

PROTOCOL

1. Prepare the Complete Hydrolysis Reference (1×)

Dilute the supplied 100× CHR stock 1:100 in Reaction Buffer. Use either preparation below:

Preparation	100× CHR stock	Reaction Buffer	Final 1× volume
Full-vial preparation	50 µL	4950 µL	5 mL
Small-volume preparation	10 µL	990 µL	1 mL

Mix gently. Aliquot the prepared 1× reference into appropriate single-use volumes. Store the prepared aliquots and any remaining unused 100× stock at –20°C. Avoid repeated freeze-thaw cycles. Include CHR wells for each assay for activity quantification.

2. Prepare the Chromogenic Substrate Working Solution (1 mM)

Dilute the 100 mM substrate stock 1:100 with Reaction Buffer immediately before the assay. For example, combine 10 µL substrate stock with 990 µL Reaction Buffer to prepare 1 mL of 1 mM working solution.

Dispense 50 µL of the resulting 1 mM substrate working solution per well, corresponding to 50 nmol substrate per reaction. Protect substrate solutions from unnecessary light exposure and use freshly prepared working solution.

3. Prepare Test Samples

For purified trypsin, a concentration of 2 µg/mL is recommended for the 50 µL sample used in each assay reaction, prepared by dilution in the supplied Reaction Buffer. For example, a 1 mg/mL trypsin sample requires a 500-fold dilution to reach this concentration.

For non-purified or complex samples containing trypsin, evaluate several dilutions experimentally. Select a dilution producing a clearly measurable, approximately linear initial increase in A405 without rapid substrate depletion or excessive sample background. Include a matched sample blank where the matrix contributes appreciably to absorbance or background drift.

4. Set Up the Assay

Well type	Add first	Initiate with
Test sample	50 µL diluted sample	50 µL 1 mM substrate working solution
Complete Hydrolysis Reference	50 µL of 1× CHR	50 µL 1 mM substrate working solution
Substrate blank	50 µL Reaction Buffer	50 µL 1 mM substrate working solution

Use a clear, flat-bottom, 96-well microplate compatible with 405 nm absorbance measurements. Run duplicate or triplicate wells where practical. Equilibrate the reagents and plate to room temperature (25°C) and perform the assay at 25°C.

5. Measure A405

Add the substrate working solution to initiate each reaction, mix briefly, and immediately monitor A405. As a suggested kinetic program, collect readings every 1 minute for 15 minutes. Use the earliest linear portion of the sample curve to determine its absorbance change over time ($\Delta A_{405}/\Delta t$). Subtract the corresponding change in the substrate blank.

Under our validated assay conditions, the Complete Hydrolysis Reference (CHR) reaches a stable absorbance plateau within approximately 5 minutes, corresponding to complete hydrolysis of the 50 nmol substrate. The time required to reach this endpoint may vary depending on experimental conditions. Users should monitor the CHR absorbance until a stable plateau is reached and record the corresponding substrate blank absorbance for activity calculations.

6. Activity Calculation

One pNA milliunit (mU) is defined as the amount of trypsin activity that hydrolyzes 1 nmol of the chromogenic substrate per minute under the specified assay conditions.

$$\text{Trypsin Activity (mU/mg)} =$$

$$[\Delta A_{405} \times 50 \text{ nmol}] / [(A_{\text{CHR}} - A_{\text{blank}}) \times \Delta t \text{ (min)} \times 0.05 \text{ mL} \times C_{\text{protein}} \text{ (mg/mL)}]$$

$$\text{Trypsin Activity (mU/mL)} =$$

$$[\Delta A_{405} \times 50 \text{ nmol}] / [(A_{\text{CHR}} - A_{\text{blank}}) \times \Delta t \text{ (min)} \times 0.05 \text{ mL}]$$

Multiply mU/mL by the dilution factor to report the activity of the original (undiluted) sample. For mU/mg, use the protein concentration of the diluted sample added to the well, do not multiply by the dilution factor again.

CALCULATION TERMS

Term	Definition
ΔA_{405}	Blank-corrected change in sample absorbance over the initial linear interval.
Δt	Corresponding time interval in minutes.
$A_{\text{CHR}} - A_{\text{blank}}$	CHR endpoint absorbance minus the matched substrate-blank absorbance at that endpoint.
C_{protein}	Protein concentration (mg/mL) in the diluted sample tested.
50 nmol / 0.05 mL	Total substrate amount / test-sample volume per standard well.

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