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Use of Phage Display Libraries to Select For B-cell Receptor-specific Peptides of Chronic Lymphocytic Leukemia Cells

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USE OF PHAGE DISPLAY LIBRARIES TO SELECT FOR B-CELL RECEPTOR-SPECIFIC PEPTIDES OF CHRONIC LYMPHOCYTIC LEUKEMIA CELLS

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By

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Richard Chou ENTITLED "Use of Phage Display Libraries to Select For B-cell Receptor-specific Peptides of Chronic Lymphocytic Leukemia Cells" BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Chou, Richard. M.S., Department of Pharmacology and Toxicology, Wright State University, 2012. Use of Phage Display Libraries to Select For B-cell Receptor-specific Peptides of Chronic Lymphocytic Leukemia Cells.

Peptides with high affinity to the B-cell receptor (BCR) fused to a toxin could be an effective therapy for Chronic Lymphocytic Leukemia (CLL) patients. We screened captured BCR of a CLL patient with peptides from 7 and 12-mer phage display libraries, using two strategies. Lymphocytes from two patients diagnosed with CLL expressing two different unmutated VH genes (VH 1-3 and VH4-34, respectively) were used. Membrane BCRs were obtained from patient CLL cells by lysis, identified by western blot, semi-quantified and screened with phage libraries. The first strategy involved using patient VH4-34 BCRs which were captured using goat anti-human IgM to deplete bound phages. Unbound-phages were positively screened for those binding to patient VH 1-3 BCRs. Several clones were randomly selected and a sequence consisting of "LLPPAR_" peptide was found in both libraries. A phage clone displaying peptide "LLPPARE" was identified to bind to goat anti-human IgM. By including more goat anti-human IgM negative selections, we identified 3 different phages displaying peptides "GFTFMPA", "QSRPLLP" and "GLPCCSS". Clones "GFTFMPA" and "GLPCCSS" showed binding

to goat anti-human IgM, while “QSRPLLP” did not. Phage clone “QSRPLLP” showed no binding to human serum IgM but showed binding to both patients’ BCRs. “QSRPLLP” peptide binds a common BCR molecule region present in both patients but not present in human serum IgM. This data suggests that it is possible to use a peptide-phage display library to select peptides unique for the BCRs of CLL patients. However, the critical component in making this process patient-specific resides in enhanced discrimination in phage selection.

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SYMBOLS AND ABBREVIATIONS

7-mer …….. 7 amino acid long peptide

12-mer …….. 12 amino acid long peptide

Abs……. Absorbance

Amp…….Amplification

APC……....Allophycocyanin

BCR……B-cell receptor

BSA…….…..Bovine serum albumin

EDTA…….Ethylenediaminetetraacetic acid

Fc region……..……Fragment, Crystallizable region

FCS……...……Fetal calf serum

FITC……..…Fluorescein isothiocynate

FSC-H……..…….Foward Scatter-Height

HRP……..……Horseradish peroxidase

IgM…….Immunoglobulin M

IPTG…….Isopropyl β-D-1-thiogalactopyranoside

kDa……...kilo Dalton

mIgM……membrane-IgM

ml…….milliliter

ng…….nanograms

O.D……...…….Optical Density

PBMC…..…….Peripheral Blood Mononuclear Cell

PBS……..…Phosphate Buffered Solution

PE……Phycoerythrin

PE-Cy7……..……Phycoerythrin-Cyanine 7

pfu……..……Plaque forming unit

PVDF…….....Polyvinylidene fluoride

SDS-PAGE……..…Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SSC-H.……Side Scattered-Height

TBST…..……Tris-buffered saline (Tween-20)

TMB substrate……..……3,3’,5,5’-Tetramethylbenzine substrate

VH…….Variable Heavy

VL……...Variable Light

X-Gal……..5-bromo-4-chloro-indolyl-β-D-galactopyranoside

ACKNOWLEDGEMENT

I owe a great deal of thanks to my mentor and thesis advisor Dr. Osvaldo Lopez, my co-advisor Dr. Cheryl Conley, Dr. David Cool, my fellow research team members, staff of the department of Pathology and the department of Pharmacology and Toxicology. I will like to thank R. Raju, MD. and M. Owens, RN, OCN, CRC at Kettering Innovation Center, Kettering Hospital, Dayton OH.

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DEDICATION

I dedicate this work to my dear wife, my mother, family, and friends who have supported me throughout my life.

INTRODUCTION

B-Cell Chronic Lymphocytic Leukemia (CLL) is the most common adult leukemia in Western countries [1]. Most patients are asymptomatic or present with reduced tolerance to exercise and fatigue, autoimmune hemolytic anemia, and sometimes with lymphoadenopathy. The laboratory diagnostic hallmark is the presence of small monoclonal lymphocytosis (greater than 5,000/μL) with infiltration into bone-marrow and secondary lymphoid organs [2]. CLL cells express CD5, CD19, CD23, and low levels of surface immunoglobulin and CD79b (the Ig-coreceptor) [3]. The increase in cell numbers over time is a dynamic process between cell proliferation and apoptosis[4]. CLL cells do not secrete antibodies, resulting in hypogammagobulinemia, which contributes to susceptibility to major infections and accounts for about 50% of deaths [5-7].

CLL has a heterogeneous clinical course [8] which can be divided into three phases [9]: low risk stage patients with little or no clinical disease, intermediate-risk stage, with a period of inactivity of the disease and longer survival time (24.4 years after diagnosis), and high-risk stage patients with an aggressive course resulting in a decrease in quality of life and early death (median 9.7 years after diagnosis) [10]. Clinical stage systems were developed to describe the disease progression [11, 12]. A higher stage indicates worse prognosis.

There is currently no cure for CLL. The treatment for cases with poor prognosis of CLL is the use of alkylating agents, purine analogs, and anti-CD20 and anti-CD52 antibodies. The addition of humanized anti-CD52 or chimeric anti-CD20 to purine analogs showed a prolongation of progression free survival in relapsed or refractory patients [2]. These treatments can induce remission that can be consolidated in some cases with further therapy. The problems associated with these treatments are 1) increased immunosuppression causing an increase in major infections [13], and 2) selection of sub-clones with increased anti-apoptotic mutations [14]. In addition, the population with the more aggressive form of the disease will inevitably relapse in less than 4 years. Because of the adverse effects associated with therapy, physicians often use a "wait and see" strategy; follow the course of the disease, and treat patients at higher stages. Therefore, it is advised not to treat the patients in lower stages of the disease [15].

New therapies under investigation or entering clinical trials are also based on chemo- or immune-therapy but normal cells are also sensitive to these drugs [16]. Thus, these therapies are comparable to those currently in use in that they target an antigen shared by CLL and normal cells.

In the last ten years the prognosis of the disease had been improved. Up-regulation of molecules involved in activation such as CD38 [17] or Zap 70 [18] are used as surrogates of replication [15]. High-level expression of these genes correlates well with high-risk CLL but are not helpful to assess the prognosis at early stages [19].

Deletions in chromosomes 17 and 11 correlate with poor prognosis and refraction to therapy, while deletions in chromosome 13 correlate with good prognosis [20]. Most

patients developing genetic abnormalities have been treated before, suggesting a selection of these clones due to treatment [14]. Nevertheless, a retrospective study of patients without previous therapy showed a clonal evolution to genetic abnormalities with poor prognosis [21]: patients with high Zap 70 expression of unmutated variable heavy chain gene (U-VH) evolved to chromosome deletion 17 and 11. Interestingly, patients with mutated variable heavy chain gene (M-VH) evolved to deletion 13 (good prognosis).

The observation that some VH genes from CLL were unmutated while others were mutated [22] led to the study of the relation between mutated and non-mutated genes with the risk-level of CLL. Patients with less than 2% mutations in the VH (U-VH) are more likely to have a poor course of the disease compared to patients with more than 2% mutations (M-VH) [23]. Recently, in a study of almost 2,000 patients, it was shown that patients with unmutated VH and usage of a particular set of D-genes are more prone to high-risk disease [24]. VH-mutation status is the only intrinsic prognostic factor independent of the disease stage. Patients with U-VH are at higher risk of a major infection and death by infections than patients with M-VH genes [6]. This is due to a deregulation associated with high numbers of CLL cells (almost 90% of circulating lymphocytes) and a dramatic decrease in number and function of T-cells and NK-cells [25].

In a recently published retrospective study it was shown that virtually all patients with monoclonal B-cell lymphocytosis that will become CLL already have the leukemic clones in circulation from 3-5 years before a clinical diagnosis of CLL [26]. However, only a small percentage of patients progress to CLL. The reason for disease progression is not understood. The etiology of this B-cell population and the mechanism leading to

this leukemia are also unknown. Until recently, this leukemia was considered an accumulation of small naïve lymphocytes with a defect in apoptosis [27], but the findings of hypermutated clones plus the combination of expression of cell markers led researchers to postulate that CLL derives from innate-like "antigen experienced" lymphocytes derived from the human counterpart of the murine "innate-like" B-cell populations [28]. Cross-reaction with auto-antigens, environmental antigens and/ or infection may play a role in selecting B-cell clones that will become neoplastic in CLL cells [24, 29, 30]. A recent study of the VH sequences of 1,577 patients showed that CLL display a restricted repertoire of Ig gene rearrangements, adding more evidence to antigen selection and induction in the development of CLL cells [3].

Clinical and experimental evidence strongly suggest that autoantigens are involved in the maintenance of activation of the cells originating CLL. Autoimmunity is a well-known complication of CLL [31-33]. The majority of CLL cells are kappa light chain restricted and 25% of these react to an anti-idiotype anti-kappa antibody [34, 35]. Naturally occurring CD5+ B-cells, with a restricted expression of VH genes, resembled auto-antibody producing CLL cells [36]. The study of the antibodies secreted by CLL cells after induction with phorbol myristate acetate (PMA) showed high degree of autoreactivity to self antigens such as IgG and single and double stranded DNA [37]. Mutated VH genes from CLL cells were reverted *in vitro* to the unmutated germline and showed reactivity to autoantigens [38]. Around 10% of CLL cells use VH gene 1-69, of which around 80% do not have mutations, and present stereotyped usage of CDR3s, indicating similar binding properties [39]. Hepatitis C virus drives the proliferation of "innate-like" B-cell clones using VH1-69 with polyreactive properties [40]. Using

molecular modeling of several CLL antibodies, it was shown that they might recognize the same antigen [41, 42]. Innate-like B-cells can receive help directly from dendritic cells [43] or from NKT-cells through the CD1 molecule [44] without need of T-cell help.

Thus, it is becoming clear that innate-like B lymphocytes are activated by an environmental agent (e.g. viral infection) and continue proliferating by engaging autoantigens through their B-cell receptors (BCRs). Those CLL cells that do not mutate their VH might receive more stimulation by autoantigens, and thus have higher proliferation rate, which increases the risk for poor prognosis. The combined results suggest that the BCR is a possible therapeutic target against CLL.

The BCRs are transmembrane proteins located on the outer surface of a B-cell and are involved in antigen recognition. BCRs are tetramers made of two heavy and two light chains. The light chain consists of two domains, the constant and variable domains. Each heavy chain consists of four constant and one variable domains in the IgM isotype and three constant and one variable domain in the IgD isotypes. Both isotypes are expressed on the cell membrane of a B cell carrying the same heavy and light variable domains with either IgM or IgD constant regions. Each BCR has two antigen binding sites formed by the hypervariable regions of the variable heavy (VH) and variable light chains (VL). After the B cell is activated, it differentiates into a plasma cell and the BCR is secreted as an IgM antibody. Indeed, the BCR of CLL cells was targeted in the past. Anti-idiotype antibodies were popular and used often in the 1980s. For example, an anti-idiotype vaccine was developed against Hepatitis B [45]. Anti-idiotype antibodies were used as therapy against CLL cells [46, 47] . A partial remission was reported in a patient receiving therapy for one year, however, subsequent resistance to therapy occurred [48].

Based on the published data, it is not clear whether the resistance was due to antigen modulation of the idiotype on the BCR or an immune response against the anti-idiotype (a mouse antibody). These antibodies were not conjugated to toxins or radionuclides, which suggests that the mechanism of clearance of the CLL cells was immune-mediated. New technologies allow for the successful use of variable fragments (Fv) of antibodies conjugated to toxins against molecules on the cell membrane of leukemias and lymphomas [49].

Anti-idiotype therapy is not currently used because the technique to produce and screen anti-idiotype antibodies is laborious, and the target for the anti-idiotype antibody was not well characterized, the anti-mouse response was problematic, and the role of the network of anti-idiotype-idiotype complexes was not defined.

Another strategy to target the BCR of CLL cells is to discover ligands with high affinity to the BCR. For example, a powerful technique was developed by Dr. G. Smith's lab at U. of Missouri using peptides displayed on M13 bacteriophages and encoded in the phagemids [50]. Many peptides obtained with phage display technology are used in imaging and as prospective for anti-cancer applications [51]. This strategy also has application for targeting BCRs of CLL cells. These authors selected nine-mer peptides specific to the BCR of CLL cells from a patient. Using flow cytometry, they demonstrate that these phages bound to the patient's CLL cells with intensities more than 10 times higher compared to phage binding to CLL cells from other patients. The authors of this work did not characterize the VH usage of the patients, the binding affinities of the peptides to the BCR, the binding capabilities of the peptides against normal B-cells, nor

the CLL cell killing properties of the peptide [52]. However, this preliminary work shows that it is possible to select for peptides with specificity to the BCR of CLL cells.

Based on new research demonstrating that unmutated VHs BCRs use similar rearrangements which seem to recognize the same antigens, and prior work indicating that BCRs can be used as a target for therapy, we targeted the BCRs of CLL cells with unmutated VHs using peptides mimicking the antigen binding to the binding pocket. By screening a second phage display library around the motif found in the first selection, this process resembles the mechanism of affinity maturation occurring with BCRs in the germinal center, but in this case, we selected peptides with the highest affinity for the BCR of the CLL cells.

HYPOTHESIS:

Using a 7-mer or 12-mer Phage Display Peptide Library, it is possible to obtain peptides binding specifically to the binding pocket of B-cell receptor of CLL.

RATIONALE AND SIGNIFICANCE OF STUDY

The approach of targeting the BCR has the great advantage of low toxicity because only the CLL cells are marked for destruction. On the other hand, this strategy cannot be used as a generic therapy since BCRs differ from one CLL patient to the other. Until recently, the policy of the pharmaceutical industry was to have one medicine per disease; however clinical evidence, corroborated by genetic evidence indicated that this strategy is not optimum. Thus, the strategy of "one fits all" is under scrutiny by the scientific community and the pharmaceutical industry [53, 54], as the ability to genotype patients has become common and provides a basis for individualized therapy [55]. At this time, it is not advised to make therapeutic decisions based on the rate of VH mutation because of the drawbacks of the current therapy [2]. A therapy specifically targeting the CLL cell with low toxicity could be used soon after diagnosis with CLL patients with poor prognosis, and will have a significant social impact on many patients with CLL before progression to higher stages.

CHAPTER 1: Characterization of cells from CLL patients

1.1 Materials /Methods:

Subjects

Peripheral blood sample from healthy subjects and two untreated individuals diagnosed with CLL were investigated in this study. In all cases, patients provided informed consent for use of peripheral blood in this investigation.

Peripheral blood mononuclear cells isolation

Peripheral blood from CLL patients and healthy subjects was obtained using EDTA tubes and stored at 4^0C. In a biosafety cabinet, the patient blood was diluted 1:2 with 2mM EDTA in HBSS pH 7.4 in a sterile 15 mL conical tube. Six milliliters of diluted blood was gently layered on top of 2 mL Histopaque 1077 (Sigma-Aldrich). Each 15 mL conical tube was then centrifuged (with centrifuge brake switched "OFF") at 2000 RPM for 20 minutes at room temperature. The peripheral blood mononuclear cell (PBMC) buffy coat layer was collected using a Pasteur pipette, and added to cold 10 mL 1% BSA, 2mM EDTA in HBSS pH 7.4. The cells were washed 3X with 10 mL 1% BSA, 2mM EDTA in HBSS pH 7.4 at 1000 RPM for 5 minutes. After the final rinse, the cells were resuspended in 5 mL 1% BSA, 2mM EDTA in HBSS pH 7.4 and counted. For cell quantification, 10 microliters of cell suspension was added to 80 microliters of HBSS pH 7.4 and an additional 10 microliters of trypan blue (Invitrogen

Cat. No 15250-061) was added to give a 1:10 dilution. Cells were quantified using a hemocytometer and a light microscope [56]:

Cryo-preservation of isolated PBMCs from CLL patients

CLL peripheral blood mononuclear cells were frozen in 1.5 mL cryovials at a concentration of 1-2 x 10^7 cells per vial in 2% DMSO, 10% FCS in RPMI. Cryovials were stored at -70^0 C overnight and then transferred to liquid nitrogen.

Freezing of CLL cells in Tri-Reagent

Obtained CLL PBMCs were pelleted at 800 xg and resuspended in TE pH 8.0 (IDTE lot no. 11-05-01-10) to a concentration of 5x10^6 cells per 250 microliters. The cell suspension, 250 microliters, was aliquoted into 1.5 mL cryovials followed by addition of 750 microliters of Tri Reagent (Molecular Research Center, Inc. Cat No. TR118). Tryzol samples were kept frozen at -70^0C.

RNA isolation from PBMCs

Frozen 1.5 mL cryovial Tryzol sample was quickly thawed and incubated at room temperature for 5 minutes. After 5 minutes incubation, 1 mL Tryzol sample was transferred into a 1.5 mL microcentrifuge tube with the addition of 250 microliters of chloroform. Tube was capped and vigorously shaken for 15 seconds and incubated for 5 minutes at room temperature. Sample was then centrifuged at 12000 RPM for 15 minutes at 4^0C. Using RNase-free tips, the upper aqueous layer was carefully transferred into a newly labeled 1.5 mL microcentrifuge tube containing 700 microliters of isopropanol and incubated for 10 minutes at room temperature. After 10 minutes of incubation, the sample

was centrifuged at 12000 RPM to pellet RNA. Pelleted RNA was washed with 750 microliters of 75% ice-cold ethanol at 7500 RPM for 5 minutes at 4^0. RNA pellet was air-dried and dissolved in 20 microliters of RNase-free water (GIBCO ultra pure) containing 1 microliter of rRNasin (Promega N251A). RNA concentration was determined using a spectrophotometer (Jenway Genova) at excitation wavelengths of 260 nm / 280 nm. RNA concentration was calculated using the formula below[56]:

[RNA] = Reading at 260 nm x dilution factor x 40 micrograms/ microliter

Reverse-transcription on RNA from patients "P" and "E"

RT-PCR tube was loaded with 3 micrograms of RNA, 1 microliter 7.5 micromolar IgM 342-356 reverse primer (Table 1a), 4 microliters of 2mM dNTPs (NEB # N0447S) and DNase/ RNase-free distilled water (GIBCO Ultra pure), to bring to a final volume of 16 microliters. Tube was incubated at 65^0C for 5 minutes, placed on ice for 2 minutes and briefly centrifuged for 5 seconds. After centrifugation, tube received 1 microliter rRNasin (Promega N251A), 2 microliters M-MuLV Transcriptase Reaction buffer (M-MuLV 10X buffer, NEB Cat. No. B0253S) and 1 microliter M-MuLV Reverse transcriptase (RT-M-MuLV, NEB Cat. No. M0253S). Tube was incubated at 42^0C for 60 minutes and then incubated at 65^0C for 20 minutes. Obtained cDNA was stored at -20^0C.

PCR on cDNA from patients "P" and "E" using Variable Heavy chain primers

Five PCR tubes were labeled P-1-M, P-2-M, E-1-M, E-2-M and Control. Each PCR tube received 22 microliters of DNase/ RNase-free distilled water (GIBCO Ultra pure), 5 microliters of $Vent_R$ (10x) ThermoPol Reaction buffer (NEB Cat. No. B9004S, lot No. 0010906), 5 microliters of 2mM dNTPs (NEB Cat. No. N0447 S) and 3

microliters of 15 mM IgM 336-356 primer (Table 1b). Tubes P-1-M, E-1-M and Control each received 9 microliters of "primer cocktail 1"containing 3 microliters of each of the following primers: VH_1FR_1, VH_2FR_1, and VH_3FR_1 (Table 1c). Tubes P-2-M and E-2-M each received 9 microliters of "primer cocktail 2"containing 3 microliters of each of the following primers: VH_4FR_1, VH_5FR_1, and VH_6FR_1 (Table 1c). Tubes P-1-M and P-2-M received 5 microliters of cDNA from patient "P", while tubes E-1-M and E-2-M received cDNA from Patient "E". The samples were heated for 2 minutes at 94^0C in a PCR cycler (Labnet Multi Gene II) and immediately place on ice for 2 minutes and briefly centrifuged for 5 seconds. Each tube, including the Control tube received 1 microliter of $Vent_R$ DNA Polymerase M0254S (NEB lot No. 0310906). PCR tubes were run in a PCR cycler (Labnet Multi Gene II) for 35 cycles with a 45 second denaturation at 94^0C, 45 second annealing at 60^0C, and 1 minute extension at 72 ^{0}C.

Table 1a. IgM Reverse primer used in reverse transcription for cDNA production

Reverse Primer	**Primer sequence**
IgM-REV 342-356	5'-CAG GAC AAA GTG ATG-3'

Table 1b. **IgM Reverse primer sequence for sequencing of PCR products**

Reverse Primer	**Primer sequence**
IgM-REV 336-356	5'-CAG GAC AAA GTG ATG GAG TCG-3'

Table 1c. Primer sequences for variable heavy chain gene families

IGH	Primer sequence
VH_1FR_1 (1-2) (-252)	5'- GGCCTCAGTGAAGGTCTCCTGCAAG- 3'
VH_2FR_1 (2-5) (-284)	5'- GTCTGGTCCTACGCTGGTGAAACCC- 3'
VH_3FR_1 (3-7) (-256)	5'- CTGGGGGGTCCCTGAGACTCTCCTG- 3'
VH_4FR_1 (4-4) (-256)	5'- CTTCGGAGACCCTGTCCCTCACCTG- 3'
VH_5FR_1 (5-51) (-255)	5'- CGGGGAGTCTCTGAAGATCTCCTGT- 3'
VH_6FR_1 (6-1) (-263)	5'- TCGCAGACCCTCTCACTCACCTGTG- 3'

Electrophoresis of Variable Heavy chain VH FR_1 PCR products

Ten microliters of each PCR product (P-1-M, P-2-M, E-1-M, E-2-M and Control) was added to 2 microliters of Loading Dye (NEB Cat. No B7021S) and separated using a 1.8% Agarose gel (Nusieve 3:1 Agarose) in 40 ml of TBE (GIBCO BRL Ultra Pure Cat No. 15546-013) with 2 microliters of Ethidium Bromide in TBE at 90 mV. Seven microliters of 100bp DNA ladder (Promega Cat. No. G210A) was added to 3 microliters of TE pH 8.0 (IDTE lot no. 11-05-01-10) and 2 microliters of Loading Dye (NEB Cat. No B7021S) and run along the PCR samples. DNA fragments of approximately 500bp were excised from the agarose gel and extracted using GeneJET ™ gel extraction kit (Fermentas Cat. No. K0691, Lot No. 00057032) and each resuspended in 50 microliters

of Elution Buffer. Each extracted DNA fragment was added to reverse primer IgM 336-356 for sequencing at Retrogen, Inc (San Diego, CA).

Flow cytometry

Isolated PBMCs from healthy donor, CLL patients "E" and "P" were divided into 1.5 ml microcentrifuge tubes, with a total amount of 1 x 10^5 cells used per reaction tube, for subsequent staining with fluorophore conjugated antibodies. The cells were pelleted at 2000RPM for 5 minutes at 4^0C and resuspended in 100 microliters of one of the following antibody dilution cocktails in HBSS 1%BSA, 2mM EDTA: **Group 1** cocktail of anti-CD19 APC, anti-CD5 PE-Cy7, anti-Lambda PE, anti-Kappa FITC. **Group 2** coctail of anti-CD19 APC, anti-CD5 PE-Cy7 and anti-IgM biotin with streptavidin FITC. **Group 3** cocktail of mouse IgG isotype controls. The cells were incubated in the dark on ice for 30 minutes with gentle agitation every 10 minutes. After a 30 minute incubation period, each sample was washed 3 times with 500 microliters of HBSS 1%BSA, 2mM EDTA. After the final rinse, each cell pellet was resuspended in 200 microliters of HBSS containing 1% paraformaldehyde and stored at 4^0C in darkness. Samples were run on an Accuri-C6 flow cytometer and analyzed using Accuri C-6 CFLOW PLUS software.

1.2 Results:

Peripheral blood mononuclear cells isolation and RT-PCR analysis on CLL cells mRNA

Peripheral blood mononuclear cells were isolated from a healthy volunteer, CLL patients "E" and "P" by density gradient centrifugation as shown in Figure 1.

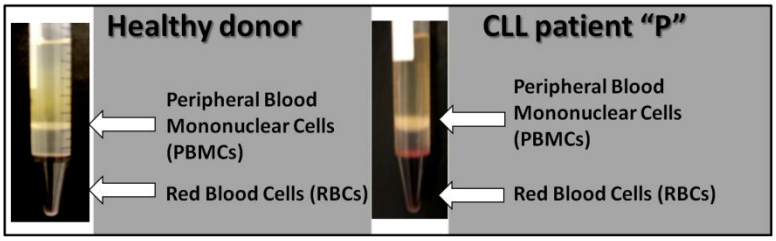


Figure 1. PBMCs separation from CLL patient "P" and healthy volunteer peripheral blood, using Histopaque 1077.

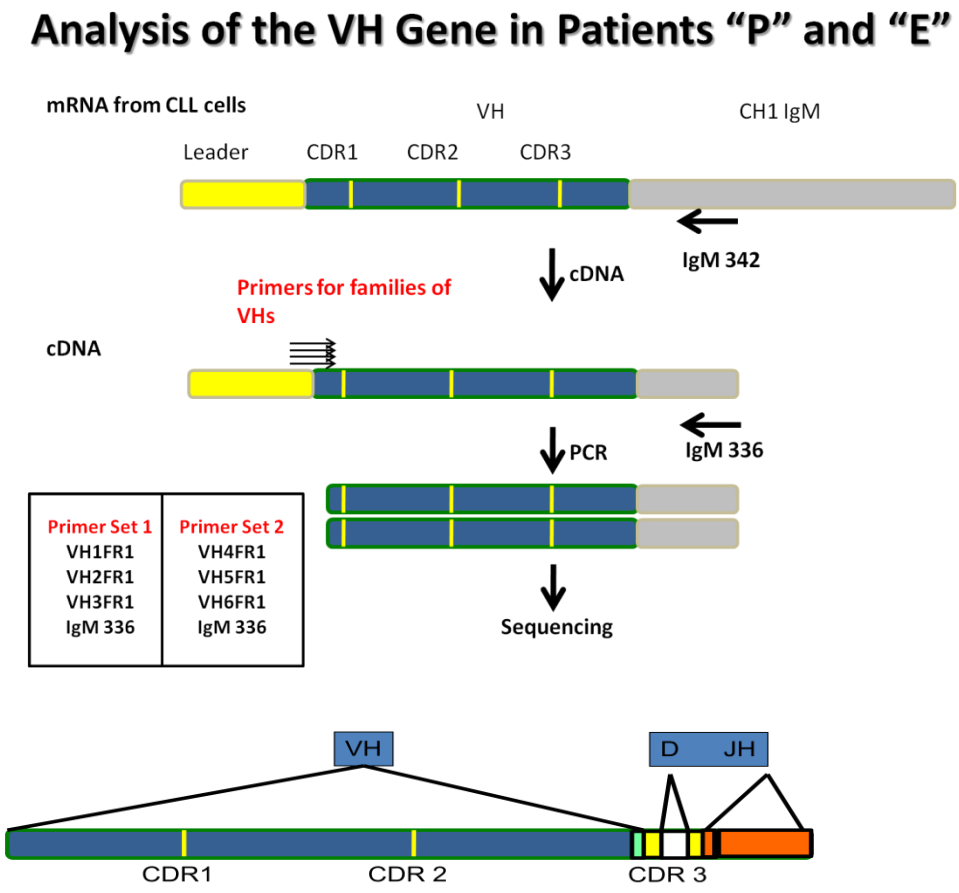
As shown in Table 2, CLL patients "P" and "E" total cell counts were greater than 5 x 10^6 PBMCs per milliliter. Patient "P" had 1.5 x 10^8 PBMC per milliliter of blood, while highest count for CLL patient "E" was 3.3 x 10^7 cells per milliliter of blood.

Table 2. PBMC counts of healthy volunteer, CLL patients "P" and "E".

Patient	PBMCs per mL of Blood (x10^7 cells)
Healthy donor	0.5
Patient "P"	15
Patient "E"	3.3

RT-PCR on RNA isolated from CLL PBMCs

Frozen 1.5 ml cryovial Tryzol samples from CLL patients "E" and "P" were quickly thawed and incubated at room temperature for 5 minutes. RT-PCR was performed on CLL patients "E" and "P" Tryzol samples (Figure 2), using the procedure in "Materials and Methods" and reverse-transcribed using IgM 342-356 reverse primer (Table 1a). Obtained cDNA from patients "P" and "E" were PCR-amplified using Variable Heavy Chain Forward primers (Table 1c) and IgM 336-356 reverse primer (Table 1b).



<u>Figure 2</u>. Schematic Representation of RT-PCR Amplification of cDNA using Variable Heavy Chain Framework 1 (VH FR) Gene Primers.

Using a 1.8% Agarose gel (Nusieve 3:1 Agarose), the CLL patients Variable Heavy Chain PCR products were separated (Figure 3), excised, extracted and purified using GeneJET ™ gel extraction kit (Fermentas Cat. No. K0691, Lot No. 00057032).

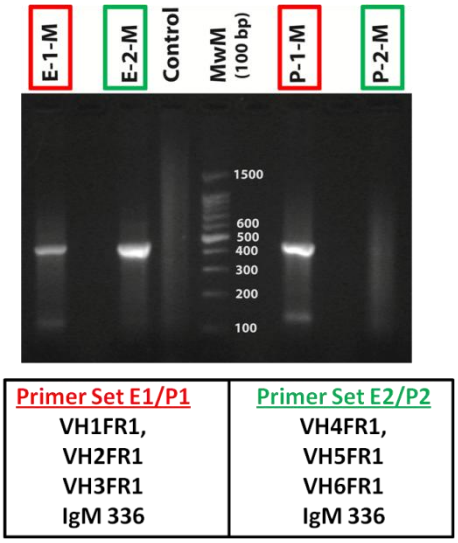
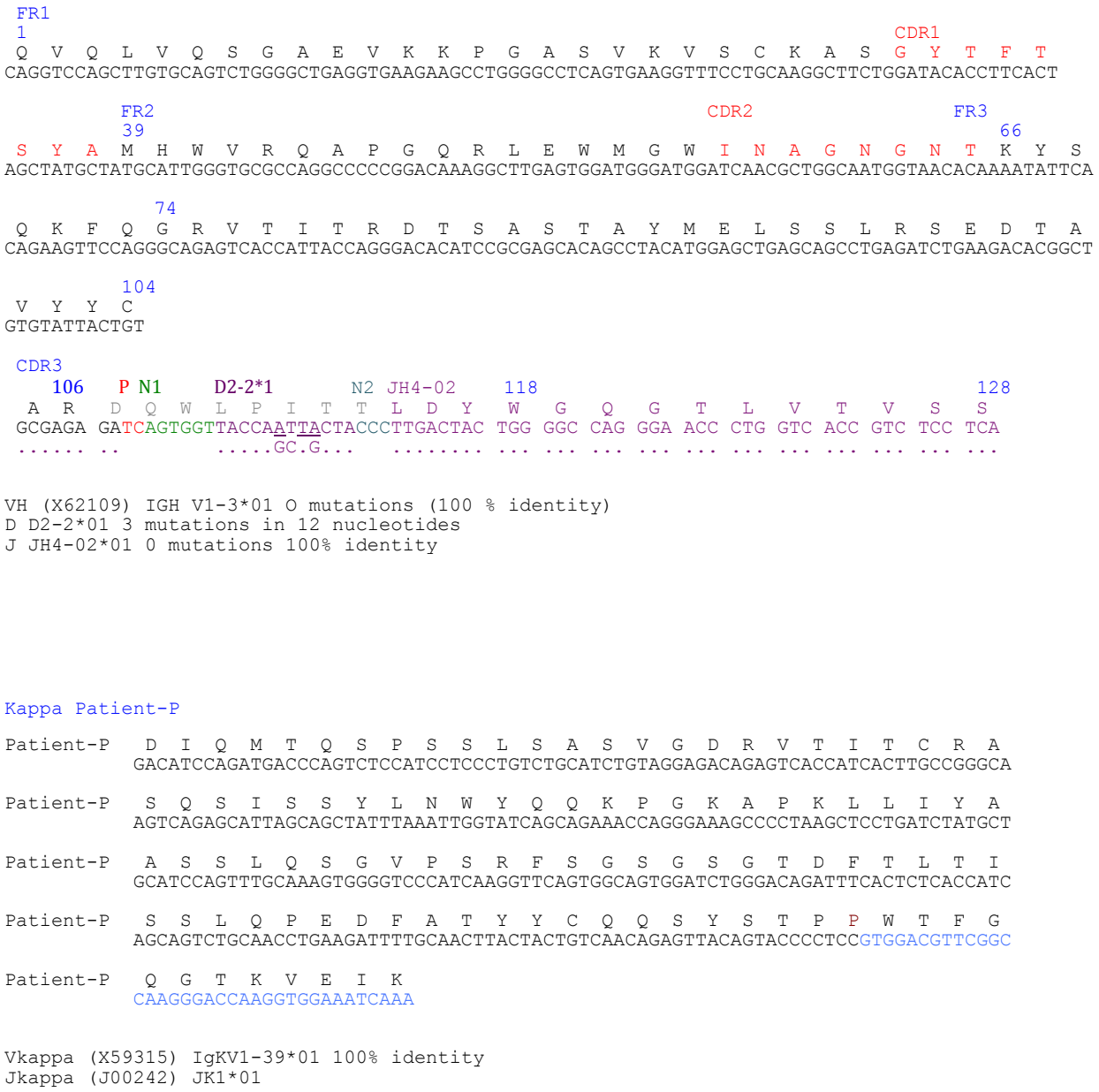


Figure 3. DNA Gel Electrophoresis of RT-PCR IGH Gene Products. VH_1FR_1 (1-2) (-252), VH_2FR_1 (2-5) (-284), VH_3FR_1 (3-7) (-256), VH_4FR_1 (4-4) (-256), VH_5FR_1 (5-51) (-255), VH_6FR_1 (6-1) (-263) primers and IgM-REV 336-356 primers were used the the RT-PCR. Notation: "E-1-M" (CLL Patient "E"-Primer set 1- Reverse Primer IgM 336).

Each extracted DNA fragment was added to reverse primer IgM 336-356 and sent for sequencing at Retrogen, Inc (San Diego, CA). Analysis showed CLL Patient "P" used VH 1-3*01with no mutations (100 percent identical), D2-2*01 with 3 mutations out of 12 nucleotides and JH4-02*01 with no mutations (100 percent identical) (Figure 4). Analysis of Variable Light Chain showed that CLL patient "P" used VK1-39*01 with no mutations (100 percent identical) and JK1*01. CLL patient "E" used VH4-34*01 with 2 mutations out of 285 nucleotides (99.30 percent identical), a possible D2-2*01 with 4 mutations out of 16 nucleotides and JH6-02*01 with 1 mutation out of 58 nucleotides (Figure 5). Analysis of Variable Light Chain showed that CLL patient "E" used a VK5*02.

Patient "P"



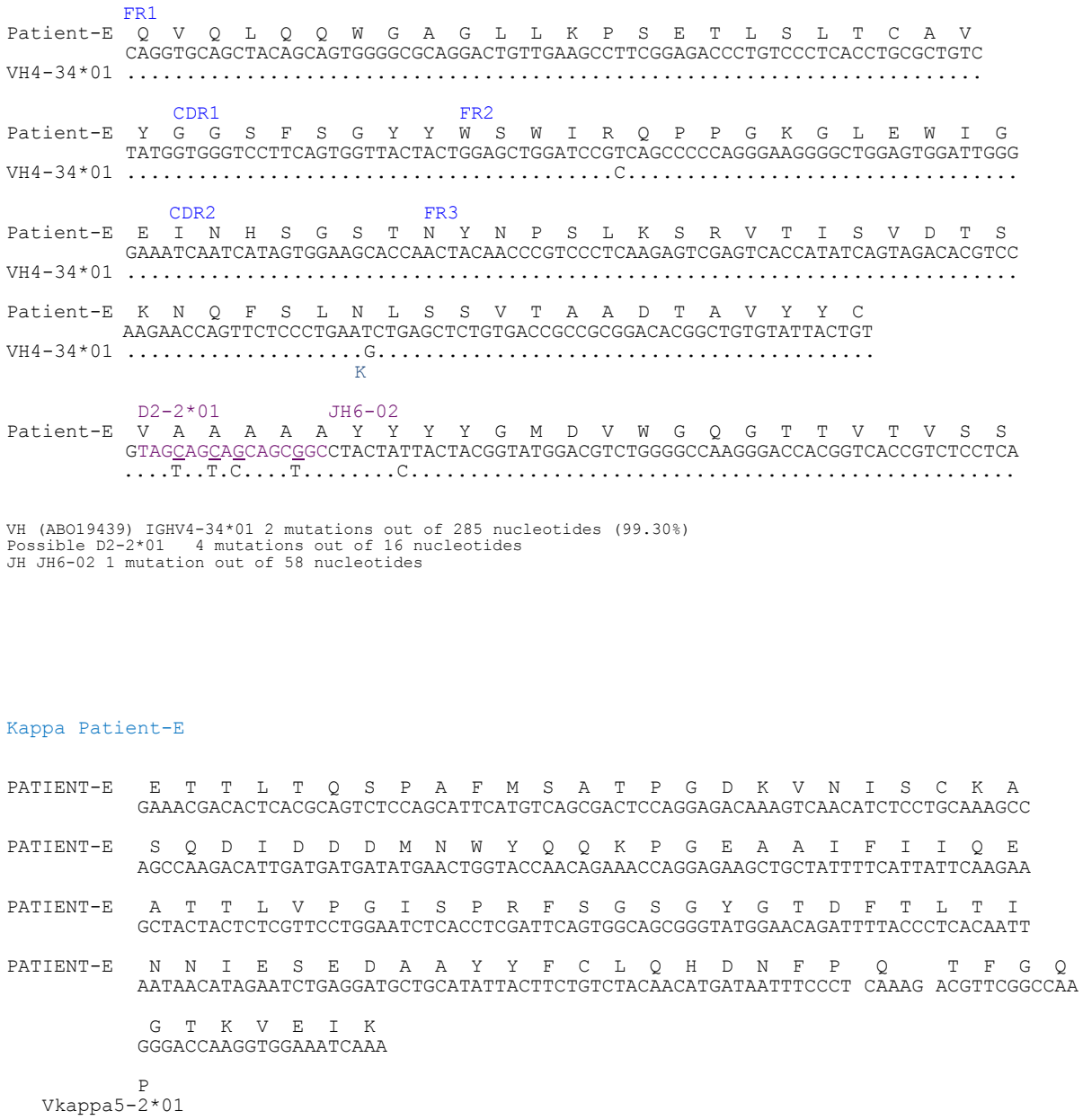
VH (X62109) IGH V1-3*01 0 mutations (100 % identity)
D D2-2*01 3 mutations in 12 nucleotides
J JH4-02*01 0 mutations 100% identity

Kappa Patient-P

Vkappa (X59315) IgKV1-39*01 100% identity
Jkappa (J00242) JK1*01

<u>Figure 4.</u> Variable Heavy and Light Chains Sequence Analysis of CLL Patient "P". RT-PCR products were sequenced and the germline usage was analyzed.

Patient “E”



VH (ABO19439) IGHV4-34*01 2 mutations out of 285 nucleotides (99.30%)
Possible D2-2*01 4 mutations out of 16 nucleotides
JH JH6-02 1 mutation out of 58 nucleotides

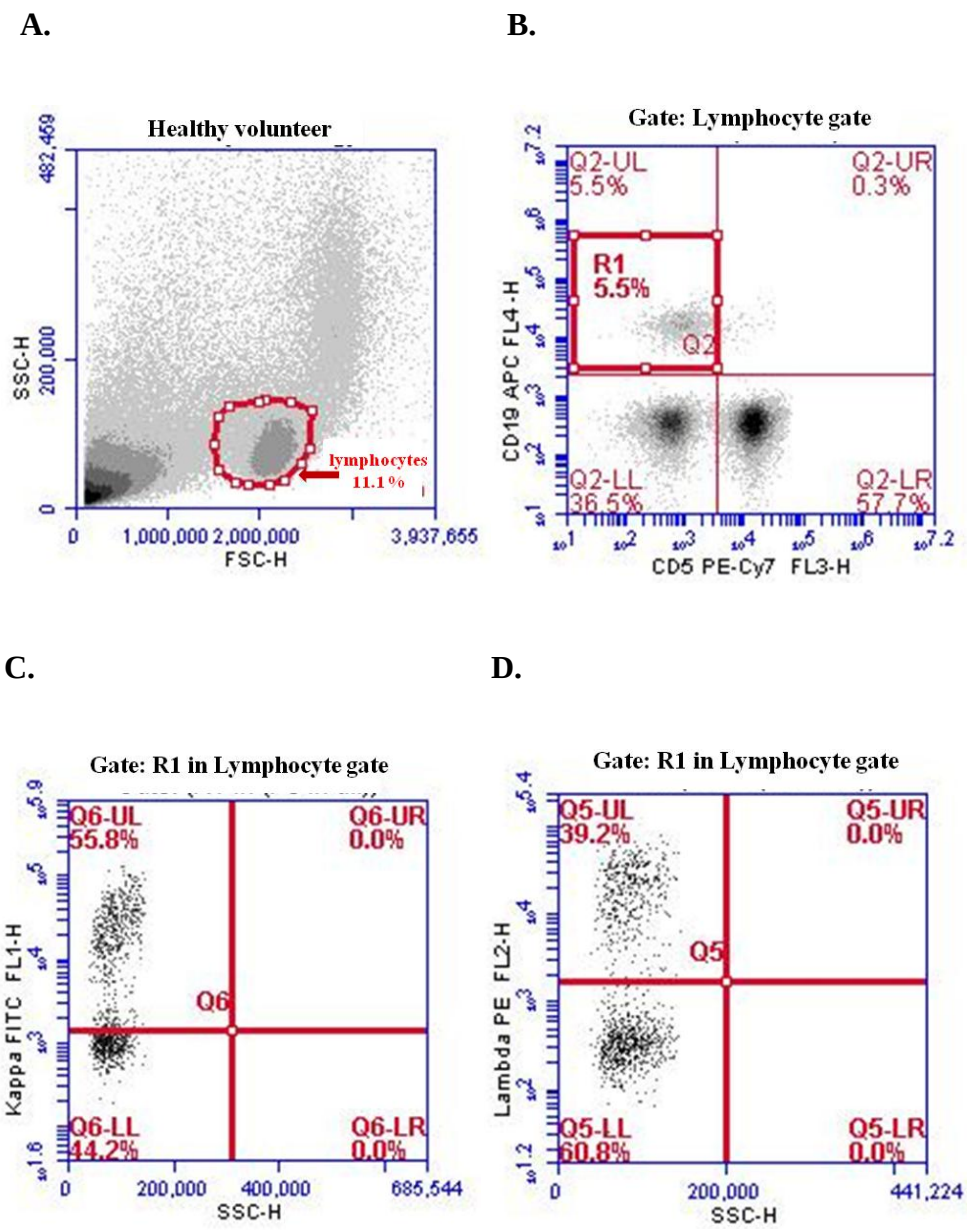
<u>Figure 5.</u> Variable Heavy and Light Chains Sequence Analysis of CLL Patient “E”. RT-PCR products were sequenced and the germline usage was analyzed.

Flow cytometry analysis

In order to characterize patients “E” and “P” B-cells, immunophenotying was performed on isloated PBMCs. Isolated PBMCs from a healthy donor, and cells from CLL patients “E” and “P” were stained with the following antibody dilution: Group 1 cocktail of anti-human CD19 APC, anti-human CD5 PE-Cy7, anti-human lambda PE,and or anti-human kappa FITC, Group 2 cocktail of anti-human CD19 APC, anti-human CD5 PE-Cy7 and anti-human IgM biotin with streptavidin FITC, Group 3 cocktail of mouse IgG isotype controls, pelleted and resuspended in 200 microliters of HBSS containing 1% paraformaldehyde.

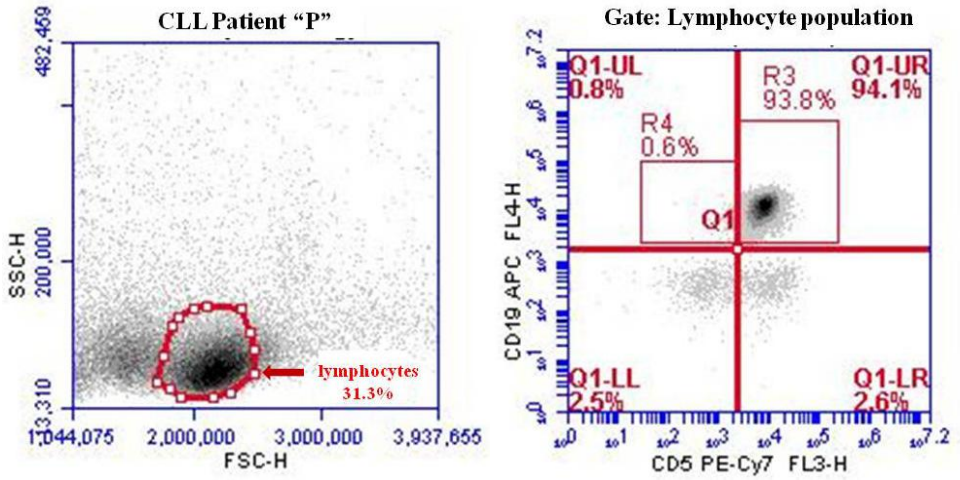
Group 1 samples (mouse anti-human CD19 APC, CD5 PE-Cy7, lambda PE,and kappa FITC stained PBMCs) were then run on an Accuri-C6 flow cytometer and analyzed using Accuri C-6 CFLOW PLUS. As shown in Figure 6, by gating on the “lymphocyte” population within the healthy volunteer sample, the CD19 and CD5 positive population within the “lymphocyte” gate was 5.5% with a kappa to lambda ratio of 3:2 (55.8% kappa positive to 39.2% lambda positive).

By gating on the lymphocyte population within the sample, the percent of $CD5^+$ $CD19^+$ cells in patient “P” was 94.1% of which 89.9% of the cells were positive for kappa light chain (Figure 7). The $CD5^+$ $CD19^+$ within patient “E” was 88.9% of which 65.2% of the cells were positive for kappa light chain and 26.1% positive for Lambda light chain (Figure 8).

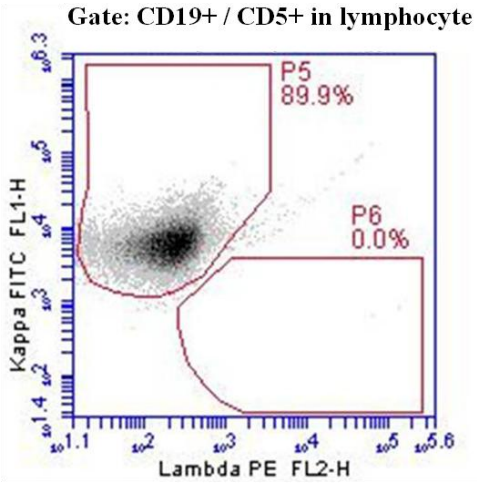


<u>Figure 6.</u> Flow Cytometry Analysis of PBMCs from Healthy Donor Peripheral Blood. Cells were stained with anti-human CD19-APC, CD5 Pe-Cy7, anti-kappa FITC and anti-lambda PE. ***A.*** Diagram shows the "lymphocyte" gate on a side scatter (SSC-H) versus forward scatter (FSC-H) plot. ***B.*** The diagram shows the CD19 positive B-cells (R1) on a density plot of CD5 Pe-Cy7 versus CD19-APC positive stained cells within "lymphocyte gate". ***C.*** Graph shows a density plot of B-cells kappa light chain usage. ***D.*** Graph shows a density plot of B-cells lambda PE light chain usuage.

A. **B.**

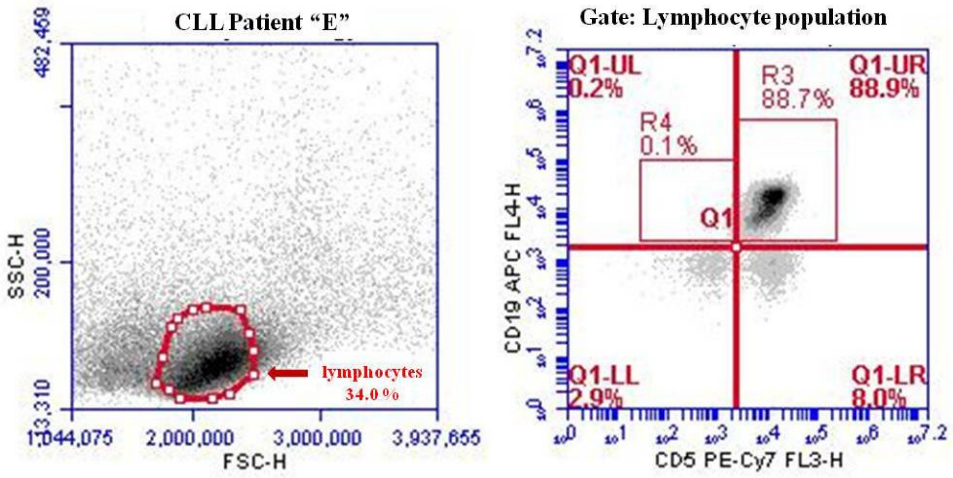


C.

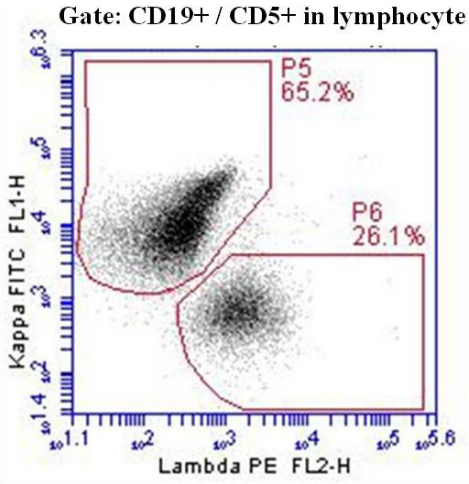


<u>Figure 7.</u> Flow Cytometry Analysis of PBMCs from CLL Patient "P" Peripheral Blood. Cells from CLL patient were stained with anti-human CD19-APC, CD5 Pe-Cy7, anti-kappa FITC and anti-lambda PE. ***A.*** The diagram is the side scatter (SSC-A) versus forward scatter (FSC-A) plot with a lymphocyte gate. ***B.*** The diagram is a density plot of CD5 Pe-Cy7 versus CD19-APC positive stained cells gated on the "lymphocyte gate". ***C.*** Diagram is a density plot showing analysis of CD19+/CD5+ cells from CLL patient light chain usage after staining with anti-kappa FITC and anti-lambda PE .

A. **B.**

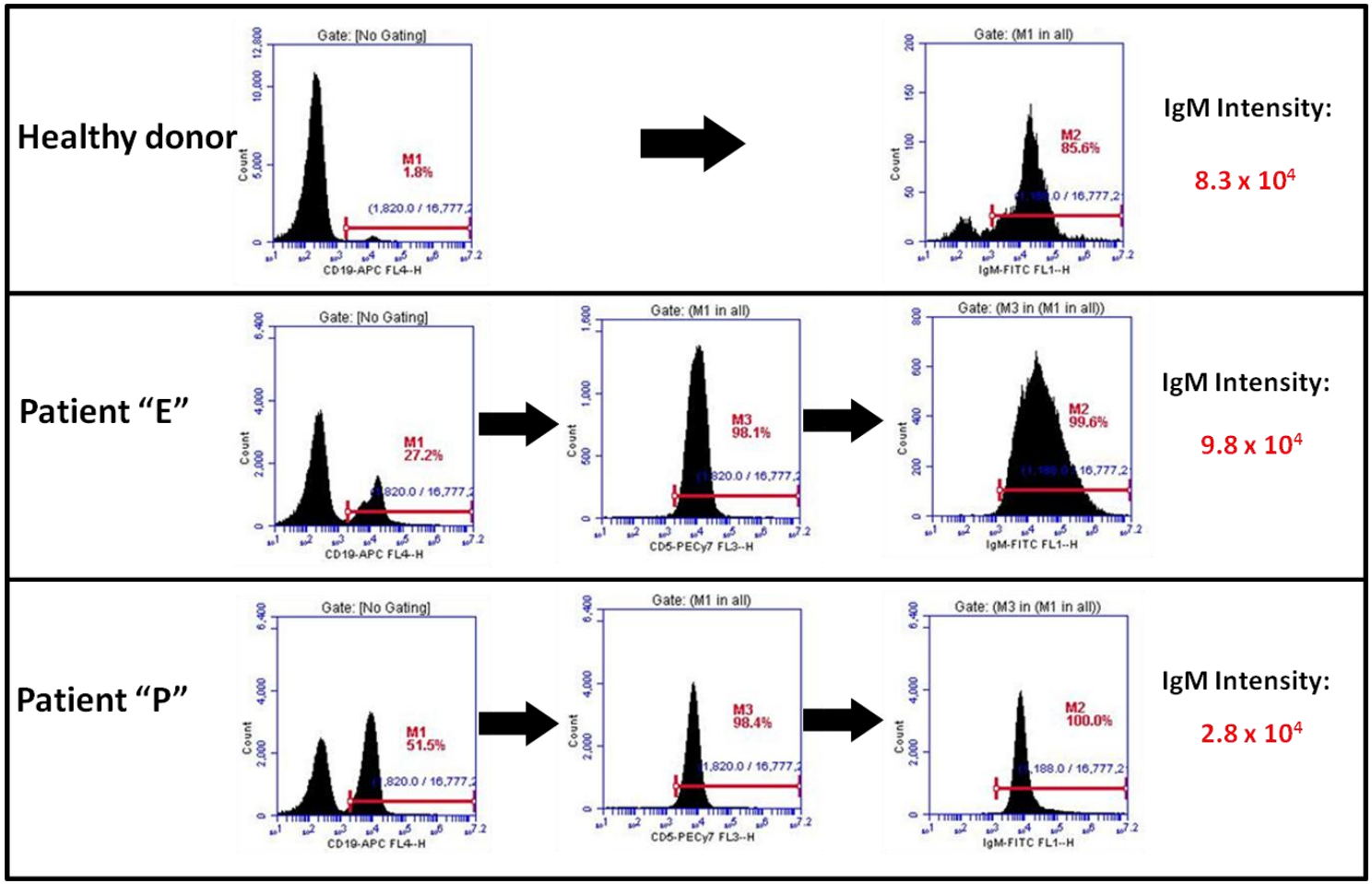


C.



<u>Figure 8.</u> Flow Cytometry Analysis of PBMCs from CLL Patient "E" Peripheral Blood. Cells from CLL patient were stained with anti-human CD19-APC, CD5 Pe-Cy7, anti-kappa FITC and anti-lambda PE. ***A.*** Diagram is the side scatter (SSC-A) versus forward scatter (FSC-A) plot with a lymphocyte gate. ***B.*** The diagram is a density plot of CD5 Pe-Cy7 versus CD19-APC positive stained cells in a "lymphocyte gate". ***C.*** Diagram is a density plot showing analysis of CD19+/CD5+ cells from CLL patient light chain usage after staining with anti-kappa FITC and anti-lambda PE.

To compare cell surface IgM expression on B-cells, **Group 2** samples (mouse anti-human CD19 APC, CD5 PE-Cy7, IgM biotin with streptavidin FITC stained PBMCs) were analyzed using Accuri C-6 CFLOW PLUS. As shown in Figure 9, healthy volunteer B-cells were identified by using CD19 from which marker "M1" (CD19 positive population) was applied to the IgM plot to determine the mean IgM fluorescence intensity "M2". Mean IgM fluorescence intensity "M2" of CLL cells in patients "E" and "P" was determined by gating on the "M3" (CD5 positive cells) within "M1" (CD19 positive cells) on the IgM histogram plot. The mean IgM fluorescence intensities for the healthy volunteer, and CLL patients "E" and "P" were "8.3×10^4 ", "9.8×10^4" and "2.8×10^4", respectively, indicating that CLL patient "P" had much lower expression of IgM on $CD5^+$ $CD19^+$ B-cells and probably at late stage CLL, while patient "E" IgM expression was similar to the healthy donor B-cells and is probably at early stage of the disease.



<u>Figure 9.</u> Flow CytometryAanalysis of Cell Surface IgM Expression on Healthy Donor B-cell and CLL Patients "P" and "E" B-cells. Peripheral blood PMBCs were stained with mouse anti- human CD19, CD5 and IgM antibodies. B-cells were identified using CD19 expression and CLL cells identified with co-expression of CD5 and CD19.

CHAPTER 2: Semi-quantification of isolated BCRs from patients

2.1 Materials /Methods:

Before patient BCRs could be targeted using the phage display library, the isolated PMBCs from each patient had to be lysed, using a mild detergent to release all surface proteins including the BCRs without denaturing them, and total protein concentration determined and patient BCRs semi-quantified, as explain below:

Protein extraction from isolated CLL patient PBMCs

Patient PBMCs, 2 x 10^7 cells, were lysed overnight in 0.9 mL containing TBS, 1% Tween-20, 10 µl of 0.5 M EDTA and 20 µl of HALT Protease Inhibitor Cocktail (Thermo Scientific, Cat. No. 78430, Lot No. KG134034). Tubes were centrifuged at 14,000 RPM for 30 seconds, protein supernate transferred into microcentrifuge tubes and the pellet discarded. A solution was prepared with 4.5 mL of TBS plus 90 µl of HALT buffer and 45 µl of 0.5 M EDTA of which 0.9 mL of this solution was added to the 300 µl of the protein supernatant and 250 µl aliquoted per tube and stored at -70^0C.

Total protein quantification of CLL patient PBMC cell extracts

Two hundred microliters of protein assay dye reagent concentrate (Bio-Rad, Cat. No.500-0006) was added to dilutions of known concentration of BSA standard to make the following final concentrations in dd: 0.5 µg/ ml, 1 µg/ ml, 2 µg/ ml, 5 µg/ ml, 7

μg/ ml and 10 μg/ ml. Patient "P" and "E" samples were diluted in 0.8 ml distilled μl protein assay dye reagent concentrate. Two hundred microliters of dilutions were transferred into a well on a 96-well plate (Costar). The plate was read at 595 nm using Elx 800 biotek plate reader. A standard curve obtained from the BSA standard and the optical densities obtained for Patients "P" and "E" wells were used to extrapolate the concentrations of patients "P" and "E".

SDS-PAGE and western blot analysis of CLL patient protein lysates

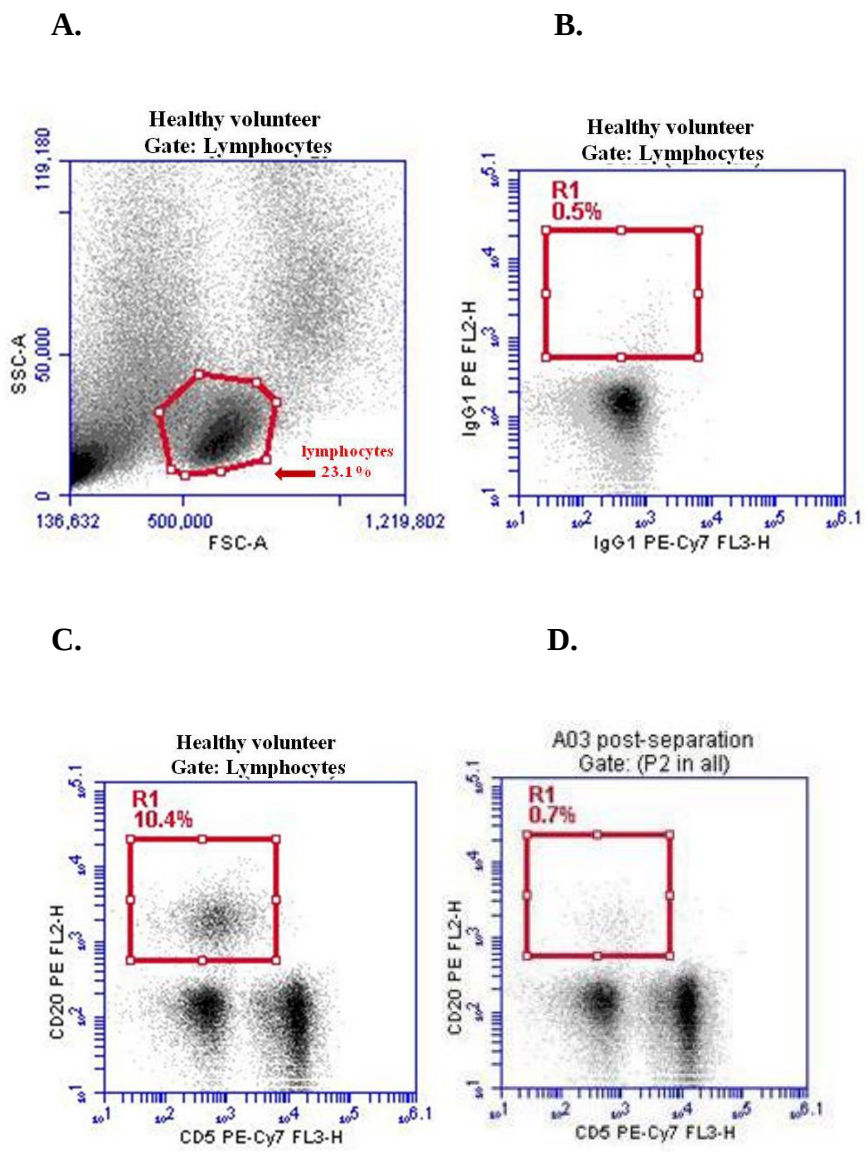
SDS-PAGE and Western blot analysis were then performed on patients "E" and "P" cell lysates, as mentioned below to determine if complete BCRs, based on size and staining with goat anti-human IgM, could be identified. In determining the presence of BCRs within cell lysates, 0.2 micrograms and 0.02 micrograms of each patient's lysate, and standard human serum IgM were each diluted in a sample loading buffer containing β-mercaptoethanol and heated at 100 ^{0}C for 5 min prior to loading in a 15% Tris-HCl sodium dodecyl sulfate-polyacrylamide gel (Bio-Rad Criterion ™ Precast Gel, Cat# 345-0019). The separated proteins were then transferred overnight onto an immuno-blot PVDF membrane (Bio-Rad Criterion Gel Blotting Sandwiches, Lot # BR7316003B). The membrane was blocked overnight with PBS containing 1% non-fat milk, incubated with goat anti-human IgM perioxidase labeled (1:1000 dilution, Cat # 074-1003; KPL), incubated with Fluorescein (FITC) conjugated AffiniPure Rabbit Anti-goat antibody (1:500, Lot # 48485; Jaskson ImmunoResearch Lab, INC.). The bands were detected using anti-FITC alkaline phosphatase (1:1000 dilution) and an enhanced chemifluorescence (ECF) reagent before exposure to 473 nm laser using an Image Reader FLA-5100 Fuji Film Reader and image captured.

2.2 Results:

Depletion and lysis of B cell-depleted PBMCs from a healthy donor

B-cell depletion of a healthy donor PBMCs was performed by incubating with biotinylated mouse anti-human CD19 antibodies, washing, incubating with streptavidin-coated magnetic beads and allowed to pass through a column which was attached to a magnet. CD19 positive B-cells remained attached to the column while the eluate was depleted of B-cells. Flow cytometery was performed on the PBMCs before and after magnetic bead separation by staining with mouse anti-human CD20 FITC and CD5 PE antibodies. To determine background binding pre-separated cells were incubated with anti-mouse IgG1 FITC and IgG1 PE antibodies. As shown in Figure 10 C and D, isolated PBMCs from the healthy volunteer was depleted of CD19 positive B-cells. Before B-cell depletion, CD20 positve B-cell count was 10.4% and after B-cell depletion, B-cell count reduced to 0.7%. B-cell depleted PBMCs were lysed as mentioned in "Materials and Methods", and protein concentration determined to be 1.23µg/µl.

To determine any non-specific binding from cell lysate non-BCR proteins, within the ELISA semi-quantification wells for patient BCRs, B-cell depleted PBMCs were lysed and spiked with known concentrations of human serum IgM. The B-cell depleted PBMC lysate, spiked with known concentrations of IgM, was used to, as a standard, generate a curve and compared to a standard curve which was obtained by not using B-cell depleted PBMC lysate (Figure 13 B and C).



<u>Figure 10.</u> Depletion of CD19 Positive B-cells from PBMC of a Healthy Volunteer, using mouse anti-human CD19 magnetic bead-bound antibodies and stained with mouse anti-human CD20 PE and CD5 PECy7 antibodies. ***A.*** The "lymphocyte" gate on a side scatter (SSC-A) versus forward scatter (FSC-A) plot . ***B.*** A density plot of isotype controls, anti-mouse IgG1 FITC and IgG1 PE-Cy7 antibodies, used to determine non-specific binding. ***C.*** Shows CD20 versus CD5 Pe-Cy7 positive stained cells within "lymphocyte gate" before CD19 depletion. ***D.*** Shows CD20 versus CD5 Pe-Cy7 positive stained cells wihtin "lymphocyte gate" after CD19 depletion. R1 is the CD20 postive B-Cells population.

Protein extraction and quantification of isolated CLL patient PBMCs

CLL patient PBMCs were isolated, digested and total protein quantified against BSA standard (Figure 11) as described in "Materials and Methods". CLL patient "P" protein concentration was calculated to be 1.78 µl/ µg, while protein concentration of CLL patient "E" was 1.96 µl/µg (Table 3).

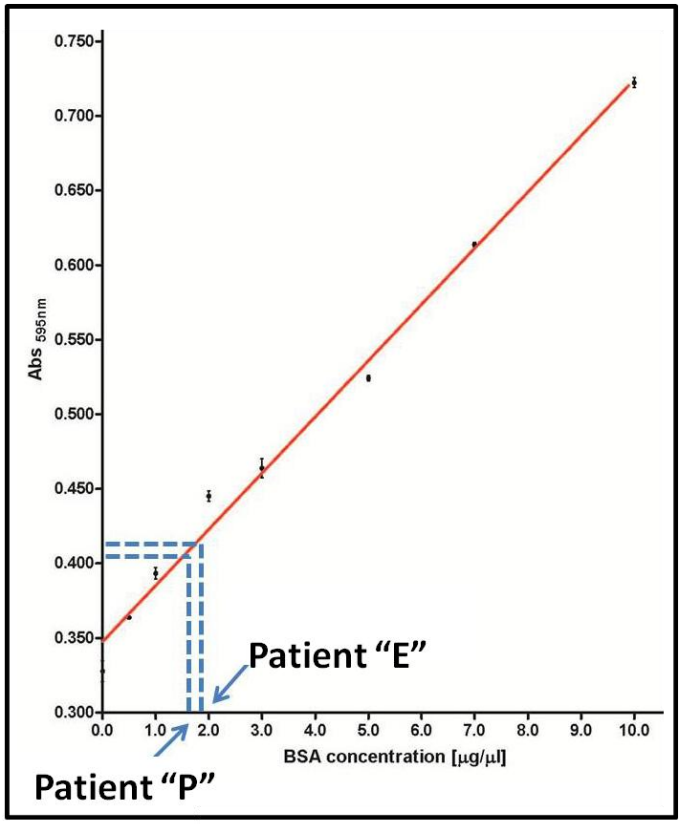


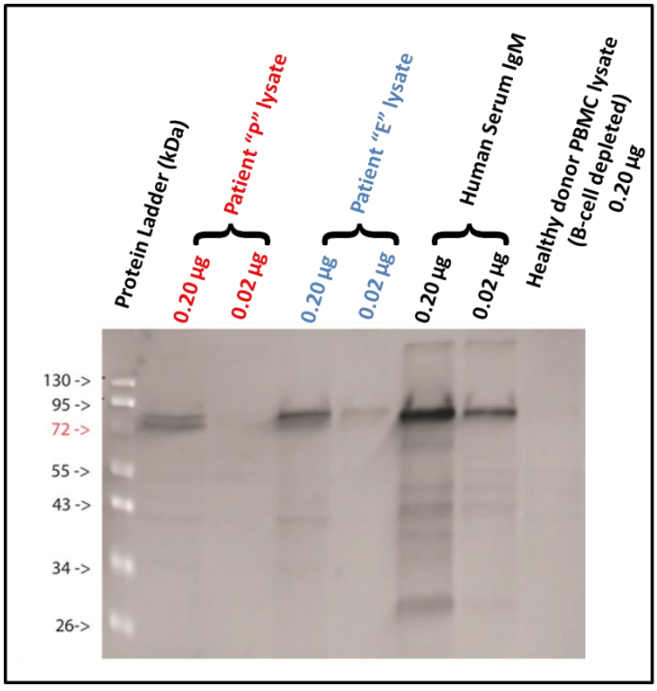
Figure 11. BSA Standard Line of Best-fit Graph. BSA standard was used for extrapolation of CLL patient total protein cell extracts concentration (R^2 0.9889).

Table 3. Total Protein Concentration within CLL Patient PBMC Lysate.

Patient	Protein concentration [µg/ µl]
"P"	1.78
"E"	1.96

SDS-PAGE and western blot analysis of CLL patient cell lysate

In order to further determine the presence of IgM within the protein lysates, SDS-PAGE and Western blotting, described in "Materials and Methods", were performed on patient protein lysates and healthy donor lysate (B-cell depleted). Lysates were separated using a 15% Tris-HCl sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred overnight onto an immuno-blot polyvinylidene difluoride membrane and IgM was detected using goat anti-human IgM antibody. Analysis of 0.2 µg and 0.02 µg of standardized IgM from human serum, patients "E" and "P" protein lysates showed a band at approximately 72 kDa showing the presence of IgM molecule and no band identified in healthy donor B-cell depleted PBMC cell lysate (Figure 12).



<u>Figure 12.</u> Western Blot Analysis for IgM Detection within Protein Lysates of CLL cells from patients "E" and "P". Western blot membrane showing distinct bands of IgM within IgM standard from human serum, CLL patients "P" and "E" PBMC lysates, and by staining with goat anti-human IgM antibody. Healthy donor B-cell depleted PBMC lysate showing no IgM band present.

BCR semi-quantification of isolated CLL patient PBMCs protein extracts

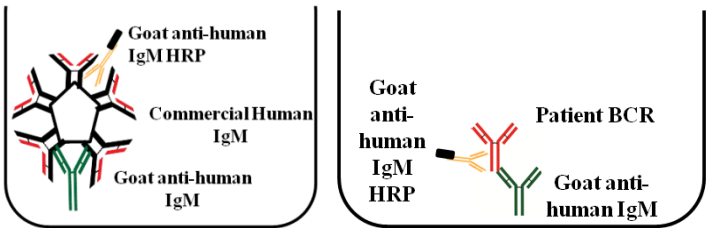
As shown in Figure 13A, two 96-well ELISA plates (CLL patient “E” and “P” labeled plates) were sensitized with unconjugated goat anti-human IgM antibody, Fc 5µ fragment specific, in bicarbonate buffer.

Each ELISA plate received 2-fold serial dilutions of lysate, obtained from PBMCs depleted of B-cells, but spiked with human serum IgM standard of known concentrations. IgM standard from serum was added to B-cell depleted PBMC healthy donor cell lysate before incubating with goat anti-human IgM to account for any non-specific binding due to other proteins within PBMC lysate. Two-fold serial dilutions of IgM from Human Serum (IgM standard) were also added to each plate in duplicates. Two-fold serial dilutions of patients “P” and “E” samples were tested in triplicates.

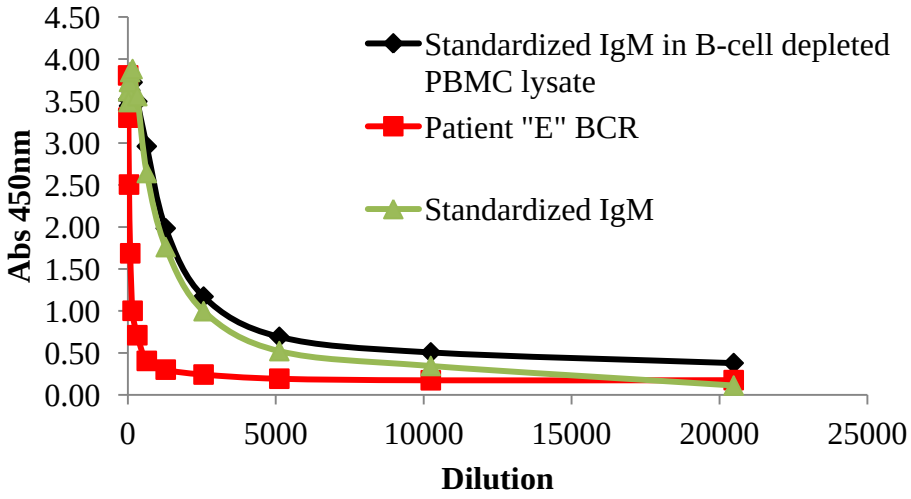
The ELISA plates were read at 450 nm using a spectrophotometer and the absorbance recorded and plotted for each ELISA plate. As shown in Figure 13 B and C, absorbance values of CLL patient “E” dropped faster as compared to patient “P” lysate wells indicating a lower concentration of captured BCRs present within patient “E” PBMC lysate.

Using each CLL patient’s plate “B cell-depleted lysate spiked with known concentrations of IgM from human serum”, standard curves with line of best fit were generated for each plate and used to determine IgM concentrations for patients “E” and “P” (Figure 14). BCR concentration in patients "E" and “P” lysate were determined, using the best-fit values from the standard curves, to be 0.048 µg/ µl and 0.180 µg/ µl, respectively (Table 4).

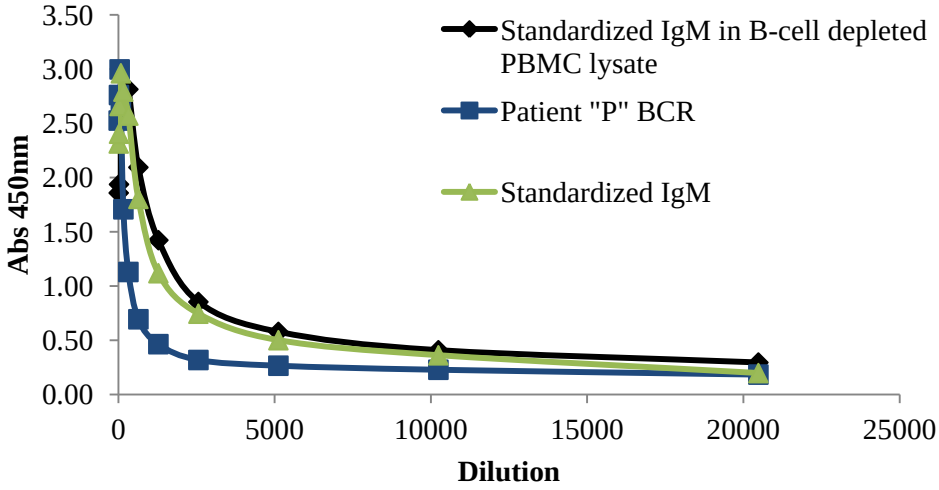
A.



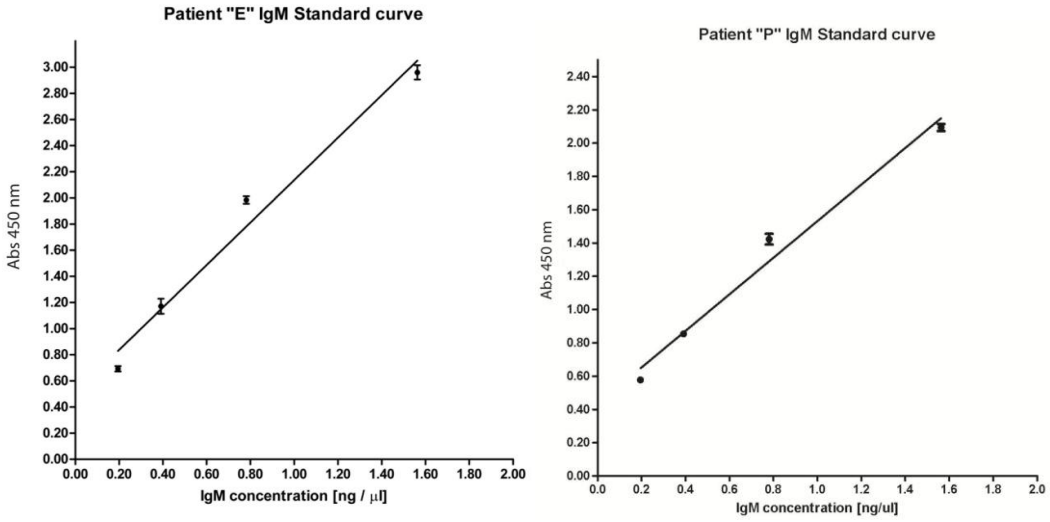
B.



C.



<u>Figure 13.</u> CLL Patients "E" and "P" cell lysate BCR Semi-Quantification. *A.* Schematic representation of method used to semi-quantify patient BCRs by extrapolating from a Human Serum IgM standard curve. ***B*** and ***C.*** Graphs showing the absorbance of serially diluted patient lysate, Standardized IgM and serial dilutions of Standardized IgM added to B cell-depleted PBMC lysate.



<u>Figure 14.</u> Human Serum IgM Standard curves used to Semi-quantify concentrations of BCRs present in CLL Patients "P" and "E"cell lysates. Standard curves, for each CLL patient ELISA plate, obtained from B cell depleted-PBMC lysate spiked with known concentrations of Human Serum IgM (ng/ µl) and used to extrapolate CLL patients "E" and "P" BCR concentrations. ***Left panel:*** Graph of CLL patient "E" BCR standard quantification plate (R^2 of 0.9718). ***Right panel*:** Graph of CLL patient "P" BCR standard quantification plate (R^2 of 0.9784).

Table 4. Semi-Quantification of BCRs in Patient "E" and "P" cell lysate.

Patient	**Mean BCR concentration (µg/µl)**
"E"	0.048
"P"	0.180

Detection of kappa and lambda light chain in human serum IgM standard by capturing using goat anti-human kappa or lambda

To determine the presence of kappa light chain on CLL patient BCRs within the cell lysates, two methods of detection (Figure 15 and 18) were optimized using human serum IgM Standard of known concentrations. As shown in Figure 15, ELISA plate was sensitized with goat anti-human kappa or goat anti-human lambda in 50 µl $NaHCO_3$ pH 8.6, for 1 hour at room temperature, washed and then blocked overnight at 4^0C with 5% non-fat milk in PBS (0.1%Tween-20). Wells were then washed and incubated for 1hour with 2-fold serial dilutions of known concentrations of human IgM standard. All wells were washed and incubated for 1 hour at room temperature with horseradish peroxidase conjugated goat anti-human IgM. Wells were then washed, TMB substrate added and the reaction stopped with 1M sulfuric acid and read at 450nm. Absorbance versus IgM concentration curves were generated (Figure 16A and Figure 17A) from which lines of best-fit were obtained. Kappa and lambda detection assays had R^2 values of 0.9333 and 0.9224, respectively (Figure 16B and 17B).

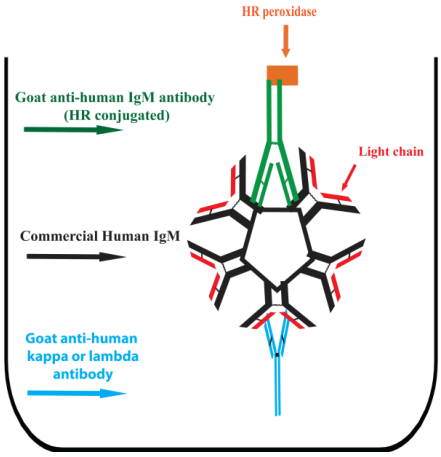
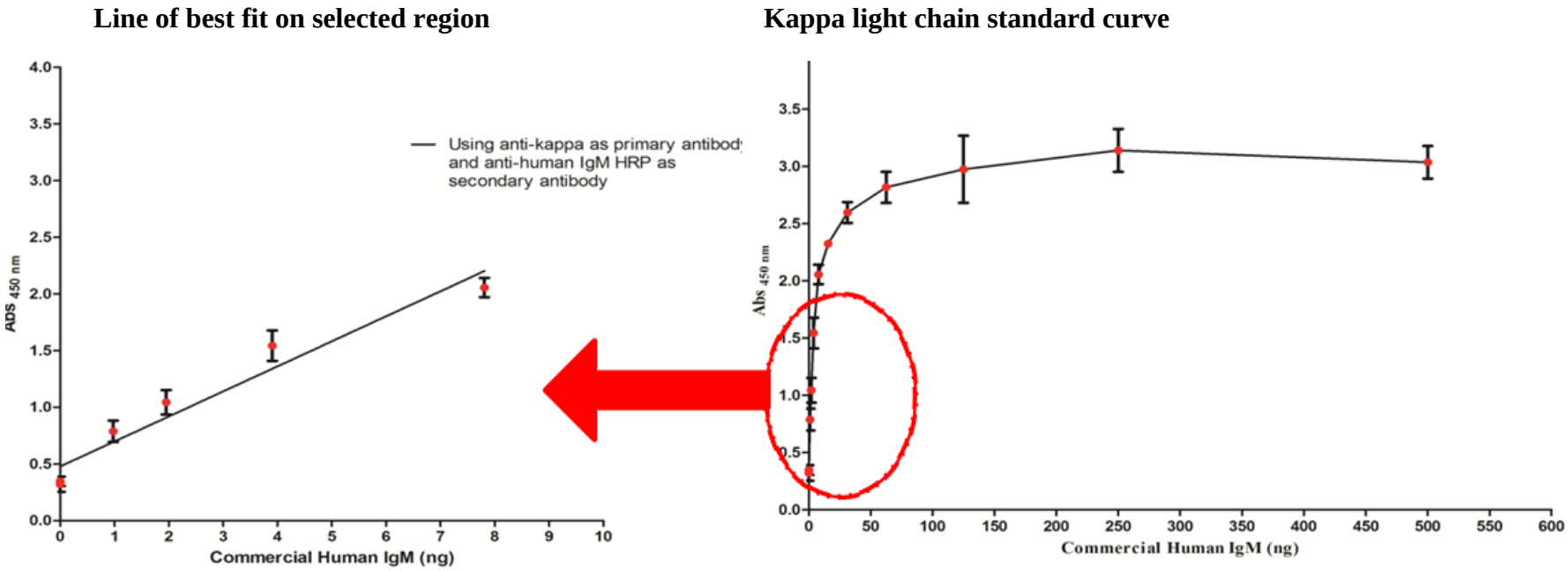
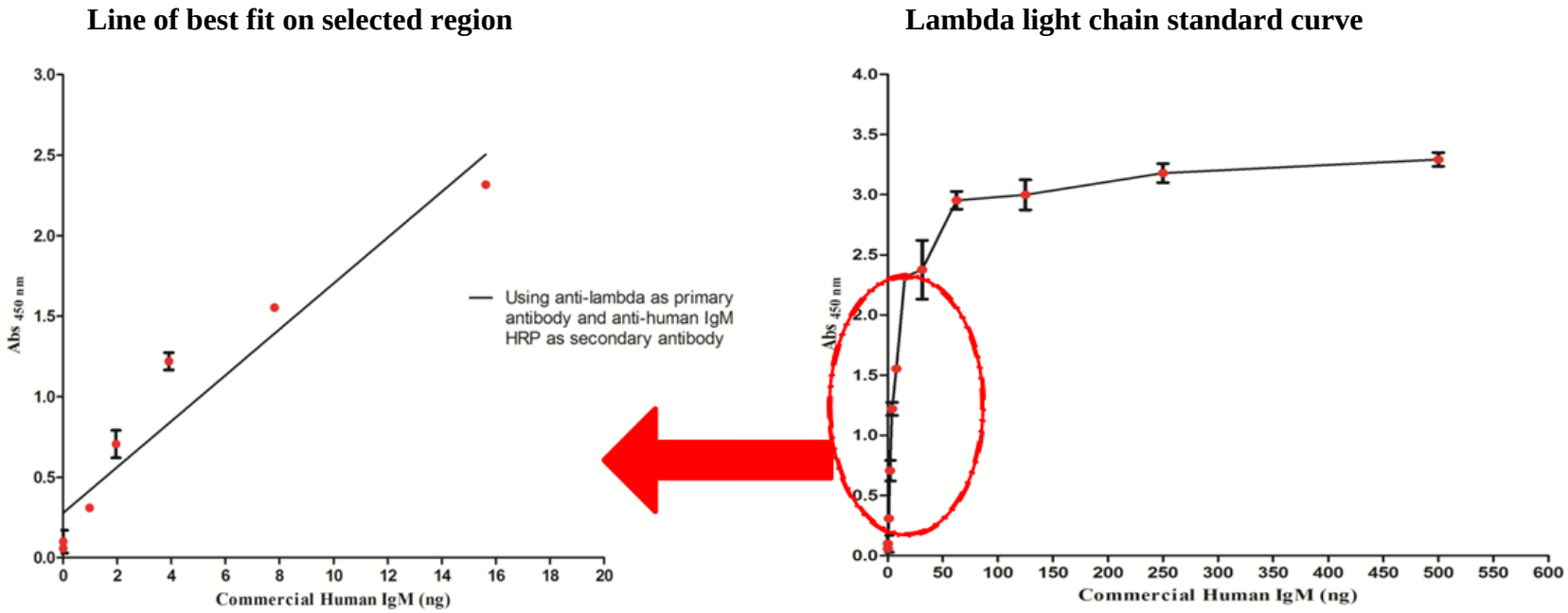


Figure 15. "Schematic Representation 1" of Human IgM Light Chain Detection. human IgM captured using goat anti-human kappa or lambda antibody and then detected using horseradish conjugated (HR) goat anti-human IgM.



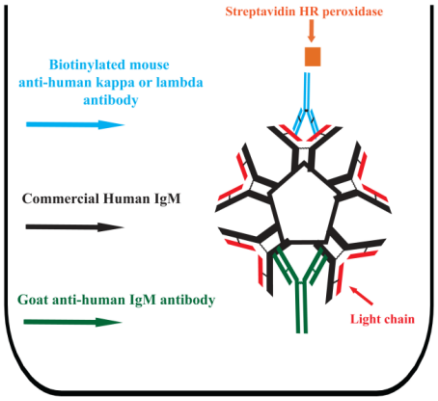
<u>Figure 16.</u> IgM Kappa Light Chain Detection in Human IgM from "Schematic Representation 1". Human IgM was captured using goat anti-human kappa antibody (primary antibody) and anti-human IgM HRP conjugated used as secondary antibody. ***Left panel.*** The line of best fit used to estimate concentration of kappa light chain within Human IgM standard (R^2 of 0.9333). ***Right panel.*** Absorbance values of kappa light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.



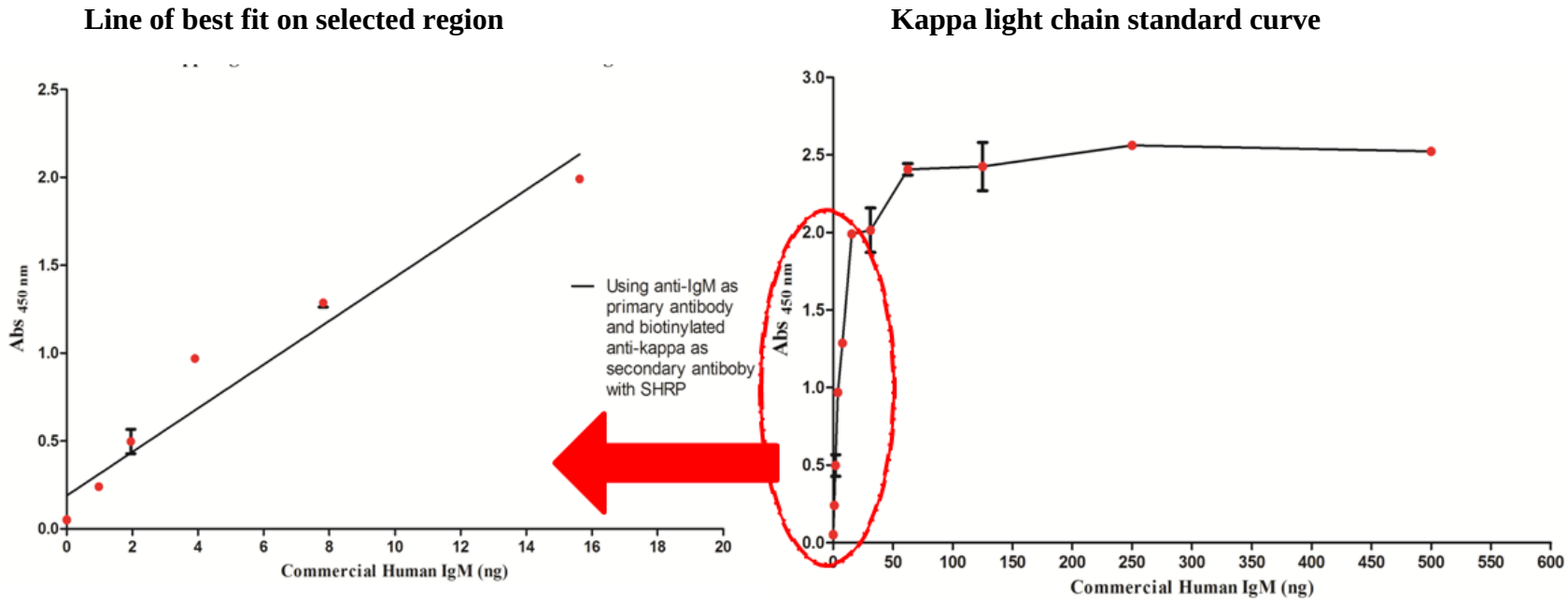
<u>Figure 17.</u> IgM Lambda Light Chain Detection in Human IgM from "Schematic Representation 1". Human IgM was captured using goat anti-human lambda antibody (primary antibody) and anti-human IgM HRP conjugate used as secondary antibody. ***Left panel.*** The line of best fit used to estimate concentration of kappa light chain within Human IgM standard (R^2 of 0.9224). ***Right panel.*** Absorbance values of lambda light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.

Detection of Kappa and Lambda Light Chain in Human Serum IgM Standard by capturing using goat anti-human IgM

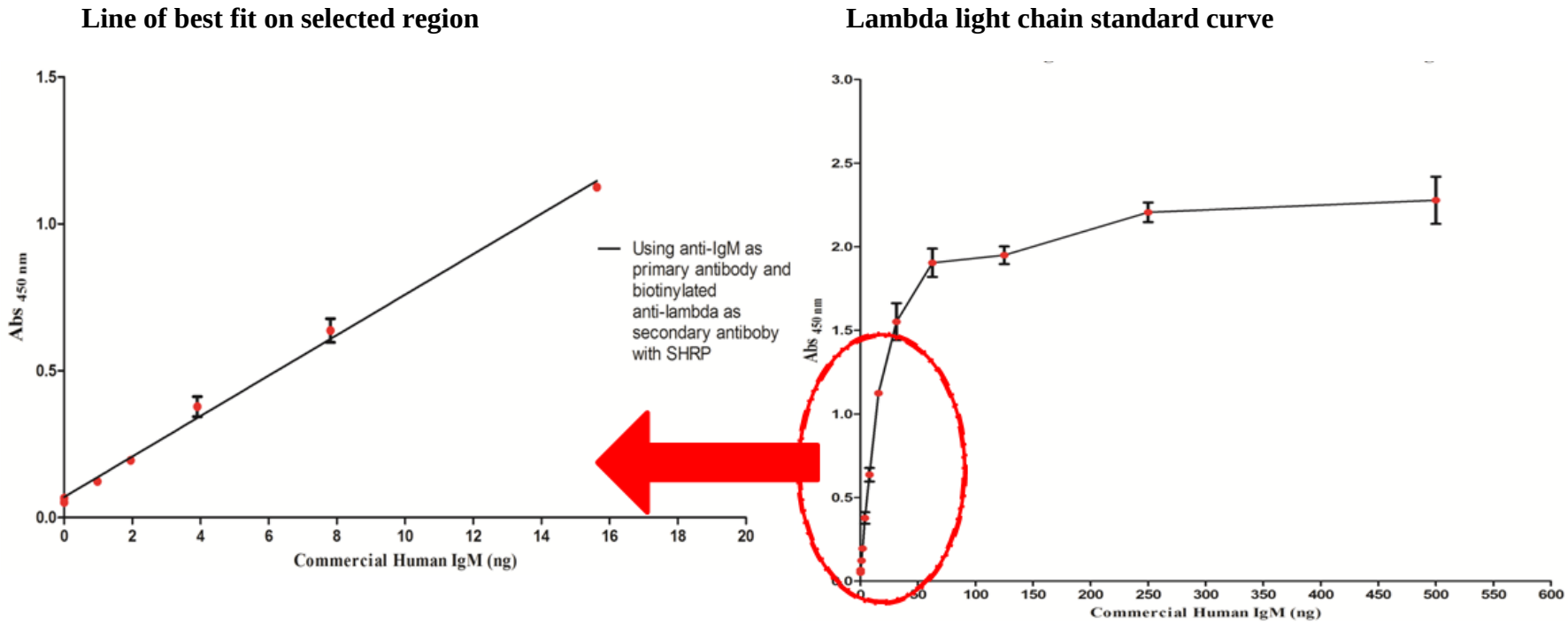
As shown in Figure 18, wells in an ELISA plate were sensitized with 0.5 µg goat anti-human IgM in 50 µl $NaHCO_3$ pH 8.6 for 1 hour at room temperature, washed and then blocked overnight at 4^0C with 5% non-fat milk in PBS (0.1%Tween-20). Wells were then washed and incubated for 1hour with 2-fold serial dilutions of known concentrations of human IgM standard for anti-kappa semi-quantification. All wells were washed, incubated for 1 hour at room temperature with either biotinylated mouse anti-human kappa antibody for kappa chain detection or with biotinylated mouse anti-human lambda antibody for lambda chain detection. All wells were then washed and incubated for 1 hour at room temperature with streptavidin horseradish peroxidase. Wells were then washed and TMB substrate added, reaction stopped with 1 M sulfuric acid and read at 450nm. Absorbance versus IgM concentration curves were generated (Figure 19A and 20A) from which lines of best-fit were obtained. Kappa and lambda detection assays had R^2 values of 0.9456 and 0.9946, respectively (Figure 19B and 20B).



<u>Figure 18.</u> "Schematic Representation 2" of Human IgM Light Chain detection. Human IgM captured using goat anti-human IgM antibody and then light chain detected using mouse anti-human kappa or lambda IgM.



<u>Figure 19.</u> IgM Kappa Light Chain Detection in Human IgM from "Schematic Representation 2". Human IgM was captured using goat anti-human IgM antibody (primary antibody) and biotinylated mouse anti-human kappa used as secondary antibody with SHRP. ***Left panel.*** The line of best fit used to estimate concentration of kappa light chain within Human IgM standard (R^2 of 0.9456). ***Right Panel.*** Absorbance values of kappa light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.



<u>Figure 20.</u> IgM Lambda Light Chain Detection in Human IgM from "Schematic Representation 2". Human IgM was captured using goat anti-human IgM antibody (primary antibody) and biotinylated mouse anti-human lambda used as secondary antibody with SHRP. ***Left panel.*** The line of best fit used to estimate concentration of lambda light chain within human IgM standard (R^2 of 0.9946). ***Right panel.*** Absorbance values of lambda light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.

IgM Kappa and Lambda Light Chain Detection within CLL Patient "P" and "E" cell lysate by capturing using goat anti-human IgM antibody

After optimized methods were compared, and "Method 2" was used to semi-quantify the light chain concentrations within CLL patients "P" and "E" cell lysates. "Method 2" was repeated using IgM standard, as shown in Figure 21. The ELISA wells were sensitized with 0.5 µg goat anti-human IgM in 50 µl $NaHCO_3$ pH 8.6, for 1 hour at room temperature, washed and then blocked overnight at 4^0C with 5% non-fat milk in PBS (0.1%Tween-20). Wells were then washed and incubated with 2-fold serial dilutions of known concentrations of human IgM standard for anti-kappa semi-quantification. In parallel, goat anti-human IgM sensitized wells were also incubated, for 1 hour at room temperature, with 2-fold serial dilutions, starting with 2 µl, of CLL patients "E" and "P" lysates in blocking buffer. All wells were washed, incubated for 1 hour at room temperature with biotinylated mouse anti-human kappa antibody for kappa light chain detection. The ELISA to quantitate IgM lambda concentration within patient lystate was performed as described above except a biotinylated mouse anti-human lambda antibody was used for lambda light chain detection on IgM. All wells were then washed and incubated for 1 hour at room temperature with streptavidin horseradish peroxidase. Wells were then washed and TMB substrate added, reaction stopped with 1M sulfuric acid and read at 450nm. Absorbance versus IgM concentration curve was generated for kappa detection within IgM Standard (Figure 22A) from which a line of best-fit was obtained. Kappa light chain detection assay had an R^2 value of 0.9650 (Figure 22B). The kappa light chain of CLL patients "P" and "E" were semi-quantified to be 4.81 µg/ µl and 2.11 µg/ µl, respectively (Table 5). Absorbance versus IgM concentration curve was also

generated for lambda detection within IgM Standard (Figure 23A) from which a line of best-fit was obtained. Lambda light chain detection assay had an R^2 value of 0.9909 (Figure 23B). The lambda light chain within CLL patients “P” and “E” were semi-quantified to be 0.06 µg/ µl and 0.56 µg/ µl, respectively. These results support the flow cytometry light chain analysis of CLL patients “P” and “E” with patient “E” having lower lambda positive $CD5^+/CD19^+$ population.

Total protein concentrations within patient lysates, using "BSA" as standard, were 1.78 µg/ µl and 1.96 µg/ µl for Patients “P” and “E”, respectively (Table 3). ELISA, using serum IgM as standard, by capturing patient IgM with goat anti-human IgM (Fc 5µ fragment specific) and then detecting with goat anti-human IgM (µ fragment specific) horseradish peroxidase labeled was 0.180 µg/ µl and 0.048 µg/ µl for patients “P” and “E”, respectively (Table 4). However, ELISA by capturing patient IgM with goat anti-human IgM (Fc 5µ fragment specific) and then incubating with biotinylated mouse anti-human kappa/lambda antibody yielded kappa concentration of 4.81 µg/ µl (+/- 0.54) and 2.11 µg/ µl (+/- 0.35) for patients “P” and “E”, respectively. In addition, Lambda detection yielded 0.06 µg/ µl (+/- 0.05) and 0.56 µg/ µl (+/- 0.20) for patients “P” and “E”, respectively (Table 5 and 6). The higher concentrations observed of kappa and lambda between the ELISAs could be a result of repeated freeze thawing leading to a more concentrated cell lysate or much more efficient binding with the mouse anti kappa/ lambda antibodies. Despite the difference in concentrations between the ELISAs, it can still be concluded that patient “P” has all kappa, while patient “E” has both kappa and lambda, with kappa being more than lambda in patient “E” lysate. Also, Human IgM from Serum would have been a much better standard instead of BSA.

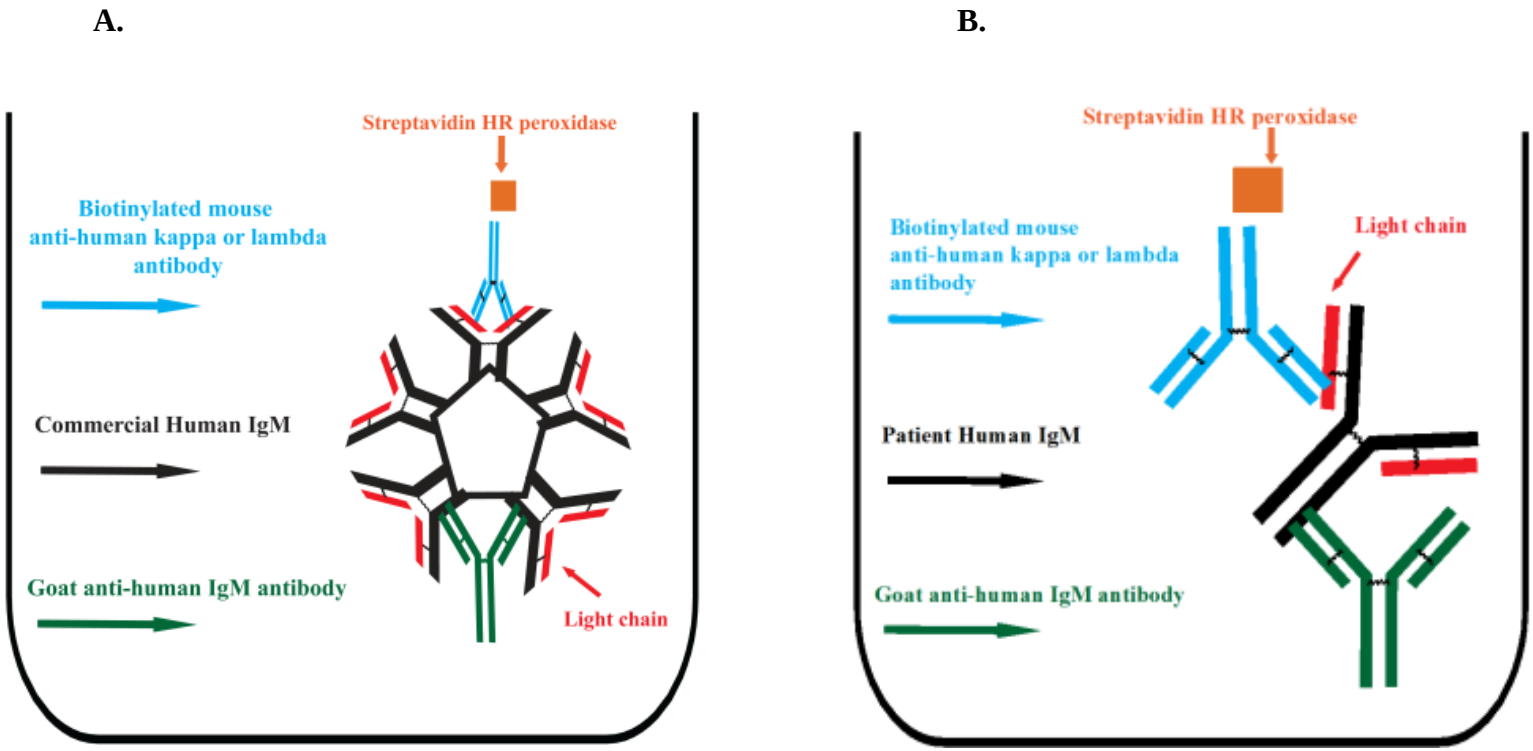


Figure 21. Schematic Representation of CLL Patients "P" and "E" BCR Light Chain detection. ***A.*** Human IgM captured using goat anti-human IgM antibody and then light chain detected using mouse anti-human Kappa or Lambda IgM antibody. ***B.*** CLL patients "P" and "E" BCRs were captured using goat anti-human IgM antibody (Model 2) and then light chain detected using mouse anti-human kappa or lambda IgM antibody.

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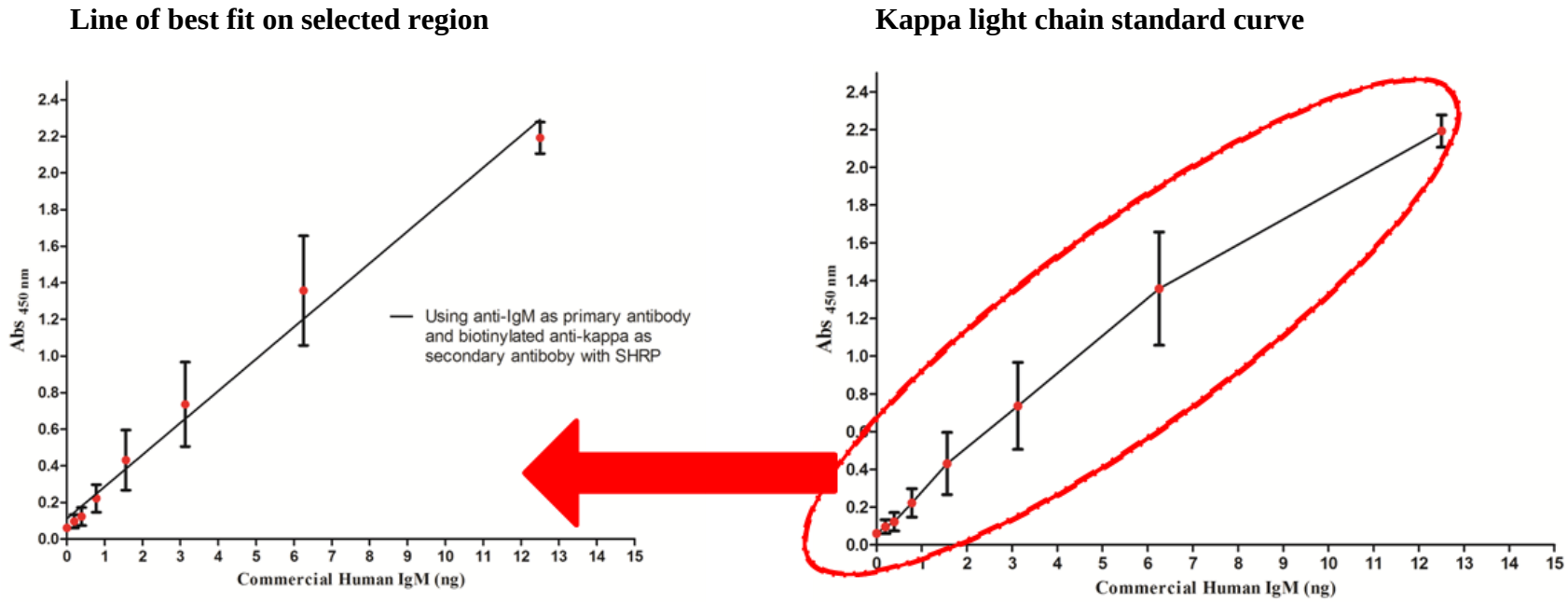
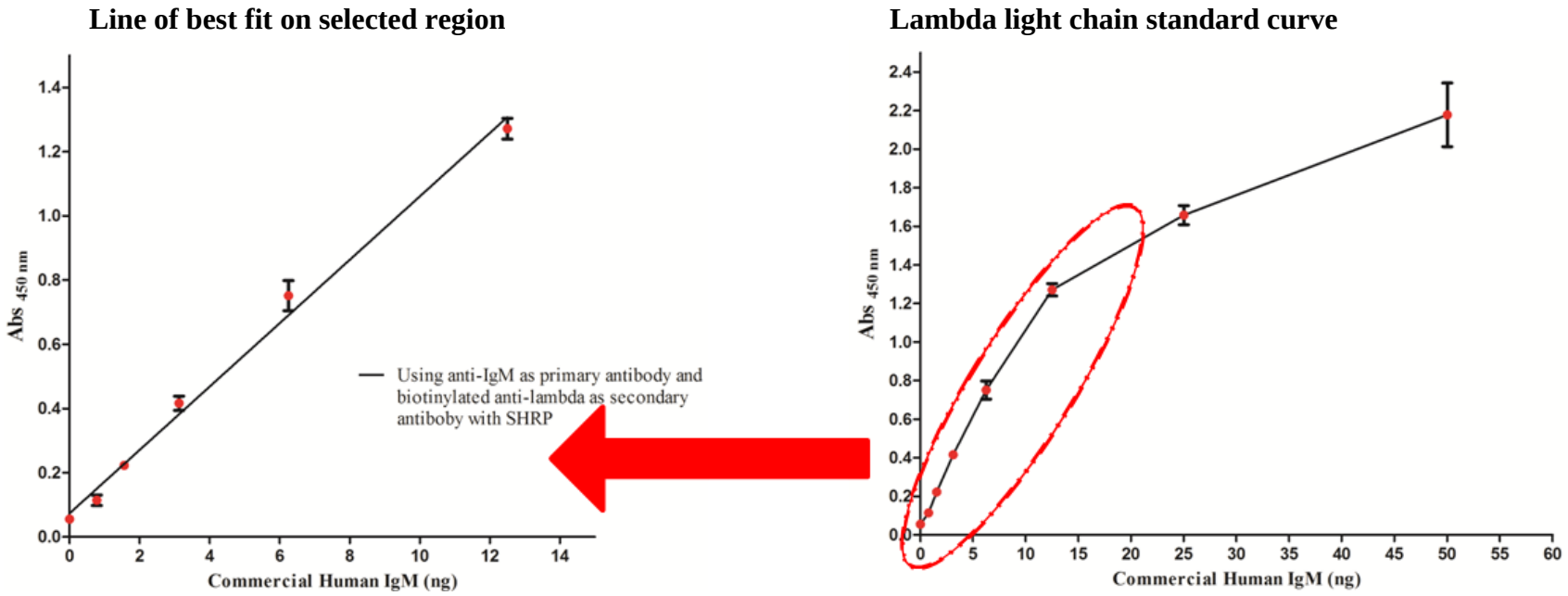


<u>Figure 22.</u> IgM Kappa Light Chain in Human IgM for Semi-quantification of CLL Patients "P" and "E" BCR Concentration. Human IgM was captured using goat anti-human IgM antibody (primary antibody) and biotinylated mouse anti-human kappa used as secondary antibody with SHRP. ***Left panel.*** The line of best fit used to estimate concentration of kappa light chain within Human IgM standard (R^2 of 0.9650). ***Right panel.*** Absorbance values of kappa light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.



<u>Figure 23.</u> IgM Lambda Light Chain in Human IgM for Semi-quantification of CLL Patients "P" and "E" BCR Concentration. Human IgM was captured using goat anti-human IgM antibody (primary antibody) and biotinylated mouse anti-human lambda used as secondary antibody with SHRP. ***Left panel.*** The line of best fit used to estimate concentration of lambda light chain within Human IgM standard (R^2 of 0.9909). ***Right panel.*** Absorbance values of lambda light chain detection from 2-fold serial dilutions of IgM (ng) from which linear slope section was taken.

Table 5. CLL patients Kappa light chain concentration determination using anti-human IgM to capture BCRs from Patient lysates (µg/µl).

Patient	Minimum	Maximum	Mean	Std. Error (+/-)	Lower 95% CI of mean	Upper 95% CI of mean
"P"	2.94	5.93	4.81	0.54	3.30	6.31
"E"	1.43	2.98	2.11	0.35	0.99	3.23

Table 6. CLL patients Lambda light chain concentration determination using anti-human IgM to capture BCRs from patient lysates (µg/µl).

Patient	Minimum	Maximum	Mean	Std. Error (+/-)	Lower 95% CI of mean	Upper 95% CI of mean
“P"	0.01	0.11	0.06	0.05	-0.56	0.69
"E"	0.03	0.94	0.56	0.20	-0.06	1.18

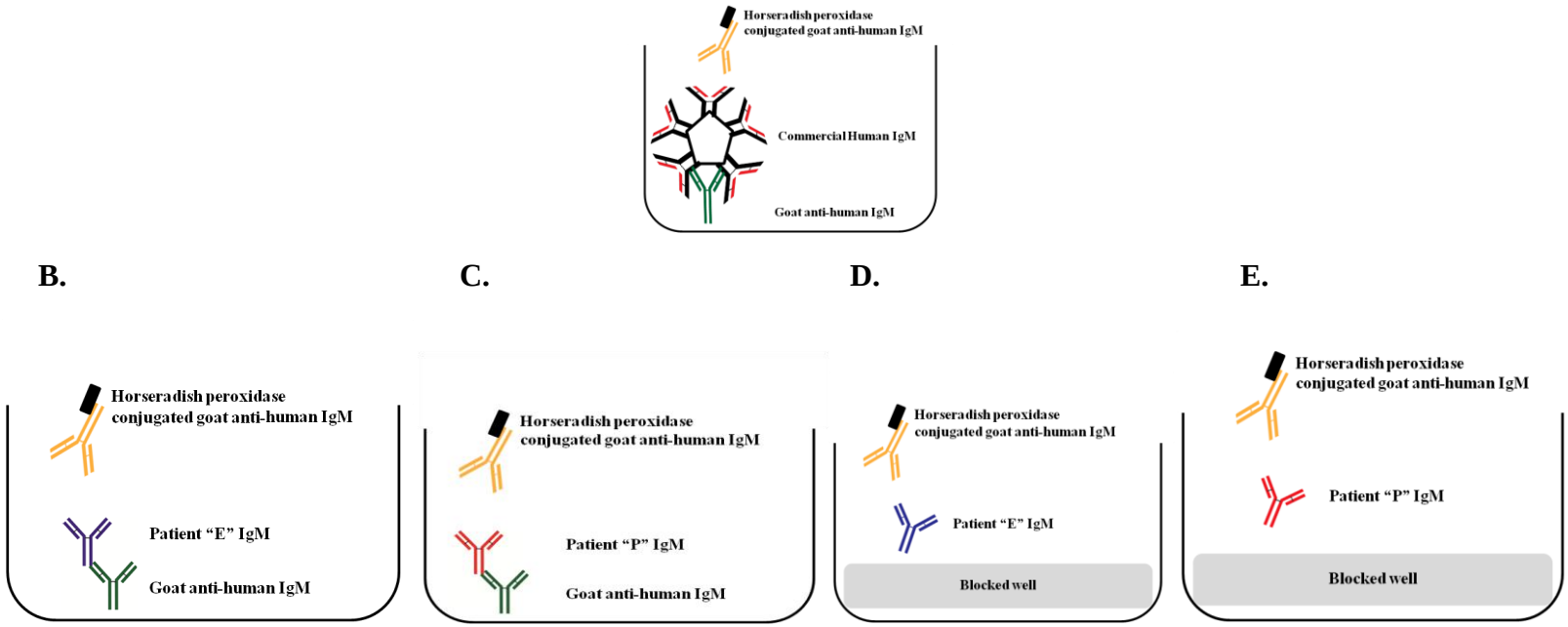
CHAPTER 3: Use of 7-mer and 12-mer Phage Display Library to select peptides with high affinity to BCR isolates from CLL cells

3 Materials / Methods:

3.1 Can captured CLL patient BCRs withstand Phage Display Library surfacing panning conditions?

A qualitative ELISA was performed to determine whether patient BCRs which were captured onto goat anti-human IgM sensitized well could withstand the surface panning conditions used in the selection process. Patients “E” and “P” wells were performed using 50 µl and 200 µl, respectively. The volumes were used because, in the phage selection process, 50 µl of Patient “E” unbound phages are transferred into Patient “P” well and the empty patient “E” wells are rinsed 3 times with 50 µl each wash and then transferred into Patient “P” well giving a total volume of 200 µl in Patient “P” well. As shown in Figure 24, wells “A” and “B” were sensitized with 1.6 micrograms of goat anti-human IgM in 50 µl of 0.1M $NaHCO_3$ pH 8.6 solution. Well “C” was sensitized with 1.6 micrograms of goat anti-human IgM in 200 µl of 0.1M $NaHCO_3$ pH 8.6 solution. The plate was incubated for 1 hour at room temperature and then wells “A” and “B” were washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS, while well “C” was washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS. Wells A,“B”, “C”, “D”, and “E” were then blocked with 300 µl of 5% BSA in 0.1M $NaHCO_3$ pH 8.6,

covered and incubated overnight at 4^0C. After overnight blocking with 5% BSA in 0.1M $NaHCO_3$ pH 8.6, the wells "A" and "B" were washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS, while well "C" was washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS. As a positive control, 100 µl (0.08 micrograms) of IgM from human serum was added to well "A" and incubated for 1 hour at room temperature. Twenty microliters of patient "E" lysate was added to 80 µl of 0.1M $NaHCO_3$ pH 8.6 and 50 µl of this was added to wells "B" and "D" and incubated for 1 hour at room temperature. Eight microliters of patient "P" lysate was added to 320 µl of 0.1M $NaHCO_3$ pH 8.6 and then 200 µl of this was added to wells "C" and "E" and incubated for 1 hour at room temperature. After 1 hour incubation, the well "A" was washed 3 times with 100 µl per wash of 0.05% Tween-20 in TBS. Wells "B" and "D' were washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS, while wells "C" and "E" were washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS. Then the well "A" was washed 3 times with 100 µl per wash of TBS. Wells "B" and "D" were washed 3 times with 50 µl per wash of TBS, while wells "C" and "E" were washed 3 times with 200 µl per wash of TBS. 50 µl of 1:1000 dilution of *peroxidase*-labeled affinity purified goat anti-human IgM (µ) antibody (KPL Cat No. 074-10003) in 5% BSA in 0.1M $NaHCO_3$ pH 8.6 was added to all the wells ("A", "B", "C", "D" and "E") and incubated for 1 hour at room temperature. The wells were then rinsed 3 times with 300 µl per wash of 0.05% Tween-20 in TBS and rinsed again with 300 µl of TBS. The plate was allowed to incubate for about 10 minutes at room temperature and the reaction stopped with 50 µl of 1M H_2SO_4 and a picture of the wells taken with a digital camera.



<u>Figure 24.</u> Schematic Representation of ELISA Performed to Determine Whether Patient BCRs Captured Onto Goat Anti-Human IgM Sensitized Well Could Withstand the Surface Panning Conditions Used in the Phage Display Library Selection Process.

3.2 Strategy 1: 7-mer and 12-mer First Round Panning on CLL Patient BCRs

As illustrated in Figure 26 and detailed below, in separate wells, BCRs from CLL patients "P" and "E" were captured using goat anti-human IgM. Patient "E" BCR-captured well was used as the "Negative" subtraction plate to remove, from the Phage Display Peptide Library, phages binding to regions of the B-cell receptor. Since BCRs from patients "E" and "P" differed in the binding pocket, the remaining phages were incubated with the "Positive" selection patient "P" BCR-captured well. The unbound phages were discarded by washing 10 times with TBS (0.05% Tween). Patient "P" BCR-bound phages, targeting the binding pocket, were eluted and amplified and the panning repeated with amplified phages.

3.2.1 Sensitization of wells with anti-human IgM for "Negative subtraction" and "Positive Selection" plate

Goat anti-human IgM, 1.6 µg in 50 µl 0.1M sodium bicarbonate pH 8.6 (Pierce lot # KH1248781), was added to sensitize the "Negative subtraction" wells on a 96-well plate. Incubated for 1 hour at room temperature and then washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS and then 3 times with 50 µl of TBS. "Positive selection" wells on the plate were sensitized with the 200 µl (1.6 µg) of goat anti human IgM. Incubated for 1 hour at room temperature, washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS. and then washed 3 times with 200 µl per wash of TBS. The plates were then blocked with 300 µl of 5% BSA in 0.1M $NaHCO_3$ pH 8.6, covered and incubated overnight at 4^0C.

3.2.2 Capturing of patient "E" BCRs on "Negative subtraction" plate

After overnight blocking with 5% BSA in 0.1M $NaHCO_3$ pH 8.6, the "Negative subtraction" plates were washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS. The plates were then washed 3 times with 50 µl of TBS. Twenty microliters of patient "E" lysate was added to 80 µl of 0.1M $NaHCO_3$ pH 8.6 and 50 µl of this was added to each goat anti-human IgM sensitized well on the "Negative subtraction" plate and incubated for 1 hour at room temperature. After 1 hour incubation, wells were washed 3 times with 50 µl per wash of 0.05% Tween-20 in TBS. Plates were then washed 3 times with 50 µl of TBS.

3.2.3 Capturing patient "P" BCRs on "Positive selection" plate

After overnight blocking with 5% BSA in 0.1M $NaHCO_3$ pH 8.6, the "Positive selection" plates were washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS and then washed 3 times with 200 µl of TBS. Eighty microliters of patient "P" lysate was added to 320 µl of 0.1M $NaHCO_3$ pH 8.6 and then 200 µl of this was added to each goat anti-human IgM sensitized well on the "Positive selection" plate and incubated for 1 hour at room temperature. After 1 hour incubation, wells were washed 3 times with 200 µl per wash of 0.05% Tween-20 in TBS and then washed 3 times with 200 µl of TBS.

3.2.4 Phage Display Library (7 and 12-mer) incubation in "Negative subtraction" plate

Ten microliters of 7-mer Phage Display Library (E8100S, lot No. 0100908) or 12-mer (E8110S, Lot No. 0170909) Phage Display Peptide library was added to 40 µl of 0.05% Tween-20 in TBS. Diluted 7-mer or 12-mer Phage Display Peptide library was added to the "Negative subtraction" plate well and incubated at room temperature for 1

hour on a rocking plate. After 1 hour incubation, the plate was inclined and the 50 µl of unbound phages within the well were transferred into a microcentrifuge tube. The well was washed 3 times with 50 µl of 0.05% Tween-20 in TBS and the washes were added to the initial 50 µl of subtracted unbound phages to give a final volume of 200 µl. Five microliters of the subtracted unbound phages was kept for titration (*P7R1-IN* and *P12R1-IN*) and the remaining 195µl "Subtracted 7 and 12-mer phage display libraries" was kept for "Positive selection" plate.

3.2.5 Subtracted P7R1-IN and P12R1-IN incubation in "Positive selection" plate

One hundred and ninety five microliters of "Subtracted Phage Display Peptide library" (*P7R1-IN* or *P12R1-IN*) library was added to the "Positive selection" well and incubated for 1 hour at room temperature on a rocking plate. After 1 hour incubation, the non-binding phages were rinsed off by emptying the wells and then tapping the plate onto paper towels. The well was washed 10 times with 200 µl of 0.05% Tween-20 in TBS per wash. The bound phages remaining in the well were eluted with 15 minutes gentle rocking with 200 µl of elution buffer (0.2 M Glycine, HCl pH 2.2, 1% BSA). After 15 minute incubation with the elution buffer, the 200 µl of eluted phages were transferred into a 1.5mL microcentrifuge tube containing 30 µl of 1M Tris-HCl pH 9.1. After neutralization, 5 µl of the eluate was kept for titration (*P7R1-OUT* and *P12R1-OUT*) and remaining 225 µl was amplified by incubating all the eluate in 4 ml of *E. coli* ER2738 in log phase (O.D. of 0.05) within a 50 ml Erlenmeyer flask for 4.5 hours at 37^0C in a shaker at 260 RPM.

3.2.6 Amplification of eluted 7 and 12-mer first round phages

After 4.5 hour incubation, the 4 mL of the amplified product was aliquoted into 1.5 mL microcentrifuge tubes and centrifuges at 12000 RPM for 10 minutes at 4^{o}C to pellet the bacteria and leave phage-containing supernatant. The phage-containing supernatant was transferred into microcentrifuge tubes and centrifuged again at 12000 RPM for 10 minutes at 4^{o}C to pellet any residual bacteria remaining within the supernatant. After the last centrifugation, 1.2 mL of supernatant was transferred into a microcentrifuge tube containing 200 µl of 20% PEG 8000 (Polyethylene Glycol 8000), 2.5 M NaCl labeled *P7R1-OUT Amp* and *P12R1-OUT Amp*. Contents were mixed by tilting and then allowed to precipitate horizontally overnight at 4^{0}C.

3.2.7 Precipitation and concentration of first round amplified phages

After overnight incubation at 4^{o}C, the Phages are precipitated by the PEG. The Phage-PEG precipitate was pelleted by centrifuging the microcentrifuge tubes at 12000 RPM for 15 minutes at 4^{0}C. The supernate was decanted into bleach, leaving behind the phage-PEG pellet at the bottom of the microcentrifuge tube. Residual supernatant was removed, by using a barrier-tip 200 microliter pipette, after the tubes were centrifuged briefly at 12,000 RPM for 10 minutes. The phage-PEG pellets were concentrated through resuspension in a final volume of 200 µl and transferred into a new 1.5 mL microcentrifuge tube labeled *P7R1-OUT Amp* and *P12R1-OUT Amp*.

3.2.8 Titration of first round 7 and 12-mer phages

E. coli ER2738 was grown to reach a log phage (O.D. 600 nm = 0.4 - 0.5) in LB tetracycline within a 50 ml Erlenmeyer flask placed in a 37 ^{o}C shaker at 250 RPM. The phage stocks (*P7R1-IN, P7R1-OUT, P7R1-OUT Amp, P12R1-IN, P12R1-OUT* and *P12R1-OUT Amp*) were each serially diluted by adding 1µl of phage stock to 99 µl of LB and vortex to mix to give a 100 fold dilution. The 100 fold dilution was then added to 900 µl of LB to give a 1000 fold dilution. Subsequent 1:10 serial dilutions were carried out to reach a dilution of 10^{12}. 100 µl of each serial phage dilution were each added to 200 µl of *E. coli* ER2738 (O.D. 600 = 0.4 - 0.5) in LB. The phage bacteria mixture was then added to phage top agar tubes and plated on IPTG/Xgal plates for "blue/white screening". Control plates containing only 200 µl of bacteria with no phages were added to phage top agar tubes and plated on IPTG/Xgal plates. IPTG/Xgal plates were incubated at 37^{o}C no more than 18 hours. After incubation at 37^{0}C, the plates are removed and the number of pfu counted and titers determined using the formula below [56]:

$$\frac{\text{pfu} \quad \text{x} \quad \text{dilution factor}}{\text{Amount plated in microliters}}$$

3.3 Phages 7-mer and 12-mer Second Round Panning on Patient BCRs

3.3.1 P7R1-Out Amp and P12R1-Out Amp incubation in "Negative subtraction" plate

"Negative subtraction" and "Positive selection" plates were each prepared as described in "Materials and Methods Sections 3.2.1 to 3.2.3" with goat anti-human IgM and patients "E" and "P" BCRs.

Ninety five microliters of *P7R1-Out Amp* was added to 5 µl of 10% Tween-20 in TBS. In the case of *P12R1-Out Amp*, 10 µl was added to 40 µl of 0.5% Tween-20 in TBS. Fifty microliters of diluted *P7R1-Out Amp* or *P12R1-Out Amp* suspension was added to the "Negative subtraction" well and incubated at room temperature for 1 hour on a rocking plate. After 1 hour incubation, the plate was inclined and the 50 µl of unbound phages within the well were transferred into a microcentrifuge tube. The well was washed 3 times with 50 µl of 0.5% Tween-20 in TBS and the washes were added to the initial 50 µl of Subtracted unbound phages to give a final volume of 200 µl. Five microliters of the subtracted unbound phages was kept for titration (*P7R2-IN* and *P12R2-IN*) and the remaining 195 µl "Subtracted 7-mer or 12-mer phage display library" was kept for "Positive selection" plate.

3.3.2 Subtracted P7R2-IN and P12R2-IN incubation in "Positive selection" plate

One hundred and ninety five microliters of "Subtracted 7-mer Phage Display Peptide library" (*P7R2-IN*) library was added to the "Positive selection" well and incubated for 1 hour at room temperature on a rocking plate. After 1 hour incubation, the non-binding phages were rinsed off by emptying the wells and then tapping the plate onto

paper towels. The well was then washed 10 times with 200 µl of 0.05% Tween-20 in TBS per wash. The bound phages remaining in the ELISA well were eluted with 15 minutes gentle rocking with 200µl of elution buffer (0.2 M Glycine, HCl pH 2.2, 1% BSA). After 15 minute incubation with the elution buffer, the 200 µl of eluted phages were transferred into a 1.5mL microcentrifuge tube containing 30 µl of 1M Tris-HCl pH 9.1. After neutralization, 5 µl of the eluate was kept for titration (*P7R2-OUT* and *P12R2-OUT*) and the remaining 225 µl of the eluate was amplified by incubating all the eluate in 4 ml of *E. coli* ER2738 in log phase (O.D. of 0.05) within a 50 ml Erlenmeyer flask for 4.5 hours at 37^0C in a shaker at 260 RPM.

After neutralization, the remaining 225 µl of the eluate (*P7R2-OUT* and *P12R2-OUT*) was amplified, precipitated and concentrated as described in "Materials and Methods Section 3.2.6 to 3.2.7" and labeled *P7R2-OUT Amp* and *P12R2-OUT Amp*.

Phages were then titrated as described in "Materials and Methods Section 3.2.8" and labeled *P7R2-IN, P7R2-OUT, P7R2-OUT Amp, P12R2-IN, P12R2-OUT* and *P12R2-OUT Amp*.

3.4 Phages 7 and 12-mer third round panning on patient BCRs

3.4.1 P7R2-Out Amp and P12R2-Out Amp incubation in "Negative subtraction" plate

"Negative subtraction" and "Positive selection" plates were each prepared as described in "Materials and Methods Sections 3.2.1 to 3.2.3" with goat anti-human IgM and patients "E" and "P" BCRs.

10^{11} phages from *P7R2-Out Amp* or *P12R2-Out Amp*, in 0.05% TBST, were incubated in "Negative subtraction" as described in "Section 3.3.1". Unbound phages ((*P7R3-IN* and *P12R3-IN*) were collected, titrated and then kept for "Positive selection" plate.

3.4.2 Subtracted P7R3-IN and P12R3-IN incubation in "Positive selection" plate

Positive selection was performed as described in "Section 3.3.2" using *P7R3-IN* and *P12R3-IN.* Eluted phages *P7R3-OUT* and *P12R3-OUT* were titrated, amplified as described in "Section 3.2.7" and labeled *P7R3-OUT Amp* and *P12R3-OUT Amp* and titrated as described in "Section 3.2.9".

3.5 Growing E. coli culture overnight

In a 50 mL falcon tube, 10 µl of tetracycline (20 mg/ml) was added to 10 ml LB broth. 1mL of the LB tetracycline was transferred into a 50 ml falcon tube and inoculated with an *E. coli* ER2738 clone, using a sterile pipette tip to pick an isolated colony from a streaked plate. The tube was placed in a shaker at 37^0C at 260 rpm overnight.

3.6 Amplification of selected clones of first, second and third round 7 and 12-mer selections

The number of plaques on an IPTG/Xgal plate with less than 100 blue plaques was counted for first, second, and third round "OUT". An overnight culture of bacteria was diluted 1:100 in LB tetracycline medium and 2 mL of the diluted culture was dispensed into 5 ml polystyrene culture tubes, one for each clone to be characterized. Ten of the well separated blue plaques from each plate were selected, using a sterile 200 microliter pipette tip and placed in the 2 ml LB tetracycline diluted *E. coli* culture (O.D. 0.01 - 0.05). The polystyrene tubes were incubated at 280 rpm for 4.5 to 5 hours at 37 ^{0}C. After incubation, 1.4 mL of each culture was transferred into separated 1.5 ml microcentrifuge tubes. The tubes were centrifuge at 14,000 RPM for 30 seconds at 4^{o}C to pellet the bacteria and leave the phages in supernatant. 1.2 µl of the supernate was transferred into a microcentrifuge tube containing 200 µl of 20% PEG8000, 2.5 M NaCl. The contents of the tubes were mixed by tilting. The phages were allowed to precipitate overnight at 4^{0}C positioned horizontal. After overnight incubation at 4^{o}C, the phages are precipitated by the PEG. The phage-PEG precipitate was pelleted by centrifuging the microcentrifuge tubes at 12000 RPM for 15 minutes at 4^{0}C. The supernatant was decanted, with phage-PEG pellet remaining at the bottom of tube. Residual supernatant was removed by using a barrier-tip 200 microliter pipette after the tubes were centrifuged briefly. The phage-PEG pellets were concentrated through resuspension in a final volume of 50 µl and transferred into a new microcentrifuge tube.

3.7 Phage DNA extraction from amplified clones

Forty microliters of the concentrated phage clones were each added to 460 µl of TBS in a 1.5 mL microcentrifuge tube containing 200 ul of 20% PEG 8000, 2.5 M NaCl. Tubes were inverted several times to mix contents of the tube and left to stand for 15 minutes at room temperature. Tubes were centrifuged at 13000 RPM for 10 minutes at 4^0C and the supernatant discarded. Tubes were centrifuged again, briefly, to discard remaining supernatant. Each pellet was thoroughly resuspended in 100 µl of Iodide Buffer by vigorously tapping the tube. Two hundred and fifty microliters of 96% ethanol was added to each tube, mixed and incubated for 15 minutes at room temperature. Tubes were then centrifuged at 13,000 RPM for 10 minutes at 4^0C and the supernate discarded. The pellet was then washed with 500 microliters of 70% ice-cold ethanol without disturbing the pellet. Tubes were centrifuged at 12,000 RPM for 10 minutes and the supernatant was discarded. The pellet was left to air-dry for 5 to 10 minutes at room temperature and the pellet was then resuspended in 20 µl of 1X TE pH8.0. Ten microliters of each extracted phage clone DNA added to 2 µl of 6x loading Dye (NEB, Cat No. B7021S) and run against 0.5 micrograms of M13 sDNA (N4040S Lot. No. 0130907) in a 0.7% agarose I gel (Figure 28). DNA was sent with sequencing primer (96GIII) to Retrogen, Inc (San Diego, Ca) for sequencing to determine the peptide expressed on each phage coat protein (Figure 29).

3.8 Strategy 2: Phages 7-mer first round panning on CLL patient BCRs

To reduce the possibility of selection for phages binding to goat anti-human IgM antibody, in the "positive selection" plate, used to capture the patient BCRs, a new approach was devised. As shown in Figure 35 and detailed below, for the first round selection, wells were sensitized with goat-anti-human IgM and incubated with 7-mer Phage Display Library (10^{11} phages) to remove any phages binding to the goat anti-human IgM. Patient "E" BCRs were then captured using goat anti-human IgM onto an ELISA plate and then incubated with the subtracted phage library to remove, from the supernate, any phages binding to CLL patient "E" BCRs. Patient "P" BCRS were then captured on the plate using goat anti-human IgM and then incubated with the subtracted phage library coming out patient "E" subtraction well. Unbound phages were washed off and bound phages were eluted, titrated, amplified using *E. coli* ER2738, precipitated and then titrated. In the second round selection, 10^7 amplified phages were incubated in 7 successive goat anti-human IgM subtractions then transferred to patient "E" BCR captured well and then incubated in patient "P" BCRs captured well. Bound phages were eluted, titrated, amplified, precipitated and then titrated for the third round selection. This was repeated again for the third round selection.

3.8.1 Sensitization of wells with anti-human IgM for "Negative Subtraction" plates

Thirty two microliters (32µg) of goat anti-human IgM was added to 768 µl of 0.1 M $NaHCO_3$ pH8.6 to give a concentration of 0.04µg goat anti-human IgM per µl. Wells 1, 2, 3 and 4 where each sensitized with 2 µg goat anti-human IgM in volumes of 50 µl, 100 µl, 150 µl and 200 µl, respectively. Plates were incubated for 1 hour at room

temperature. After 1 hour incubation, Wells 1, 2, 3 and 4 were washed 6 times with TBST in volumes of 50 µl, 100 µl, 150 µl and 200 µl, respectively. Wells were then blocked with 300 µl blocking buffer (0.1 M $NaHCO_3$ pH8.6, 5mg/ml BSA) and incubated at 4^0C overnight.

3.8.2 Phage Display Library 7-mer incubation in "Negative Subtraction" plate

Ten microliters of 7-mer Phage Display Library (10^{11}pfu) was added to 40 µl of TBST. The 50 µl goat anti-human IgM sensitized well was washed 6 times with 50 ul TBST and incubated with the Phage Display library for 1 hour at room temperature. The 100 µl goat anti-human IgM sensitized well was washed 6 times with 100 µl TBST and phages were added to the 50 µl well. After seven subtractions using anti-human IgM sensitized wells, unbound phages were incubated in patient "E" BCR captured wells (using goat anti-human IgM).

3.8.3 Capturing patient "P" BCRs on anti-human IgM "Positive Selection" plate

The 200 µl goat anti-human IgM sensitized well was washed 6 times with 200 µl of TBST and incubated at room temperature, with 200 µl of diluted patient "P" cell lysate after adding 40 µl patient "P" cell lysate to 160 µl TBS.

3.8.4 Subtracted 7-mer phages (I7R1-IN) incubation in "Positive Selection" plate

Patient "P" BCR-bound well was then washed 6 times with 200 µl of TBST and incubated with the phages after their incubation in the 150 µl patient "E" well for 1 hour at room temperature. After incubation of the phages in the patient "P" well, the well was washed 10 times with TBST. Two hundred microliters of Glycine HCL pH 2.2 was added

for 15 minutes to elute the phages bound to patient “P” BCRs. Supernate containing eluted phages were then neutralized with 30 µl of Tris-HCl pH 9.1 and then amplified with 20 ml of *E. coli* at 0.01-0.05 O.D. in a shaker at 265 RMP at 37 ^{0}C for 4 ½ hours, precipitated and the titer determined (*I7R1-Out Amp*).

3.9 Phage Display Library 7-mer second round panning on CLL patient BCRs

3.9.1 Sensitization of wells with anti-human IgM for "Negative Subtraction" plates

Wells were sensitized with goat anti-human IgM (1µg per well) using 17 µl of goat anti-human IgM with 1.683 ml of 0.1M $NaHCO_3$ pH 8.6 resulting in 1ug per 100 µl. One hundred microliters (1µg goat anti-human IgM) of above was then added to each of 7 wells and incubated for 1 hour at room temperature. After 1 hour incubation at room temperature, the wells were washed 6 times with 100 µl TBST and then blocked with 300 µl blocking buffer (0.1 M $NaHCO_3$ pH8.6, 5mg/ml BSA) and incubated overnight at 4^oC.

3.9.2 Capturing patient "E" BCRs on anti-human IgM "Negative Subtraction" plate

Patient "E" well was sensitized with 2 µg (goat anti-human IgM) per 100 µl. 10 µl of patient "E" lysate was added to 40 ul TBS and 50 ul TBST(0.05% Tween-20) and incubated, 1 hour at room temperature, in patient "E" well after washing the well 6 times with TBST.

3.9.3 Phages I7R1-out Amp incubation in "Negative Subtraction" plate

The 1 µg per 100 µl goat anti-human IgM sensitized wells were washed 6 times with TBST and incubated with *I7R1-Out Amp* (10^7 phages in TBST) for 1 hour at room temperature and then transferred from one well into the next for subtraction of all anti-IgM binding phages. After the last incubation of the phages in the 7^{th} anti-human IgM well, the phages were transferred to the patient "E" BCR-captured well and incubated for 1 hour at room temperature.

3.9.4 Capturing patient "P" BCRs on anti-human IgM "Positive Selection" plate

Patient "P" well was sensitized with 2 µg (goat anti-human IgM) per 100 µl. The 100 µl goat anti-human IgM sensitized well was washed 6 times with 100 µl of TBST and incubated at room temperature, with 100 µl of diluted patient "P" cell lysate after adding 40 µl patient "P" cell lysate to 60 µl TBS.

3.9.5 *Subtracted 7-mer phages (I7R2-IN) incubation in "Positive Selection" plate*

Patient "P" well was washed 6 times with TBST after which phages from patient "E" well were transferred into patient "P" well and incubated for 1 hour at room temperature. The well was washed 10 times with 100 µl TBST. After the last wash, 100 µl of Glycine HCl pH 2.2 was added to the well for 15 minutes to elute bound phages and then transferred to a microcentrifuge tube containing 15 µl of Tris-HCl pH 9.1. After neutralization, 15 µl of the phages was kept in an eppendorf tube for titration (*I7R2-OUT*). The rest of the eluted phages were then amplified for 4 ½ hours with 20 ml of *E. coli* at an O.D. of 0.01-0.05, precipitated overnight in PEG and then concentrated in 200µl of TBS (*I7R2-OUT Amp*).

3.10 Phage Display Library 7-mer third round panning on CLL patient BCRs

3.10.1 Phages I7R2-Out Amp incubation in "Negative Subtraction" plate

Wells were sensitized with goat anti-human IgM for "Negative subtraction" plate as described in "Materials and Methods Section 3.9.1". Patient "E" BCRs were captured as described in "Materials and Methods Section 3.9.2"

I7R2-Out Amp (10^7 phages in TBST) were subtracted in 7 successive goat anti-human IgM wells as described in "Section 3.9.3".

3.10.2 Subtracted 7-mer phages (I7R3-IN) incubation in "Positive Selection" plate

Patient "P" BCRs were captured onto "Positive selection" plate as described in "Materials and Methods Section 3.9.4", incubated with *I7R3-IN* as described in "Section 3.9.5", and bound phages eluted and labeled *I7R2-OUT*.

3.2 Results:

Captured BCRs withstand Phage Display Library surface panning conditions: BCR detection before panning

In order to qualitatively determine if BCRs could be captured onto a well in a 96-well plate, the wells were sensitized with goat anti-human IgM and then incubated with either IgM standard or cell lysates from CLL patients "E" or "P". IgM was then detected using *peroxidase*-labeled affinity purified goat anti-human IgM (μ) antibody. As shown in Figure 25, a more intense color change was observed in IgM standard and cell lysates from patients "E" and "P" wells as compared to the negative control wells indicating capture of serum IgM standard or patient BCRs from cell lysates.

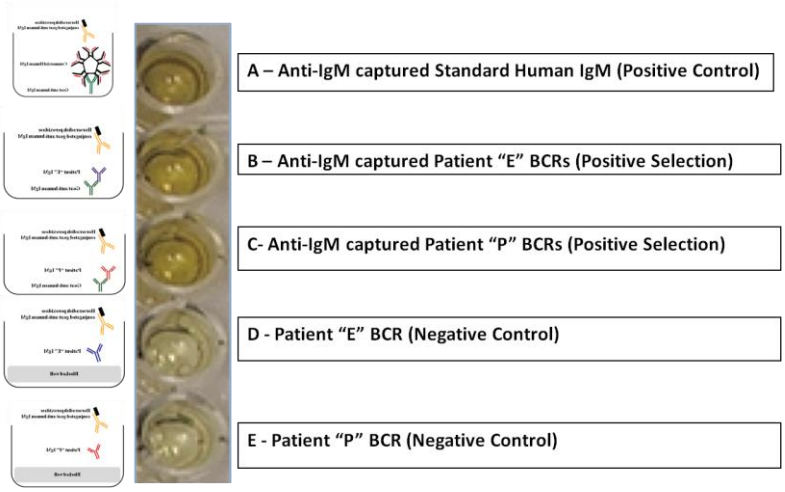


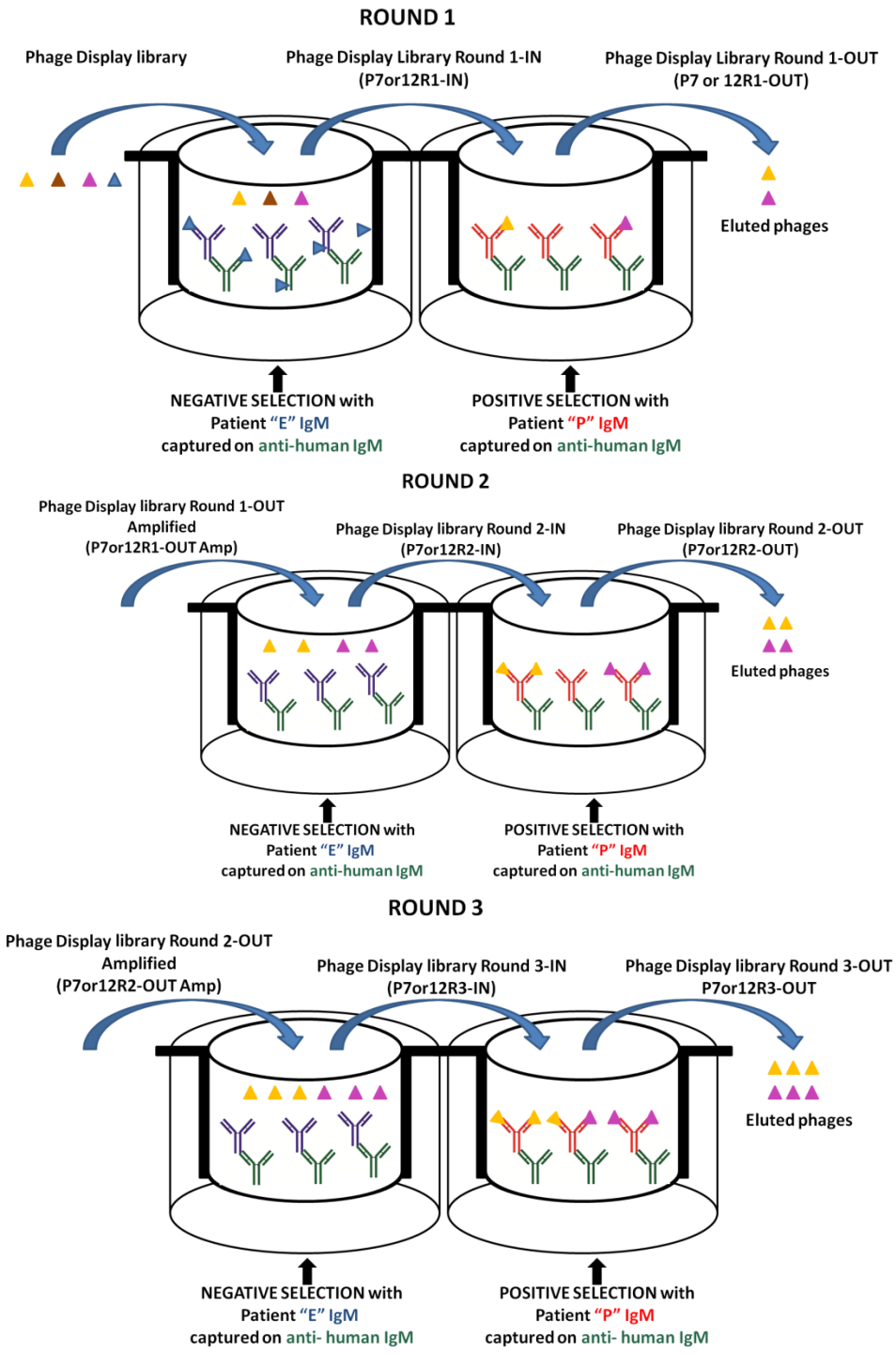
Figure 25. BCR Detection before Phage Display Library Surface Panning. ELISA wells indicating detection of captured BCRs from CLL patients "P" and "E" cell lysates before panning with Phage Display Library. IgM from Human serum (Standardized Human IgM) is used as a positive control.

Selection Strategy 1:

7-mer Phage Display Library first, second and third round panning on CLL patient BCRs

As illustrated in Figure 26, in separate wells, BCRs from CLL patients "P" and "E" were captured using goat anti-human IgM, incubated for 1 hour at room temperature and washed 3 times with 0.05% Tween-20 in TBS. Patient "E" BCR-captured well was used as the "Negative" subtraction plate to remove 7-mer phages binding to regions other than the antigen binding site of the B-cell receptor. Remaining phages were incubated with the "Positive" selection patient "P" BCR-captured well for 1 hour at room temperature and unbound phages were discarded by washing 10 times with TBS (0.05% Tween). BCR-bound 7-mer phages were eluted, amplified and titrated, using *E. coli* 2738 (Figure 27). Three rounds of selection were performed. The ratios of *P7R1- IN*: *P7-R1-OUT* from the first, second and third round selections were determined. As shown in Table 7, the ratio of *P7R1- IN: P7-R1-OUT, P7R2- IN: P7-R2-OUT* and *P7R3- IN: P7-R3-OUT* showed enrichment from the first to the third round selection.

After elution, the phages (*P7R3-OUT*) were amplified, using *E. coli* ER2738, separated from the bacteria by centrifugation and precipitated overnight, using 20% PEG 8000 (Polyethylene Glycol 8000), as described in "Materials and Methods". The Phage-PEG precipitates were pelleted by centrifuging the microcentrifuge tubes at 12000 RPM for 15 minutes at 4^0C and concentrated through resuspension in a final volume of 200 µl. The tube was labeled *P7R3-OUT Amp* and titer determined using *E. coli* ER2738.



<u>Figure 26.</u> Schematic representation of three rounds of panning using Phage Display Peptide Library on CLL Patient membrane-bound IgM (BCRs) from lysates captured on goat anti-human IgM antibody.

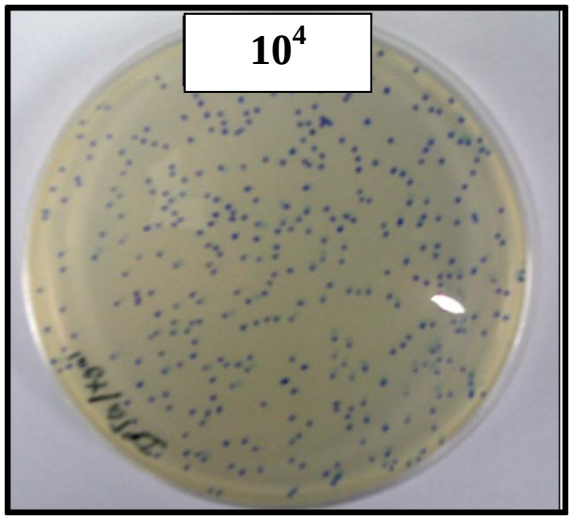


Figure 27. Phage Titering Plate. Plate shows blue plaques from 10^4 dilution of phage stock used to infect *E. coli* ER2738 on IPTG/ Xgal LB plates.

Table 7. Titration of eluted 7-mer phages from Strategy 1

P7 library	Phages/ul	RATIO (IN:OUT)
P7 library	3.00×10^{11}	
P7R1-IN	6.10×10^{8}	
P7RI-OUT	**2.24×10^{2}**	**3.00×10^{6}**
P7R1OUT AMP1	1.83×10^{8}	
P7R2-IN	6.20×10^{8}	
P7R2-OUT	**4.60×10^{4}**	**1.00×10^{4}**
P7R2-OUT AMP1	5.30×10^{9}	
P7R3-IN	1.09×10^{9}	
P7R3-OUT	**1.32×10^{6}**	**8.00×10^{2}**
P7R3-OUT AMP1	7.00×10^{8}	

12-mer Phage Display Library first, second and third round panning on CLL patient BCRs

In separate wells, BCRs from CLL patients "P" and "E" lysates were captured using goat anti-human IgM, incubated 1 hour at room temperature and washed 3 times with 0.05% Tween-20 in TBS. Patient "E" BCR-captured well was used as the "Negative" subtraction plate to remove any12-mer phages binding to regions other than the antigen binding site of the B-cell receptor. Remaining phages were incubated with the "Positive" selection patient "P" BCR-captured well for 1 hour at room temperature and unbound phages were discarded by washing 10 times with TBS (0.05% Tween). BCR-bound 12-mer phages were eluted, titrated and amplified using *E. coli* ER2738. Ratio of *P12R1- IN: P12-R1-OUT* from the first, second and third round selections were determined. Ratios of *P12R1- IN: P12-R1-OUT, P12R2- IN: P12-R2-OUT* and *P12R3-IN: P12-R3-OUT* showed enrichment from the first round to the third round selection (Table 8) but less when compared to 7-mer selection (Table 7).

After elution, the phages (*P12R3-OUT*) were amplified, using *E. coli* ER2738, separated from the bacteria by centrifugation and precipitated overnight, using 20% PEG 8000 (Polyethylene Glycol 8000), as described in "Materials and Methods". The Phage-PEG precipitates were pelleted by centrifuging the microcentrifuge tubes at 12000 RPM for 15 minutes at 4^0C, concentrated through resuspension in a final volume of 200 µl. The tube was labeled *P12R3-OUT Amp* and titer determined using *E. coli* ER2738.

Table 8.Titration of eluted 12-mer phages from Strategy 1.

P12 Library	Phages/ul	RATIO (IN:OUT)
P12 Library	3.00×10^{11}	
P12R1-IN	8.30×10^{7}	
P12R1-OUT	**1.72×10^{3}**	**5.00×10^{4}**
P12R1-OUT AMP1	8.40×10^{9}	
P12R2-IN	2.30×10^{8}	
P12R2-OUT	**1.42×10^{4}**	**2.00×10^{4}**
P12R2-OUT AMP1	7.10×10^{9}	
P12R3-IN	1.19×10^{9}	
P12R3-OUT	**2.73×10^{6}**	**4.00×10^{2}**
P12R3-OUT AMP1	8.10×10^{8}	

Amplification of selected phage clones of first, second and third round 7 and 12-mer selections

The number of plaques on an IPTG/Xgal plates with less than 100 blue plaques were counted for first, second, and third round "OUT". Approximately, 10 of the well-separated blue plaques from each plate (*P7R2-OUT*, *P7R3-OUT*, *P12R2-OUT* and *P12R3-OUT*) were each separately isolated, using a sterile 200 microliter pipette tip and placed in the 2 ml LB tetracycline diluted *E. coli* culture (O.D. 0.01 - 0.05) for amplification. The phage clones were then precipitated, and phage DNA extracted as described in "Materials and Methods". Extracted phage DNA samples were run with M13 sDNA in a 0.7% agarose I gel. As shown in Figure 28, the quantity of the phage DNA in each sample was estimated by using DNA gel electrophoresis with M13 sDNA as the control.

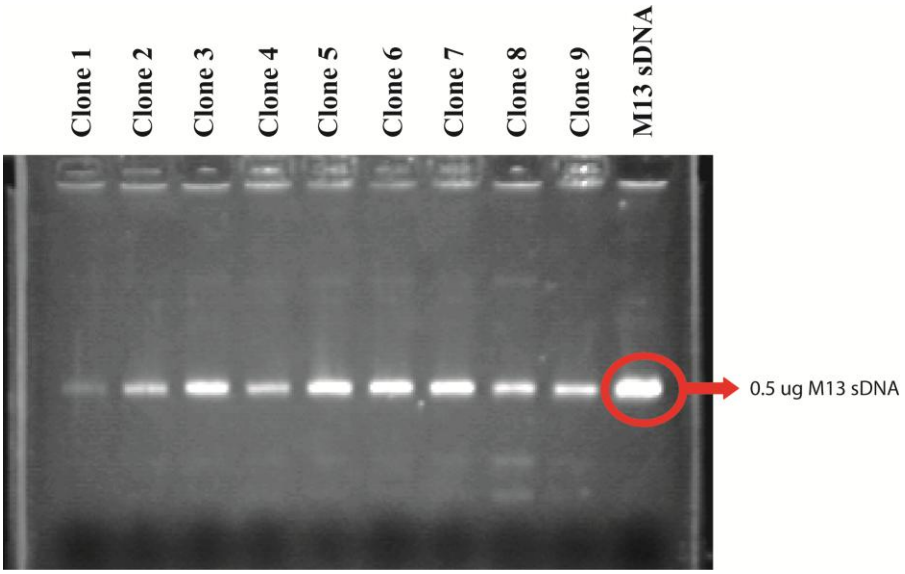
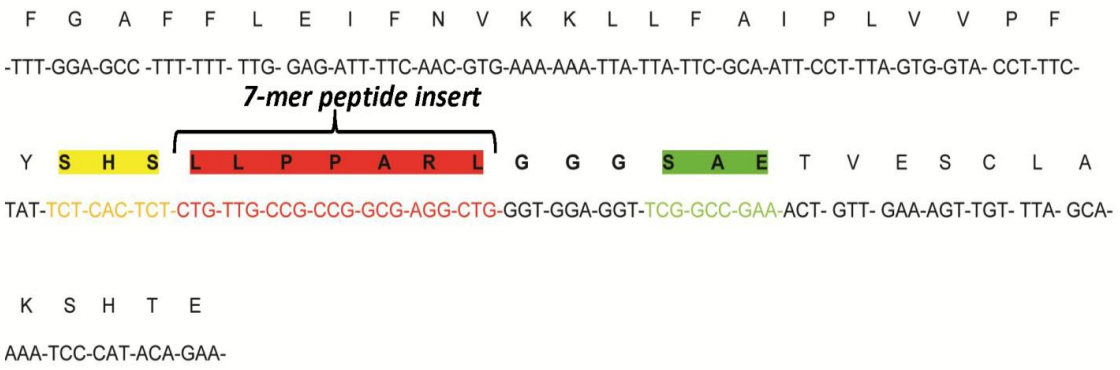


Figure 28. DNA-gel electrophoresis image of extracted sDNA phage from isolated phage clones and M13 sDNA control. M13 sDNA is used as a ladder and semi-quantification of concentration of extracted phage clones sDNA.

The sDNA phage clone samples were sent together with sequencing primer (96GIII) to Retrogen, Inc (San Diego, Ca) for sequence analysis to determine the peptide expressed on each phage coat protein. As shown in Figure 29, the 7-mer or 12-mer peptides are flanked by "SHS" and "GGG-SAE" sequences.

7-mer phage clone



12-mer phage clone

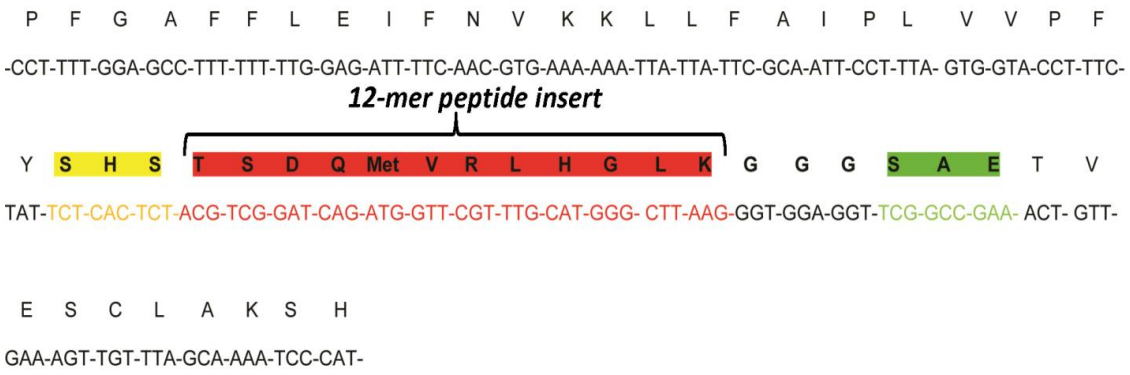


Figure 29. Sequence analysis of sDNA phage clones translated. Translation of a 7-mer and 12-mer phage clone showing sequence of peptide expressed on phage coat protein. Peptide is flanked by "SHS" and "GGG-SAE" sequences. ***Top:*** 7-mer phage clone translation. ***Bottom:*** 12-mer-phage clone translation.

As shown in Table 9a and b, translated sDNA from 7-mer phages of second and third round selections showed a consensus "LLPPAR_" sequence. "LLPPAR_" sequence was also found in sDNA from 12-mer phage clones of second and third round selections (Table 10a and b).

Table 9a. Amino acid sequences of second round isolated 7-mer phages from Strategy 1.

Clone	Amino acid sequence
P7R2 Clone 1-A	S T W T S V I
P7R2 Clone 2-B	L L P P A R L
P7R2 Clone 3-C	Q L L L S P T
P7R2 Clone 4-D	L L P P A R L
P7R2 Clone 5-E	L L P P H R L
P7R2 Clone 6-F	L L S P A R L
P7R2 Clone 7-G	L L P P A R L
P7R2 Clone 8-H	L Q P F Q A R
P7R2 Clone 9-I	L L P P S R L
P7R2 Clone 10-J	L L P P A R I

Table 9b. Amino acid sequences of third round isolated 7-mer phages from Strategy 1.

Clone	Amino acid sequence
P7R3 Clone 2-A	L L S P A R L
P7R3 Clone 3-B	L L P P H R I
P7R3 Clone 4-C	G L T F A X X
P7R3 Clone 5-D*	L L P P A R E
P7R3 Clone 6-E	L L P P H R L
P7R3 Clone 7-F	L L P P A R F
P7R3 Clone 8-G	L L P P H R L
P7R3 Clone 9-H	L L P P A R L
P7R3 Clone 10-I	L L P P A R D
P7R3 Clone 11-J	L L P P A R L

"X" – denotes missing amino acid from difficulty in translating nucleotide sequences.

Table 10a. Amino acid sequences of second round isolated 12-mer phages from Strategy 1.

Clone	Amino acid sequence
P12R2 Clone 1-A	T S D Q M V R L H G L K
P12R2 Clone 2-B	G L L T P H R E T L T T
P12R2 Clone 3-C	L L S P H R F N I Y P E
P12R2 Clone 4-D	W G K A W L I P Y P H S
P12R2 Clone 5-E	L L P P A R E L E N Y N
P12R2 Clone 6-F	V P Y T L L M P H R V T
P12R2 Clone 7-G	G L T F S H P T L Q L R
P12R2 Clone 8-H	L I P P H R D H L G S H

Table 10b. Amino acid sequences of third round isolated 12-mer phages from Strategy 1.

Clone	Amino acid sequence
P12R3 Clone 2–A	L L S P A R C T P Y C V
P12R3 Clone 3-B	S L L Q P A R E Y L A M
P12R3 Clone 4-C	Y L E P A R Q Q V Q R X
P12R3 Clone 5-D	L L P P H R T M G T G X
P12R3 Clone 6-E	A H F P P C C R T S N X
P12R3 Clone 7-F	T L I Q P H R L Q Q L X
P12R3 Clone 8-G	S L L P P H R L H Y S F
P12R3 Clone 9-H	Q Q P V S P A D L F I X
P12R3 Clone 10-I	N L L P P A R L Q L A X
P12R3 Clone 11-J	A S H D E I W L X V X X

"X" – denotes missing amino acid from difficulty in translating nucleotide sequences.

Concentration of amplified phage clone "LLPPARE" by phage ELISA

Phage Clone "LLPPARE" and M13 were amplified and relative titers determined. As illustrated in Figure 30A and B, serial dilutions of P7R3 Clone 5-D (LLPPARE) and M13 phages starting with 1.25 x 10^9 pfu in $NaHCO_3$ pH 8 were prepared and used to sensitize ELISA wells. The plate was incubated for 1 hour at room temperature, washed 6 times, blocked with blocking buffer and incubated overnight at 4^0C. All wells were washed 10 times and incubated with biotinylated mouse anti-M13 antibody and then with streptavidin horseradish peroxidase (SHRP) biotin. 1-Step™ Ultra TMB-ELISA substrate was added to each well and the reaction stopped using 1M sulfuric acid. The plate was read at 450 nm. As shown in Figure 30 C, amplified "LLPPARE" and M13 had same titers and could be used to determine binding to patient-captured BCRs.

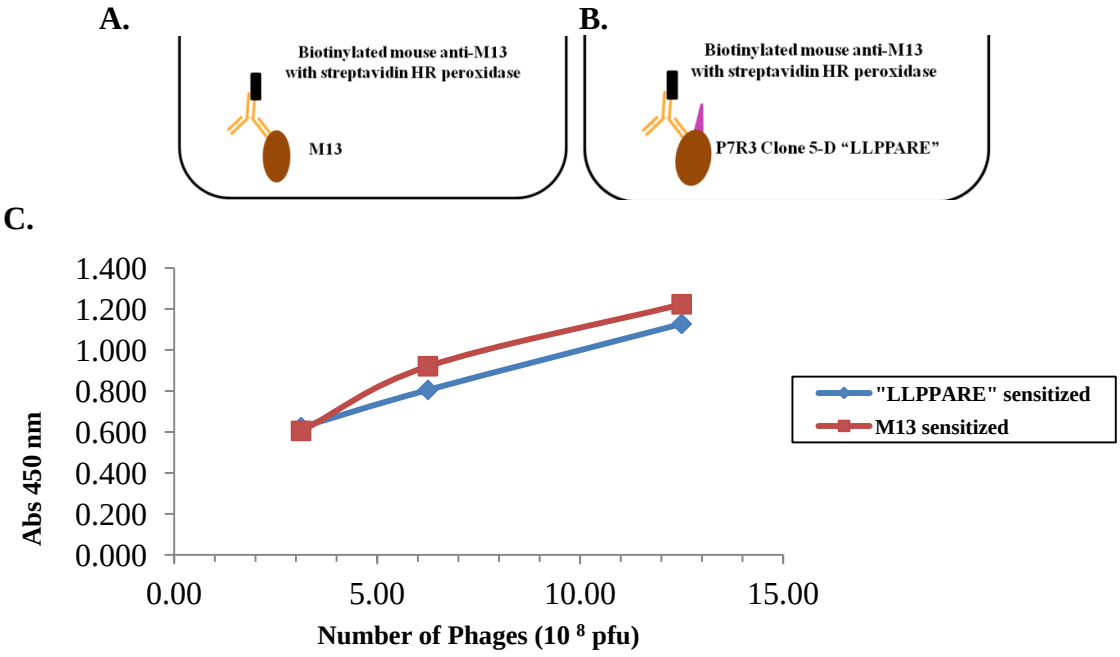
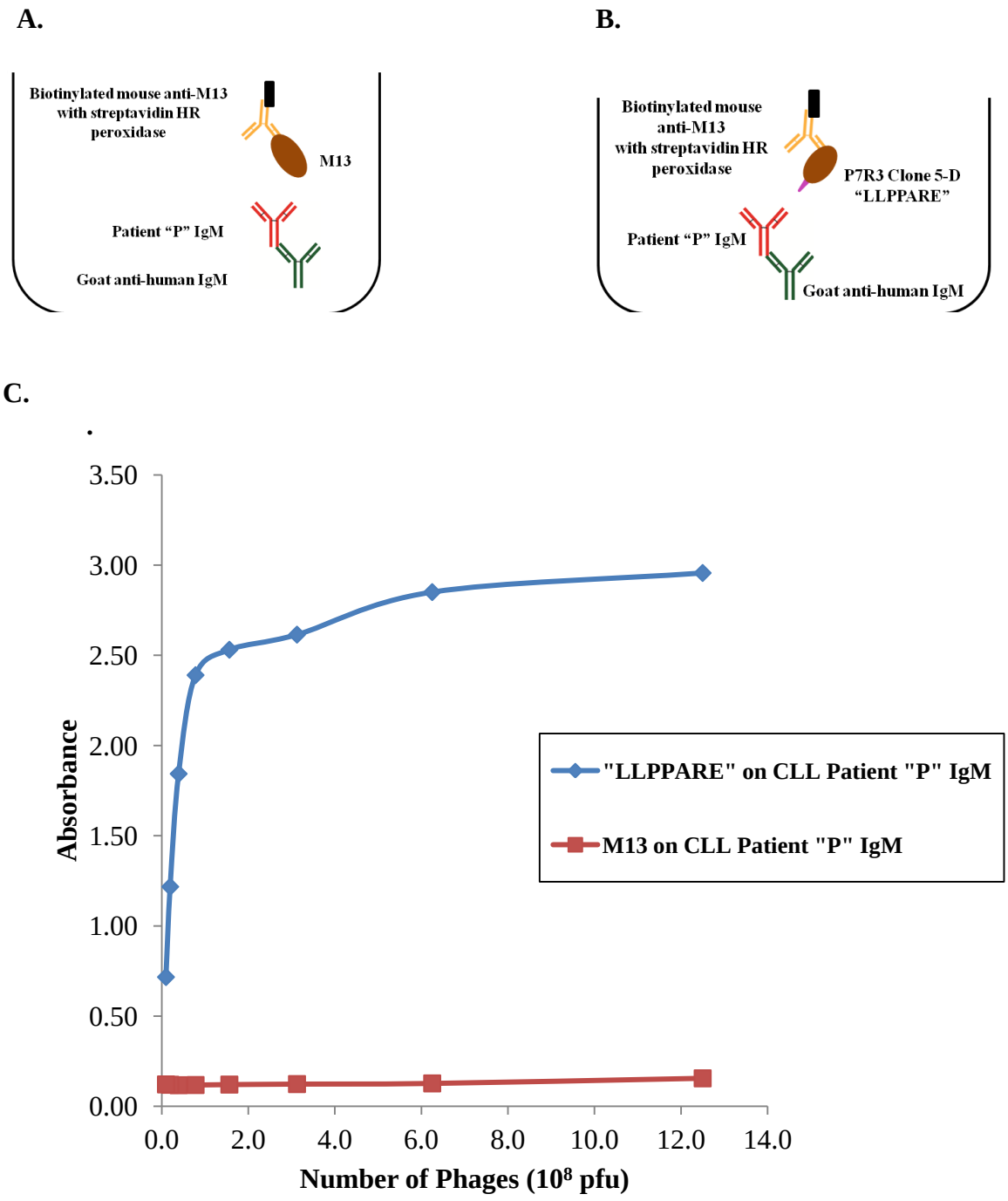


Figure 30. Concentration of Amplified Phage Clone "LLPPARE" by Phage ELISA. ***A*** and ***B***. Diagrams showing wells to detect serially diluted phage clone M13 and "LLPPARE", respectively. ***C***. Graph showing relative concentrations of "LLPPARE" and M13 wildtype phages.

Using P7R3 clone 5-D "LLPPARE" on CLL patient "P" BCR

As illustrated in Figure 31 A and B, wells in an ELISA plate were sensitized with 1.2 µg goat anti-human IgM per well. The ELISA plate was incubated for 1 hour at room temperature, washed 6 times and blocked with blocking buffer and incubated overnight at 4^0C. CLL patient "P" BCR were captured on goat anti-human IgM sensitized plate, washed and incubated, for 1 hour at room temperature, with serial dilutions of P7R3Clone 5-D (LLPPARE) or M13 phages starting with 1.25×10^9 pfu. All wells were washed 10 times and incubated with biotinylated mouse anti-M13 antibody and then with streptavidin horseradish peroxidase (SHRP) biotin. 1-Step™ Ultra TMB-ELISA substrate was added to each well and incubated at room temperature , reaction stopped using 1M sulfuric acid and read at 450 nm using an ELISA plate reader. As shown in Figure 31C, "LLPPARE" shows higher binding to patient "P" BCR-captured well as compared to the M13 phages. However, it is not clear whether "LLPPARE" binds to CLL patient "P" BCR or goat anti-human IgM in the wells.



<u>Figure 31</u>. "LLPPARE" binds Patient "P" BCRs captured using goat anti-human IgM. ***A*** and ***B.*** Diagrams showing detection method for binding of phages by incubating phage clone M13 and "LLPPARE" on patient "P" BCRs which were captured onto goat anti-human IgM sensitized wells. ***C.*** Graph showing the absorbance values of the Phage ELISA to investigate binding of serial dilutions of "LLPPARE" and M13 phage clones against CLL patient "P" BCRs which were captured using goat anti-human IgM.

Does "LLPPARE" bind to goat anti-human IgM antibody or patient BCR?

As illustrated in Figure 32A, B, C and D, wells were sensitized with goat anti-human IgM (1.2 µg), blocked and incubated at 4^0C overnight. Wells were washed 6 times and incubated with serial dilutions of IgM from human serum, CLL patients "P" and "E" cell lysates and as a control some wells were left without Human IgM. 1.25×10^9 pfu of phage clone "LLPPARE" was added to each well and incubated for 1 hour at room temperature. Wells were washed 10 times, incubated with biotinylated mouse anti-M13 and streptavidin horseradish peroxidase. 1-Step™ Ultra TMB-ELISA substrate was added to each well incubated at room temperature, reaction stopped using 1M sulfuric acid and read at 450 nm using an ELISA plate reader and graphed. As shown in Figure 32E, there was no change in using serial dilutions of Human IgM, CLL patients "E" or "P" lysates, as compared to the control wells which did not receive IgM of any kind. "LLPPARE" binds to the goat anti-human IgM wells at the same absorbance as the serum IgM or BCR-captured wells, suggesting that "LLPPARE" binds goat anti-human IgM antibody. After performing a database search of "LLPPARE", it was found to be located on the hydrophilic region of the CH4 domain of the human IgM molecule (Figure 34). "LLPPARE" was therefore selected for by the goat anti-human IgM antibodies which were used to capture patient BCRs onto the wells.

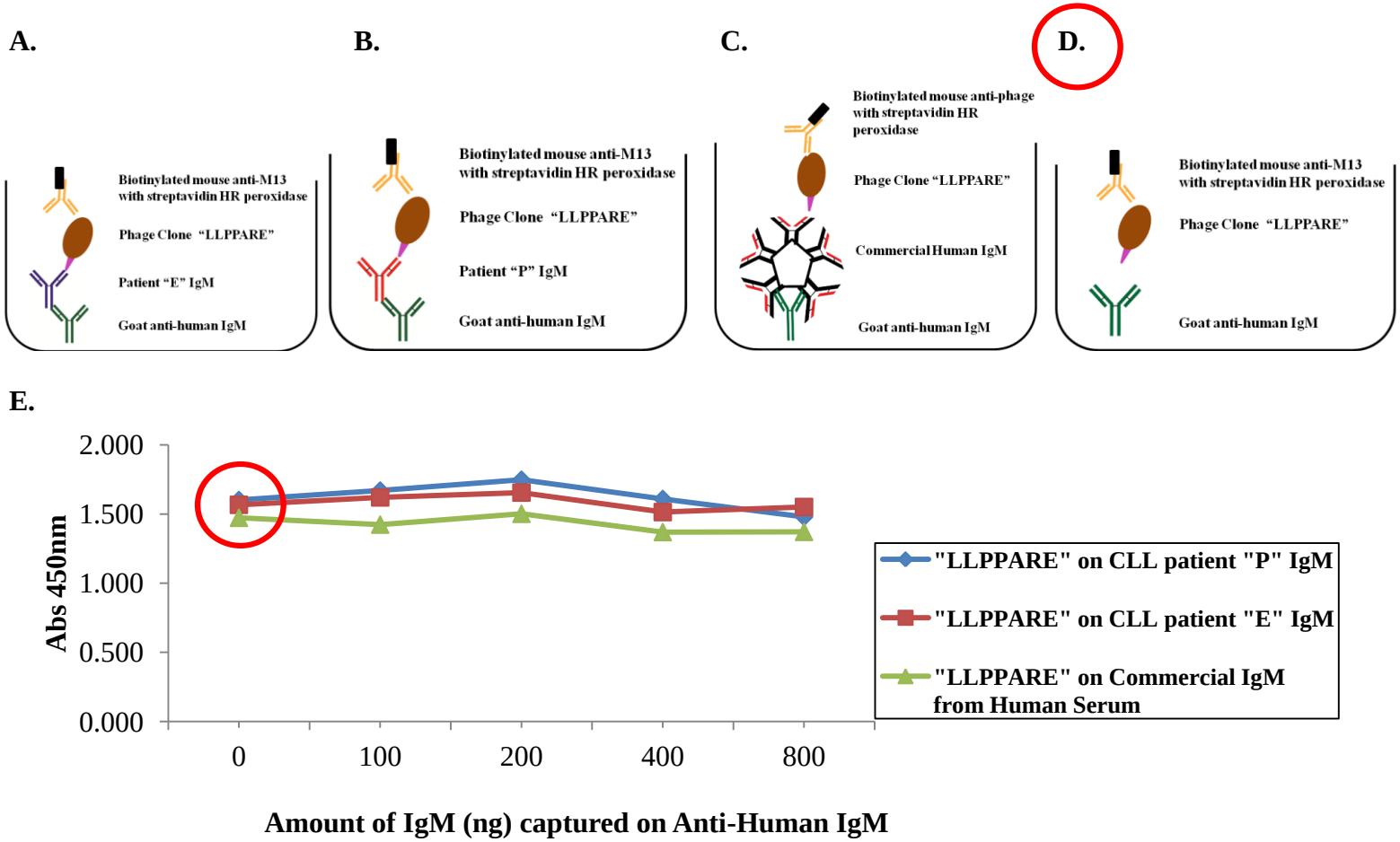


Figure 32. "LLPPARE" binds goat anti-human IgM. ***A, B, C*** and ***D.*** Diagrams showing the detection, using biotinylated anti-phage antibody, of phage clone "LLPPARE" binding to patient "P", "E"BCRs and Human IgM (captured onto goat anti-human IgM sensitized wells) and goat anti-human IgM, respectively. ***E.*** Graph showing the absorbance from incubation of "LLPPARE" (1.25 x 10^9 pfu) phages onto dilutions of BCRs from patient "E", "P" lysates and Human standard captured using goat anti-human IgM.

As a control to detect the presence of serum IgM or patient BCRs in dilution, used in the above experiment, wells were sensitized with 1.2 µg of anti-human IgM and incubated with the same dilutions of serum IgMs or BCRs used for the phage wells to detect the presence of IgM within the prepared lysates. Wells were incubated with the IgMs, washed 6 times and incubated with goat anti-IgM HRP. IgMs were detected using goat anti-human IgM peroxidase starting with patient "P", "E" and Human IgM wells. As shown in Figure 33, the presence of IgM captured using goat anti-human IgM was confirmed to be present in serial dilutions of lysates and human serum IgM .

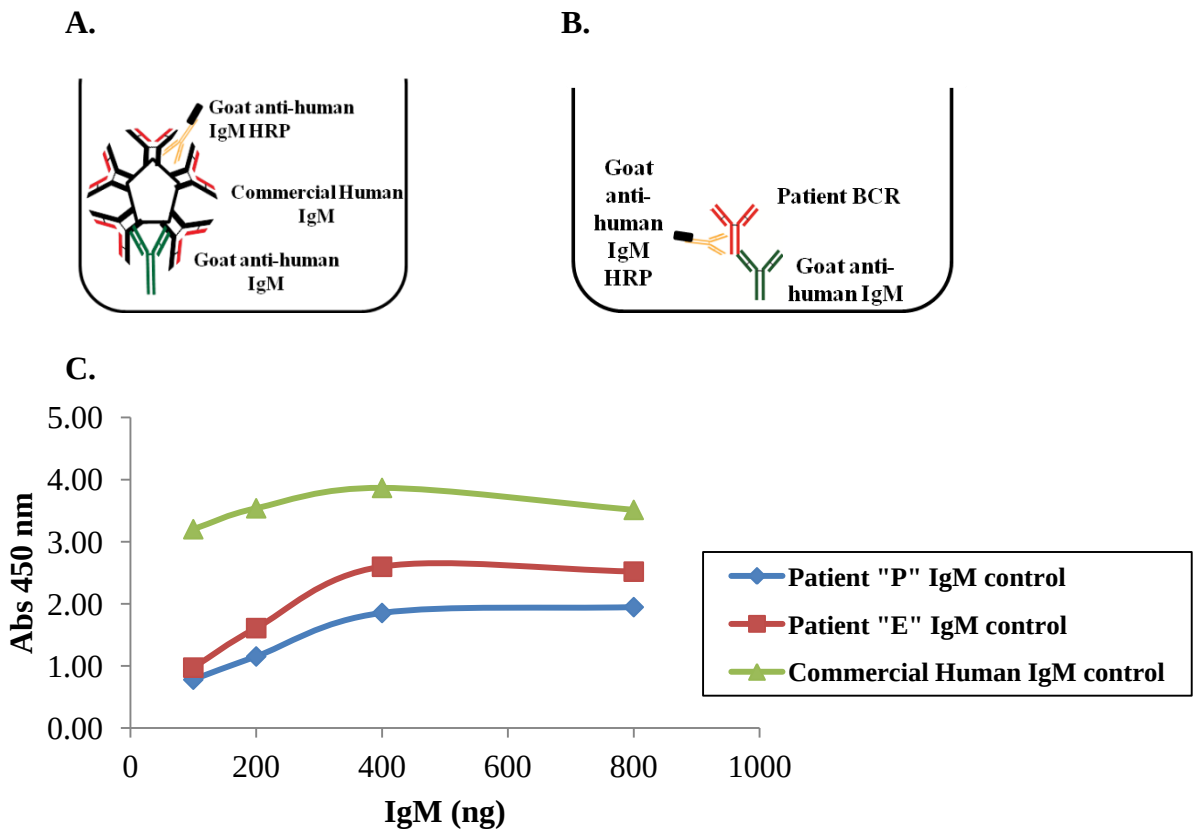


Figure 33. IgM detection within IgM Standard from human serum and BCR detection within CLL patients "P" and E" cell lysates. ***A*** and ***B***. Diagrams showing IgM detection within serum IgM and patients "E" and "P" cell lysates used in testing the binding of "LLPARE" clone. ***C.*** Graph shows binding from serially diluted IgM from human serum, patients "P" and E" cell lysates onto goat anti-human IgM.

ProtScale

User-provided sequence:

```
        10          20          30          40          50          60
GVALHRPDVY LLPPAREQLN LRESATITCL VTGFSPADVF VQWMQRGQPL SPEKYVTSAP

        70          80          90
MPEPQAPGRY FAHSILLTVS EEEWNTGETY TCV
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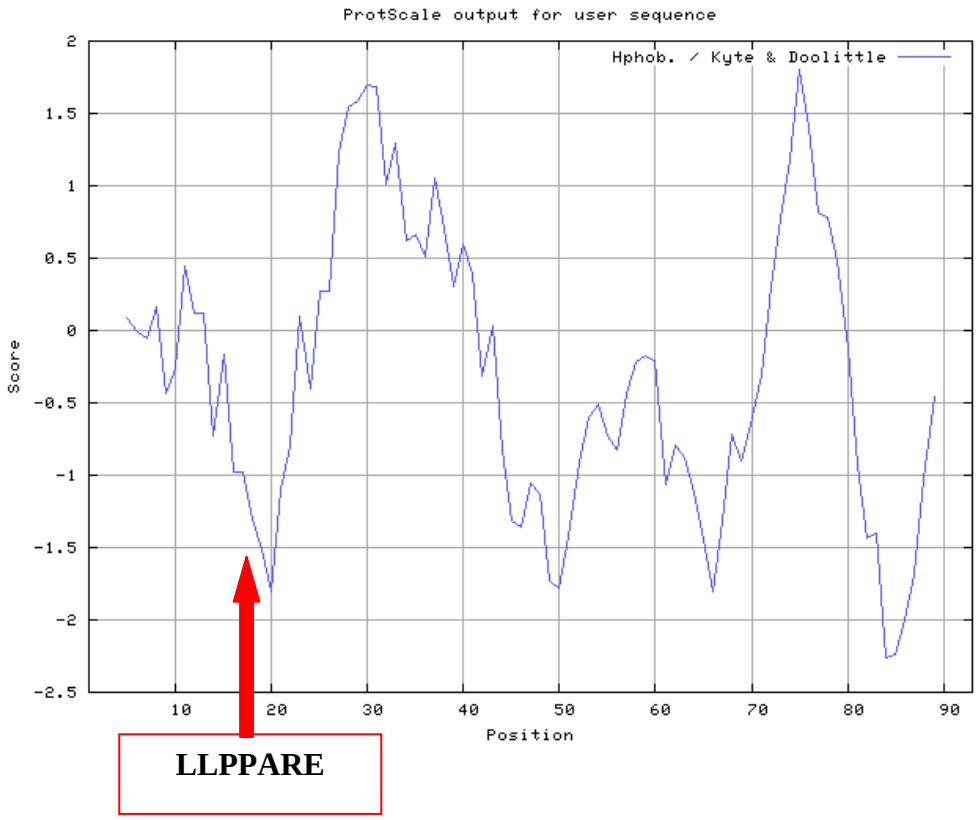


Figure 34. Hydrophobicity plot of "LLPPARE" sequence in CH4 region of human IgM molecule (Fc region). Diagram shows hydrophobicity of CH4 region of Human IgM using Kyte & Doolittle (http://web.expasy.org/cgi-bin/protscale/protscale.pl?1)

Selection Strategy 2:

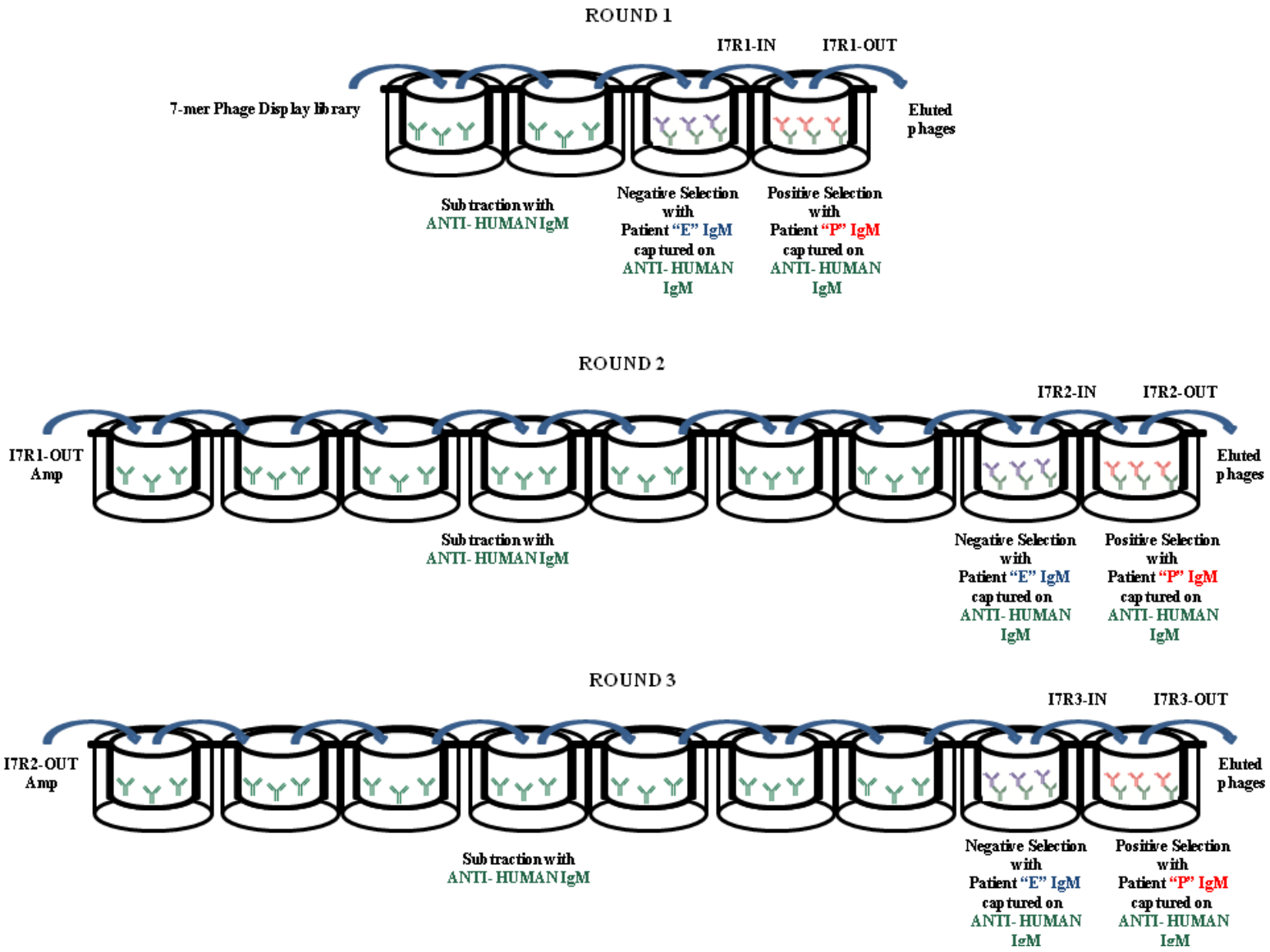
7-mer Phage Display Library three rounds of panning on CLL patient BCRs

In order to reduce the possibility of phages binding to goat anti-human IgM antibody used to capture the patient BCRs, a new approach was devised. As shown in Figure 35, for the first round selection, wells were sensitized for 1 hour at room temperature with 2 μg goat-anti-human IgM in $NaHCO_3$ pH 8.6, washed 6 times and blocked overnight at 4^0C. 7-mer Phage Display Library (10^{11} phages) in TBST (0.05% Tween-20) was incubated with 2 goat anti-human IgM sensitized wells to remove any phages binding to the goat anti-human IgM. Patient "E" BCRs were captured using goat anti-human IgM and then incubated with the subtracted phage library to remove, from the supernatant, any phages binding to CLL patient "E" BCRs. Patient "P" BCRS were then captured on the plate using goat anti-human IgM and then incubated with the subtracted phage library coming out patient "E" subtraction well (*I7R1-IN*). Phages were incubated, in patient "P" well, for 1 hour at room temperature and then unbound phages were washed off using 10 washes with TBST (0.05% Tween-20). Bound phages were eluted, titrated (*I7R1-OUT*), amplified using *E. coli* 2738, precipitated and then titrated (*I7R1-Out Amp*) for the second round selection.

In the second round selection, 7 wells were sensitized with 1 μg goat anti-human IgM in 0.1M $NaHCO_3$ pH 8.6 for 1 hour at room temperature and then blocked overnight at 4^0C. *I7R1-Out Amp* (10^7 phages in TBST 0.5% Tween-20) was incubated for 1 hour at room temperature and then transferred from one well into the next for 7 successive goat anti-human IgM subtraction of all anti-IgM binding phages. After the last incubation of

the phages in the 7th anti-human IgM well, the phages were transferred to patient "E" BCR well captured using goat anti-human IgM and then incubated for 1 hour at room temperature. Patient "P" BCRs were then captured on the plate using goat anti-human IgM and then incubated with the subtracted phage library coming out patient "E" subtraction well (*I7R2-IN*). Phages were incubated, in patient "P" well, for 1 hour at room temperature and then unbound phages were washed off using 10 washes with TBST (0.05% Tween-20). Bound phages were eluted, titrated (*I7R2-OUT*), amplified using *E. coli* 2738, precipitated and then titrated (*I7R2-Out Amp*) for the third round selection.

In the third round selection, 7 wells were sensitized with 1 μg goat anti-human IgM in 0.1M $NaHCO_3$ pH 8.6 for 1 hour at room temperature and then blocked overnight at 4^0C. *I7R2-Out Amp* (10^7 phages in TBST 0.5% Tween-20) was incubated for 1 hour at room temperature and then transferred from one well into the next for 7 goat anti-human IgM subtraction of all anti-IgM binding phages. After the last incubation of the phages in the 7th anti-human IgM well, the phages were transferred to patient "E" BCR well captured using goat anti-human IgM and then incubated for 1 hour at room temperature. Patient "P" BCRs were then captured on the plate, using goat anti-human IgM, and then incubated with the subtracted phage library coming out of patient "E" subtraction well (*I7R3-IN*). Phages were incubated in patient "P" well for 1 hour at room temperature and then unbound phages were washed off using 10 washes with TBST (0.05% Tween-20). Bound phages were eluted and titrated (*I7R3-OUT*). As shown in Table 11a, there was enrichment between second round and third round selection titers. First round selection could not be used in the comparison because of the high number of phages (10^{11} pfu) used in the selection.



<u>Figure 35.</u> Strategy 2 schematic representation of three rounds of panning using 7 Phage Display Library on CLL Patient BCRs captured on goat anti-human IgM antibody.

Table 11a. Titration of eluted 7-mer phages from Strategy 2 phage selection.

P7 library	Phages/ul	RATIO (IN:OUT)
I7R1-IN	8.30×10^8	
I7RI-OUT	**2.60×10^3**	**3.19×10^5**
I7R1OUT AMP1	4.60×10^9	
I7R2-IN	1.50×10^5	
I7R2-OUT	**8.30×10^1**	**1.81×10^3**
I7R2-OUTAMP1	5.50×10^8	
I7R3-IN	1.10×10^5	
I7R3-OUT	**4.60×10^2**	**2.39×10^2**

Amplification of selected clones of third round 7-mer selection

The number of plaques on an IPTG/Xgal plates with less than 100 blue plaques were counted for the third round "OUT". Approximately, 10 of the well-separated blue plaques from each plate (*I7R3-OUT*) were each separately isolated, using a sterile 200 microliter pipette tip and placed in the 2 ml LB tetracycline diluted *E. coli* culture (O.D. 0.01 - 0.05) for amplification. The phage clones were then precipitated, and phage DNA extracted as described in "Materials and Methods". Extracted phages were run with M13 sDNA in a 0.7% agarose I gel. The sDNA phage clone sample were sent together with sequencing primer (96GIII) to Retrogen, Inc (San Diego, Ca) for sequence analysis to determine the peptide expressed on each phage coat protein.

As shown in Table 11b, the translated sDNA from 7-mer phage clones of the third round selection showed a consensus "GFTFMPA" sequence and two other sequences "QSRPLLP" and "GLPCCSS".

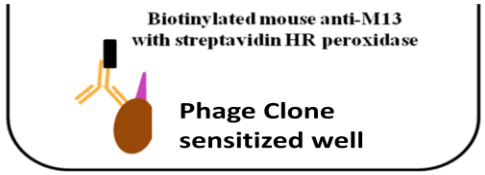
Table 11b. Amino acid sequences of Third Round isolated 7-mer phages from Strategy 2.

Name	Amino acid sequence
I7R3 Clone 1-A	GFTFMPA
I7R3 Clone 2-B	GFTFMPA
I7R3 Clone 3-C	GFTFMPA
I7R3 Clone 4-D	GFTFMPA
I7R3 Clone 5-E	QSRPLLP
I7R3 Clone 6-F	GLPCCSS
I7R3 Clone 7-G	GFTFMPA
I7R3 Clone 8-H	GFTFMPA
I7R3 Clone 9-I	GFTFMPA
I7R3 Clone 10-J	GFTFMPA

Determining concentrations of amplified clones selected in Strategy 2 before testing their binding to goat anti-human IgM

Before proceeding to test binding of phage clones "GFTFMPA", "QSRPLLP", "GLPCCSS", and "LLPPARE", their relative concentrations were determined. As illustrated in Figure 36A, starting with 10^{10} phages, the phage clones were serially diluted (2-fold), used to sensitize an ELISA well, incubated for 1 hour at room temperature, washed and blocked overnight at 4^0C. The ELISA plate was then washed 10 times with TBST and then incubated with biotinylated mouse anti-phage antibody and then streptavidin peroxidase. TMB-ELISA substrate was added to each well, incubated at room temperature, the reaction stopped using 1M H_2SO_4 and absorbance read at 450 nm. Phage clones (GFTFMPA, QSRPLLP, GLPCCSS, and LLPPARE) dilution optical densities were comparable (Figure 36B) and used in anti-IgM binding determination experiment.

A.



B.

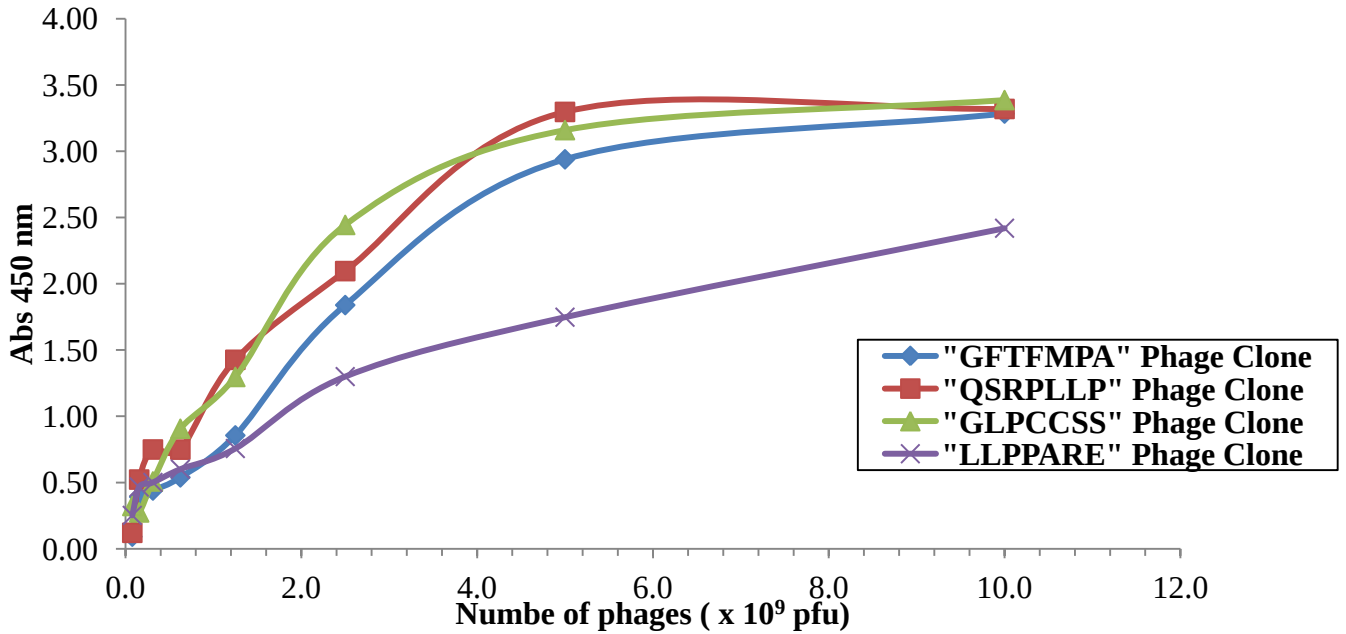
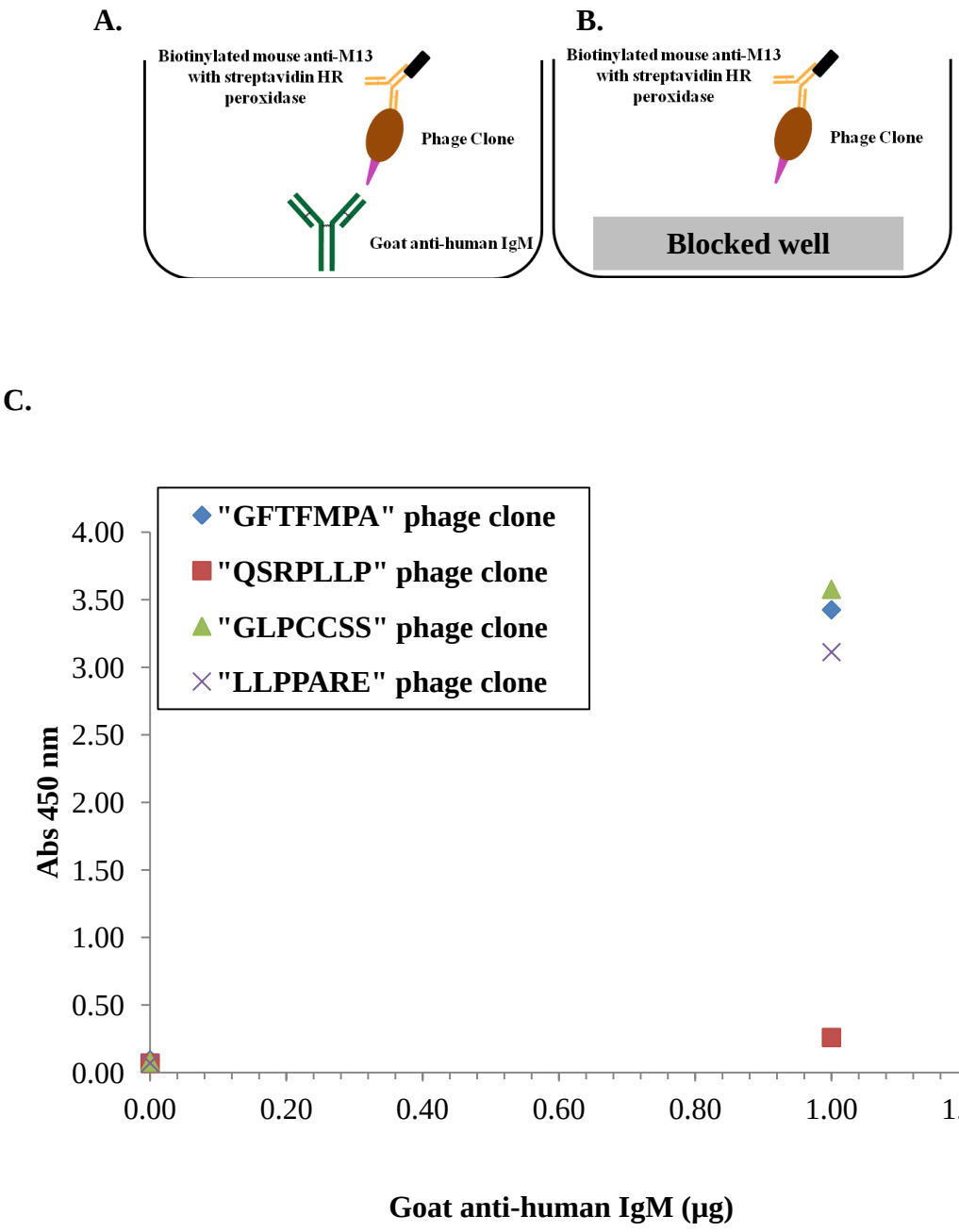


Figure 36. Concentration of Amplified Phage clones "GFTFMPA", "QSRPLLP", "GLPCCSS" and "LLPPARE" by Phage ELISA. ***A.*** Diagram showing detection method for relative concentrations of phage clones. ***B.*** Graph showing the relative concentrations of Phage clones "GFTFMPA", "QSRPLLP", "GLPCCSS" and "LLPPARE".

Determining whether clones selected in Strategy 2 bind to goat anti-human IgM

As illustrated in Figure 37A, ELISA wells were sensitized with 1 μg goat anti-human IgM per well and incubated at room temperature for one hour. Wells were washed 6 times with TBST and blocked with blocking buffer (0.1M $NaHCO_3$ pH 8.6, 5 mg/ml BSA) overnight at 4^0C. For a negative control, empty wells received no goat anti-human IgM but were blocked overnight at 4^0C (Figure 37B). Wells were then washed and incubated with 10^{10} pfu of each Phage clone "GFTFMPA", "QSRPLLP", "GLPCCSS", or "LLPPARE". The plate was incubated for 1 hour at room temperature, washed 10 times with TBST, incubated with mouse anti-phage biotin with final addition of streptavidin peroxidase. TMB-ELISA substrate was added to each well, incubated at room temperature, the reaction stopped using 1M sulfuric acid and absorbance read at 450 nm.

As shown in Figure 37C, phage clones "GFTFMPA", "GLPCCSS" and "LLPPARE" each showed higher binding to goat anti-human IgM. In contrast, phage clone "QSRPLLP" did not show higher binding to the goat anti-human IgM antibody and was similar to the negative control absorbance for non-specific binding (Figure 37C). Background optical density of each clone was below absorbance value of 0.5 O.D indicating that the optical densities obtained for "GFTFMPA", "GLPCCSS" and "LLPPARE" was due to binding to goat anti-human IgM. In addition, "LLPPARE" phage clone was used as a control and showed binding to the goat anti-human IgM.



<u>Figure 37.</u> "GFTFMPA", "GLPCCSS" and "LLPPARE" Phage Clones Bind to Goat Anti-Human IgM. ***A.*** Phage clones serially diluted and incubated with goat-anti human IgM to test binding. ***B.*** As a negative control, the phage clones were only incubated with blocked well to determine non-specific binding. ***C.*** Graph showing the binding from incubation of 10^{10} pfu phages clones "GFTFMPA", "QSRPLLP", "GLPCCSS" and "LLPPARE" with goat anti-human IgM sensitized wells.

Using IgM from human serum captured on anti-human IgM to determining binding of selected clones "GFTFMPA", "QSRPLLP" and "LLPPARE"

To determine whether selected phage clones "GFTFMPA", "QSRPLLP" and "LLPPARE" bind to IgM standard from human serum, and ELISA was performed as illustrated in Figure 38A and B. ELISA wells were sensitized with 1 µg of goat anti-human IgM, washed and blocked overnight at 4^0C. Wells were then incubated with 2-fold serial dilutions of human IgM standard (starting with 800 ng). Wells were then incubated with each phage clone (1.25×10^9pfu per well) for 1 hour at room temperature and washed 10 times with TBST and then incubated with biotinylated mouse anti-phage antibody followed by streptavidin horseradish peroxidase. 1-Step™ Ultra TMB-ELISA substrate was added to each well, incubated at room temperature, stopped with 1M sulfuric acid and absorbance read at 450 nm. As shown in Figure 38C, "GFTFMPA" and "LLPPARE" showed higher binding at an optical density of 3.764 to Human IgM captured using goat anti-human IgM and this could be the due to the binding of these phage clones to goat anti-human IgM. However, "QSRPLLP" showed no binding to the captured IgM with a maximum optical density of 0.359. This shows that "QSRPLLP" does not bind to IgM from human serum.

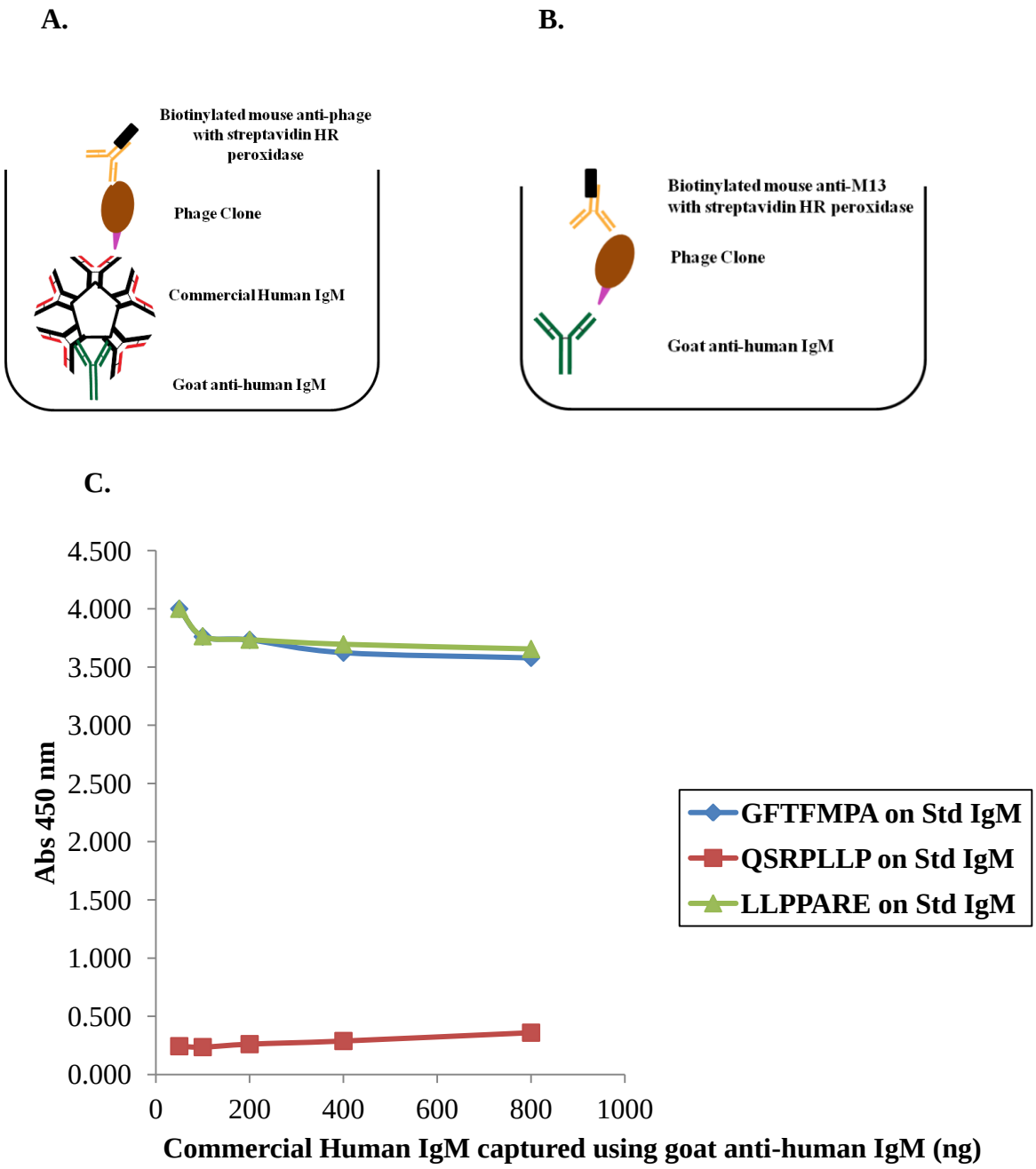


Figure 38. "QSRPLLP" does not bind Serum IgM. ***A.*** Diagram showing the order of goat-anti-human IgM, IgM from human serum and phage clones "GFTFMPA", "QSRPLLP" and "LLPPARE". ***B***. Binding of phage clones to goat anti-human IgM wells with no IgM present ***C.*** Graph showing absorbance from binding of Clones "GFTFMPA", "QSRPLLP" and "LLPPARE" on serially diluted human IgM standard captured using goat anti-human IgM.

Binding of phage clone "QSRPLLP" to patients "P" and "E" BCRs captured on goat anti-human IgM

AS illustrated in Figure 39A and B, binding of Phage Clone "QSRPLLP" was tested against CLL patients "P" and "E" BCRs which were captured onto an ELISA plate using goat anti-human IgM. Binding was determined by using 2-fold serial dilutions of patients "P" and "E" BCR-containing lysates captured on 1 µg goat anti-human IgM. Wells were washed and incubated with "QRSPLLP" phage clone (1.25×10^9pfu per well) for 1 hour at room temperature, washed, incubated with biotinylated mouse anti-phage antibody and then incubated with streptavidin horseradish peroxidase. 1-Step™ Ultra TMB-ELISA substrate was added to each well, incubated at room temperature, stopped with 1M sulfuric acid and absorbance read at 450 nm. As shown in Figure 39C, "QSRPLLP" phage clone showed the same optical density from binding to CLL patient "P" and "E" BCRs captured wells with a maximum optical density of 0.78 O.D. with both CLL patients. As a control, "QSRPLLP" phage clone was incubated with goat anti-human IgM sensitized well only. The results showed that "QSRPLLP" was binding to BCR of Patients "E" and "P" and not the result of background binding.

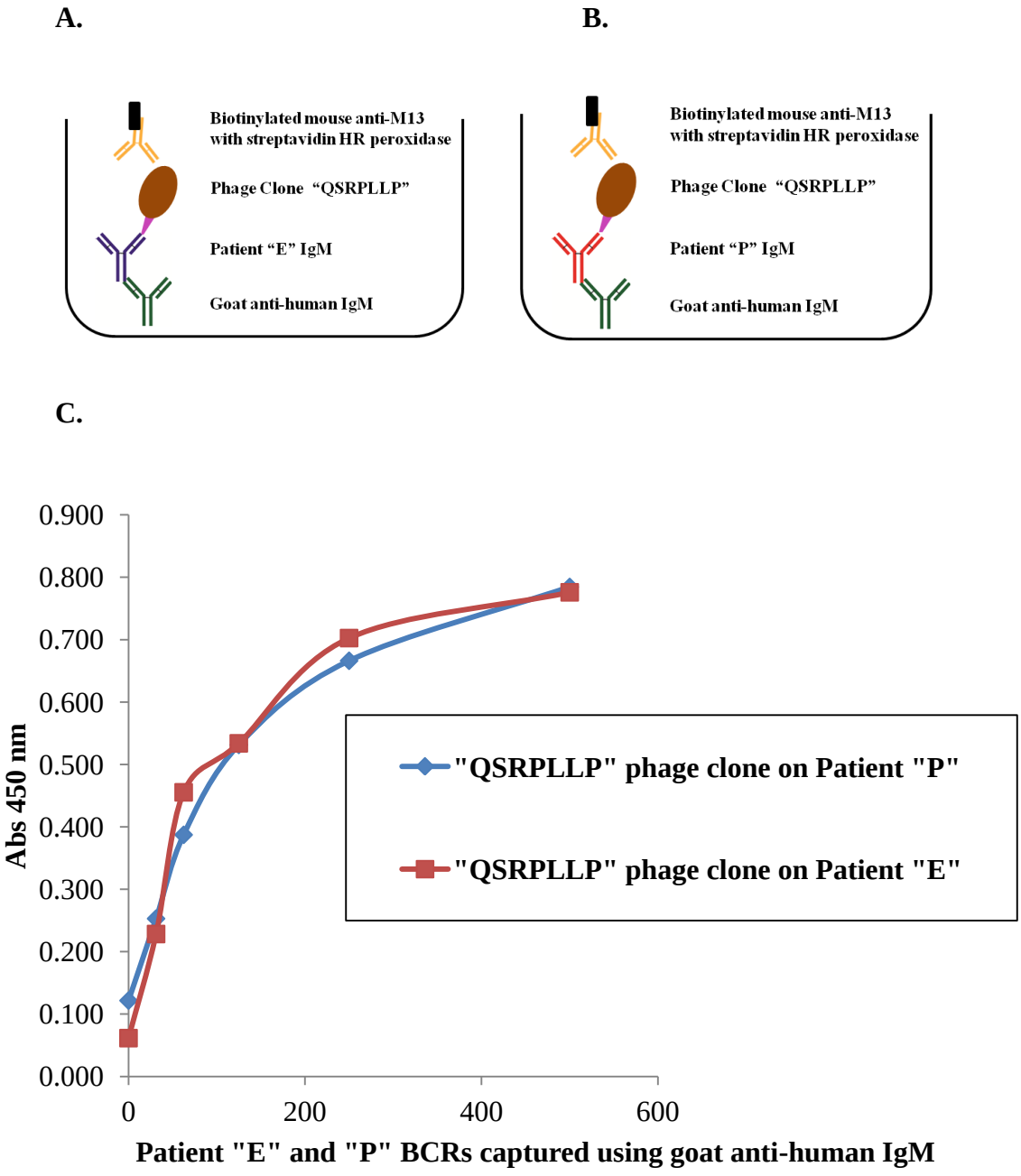


Figure 39. Binding of Phage Clone "QSRPLLP" to Patients "P" and "E" BCRs captured using goat anti-human IgM antibody. ***A*** and ***B.*** Diagrams showing the order of goat-anti-human IgM, patients "E" or "P" BCRs and phage clones on an ELISA plate used to determine binding of "QSRPLLP" clone on patients "E" and "P" BCRs captured using goat anti-human IgM. ***C.*** Graph showing absorbance from binding of Phage Clone "QSRPLLP" on serially diluted patient "E" and "P" BCRs captured using goat anti-human IgM**.**

As illustrated in Figure 40A, BCR concentration within CLL patients "E" and "P" lysates were also determined by capturing BCRs from serially diluted patient lysates, using goat anti-human IgM and then incubated with horseradish peroxidase labeled goat ant-human IgM. 1-Step™ Ultra TMB-ELISA substrate was added to each well, incubated at room temperature, stopped with 1M sulfuric acid and read at 450 nm. As shown in Figure 40B, concentrations of BCRs present in CLL patients "E" and "P" lysates decreased with increased dilution of lysates. In addition, both had an initial maximum optical density of 3.7.

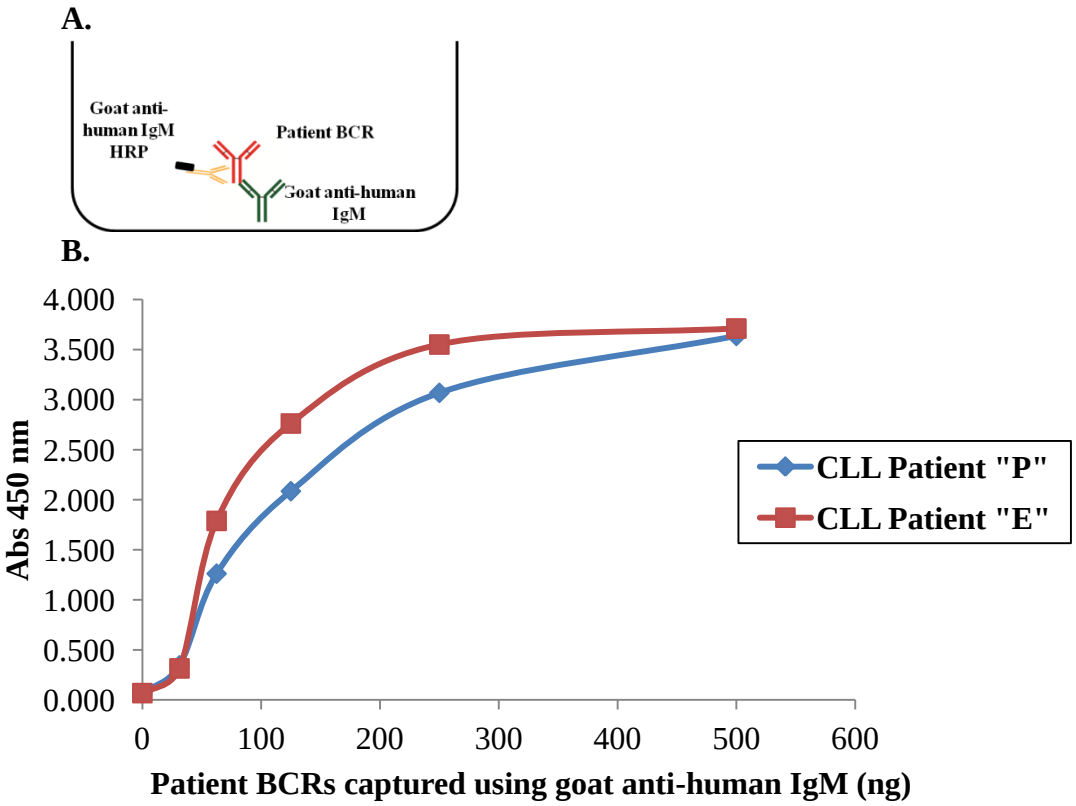


Figure 40. Detection of CLL Patients "P" and "E" BCRs captured using goat anti-human IgM. ***A.*** Schematic representation of ELISA used to determine presence of BCRs within lysate used to determine binding of "QSRPLLP" phage clone. ***B.*** CLL patients "P" and "E" lysates serially diluted, and their BCRs captured onto an ELISA plate using goat anti-human IgM and then detected using horseradish peroxidase-conjugated goat anti-human IgM.

Identification of apoptotic cells within Patients "P" and "E" PBMCs

Frozen patient "P" PBMC cryovial was quickly thawed and washed with 10% FCS in RPMI. The patient cells were resuspended in 1ml flow buffer (PBS, 2mM EDTA, 1% BSA). Hundred thousand cells were aliquoted per flow tube. Patient "P" cells were stained for Annexin-V and propidium iodide and incubated for 1 hour on ice. The cells were then washed 3 times with flow buffer and then fixed with 1% paraformaldehyde in PBS and read using an Accuri C6 flow cytometry. As shown in Figure 41, FCS versus SSC shows two distinct lymphocyte populations, regions "R3" and "R4". Annexin-V and propidium iodide-stained cells were excluded from the FCS and SSC plot by using Markers "M1" and "M2" from the histograms of propidium iodide and Annexin-V plots, respectively. Region "R3" was the only lymphocyte population remaining after excluding Annexin-V and propidium iodide stained cells (Figure 41).

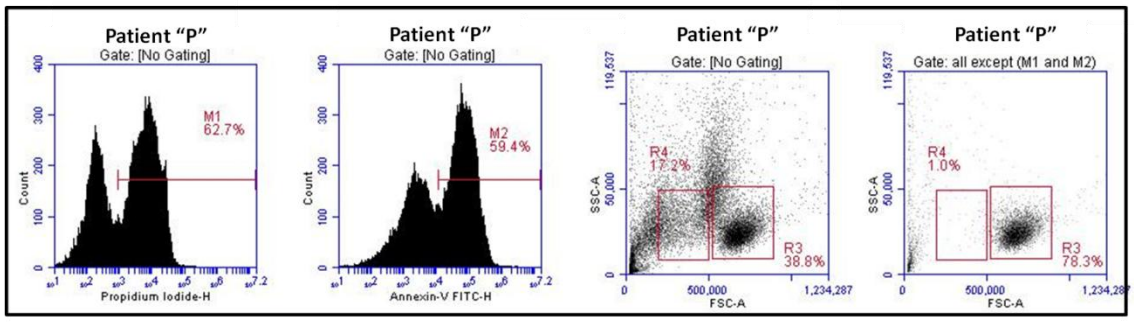
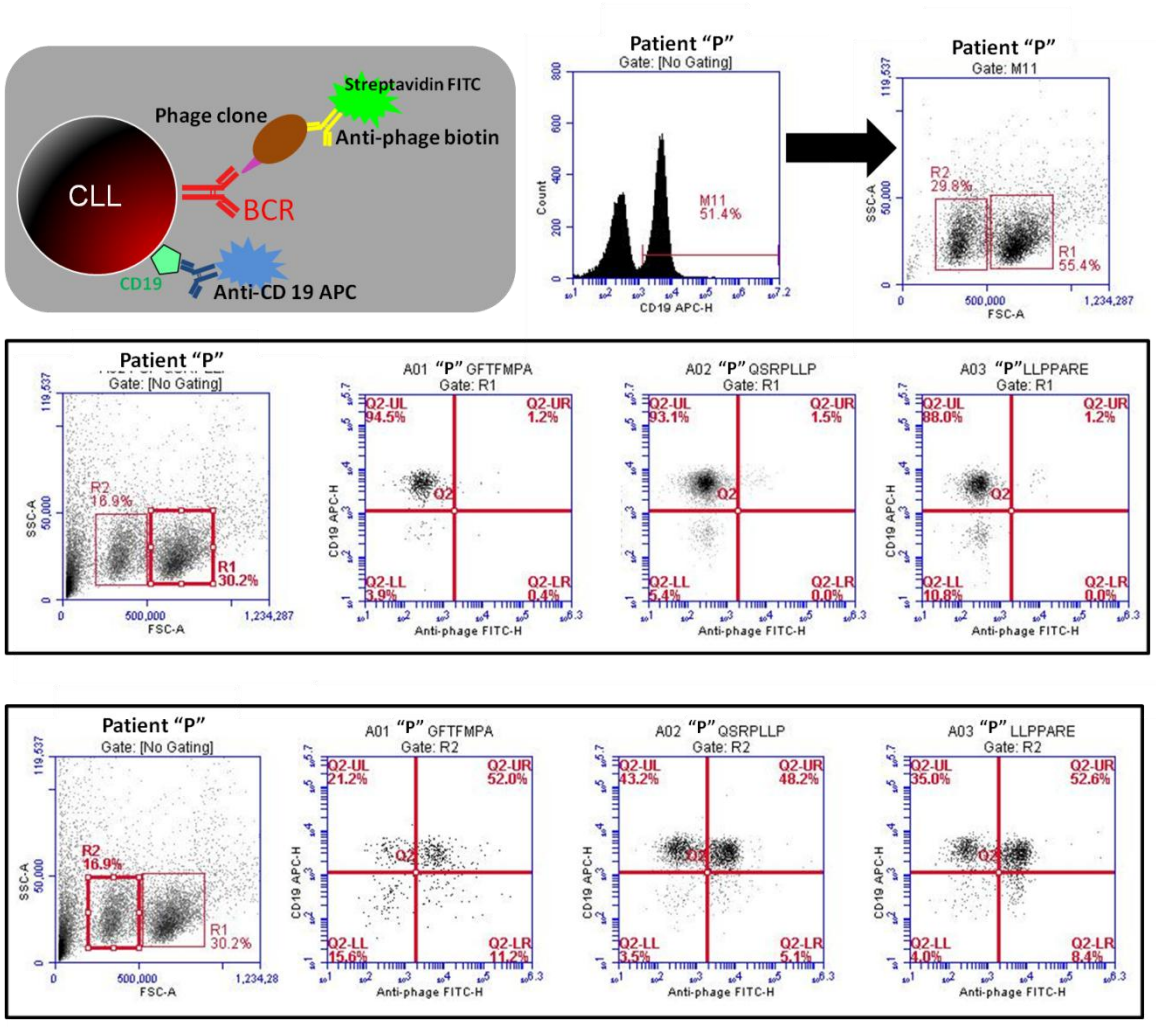


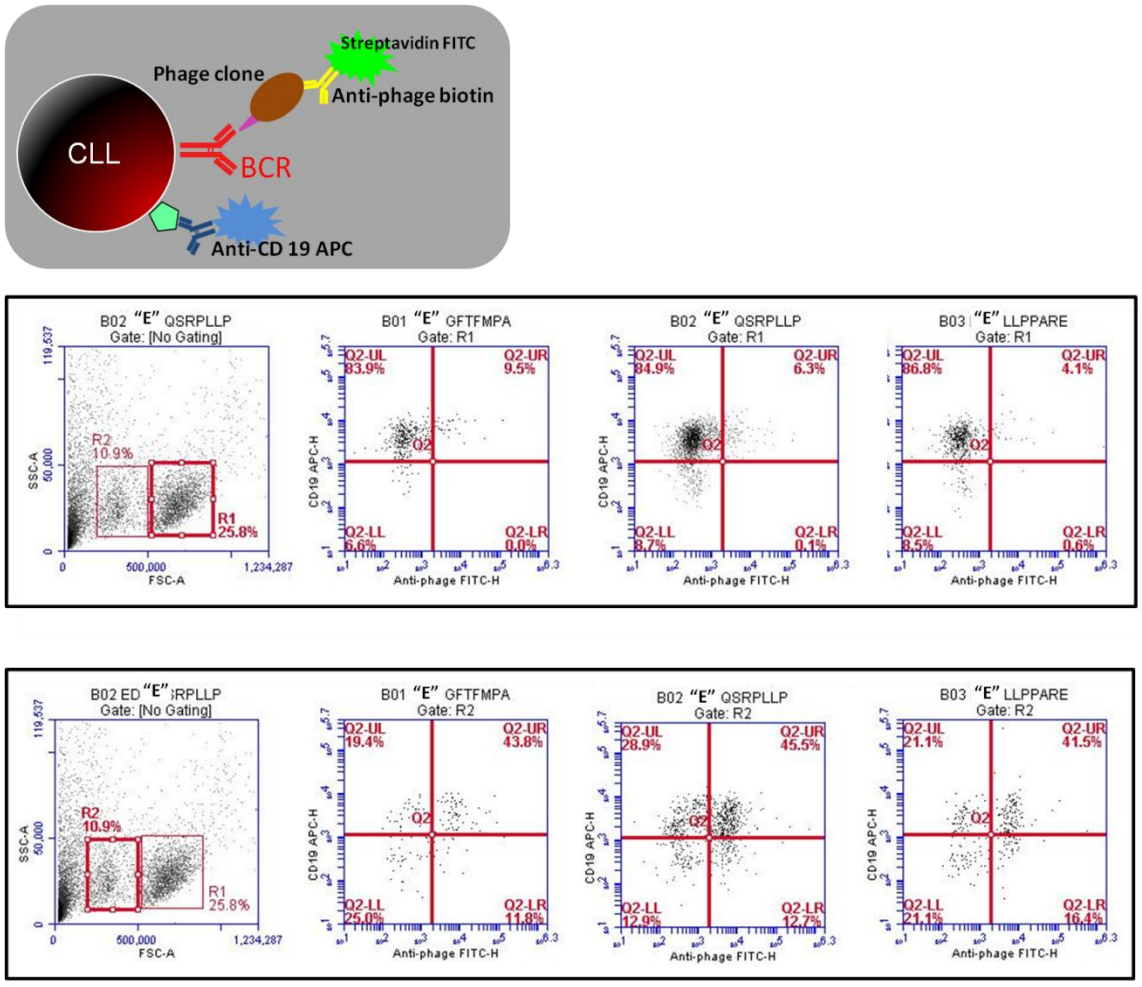
Figure 41. Flow Cytometry Distinguishing the Two B-lymphocyte Regions Observed in CLL Patients. PBMCs from CLL patient "P" were incubated with stained for Annexin-V and propidium iodide location of apoptotic cells.

Testing phage clones "GFTFMPA", "QSRPLLP" and "LLPPARE" on CLL patients "P" and "E" PBMCs

Phage clones "GFTFMPA", "QSRPLLP" and "LLPPARE" were each incubated against CLL patient "E' and "P" PBMCs to determine binding to CLL B-cells. Patients "P" and "E" frozen PBMC cryovials was quickly thawed and washed with 10% FCS in RPMI. The patient cells were each resuspended in 1ml flow buffer (PBS, 2mM EDTA, 1% BSA). Fifty thousand cells were aliquoted per flow tube. Patient "P" and "E" cells were separately incubated for 1 hour on ice with each of the clones "GFTFMPA", "QSRPLLP" and "LLPPARE". The cells were then washed 3 times with flow buffer and incubated for 30 minutes on ice with mouse anti-phage biotin. Cells were washed 3 times with flow buffer and then incubated for 30 minutes on ice with Streptavidin-FITC and anti-CD19 antibodies. Cells were washed 3 times with flow buffer and then fixed with 1% paraformaldehyde in PBS and read using an Accuri C6 flow cytometry. As shown in Figure 42 and 43, B cells were identified using anti- CD19 antibodies and two regions "R1" and "R2" were created. By gating on Region "R1" and looking at CD19 versus Anti-phage FITC for the three phage clones incubated with patients "E" and "P" PBMCs, no change was observed indicating that phage clones "GFTFMPA", "QSRPLLP" and "LLPPARE" did not bind to the exposed region of surface BCR on B cells.



<u>Figure 42</u>. Flow Cytometry Testing the Binding of Phage Clones "GFTFMPA", "QSRPLLP" and "LLPPARE" on CLL Patient "P" PBMCS.



<u>Figure 43.</u> Flow Cytometry Testing the Binding of Phage Clones "GFTFMPA", "QSRPLLP" and "LLPPARE" on CLL Patient "E" PBMCS.

DISCUSSION

Phage display peptide libraries have been used to target BCRs of CLL cell. In 2002, Buhl et al. discovered 9-mer peptides which were specific to the BCR of CLL cells from a patient [52]. They did not perform a negative selection and bound the monomeric IgM to protein A sepharose. Using flow cytometry, they showed that these phages bound to the patient's CLL cells with intensities more than 10 times higher compared to phage binding to CLL cells from other patients. However, there was not further work published by this group using this technology.

An investigated alternate method to targeting the BCRs of CLL cells was to express the VH and VL segments of CLL clones as human IgGs in a mammalian vector, incubating them with a phage display library and then capturing the peptide-CLL mAb complex using Protein A or G agarose beads [57]. However, this methodology is cumbersome. The aim of our strategy was to allow for a rapid selection of peptides binding specifically to patient-specific CLL cells to benefit many CLL patients in a timely manner.

This work provides an attempt to select patient-specific peptides from phage display libraries binding to BCRs of B-CLL cells. We worked with CLL cells from two patients. The VH and VL genes from each patient were determined. Sequence homology to germline VH greater than 98% was considered unmutated (U-VH) and less than 98% homology was considered mutated (M-VH). CLL cells from patient "P" use VH 1-3* 01

gene, D 2-2* 01 gene and JH 4-02* 01 gene with no mutations. In addition, CLL cells from patient "P" used variable light chain Vk 1-39* 01 gene and JK1 * 01 gene with no mutations (Figure 4). Thus, the VH and VL portions of the immunoglobulin are identical to the germline with no mutations. CLL cells from patient "E" use VH 4-34* 01 gene with 2 mutations out of 285 nucleotides (99.30 percent identical), a possible D2-2* 01 gene with 4 mutations out of 16 nucleotides and JH 6-02* 01 gene with 1 mutation out of 58 nucleotides. Also, CLL cells from patient "E" use variable light chain VK5* 02 gene (Figure 5). Thus, CLL cells from patient "E" show less than 2 percent mutation. Patients with U-VH have a poorer prognosis compared to M-VH patients [23]. Therefore, based on the molecular analysis of the VH genes, both patients are included in the poor prognosis subgroup for CLL.

Flow cytometry analysis of PBMCs from patients "P" and "E" peripheral blood $CD5^+/CD19^+$ lymphocytes (Figure 6) showed that 93.8% of the lymphocytes in patient "P" PMBCs were $CD5^+/CD19^+$ and using a kappa light chain (Figure 7), confirming clonality. However, CLL cells from patient "E" $CD5^+/CD19^+$ showed that 65.2% of these cells are $kappa^+$ and 26.1% $lambda^+$ (Figure 8). B-CLL with 2 or more distinct B-cell clonal populations has been identified in some patients [58, 59]. CLL cells from patient "P" showed a decrease in BCR expression compared to patient "E" $CD5^+/CD19^+$ cells and CD19 B-cells from healthy donors (Figure 9). This low expression is consistent with CLL, while patient "E" still had surface BCR expression similar to CD19 B-cells from healthy donors. These results are consistent with an early stage in the disease of patient E.

To obtain membrane-bound IgM from both patients, PBMCs were lysed using a mild detergent. The membrane-bound IgMs were released from B cell surfaces and IgM

presence confirmed by Western blot (Figure 12). The membrane-bound IgMs in the patient lysates were semi-quantified against standardized IgM from human serum, by capturing with goat anti-human IgM and identifying with anti-human IgM HRP (Figure 13). Presence of BCRs in patient "P' was determined to be about four times more concentrated than patient "E" cell lysate (Table 4). This is consistent with the higher PBMC count obtained during PBMC isolation with patient "P" compared to "E" (Table 2).

BCR light chain concentrations were also semi-quantified by capturing the membrane-bound IgMs with goat anti-human IgM and using a mouse anti-human kappa or lambda monoclonal antibody for detection. Patient "P" had only kappa, while patient "E" had both kappa and lambda light chains still present (Tables 5 and 6). The low concentration of BCR lambda light chain in patient "P" cell lysate further supports the monoclonal $CD5^+/CD19^+$ B-cell population identified in flow cytometry data of kappa versus lambda light chain on cell surface of $CD5^+/CD19^+$ B-cells (Figure 7). In addition, the presence of both kappa and lambda in patient "E" is in agreement with the kappa versus lambda flow data (Figure 8) BCRs within cell lysates. Therefore, the method used to solubilize membrane-bound IgM was successful and yielded a high concentration of intact membrane-bound IgM.

Membrane-bound IgMs from patient "P" and "E" were then used to select for peptides binding to the antigen binding site of the BCR for patient "P" cells. BCRs from patient "E" were used to subtract peptides binding to regions of BCR molecule present in both lysates. For this purpose, we used the following strategy (Figure 26): Patient "E" BCRs were captured with goat anti-human IgM and this well provided negative selection

of the peptide-Phage Display Libraries. The remaining unbound phages were then incubated in a well containing patient "P" BCRs to positively select for phages binding to patient "P" BCR antigen binding pocket. Two different peptide libraries displaying 7 and 12-mer peptides were used. Results from Strategy 1 showed enrichment in eluted phages in both the 7-mer and 12-mer selections (Table 7 and 8). Eight to ten clones were selected and a sequence consisting of a "LLPPAR_" peptide was found in both 7 and 12-mer libraries (Table 9b). Phages displaying peptide "LLPPARE" showed higher binding, compared to M13 phages, when incubated with patient "P" BCRs which were captured using goat anti-human IgM (Figure 31C). To determine whether phages displaying peptide "LLPPARE" were binding to patient "P" BCRs or the goat anti-human IgM in the well, phages displaying peptide "LLPPARE" were tested for binding to only goat anti-human IgM, and then tested against patients "E", "P" BCRs and human serum IgM captured using goat anti-human IgMs (Figure 32A, B, C and D). The results showed that goat anti-human IgM well had the same optical density as the IgM and patient BCR-captured wells confirming that phages displaying peptide "LLPPARE" were binding to goat anti-human IgMs in the well (Figure 32E) rather than to the BCR from patient "P". Database sequence search of "LLPPARE" showed an identical sequence was located on the CH4 hydrophilic region of human IgM (Figure 34). Thus, "LLPPARE" may be the core of an immunodominant epitope for goat antibodies on the IgM molecule.

To remove phages with affinity for the goat anti-human IgM, a new strategy was devised. We used two goat anti-human IgM incubations of the peptide-Phage Display library in the first round and seven goat anti-human IgM incubations in the second and third rounds, prior to positive selection for phages binding to patient "P" captured BCRs

(Figure 35). In addition, the input phages in second and third round were also reduced from 10^{11} to 10^{7}, after the first round selection. The rationale for this decrease in input of phages is to ensure that majority of the goat anti-human IgM binding phages were removed from the pool in the successive anti-human IgM subtraction wells. Enrichment of phages was observed (Table 11a). After the second and third round selection, sequencing of the selected phage clones from the positive selection showed 3 different phage displaying peptides “GFTFMPA”, “QSRPLLP” and “GLPCCSS” (Table 11b).

Phages displaying peptides “GFTFMPA”, “QSRPLLP”, “GLPCCSS” and “LLPPARE” were then tested for binding to goat anti-human IgM (Figure 37A). The goat anti-human IgM-binding “LLPPARE” phage clone was used as a control. Phage clones displaying peptides “GFTFMPA” and “GLPCCSS” showed binding to goat anti-human IgM. However, phage clones displaying peptide “QSRPLLP” did not show binding to goat anti-human IgM antibody (Figure 37C).

Phages displaying peptides “GFTFMPA”, “QSRPLLP” and “LLPPARE” were then tested for binding to IgM from human serum which was captured using goat anti-human IgM. The results showed significantly higher binding of phage clones displaying peptides “GFTFMPA” and “LLPPARE”, as expected due to the presence of goat anti-human IgMs used to sensitize the wells. However phage clones displaying “QSRPLLP” peptide showed no binding to IgM from human serum (Figure 38).

Phage clones displaying “QSRPLLP” peptide were then tested for binding to patient “P” and “E” BCRs which were captured using goat anti-human IgM. The phage clones displaying “QSRPLLP” peptides showed same binding to patient “P” and “E”

BCR-captured wells (Figure 39). Phage clones displaying “QSRPLLP” peptide were therefore binding to a common region of the BCR molecule in patients “P” and “E” which is not present in IgM from human serum.

Phage clones displaying peptide “QSRPLLP” were then tested by flow cytometry for binding to intact PMBCs from CLL patients “P” and “E” and analyzed. Phage clones displaying peptides “GFTFMPA” and “LLPPARE” were used as negative controls. Phages displaying “QSRPLLP” peptides did not bind to patients “P” or “E” B-cells (Figures 42 and 43). This suggests that the “QSRPLLP” peptide does not bind to exposed regions of membrane-bound BCRs of CLL patient “P” or “E” cells. These results suggest that “QSRPLLP” peptide binds a portion of the BCR from patients “P” and “E” which is not accessible on cells and not present on serum IgM. The binding of “QSRPLLP” peptide-phage clone to patients “P” and “E” BCRs using an ELISA method suggest that it is possible to use a Phage Display Peptide Library to select for peptides unique to the BCRs of patients with CLL.

The two strategies used in our work showed that the strategy used for negative selection is the critical component in the search for peptides binding specifically to the antigen binding site of the BCRs. This can be improved by increasing the number of subtractions with BCRs from CLL cells from another patient. Alternative, since CLL cells secret polyreactive immunoglobulins that bind self antigens with low binding affinities [38], it is possible that the BCR antigen binding site is bound to self antigens. Thus, the antigen binding site of the BCR could be blocked and this would hinder the selection of peptides binding to the antigen binding site of the targeted BCR. A solution to this problem would be to reduce the pH of captured BCRs, without compromising the

structural integrity of the BCR, thereby detaching any bound self antigens and then incubating with the phage display peptide library.

Targeting the BCR of CLL cells will ensure that only those cells are affected and this approach still remains a possible target for treatment of CLL. Discovered patient-specific peptides can then be fused with a toxin and used to treat patients with CLL [16, 49].

An optimized selection strategy for peptides binding to the antigen binding site of the BCRs would allow for patients, diagnosed with CLL and with a poor prognosis, to be treated early. The use of the peptide-toxin complex would have an immunotherapeutic advantage in killing CLL cells with less non-specific toxicity.

Sequence analysis reveals the use of similar restricted repertoire of immunoglobulin V_H DJ_H and V_LJ_L genes among CLL patients and shows that some CLL patients have similar stereotypic HCDR3 and LCDR3 motifs [3]. The antigen binding sites of the BCRs, therefore, recognize the same antigenic epitope [24, 29, 41, 60] and a peptide targeting the antigen binding site of a patients' BCR could also be tested on patients expressing the same restricted repertoire of Ig genes.

APPENDIX A

Preparation of reagents used in this study:

- Tris 1 M pH 9.1- Add 6 grams of Tris base (MW 121 grams per mole) to 40 mL of filtered distilled deionized water. Mix well until dissolved. Add 1 M HCl acid to adjust to pH 9.1. Add filtered distilled deionized water to bring to a final volume of 50 mL.

- Glycine 0.2 M, HCl pH 2.2, 1% BSA- Dissolve 1.5 grams of glycine (MW 75.07) to 50 mL of filtered distilled deionized water. Mix well. Add 1 M HCl to adjust to pH 2.2. Add 100 milligrams BSA and dissolve it. Add filtered distilled deionized water to bring to a final volume of 100 mL.

- 20% PEG 8000 (Poly Ethylene Glycol 8000), 2.5 M NaCl- Dissolve 14.6 grams of NaCl in 60 mL of filtered distilled deionized water using a stirrer and heat. Add filtered distilled deionized water to bring volume to 80 mL. Add 20 grams of PEG. Autoclave and mix well to combine separate layers while still warm. Store at room temperature.

- 1% BSA, 2 mM EDTA in HBSS pH 7.4- Dissolve 0.4 grams of BSA in 40 mL of HBSS pH 7.4. Add 160 microliters of 0.6 M EDTA.

- 1% Paraformaldehyde (PFA) in HBSS- Add 1 mL of 16% PFA solution to 15 mL of HBSS pH 7.4

- LB Broth- Dissolve 12.5 grams of LB broth powder in 500 mL of filtered distilled deionized water. Add a magnetic stirrer and autoclave.

- Tetracycline Stock (suspension) - 20 mg/mL in 1:1 Ethanol: Water. Store at -20^0C. Vortex before using.

- LB Tetracycline plates- Add 15 grams to 1 liter of LB medium, autoclave and cool to <70 ^{o}C. Add 1mL Tetracycline Stock and pour onto Petri dishes. Store plates at 4^0C. Do not use plates if they are brown or black.

- IPTG/Xgal Stock. Mix 1.25 grams IPTG (isopropyl-β-D-galactosidase) and 1 gram Xgal (5-Bromo-4-chloro-3-indolyl-β-D-galactoside) in 25 mL DMF (dimethyl formamide). Store solution at -20^{0}C.
- IPTG/ Xgal LB Plate Agar- Dissolve 12.5 grams of LB broth powder and 7.5 grams of bacteriological agar in 500 mL of filtered distilled deionized water. Add a magnetic stirrer and autoclave. Pour 20 mL into each petri dish and allow it to cool and solidify.

- Phage top agar- Dissolve 12.5 grams of LB broth powder and 3.5grams of bacteriological agar in 500 mL of filtered distilled deionized water. Add a magnetic stirrer and autoclave. Pour 3 mL to sterile glass tubes to be used for phage titrations.

- Iodide buffer (10 mM Tris HCl pH 8, 1 mM EDTA, 4 M Sodium Iodide). Weigh 29.978 grams of Sodium Iodide and add to a 250 mL glass beaker. Add 100 microliters of 0.5 M EDTA solution. Add 500 microliters of 1 M Tris solution. Bring volume up to 50 mL using filtered distilled deionized water

- TBS- 50 mM Tris-HCL (pH7.5), 150 mM NaCl. Autoclave and store at room temperature.

- Blocking Buffer for phage panning- 0.1 M $NaHCO_3$ (pH 8.6), 5 mg/ml BSA. Filter sterilize and store at 4^{0}C.

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