APPLICATION NOTE

Quantitative characterization of enzymatic nucleic acid modifications: mRNA capping, phosphorylation, and adenylation LabChip GX/GXII Touch systems.

LabChip™ GX/GXII Touch™ Systems

Introduction

The success of messenger RNA (mRNA) vaccines against the SARS-CoV-2 virus has opened the door to a whole new era of mRNA- based therapeutics and vaccines. Research and investment in this space have ramped up dramatically, including variations on mRNA vaccines such as self-amplifying mRNA and circular mRNA. Meanwhile, quality control tests are essential to ensure the products' safety and efficacy. For example, the 5′-capping of the mRNA molecule is critical to its integrity upon delivery to the target site and overall immunogenicity. The characterization of capping efficiency is an important quality parameter. However, mRNA capping analysis involves multiple steps, including the generation of pre-defined short fragments (less than 100 nt) from the 5′ end of the kilobase-long synthetic mRNA using enzyme cleavage. The cleaved small fragments are analyzed by either urea-PAGE gel or capillary electrophoresis which is one of the analytical technologies used for mRNA vaccine and drug development[1,2].

The LabChip™ GXII Touch™ Protein Characterization System and the LabChip GX Nucleic Acid Analyzer can be used to ensure the integrity of mRNA products based on intercalating dye detection. By utilizing fluorescently labeled oligonucleotide substrates[3], our microfluidic platform can also offer a simple solution to evaluate enzyme activities in mRNA vaccine and drug development. In this application note, we are demonstrating the use of the FFA120 Assay Reagent Kit run on the LabChip GX/GXII Touch systems together with DNA 5K/RNA/CZE chip to characterize mRNA capping efficiency with a simplified workflow (Figure 1). In addition, we demonstrate the platform's capability to detect nucleic acid phosphorylation and adenylation, both of which are critical in many molecular assays, including NGS (next-generation sequencing) library preparation (Figure 2).

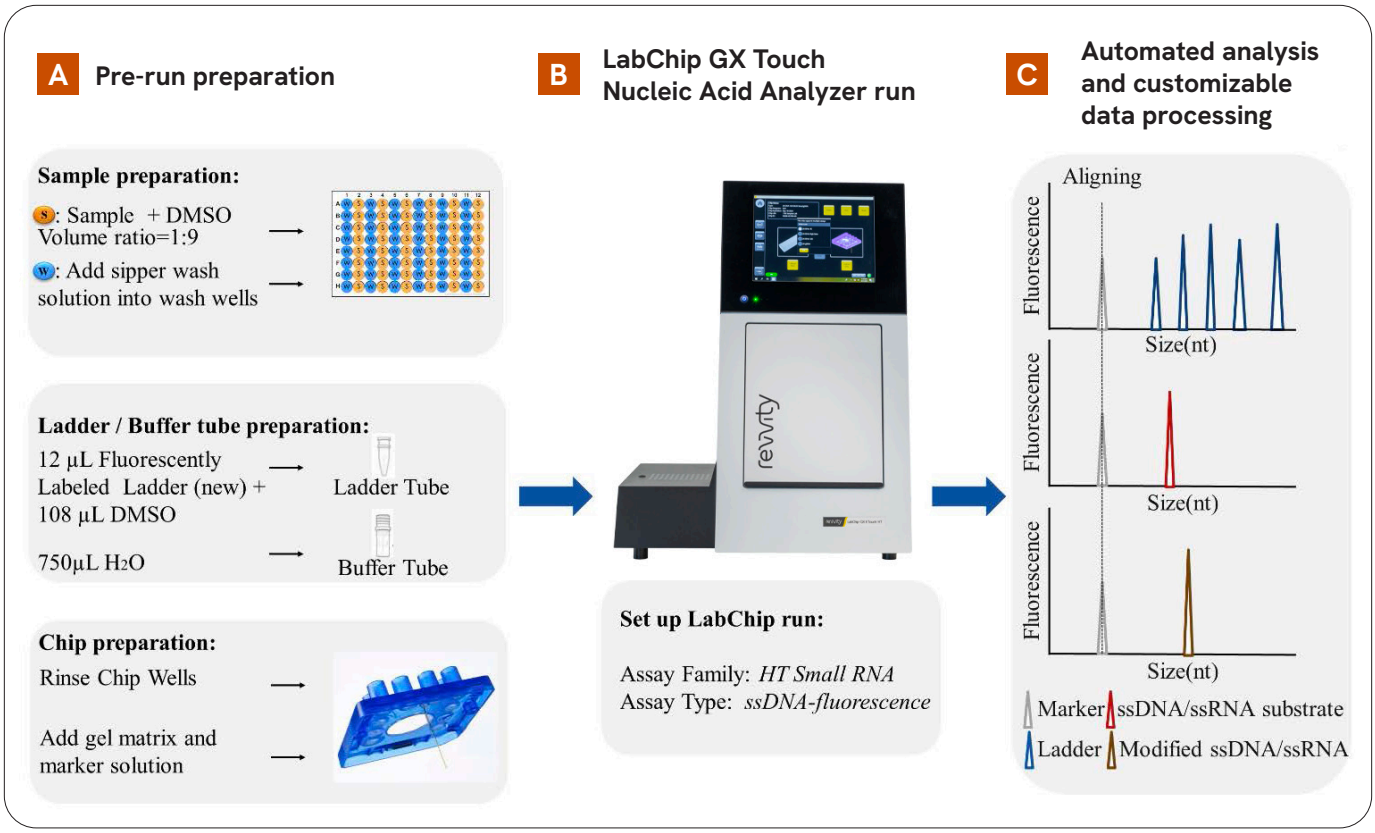

Figure 1: Workflow for LabChip fluorescence fragment analysis. (A) Pre-run preparation (with an example of sample run by row); (B) LabChip GX/GXII Touch Systems run; (C) Data analysis in LabChip GXII Reviewer software, Lower Marker is used for sample aligning.

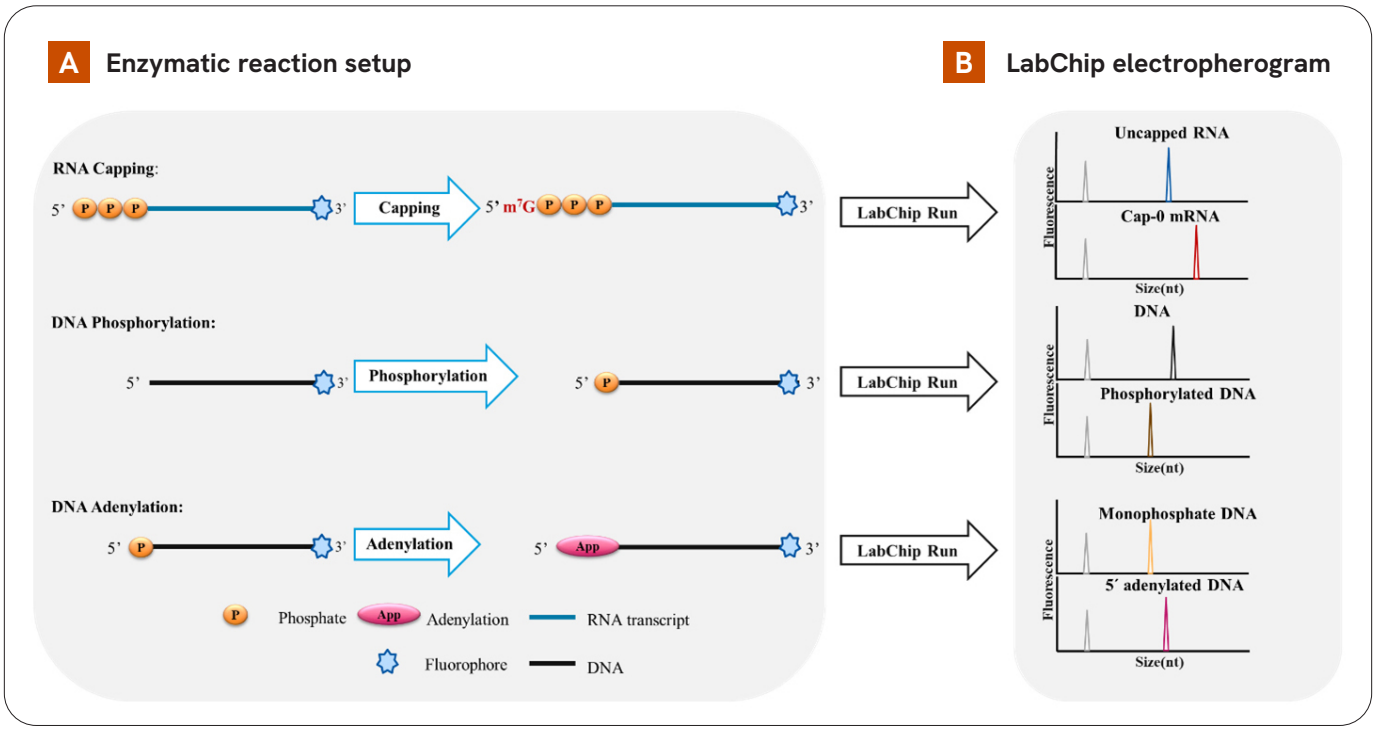

Figure 2: Workflow of determining the efficiency of nucleic acid modification reaction using fluorescence fragment analysis on LabChip GX/GXII Touch Systems. (A) Enzymatic reaction setup example of RNA Capping, DNA Phosphorylation, and DNA Adenylation. (B) LabChip electropherogram data example of RNA Capping, DNA Phosphorylation and DNA Adenylation.

Materials and methods

Oligonucleotides used in the studies are listed in Table 1. DNA phosphorylation was evaluated using T4 Polynucleotide Kinase (NEB) and T4 Polynucleotide Kinase (3′ phosphatase minus) (NEB). The phosphorylation reaction was performed at 37°C for 60 minutes with 0.2 µM oligo substrate (DNA1) and 0.5 µL (5 units) enzyme in a 25 µL reaction and then heat-inactivated by a 20-minute incubation at 65°C following the product manual. A reaction mixture, with oligo substrate (DNA1) but without the enzyme, was prepared as the negative control. Monophosphate DNA (p-DNA1) was used as the positive control.

DNA adenylation was studied using 5′ DNA Adenylation kit (NEB). Briefly, 2 µM p-DNA1 substrate, 2 µM Mth RNA Ligase (RNA ligase MthRnl from *Methanobacterium thermoautotrophicum*), and 0.5 mM ATP were used for a 20 µL reaction at 65°C for 1 hour, and a 5-minute incubation at 85°C was followed for enzyme inactivation. Mth RNA Ligase was replaced by water for the negative control sample.

RNA capping efficiency study was performed using Vaccinia Capping System (NEB). 0.5 µM triphosphate RNA (ppp- RNA1) was incubated with three concentrations of Vaccinia virus Capping Enzyme (VCE, 1 nM, 10 nM, and 45 nM) at 37°C for 30 minutes in a 20 µL reaction with 1X RNA capping buffer, 100 µM SAM and 0.5 mM GTP. The reaction was stopped by the addition of EDTA to 5 mM and heated at 70°C for 10 minutes. A reaction mixture, with uncapped RNA substrate (ppp-RNA1) and without VCE, was prepared as the negative control. And a 1:1 ratio mixture of uncapped RNA (ppp-RNA1) and Cap0-mRNA (N-7mGpppRNA1) was used as a positive control.

All enzymatic reaction products as described above were diluted with nuclease-free water to a final concentration of 200 nM (before dilution with DMSO) for fragment analysis on LabChip GX Touch Nucleic Acid Analyzer (Revvity). Pre-run preparation of the instrument, chip, and FFA120 Assay Reagent Kit (Figure 1) was conducted following the fragment analysis evaluation user guide. Data analysis was performed using LabChip GX Reviewer software.

Table 1: Sequences of single-stranded DNA and single-stranded RNA oligos[1]

Name	Type	Sequences (5′ – 3′)	Study	Vendor
DNA1	DNA	GTAGAACTTCGTCGAGTACGCTCAA[Cy5]	Phosphorylation, adenylation	IDT
p-DNA1	DNA	[Phos]GTAGAACTTCGTCGAGTACGCTCAA[Cy5]	Phosphorylation, adenylation	IDT
ppp-RNA1	RNA	[Tri-Phos]GUAGAACUUCGUCGAGUACGCUCAA[Cy5]	Capping	Bio-Synthesis
N-7mGpppRNA1	RNA	[N-7mGppp]GUAGAACUUCGUCGAGUACGCUCAA[Cy5]	Capping	Bio-Synthesis

Results

mRNA enzymatic capping efficiency study

In the eukaryotic cell, 5′ end mRNA capping is an essential modification which protects the mature mRNA from degradation and serves a role in nuclear export and translation initiation. *In vitro* mRNA vaccine production, the 5′-capping of the mRNA is critical to its integrity upon delivery to the target site and overall immunogenicity, which underscore the importance of capping methods or enzymes with higher capping efficiency. This study was designed to evaluate the LabChip GX/GXII Touch Systems' capability to characterize mRNA capping system from Vaccinia virus by differentiating the uncapped and capped structures.

The Vaccinia Capping System is comprised of three enzymatic activities (RNA triphosphatase, guanylyl-transferase, guanine N7-methyltransferase) that are necessary for the formation of the complete Cap-0 structure, m7Gppp5′N, using GTP and the methyl donor Sadenosylmethionine. As shown in Figure 3, uncapped RNA is ~ 25 nt and capped RNA product is ~34 nt. Without VCE, only one peak, the triphosphate RNA substrate (ppp-RNA1), is detected. The Cap0-mRNA (N-7mGpppRNA1) peak starts to be detected when 1nM VCE is added to the reaction and its peak area percentage gradually increases to 95.83% and 98.07% when VCE concentration reaches 10 nM and 45 nM, which indicates RNA capping yield and efficiency. Our results show that the efficiency of VCE RNA capping system is enzyme dose-dependent and that LabChip GX/GXII Touch systems together with the FFA120 Assay Reagent Kit can be applied to mRNA capping efficiency characterization.

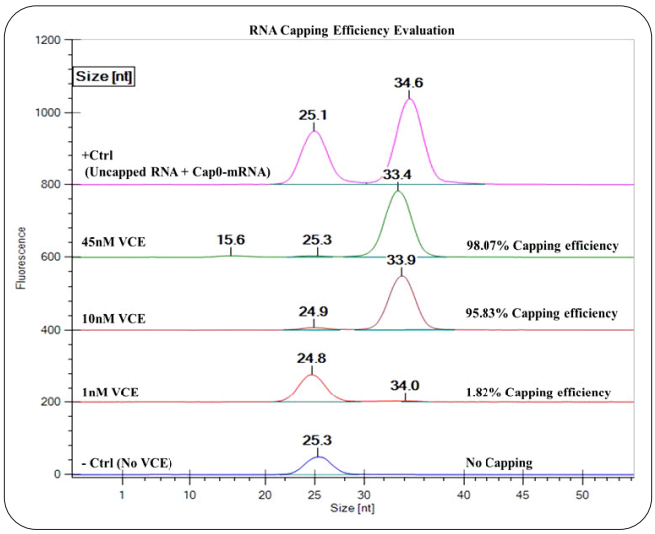

Figure 3: RNA capping efficiency evaluation using fluorescence fragment analysis on LabChip GX/GXII Touch systems. + Ctrl (positive control): a 1:1 ratio mixture of uncapped RNA and Cap0-mRNA. -Ctrl (negative control): uncapped RNA without VCE enzyme mix. Capping efficiency (peak percentage) is automatically calculated by the software, dividing the peak area of capped RNA by the sum of peak areas of uncapped and capped RNA.

Phosphorylation study

The status of DNA and RNA strand 5′ end and 3′ end phosphorylation impacts many molecular processes (ligation, cloning, NGS library construction, etc.). Monitoring the phosphorylation status modified by an enzyme is challenging since this modification does not result in an oligo size difference. However, phosphorylation does alter the net charge of oligonucleotides, which gives microfluidic chips, separation of oligonucleotides based on charge-to-mass ratio, the potential to detect this modification. This study was designed to evaluate LabChip GX/GXII Touch systems' capability to differentiate zero, one, and more than one phosphorylation of 5′ end of single-stranded DNA.

As shown in Figure 4, DNA1 substrate without enzyme shows one peak at 31.6 nt, whereas the positive control (p-DNA1), monophosphate singlestranded DNA (ssDNA), shows one peak at 26.0nt. Interestingly, the incubation with T4 PNK minus (modified T4 PNK protein without 3′ phosphatase activity, with full kinase activity) generated one peak at 21.2 nt with a 100% efficiency, and the T4 PNK reaction resulted in two peaks: one at 25.5 nt, the monophosphate DNA (p-DNA1), and one at 21.0 nt. Since there are no commercial diphosphate and triphosphate DNA controls available, there is no conclusion for this additional peak (~21 nt) as diphosphate or triphosphate or a mixture of both. These results indicate that T4 PNK minus has a different efficiency of DNA phosphorylation compared to T4 PNK and demonstrates that one or more than one phosphorylation modifications of a 25nt ssDNA could be separated and detected by LabChip GX/GXII Touch systems.

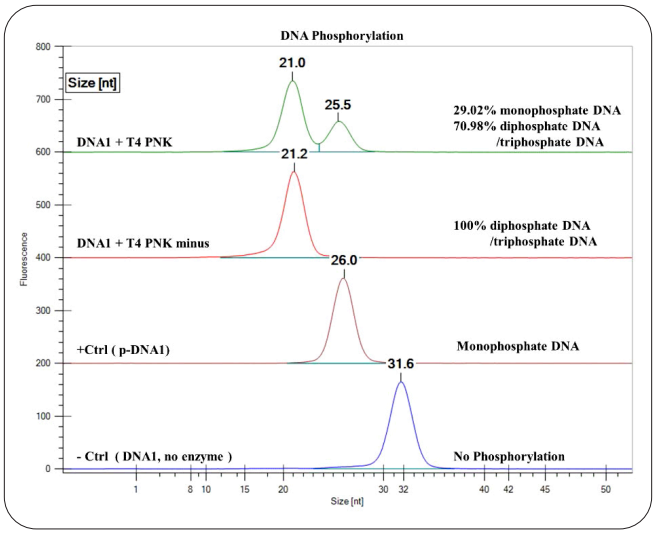

Figure 4: DNA phosphorylation study using fluorescence fragment analysis on LabChip GX/GXII Touch. DNA1: the ssDNA substrate. T4 PNK: T4 Polynucleotide Kinase. T4 PNK minus: T4 Polynucleotide Kinase 3′Phosphatase minus. -Ctrl (Negative Control): ssDNA substrate without enzyme. +Ctrl (Positive Control): monophosphate ssDNA (p-DNA1) without enzyme. Phosphorylation efficiency (peak percentage) is quantified automatically by the software, dividing the peak area of phosphorylated DNA by the sum of peak areas.

Adenylation study

Ligation is a critical step in many modern molecular biology workflows. The adenylation step, transferring the adenyl group to the 5′-phosphorylated end of the "donor" strand, is important for ligation efficiency. For example, adenylated DNA linkers can be used for 3′-end ligation of RNA in cDNA library preparation for NGS. This study was designed to evaluate LabChip GX/GXII Touch systems capability to differentiate the phosphorylated DNA strand (p-DNA) relative to the adenylated DNA (App-DNA).

Adenylated oligonucleotide is known to run about 1 base slower on a denaturing 15% or 20% polyacrylamide gel[4]; additionally, adenylated oligonucleotide shifts to slower migration time on capillary electrophoresis instrument[5]. As shown in Figure 5, the ssDNA substrate (p-DNA1) shows one peak at 23.5 nt whereas the adenylation reaction shows two peaks: one peak at 23.1 nt, the p-DNA1 substrate, and another peak at 27.7 nt, which is identified as the adenylated DNA (App-DNA1). In summary, the results demonstrate that LabChip GX/GXII Touch systems can provide enough resolution to differentiate a phosphorylated DNA strand (p-DNA) from an adenylated DNA (App-DNA) with a short oligo as 25 nt.

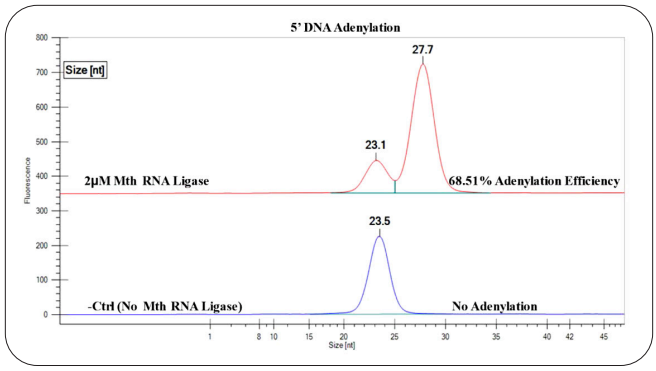

Figure 5: 5′ DNA adenylation study using fluorescence fragment analysis on LabChip GXII Touch. -Ctrl (Negative Control): monophosphate ssDNA substrate (p-DNA1) without Mth RNA Ligase. Adenylation efficiency (peak percentage) is determined automatically by the software, dividing the peak area of adenylated DNA (App-DNA1) by the sum of peak areas of unadenylated DNA (p-DNA1) and adenylated DNA (App- DNA1).

Summary

We have demonstrated that the LabChip GX/GXII Touch Systems together with the FFA120 Assay Reagent Kit can provide around 9 nt, 5 nt, 4 nt separation for mRNA capping, single phosphorylation and adenylation, respectively. With this resolution, the LabChip GX/GXII Touch Systems can support quantitative enzymatic study for nucleic acid modification. In addition, it can provide fragment analysis up to 120 nt, with the resolution of single phosphorylation modification on both DNA and RNA (RNA data not shown here), and adenylation (App) on both DNA and RNA (RNA data not shown here). This resolution depends on fragment size. Smaller size fragments show more obvious migration differences on microfluidic chips with phosphorylation or adenylation modification. For example, a single phosphorylation modification can be visible with a DNA fragment up to 60 nt (data not shown here). Additionally, the LabChip GXII Reviewer software provides multiple functions to support high throughput data analysis automation: size calling, visualization, peak percentage calculation, sample information batch import, peak table export, etc. To avoid run-to-run size-calling variation with small size fragments, we suggest proper run controls in the same or similar buffer should be included in each run as experimental design.

Traditional approaches for nucleic acid metabolic enzyme characterization studies are limited by the cost of high throughput capillary electrophoresis instrumentation and are typically operated by sequencing core facilities. With the new reagent developed on the LabChip GX/GXII Touch Systems to support 96-well or 384-well platform, scientists can perform similar analytical studies as a costefficient, high throughput solution.

References

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