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A simple approach to identify functional antibody variable genes in murine hybridoma cells that coexpress aberrant kappa light transcripts by restriction enzyme digestion

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Abstract

Background: Specific binding to target protein epitopes by a mouse monoclonal antibody (mAb) relies on its variable domains. However, the isolation of functional variable gene transcripts is sometimes hindered by co-expression of aberrant transcripts in hybridoma cells.

Objective: To develop general strategies for identifying the functional variable transcripts of both heavy (V_H) and kappa light (V_κ) chains from mouse hybridomas.

Methods: V_H and V_κ genes of anti-dengue hybridoma clones were PCR-amplified using set of degenerate primers covering all mouse immunoglobulin families. V_κ amplicons were additionally digested with *Bci*VI to eliminate aberrant V_κ transcripts. The productive V_H and V_κ sequences were identified by Immunogenetics (IMGT) database analysis and cloned into a dual human IgG expression vector to generate chimeric antibodies (chAbs) in mammalian cells. The reactivity of chAbs was tested by immunoblot and immunofluorescent assays.

Results: Among 17 tested hybridoma clones, 400 bp V_κ amplicons were obtained using eight different V_κ primers. Amplicons from productive V_κ transcripts are resistant to *Bci*VI digestion, whereas *Bci*VI-digested amplicons indicated aberrant V_κ transcripts. 500-bp productive V_H amplicons could be obtained from all clones using a set of five V_H primers. The productive V_H/V_κ genes of three anti-dengue NS1 mAbs (m2G6, m1F11 and m1A4) were cloned and mouse-human chAbs were generated. The binding reactivities of the chAbs to dengue NS1 were similar to the original mAbs.

Conclusions: A general protocol to identify productive/functional V_H and V_κ genes was demonstrated. The method is useful for producing chAbs and genetic archiving of valuable hybridoma cell lines.

Key words: Aberrant variable transcripts, chimeric antibody, dengue virus, functional variable transcripts, mouse hybridoma cells

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Introduction

Hybridoma technology is used to produce monoclonal antibodies (mAbs) by fusion of murine B cells and myeloma cells. However, some hybridoma clones may lose the ability to produce mAb due to genetic instability and/or inappropriate culturing and storage conditions. Thus, archiving of variable genes should be performed for safekeeping of valuable clones. In addition, mouse variable genes can be fused to the constant

region of an immunoglobulin gene derived from another species in order to generate chimeric antibodies (chAbs), which are useful for various applications. For example, chAbs are used therapeutically in humans, in which the variable genes are fused to the human immunoglobulin backbone to minimize irrelevant immunogenicity. To date, a number of chAbs have been used for treatment of disease, e.g. Rituxan (rituximab) for rheumatoid arthritis and Remicade (Infliximab) for auto-immune diseases.[1,2]

For the generation of chAbs, it is necessary to clone genes encoding V_H and V_κ of target mAbs from mouse hybridoma cell lines. However, aberrant immunoglobulin mRNAs are found in hybridoma cells derived from MOPC21 myeloma fusion partners, which interfere with the cloning of the desired variable gene transcripts. Aberrant V_κ sequences frequently contain a premature stop codon at a position 105 and the codon replacement of cysteine to tyrosine at position 23.[3]

Several strategies have been applied to eliminate aberrant V_κ transcripts, such as peptide sequencing and antisense directed RNase H to digest the aberrant V_κ mRNA, but these strategies are ineffective.[4] However, most aberrant V_κ sequences contain recognition sites for some restriction endonucleases.[5] A *Bci*VI site, for example, is commonly located in aberrant transcript sequences (96%). Therefore, *Bci*VI-sensitive aberrant V_κ can be distinguished from the *Bci*VI-resistant sequences that encode functional V_κ. However, since the aberrant V_κ transcripts in most myeloma partners are abundant and preferentially amplified, a rare functional V_κ genes are then hardly to be recovered after *Bci*VI digestion, which still allowed low efficiency in downstream cloning step.[6]

We have generated several mouse hybridoma clones producing mAbs against dengue virus proteins, which are useful for immunopathology of dengue diseases, diagnostics and dengue vaccine development. We wished to clone V_H and V_L genes from these valuable hybridoma clones as genetic archive in case the cells lose viability. Moreover, the cloned immunoglobulin genes are required to generate chAbs. The presence of aberrant V_κ transcripts in these clones could prevent the isolation of the desired functional genes. In this study, we proposed a strategy to select functional variable transcripts from murine hybridoma clones by using two sets of specific primers covering most of the mouse immunoglobulin genes to selectively amplify different V_H or V_κ gene sequences. PCR products from aberrant V_κ transcripts can be identified by *Bci*VI digestion. Functional transcript sequences of variable domains could be selectively amplified from 17 hybridoma cell lines producing antibodies against dengue virus proteins (NS1, nonstructural protein 1; E, envelope; and prM, pre-membrane). Functional V_H and V_κ genes of three hybridoma cell lines with the human IgG1 constant region were assembled to generate chAbs. The functional properties of the chAbs were compared with the corresponding original mAbs.

Methods

Hybridoma cells and viruses

Seventeen mouse hybridoma cell lines producing mAbs against dengue prM, E and NS1 proteins were used in this study. Most of them were generated from X63-Ag8.653 myeloma fusion partner, except 4G2 and 2H2 which were from X-63-Ag8.[7-10] Their characteristics are summarized in **Table S1**. C6/36 or BHK cells were infected with prototypic strains of four dengue viruses, DENV1 (Hawaii), DENV2 (16681), DENV3 (H87), and DENV4 (H241) and used for expression of antigens in immunoassays.

RNA isolation and cDNA synthesis

Total RNA was isolated from hybridomas with the RNeasy mini kit (Qiagen, Germany) and cDNA was synthesized with oligo (dT)20 using Superscript III reverse transcriptase (Life Technologies, USA) according to the manufacturers' protocols.

PCR amplification of V_H and V_κ genes

V_H and V_κ transcripts were amplified from hybridoma cDNAs using Phusion high fidelity DNA polymerase (NEB, USA). Eight sense degenerate primers binding to leader sequences of V_κ regions and an antisense primer binding to the constant kappa light chain region were designed (**Table S2**). PCRs were carried out for 50 cycles with 10 s denaturing (98°C), 10 s annealing (55°C), 30 s extension (72°C) and a final extension of 5 min (72°C). The size of V_κ PCR product was about 400 base pairs (bp). For V_H amplification, five sense primers located upstream of the V_H regions or the highly conserved sequences at the beginning of the FR1 region and six antisense primers located in the constant regions of IgG1, IgG2a and IgM were used (**Table S2**). To enhance yield of V_H product, a primary PCR was performed with the V_H sense and outer antisense primers for 35 cycles with 10 s denaturing (98°C), 10 s annealing (55°C), 40 s extension (72°C) and a final extension of 5 min (72°C). The primary PCR product was re-amplified with the same PCR protocol in a semi-nested PCR using inner antisense primers and the same sense primer (**Figure S5**). The size of the V_H semi-nested PCR product was about 500 bp.

Identification of aberrant variable gene transcripts and gene cloning

The V_κ PCR products were subsequently digested with *Bci*VI (NEB) restriction enzyme. The 400 bp *Bci*VI-resistant V_κ as well as the 500 bp V_H PCR products were cloned into the pGEM-T TA cloning vector (Promega). Plasmids containing V_H or V_κ gene were purified from transformed *E. coli* strain DH5α bacteria and verified by DNA sequencing. Plasmids with cloned V_H or V_κ sequences were analyzed with BLAST and the IMGT V-QUEST database tool (http://www.imgt.org/IMGT_vquest/vquest).[11]

Construction of dual antibody expression plasmid

The dual expression plasmid containing HER2/neu receptor specific humanized IgG1/κ antibody isotype (pVITRO1-hTrastuzumab-IgG1κ), a gift from Andrew Beavil (Addgene plasmid # 61883), was used. Variable domains of the HER2/neu receptor in the dual plasmid were replaced with V_H and V_κ genes derived from mAbs using the polymerase incomplete primer extension (PIPE) technique.[12] Briefly, pVITRO1-hTrastuzumab-IgG1κ was amplified to obtain two linearized plasmid fragments by PCR using two vector-specific primer pairs (Linear_Kfwd/Linear_Hrev and Linear_Hfwd/Linear_Krev; (**Table S3**). V_H and V_κ genes cloned in pGEM-T plasmid vector were amplified with specific primer pairs for each mAb

Table S1. Characteristics of 17 mAbs generated from mouse hybridoma cells.

No.	Hybridoma cells	mAbs	Dengue protein specificity	Isotype	Serotype specificity	Reference(s)
1	D2NS1 (2G6)	2G6	NS1	IgG2a	DENV2	[10]
2	NS1-3F (1F11)	1F11	NS1	IgG2a	DENV1–4	[9]
3	D2NS1 (1A4)	1A4	NS1	IgG2a	DENV2	[10]
4	D2NS1 (1B10)	1B10	NS1	IgG1	DENV2	[10]
5	D1NS1 (84B)	84B	NS1	IgG1	DENV1	[10]
6	D3NS1 (5F3)	5F3	NS1	IgG1	DENV1,3	[10]
7	NS1-1F (2E11)	2E11	NS1	IgM	DENV1–4	[9]
8	D2NS1 (3A2)	3A2	NS1	IgG1	DENV1,2	[10]
9	D3NS1 (46A)	46A	NS1	IgG2a	DENV3	[10]
10	NS1-2S (2C5)	2C5	NS1	IgM	DENV2	[9]
11	D2NS1 (4D2)	4D2	NS1	IgG1	DENV1–4	[10]
12	D2NS1 (1D5)	1D5	NS1	IgG1	DENV2	[10]
13	D2NS1 (4D4)	4D4	NS1	IgG2a	DENV1,2,4	[10]
14	NS1-4F (2E3)	2E3	NS1	IgG1	DENV1–4	[9]
15	D2NS1 (1B2)	1B2	NS1	IgG1	DENV2	[10]
16	4G2	4G2	E	IgG2a	DENV1–4	[8,7]
17	2H2	2H2	prM	IgG2a	DENV2	[8,7]

Table S2. Oligo nucleotide primers used for the amplification of murine V_H and V_κ.

No.	Primer	5′→3′ sequence	Length (bp)	Reference(s)
Leader heavy chain sense primers				
1	5′MsVHE	GGGAATTCGAGGTGCAGCTGCAGGAGTCTGG	31	[19]
2	5′LVh1	ACTACAGGTGTCCASTSC	18	Modified from [20]
3	5′LVh2	CCAAGCTGTRTCCTDTCC	18	Modified from [20]
4	5′LVh5,6,7	TTTAAAWAGTRTCCAGWGT	19	Modified from [20]
5	5′LVh3,14	CTTCCTGATGGCAGYSSTT	19	Modified from [20]
Constant heavy chain anti-sense primers				
1	3′Cg1_outer	GGAAGGTGTGCACACCGCTGGAC	23	[19]
2	3′Cg2a_outer	GGAAGGTGTGCACACCACTGGAC	23	[19]
3	3′Cm_outer	AGGGGGCTCTCGCAGGAGACGAGG	24	[19]
4	3′Cg1_inner	GCTCAGGGAAATAGCCCTTGAC	22	[19]
5	3′Cg2a_inner	GCTCAGGGAAATAACCCTTGAC	22	[19]
6	3′Cm_inner	AGGGGGAAGACATTTGGGAAGGAC	24	[19]

DNA degeneracy codes: S = C or G; R = A or G; D = A or G or T; W = A or T; Y = C or T; K = G or T

Table S2. (Continued)

No.	Primer	5′→3′ sequence	Length (bp)	Reference(s)
Leader light chain sense primers				
1	5′LVk3	TGCTGCTGCTCTGGGTTCCAG	21	[19]
2	5′LVk4	ATTWTCAGCTTCCTGCTAATC	21	[19]
3	5′LVk5	TTTTGCTTTTCTGGATTYCAG	21	[19]
4	5′LVk6	TCGTGTTKCTSTGGTTGTCTG	21	[19]
5	5′LVk689	ATGGAATCACAGRCYCWGGT	20	[19]
6	5′LVk14	TCTTGTTGCTCTGGTTYCCAG	21	[19]
7	5′LVk19	CAGTTCCTGGGGCTCTTGTTGTTC	24	[19]
8	5′LVk20	CTCACTAGCTCTTCTCCTC	19	[19]
Constant light chain anti-sense primers				
1	3′MCk	GATGGTGGGAAGATGGATACAGTT	24	[19]

DNA degeneracy codes: S = C or G; R = A or G; D = A or G or T; W = A or T; Y = C or T; K = G or T

Table S3. Primers used for PIPE cloning.

No.	Primer	5′→3′ sequence	Length (bp)	Reference(s)
1	Linear_Kfwd	CGTACGGTGGCGGCGCCATCTGTCTTCATCTTCCCGCCAT	40	[12]
2	Linear_Krev	ACCGCGGCTAGCTGGAACCCAGAGCAGCAGAAACCCAATG	40	[12]
3	Linear_Hfwd_IgG1	GCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCT	40	[12]
4	Linear_Hrev	GGAGTGCGCGCCTGTGGCGGCCGCCACCAAGAAGAGGATC	40	[12]
5	2G6VK_fwd	GGGTTCCAGCTAGCCGCGGTgaaacaactgtgacccagtc	40	In this study
6	2G6VK_rev	GATGGCGCCGCCACCGTACGtttcagttccagcttggtcc	40	In this study
7	2G6VH_fwd	CCGCCACAGGCGCGCACTCCcaggtccaactgcagcagtc	40	In this study
8	2G6VH_rev	GATGGGCCCTTGGTGCTAGCtgaggagactgtgagagtgg	40	In this study
9	1F11VK_fwd	GGGTTCCAGCTAGCCGCGGTgatgttgtgatgacccagac	40	In this study
10	1F11VK_rev	AGATGGCGCCGCCACCGTACGtttcagctccagcttggtc	40	In this study
11	1F11VH_fwd	CCGCCACAGGCGCGCACTCCgaggtccagctgcagcagtc	40	In this study
12	1F11VH_rev	GATGGGCCCTTGGTGCTAGCtgaggagacggtgactgagg	40	In this study
13	1A4-1VK_fwd	GGGTTCCAGCTAGCCGCGGTgacattgtcatgacccagtc	40	In this study
14	1A4-1VK_rev	AGATGGCGCCGCCACCGTACGtttcagctccagcttggtc	40	In this study
15	1A4-1VH_fwd	CCGCCACAGGCGCGCACTCCgaggttcagctgcagcagtc	40	In this study
16	1A4-1VH_rev	GATGGGCCCTTGGTGCTAGCtgcagagacagtgaccagag	40	In this study

Uppercase letters indicate expression vector sequences and lower case letters indicate antibody sequences.

(e.g., 2G6VH-fwd/rev and 2G6VK-fwd/rev; **Table S3**). Four PCR fragments with overlapping ends were mixed and annealed to make circular plasmids at 50°C for 1 hour, and then treated with the restriction enzyme *Dpn*I (NEB). The assembled PCR products were transformed into *E. coli* strain DH5α. The pVITRO1 plasmids containing the cloned V_H and V_κ genes for production of antibodies against dengue proteins were validated by nucleotide sequencing (Macrogen).

Transfection and recombinant antibody production

Two μg of a plasmid encoding V_H and V_κ transcripts was transfected into human embryonic kidney cell line 293T (ATCC, USA) with Lipofectamine 2000 (Thermo Fisher, USA). Supernatants containing chAbs were harvested to assay for expression.

Characterization of chAbs

The obtaining chAbs were characterized for the presence of human immunoglobulin and their specific reactivity to dengue NS1. The human immunoglobulin of chAbs were tested by dot blot analysis using HRP-conjugated goat anti-human immunoglobulins (Jackson Immuno Research, USA), specific to human immunoglobulin. To determine specific binding reactivity in comparison with mAbs, DENV1 to 4 -infected cell lysates or supernatants were tested with chAbs by dot blot assay and Western blot analysis. For secondary or conjugated antibody, HRP-conjugated goat anti-human immunoglobulins was used followed by chAbs, whereas, HRP-conjugated anti-mouse Igs (Dako, USA) was followed by mAbs. Antibody reactivity on DENV1 to 4 -infected cells was also tested by immunofluorescent assay (IFA), in which anti-E mAb (m4G2) was used to confirm dengue virus infection.

Competitive binding ELISA

The purified NS1 protein from DENV2 infected Vero cells (10 ng) was coated on ELISA wells. The blocking mAb was initially added to NS1-coated ELISA wells from 0.17 to 100 µg/ml (100 µl) for 1 hour. PBS was used as negative control. The chAb was subsequently added to those ELISA wells to obtain a final concentration of 6 µg/ml for another 1 hour. The bound chAb-NS1 complex was detected with goat-anti-human IgG-HRP (dilution 1:1000; P214, Dako). The TMB substrate (Thermo Fisher Scientific, USA) was used to develop reaction colors and OD reading at 450 nm (A450) was measured by ELISA reader. The percentage of blocking (% blocking) were calculated as followed:

% Blocking of chAb = (A450 of control well - A450 of the test well) × 100/A450 of control well Where control well is A450 obtained from chAb without blocking antibody (no mAb). Test well is A450 obtained from chAb reactivity in the presence of blocking mAb (+mAb).

Results

Coexpression of functional and aberrant V_κ transcripts in murine hybridoma cells

Three hybridoma clones (2G6, 1F11 and 1A4) producing mAbs against dengue NS1 proteins were selected for initial characterization of V_κ genes. V_κ PCR products of the expected size (400 bp) were obtained using a mixture of leader light chain primers and a constant kappa light chain primer (**Table S2**). Analysis of cloned nucleotide sequences indicated that only the 2G6 clone V_κ sequence could produce functional antibody protein. In contrast, V_κ sequences from clones 1F11 and 1A4 contained a stop codon at a position 105 and Tyr at position 23, the hallmarks of aberrant V_κ genes. These aberrant V_κ sequences were identical to those of several hybridoma clones derived from the X63-Ag8.653 myeloma cell line as previously reported.[3,13] As a *Bci*VI recognition site is commonly found in aberrant V_κ genes, we hypothesized that mouse hybridoma clones with functional or aberrant V_κ genes could be distinguished by digestion of V_κ gene PCR products with *Bci*VI. As expected from sequence analysis, the PCR product from clone 2G6 was resistant to *Bci*VI treatment, but an additional digested 200 bp DNA fragment was observed from the 1F11 and 1A4 clones producing aberrant V_κ (**Figure 1B**, lanes 1-3), indicating the presence of aberrant V_κ transcripts. Although V_κ gene PCR products of the expected size (400 bp) were obtained from all 17 clones (**Figure 1A**), *Bci*VI-digested products (200 bp) were obtained from V_κ amplicons derived from most of the tested clones, except 2H2 and 1B10 (**Figure 1B**). These results suggest that aberrant V_κ genes are present in the majority of our mouse hybridoma clones. However, the presence of 400 bp bands resistant to *Bci*VI treatment in addition to digested 200 bp bands for all clones (**Figure 1B**) suggested the coexpression of functional and aberrant V_κ transcripts, perhaps from different genes.

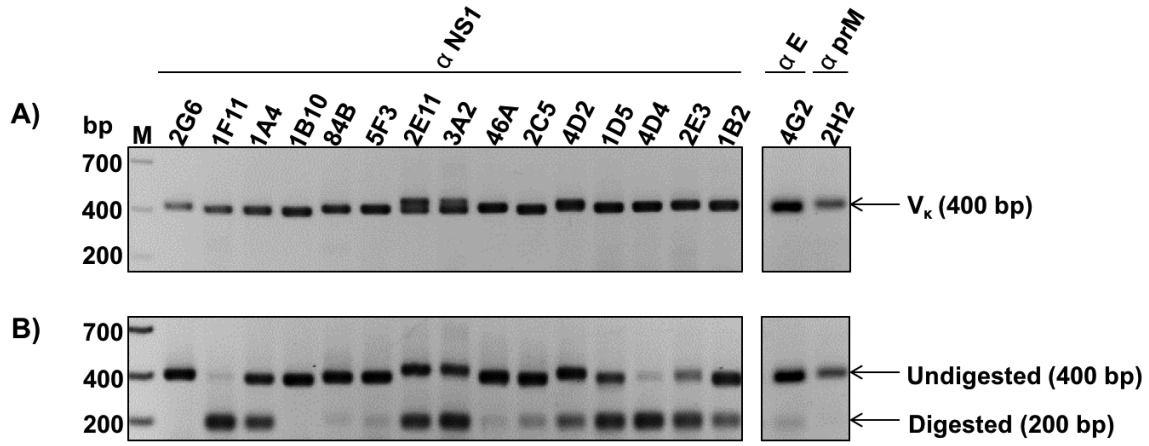

Figure 1. Identification of aberrant V_κ transcripts in hybridoma cells. The V_κ transcripts derived from first strand cDNAs were amplified using a mixture of eight sense primers binding to the leader light chain and the anti-sense constant kappa light chain primer. The V_κ PCR products were (A) left untreated or (B) treated with *Bci*VI prior to separation by agarose gel electrophoresis. ZipRuler express DNA ladder 2 (Thermo Fisher) was separated in the lane labeled M. Amplicons of the size expected for V_κ transcripts are marked as V_κ (400 bp). Amplicon undigested with *Bci*VI corresponds to putatively productive V_κ transcript (marked as Undigested (400 bp)), whereas *Bci*VI digested amplicon corresponds to putatively aberrant V_κ transcript (marked as Digested (200 bp)). PCR products of anti-NS1 antibody clones were separated in lanes 2-15 (clone identifiers shown above the lanes), whereas PCR products of anti-E (4G2) and anti-prM (2H2) antibody clones were separated in lane 16 and 17, respectively.

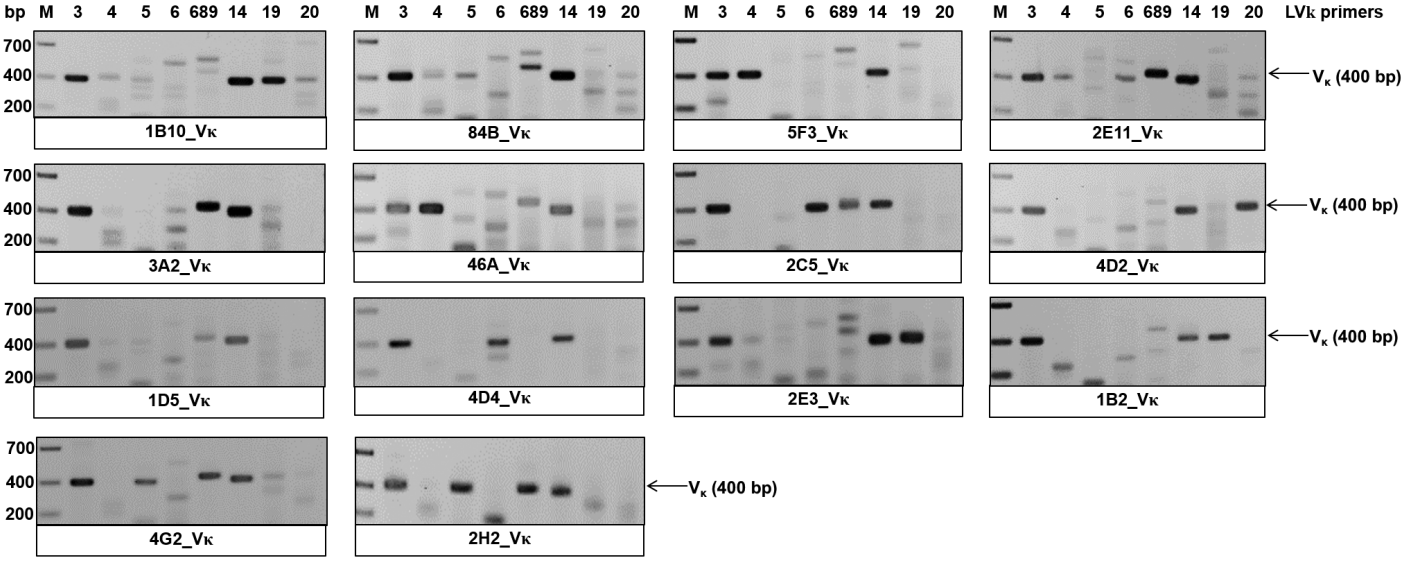

Figure S1. Amplification of $V_κ$ transcripts from a panel of hybridoma cells. V_H amplicons derived from first strand cDNAs were obtained from 14 hybridoma clones by PCR with eight individual sense primers (LVk primers; **Table S2**) and separated by agarose gel electrophoresis (clones indicated in each panel below the lanes). Amplicons of the size expected for $V_κ$ sequences are marked as $V_κ$ (400 bp). ZipRuler express DNA ladder 2 (Thermo Fisher) was separated in the lane labeled M.

Functional $V_κ$ genes from clones coexpressing aberrant $V_κ$ genes

From the coexpression of functional and aberrant $V_κ$ transcripts, we hypothesized that the functional $V_κ$ transcripts could be selectively PCR-amplified using different sense primers binding to variable regions. Each of eight different sense primers (LVk) with a constant kappa light chain anti-sense primer were initially tested for PCR amplification using cDNA from three clones (1F11, 1A4 and 2G6). The 400 bp $V_κ$ PCR product was obtained by some, but not all individual primers from each clone (**Figure 2A**). Subsequent treatment with *Bci*VI was performed to distinguish functional $V_κ$ genes PCR products (resistant to digestion) from aberrant $V_κ$ genes (sensitive to digestion) (**Figure 2B**). The *Bci*VI-resistant products from 2G6 (obtained using primer LVk20), and these from 1F11 and 1A4 (obtained using primer LVk6) were cloned. Sequencing of the cloned $V_κ$ genes demonstrated that all cloned genes encoded functional $V_κ$ as expected. The same experimental protocol was applied to the other hybridoma clones, and *Bci*VI-resistant $V_κ$ amplicons were obtained using different sense primers (**Figure S2**). Sequencing of these *Bci*VI-resistant $V_κ$ gene products showed that all encoded functional $V_κ$ gene sequences. A few exceptions were found in clones 4G2 and 2H2 in which these

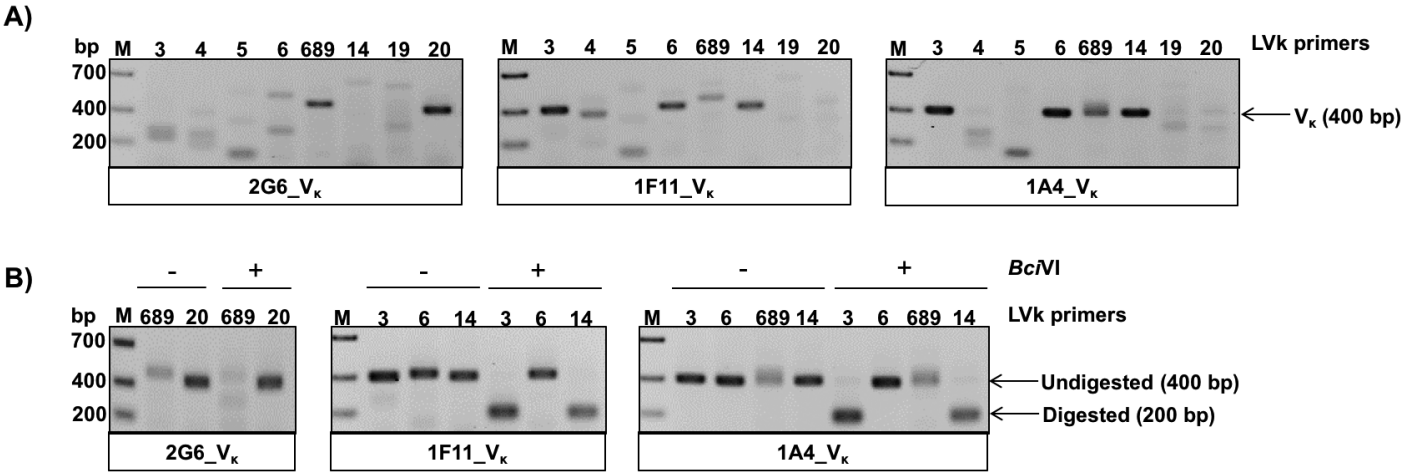

Figure 2. Identification of aberrant $V_κ$ transcripts by *Bci*VI digestion. (A) PCR products derived from first strand cDNAs of hybridoma clones 2G6, 1F11 and 1A4 were generated with eight different sense primers (LVk primers) and separated by agarose gel electrophoresis. Amplicons of the size expected for $V_κ$ transcripts are marked as $V_κ$ (400 bp). (B) $V_κ$ transcripts amplified with the indicated primers were either left untreated (-) or digested with *Bci*VI (+) prior to agarose gel electrophoresis. Amplicon undigested with *Bci*VI corresponds to putatively productive $V_κ$ transcript (marked as Undigested (400 bp)), whereas *Bci*VI digested amplicon corresponds to putatively aberrant $V_κ$ transcript (marked as Digested (200 bp)). Lane M indicates ZipRuler express DNA ladder 2 (Thermo Fisher) (bp).

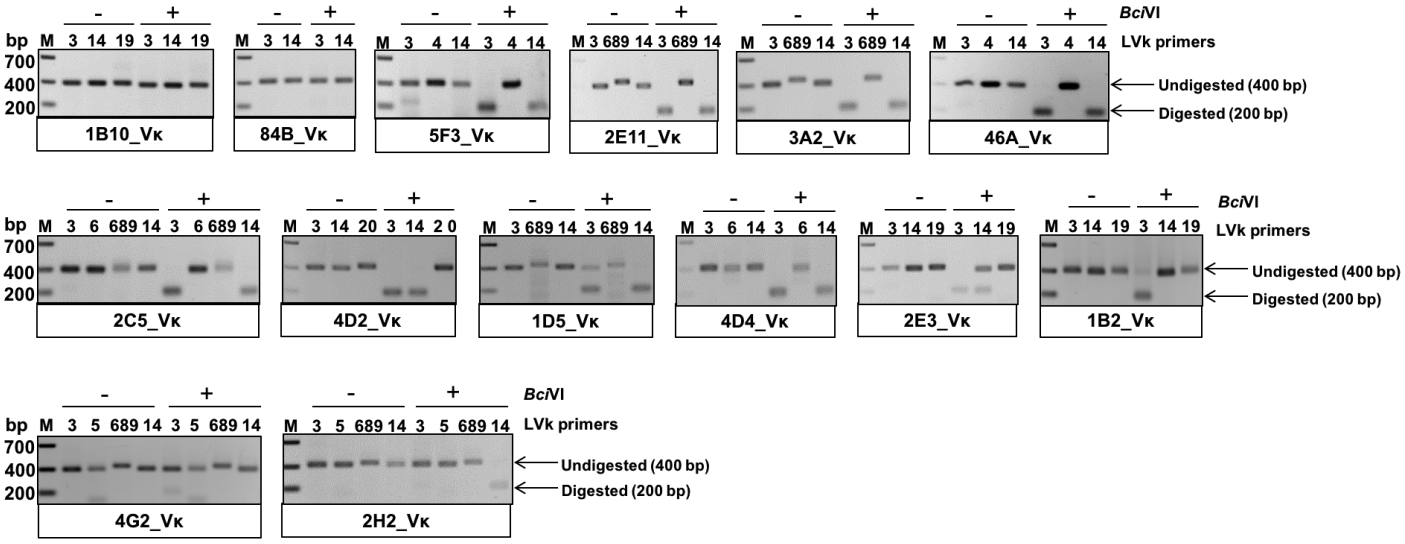

Figure S2. *Bci*VI digestion of amplified V_κ transcripts. V_κ transcripts derived from 14 hybridoma cells were PCR-amplified with different sense primers (LVk primers; **Table S2**) and separated by agarose gel electrophoresis (clones indicated in each panel below the lanes). The PCR products were either left untreated (-) or treated with *Bci*VI (+), prior to electrophoresis. Amplicon undigested with *Bci*VI corresponds to putatively productive V_κ gene (marked as Undigested (400 bp)), whereas *Bci*VI- digested amplicon corresponds to putatively aberrant V_κ gene (marked as Digested (200 bp)). Lane M represents standard DNA markers (bp).

Table S4. Summary of productive V_κ sequences derived from 17 hybridoma clones.

No.	Hybridoma cell line	Sense primers (LVk) producing 400-bp V_κ amplicons	Sense primers (LVk) producing *Bci*VI-resistant V_κ PCR amplicons*	Functional Ig_κ sequences identified from IMGT database analysis	
				From direct sequencing of PCR products	From TA cloning transformants: No. of functional Ig_κ / total no. of tested colonies (%)
1	2G6	689, 20	**20**	Nd†	2 / 2 (100)
2	1F11	3, 6, 14	**6**	Nd	2 / 2 (100)
3	1A4	3, 6, 689, 14	**6** 689	Nd **Yes**	3 / 3 (100) Nd
4	1B10	3, 14, 19	**3** 14 19	Nd **Yes** **Yes**	4 / 4 (100) Nd Nd
5	84B	3, 14	**3** 14	Nd **Yes**	3 / 3 (100) Nd
6	5F3	3, 4, 14	**4**	Nd	3 / 3 (100)
7	2E11	3, 689, 14	**689**	Nd	1 / 1 (100)
8	3A2	3, 689, 14	**689**	Nd	2 / 2 (100)
9	46A	3, 4, 14	**4**	Nd	3 / 3 (100)
10	2C5	3, 6, 689	**6** 689	Nd Nd	3 / 3 (100) 3 / 3 (100)
11	4D2	3, 14, 20	**20**	Nd	3 / 3 (100)
12	1D5	3, 689, 14	**689**	Nd	3 / 3 (100)
13	4D4	3, 6, 14	**6**	Nd	3 / 3 (100)
14	2E3	3, 14, 19	**19**	Nd	3 / 3 (100)
15	1B2	3, 14, 19	14 **19**	**Yes** Nd	Nd 3 / 3 (100)

* Sense primers selected for amplifying V_κ PCR products for PIPE cloning are highlighted in bold
†Nd, Not done.

Table S4. Summary of productive V_κ sequences derived from 17 hybridoma clones.

No.	Hybridoma cell line	Sense primers (LVk) producing 400-bp V_κ amplicons	Sense primers (LVk) producing *Bci*VI-resistant V_κ PCR amplicons*	Functional Ig_κ sequences identified from IMGT database analysis	
				From direct sequencing of PCR products	From TA cloning transformants: No. of functional Ig_κ / total no. of tested colonies (%)
16	4G2	3, 5, 689, 14	5	**Yes**	Nd
			689	Nd	0 / 3 (0) (All were identified as endogenous V_κ from myeloma)
			14	Nd	3 / 3 (100)
17	2H2	3, 5, 689, 14	3	**Yes**	Nd
			5	Nd	2 / 2 (100)
			689	Endogenous V_κ from myeloma	Nd

* Sense primers selected for amplifying V_κ PCR products for PIPE cloning are highlighted in bold
†Nd, Not done.

V_κ gene products amplified by primer LVk689, but not the others, were identified as endogenous V_κ genes from myeloma cells (**Table S4**). These results suggested that the protocol for obtaining functional V_κ genes is reproducible among different mouse hybridoma clones.

Amplification of V_H transcripts

PCR amplification of the V_H gene was initially tested from three hybridoma clones (2G6, 1F11 and 1A4). 500 bp V_H gene products were obtained by semi-nested PCR using different V_H primer pairs (**Figure 3**). Sequencing analysis indicated that all cloned V_H genes encoded functional antibody proteins. V_H transcripts of the other 14 hybridoma clones were PCR amplified in the same manner (**Figure S3**) and verified by sequencing analysis. Functional V_H transcripts were obtained from all hybridoma clones, except 1B2 of which the aberrant sequence was generated by a frameshift at the CDR3 region. The aberrant V_H sequence from 1B2 is not described in previous reports.[6,14] Therefore, we deposited this novel aberrant V_H sequence in the GenBank database (GenBank accession no. MF174854).

Generation and functional testing of chAbs

To verify the functionality of cloned V_H and V_κ genes, chAbs containing mouse variable region and human IgG1 constant region were generated. The putatively functional V_H and V_κ genes obtained from mouse hybridoma clones 2G6, 1F11 and 1A4 were sub-cloned into the dual expression plasmid pVITRO/IgG1κ. Recombinant plasmids were transfected to 293T cells. Three transfected cell lines were established that secreted chAbs to dengue NS1 protein, designated as ch2G6/IgG1κ, ch1F11/IgG1κ, and ch1A4/IgG1κ. The human IgG1 constant region of these chAbs cross-reacted with anti-human immunoglobulins (Igs)-HRP, but not with anti-mouse Igs-HRP, by dot blot assay (**Figure S4A**). In addition, by competitive binding ELISA, we showed that NS1 binding of ch1F11 and ch1A4 Abs can be blocked by their original mAbs, but not by other clones (**Figure S4B**). This result indicated the same recognition epitope on NS1 protein of original mAb and its corresponding chAb. Functional assays were performed to test the activity of chAbs against dengue NS1 proteins in comparison with the original mouse mAbs (m2G6, m1F11 and m1A4). By immunoblotting, the

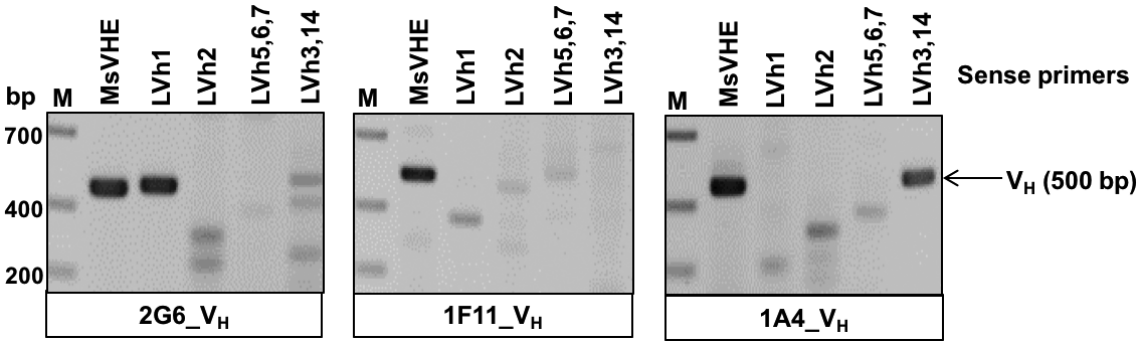

Figure 3. Amplification of V_H transcripts in hybridoma cells. V_H amplicons derived from first strand cDNAs of hybridoma clones 2G6, 1F11 and 1A4 were obtained by PCR with five different sense primers. The PCR products were separated by agarose gel electrophoresis. Amplicons of the size expected for V_H transcripts are marked as V_H (500 bp). Lane M indicates ZipRuler express DNA ladder 2 (Thermo Fisher) (bp). The reactions with different sense primers are indicated above the lanes.

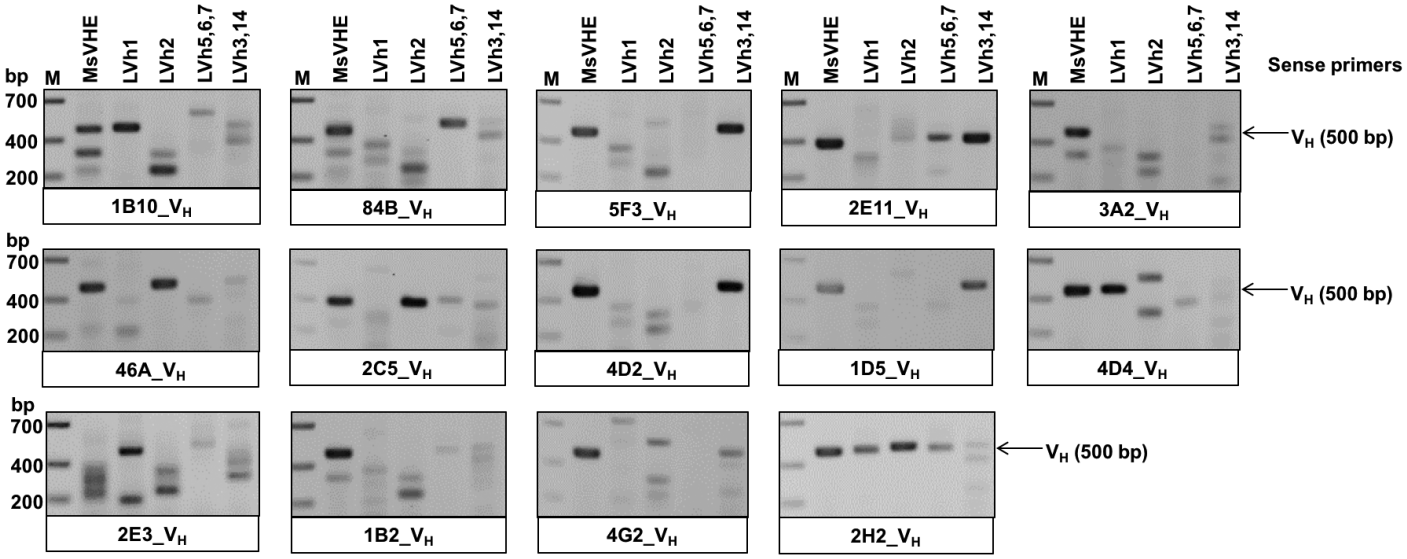

Figure S3. Amplification of V_H transcripts from a panel of hybridoma cells. V_H amplicons derived from first stand cDNAs of 14 hybridoma clones were obtained by PCR with five different sense primers (**Table S2**). The PCR products were separated by agarose gel electrophoresis (clones indicated in each panel below the lanes). Amplicons of the size expected for V_H transcripts are marked as V_H (500 bp). Lane M indicates ZipRuler express DNA ladder 2 (Thermo Fisher) (bp). The reactions with different sense primers are indicated above the lanes.

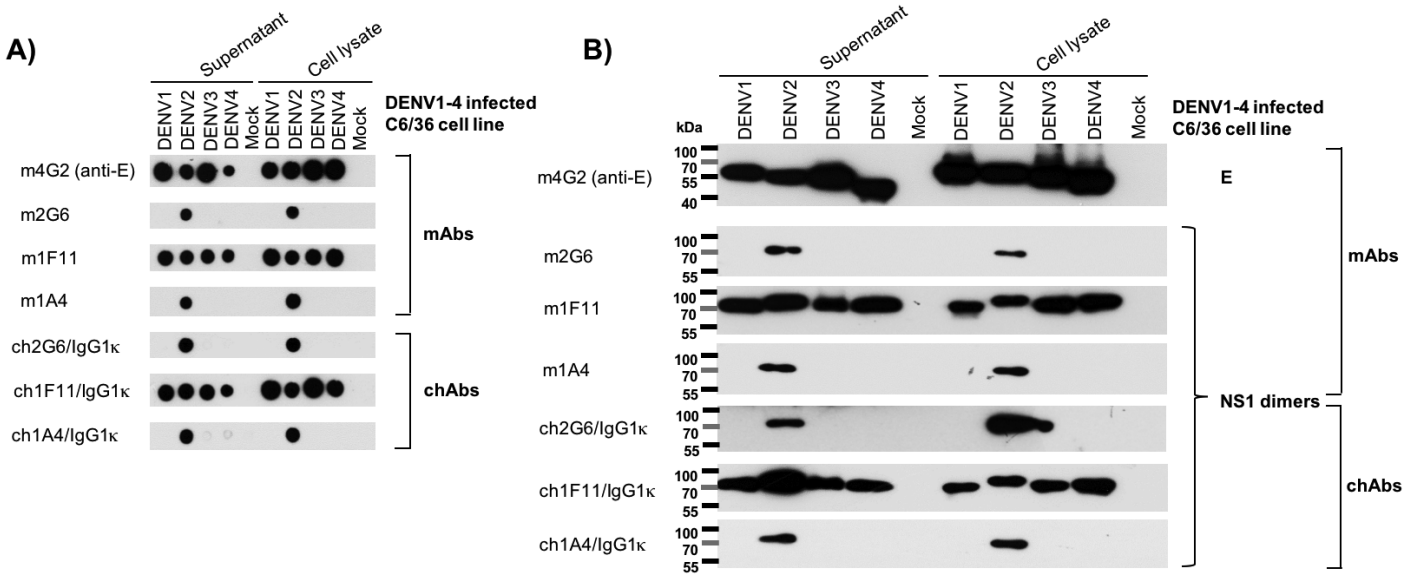

Figure 4. Functional testing of chimeric antibodies. C6/36 cells were infected with one of four serotypes of dengue virus (DENV1–4). Supernatants and cell lysates of infected and mock-infected cells were analyzed by (A) dot blot and (B) Western blot assays by probing with either murine antibodies (mAbs) or chimeric antibodies (chAbs) specific to the NS1 protein (2G6, 1F11 and 1A4). Anti-E (m4G2) was used as a positive control to detect the dengue virus E antigen in infected cells. The migrations of protein markers (PageRuler prestained protein ladder; Thermo Fisher) are indicated on the left of the blots in (B).

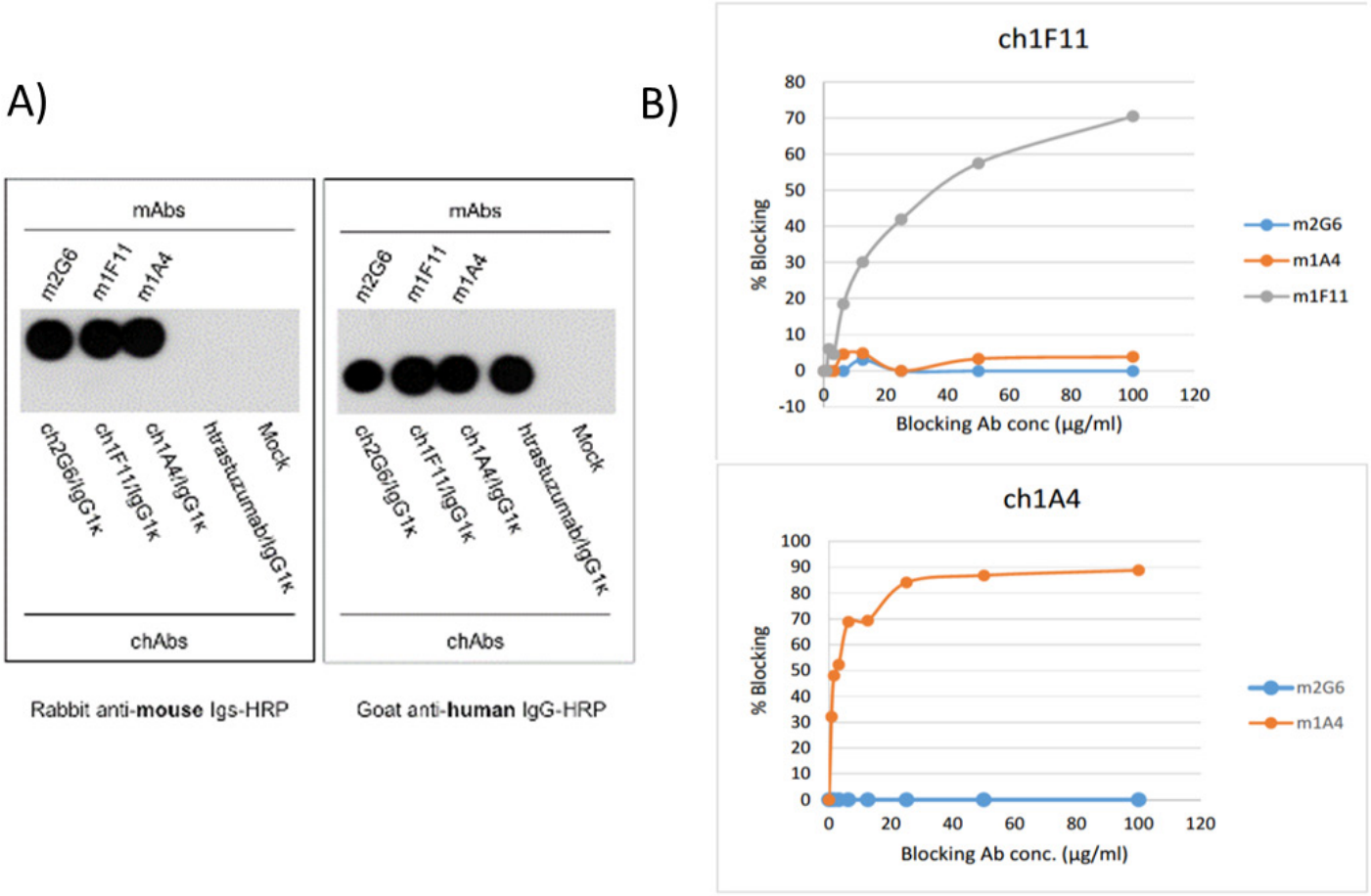

Figure S4. A) Detection of human IgG1 constant region in chimeric antibodies. Chimeric (ChAb) and mouse (mAb) antibodies were dotted onto a nitrocellulose membrane for a dot blot assay. Either HRP-conjugated rabbit anti-mouse Igs or HRP-conjugated goat anti-human IgG was added to detect host isotype specificity. **B) The original mAb blocks NS1 binding of corresponding chAb by a competitive binding ELISA.** The blocking mAb (m2G6, m1F11or m1A4) was initially bound to DENV2 NS1-coated ELISA wells at various concentrations. The chAb (ch1A4 or ch1F11) was subsequently added to compete NS1 binding with those blocking mAbs. The percentage of blocking of ch1A4 or ch1F11 were analyzed and plotted against various concentration of blocking mAb. The higher efficient blockage (% blocking) suggested higher degree of overlapped epitope, or even the same epitope, as recognized by two competitive antibodies (ch1A4/m1A4 or ch1F11/m1F11). In contrast, lower % blocking suggested the discrete or non-overlapped epitopes of the two antibodies (ch1A4/m2G6 or ch1F11/m1A4 and ch1F11/m2G6).

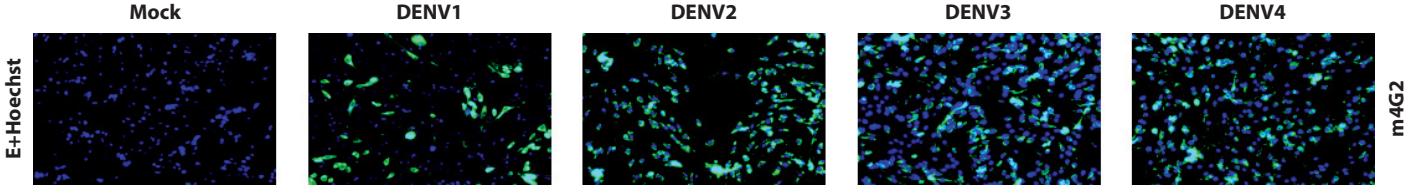

Figure 5. Immunofluorescent cell staining by recombinant antibodies. BHK cells were infected with one of four serotypes of dengue virus (DENV1–4). NS1 staining (green) was performed with either a mouse monoclonal antibody (mAb) (2G6, 1F11 or 1A4), followed with goat anti-mouse IgG-Alexa 488 or their chimeric counterpart (chAb/IgG1κ), followed with goat anti-human IgG-Alexa 488. Infectivity was confirmed by staining the dengue virus E protein with m4G2 (green). Hoechst33342 dye (blue) was used to stain cell nuclei. Cell staining was observed under EVOS cell imaging systems, at magnification of 20 **(20x).**

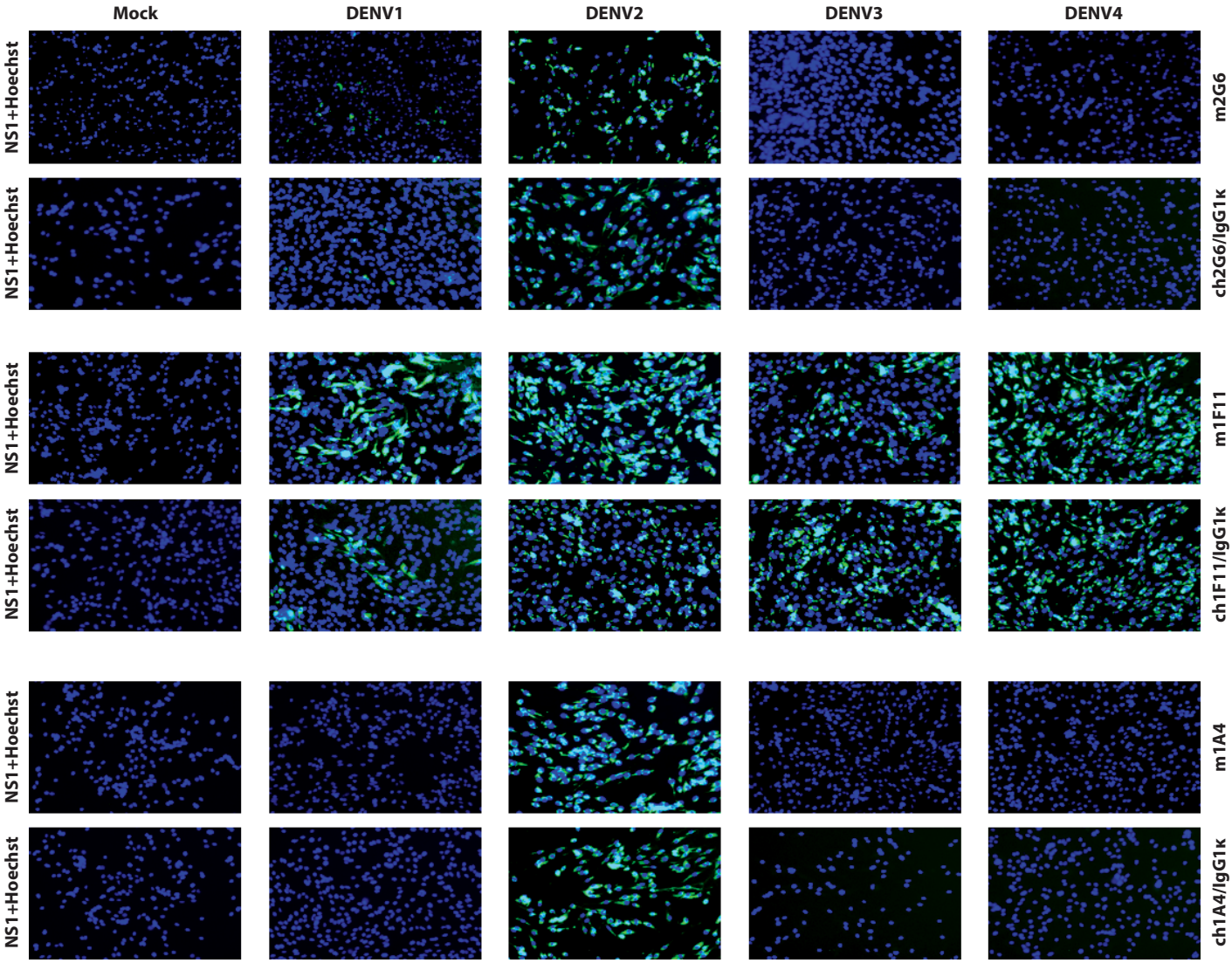

Figure 5. (Continued)

ch2G6/IgG1κ and ch1A4/IgG1κ chAbs reacted with DENV2 NS1 proteins, similar to the corresponding m2G6 and m1A4 antibodies, whereas the ch1F11/IgG1κ chAb recognized NS1 proteins of all four dengue serotypes, similar to the corresponding m1F11 (**Figure 4**). The three chAbs were tested for NS1 detection in dengue-infected fixed cells by IFA (**Figure 5**). Similar staining patterns to the original mouse hybridoma clones were found, in which the ch1F11 antibodies bound to NS1 proteins located in the cytoplasm of all dengue-infected cells, whereas ch1A4 antibodies recognized NS1 in DENV2-infected cells. Moreover, the ch2G6 antibodies strongly detected NS1 proteins in DENV2-infected cells, with weaker detection in DENV1-infected cells. None of the chAbs showed non-specific binding to mock-infected cells.

Discussion

The occurrence of endogenous aberrant variable transcripts, in particular V_κ gene transcripts in murine hybridoma cells presents an obstacle for manipulation of antibodies. Several strategies, including restriction enzyme digestion, for example *BCi*VI, to eliminate aberrant transcripts have not been fully effective because these transcripts may be expressed at similar or higher levels than the functional ones. To our experience, we initially amplified 1F11 V_κ PCR products using the mixed primers covering all mouse immunoglobulin families and directly cloned to TA cloning vector without *BCi*VI digestion, but all selected transformants contained only undesirable aberrant transcripts. Another attempt was done according to Ding's method by using the same 1F11 V_κ PCR products, but followed with BCiVI digestion.[6] The proportion of *BCi*VI-resistant PCR product (supposed to be functional V_κ) was much lower than the digested one (supposed to be aberrant V_κ) as demonstrated by agarose gel electrophoresis (**Figure 1B**, lane 3 from the left). The fainted 1F11 V_κ band was hardly extracted from gel and cloned into TA cloning vector. Unfortunately, none of transformants was obtained though it had been done several times. This might be due to the insufficient amount of the recovered *BCi*VI-resistant V_κ gene. Based on the hypothesis that amplification of the less proportion of gene encoding functional V_κ antibodies should be enriched by particular primer pairs among various mouse immunoglobulin V_κ gene families. Here, we therefore demonstrated an alternative strategy to isolate functional transcripts from murine hybridoma clones by selective PCR amplification with eight individual

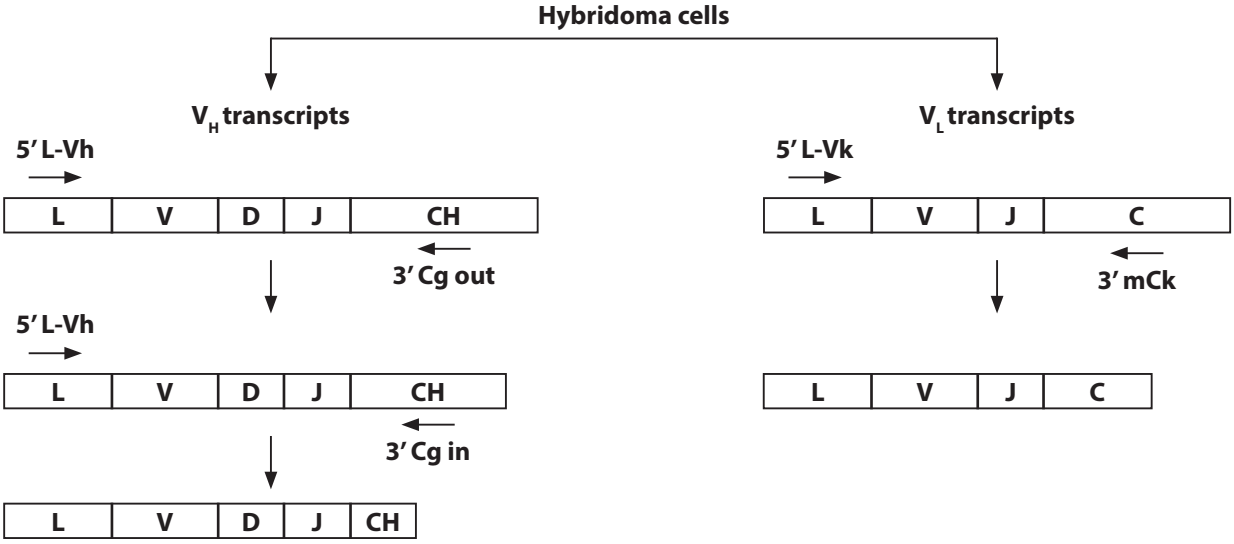

Figure S5. The location of primers on immunoglobulin transcripts. Variable transcripts of heavy chain (V_H) and light chain (V_L) extracted from hybridoma cells were amplified by PCR. The locations of forward primers and reverse primers were shown. The forward primers 5' LVH and 5' LVk were bound on leader sequences (L) of V_H and V_L, respectively. 3' Cg out and 3' Cg in were reverse outer and inner primers bound on the constant region of V_H (CH) whereas 3'mCk was bound on the constant region of V_L (C). V, D and J are the domains on immunoglobulin.

primers, instead of the mixed primers, followed by *BCi*VI treatment to differentiate the functional V_κ gene from the aberrant one. By this strategy, the *BCi*VI-resistant, functional V_κ PCR products were much easily obtained and could be directly cloned. Interestingly, those functional V_κ genes of each mouse hybridoma cell lines were found in all selected transformants (**Table S4**), suggesting highly success rate of V_κ gene cloning.

Mouse immunoglobulin (Ig) genes are highly diverse in sequence.[13,15] In this study we showed that 14 of 17 hybridoma clones, except 2G6, 1B10 and 2H2, contained *Bci*VI-aberrant V_κ genes when the mixed V_κ primers were used (as shown by *Bci*VI-digested products in **Figure 1B**). Some of them obviously demonstrated high proportion of aberrant V_κ over functional V_κ (clone 1F11, 4D4, 2D3, **Figure 1B**). It is suggested that the expression of aberrant V_κ is predominant in mouse hybridoma clones. This observation is concordant with other studies describing preferential amplification of aberrant V_κ cDNAs from mouse hybridoma cells despite using different sets of primers.[5,6] Nevertheless, we demonstrated that *Bci*VI-resistant amplicons could be obtained according to our selective primers strategy and functional V_κ genes were cloned for all 17 hybridoma clones in this study (**Table S4**).

Noted that *Bci*VI-resistant amplicons were obtained using sense primers LVk5, LVk6, LVk19, LVk20 and LVk689 that varied among hybridoma clones; however, *Bci*VI-sensitive amplicons were obtained using primers LVk3 and LVk14 (**Figure 2 and S2**). The latter two primers shared 17/21 nucleotides to each other, and are most similar to the leader sequence of the aberrant V_κ transcript reported previously, explaining why aberrant V_κ gene are frequently amplified by these two primers.[3,16] Being different from the others, the *Bci*VI-resistant amplicon obtained from clones 4G2 and 2H2 using primer LVk689 (**Table S4**) was identified as endogenous MOPC21 kappa light chain transcript (GenBank accession no.V00810).[17] In the early days of hybridoma technology development, mouse myeloma P3-X63-Ag8 derived from MOPC21 tumor cells was used for fusion with mouse B-cells. This immortal myeloma expresses and secretes MOPC21 immunoglobulin, which interferes with hybridoma clone immunoglobulin production.[18] Therefore, we suspect that these endogenous V_κ transcripts are produced from the parental mouse myeloma fusion partner (P3-X63-Ag8) which was used to generate 4G2 and 2H2 hybridoma clones.[7,8] The LVk689 primer sequence matches the V region of MOPC21 Ig kappa light chain, and thus is expected to prime amplification of endogenous MOPC21 transcripts that may be present. In contrast, subclones of P3-X63-Ag8, such as Sp2/0-Ag14, and P3-X63-Ag8.653, were later developed to eliminate the secretion of this endogenous immunoglobulin and are still commonly used for generating hybridomas.[18] No MOPC21 or endogenous immunoglobulin transcript sequences were obtained from the other 15 hybridoma clones (**Table S4**), as they were generated from a non-secreting immunoglobulin myeloma fusion partner P3-X63-Ag8.653.[9,10]

For V_H genes, 500 bp amplicons of functional transcripts were obtained from one or more primer pairs from all hybridoma cells used in this study. The one notable exception is the aberrant 1B2 V_H transcript, which contains a premature stop codon at the beginning of CDR3, leading to a non-productive heavy chain variable region. In general, aberrant V_H transcripts are rarely reported. V_H aberrant sequences are more diverse than aberrant V_κ. Two aberrant V_H transcripts (abVH-HF3, GenBank accession no. EU121635; abVH-HF4, GenBank accession no. EU121634) in the myeloma fusion partner P3-X63-Ag8.653 were previously reported by Yazad Irania *et. al.*[14] The aberrant V_H transcripts abVH-HF3 and abVH-LF8 (GenBank accession no. HM046413.1) are 98% identical and share the same change in the reading frame in the VDJ joining region encoding CDR3 (different from 1B2 V_H), whereas abVH-HF4

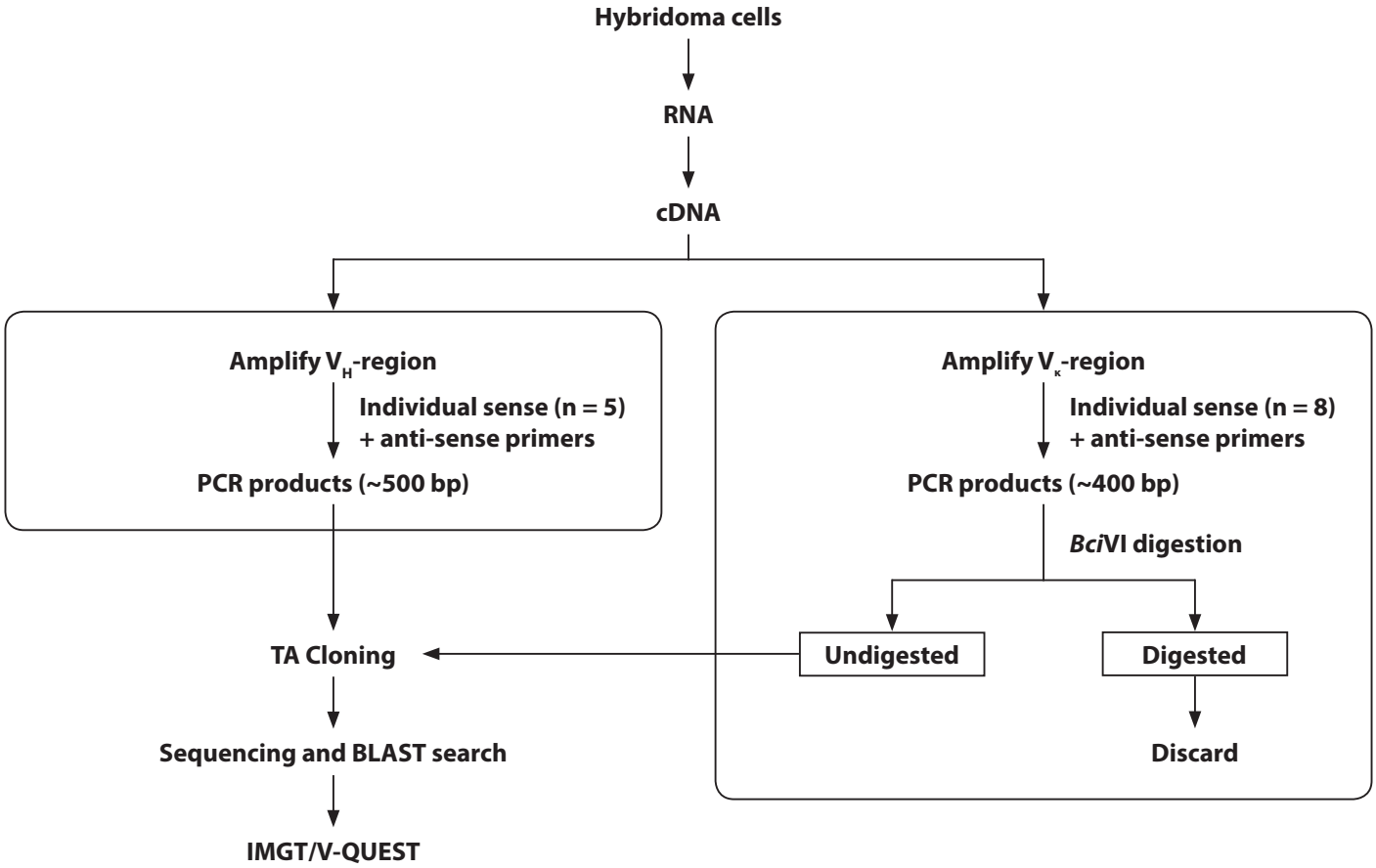

Figure 6. V_H and V_κ amplification strategy. Murine hybridoma cells were used as sources of immunoglobulin variable domains V_H and V_κ. RNA was extracted and reverse transcribed to generate first strand cDNA. V_H was amplified with a panel of five sense primers together with a common antisense primer in separate reactions, and the 500 bp PCR products were cloned into the TA plasmid. V_κ was amplified with eight individual sense primers and a common antisense primer in separate reactions, and the 400 bp PCR products were digested with *Bci*VI. Digested amplicons were identified as aberrant V_κ transcripts and only undigested amplicons of putatively productive V_κ transcripts were cloned into the TA plasmid. Plasmids containing V_H or V_κ sequences were analyzed with the IMGT database tool.

harbors a 50 bp deletion in the same region.[6,14] The diversity of aberrant V_H transcripts hindered the development of general strategies to select the functional gene for further cloning step.

The putatively productive variable genes cloned from PCR products were verified as functional chAbs with the human IgG1κ backbone. The binding properties of ch2G6, ch1F11 and ch1A4, as well as recognition epitopes are indistinguishable from the original mAbs by various assays (**Figure 4 and 5**, **Figure S4B**). These results validated our protocol, as summarized in **Figure 6** for identifying functional antibody transcripts from mouse hybridoma clones which coexpress aberrant genes.

In conclusion, we have developed a simple method for cloning variable gene transcripts for both V_H and V_κ that is reproducible in different mouse hybridoma cells by using 8 individual primer pairs covering most of the mouse immunoglobulin genes. Screening of *Bci*VI-resistant V_κ PCR product is a quick and easy way to identify functional V_κ transcripts and reduce the tedious downstream steps of cloning and selection. This approach is promising for the generation of chAbs as well as genetic archiving of valuable mouse hybridoma clones, which is beneficial for therapeutics and other medical applications.

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