ab222941 - Hydroxyproline Assay kit (Colorimetric)

For the sensitive and accurate measurement of Hydroxyproline in tissue homogenates and biological fluids.

For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit: www.abcam.com/ab222941
(use www.abcam.cn/ab222941 for China, or www.abcam.co.jp/ab222941 for Japan)

Materials Supplied:

Item	Quantity	Storage temperature (before preparation)	Storage temperature (after preparation)
Chloramine T Concentrate	600 µL	-20°C	-20°C
DMAB Concentrate	5 mL	-20°C	-20°C
Developer Solution I	5 mL	-20°C	-20°C
Hydroxyproline Standard	100 µL	-20°C	-20°C
Oxidation Buffer	10 mL	-20°C	-20°C
Microplate Sealing Film	1 unit		

PLEASE NOTE: Developer Solution I was previously labelled as Developer Solution. The composition has not changed.

Storage and Stability: Store kit at -20°C (store Developer Solution I at 4°C) in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt.

Materials Required, Not Supplied

These materials are not included, but will be required to successfully perform this assay:

- Microplate reader capable of measuring absorbance at OD 560 nm
- Distilled water (dH_2O)
- 10N Sodium hydroxide (NaOH)
- 10N Hydrochloric acid (HCl)
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Pressure-tight 1.5-2.0 mL screw top polypropylene tubes
- 96 well plate clear flat bottom
- Dounce or ultrasonic probe homogenizer (if using tissue)
- Hot plate or oven set at 120 °C

For urine samples:

- 4 mg Activated charcoal

Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

Chloramine T Concentrate (600 µL)

Ready to use as supplied (provided as concentrated solution in ddH_2O). Warm to room temperature and vortex to ensure it is fully re-suspended before use. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C protected from light. Do not perform more than 2 freeze/thaw cycles.

Developer Solution I (5 mL)

Ready to use as supplied. Equilibrate to room temperature before use. After use, promptly retighten cap to minimize adsorption of airborne moisture. Store at 4°C.

DMAB Concentrate (5 mL, in DMSO):

Ready to use as supplied. Warm to room temperature and mix by vortexing. After use, promptly retighten cap to minimize adsorption of airborne moisture.

∆ **Note**: DMSO tends to be solid when stored at -20°C or 4°C. Repeat this step every time concentrate is needed.

Aliquot so that you have enough volume to perform the desired number of assays. Store at - 20°C protected from light. Do not perform more than 5 freeze/thaw cycles.

Oxidation Buffer (10 mL):

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C.

Hydroxyproline Standard (100 µL, 1 mg/mL in ddH_2O):

Ready to use as supplied. Equilibrate to room temperature prior to use. Aliquot so that you have enough volume to perform the desired number of assays. Store at -20°C. Do not perform more than 5 freeze/thaw cycles.

Microplate sealing film (1 unit):

Ready to use as supplied. Equilibrate to room temperature before use. Do not reuse and discard after using.

1. Standard Preparation

– Always prepare a fresh set of standards for every use.

– Discard working standard dilutions after use as they do not store well.

1. Prepare 200 µL of 0.1 µg/µL standard by diluting 20 µL of the provided standard (1 mg/mL solution) in 180 µL of ddH_2O.
2. Using 0.1 µg/µL Hydroxyproline (Hyp) standard, prepare standard curve dilution as described in the table in a microplate or microcentrifuge tubes:

Standard#	Hyp Standard (µL)	ddH_2O (µL)	Final volume standard in well (µL)	End amount Hyp in well (µg)
1	0	30	10	0
2	6	24	10	0.2
3	12	18	10	0.4
4	18	12	10	0.6
5	24	6	10	0.8
6	30	0	10	1

Each dilution has enough amount of standard to set up duplicate readings (2 x 10 µL).

2. Sample Preparation

General sample information:

– We recommend performing several dilutions of your sample to ensure the readings are within the standard value range.

– We recommend that you use fresh samples. If you cannot perform the assay at the same time, we suggest that you snap freeze your samples in liquid nitrogen upon extraction and store them immediately at -80°C. When you are ready to test your samples, thaw them on ice and proceed with the Sample Preparation step. Be aware however that this might affect the stability of your samples and the readings can be lower than expected.

Tissue lysates:

2.1.1 Harvest the amount of tissue necessary for each assay (initial recommendation ~10- 100 mg).
2.1.2 Add 100 µL of dH_2O for every 10 mg of tissue and thoroughly homogenize with a glass bead (Dounce) or ultrasonic probe homogenizer.
2.1.3 Transfer 100 µL of sample homogenate to a pressure-tight, screw-capped polypropylene vial.
2.1.4 Add 100 µL of 10 N concentrated NaOH (not provided) to tissue homogenate. Ensure the cap is securely tightened and heat at 120°C for 1 hour.

∆ **Note**: Extremely tough samples (containing bone or exoskeletal tissue) may require heating for longer for complete hydrolysis.

2.1.5 Following alkaline hydrolysis, place vial on ice.
2.1.6 Allow vial to cool briefly before opening cap and adding 100 µL of 10 N concentrated HCl (not provided) to neutralize residual NaOH. Vortex vial.
2.1.7 Centrifuge vial at 10,000 x *g* for 5 minutes to pellet any insoluble debris that may remain following hydrolysis.
2.1.8 Collect supernatant and transfer to a new tube.
2.1.9 Keep on ice.

∆ **Note**: Hydrolysates of certain samples, such as fatty tissues, may contain lipid debris that is difficult to pellet by centrifugation. Take care when pipetting hydrolyzed samples to avoid transferring these insoluble globules.

Serum:

2.2.1 Mix serum with equal volume of 10 N concentrated NaOH (ie. 100 µL serum + 100 µL of 10N NaOH) in pressure-tight, screw-capped polypropylene vial. Mix well by pipetting up and down.
2.2.2 Hydrolyze mix at 120°C for 1 hour.
2.2.3 Cool vial on ice.
2.2.4 Neutralize hydrolysate by adding an equivalent volume of 10 N concentrated HCl to NaOH added (ie, 100 µL). Vortex vial.
2.2.5 Centrifuge vial at 10,000 x *g* for 5 minutes to pellet any insoluble debris that may remain following hydrolysis.
2.2.6 Collect supernatant and transfer to a new tube.
2.2.7 Keep on ice.

Urine:

2.3.1 Mix serum with equal volume of 10 N concentrated NaOH (ie. 100 µL serum + 100 µL of 10N NaOH) in pressure-tight, screw-capped polypropylene vial. Mix well by pipetting up and down.
2.3.2 Hydrolyze mix at 120°C for 1 hour.
2.3.3 Cool vial on ice.
2.3.4 Neutralize hydrolysate by adding an equivalent volume of 10 N concentrated HCl to NaOH added (ie, 100 µL). Vortex vial.
2.3.5 Decolorize samples by adding 4 mg of activated charcoal to the neutralized hydrolysate. Vortex vial.
2.3.6 Centrifuge vial at 10,000 x *g* for 5 minutes to remove precipitate and activated charcoal.
2.3.7 Collect clarified supernatant and transfer to a new tube.

∆ **Note**: We suggest using different volumes of sample to ensure readings are within the standard curve range.

3. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

Reaction wells set up:

- Standard wells = 10 µL standard dilutions
- Sample wells = 2-10 µL samples (adjust volume to 10 µL/well with ddH_2O)

Reaction mix:

3.2.1 Evaporate the sample hydrolysate and standard curve wells to dryness by heating the plate at 65°C on a hot plate/dry heat block or microplate incubator.

Δ **Note**: To prevent warping/etching of the plastic, do not expose microplate to extreme temperatures (>85°C).

3.2.2 Following evaporation of the hydrolysates, a crystalline residue will be left in the well. Gentle shaking will help dissolve the crystals in Oxidation Reagent Mix more quickly.

3.2.3 Prepare 100 µL of Oxidation Mix for each reaction. Prepare a master mix to ensure consistency

Δ **Note**: Once diluted and exposed to light and air, Chloramine T is only stable for 1-2 hours. Discard after diluting.

Component	Reaction Mix (µL)
Chloramine T concentrate	6
Oxidation Buffer	94

3.2.4 Add 100 µL of the Oxidation Reagent Mix to each well and mix by pipetting up and down. Then incubate the plate at room temperature for 20 minutes.

Δ **Note**: Hydrolysates from certain samples may impart a faint yellow tint to the Oxidation Reagent Mix. This slight colorization usually dissipates upon addition of Developer and does not interfere with the assay.

3.2.5 Add 50 µL of Developer to each reaction well and mix by pipetting up and down. Then incubate the plate at 37°C for 5 minutes.

3.2.6 Add 50 µL of DMAB Concentrate solution to each reaction well and mix contents thoroughly by pipetting.

3.2.7 Seal the plate with the provided microplate sealer film and incubate at 65°C on a hot plate/dry heat block or microplate incubator for 30 minutes.

Δ **Note**: The reaction with DMAB is temperature sensitive and chromophore development is expected to peak at ~30 min, when incubated at 65°C. Higher or lower incubation temperatures will require shorter or longer incubation times, respectively. The signal is expected to decay after this time, due to degradation of the chromophore.

Measurement:

3.3.1 Remove the plate from the heat source.

3.3.2 Immediately, measure absorbance at OD 560 nm on a microplate reader.

4. Calculations

- Samples producing signals greater than that of the highest standard should be further diluted in appropriate buffer/solution and reanalyzed, then multiply the concentration found by the appropriate dilution factor.

Measurement of Hydroxyproline in the sample:

4.1.1 Subtract the mean absorbance value of the blank (Standard #1) from all standards and sample readings. This is the corrected absorbance.

4.1.2 Average the duplicate reading for each standard and sample.

4.1.3 Plot the corrected absorbance values for each standard as a function of the final amount of hydroxyproline.

4.1.4 Calculate the equation of the standard curve using a linear regression and determine the slope.

4.1.5 Interpolate the amount of hydrolyzed hydroxyproline (B) in the sample wells by using the linear standard curve equation.

4.1.6 Concentration of Hydroxyproline (µg/µL) in the test samples is calculated as:

$$Hydrolyzed\ Hydroxyproline\ concentration = \frac{B}{V} * D$$

Where:

B = amount of hydrolyzed hydroxyproline in the sample well calculated from standard curve (µg).

V = sample volume added in the sample wells (µL).

D = sample dilution factor if sample is diluted to fit within the standard curve range.

4.1.7 Concentration of Hydroxyproline in the original sample (homogenate or biological fluid) is calculated as:

Total Hydroxyproline = Hydrolyzed Hydroxyproline x 3

Where:

Hydrolyzed Hydroxyproline = amount obtained from Step 4.1.5

3 = sample dilution fold that occurs during the generation of the hydrolysate

Problem	Reason	Solution
Assay not working	Use of ice-cold buffer	Buffers must be at assay temperature
	Plate read at incorrect wavelength	Check the wavelength and filter settings of instrument
	Use of a different microplate	Colorimetric: clear plates Colorimetric: black wells/clear bottom plates Luminometric: white wells/clear bottom plates
Sample with erratic readings	Cells/tissue samples not homogenized completely	Use Dounce homogenizer, increase number of strokes
	Samples used after multiple free/ thaw cycles	Aliquot and freeze samples if needed to use multiple times
	Use of old or inappropriately stored samples	Use fresh samples or store at - 80°C (after snap freeze in liquid nitrogen) till use
	Presence of interfering substance in the sample	Check protocol for interfering substances; deproteinize samples
Lower/higher readings in samples and standards	Improperly thawed components	Thaw all components completely and mix gently before use
	Allowing reagents to sit for extended times on ice	Always thaw and prepare fresh reaction mix before use
	Incorrect incubation times or temperatures	Verify correct incubation times and temperatures in protocol
Standard readings do not follow a linear pattern	Pipetting errors in standard or reaction mix	Avoid pipetting small volumes (< 5 µL) and prepare a master mix whenever possible
	Air bubbles formed in well	Pipette gently against the wall of the tubes
	Standard stock is at incorrect concentration	Always refer to dilutions described in the protocol
Unanticipated results	Measured at incorrect wavelength	Check equipment and filter setting
	Samples contain interfering substances	Troubleshoot if it interferes with the kit
	Sample readings above/ below the linear range	Concentrate/ Dilute sample so it is within the linear range

Technical Support

For all technical or commercial enquiries please go to:

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