Supplementary figures/tables

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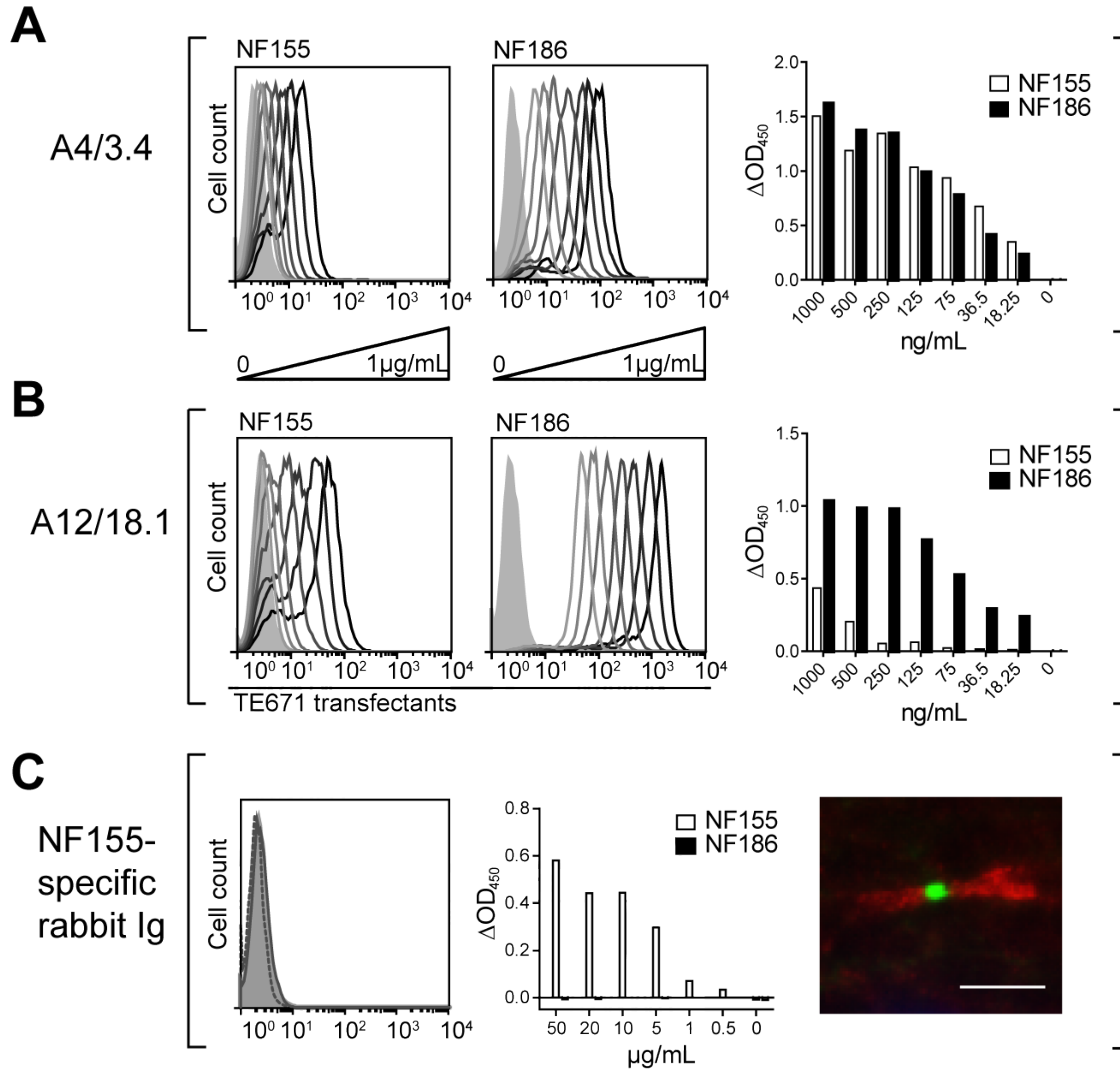
Figure e-2: Serum screening by flow cytometry and features of anti-NF155 antibodies in a CIDP patient

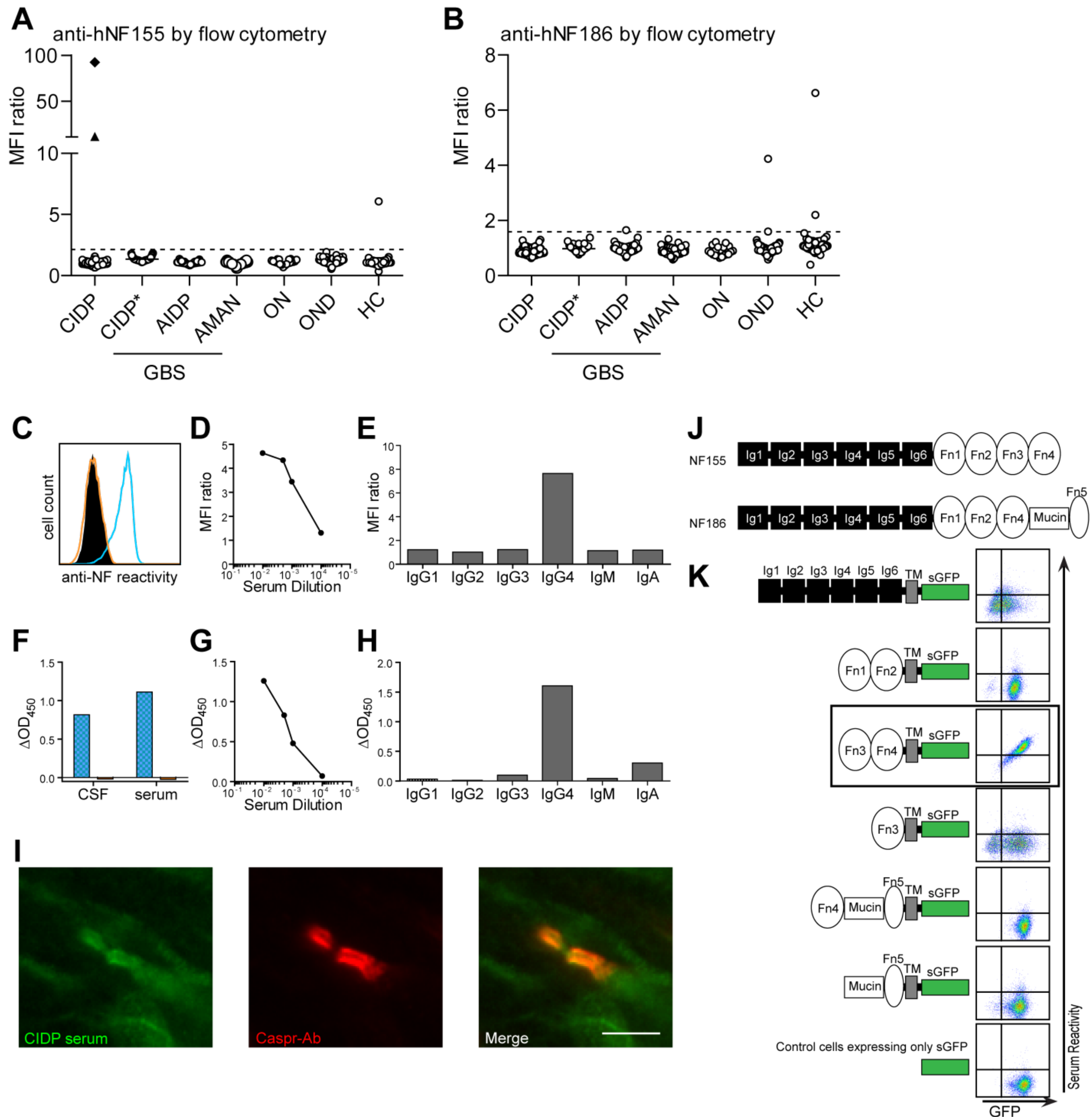
Figure e-3: NF155 reactivity in a CIDP patient benefiting from plasma exchange

Figure e-1: **Validation of human NF155 and NF186 antibody detection assays with NF-specific antibodies**



A dilution series was done with both mAbs A12/18.1 (**A**) and A4/3.4 (**B**) using flow cytometry and ELISA. Concentration of mAbs used in ng/mL: 1000, 500, 250, 125, 75, 36.5, and 18.25. Optical density was read at 450 nm for ELISA reactivity. (**C**) NF155-specific purified rabbit antibodies were tested for reactivity to NF155 (solid line) and NF186 (dashed line) on transfectants as compared to mock transfectants (filled) by flow cytometry (left). The antibodies were also tested by ELISA (middle). On tissue sections (right), the rabbit antibodies (red) stain paranodes (green = A12/18.1). The scale bar represents 10 μm.

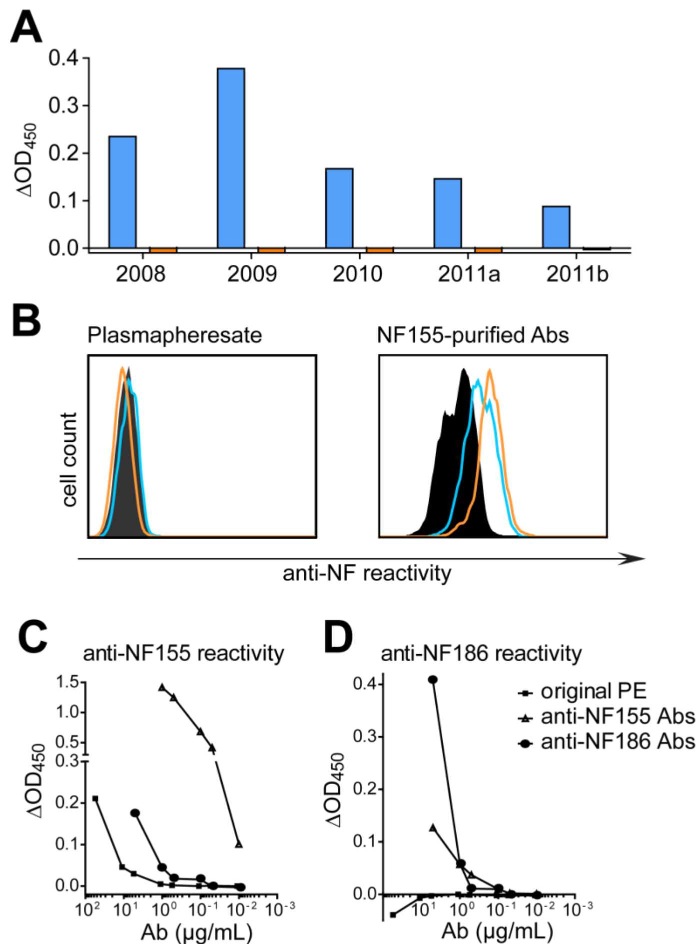
Figure e-2: Serum screening by flow cytometry and features of anti-NF155 antibodies in a CIDP patient



Serum samples from patients with CIDP (n = 117), AIDP (n = 65), AMAN (n = 50), ON (n = 20), and controls OND (n = 61), HC (n = 77) were tested for autoantibodies to NF155 and NF186 by flow cytometry. Mean fluorescence intensities (MFI) measured with flow cytometry are shown as the MFI ratios between NF155 (**A**) or NF186 (**B**) transfectants and mock transfectants. The cutoff (dashed line) represents the mean of OND group plus four SDs. Two CIDP samples marked with special symbols (▲,◆) showed reactivity by both ELISA and flow cytometry to NF155 (more details in Figure 3

and e-2C-K). Reactivity to NF155 in a CIDP sample was seen by flow cytometry (**C,D,E**) up to a dilution of 1:10 000 (**D**). (**C**) Reactivity to NF155 (blue line) was compared with reactivity to NF186 (orange line) and to control cells (black, filled). (**E**)The NF155 reactivity was mediated by IgG4. Reactivity to human NF155 was also seen by ELISA in serum and cerebrospinal fluid. Serum reactivity by ELISA (**F,G,H**) was seen up to a dilution of 1:10000 (**G**) by IgG4 and weakly by IgG3 and IgA (**H**). Serum staining (**I**, left) colocalizes with Caspr staining (**I**, middle) on longitudinally cut rat spinal cord sections. The scale bar represents 10 µm. (**J**) NF186 differs from NF155 by substitution of fibronectin (Fn) domains Fn3-Fn4 with Fn4-Mucin-Fn5. (Ig = immunoglobulin-like domain; Fn = fibronectin type III domain) A scheme of super green fluorescent protein (sGFP) fusion truncated NF variants is shown beside the corresponding serum reactivity by flow cytometry (**K**). Reactivities to truncated NF variants and to negative control cells are shown as sGFP intensity versus serum reactivity. The fragment recognized by both NF155-reactive serum samples is boxed.

Figure e-3: **NF155 reactivity in a CIDP patient benefiting from plasma exchange**



Anti-NF155 reactivity by ELISA persist over years in one CIDP patient, and after affinity purification, reactivity is seen by ELISA and flow cytometry to both NF155 and NF186. (**A**) By ELISA, reactivity to NF155 (blue), but not NF186 (orange) was found in serum and PE samples taken from 2008, 2009, 2010, and two time points (February (a) and October (b)) in 2011. Reactivities were measured at 5 µg/mL Ig concentration for each time point. (**B**-**D**) Anti-NF antibodies were purified from PE material from 2011a against NF155 and NF186. By flow cytometry (**B**), original PE material was not reactive to either NF (NF155, blue line; NF186, orange line; control cells, black, filled), but NF155-purified antibodies showed binding to both isoforms. By ELISA (**C**,**D**), serial dilution of PE material and purified antibodies was done. (**C**) NF155 was recognized by the original PE material (▪) and by antibodies from both NF columns (NF155 ▴; NF186 ●). (**D**) NF186 was recognized by only purified antibodies from both NF columns but not by the original PE material.

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Appendix e-1: Additional patient information

We included patients with 119 chronic inflammatory demyelinating polyneuropathy (92 (male) / 27 (female); average age 61 [age range 9-90]), 65 acute inflammatory demyelinating polyneuropathy (42/23; 50 [1-84]), 50 acute motor axonal neuropathy (29/21; 47 [14-83]), and 20 other neuropathies (19/1; 63[20-78]). The other neuropathy group includes patients with diabetic polyneuropathy, monoclonal gammopathy, polyneuropathy from vitamin B12 deficiency, and polyneuropathy with unclear origins. The control group consisted of 63 patients with other neurological diseases (4/48; 42 [21-80]) and 77 healthy donors (0/47; 46 [14-84]). The other neurological disease group includes patients with sensory symptoms, headache, migraine, vertigo, neurastenia, pain, cancer, and vascular problems. Plasma exchange material was obtained from 41 chronic inflammatory demyelinating polyneuropathy patients (30/10; 59 [28-83]) and 4 patients with relapsing-remitting multiple sclerosis.

Appendix e-2: Complete NF155 and NF186 cloning

The complete cDNAs of NF155 and NF186 were generated stepwise. First, the cDNAs was prepared from human brain RNA using reverse-transcription primers specific to the cytosolic

region (CAGAGATGTCTGACTGC) and to the third immunoglobulin-like (Ig) domain (ATGTCTGGTGTTGGGAC) using Superscript III reverse transcriptase (Invitrogen). Second, three fragments were amplified using Taq DNA polymerase (Roche): fragment 1 spanned the signal sequence and the first two Ig domains (Ig1,Ig2) and was amplified with primers TAT GTC GAC ATG GCCAGGCAGCCACCG and ACATGAAGCTTGGTGTTCTTTC; fragment 2 spanned the third and fourth fibronectin type-III (Fn3, Fn4) domains to the cytoplasmic region and was amplified with primers GGTTACTCCGGAGAAGATTATC and AGTAGATCTTCAGCCGCGACTCCTCTTGATG; fragment 3 spanned the Fn4, mucin, and Fn5 domains to the cytoplasmic region and was amplified with primers GGTGAATCCGGAGAAGATTTACCCAG and AGTAGATCTTCAGCCGCGACTCCTCTTGATG. For soluble NF155 and NF186, the transmembrane domain was replaced by a polyhistidine tag using primer CAGTAGATCTTAGTGATGGTGATGGTGATGGCCCTGGGTGGCGATGTC. Fragment 1 was common to both isoforms, Fragment 2 was NF155-specific and Fragment 3 was NF186-specific. A cDNA fragment containing Ig3-Ig4-Ig5-Ig6 and Fn1-Fn2 domains common to both isoforms was excised from cDNA clone IRAKp961P2270Q (GenomeCube; Nottingham, UK) using restriction enzymes HindIII and AvrII (NEB Biolabs; Ipswich, USA) To assemble the fragments, pcDNA6.1v5 His C (Invitrogen) was used as a surrogate vector. First, the vector and Fragment 1 were cut with EcoRI and HindIII, and then they were ligated together using T4 DNA Ligase (Invitrogen). Next this ligation product was cut with HindIII and NheI, and ligated to the cDNA fragment with ends digested with HindIII and AvrII. The product was then cut with BsimI and BglII, and either Fragment 2 or Fragment 3 was inserted, producing essentially the cDNA insert of the entire NF155 and NF186. Both full NF's were excised from the vector using SalI and BglII, and ligated into a vector appropriate for each expression system used. For cell surface expression of the complete neurofascins in TE671 cell line, the full NF cDNA was inserted into plasmid pRSV5neo using SalI and BamHI sites. For soluble NF production in HEK293-EBNA cells, the NF constructs containing the *C*-terminus polyhistidine

tag were inserted into plasmid pTT5 digested with PmeI and BamHI by first making the SalI site of the insert blunt-ended with DNA polymerase I Klenow fragment (Invitrogen).

Appendix e-3: NF-truncation mutant cloning

We produced six truncation mutants of NF155 and NF186, all fused to superGFP (sGFP) at the *C*-terminus, for stable cell surface expression on TE671 cell line. First, we amplified by PCR the sGFP using primers GCTCTAGAGCCATCGATGGCGGAGGTGGCAGCATGGTGAGCAAGGGCGAG and GCTCTAGAGCTTAGTG ATGGTGATGGTGATG from a pre-existing sGFP-containing vector. We then cut this PCR product and the plasmid pRSV5neo vector with XbaI, ligated them together and chose a clone with the correct insert orientation for expression. To produce the constructs, a combination of restriction enzymes and overlap-extension-PCR [1] using KOD DNA polymerase (Novagen) was used. Each construct contained a signal sequence and a transmembrane domain for surface expression, and in addition fusion to the sGFP in the cytosolic region for detection. 1) For NF-Ig construct covering the six Ig-domains, the fragment containing the signal sequence and the six Ig-domains was excised from the complete NF155 construct using SalI and NheI and the transmembrane domain fragment was PCR-amplified with primers ACGCGTCGACTCTCTAGCTAGCTGATCAGGCCACTAACAACCAAGCGGACATCG and GCCATCGATGGCGCCGCGACTCCTCTTGA. The PCR product and the pRSV5neo-sGFP vector were both digested with SalI and ClaI, and subsequently ligated together. The ligation product was then cut with SalI and NheI, and the fragment containing the Ig-domains was inserted. 2) For NF-Fn1Fn2 construct covering Fn1 and Fn2 domains, three PCR products were produced to separately cover the signal sequence, the Fn1-Fn2 domains, and the

transmembrane domain, using primers TATGTCGACATGGCCAGGCA and AACGGTTAGTCGGCTGCGTCAGCTCATTC, primers GACGCAGCCGACTAACCGTTTGGCTGCCCT and TCTTCTCCGGAGTAACCGATG, and primers GTTACTCCGGAGAAGATTACACCAACAACCAAGCGG and GCCATCGATGGCGCCGCGACTCCTCTTGA respectively. The Fn1-Fn2 and transmembrane domain fragments were ligated through a MroI site. This ligation product was PCR-amplified, and then joined to the signal sequence fragment by overlap-extension PCR. The PCR product was inserted into the pRSV5neo-sGFP vector through SalI and ClaI restriction sites, as it was for the following inserts described. 3) The NF-Fn3Fn4 construct required overlap-extension PCR to join the signal sequence PCR product (primers TATGTCGACATGGCCAGGCA and AATCTTCTCC CGGCTGCGTCAGCTCATTC) and the Fn3-Fn4, and transmembrane domains PCR product (primers GACGCAGCCG GGAGAAGATTATCCCAGGG and GCCATCGATGGCGCCGCGACTCCTCTTGA). 4) The NF-Fn4MucFn5 insert required overlap-extension PCR to join the PCR product for the signal sequence (primers TATGTCGACATGGCCAGGCA and AATCTTCTCC CGGCTGCGTCAGCTCATTC) and the PCR product for Fn4-Muc-Fn5, and transmembrane domains (primers GACGCAGCCG GGAGAAGATTTACCCAGTGC and GCCATCGATGGCGCCGCGACTCCTCTTGA). 5) The NF155-specific NF-Fn3 fragment was produced by joining 3 PCR products together: the signal sequence (same as for NF-Fn3Fn4), the Fn3 domain (primers GACGCAGCCG GGAGAAGATTATCCCAGGG and TGTTGGTGTA TACTCCTTCCGGGGTGGTG), and the transmembrane domain (primers GGAAGGAGTA TACACCAACAACCAAGCGGA and GCCATCGATGGCGCCGCGACTCCTCTTGA). The Fn3 domain fragment was joined to the transmembrane domain though a BstZ17I restriction site, and this ligation product was joined with the signal sequence by overlap-extension PCR. 6) For the fragment NF-MucFn5, two PCR fragments were joined by overlap-extension PCR: the signal sequence fragment (primers TATGTCGACATGGCCAGGCA and CTTCATTCGG GGGCTGCGTCAGCTCATTC)

and the Muc-Fn5 fragment (primers GACGCAGCCCCCGAATGAAGCTACTCCAAC and GCCATCGATGGCGCCGCGACTCCTCTTGA).

Appendix e-4: Soluble NF155 and NF186 expression in HEK293-EBNA cells

To generate soluble human neurofascin, the plasmids pTT5-NF155sol and pTT5-NF186sol were transiently transfected into HEK293-EBNA suspension cells [2]. The cells were grown in suspension in Freestyle 293 Expression Medium (Gibco; Darmstadt, Germany) supplemented with 1% Pluronic F-68 (10%) (Gibco) and 25 mg/L Geneticin (Gibco) in at 37°C, 7.9% CO_2, 70% humidity, and shaken at 105 rpm. To transfect 100 mL of cells, 100 µg of plasmid DNA and 200 µL of jetPEI DNA (Polyplus Transfection) was mixed in Optipro SFM (Gibco) and added dropwise into the cell suspension. After 24 h, Bacto TC Lactalbumin hydrolysate (BD Biosciences) solution was added to 0.5% final concentration. After 96 h, the cell culture was harvested. The entire cell culture was ultracentrifugated at 10000 rpm for 15 min. The supernatant was adjusted to pH 8.0, and loaded onto a Ni-NTA (Qiagen) column (Sigma-Aldrich), typically by gravity flow at 4°C overnight. The bound protein was eluted with PBS containing 250 mM imidazol, and then concentrated, buffer-exchanged in PBS and stored in 1mL aliquots at -20°C. The concentration of the protein preparation was measured either by the nanodrop (Peqlab; Erlangen, Germany) or by using a bicinchoninic acid assay (Thermo Scientific).

Appendix e-5: Western blot analysis

For Western blotting, protein samples were mixed in LDS buffer (Invitrogen), brought to 95°C for 5 min, and separated on 4-12% NuPAGE gels (Invitrogen). Subsequently the separated protein

samples were blotted onto PVDF membrane (GE Healthcare). Blots were blocked in 5% skimmed milk in phosphate-buffered saline containing 0.05% Tween-20, and incubated overnight with 200 ng/mL of purified patient antibodies diluted in the blocking solution. Anti-human-Ig-peroxidase (1:7000; JacksonImmuno) was used for detection by chemiluminescence.

References

1. Heckman KL, Pease LR. Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protocols 2007; 2(4):924-932.

2. Durocher Y, Perret S, Kamen A. High-level and high-throughput recombinant protein production by transient transfection of suspension-growing human 293-EBNA1 cells. Nucleic Acids Research 2002; 30(2):e9.