PRECICE® Nucleotides Assay Kit

For rapid measurement of IMP, inosine and hypoxanthine in muscle

Ref: 0700-03

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

I. Principle of PRECICE® Nucleotides Assay Kit

The **"PRECICE® Nucleotides Assay"** is a first microplate assay for assessment of quality of seafood freshness. The enzymes provided with kit allow specific conversion of three major ATP catabolites - IMP, inosine (Ino) and hypoxanthine (Hx) to $NADH_2$. The quantification of nucleotides is done by measuring sample absorbance at 340nm. **PRECICE® K (IMP) 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).

	Auto-absorbance of fish extract and co-factors	**Blank**
Reaction buffer + co-factors 1 and 2		
Enzyme mix 1	**IMP** ⟶ **NADH**	$Absorbance_{340}1$
Enzyme mix 2 + co-factor 3	**IMP + Hx** ⟶ **NADH**	$Absorbance_{340}2$
Enzyme Mix 3	**IMP + Hx + Ino** ⟶ **NADH**	$Absorbance_{340}3$

In each sample, **IMP** is totally converted to $NADH_2$ by specific dehydrogenases found in **Enzyme Mix 1,** while enzymes found in **Enzyme Mix 2** convert both **IMP** and **Hx,** and **Enzyme Mix 3** converts to $NADH_2$ all three major ATP catabolites - **IMP** + **Ino** + **Hx**. The $NADH_2$ 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. The absorbance in a long UV range (340nm) allows using common consumable and running the assay on a 96-well plate. For each sample, **IMP** (%), indicator of ultrafreshness, **Hx** (%) indicator of alteration and **Ino** (%) are calculated as follows:

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

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

II. Equipments required *(not provided)*

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

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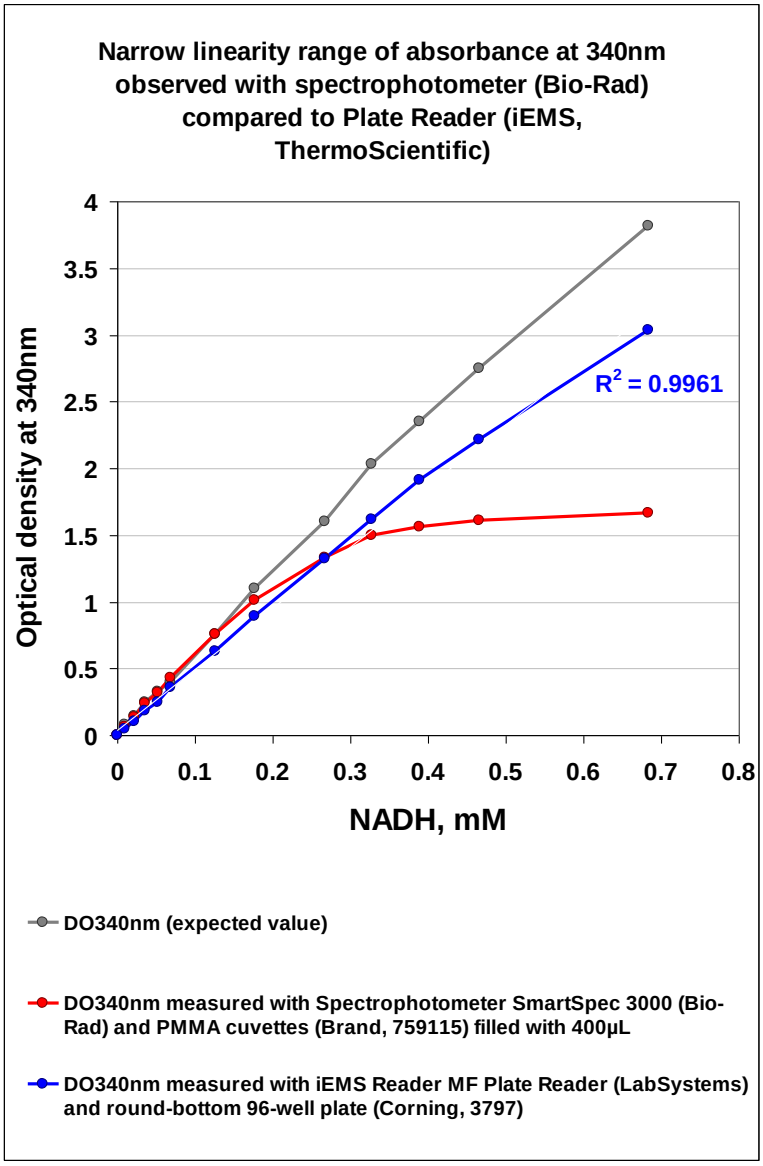
III. OPTIONAL: Defining the linearity range of absorbance at 340nm of your instrument



In "PRECICE® K" 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 of plate reader.

We strongly recommend using plate reader because this instrument has a larger linearity range compared to a spectrophotometer and allows simultaneous reading of 96 samples without additional pipeting and liquid handling. Alternatively, 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 400µL for absorbance reading, which is convenient since each 200µL assay must be diluted 2x or 4x before absorbance reading with a spectrophotometer.

IV. Kit Content (one 96-well plate, 22 samples)

1) **"Extraction buffer ",** for preparing 1L of extraction solution**.**

2) **"Reaction buffer"** (provided in 15-ml tube)

3) **"Enzyme mix 1"**, lyophilized in 15-ml glass vial, for **IMP** quantification

4) **"Enzyme mix 2"**, lyophilized in 10-ml glass vial, for **Hypoxathine** + **Inosine** quantification

5) **"Enzyme mix 3"**, lyophilized in 10-ml glass vial, for **IMP** + **Hypoxathine** + **Inosine** quantification

6) 1 tube **"Cofactor 1"** (powder)

7) 1 tube **"Cofactor 2"** (powder)

8) 1 tube **"Cofactor 3"** (powder)

9) Transparent empty microplate (round-bottom 96-well plate Corning, Costar® ref. 3797) pre-filled with 50nmols of IMP, inosine and hypoxanthine (supplied dried)

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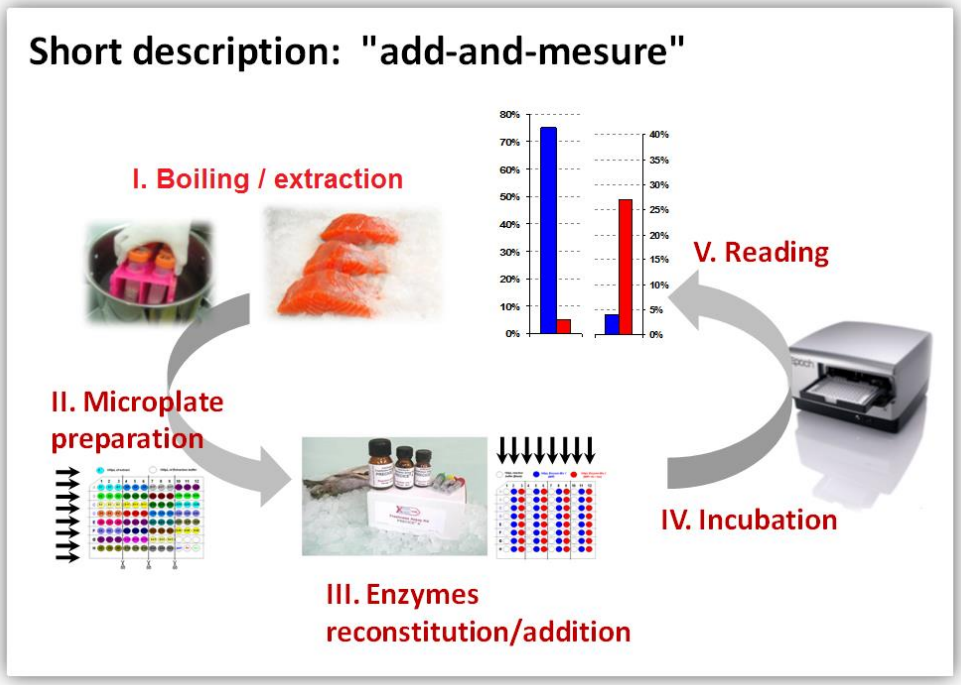
V. Storage

PRECICE® K-Freshness Assay Kit must be stored at -20°C until used. Enzyme mixes must be freshly prepared before performing the assays. Once prepared, reagent and enzyme solutions are not stable enough for storage.



VI. Sample preparation

VI.1. ***Before starting:*** Thaw **"Reaction buffer"** tubes at room temperature (**1h 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. Thaw **"Extraction buffer".**
VI.2. To prepare extraction buffer, quantitatively transfer the content of **"Extraction buffer concentrate"** vial into a bottle with 1L of deionized water.

VI.3. Weight 2-5g of muscles into a 50-ml polypropylene tube with screw caps resistant to heating (ex. polypropylene tubes from Corning ref. 430828), add 8-10*volumes of extraction buffer per gram of muscle (see Table 1)**,** tightly close the tube to avoid evaporation, and put them into a boiling water bath for 20min. Be sure that the tubes are put in the water deeply enough to cover all the muscle.

***Table 1.**

Raw material	**Recommended dilution**
Fish fillet	10 volumes of extraction buffer per gram
Shrimps	8 volumes per gram
Scallop	4 volumes per gram
Frog legs	1 volume per gram

Nucleotide concentration in muscle is ~5-10mM. The recommended dilutions allow to obtain ~300µM concentration of nucleotide in extract, that, once converted to $NADH_2$, would correspond to less than 1.5 Absorbance Unit, within linearity range of common plate reader. This dilution also helps to avoid depletion of cofactors that would lead to incomplete enzymatic reactions and underestimated values.

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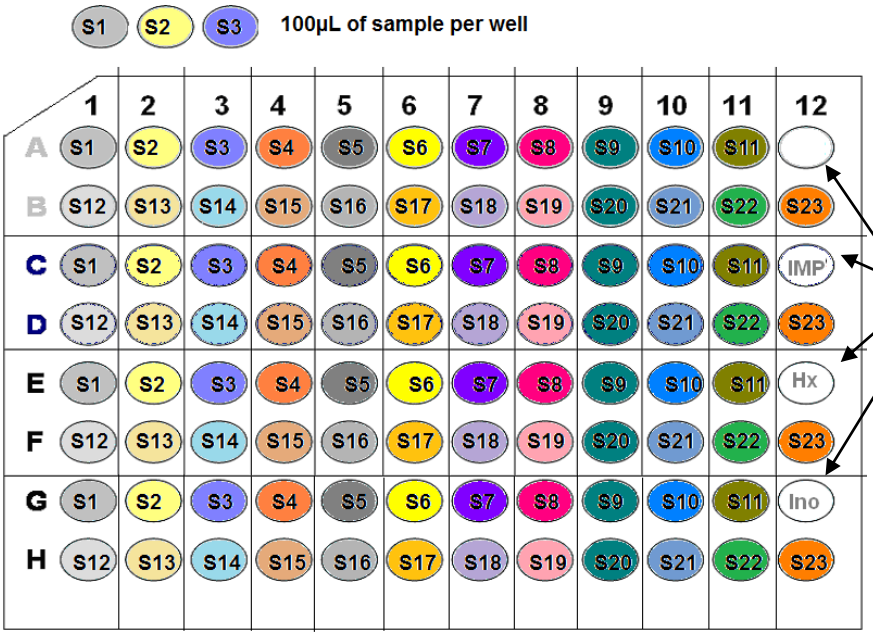
VI. 4. Filter the exudates:

- 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 the exudates into a clean tube.

IMPORTANT: Do not filter hot or warm samples!

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



VII.1. The microplate provided with the kit is pre-filled with nucleotides (wells C12, E12 and G12 closed with adhesive films). Peel off a band of film covering wells with standards before use, add 100µL of **"Extraction buffer"** to wells A12, C12, E12 and G12H10-H12 for standards solubilization.

100µL of Extraction buffer per well

VII.2. Dispense 100µL of diluted filtered extracts in a 96-well plate, as shown in the following scheme.

VIII. Preparation of reaction mixtures

VIII. 1. To solubilize lyophilized enzymes, add 200µL of deionized water to glass vial **"Enzyme Mix 1"** and 100µL of deionized water to 7-ml vial **"Enzyme mix 2"** Agitate gently until complete dissolution of the powder in all three vials.

VIII. 2. Quantitatively transfer **cofactors 1** and **2** to **"Reaction buffer"**. To do so:

- pipet 1ml of **"Reaction buffer"** to each tubes and mix them by inverting or pipeting up and down until dissolved,
- transfer by pipeting the content of all two small tubes back into a 15-ml tube with **"Reaction buffer"**,
- repeat to be sure that all reagent and enzymes of the small tubes are recovered.
- mix the complete **"Reaction buffer"** then containing cofactors 1 et 2 by gently inverting. Avoid bubbles.

VIII. 3. Pipet 8.4 mL of the so prepared **"Reaction buffer"** into a glass vial **"Enzyme mix 1"**. Mix gently by inverting (do not vortex, avoid bubbles). **"Enzyme mix 1"** is ready.

VIII. 4. Transfer 5.6 mL of the **"Enzyme mix 1"** into a glass vial with **"Enzyme mix 2"**. Quantitatively transfer the content of the tube with **cofactor 3** to **"Enzyme mix 2".** To do so, pipet 1ml of **"Enzyme mix 2"** to the tube with **cofactor 3,** mix by inverting. Avoid bubbles. **"Enzyme mix 2"** is ready.

VIII. 5. Transfer 2.8 mL of so prepared **"Enzyme mix 2"** into the vial with **"Enzyme mix 3"** (5-ml)**.** Mix gently by inverting (do not vortex, avoid bubbles). **"Enzyme mix 3"** is ready.

You have:
2.8 ml of **"Reaction mix"** for Blank measurement
2.8 ml of **"Enzyme mix 1"** for IMP measurement
2.8 ml of **"Enzyme mix 2"** for IMP + hypoxanthine (Hx) measurement
2.8 ml of **"Enzyme mix 3"** for IMP + Inosine + hypoxanthine measurement

IX. Microplate filling with reaction mixture

IX. 1. Dispense 100µL of **"Reaction mix" (Blank)** into the wells of line A and B; 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.

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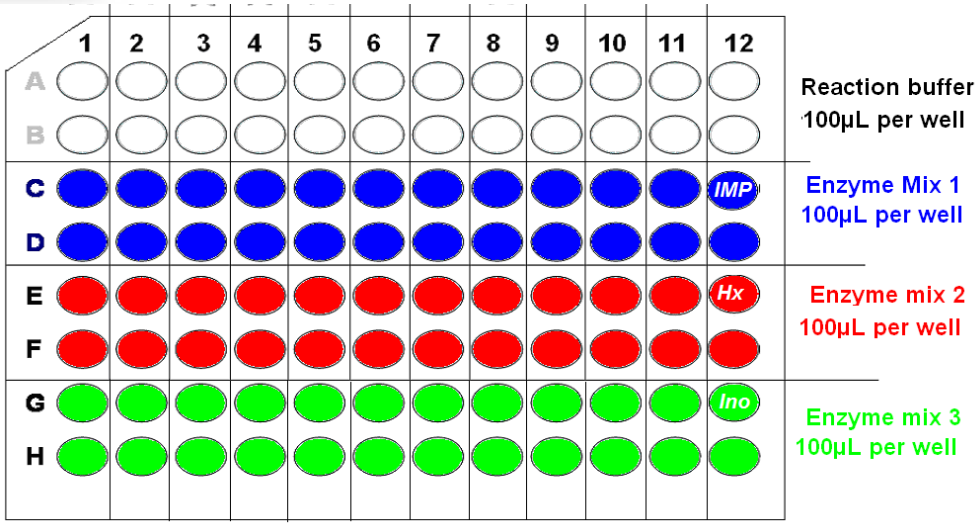

IX. 2. Agitate for 2 min at 1000rpm. Incubate for 30min. Agitate again the plate before reading the absorbance at 340nm.

IX. 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).

X. 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"**

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