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RESEARCH PAPER

# A Rapidly New-typed Detection of Norovirus Based on $F_0F_1$-ATPase Molecular Motor Biosensor

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**Abstract** In order to adapt port rapid detection of food borne norovirus, presently we developed a new typed detection method based on $F_0F_1$-ATPase molecular motor biosensor. A specific probe was encompassed the conservative region of norovirus and $F_0F_1$-ATPase within chromatophore was constructed as a molecular motor biosensor through the "ε-subunit antibody-streptomycin-biotin-probe" system. Norovirus was captured based on probe-RNA specific binding. Our results demonstrated that the Limit of Quantification (LOQ) is 0.005 ng/mL for NV RNA and also demonstrated that this method possesses specificity and none cross-reaction for food borne virus. What's more, the experiment used this method could be accomplished in 1 h. We detected 10 samples by using this method and the results were consistent with RT-PCR results. Overall, based on $F_0F_1$-ATPase molecular motors biosensor system we firstly established a new typed detection method for norovirus detection and demonstrated that this method is sensitive and specific and can be used in the rapid detection for food borne virus.

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**Keywords:** norovirus, molecular motor biosensor, detection, $F_0F_1$-ATPase

## 1. Introduction

Norovirus (NV), a member of the goblet virus family and norovirus genus, has the properties of high morbidity, low infective dose and strong resistance. This virus was discovered in America in 1972 for the first time [1]. Norovirus is the leading cause of human acute viral diarrhea pathogens and is the B class pathogen by World Health Organization (WHO) [2-7]. This virus is transmitted through food, water, and daily life activities. Among them, contaminated food is one of the important transmitted routes [8]. Therefore, the detection of food borne norovirus has become very important.

Currently used detection methods for NV are electron microscopy examinations including direct electron microscopy (EM) and immunoelectron microscopy (IEM) [9-12]. Generally, the electron microscopy examinations require high concentration of virus particles. In the recent years, there have been some quick and easy detection methods such as PCR, ELISA, and gene chip technology [13-17]. Although these detected technologies have certain validity, the detection time is still longer.

Molecular motor biological sensing based technology has incomparable advantages compared the traditional methods [18-20]. $F_0F_1$-ATPase, one of important molecular motor, is a rotating biological molecular motor and is responsible for the biological energy conversion *in vivo*. $F_0F_1$-ATPase consists of two parts, $F_0$(ab2cn) embedded in the membrane and $F_1$($\alpha3\beta3\gamma\delta\varepsilon$) in membrane periphery [21-23]. Many researches have used $F_0F_1$-ATPase molecular

motor biosensor to detect bird flu virus, mouse hepatitis virus, chemical pollutants, and pathogenic bacteria [24-27].

In this study, we developed a new method for rapid and precise detection of norovirus based on $F_0F_1$-ATPase molecular motor biosensor system. We used the new method in the food-borne norovirus detection. To our best knowledge, these results are first application of $F_0F_1$-ATPase biosensor in food-borne norovirus detection.

## 2. Materials and Methods

### 2.1. Chemicals and materials

Freeze-dried live attenuated hepatitis A vaccine was purchased from Zhejiang Pukang Biotechnology Co., Ltd. National Institute for Viral Disease Control and Prevention, Chinese Center for Disease Control and Prevention provided the rotavirus and norovirus samples. Biotin-AC5-Sulfo-Osu was purchased from Dojindo (Japan). Streptavidin and adenosine diphosphate (ADP) were purchased from Sigma (St. Louis, MO, USA). N-(fluorescein-5-thiocarbamoyl)-1,2-dihexadecanoyl-sn-glycero-3-phosphoethanolamine, triethylammonium salt (F-DHPE) was purchased from invitrogen (Carlsbad, CA, USA).

### 2.2. RNA extraction

NV RNA was extracted using RNA extraction kit (Qiangen, Valencia, CA, USA) following manufacture's protocol and was then used as the target of the molecular motor biosensor. After analyzing the concentration and purity of RNA solution by spectrophotometer, the RNA sample was stored at -20°C. RNA samples were thawed at room temperature and vortexed before use.

### 2.3. Synthesizing and labeling of probes

Based on bioinformatics analysis, we designed a probe for NV. The sequences used were: NV, 5' – AGGAGATYG CGATCYCCTGTCCAYA – 3'. The probe was labeled by biotin in the 5' end. The NV probe was purchased from Takara (Kyoto, Japan). The probe's location of NV is in the highly conserved region of joint between ORF1 and ORF3.

### 2.4. Preparation of chromatophore and labeling of F-DHPE

Chromatophores were prepared from the cells of *Thermomicrobium roseum (T. roseum)* by shaking at 150 rpm, culturing for 24 h at 60°C, and were harvested by centrifugation at 4,000 rpm at 4°C for 30 min. The bacterial pellets were resuspended in extracting buffer (20 mM Tris–Cl, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, pH 8.0), and were harvested by centrifugation at 6,000 rpm at 4°C for 10 min. The supernatant was removed and then resuspended in extracting buffer. The suspension with PMSF (final concentration 1 mM) was sonicated on ice for 30 min (5 sec on, 8 s off), causing the plasma membrane to break and invert to form vesicles with the $F_0F_1$-ATPases inside out. The suspension was then centrifuged at 25,000 rpm (4°C for 30 min) and the supernatant was transferred to a new tube and further centrifuged at 145,000 rpm at 4°C for 1 h. The pellets as chromatophores were resuspended in extraction buffer with 50% glycerol, frozen immediately in liquid nitrogen, and then stored at −80°C. The concentration of chromatophores in the samples was determined spectrophotometrically at 880 nm.

The fluorescence probe F-DHPE was labeled onto the surface of chromatophores [28]. Aliquots of 10 μL F-DHPE (200 mg/mL, dissolved in ethanol) were mixed with 200 μL chromatophores, and incubated for 15 min in dark with gentle shaking at room temperature. 10 mM phosphate buffered saline (PBS, pH 7.4) was added to a volume of 1.3 mL. Labeled chromatophores were then harvested by centrifugation at 30,000 rpm (4°C for 15 min). After removing free F-DHPE by washing with 10 mM PBS and centrifugation at 10,000 rpm (4°C, 15 min) three times, the resulting pellets were resuspended in 200 μL PBS. The labeled chromatophores are called fluorescent chromatophores hereafter.

### 2.5. Preparation of anti-epsilon subunit antibody and labeling with biotin

The ε-subunit of $F_0F_1$-ATPase from *T.* roseum was expressed in *E. coli* BL21 and purified commercially. Approximately, the DNA fragment encoding the peptide of ε-subunit was sub-cloned to pET22b(+) to generate the expression construct pET22B(+)/ε-subunit with an N-terminal 6×His tag. *E. coli* BL21 transformation and isopropyl β-D-thiogalatoside (IPTG) inducing following methods specified by the manufacturer. ε-subunit expressed in *E. coli* was purified using a Ni-NTA sepharose column according to the manufacturer's protocols, and analyzed using SDS-polyacrylamide gel electrophoresis. The antibody was prepared as described previously [29], purified by precipitation with 33% $(NH_4)_2SO_4$, and stored at -20°C until use.

The ε-subunit antibody was labeled with biotin-AC5-Sulfo-Osu on the N end as follows: 2 μL of 2 μM biotin-AC5-Sulfo-Os was added in 20 μL ε-subunit antibody at room temperature for 30 min.

### 2.6. Construction of $F_0F_1$-ATPase molecular motor biosensor

We incubated a mixture of 2 μL of biotin (biotin-AC5-Sulfo-Os, 2 μM) and 20 μL ε-subunit antibody for 30 min at room temperature. Briefly, the $F_0F_1$-ATPase molecular motor biosensor was constructed as follows: Aliquots of

40 μg of biotin-labeled ε-subunit antibody were added to 200 μL fluorescent chromatophores and PBS was added to make the final volume 1 mL. After incubating at 37°C for 1 h, PBS was added to make the final volume 1.4 mL. The pellets were then harvested by centrifugation at 30,000 rpm at 4°C for 10 min and resuspended in 500 μL PBS. Then 2 μL (2 mg/mL) of avidin and PBS were added to make the final volume 1 mL. The mixture was shaken at 50 ~ 100 rpm at room temperature for 10 min. The final volume was made to 1.4 mL by adding PBS. The pellets were harvested by centrifugation at 30,000 g at 4°C for 10 min and resuspended in 500 μL PBS. Subsequently, a volume of 2 μL (2 μM) of biotin-labeled probe and PBS was added to a final volume of 1 mL. The mixture was shaken at 50 ~ 100 rpm at room temperature for 10 min, PBS was added to a final volume of 1.4 mL, the pellets were harvested by centrifugation at 30,000 g at 4°C for 10 min and chromatophores were resuspended in 150 μL glycerol (30%, V/V). The prepared $F_0F_1$-ATPase molecular motor biosensors were called Chro NV in the following test and stored at –20°C before use.

### 2.7. Construction of molecular motor detection method

Chro NV was diluted with synthetic buffer (0.1 mM Tricine, 10% glycerol, 5 mM $NaH_2PO_4$, 5 mM $MgCl_2$, pH 9.0). A 10 μL sample was mixed and vortexed. The buffer solution was used as blank. After adding 30 μL of start buffer (start buffer was prepared with 1.6 M ADP and synthetic buffer (1:3, v/v) before use), the reaction system was incubated for 10 min at room temperature. The fluorescence signal was measured after adding 200 μL of PBS using 96 well plates and microplate reader. The results were calculated from fluorescence value using following formula: $((A_{(+)}-A_{(-)})/A_{(-)})\times 100$ ($A_{(+)}$: sample fluorescence, $A_{(-)}$: fluorescence of water).

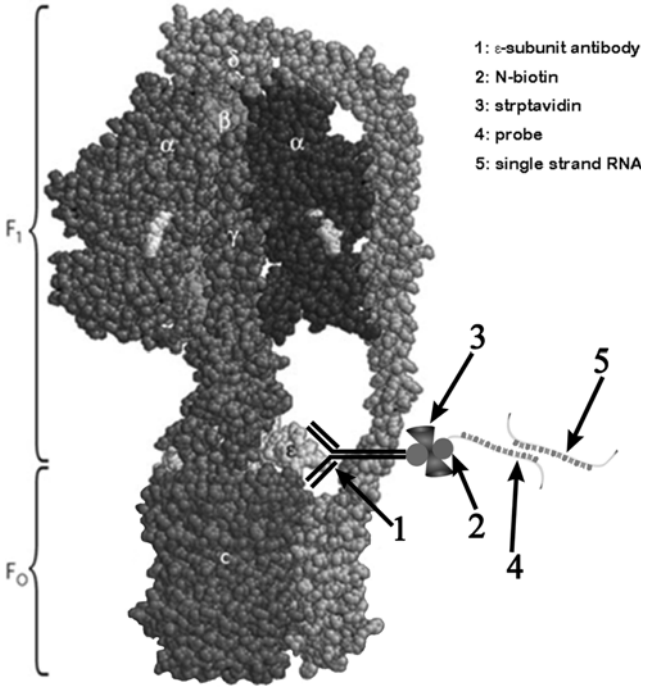


**Fig. 1.** Schematic of $F_0F_1$-ATPase molecular motor biosensor.

### 2.8. Statistical analyses

All experimental data were shown as the mean ± standard deviation. Statistical significance was evaluated with SPSS software. Experimental differences of multiple groups (>3 groups) were analyzed using the two-tailed Student's *t*-text. *P* values<0.05 was considered statistically significant.

## 3. Results and Discussion

### 3.1. The construction of $F_0F_1$-ATPase molecular motor biosensor

F-DHPE is known as a lipid fluorescent probe and can be embedded into the phospholipid molecular layers. In our study, F-DHPE was labeled into the phospholipid molecular layers to point out the synthetic efficiency of ATP. Here, we successfully connected "ε-subunit antibody-streptomycin-biotin-probe" system to $F_0F_1$-ATPase and constructed an $F_0F_1$-ATPase molecular motor biosensor. Generally, fluorescence intensity without conjugation is significantly higher than with conjugation. However, because of Brownian motion, the fluorescence intensity is often lower than that of without conjugation. In other words, the significant differences in fluorescence intensity compared with negative control suggest the successful capture of target molecule. A schematic diagram of $F_0F_1$-ATPase molecular motor biosensor is shown in Fig. 1.

$F_0F_1$-ATPase, as an ATP synthase, has faster rotary properties in itself [21]. In our study, according to $F_0F_1$-ATPase rotation property, we established the coupling between the catalytic site and proton transferring site. Additionally, F-DHPE can be embedded into the phospholipid molecular layer. During ATP synthesis, protons are pumped out of the chromatophores, resulting in an increase in the concentration of $H^+$ out of the chromatophores. Thus, the pH out of the chromatophores will decrease, and the fluorescence probe F-DHPE is expected to detect the pH decrease.

### 3.2. Establishment of the molecular motor biosensor reaction system

Firstly, Chro NV was diluted by concentration gradient. In the different concentration conditions, the fluorescence value of $H_2O$ and virus and the fluorescence difference are shown

**Table 1.** Fluorescence values of $H_2O$ and NV RNA under different concentrations of Chro NV

| Dilution (fold) | Chro RV (mg/mL) | Fluorescence values of $H_2O$ | Fluorescence values of RV | Fluorescence difference (%) |
|---|---|---|---|---|
| 100 | 0.0156 | 69449.67 | 78449.67 | 13.0 |
| 90 | 0.0173 | 79171.33 | 93833.67 | 18.5 |
| 80 | 0.0195 | 86763.33 | 100086 | 15.4 |
| 70 | 0.0223 | 102883 | 126549.7 | 23.0 |
| **60** | **0.026** | **131176.3** | **171176.3** | **30.5** |
| 50 | 0.0312 | 170249.7 | 210230 | 23.5 |
| 40 | 0.039 | 230048.7 | 260048.7 | 13.0 |
| 30 | 0.0519 | 377513.7 | 430846.3 | 14.1 |
| 20 | 0.0779 | 648848.7 | 705515.3 | 8.7 |
| 10 | 0.1558 | 1471912 | 1598579 | 8.6 |

**Table 2.** Fluorescence values of $H_2O$ and NV under different concentrations of NV RNA with 60 fold dilution of Chro NV

| NV RNA (ng/μL) | Fluorescence values of $H_2O$ | Fluorescence values of RV | Fluorescence difference (%) |
|---|---|---|---|
| 0.1 | 133666 | 153292 | 12.8 |
| 0.2 | 133666 | 161373 | 17.2 |
| 0.3 | 133666 | 159061 | 16.0 |
| 0.4 | 133666 | 163073 | 18.0 |
| 0.5 | 133666 | 154073 | 13.2 |
| 0.6 | 133666 | 153073 | 12.7 |
| **0.7** | **133666** | **168193** | **20.5** |
| 0.8 | 133666 | 149061 | 10.3 |
| 0.9 | 133666 | 153073 | 12.7 |
| 1 | 133666 | 152673 | 12.4 |

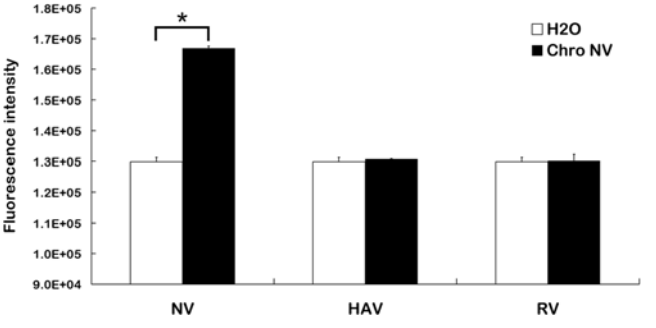


**Fig. 2.** Cross-reactivity analyses of Chro NV with RV, HAV, and NV detection. *: Fluorescence values of samples are significantly different than $H_2O$ ($p < 0.05$).

in Table 1. From the results, we found that the fluorescence intensity of final reaction system was inversely proportional to the dilution ratio. Because along with the concentration of molecular motor biosensor increasing, the reaction system has more ATPase, and therefore fluorescence intensity is larger. Based on the principle of maximum differentials between molecular motor and $H_2O$, we finally chose 0.0260 mg/mL (60 fold dilution) as the final concentration of Chro NV. Meanwhile, we explored the optimum reaction concentration of virus RNA (Table 2). We found that the fluorescence values of different concentrations of RNA were all higher than the control ($H_2O$), especially 0.7 ng/μL of NV RNA indicating that the detection of $F_0F_1$-ATPase molecular motor biosensor is effective and the optimum concentration of RNA is 0.7 ng/μL of NV RNA.

### 3.3. Specificity and sensitivity of chro NV assay

In order to measure the cross-reactivity of $F_0F_1$-ATPase molecular motor biosensor in the detection, we further tested the specificity of Chro NV with other common food borne viruses (*e.g.,* rotavirus (RV) and hepatitis A virus (HAV)) (see Fig. 2). We found that the molecular-biosensor fluorescence intensity of NV was significantly higher than the fluorescence intensity of $H_2O$, however, the fluorescence intensity of RV and HAV was unchanged compared with the control indicating that the Chro NV molecular motor biosensor is specific to the RV detection and there were not cross reactions with other viruses tested.

To estimate the sensitivity of our assay method, we measured the fluorescent intensity of various concentrations of norovirus RNA (Fig. 3). We found that the concentration of Chro NV in the range of 0.005 ~ 100 ng/mL had significantly higher fluorescence than the control but the concentration less than 0.001 ng/mL, indicating that the LOQ of Chro NV is the concentration of 0.005 ng/mL.

### 3.4. Validation of the assay method with actual samples

In the above experiments, we proved that $F_0F_1$-ATPase molecular motor assay method was effective and specific. We then applied the same assay method to measure the NV in real samples. To this end, we randomly chose 10 known negative/positive samples as test samples to perform molecular motor biosensor detection and the results were compared with the results of RT-PCR detected method (Fig. 4). As seen the figure, the result of molecular motor detection was similar to the result of RT-PCR indicating that our new assay method can be used for the daily

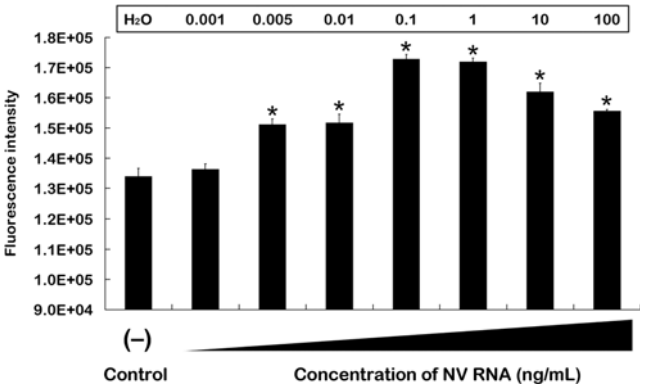


**Fig. 3.** Sensitivity analyses of Chro NV detection under different concentrations of NV RNA. *: Fluorescence values of samples are significantly different than $H_2O$ ($p < 0.05$). Some concentrations of NV RNA are circled at the top of the chart.

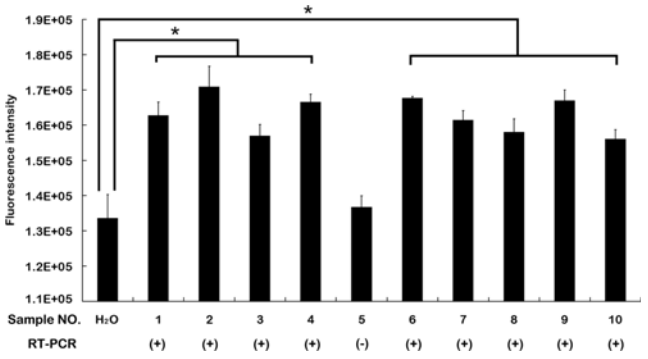


**Fig. 4.** Real food sample analysis using molecular motor detection method. *: Fluorescence values of samples are significantly different than $H_2O$ ($p < 0.05$). Results of RT-PCR detection method is noted in the bottom.

inspection of food-borne noroviruses.

Food borne norovirus has seriously threatened food safety in recent years [1,3]. Generally norovirus in food is found in trace level and only 10 ~ 100 virus units can cause the infection. Considering the large-scale food import-export trade, it requires screening of large number of samples. Thus, it is very necessary to develop an effective, rapid, precise, and sensitive detection method. In this study, we successfully developed an effective detection method for food borne norovirus by using $F_0F_1$-ATPase molecular motors biosensor. We hope that besides providing scientific basis for clinical researches, norovirus detection based on the $F_0F_1$-ATPase biosensor could also provide better solutions for virus detection in import-export food detection. Our method does not need complicated facilities such as PCR amplication, electrophoresis and hybridization follow-up processing, it greatly reduced the risk of contamination [24,30]. Along with the progress of micromachining technology and nanotechnology, the molecular motor biosensor has been largely applied in the various detection fields [31-35]. Further miniaturization and portability of our method would allow expanding this technology to wider applications.

## 4. Conclusion

In this research, we have successfully used the chromatophores extracted from thermophilic bacteria to construct a new molecular motor biosensor and applied it to detect NV. In our studies, we systematically investigated the specificity, sensitivity, and stability of $F_0F_1$-ATPase biosensor. The results revealed that the new assay method is an effective detection method for norovirus. Additionally, this method is low-cost due to large volume production of $F_0F_1$ATPase molecular motors biosensor and the test can be completed in only 1 h.

In summary, based on $F_0F_1$-ATPase molecular motors biosensor system our works have firstly established a new typed detection method for norovirus detection. We propose these findings might provide better solutions for clinical diagnosis, food detection and disease prevention of food-borne virus in the future.

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## Nomenclature

NV : Norovirus
RV : Rotavirus
HAV : Hepatitis A virus
LOQ : Limit of Quantification
WHO : World Health Organization
EM : Directelectromicroscope
IEM : Immuneelectromicroscope

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