00	3.38	470.00	3.76	3.95
0.48	480.00	3.46	480.00	3.84	4.03
0.49	490.00	3.53	490.00	3.92	4.12
0.50	500.00	3.60	500.00	4.00	4.20
0.51	510.00	3.67	510.00	4.08	4.28
0.52	520.00	3.74	520.00	4.16	4.37
0.53	530.00	3.82	530.00	4.24	4.45
0.54	540.00	3.89	540.00	4.32	4.54
0.55	550.00	3.96	550.00	4.40	4.62
0.56	560.00	4.03	560.00	4.48	4.70
0.57	570.00	4.10	570.00	4.56	4.79
0.58	580.00	4.18	580.00	4.64	4.87
0.59	590.00	4.25	590.00	4.72	4.96
0.60	600.00	4.32	600.00	4.80	5.04
0.61	610.00	4.39	610.00	4.88	5.12
0.62	620.00	4.46	620.00	4.96	5.21
0.63	630.00	4.54	630.00	5.04	5.29
0.64	640.00	4.61	640.00	5.12	5.38
0.65	650.00	4.68	650.00	5.20	5.46
0.66	660.00	4.75	660.00	5.28	5.54
0.67	670.00	4.82	670.00	5.36	5.63
0.68	680.00	4.90	680.00	5.44	5.71
0.69	690.00	4.97	690.00	5.52	5.80
0.70	700.00	5.04	700.00	5.60	5.88

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
0.71	710.00	5.11	710.00	5.68	5.96
0.72	720.00	5.18	720.00	5.76	6.05
0.73	730.00	5.26	730.00	5.84	6.13
0.74	740.00	5.33	740.00	5.92	6.22
0.75	750.00	5.40	750.00	6.00	6.30
0.76	760.00	5.47	760.00	6.08	6.38
0.77	770.00	5.54	770.00	6.16	6.47
0.78	780.00	5.62	780.00	6.24	6.55
0.79	790.00	5.69	790.00	6.32	6.64
0.80	800.00	5.76	800.00	6.40	6.72
0.81	810.00	5.83	810.00	6.48	6.80
0.82	820.00	5.90	820.00	6.56	6.89
0.83	830.00	5.98	830.00	6.64	6.97
0.84	840.00	6.05	840.00	6.72	7.06
0.85	850.00	6.12	850.00	6.80	7.14
0.86	860.00	6.19	860.00	6.88	7.22
0.87	870.00	6.26	870.00	6.96	7.31
0.88	880.00	6.34	880.00	7.04	7.39
0.89	890.00	6.41	890.00	7.12	7.48
0.90	900.00	6.48	900.00	7.20	7.56
0.91	910.00	6.55	910.00	7.28	7.64
0.92	920.00	6.62	920.00	7.36	7.73
0.93	930.00	6.70	930.00	7.44	7.81
0.94	940.00	6.77	940.00	7.52	7.90
0.95	950.00	6.84	950.00	7.60	7.98
0.96	960.00	6.91	960.00	7.68	8.06
0.97	970.00	6.98	970.00	7.76	8.15
0.98	980.00	7.06	980.00	7.84	8.23
0.99	990.00	7.13	990.00	7.92	8.32
1.00	1,000.00	7.20	1,000.00	8.00	8.40
1.01	1,010.00	7.27	1,010.00	8.08	8.48
1.02	1,020.00	7.34	1,020.00	8.16	8.57

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
1.03	1,030.00	7.42	1,030.00	8.24	8.65
1.04	1,040.00	7.49	1,040.00	8.32	8.74
1.05	1,050.00	7.56	1,050.00	8.40	8.82
1.06	1,060.00	7.63	1,060.00	8.48	8.90
1.07	1,070.00	7.70	1,070.00	8.56	8.99
1.08	1,080.00	7.78	1,080.00	8.64	9.07
1.09	1,090.00	7.85	1,090.00	8.72	9.16
1.10	1,100.00	7.92	1,100.00	8.80	9.24
1.11	1,110.00	7.99	1,110.00	8.88	9.32
1.12	1,120.00	8.06	1,120.00	8.96	9.41
1.13	1,130.00	8.14	1,130.00	9.04	9.49
1.14	1,140.00	8.21	1,140.00	9.12	9.58
1.15	1,150.00	8.28	1,150.00	9.20	9.66
1.16	1,160.00	8.35	1,160.00	9.28	9.74
1.17	1,170.00	8.42	1,170.00	9.36	9.83
1.18	1,180.00	8.50	1,180.00	9.44	9.91
1.19	1,190.00	8.57	1,190.00	9.52	10.00
1.20	1,200.00	8.64	1,200.00	9.60	10.08
1.21	1,210.00	8.71	1,210.00	9.68	10.16
1.22	1,220.00	8.78	1,220.00	9.76	10.25
1.23	1,230.00	8.86	1,230.00	9.84	10.33
1.24	1,240.00	8.93	1,240.00	9.92	10.42
1.25	1,250.00	9.00	1,250.00	10.00	10.50
1.26	1,260.00	9.07	1,260.00	10.08	10.58
1.27	1,270.00	9.14	1,270.00	10.16	10.67
1.28	1,280.00	9.22	1,280.00	10.24	10.75
1.29	1,290.00	9.29	1,290.00	10.32	10.84
1.30	1,300.00	9.36	1,300.00	10.40	10.92
1.31	1,310.00	9.43	1,310.00	10.48	11.00
1.32	1,320.00	9.50	1,320.00	10.56	11.09
1.33	1,330.00	9.58	1,330.00	10.64	11.17
1.34	1,340.00	9.65	1,340.00	10.72	11.26

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
1.35	1,350.00	9.72	1,350.00	10.80	11.34
1.36	1,360.00	9.79	1,360.00	10.88	11.42
1.37	1,370.00	9.86	1,370.00	10.96	11.51
1.38	1,380.00	9.94	1,380.00	11.04	11.59
1.39	1,390.00	10.01	1,390.00	11.12	11.68
1.40	1,400.00	10.08	1,400.00	11.20	11.76
1.41	1,410.00	10.15	1,410.00	11.28	11.84
1.42	1,420.00	10.22	1,420.00	11.36	11.93
1.43	1,430.00	10.30	1,430.00	11.44	12.01
1.44	1,440.00	10.37	1,440.00	11.52	12.10
1.45	1,450.00	10.44	1,450.00	11.60	12.18
1.46	1,460.00	10.51	1,460.00	11.68	12.26
1.47	1,470.00	10.58	1,470.00	11.76	12.35
1.48	1,480.00	10.66	1,480.00	11.84	12.43
1.49	1,490.00	10.73	1,490.00	11.92	12.52
1.50	1,500.00	10.80	1,500.00	12.00	12.60
1.51	1,510.00	10.87	1,510.00	12.08	12.68
1.52	1,520.00	10.94	1,520.00	12.16	12.77
1.53	1,530.00	11.02	1,530.00	12.24	12.85
1.54	1,540.00	11.09	1,540.00	12.32	12.94
1.55	1,550.00	11.16	1,550.00	12.40	13.02
1.56	1,560.00	11.23	1,560.00	12.48	13.10
1.57	1,570.00	11.30	1,570.00	12.56	13.19
1.58	1,580.00	11.38	1,580.00	12.64	13.27
1.59	1,590.00	11.45	1,590.00	12.72	13.36
1.60	1,600.00	11.52	1,600.00	12.80	13.44
1.61	1,610.00	11.59	1,610.00	12.88	13.52
1.62	1,620.00	11.66	1,620.00	12.96	13.61
1.63	1,630.00	11.74	1,630.00	13.04	13.69
1.64	1,640.00	11.81	1,640.00	13.12	13.78
1.65	1,650.00	11.88	1,650.00	13.20	13.86
1.66	1,660.00	11.95	1,660.00	13.28	13.94

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
1.67	1,670.00	12.02	1,670.00	13.36	14.03
1.68	1,680.00	12.10	1,680.00	13.44	14.11
1.69	1,690.00	12.17	1,690.00	13.52	14.20
1.70	1,700.00	12.24	1,700.00	13.60	14.28
1.71	1,710.00	12.31	1,710.00	13.68	14.36
1.72	1,720.00	12.38	1,720.00	13.76	14.45
1.73	1,730.00	12.46	1,730.00	13.84	14.53
1.74	1,740.00	12.53	1,740.00	13.92	14.62
1.75	1,750.00	12.60	1,750.00	14.00	14.70
1.76	1,760.00	12.67	1,760.00	14.08	14.78
1.77	1,770.00	12.74	1,770.00	14.16	14.87
1.78	1,780.00	12.82	1,780.00	14.24	14.95
1.79	1,790.00	12.89	1,790.00	14.32	15.04
1.80	1,800.00	12.96	1,800.00	14.40	15.12
1.81	1,810.00	13.03	1,810.00	14.48	15.20
1.82	1,820.00	13.10	1,820.00	14.56	15.29
1.83	1,830.00	13.18	1,830.00	14.64	15.37
1.84	1,840.00	13.25	1,840.00	14.72	15.46
1.85	1,850.00	13.32	1,850.00	14.80	15.54
1.86	1,860.00	13.39	1,860.00	14.88	15.62
1.87	1,870.00	13.46	1,870.00	14.96	15.71
1.88	1,880.00	13.54	1,880.00	15.04	15.79
1.89	1,890.00	13.61	1,890.00	15.12	15.88
1.90	1,900.00	13.68	1,900.00	15.20	15.96
1.91	1,910.00	13.75	1,910.00	15.28	16.04
1.92	1,920.00	13.82	1,920.00	15.36	16.13
1.93	1,930.00	13.90	1,930.00	15.44	16.21
1.94	1,940.00	13.97	1,940.00	15.52	16.30
1.95	1,950.00	14.04	1,950.00	15.60	16.38
1.96	1,960.00	14.11	1,960.00	15.68	16.46
1.97	1,970.00	14.18	1,970.00	15.76	16.55
1.98	1,980.00	14.26	1,980.00	15.84	16.63

Tissue Weight (g)	Tissue Weight (mg)	GL buffer (ml)	Proteinase K (µl)	G3 Buffer (ml)	Ethanol (ml)
1.99	1,990.00	14.33	1,990.00	15.92	16.72
2.00	2,000.00	14.40	2,000.00	16.00	16.80

Annex 2: Pico Green Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	2 ng/µl	2 ng/µl	Right Thigh 1	Right Calf 1	Brain 1	Heart 1	Lung 1	Liver 1	Kidney 1	Spleen 1		
B	1 ng/µl	1 ng/µl	Right Thigh 1	Right Calf 1	Brain 1	Heart 1	Lung 1	Liver 1	Kidney 1	Spleen 1		
C	0.5 ng/µl	0.5 ng/µl	Right Thigh 2	Right Calf 2	Brain 2	Heart 2	Lung 2	Liver 2	Kidney 2	Spleen 2		
D	0.2 ng/µl	0.2 ng/µl	Right Thigh 2	Right Calf 2	Brain 2	Heart 2	Lung 2	Liver 2	Kidney 2	Spleen 2		
E	0.1 ng/µl	0.1 ng//µl	Right Thigh 3	Right Calf 3	Brain 3	Heart 3	Lung 3	Liver 3	Kidney 3	Spleen 3		
F	0.05 ng/µl	0.05 ng/µl	Right Thigh 3	Right Calf 3	Brain 3	Heart 3	Lung 3	Liver 3	Kidney 3	Spleen 3		
G	0.02 ng/µl	0.02 ng/µl	Right Thigh 4	Right Calf 4	Brain 4	Heart 4	Lung 4	Liver 4	Kidney 4	Spleen 4		
H	0 ng	0 ng	Right Thigh 4	Right Calf 4	Brain 4	Heart 4	Lung 4	Liver 4	Kidney 4	Spleen 4		

Note: Within each tissue DNA sample, the number (1-4) represents replicates of each biologic sample. For example, A4-A12 and B4-B12 are technical replicates from the same animal (animal 1).

Annex 3: qPCR Assay Plate Setup

	1	2	3	4	5	6	7	8	9	10	11	12
A	200 ng Standard	200 ng Standard	Right thigh 1	Right calf 1	Heart 1	Lung 1	Brain 1	Liver 1	Kidney 1	Spleen 1		
B	20 ng Standard	20 ng Standard	Right thigh 1	Right calf 1	Heart 1	Lung 1	Brain 1	Liver 1	Kidney 1	Spleen 1		
C	2 ng Standard	2 ng Standard	Right thigh 2	Right calf 2	Heart 2	Lung 2	Brain 2	Liver 2	Kidney 2	Spleen 2		
D	0.2 ng Standard	0.2 ng Standard	Right thigh 2	Right calf 2	Heart 2	Lung 2	Brain 2	Liver 2	Kidney 2	Spleen 2		
E	0.02 ng Standard	0.02 ng Standard	Right thigh 3	Right calf 3	Heart 3	Lung 3	Brain 3	Liver 3	Kidney 3	Spleen 3		
F	0.002 ng Standard	0.002 ng Standard	Right thigh 3	Right calf 3	Heart 3	Lung 3	Brain 3	Liver 3	Kidney 3	Spleen 3		
G	0.0002 ng Standard	0.0002 ng Standard	Right thigh 4	Right calf 4	Heart 4	Lung 4	Brain 4	Liver 4	Kidney 4	Spleen 4		
H	0 ng Standard	0 ng Standard	Right thigh 4	Right calf 4	Heart 4	Lung 4	Brain 4	Liver 4	Kidney 4	Spleen 4		

Note: Within each tissue DNA sample, the number (1-4) represents replicates of each biologic sample. For example, A4-A12 and B4-B12 are technical replicates from the same animal (animal 1).