Sensitive Detection of Human Cells in Murine Organ DNA by qPCR

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1 ABSTRACT

Cell therapy products have been broadly used for new generation of gene therapy treatments in various diseases. During assessment and safety profile generation, the biodistribution and cell fate is crucial for regulatory purposes. Multiple different human specific sequences have been used to detect human DNA in rodent (e.g hTERT). Human ALU (hALU) genomic repeated sequences are a perfect target as they are species specific and the number of repeats makes the detection sensitive and accurate.

Method: Using in-house designed primers and probe, we have developed and validated a sensitive quantitative polymerase chain reaction (qPCR) method to enable the detection of human DNA by detecting the hALU sequences in DNA extracted from mouse blood and 23 different tissues: brain, lungs, heart, liver, spleen, bone marrow, stomach, cecum, duodenum, ileum, jejunum, rectum, colon, prostate, testes, uterus, ovaries, tail, mammary glands, pancreas, draining lymph nodes, thymus, skeletal muscle from the thigh and kidneys. While validation criteria guidelines from regulatory agencies for gene therapy qPCR assay are incomplete, guidelines for bioanalytical validation are often referred to. Therefore, the following validation parameters were validated: quantification range, sensitivity, specificity, matrix interference, extraction recovery, stability of analyte in mouse blood (short and long term), stability of tissue homogenate, stability of extracted analyte in murine DNA as well as mouse strain equivalence (BALB/c, C57BL/6J, CD1, NSG and NCG).

Results: The quantification range of this assay was determined to have an upper limit (ULOQ) of 200,000 pg and a lower limit (LLOQ) of 12.8 pg with a limit of detection (LOD) of 1.6 pg of human gDNA per µg of murine gDNA which represent less than 1 human cell (0.3 cells). The validated assay was shown to be specific with no matrix interference from the mouse genome in any of the 23 organs tested. The extraction recovery of the test item was evaluated by spiking a known amount of hPBMC cells in organ homogenates of 5 different individuals. Extraction recovery ranged with an average efficiency between 7.4 to 75.4% with the most challenging organs being the blood (7.4%) and the thymus (12.5%). The stability was evaluated in blood over 193 days and across multiple freeze-thaw cycles. To demonstrate the stability of the test items in tissue, liver homogenates were used as a representative organ and spiked with hPBMC cells and stability was assessed over 39 days. All analysis of spiked blood and tissue homogenates showed a recovery compared to day 1 (D1) within 30%.

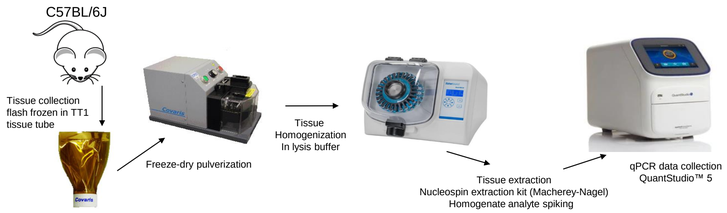
2 METHOD

1- Primers and probe were designed based on M.Creane *et al. (Cytotherapie, 2016)* to bind to L35531 ALU sequence. The reverse primer was designed in a conserved region (bold). Forward and reverse primers are double underlined and FAM labelled probe corresponds to the underline sequences.

CATGAGGTCAGGAGATCGAGACCATCCTGGCTAACAAGGTGAAACCCCGTCTCTACTAAAAATACAAAAAATTAG
CCGGGCGCGGTGGTGGGCATCTGTAGTCCCAGCTACTCGGGAGGCTGAGGCAGGAGAATGGTGTGAACCCGGG
AAGCGGAGCTTGCAGTGAGCCGAGATTGCGCCACTGCAGTCCGCAGTCTGGCCTGGGCGACAGAGCGAGACTCC
GTCTCAAAAAAAAAAAACAAAAAAAAACCATGCATGGTGCATCAGCAGCCCATGCCTCTGGCCAGGCATGGCGAG

2- Taqman Fast advance master mix was used to carry all qPCR reactions with the following cycle parameters: Polymerase activation 95°C for 120 seconds followed by 35 cycles of 95°C for 1 second (denaturation) and 60°C for 20 seconds (annealing/extension).

3- Tissue collection, DNA extraction, positive controls and DNA spiking: Tissue extraction was performed on pulverized flash frozen tissues. Extracted human PBMC genomic DNA was used to generate standard curves and positive controls. When needed, human PBMC cells (analyte) were used for spiking purposes. Blood and the following tissues were collected: brain, lungs, heart, liver, spleen, bone marrow, stomach, cecum, duodenum, ileum, jejunum, rectum, colon, prostate, testes, uterus, ovaries, tail, mammary glands, pancreas, draining lymph nodes, thymus, skeletal muscle from the thigh and kidneys.



4 DISCUSSION

The designed primers and probe used to detect hALU sequences were validated in 5 different mouse strains over 23 different organs. This assay was thoroughly validated using over 8 different parameters. One aspect of qPCR validation that is extremely difficult to assess because of variability between individuals and variability in most extraction methods, is the efficiency of extracting the test item from different tissues as well as the stability of the test item in those tissues. The combined results from extraction recovery, the stability in blood as well as the stability in spiked homogenates addresses these questions in a novel fashion. Our tissue processing also permits a better representativity of the whole organ as the freeze-dry pulverization homogenizes the tissue sections instead of extracting from one piece of the organ. The extremely low LOD of this assay enables the detection of less than one human cell (0.3 cells) per µg of mouse genomic DNA. To our knowledge, a qPCR assay to detect human cells over 23 different tissues in 5 different mouse strains has never been validated as extensively with this much sensitivity. With such specificity and sensitivity, our validated hALU assay is suitable for any human cell therapy product in a mouse study.

3 Validation Results

A. qPCR Standard Curve Fit in 5 Different Mouse Strains

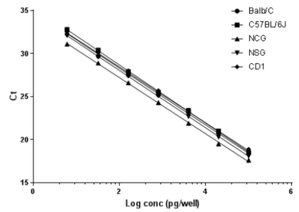


Figure 1. Standard curve was prepared with serial dilutions (1/5) of DNA extracted from hPBMC cells from 100,000 pg/well to 6.4pg/well (1 cell) in presence of 500ng of murine gDNA matrix. Genomic DNA was extracted from each strain listed and used as matrix (500ng/well). Standard curve in all matrices are within acceptance criteria. All strains are comparable except for NCG matrix background which seems slightly more efficient than other matrices. All other assays were preformed using Balb/C commercial mouse gDNA for standard and QC preparation.

B. Precision and Accuracy

Concentration (pg/well)	Intra-assay		Inter-assay	
	CV(%)	RE(%)	CV(%)	RE(%)
100,000.00	3.1 to 18.3	-6.2 to 22.3	13.5	3.3
75,000.00	0.5 to 17.7	-4.5 to 26.2	13.6	5.1
1,000.00	3.6 to 10.8	-3.3 to 19.2	8.9	4.7
65.00	3.5 to 10.2	-18.1 to 8.1	11.4	-6.4
6.40	2.6 to 20.0	-31.6 to 7.1	20.1	-10.8

Table 1. Precision and accuracy samples were prepared with dilutions of DNA extracted from hPBMC cells in 500ng of mouse matrix. Intra-assay CV(%) and RE(%) correspond to the spread over 6 runs performed by 2 different analysts. Inter-assay CV(%) and RE(%) correspond to the variation and error on the overall mean concentration of the same 6 runs.

C. Extraction Recovery (%), Specificity and Matrix Interference (qPCR Inhibition)

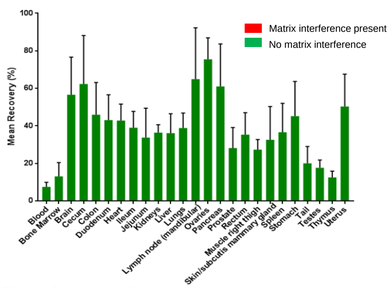


Figure 2. Human gDNA extraction mean recovery (%) per organ. Extraction recovery was performed using 5 different individuals for each organ. Specificity (data not shown) and matrix interference were tested on all individuals for each organ and assay was specific with no interference in all organs for all individuals.

D. Stability

Stability Conditions	Recovery (%) to D1
Extracted hgDNA 2-8°C (20h)	85
Extracted hgDNA -80°C (95days)	95
Extracted gDNA 3 freeze-thaw cycles	125
hPBMC cells in tissue homogenate (39 days)	126
hPBMC cells in blood (193 days)	128

Table 2. To generate extracted hgDNA for stability evaluation, tissue homogenates were spiked with hPBMC cells and total DNA was extracted. Extracted total DNA (human and murine) was used for stability assessment at 2-8°C, long term and over 3 freeze-thaw cycles. For homogenate and blood stability, hPMBC cells were spiked, stored at -80°C and total DNA was extracted on the day of stability analysis.

E. Lower Limit of Detection

	LLOQ/2	LLOQ/4	LLOQ/8
Target concentration (pg/well)	**3.20**	**1.60**	**0.80**
Mean Ct value Replicate 1	33.1	33.5	34.5
Mean Ct value Replicate 2	33.0	33.3	34.9
Mean Ct value Replicate 3	32.9	33.7	34.3
Mean Ct value Replicate 4	32.8	34.0	34.4
Mean Ct value Replicate 5	33.3	33.7	33.8
Mean Ct value Replicate 6	32.9	34.1	34.3
Mean Ct value Replicate 7	33.4	33.7	34.4
Mean Ct value Replicate 8	32.8	33.8	34.9
Overall mean Ct	***33.0***	***33.7***	***34.4***
Overall SD	0.2	0.3	0.3

Table 3. Lower limit of detection (LOD) is defined as the lowest concentration where 95% of the replicates are detectable. All replicates are loaded in triplicate. For our ALU qPCR assay the LOD is established at 0.8 pg/500ng which corresponds to 1.6pg/µg of total mouse gDNA or 0.3 human cells.

F. K_2EDTA vs K_3EDTA Anti-coagulant Use

The question of which anti-coagulant should be used for qPCR blood sample collection arose. In order to compare both, blood was collected in either K_2 or K_3-EDTA and the analyte was spiked in each. Extraction was performed and samples were assessed with our ALU qPCR assay. The recovery in K_2-EDTA was 29% and in K_3-EDTA was 38.8%. Considering that the difference between both anti-coagulant was of 11%, both anti-coagulant agent were considered acceptable for blood qPCR sample collection in the present assay.