PRECICE® Nucleotides Assay Kit

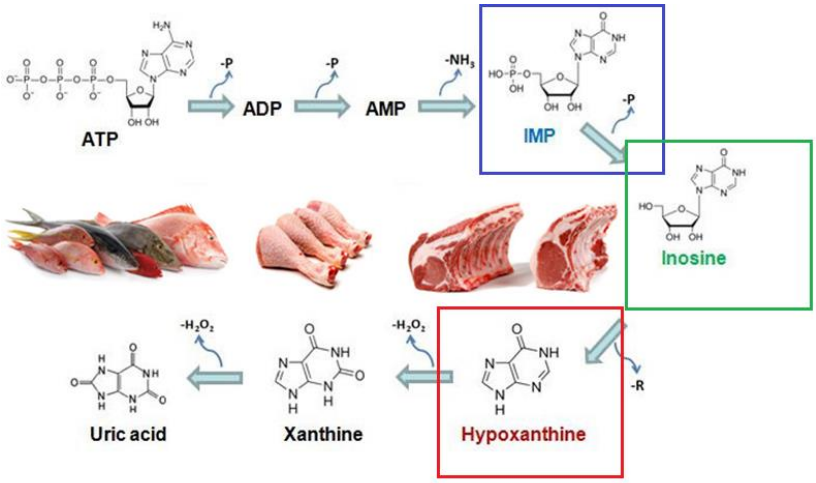
For measurement of three major ATP breakdown products (IMP, inosine and hypoxanthine) in seafood

Ref. K0700-003-22 (for microplate reader)

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 + $NADH_2$

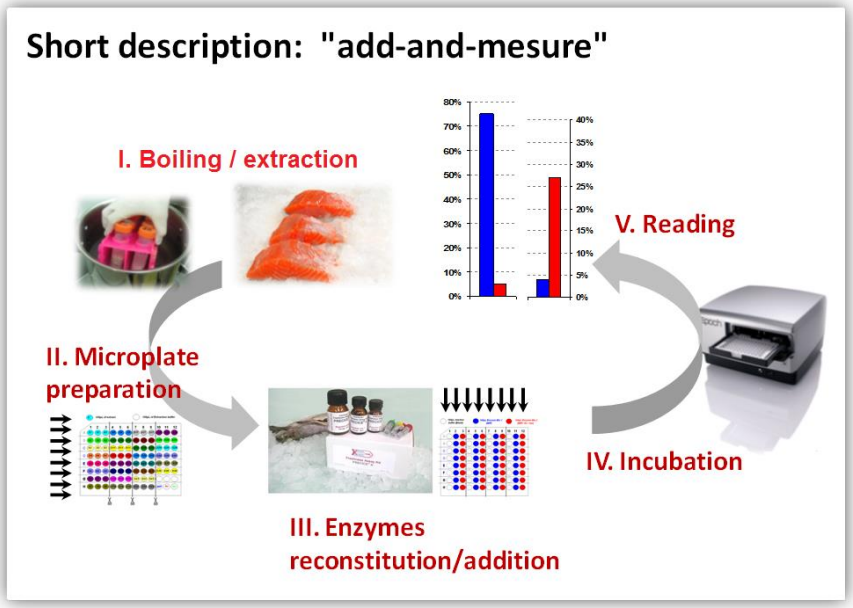
The reactions catalysed by these enzymes are:

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

III. Storage

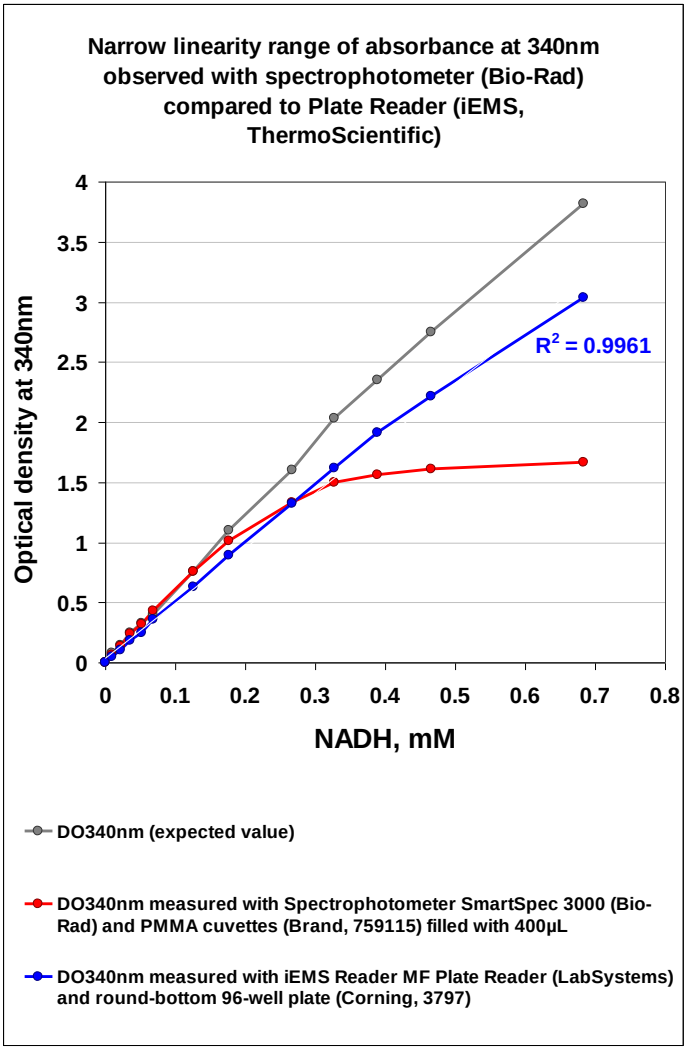
All enzymes are provided in lyophilized stable form and are transported at room temperature. After reception, **"PRECICE® Nucleotides Assay Kit"** must be stored at -20°C (stable for 12 months). Enzyme solutions must be freshly prepared before performing the assays. Once solubilized, reagent and enzyme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

IV. Short description



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

1) Deionized water ;
2) Boiling water bath;
3) Precision scale ;
4) 50-ml tubes with screw caps resistant to heating (ex. polypropylene tubes from Corning ref. 430828);
5) 0.2µm non-sterile filters (ex. Sartorius);
6) 10mL non-sterile syringes (ex. Braun);
7) Plate agitator;
8) Plate reader fitted with a 340nm filter (ex. Labsystems iEMS Reader MF (Thermo), Epoch (BioTec); PerkinElmer)



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 the standard solutions of $NADH_2$ 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.

VII. Kit components:

1) **"Extraction buffer concentrate",** for preparing 0.4L of extraction solution
2) **"Reaction buffer"** (provided in 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) Transparent empty microplate (round-bottom 96-well plate Corning, Costar® ref. 3797) prefilled with nucleotide (well H12)

VIII. Sample extraction

Before starting:

*a) 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.*

b) Turn on boiling water bath in advance

1. Thaw **"Extraction buffer concentrate"**, quantitatively transfer the content of **"Extraction buffer concentrate"** vial into a bottle with 0.38L of deionized water.
2. Mince* 50-100g of each sample
3. Weight 2g of minced flesh into a separate 50-ml polypropylene tube.

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

4. Add 20 mL of extraction buffer to minced samples, tightly close to avoid evaporation, and put immediately the tubes into boiling water for 20min. Be sure that the tubes are put in the water deeply enough.
5. After 20 min, take off the tubes and put them into tap water for fast cooling.

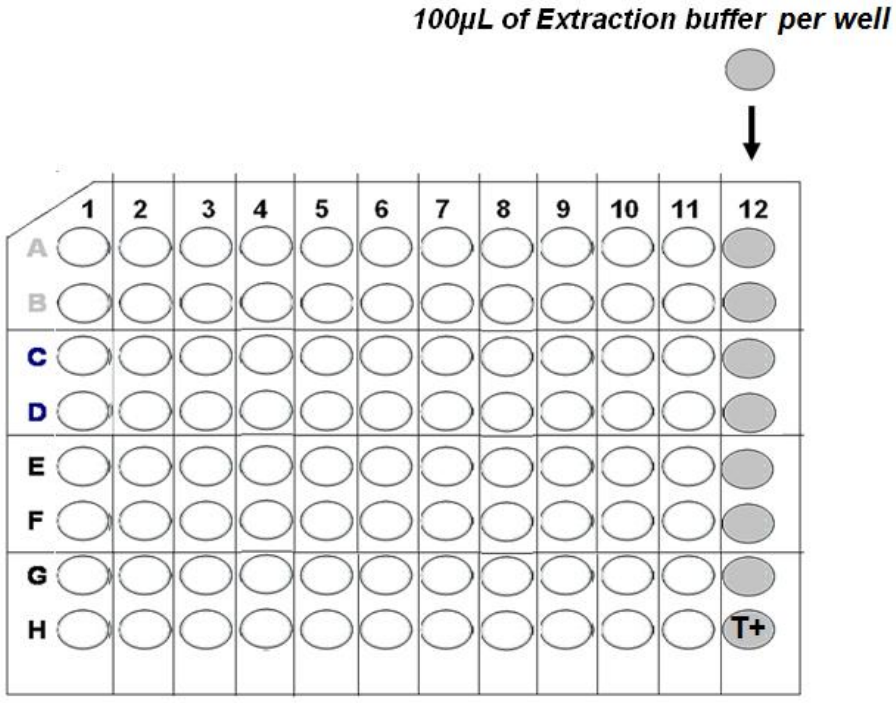
6. Filter the exudates:
   - Take off the plunger from syringe
   - Fix 0.2µm filter on the barrel
   - Carefully transfer the exudate to the barrel
   - Insert the plunger and filter 1.5-2mL of exudates into a clean 2-mL tube

IMPORTANT: Do not filter hot or warm samples!

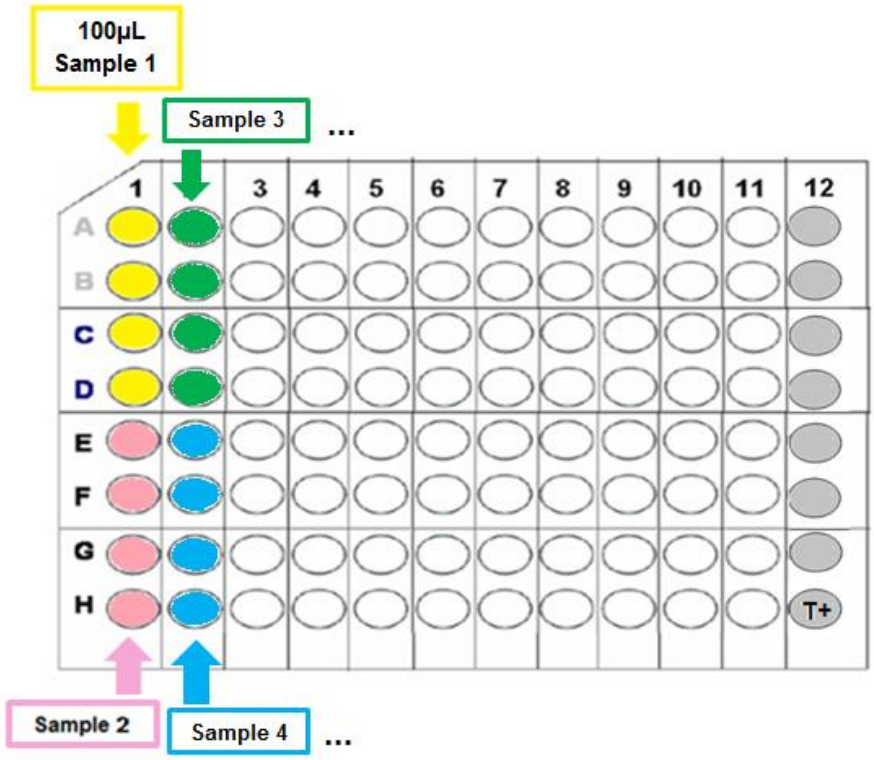
IX. Microplate filling with extracts (22 samples per plate)

The microplate provided with the kit is pre-filled with nucleotides (wells H12 closed with adhesive film).

1. Peel off a film covering well with standards before use, add 100µL of "Extraction buffer" to wells of column 12.



2. Dispense 100µL of filtered extracts in four wells of a 96-well plate, as shown in the following scheme. Avoid the bubles.



X. 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".***

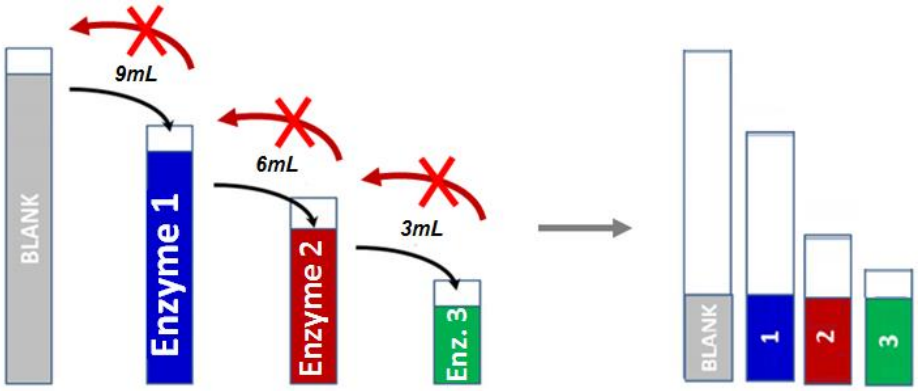
1. Transfer the content of **"Reaction buffer"** vial to 15-ml **"Blank"** tube pre-filled with powder. Mix well by inverting repeatedly several times. Visually ensure that all contents are dissolved. Avoid foaming and do not vortex.

2. Transfer 9 mL of Blank to **"Enzyme 1"** , close and mix well by inverting.

3. Transfer 6 mL of **"Enzyme 1"** to **"Enzyme 2"**, close and mix well by inverting.

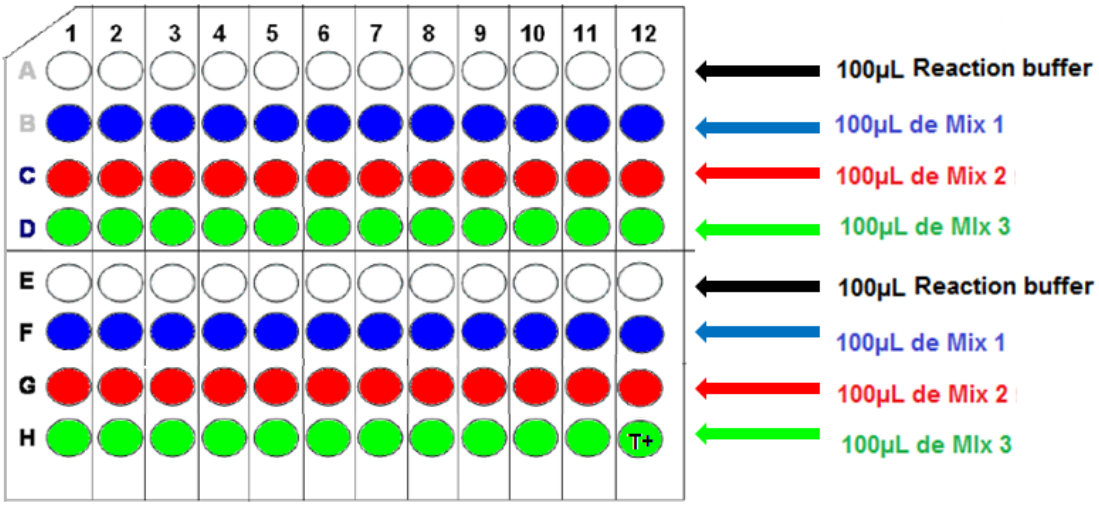
4. Transfer 3 mL of **"Enzyme 1"** to **"Enzyme 2"**, close and mix well by inverting.

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



XI. Microplate filling with reaction mixture

1. Dispense 100µL of **"Reaction mix" (Blank)** into the wells of line A; 100µL of **"Enzyme mix 1"** – of line C and D, 100µL of **"Enzyme mix 2"** - lines E and F and 100µL of **"Enzyme mix 3"** – lines G and F as shown below.



2. Agitate for 2 min at 1000rpm. Incubate for 30min. Agitate again the plate before reading the absorbance at 340nm.
3. Program plate reader for single absorbance reading and read OD340 for whole plate. (Optional: You can follow Optical Density of the reaction at 340nm by programming plate reader for kinetics).

XII. Calculate nucleotide concentrations:

For each sample calculate nucleotide concentrations as follows:

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

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

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

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

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