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A novel IgE-binding epitope of cat major allergen, Fel d 1

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ABSTRACT

Information on the antigenic repertoire, especially the IgE-binding epitopes of an allergen is important for understanding the allergen induced immune response and cross-reactivity, as well as for generating the hypoallergenic variants for specific component resolved immunotherapy/diagnosis (CRIT and CRD). Data on the IgE-binding epitopes of cat allergens are scarce. In this study, a novel IgE-binding epitope of the cat major allergen, Fel d 1, was identified. Mouse monoclonal antibody (MAb) specific to the Fel d 1 was produced. Computerized intermolecular docking was used for determining the residues of the Fel d 1 bound by the specific MAb. The presumptive surface exposed residues of the Fel d 1 intrigued by the MAb are located on the chain 1. They are: L34 and T37 (helix 1); T39 (between helices 1 and 2); P40, E42 and E45 (helix 2); R61, K64, N65 and D68 (helix 3); and E73 and K76 (helix 4). The MAb competed efficiently with the cat allergic patients' serum IgE for Fel d 1 binding in the competitive IgE binding assay, indicating allergenicity of the MAb epitope. The newly identified allergenic epitope of the Fel d 1 is useful in a design of the CRIT and CRD for cat allergy.

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1. Introduction

Cat is an abundant source of allergenic proteins which sensitize about 5–20% of people [1,2]. The allergens may be from the pet's hair, dander, saliva, and/or urine [3,4]. It is difficult for the cat owners to avoid the allergens as the offending proteins can cling to various materials and surfaces in the house and retain there for a long time after removal of the pets [2]. The allergens are airborne, thus they may be distributed to the places without the pet. Non-cat keepers can be sensitized by the allergen also.

Eight proteins have been recognized as cat allergens [5]. Among them, Fel d 1 (a uteroglobin-like protein of the secretoglobulin superfamily) is regarded as the cat's major allergen, as more than 80% of the cat allergic subjects have specific IgE to this protein [6]. Fel d 1 is produced by sebaceous glands and squamous epithelial cells which were then transferred to the animal pelt by licking and grooming [7,8]. Each molecule of the Fel d 1 is composed of two 18–

kDa heterodimers; each heterodimer consists of chains 1 and 2 linked together by three disulfide bonds. The two chains form eight anti-parallel helices (H1–H4 in chain 1 and H5–H8 in chain 2) [9]. Fel d 1 often forms tetramers (dimers of the heterodimers; 38 kDa) [9,10]. Polymorphisms of this allergen have been described [11]. Three IgE-binding epitopes of the Fel d 1 have been recognized; two on the chain 1 and another one on the chain 2 [9,12]. In this study, a novel allergenic Fel d 1 conformational epitope was identified and characterized.

2. Materials and methods

2.1. Cat hair extract and recombinant Fel d 1

To each gram of the cat's hair, 20 mL of PBS containing 0.1% Tween-20 (PBST) were added. The preparation was sonicated at 4 °C, 30 min, before filtering through a cell strainer and centrifuged. The supernatant was concentrated and the protein content was determined (Bradford). The CE was kept at –80 °C until use.

For recombinant Fel d 1 (rFel d 1), a transformed BL21 (DE3) *E. coli* carrying *Fel d 1*-plasmid [13] was grown under 0.4 mM IPTG induction. The bacterial cells were added with 4% glycerol in 10 mM

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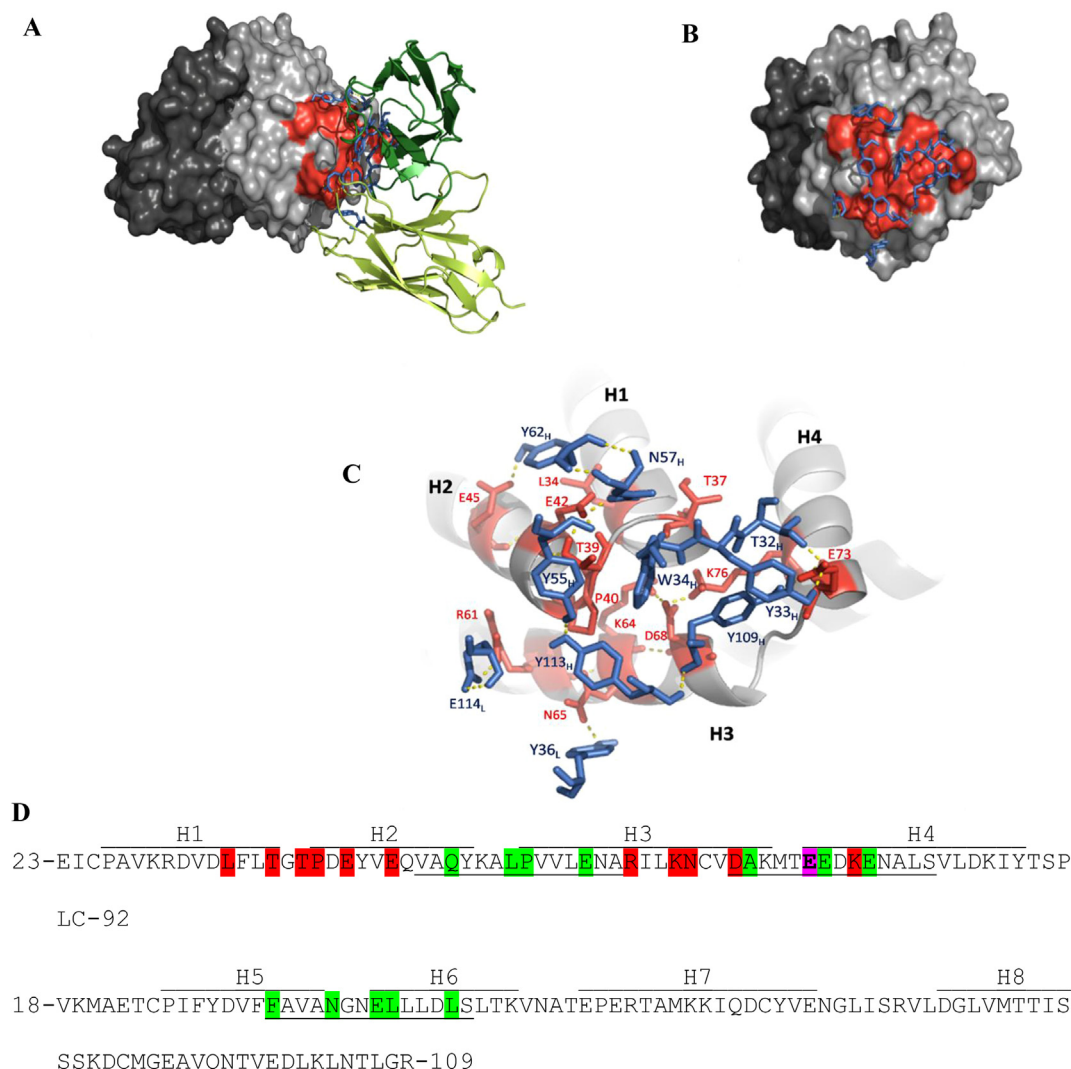


Fig. 2. Computerized interaction of Fel d 1 with variable domains of the MAbC48 and the presumptive residues of the Fel d 1 bound by the MAb. (A) The antibody uses CDRs 1–3 of VH (dark green) and CDR1 of VL (light green) in binding to the spatially juxtaposed areas (red) of the Fel d 1 chain 1 (light grey surface). (B) Residues of the MAb (blue) that involved in the interaction with the conformational epitope (red) on Fel d 1 chain 1 (light grey surface). (C) Residues of the MAb (blue) that interacted with residues of the Fel d 1H1, H2, H3 and H4 (red). (D) Amino acid sequences of Fel d 1 chains 1 (P30438/23–92) and 2 (P30440/18–109). Residues that formed the conformational epitope of the MAbC48 are in red boxes. Underlined residues are sequences of the previously reported three IgE binding peptides of Fel d 1 (from N terminal: peptides 1, 2 and 3, respectively) characterized by van Milligen et al. [12]. Amino acids of these three IgE binding peptides that are surface exposed are shown in green boxes [9]. E73 in helix 4 of chain 1 (pink box) is the surface exposed residue located in the predicted epitope of the MAbC48 as well as the IgE binding peptide-2 described by van Milligan et al. [12]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tissue culture plates (200 μ L/well) and incubated at 37 $^{\circ}$ C in a humidified CO₂ incubator. Hybrid cells were cloned by limiting dilution method. Culture supernatants of individual hybridomas were tested against the native Fel d 1 and CE as well as heterologous antigens including native Can f 1 (dog major allergen), crude extracts of cockroaches, i.e., American (*Periplaneta americana*) and German (*Blattella germanica*), house dust mites (*Dermatophagoides farinae* and *D. pteronyssinus*), and various bacteria by indirect ELISA. Isotypes of the Fel d 1-specific-MAb were determined using commercial typing kit (Thermo scientific).

2.3. Indirect ELISA and western blot analysis (WB)

Indirect ELISA [14] was used for screening for the presence of antibody to the homologous antigen in spent culture fluids of the growing hybrid cells and for determining antigenic specificity and titers of the MABs. Antigen (1 μ g in 100 μ L of 0.05 M carbonate-

bicarbonate buffer, pH 9.6) was used to coat wells of an ELISA plate. The antigen coated-wells were washed with PBST and blocked with 300 μ L of 1% BSA in PBS for 1 h. After removing the excess blocking protein, the wells were added with the antibody preparations and kept at 37 $^{\circ}$ C for 1 h. Wells filled with only PBS served as blank. All wells were washed and 100 μ L of anti-isotype-horseradish peroxidase conjugate (Southern Biotech) were added. The plate was kept at 37 $^{\circ}$ C for 1 h, washed, and added with ABTS chromogenic substrate (KPL). The reaction was stopped by adding 100 μ L of 1% SDS. OD_{450nm} of the content of each well was determined against the blank. OD_{450nm} $\geq$ 0.05 was taken as positive ELISA result.

Antigenic specificity of the specific MAb was determined by WB. Antigen was mixed with β -mercaptoethanol, boiled for 5 min and subjected to 12% SDS-PAGE. The separated components were electroblotted onto nitrocellulose membrane (NC). After blocking the empty sites on the NC blot with 3% skim milk, the membrane

Table 1

Residues and regions of the Fel d 1 bound by residues and domains of the Fel d 1-specific-MAbC48.

Fel d 1 chain 1		MAbC48		Interaction
Residue	Domain	Residue	Domain	
L34	H1	Y62	CDR2/VH	π -effects
T37	H1	W34	CDR1/VH	H-bond
T39	Between H1–H2	W34	CDR1/VH	π -effects
		Y55	FR2/VH	π -effects
P40	H2	W34	CDR1/VH	π -effects
		Y113	CDR3/VH	π -effects
E42	H2	N57	CDR2/VH	H-bond
E45	H2	Y62	CDR2/VH	H-bond
R61	H3	E114	CDR3/VL	Salt-bridge
K64	H3	W34	CDR1/VH	π -effects
N65	H3	Y36	CDR1/VL	H-bond
D68	H3	W34	CDR1/VH	π -effects
E73	H4	T32	CDR1/VH	H-bond
		Y33	CDR1/VH	H-bond
		Y109	CDR3/VH	H-bond
K76	H4	W34	CDR1/VH	π -effects

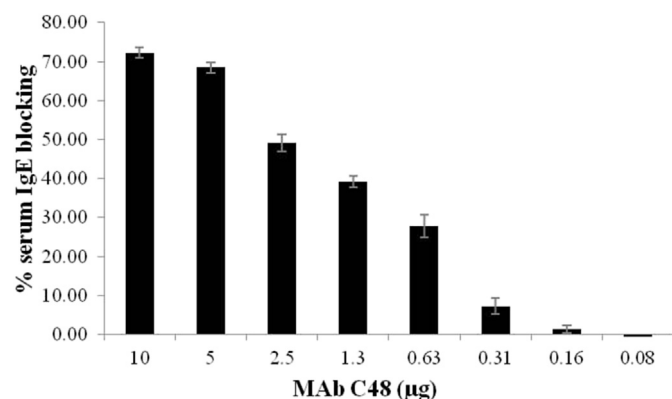


Fig. 3. Efficacy of the MAbC48 in blocking the binding of the cat allergic patients' serum IgE to rFel d 1. At 10 µg, the MAb blocked 72.32% of the serum IgE binding to the rFel d 1 (higher amounts of the MAb were not tested). Control MAb did not have any IgE blocking activity (not shown).

was cut vertically into strips and individual strips were incubated with antibody preparations. The antigen-antibody reactive bands were revealed by using anti-isotype-alkaline phosphatase conjugate (Southern Biotech) and BCIP/NBT substrate (KPL).

2.4. Determination of the amino acid sequence, complementarity determining regions (CDRs), and framework regions (FRs) of the Fel d 1-specific-MAb

Total RNA was extracted from hybridoma cells using Total RNA Mini Kit (Geneaid, Taiwan). First strand cDNA was synthesized by using RevertAid First Strand cDNA Synthesis kit (Thermo Scientific) and anchored oligo dT primers (Thermo Scientific). The reaction was set at 42 °C for 60 min and terminated at 70 °C for 5 min. Based on the isotype of the MAb, the oligonucleotide primers were designed and synthesized [15]. The primer sequences are listed in Supplemental Table 1. The *Igh* and *Igk* gene transcripts of the hybrid cells were amplified separately using 1 µL of the first strand cDNA as template. The PCR mixture (20 µL) contained 200 nM of each primer or total primer mix, 200 µM of dNTP, and Phusion Hot Start II DNA polymerase (Thermo Scientific). The thermal cycling was 98 °C for 30 s followed by 35 cycles of 98 °C for 10 s, 50 °C for 10 s and 72 °C for 10 s and final extension at 72 °C for 5 min. The PCR products were separated on 1% agarose gels. DNA amplicons of the

expected sizes were purified from the gels and cloned into pJET1.2 by using CloneJET PCR Cloning kit (Thermo Scientific). The ligated DNA was introduced to JM109 *E. coli*. The recombinant plasmid was extracted from appropriately transformed JM109 clone and sequenced. The DNA sequence was analyzed for CDRs and FRs by using integrative database of germ-line variable genes from the immunoglobulin loci of human and mouse, VBASE2 [16].

2.5. Specific epitope of the Fel d 1-specific-MAb

Computerized homology modeling and intermolecular docking were used to determine the Fel d 1 and the specific MAb interactive residues in order to reveal the epitope. The VH and VL amino acid sequences of the MAb were subjected to homology modeling by the I-TASSER server service [17]. The quality of the 3D structural model predicted by the I-TASSER was subsequently refined in order to make them become near to the native state by using the high-resolution protein structure refinement, ModRefiner [18] and the fragment-guided molecular dynamics (FG-MD) simulation [19]. The 3D structure of the Fel d 1 from protein data bank (PDB 2EJN) was used. The refined model of the scFv and the Fel d 1 structure were docked according to the Fast Fourier Transform (FFT)-based program, i.e., PIPER. The antibody mode available on the automated ClusPro 2.0 protein-protein docking server was used for the docking [20,21]. The largest cluster size with a minimal local energy and a near native state of the protein conformation was chosen. The Pymol software (The PyMOL Molecular Graphics System, Schrodinger, LLC) was used for building and visualizing the intermolecular interaction.

2.6. Serum samples of cat allergic subjects

This work received approval from the Ethic Committee of Faculty of Medicine Siriraj Hospital, Mahidol University. Twenty patients with clinically diagnosed allergic rhinitis (AR) and positive skin prick test to the cat hair extract were included. They were 3 males and 17 females. Their ages ranged from 15 to 71 years old.

2.7. Competitive IgE binding ELISA

This assay was used for determining allergenicity of the epitope of the Fel d 1-specific-MAb. In doing so, wells of an ELISA plate were coated individually with 1 µg of rFel d 1 in 100 µL of coating buffer and the plate was incubated at 37 °C until dry. All wells were blocked with a blocking solution for 1 h and washed with PBST. Pooled serum IgE (50 µL each of 20 cat allergic patients' sera) mixed with varying amounts (0.078–10 µg) of Fel d 1-specific-MAb or control MAb were added (100 µL) in duplicates into the rFel d 1 coated wells and kept at 37 °C for 1 h. Wells added with cat allergic patients' serum pool alone or MAb alone were non-competitive and background controls, respectively; wells added with PBS were blank. All wells were then washed and 100 µL of biotinylated mouse-anti-human IgE antibody (Southern Biotech) were added. The plate was incubated at 37 °C for 1 h, washed, added with streptavidin-HRP conjugate (Southern Biotech), and kept further for 60 min. ABTS chromogenic substrate (KPL) was added and kept in darkness for 30 min. The wells added with MAb only were filled with anti-isotype-HRP conjugate and detected with the same substrate. Percent IgE blocking was: $100 - [(OD_{405nm} \text{ of patients' serum pool mixed with MAb}) / (OD_{405nm} \text{ of patients' serum pool})] \times 100$.

3. Results

3.1. *Fel d 1-specific-MAB*

From a total of 1023 tissue culture wells containing the fused cells, growing hybrids were found in 214 wells but only culture fluids of 3 wells showed positive indirect ELISA to rFel d 1 and CE. Culture supernatant in one of the wells reacted only to native Fel d 1 (titer 1:1280) and CE and did not react to any of the heterologous antigens. Therefore, cells in this well were cloned and finally 6 hybridomas were established. MAb of a subclone C48 (MAbC48) which grew faster than the others was subsequently used. The MAbC48 immunoglobulin was $\gamma 1, \kappa$. At the maximal phase of the cell growth, the culture supernatant gave indirect ELISA titer 1:512 against the rFel d 1. The IgG1 was purified from the culture supernatant by using HiTrap Protein-G HP column (GE Healthcare) and adjusted to 1 mg/mL of PBS.

3.2. *Amino acid sequence, complementarity determining regions (CDRs), and framework regions (FRs) of the Fel d 1 specific-MAB*

The nucleotides, deduced amino acid sequences, CDRs, and FRs of the MAbC48 VH and VL domains are shown in Fig. 1A and B, respectively. The VH belonged to the mouse IgH-VJ558 VH1 family while the VL was Igk V21 subgroup.

3.3. *Epitope and presumptive residues of the Fel d 1 bound by the specific-MAB*

The MAbC48 model was predicted by the I-TASSER and the most accurate data were extracted from the best scores for estimating the modeled quality. The molecular docking between the MAbC48 with the Fel d 1 performed by the ClusPro 2.0 server was predicted in four separate modes (balance, electrostatic-favored, hydrophobic-favored and Van der Waals + Electrostatic). The Fel d 1-MAbC48 interactive complex with the lowest energy score average (−364.3 kcal/mol) was collected from the antibody mode. According to the docking output, the MAbC48 used all CDRs of the VH and CDR1 of the VL to bind to a surface exposed conformational epitope formed by the Fel d 1 spatially juxtaposed amino acids L34, T37 of helix 1; T39 (between helices 1 and 2); P40, E42, and E45 of helix 2; R61, K64, N65, and D68 of helix 3; and E73 and K76 of helix 4. Fig. 2 and Table 1 give details of the interaction of the two parties.

3.4. *Allergenicity/IgE binding ability of the epitope of the Fel d 1 specific-MAB*

The ability of the MAbC48 and control MAb in competing with the serum IgE for binding to the fixed amount of the rFel d 1 is shown as the percent serum IgE blocking in Fig. 3. The MAbC48 at 0.16–10 μ g blocked the binding of the patients' serum IgE to the rFel d 1 in a dose-dependent manner, i.e., from 1.01 to 72.32%, respectively, while the control MAb could not do so. The overall results indicated that the epitope of the MAbC48 is allergenic (induced specific IgE generation) in humans.

4. Discussion

Information on repertoire of antigenic sites, especially the IgE binding epitopes of a particular allergen is necessary not only for understanding the immune responses to the allergen and the allergen cross-reactivity, but also for generating engineered hypo-allergenic variants of the protein or a defined allergen molecule for a more efficacious specific component resolved immunotherapy or personalized allergen vaccine design [22–28]. Several strategies

have been applied to identify the B cell epitopes of allergens including: use of overlapping synthetic peptides [12]; mimotope mapping using phage display peptide library [27], peptide microarray immunoassay [29], X-ray crystallographic and NMR-based techniques [27,30–33], computational (*in silico*) methods [34,35], and specific monoclonal antibody binding and IgE inhibition assay [36,37]. The peptide based-methods have limitations as most of the IgE binding epitopes are conformational (formed by spatially juxtaposed regions upon a proper protein folding). The crystallographic approach is sometimes not feasible for determining the allergenic epitopes that locate in the surface exposed, hydrophilic disorder, and flexible regions of the protein. IgE complexing allergens are relatively stable for structural analysis of the epitopes as well as the corresponding paratopes [27]. Nevertheless, the conformational changes of the allergens do occur after antibody binding, not only the antibody interacting side chains but also the backbone structure [34,38].

Data on the IgE binding epitopes of the cat allergens are scarce. Thus far, there have been only three IgE-binding epitopes for Fel d 1 identified by using overlapping synthetic peptides [12]. These peptides encompassed residues 25–38 and 46–59 of chain 1 (peptides 1 and 2, respectively) and 15–28 of chain 2 (peptide 3) (underlined in Fig. 2D). Later, the crystal structure of the Fel d 1 and the amino acids in the three IgE binding peptides that exposed on the molecular surface were revealed [9] as shown in green letters in Fig. 2D. In this study, the Fel d 1 specific monoclonal antibody, IgE inhibition assay, and a computerized intermolecular docking between the existing 3D structure of the Fel d 1 and the modeled MAbC48 scFv were used to characterize a novel B cell epitope of the Fel d 1. It was found that the MAb bound to a conformational epitope on chain 1 of Fel d 1 consisting of surface exposed residues L34 and T37 of helix 1; T39 (between helix 1 and 2); P40, E42, and E45 of helix 2; R61, K64, N65 and D68 of helix 3; and E73 and K76 of helix 4. This epitope is an IgE-binding epitope as determined by the ability of the MAb to block the Fel d 1 binding of the allergic patients' serum IgE by as much as 72.32% (when 10 μ g of the MAb was used; higher concentrations were not tested). Residues that formed the MAbC48 epitope except the E73 of helix 4 (Fig. 2D, pink box) are different from the surface exposed residues of the previously reported IgE-binding epitopes [9] indicating that the MAbC48 epitope is a novel IgE-binding epitope of Fel d 1.

In conclusion, a mouse MAb that binds specifically to a novel epitope of cat potent allergen, Fel d 1, was produced. Epitope of the MAb consists of surface exposed residues located on helices 1, 2, 3 and 4 of the Fel d 1 chain 1 that are spatially juxtaposed upon the protein folding. The MAb competed efficiently with the cat allergic patients' serum IgE for binding to Fel d 1, indicating that the epitope is allergenic.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.bbrc.2016.01.099>.

Transparency document

Transparency document related to this article can be found online at <http://dx.doi.org/10.1016/j.bbrc.2016.01.099>.

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Supplemental Table 1. Oligonucleotide primer sequences used for amplification of the
hybrodoma *vh* and *vl* (v©) chains

Gene	Primer name	Sequence
<i>Igh</i>	5' MsVHE	5'-GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG-3'
	3' C γ 1 outer	5'-GGAAGGTGTGCACACCGCTGGAC-3'
<i>Igk</i>	5' L-V κ _3	5'-TGCTGCTGCTCTGGGTTCAG-3'
	5' L-V κ _4	5'-ATTWTCAGCTTCCTGCTAATC-3'
	5' L-V κ _5	5'-TTTTGCTTTTCTGGATTYCAC-3'
	5' L-V κ _6	5'-TCGTGTTKCTSTGGTTGTCTG-3'
	5' L-V κ _6,8,9	5'-ATGGAATCACAGRCYCWGGT-3'
	5' L-V κ _14	5'-TCTTGTTGCTCTGGTTYCCAG-3'
	5' L-V κ _19	5'-CAGTTCCTGGGGCTCTTGTTGTTC-3'
	5' L-V κ _20	5'-CTCACTAGCTCTTCTCCTC-3'
	3' mC κ	5'-GATGGTGGGAAGATGGATACAGTT-3'