Establishment of PALSAR method for quantification of human cells in mouse kidney.

SEKISUI

Masako Osawa, Funa Ogawa, Koichi Shibusawa and Akira Ideno

Analytical Technology Group for Drug Development Research & Development, Sekisui Medical Co., Ltd.,

E-mail：masako.osawa@sekisui.com

Background

qPCR and ddPCR are currently the most popular quantification methods for nonclinical biodistribution studies of mesenchymal stem cell (MSC) therapeutics. Advantage of the PCR-based method is high sensitivity and specificity. On the other hand, since the method usually require the extraction and purification steps to avoid matrix effects, it is necessary to calculate recovery rate during extraction steps for each tissue. Therefore, there is a demand for a quantification method directly measuring from homogenate with high sensitivity and specificity.

Purpose

We aimed to quantify the number of human cells directly in an animal tissue lysate by using PALSAR method without sample extraction.

Strategy

① Measurement of Alu elements

✓ Alu elements are primate-specific short interspersed elements (SINEs), containing over one million copies dispersed throughout the human genome.

✓ By using Alu-model sequence determined from 46 Alu subfamilies, we can measure Alu elements sensitively even by ELOSA-based assay.

Citing：Kodai Funakoshi et al."Highly sensitive and specific Alu-based quantification of human cells among rodent cells" Scientific Reports | 7: 13202 | DOI:10.1038/s41598-017-13402-3

② Fragmentation of genomic DNA by ultrasonicator

✓ By fragmentation of human cell genomic DNA (gDNA), probes and gDNA fragment are hybridized more effectively.

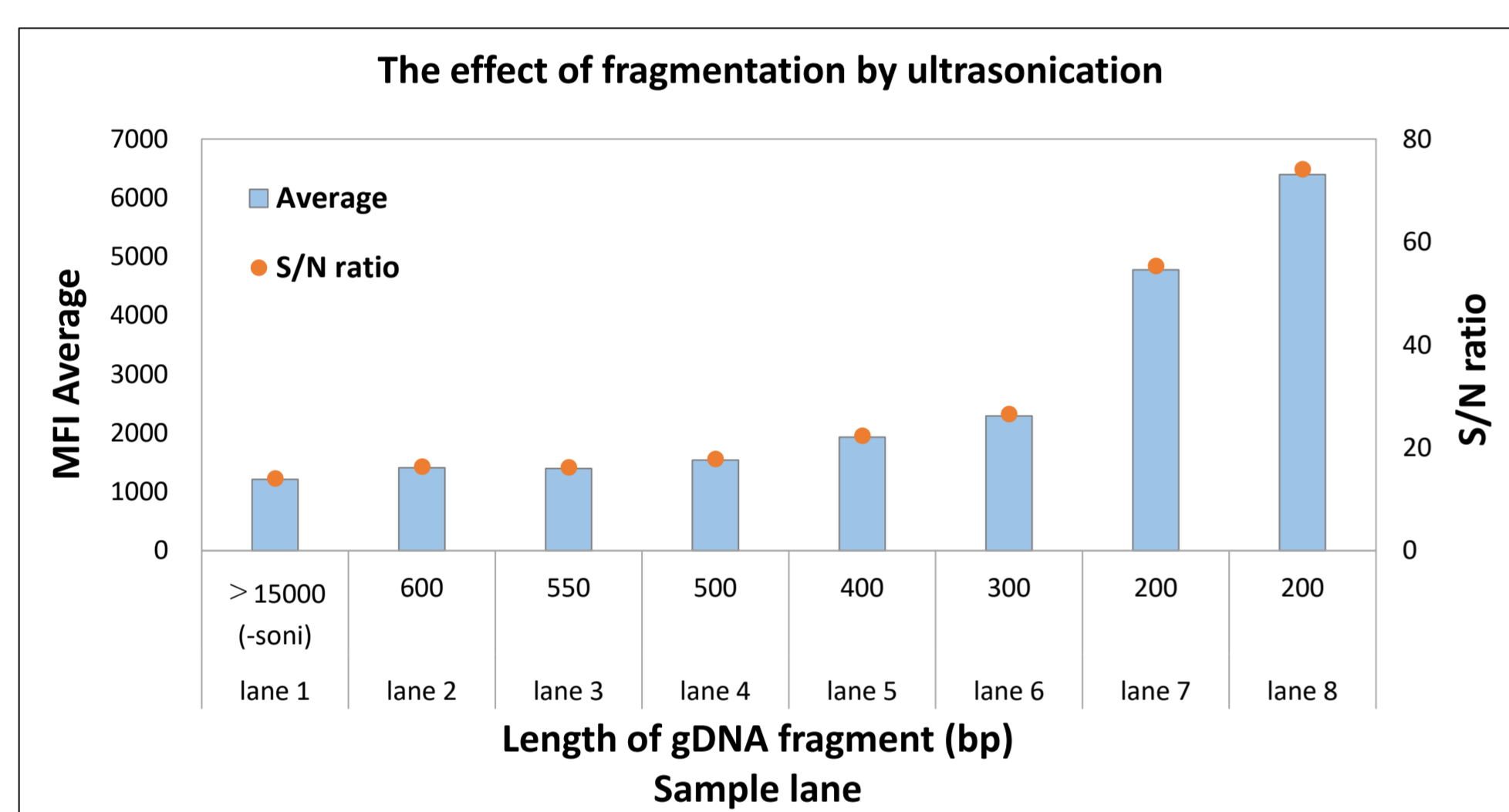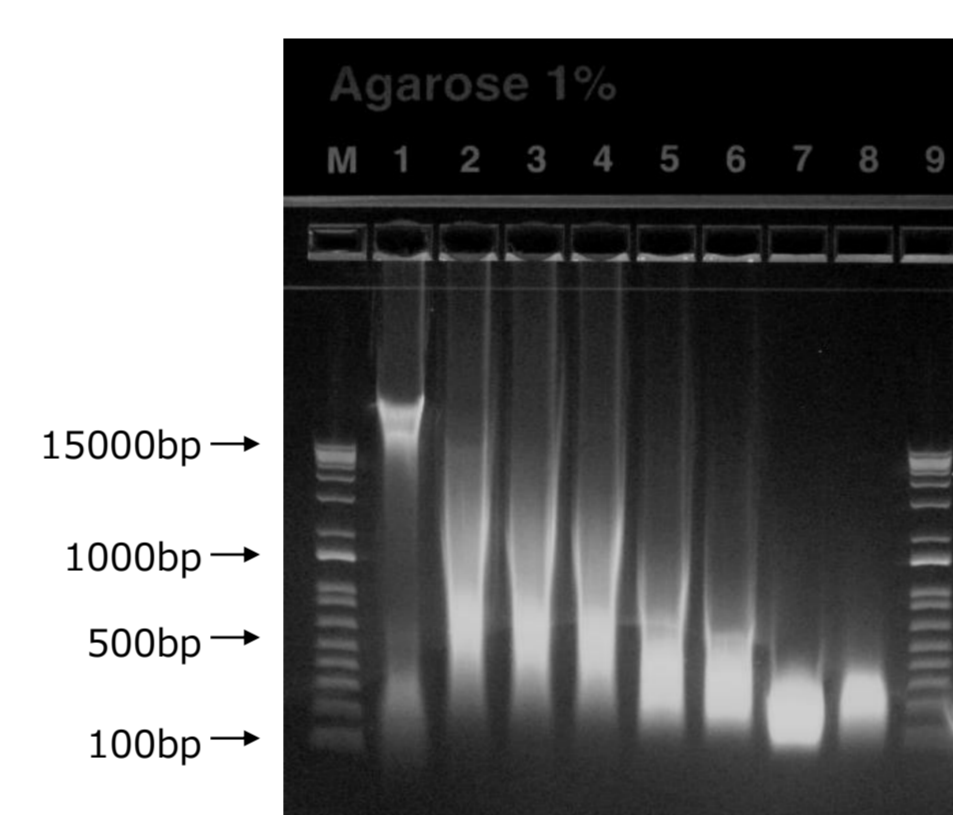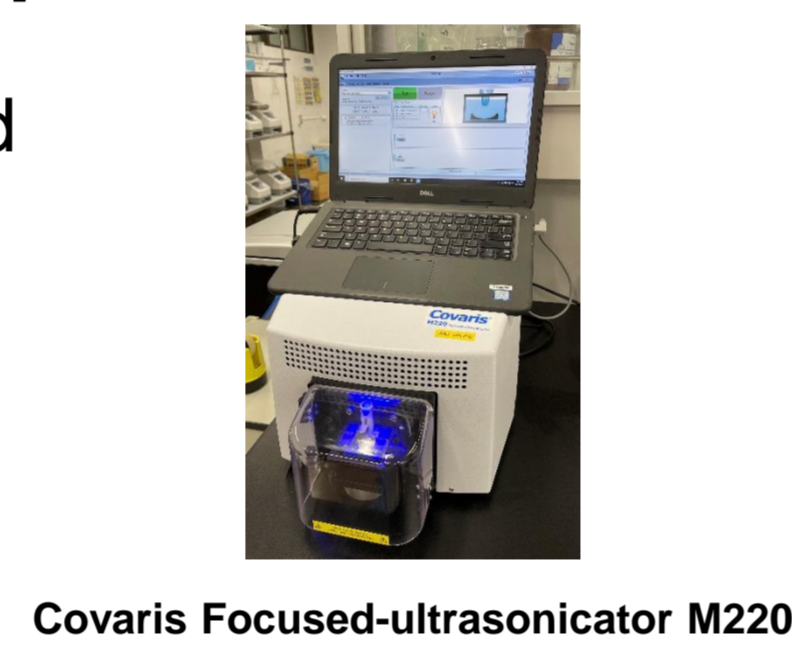✓ We use ultrasonicator, and gDNA is fragmented about 200 bp.

Covaris Focused-ultrasonicator M220

The effect of fragmentation by ultrasonication

Figure 1. Correlation between Median fluorescence intensity (MFI) and length of genomic DNA (gDNA) fragment

Materials & Methods

Materials

Human cell line：Human Embryonic Kidney cells 293 (HEK293 cell)

Animal line：Mouse (B6J)

Target sequence：Alu-model element

Methods

Step 1. Dissolution

Human cell HEK293 and mouse tissue were soaked in lysis buffer and the cells were then dissolved by heat treatment.

✓ Initial concentration of human cell：1,000,000 cells/mL lysis buffer
✓ Initial content of mouse tissue：200 mg/mL lysis buffer

Step 2. Preparation of cell lysate mixture

Human cell lysate and 0.01 % Tween solution, or mouse tissue lysate were mixed and we prepared cell lysate mixture. The final number of human cell was 50, 20, 10, 5, 2, 1 and 0.5 cells/reaction.

Step 3. Fragmentation

gDNA in cell lysate mixture were fragmented by ultrasonicator.

Step 4. Measurement Alu elements by PALSAR

Alu element was measured using PALSAR method, and the human cell number in the lysate was estimated.

1. Hybridization gDNA to capture probe and assist probe
2. PALSAR reaction
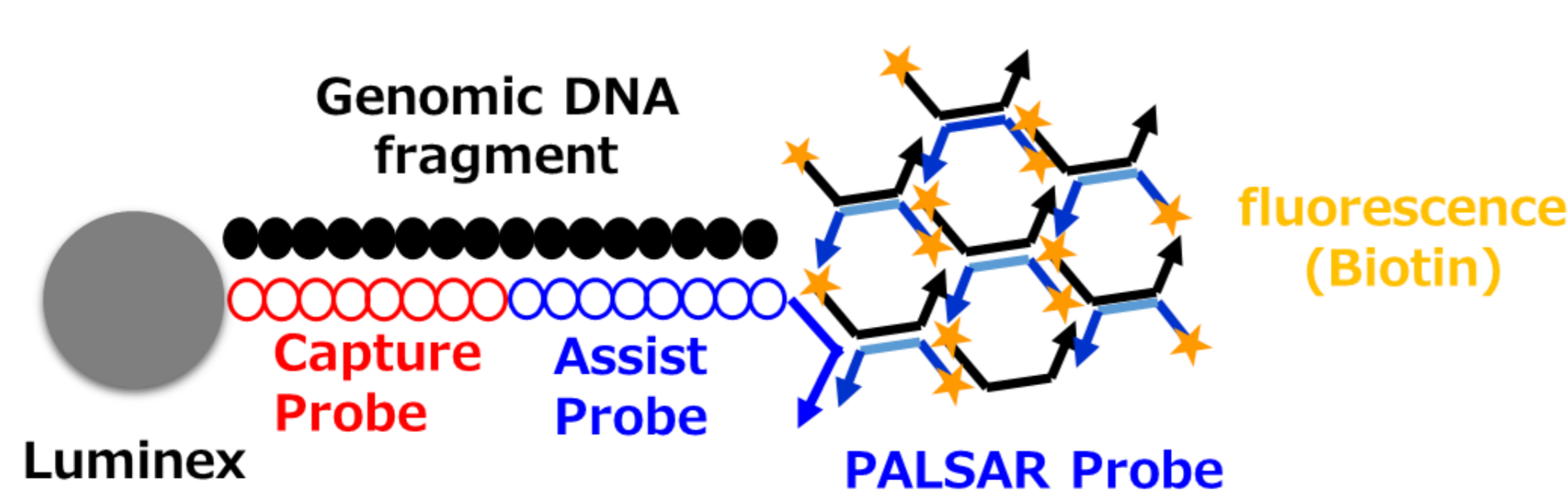3. Fluorescence detection by Luminex 100/200 system

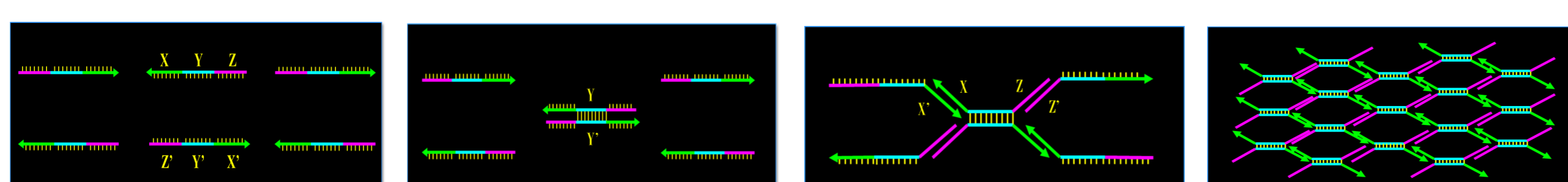Figure 2. The measurement model of Alu elements by PALSAR method

Figure 3. Schematic presentation of self-assembly formation with a pair of HCPs

Result

Assay validation

The quantitative range from 100 (LLOQ) to 10000 (ULOQ) cells/ mL was obtained in 0.01% Tween 20 solution. The five QC levels showed good precision, accuracy and total error within 10.3 %CV, ± 9.4 %RE and 13.0 %TE, respectively.

Nominal concentration (cells/rxn)	1st assay				2nd assay				3rd assay			
	Mean observed concentration (cells/rxn)	Coefficient of variation (%)	Relative error (%)	Total error (%)	Mean observed concentration (cells/rxn)	Coefficient of variation (%)	Relative error (%)	Total error (%)	Mean observed concentration (cells/rxn)	Coefficient of variation (%)	Relative error (%)	Total error (%)
0.50	0.54	5.6	7.4	13.0	0.48	8.4	-3.6	12.1	0.49	4.3	-1.2	5.5
1.00	1.06	4.1	5.8	9.9	1.01	2.7	1.3	4.1	0.97	5.1	-3.4	8.6
5.00	5.04	10.3	0.9	11.2	5.18	1.5	3.7	5.1	5.03	6.2	0.5	6.7
20.00	21.17	4.4	5.8	10.2	21.70	2.5	8.5	11.0	21.58	4.5	7.9	12.4
50.00	45.32	1.3	-9.4	10.7	46.07	4.6	-7.9	12.4	45.96	2.9	-8.1	10.9

Table1. inter- and intra-assay precision for quantification of dissolved HEK293 in 0.01% Tween 20 solution (Log-Log analysis)

Cross reactivity between human cell and mouse gDNA

After extracted HEK293 gDNA and mouse gDNA were mixed and fragmented, the human cell number in the mixture was estimated. The cross-reactivity between the genomes was also low at 0.77% in the presence of a 20,000 fold dose of the mouse genome.

Concentrasion of mouse gDNA (ng/µL)	HEK293 gDNA (ng/µL)	Ratio of HEK293 gDNA to mouse gDNA (%)	Mean observed concentration (ng/µL)	Coefficient of variation (%)	Relative error (%)	Cross-reactivity (%)
100	0.1	0.1	0.093	0.21	-6.6	0.93
	0.01	0.01	0.009	6.23	-8.9	0.84
	0.005	0.005	0.005	2.28	-8.6	0.77
	0	-	0.001	81.75	N.D**	-
10	0.1	1	0.110	2.25	10.0	N.D**
	0.01	0.1	0.011	6.90	9.1	N.D**
	0.005	0.01	0.005	2.31	4.4	N.D**
	0	-	N.D*	N.D**	N.D**	-
1	0.01	1	0.010	2.55	-1.7	N.D**
	0.005	0.5	0.005	0.34	-1.8	N.D**
	0	-	N.D*	N.D**	N.D**	-

Table2. Cross reactivity between HEK293 and mouse gDNA in 0.01% Tween 20 solution
N.D* ：Below the background
N.D** ：Not calculable

Demonstration of calibration curve

In mouse kidney and lung homogenates, the calibration curve demonstrated recovery within ± 10 %. In the different kidney content in lysate, the same quantitative range from 100 to 10000 cells/ mL was obtained.

	Concentration cells/mL	Concentration cells/g organ	Mean Signal	SD	CV (%)	Relative error (%)
100mg/mL kidney	100	1200	314.0	9.2	2.9	6.9
	200	2400	495.8	13.1	2.6	-8.1
	500	6000	1161.3	211.1	18.2	-5.3
	1000	12000	2380.0	65.8	2.8	3.2
	2000	24000	4560.0	253.9	5.6	5.0
	5000	60000	10264.5	230.5	2.2	3.5
	10000	120000	17514.3	49.1	0.3	-5.0
20mg/mL kidney	100	6000	328.5	4.9	1.5	-1.1
	200	12000	573.3	64.0	11.2	3.0
	500	30000	1226.5	78.5	6.4	1.4
	1000	60000	2058.0	0.7	0.0	-8.9
	2000	120000	4310.5	216.4	5.0	4.1
	5000	300000	9923.5	336.6	3.4	5.7
	10000	600000	16903.8	1130.3	6.7	-3.7
20mg/mL lung	100	6000	301.0	15.6	5.2	2.5
	200	12000	513.8	23.0	4.5	-3.3
	500	30000	1232.0	37.5	3.0	0.6
	1000	60000	2246.5	321.7	14.3	-4.3
	2000	120000	4646.0	9.9	0.2	5.1
	5000	300000	10316.0	425.7	4.1	2.4
	10000	600000	17927.8	498.9	2.8	-3.0

Table3. Calibration curve of the number of HEK293 in mouse kidney and lung homogenates

100mg/mL mouse kidney

$y = -0.0373x^2 + 1.2513x - 1.1321$
$R^2 = 0.9986$

20mg/mL mouse kidney

$y = -0.0212x^2 + 1.1325x - 1.5958$
$R^2 = 0.9991$

20mg/mL mouse lung

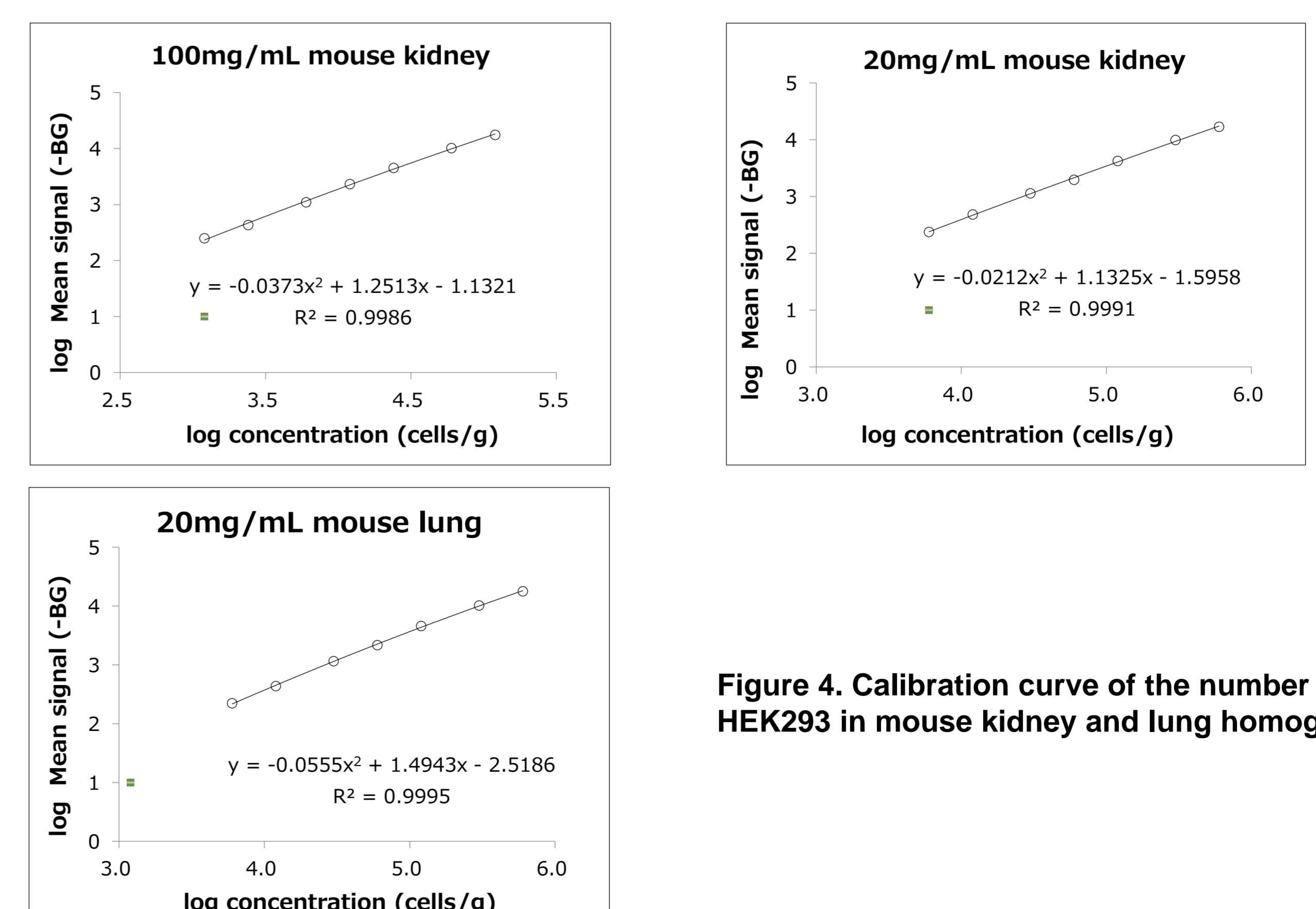$y = -0.0555x^2 + 1.4943x - 2.5186$
$R^2 = 0.9995$

Figure 4. Calibration curve of the number of HEK293 in mouse kidney and lung homogenates

Conclusion

The quantification of human cells in mouse kidney and lung homogenates was performed with high sensitivity and good reproducibility without extracting genomic DNA. PALSAR method is expected to appropriately evaluate the pharmacokinetics of MSC therapeutics and to contribute to drug discovery