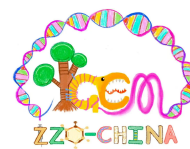


Determination of HCR Amplification Fold

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Contents

1	Determination of HCR Amplification Fold	2
1.1	Materials	2
1.2	Protocol	2



1 Determination of HCR Amplification Fold

1.1 Materials

- TH1 hairpin probe (2.5 μ M stock)
- TH2 hairpin probe (2.5 μ M stock)
- Trigger strand (250 nM or higher stock)
- DEPC-treated water (RNase/DNase-free)
- Microcentrifuge tubes (1.5 mL, RNase/DNase-free)
- Constant-temperature incubator
- Fluorescence microplate reader (Ex = 490 nm, Em = 520 nm; detection temperature 37 °C)
- 384-well or 96-well microplate
- 10 \times CRISPR reaction buffer (with Mg²⁺)
- Cas12a protein (1 μ M)
- crRNA (250 nM)
- F-Q probe (10 μ M)
- Ice box and precision pipettes with sterile tips

1.2 Protocol

1.2.1 Prepare HCR Reaction

Purpose: To perform HCR amplification under optimal conditions and subsequently measure amplification fold via CRISPR fluorescence readout.

HCR Reaction System (20 μ L total volume per tube):

Component	Stock Concentration(μ M)	Volume(μ L)	Final Concentration(μ M)
TH1	2.5	8	1
TH2	2.5	8	1
Trigger	250	4	50

1. Mix gently and incubate at 25 °C for 10 minutes to complete the HCR reaction.



2. Place tubes on ice after incubation.

1.2.2 Build CRISPR Standard Curve

Prepare non-hybridized TH2 at concentrations of 1, 5, 50, 100, and 500 nM. These will serve as standards for RFU–concentration calibration.

Run CRISPR reactions for each concentration to generate a standard curve for equivalent concentration conversion.

1.2.3 CRISPR Readout

CRISPR Detection System (40 μ L total volume per well):

Component	Concentration(μ M)	Volume(μ L)
DEPC-treated water	—	13
10 \times CRISPR Buffer	—	4
crRNA	1	1
F-Q probe	10	1
Cas12a protein	1	1
Sample (HCR product or standard)	—	20 μ L (final 40 μ L)

Incubate reactions at 37 $^{\circ}$ C and measure fluorescence every 0.5 minutes for 30 minutes using Ex = 490 nm, Em = 520 nm.

1.2.4 Data Analysis

1. Plot RFU (30 min endpoint) versus known concentration for the standard curve and fit using linear regression.
2. Convert RFU of HCR samples into equivalent target concentration (C_{eq}) using the fitted curve.
3. Calculate amplification fold as Amplification Fold = C_{eq} / C_{true} , where C_{true} is the actual trigger concentration (e.g., 1, 5, 50 nM).
4. Report mean \pm SD from ≥ 3 replicates.
5. Determine positive threshold as NTC mean + 3SD; samples below threshold are considered invalid.