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Author	Sungeun Hwang1, Wonhee Lee2, Yoonseok Lee1,3
Affiliation	1 School of Biotechnology, Hankyong National University, Gyeonggi-do, Anseong, 17579, Korea 2 Biattic Inc., Anyang 14059, Korea 3 Center for Genetic Information, Hankyong National University, Gyeonggi-do, Anseong, 17579, Korea
ORCID (for more information, please visit https://orcid.org)	Sungeun Hwang (https://orcid.org/0000-0003-3555-1286) Wonhee Lee (https://orcid.org/0000-0003-1339-0207) Yoonseok Lee(https://orcid.org/0000-0003-4803-5192)
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CORRESPONDING AUTHOR CONTACT INFORMATION

6

For the corresponding author (responsible for correspondence, proofreading, and reprints)	Fill in information in each box below
First name, middle initial, last name	Yoonseok Lee
Email address – this is where your proofs will be sent	yoonseok95@hknu.ac.kr
Secondary Email address	Yoonseok95@naver.com
Address	Center for Genetic Information, Hankyong National University, 327 Jungang-ro, Anseong-si, Gyeonggi-do, Anseong, 17579, Republic of Korea
Cell phone number	010-3814-8660
Office phone number	031-670-5332
Fax number	0508-903-8660

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Abstract

Bovine Viral Diarrhea (BVD) is a single-stranded, positive-sense ribonucleic acid (RNA) virus belonging to the genus Pestivirus of the Flaviviridae family. BVD frequently causes economic losses to farmers. Among bovine viral diarrhea virus (BVDV) strains, BVDV-1b is predominant and widespread in Hanwoo calves. Reverse-transcription polymerase chain reaction (RT-PCR) is an essential method for diagnosing BVDV-1b and has become the gold standard for diagnosis in the Republic of Korea. However, this diagnostic method is time-consuming and requires expensive equipment. Therefore, Clustered Regularly Interspaced Short Palindromic Repeats-Cas (CRISPR-Cas) systems have been used for point of care (POC) testing of viruses. Developing a sensitive and specific method for POC testing of BVDV-1b would be advantageous for controlling the spread of infection. Thus, this study aimed to develop a novel nucleic acid detection method using the CRISPR-Cas13 system for POC testing of BVDV-1b. The sequence of the BVD virus was extracted from NCBI (NC_001461.1), and the 5' UTR region, commonly used for detection, was selected. CRISPR RNA (crRNA) was designed using the Cas13 design program and optimized for the expression and purification of the LwCas13a protein. Madin Darby bovine kidney (MDBK) cells were infected with BVDV-1b, incubated, and the viral RNA was extracted. To enable POC viral detection, the compatibility of the CRISPR-Cas13 system was verified with a paper-based strip through collateral cleavage activity. Finally, a colorimetric assay was used to evaluate the detection of BVDV-1b by combining the previously obtained crRNA and Cas13a protein on a paper strip. In conclusion, the CRISPR-Cas13 system is highly sensitive, specific, and capable of nucleic acid detection, making it an optimal system for the early point-of-care testing of BVDV-1b.

Keywords: BVDV-1b, CRISPR-Cas13 system, Point-of-care testing, Korean native cattle, nucleonic acid detection

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Introduction

30 Bovine viral diarrhea (BVD) is an economically significant disease in cattle and is found in most countries worldwide.
31 Infection and death associated with BVD lead to significantly reduced reproductive performance and increased premature
32 culling. These clinical signs are especially pronounced when one or more BVD carriers are present in a herd. Animals
33 that develop acute diarrhea and fever may die or have long, costly recovery periods with decreased production and growth
34 performance. Over the past 10 years, BVD has occurred frequently, causing economic losses to farmers in the domestic
35 livestock industry (Kim et al. 2016). The major pathogens causing calf diarrhea in these reports were viruses (bovine
36 coronavirus (BCV), bovine rotavirus group A (BRV), and bovine viral diarrhea virus (BVDV)), bacteria (*Escherichia*
37 *coli* K99 and *Salmonella* spp.), and protozoa (*Cryptosporidium parvum* and *Eimeria* spp.). Some of these agents are
38 detected not only in diarrheic calves but also in normal calves (Jeong et al. 2012). BVDV is predominant in most parts
39 of the world, with a high prevalence, persistence, and clinical consequences (Chae et al. 2021). BVDV is an enveloped,
40 positive-sense, linear, single-stranded RNA virus (12.5kb) of the genus *Pestivirus* in the family *Flaviviridae*. BVDV can
41 be divided into two types (BVDV1 and BVDV2) based on sequence similarity of the 5' untranslated region (UTR) in the
42 viral genome (Cho et al. 2008). BVDV transmission occurs both horizontally and vertically, with persistently and
43 transiently infected animals excreting the virus. The virus is transmitted via direct contact, bodily secretions, and
44 contaminated fomites, and can persist in the environment for more than two weeks. Persistently infected animals are
45 the most important source of the virus, continuously excreting a viral load 1000 times higher than that shed by acutely
46 infected animals (Hou et al. 2020). Laboratory testing for identifying BVDV has typically been performed with
47 methods such as enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR). However, these
48 are costly and require precise instruments, making it imperative to establish a fast, accurate, and efficient method for
49 detecting BVDV (Adams et al., 2015; Lee et al. 2020; Yao et al. 2021). Quick and accurate point-of-care (POC) testing
50 for BVDV would greatly enhance diagnostic capacity, leading to improved quarantine and disease prevalence control.
51 There are several POC RNA detection technologies that do not require special instruments, such as reverse
52 transcription recombinase polymerase amplification (RT-RPA) (Piepenburg. et al. 2006) and reverse transcription-
53 loop-mediated isothermal amplification (RT-LAMP) (Notomi. et al. 2000). RT-RPA and RT-LAMP are highly
54 sensitive methods for detecting viral RNA; however, they are prone to non-specific amplification under isothermal
55 conditions. This can lead to false positive results when used for viral RNA detection (Rabe & Cepko. 2020). This
56 problem worsens when non-sequence-specific probes, such as pH-sensitive dyes, are used. The use of sequence-
57 specific probes, such as hybridization-based fluorescent oligonucleotide probes, can improve detection sensitivity

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(Qian et al., 2020). Diagnostic methods have been developed based on clustered regularly interspaced short palindromic repeats (CRISPR), which utilize the collateral cleavage activity of bystander nucleic acid probes by RNA-guided CRISPR-associated 12/13 (Cas12/13) nucleases (Gootenberg et al. 2018). CRISPR diagnostic methods are both highly sensitive (at the attomolar level) and specific (down to the single-nucleotide level) (Gootenberg et al. 2017). The readout of Cas-mediated nucleic acid probe cleavage can be detected using fluorescence or the lateral-flow strip method. The latter is advantageous because the strips are portable, and the results can be easily read with the naked eye (Myhrvold et al. 2018). Cas13a, previously known as a single-effector RNA-guided ribonuclease (RNase), can detect the presence of an RNA target using CRISPR RNAs (crRNAs), providing a platform for specific RNA sensing (Kellner et al. 2019). Several studies have reported that Cas13a-based molecular detection systems can detect both Zika and Dengue viruses (Gootenberg et al. 2017). Recently, a nucleic acid detection method based on CRISPR-Cas13a was developed as a new system for the early diagnosis of BVDV (Yao et al. 2021). This study aimed to establish a nucleic acid detection method based on the lateral flow strip method for early POC detection of BVDV-1b using CRISPR-Cas13a. To improve the accuracy and performance of POC diagnosis in testing settings with limited resources, we designed a crRNA in the 5'UTR conserved region and a sequence-specific primer to provide detection.

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Materials and Methods

BVDV-1b RNA extraction and cDNA synthesis

(1) BVDV-1b RNA extraction

MDBK cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin, and streptomycin at 37℃ with 5% CO_2. MDBK cells were obtained from Korean Cell Line Bank, and BVDV-1b cells were obtained from the Korea Veterinary Culture Collection (KVCC). MDBK cells at 80-90% confluency were washed twice with phosphate-buffered saline (PBS) and then infected with BVDV-1b diluted to a concentration of 10^2–10^3 using DMEM. The cells were then incubated at 37℃ with 5% CO_2 for 60-90 minutes to allow the virus to adsorb onto them. Infected MDBK cells were cultured for more than 48 h in DMEM supplemented with 10% FBS. The cells were observed daily for cytopathic effects (CPE). The cells were lysed by three repeated freeze-thaw cycles, and the lysate was centrifuged for 10 minutes at 1,600 rpm in a 15 mL tube. The supernatant was aspirated and stored at -70℃ until further use. The viral RNA of BVDV-1b was extracted using the Viral Gene-spin Viral DNA/RNA Extraction Kit (iNtRON, Burlington, USA), following the manufacturer's instructions.

87 (2) cDNA synthesis.

88 The extracted viral RNA of the BVDV-1b was reverse transcribed into complementary DNA (cDNA) using the
89 AccuPower® RT Master Mix (Bioneer, Daejeon, Korea), following the manufacturer's instructions. The template
90 RNA, forward primer (5’-GCCATGCCCTTAGTAGGACT-3’), reverse primer (5’-T7 promoter sequence-
91 CGAACCACTGACGA CTACCC-3’), RNase inhibitor, and ddH_2O were combined in a 1.5 mL tube. The mixture
92 was incubated at 70℃ for 5 min and then placed on ice. AccuPower® RT Master Mix and DNA polymerase (Bioneer,
93 Daejeon, Korea) was added to the PCR tube to achieve a final volume of 50 µl. The RT-PCR reaction was performed
94 by incubating the mixture at 42℃ for 60 minutes, followed by 5 minutes at 95℃ and then 35 cycles of DNA
95 amplification, which consisted of 30 seconds at 95℃, 30 seconds at 54-67℃, and 1 min at 72℃. A final extension
96 step of 5 minutes at 72℃ was included. The cDNA was stored at -20℃ until use.

97

98 **Design of crRNA, forward/reverse primer, direct repeat sequence and design**

99 The 5’UTR region of the BVDV FASTA sequence (NC_001461.1) was obtained from NCBI. Forward and reverse
100 primers were designed using the NCBI Primer-BLAST. For detection reactions involving Cas13, a T7 promoter
101 sequence must be added to the 5′ end of the forward or reverse primer to enable T7 transcription. The gRNA sequence
102 was designed using the Cas13 online platform (https://cas13design.nygenome.org). The 5′ end of the gRNA sequence
103 is then combined with a direct repeat (DR) sequence to form a complete crRNA (Kellner et al. 2019). The primers and
104 crRNAs were designed as shown in Table 1.

105

106 **Expression and purification of LwCas13a**

107 (1) Expression of LwCas13a

108 The pC013-Twinstrep-SUMO-huLwCas13a plasmid (#90097 Addgene, Watertown, USA) was transformed into
109 Rosetta™2 (DE3) pLysS Single Competent Cells (Novagen™ Sigma-Aldrich, St. Louis, MO, USA). The transformed
110 cells were plated on an LB agar plate and incubated for 24 hours at 37℃. The following day, a single colony was
111 isolated and cultured in 25 mL of TB medium with 12.5 µL of ampicillin at 300 rpm for over 9 hours at 37℃ until an
112 OD_{600} of 0.4-0.6 was reached. The culture was then cooled at 4℃ for 30 minutes. After adding 62.5 µL of 0.2 M
113 Isopropyl β-D-1-thiogalactopyranoside (IPTG) (Sigma-Aldrich, St. Louis, MO, USA), the cells were incubated in
114 shaking incubators at 300 rpm for 16 hours at 21℃. After 16 hours of cultivation, the cells were centrifuged at 5,000
115 rpm for 15 minutes at 4℃. The cell pellet was then stored at -80℃ until it was used.

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(2) Purification of LwCas13a

The cell pellet was resuspended in 20 mL of supplemented lysis buffer (composed of 50 mL of lysis buffer, 1 tablet of cOmplete Ultra EDTA, 0.05 g of lysozyme, and 0.5 μL of benzonase) by stirring the mixture on ice for 30 min. The resuspension was sonicated using a Vibra cell (Sonics, Connecticut, USA) for 10 min with a 1-second on and 2-second off cycle at a 30% pulse amplitude. The lysate was cleared by centrifugation at 10,000 rpm for 30 minutes at 4℃, after which the supernatant was transferred to a new 50 mL conical tube. The supernatant was added to 1.25 mL of Strep-Tactin Superflow Plus resin (Qiagen, Hilden, Germany), and the mixture was gently shaken at 4℃ for 2 hours to bind the Twinstrep-SUMO-huLwCas13a protein to the resin. The glass Econo-column® (BIO-Rad, Hercules, USA) was prepared by washing the column with cold lysis buffer (4 mL of Tris-HCl (1M, pH8.0), 20 mL of NaCl (5M), and 0.2 mL of DTT (1M), adjusted to a final volume of 200 mL with ddH_2O) on ice. The solution was poured and allowed to flow through the column. The column with the binding resin was washed by adding 18.75 mL of cold lysis buffer. Then, 3.75 mL of SUMO protease cleavage solution (3 μL of SUMO-protease, 7.5 μL of IGEPAL® CA-630, and 5 mL of lysis buffer) was added to the column and it was covered. The mixture was gently shaken at 4℃ for 16 h. The SUMO cleavage sample was collected in a new 50 ml conical tube, and 1.5 mL of glycerol was added. The LwCas13a protein was stored at −20℃.

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Colorimetric-based lateral-flow detection assay

For each Cas13-based detection reaction, a pre-cooled 1.5 mL microcentrifuge tube was used, and the following components were added:3.5 μL DEPC-treated water, 1.2 μL Tris-HCl (1M stock, pH 8), 6 μL $MgCl_2$ (20 mM stock), 0.8 μL NaCl (1M stock), 2 μL BVDV-1b cDNA, 2 μL LwCas13a, 1 μL Murine RNase inhibitor (40 U/μL stock; New England Biolabs, Massachusetts, USA), 0.6 μL T7 RNA polymerase (50 U/μL stock; Bioneer, Daejeon, Korea), each 0.5 μL rNTPs (ATP,GTP,CTP,UTP) (10 mM stock; Bioneer, Daejeon, Korea), and 0.4 μL RNA reporter (10 μM stock; Bioneer, Daejeon, Korea). And then, a 0.2 mL reaction tube containing 0.5 μL crRNA (1 μM stock) was added to the mixture to achieve a final volume of 20 μL. A negative control (NC) was prepared without crRNA. The reaction tubes were incubated at 37℃ for 6 hours to allow reaction of T7 transcription and collateral cleavage at the same time. To analyze the Cas13-based reaction using a lateral-flow strip, 80 μL of HybriDetect assay buffer was added to each reaction tube containing Cas13 enzyme and mixed thoroughly. And then, 25 μL of the detection solution was dropped onto the sample spot of a HybriDetect lateral-flow strip (Milenia Biotec, Gießen, Germany) allowing the reaction to

145 flow into the strips. We used gold nanoparticles (GNPs) provided by HybriDetect lateral-flow strip (Milenia Biotec,
146 Gießen, Germany).

147
Results

148 **The genome structure of BVDV-1b and its 5' UTR for crRNA design**

149 The RNA genome of BVDV-1b is approximately 12.5 kb in length containing a single open reading frame (ORF)
150 flanked by a 5' and 3' UTR (Deng et al. 1992; Colett et al. 1988; Zhang et al. 2014). As shown in Figure 1a, Five
151 structural proteins of BVDV (nucleocapsid; C/BVDV-encapsulated glycoprotein; Erns, E1, E2/ ion channel-related
152 protein; P7) were present in the N-terminal region. Six nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and
153 NS5B) are located in the C-terminal region. The BVDV protein sequence contained NH2-Npro-C-Erns-E1-E2-P7-
154 NS2-NS3-NS4ANS4B-NS5A-NS5B-COOH. BVDV presentation (processing) begins with structural/non-structural
155 protein degradation by Npro (autoprotease) in the N-terminal region. The 5' UTR of BVDV-1b was selected as the
156 molecular detection region due to sequence conservation. BVDV was classified into BVDV-1 and BVDV-2 based on
157 the nucleotide sequence of the respective 5' UTR region. As a result of observing MDBK cells infected with BVDV-
158 1b after 48 hours, the biotype of the virus was non-cytopathic (ncp).

159

160 **cDNA synthesis and design of crRNA, forward/reverse primer, direct repeat sequence, and RNA reporter**

161 The cDNA was synthesized by RT-PCR targeting the 5' UTR region of BVDV-1b, resulting in a 101 bp product
162 (Figure 1b). As shown in Figure 3a, the crRNAs did not overlap with the primers. The crRNAs were designed similarly.
163 The sequences of the primers and crRNA were designed relative to the target cDNA and transcribed RNA. A T7
164 promoter added to the 5′ end of the primer, and the crRNA sequence was the reverse complement of the target site in
165 the transcribed RNA. The DR of the LwCas13a crRNA was located at the 5' end of the spacer sequence, forming a
166 complex with the Cas13 enzyme (Figure 3b).

167 The HybriDetect lateral flow strip (Milenia Biotec, Gießen, Germany) used for the detection assays consisted of a
168 biotin ligand on the control line and an anti-rabbit antibody on the test line. Gold nanoparticles were coated on the
169 sample spot of the strip, which produced a color. The gold nanoparticle (GNP) can bind to FAM or the Anti-rabbit
170 antibodies on the test line. As a colorimetric-based lateral-flow detection assay (LFDA) probe, an RNA reporter was
171 labeled with FAM at the 5′ end of the poly U sequence and Biotin at the 3′ end (Figure 3c). Thus, the biotin of the

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172 RNA reporter bound to the biotin ligand on the control line of the strip, and the FAM of the RNA reporter bound to
173 the GNP (Figure 4a).
174

175 **Expression and Purification of LwCas13a Protein**

176 The pC013-Twinstrep-SUMO-huLwCas13a plasmid (#90097 Addgene, Watertown, USA) was digested into
177 fragments using Xho I. Rosetta^{TM} (DE3) was transformed with the plasmid, and single colonies were obtained for the
178 analysis of LwCas13a protein expression. The purified LwCas13a protein showed a single band on SDS-PAGE with
179 a molecular weight of 138.5 kDa (Figure 4d), indicating successful purification, and was expressed primarily in the
180 supernatant (Yao et al. 2021).
181

182 **Colorimetric-based lateral-flow detection assay**

183 To confirm the collateral cleavage activity of Cas13, we performed a colorimetric lateral-flow detection assay (LFDA).
184 The results of the colorimetric-based lateral-flow detection assay (LFDA) using the CRISPR-Cas13 system for
185 detecting BVDV-1b are shown in Figures 4b and 4c.

186 First, we tested the RNA reporter at a concentration of 0 μM, and only a band appeared on the test (T) line (Figure
187 4b). The gold nanoparticles (GNP) did not bind to the control (C) line without the RNA reporter. The Biotin-FAM
188 RNA reporter specifically binds to GNP. To avoid false positive and high dose hook effect (a state of antigen excess
189 relative to the antibody proves, resulting in falsely lowered values), we tested the RNA reporter at concentrations of
190 2 μM, 1 μM, 0.2 μM, 0.15 μM, 0.1 μM, and 0.05 μM using a lateral-flow detection assay (LFDA) with a paper strip.
191 All lateral flows with paper strips contained test (T) and control (C) bands. We found that a concentration of 0.2 μM
192 resulted in a true-negative readout.

193 Secondly, we verified that all four crRNAs could effectively detect BVDV-1b. As LwCas13a showed RNase collateral
194 activity, we designed a Cas13a-responsive RNA reporter that remained susceptible to ssRNA-mediated cleavage
195 (Figure 3c). The RNA reporter was labeled with biotin and FAM, allowing it to be captured on a lateral flow strip
196 with a biotin ligand and detected using anti-FAM antibodies conjugated to gold nanoparticles (biotin-ligand-FAM-
197 GNP). In the true-negative control (no crRNA), all the intact biotin-FAM-GNPs bound to the biotin ligand were
198 captured in the control (C) line. In a positive sample, the reporter is cut, releasing the FAM-containing fragment to be
199 captured by a second line of antibodies resulting in a "test band." Specific detection of the RNA target cleaves the
200 biotin-FAM reporter, allowing the production of two colored bands in the test (T) line (where anti-rabbit IgG binds to

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excess anti-FAM-GNPs). We tested the detection of BVDV-1b using a colorimetric lateral flow detection assay (LFDA) with the CRISPR-Cas13 system (Figure 4c). True-positive samples produced two colored bands in the control (C) and test (T) bands, which were distinguishable from the true-negative controls (main band at C). Analysis of BVDV-1b showed the specificity of the BVDV-1b gene detection.

Discussion

BVDV-1b is an economically significant viral disease in the national cattle industry. BVDV has spread quickly throughout farms, making prevention and control of the virus challenging (Gates et al. 2014; Pinior et al. 2017). Therefore, early POC testing is essential to prevent and control the spread of BVDV-1b. The nucleic acid detection method has the advantage of high sensitivity and is more sensitive and specific than the BVDV-1b antibody detection method. As a result, RT-PCR of viral RNA has been widely used in various countries to detect BVDV-1b. RT-PCR is regarded as the essential method for the diagnosis of BVDV-1b and has become the gold standard for diagnosis in the Republic of Korea. Therefore, Diagnosing BVDV-1b using RT-PCR has a limited ability to prevent and control the spread of BVDV-1b throughout the farm, it requires expensive equipment, such as a thermal cycler, and skilled personnel to conduct the experiments. POC testing has become an excellent supplement to the gold standard for diagnosis using RT-PCR in standard laboratories. Recently, a nucleic acid detection method based on CRISPR-Cas13a was developed as a new system for the early diagnosis of BVDV (Yao et al. 2021). According to several studies, nucleic acid detection based on the CRISPR-Cas13 system is a novel strategy for developing detection of RNA viruses for POC testing (Patchsung et al. 2020; Myhrvold et al. 2018; Gootenberg et al. 2017, 2018). Patchsung et al. (2020) validated the SHERLOCK method on 154 clinical COVID-19 samples and found it to be 100% specific, 96% sensitive with a fluorescence readout, and 88% sensitive with a lateral-flow readout. This method was able to detect SARS-CoV-2 in RNA extracts from nasopharyngeal and throat swab samples, including sputum samples, without any cross-reactivity to other common human coronaviruses, and was able to detect the virus in asymptomatic cases (Patchsung et al. 2020). Myhrvold et al. (2018) have validated the ability of SHERLOCK to detect DENV and ZIKV infections. All RT-PCR-positive ZIKV and DENV RNA samples were confirmed to be positive for ZIKV and DENV after 1hour of detection (Myhrvold et al. 2018).

BVDV strain was classified based on a variety of genetic variance in the 5′ UTR. Among the BVDV strains, BVDV-1b occurs frequently in the Republic of Korea. This information was obtained from the Animal and Plant Quarantine

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229 Agency. We found that all crRNAs detected were BVDV-1b. Hence, the nucleonic acid detection method using the
230 CRISPR-Cas13 system could be useful for POC testing of BVDV-1b as a novel strategy in the Republic of Korea.
231 To meet the World Health Organization's "ASSURED" criteria for diagnostic tests – affordable, sensitive, specific,
232 user-friendly, rapid, equipment-free, and deliverable –, several challenges must be addressed. Ideal POC testing
233 products must be characterized by miniaturization, automation, visualization of results, and rapid, high-precision, and
234 high-throughput detection. Several problems are associated with POC testing using the CRISPR-Cas13 system. First,
235 the SHERLOCK reagents, which are detection reagents from BVDV-1b, must be freeze-dried not only for cold chain
236 transportation but also for long-term storage or easy reconstitution on paper strips for field applications. Another
237 problem that combines LwCas13a collateral cleavage activity with lateral flow readings is disruption of the FAM-
238 biotin reporter gene. There is increasing evidence that combining Csm6 with LwCas13a detection can improve the
239 test stability and reduce the possibility of false-positive readings (Gootenberg et al. 2018).
240 As shown in Figure 4b and 4c, a faint line was observed on the lateral flow paper strip. Kim et al. (2019) observed
241 incomplete removal of the test line from the colorimetric assay results of the negative control, which could lead to
242 false-positive results. When using the traditional colloidal gold test strip for immunological testing, false-positive
243 results are commonly observed, and these results may have serious consequences that affect clinical diagnosis and
244 subsequent control measures (Kim et al. 2019). To reduce the false-positive rate of the ERASE assay, Li et al. (2021)
245 adjusted its interpretation mode and considered the disappearance of the T-band as the positive threshold. Beatrice et
246 al. (2022) empirically determined that positive samples exhibit band intensity ratios greater than 0.2 compared to
247 negative samples.
248 CRISPR-based nucleic acid detection, multi-silicon microfluidic chips, and multi-isothermal PCR-based molecular
249 diagnostic methods are being integrated into lab-on-a-chip platforms that utilize microfluidic and biosensor
250 technologies and are emerging as ideal platforms for POC diagnostics. Separate nucleic acid amplification steps
251 increase the assay time and complexity and introduce a cross-contamination risk during sample transfer to the CRISPR
252 reaction. Therefore, integrating CRISPR assay procedures into user-friendly devices, such as microfluidic chips or
253 lateral flow assays, allows for the provision of qualitative and quantitative readouts (Huang et al. 2023).
254 In this study, cDNA was synthesized using primers designed for BVDV-1b obtained from the Korea Veterinary
255 Culture Collection (KVCC). The crRNAs were designed to have high specificity for the target pathogen. The Cas13
256 enzyme was expressed and purified. For detection, the BVDV-1b target cDNA, crRNA, Cas13 enzyme, and RNA
257 reporter were subjected to collateral cleavage reaction at 37℃. Lateral flow cytometry analysis showed that crRNAs

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258 1, 2, 3, and 4 were positive for BVDV-1b. If CRISPR-Cas13-based detection is conducted, BVDV-1b can be rapidly
259 and accurately diagnosed, even in samples collected under harsh conditions with a high risk of contamination. We
260 propose that our results could serve as a new POC test at the DNA level for detecting nucleic acid of the BVDV-1b,
261 which frequently occurs in the Republic of Korea. In the next study, we will conduct experiments using clinical
262 samples.

263

264

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Table

Table 1. Sequence of primers, crRNA, and Reporter used in this study.

Name	Sequence (5'→ 3')
BVDV-1b primer	Forward: GCCATGCCCTTAGTAGGACT
	Reverse: (T7 promoter sequence)/CGAACCACTGACGACTACCC
crRNA 1	(DR seq)/CAGUGGUGAGUUCGUUGGAUGGC
crRNA 2	(DR seq)/ACAGUGGUGAGUUCGUUGGAUGGp
crRNA 3	(DR seq)/AGUGGUGAGUUCGUUGGAUGGCU
crRNA 4	(DR seq)/GGGGUAGCAACAGUGGUGAGUUC
DR seq	GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAAC
RNA reporter	FAM/ UUUUUUUUUUU /Biotin

BVDV: Bovine Viral Diarrhea Virus, crRNA: CRISPR RNA, DR seq: Direct repeat

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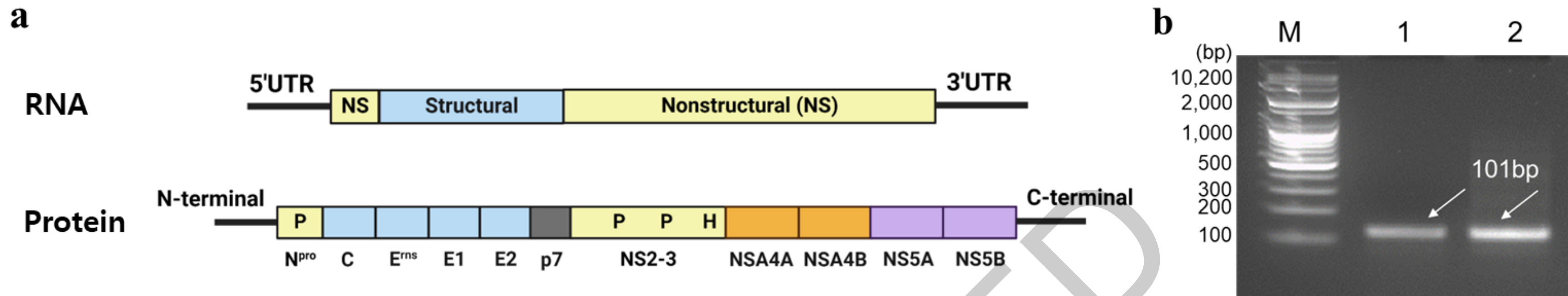

Figure 1. The Open Reading Frame of BVDV-1b. a, The ORF encodes a precursor polyprotein of about 3,900 amino acids, which is subsequently processed by viral or cellular proteases into 11 or 12 individual proteins including Npro, C, Erns, E1, E2, p7, NS2/3, NS4A, NS4B, NS5A, and NS5B from the N terminus to the C terminus (Colett MS et al. 1988; Tautz N et al. 1997; Rumenapf T et al. 1993). The C, Erns, E1, and E2 are four structural proteins, and the remains are nonstructural viral proteins (Rumenapf T et al. 1993; Lindenbach BD et al. 2003). Out of the four structural proteins, E2 has a mass of 55 kDa and is classified as a type I transmembrane protein, which is associated with virus entry, viral pathogenicity, and immunity. Erns is a structural glycoprotein that possess the intrinsic ribonuclease activity involved in virus attachment and entry into target cells. **b,** Electrophoresis of cDNA from BVDV-1b by RT-PCR using designed primers. RT-PCR product size 101bp. M, DNA ladder; 1 and 2, BVD virus target cDNA.

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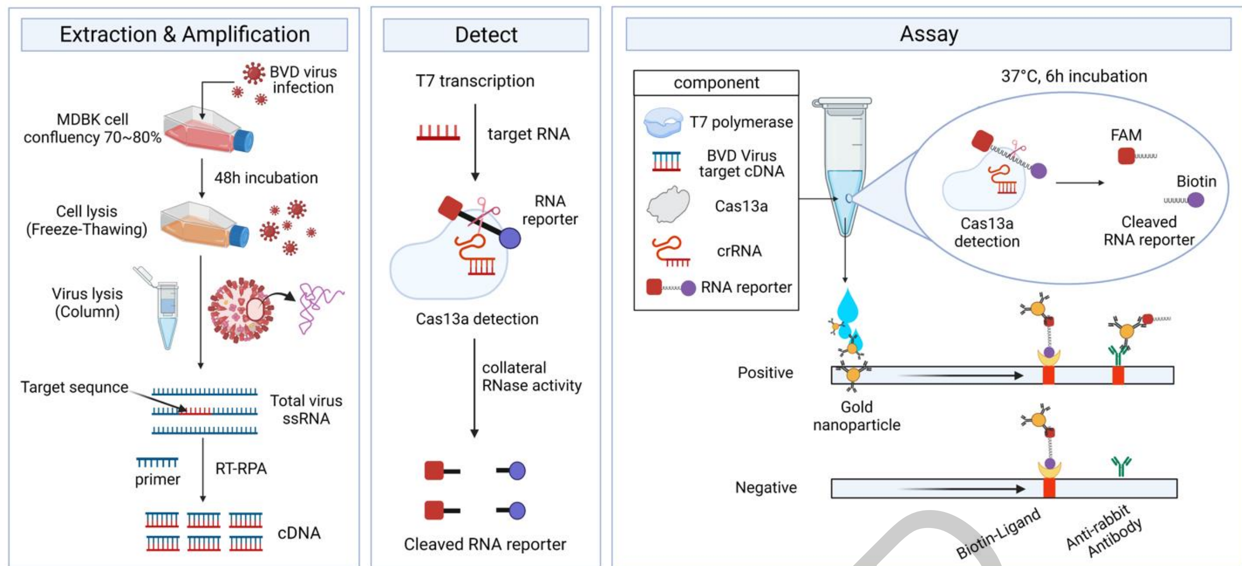

Figure 2. Detection of BVDV-1b RNA. Cas13 complex and collateral activity experimental workflow. A BVDV-1b RNA region of interest is amplified to DNA by RT–PCR, then converted to RNA by T7 transcription. Cognate binding of Cas13a–crRNA complex to amplified RNA targets triggers collateral activity of Cas13a, which cleaves RNA reporters. Cleaved RNA reporters can be captured on a colorimetric lateral-flow strip. Predicted colorimetric outcomes for negative and positive samples.

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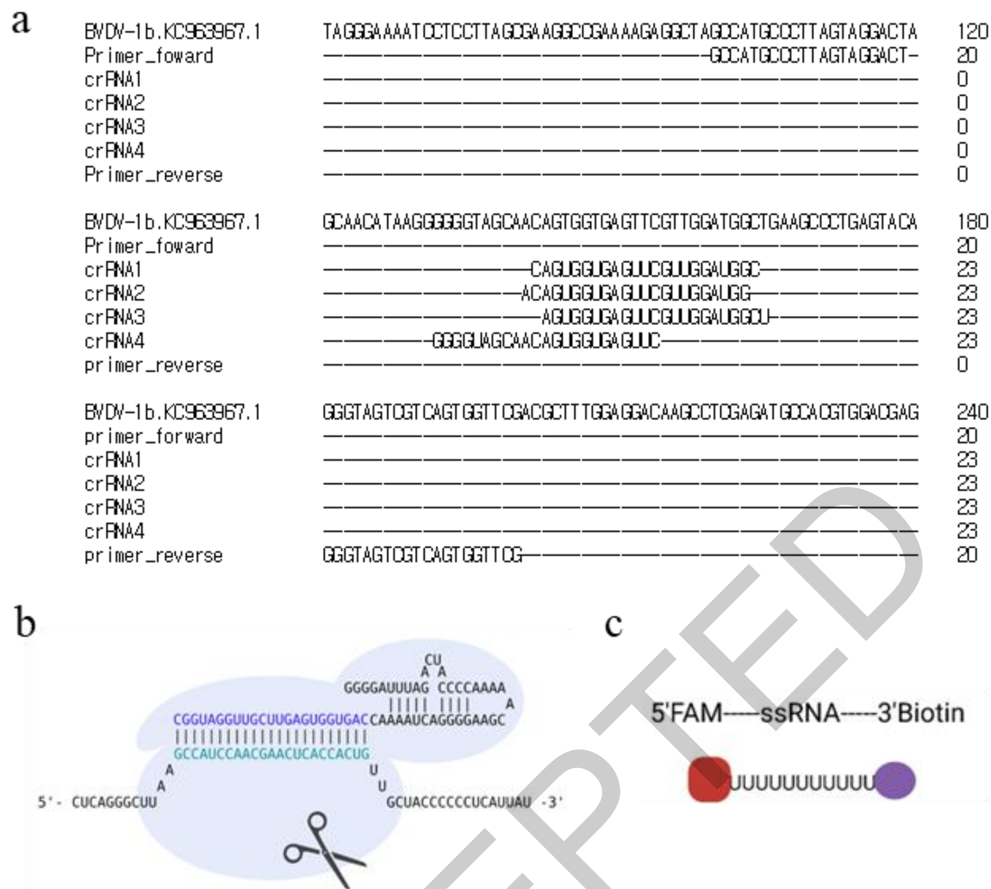

Figure 3. Specificity for BVDV-1b of the CRISPR-Cas13 system. a, Alignment of primer and crRNA designed for BVDV-1b. **b,** Schematic of Cas13 enzyme activity with crRNA1. **c,** Schematic of the designed RNA reporter. FAM and Biotin are labeled at both ends of the ssRNA.

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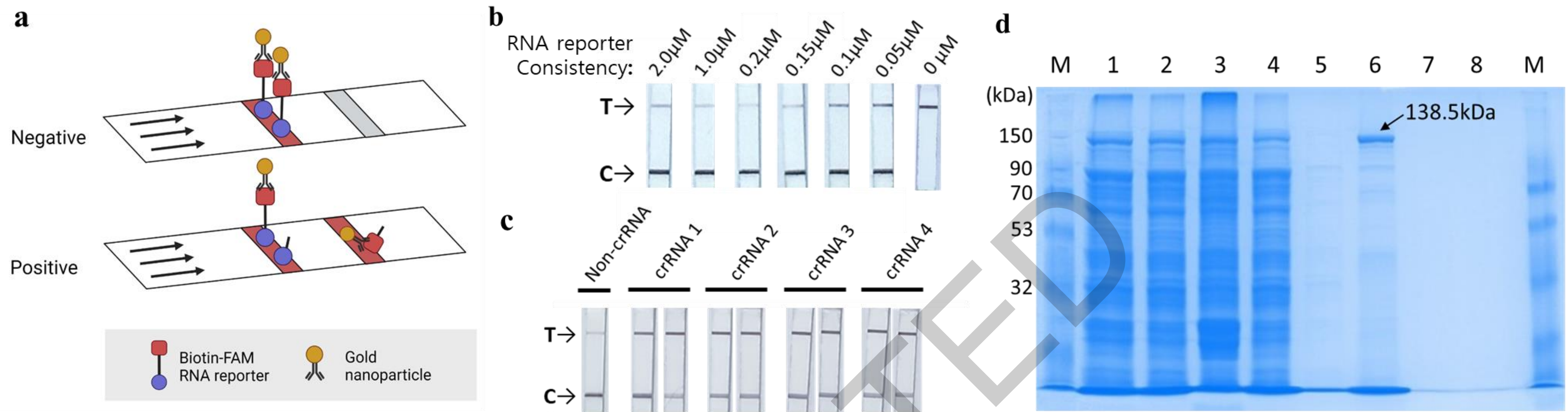

Figure 4. Lateral flow detection assay for point-of-care test. a, Lateral-flow strip result schematic diagram. In the case of a negative result, one band appears, in the case of a positive result, two bands appear. **b,** Lateral-flow strip results according to RNA reporter concentration. **c,** Colorimetric-based lateral-flow detection assay results for BVDV-1b; T, test-line; C, control-line. **d,** Coomassie Blue-stained SDS-PAGE gel of LwCas13a protein. The progress of protein purification is shown, and we confirmed the presence of LwCas13a with a molecular weight of 138 kDa. M, Protein marker; 1, Cell lysate; 2, Supernatant of centrifuged cell lysate; 3, Pellet obtained after centrifugation of cell lysate; 4, Flow-through following Strep-Tactin binding; 5, Strep-Tactin resin before SUMO cleavage; 6, Eluted fraction post SUMO cleavage; 7 and 8, Washing column.

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