39 Ultrasensitive quantitation assay of oligonucleotide therapeutics based on the signal amplification technology, "PALSAR method"

Akane Omori, Koichi Shibusawa, Kazuhiro Takeuchi, Akira Ideno
Tokai Research & Development Center, Research & Development, SEKISUI MEDICAL CO., LTD. 2117 Muramatsu, Tokai, Ibaraki 319-1182, Japan

Introduction

Recently, development of oligonucleotide therapeutics is accelerated in pharmaceutical industry, and the needs for their ultrasensitive quantitation methods are greatly increased. Conventional approaches, such as hybridization, LC-MS/MS, PCR are used in the analysis. Hybridization is commonly used, yet sensitivity is insufficient. Sensitivity of LC-MS/MS is improved by instrumental advancement, but still not enough. PCR and qRT-PCR are also known as sensitive detection methods; however, the high variability of measured values is a problem. To solve these problems, we aimed to develop an ultrasensitive hybridization assay method without nucleic acid amplification procedure.

PALSAR

PALSAR (Probe Alternation Link Self-Assembly Reaction) is a signal amplification technology, based on self-assembly of a DNA probe pair. PALSAR does not need DNA/RNA polymerases and ligases. In this study, we assayed by Luminex-PALSAR method, a combination of Luminex technology enabling multiplex detection (xMAP) and PALSAR.

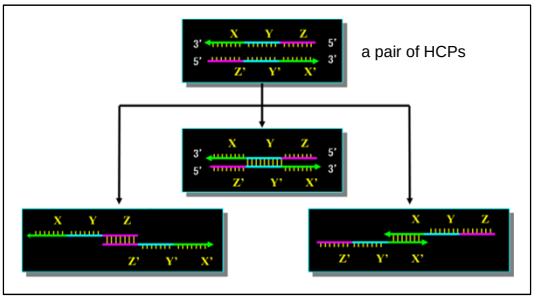


Figure 1. Principle of PALSAR

Honeycomb probes (HCPs) are DNA probes and have 3 regions, X, Y, Z for the one probe, and X', Y', Z' for the other probe. The regions X and X', Y and Y', and Z and Z' are complementary to each other, therefore three kinds of dimers will be formed.

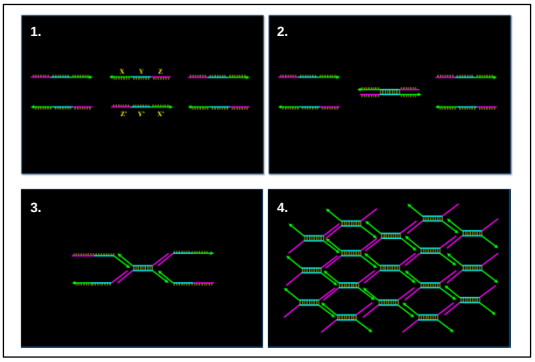
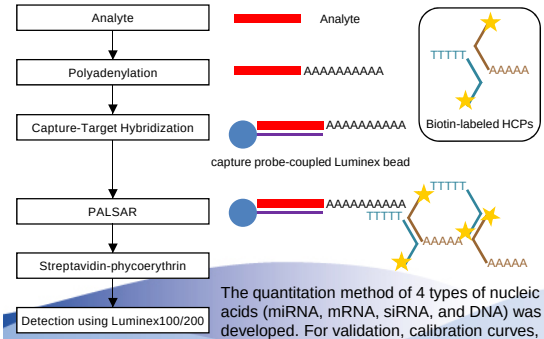


Figure 2. Illustrations of PALSAR

(1) A pair of HCPs is used for PALSAR. (2) One of the 3 regions in the probe is hybridized with the complementary region of the other probe. (3) The rest of the regions in the probes are hybridized. (4) A self-assembly is formed by PALSAR.

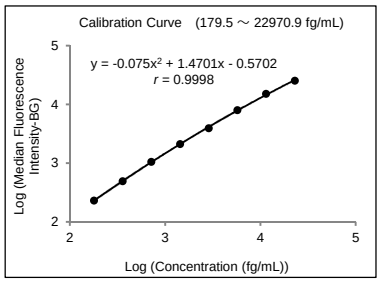
Workflow



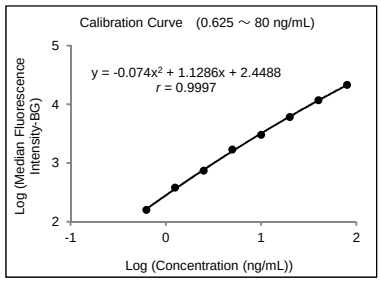
The quantitation method of 4 types of nucleic acids (miRNA, mRNA, siRNA, and DNA) was developed. For validation, calibration curves, intra-day reproducibility, and inter-day reproducibility were studied.

Results

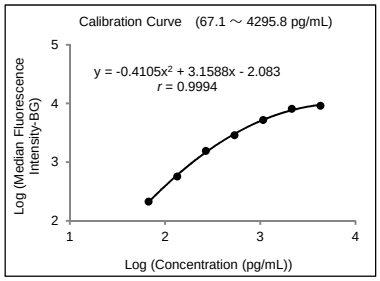
(A) miRNA



(B) mRNA



(C) siRNA



(D) DNA

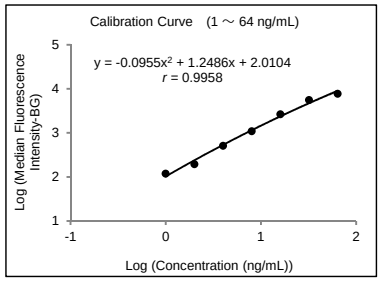


Figure 3. Representative calibration curves

(A) 179.5 to 22970.9 fg/mL of miRNA (22 bases) (custom-synthesized by Takara Bio, HPLC grade) was prepared with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). (B) 0.625 to 80 ng/mL of mRNA (1,380 bases) (purchased from NIPPON GENE) was prepared with nuclease-free water. (C) 67.1 to 4295.8 pg/mL of siRNA (19 bp with dTdT 3' overhang) (custom-synthesized by Nihon Gene Research Laboratories, HPLC grade) was prepared with nuclease-free water. (D) 1 to 64 ng/mL of phosphorothioated DNA (20 bp) (purchased from Gene Design) was prepared with nuclease-free water.

Table 1. Assessment of calibration curve of miRNA

Nominal concentration (fg/mL)	RE (%)						*r*					
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
179.5	3.9	4.8	-4.0	0.4	3.1	-1.5	0.9997	0.9995	0.9991	0.9998	0.9991	0.9997
358.9	-5.1	-5.0	5.2	-0.9	-0.2	4.3						
717.8	-2.1	-5.0	6.7	1.7	-2.0	0.1						
1435.7	1.8	5.2	-3.7	0.4	-8.8	-6.1						
2871.4	3.2	-4.2	-11.6	-4.8	-1.6	1.0						
5742.7	-3.0	5.7	7.8	1.9	13.7	-0.4						
11485.4	5.1	3.8	4.9	5.4	7.5	7.8						
22970.9	-3.1	-5.0	-2.8	-3.1	-9.3	-4.7						

A series of standard solutions for calibration curve was prepared as described in Figure 3. Assay of this study was performed for 6 days.

Table 2. Intra-day and inter-day reproducibility of miRNA

<Intra-Day Reproducibility>

n	Nominal concentration (fg/mL)				
	179.5	358.9	2871.4	11485.4	22970.9
	Observed concentration (fg/mL)				
1	209.2	379.6	2736.3	11656.4	22062.4
2	191.0	364.1	2845.3	13156.2	22195.7
3	196.6	323.4	2722.4	11493.5	22279.0
Mean	198.9	355.7	2768.0	12102.0	22179.0
SD	9.3	29.0	67.3	916.6	109.3
RE (%)	10.8	-0.9	-3.6	5.4	-3.4
CV (%)	4.7	8.2	2.4	7.6	0.5
TE (%)	15.5	9.1	6.0	13.0	3.9

<Inter-Day Reproducibility>

n=18	Nominal concentration (fg/mL)				
	179.5	358.9	2871.4	11485.4	22970.9
	Observed concentration (fg/mL)				
Mean	175.3	334.0	2677.8	11581.9	21833.5
SD	20.7	26.7	214.2	743.0	539.6
RE (%)	-2.3	-6.9	-6.7	0.8	-5.0
CV (%)	11.8	8.0	8.0	6.4	2.5
TE (%)	14.1	14.9	14.7	7.2	7.5

179.5 to 22970.9 fg/mL of miRNA was prepared for quality control samples. Assay of this study was performed in triplicate. For inter-day reproducibility assessment, assay was performed for 6 days.

Conclusions

Luminex-PALSAR method, which has been developed and evaluated in this study, is able to quantitate various types of nucleic acids with ultra-sensitivity. Calibration ranges of nucleic acids are verified as follows: 179.5 to 22970.9 fg/mL miRNA; 0.625 to 80 ng/mL mRNA; 67.1 to 4295.8 pg/mL siRNA; 1 to 64 ng/mL DNA. This method can be expected to be a powerful technique for ultrasensitive quantitation of oligonucleotide therapeutics.

Correspondence : Akane Omori
akane.omori@sekisui.com

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We have no financial relationship to disclose for our presentation contents.