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Ultrasensitive Detection of Echinococcus multilocularis Circulating Free DNA in Plasma Using a One-Pot CRISPR/Cas12a-Rolling Circle Amplification Assay

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We use this protocol and it's working

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Disclaimer

Abstract

This protocol describes a one-pot, isothermal detection system based on CRISPR/Cas12a and Rolling Circle Amplification (RCA) for the ultrasensitive and specific detection of Echinococcus multilocularis-derived circulating free DNA (cfDNA) in human plasma. The entire reaction is completed within 30-60 minutes at 37°C, with a detection limit as low as 1.41 aM. It requires no complex instrumentation and is suitable for the early non-invasive diagnosis and point-of-care testing of alveolar echinococcosis.

Materials

2. Materials 26 Reagents

2.1 Samples and Nucleic Acids
- cfDNA extracted from human plasma (recommended kit: QIAamp MinElute cfDNA Kit or equivalent)
- Synthetic E. multilocularis Em-28S cfDNA standard (for standard curve and sensitivity testing)
- Nuclease-free water

2.2 Enzymes and Reaction Reagents
- T4 DNA Ligase (1000 U/μL, e.g., NEB)
- Phi29 DNA Polymerase (0.1 U/μL, e.g., Thermo Fisher Scientific)
- Cas12a (LbCas12a) protein (e.g., from Integrated DNA Technologies or NEB)
- crRNA (designed against the Em-28S fragment, sequence as per original paper, synthesized by a vendor)

2.3 Probes and Reporters
- Padlock Probe (sequence as per S4 Table in the original paper, 5'-phosphorylated)
- Fluorescent Reporter Probe (e.g., FAM-TTATT-BHQ1)

2.4 Buffers
- 10x T4 DNA Ligase Reaction Buffer
- 10x Phi29 DNA Polymerase Reaction Buffer
- NEBuffer 2.1 (or other compatible Cas12a reaction buffer)

2.5 Equipment
- Constant temperature metal bath or PCR machine (capable of maintaining 37°C)
- Real-time Fluorescence Quantitative PCR instrument (for real-time monitoring, e.g., MA-6000)
- OR a portable blue/UV light source (for visual end-point detection)
- Microcentrifuge
- Vortex mixer
- Pipettes and nuclease-free tips

Additional Reagents:
- dNTP Mix (25 mM)
- BSA (10 mg/mL)
- ATP (10 mM)
- DTT (1 M)

Safety warnings

Critical: Prepare the reaction mix on ice to prevent non-specific amplification.

No or Weak Signal: Check enzyme activity; confirm the reaction temperature is correctly set at 37°C; optimize concentrations of the padlock probe and crRNA.

High Background Signal: May indicate reagent contamination. Use fresh batches of reagents and ensure a clean working environment.

Brief Principle

1 Hybridization and Ligation: A padlock probe, designed to target a specific fragment of the E. multilocularis 28S rDNA gene, hybridizes to the target cfDNA and is circularized by T4 DNA Ligase.

2 Rolling Circle Amplification: Phi29 DNA polymerase uses the circularized probe as a template for isothermal RCA, generating long tandem repeat DNA products.

3 CRISPR/Cas12a Detection and Signal Amplification: The pre-mixed Cas12a/crRNA complex recognizes and binds to specific sequences within the RCA product, activating its "trans-cleavage" activity. This indiscriminately degrades fluorescent reporter molecules (e.g., FAM-BHQ1), producing a fluorescent signal. Cas12a's "cis-cleavage" activity also cleaves the long RCA products, generating new primers to initiate secondary RCA cycles, resulting in exponential signal amplification.

Procedure

4 Critical: Prepare the reaction mix on ice to prevent non-specific amplification.

5 Step 1: Reaction Setup (Perform on ice)
In a sterile, nuclease-free PCR tube, add the following components in the order listed to prepare a 25 µL one-pot reaction mix:
Nuclease-free Water to 25 µL
10x Phi29 Reaction Buffer 2.5 µL, 1x final concentration
Padlock Probe (100 nM) 2.5 µL, 10 nM final concentration
dNTP Mix (25 mM) 0.5 µL, 0.5 mM final concentration
BSA (10 mg/mL) 2.0 µL, 0.8 mg/mL final concentration
DTT (1 M) 0.5 µL, 20 mM final concentration
Fluorescent Reporter Probe (10 µM) 1.0 µL, 400 nM final concentration
Cas12a protein (1 µM) 1.0 µL, 40 nM final concentration
crRNA (10 µM) 1.0 µL, 400 nM final concentration
T4 DNA Ligase (1000 U/µL) 0.5 µL, 20 U/µL final concentration
Phi29 DNA Polymerase (0.1 U/µL) 0.1 µL, 0.0004 U/µL final concentration
Template (cfDNA or Standard) 5.0 µL

6 Step 2: Reaction Incubation and Signal Detection
Gently vortex the reaction mix and briefly centrifuge to collect the liquid at the bottom of the tube.
Place the reaction tube in a pre-heated (37°C) real-time PCR instrument or a constant temperature metal bath.

Real-time Fluorescence Detection (Recommended): Incubate at 37°C and acquire fluorescence signal (FAM channel) every 30 seconds for 50-60 minutes.
Visual Observation (End-point): After incubating at 37°C for 50 minutes, place the tube under a blue or UV light. A distinct green fluorescence indicates a positive result.

7 Step 3: Data Analysis
Real-time Fluorescence Data: Use the instrument's software to analyze the amplification curves. Set a threshold line; the time (Ct value) at which the fluorescence signal crosses the threshold has a linear relationship with the logarithm of the template concentration, allowing for quantitative analysis.
Visual Observation: Record results directly as positive or negative.

Expected Results

8 Sensitivity: Using serially diluted synthetic standards (100 pM to 1 aM), the assay should reliably detect concentrations as low as 1.41 aM.
Specificity: The assay should be highly specific for the E. multilocularis Em-28S sequence. There should be no cross-reactivity with cfDNA or miRNAs from non-target parasites (e.g., Taenia solium, Clonorchis sinensis).
Clinical Validation: When testing clinical samples including healthy controls, other liver disease controls, and AE patients, the assay should demonstrate high sensitivity (approx. 87.5%) and high specificity (approx. 96.9%).
Amplification Curves: Positive samples should show a characteristic "S"-shaped fluorescence amplification curve, while the negative control (e.g., no-template control) should yield a flat curve.

Notes & Troubleshooting

9 Negative Controls are Crucial: Always include a no-template control (NTC) in each run to monitor contamination.
Prevent Contamination: Physically separate reagent preparation, sample handling, and amplification areas. Use filter tips.
No or Weak Signal: Check enzyme activity; confirm the reaction temperature is correctly set at 37°C; optimize concentrations of the padlock probe and crRNA.
High Background Signal: May indicate reagent contamination. Use fresh batches of reagents and ensure a clean working environment.