APPLICATION NOTE

CRISPR-Cas9 Genome-editing efficiency measured accurately using fluorescently labeled single-stranded DNA for fragment analysis.

LabChip™ GX/GXII Touch™ Systems

Introduction

Nucleic acid metabolism, including DNA/RNA synthesis, ligation, repair, cleavage, and degradation, can be characterized by changes in nucleic acid size and structure. The analysis of these changes brought about by various enzymatic activities is fundamental to enzyme discovery, characterization, and engineering studies.

The LabChip™ GX Touch™ Nucleic Acid Analyzer and LabChip GXII Touch™ Protein Characterization System are well-established microfluidic systems for seamless nucleic acid analysis, providing quantitation, sizing (molecular weight) and purity (%) of RNA or DNA. Most commercially available assays for nucleic acid analysis utilize nonspecific intercalating dyes limiting the scope of nucleic acid metabolism analysis. For instance, the use of an intercalating dye cannot provide strand-specific information nor differentiate substrate strand from other nucleic acid species. The fluorescence emitted by the intercalating dye is a result of both length and quantity of oligonucleotides; peaks in electropherogram cannot directly reflect the molar ratio (or percentage) of analyzed fragments without calibration. In this application note, we present the LabChip fragment analysis assay which removes this limitation and enables further applications of the LabChip GX/GXII Touch systems. The principles of this approach can be applied to nearly any enzymatic system that acts on a fluorescently labeled oligonucleotide substrate.

In the LabChip fragment analysis assay, the intercalating dye is removed from the microfluidic gel matrix formulation. Single-stranded substrates of interest are fluorescently labelled at a single nucleotide (5′ end, 3′ end or internal nucleotide) by end users. Denaturing is performed for single-stranded nucleotide analysis to minimize the impact of secondary structures on fragment size distributions.

The size and quantity of substrates, intermediates, and products are automatically aligned and analyzed using fluorescently labeled single- stranded DNA (ssDNA) ladder (Fig. 1). The relative quantification (fragment percentage) is only associated with the copy number or molarity of nucleotide substrates avoiding size-related bias caused by intercalating dye.

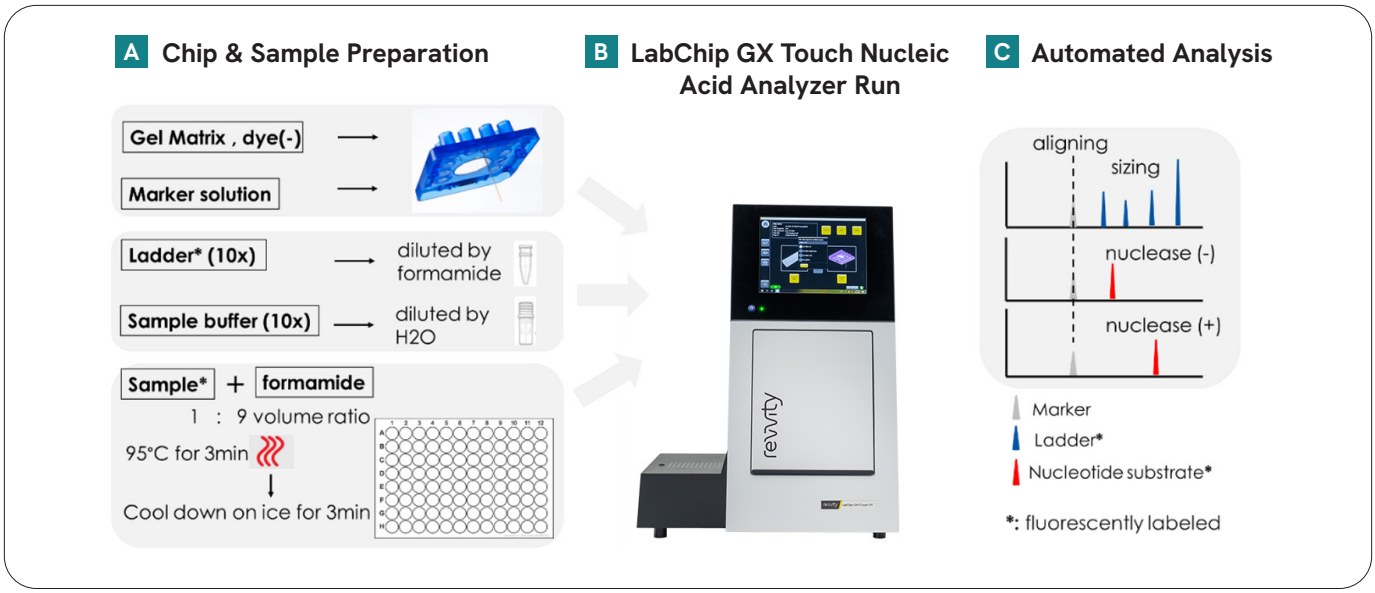

Figure 1: Workflow for LabChip fragment analysis. (A) Reagent preparation; (B) LabChip GXII Touch run; (C) Data analysis in LabChip GXII Reviewer software, Lower Marker in gel is used for aligning different samples.

This application note demonstrates how the LabChip fragment analysis assay can screen nucleases and single-guide RNAs (sgRNAs) with higher editing efficiency in *in vitro* CRISPR-Cas9 genome editing. Our product portfolio not only allows researchers the ability to optimize genome editing assay conditions but also offers a solution for high throughput sgRNA or engineered Cas9 variant screening and quality assessment of CRISRP-Cas system to industry experts (Fig. 2).

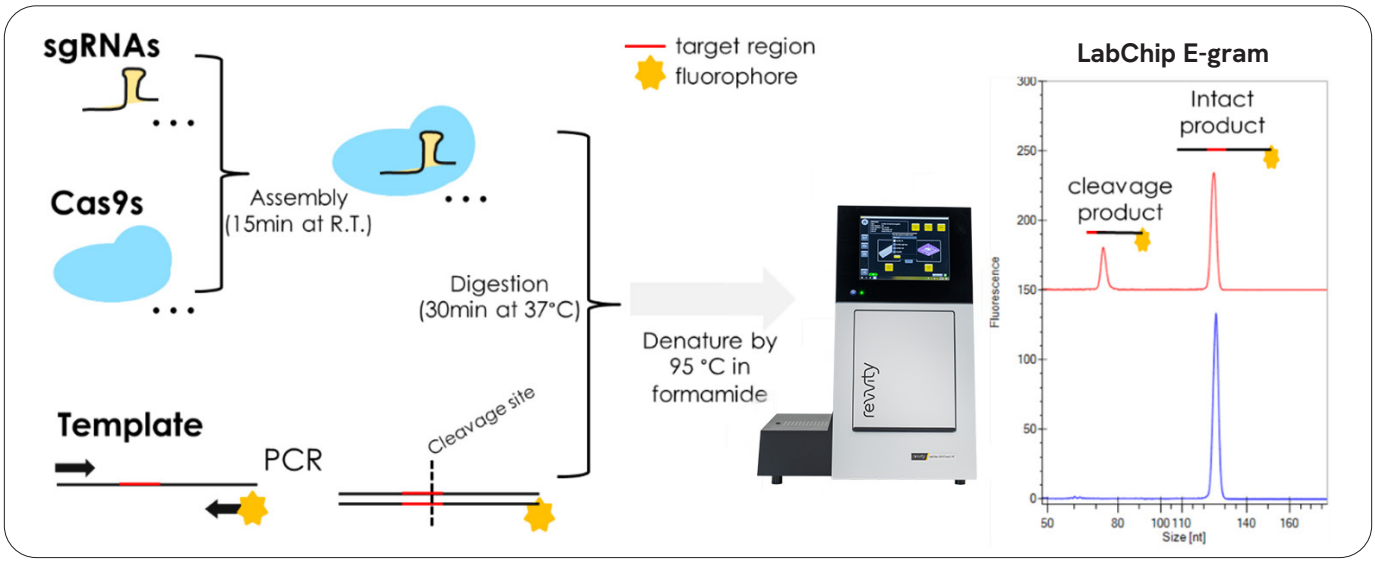

Figure 2: Workflow of determining efficiency of *in vitro* CRISPR-Cas9 genome editing using fragment analysis on LabChip GX/GXII Touch systems. R.T.: room temperature. e-gram: electropherogram.

Materials and methods

Primers including fluorescently labeled oligos, ssDNA template, Spy Cas9 enzyme (Revvity, cat# CAS12205) and sgRNAs (Revvity) (Table 1). The dsDNA fragment that contains the CRISPR target region is prepared by PCR. PCR with 500 nM of each primer, and 100 nM of template is conducted at 37°C/2 min, 94°C/10 min, 30 cycles (94°C/10 sec, 60°C/10 sec, 65°C/15 sec). Certain molar ratio of Spy cas9 and sgRNA is preassembled at room temperature (about 25°C) for 15 min in a 24 µL reaction volume.

6 µL PCR products are then directly added into preassembled ribonucleoprotein (RNP) complex and incubated at 37°C for another 30 min with final 100 mM NaCl, 50 mM Tris-HCl, 10 mM MgCl2, 100 µg/ mL BSA. The digestion products are mixed with Hi-Di™ formamide (ThermoFisher Scientific, cat# 4311320) by 1:9 ratio (i.e. 3 µL digestion product: 27 µL Hi-Di™ formamide), heated at 95°C for 3 min for denaturation, then immediately chilled on ice for another 3 min before analysis on LabChip GXII Touch system. Fluorescently labeled ladder and gel marker is used for peak sizing alignment and quantification.

Table 1: Sequences of primer, target DNA and sgRNAs

Name	Sequences (5′ – 3′)
Primer_F	CACAATCAAATGACACAGAC
Primer_R	/ATTO633/TCAGTGTTATTGTCTCATGA
DNA_template	CACAATCAAATGACACAGACTGTGTGTAGTGTGAAAGTTAATCGAACTGTTGGAATCTGCACGTTCAGCAT AGCTTGCATACAAGAGCGCTCATGAGACAATAACACTGA
SgRNA 1	gugaaaguuaaucgaacuguGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAA AGUGGCACCGAGUCGGUGCUUUU
SgRNA 2	gugaaaguuaaucgaacuguGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAA AGUGGCACCGAGUCGGUGCUUUU

Reverse primer is labeled with ATTO633. Cas9 target region is highlighted by green. Scaffold sequences in sgRNA are in capital. Red highlighted bases are modified by 2′O-methyl nucleotides and phosphorothioate linkages.

Results

CRISPR-Cas systems consist of two general components: an engineered nuclease with modular DNA or RNA-binding domain and a sgRNA functioning as a guide for the engineered nuclease. Here we demonstrate how we use the LabChip fragment analysis assay to find the optimal cleavage efficiency of these two components in genome editing.

Molar ratio of Cas9 and sgRNA affects cleavage efficiency

The RNA-guided Cas9 cuts both strands of DNA at a specific target sequence resulting in two fragments. On our single end fluorescently labeled DNA substrate, the cleavage efficiency (%) can be calculated by peak area of cleaved product (short size) divided by sum of peak areas of cleaved and intact product. We first evaluated whether adding more sgRNAs can enhance RNP complex genome editing. As shown in Figure 3, 270 nM : 500 nM of Cas9 and sgRNA significantly enhances genome editing increasing cleavage efficiency from 27% to 64% compared to 270 nM : 100 nM of Cas9 and sgRNA ratio.

Synthetic sgRNAs with different modifications and Cas9 nucleases from different vendors show different cleavage efficiency

We further quantitatively characterized synthetic sgRNAs with different modifications and Cas9s from different vendors. In sgRNA 1 and sgRNA 2, the CRISPR RNA (crRNA) sequence and scaffold RNA sequence are the same; sgRNA 1 has two 2′O-methyl nucleotides and phosphorothioate linkages in the backbone on both 5′ and 3′ end, while sgRNA 2 contains three such modifications on both 5′s and 3′ end (Table 1). Our data indicated that a >95% cleavage efficiency can be achieved by combining sgRNA 2 at 500 nM with Cas9 (Revvity), the optimal genome editing condition we have tested so far (Fig. 3).

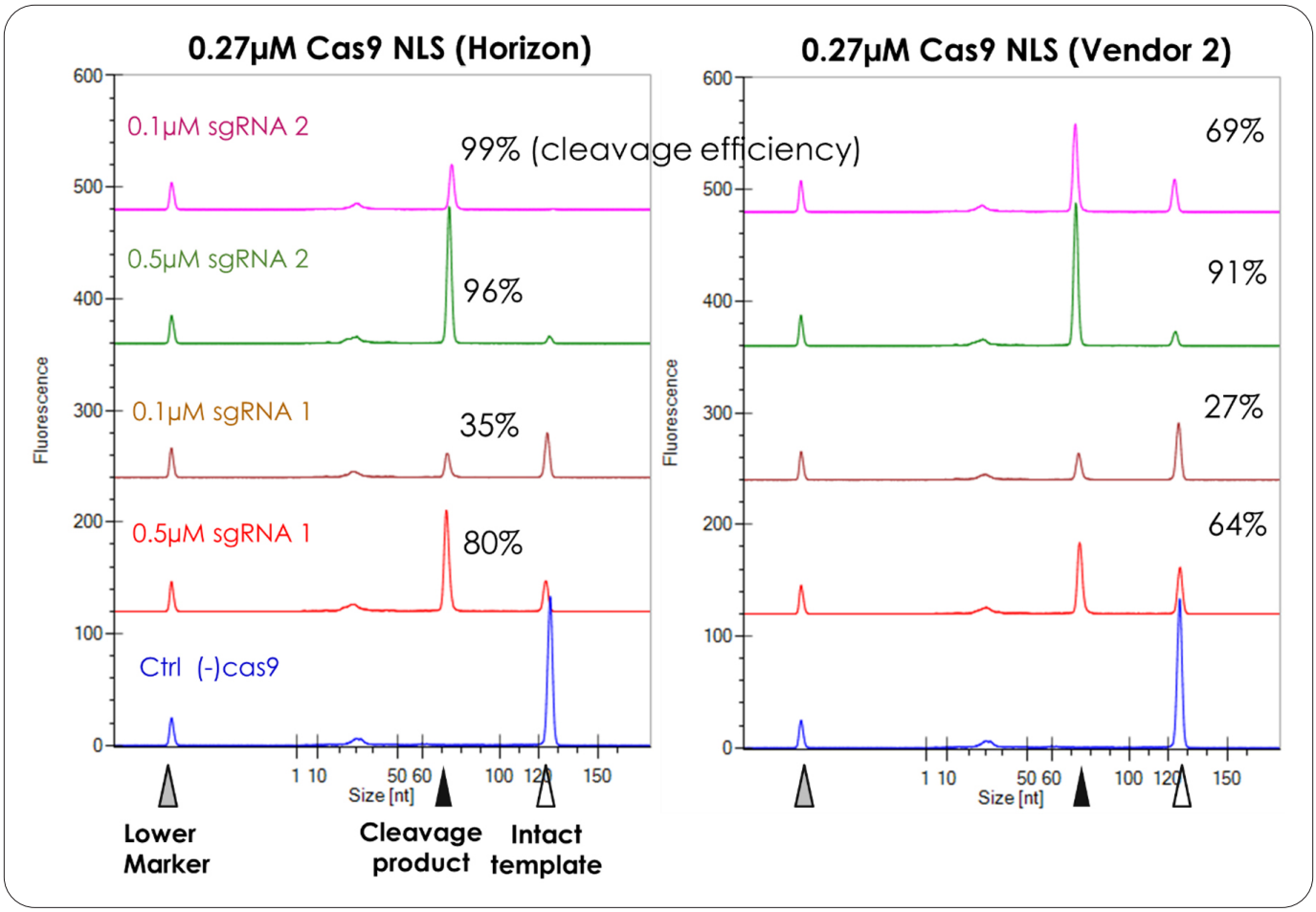

Figure 3: LabChip fragment analysis of *in vitro* genome editing. DNA template was treated by CRISPR-Cas complex 37°C for 30 min. Intact DNA template is ~120 bp, cleavage product is ~70 bp. Data shown here is one representative result from triplicates. NLS: nuclear localization sequence. Different colors represent different genome editing conditions.

Discussion

In this novel LabChip fragment analysis assay, fluorescently labeled oligonucleotide substrate (Table 2) removes intercalating dye staining interferences from non-relevant nucleic acid species, reaches detection sensitivity as low as 2 pg/µL (data not shown). Additionally, formamide denaturation of single-stranded fluorescently labeled nucleotide can provide strand-specific information in certain nucleic acid catalytic reactions, e.g. primer extension of DNA polymerase or one strand cut of nicking enzymes.

Furthermore, this new assay can be applied to any enzyme analysis that utilizes a fluorescently labeled oligo substrate, such as examination of strand displacement activity, a critical enzyme feature exemplified by Bst DNA polymerase (large fragment) for field-deployable, isothermal amplification nucleic acid detection; or (b) screening and optimization of engineered reverse transcriptase and prime editing guide RNAs (pegRNAs) in Prime Editor machinery—one versatile and precise genome editing method without double-strand breaks or donor DNA (1).

Table 2: Recommended oligonucleotide labeling dyes for end users on LabChip GX/GXII Touch instruments.

Dye	Excitation (nm)	Eminssion (nm)	Label site	Recommended Conc.*
ATTO 633	635	653	5′, Int, 3′	0.5 nM to 15 nM
Alexa 647	650	670	5′, Int, 3′	1 nM to 20 nM
LC640	620	635	5′, Int, 3′	1 nM to 20 nM
Cy5	648	668	5′, Int, 3′	2 nM to 40 nM

*This concentration is the sample loading concentration on LabChip GX/GXII Touch instruments.

Summary

We have demonstrated using the LabChip GX/GXII Touch instruments for accurate quantitation of CRISPR-Cas9 genome-editing efficiency. This new assay is a high throughput, simple setup, and cost-efficient solution compared to traditional capillary electrophoresis for characterization, discovery, and quality control of nucleic acid metabolic enzymes.

References

1. Anzalone, A et.al (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576(7785):149-157.