HyTest

Cardiac troponin I

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Abbreviations

AMI	Acute myocardial infarction
cc	cell culture; produced *in vitro* (in the Cat or MAb name)
cTnI	cardiac troponin I
cTnT	cardiac troponin T
HAMA	human anti-mouse antibody
hs-cTn	high-sensitivity cardiac troponin
MAb	monoclonal antibody
skTnI	skeletal troponin I
skTnT	skeletal troponin T
Tn	Troponin
TnC	Troponin C

Introduction

Troponin I is a subunit of the troponin complex (Tn), which is a heteromeric protein that is bound to the thin filament. The troponin complex plays an important role in the regulation of skeletal and cardiac muscle contraction. The complex consists of three subunits: troponin T (TnT), troponin I (TnI) and troponin C (TnC). These subunits are held together by non-covalent interactions. TnT is the tropomyosin-binding subunit that regulates interaction between the troponin complex and the thin filament. The TnI subunit is responsible for inhibiting actomyosin formation at low intracellular Ca^{2+} concentrations. The TnC subunit binds Ca^{2+} ions during the excitation of the muscle and changes the conformation of the troponin complex, thus enabling the formation of actomyosin complex and the subsequent muscle contraction (1).

In human beings, TnI and TnT are each presented by three isoforms. Two different skeletal muscle isoforms of both TnI and TnT (skTnI and skTnT) are expressed, one in slow twitch skeletal muscle and one in fast twitch skeletal muscle. The third isoform of both TnI and TnT (cTnI and cTnT) is typical for the cardiac muscle. While cTnI is presented exclusively in heart tissue (2), cTnT is probably less specific and can be transiently expressed in some forms of diseased skeletal muscles (3).

In the late 1980s, cTnI (4), and later cTnT (5), were proposed as markers of cardiac cell death. Both proteins are now widely used and established as the guideline recommended markers in order to assist in the diagnosis of acute myocardial infarction (AMI) (6-9), as well as markers of myocardial injury in clinical pathologies, such as postsurgery myocardium trauma, chemotherapy cardio-toxicity and many other diseases related to cardiac muscle injury.

Antibodies that are specific to different epitopes of cTnI

At HyTest, we have been working with cTnI antibodies for more than 20 years and during this time we have generated and analyzed thousands of cTnI-specific antibodies. The best of these are manufactured for sale. Our antibody selection includes antibodies that are specific to different epitopes of the cTnI molecule (see Figure 1).

HyTest antibodies are widely used in commercial cTnI assays that are based on different types of platforms, e.g. ELISAs, turbidimetry, lateral flow and magnetic particles. The antibodies are also used in research applications such as Western blotting, immunohistochemistry, and many others.

Antibodies that are available in different formats

Currently, all of our antibodies are available as *in vivo*-produced forms. Several antibodies are also manufactured *in vitro* and we suggest selecting the *in vitro*-produced form for immunoassay development, if available. Furthermore, we can now offer a few antibodies as chimeric recombinant proteins. These MAbs consist of the original mouse derived variable regions and human derived constant regions. The chimeric MAbs help to avoid false negative and false positive results that are caused by human anti-mouse antibodies (HAMA) in immunoassays (see page 9).

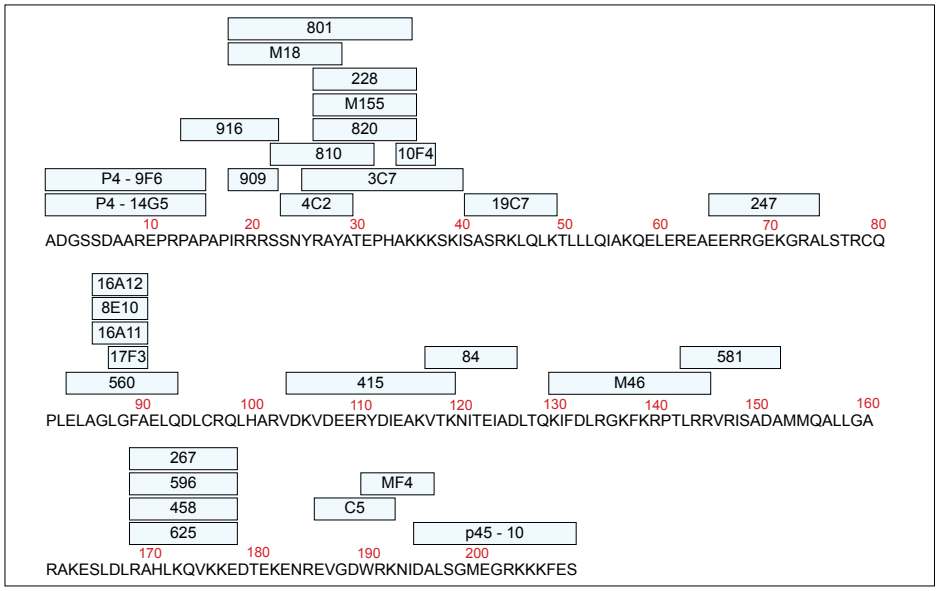

Figure 1. Epitope mapping of HyTest anti-cTnI monoclonal antibodies. We offer more than 30 specially selected antibodies that are specific to various epitopes along the cTnI molecule. The epitope specificity of all of the MAbs has been precisely determined either by the SPOT technique or by other methods that utilize different peptide libraries.

High-sensitivity cTn assay concept

In the late 1990s, the contemporary cTnI (and cTnT) assays were able to detect cTn from the blood of patients at ng/ml (µg/l) levels. In practice, these assays allowed for the reliable detection of cTn just 3 to 6 hours following the onset of ischemic symptoms such as chest pain. This meant that cTns were considered to be rather moderate, late markers of AMI. In contrast, recent high-sensitivity cTn (hs-cTn) assays, the detection limit of which is pg/ml (ng/l) rather than ng/ml, have made it possible to identify any myocardial injury, including those of AMI patients, within 1 to 3 hours; this represents a potential 3 hour time saving to ensure more rapid patient management. The hs-cTn assays have made cTn early markers of AMI.

The current generation of commercially available hs-cTn assays are approximately 1,000 times more analytically sensitive (10 ng/l vs. 10 ng/ml) than the first cTnI assay described by Cummings in 1987 (4). hs-cTn assays are able to detect minor cardiac injury events from a long list of pathologies that cause myocardial tissue necrosis or cell death (8).

The hs-cTn assay concept, its analytical characteristics and what should be known when implementing these assays in clinical practice are all well described in articles and reviews that have been recently published (10-15).

High sensitivity cardiac troponin assays

The hs-cTn assay is an assay that meets the following two criteria (16):

1. 99th percentile upper reference limit (URL) measured with an analytical imprecision (% CV; coefficient of variation) of ≤10%

   and

2. Measures concentrations at ≥ the limit of detection (LoD) in ≥ 50% of healthy (normal) subjects

Factors that influence cTnI measurements

cTnI is a very challenging analyte that features a complicated "biochemical character". We have spent years studying cTnI in order to better understand its biochemical characteristics and posttranslational processing. Our knowledge has provided us with a better understanding of what antibody requirements are necessary to develop a sensitive, quantitative immunoassay enabeling precise measurement in blood.

While cTnI is considered to be the gold standard for the diagnosis of cardiac muscle cell injury, there is currently no standardization between the many different diagnostic assays designed for the quantitative measurements of cTnI in human blood. Therefore, a blood sample often gives varying cTnI concentrations when it is analyzed with different commercial cTnI assays.

The most common reason for the discrepancy between the different assay measurements is the difference in the epitope specificities of the antibodies that are used in the different assays. Several of the multiple factors that have an influence on the measurements include: Proteolytic degradation, complexing of cTnI with other proteins, heparin in the sample tube, as well as cTnI-specific autoantibodies and heterophile antibodies that might be present in the blood of a patient. Different monoclonal and polyclonal antibodies that are utilized in assays are sensitive to these factors in different degrees.

Cardiospecificity

Three different isoforms of TnI can be found in human beings. cTnI isoform is cardio-specific and two different skTnI isoforms are expressed in skeletal muscle. The three proteins are highly homologous: The sequence identity between cTnI and slow skTnI is approximately 52% and the sequence identity between cTnI and fast skTnI is 46%. Figure 2 shows the sequence similarity between cTnI and skTnI proteins. Apart from the extension in the N-terminus of cTnI, only very short fragments are unique to cTnI. As a result of this, the development of cTnI antibodies with no cross-reaction with skeletal isoforms is a challenging task. Cross-reactivity should be taken into account when designing an immunoassay.

The high-sensitivity cTnI assay concept imposes special requirements as regards the cardio-specificity of the antibodies. Indeed, even low (0.1% or less) cross-reaction with skeletal TnI isoforms in a high-sensitivity assay can result in false positives and misleading results if the concentration of skTnI in the blood of a patient is increased.

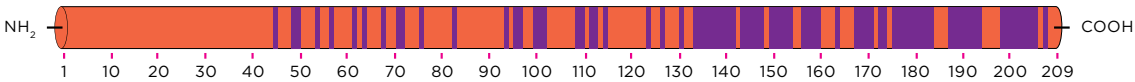

Figure 2. Sequence similarity between cTnI and two skeletal muscle forms of TnI. Parts that are unique to cTnI are marked in orange.

cTnI is a challenging analyte to be quantitatively measured

When detecting cardiac troponin I in the blood of patients, several factors can influence the quantitative measurement of this cardiac marker by altering the availability of epitopes for antibody binding. These factors include, for example, phosphorylation, proteolytic degradation, or the blocking of the epitopes by autoantibodies.

The influence of these factors on the interaction of antibodies with cTnI is multidimensional. For instance, it is well established that purified cTnI is highly susceptible to proteolytic degradation. In contrast, in the Tn complex, the central part of cTnI closely interacts with TnC and this interaction protects cTnI from proteolysis. Consequently, the epitopes that are located at the central part of the cTnI are significantly more stable than the epitopes which are located at the terminal parts of the molecule. However, while TnC interaction renders the central part more stable, TnC also competes with antibodies for the ability to bind with cTnI. Therefore, antibodies specific to only a few epitopes located in the central part of cTnI molecule are able to recognize cTnI in the blood of patients as the majority of cTnI is found in complex with TnC.

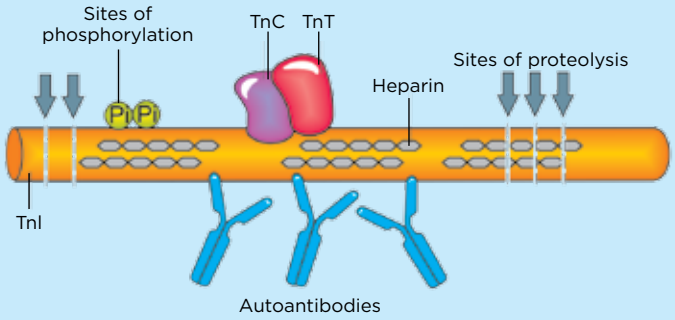

Figure 3. Schematic presentation of factors that can influence precise measurements of cTnI circulating in human blood.

Complex of cTnI with TnC and cTnT

In cardiomyocytes, cTnI forms a ternary troponin complex with cTnT and TnC. Many scientific groups have shown that, in the blood of AMI patients, cTnI is still in complex with TnC, whereas the information about the existence of the ternary cTnI-cTnT-TnC complex in the blood of patients is contradictory.

TnC binding changes the conformation of cTnI and part of the cTnI surface is shielded by TnC. Hence, the immunological properties of cTnI in free form and in complex are different. Antibodies that have been raised against the epitopes on the regions closed by TnC binding might not be able to recognize cTnI in a protein complex in clinical samples. As most (if not all) cTnI molecules in blood are found in complex with TnC, it is important that the cTnI-specific antibodies that are utilized in assays are capable of recognizing the protein in the cTnI-TnC binary complex.

Proteolytic degradation

cTnI is known to be an extremely unstable molecule that easily undergoes proteolytic degradation. The most stable part of cTnI is located between amino acid residues 30 and 110 (17). It is thought that, both in necrotic tissue and in blood, this part of the cTnI molecule is protected from the endogenous proteases by TnC.

The information about the level of the degradation of cTnI in the blood of a patient is somewhat contradictory. However, it seems likely that the N-terminal and C-terminal parts of the cTnI molecule that are not protected by TnC are at least partially truncated, especially in the samples collected 20 hours or more following the onset of symptoms.

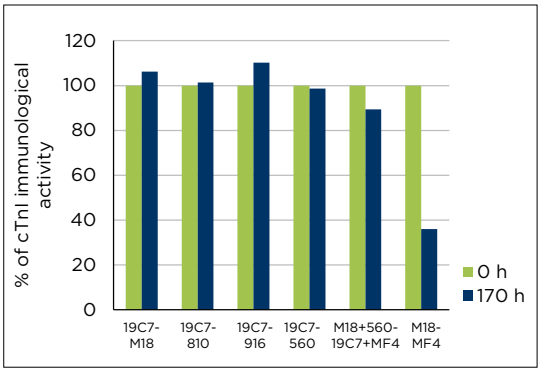

Figure 4. Effect of proteolytic degradation. The best two-site combinations of anti-cTnI specific to the stable part of the cTnI molecule tested with cTn complex (Cat.# 8T62) before and after incubation for 170 hours with the mixture of endogenous proteases from human cardiac tissue. The M18-MF4 control assay is sensitive to the proteolytic degradation of cTnI.

Phosphorylation

Serines 22 and 23 of cTnI can be phosphorylated by protein kinase A *in vivo*. This means that four forms of the cTnI protein (one dephosphorylated, two monophosphorylated and one bisphosphorylated) can coexist in the cell and appear circulating in blood after MI (18).

Phosphorylation of cTnI changes the conformation of the protein and modifies its interaction with other troponins. It also modifies the interaction with some anti-cTnI antibodies, such as 22B11. MAb 22B11 only recognizes the dephosphorylated form of cTnI and does not react with mono- or bisphosphorylated forms of the cTnI antigen. 22B11 can be used for quantitative measurements of dephosphorylated cTnI in sandwich immunoassays. It can also be used for the qualitative or semi-quantitative immunodetection of dephosphorylated cTnI in Western blotting.

Effect of heparin

Heparin is widely used in clinical practice as an anticoagulant. Almost all patients with suspected AMI receive heparin in the first few minutes following admission. In addition, blood samples are often collected into heparin tubes. Heparin is a negatively charged molecule and it can readily interact with cTnI, which is a highly positively charged protein with pI ~9.9. We have shown that some anti-cTnI MAbs are sensitive to the presence of heparin in a sample that can result in a lower response with the samples containing heparin (see Figure 5) (19).

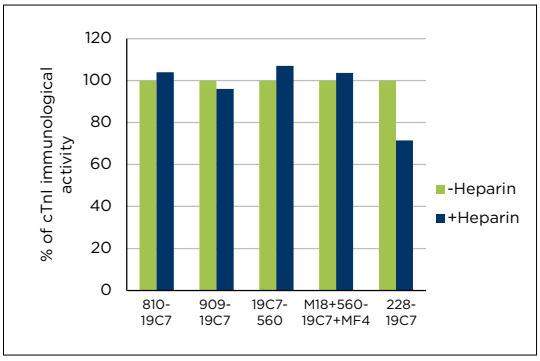

Figure 5. Sensitivity to heparin. Different MAb combinations were tested either in the presence (5 IU/ml) or absence of heparin. Native cTnI (50 ng/ml) was used as an antigen. A significant decrease in the immunoreactivity in the presence of heparin indicates that MAb 228 is sensitive to heparin.

Autoantibodies

Autoantibodies against cTnI are found in the blood of both patients with heart disease and seemingly healthy individuals (20-22). Autoantibodies have been shown to negatively influence the recognition of cTnI by some assays. If the concentration of autoantibodies is high, it can result in a significant underestimation in the amount of cTnI measured in the blood specimen of a patient.

Heterophile antibodies and the HAMA effect

Heterophile antibodies arise when people are exposed to different animals or products derived from animals. These antibodies are typically human anti-mouse (HAMA), anti-rabbit, anti-goat, anti-sheep, anti-cow, anti-pig, anti-rat or anti-horse. In immunodiagnostics, the problem is most commonly associated with HAMA due to the fact that most diagnostics assays use mouse derived antibodies.

In diagnostic tests, HAMA might cause false positive or false negative results (23-24). False positive results can cause delays in making a correct diagnosis and indeed even in unnecessary hospital admissions if the test is used for the diagnosis of life-threatening conditions such as acute myocardial infarction (25).

Troponin assays are particularly susceptible to HAMA due to low cut-off value requirements and because the levels of cTnI even in the plasma of AMI patients are very low. A study of subjects investigated with cardiac troponin I due to suspected myocardial infarction found that HAMA caused false positives in 5.5% of subjects with raised cTnI and 14% of subjects with raised cTnI and normal creatine kinase (26).

A powerful tool to solve the issue with HAMA in diagnostics tests is the use of chimeric or fully humanized antibodies. A few of our anti-cTnI antibodies have now been converted to chimeric proteins by changing the antibody constant regions from mouse to human derived sequences. When tested with HAMA containing serum samples, the results showed that the HAMA effect was blocked by the use of chimeric cTnI antibodies Rec19C7 and Rec16A11 (see Figure 6).

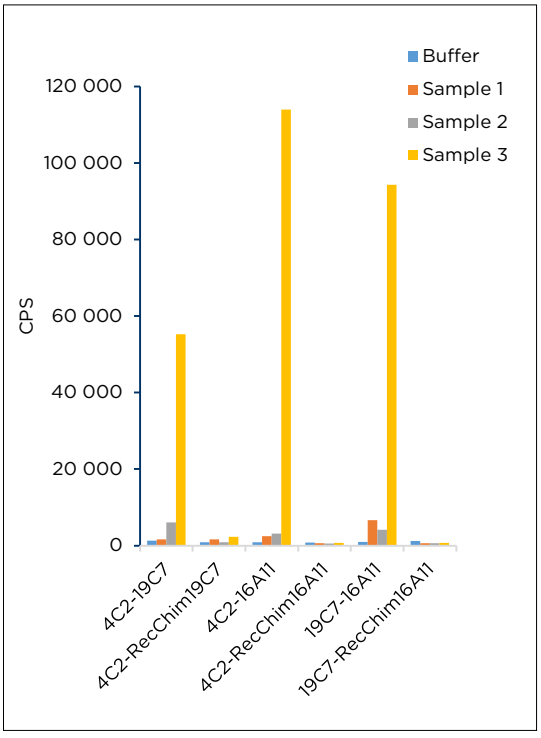

Figure 6. Chimeric antibodies mitigate the HAMA effect. The performance of chimeric and native 19C7 and 16A11 in the presence of HAMA was tested with three serum samples featuring varying HAMA concentrations: 807 ng/ml in Sample 1, 1388 ng/ml in Sample 2 and 6220 ng/ml in Sample 3. Buffer without serum was used as a control. Antibody pairs that were compared are indicated in the picture.

cTnI assay development and pair recommendations

We can recommend several different antibody combinations for the development of cTnI immunoassays (see Table 1). All of the suggested capture-detection pairs have been selected following the thorough testing of hundreds of various antibody pair combinations by using our in-house sandwich fluoroimmunoassay method. The recommended pairs all demonstrate good kinetics, low background, high analytical sensitivity and high reproducibility. In addition, all suggested antibody combinations have been tested with blood samples from AMI patients and were shown to successfully recognize cTnI circulating in the blood of patients. Our results have also been confirmed by assay manufacturers which are utilizing HyTest antibodies in their commercial high sensitivity cTnI assays.

It is important to note that it is not possible to recommend only one "best pair" for the development of a cTnI immunoassay. The reason for this is that antibodies can perform differently depending on the assay platform. For example, MAbs that show excellent performance in ELISA could also be ideal in an assay that utilizes magnetic particles but then behave in a less than optimal way in a lateral flow format, or vice versa. A good, optimized assay is always the sum of all assay components and variables: antibodies, assay platform, buffers, label, incubation times, etc.

TABLE 1. Antibody pair recommendations for quantitative cTnI sandwich immunoassays.

Assay type	Capture	Detection
1 + 1	19C7cc	16A11cc
	19C7cc	560cc
	625	19C7cc
	560cc	458
	4C2cc	19C7cc
2 + 1	19C7cc + MF4cc	7B9cc (specific to TnC)
2 + 2	916 + 560cc	19C7cc + MF4cc
	801 + 560cc	19C7cc + MF4cc
	909 + 560cc	19C7cc + MF4cc
	M18 + 560cc	19C7cc + MF4cc

Multi-MAb approach

In addition to the "traditional" 1+1 assay format, in which there is one capture and one detection antibody, we also recommend testing a multi-MAb approach. In this approach, there are two or even three capture antibodies, and one or more detection antibodies. In our experience, additional antibodies usually help to improve the assay analytical sensitivity. More importantly, this approach helps to reduce the negative effect of both the various posttranslational modifications of cTnI and the assay interfering factors (such as heparin) on the performance of the assay.

All of the two-site antibody combinations that we recommend demonstrate:

- High or very high analytical sensitivity
- No cross-reaction with either skeletal TnI isoform
- Good recognition of cTnI in either the free and complexed (with TnC) format
- Low susceptibility to partial proteolysis of the cTnI molecule
- Either no or low susceptibility to the presence of heparin in the sample
- Either no or low susceptibility to phosphorylation
- Low susceptibility to the presence of autoantibodies in the sample

cTnI combinations in lateral flow assays

Tests based on the lateral flow platform have been popular in diagnostics since their introduction in the late 1980s. Table 2 shows a list of antibody combinations that work well in lateral flow immunoassays. Please note that other combinations may work as well or even better.

TABLE 2. Antibody pair combinations that perform well in the lateral flow format in different assay types.

Assay type	Capture	Detection
1 + 1	20C6 (specific to cTn complex)	560cc
	560cc	20C6
	20C6	7B9cc (specific to TnC)
	19C7cc	560cc

Assay type	MAb1	MAb2	MAb3
1 + 2 or 2 + 1	4C2cc	7B9cc	20C6
	19C7cc	560cc	20C6
	560cc	7B9cc	20C6
	19C7cc	267	4T21/2
	19C7cc	560cc	4T21/2

Assay type	MAb1	MAb2	MAb3	MAb4
2 + 2	4C2cc	560cc	7B9cc	20C6

Assays that detect cTnI in complex forms

Both ourselves and others have shown that the vast majority of cTnI in the blood of AMI patients is found as a binary complex with TnC (17, 27, 28). Free cTnI is either present in minor quantities or can be totally undetectable (28). For this reason, it would be possible to detect cTnI by using antibody pairs that are able to recognize the cTn complex instead of cTnI alone.

We provide two different concepts for this purpose.

cTnI assays that utilize anti-TnC antibodies use one MAb that is specific to cTnI and one MAb that is specific to TnC (see Figure 7). TnC is not phosphorylated, it is not cleaved by proteases, nor is it susceptible to the presence of heparin or autoantibodies in the sample. Such an approach would help to improve analytical sensitivity, precision and the reproducibility of quantitative cTnI immunoassays. We provide two MAbs (Cat.# 4T27 and 4T27cc) that recognize TnC.

cTnI assays that utilize anti-cTn complex antibodies use one MAb that is specific to the cTn complex and one MAb that is specific to either cTnI or TnC (see Figure 8). We provide two monoclonal antibodies that are specific to the cTn complex (Cat.# 4TC2). Based on customer feedback, the anti-cTn complex antibodies are often used in lateral flow immunoassays.

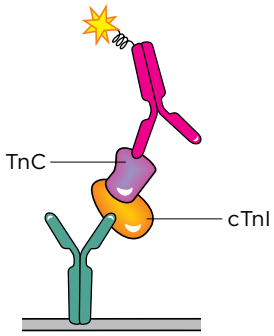

Figure 7. Schematic presentation of a cTnI immunoassay utilizing an anti-TnC antibody. In this assay type the capture antibody (turquoise) is specific to cTnI and the detection antibody (pink) is specific to TnC.

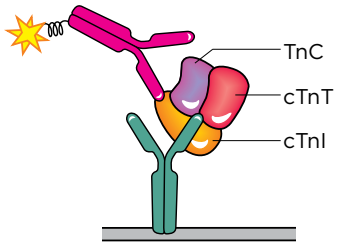

Figure 8. Schematic presentation of a cTnI immunoassay utilizing an anti-cTn complex antibody. In this assay type the capture antibody (turquoise) is specific to cTn complex and the detection antibody (pink) is specific to cTnI.

Heterogeneity of cTnI forms in human blood and assay standardization

The heterogeneity of cTnI forms in human blood and the difference in the epitope specificity of the antibodies that are utilized in different assays means that results obtained using different assays can differ greatly. In the first generation of cTnI assays, the concentration of cTnI measured with one assay could be 10 to 1,000 times greater if analyzed with another cTnI assay. As a result of close collaboration between national and international organizations, scientists, clinical practitioners and industrial assay manufacturers, the between assay agreement is nowadays much better. However, cTnI standardization has not been achieved.

Steps towards the standardization of the cTnI assays include:

- The introduction of the international cTnI standard (SRM 2921). This standard was developed by the National Institute of Standards and Technology (NIST) with material prepared by HyTest.
- Gradual "standardization" of the epitopes detected in commercial assays. Figure 9 shows the epitope specificities of the commercial assays that are currently on the market. Most assays utilize antibodies that are specific to three regions of the cTnI molecule: 23–43, 41–56 and 83–93.

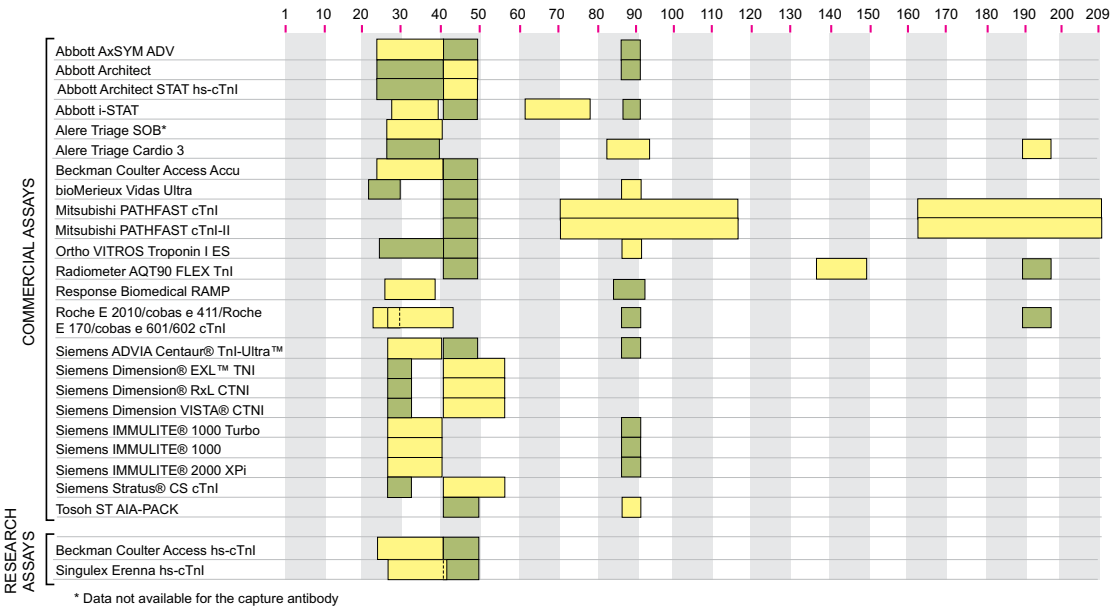

Figure 9. Epitopes of the antibodies utilized in commercial cTnI immunoassays. Green and yellow bars represent epitopes that are recognized by capture and detection antibodies respectively. This figure is based on the information available on the International Federation of Clinical Chemistry (IFCC) website www.ifcc.org (Troponin assay analytical characteristics, version October 2013). Printed with permission.

Antibodies for cTnI or cTnI fragments detection by Western blotting

All of our anti-cTnI MAbs recognize human cTnI (or cTnI fragments, if they contain the epitope that a certain MAb is specific to) in Western blotting. For improved sensitivity in Western blotting, we recommend using one of the following MAbs: 19C7, 16A11 or MF4.

Antibodies for the detection of cTnI from different animal species

New drug testing and the evaluation of new surgery approaches are often carried out on experimental animals. The effects of new therapeutic or surgery technology on cardiac function and on cardiac myocyte viability are important and these can be studied by cTnI measurements in animal blood.

Several of our anti-cTnI MAbs detect cTnI of various animal species in Western blotting (see Table 3). We have also tested the ability of selected antibody combinations to detect native purified animal cTnI in two-site combinations. Table 4 lists the combinations which, according to our studies, could be used for developing an immunoassay that detects cTnI in different animals. Calibration curves for one of these pairs, M155-19C7, are shown in Figure 10.

TABLE 3. Cross-reactivity of anti-cTnI MAbs with antigens from different animal species in Western blotting.

MAb	Human	Bovine	Por-cine	Goat	Canine	Rabbit	Cat	Rat	Mouse	Fish
4C2	++	++	++	++	++	++	+	++	++	-
19C7	++	++	+	++	+	++	++	++	+	++
8E10	+	+	+	+	+	+	+	-	-	-
16A11	+	+	+	+	+	+	+	-	-	-
C5	++	++	++	++	++	++	++	++	++	++
MF4	+	+	+	+	+	-	+	+	+	-
22B11	++	-	+	-	-	-	-	-	-	-
247	++	++	++	++	++	+	++	++	++	N/A
10F4	++	++	++	++	++	++	++	++	+	N/A

TABLE 4. Two-site MAb combinations for the immunodetection of cTnIs from different animal species.

		Detecting MAb													
		19C7					M155					MF4			
		B	C	M	R	Rb	B	C	M	R	Rb	B	C	M	R
Coating MAb	9F6			•	•	•									
	4C2		•	•	•	•									•
	801			•											
	M155	•	•	•	•	•									
	19C7											•	•	•	•
	625	•	•	•	•	•									
	MF4			•			•	•	•	•	•				

B: Bovine, C: Canine, M: Mouse, R: Rat, Rb: Rabbit

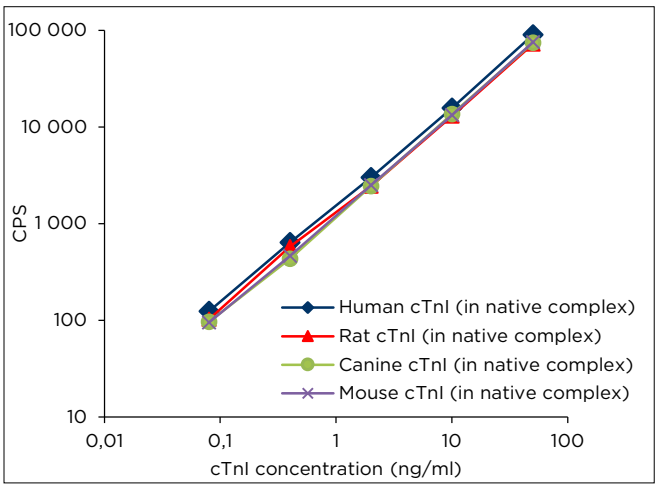

Figure 10. cTnI calibration curves for human, mouse, rat and dog (canine) Tn complexes. M155 was used as the capture and 19C7 was used as the detection antibody. This MAb combination gives equal response with antigens from different animal species.

Cardiac troponin I and troponin complex

HyTest's scientists have been working with cardiac troponin I for more than 20 years. During this time, they have obtained a profound understanding of the development, production and purification of different forms of this protein.

Native human cardiac troponin I

HyTest's cTnI (Cat.# 8T53) is purified from human cardiac muscle tissue by immuno-chromatography followed by an additional ion-exchange chromatography step. The purified preparation contains a small amount (<5%) of cTnI proteolytical fragments that retain the cTnI's immunological activity. According to immunological and mass spectral studies, the N-terminal alanine of native cTnI is acetylated. The preparation contains a mixture of differentially phosphorylated and dephosphorylated cTnI. SDS-PAGE of the purified cTnI is shown in Figure 11.

cTnI that is completely phosphorylated or dephosphorylated after purification is also available under Cat.# 8T53ph and 8T53dp respectively.

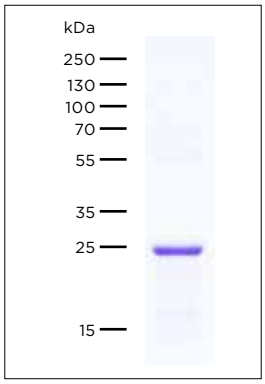

Figure 11. Native cardiac TnI. 1 µg of purified human native cardiac TnI (Cat.# 8T53) was run in 10-20% SDS-PAGE under reducing conditions.

Recombinant human cardiac troponin I

Our recombinant human cardiac troponin I (Cat.# 8RT17) is produced by expressing the TNNI3 gene in *E. coli*. The recombinant troponin I contains one additional Met residue at its N-terminus (due to *E. coli* expression) and it is not phosphorylated on residues Ser23 and Ser24. In SDS-PAGE, this protein migrates as a single band (see Figure 12). This highly purified protein can be used as a calibrator for immunoassays, as an immunogen for antisera production and as a mass cTnI standard.

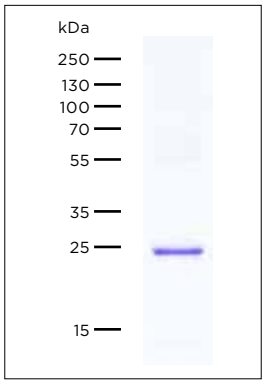

Figure 12. Recombinant cardiac TnI. 1 µg of human recombinant cardiac TnI (Cat.# 8RT17) was run in 10-20% SDS-PAGE under reducing conditions.

Human cardiac troponin complexes (I-C and I-T-C)

In the cardiac troponin complex, the troponin subunits are non-covalently attached to each other. The strongest interaction has been demonstrated between cTnI and TnC. This interaction is Ca^{2+} dependent and this should be noted if using serum samples containing EDTA.

cTnI is extremely unstable in its free form and it demonstrates significantly better stability in complex with TnC (I-C) or in ternary cTnI-cTnT-TnC (I-T-C) complex. These two forms of the protein are preferable as a material for the preparation of protein standards and calibrators (17). Figure 13 shows the stability of purified cTnI in I-T-C complex and in free form when incubated at 4°C.

In the native troponin complex supplied by HyTest, cTnI is presented in the same form that it can be detected in the blood of AMI patients. The purification of the troponin complex is performed under mild conditions without treatment using urea containing buffers. The concentration is precisely determined for each of the three components in the complex. In SDS-PAGE, the purified troponin complex migrates as three major bands: cTnT, cTnI and TnC (see Figure 14).

Advantages of native troponin complex over purified cTnI include:

- Antigen is in the same form as in AMI blood samples
- Unchanged tertiary structure
- Unchanged antibody binding sites
- High cTnI stability
- Ideal for the preparation of a cTnI calibrator and standards

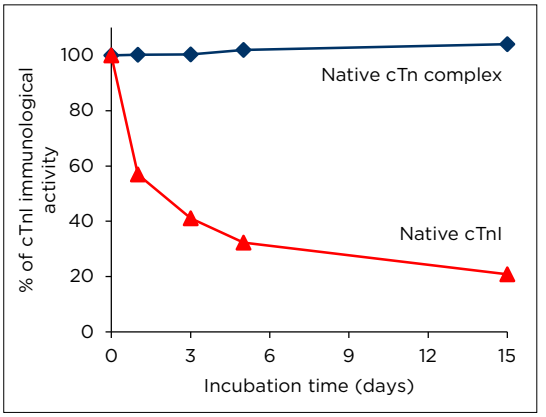

Figure 13. Comparison of the stability of different forms of purified cTnI. Native troponin complex (Cat.# 8T62) or native cTnI (Cat.# 8T53) was dissolved in normal human serum (the final concentration of cTnI was 30 ng/ml) and incubated for several days at 4°C. The immunoreactivity of the samples was measured at the indicated time points. When dissolved in serum, cTnI remains highly stable in troponin complex whereas the purified free cTnI quickly loses its immunoreactivity.

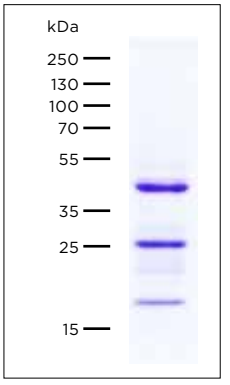

Figure 14. Native cardiac troponin complex. Purified troponin complex (Cat.# 8T62; the amount of cTnI was 1 µg) was run in 10-20% SDS-PAGE under reducing conditions.

In 2004, HyTest's troponin I-T-C complex was selected by the American Association for Clinical Chemistry (AACC) Standardization Subcommittee for use by assay manufacturers as reference material in troponin I assays. The certified reference material SRM 2921 is only available from the National Institute of Standards and Technology (NIST). For more information, please visit www.nist.gov.

Cardiac troponin T (cTnT)

In human beings, cardiac troponin T is encoded by the TNNT2 gene. Ten different isoforms of cTnT have been identified, which result from alternative splicing of the mRNA transcript. Some of these isoforms are characteristic to the embryonic state of heart development, some are characteristic to normal adult heart tissue, while other forms have been associated with different cardiac pathologies. The major isoform found in normal adult human heart tissue (isoform 6 or TnT3) is 287 amino acids long with a calculated molecular weight of 34.6 kDa.

Similarly to cTnI, the cardiac isoform of TnT is widely used as a marker of myocardial cell injury. cTnT has the same release kinetics into the bloodstream and the same sensitivity for minor myocardial injury (necrosis) as cTnI.

At HyTest, we provide MAbs that are suitable for the development of immunoassays for diagnostic purposes as well as several MAbs that are recommended for research use (see Figure 15).

Monoclonal antibodies for high-sensitivity cTnT assays

Our *in vitro* produced anti-cTnT MAbs (Cat.# 4T19cc) can be used for the development of an immunoassay with superior sensitivity (limit of detection better than 0.3 ng/l) and high specificity (no cross-reaction to cTnI or to skeletal isoforms of TnT up to 30 µg/l).

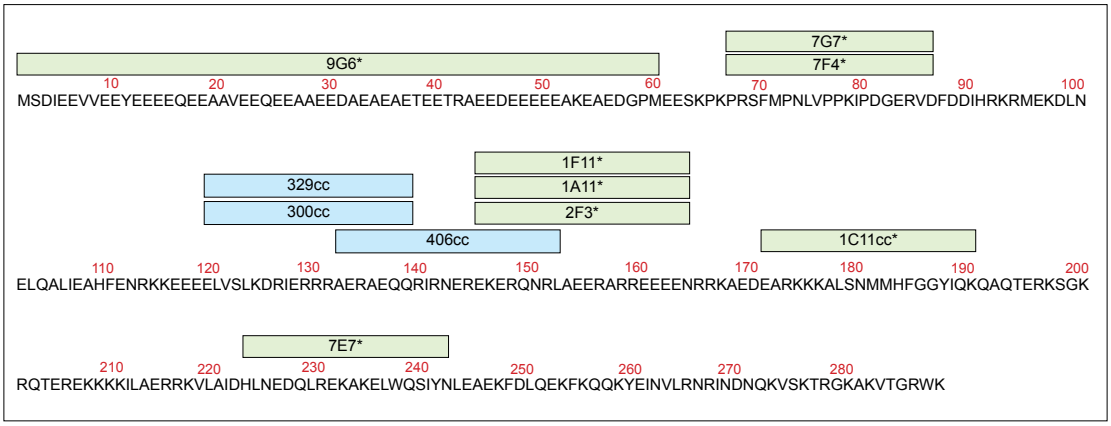

Figure 15. Epitope mapping of HyTest anti-cTnT monoclonal antibodies. We offer antibodies for the development of high-sensitivity cTnT assays as well as for research purposes (marked with *).

The ability of the antibody pairs 329cc-406cc and 406cc-300cc to recognize cTnT in the blood of AMI patients has been studied with over 80 serum and plasma samples. The antibody pairs demonstrate a good correlation with a commercially available hs-cTnT assay. Results of the analysis of 38 serum samples are provided in Figure 16.

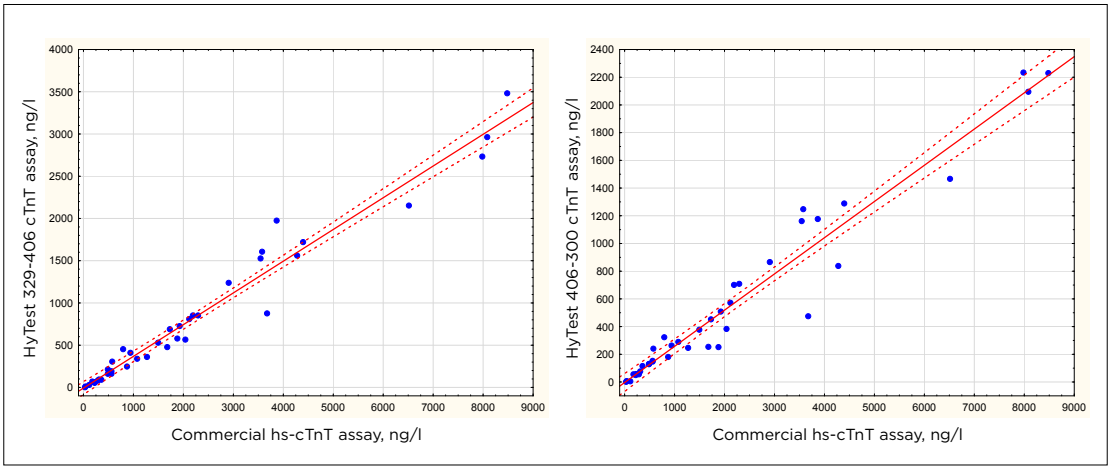

Figure 16. HyTest immunoassays show good correlation to a commercially available hs-cTnT assay. The concentration of cTnT in 38 serum samples obtained from AMI patients was determined by using two immunoassays that utilized HyTest antibodies (capture-detection pairs 329-406 and 406-300) and a commercially available hs-cTnT assay.

Antibodies for research purposes

We offer several MAbs that are recommended for research purposes. They also cross-react with cTnT proteins from different animal species (see Table 5).

TABLE 5. Cross-reactivity of anti-cTnT MAbs with antigens from different animal species in Western blotting.

MAb	Human	Bovine	Porcine	Goat	Canine	Rabbit	Cat	Rat	Mouse	Fish
7F4	++	N/A	++	N/A	-	-	-	N/A	N/A	-
1F2	+	+	-	+	+	+	+	-	-	+
7G7	+	+	-	-	-	-	-	-	-	-
2F3	++	+	++	++	+	+	+	+	+	+
1A11	++	++	++	++	+	+	+	+	++	+
2G3	++	+	+	+	+	+	+	+	+	-
1F11	++	++	++	++	+	+	+	+	+	+
7A9	+	+	+	+	+	+	-	-	-	-

Native human cTnT

HyTest cTnT (Cat.# 8T13) is purified from human cardiac muscle tissue by immunoaffinity chromatography followed by an additional ion exchange chromatography step. In SDS-PAGE, the purified protein migrates as a single band (see Figure 17).

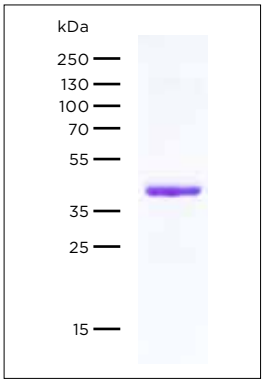

Figure 17. Native cardiac TnT. 1 µg of human native cardiac TnT (Cat.# 8T13) was run in 10–20% SDS-PAGE under reducing conditions.

Recombinant human cTnT

Isoform 6 (which is also known in the literature as TnT3) is the major isoform of troponin T that is presented in normal adult human heart tissue.

Our recombinant human cTnT (Cat.# 8RTT5) is produced in *E. coli* by expressing a gene encoding for the 288 amino acid long isoform 6 (TnT3) of cTnT. This isoform is the main isoform of cTnT in normal adult human heart tissue. The protein has an additional Met residue at its N-terminus. In SDS-PAGE, the purified recombinant cTnT migrates as a single band (see Figure 18).

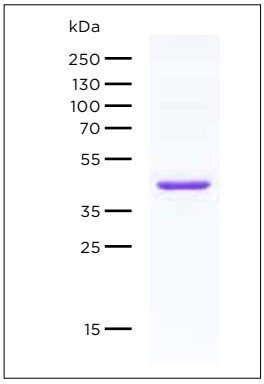

Figure 18. Recombinant cardiac TnT. 1 µg of human recombinant cardiac TnT (Cat.# 8RTT5) was run in 10–20% SDS-PAGE under reducing conditions.

Recombinant human slow and fast skTnT

The recombinant slow skeletal TnT (Cat.# 8RST2) and fast skeletal TnT (Cat.# 8RFT4) are ideal for studying immunoassay cross-reactivity to these isoforms.

Troponin C (TnC)

Two forms of troponin C (TnC) are expressed in human muscles. One is typical for slow skeletal muscles and myocardium, while the other is typical for fast skeletal muscles. The TnC present in cardiac muscle consists of 161 amino acid residues. It has a molecular weight of 18.4 kDa and a theoretical pI of 4.05.

TnC forms high affinity complex with cTnI and in the blood of AMI patients the majority of cTnI is found in complex with TnC. TnC protects cTnI from protease cleavage and can therefore be used as a natural stabilizer of cTnI in water solutions (17).

Native human TnC from cardiac muscle

HyTest TnC (Cat.# 8T57) is purified from human cardiac muscle tissue by immunoaffinity chromatography followed by an additional ion-exchange chromatography step. In SDS-PAGE, the purified protein migrates as a single band (not shown).

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Selected troponin articles from HyTest scientists

Katrukha AG, Bereznikova AV, Esakova TV, Filatov VL, Bulargina TV and Gusev NB. **A new method of human cardiac troponin I and troponin T purification.** Biochem. Mol. Biol. Int. 1995, 36:195-202.

Protocols for the purification of endogenous troponin I and T are described. The affinity purification method based on our monoclonal antibody C5 could be utilized for the purification of troponin I molecules from various animal species as well.

Katrukha AG, Bereznikova AV, Esakova TV, Pettersson K, Lövgren T, Severina ME, Pulkki K, Vuopio-Pulkki LM and 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):1379-1385.

In this study we have shown for the first time that cTnI molecule is not released in the blood stream in a free form (as it was thought previously) but complexed with TnC. Different cTnI-specific antibodies vary in their ability to recognize cTnI in free and complexed forms. We have suggested using for the assay development antibodies that are not affected by cTnI – TnC complex formation and equally recognize both – free and complexed cTnI forms.

Filatov VL, Katrukha AG, Bereznikova AV, Esakova TV, Bulargina TV, Kolosova OV, Severin ES and Gusev NB. **Epitope mapping of anti-troponin I monoclonal antibodies.** Biochem. Mol. Biol. Int. 1998, 45(6):1179-1187.

We describe here the development of monoclonal antibodies using purified cTnI or troponin complex as immunogen. Epitope specificities of 31 antibodies are determined by using the SPOT technique. The generation of antibodies that recognize both isolated cTnI and cTnI in troponin complex allows for a reliable detection of cTnI in clinical samples.

Katrukha AG, Bereznikova AV, Filatov VL, Esakova TV, Kolosova OV, Pettersson K, Lövgren T, Bulargina TV, Trifonov IR, Gratsiansky NA, Pulkki K, Voipio-Pulkki LM and Gusev NB. **Degradation of cardiac troponin I: implication for reliable immunodetection.** Clin. Chem. 1998, 44(12):2433-2440.

In this study we showed that both N-terminal and C-terminal regions of cTnI were rapidly proteolytically degraded in necrotic tissue incubated at 37°C and in serum incubated at 23°C. The most stable part of cTnI was located between amino acid residues 30 and 110. We suggest that antibodies specific to the epitopes that are most resistant to proteolysis should be utilized when developing cTnI immunoassays.

Filatov VL, Katrukha AG, Bulargina TV and Gusev NB. **Troponin: structure, properties, and mechanism of functioning.** Biochemistry. 1999, 64(9):969-985.

REVIEW. The review summarizes what is known about the structure and function of troponin complex components. Data on phosphorylation of troponin I and troponin T are viewed.

Katrukha A, Bereznikova A, Filatov V and Esakova T. **Biochemical factors influencing measurement of cardiac troponin I in serum.** Clin. Chem. Lab. Med. 1999, 37(11-12):1091-1095.

Effect of complex formation, the unstable nature of cTnI, phosphorylation, as well as other factors that influence the recognition of cTnI in human serum are discussed. We concluded that all of these factors should be considered during the selection of the antibodies for the assay development.

Katrukha A, Bereznikova A and Pettersson K. **New approach to standardization of human cardiac troponin I (cTnI).** Scand. J. Clin. Lab. Invest., Suppl. 1999, 230:124-127.

In this study, we compared the results from six different cTnI assays by measuring the cTnI concentration in a total of 21 clinical samples. All of the samples were analyzed in all of the assays using a set of different calibrators. The lowest between-manufacturer bias was obtained when using a heart tissue derived native troponin complex as the calibrator. Our conclusion is that in order to reduce assay-to-assay variation, the native troponin complex should be used as the calibrator.

Katrukha AG. **Antibody selection strategies in cardiac troponin assays.** Cardiac Markers, 2003, 2nd edition, Edited by Alan HB. Wu. 173-185.

In this specific chapter of the book, the biochemical properties of troponin I (and T) are viewed and parameters affecting antibody selection for the assay development are discussed.

Vylegzhanina AV, Katrukha IA, Kogan AE and Bereznikova AV. **Epitope Specificity of Anti–Cardiac Troponin I Monoclonal Antibody 8I-7.** Clin. Chem. 2013, 59(12):1814-1816.

In this letter to the editor, we showed data relating to the epitope specificity of anti-cTnI MAb 8I-7. Our results also indicated that this MAb cross-reacts with skeletal troponin I.

Vylegzhanina AV, Kogan AE, Katrukha IA, Antipova OV, Kara AN, Bereznikova AV, Koshkina EV, Katrukha AG. **Anti–Cardiac Troponin Autoantibodies Are Specific to the Conformational Epitopes Formed by Cardiac Troponin I and Troponin T in the Ternary Troponin Complex.** Clin. Chem. 2017, 63(1), 343-350.

In this article, we investigated the epitope specificity of troponin autoantibodies that prevent an efficient detection of cTnI by such MAbs that bind to epitopes commonly utilized in commercial assays. The autoantibodies investigated were specific to the conformational epitopes found in the troponin I-T-C ternary complex but not in the binary I-C complex or free cTnI. On the other hand, the ternary I-T-C complex was one of the main cTnI forms only in the early samples of acute myocardial infarction patients. This means that if the blood of an AMI patient contains autoantibodies then the risk of obtaining falsely low troponin levels is higher in samples that are taken shortly after the onset of the AMI.

Katrukha IA, Kogan AE, Vylegzhanina AV, Serebryakova MV, Koshkina EV, Bereznikova AV, Katrukha AG. **Thrombin-Mediated Degradation of Human Cardiac Troponin T.** Clin Chem. 2017, 63(6):1094-1100.

In this article, our researchers investigated in more detail the proteolytic degradation of cTnT. The results suggest that the 29 kDa fragment present in serum samples is formed during the sample preparation and that it is caused by the cleavage of cTnT by thrombin, a serine protease that is involved in the coagulation cascade.

Patents

HyTest holds patents for 'Method and kit for the diagnosis of troponin I' (US7285418 and EP0938678).

Ordering information

Troponin I (TnI)

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
Troponin I cardiac	4T21	P4-14G5	IgG1	EIA, WB, a.a.r. 1-15
		P4-9F6	IgG1	EIA, WB, a.a.r. 1-15
		916	IgG3	EIA, WB, a.a.r. 13-22
		909	IgG1	EIA, WB, a.a.r. 18-22
		M18	IgG1	EIA, WB, a.a.r. 18-28
		801	IgG3	EIA, WB, a.a.r. 18-35
		810	IgG1	EIA, WB, a.a.r. 22-31
		4C2	IgG2a	EIA, WB, a.a.r. 23-29
		3C7	IgG1	EIA, WB, a.a.r. 25-40
		228	IgG1	EIA, WB, a.a.r. 26-35
		M155	IgG1	EIA, WB, a.a.r. 26-35
		820	IgG1	EIA, WB, a.a.r. 26-35
		10F4	IgG2a	EIA, WB, a.a.r. 34-37
		19C7	IgG2b	EIA, WB, a.a.r. 41-49
		247	IgG1	a.a.r. 65-74, only free cTnI
		560	IgG1	EIA, WB, a.a.r. 83-93
		16A12	IgG1	EIA, WB, a.a.r. 86-90
		8E10	IgG1	EIA, WB, a.a.r. 86-90
		16A11	IgG1	EIA, WB, a.a.r. 86-90
		17F3	IgG1	EIA, WB, a.a.r. 87-90
		415	IgG1	EIA, WB, a.a.r. 104-119
		84	IgG1	EIA, WB, a.a.r. 117-126
		M46	IgG1	EIA, WB, a.a.r. 130-145, <10 % C/r with skeletal troponin I
		581	IgG1	EIA, WB, a.a.r. 143-152
		625	IgG1	EIA, WB, a.a.r. 169-178
		458	IgM	EIA, WB, a.a.r. 169-178
		596	IgG1	EIA, WB, a.a.r. 169-178, <10 % C/r with skeletal troponin I
		267	IgG2a	EIA, WB, a.a.r. 169-178, <10 % C/r with skeletal troponin I
		C5	IgG2b	EIA, WB, a.a.r. 186-192, >50 % C/r with skeletal troponin I
		MF4	IgG1	EIA, WB, a.a.r. 190-196
		p45-10	IgG1	EIA, WB, a.a.r. 195-209
Troponin I cardiac, *in vitro*	4T21cc	4C2cc	IgG2a	EIA, WB, a.a.r. 23-29
		M155cc	IgG1	EIA, WB, a.a.r. 26-35
		19C7cc	IgG2b	EIA, WB, a.a.r. 41-49
		560cc	IgG1	EIA, WB, a.a.r 83-93
		16A12cc	IgG1	EIA, WB, a.a.r. 86-90
		16A11cc	IgG1	EIA, WB, a.a.r. 86-90
		MF4cc	IgG1	EIA, WB, a.a.r. 190-196
Recombinant chimeric anti-cTnI	RC4T21	RecChim19C7	IgG1	EIA
		RecChim16A11	1gG1	EIA
Troponin I cardiac, phosphorylated form	4T45	1G11	IgG2b	EIA, WB, a.a.r. N/A
Troponin I cardiac, dephosphorylated form	4T46	22B11	IgG2b	EIA, WB, a.a.r. 20-24
Troponin complex, human native cardiac	4TC2	20C6	IgG2b	EIA
		Tcom8	IgG1	EIA
Troponin I skeletal muscle	4T20	12F10	IgG2b	EIA, WB
		7G2	IgG2b	EIA, WB

Troponin I (TnI)

POLYCLONAL ANTIBODY

Product name	Cat. #	Host Animal	Remarks
Troponin I cardiac	4T21/2	goat	EIA

HUMAN ANTIGENS

Product name	Cat. #	Purity	Source
Troponin I cardiac, human	8T53	>98%	Human cardiac muscle
Troponin I cardiac, human, recombinant	8RTI7	>95%	Recombinant
Troponin I cardiac, dephosphorylated	8T53dp	>95%	Human cardiac muscle
Troponin I cardiac, phosphorylated	8T53ph	>95%	Human cardiac muscle
Troponin complex (I-C)	8IC63	N/A	Human cardiac muscle
Troponin complex (I-T-C), human	8T62	N/A	Human cardiac muscle
Troponin complex (I-T-C), artificial	8T62a	N/A	Human cardiac muscle
Troponin I cardiac Calibrator Set	8T60	N/A	Suggested range 0-100 ng/ml
Troponin I cardiac Diversity Kit	K01	N/A	Different forms of human cardiac troponin I
Troponin I skeletal muscle, human	8T25	>95%	Human skeletal muscle

ANIMAL ANTIGENS

Product name	Cat. #	Purity	Source
Troponin I cardiac, bovine	8T53b	>98%	Bovine cardiac muscle
Troponin I cardiac, canine	8T53c	>98%	Canine cardiac muscle
Troponin I cardiac, mouse	8T53m	>98%	Mouse cardiac muscle
Troponin I cardiac, porcine	8T53p	>98%	Porcine cardiac muscle
Troponin I cardiac, rabbit	8T53rb	>98%	Rabbit cardiac muscle
Troponin I cardiac, rat	8T53r	>98%	Rat cardiac muscle
Troponin complex (I-T-C), bovine	8T62b	N/A	Bovine cardiac muscle
Troponin complex (I-T-C), canine	8T62c	N/A	Canine cardiac muscle
Troponin complex (I-T-C), mouse	8T62m	N/A	Mouse cardiac muscle
Troponin complex (I-T-C), porcine	8T62p	N/A	Porcine cardiac muscle
Troponin complex (I-T-C), rabbit	8T62rb	N/A	Rabbit cardiac muscle
Troponin complex (I-T-C), rat	8T62r	N/A	Rat cardiac muscle
Troponin I skeletal muscle, bovine	8T25b	>95%	Bovine skeletal muscle
Troponin I skeletal muscle, canine	8T25c	>95%	Canine skeletal muscle
Troponin I skeletal muscle, mouse	8T25m	>95%	Mouse skeletal muscle
Troponin I skeletal muscle, porcine	8T25p	>95%	Porcine skeletal muscle
Troponin I skeletal muscle, rabbit	8T25rb	>95%	Rabbit skeletal muscle
Troponin I skeletal muscle, rat	8T25r	>95%	Rat skeletal muscle

DEPLETED SERUM

Product name	Cat. #	Source
Troponin I free serum	8TFS	Pooled normal human serum

WESTERN BLOT SET

Product name	Cat. #	Remarks
Cardiac troponin I Western blot set	9CT4	Contains anti-cTnI MAb 4C2 and human cTnI antigen. For cTnI immunodetection in Western blotting and DOT blot, using cTnI sample as internal standard or calibrator.

Troponin T (TnT)

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
Troponin T cardiac	4T19	9G6	IgG1	EIA, WB, a.a.r. 1-60
		7F4	IgG2b	EIA, WB, a.a.r. 67-86
		7G7	IgG1	EIA, WB, a.a.r. 67-86
		2F3	IgG2b	EIA, WB, a.a.r. 145-164
		1A11	IgG2b	EIA, WB, a.a.r. 145-164
		1F11	IgG2b	EIA, WB, a.a.r. 145-164
		1C11	IgG1	EIA, WB, a.a.r. 171-190
		7E7	IgG1	EIA, WB, a.a.r. 223-242
Troponin T cardiac, *in vitro*	4T19cc	1C11cc	IgG1	EIA, WB, a.a.r. 171-190
		300cc	IgG1	EIA, a.a.r. 119-138
		329cc	IgG1	EIA, a.a.r. 119-138
		406cc	IgG2b	EIA, a.a.r. 132-152

HUMAN ANTIGENS

Product name	Cat. #	Purity	Source
Troponin T cardiac, human	8T13	>98%	Human cardiac muscle
Troponin T cardiac, human, recombinant	8RTT5	>95%	Recombinant
Troponin T skeletal muscle, human	8T24	>95%	Human skeletal muscle
Troponin T fast skeletal, human, recombinant	8RFT4	>95%	Recombinant
Troponin T slow skeletal, human, recombinant	8RST2	>95%	Recombinant
Troponin complex (I-T-C), human	8T62	N/A	Human cardiac muscle
Troponin complex (I-T-C), artificial	8T62a	N/A	Human cardiac muscle

ANIMAL ANTIGENS

Product name	Cat. #	Purity	Source
Troponin T cardiac, bovine	8T13b	>98%	Bovine cardiac muscle
Troponin T cardiac, canine	8T13c	>98%	Canine cardiac muscle
Troponin T cardiac, mouse	8T13m	>98%	Mouse cardiac muscle
Troponin T cardiac, porcine	8T13p	>98%	Porcine cardiac muscle
Troponin T cardiac, rabbit	8T13rb	>98%	Rabbit cardiac muscle
Troponin T cardiac, rat	8T13r	>98%	Rat cardiac muscle
Troponin T skeletal muscle, bovine	8T24b	>95%	Bovine skeletal muscle
Troponin T skeletal muscle, canine	8T24c	>95%	Canine skeletal muscle
Troponin T skeletal muscle, mouse	8T24m	>95%	Mouse skeletal muscle
Troponin T skeletal muscle, porcine	8T24p	>95%	Porcine skeletal muscle
Troponin T skeletal muscle, rabbit	8T24rb	>95%	Rabbit skeletal muscle
Troponin T skeletal muscle, rat	8T24r	>95%	Rat skeletal muscle

POLYCLONAL ANTIBODY

Product name	Cat. #	Host Animal	Remarks
Troponin T cardiac	4T19/2	goat	EIA, WB, IHC, IP

Troponin C (TnC)

MONOCLONAL ANTIBODIES

Product name	Cat. #	MAb	Subclass	Remarks
Troponin C	4T27	1A2	IgG2a	EIA, WB
		7B9	IgG1	EIA, WB
Troponin C, *in vitro*	4T27cc	7B9cc	IgG1	EIA, WB
Troponin complex, human native cardiac	4TC2	20C6	IgG2b	EIA
		Tcom8	IgG1	EIA

HUMAN ANTIGEN

Product name	Cat. #	Purity	Source
Troponin C, human	8T57	>98%	Human cardiac muscle
Troponin C slow skeletal/cardiac, human, recombinant	8RSC4	>95%	Recombinant
Troponin C skeletal isoform 2, human, recombinant	8RKC3	>90%	Recombinant
Troponin complex (I-C)	8IC63	N/A	Human cardiac muscle
Troponin complex (I-T-C), human	8T62	N/A	Human cardiac muscle
Troponin complex (I-T-C), artificial	8T62a	N/A	Human cardiac muscle

ANIMAL ANTIGENS

Product name	Cat. #	Purity	Source
Troponin C, bovine	8T57b	>98%	Bovine cardiac muscle
Troponin C, canine	8T57c	>98%	Canine cardiac muscle
Troponin C, mouse	8T57m	>98%	Mouse cardiac muscle
Troponin C, porcine	8T57p	>98%	Porcine cardiac muscle
Troponin C, rabbit	8T57rb	>98%	Rabbit cardiac muscle
Troponin C, rat	8T57r	>98%	Rat cardiac muscle

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