Recombinant nanobodies detecting the human cardiac troponin proteins by ELISA

Bernardo Bollen Pinto[1], Philippe Hammel[2], Marianne Paolini Bertrand[3], Bernhard Walder[1], Nicolas Vuilleumier[4], Oliver Hartley[3]

[1]*Division of Anaesthesiology, Department of Anaesthesiology, Pharmacology, Intensive Care and Emergency Medicine, Geneva University Hospitals, 4 Rue Gabrielle-Perret-Gentil, CH-1211, Geneva, Switzerland*
[2]*Geneva Antibody Facility, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, CH-1211, Geneva, Switzerland;*
[3]*Department of Pathology and Immunology, Faculty of Medicine, University of Geneva, 1 rue Michel Servet, CH-1211, Geneva, Switzerland*
[4]*Division of Laboratory Medicine, Department of Diagnostics and of Medical Specialties, Geneva University Hospitals, 4 Rue Gabrielle-Perret-Gentil, CH-1211, Geneva, Switzerland;*

Abstract

We tested the capacity of antibodies RB733, RB734, RB766, RB767, RB768, RB769, RB770, RB771, RB772 and RB773 to recognize by ELISA three different molecular forms of the human cardiac troponin T isoform 6 (cTnT): an N-terminal peptide (Nter-cTnT), the full-length cTnT protein (FL-cTnT) and the cardiac troponin I-T-C complex (ITC-complex). Our results show that RB734 specifically recognizes the N-terminal region of cTnT as well as the full-length protein alone and the ITC-complex. RB767, RB770, RB771, RB772 and RB773 recognize the full-length cTnT protein alone or in the ITC-complex but not the N-terminal peptide. RB766, RB768 and RB769 recognize the ITC-complex but not cTnT or cardiac troponin I (cTnI) proteins.

Introduction

Cardiac troponin T (cTnT, UniProt P45379-6) is a ~37kDa cytosolic protein essential for cardiomyocyte contraction and relaxation. It forms a complex (ITC-complex) with the cTnI and cTnC proteins. Detection by ELISA of circulating cTns in blood samples is the gold standard for the diagnosis of myocardial injury/infarction (Thygesen *et al*., 2019). Clinically available assays use antibodies that recognize epitopes in the central region of cTnI or cTnT proteins (Apple *et al*, 2017), which are present in the cTn ITC-complex, the intact cTnI/T proteins and some truncated forms. These antibodies are not able to discriminate between the different molecular forms of cTnI/T. Selective identification of different cTnT molecular forms may help to differentiate between myocardial infarction and non-ischemic causes of myocardial injury (Juhani Airaksinen *et al.*, 2022). Here we describe the capacity of ten new recombinant antibodies to selectively detect different molecular forms of the cTnT protein: Nter-cTnT, FL-cTnT and the ITC-complex by ELISA.

Materials & Methods

Antibodies: ABCD_RB733, ABCD_RB734 and ABCD_RB766-773 (ABCD nomenclature, https://web.expasy.org/abcd/) nanobodies were discovered by the Geneva Antibody Facility https://www.unige.ch/medecine/antibodies). They were produced as mini-antibodies with the antigen-binding VHH portion fused to a human IgG1 Fc. The antibodies and the antigens against which they were originally selected are listed in Table 1. HEK293 suspension cells (growing in HEK TF medium, Xell#861-0001, supplemented with 0.1% Pluronic F68, Sigma#P1300) were transiently transfected with the vector coding for each mini-antibody. Supernatants were collected after 4 days (see Table 1 for individual yields).
The mouse monoclonal antibody 9G6, which recognizes the N-terminal region of the cTnT protein (residues 2-61) was purchased from HyTest (#4T19) and used as control.

Table 1: Clone name, antigen, epitope and production yields of the antibodies used in this study

ABCD	Antigen	Yield (mg/L)
RB733	Nter-cTnT(1-66)	80
RB734	Nter-cTnT (1-66)	80
RB766	ITC-complex	60
RB767	ITC-complex	60
RB768	ITC-complex	70
RB769	ITC-complex	70
RB770	ITC-complex	90
RB771	ITC-complex	60
RB772	FL-cTnT	100
RB773	FL-cTnT	50

Antigens: The N-terminal cTnT peptide (Nter-cTnT) encompasses the first 66 residues of the cTnT protein (MSDIEEVVEEYEEEEQEEAAVEEQEEAAEEDAEA EAETEETRAEEDEEEEEAKEAEDGPMEESKPK). It was synthetized and C-terminally biotinylated by the UNIGE PPR2P Core Facility. The recombinant human cTn ITC-complex (ITC-complex, purity >95%) and the full-length cTnT protein (FL-cTnT, purity >95%) were purchased from HyTest (#8ITCR and #8RTT5 respectively). The human cTnI protein was purchased from Sigma (#T9924). CTnI and an irrelevant N-biotinylated peptide (KFSTFDRDNDKWEENC) from the zebrafish fibrinogen alpha protein (UniProt Q6DHS2) were used as negative controls.

Protocol: All procedures were carried out at room temperature. Biotinylated peptides (Nter-cTnT and Q6DHS2) at saturating concentration (10 pmol/well) were immobilized on streptavidin-coated ELISA plates (Pierce #15124) for 30 min. FL-cTnT, cTnI and ITC-complex (50 ng/well) were directly coated on ELISA plates (Pierce #15124). Each well was rinsed three times with 100 µl of washing buffer (PBS + 0.5% (w/v) BSA + 0.05% (w/v) Tween20), then incubated for 1 hour with 50 µl of RB antibody-containing supernatant diluted in washing buffer (Fig. 1). After rinsing 3 times (100 µl washing buffer), wells were incubated with horseradish peroxidase-coupled goat anti-human IgG (Sigma #1721050, dilution 1:1000, 50 µl per well) for 30 min. After 3 rinses, Tetramethylbenzidine (TMB) substrate (Sigma #T5569) was added (50 µl per well). The reaction was stopped by the addition of 25 µl of 2 M H_2SO_4. The absorbance (OD) was measured at 450 nm, and the absorbance at 570 nm was subtracted.

Results

The RB734 antibody, raised against a cTnT N-terminal peptide, recognized both the antigenic peptide and the full-length cTnT, as well as the ITC-complex (Fig. 1 and 2). As expected, it did not bind to an irrelevant peptide (Fig. 1) or the cTnI protein (Fig. 2). Remarkably, RB734 exhibits a binding profile similar to that of the commercial antibody 9G6 (Table 2).

RB733 recognized cTnT (N-terminal peptide and full-length protein) and the ITC-complex only when used at the highest concentration (Fig. 1 and 2). At this concentration, it also showed affinity towards the cTnI protein (Fig. 2), indicating the possibility of some nonspecific binding.

Antibodies RB767, RB770, RB771, RB772, and RB773 specifically recognized the full-length cTnT protein but not the N-terminal cTnT peptide (Fig. 1 and 2, Table 2), suggesting that they recognize distinct non-N-terminal epitopes on the cTnT protein. Furthermore, all of these antibodies bound to the ITC-complex (Fig. 1).

Antibodies RB766, RB768, and RB769 recognized the ITC-complex against which they were developed, but did not bind to the cTnT protein (full-length and N-terminal peptide) (Fig. 1 and 2). These three antibodies displayed weak binding to cTnI, but only at the highest concentration of the antibody (Fig. 2). These findings suggest that RB766, RB768, and RB769 may recognize the ITC-complex either through the cTnC protein or via a complex conformational epitope, possibly located on the cTnI protein, primarily found within the ITC-complex. Further experiments will be required to thoroughly characterize the specificity of these antibodies and their ability to specifically detect their targets within complex mixtures, such as blood samples.

Table 2: Summary of ELISA results.
(+) and (-) indicate respectively the presence or absence of binding between the indicated antibodies and targets.

	Control (Q6DHS2)	Control (cTnI)	Nter-cTnT	FL-cTnT	ITC-complex
RB733	-	+	+	+	+
RB734	-	-	+	+	+
RB766	-	-	-	-	+
RB767	-	-	-	+	+
RB768	-	-	-	-	+
RB769	-	-	-	-	+
RB770	-	-	-	+	+
RB771	-	-	-	+	+
RB772	-	-	-	+	+
RB773	-	-	-	+	+
9G6	-	-	+	+	+

References

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Conflict of interest

BBP participated in Roche Diagnosis Advisory Board on post-operative myocardial injury and received an unrestricted grant from Basel University/Roche Diagnosis unrelated to the present work. PH is a founder and shareholder of ABCD Antibodies SA.

The remaining authors declare no conflict of interest.

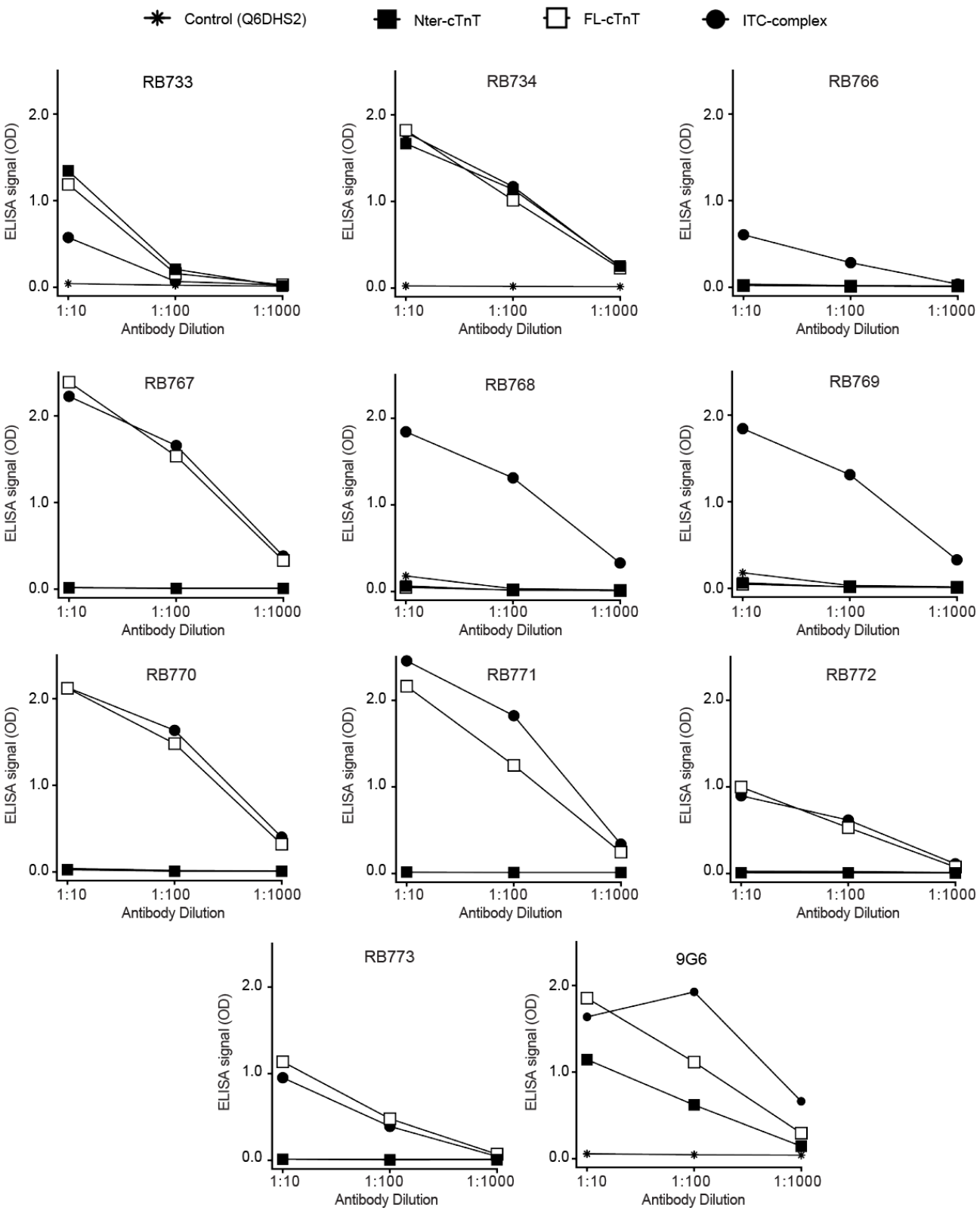

Fig. 1. Binding of RB and 9G6 antibodies to the Nter-cTnT peptide, the FL cTnT protein and the ITC-complex as detected by ELISA. 'Control' indicates the binding of the tested antibodies to the negative control peptide (Q6DHS2).

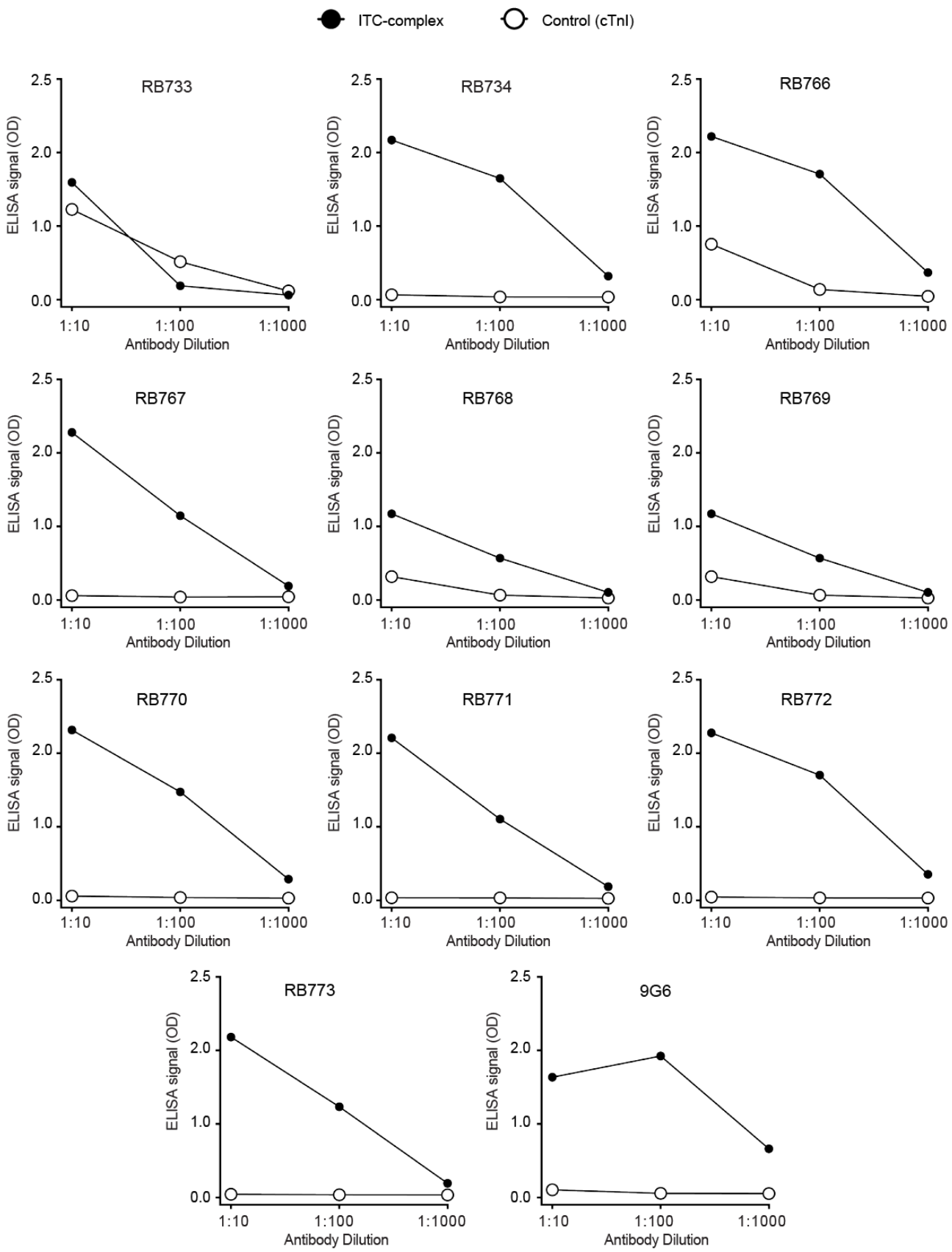

Fig. 2. Binding of 9G6 and RB antibodies to the ITC-complex and the cTnI protein (control) as detected by ELISA.