Collagen Type II Cleavage
ITEM # 60-1017

INSTRUCTIONS FOR USE

IBEX C2C Human Urine Sandwich Assay, or IB-C2C-HUSA™, is a colour-monitoring immunoassay optimized for the measurement of C2C neoepitope fragments present in human urine samples. This product is not suitable for testing serum. This procedure has been developed for *in-vitro* research use only and should be performed by trained professionals.

BACKGROUND

Joint cartilage is composed of a type II collagen-based fibrillar network complexed to proteoglycans. Type II collagen consists of three (3) identical α-chains arranged in a triple helix that form fibrils. Within this fibrillar meshwork resides the large aggregating proteoglycan aggrecan. In arthritis, type II collagen is extensively cleaved and destroyed by the activity of collagenases, which results in loss of type II collagen (Billinghurst *et al.* 1997). Different collagenases, namely MMP-1, MMP-8 and MMP-13, can cleave type II collagen. Fragments containing the neoepitope created at the C-terminus of the three quarter piece of type II collagen cleavage product (C2C neoepitope) are recognized by IBEX C2C antibodies which are specific to type II collagen.

It has been reported that a 45-mer peptide is the most abundant C2C neoepitope fragment in human urine samples (Nemirovskiy *et al,* 2007). IB-C2C-HUSA™ sandwich immunoassay measures, in human urine samples, fragments longer than 20 amino acids such as the OA pathology-related 45 mer peptide containing the C2C neoepitope (Poole *et al.* 2016). Initial studies with this assay reveal that it detects the pathology-related cartilage collagen peptide or peptides in urine although little or no reactivity is seen in serum (Cibere *et al.* 2006; Cibere *et al.* 2009). There was no correlation between the serum C2C assay and the C2C-HUSA urine assay, revealing their distinctness. A previous study with the C2C competitive assay also revealed a lack of correlation between serum and urine measurements of C2C in the same patient (Cibere *et al.* 2009).

The C2C urinary peptide is progressively increased with onset and progression of cartilage degeneration (Nemiroskiy *et al.* 2007), as seen in a population-based cohort examined radiologically and by MRI (Poole *et al*. 2016). It was discovered that baseline values are associated with the progression of cartilage degeneration in knee OA over three (3) subsequent years: baseline uC2C levels were also higher in progressors versus non-progressors (Poole *et al.* 2016). Furthermore, in an OA initiative head to head assessment of 18 biomarkers, C2C-HUSA was one of 8 biomarkers that significantly predicted case status and one of only 2 biomarkers, the other one being CTX-II, that predicted individual group status, including pain worsening, joint space loss and their combination (Kraus *et al.* 2016). Tamm *et al*. (2014) also observed positive correlations with symptoms as well as joint function. These correlations were strongest when C2C-HUSA was expressed per creatinine.

In a recent study of adolescent and adult volleyball athletes, uC2C levels were reduced in adolescent with closed growth plates compared to those with open growth plates. In adults, uC2C as well as uCTX-II levels, showed a marked reduction from adolescent levels (Boeth *et al.* 2017).

IB-C2C- HUSA™ alone or in combination with C1,2C and CPII assays may provide additional important data to clinical information or drug development.

PRINCIPLE OF THE ASSAY

IB-C2C-HUSA™ is based on the direct or 'antigen sandwich' principle. The assay uses a 96-well microplate (12 removable strips of 8 wells). The solid phase is coated with monoclonal antibodies that effectively capture the C2C fragments. The Tracer antibody, conjugated to HRP, provides further specificity to the assay by binding to C2C neoepitope.

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Out-of-plate pre-dilution method is recommended for this assay. In a mixing plate, the orange-coded Matrix buffer (30 µL) is first added to each well. Upon addition of standards and samples (30 µL), the orange colour will change visually, confirming the addition and thus minimizing human errors due to repeated pipetting. Dilution buffer (60 µL) is then added to each well. After gentle mixing, the pre-mix solutions are transferred to the coated microplate (100 µL / well). An incubation step [1 hour at room temperature (RTºC), no shaking] allows the specific capture of C2C fragments to the solid phase. After removing unwanted residues through washing, Tracer Ab-HRP is added (100 µL /well) to specifically bind to any C2C complexes captured to the solid phase. After removing unbound tracer, TMB substrate solution (100 µL /well) is then added to reveal colour intensity proportional to the amount of the C2C fragments present in the test sample. Based on the values obtained with the standard set included in the assay, the content of C2C fragments may be estimated.

MATERIALS

Specimen collection

Second morning void urine samples is recommended. The urine samples should be stored at 4ºC for less than 24 hours but they should be stored frozen (-70ºC) for longer storage terms. Prior to use, urine samples should be thawed gradually to room temperature; the samples should be shaken and sedimentation allowed for a minimum of 30 minutes or centrifugation at 500 g for 5 minutes.

Supplied Reagents

Kit components	Format	Quantity
Coated microplate	Ready-to-use	12 strips of 8 wells (96 wells)
Matrix buffer	Ready-to-use	min. 3.5 mL
Standard set	Ready-to-use	7 x min. 130 µL/vial
Positive control - 1	Ready-to-use	min. 130 µL/vial
Positive control - 2	Ready-to-use	min. 130 µL/vial
Dilution buffer	Ready-to-use	min. 20 mL
Tracer Ab-HRP	Concentrated	min. 310 µL
TMB	Ready-to-use	min. 14 mL
Stop solution	Ready-to-use	min. 14 mL
Wash buffer (50x concentrate)	Concentrated	2 x min. 25 mL
Mixing plate	Ready-to-use	1 x 96 wells

Description

- Coated microplate: 12 strips of 8 breakable wells coated with monoclonal antibodies. The coated microplate has been sealed into Zipper aluminum pouch with desiccant. Tightly close unused strips in the zipper pouch with desiccant to prevent moisture exposure and store at 2-8ºC.
- Matrix buffer: 1 vial ready-to-use orange colour-coded reagent to be added to each well.

- Standard set: A set of seven (7) vials of ready-to-use, blue colour-coded standards with known concentration of C2C peptide fragments:

  Standard 1 = 5000 pg/mL
  Standard 2 = 2500 pg/mL
  Standard 3 = 1250 pg/mL
  Standard 4 = 625 pg/mL
  Standard 5 = 312 pg/mL
  Standard 6 = 156 pg/mL
  Standard 7 = 0 pg/mL

  Store any unused portion at 2-8ºC.

- Positive control - 1 (PC-1): 1 vial ready-to-use High level PC. Store any unused portion at 2-8ºC.

- Positive control - 2 (PC-2): 1 vial ready-to-use Low level PC. Store any unused portion at 2-8ºC.

- Dilution buffer: 1 vial blue colour-coded ready-to-use Dilution buffer. Used as: assay buffer to be added to the Mixing plate; diluent of concentrated Tracer Ab-HRP. This Dilution buffer may also be used for pre-dilution of samples, should this be necessary. Store any unused portion at 2-8ºC.

- Tracer Ab-HRP: 1 vial (min. 310 µL) concentrated Tracer Ab-HRP conjugate to be diluted 1:40 with Dilution buffer. Store any unused concentrated Tracer at 2-8ºC.

- TMB: 1 vial ready-to-use Tetra methylbenzidine (TMB) in a proprietary buffer. Keep away from direct sunlight. Store any unused portion at 2-8ºC.

- Stop solution: 1 vial ready-to-use Stop solution (diluted sulphuric acid). Handle all acid solutions with caution. Store any unused portion at 2-8ºC.

- Wash buffer (50x concentrate): 2 vials concentrated Wash solution containing buffered saline with a non-ionic detergent and a preservative.

- Mixing plate: 1 polypropylene non coated 96 well round bottom mixing plate.

Required Equipment not supplied

- Pipette capable of accurately dispensing 30 µL
- Multichannel pipettes capable of accurately dispensing 30-100 µL and 350 µL
- Graduated cylinders for dilution of concentrated wash buffer 50x
- Distilled or deionized water
- Vortex mixer
- Automatic washer or equivalent plate washing system
- ELISA plate reader suitable for 96-well plate formats and capable of measuring at 450 nm and 650 nm.
- 2-8°C environment(s) for product storage

WARNINGS AND PRECAUTIONS

This kit is intended for *in-vitro* research use by trained professionals only.

Storage

Store all kit reagents at 2-8°C. Do not freeze. Store any unused portion at 2-8°C. Seal the pouch containing unused coated wells (with desiccant) carefully. Close all vials well.

Kit stability

The kit is currently granted a 6-month shelf life. Once opened and stored appropriately, the kit reagents are stable for 1 month or up to the expiry date stated on the box, whichever comes first.

Frozen kit

While the kit should not be frozen, a kit that has been accidentally frozen once still performs as usual.

ASSAY PROCEDURE

The following procedure must be followed in order to minimize errors / variation and to optimize reliable results.

Reagent preparation:

1. Bring all kit components and samples to room temperature (18 – 25ºC) before use for at least 30 minutes.

2. Wash solution: Dilute the Wash buffer (50x) 1:49 (v/v) with deionized water. For example, in a graduated cylinder, mix deionized water with 20 mL Wash buffer (50x) to a final volume of 1000 mL. Crystallization may occur at the bottom of the 50X concentrate vials. Prior to its use, simply dissolve the crystals by immersing the vials in warm water for a few minutes and mix well. Unused portion should be stored at 2-8ºC and may be used within one week.
   Note: It is important to always follow the same washing method (manual or automated) in order to minimize variation in OD signals.

3. Coated microplate: Remove required strips from pouch. Return unused strips and desiccant to original pouch. Seal pouch carefully after each use. Store at 2-8ºC.

4. Standard set: All standards should be assayed in duplicate.

5. Urine samples: We recommend testing all samples in duplicate. Occasionally, some samples may require additional dilution prior to testing, in which case, optimal dilution factor for such samples has to be pre-determined.

6. Tracer Ab-HRP: Prepare the Tracer Ab-HRP working solution before use by diluting the Tracer Ab-HRP concentrate 1:40 with Dilution buffer. For example: add 300 µL of Tracer-Ab-HRP concentrate to 12 mL of Dilution buffer; this is sufficient for testing 1 full microplate.

Test procedure:

1. Recommended positions of C2C standards and PC on Coated microplate:

	1	2	3
A	Std 1	Std 1	PC-2
B	Std 2	Std 2	PC-2
C	Std 3	Std 3	
D	Std 4	Std 4	
E	Std 5	Std 5	
F	Std 6	Std 6	
G	Std 7	Std 7	
H	PC-1	PC-1	

2. Using a multi-channel pipette, dispense 30 µL of orange colour-coded Matrix buffer into each well of the Mixing plate..
3. Dispense 30 µL of each Standard and Positive control into respective wells, using a separate pipette tip for each addition (including replicates). Dispense 30 µL of each test sample into individual wells beginning with well C3, using a separate pipette tip for each addition. Rinse tips by gently pipetting up and down at least 3 times. Orange colour will change confirming the addition of the Standard, PC or sample. Different samples may show different colour change.
4. Using a multi-channel pipette, dispense 60 µL of Dilution buffer into each well.
5. Mix by gently tapping the side of the Mixing plate.
6. Using a multi-channel pipette, transfer 100 µL of the mixture from each well of the Mixing plate to the corresponding wells of the Coated microplate. Change pipette tips after each transfer.
7. Incubate at room temperature (18-25ºC) for 1 hour (without agitation).
8. Aspirate and wash 5 times with 350 µL/well diluted Wash buffer (1x). Automated washers should be adjusted to fill each well completely without overfilling and without soaking time. Wash instrument and procedure should be verified for IB-C2C-HUSA™ prior to any study.
9. Add 100 µL of diluted Tracer Ab-HRP to each well.
10. Incubate at room temperature (18-25ºC) for 30 minutes (without agitation).
11. Aspirate and wash 5 times with 350 µL/well diluted Wash buffer (1x).
12. After the final wash, gently tap the microplate upside down on absorbent paper to remove any remaining liquid, taking care not to dislodge strips from the holder.
13. Dispense 100 µL of TMB into each well. Protect the microplate from direct strong light sources to avoid an increased background signal (undesired oxidation of TMB).
14. Incubate the microplate at room temperature (18-25ºC) for 30 minutes.
15. Stop the reaction by dispensing 100 µL of Stop solution into each well.
16. After adding the Stop solution, read the absorbance on a microplate reader at 450 nm and optionally at 650 nm for background value due to the microplate alone. Use the net absorbance values (OD 450 nm – OD 650 nm) for all further calculations. Absorbance must be read within 30 minutes for accurate measurement.

RESULTS

Calculation of results

A four- or five-parameter logistic curve fit (4PL or 5PL) can be used. Alternatively, plot the mean absorbance values of the standards (Y axis) against the corresponding C2C concentrations (X axis) and determine the C2C concentration of the controls and samples by interpolation. This is the C2C concentration (pg/mL) in each reaction well. Multiply this value with the dilution factor to obtain the C2C concentration present in the samples/controls.

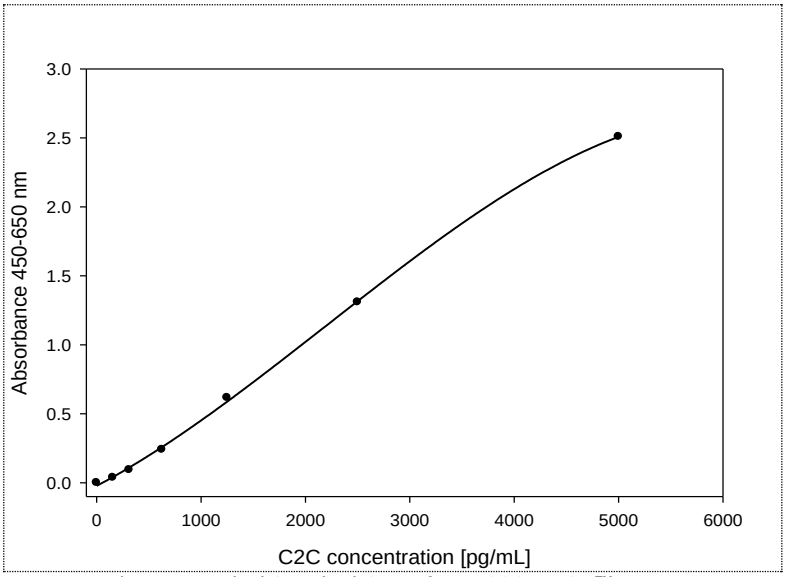

Figure 1: Typical Standard Curve for IB-C2C-HUSA™ Assay
(Not for use in calculation of actual results)

Creatinine correction

We recommend that C2C concentration obtained in test should be corrected with creatinine concentration.

Creatinine concentration (mmol/L) in urine samples can be determined using any creatinine (urinary) colorimetric assay kit. Perform the correction using the following equation:

$$\text{Corrected uC2C value (ng/mmol)} = \frac{\text{urine C2C (ng/L)}}{\text{Creatinine (mmol/L)}}$$

Note: conversion of uC2C value obtained in test from pg/mL to ng/L should be done prior to using the above equation.

TEST PERFORMANCE

Detection limit

The lower limit of quantification is 70 pg/mL

Specificity

No cross reactivity with corresponding neoepitope peptides derived from collagen I and collagen III is detected.

Spiking recovery
Human urine samples were spiked with different known amounts of C2C and assayed.

Urine sample	Observed [pg/mL]	Expected [pg/mL]	Recovery (%)
1	467	-	-
	4184	4217	99.2
	2363	2467	95.7
2	1704	-	-
	5284	5454	96.8
	3717	3704	100.3

Linearity dilution
Urine samples were diluted with Dilution buffer and assayed.

Sample ID	Dilution Factor	net OD 450-650nm	Conc [pg/mL]	Corrected conc [pg/mL]
h-u001	neat	0.518	1954	1954
	2	0.262	1021	2041
	4	0.128	535	2138
h-u002	neat	0.643	42408	2408
	2	0.309	1192	2383
	4	0.141	580	2320
	8	0.080	308	2464
h-u003	neat*	1.004	3722	3722
	2	0.909	3378	6756
	4	0.526	1982	7926
	8	0.236	928	7422

*: This sample exhibits a hook effect at neat concentration. Additional dilution corrects the problem.

Precision

Intra-lot

Sample	Mean (pg/mL)	SD (pg/mL)	CV (%)
1	4931	16	< 1.0
2	2771	63	2.3
3	1093	39	3.6
4	592	22	3.7

Inter-lot

Sample	Mean (pg/mL)	SD (pg/mL)	CV (%)
1	4922	20	< 1.0
2	2732	81	3.0
3	1110	41	3.7
4	612	39	6.4

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