Application of droplet digital PCR to detect human Alu DNA elements in biodistribution assays in pre-clinical studies using rodents

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Abstract

Biodistribution and engraftment assays for administered human cells in animal organs must be performed to ensure the safety and efficacy of the human cells in preclinical studies. Quantitative real-time polymerase chain reaction (qRT-PCR) is currently the standard method used to detect *Alu* DNA elements. The recently developed droplet digital PCR (ddPCR) method has been adopted in many biological fields, in which the small quantities of DNA must be detected. In this study, we evaluated whether ddPCR could replace qRT-PCR in biodistribution research. We applied ddPCR to detect human *Alu* DNA elements using various concentrations of genomic DNA (gDNA) extracted from human mesenchymal stem cells spiked with 100 ng of rat gDNA. We found that ddPCR could specifically detect human DNA and exhibited linearity within a concentration range of 10 ag–100 pg/20 μ l. ddPCR was far more sensitive than qRT-PCR in measuring *Alu* elements, with a reliable lower limit of detection of 100 ag DNA/20 μ l reaction. Additionally, the copy numbers of *Alu* elements were quantified using ddPCR in an *ex vivo* study examining DNA samples extracted from mouse spleen and lymph nodes that had been spiked with human natural killer cells; no human DNA was detected using qRT-PCR in this assay. Thus, we established a ddPCR method for detecting human gDNA in bio-distribution assays of human cells in rodents in preclinical studies.

Introduction

In recent years, different types of *ex vivo* expanded autologous or allogenic cells, such as natural killer (NK) cells¹ and chimeric antigen receptor-engineered T-cells², have been applied clinically as a form of cell therapy. Mesenchymal stem cells (MSCs) in particular have been widely applied as a treatment module for regenerative medicine and chronic inflammatory diseases³. In pre-clinical studies, the bio-distribution, engraftment, and persistence of human cells within animal models in which the cells are implanted must be evaluated^{4,5}, as well as the safety and activity of the cells^{4,6}.

To evaluate the bio-distribution of administered MSCs in experimental animals, many *in vivo* and *ex vivo* methods have been developed⁷. Methods for the *ex vivo* detection of human cells in animal tissues include immunohistochemical staining and *in situ* hybridization; however, the most sensitive and accurate method is to detect human DNA markers within animal tissues such as *Alu* elements, primate-specific short interspersed elements⁸, for which quantitative real-time polymerase chain reaction (qRT-PCR) is the standard detection method^{5,9–11}.

Although qRT-PCR is a powerful technique that enables the accurate and sensitive quantification of specific DNA sequences, there are some technical disadvantages. For example, external standard calibration curves based on reference standard materials (RSMs), which are time-consuming to generate and maintain, must be generated to quantify qRT-PCR signals for each experiment. Additionally, the cycle threshold (Ct) value may vary depending on the structure of the RSM. The secondary structure of reference plasmid DNA can affect qRT-PCR titration assays. For example, the supercoiled conformation of circular plasmid DNA suppresses primer annealing to the DNA template, resulting in regression line shift and titer overestimation¹². Furthermore, the sensitivity of qRT-PCR is affected by various PCR inhibitors¹³.

Recently, a new highly sensitive DNA detection system, digital PCR (dPCR), has been developed¹⁴. In contrast to qRT-PCR, target DNA sequences are directly quantified in dPCR without the need to generate a standard curve using an RSM. This method is highly sensitive and precise, and is thus applied in many biological fields for the detection of rare DNA sequences such as microRNAs in clinical samples¹⁵, rare mutations in cell-free DNA samples¹⁶, chromosome abnormalities¹⁷, and loaded pathogens such as virus and bacteria¹⁸. However, dPCR has not yet been applied to identify *Alu* elements in biodistribution studies of human cells administered into experimental animals.

Droplet digital PCR (ddPCR)¹⁹ is a recently developed and commonly used dPCR method, in which the PCR reaction is performed in numerous nanoliter volume lipid droplets. In the present study, we evaluated the performance of ddPCR for identifying and quantifying *Alu* elements in biodistribution studies of human cells in experimental animals.

Results

Specificity First, we examined the specificity of ddPCR for the *Alu* element and evaluated its ability to differentiate between human DNA (hDNA) and matrix rodent DNA. We compared the ddPCR results of the NC and NTC groups to those of the experimental group, which contained 100 ng of human Wharton's jelly-derived MSC DNA and 100 ng of rat DNA per reaction mixture. A high *Alu* DNA copy number (1.8×10^4 copies/total reaction mixture) was detected in the experimental group, while negative results (0–0.03 copies/total reaction mixture) were obtained in both the NC and NTC groups (Fig. 1), indicating that no false-positive results were generated by ddPCR.

Detection limit Next, we determined the detection limit of the ddPCR method for identifying *Alu* elements. We calculated the dose range of hDNA in 20 μ l of reaction mixture containing 100 ng of rat DNA. A single copy of the human genome has an average mass of 6.46 pg²⁰ and contains approximately 1,090,000 copies of *Alu* elements²¹. Therefore, one *Alu* element copy weighs 5.9 attograms (ag), and 10 ag of hDNA contains 1.69 copies of *Alu* elements. Based on this calculation, hDNA was serially diluted and added to the ddPCR mixture at concentrations ranging from 10^{-1} to 10^4 ag/20 μ l. Each mixture also contained 100 ng of rat DNA. For the ddPCR mixtures containing 10^{-1} to 10^4 ag/20 μ l hDNA, 1.4, 9.2, 104, and 1024 copies of *Alu* elements were detected by ddPCR, respectively (Figure 2A). These values displayed linearity and strong correlation ($R^2 = 0.9974$). The *Alu* element copy number detected in the 10 ag/20 μ l concentration ddPCR mixture (1.4 copies/total reaction mixture) closely matches that calculated theoretically for the same hDNA concentration (1.69); this suggests that the copy number obtained via ddPCR is reliable. However, because the 1 and 10^{-1} ag/20 μ l mixtures measured as 1.4 and 1.2 copies/total reaction mixture, respectively, the limit of reliable values is thought to be 9.2 copies of 100 ag.

Linearity The results of biodistribution assays must be linear^{22,23}. As shown in Figure 2, the ddPCR results were linear within the range of 10^{-1} – 10^4 ag/20 μ l. To expand this range, we performed ddPCR using 10-fold dilutions of hDNA at concentrations between 1 fg/20 μ l and 10^2 ng/20 μ l (Fig. 3). The results were linear across concentrations from 1 fg/20 μ l to 10^2 pg/20 μ l ($R^2 = 0.9397$). Taken together, our results indicate that ddPCR exhibited linearity over a broad range of concentrations, from 10 ag to 100 pg/20 μ l, for the detection of *Alu* elements (Figures 2, 3).

Comparison with qRT-PCR data Next, we compared our ddPCR results with those obtained by qRT-PCR, the method currently recommended for biodistribution assays. We generated qRT-PCR Ct values for *Alu* elements amplification. Due to potential matrix effects driven by xenogeneic DNA, we performed the qRT-PCR reactions both in the absence (Fig. 4A) and presence (Fig. 4B) of rat DNA.

In the absence of rat DNA, the qRT-PCR Ct values were linear within an hDNA concentration range of 10 fg–100 pg/20 μ l (Fig. 4A); the Ct value at 10 fg/20 μ l was 30.95. In the presence of rat DNA, the Ct values were linear within a range of 10 fg–10 pg/20 μ l (Fig. 4B); the Ct value at 10 fg/20 μ l was 33.95, which was higher than that in the absence of rat DNA. This higher Ct value, by over 3, was observed at all concentrations of hDNA (Fig. 4C, Table 1), revealing that the spiked rat DNA exerted a matrix effect.

To determine whether rat DNA also exerted an inhibitory effect during ddPCR, ddPCR was performed using an hDNA concentration range of 10^1 – 10^4 fg DNA/20 μ l in the absence (Fig. 4D) and presence (Fig. 4E) of 100 ng rat DNA; the detected copy numbers for both sample types were similar.

Ex vivo application The results of our *in vitro* experiments suggested that ddPCR is an effective method to evaluate biodistribution. Therefore, we tested the ddPCR method *ex vivo* using SCID mice that were intravenously injected with human NK cells and sacrificed after 10 days. First, the spleen and inguinal nodes were harvested, paraffin-embedded, and immunostained for the hNK cell marker CD56. As shown in Figure 5 (left panel), a few CD56-positive cells were identified in both organs. We then performed ddPCR using 100 ng of tissue DNA. The *Alu* element copy numbers calculated by ddPCR were 636 and 128 copies/100 ng of gDNA for the spleen and lymph nodes, respectively (right panel). However, qRT-PCR failed to detect hDNA in either organ (data not shown).

Discussion

In this study, we explored the effectiveness of ddPCR for the detection of *Alu* elements in biodistribution studies, and for detecting small quantities of hDNA in animal tissues, which are requirements for pre-clinical studies. Our results indicated that ddPCR met the specificity and linearity requirements for DNA detection. Notably, the detection limit of ddPCR was significantly lower than that of qRT-PCR. We then applied ddPCR to detect hDNA in SCID mouse organs following intravascular injection with hNK cells and were able to detect *Alu* elements that were not detected by qRT-PCR.

Prior to ddPCR, the template DNA was digested with the restriction enzyme *MseI*, an endonuclease that recognizes the sequence 5'-TTAA-3' and cleaves between the two T residues²⁴. The theoretical probability of the TTAA sequence occurring throughout the human genome is 4^{-4} (1/256). Because the human genome size is approximately 6.32 Gb²⁰, the theoretical number of fragments yielded by *MseI* is 24,687,547, far exceeding the total number of *Alu* elements in the human genome, which is 1,090,000²¹. This suggests that *MseI* treatment caused the *Alu* elements to be distributed into the lipid droplets much more evenly, even when the hDNA concentrations within the samples were extremely low. Therefore, this improved the precision of the experiment.

Previous studies comparing ddPCR to qRT-PCR revealed that ddPCR was more precise and accurate, both within and between assays, as well as between different laboratories^{19,25-27}. Regarding the sensitivity and limit of detection, the results of previous studies were dependent

on the detection target and sample source, with some studies suggesting that ddPCR is less sensitive compared to qRT-PCR^{25,28}. However, in more recent studies, the sensitivity of ddPCR has been reported to be higher than that of qRT-PCR. In a study measuring *Bifidobacterium* and *Lactobacillus* 16S RNA gene copy numbers in breast milk, ddPCR was 10-fold more sensitive than qRT-PCR²⁹. Furthermore, ddPCR was 100-fold more sensitive than qRT-PCR when detecting the microRNA miR-29a in urine²⁷. In the present study, the limit of detection was between 9.2 copies at 100 ag and 1.4 copies at 10 at, which is 1000-fold lower than the 10 fg limit of detection obtained with qRT-PCR. This was similar to the previous finding that ddPCR is 1000-fold more sensitive than qRT-PCR when detecting FAdV-4 virus contaminants in various samples including in vaccines³⁰. Additionally, the Zika virus standard plasmid could be detected by ddPCR to a detection limit of 1 copy/ μ l, which was not detectable with qRT-PCR³¹.

In our *ex vivo* experiment, 31.8 and 6.4 copies of *A/u* elements per 5 ng DNA/ μ l were detected in DNA samples extracted from the spleen and lymph nodes of mice injected with human NK cells, respectively. This suggested that 3.77 ag ($31.8 \times 20 \times 6.46/1,090,000$) and 0.76 ag ($6.4 \times 20 \times 6.46/1,090,000$) of hDNA were present in the 100 ng DNA per PCR mixture for the spleen and lymph node samples, respectively. Given that the detection limit for qRT-PCR was 10 fg of hDNA, this explains why we were unable to detect hDNA using qRT-PCR in our *ex vivo* experiment; the NK cell hDNA observed in the tissue sections was diluted with mouse DNA to a concentration below the detection limit of qRT-PCR. Or, the qRT-PCR sensitivity in our laboratory might not be enough. Previously reported qRT-PCR sensitivities include 1 pg DNA/reaction³², 0.2 pg DNA³³, and 0.1–0.01 pg DNA¹⁰. The lowest reported detection limit is 0.01 pg⁹. Even with this level of sensitivity, hDNA could not be detected in our DNA samples extracted from the lymph nodes.

Currently, we do not have criteria to define how many human cells would be meaningfully interpreted as being present in animal organs in preclinical studies, and further studies are needed to develop such criteria.

In our qRT-PCR experiments, the Ct values were higher in the presence of rat DNA (Figure 4C). A similar phenomenon has been observed in the presence of a high amount of porcine DNA³². It is not currently clear why the presence of xenogeneic DNA reduces the amplification efficiency of hDNA. One possible explanation is the presence of DNA inhibitors in the PCR mixture; various substances can act as PCR inhibitors¹³. This phenomenon did not occur in the ddPCR experiments, which are not affected by PCR inhibitors^{35,36}. This is because reductions in signal driven by PCR inhibitors do not affect the binary positive/negative readings for each micro-well in ddPCR. This highlights another advantage of the ddPCR method compared to qRT-PCR.

Conclusion

In this study, we developed and validated a highly sensitive and specific ddPCR system to study hDNA biodistribution in rodent gDNA mixtures. ddPCR could potentially replace the present standard detection method, qRT-PCR, due to its higher sensitivity, lower sensitivity to PCR inhibitors, and the lack of requirements for RSMs.

Methods

Human mesenchymal stem cells (hMSCs) Wharton's jelly-derived MSCs were developed as previously described³⁷ and maintained in low-glucose DMEM (Hyclone, South Logan, UT, USA) supplemented with 10% fetal bovine serum (Hyclone), 100 U/mL penicillin, 100 μ g/mL streptomycin, and 0.25 μ g/mL amphotericin (Welgene Inc., Gyeongsan, Korea). The study protocol was approved by the Institutional Review Board of Seoul National University (SNU-J-1511-005-715).

Human NK cells Human NK (hNK) cells were kindly gifted by Chaok Yim (S Bio Co. Ltd.). The hNKs were immediately injected intravenously into 6–7-week-old NOG mice without any maintenance.

Animals Adult Sprague-Dawley rats were purchased from Koatech (Pyeongtaek, Korea). The animals were kept at the animal facility of Seoul National University College of Medicine under specific pathogen-free conditions. All animal experiments were approved by the IACUC of Seoul National University (SNU-151228-4-1). For rat DNA preparation, rats were anesthetized with Zoletil (Virbac, intraperitoneally, 30 mg/kg) and Rompun (Bayer Korea, intraperitoneal, 10 mg/kg). Following anesthesia, organs such as the liver, pancreas, testis, and brain were harvested and immediately subjected to DNA extraction as described below.

NOG mice (6–7 weeks old) were purchased from Koatech (Pyeongtaek). The animals were kept at the animal facility of the laboratory animal center at the Osong Medical Innovation Foundation (KBIO) under specific pathogen-free conditions. All animal experiments were approved by Osong Medical Innovation Foundation (KBIO-IACUC-2021-163-1). Twenty million hNK cells were injected intravenously into the NOG mice, which were sacrificed 10 days after injection.

DNA extraction DNA was extracted from twelve types of rodent organ. Genomic DNA (gDNA) was extracted using a DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. The gDNA concentration was determined using a Thermo Scientific NanoDrop One spectrophotometer (Thermo Scientific Inc., Waltham, USA). DNA samples were kept at -80°C until use.

qRT-PCR qRT-PCR to detect *Alu* elements as a human-specific marker was performed using a Thermo Scientific QuantStudio Real-Time System. The following primers were synthesized (Bioneer, Daejeon, Korea) and used in the qRT-PCR reaction: human *Alu*, YB8-ALU-S68: 5'-GTCAGGAGATCGAGACCATCCT-3' (position 68–90) and YB8-ALU-AS244: 5'-AGTGGCGCAATCTCGGC-3' (position 244–227)³⁸. All TaqMan assays were performed using the following PCR conditions: denaturation at 95°C for 5 min, followed by 40 cycles of denaturing at 95°C for 30 s and annealing at 60°C for 20 s. Reactions were performed in duplicate. We used PowerUp SYBR Green Master Mix (Applied Biosystems, Cat No a25741) as the fluorescent reporter system for qRT-PCR instead of the FAM-probe, which we used in ddPCR; a pilot study revealed that the results obtained using both fluorescent reporter systems for qRT-PCR were equivalent (data not shown).

ddPCR ddPCR was performed on a QX200 AutoDG Droplet Digital PCR System (Bio-Rad, Hercules, CA, USA). The same primers were used for ddPCR as those used for qRT-PCR. The following probe was used for ddPCR: YB8-ALU-167, 5'-FAM-AGCTACTCGGGAGGCTGAGGCAGGA-TAMRA-3' (position 167–192)³⁸. PCR was performed using the following amplification conditions: 10 min at 95°C , 40 cycles of 30 s at 95°C and 60 s at 56°C , and then 10 min at 98°C . Amplified DNA was stored at 4°C . Each ddPCR analysis batch contained a set of samples, negative template control (NTC), which contained mixture, probes, primers, and 100 ng of gDNA extracted from rat liver, and negative control (NC), which contained mixture, probes, primers, and diethylpyrocarbonate-treated water. DNA extracted from human cells was spiked into rat liver DNA. All DNA samples were treated with the restriction enzyme *MseI* (1 unit/20 μl) prior to droplet generation, which did not cut the primers, probe, or *Alu* sequence. This was to reduce the potential increase in mixture viscosity of the mixture caused by the large amount of DNA present (>100 ng DNA), which could influence the average volume of the droplets¹⁹. The PCR products were transferred to a QX200 Droplet Reader for analysis. The ddPCR data were analyzed using QX200 Droplet Reader control software QuantaSoft 1.7.4 (Bio-Rad, Hercules, CA, USA) and presented as copy number/ μl .

Histology Severe combined immunodeficient (SCID) mice were intravenously administered with 20 million hNK cells and then sacrificed 10 days post-injection. The spleen and inguinal lymph nodes were harvested, fixed in 4% paraformaldehyde solution, and embedded in paraffin. Tissue sections of 4 μl thickness were sliced, mounted on glass slides, and subjected to immunohistochemical staining to detect human CD56 (NK cell marker). Before staining, the sections were incubated in Antigen Retriever Citrate Buffer, pH 6.0 (Sigma, Catalog # C9999) for 20 min at 100°C . Non-specific antibody binding was blocked by incubating the sections with 15% normal goat serum (Gibco, Dublin, Ireland) in phosphate buffered saline for 1 h at room temperature. Sections were incubated with anti-human CD56 antibody (1:100 dilution, clone 123C3.D5, Cell Marque, Cat. No. 156M-85, Rocklin, CA, USA) overnight at 4°C , then with anti-mouse-HRP antibodies (1:200 dilution, Sigma, Cat. No. A9044-2ML) for 1 h at room temperature. The final staining step was performed using 3,3'-diaminobenzidine solution. Sections were counter-stained with hematoxylin and then mounted with Vector mounting medium (Vector Labs).

Data analysis DNA copy numbers detected by ddPCR were calculated using QuantaSoft version 1.7.4 (Bio-Rad, Hercules, CA, USA) software. Linear regression curves for the DNA concentrations measured by ddPCR and the Ct values obtained by qRT-PCR were calculated using GraphPad Prism Version 6 (GraphPad Prism Software Inc., San Diego, CA, USA).

Data availability

The data of the present study are available from the corresponding author on request.

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Declarations

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Author contributions

J.H.K.¹ and Y.-I. H. raised the idea and designed the study. J.H.K.² and C.H. J. discussed the study design. J.H.K.¹ collected and analyzed the data. All authors are involved in data analysis. Y.-I. H. wrote the manuscript. J.H.K.¹, J.H.K.², and C.H. J. edited the manuscript and approved the final version.

Competing interests

The authors declare that there is no competing interest.

Additional information

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Table

Table 1. Ct values of qRT-PCR in the presence and absence of rat DNA.

hDNA concentraion (fg)	without rat DNA	with rat DNA	increases
1	33.32	34.25	0.93
10	30.95	33.95	3.00
100	27.26	31.02	3.76
1,000	23.96	27.94	3.96
10,000	20.45	24.07	3.62
100,000	17.21	21.04	3.83
1,000,000	26.64	n.d.	n.d.

n.d. = not determined

Figures

Figure 1

Figure 1

A representative profile of the ddPCR reactions showing the specificity of the ddPCR method for *Alu* elements. NTC, negative template control (no human DNA and 100 ng of rat DNA); NC, negative control (no human DNA or rat DNA); PC, positive control (100 ng of human DNA and rat DNA). This experiment was repeated three times.

Figure 2

Figure 2

ddPCR reaction profiles at lower concentrations of hDNA. A representative reaction profile (A) and graph (B) are shown. NTC, negative template control (no human DNA and 100 ng of rat DNA); NC, negative control (no human DNA or rat DNA); hDNA, human DNA.

Figure 3

ddPCR reaction profiles at higher concentrations of hDNA. A representative reaction profile (A) and graph (B) are shown. NTC, negative template control (no human DNA and 100 ng of rat DNA); hDNA, human DNA.

log ₁₀ X fg	UC-MSC	+ pancreas
1	30.946	33.94955635
2	27.263	31.02162806
3	23.96	27.94381269
4	20.452	24.07140223
5	17.208	21.0404199

Figure 4

Figure 4

Comparison of the qRT-PCR and ddPCR methods. Human DNA extracted from umbilical cord-derived mesenchymal stem cells was subjected to qRT-PCR to detect *A/u* elements in the absence (A) or presence (B) of rat DNA. Plotted Ct values for each experiment (C). Similar experiments were performed using ddPCR to detect *A/u* elements in the absence (D) or presence (E) of rat DNA. The measured copy numbers for each experiment are plotted in (F). NTC, negative template control (no human DNA and 100 ng of rat DNA); hDNA, human DNA; rDNA, rat DNA.

Figure 5

Figure 5

Ex vivo detection of human natural killer (NK) cells in NOG mice 10 days after injection. Immunohistochemical staining for CD56 showed some residual cells in the spleen (left upper panel) and lymph node (left lower). ddPCR measured 39.5 and 11.1 copies of *Alu* elements per 5 ng of genomic DNA extracted from the spleen and lymph nodes, respectively.