

HyTest NEWS



hsTnI MAbs™



High sensitivity cTnI assay concept

In 2007 scientific and medical societies are celebrating the 20th anniversary of the first troponin I assay described in literature (Cummins B, Auckland ML, Cummins P. Cardiac-specific troponin-I radioimmunoassay in the diagnosis of acute myocardial infarction. *Am Heart J.* 1987 113(6):1333-44.). Now we can ascertain that over the two decades concept of troponin I assay transformed greatly. Current generations of commercially available assays have sensitivity of almost 1000-fold higher (10 pg/ml vs. 10 ng/ml) than that of the experimental assay described by Dr. Cummins in 1987. New monoclonal antibodies specific to several selected epitopes are able to recognize all known modifications (free and complexed, phosphorylated, partially digested by proteases and others) of the antigen circulating in blood. Special forms of the antigen used as standards or calibrators helped to improve correlations between different assays more than 10-fold. All these changes make troponin I assay more precise and reliable tool in clinical practice.

High sensitivity of new generations of cTnI assays helps to register even minor events resulting in necrosis or apoptosis of myocardial tissue. But that is not true for every sample. Presence of cTnI-specific autoantibodies (Eriksson S, Hellman J, Pettersson K. Autoantibodies against cardiac troponins. *N Engl J Med.* 2005 6;352(1):98-100.) in blood of some patients makes detection of the antigen almost impossible. Autoantibodies, competing with the assay antibodies for the binding sites on the antigen surface, make troponin I "invisible" for the diagnostic assays. Effect of autoantibodies becomes even more prominent in the cases of minor events, when antigen concentration in human blood is very low. By testing of multiple blood samples our specialists

were able to discriminate the major epitopes recognized by autoantibodies. These data helped us to select monoclonal antibodies that could be used for the development of cTnI assays, which are not or almost not affected by the presence of autoantibodies in the sample.

So it becomes more and more evident that it is necessary to keep in mind that highly sensitive troponin I assay should utilize antibodies specific to the epitopes that are not affected by numerous modifications, which could occur with the antigen in human blood. Such an assay concept is described in following pages.

Cardiac isoform of Troponin I is a golden marker of cardiac muscle cell damage and death today. Different diagnostic platforms were designed for quantitative measurements of cTnI in human blood and are used extensively in big hospitals and small diagnostic laboratories. But still there is no between assay agreement and it often happens that one and the same blood sample gives different values when being analyzed by different cTnI assays.

The most common reason for the discrepancy in the assay measurements is difference in the epitope specificity of the antibodies used in different assays. Now we know that multiple factors are influencing cTnI measurements. Among them are posttranslational modifications (proteolytic degradation (1), phosphorylation (2)), complexing with other molecules (troponin C (3), heparin (2)) and cTnI-specific autoantibodies circulating in patients' blood (4). Different mono- and polyclonal antibodies, utilized in assays, are sensitive to these factors in different degree.

1. Katrukha AG, Bereznikova AV, Filatov VL, Esakova TV, Kolosova OV, Pettersson K, Lovgren T, Bulargina TV, Trifonov IR, Gratsiansky NA, Pulkki K, Voipio-Pulkki LM, Gusev NB. Degradation of cardiac troponin I: implication for reliable immunodetection. *Clin Chem.* 1998; 44(12):2433-40.
2. Katrukha A, Bereznikova A, Filatov V, Esakova T. Biochemical factors influencing measurement of cardiac troponin I in serum. *Clin Chem Lab Med.* 1999;37(11-12):1091-5.
3. Katrukha AG, Bereznikova AV, Esakova TV, Pettersson K, Lovgren T, Severina ME, Pulkki K, Vuopio-Pulkki LM, Gusev NB. Troponin I is released in bloodstream of patients with acute myocardial infarction not in free form but as complex. *Clin Chem.* 1997;43(8):1379-85.
4. Eriksson S, Halenius H, Pulkki K, Hellman J, Pettersson K. Negative interference in cardiac troponin I immunoassays by circulating troponin autoantibodies. *Clin Chem.* 2005, 51(5):839-47.

HyTest specialists have been involved in cTnI studies for more than 15 years. We generated and tested several thousands of monoclonal antibodies specific to different regions of cTnI molecule; we tried dozens of hundreds of different two-site MAb combinations in order to find the best one for precise cTnI immunoassay. Summarizing results of our studies we can conclude that on this moment it is impossible to have one antibody pair (one capture and one detection antibody), which would be absolutely insensitive to all known cTnI modifications and interferences. According to our opinion MAb combinations, which could be used for the development of precise cTnI immunoassay, should utilize two monoclonal antibodies as capture (plate or particle coating) and two MAbs for detection (conjugated with the specific label). We call such an approach as 2+2 concept. In these assays monoclonal antibodies should be selected in such a way, that if one of the MAbs (capture or detection) is sensitive to the presence of some factor in the sample, then the other MAb should be insensitive to the same factor. Thus the effect of negative or positive interference is minimized. Also one important parameter should be considered: antibodies utilized in the assay should be specific to the cardiac isoform of the protein and should not have crossreaction with the two skeletal isoforms.

Today HyTest can suggest several combinations of monoclonal antibodies useful for the development of cTnI assays according to 2+2 concept. Such assays would be cardiac specific and almost insensitive to all known factors that could affect cTnI measurements. Moreover, while selecting antibodies we also considered the fact that new generation of cTnI assays should display high sensitivity and antibody combinations could be used in point-of-care

diagnostics platforms. So, assays described in Table 1 have good kinetics and they recognize standard preparation of antigen (cTnI in troponin complex) with sensitivity better than 50 pg/ml.

Table 1. The best HyTest 2+2 combinations of monoclonal antibodies.

Capture antibodies	Detection antibodies
MF4 + 560	19C7 + 267
M18 + 560	19C7 + MF4
M18 + 560	19C7 + 267

Epitope specificity of antibodies:
M18₁₈₋₂₈, 19C7₄₁₋₄₉, 560₈₃₋₉₃, 267₁₆₉₋₁₇₈, MF4₁₉₀₋₁₉₆

Factors influencing cTnI measurements are schematically presented in Fig. 1. Antibodies specific to different parts of the molecule are sensitive to these factors in different degree. For instance, it is well-known fact that purified cTnI is very susceptible to proteolytic degradation. But in troponin complex central part of the cTnI closely interacts with troponin C (TnC) and TnC protects cTnI from proteolytic degradation. As a result, the epitopes located on the central part of the cTnI are significantly more stable than epitopes located at the terminal parts of the molecule. But at the same time TnC competes with antibodies for binding with cTnI and only few antibodies, specific to the central part of cTnI can recognize complexed form of the antigen.

In figures 2–6 we are comparing new assays designed in accordance to 2+2 concept with conventional assays sensitive to different factors, thus illustrating the insensitivity of new assays to these factors.

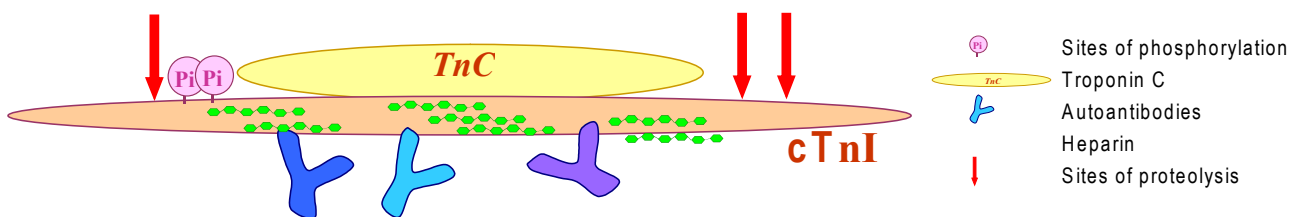


Figure 1. Factors influencing cTnI measurements.



A. Cardiospecificity

Three isoforms of cTnI are described for humans – one is expressed in cardiac muscle (cardiac TnI) and two others are essential for slow and fast skeletal muscles (slow skTnI and fast skTnI, respectively). Identity in sequence between cTnI and slow skTnI is about 40% and is less but still significant for fast skTnI. So, all antibodies selected for cTnI assay should be tested to have no crossreaction with skeletal isoforms of the protein. As it can be seen from Fig. 2, being tested even with high concentrations of skTnI (50 ng/ml) new assays produce no response, thus indicating their extremely high cardiospecificity.

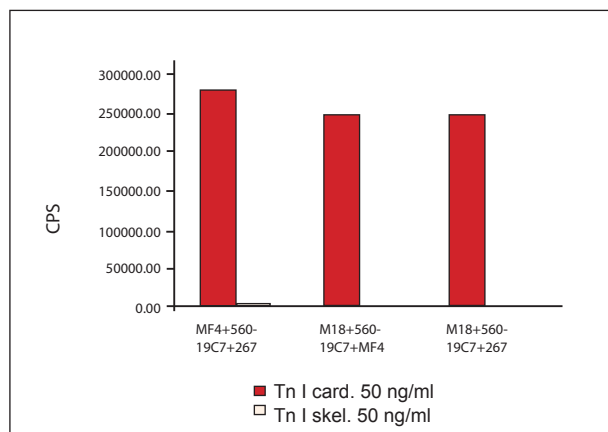


Figure 2. Testing of 2+2 assays with cardiac and skeletal isoforms of TnI.

B. Sensitivity to heparin

cTnI is a highly basic molecule with pI ~9.9. It easily forms complexes with molecules with negative charge. Heparin is widely used in clinical practice as an anticoagulant and almost all patients with suspected acute myocardial infarction (AMI) receive heparin during the first minutes after admission. It was shown that some anti-cTnI MAbs are sensitive to the presence of heparin in the sample and give lower response with the samples containing heparin (MAb 228 in the pair 228-MF4 in Fig. 3). MAbs, utilized in 2+2 concept are not sensitive to the presence of heparin.

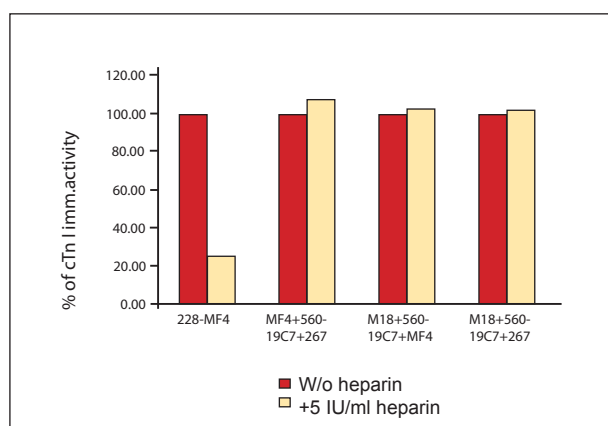


Figure 3. MAb combinations tested with cTnI (concentration 50 ng/ml) in the presence (5 IU/ml) and absence of heparin.

C. Sensitivity to proteolytic degradation

cTnI is known as an extremely unstable molecule. We incubated native troponin complex for 116 hours with endogenous tissue protease cocktail and tested it in 2+2 assays. In all 2+2 assays antigen demonstrated significantly better stability in comparison with the control assay utilizing antibodies 9F6 and p45-10 with the epitopes located at the terminal parts of the molecule (assay is sensitive to the proteolytic degradation of the antigen).

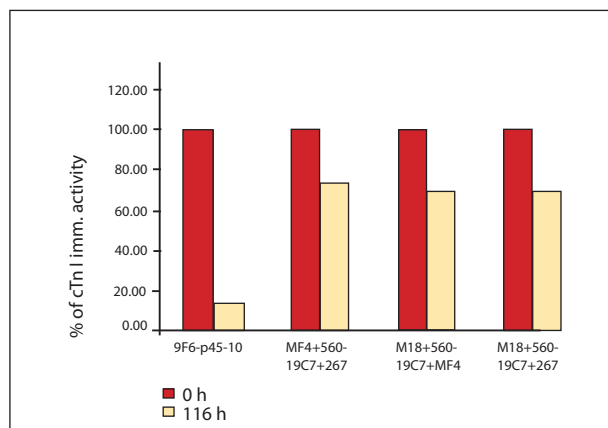


Figure 4. Testing of new assays with degraded antigen.

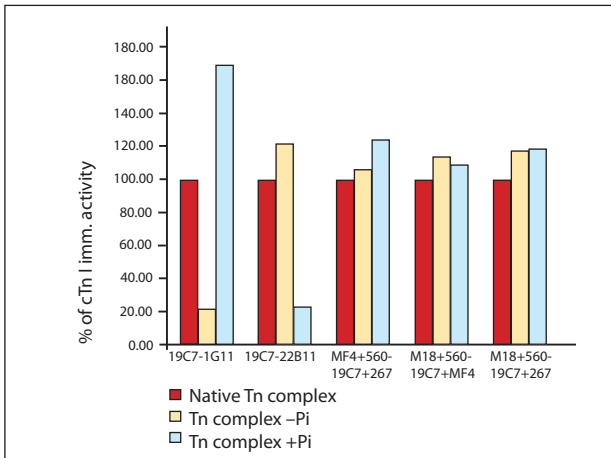


Figure 5. Testing of new assays with native troponin complex, artificially phosphorylated troponin complex and artificially dephosphorylated troponin complex (Note: MAb 1G11 recognizes only phosphorylated cTnI and MAb 22B11 is specific only to the dephosphorylated antigen).

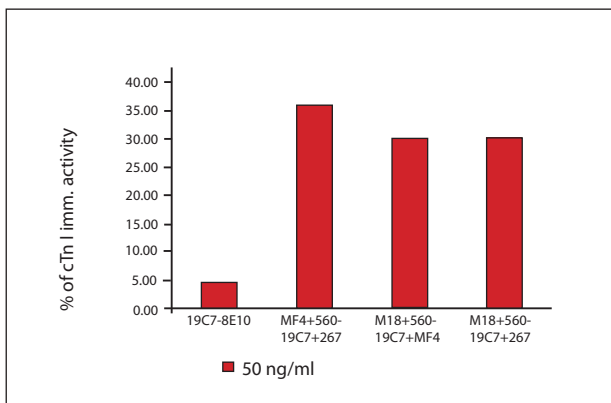


Figure 6. Antigen was spiked into the serum sample with high level of autoantibodies and the signal was compared with the antigen spiked in the same concentration (50 ng/ml) into buffer (100% of activity) (Note: Assay 19C7-8E10 is sensitive to the presence of autoantibodies in the sample).

D. Sensitivity to phosphorylation (protein kinase A)

Two serines in the 22 and 23 positions could be phosphorylated by protein kinase A *in vivo*, so four forms of protein (one dephospho-, two monophospho- and one bisphospho-) can coexist in the cell. Phosphorylation of cTnI changes the conformation of the protein and modifies its interaction with other troponins as well as the interaction with some anti-cTnI antibodies (MAbs 1G11 and 22B11 in pairs 19C7-1G11 and 19C7-22B11 in Fig. 5). According to the latest findings significant part of cTnI released into the patient's blood stream is phosphorylated. MAbs utilized in 2+2 concept are not sensitive to phosphorylation status of cTnI.

E. Sensitivity to the presence of autoantibodies

Recent studies revealed that some blood samples (5-10% of total population) contain high level of autoantibodies specific to cTnI. Antigen concentration in such samples can be significantly underestimated or antigen becomes undetectable (in case of low concentration of cTnI in the sample). In 2+2 concept we utilized at least one capture and one detection MAb, which are in less degree sensitive to the presence of autoantibodies. As a result new assays can detect antigen even in the samples with high content of autoantibodies.

Ordering information:

Product	Cat.#	MAb	Subclass	Epitope	Application
Anti-cTnI	4T21	M18	IgG1	a.a.r. 18-28	EIA, WB
Anti-cTnI	4T21	19C7	IgG2b	a.a.r. 41-49	EIA, WB
Anti-cTnI	4T21	560	IgG1	a.a.r. 83-93	EIA, WB
Anti-cTnI	4T21	267	IgG2a	a.a.r. 169-178	EIA, WB <10% C/r with skeletal TnI
Anti-cTnI	4T21	MF4	IgG1	a.a.r. 190-196	EIA, WB

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