Electronic Supplementary Information for

A Novel Biosensor for Highly Sensitive DNA Damage Detection Using TdT and CRISPR-Cas12a

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1.Materials and methods

1.1 Oligonucleotides and Reagents

All DNA and RNA oligonucleotides were synthesized by Sangon Biotech (Shanghai,China), and their sequences are listed in the Supplementary Information (Table S1). LbCas12a, TdT, Nt.AlwI, SnaB I, Buffer 1, Buffer 2.1, Buffer 3.1, Buffer 4, and other reagents were purchased from New England Biolabs (NEB, USA). The DNA extraction kit (DP304) was obtained from TIANGEN Biotech (Beijing, China).

1.2 Source of Samples

Semen samples were collected between June 2024 and November 2024 from patients attending the Reproductive Medicine Center at the General Hospital of Ningxia Medical University and the Yinchuan Maternal and Child Health Hospital. Written informed consent was obtained from all participants, and ethical approval was granted by the Ethics Committee of the General Hospital of Ningxia Medical University (Approval No. KYLL2022-1081). The criteria for normal semen parameters were based on the WHO Laboratory Manual for the Examination and Processing of Human Semen (6th Edition)[1], which defines: semen volume ≥1.5 mL, total sperm count ≥39 × 10^6/mL, progressive motility ≥32%, and total motility ≥40%. Asthenozoospermia was defined as progressive motility <32%. The recurrent pregnancy loss (RPL) group was defined according to guidelines from the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM)[2], which specify two or more consecutive pregnancy losses. Inclusion criteria required couples to have conceived naturally within one year prior to the study and experienced spontaneous miscarriage during the first trimester. Female factors, such as chromosomal abnormalities, antiphospholipid antibody disorders, and infections, were excluded. The healthy fertile male control group consisted of men with confirmed fertility, defined as having fathered at least two full-term live births, the most recent of which occurred within one year prior to enrollment. None of the participants in the control group had a history of recurrent pregnancy loss.

1.3 DNA Extraction

DNA was extracted from semen samples using the DP304 Cell Genome Extraction Kit (TIANGEN Biotech, Beijing, China) in accordance with the manufacturer's protocol [3].

1.4. MDB Detection

1.41 Breakpoint Extension

DNA was adjusted to a concentration of 5 ng/μL for breakpoint detection. A 15 μL reaction mixture was prepared, containing 1× TdT buffer, 1× $CoCl_2$, 0.1 mM dATP, 0.5 U TdT, and the substrate DNA. The reaction was incubated at 37°C for 30 minutes, followed by heat inactivation at 85°C for 10 minutes.

1.42 Fluorescence Reporting

A total reaction volume of 50 μL was prepared by adding a Cas12a/crRNA system to the 15 μL reaction mixture from the previous step. The Cas12a/crRNA system consisted of 100 nM Cas12a, 200 nM crRNA, magnesium chloride, a fluorescent probe, and 1× NEBuffer. The final reaction was conducted at 37°C. The PCR program was set as follows: 37°C for 30 seconds, repeated for 60 cycles.

1.43 Calculation Methods

We constructed a linear equation using standard concentrations of single-stranded DNA (ssDNA) to correlate the number of 3'-OH groups with fluorescence values. This equation exhibited excellent linearity within the range of 0.001–0.2 nM. Using this linear equation, the fluorescence value (Y) obtained after adding sperm DNA into the system was substituted into the equation to calculate the corresponding number of 3'-OH groups (X) for the sperm sample. Based on the value of X (C_0), further analysis was performed to determine the Mean DNA Breaks (MDB) for individual sperm DNA.

$$n \times m \times NA \times (M_0 \times N)^{-1} = C_0 \times V_0 \times NA$$

Where:

n: Mean number of sperm DNA breakpoints (MDB)

m: Total mass of sperm in the reaction system (g)

C_0: Concentration of single-stranded DNA (ssDNA) (nM)

NA: Avogadro's constant, which is 6.02×10^{23}

M_0: Average molecular weight of a single base, 325 g/mol

N: Number of bases contained in a complete sperm cell DNA, which is 15×10^{8}

V_0: Total volume of the reaction system (L)

The left side of the formula ($n \times m \times NA \times (M_0 \times N)^{-1}$) represents the total number of 3'-OH

(i.e., breakpoints) contained in the sperm DNA sample with mass m.

The right side of the formula ($C_0 \times V_0 \times NA$) represents the number of 3'-OH at the end of a single-stranded DNA at concentration C_0.

1.5 ROS and Sperm

Sperm cells were incubated in a culture medium containing 100 μM hydrogen peroxide for 15, 30, 45, and 60 minutes. After incubation, DNA was extracted from the sperm cells and subjected to breakpoint detection as described in Section 1.4.

1.6 SSCs (Spermatogonial Stem Cells) MDB Detection

Spermatogonial stem cells (SSCs), previously cultured by the research group, were used in this experiment [4]. The SSCs were incubated at 42°C for 30 minutes and 60 minutes, after which cellular DNA was extracted. The extracted DNA was adjusted to a concentration of 10 ng/μL and subjected to breakpoint detection using the same method described in the "MDB Detection" section (Section 1.4).

1.7. Statistical Analysis.

All sperm parameters were expressed as mean ± standard deviation (SD), with statistical significance defined as $P \leq 0.05$. The mean number of sperm DNA breaks (MDB) per sperm was compared between the normal sperm group and the asthenospermia group, as well as between the healthy male group and the Recurrent pregnancy loss group, using an unpaired Student's t-test. Data analysis was performed with SPSS software version 25.0. For comparisons across multiple groups, one-way analysis of variance (ANOVA) was used, followed by Tukey’s post hoc test to evaluate significant differences.

Table S1. Sequences of the oligonucleotide probes in the biosensor

Name	Sequence (5'→ 3')
Target-1	AGTGTAGAGGGTTAGAGTTTGGAAAGTAAAGTAAAGAGGTGT
S-001	AAA AAA AAA AAA AAA AAA AAA AAA
crRNA	UAAUUUCUACUAAGUGUAGAUUUUUUUUUUUUUUUUUUUU
Probe-1	FAM-TTTTTT-BHQ-1
Probe-2	FAM-TTT ATT ATT T-BHQ-1
Probe-3	FAM-TTT ATT TAA TTA TAT-BHQ-1
Probe-4	FAM-TTT ATT TAT TTT ATT TTT AT-BHQ-1
Probe-5	FAM-TTT ATT TAT TTT ATT TTT ATT TTT A-BHQ-1
Probe-6	FAM-TTT ATT TAT TTT ATT TTT ATT TTT AAT TAA-BHQ-1
99bp-1	GAG AAG GAT CAG ACT GTA TCA GGT TGT GAG TAG TAG GAA ATG TAG AAG GGA TCA CAT GGA ATG AAA GTA AAG TAA AGA GTT GTG GTA TGG TTA GTT CAT
99bp-2	ATG AAC TAA CCA TAC CAC AAC TCT TTA CTT TAC TTT CAT TCC ATG TGA TCC CTT CTA CAT TTC CTA CTA CTC ACA ACC TGA TAC AGT CTG ATC CTT CTC

Table S2. Experimental Results on the Recovery Rate of Sperm DNA Breakpoint Detection by the biosensor (n=10)

Name	Recovery Rate (%)	Name	Recovery Rate (%)
NO.1	93.34	NO.6	89.01
NO.2	85.96	NO.7	106.5
NO.3	102.91	NO.8	86.78
NO.4	97.43	NO.9	107.12
NO.5	104.42	NO.10	106.56

Table S3. The MDB of sperm DNA with 25 repeats.

Test	1	2	3	4	5	Mean	Standard deviation	Intraday RSD (%)
Day 1	6.37	6.21	6.33	6.04	6.15	6.22	0.12	1.93
Day 2	6.23	6.52	6.86	7.01	6.31	6.59	0.30	4.62
Day 3	6.39	6.7	6.35	6.25	6.11	6.36	0.20	3.07
Day 4	6.79	7.18	6.44	6.53	7.30	6.85	0.34	4.99
Day 5	6.53	7.11	6.57	7.04	6.29	6.71	0.32	4.70

For the same sample, a total of 25 repeated experiments were conducted over 5 days, and the interday relative standard deviation (RSD) was calculated to be 3.48%.

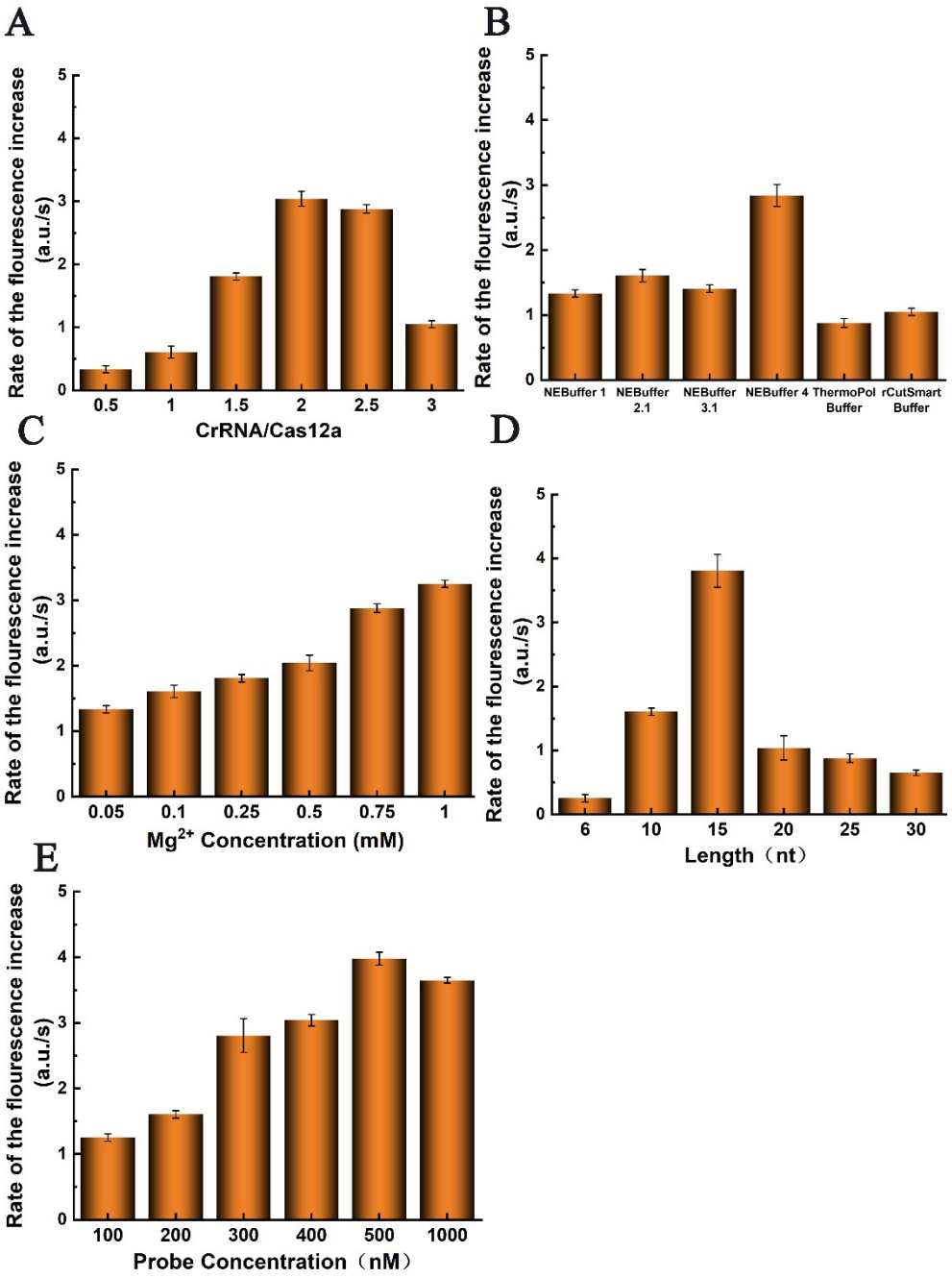

Figure S1. Optimization of the Cas12a Reaction System

Optimization of reaction conditions using S-001 (AAA AAA AAA AAA AAA AAA AAA AAA, 1 nM) as the substrate strand for binding with crRNA.

(A) Effect of Cas12a/crRNA ratios on activity. (B) Effect of buffer types on reaction rate.

(C) Effect of Mg^{2+} concentration on activity. (D) Effect of probe length on activity.

(E) Effect of probe concentration on activity.

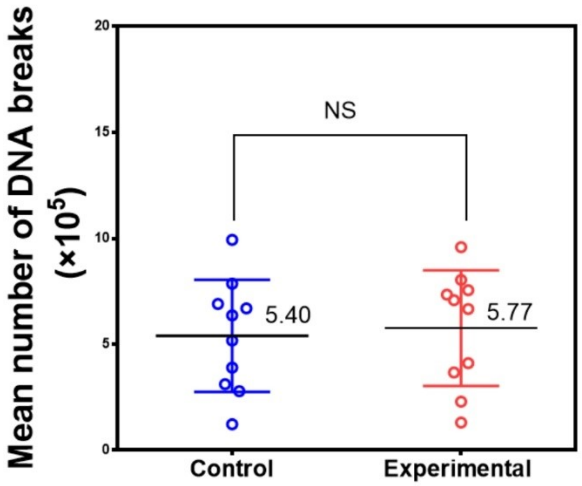

Figure S2. Comparison of MDB (Mean number of DNA Breaks) values in sperm samples subjected to one or two rounds of extraction. The difference was not significant (NS).

References

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