PRECICE® IMP, Inosine and Hypoxanthine Assay Kit (Freshness Test)

For 6 samples (microplate reader) Ref. K0700-003-06

Introduction - Post mortem ATP catabolism

Fish muscle is particularly rich in ATP, which is a most abundant muscle metabolite after amino acids. Post-mortem transformation of ATP in fish muscle is a well-documented process that has significant implications for food quality and shelf life. The degree to which ATP breaks down into IMP, inosine, and eventually hypoxanthine is a key indicator of fish freshness (Saito et al 1959).

In freshly caught fish, ATP is predominant nucleotide, but as the fish is stored, ATP breaks down through a series of enzymatic reactions:

1) ATP to IMP: Initially, ATP is broken down to IMP. This process is relatively quick and indicates very fresh fish. IMP is a predominant nucleotide in extra-fresh fish during first hours after slaughtering (Wang D et al 1998).
2) IMP to Inosine: IMP is then hydrolyzed to inosine by autolytic enzymes;
3) Inosine to Hypoxanthine: Finally, inosine is converted into hypoxanthine by both autolytic and bacterial enzymes. Fish muscle lacks hypoxanthine-hydrolyzing enzymes like xanthine oxidase, leading to the gradual accumulation of hypoxanthine as the fish spoils. This hypoxanthine buildup coincides with the production of volatile amines, which are responsible for the characteristic odor of spoiled fish (Dalgaard et al., 1993).

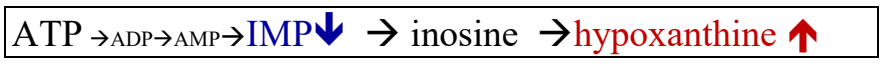


Figure 1: Post-Mortem ATP Degradation Pathway in Fish Muscle.

Relative molar concentrations of nucleotides (K_i value (Karube et al, 1984) , H-value (Huong et al, 1992) and F_r value (Gill et al. 1987)) are reliable indicators of fish freshness. They are not influenced by extraction efficiency or variations in muscle ATP between different species (Luong et al, 1992).

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

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

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

Given that the rate of ATP degradation can vary greatly among different species, it is essential to establish nucleotide criteria for high, medium, or low freshness, and spoilage for each species or product. This can be achieved by comparing nucleotide values with the sample's history (such as catch date and storage temperature), or with sensory analysis, microbiological assessments, and total volatile nitrogen measurements. This approach ensures more accurate and species-specific freshness indicators.

Principle

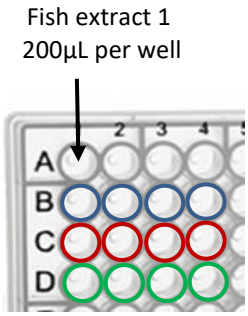
The **PRECICE® IMP, Inosine, and Hypoxanthine Assay Kit** is a colorimetric enzymatic assay designed for the quantitative analysis of three nucleotides: IMP, inosine, and hypoxanthine, in a single sample. It is suitable for use with fresh, frozen, and cooked fish and seafood products, including canned items

The **PRECICE® IMP, Inosine, and Hypoxanthine Assay Kit** is developed in a 96-well plate format. The microplate wells come prefilled with specific enzymes:

- **Line B**: Enzymes specific for IMP
- **Line C**: Enzymes specific for IMP and hypoxanthine
- **Line D**: Enzymes specific for IMP, hypoxanthine, and inosine

Once 200µL of cooked, diluted and clarified fish extract are added to four wells of the samez column, the enzymes convert IMP, inosine, and hypoxanthine present in the sample to $NADH_2$, which is then measured at 340 nm using a microplate reader.

Ligne A, empty	Blank
Ligne B, filled with enzyme 1	IMP ⟶ $NADH_2$
Ligne C filled with enzymes 1 & 2	IMP + Hx ⟶ $NADH_2$
Ligne D filled with enzymes 1, 2 & 3	IMP + Hx + INO ⟶ $NADH_2$



Specificity

No interference with other nucleotides 5'NMP (AMP, GMP, CMP or UMP), nucleosides (adenosine, guanosine, cytidine or uridine) or heterocyclic bases (adenine, guanine, cytosine or uracyl).

Linearity range:

For each nucleotide (IMP or hypoxanthine or inosine) the assay is linear between 0.1AU-1.6AU, corresponding to 0.2mmols/kg to 6mmols/kg of nucleotide after 20-fold dilution.

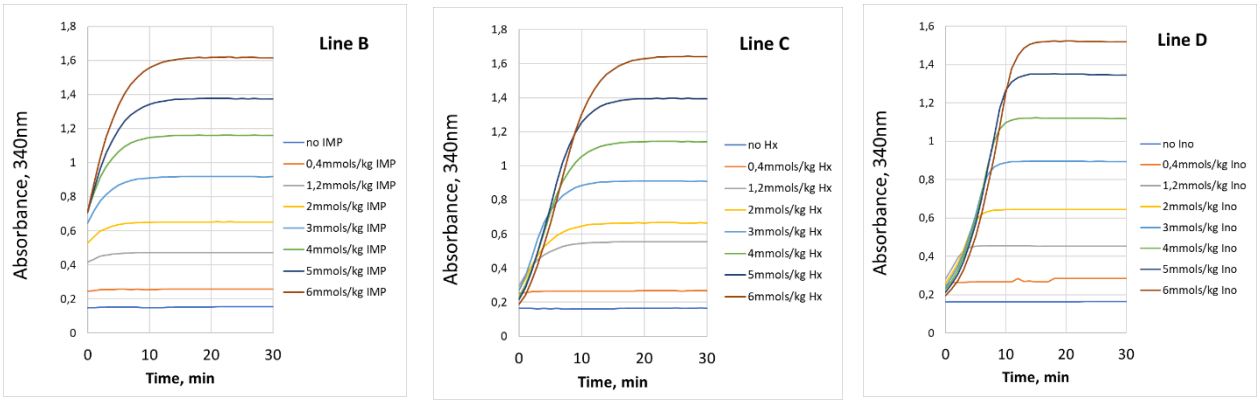


Figure 2. Increase in absorbance at 340nm in the presence of indicated concentrations of IMP (Line B), hypoxanthine (Line C) and inosine (Line D). The reactions were followed at 30°C using PRECICE® microplate (30°C, 200µL per well) and Labsystems iEMS Reader (Thermo).

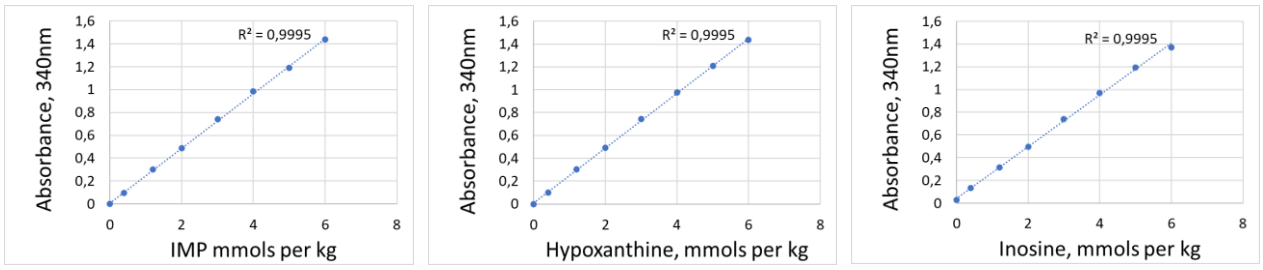


Figure 3. Calibration curves showing the linearity of PRECICE® IMP, Inosine, and Hypoxanthine Assay Kit in 0.2mmols/kg to 6mmols/kg range of IMP, hypoxanthine and inosine obtained using PRECICE® microplate (30°C, 200µL per well) and Labsystems iEMS Reader (Thermo) at 30°C.

Accuracy, Precision and Recovery:

Added nucleotide (mmol/kg)	Recovered (mmol/kg, mean of 6)	Recovery, %	Repeatability, RSD %	Added nucleotide (mmol/kg)	Recovered (mmol/kg, mean of 6)	Recovery, %	Repeatability, RSD %	Added nucleotide (mmol/kg)	Recovered (mmol/kg, mean of 6)	Recovery, %	Repeatability, RSD %
IMP, 1,0mmols/kg	1,025+/- 0,019	103%	2%	Hypoxanthine, 1mmols/kg	1,022+/- 0,043	102%	4%	Inosine, 1mmols/kg	1,022+/- 0,043	88%	4%
IMP, 2,0mmols/kg	1,982 +/- 0,059	99%	3%	Hypoxanthine, 2mmols/kg	1,960 +/- 0,064	98%	3%	Inosine, 2mmols/kg	1,960 +/- 0,064	85%	3%
IMP, 3,0mmols/kg	2,933+/- 0,042	98%	1%	Hypoxanthine, 3mmols/kg	2,837+/- 0,144	95%	5%	Inosine, 3mmols/kg	2,837+/- 0,144	84%	4%
IMP, 3,5mmols/kg	3,369+/- 0,088	96%	3%	Hypoxanthine, 3,5mmols/kg	3,336+/- 0,114	95%	3%	Inosine, 3,5mmols/kg	2,871+/- 0,114	82%	4%

Test time: 30min

Kit composition (for 6 samples):

1. ***"Reaction Buffer"*** (8mL; pH8.5);
2. "***Cofactors***" (powder)
3. ***Microplate*** (7 columns, filled with enzymes, lyophilized, sealed).

The kit is shipped at room temperature
After reception the kit should be stored sealed, at -20°C.
Stable 1 year if stored sealed and at -20°C.
Important: use the kit immediately after opening.

II. Material and equipment required for the analysis:

1. Deionized water ;
2. Boiling water bath or microwave;
3. 50-ml heat-resistant tubes with screw caps (ex. PP tubes from Corning ref.430828);
4. 1,5 mL tubes;
5. Scale ;
6. Minicentrifuge for 1,5ml tubes;
7. Micropipette for 200µL and 1mL Research Pro is strongly recommended;
8. Plate reader fitted with a 340nm filter and temperature control (Ex. Epoch, BioTek)

Before starting:
Turn on boiling water bath in advance;
Thaw **"Reaction Buffer"**;
Take microplate from -20°C (do not open immediately to avoid condensation);

III. Nucleotides extraction from fish by boiling (for frozen samples)

1. Weight 15-25g of fish muscle into a separate 50-mL polypropylene tube, note exact weight. Do it with all samples to be measured;
2. Add deionized water (1V per gram of fish muscle), tightly close the tube and put immediately into boiling water bath for 20min.
3. Take the tubes off waterbath and let them to cool down. To release all nucleotides in water, blender cooked samples for 15-30 sec. Pour ~1mL of bolied extract to a clean 1.5mL tube and clarify by centrifugation at 13000rpm, 5min;

Optional: nucleotides can be also extracted from raw fish using a household microwave oven.

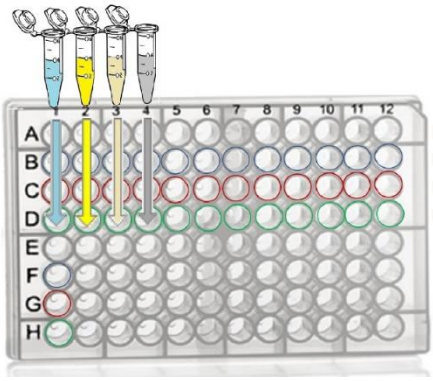
- *Weight 30-50g of fish muscle in 200-300mL dish compatible with microwave and note exact weight,*
- *Heat for 1 min at maximum power,*
- *Blender cooked samples in 1 volume of deionized water per gram for 15-30 sec,*
- *Clarify by centrifugation at 13000rpm, 5min*

IV. Reagents:


1. Add the totality of ***"Reaction Buffer"*** to "***Cofactors"*** tube. To do so, pour the content of glass vial to the tube and transfer remaining liquid at the bottom to the tube from vial to the tube using micropipette. Close, mix by inverting until complete dissolution of powder.
2. Dispense 900µL of "***Reaction Buffer"*** with cofactors to clean 1.5mL numbered tubes.
3. Add 100*µL of clarified extracts to 900µL of "***Reaction buffer"***, close, mix and centrifuge again at maximal speed for 5 min. Usually we use 100µL of fish extract and 900µL of buffer

**Most accurate results are obtained when OD of ligne D is 1.5-2.1. However, nucleotides concentration may slightly vary between species and different type of muscles. To choose optimal dilution, realize first experiment with 80µL of extract per 900µL. If OD_{340} in ligne D is <1.5, increase the volume of fish extract per mL. If $OD_{340\ ligne\ D}$ >2.1, decrease the volume of fish extract per mL. Contact us contact@novocib.com if you need more information.*

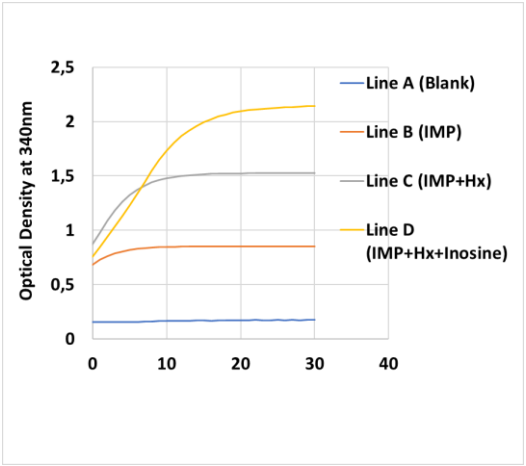
V. Microplate preparation



1. Using electronic pipette, dispense 200µL of each diluted fish extract to 4 wells of the same column (example: 200µL of Sample 1 to A1,B1,C1 and D1). Respect the order to avoid the contamination between well of the same column and avoid bubbles*.

IMPORTANT: *Start **always** with line A well (figure below) and go to the lines B, C and D. Dispensing in this direction you can touch the wells with the same tip with no risk of cross-contamination.*

Once microplate is filled, inspect for the absence of air bubbles that could disturb reading. Air bubbles can be easily removed from microplate by short blowing microplate with hair dryer.



2. Shake microplate for 1min, insert it into microplate reader pre-heated at 30°C and incubate for 30min. During this time, optical density signals achive plateau. After 30-min incubation agitate microplate for 1 min again and read Optical Density (single reading mode).

***IMPORTANT:** *Rigorous prolonged shaking is essential for enzymes solubilization and reactions. It helps to avoid elevated products concentrations at the bottom of the well.*

VI. Results

The absolute concentrations of the three nucleotides ([IMP], [inosine], and [hypoxanthine]) measured with the kit can be calculated using following formula and expressed in µmol/g.

$$\textbf{[IMP], µmol/g} = \frac{(\text{Abs B1} - \text{Abs A1}) * \text{Df}}{4{,}8}$$

$$\textbf{[Hx], µmol/g} = \frac{(\text{Abs C1} - \text{Abs B1}) * \text{Df}}{4{,}8}$$

$$\textbf{[Ino], µmol/g} = \frac{(\text{Abs D1} - \text{Abs C1}) * \text{Df}}{4{,}8}$$

$$\textbf{[IMP] + [Ino] + [Hx], µmol/g} = \frac{(\text{Abs D1} - \text{Abs A1}) * \text{Df}}{4{,}8}$$

Abs A1 states for absorbance at 340 nm in well A1 ;

Abs B1 - absorbance at 340 nm in well B1 ;

Abs C1 - absorbance at 340 nm in well C1 ;

Abs D1 - absorbance at 340 nm in well D1 ;

4,8 - absorbance at 340 nm of 200µL of 1 mM NADH solution in roud bottom microplate (l = 0,77 cm) Corning Ref. 3797;

Df - dilution factor = 20 (100µL of bolied extract, prepared by adding 1 volume of water per 1g, have been added to 900µL of reaction buffer).

Since each of the three nucleotides is converted into the same NADH substance measured through its absorbance at 340 nm, the relative molar rates of IMP or hypoxanthine can be calculated directly from absorbance values using formulas:

$$\textbf{IMP, molar \%} = \frac{(\text{Abs B1} - \text{Abs A1})}{(\text{Abs D1} - \text{Abs A1})} = \frac{([\text{IMP}])}{([\text{IMP}]+[\text{inosine}]+[\text{hypoxanthine}])}$$

$$\textbf{Hx, molar \%} = \frac{(\text{Abs C1} - \text{Abs B1})}{(\text{Abs D1} - \text{Abs A1})} = \frac{([\text{Hypoxanthine}])}{([\text{IMP}]+[\text{inosine}]+[\text{hypoxanthine}])}$$

$$\textbf{K}_\textbf{i}\textbf{, molar \%} = \frac{(\text{Abs D1} - \text{Abs B1})}{(\text{Abs D1} - \text{Abs A1})} = \frac{([\text{inosine}]+[\text{hypoxanthine}])}{([\text{IMP}]+[\text{inosine}]+[\text{hypoxanthine}])}$$

Abs A1 states for absorbance at 340 nm in well A1 ;

Abs B1 - absorbance at 340 nm in well B1 ;

Abs C1 - absorbance at 340 nm in well C1 ;

Abs D1 - absorbance at 340 nm in well D1 ;

To calculate absolute (mM) and relative concentrations of each nucleotides, you can also paste absorbance data into **Excel table** available at contact@novocib.com upon request.

References:

[1] Saito, T., Aarai, K. and Matsuyoshi, M. (1959) A New Method for Estimating the Freshness of Fish. Bulletin of the Japanese Society of Scientific, 24, 749-750.

[2] Peter Howgate (2006) Review of the kinetics of degradation of inosine monophosphate in some species of fish during chilled storage. International Journal of Food Science & Technology (2006), 41(4):341–353

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[4] Watanabe E, Toyama K, Karube I, Matsuoka H, Suzuki S. 1984. Enzyme sensor for hypoxanthine and inosine determination in edible fish. Applied Microbiology and Biotechnology 19(1):18–22

[5] Hong H, Regenstein IM, Luo Y. 2017. The importance of ATP-related compounds for the freshness and flavour of post-mortem fish and shellfish muscle: a review. Critical Reviews in Food Science Nutrition 57(9):1787–1798.