PRECICE® ATP Breakdown Assay Kit

for rapid measurement of IMP in muscle tissues

Ref: 0700-001

I. Principle of PRECICE® ATP Breakdown Assay Kit

PRECICE® ATP Breakdown Assay Kit provides an enzymatic tool for measuring relative IMP content of fish flesh samples by spectrophotometry. The kit is developed for 96-well plate format and allows simultaneous analysis of 31 samples per plate.

PRECICE® ATP Breakdown 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). In each sample, **IMP** is totally converted to NADH by specific dehydrogenases found in **Enzyme Mix 1,** while enzymes found in **Enzyme Mix 2** convert to NADH all three ATP catabolites - **IMP** + **Ino** + **Hx**. The NADH 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.

Reaction buffer + co-factors 1 & 2	Auto-absorbance of fish extract and co-factors	**Blank**
Enzyme mix 1	**IMP** ⟶ **NADH**	**$Absorbance_{340}1$**
Enzyme Mix 2 + co-factors 3 & 4	**IMP**, **Ino**, **Hx** ⟶ **NADH**	**$Absorbance_{340}2$**

For each sample, relative content of IMP (%) is calculated as follows:

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

The enzymatic reactions used in **PRECICE® ATP Breakdown Assay Kit** are:

- ✓ highly specific and selective toward each particular nucleotides;
- ✓ irreversible;
- ✓ fast;
- ✓ converts all nucleotides to common denominator–NADH.

As a consequence, **PRECICE® ATP Breakdown Assay Kit** offers following advantages:

- ❖ simple ("add-and'mesure");
- ❖ does not require chemical extraction (the enzymes are added directly to filtered "bouillon");
- ❖ convenient (96-well plate);
- ❖ reliable (ratio instead of absolute concentration is mesured).

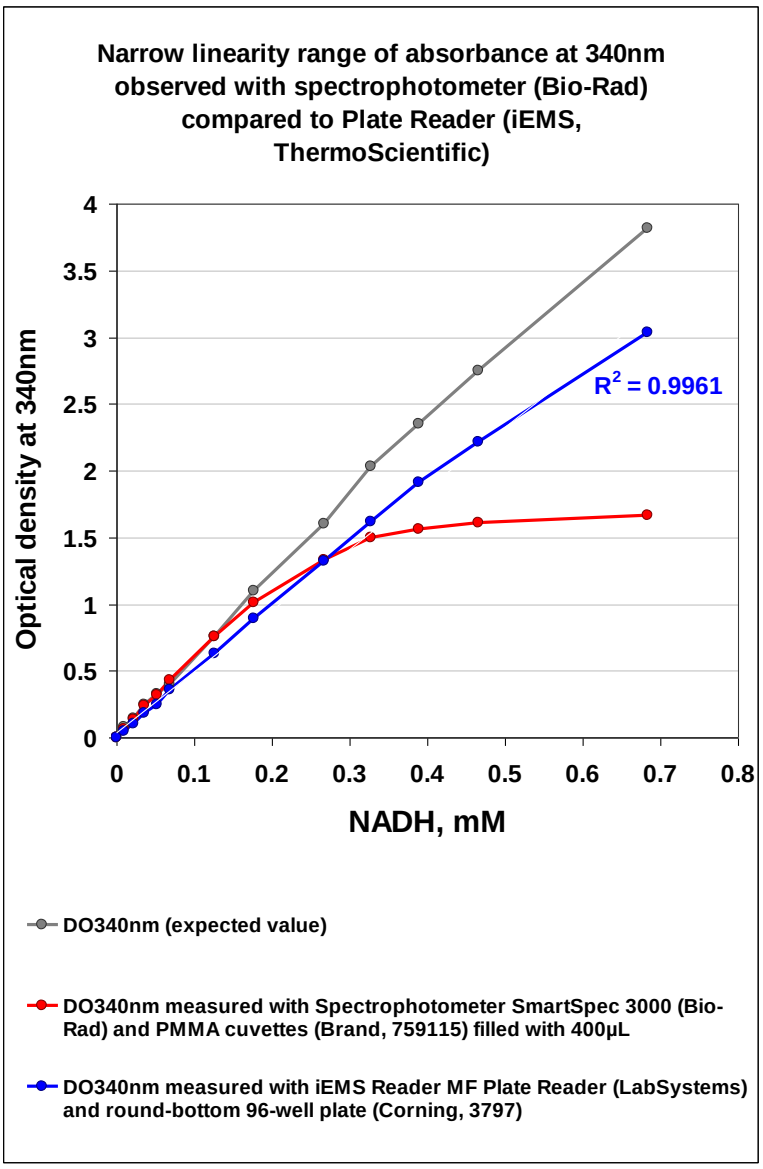
II. Equipments required *(not provided)*

For samples extraction by boiling

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 filter 340nm (ex. Labsystems iEMS Reader MF (Thermo), Epoch (BioTec); PerkinElmer)

We strongly recommend using plate reader because this instrument has a larger linearity range compared to a spectrophotometer (see below) 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.



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

In **PRECICE® K (IMP) Assay Kit** kit, the quantification of ATP-degradation products totally relies on the NADH 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 that can be used for the calibration of spectrophotometer of plate reader.

IV. Kit Content (one 96-well plate, 31 samples)

1) **"Extraction buffer concentrate",** for preparing of 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 **IMP** + **Hypoxathine** + **Inosine** quantification

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

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

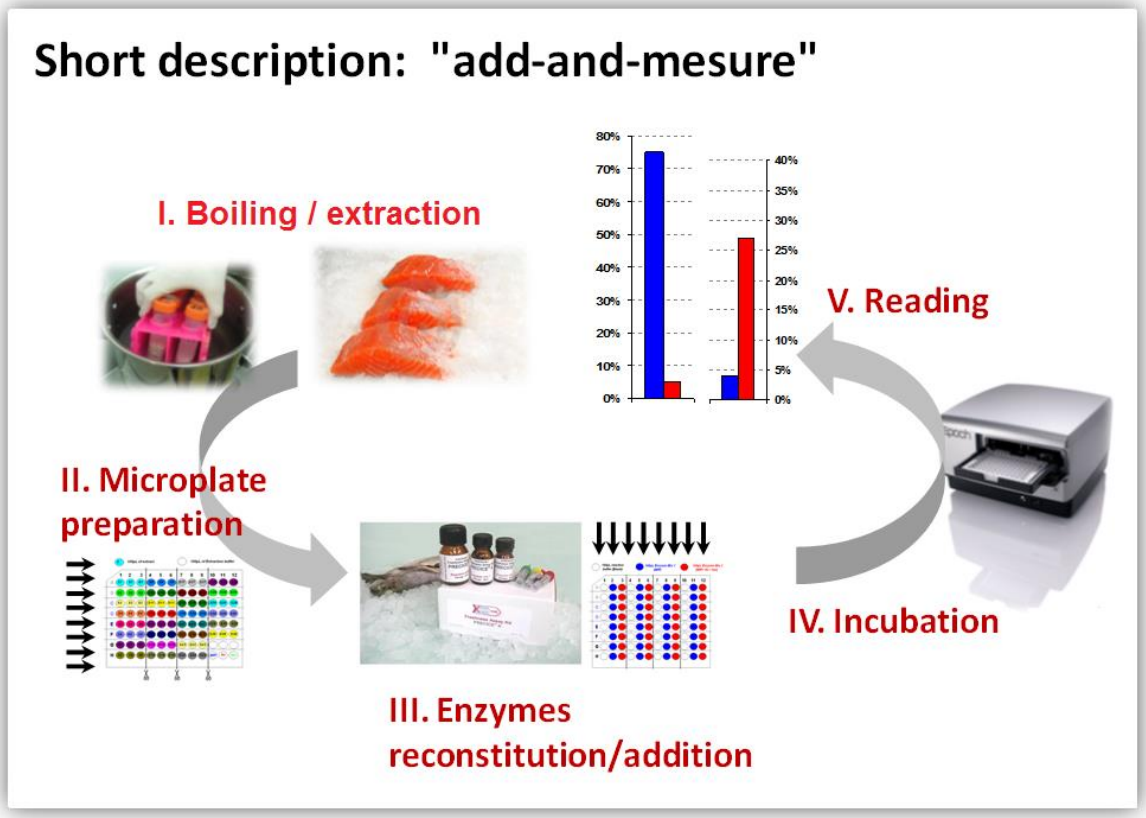
8) 1 tube **"Cofactor 3"** (powder)

9) 1 tube **"Cofactor 4"**(lyophilized)

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

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.

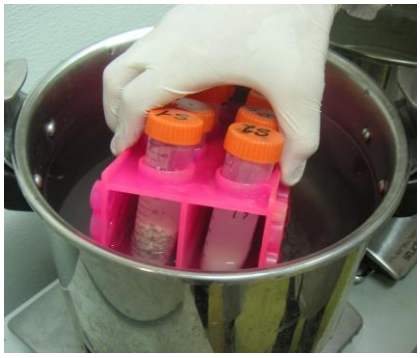


***Table 1.**

Raw material	**Recommended dilution**
Fish fillet	10 volumes of extraction buffer per gram
Shrimps	8 volumes of extraction buffer 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, 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 reaction and underestimated values. ***For additional information contact us directly at contact@novocib.com***	

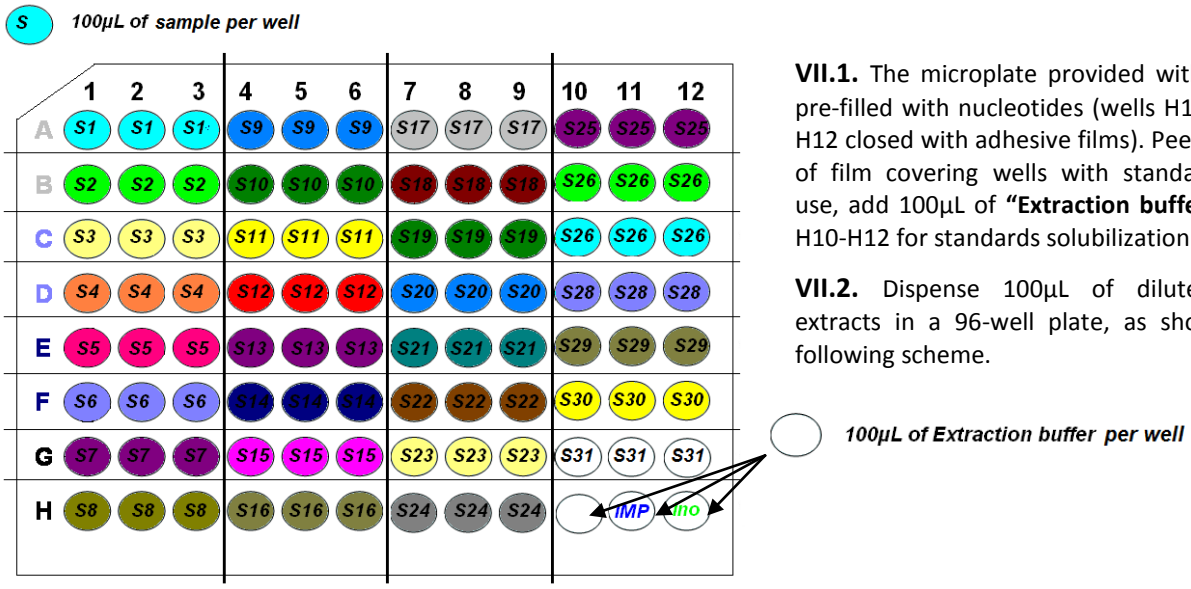
VI.4. Take off the samples from boiling bath and let them cool down to room temperature (25°C) before filtering. To accelerate cooling process, put the tubes into a wather bath at room temperature. Avoid to move /shake the tubes as much possible after boiling so that the liquid around the sample (exudates) stays as clear as possible.

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: Never filter hot or warm samples!

VII. Microplate filling with extracts (31 samples per plate)



VII.1. The microplate provided with the kit is pre-filled with nucleotides (wells H10, H11 and H12 closed with adhesive films). Peel off a band of film covering wells with standards before use, add 100µL of **"Extraction buffer"** to wells H10-H12 for standards solubilization.

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 7.5 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 3.75 mL of the **"Enzyme mix 1"** into a glass vial with **"Enzyme mix 2"**. Quantitatively transfer **cofactor 3** and **cofactor 4** to **"Enzyme mix 2"** as described in chapter IX.1. .

You have: 3.75 ml of **"Reaction mix"** for Blank measurement
3.75 ml of **"Enzyme mix 1"** for IMP measurement
3.75 ml of **"Enzyme mix 2"** for IMP+Inosine+hypoxanthine measurement

IX. Microplate filling with reaction mixture

IX. 1. Dispense 100µL of **"Reaction mix" (Blank)** into the wells of columns 1, 4, 7 and 10 as indicated in the following scheme. Dispense 100µL of **"Enzyme mix 1"** (columns 2, 5, 8 and 11) and 100µL of **"Enzyme mix 2"** (columns 3, 6, 9 and 12) as shown below.

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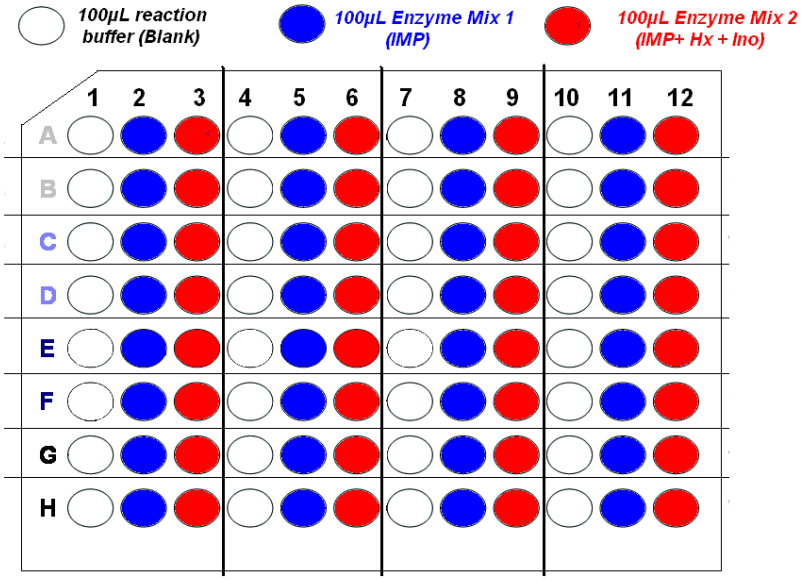

IX. 2. Agitate for 2 min at 1000rpm. Incubate for 30min. Agitate again the plate before reading the absorbance at 340nm

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).

Alternatively, absorbance reading with a spectrophotometer can be done in disposable cuvettes (PMMA, 1.5ml ref. 759115, Brand). Read the "Blank" with an empty cuvette. Then fill it with the 400µL of deionized water, add 200µL of well content. Read the absorbance at 340nm.

X. Calculate IMP ratio and K-value

For each sample calculate %IMP and K-value as follows:

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

where:

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

References:

1. Saito, T, Arai, K.I. and Yajima T. (1959) Changes in purine nucleotides of red lateral miscle of rainbow trout Nature 184: 1415
2. Saito, T, Arai, K.I. and Tanaka, T. (1958) Changes in adenine nucleotides in squid muscle. Nature 181: 127-1128