A Non-enzymatic, Isothermal Amplification Sensor for Quantifying the Relative Abundance of *Akkermansia Muciniphila*

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Materials and methods

Reagents

HEPES, $MgCl_2$, KCl, NaCl, tris (aminomethane), and sodium citrate were purchased from Sinopharm Chemical Reagent Co., Ltd. in Shanghai, China. High-fat feed were purchased from Research Diets Corporation, USA. All chemicals were used without further purification. Gel Red was purchased from Biotium in California, USA. C57BL/6J mice were supplied by Beijing Vital River Laboratory Animal Technology Co., Ltd. Antibiotics were purchased from Sangon Biotechnology Co. Ltd. in Shanghai. All oligonucleotides were synthesized and purified in the same company. The DNA hairpin probe H2 was labelled with the fluorescent molecule FAM in the middle and the quenching agent molecule BHQ1 at the 3' end. 20 mM HEPES buffer solution (pH 7.4, 8 mM Mg^{2+}, 10 mM K^+, 200 mM Na^+) was used for DNA hybridization. All solutions were prepared with ultrapure Millipore water (18.2 MΩ cm).

Table 1: DNA sequences used in this study.

ssDNAs	Base sequence (5'-3')
T	CCTTGCGGTTGGCTTCAGATACT
M	CGACATCTAACCTGGGACACTGACAGTATCTGAAGC CAACCGCAAGGGTCAGTGTCC
H1	GTCAGTGTCCCAGGTTAGATGTCGCCATGTGTAGAC GACATCTAACCTGGGTAGGGCGGGTT
H2	AGATGTCGTCTACACATGGCGACATCTAACCTCCCCCATGTGTAG A
M2	CCTTACGGTTGTCTTCAGATACT
M4	CATTGCGGTTTGCTGCAGCTACT
M6	ACTTGCTGTTAGTTTCCGGTACT
reference	Forward TGSTGCAYGGYTGTCGTCA

	reverse	ACGTCRTCCMCACCTTCCTC
MUC-1437	Forward	CAGCACGTGAAGGTGGGGAC
	reverse	CCTTGCGGTTGGCTTCAGATACT

Target-triggered enzyme-free amplification recycling

The target-triggered enzyme-free amplification recycling was performed using hairpin probes (M, H1, and H2) and target DNA (T) in a HEPES buffer. Firstly, M, H1, and H2 were heated to 95°C for 5 minutes each and then slowly cooled to 4°C to form the hairpin structures. Secondly, T was added to the mixture of M (1.5 μM), H1 (1.5 μM), and H2 (1.5 μM) in the HEPES buffer at 4°C for a 2-hour target-triggered enzyme-free amplification recycling.

Gel electrophoresis verification

Gel electrophoresis samples including H1 (1.5 μM), H2 (1.5 μM), and T (1.5 μM) were reacted in the HEPES buffer at 4°C for 2 hours. Then, 10 μL of these samples were mixed with 2 μL of loading buffer and loaded into a 15% natural polyacrylamide gel. Electrophoresis was conducted in 1× TBE buffer (89 mM Tris, 89 mM boric acid, 2.0 mM EDTA, pH 8.3) at 120V for 80 minutes. After staining with Gel Red, the gels were imaged using a FluorChem FC3 under 365 nm light.

Fluorescence assay

In the HEPES buffer, T was added to a mixture containing M (1.5 μM), H1 (1.5 μM), and H2 (1.5 μM) and hybridized at 4°C for 2 hours. The target DNA strand T was set at different concentrations (1 aM to 500 nM), and in the control group, T was replaced with an equal amount of ultrapure Millipore water and processed the same way. The excitation-emission of the labeled fluorophore FAM was then detected using ex/em = 494 nm/520 nm. All experiments were repeated at least three times.

Target-specific assays

To verify the specificity of the method, the bases of the target DNA (T) were mutated and three sets of sequences were designed, with two base mutations (M2), four base mutations (M4), and six base mutations (M6) from the target sequence as controls. T and M2, M4, M6 were placed in the reaction system described above and reacted at 4°C for 2 hours, respectively, and the fluorescence intensity signal was detected. All experiments were repeated at least three times.

Modelling process

Eighteen male mice were used: C57BL (Beijing Vital River Laboratory Animal Technology Co., Ltd.). At the time of test, the mice were 6-8 weeks old and had body weights of 22.7 ± 0.8 g (mean ± SEM). The mice were adaptively fed for one week. All mice were randomly divided into three groups, namely control group, the antibiotic group (ATB) and the high-fat diet group (HFD). Each group consisted of six mice. All mice were housed in static cages at room temperature with free access to food and water. The ATB group received a normal diet, and the gavage dose of antibiotics for each mouse is as follows: metronidazole is 0.05 g/kg/day, neomycin sulfate is 0.05 g/kg/day, ampicillin is 0.05 g/kg/day, and vancomycin is 0.025 g/kg/day. The administration of HFD is free eating and feeding is continued for twelve weeks. Record the weight changes of mice every week (Fig. S1). All animal experiments were approved by the Institutional Animal Care and Use Committee of Zhejiang Provincial People's Hospital (Approval No.: 20230801142554866622).

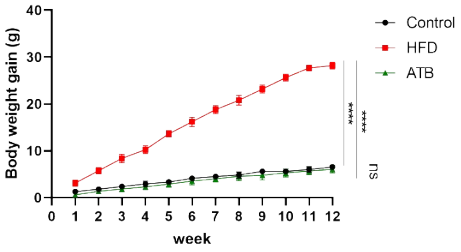

Figure S1: The body weight information for mice fed by a normal diet (control), antibiotic group (ATB) and the high-fat diet group (HFD). *P＜0.05.

Analysis of fecal samples from mice

Fecal genomic DNA was extracted from mice in the normal, high-fat diet, and antibiotic groups using the SPINeasy DNA Kit for Feces. The extracted genomic DNA was then replaced with 10× SSC buffer (300 mM NaCl, 30 mM $Na_3Citrate\text{-}2H_2O$). The buffer was replaced, and the concentrations were measured and diluted to 0.005 ng/μL, 0.002 ng/μL, and 0.001 ng/μL, respectively. The treated genomic DNA was mixed with 1.5 μM M at 55°C for 1 hour, kept at 4°C for 1 hour, and then mixed with equal amounts of H1 (1.5 μM) and H2 (1.5 μM).

qPCR assay

qPCR amplification and detection were performed with optical-grade 96-well plates in an LightCycler96 - PCR sequence detection system (Roche, Switzerland). The analysis was performed using Taq Pro Universal SYBR qPCR Master Mix (Vazyme). The primer sequences are shown in the table above. Each PCR was performed in a final volume of 20 μL. PCR conditions were as follows: 3 min at 95°C, followed by 40 cycles of 95°C for 10 sec and 30 sec at 60°C. Analysis of melting curves was made to distinguish the specific PCR product from primer-dimers or other artifact. Subsequently, obtained data were normalized to 16S rRNA gene sequence of *A. muciniphila* as a housekeeping standard. Fold changes were analyzed using the $2^{-\triangle\triangle CT}$ method.