

PRECICE[®] K- and H-values Freshness Assay Kit
 for measurement of relative content of IMP ratio (% IMP) and of Hypoxanthine ratio (%Hx) in fish and meat samples

I. Measuring ATP degradation products for freshness quantification

ATP (adenosine triphosphate) is the major energy currency in cell. Its content is particularly high in muscle tissue where ATP is used to power contraction. As soon as an animal dies, cell respiration stops as well as ATP formation. As soon as the *rigor mortis* stage is reached, within minutes to few hours after the animal has been slaughtered, ATP pool is progressively and sequentially depleted through a succession of reactions, as shown on the figure 1. *Post-mortem* ATP depletion is mainly due to autolytic enzymes, even if the microbial flora, when developed enough, may contribute to the process. This makes the level of ATP degradation products an excellent criterion for fish freshness measurement and storage age, before microbial spoilage starts and the corresponding traditional techniques for spoilage measurement become relevant.



Figure 1: Post-mortem ATP degradation in fish and subsequent formation of IMP, Inosine (Ino) and Hypoxanthine (Hx)

In the late 1950's, a Japanese research team (Saito *et al.*) proposed a new concept, called "K value" (or "K Factor"), for the indication of the freshness of fish flesh. The K value is based on ATP breakdown and the subsequent formation of its by-products, namely adenosine diphosphate (ADP) and monophosphate (AMP), inosine monophosphate (IMP), inosine (Ino) and, at a later stage, hypoxanthine (and Hx). K-value is expressed as a percentage of the content of the last two final compounds of the ATP catabolic pathway, *i.e.* Ino and Hx, over the total content of ATP and its degradation by-products: ATP, ADP, AMP, IMP, Ino and Hx.

$$\text{K-value}(\%) = \frac{(\text{Ino} + \text{Hx})}{(\text{ATP} + \text{ADP} + \text{AMP} + \text{IMP} + \text{Ino} + \text{Hx})}$$

However, because ATP decomposes very quickly to IMP in most animals, a simplified K value (generally called K_i or K' value) was soon proposed by Karube *et al.* (1984) and is currently considered as equivalent to the original 6-parameter equation for K-value.

$$\text{K}_i \text{-value}(\%) = \frac{(\text{Ino} + \text{Hx})}{(\text{IMP} + \text{Ino} + \text{Hx})}$$

Since IMP content, when expressed as a percentage of the three ATP degradation products (namely IMP, Ino and Hx) is directly linked to the K-value formula :

$$\text{K}_i(\%) = 100\% - \% \text{IMP}$$

it may appear more convenient to consider %IMP as an equivalent freshness marker for which, the higher %IMP, the fresher the fish.

$$\text{IMP ratio} (\% \text{IMP}) = \frac{\text{IMP}}{\text{IMP} + \text{Ino} + \text{Hx}}$$

The measurement of H-value (or Hx ratio) has been proposed as freshness indexes of meat and has been considered an excellent index of meat aging and quality (Batlle et al., 2001; Tsai, Cassens, Briskey, & Greaser, 1972; Yano et al., 1995, A.S. Hernández-Cázares et al. 2010).

In fish muscle, elevated hypoxanthine ratio was found to correlate with the proliferation of spoilage bacteria *Pseudomonas* (Surette et al, 1988), *Shewanella putrefaciens* and *Photobacterium phosphoreum* (van Spreekens 1977) and was found to be in direct correlation with TMA content (Dalgaard, 1993).

$$\text{H-value Hx ratio (\%)} = \frac{\text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}}$$

II. Principle of PRECICE® K- and H- values Freshness Assay Kit

PRECICE® K- and H- Freshness Assay Kit provides an enzymatic tool for measuring the K- and H-values of fish flesh samples by spectrophotometry. The kit is developed for 96-well plate format and allows rapid screening of numerous samples (22-33 samples per plate).

PRECICE® K- and H- Freshness Assay Kit is based on the use of original recombinant enzymes of nucleotide metabolism that allow a simple and reliable quantification of IMP, Ino and Hx by measuring absorbance at 340nm (patented). In each fish sample, IMP, or IMP + Hx or IMP + Ino + Hx are totally converted to NADH by specific dehydrogenases contained in Enzyme Mix 1 or in Enzyme Mix 2 or Enzyme Mix 3. The NADH formed can be quantified by measuring specific absorbance at 340nm. The non-specific absorbance of sample value due to the presence of fish extract and co-factors is measured as a Blank and subtracted from experimental values.

	Absorbance at 340
<div style="border: 1px solid black; padding: 5px; width: fit-content; margin: 0 auto;"> Reaction mixture without enzymes </div>	Auto-absorbance of fish extract and co-factors
<div style="border: 2px solid red; padding: 5px; width: fit-content; margin: 0 auto;"> Enzyme mix 1 NADH ← IMP </div>	IMP content
<div style="border: 2px solid green; padding: 5px; width: fit-content; margin: 0 auto;"> Enzyme mix 2 Hx → NADH ← IMP </div>	IMP + Hx content
<div style="border: 2px solid blue; padding: 5px; width: fit-content; margin: 0 auto;"> Enzyme mix 3 Ino → Hx → NADH ← IMP </div>	IMP+ Ino + Hx content

III. Equipments required (not provided)

For samples extraction by boiling

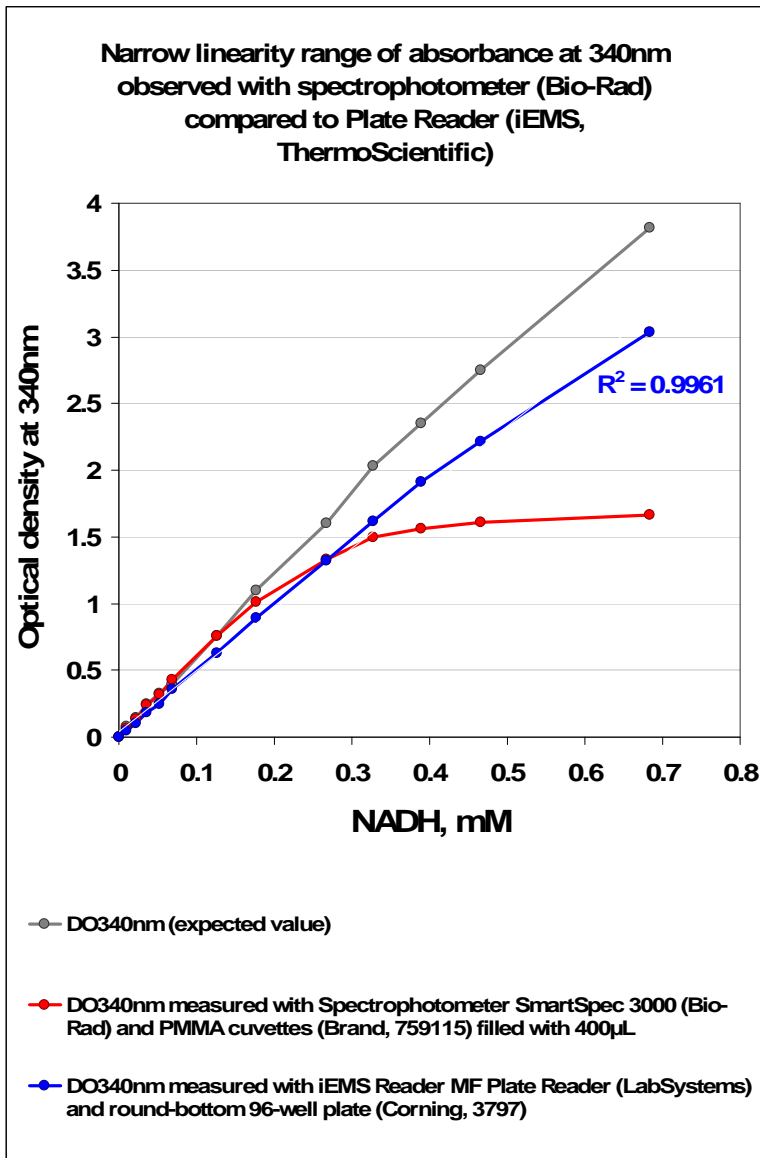
- 1) Boiling water bath
- 2) 50-ml tubes or bottles with screw caps resistant to heating (ex. polypropylene tubes from Corning ref. 430828).
- 3) Folded paper filter for slow filtration



- 4) Plate agitator
- 5) Plate reader fitted with a filter 340nm (ex. Labsystems iEMS Reader MF (Thermo), Epoch (BioTec); PerkinElmer)

We strongly recommend using plate reader because this instrument has a larger linearity range compared to a spectrophotometer (see below) and allows simultaneous reading of 96 samples without additional pipeting and liquid handling. Alternatively, the results can be read with a spectrophotometer by measuring absorbance at 340nm in disposable cuvettes (PMMA, 1.5ml ref. 759115, Brand). These cuvettes have to be filled at least with 400 μ L for absorbance reading, which is convenient since each 200 μ L assay must be diluted 2x or 4x before absorbance reading with a spectrophotometer.

IV. Defining the linearity range of absorbance at 340nm of your instrument



In "PRECICE K" kit, the quantification of ATP-degradation products totally relies on the NADH absorbance. The linearity range of the instrument used for the quantification is of **critical importance** and should be checked before starting the experiments. NovoCIB provides upon request the standard solutions of NADH that can be used for the calibration of spectrophotometer of plate reader.

Solution	1	2	3	4	5	6	7	8	9	10	11	12	13
NADH, mM	0	0.01	0.02	0.04	0.05	0.07	0.13	0.18	0.27	0.33	0.39	0.47	0.68
Optical Density at 340nm (expected)	0	0.08	0.14	0.25	0.33	0.4	0.76	1.1	1.6	2.03	2.35	2.75	3.82
OD ₃₄₀ measured with 400µL in disposable PMMA cuvettes (Brand, ref. 759115, 1.5ml) and spectrometer SmartSpec 3000 (BioRad)	0.002	0.062	0.132	0.236	0.32	0.4	0.431	0.753	1.014	1.333	1.497	1.565	1.61
OD ₃₄₀ measured with 200µL in round-bottom 96-well plate (Corning, ref 3797) and Plate Reader iEMS Labsystems (ThermoScientific)	0.10	0.14	0.21	0.27	0.34	0.41	0.72	0.98	1.33	1.68	1.96	2.23	2.97

V. Kit Content for analysis of 11 samples in duplicate

- 1) Extraction buffer (20x), 30ml
- 2) "Reaction buffer" (11.2 mL), in 15-ml tube
- 3) "Enzyme mix 1", lyophilized in 15-ml glass vial, for IMP quantification
- 4) "Enzyme mix 2", lyophilized in 10-ml glass vial, IMP+hypoxanthine quantification
- 5) "Enzyme mix 3", lyophilized in 5-ml glass vial, for IMP+hypoxanthine+Inosine quantification
- 5) 1 tube "Cofactor 1" (powder)
- 6) 1 tube "Cofactor 2" (powder)
- 7) 1 tube "Cofactor 3" (powder)
- 8) 1ml-tips with filter plug, numbered, 16, provided with recipient tubes
- 9) Transparent empty microplate (round-bottom 96-well plate Corning, Costar[®] ref. 3797) pre-filled with 30nmols of IMP, hypoxanthine and inosine (supplied dried)

VI. Storage

PRECICE[®] K-Freshness Assay Kit must be stored at -20°C until used. Enzyme mixes must be freshly prepared before performing the assays. Once prepared, reagent and enzyme solutions are not stable enough for storage.

VII. Description of the procedure

Thaw the "Extraction buffer, 20x" and "Reaction buffer" tubes at room temperature (**1h in advance**). DO NOT HEAT! Since the rate of enzymatic reaction depends on the temperature, it is important to completely thaw the "Reaction buffer" and to equilibrate it at room temperature.

VIII. Fish sample preparation

VIII.1. Prepare "Extraction buffer, 1x" by adding 19 volumes of deionized water to 1 volume of "Extraction buffer, 20x" (ex 190ml of water to 10ml of **Extraction buffer, 20x**).

VIII.2 Weight 3-5g of fish muscles into labelled tubes, add 5 volumes of "**Extraction buffer, 1x**", tightly close the tube to avoid evaporation, and put them into a boiling water bath for 20min. Be sure that the tubes are put in the water deeply enough to cover all the muscle. The extraction is more efficient with fine slices of fish (ou haché ou emincés).

VIII.3 Use folded paper filter (for slow filtration) for filtering of boiled extracts.

Important: Since the quantification of IMP and IMP+Hx+Inosine is done by measuring absorbance, the fish extracts must be totally transparent to avoid any interference due to the presence of solid particles.









































































































IX. Microplate preparation

For 11 samples in duplicate

IX.1. The microplate provided with the kit is pre-filled with nucleotides and is closed with adhesive films. Peel off a band of film covering plate before use, add 100µL of 11 filtered fish extracts into 8 wells in a 96-well plate, as shown in the following scheme.

IX.2. Add 100µL of "Extraction buffer 1x" per well in column 12 for standards solubilization.

Samples (100µL per well)

	 1	 2	 3	 4	 5	 6	 7	 8	 9	 10	 11	Extraction buffer 1x. 100µL/well
A												
B												
C												IMP
D												
E												Hx
F												
G												Ino
H												

X. Preparation of reaction mixtures

X. 1. Add the **cofactors 1 and 2** (small tubes) to 11.2 mL of "Reaction buffer" provided in 15-ml tube. To do so :

- pipet 1ml of "Reaction buffer" to each tubes and mix them by inverting or pipeting up and down until dissolved,
- transfer by pipeting the content of all two small tubes back into a 15-ml tube with "Reaction buffer",
- repeat to be sure that all reagent and enzymes of the small tubes are recovered.
- mix the complete "Reaction buffer" then containing cofactors 1 et 2 by gently inverting. Avoid bubbles.












X. 2. Pipet 8.4 mL of the so prepared "Reaction buffer" into a glass vial "Enzyme mix 1" (15-ml) to dissolve the enzymes. Mix gently by inverting until complete dissolution of lyophilized enzymes (do not vortex, avoid bubbles). "Enzyme mix 1" is ready.

X. 3. Transfer 5.6 mL of the "Enzyme mix 1" into a glass vial with "Enzyme mix 2" (10-ml). Add the content of the tube with cofactor 3 to "Enzyme mix 2". To do so, pipet 1ml of "Enzyme mix 2" to the tube with cofactor 3, mix by inverting, avoid bubbles. "Enzyme mix 2" is ready.

X. 4. Transfer 2.8 mL of the so prepared "Enzyme mix 2" into the 5-mL glass vial with "Enzyme mix 3" (5-ml). Mix gently by inverting until complete dissolution of lyophilized enzymes (do not vortex, avoid bubbles). "Enzyme mix 3" is ready.

You have: 2.8 ml of "Reaction mix" for Blank measurement
 2.8 ml of "Enzyme mix 1" for IMP measurement
 2.8 ml of "Enzyme mix 2" for IMP+hypoxanthine (Hx) measurement
 2.8 ml of "Enzyme mix 3" for IMP+Inosine+hypoxanthine measurement

X. 5. Add 100µL of "Reaction mix", "Enzyme mix 1", "Enzyme mix 2" and "Enzyme mix 3" as indicated in the following scheme to the plate with fish extract and into the wells prefilled with standards (lane 12).

	 1	 2	 3	 4	 5	 6	 7	 8	 9	 10	 11	Standards	
A	○	○	○	○	○	○	○	○	○	○	○	○	Reaction buffer 100µL per well
B	○	○	○	○	○	○	○	○	○	○	○	○	
C	●	●	●	●	●	●	●	●	●	●	●	● IMP	Enzyme Mix 1 100µL per well
D	●	●	●	●	●	●	●	●	●	●	●	●	
E	●	●	●	●	●	●	●	●	●	●	●	● Hx	Enzyme mix 2 100µL per well
F	●	●	●	●	●	●	●	●	●	●	●	●	
G	●	●	●	●	●	●	●	●	●	●	●	● Ino	Enzyme mix 3 100µL per well
H	●	●	●	●	●	●	●	●	●	●	●	●	

X. 6. Incubate for 30 min -1 h at room temperature, agitate before or during incubation.

XI. Reading optical density at 340nm

XI.1. Insert the microplate for sample analysis into the reader, shake the plate for 2 min and read the absorbance at 340nm. Record the data

Alternatively, absorbance reading with a spectrophotometer can be done in disposable cuvettes (PMMA, 1.5ml ref. 759115, Brand).

XI.2. Read the "Blank" with an empty cuvette. Then fill it with the 200µL of the microplate well, then add 200µL of deionized water to get a 2x dilution. Read the absorbance at 340nm. If the absorbance read exceeds 0.8, add 400µL of deionized water to get a 4x dilution and read the absorbance.

XII. Calculate %IMP and K-value (=100%-%IMP)

For each assay, %IMP (=IMP/(IMP+Hx+Ino)) and K-value are calculated according to the following formulas:

$$\text{IMP (\%)} = \frac{\text{Abs}_{\text{Mix1}} - \text{Abs}_{\text{Blank}}}{\text{Abs}_{\text{Mix3}} - \text{Abs}_{\text{Blank}}} \quad \text{and} \quad \text{K-value (\%)} = 100\% - \% \text{IMP}$$

where: **Abs_{Mix1}** is the absorbance of the assay in the well containing **"Enzyme mix I"**
Abs_{Mix3} is the absorbance of the assay in the well containing **"Enzyme mix 3"**
Abs_{Blank} is the absorbance of the assay in the well containing **"Reaction buffer"**

XI. Calculate H-value (%Hx)

For each sample, H-value =Hx/(IMP+Hx+Ino) is calculated according to the following formulas:

$$\text{Hx -value (\%)} = \frac{\text{Abs}_{\text{Mix2}} - \text{Abs}_{\text{Mix1}}}{\text{Abs}_{\text{Mix3}} - \text{Abs}_{\text{Blank}}}$$

where: **Abs_{Mix1}** is the absorbance of the assay in the well containing **"Enzyme mix I"**
Abs_{Mix2} is the absorbance of the assay in the well containing **"Enzyme mix II"**
Abs_{Mix3} is the absorbance of the assay in the well containing **"Enzyme mix 3"**
Abs_{Blank} is the absorbance of the assay in the well containing **"Reaction buffer"**