PRECICE® Freshness Assay Kit

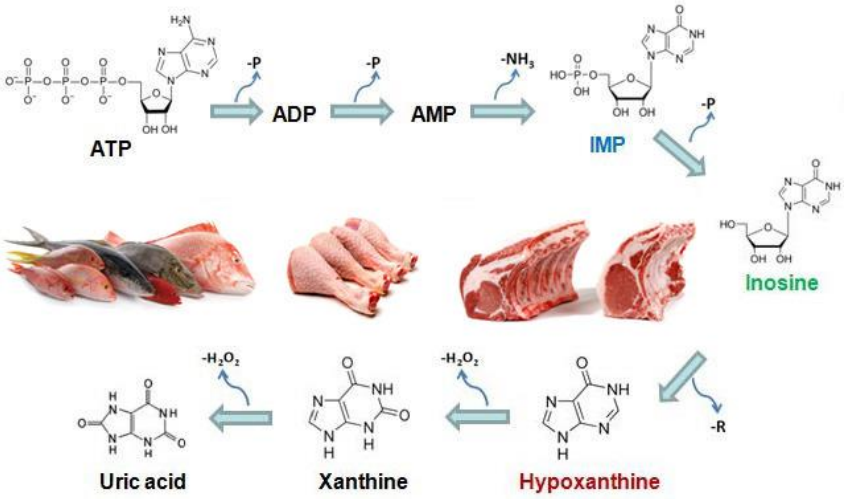
For measurement of IMP, inosine and hypoxanthine with cuvettte spectrophotometer

Ref: 0700-04-10 samples

Key words: K-value, ATP nucleotide catabolites; ATP breakdown products

I. ATP catabolism

The content of ATP is particularly high in muscle where it provides the energy for contraction. As soon as an animal is slaughtered, cell respiration stops as well as ATP formation. Post-mortem ATP degradation leads to the formation of three major nucleotide catabolites: IMP (5-24h), Inosine (days), and Hypoxanthine (weeks).



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. The K-value measures how far ATP degradation has progressed within the tissue. It is expressed as a percentage of the content of the last two final compounds of the ATP catabolic pathway (Ino and Hx) over the total content of ATP and its degradation by-products: ATP, ADP, AMP, IMP, Ino and Hx. Because ATP decomposes very quickly to IMP in most animals, a simplified K value (generally called Ki- or K'-value) was proposed.

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

The K-value has been recognized for several decades as the most effective and objective indicator of the freshness of fish and seafood products, as well as of meat (beef, pork, lamb and poultry). **The lower the K-value, the fresher the fish.** In difference to volatile amines (TVA) appearing at a medium- or a late-stage of spoilage, nucleotides allow the detection of very beginning of the spoilage process.

"PRECICE® Freshness Assay" is a first assay for routine physico-chemical assessment of quality of food freshness.
The enzymes provided with the kit allow specific conversion of three major ATP catabolites - IMP, inosine (Ino) and hypoxanthine (Hx) to NADH2. The quantification of nucleotides is done by measuring sample absorbance at 340nm.

The reactions catalysed by these enzymes:

- are highly specific and selective toward each particular nucleotides;
- fast and irreversible;
- convert three different nucleotides (IMP, inosine and hypoxanthine) to one common denominator (NADH2).

References

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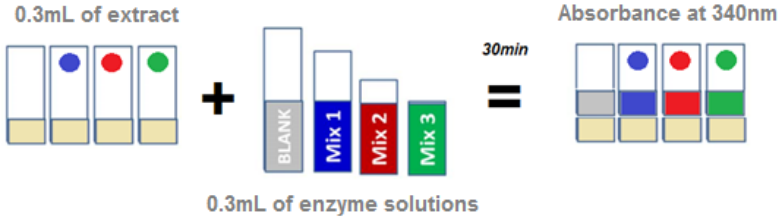
2. Saito, T, Arai, K.I. and Tanaka, T. (1958) Changes in adenine nucleotides in squid muscle ***Nature*** 181: 127-1128
3. Batlle, N., Aristoy, M. C., & Toldrá, F. (2000). Early post-mortem detection of exudative pork meat based on nucleotide content. ***Journal of Food Science***, 65(3), 413–416.
4. Batlle, N., Aristoy, M. C., & Toldrá, F. (2001). ATP metabolites during aging of exudative and nonexudative pork meats. ***Journal of Food Science***, 66(1), 68–71.
5. K. Saito a, A. Ahhmed b, H. Takeda b, S. Kawahara b, M. Irie b, M. Muguruma Effects of a humidity-stabilizing sheet on the color and K-value of beef stored at cold temperatures. ***Meat Science*** 75 (2007) 265–272
6. Luong, J.H.T., Male, K.B., Masson, C., & Nguyen, A.L. 1992. Hypoxanthine ratio determination in fish extract using capillary electrophoresis and immobilized enzymes. ***J. Food Sci***., 57, pp. 77 - 81.

II. Short description

1) **Boil minced fish muscle in extraction buffer, cool and filter the extract;**
2) **Fill 4 cuvettes with 0.3mL of extract;**



3) **Prepare enzymatic solutions, add 0.3mL of each mix to cuvettes pre-filled with extract, shake gently and incubate for 30 min ;**
4) **Read the absorbance.**

III. Principle

In each sample, **IMP, hypoxanthine** and **inosine** are converted to $NADH_2$ by **Enzyme 1, Enzyme 2** and **Enzyme 3,** respectively. $NADH_2$ formed is 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.

White Cuvette	Reaction buffer + co-factors	Auto-absorbance of fish extract and co-factors	Blank
Blue Cuvette	Enzyme 1	IMP ⟶ NADH	$Absorbance_{340}1$
Red Cuvette	Enzyme 1 + Enzyme 2	IMP + Hx ⟶ NADH	$Absorbance_{340}2$
Green Cuvette	Enzyme 1 + Enzyme 2 +Enzyme 3	IMP + Hx + Ino ⟶ NADH	$Absorbance_{340}3$

For each sample, **Ki-** value and relative contents of **IMP** or **Hypoxanthine (H-value)** can be calculated as follows:

$$Ki\ (\%) = \left(1 - \frac{Absorbance_{340}1}{Absorbance_{340}3}\right) \times 100$$

$$IMP\ (\%) = \frac{Absorbance_{340}1}{Absorbance_{340}3} \times 100$$

$$Hx\ (\%) = \frac{Absorbance_{340}2 - Absorbance_{340}1}{Absorbance_{340}3} \times 100$$

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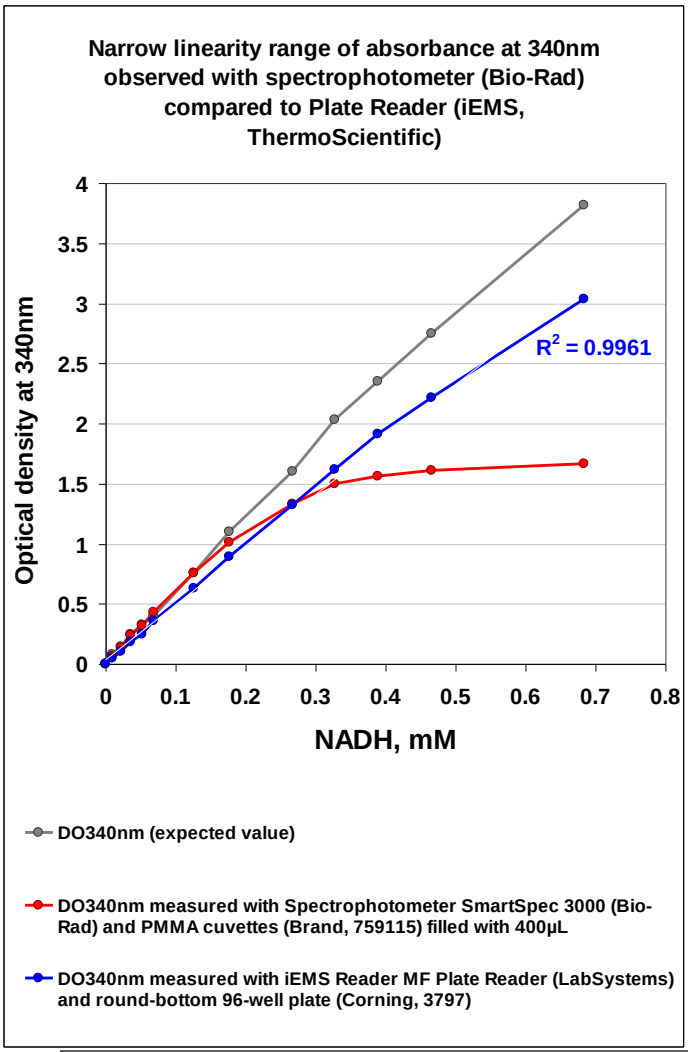
IV. Storage

All enzymes are provided in lyophilized stable form and are transported at room temperature. After reception, **"PRECICE® Freshness Assay Kit"** must be stored at -20°C until used (stable for 6 months if stored at-20°C). Enzyme mixes must be freshly prepared before performing the assays. Once prepared, reagent and enzyme solutions are not stable and should be used immediately.

V. Material and equipment required for the analysis of 10 samples *(not provided)*

1) Deionized water, 0.95L
2) Boiling water bath
3) Precision scale (0.1g)
4) Twenty (20) 50-ml tubes with screw caps resistant to heating (ex. polypropylene tubes from Corning ref. 430828)
5) Ten (10) 0.2µm filters (ex. Sartorius)
6) Ten (10) non-sterile syringes (ex. Braun, 5ml)
7) Forty (40) PMMA cuvettes for spectrophotometer (ex. ref. 759115, Brand, 1.5ml).
8) Spectrophotometer fitted with a 340nm filter
9) Pipette and tips for 1mL
10) Ten (10) 2-mL tubes for filtered extracts
11) NADH calibration solutions

VI. Defining the linearity range of absorbance at 340nm of your instrument *(recommended)*



In "PRECICE®" kit, the quantification of ATP-degradation products totally relies on the $NADH_2$ 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 ***"NADH calibration solutions"*** that can be used for the calibration of spectrophotometer or plate reader.

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 500µL for absorbance reading (the volume depends on the spectrophotometer) .

VII. Kit components:

1) **"Extraction buffer concentrate",** for preparing 1L of extraction buffer
2) **"Reaction buffer"** (provided in 22-mL glass transparent vial)
3) **"Blank"** (15-ml PP tube with orange cap prefilled with cofactors powder)
4) **"Enzyme 1"**, lyophilized in 15-ml glass amber vial, for **IMP** quantification
5) **"Enzyme 2"**, lyophilized in 7-ml glass vial, for **IMP** + **Hypoxathine** quantification
6) **"Enzyme 3"**, lyophilized in 4-ml glass vial, for **IMP** + **Hypoxathine** + **Inosine** quantification
7) 4 labelled cuvettes for spectrophotometer for negative control

VIII. Sample extraction

Before starting: *Thaw* ***"Reaction buffer"*** *tubes at room temperature several hours* ***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.*

1. Thaw **"Extraction buffer concentrate"**, quantitatively transfer the content of **"Extraction buffer concentrate"** vial into a bottle with 0.95L of deionized water.

2. Heat boiling water bath.
3. Mince* 50-100g of each sample and weight 2g of minced flesh into a separate 50-ml polypropylene tube, put on ice.

** Since nucleotides content may vary depending on type of tissue, mincing 100g fish muscle results in more representative results. However, rapid extraction and analysis can be done also with piece of fish flesh.*

4. Add 28 mL of extraction buffer (14* volumes per gram) of minced sample, tightly close to avoid evaporation, and put the tubes into boiling water for 20min. Be sure that the tubes are put in the water deeply enough.
   ** The volume of extraction buffer per gram depends on*
   - linearity range of your spectrophotometer;
   - nucleotide concentration if fish muscle (depends on species).
   Please, fill free to write us at contact@novocib.com if you have any questions.
5. After 20 min, take off the tubes and put them into tap water for fast cooling.

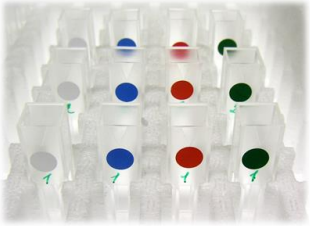
6. Filter the exudates:
   - Take off the plunger from syringe
   - Fix 0.2µm filter on the barrel
   - Carefully transfer the exudate to the barrel
   - Insert the plunger and filter 1.5-2mL of exudates into a clean 2-mL tube

IMPORTANT: ***Do not filter hot or warm samples!***

7. Fill 4 supplied cuvettes with 0.3**mL of diluted extraction buffer.

   The volume of extract depends on type of spectrophotometer and the volume of cuvette. We recommend to check minimal volume of cuvette in advance using* ***"NADH calibration solutions".

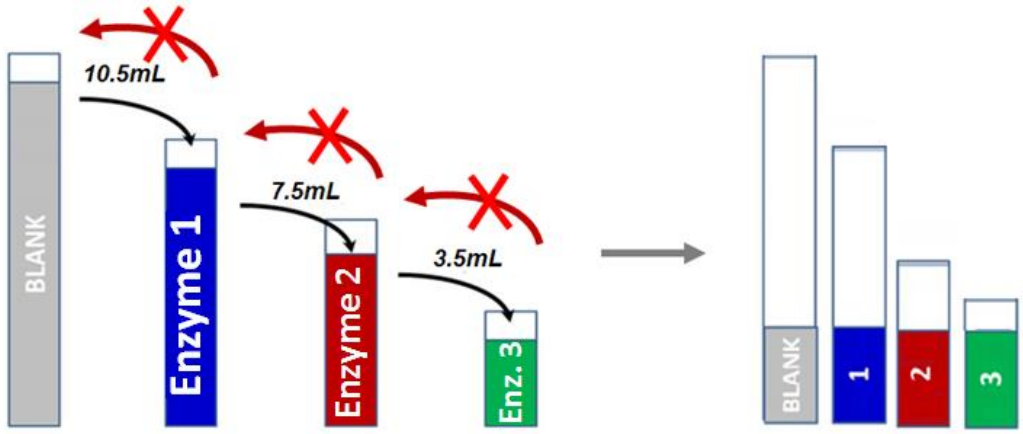
8. Label 40 cuvettes as white (for **"Blank"**), blue (**"Enzyme 1"**), red (**"Enzyme 2"**) and green (**"Enzyme 3"**) and sample number. To avoid error, we recommend to use colored stickers as shown below.



9. Fill four cuvettes (one white, one blue, one red and one green) with 0.3mL of the same filtered extract.

IX. Prepare enzymatic solutions

IMPORTANT: ***To avoid contamination of "Blank" with "Enzyme 1", do not touch "Enzyme 1" vial with the tip during liquid transfer. Similarly, avoid touching "Enzyme 2" vial with the tip during liquid transfer from "Enzyme 1" and "Enzyme 3" while transferring "Enzyme 2".***



1. Transfer the content of **"Reaction buffer"** vial to 15-ml **"Blank"** tube. Mix well by inverting repeatedly several times. Visually ensure that all contents are dissolved. Avoid foaming and do not vortex.

2. Transfer 10.5 mL of Blank to **"Enzyme 1"** , close and mix well by inverting.

3. Transfer 7 mL of **"Enzyme 1"** to **"Enzyme 2"**, close and mix well by inverting.

4. Transfer 3.5 mL of **"Enzyme 1"** to **"Enzyme 2"**, close and mix well by inverting.

   You have prepared: 3.5 ml of **"Blank"** for blanking for every sample
   3.5 ml of **"Enzyme 1"**
   3.5 ml of **"Enzyme 2"**
   3.5 ml of **"Enzyme 3"**

5. Add 0.3mL of **"Blank"** to eleven white cuvettes already filled with 0.3mL of samples;
   Add 0.3mL of **"Enzyme 1"** to eleven blue cuvettes;
   Add 0.3mL of **"Enzyme 2** to eleven red cuvettes;
   Add 0.3mL of **"Enzyme 3"** to eleven green cuvettes. Shake gently and wait for 30 min until reaction is completed.

6. Program spectrophotometer for absorbance reading at 340nm.
   For each sample or negative control :
   Insert **"Blank-N"** cuvette and make blanking (N is a sample number)**;**
   Insert **"Blue-N"** cuvette and measure **Absorbance$_{340}$1** (**IMP** converted to NADH). Note the value.
   Insert **"Red-N"**cuvette and measure **Absorbance$_{340}$2** (**IMP+hypoxanthine** converted to NADH). Note the value.
   Insert **"Green-N"** cuvette and measure **Absorbance$_{340}$3** (**IMP** + **Hypoxathine** + **Inosine** converted to NADH). Note the values.
   Do this for all samples and negative control.

7. To calculate K-value, relative and absolute concentrations of each nucleotide, use the formula cited in **Section II. Excel tables** allowing easy calculations of relative and absolute concentrations of each nucleotide are available at contact@novocib.com upon request.