PRECICE® Nucleotides Assay Kit

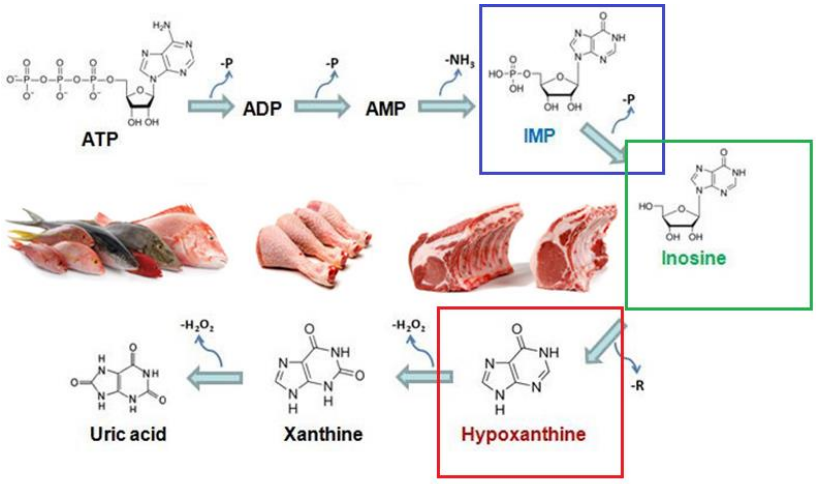
For measurement of three major ATP breakdown products (IMP, inosine and hypoxanthine) in seafood

Ref: 0700-04-10 (for spectrophotometer)

I. Introduction

Post mortem ATP catabolism in fish musle: Fish muscles are particularly rich in ATP, second abundant muscle metabolite after amino acids, where it provides the energy for contraction. In a live animal, muscle ATP hydrolysed to ADP is constantly resynthesized by mitochondiral respiratory chain, AMP and IMP concentration is low.

After death, ATP continues to be hydrolysed to ADP by contracting muscles, but its resynthesis by mitochondrial ATP-synthetase becomes impossible because of respiratory arrest. ATP is resynthesized by less efficient pathways (anaerobic glycolysis, creatine kinase and myoadenylate kinase), its concentration drops leading to gradual accumulation of ADP. ADP is rapidly dephosphorylated to AMP and deaminated to IMP by muscle AMP-deaminase. IMP is a predominant nucleotide in in-rigor muscle (Wang D et al 1998). Postmortem degradation of IMP to inosine and hypoxanthine results from both autolytic and bacterial enzymes. The rate of IMP degradation varies considerably between fish species and depends on handling and storage conditions (Surette et al., 1988).



While measuring absolute concentration of single nucleotide degradation product (e. g. hypoxanthine) was found to be not appropriate to determine the freshness quality of seafood, measuring relative concentrations provides a method for chemical assessment of fish freshness.

Measuring nucleotides can be of particular interest for assessment of the freshness of seafood products stored under modified atmosphere or transformed products for which the assessment of volatile amines can not be applied.

PRECICE® Nucleotides Assay Kit allows accurate measurement of concentrations of IMP, inosine and hypoxanthine in fresh, frozen and cooked fish and seafood products. These data can be used to calculate K_i value (Karube et al, 1984) , H-value (Huong et al, 1992) and F_r value (Gill et al. 1987).

$$K_i(\%) = \left[\frac{\text{Ino} + \text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}}\right] \times 100$$

$$H(\%) = \left[\frac{\text{Hx}}{\text{IMP} + \text{Ino} + \text{Hx}}\right] \times 100$$

$$F_r(\%) = \left[\frac{\text{IMP}}{\text{IMP} + \text{Ino} + \text{Hx}}\right] \times 100$$

References

1. Wang, D., Tang, L.R., Correia, L.R., Gill, T.A. 1998. Postmortem changes of cultuvated salmon and their effects on salt uptake. ***J.Food Sci.*** 63, pp. 634-637.
2. Surette, M.E., Gill, T.A., LeBlanc, P.J.1988 Biochemical basis of postmortem nucleotide catabolism in cod (Gadus morhua) and its relationship to spoilage ***J. Agric. Food Chem.*** 36, pp.19-22
3. Karube, I., Matsuoka, H., Suzuki, S., Watanabe, E., Toyama, T. Determination of fish freshness with an enzyme sensor system. 1984 ***J. Agric. Food Chem.*** 32, pp.314-319
4. Gill, T.A. Thompson, J.W., Gould, S.& Sherwood, D. 1987 Charcaterisation of quality deterioration of yellow fin tuna. ***J.Food Sci. 52***, pp. 580-583
5. 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. Principle

IMP is oxidized to XMP by **"Enzyme 1"** in the presence of NAD (Reaction 1). The amount of NADH formed in the above reaction is stoichiometric to the amount of IMP. NADH formation is measured as an increase in the absorbance at 340 nm.

Enzyme 1

(Reaction 1) IMP + NAD $\longrightarrow$ XMP + $NADH_2$

Hypoxanthine (Hx) is oxidized to uric acid by **"Enzyme 2"** in the presence of NAD (Reaction 2). The amount of NADH formed in the above reaction is stoichiometric to the amount of hypoxanthine and is measured as an increase in the absorbance at 340 nm.

Enzyme 2

(Reaction 2) Hx + NAD $\longrightarrow$ UA + $NADH_2$

Inosine (Ino) is hydrolyzed to first to hypoxanthine by **"Enzyme 3"**, hypoxanthine is oxidized to uric acid by Enzyme II in the presence of NAD (Reaction 3). The amount of NADH formed in the above reaction is stoichiometric to the amount of inosine and is measured as an increase in the absorbance at 340 nm.

Enzyme 3

(Reaction 3) Inosine + Pi $\longrightarrow$ Hx+NAD $\longrightarrow$UA + $NADH_2$

The reactions catalysed by these enzymes are:

- irreversible;
- specific and selective toward each particular nucleotides;
- convert three different nucleotides (IMP, inosine and hypoxanthine) to one common denominator ($NADH_2$).

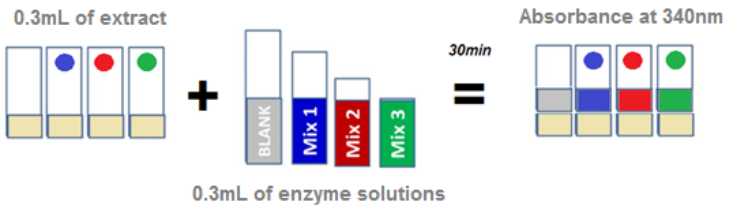
III. Storage

All enzymes are provided in lyophilized stable form and are transported at room temperature. After reception, **"PRECICE® Nucleotides Assay Kit"** must be stored at -20°C (stable for 12 months). Enzyme solutions must be freshly prepared before performing the assays. Once solubilized, reagent and enzyme solutions are not stable and should be used immediately.

IV. Short description (for spectrophotometer)

1) Boil fish muscle and filter the extract

2) Fill 4 cuvettes with 0.3mL of extract; Add 0.3mL of solubilized enzymes, incubate for 30 min ;

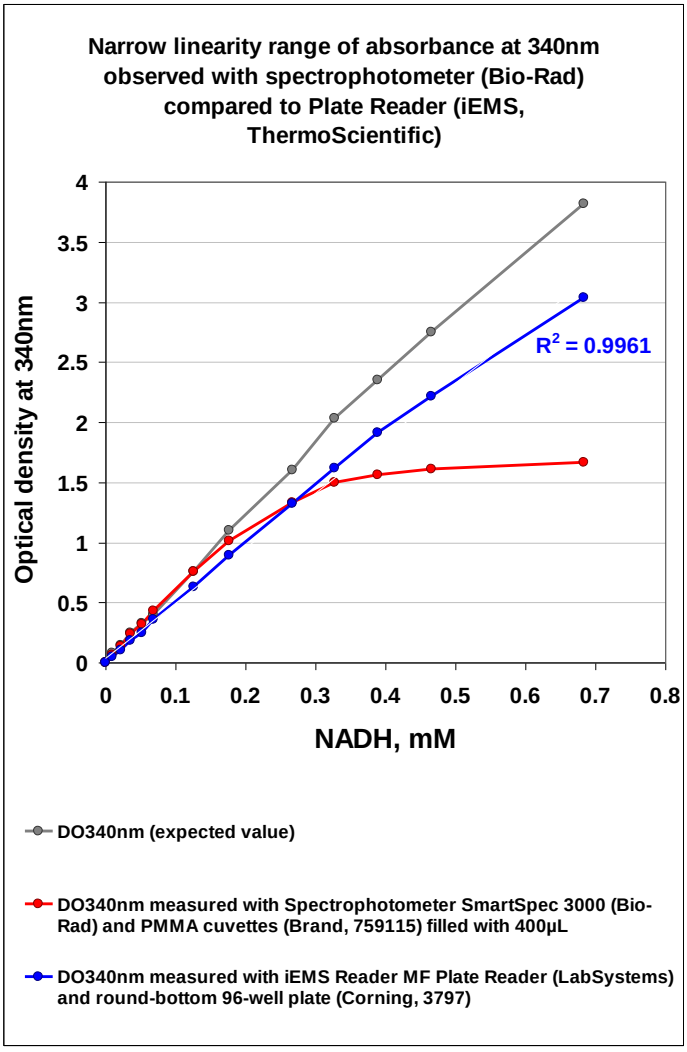


3) Read the absorbance at 340nm.

V. Material and equipment required for the analysis of 10 samples with spectrophotometer *(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.*

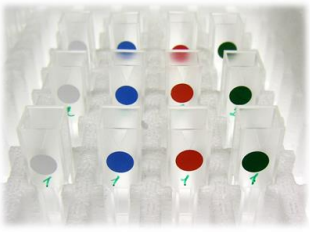
1. Thaw **"Extraction buffer concentrate"**, quantitatively transfer the content of **"Extraction buffer concentrate"** vial into a bottle with 0.95L of deionized water.
2. Heat boiling water bath.
3. Mince* 50-100g of sample and weight 2g of minced flesh into a separate 50-ml polypropylene tube, put 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!***



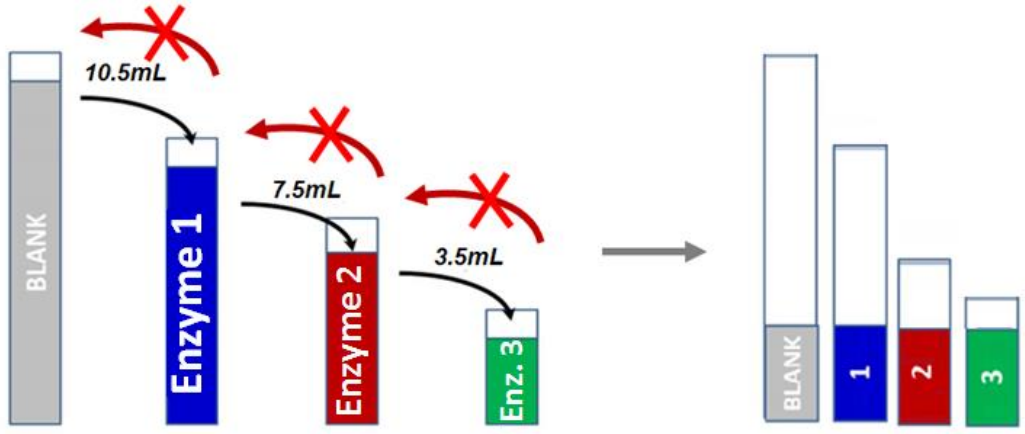
7. Fill 4 supplied cuvettes with 0.3**mL of diluted extraction buffer.

   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 $NADH_2$). Note the value.
   Insert **"Red-N"**cuvette and measure **Absorbance$_{340}$2** (**IMP+hypoxanthine** converted to $NADH_2$).
   Note the value.
   Insert **"Green-N"** cuvette and measure **Absorbance$_{340}$3** (**IMP** + **Hypoxathine** + **Inosine** converted to $NADH_2$).
   Note the value.
   Do this for all samples and negative control.

7. Calculate nucleotide concentrations:

   For each sample calculate nucleotide concentrations as follows:

$$\text{IMP (\%)} = \frac{\text{Absorbance}_{340}1 - \text{Blank}}{\text{Absorbance}_{340}3 - \text{Blank}} \times 100$$

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

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

Blank is the absorbance of the assay in the well containing **"Reaction buffer"**
$Absorbance_{340}1$ is the absorbance of the assay in the well containing **"Enzyme mix 1"**
$Absorbance_{340}2$ is the absorbance of the assay in the well containing **"Enzyme mix 2"**
$Absorbance_{340}3$ is the absorbance of the assay in the well containing **"Enzyme mix 3"**

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