CATALOGUE #: **K01**

PRODUCT NAME: **HyTest cTnI *Diversity* kit**

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1. Background

Multiple cardiac troponin I (cTnI) forms can be detected in human blood. Such posttranslational modifications as phosphorylation, proteolysis, and complex formation with troponin C (TnC) were described in literature for the cTnI molecule. The antibodies (mono- or polyclonal), used for the assay development should recognize different cTnI forms, presented in the blood, with the same efficiency.

HyTest cTnI *Diversity* kit includes four pairs of antigen preparations (normal/modified) to be used in the testing of assay susceptibility to different cTnI modifications - complex formation, phosphorylation, proteolysis, and presence of heparin in the sample.

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2. Effect of complex formation on troponin I assay

cTnI is a component of troponin complex, responsible for regulation of muscle contraction. Troponin complex consists of three different molecules - troponin C (TnC) troponin T (TnT) and cTnI. All three components interact with each other with different affinities. The strongest interactions were demonstrated for cTnI - TnC complex (in the presence of Ca^{2+}; K_d = $1.5x10^{-8}$ M^{-1}) and for cTnI - cTnT complex (K_d =$4.4x10^{-5}$ M^{-1}) (1). Recent studies revealed that the main part of the cTnI in the patient's blood is presented in the form of binary cTnI – TnC complex and a small part (3-10%) as a free molecule (2).

Antibodies are different in recognizing free and complexed forms of the antigen. In some cases TnC forming binary complex with cTnI makes epitopes “invisible” to antibodies. Some antibodies demonstrate better recognition of free antigen, others are specific to complexed form and yet others have equal recognition of free and complexed forms (2,3). In an assay, which utilizes antibodies specific to the free form of the antigen and not recognizing cTnI in binary complex with troponin C, the major part of cTnI in the patient’s blood sample will be missed. Thus it can be concluded that cTnI - TnC complex formation is a very important factor influencing cTnI immunochemical detection and it should be considered during antibody selection. For precise cTnI measurements antibodies utilized in the assay should equally recognize free antigen and antigen in complexed from.

HyTest cTnI *Diversity* kit includes free cardiac troponin I (Vial 1) and troponin I complexed with TnC (Vial 2). Results of evaluation by the assay insensitive (A) and sensitive (B) to the complex formation are presented in the Fig. 1.

Vial 1: “Free” cTnI
Matrix: Normal human serum
cTnI, purified from human cardiac tissue

Vial 2: cTnI-TnC binary complex
Matrix: Normal human serum
cTnI, purified from human cardiac tissue, in complex with TnC

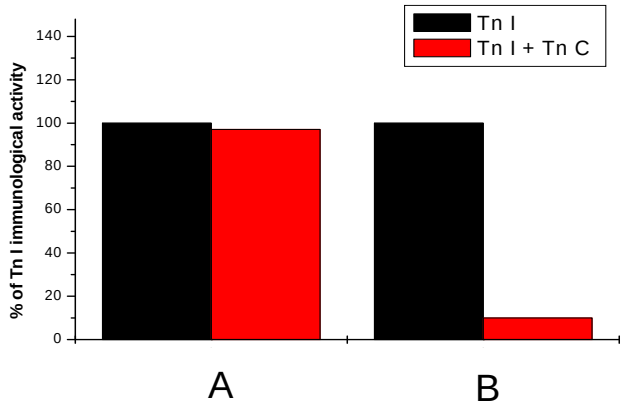

Fig. 1. Samples from Vial 1 (“free” cTnI) and Vial 2 (cTnI-TnC binary complex) were tested by two sandwich immunoassays. Assay A is insensitive to complex formation and antibodies recognize complexed form of the antigen with the same affinity as a “free” form. One of the antibodies utilized in the Assay B does not recognize the complexed form of the antigen, thus the assay can be used only for the “free” cTnI measurements. cTnI – TnC complex formation results in the significant decrease of the antigen immunoreactivity measured by Assay B.

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3. Effect of phosphorylation on troponin I assay

cTnI molecule contains two serines in the 22 and 23 positions. Both amino acid residues can be phosphorylated *in vivo* by protein kinase A (8). As a result four forms of protein (one dephospho, two monophospho and one bisphospho) can coexist in the cell. According to the recently published studies (5) the antibodies with the epitopes located close to or including the serine residues 22 and 23 are usually very sensitive to phosphorylation and do not recognize phosphorylated form of the protein. In patient's blood about 50% of the cTnI is phosphorylated. If the assay utilizes antibodies, which are sensitive to phosphorylation, at least half of the protein presented in the sample will be missed.

HyTest cTnI *Diversity* kit includes cTnI (in the form of troponin complex) purified from human cardiac tissue (vial 3) and cTnI completely phosphorylated (*in vitro)* by catalytic subunit of PKA from bovine heart (vial 4). Antigen in Vial 4 is not recognized by the antibodies, specific to dephosphorylated form of cTnI (Fig. 2).

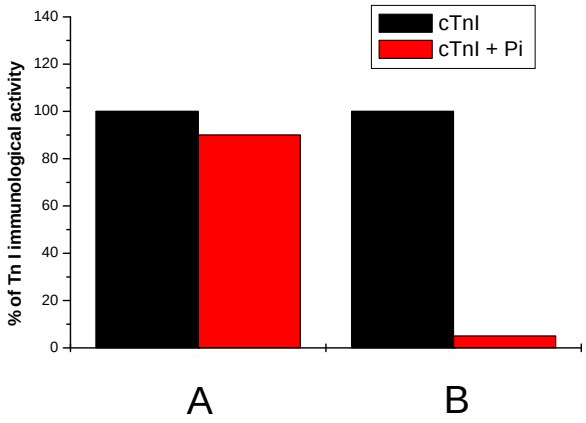

Fig. 2. Samples from Vial 3 (cTnI in ternary complex, purified from human cardiac tissue) and Vial 4 (same antigen in vitro phosphorylated by protein kinase A) were tested by two sandwich immunoassays. Antibodies utilized in the Assay A are insensitive to the antigen phosphorylation. Detection antibody in the Assay B does not recognize phosphorylated form of cTnI.

Vial 3: cTnI (-Pi)
Matrix: Normal human serum
cTnI, purified from human cardiac tissue

Vial 4: cTnI (+Pi)
Matrix: Normal human serum
cTnI, purified from human cardiac tissue, phosphorylated by protein kinase A

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4. Stability of cTnI molecule

Death of the cells in the ischemic region after blood vessel occlusion during infarction results in the lyses of all cell membrane structures, including membranes of lysosomes. As a result intracellular proteins are incubated in the protease cocktail for at least several hours. cTnI is known as a very unstable protein sensitive to the proteolytic degradation. In the necrotic tissue cTnI is rapidly cleaved by proteases and is released into the patient's blood stream as a mixture of native protein and different size proteolytic fragments (6,7).

In the *in vitro* experiments different parts of cTnI molecule revealed different stability. N- and C- terminuses were rapidly cleaved by proteases. The central part of cTnI located between amino acid residues 28 and 110 has significantly better stability because of the protection by TnC. Assays utilizing MAbs with the epitopes located on the unstable parts of the molecule failed to detect any cTnI in the late patient's samples (5-7 days after onset of the chest pain). On the other hand, assays utilizing MAbs with the epitopes located at the central stable part of the molecule were still able to detect significant amounts of the protein. Therefore in order to achieve better sensitivity and reproducibility of measurements the antibodies should recognize the stable part of cTnI molecule.

HyTest cTnI *Diversity* kit includes cTnI in ternary complex, purified from human cardiac tissue (Vial 5) and cTnI in ternary complex cleaved by proteases.

Vial 5: cTnI (troponin complex)
Matrix: Normal human serum
cTnI in troponin complex

Vial 6: cTnI (troponin complex) after proteolysis
Matrix: Normal human serum
cTnI in troponin complex after partial proteolytic cleavage

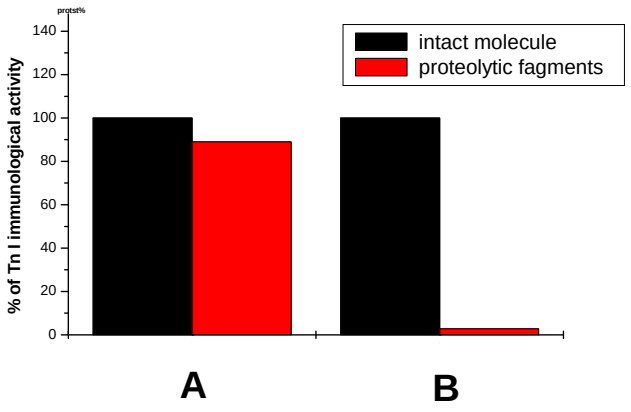

Fig.3. Samples from Vial 5 (cTnI in ternary complex, purified from human cardiac tissue) and Vial 6 (same antigen in vitro cleaved by proteases) were tested by two sandwich immunoassays. Antibodies utilized in the Assay A have the epitopes in the central (stable) part of the molecule. Antibodies of the Assay B recognize N- and C-terminal parts of the molecule.

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5. Effect of heparin on troponin I assay

cTnI molecule has a very high positive charge (pI 9.87) and consequently it easily forms complexes with negatively charged molecules. Complex between cTnI and TnC (pI 4.05) is an example of such an interaction. Plasma samples with heparin are widely used in clinics. Recent studies (5, 9) revealed that some prototype and commercially available assays show decreased concentrations of cTnI in heparin plasma compared to serum. Among the possible explanations of these observations is the electrostatic interaction between cTnI and heparin that prevents antigen – antibody interaction. At the same time there are the epitopes on the troponin surface molecule that are not affected by heparin. Antibodies recognizing these epitopes interact with the antigen with the same affinity in plasma and serum samples with heparin.

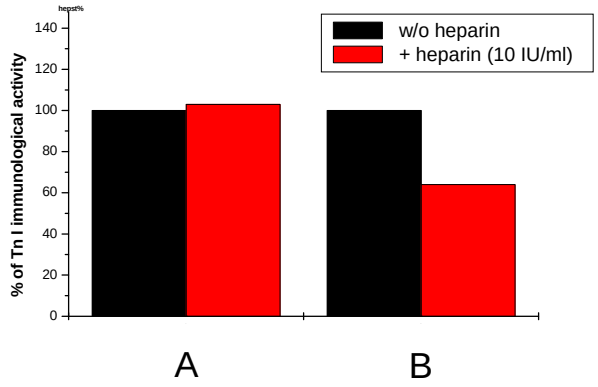

Fig.4. Samples from Vial 7 (cTnI in normal human serum) and Vial 8 (same sample in the presence of heparin) were tested by two sandwich immunoassays. Antibodies utilized in the Assay A have the epitopes that are not affected by heparin. Antibodies of the Assay B are sensitive to the presence of heparin.

HyTest cTnI *Diversity* kit includes cTnI in normal human serum (vial 7) and cTnI in the presence of heparin in matrix (vial 8).

Vial 7: cTnI (- heparin)
Matrix: Normal human serum
cTnI, purified from human cardiac tissue

Vial 8: cTnI (+ heparin)
Matrix: Normal human serum + heparin 10 IU/ml
cTnI, purified from human cardiac tissue

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6. Procedure

To prepare ready-to-use samples add 0.465 ml of deionised water to each vial with lyophilised material. Reconstituted material should be stored at +4°C no longer than 2 hours, or for one year at –70°C. Avoid repeated freezing and thawing.

7. Precautions

HyTest cTnI *Diversity* kit is for Laboratory use only. Not for clinical, household or any other uses. Reagents are containing human blood components. As all blood derivatives these reagents should be considered as potentially infectious. It is recommended that these reagents be handled using established standard laboratory working practices.

8. Storage

Lyophilised material can be stored for one year at –70°C.
Reconstituted material should be stored at +4°C no longer than 2 hours.
Avoid repeated freezing and thawing.

9. References

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