PRECICE® K-Freshness Assay Kit
for Fish Freshness measurement by "K value"

I. The "K value" concept

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.

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

Post-mortem ATP depletion is mainly due to autolytic reactions and controlled by endogenous enzymes, even if the microbial flora, when developed enough, may contribute to the process. This makes the level of ATP degradation an excellent criterion for fish freshness measurement and storage age, before microbial spoilage starts and the corresponding traditional techniques for spoilage measurement become relevant.

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 (**Hx**). 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, *i.e.* Ino and Hx, over the total content of ATP and its degradation by-products: ATP, ADP, AMP, IMP, Ino and Hx.

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

$$K\,(\%) = \frac{Ino + Hx}{IMP + Ino + Hx}$$

The K value is 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.

II. Principle

PRECICE® K-Freshness Assay Kit provides an easy enzymatic tool for measuring the K-value of fish flesh samples by spectrophotometry, in a convenient 96-well plate format. **PRECICE® K-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).

For each fish sample, **IMP** content and **IMP** + **Ino** + **Hx** content are measured by absorbance at 340nm. K value is then easily calculated from the two absorbance values obtained for a sample.

III. Assay Method

Sensitivity

PRECICE® K-Freshness Assay Kit shows an excellent sensitivity since LLOD (lowest limit of detection) and LLOQ (lowest limit of quantification) are of 4.5µM and 8.5µM, respectively. Thus, **PRECICE® K-Freshness Assay Kit** allows the detection of an IMP degradation as low as 4% (*i.e.* a K-value of 4%).

Linearity study

The correlation between absorbance level at 340nm and the concentration of **IMP**, **Ino** and **Hx** in the sample was studied using mackerel extract, to which **IMP**, **Ino** or **Hx** were added at different known concentrations. Absorbance values were corrected for the blank (mackerel extract with no added IMP, Ino or Hx).

Results show that absorbance measured using **PRECICE® K-Freshness Assay** is very closely correlated with **IMP**, **Ino** and **Hx** contents. Absorbance is linear with concentration up to 100µM concentration of **IMP**, **Ino** and **Hx**, with a correlation coefficient (ρ) > 0.998.

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The following figures show the linearity of absorbance measurement *vs.* concentration of **IMP** (Fig. 2a), **Ino** (Fig. 2b) and **Hx** (Fig. 2c).

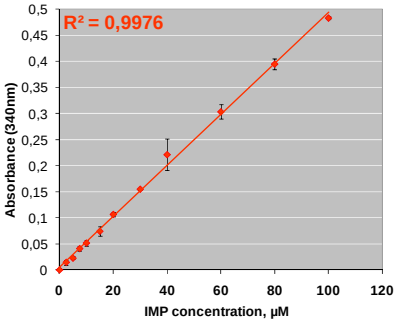

Figure 2a: Linearity of absorbance against IMP concentration in the fish sample

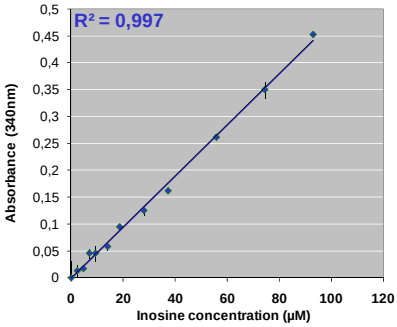

Figure 2b: Linearity of absorbance against Inosine concentration in the fish sample

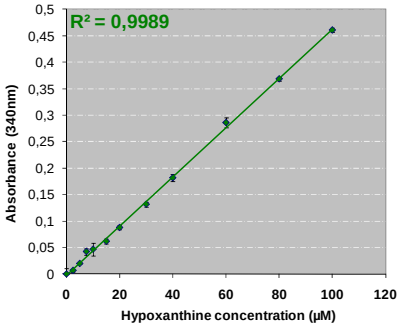

Figure 2c: Linearity of absorbance against Hypoxanthine concentration in the fish sample

IV. Kit Content

A standard PRECICE® K-Freshness Assay Kit (one 96-well plate) contains:

- 15ml vial containing **"Enzyme mix I" (orange)**, lyophilized, for **IMP** quantification
- 15ml vial containing **"Enzyme mix II" (blue)**, lyophilized, for **IMP** + **Ino** + **Hx** quantification
- 50ml vial containing 20ml of **"Reaction buffer"**
- 3 vials containing cofactors (powder)
- a transparent microplate (round-bottom 96-well plate Corning, Costar®, ref. 3797)

V. Storage

PRECICE® K-Freshness Assay Kit must be stored at -20℃ until used. Enzyme mixes a nd Reaction buffer must be freshly prepared before performing the assays.

Once prepared, reagent and enzyme solutions are not stable enough for storage.

VI. Description of the procedure

Fish sample preparation

i) Collect 3-4 g of flesh from the fish muscle (preferentially dorsal muscle). Put into a clean tube and boil in water-bath for 30 min

ii) Pipette 5 µL of the clear exudate of the sample into 6 wells of a labelled half-column (*e.g.* D1-D6): wells of rows 1 & 2 and 7 & 8 will be used for measurement of absorbance background (**Blank**), rows 3 & 4 and 9 & 10 for **IMP** quantification and rows 5 & 6 and 11 & 12 for **IMP** + **Ino** + **Hx** quantification (see Figure 3 for the preparation of micro-plate and set-up of the assays).

Preparation of microplate

The K-value of up to 16 samples can be measured in duplicate on a standard 96-well microplate.

It is strongly suggested to set up fish extract samples for **Blank**, **IMP** and **IMP** + **Ino** + **Hx** quantifications as shown on Figure 3.

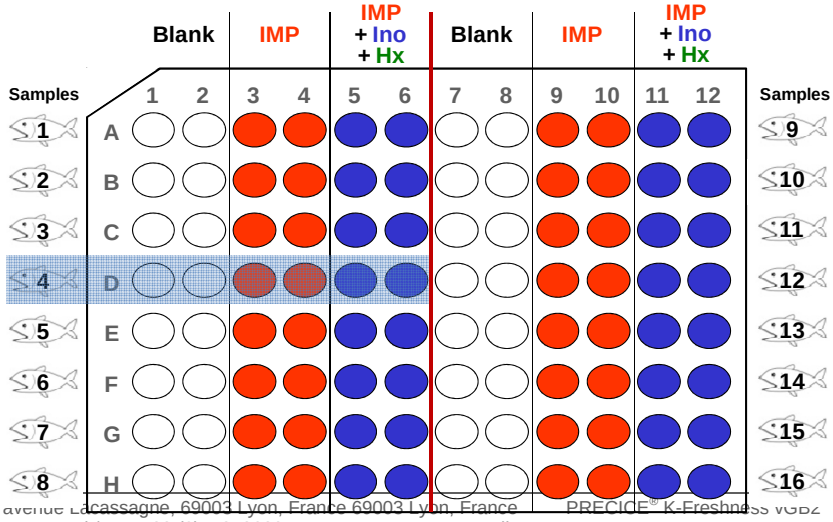

Figure 3: Arrangement on a 96-well microplate for K-value measurement of 16 fish extract samples, in duplicate.
Wells for sample 4 are shadowed as an example of arrangement of duplicate assays

Preparation of reaction mixes

i) Pour the contents of the 3 vials containing cofactors into the 50 mL vial **"Reaction buffer"**. Mix by gently flipping until cofactors are dissolved.

ii) From the **"Reaction buffer"** containing **cofactors** (prepare in i), pipette 14 mL and gently pour into the 15-mL vial **"Enzyme mix I" (orange)**. **Do not vortex**. Mix by gently flipping to avoid bubbles.
Enzyme mix I *is now ready to use for* ***IMP*** *quantification*

iii) From the **"Enzyme mix I" (orange)** prepared in ii), pipette 7 mL and gently pour into the 15-mL vial **"Enzyme mix II" (blue). Do not vortex**. Mix by gently flipping to avoid bubbles.
Enzyme mix II *is now ready to use for* ***IMP*** *+* ***Ino*** *+* ***Hx*** *quantification*

iv) In wells of rows 1 & 2 and 7 & 8 prepared for **Blank** controls in duplicate, add 200µL / well of **"Reaction buffer"** (remaider of the 50-mL vial content)

v) In wells of rows 3 & 4 and 9 & 10 prepared for **IMP** quantification, add 200µL / well of **"Enzyme mix I" (orange)** as prepared in ii).

vi) In wells of rows 5 & 6 and 11 & 12 prepared for **IMP** + **Ino** + **Hx** quantification, add 200µL / well of **"Enzyme mix II" (blue)** as prepared in iii).

vii) Insert the microplate into the reader, shake the plate for 2 min and incubate at 37°C for 60min or 25° C for 90 min. Check for the absence of air bubble in the wells and read the absorbance at 340nm. Record the data.

Calculation of K-value

For each assay, K is calculated according to the following formula:

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

where: $\text{Abs}_{\text{IMP + Ino + Hx}}$ is the absorbance in well containing **"Enzyme mix II"** for **IMP** + **Ino** + **Hx** quantification
Abs_{IMP} is the absorbance in well containing **"Enzyme mix I"** for **IMP** quantification
$\text{Abs}_{\text{Blank}}$ is the absorbance in well containing **"Reaction buffer"** for Blank absorbance background