# ENZYME-LINKED IMMUNOSORBENT ASSAYS USING RECOMBINANT EPSTEIN-BARR VIRUS ANTIGENS FOR DETECTION OF NASOPHARYNGEAL CARCINOMA IN MALAYSIAN CHINESE

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# ABSTRACT

Malaysian Chinese are highly susceptible to Epstein-Barr virus (EBV)-associated nasopharyngeal carcinoma (NPC). Because NPC is often diagnosed at a late stage, accurate EBV serologic tests might be useful to detect NPC in patients with subtle symptoms. And because EBV serological data for Malaysian Chinese are currently lacking, we sought to develop indirect enzyme-linked immunosorbent assays (ELISAs) for a case-control study of NPC in Malaysian Chinese.

Of the 165 cases and 658 controls recruited, 89.7% (148/165) prevalent NPC cases were Chinese, in which 14.2% (21/148) Chinese patients were familial NPC cases. The prevalence of family history of NPC among the Chinese controls was 0.6% (2/334), whereas none of the controls of other ethnicity ($n$ = 324) had family history of NPC. Based on individual matching in age and gender, 120 case-control pairs of Chinese descent were selected for the subsequent ELISA study.

Using recombinant DNA technology, three EBV proteins: early antigen-diffuse (EA-D), Z-encoded broadly reactive activator (ZEBRA) and viral capsid antigen (VCA) p18, were expressed in and purified from *Escherichia coli*. The EBV proteins were used as antigens for indirect ELISAs.

The antibody levels against the EBV proteins were elevated in NPC cases. Among the serologic tests, the diagnostic accuracy of ZEBRA/IgG (81.7% sensitivity, 61.7% specificity) was the highest, followed by VCA p18/IgG (95.8% sensitivity, 35.0% specificity) and EA-D/IgG (74.2% sensitivity, 48.3% specificity). To serve as useful clinical indicators for NPC, further efforts are needed to improve the accuracies of EBV serologic tests.

# ABSTRAK

Masyarakat Cina di Malaysia adalah golongan yang berisiko tinggi mengidap karsinoma nasofarinks (nasopharyngeal carcinoma, NPC) yang berkaitan dengan virus Epstein-Barr (EBV). Disebabkan NPC sering didiagnos pada peringkat lewat, ujian-ujian serologi EBV yang tepat berpotensi untuk mengesani NPC bagi pesakit yang tiada gejala jelas. Dan disebabkan data serologi EBV untuk masyarakat Cina masih terhad, kami ingin membina asai-asai imunosorben bergabung enzim (enzyme-linked immunosorbent assays, ELISAs) tidak langsung untuk kajian kes-kawalan bagi masyarakat Cina di Malaysia.

Antara 165 kes dan 658 kawalan yang telah rekrut, 89.7% (148/165) kes lazim NPC terdiri daripada kaum Cina, di mana 14.2% (21/148) pesakit Cina adalah kes NPC keluarga. Kadar kelaziman sejarah keluarga NPC di kalangan kawalan Cina adalah 0.6% (2/334), manakala kawalan-kawalan kaum lain ($n$ = 324) tidak mempunyai sejarah keluarga NPC. Dengan pemadanan individu dari segi umur dan jantina, 120 pasangan kes-kawalan yang berketurunan Cina dipilihkan untuk kajian ELISA yang seterusnya.

Dengan teknologi DNA rekombinan, protein-protein EBV, iaitu EA-D (early antigen-diffuse), ZEBRA (Z-encoded broadly reactive activator) and VCA (viral capsid antigen) p18, dieskpreskan dan ditulenkan dari *Escherichia coli*. Protein-protein EBV tersebut digunakan sebagai antigen-antigen dalam asai-asai ELISA tidak langsung.

Aras-aras antibodi terhadap protein-protein EBV bagi kes-kes NPC didapati meningkat. Antara ujian-ujian serologi, ketepatan diagnostik bagi ZEBRA/IgG (81.7% sensitiviti, 61.7% spesifisiti) adalah terbaik, diikuti oleh VCA p18/IgG (95.8% sensitiviti, 35.0% spesifisiti) dan EA-D/IgG (74.2% sensitiviti , 48.3% spesifisiti). Untuk penggunaan sebagai penunjuk klinikal NPC, usaha yang selanjutnya adalah diperlukan untuk meningkatkan ketepatan bagi ujian-ujian serologi EBV.

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# PUBLICATION AND PRESENTATIONS

## Publication

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# TABLE OF CONTENTS

# LIST OF FIGURES

# LIST OF TABLES

# LIST OF SYMBOLS AND ABBREVIATIONS

**Symbol and prefix**

| | |
|---|---|
| °C | degree Celsius |
| µg/ml | microgram per milliliter |
| µl | microliter |
| µM | micromolar |
| *A* | absorbance |
| bp | base pair |
| *CV* | coefficient of variation |
| *g* | relative centrifugal force |
| h | hour |
| kb | kilobase pairs |
| M | molar |
| min | minute |
| ml | milliliter |
| mM | millimolar |
| *n* | sample size |
| nm | nanometer |
| *p* | probability value |
| $R^2$ | coefficient of determination |
| *r* | Pearson's correlation coefficient |
| s | second |
| *SD* | standard deviation |
| U/µl | activity unit per microliter (defined by supplier) |
| v/v | volume per volume |
| w/v | weight per volume |
| kDa | kilodaltons |
| *z* | *z*-score |

**Chemical symbol and formula**

| | |
|---|---|
| B | boron |
| Ca | calcium |
| $CH_3COONa$ | sodium acetate |
| Co | cobalt |
| $CoCl_2$ | cobalt chloride |
| Cu | copper |
| Fe | iron |
| HCl | hydrochloric acid |
| $K_3EDTA$ | tripotassium EDTA |
| KCl | potassium chloride |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| $MgCl_2$ | magnesium chloride |
| $MgSO_4$ | magnesium sulfate |
| Mn | manganese |
| Mo | molybdenum |
| $Na_2CO_3$ | sodium carbonate |
| $Na_2HPO_4$ | disodium hydrogen phosphate |
| $Na_2SO_4$ | sodium sulfate |
| NaCl | sodium chloride |
| $NaH_2PO_4$ | sodium dihydrogen phosphate |
| $NaHCO_3$ | sodium hydrogen carbonate |
| $NH_4Cl$ | ammonium chloride |
| Ni | nickel |
| Se | selenium |
| Zn | zinc |

| Abbreviation | |
|---|---|
| AP | alkaline phosphatase |
| ASR | age-standardized rate |
| AUC | area under the ROC curve |
| BCIP/NBT | 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium |
| BSA | bovine serum albumin |
| C+ | moderately positive control |
| C++ | strong positive control |
| C− | negative control |
| CC | conjugate control |
| CI | confidence interval |
| C-terminus | carboxyl-terminus |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleotide triphosphate |
| EA-D | early antigen-diffuse |
| EBNA | EBV nuclear antigen |
| EBV | Epstein-Barr virus |
| EDTA | ethylenediaminetetraacetic acid |
| ELISA | enzyme-linked immunosorbent assay |
| His | histidine |
| HLA | human leukocyte antigen |
| HRP | horseradish peroxidase |
| IARC | International Agency for Research on Cancer |
| IDA | iminodiacetic acid |
| IFA | immunofluorescence assay |
| IgA | immunoglobulin A |
| IgG | immunoglobulin G |
| IMAC | immobilized metal affinity chromatography |
| IPTG | isopropyl-β-D-thiogalactopyranoside |

| | |
|---|---|
| LMP | latent membrane protein |
| LR | likelihood ratio |
| MRI | magnetic resonance imaging |
| NFDM | nonfat dry milk |
| NPC | nasopharyngeal carcinoma |
| N-terminus | amino-terminus |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PP | percentage positivity |
| PVA | poly(vinyl alcohol) |
| PVDF | poly(vinylidene difluoride) |
| PVP | poly(vinyl pyrrolidone) |
| ROC | receiver operating characteristics |
| SC1 | substrate control 1 |
| SC2 | substrate control 2 |
| SDS | sodium dodecyl sulfate |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SNR | signal-to-noise ratio |
| SOC | super optimal broth with catabolite repression |
| StAR | statistical analysis of ROC curves |
| TBE | Tris-borate-EDTA |
| TBS-T | Tris-buffered saline-Tween-20 |
| TMB | 3,3',5,5'-tetramethylbenzidine |
| Tris | Tris(hydroxymethyl)aminomethane |
| Tris-Cl | Tris base adjusted to pH with HCl |
| UMMC | University Malaya Medical Center |
| VCA | viral capsid antigen |
| WHO | World Health Organization |
| ZEBRA | Z-encoded broadly reactive activator |

# CHAPTER 1. INTRODUCTION

Global cancer statistics revealed about 84,400 incident cases of nasopharyngeal carcinoma (NPC), causing 51,600 deaths and contributing 0.7% of global cancer burden in 2008 (Jemal et al., 2011). NPC is one of the rare cancers in the world, however, NPC was the fourth most common cancer among Malaysians and the third most common cancer among males in Malaysia in 2007 (Zainal Ariffin Omar & Nor Saleha Ibrahim Tamin, 2011). Among various ethnic groups in Malaysia, Chinese and Bidayuh populations are highly susceptible to NPC (Devi et al., 2004; Pua et al., 2008; Zainal Ariffin Omar & Nor Saleha Ibrahim Tamin, 2011).

Patients having symptoms suggesting NPC may be diagnosed early by otolaryngologist. However, the majority of NPC cases may not have clear symptoms until an advanced stage (stages III and IV) (Wei & Sham, 2005). For new NPC cases in Malaysia, neck lumps were the most common symptom (42%), followed by non-specific symptoms (nasal obstruction (30%), aural problems (11%), and headache (5%)), cranial nerve (6%) and other symptoms (6%) (Pua et al., 2008). Although imaging examinations, i.e., computed tomography, magnetic resonance imaging (MRI) and positron emission tomography, can provide invaluable information about the primary and recurrent tumors, patients in developing countries, such as Malaysia, often have limited or delayed access to those imaging systems. In addition, non-invasive, reliable laboratory tests for detection of NPC are still lacking.

Because Epstein-Barr virus (EBV) is one of the causative agents of NPC (International Agency for Research on Cancer (IARC) Working Group, 1997; Young & Rickinson, 2004), and anti-EBV antibody levels were found to be elevated in NPC patients (Henle et al., 1970; Ho et al., 1976; Gan et al., 1996), EBV serologic screening programs are routinely conducted in endemic regions such as southern China, Hong Kong and Taiwan (Chien et al., 2001; Ji et al., 2007; Ng et al., 2009b; Yu et al., 2011).

Physical examinations on heck and neck are performed in parallel, providing a better quality of health care to suspected cases and individuals with family history of NPC. However, such efforts are still lacking in Malaysia.

We aim to develop and evaluate the diagnostic performances of in-house ELISAs for a case-control study of NPC in Malaysian Chinese, using recombinant EBV protein produced by *Escherichia coli*. In this study, the relationships between anti-EBV IgG levels and NPC in our case-control samples were explored. We did not study anti-EBV IgA levels because they are examined by another student in the same laboratory.

Our study samples were plasma that were obtained from prevalent NPC patients during their follow-up appointments at otorhinolaryngology clinic of University Malaya Medical Center (UMMC), and normal subjects during blood donation campaigns conducted by UMMC. Because samples from incident/recurrent NPC patients were not available to us, we wanted to determine if our in-house assays (developed in the present work) can classify the remission cases and controls satisfactory, which, in turn, (i) may provide us data to conduct an active follow-up for the patients who have strong positive EBV serologic tests before the recurrence of NPC, and (ii) may provide us a justification to acquire samples from incident and recurrent NPC patients through a multicenter collaboration in the near future. The attempt to classify remission cases from controls is a challenging task because the anti-EBV antibody levels in remission patients may be unchanged, elevated or decreased, depending on their treatment outcomes and clinical status of NPC (Lynn et al., 1985; De-Vathaire et al., 1988; Neel & Taylor, 1990; Yip et al., 1994; Baizig et al., 2012). In this context, we anticipate the diagnostic performances of in-house EBV serologic tests on the classification of incident/recurrent NPC cases and controls in the near future would be more promising than the present results.

The objectives of the present study are (i) to express and purify recombinant EBV proteins, i.e., early antigen-diffuse (EA-D), Z-encoded broadly reactive activator (ZEBRA) and viral capsid antigen (VCA) p18, from *Escherichia coli*, (ii) to develop IgG-based indirect ELISAs using the recombinant EBV proteins for a case-control study of NPC in Malaysian Chinese, and (iii) to obtain the diagnostic performances for the in-house ELISAs.

# CHAPTER 2. LITERATURE REVIEW

## 2.1. NPC

NPC is one of the head and neck cancers, arises in the epithelial lining (squamous, respiratory and transitional epithelium) of the nasopharynx (Tang et al., 2012). Besides NPC, wide range of tumors may arise in the nasopharynx. However, NPC is unique because its incidence rate is only high in certain ethnic groups and geographical regions (Curado et al., 2007; Jemal et al., 2011).

### 2.1.1. Histological subtypes

World Health Organization (WHO) classified NPC in 1978, and revised it in 1991 and 2003 (Barnes et al., 2005). The classification of NPC is based on the differentiation status of malignant squamous cells observed under light microscope (Pathmanathan et al., 1995).

In 1978 WHO classification, NPC was categorized into WHO type 1, 2 and 3, which correspond to squamous cell carcinoma, non-keratinizing carcinoma and undifferentiated carcinoma, respectively (Shanmugaratnam & Sobin, 1978).

However, conflicting points of view led to a revision of NPC classification, in which one argued that WHO type 2 and 3 have overlapping histologic patterns, and resemblance in epidemiologic and biologic features; the other considered that the existing classification is well standardized in literature, and could favor further characterization (Shanmugaratnam & Sobin, 1993). Therefore, 1991 WHO classification abolished the numerical designation by emphasizing type 1 NPC as keratinizing squamous cell carcinoma, and combining type 2 and 3 NPC as non-keratinizing carcinoma, which subdivides into differentiated and undifferentiated patterns (Shanmugaratnam, 1991).

In 2003, basaloid squamous cell carcinoma was added to 1991 WHO classification (Barnes et al., 2005). Nevertheless, 1978 WHO classification remains widely used by researchers. To synchronize with literature, here we retain the nomenclature from 1978 WHO classification.

### 2.1.2. Geographical distribution and ethnicity

In most populations, NPC is a rare cancer that account for below one of age-standardized rate (ASR) per 100,000 world population (Curado et al., 2007). However, NPC is one of the leading cancers in endemic regions such as Greenland, southern China, Hong Kong, Taiwan and Southeast Asia (Jemal et al., 2011).

In these endemic regions, certain ethnic and/or sub-ethnic groups are more susceptible to NPC, (i) Inuit in Greenland (Friborg et al., 2005), (ii) Cantonese descent of Chinese in southern China and Hong Kong (Yu & Yuan, 2002; Jia, 2008), (iii) Chinese in Taiwan, Malaysia and Singapore (Chen et al., 1988; Zainal Ariffin Omar & Nor Saleha Ibrahim Tamin, 2011; Lee et al., 2012), and (iii) Bidayuh in Sarawak (Devi et al., 2004).

It should be noted that most Chinese descent in Malaysia and Singapore share a common ancestry, having their roots in Guangdong (Canton) and Fujian provinces of southern China. Because NPC is common among Cantonese, it is well known as ‘Kwangtong tumor’ or ‘Cantonese cancer’ (Ho, 1978; Jia, 2008). However, an epidemiological study (1996-1998) in Sarawak (a province of Malaysia located in the island of Borneo) revealed that the NPC incidence rate in a native population, Bidayuh was exceptionally high (ASR for male, 31.5/100,000, and for female, 11.8/100,000), exceeding the record of Hong Kong population (ASR for male, 21.4/100,000, and for female, 8.3/100,000) (Devi et al., 2004). Therefore, the burden of NPC in Malaysia deserves extensive investigations.

### 2.1.3. Age-incidence curves

NPC incidence rate increases with age. Interestingly, low-risk (in certain areas in Indian, Japan, North America, northwest Europe and Australia) and high-risk (in certain areas or populations in Philippines, Singapore and Thailand) populations exhibit different patterns of age-incidence curves (1983–1997) (Figure 2.1) (Bray et al., 2008). Regardless of geographical area and gender, the age-incidence curves of NPC for low-risk populations demonstrated a first peak at 15–24 years and a second peak at 65–79 years. By contrast, no bimodality in the age-incidence curves of NPC for high-risk populations, which singly peaked at 45-60 years.

Notably, most NPC cases in low-risk populations were WHO type 1 but those in high-risk populations were WHO type 3. In low-risk populations, type 2 and 3 NPC cases may correspond to the first peak of incidence, while type 1 NPC cases may correspond to the second peak of incidence (Burt et al., 1992; Vaughan et al., 1996; Ayan et al., 2003; Bray et al., 2008;).

The type 1 NPC in low-risk population is plausibly associated with environmental (occupational exposures) and lifestyle (alcohol and cigarette) factors, which resulted in late sporadic NPC; the type 2 and 3 NPC in low-risk population are plausibly associated with genetic and environmental (EBV infection) factors, which resulted in familial and early sporadic NPC (Vaughan et al., 1996; IARC Working Group, 1997; Vaughan et al., 2000; Bray et al., 2008).

By contrast, the type 3 NPC in high-risk population is plausibly associated with genetic, environmental (EBV infection and occupational exposures) and lifestyle (salt-preserved foods and alcohol) factors (Bray et al., 2008; Bei et al., 2012; Jia & Qin, 2012; Tsao et al., 2012). The etiology of NPC, particularly for Malaysian Chinese, will be further discussed in Section 2.1.5.

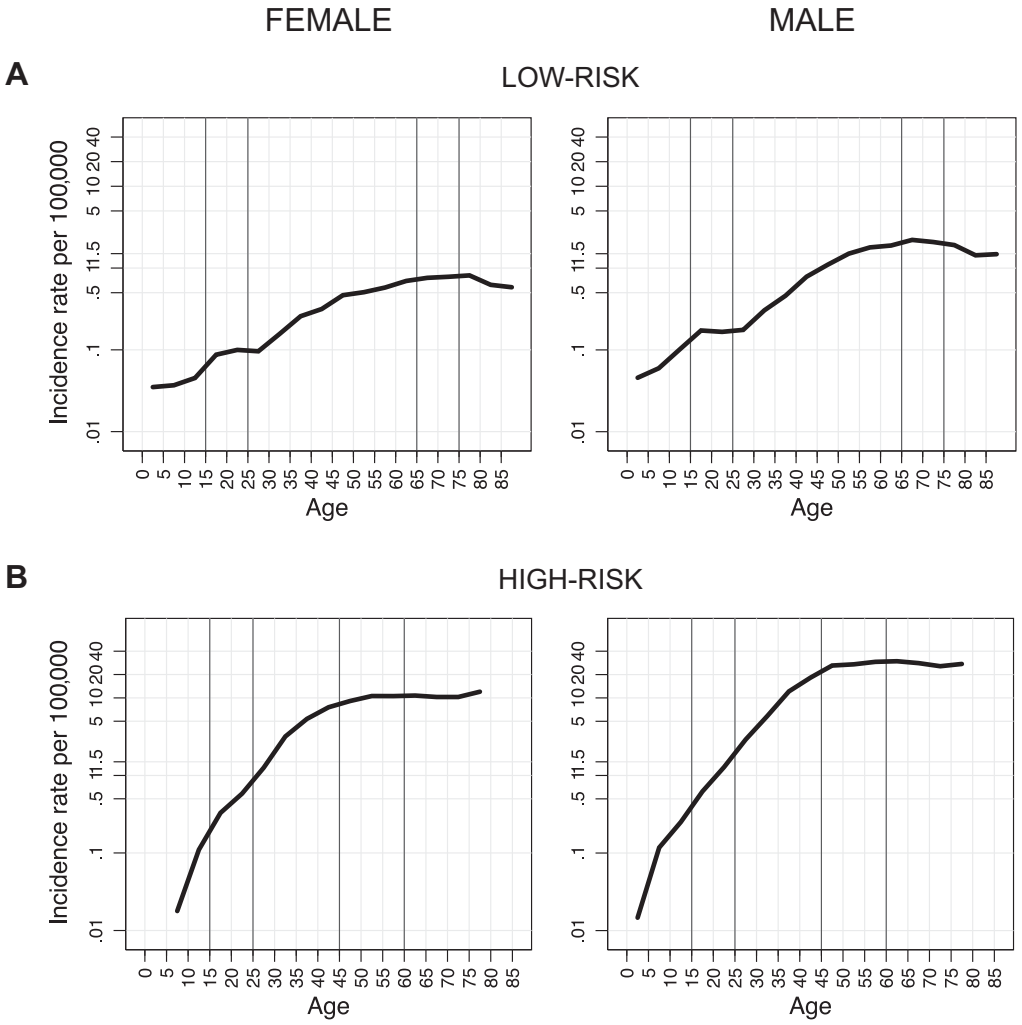


**Figure 2.1.** Aggregated age-specific incidence rates of NPC by gender in (A) low-risk and (B) high-risk populations. The rates were calculated by weighting the counts and person-years by the square root of the total population in period 1983–1997. The registry data (annual detailed dataset for Cancer Incidence in Five Continents, volume I–VIII) for low-risk populations covered certain areas in Indian, Japan, North America, northwest Europe and Australia, while for high-risk populations covered certain areas or populations in Philippines, Singapore and Thailand. Black vertical lines highlight the age boundaries of interest (adapted from Bray et al., 2008).

In Peninsular Malaysia, three major ethnic groups, Malay, Chinese and Indian, were taken into account in Malaysian Cancer Statistics 2006 (Zainal Ariffin Omar et al., 2006). It was reported that the NPC incidence rate in Chinese (ASR for male, 14/100,000, and for female, 3.8/100,000) was at least threefold higher than other ethnic groups (Malay: ASR for male, 4/100,000, and for female, 1.3/100,000; Indian: ASR for male, 1/100,000, and for female, 0.2/100,000). Regardless of ethnicity, the NPC incidence rate for male (ASR of 8.5/100,000) was also threefold higher than female (ASR of 2.6/100,000).

In 2006, the age-incidence curves of NPC for Malaysian Chinese peaked at 50–59 year (Figure 2.2). By contrast, the male populations of Malay and Indian over 70 years of age conferred the highest NPC incidence rates.

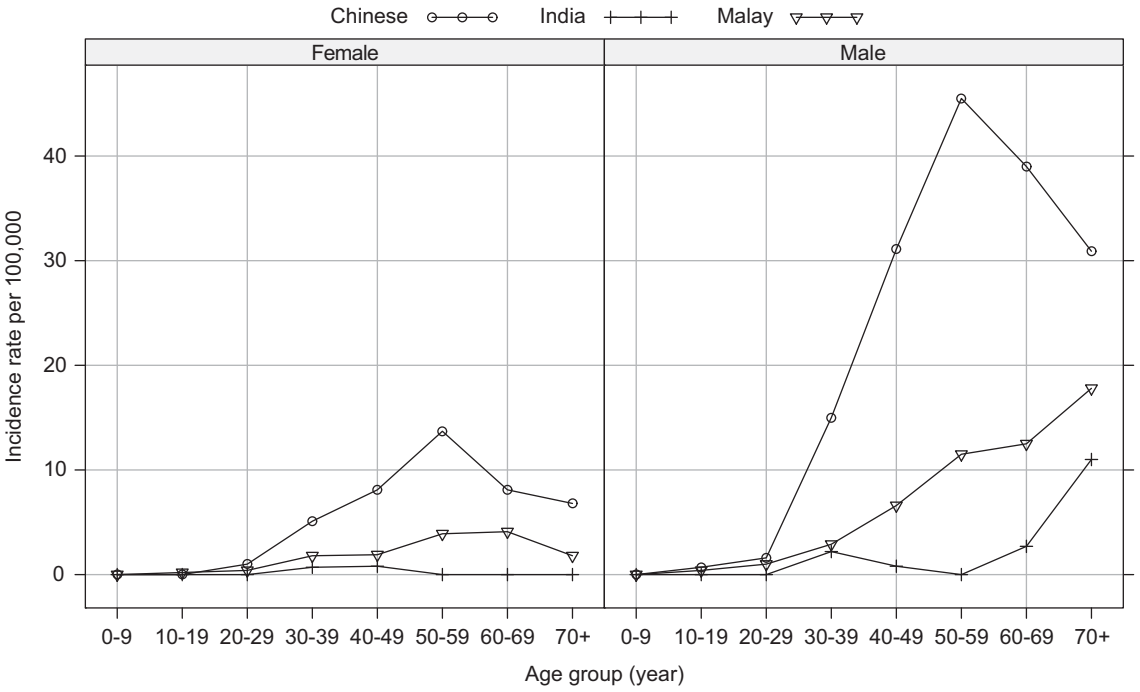


**Figure 2.2.** Age-specific incidence rate of NPC by ethnic and gender in Peninsular Malaysia 2006. Data source: Malaysian Cancer Statistics 2006 (Zainal Ariffin Omar et al., 2006).

Besides, we observed a notable similarity between the age-incidence curves of NPC in 2006 for the male populations of Malaysian Chinese and Hong Kong (Malaysian Cancer Statistics 2006 (Zainal Ariffin Omar et al., 2006); Hong Kong Cancer Statistics 2006) (Figure 2.3). Among the three regions, the NPC incidence rates by age groups were relatively lower in Taiwan (Taiwan Cancer Registry, 2006).

It should be noted that the cancer registries in Hong Kong and Taiwan did not publish NPC incidences by ethnicity. Nevertheless, 95% Hong Kong population was Chinese, according to 2006 Population By-census Office, Census and Statistics Department, Hong Kong; 98% Taiwan population in 2006 was Chinese, according to Monthly Bulletin of Interior Statistics, Taiwan. Therefore, Figure 2.3 roughly superimposes the age-incidence curves for Chinese in the three endemic regions in the same year.

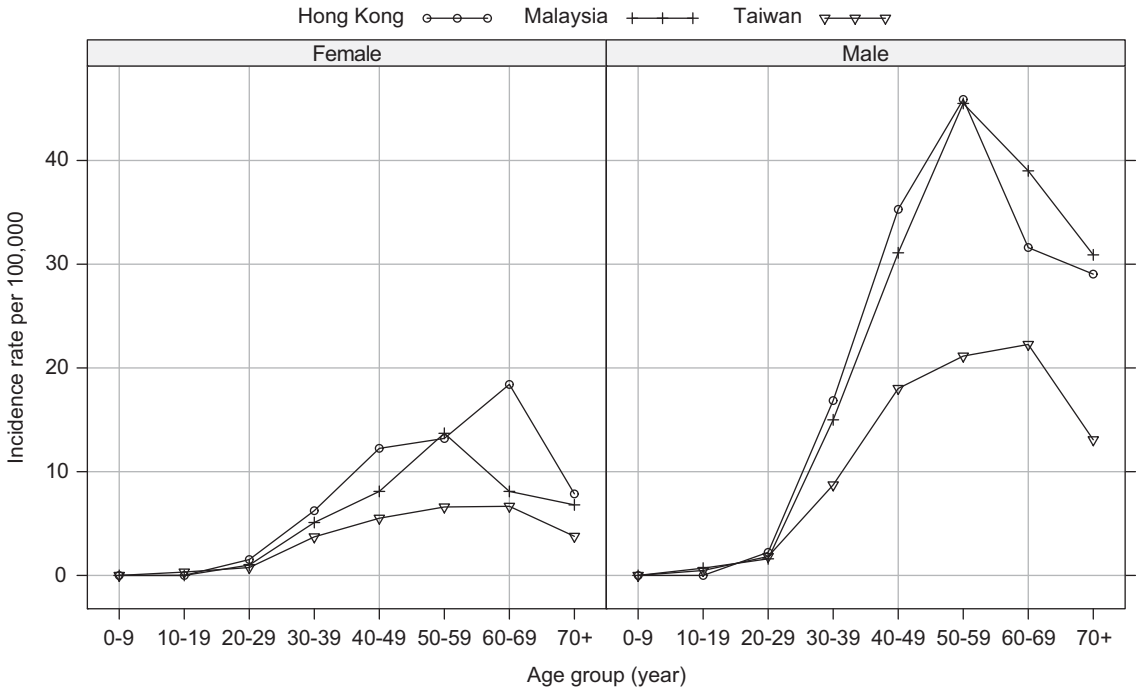


**Figure 2.3.** Age-specific incidence rate of NPC by gender, among the populations of Hong Kong, Taiwan and Peninsular Malaysia (Chinese) in 2006. Data source: Hong Kong Cancer Statistics 2006, Malaysian Cancer Statistics 2006 (Zainal Ariffin Omar et al., 2006) and Taiwan Cancer Registry, 2006.

### 2.1.4. Secular trends

The NPC incidence rates in Hong Kong, Taiwan and Singapore decreased over the past two decades (Lee et al., 2003; Hsu et al., 2006; Luo et al., 2007). Based on the four annual reports of cancer statistics available to date (The First Report of the National Cancer registry 2002 (Lim et al., 2003), Second Report of the National Cancer registry 2003 (Lim & Halimah Yahaya, 2004) and Malaysian Cancer Statistics 2006 (Zainal Ariffin Omar et al., 2006), National Cancer Registry Report 2007 (Zainal Ariffin Omar & Nor Saleha Ibrahim Tamin, 2011)), the NPC incidence rate in Peninsular Malaysia also decreased from 2002 to 2003, 2003 to 2006, and 2006 to 2007. By contrast, the NPC incidence rate in southern China remained stable over time (Jia et al., 2006).

In age-period-cohort models (1973–1997), NPC incidence rates were significantly decreased for cohorts of Chinese who were born after 1940 and 1958 in Hong Kong and Singapore, respectively (Luo et al., 2007). The observations are in agreement with the changing of lifestyles after the economic rose in Hong Kong (end of Pacific war in 1945) and Singapore (independence in 1965) (Yu & Yuan, 2002). By contrast, economic in China reformed in 1978 but only began to grow rapidly in 1990s, the impact on NPC incidence rate in 1973–1997 was not obvious (Jia et al., 2006). Because no published data across the timeline, we cannot rule out the effect of birth cohort on NPC incidence rate in Malaysia.

### 2.1.5. Etiology

Based on the epidemiological observations (geographical distribution, ethnicity, age-incidence curves and secular trends), genetic, environmental and lifestyle factors should be closely associated with NPC. The complex interactions between these factors may lead to NPC.

The environmental risk factors for NPC include occupational exposures and EBV infection (Jia & Qin, 2012; Tsao et al., 2012). Because nasopharynx is responsible in filtering small particles at upper airway (Jaeger & Blank, 2011), certain harmful inhalants are linked with NPC. A study on the etiology of NPC in Malaysian Chinese found that wood dust and industrial heat were positively associated with NPC; in contrast with other studies, no association of formaldehyde exposure with NPC was found (Armstrong et al., 2000).

Lifestyles, mainly dietary risk factors for Malaysian Chinese NPC cases were also explored, in which the consumptions of salt-preserved foods (fish, leafy vegetables, egg and root), fresh organs (pork or beef) and alcohol (beer and liquor) showed strong positive associations with NPC, whereas the consumptions of fresh fruits and vegetables showed strong negative associations with NPC (Armstrong et al., 1998). In particular, childhood consumption of salted fish conferred significant and consistent association with NPC among Chinese populations in different geographical regions (Armstrong et al., 1983; Yu et al., 1986; Yu et al., 1988; Ning et al., 1990; Yang et al., 2005; Jia et al., 2010). It was postulated that nitrosamines and nitrosamine precursors from salt-preserved foods contribute to NPC carcinogenesis.

To understand the genetic susceptibility to NPC, recently, three genome-wide association studies (high-throughput genotyping of single nucleotide polymorphisms) were performed, recruiting the NPC cases and controls of Chinese from Malaysia, Singapore, Taiwan and southern China (Ng et al., 2009a; Tse et al., 2009; Bei et al., 2010). Their most consistent results suggested that human leukocyte antigen (HLA) genes, in particular HLA-A gene, are strongly associated with NPC. In fact, the associations of HLA genes (HLA-A, HLA-B, HLA-Cw, HLA-DP, HLA-DQ, and HLA-DR genes) with NPC have been suggested by previous candidate gene studies that can be dated back to 1974, when HLA-A2 was found to be positively associated with NPC

in Singapore Chinese (Simons et al., 1974; Bei et al., 2010; Hassen et al., 2010). It is plausible that the antigen-presentation genes involve in pathogenesis of EBV in NPC, because the expression levels of HLA genes (HLA-A, HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G genes) were inversely correlated with EBV genes (genes for EBV nuclear antigens (*EBNA-1*, *EBNA-2*, *EBNA-3A*, and *EBNA-3B*), and latent membrane proteins (*LMP-1*, and *LMP-2A*)), especially *EBNA-1* in NPC tissues (Sengupta et al., 2006). However, it is currently unclear for the relationships between the polymorphisms of HLA genes, their expression levels, and the role of EBV in tumorigenesis of NPC.

## 2.2. EBV

The modes of viral entry into normal human primary epithelial cells were demonstrated by both direct infection and B-cell mediated transfer infection of EBV (Feederle et al., 2007). In particular at nasopharynx, direct infection of EBV is more plausible due to the lack of cell-to-cell contact.

Despite EBV infects and persists lifelong in 90% of world population, EBV is widely agreed as a causative agent of NPC (IARC Working Group, 1997; Young & Rickinson, 2004). EBV seroconversion was observed in Malaysian children, in which the primary infection occurred at four to five month of ages, and all have seroconverted by eight years of age (Yadav et al., 1987). Childhood EBV infection was also observed in Hong Kong and other developing countries (Kangro et al., 1994). It may be due to overcrowding and/or poor hygiene because EBV transmits easily via saliva (Hudnall & Stanberry, 2006). EBV infection in early life is subclinical, but it may involve in tumorigenesis of NPC in later life (IARC Working Group, 1997), possibly by re-infection and/or recurrent reactivations of EBV (Chien et al., 2001; Fang et al., 2009).

### 2.2.1. Serological methods

The association of EBV with NPC was shown in early findings, which employed immunofluorescence assay (IFA) for the detection of circulating antibodies against EBV proteins in NPC sera (Henle et al., 1970; Ho et al., 1976). However, the immunodetection method is (i) dependent on the batch-to-batch reproducibility of EBV antigen-expressing cells (chemically induced P3HR-1 and Raji cells to produce VCA and EA complexes, respectively), (ii) time-consuming, (iii) difficult to automate, and (iv) lack of consensus on fluorescence counting among research groups.

Because studies have shown an acceptable concordance between IFA and ELISA results (Dölken et al., 1984; Luka et al., 1984), ELISA was widely adopted in EBV serology in the past two decades (Gan et al., 1996). Recently, studies using Luminex multi-analyte profiling were conducted in southern China, using recombinant proteins (VCA-gp125, EA-D, and EBNA-1) and synthetic peptides (VCA-p18, EBNA-1, gp78, gp350/220 and BLRF1) of EBV (Gu et al., 2008a; Gu et al., 2008b; Gu et al., 2009a; Gu et al., 2009b). But this technology is not yet generalized in hospitals and diagnostic laboratories, limiting it from routine serologic test. Moreover, the overall diagnostic performances (EA-D/IgG, EA-D/IgA, EBNA-1/IgA, and VCA/IgA) of commercial ELISA kits were better than their in-house assays (Gu et al., 2008b).

ELISA is popular because (i) coating and preparation of solid phase (commonly a 96-well polystyrene microplate) is versatile and reproducible, (ii) its routine run required less technical expertise than IFA (For example, ELISA reader records optical signals for samples instead of fluorescence microscopic counting in IFA), (iv) it can be fully automated for high-throughput screening of samples, and therefore (v) cost-effective and (vi) time-saving.

We adopted indirect ELISA in the present work. The key steps of indirect ELISA (Figure 2.4) include (i) coating of the targeted antigen on a solid phase, (ii)

blocking of the remaining active surface of the solid phase after antigen immobilization, (iii) antigen-antibody binding (primary antibody from plasma), (iv) probing of primary antibody by enzyme-conjugated secondary antibody, (v) wash steps in between step i to iv, (vi) development of optical signal by enzyme-substrate reaction, and (vii) stopping of the enzyme-substrate reaction (Crowther, 2009).

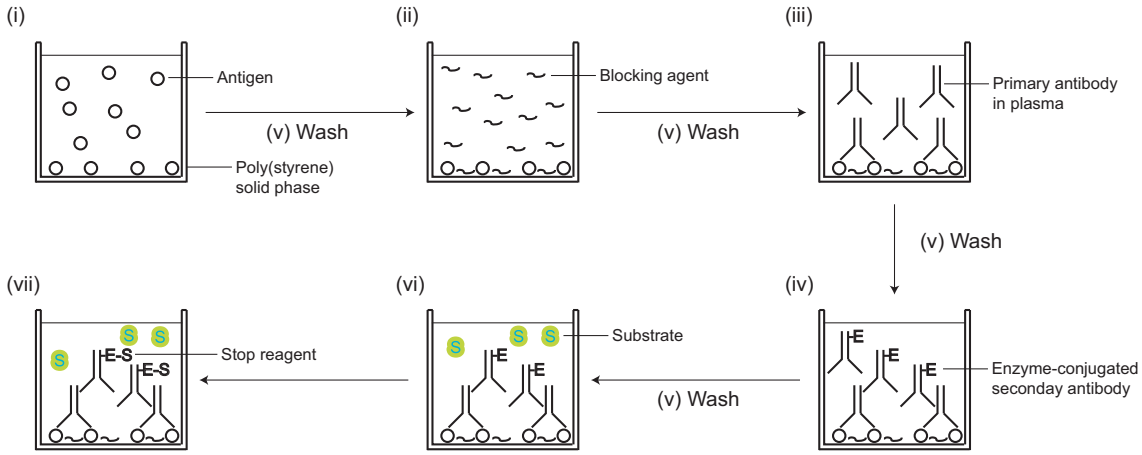


**Figure 2.4.** Key steps of indirect ELISA: (i) coating of targeted antigen on a solid phase, (ii) blocking of the remaining binding surface with blocking agent, (iii) binding of primary antibody in plasma to the immobilized antigen, (iv) probing of primary antibody by enzyme (E)-conjugated secondary antibody, (v) wash steps in between step i to iv, (vi) development of optical signal by enzyme-substrate reaction, and (vii) stopping of the enzyme-substrate reaction with stop reagent (S).

### 2.2.2 Analytical sample

Serum and plasma are extracellular components of blood for medical laboratory studies. There are three types of plasma, i.e., citrated plasma, EDTA plasma and heparinized plasma. These analytical samples should be used selectively because of the disparities in matrix components (Working Group on Preanalytical Variables, 1998).

For EBV serology, the working group suggested serum as the analytical sample of choice. Despite of the recommendation, previous studies employed sera and/or plasma for EBV serology, in which one of the studies showed that the ELISA results (IgG- and IgA-based assays, using synthetic peptides from (i) EBNA-1 of EBV, (ii) phosphoprotein pp150 of cytomegalovirus, and (iii) early and late proteins of human

papillomaviruses) for sera and EDTA plasma were identical ($n$ = 28) (Dillner et al., 1994). In the present study, we used EDTA plasma because only EDTA plasma were available.

### 2.2.3 Serologic markers

Because of re-infection and/or reactivation of EBV (Chien et al., 2001; Fang et al., 2009), previous ELISA studies (in both endemic and non-endemic regions) have found the elevated levels of anti-EBV antibodies in NPC cases, in particular type 2 and 3 NPC, and further evaluated the diagnostic and prognostic values of anti-EBV antibodies as surrogate biomarkers for NPC (Cheng et al., 1993; Mathew et al., 1994; Gan et al., 1996; Chien et al., 2001; Dardari et al., 2001, 2008; Cheng, 2002; Chan et al., 2003; Karray et al., 2005; Fachiroh et al., 2006; Hu et al., 2007; Ji et al., 2007; Paramita et al., 2007; Tedeschi et al., 2007; Ng et al., 2009b; Abdulamir et al., 2010; Fachiroh et al., 2010; Paramita et al., 2011; Yu et al., 2011). To achieve early diagnosis of NPC, EBV serologic screening and physical examinations have became routine clinical checkup for suspected cases and individuals with family history of NPC in endemic regions such as southern China, Hong Kong, and Taiwan (Chien et al., 2001; Ji et al., 2007; Ng et al., 2009b; Yu et al., 2011). But such efforts are still lacking in Malaysia.

Some EBV proteins are highly immunogenic, such as EA-D, EBNA-1, ZEBRA and VCA p18, in which anti-EBV antibodies against these proteins showed relatively strong associations with NPC (Cheng et al., 1993; Mathew et al., 1994; Gan et al., 1996; Chien et al., 2001; Dardari et al., 2001, 2008; Cheng, 2002; Chan et al., 2003; Karray et al., 2005; Fachiroh et al., 2006; Hu et al., 2007; Ji et al., 2007; Paramita et al., 2007; Tedeschi et al., 2007; Ng et al., 2009b; Abdulamir et al., 2010; Fachiroh et al., 2010; Paramita et al., 2011; Yu et al., 2011). By contrast, some EBV proteins are poorly

immunogenic, particularly the latency proteins, latent membrane proteins (LMP-1, LMP-2A) and BARF1, which are not useful biomarkers for NPC (Paramita et al., 2011).

To date, however, immunoassays for EBV-associated NPC have not yet been standardized. (i) Although VCA/IgA by IFA is known as the 'gold standard' for NPC serology, it is in discord with a recent report that screened 2444 individuals from NPC multiplex families in Taiwan, showing that the performance of EBNA-1/IgA (by ELISA) was better than VCA/IgA (by both IFA and ELISA) (Yu et al., 2011). To maximize the clinical utility of VCA/IgA, long-term monitoring (about 15-year follow-up) of its fluctuating level in a suspected subject might be necessary (Chien et al., 2001; Ji et al., 2007). This is because from the active follow-up, those who eventually developed NPC maintained high titers of VCA/IgA for three years before clinical onset (Ji et al., 2007). (ii) For ELISA, the EBV antigens were produced in various ways, ranging from the use of mammalian, insect, yeast and prokaryotic expression hosts to peptide synthesis (Cheng et al., 1991, 1993; Mathew et al., 1994; Dardari et al., 2001, 2008; Hu et al., 2007; Paramita et al., 2007; Tedeschi et al., 2007; Paramita et al., 2011). (iii) Some studies used mixtures of two antigens or putative epitopes to enhance their ELISA performances (Fachiroh et al., 2006; Fachiroh et al., 2010).

In addition, the diagnostic performances of serologic tests were varied among studies because of the discrepancies in study populations (sample size, ethnicity, and selection bias), assay setups, data analysis and interpretation, and lack of validation study. Therefore, the serological marker of choice and its cutoff value remain inconclusive (Fachiroh et al., 2010; Yu et al., 2011), hampering a comparison across ELISA studies.

Serologic profiles in many populations from endemic and non-endemic regions have been investigated. However, ELISA study that reported its diagnostic accuracy on NPC in Malaysian Chinese is currently lacking. Although Mathew et al. (1994) and

Cheng et al. (1993) reported the diagnostic accuracies of ZEBRA/IgG (75% sensitivity, 96.4% specificity) and synthetic peptides of EBNA-1/IgA (90.8% sensitivity, 92.9% specificity), respectively; they did not categorize study subjects by ethnicity (ethnicity is one of the major confounding factors). Despite the survival rate for early-stage patients is higher, most NPC cases in Malaysia were diagnosed at an advanced stage (Pua et al., 2008; Zainal Ariffin Omar & Nor Saleha Ibrahim Tamin, 2011). Therefore, the present study intends to develop indirect ELISAs that can be used to screen the high-risk population in Malaysia in the future, using the immunogenic EBV proteins, EA-D, ZEBRA and VCA p18. We do not provide comparisons between our in-house ELISAs with commercial ELISA kits because the comparisons are part of an on-going study by our colleague. We believe that a meaningful comparison across ELISA studies can be done if all ELISA studies use standardized, Food and Drug Administration-approved ELISA kits in parallel, when applicable.

# CHAPTER 3. MATERIALS AND METHODS

## 3.1. STUDY SUBJECTS

### 3.1.1. Collection of plasma

The ethical approval for this study was granted by the Medical Ethics Committee of UMMC. Volunteers were enrolled between September 2007 and April 2011.

Patients with biopsy-proven NPC were recruited at otorhinolaryngology clinic of UMMC during their follow-up appointments. A total of 165 prevalent cases (under remission) were recruited, including 148 Chinese (89.7%), 15 Malays (9.1%), and two Indians (1.2%). The ratios of male to female patients were exactly 3:1 and 2:1 in Chinese and Malays, respectively. The two Indians are males.

Meanwhile, normal subjects were recruited during the blood donation campaigns organized by UMMC. A total of 658 normal subjects were recruited, including 334 Chinese (229 males, 105 females), 154 Malays (99 males, 55 females), 168 Indians (100 males, 68 females), and two Kadazans (males).

A survey was performed to obtain their family history and individual information, i.e., age, gender, national identification, ethnicity and cancer staging (if applicable). With written consents, their blood samples were collected and managed as potentially infectious materials. About 3 ml of peripheral blood was withdrawn from each volunteer, transferred into blood collection tube containing $K_3$EDTA, and temporarily kept on ice. Upon delivered to laboratory, it was centrifuged for 10 min at 1,000 *g* at room temperature. Plasma was saved and kept at −20°C.

### 3.1.2. Individual matching

To minimize bias, we performed individual matching based on age (within 5 years), gender and ethnicity. Only Malaysian Chinese was selected for this study because the NPC cases of other ethnicity were insufficient.

Before matching, 111 male patients (mean age, 55 years; range, 19–86 years) and 37 female patients (mean age, 51 years; range, 25–72 years) were eligible. Among the Malaysian Chinese normal subjects, three of them had a NPC-affected first-degree relative ($n$ = 2) or spouse ($n$ = 1) who is male and above 40-year-old. We excluded the three normal subjects with family history of NPC. Mixed parentage subjects were excluded as well. Hence, the normal subjects that eligible for matching were 227 males (mean age, 37 years; range, 16–59 years) and 104 females (mean age, 37 years; range, 18–58 years).

After matching, 120 case-control pairs were obtained. The case group was composed of 85 males (mean age, 51 years; range, 19–63 years) and 35 females (mean age, 50 years; range, 25–63 years). The control group was composed of 85 males (mean age, 47 years; range, 19–59 years) and 35 females (mean age, 45 years; range, 25–58 years).

## 3.2. PROTEIN EXPRESSION AND PURIFICATION

### 3.2.1. Construction of prokaryotic expression vectors

The target genes of EBV, strain B95-8 (GenBank: V01555) were subcloned from yeast expression vectors, pYES2.1 (generous gifts from Prof. Sam Choon Kook) into prokaryotic expression vector, pET102/TOPO-D (Invitrogen, USA). The sizes of empty pYES2.1 and pET102 vectors are 5882 bp and 6315 bp, respectively. The target genes are *BMRF1* (1215 bp), *BZLF1* (738 bp) and *BFRF3* (531 bp), which encode full length EA-D, ZEBRA and VCA p18, respectively. Fusion protein produced by pET102

expression system contains N-terminal His-patch thioredoxin, and C-terminal V5 epitope and 6×His tags. The N-terminal tag can increase translation efficiency and solubility of the fusion protein, whereas the C-terminal tags enable the immunodetection of the fusion protein; both His-patch thioredoxin and 6×His tags allow immobilized metal affinity chromatography (IMAC)-based purification (Lu et al., 1996; Terpe, 2003).

The subcloning procedures are described in the following paragraphs. PCRs were performed using the pYES2.1 recombinant plasmids as DNA templates (Table 3.1, 3.2, and 3.3). The PCR products were purified, mixed with vector and salt solutions, and incubated at room temperature for 5 min for TOPO ligation reaction (Table 3.4).

**Table 3.1.** PCR working reaction.

| Reagent | Working reaction | |
|---|---|---|
| | Volume (µl) | Concentration |
| Sterile water | 10.8; 11.3 | - |
| 10×PCR buffer (with 20 mM $MgCl_2$) | 1.5 | (1 mM $MgCl_2$) |
| dNTPs (10 mM) | 0.3 | 0.2 mM |
| Forward primer (10 µM) | 0.6 | 0.4 µM |
| Reverse primer (10 µM) | 0.6 | 0.4 µM |
| DNA polymerase (2.5 U/µl) | 0.2 | 0.5 U/µl |
| Plasmid; cell lysate | 0.5; 1.0 | 50 ng/µl; uncharacterized |
| Total volume | 15 | |

Abbreviation: dNTP, deoxyribonucleotide triphosphate.

**Table 3.2.** Primer sequences for amplification EBV genes.

| EBV genes | Primer (5′–3′) |
|---|---|
| *BMRF1* | F: CACCATGGAAACCACTCAGACTCTC |
| | R: CTTATCGTCATCGTCAATGAGGGGGTTAAA |
| *BZLF1* | F: CACCATGATGGACCCAAACTCGAC |
| | R: CTTATCGTCATCGTCGAAATTTAAGAGATC |
| *BFRF3* | F: CACCATGGCACGCCGGCTGCCCAA |
| | R: CTTATCGTCATCGTCCTGTTTCTTACGTGC |

CACC (underlined) at the 5′ end of the forward primer (F) is required for TOPO directional ligation.
CTTATCGTCATCGTC (underlined) at the 5′ end of the reverse primer (R) introduces an enterokinase recognition site at the upstream of the V5 epitope and 6×His tags to the fusion protein.

**Table 3.3.** PCR cycling conditions.

| Cycle step | Temperature (°C) | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 94 | 5–15 min | 1 |
| Denaturation | 94 | 30 s | 25 |
| Annealing | 55 | 30 s | |
| Extension | 72 | 30 s | |
| Final Extension | 72 | 5–10 min | 1 |

**Table 3.4.** TOPO ligation reaction.

| Reagent | Volume (µl) |
|---|---|
| PCR product | 4 |
| Salt solution (1.2 M NaCl, 0.06 M $MgCl_2$) | 1 |
| pET102 vector (15 to 20 ng/µl) | 1 |

*E. coli* TOP10 competent cells (Invitrogen, USA) were transformed with the cloning reaction mixtures by heat shock method. In brief, 1 µl of the cloning reaction mixtures were added to 50 µl of the competent cells, gently mixed, and incubated on ice for 5 min. The cells were heat-shocked for 30 s at 42°C, returned to ice immediately, and grown in 250 µl of super optimal broth with catabolite repression (SOC) media at 37°C for 1 h with moderate shaking (250 rpm).

To select transformants, the bacterial cultures were grown in LB agar plate containing 100 µg/ml ampicillin at 37 °C overnight. Positive clones were identified by colony PCR (Table 3.1 and 3.3), using gene-specific forward primers (Table 3.2) and T7 reverse primer (a pET102 vector primer). Plasmids from positive clones were purified, followed by DNA sequencing. Molecular Evolutionary Genetics Analysis 4.0 (Tamura et al., 2007) was used to analyze the sequencing results.

### 3.2.2. Time course of IPTG-induced recombinant protein expression

BL21Star (DE3) (Invitrogen, USA) was used as protein expression host. The competent cells of BL21Star (DE3) were transformed with the recombinant PET102 plasmids using heat shock method (see Section 3.2.1). Their starter cultures were prepared in LB broths containing 100 µg/ml ampicillin and grown overnight at 37°C, 250 rpm.

The expression levels of the three target EBV proteins at varying time points after IPTG induction were investigated. In brief, 10 ml of fresh LB broths were seeded with 500 µl of starter cultures, and incubated at 37°C, 250 rpm. At mid-log phase, the 10 ml of fresh cultures were divided into half to assign IPTG-induced and uninduced cultures. IPTG solution (0.1 M) was added to the designated cultures at a final concentration of 1 mM.

Once the inducer was added, 500 µl was withdrawn from each culture. The aliquots were centrifuged at maximum speed for 3 min. Supernatants were discarded. Pellets were labeled as zero-hour time point and kept at −20°C. Samplings were repeated at 1 h intervals until four-hour time point. The remaining cultures were incubated overnight, followed by final samplings for overnight time point.

These *E. coli* samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (see Section 3.2.5). For Western blotting (see Section 3.2.6), samples at four-hour time point were used.

### 3.2.3. Auto-induction of recombinant protein expression

Auto-induction was used to scale-up the productions of the target proteins with minimum effort (Studier, 2005). Starter cultures were prepared by growing the freshly transformed host cells in 10 ml of MDG non-inducing minimum media containing 100 µg/ml ampicillin (Table 3.5 and 3.6), and grown overnight at 37°C, 250 rpm. Meanwhile, mock-transformed expression host was prepared, using media without antibiotic. It served as the negative control for this expression study.

Auto-induction was performed by growing the host cells (at 1000-fold dilutions of starter cultures) in 100 ml of ZYM-5052 media containing 100 µg/ml ampicillin (Table 3.5 and 3.6), and grown overnight at 37°C, 250 rpm. The auto-induced cultures were harvested for SDS-PAGE analysis and protein purification.

**Table 3.5.** Compositions of MDG and ZYM-5052 media.

| Component | MDG | ZYM-5052 |
|---|---|---|
| Tryptone | - | 1% |
| Yeast extract | - | 0.5% |
| $Na_2HPO_4$ | 25 mM | 25 mM |
| $KH_2PO_4$ | 25 mM | 25 mM |
| $NH_4Cl$ | 50 mM | 50 mM |
| $Na_2SO_4$ | 5 mM | 5 mM |
| $MgSO_4$ | 2 mM | 2 mM |
| 1000×trace metals (see Table 3.5) | 0.2× | 0.2× |
| Aspartate | 0.25% (18.8 mM) | - |
| Glycerol | - | 0.5% (54 mM) |
| Glucose | 0.5% (27.8 mM) | 0.05% (2.8 mM) |
| α-lactose | - | 0.2% (5.6 mM) |

Adapted from Studier (2005).

**Table 3.6.** Composition of 1000×trace metals for MDG and ZYM-5052 media.

| Trace metal | Concentrations (μM) |
|---|---|
| Fe | 50 |
| Ca | 20 |
| Mn, and Zn | 10 (for each solution) |
| Co, Cu, Ni, Mo, Se, and B | 2 (for each solution) |

Adapted from Studier (2005).

### 3.2.4. IMAC-based purification by membrane adsorber

We prepared the clarified lysates, i.e., the soluble fractions of cell extracts for protein purification under native conditions. In brief, the cell pellets from auto-induced cultures were resuspended in 15 ml of native purification buffer (50 mM $NaH_2PO_4$, 0.5 M NaCl, pH 8.0), and sonicated for 4 times at 12×10 s with 10 s pauses at 250 W. The lysates were clarified by centrifugation at 12,000 *g*, 4°C for 1 h.

A membrane-based purification device was assembled and charged. In brief, a 0.45 μm syringe filter (25 mm diameter) was attached to the inlet (female luer lock) of two connecting units of Sartobind IDA (iminodiacetic acid) 75 (Sartorius) to prevent clotting. The bed volume of this device is about 4.2 ml. Using a luer lock syringe, this device was equilibrated with 10 ml of equilibration buffer (0.1 M $CH_3COONa$, 0.5 M NaCl, pH 4.5), and charged with 10 ml of cobalt solution (equilibration buffer containing 0.1 M $CoCl_2$). To minimize the leaching of cobalt ion, the device was flushed with 10 bed volumes of equilibration buffer, followed by 10 bed volumes of native purification buffer.

The clarified lysates were loaded. About 1 ml of fractions were collected continuously until the end of elution. Unbound materials were washed away by 10 bed volumes of native wash buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole, pH 8.0), followed by elution at 250 mM imidazole by native elution buffer (20 mM sodium phosphate, 0.5 M NaCl, 250 mM imidazole, pH 8.0).

### 3.2.5. SDS-PAGE

Discontinuous Tris-glycine gels were prepared using Mini-PROTEAN 3 (Bio-Rad, USA) (Table 3.7). Samples were treated with Laemmli sample buffer (62.5 mM Tris-HCl buffer (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 0.01% (w/v) bromophenol blue and 5% (v/v) ß-mercaptoethanol), boiled for 10 min, and clarified by centrifugation at maximum speed for 5 min.

Supernatants of the samples (10 µl each) were applied to the vertical gels, and electrophoresed at 120 V for 90 min in running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, pH 8.3).

The gels were stained with staining solution (0.2% Coomassie brilliant blue R-250, 40% (v/v) methanol and 10% (v/v) acetic acid) for overnight. Excessive dye was removed by placing the gel in destaining solution (5% (v/v) methanol, 7% (v/v) acetic acid). The gels were then digitized on a flatbed scanner.

**Table 3.7.** Composition of discontinuous SDS-PAGE gel.

| Reagent | Volume (µl) | |
|---|---|---|
| | 10% separating gel | 4% stacking gel |
| Acrylamide/bis (30% T, 2.67% C) (w/v) | 2670 | 665 |
| 4×gel buffer | 2000 (1.5 M Tris-Cl, pH 8.8) | 1250 (0.5 M Tris-Cl, pH 6.8) |
| 10% (w/v) SDS | 80 | 50 |
| Sterile water | 3250 | 3000 |
| 10% (w/v) ammonium persulfate | 120 | 50 |
| N,N,N',N'-tetramethylethylenediamine | 8 | 5 |

Abbreviation: SDS, sodium dodecyl sulfate.

### 3.2.6. Western blotting

Protein bands in SDS-PAGE gels were transferred to 0.45 μm PVDF (poly(vinylidene difluoride)) membranes by Mini Trans-Blot cell (Bio-Rad, USA) at 50 V for 1 h 30 min. Towbin buffer (25 mM Tris, 192 mM glycine, 0.1% SDS, 20% methanol, pH 8.3) was used in the wet/tank blotting system.

The blots were blocked with milk blocking solution (KPL, USA) at 1:10 in sterile water for 1 h. Tris-buffered saline-Tween-20 buffer (TBS-T: 50 mM Tris, 150 mM NaCl, 0.05% (v/v) Tween-20, pH 7.6) were used for washing and antibody dilution. The blots were washed three times with TBS-T buffer, and incubated with anti-V5 antibody, alkaline phosphatase (AP) conjugate (Invitrogen, USA) at 1:5,000 for 1 h.

After another three wash steps, the blots were developed with BCIP/NBT (5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium) one-component phosphatase substrate (KPL, USA). The color development was halted by rinsing the blots with sterile water, and air-drying. The blots were digitized on a flatbed scanner.

For purified proteins, additional immunodetections were performed using EBV antigen-specific monoclonal or polyclonal antibodies (Table 3.8). Their secondary antibodies was goat anti-mouse IgG AP (Santa Cruz, USA) or rabbit anti-goat IgG Reserve AP (KPL, USA) antibodies, at 1:10,000.

**Table 3.8.** EBV antigen-specific monoclonal or polyclonal antibodies.

| EBV antigen | Primary antibody | | |
|---|---|---|---|
| | Clone | Host | Dilution |
| EA-D | Anti-EBV EA-D-p52/50, clone R3 (Chemicon, USA) | Mouse | 1:1,000 |
| ZEBRA | Anti-EBV Bam HI Z, clone BDI506 (Abcam, USA) | Mouse | 1:200 |
| VCA p18 | Anti-p18 of VCA, polyclonal (Virostat, USA) | Goat | 1:2,000 |

Abbreviations: EBV, Epstein-Barr virus; EA-D, early antigen-diffuse; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen.

### 3.2.7. Quantification of purified proteins

Bradford assay was performed in a microplate format. Bovine serum albumin (BSA) standard (1.39 mg/ml) was diluted from 0.1 to 0.5 mg/ml with sterile water to final volumes of 10 µl in duplicates. The pooled, eluted fractions from protein purification were diluted at 1:10 and 1:20 in duplicates.

The dye solution (Bio-Rad, USA) was diluted at 1:5 with sterile water, filtered, and added to protein solutions, 200 µl per well. The microplate was gently mixed, and incubated at room temperature for 5 min. Absorbance at 595 nm ($A_{595}$) was measured. Protein concentrations were estimated from a BSA standard curve.

## 3.3. DEVELOPMENT OF INDIRECT ELISAS

### 3.3.1. Selection of blocking agent

Blocking agent is needed for indirect ELISA in order to block the remaining binding surface of a solid phase after antigen immobilization (Crowther, 2009). To choose a blocking agent for our ELISA serology, we examined the effectiveness of two major groups of blocking agents, namely (i) commonly used blocking agents, fatty acid free BSA (Sigma, USA), nonfat dry milk (NFDM) (KPL, USA) and Hammarsten grade casein (Merck, Germany); and (ii) synthetic polymers, Ficoll PM400 (Sigma, USA), fully hydrolyzed poly(vinyl alcohol) (PVA) (Merck, Germany) and poly(vinyl pyrrolidone) (PVP) (Sigma, USA) (Rodda & Yamazaki, 1994; Studentsov et al., 2002; Huber et al., 2009). Approximate molecular weights for Ficoll (400,000), PVA (15,000) and PVP (10,000) are indicated in parentheses.

The synthetic polymers were prepared in ELISA compatible diluents, 1×phosphate-buffered saline (1×PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 1.76 mM $KH_2PO_4$, pH 7.4) and coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) (Huber et al., 2009). BSA and casein were prepared by standard protocols, using

1×PBS and alkaline hydrolysis, respectively (Pratt & Roser, 2010); 2% (w/v) NFDM stock solution was diluted at 1:20 in sterile water, according to the manufacturer's recommendation. Except NFDM, the concentration used for all blocking buffers was 4% (w/v), because most blocking agents were claimed to be effective within 5% (w/v) in ELISA studies.

This experiment is a modification of the routine ELISA serology (see Section 3.3.5, Table 3.8 versus 3.9), namely ZEBRA/IgG and VCA p18/IgG in order to estimate the sample backgrounds. All empty wells of Maxisorp poly(styrene) microplates (Nunc, Denmark) were directly coated with blocking agents, except for wells assigned to substrate control 2 (SC2) (Table 3.8) (True, 2009). Thus samples from 12 case-control pairs were assayed in the absence of immobilized antigen. Full assay controls here consist of substrate control 1 (SC1), SC2, conjugate control (CC), strong positive (C++), moderately positive (C+) and negative (C−) controls (Table 3.9) (True, 2009).

**Table 3.9.** Experimental setups for assay controls used in the comparison of blocking agents for ELISA serology.[a]

<table>
<tr><th rowspan="2">Assay control</th><th colspan="3">Incubation step</th></tr>
<tr><th>Coating</th><th>Primary antibody</th><th>Secondary antibody</th></tr>
<tr><td>SC1</td><td>Blocking buffer</td><td rowspan="2">Dilution buffer</td><td>Blocking buffer</td></tr>
<tr><td>SC2</td><td>Coating buffer</td><td>Dilution buffer</td></tr>
<tr><td>CC</td><td rowspan="4">Blocking buffer</td><td rowspan="4">Goat anti-human IgG, HRP conjugate</td></tr>
<tr><td>C++</td><td>C++</td></tr>
<tr><td>C+</td><td>C+</td></tr>
<tr><td>C−</td><td>C−</td></tr>
</table>

Adapted from True (2009).
[a] Coating buffer is 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6; dilution buffer is 1×PBS containing 0.05% (v/v) Tween-20, pH 7.4.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; SC1, substrate control 1; SC2, substrate control 2; CC, conjugate control; C++, strong positive control; C+, moderately positive control; C−, negative control; IgG, immunoglobulin G; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

It should be noted that only coated blocking agent and buffer residuals remain in SC1 prior to the addition of substrate solution, hence SC1 serves to validate the condition of quantitative substrate system. By contrast, only buffer residuals remain in SC2 prior to the addition of substrate solution. Therefore, the reading difference between SC1 and SC2 serve to monitor the non-enzymatic reactions between a blocking layer and the components of substrate system, if any. CC serves to monitor the nonspecific binding of conjugate to the solid phase upon blocking. C++, C+ and C− assay controls are three in-house plasma pools determined by checkerboard titrations (see Section 3.3.4).

### 3.3.2. Grid experiments

We performed grid experiments to optimize the blocking conditions of PVA in the absence of immobilized antigen (see Figure 3.1, Section 3.3.1 and 3.3.5). The kosmotropes and pHs of PVA solutions were given by the diluents used, 1×PBS and coating buffer. PVA blocking buffers were serially diluted from 4% to 0.25% (w/v), to include both 1% and 0.5% (w/v) that were shown to be effective in other immunoassays (Huber et al., 2009; Rodda & Yamazaki, 1994; Studentsov et al., 2002). The blocking steps were performed accordingly, under three common incubation conditions of ELISA, (i) static incubation at 4°C overnight, and shaking incubation for 2 h at (ii) 37°C and (iii) room temperature (Huber et al., 2009). Plasma pools of NPC ($n$ = 4) and normal ($n$ = 4) subjects were assayed at 1:100, 1: 400 and 1: 1,600. Different sets of plasma were used in Section 3.3.1, 3.3.2, and 3.3.3.

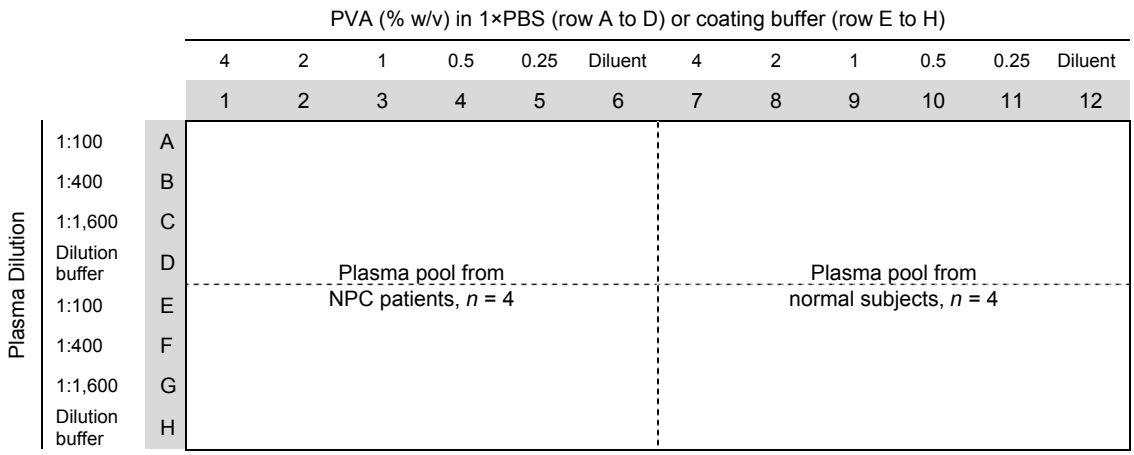


**Figure 3.1.** Microplate design for grid experiments to represent the possible combinations of PVA blocking buffers, and plasma pools from NPC patients ($n$ = 4) and normal subjects ($n$ = 4). Diluent for PVA is either 1×PBS (row A to D) or coating buffer (row E to H). Dilution buffer used to dilute the two plasma pools was 1×PBS containing 0.05% (v/v) Tween-20, pH 7.4. Gray areas indicate rows and columns of a microplate.

### 3.3.3. Comparison of signal-to-noise ratios (SNRs)

To estimate SNRs, additional 24 NPC patients were assayed with and without immobilized antigen (ZEBRA), using indirect ELISA approaches as described in both Section 3.3.1 and 3.3.5. Blocking using (i) NFDM at 1:20 in sterile water (manufacturer's recommendation), and 4% (w/v) PVA in (ii) 1×PBS or (iii) coating buffer for 2 h at room temperature was performed accordingly.

The signals of assayed samples were recorded from solid phases that coated with antigen and blocked with blocking buffers. The noises of assayed samples (sample backgrounds) were estimated from solid phases that blocked with blocking buffers, but not coated with antigen.

Using coin 1.0-18 package (Hothorn et al., 2006, 2008) for R 2.13.0 (R Development Core Team, 2011), Wilcoxon signed rank test (two-sided) was used to compare the paired data, i.e., signals and noises for 24 NPC samples. $p$-value greater than 0.05 indicates statistical significance.

### 3.3.4. Checkerboard titrations

To optimize the key reagents (concentrations of antigens, plasma, and conjugate) for ELISA serology (see Section 3.3.5), checkerboard titrations were performed in two stages (Crowther, 2009). Only two reagents can be titrated at a single stage.

At stage 1, EBV antigens were titrated against C++, C+ and C− (Figure 3.2A). The in-house assay controls were used because they could represent strong positive, moderately positive, and negative plasma samples. At stage 2, the three assay controls were titrated against conjugate at optimum dilutions of EBV proteins that obtained from stage 1 (Figure 3.2B).

Using drc 2.1-4 (Ritz & Streibig, 2005) and qpcR 1.3-4 (Ritz & Spiess, 2008) packages for R 2.13.0 (R Development Core Team, 2011), the endpoint titration curves of C++ and C+ were fitted with four-parameter logistic function in order to determine if the blocking buffer (4% (w/v) PVA in coating buffer) is compatible with other ELISA reagents, i.e., signal exhibits a sigmoidal relationship with the concentration of primary antibody in plasma.

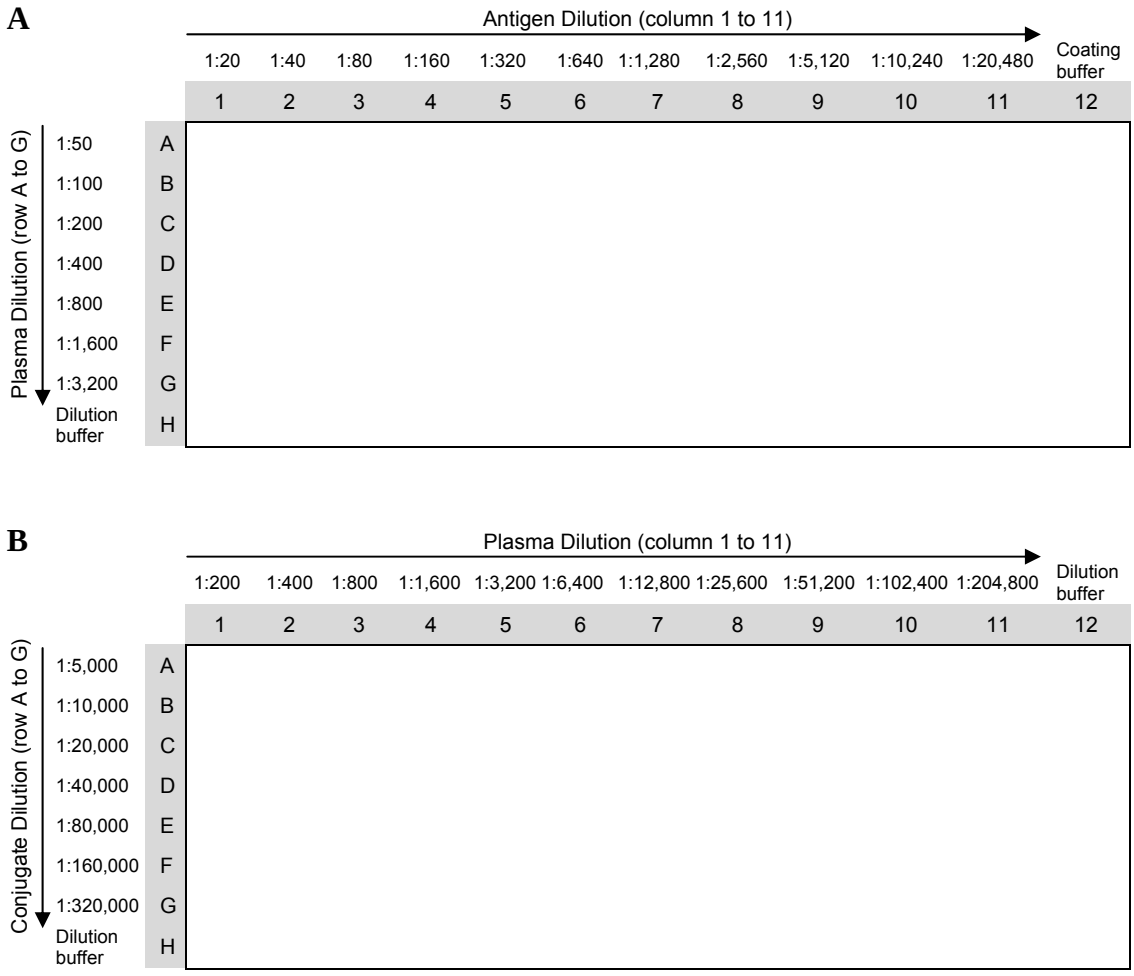


**Figure 3.2.** Microplate layouts for checkerboard titrations. (A) At stage 1, coating step, column 1 to 11 received a serially diluted EBV protein; column 12 received coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) only. During primary antibody incubation, row A to G received a serially diluted assay control, C++, C+ or C−; row H received dilution buffer (1×PBS containing 0.05% (v/v) Tween-20, pH 7.4) only. (B) At stage 2, coating step, all wells receive the optimally diluted EBV protein determined from stage 1. During primary antibody incubation, column 1 to 11 received a serially diluted assay control, C++, C+ or C−; column 12 received dilution buffer only. During secondary antibody incubation, row A to G received a serially diluted conjugate; row H received dilution buffer only. Gray areas indicate the rows and columns of microplates.

### 3.3.5. Serologic tests

The purified EBV proteins served as antigen for indirect ELISAs. Microplates were coated with antigen, except for wells assigned to SC1 and CC (Table 3.9) (True, 2009). The plates were washed three times with wash buffer (1×PBS, 0.1% (v/v) Tween-20, pH 7.4).

Remaining active sites in all wells were blocked with blocking buffer, 200 µl per well, followed by shaking incubation for 2 h at room temperature. The three wash steps were repeated. Dilution buffer (1×PBS, 0.05% (v/v) Tween-20, pH 7.4) was used to dilute plasma at 1:400. All plasma samples were assayed in triplicates, 50 µl per well. Full assay controls that consist of blank, SC1, CC, C++, C+ and C− were assayed in quadruplicates (Table 3.10) (True, 2009). Thus we screened up to 24 samples per microplate.

**Table 3.10.** Experimental setups for assay controls used in ELISA serology.[a]

<table>
<tr><th rowspan="2">Assay control</th><th colspan="4">Incubation step</th></tr>
<tr><th>Coating</th><th>Blocking</th><th>Primary antibody</th><th>Secondary antibody</th></tr>
<tr><td>SC1</td><td rowspan="2">Coating buffer</td><td rowspan="6">Blocking buffer</td><td rowspan="3">Dilution buffer</td><td>Blocking buffer</td></tr>
<tr><td>CC</td><td rowspan="5">Goat anti-human IgG, HRP conjugate</td></tr>
<tr><td>Blank</td><td rowspan="4">Antigen</td></tr>
<tr><td>C++</td><td>C++</td></tr>
<tr><td>C+</td><td>C+</td></tr>
<tr><td>C−</td><td>C−</td></tr>
</table>

Adapted from True (2009).
[a] Coating buffer is 15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6; dilution buffer is 1×PBS containing 0.05% (v/v) Tween-20, pH 7.4.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; SC1, substrate control 1; CC, conjugate control; C++, strong positive control; C+, moderately positive control; C−, negative control; IgG, immunoglobulin G; HRP, horseradish peroxidase; PBS, phosphate-buffered saline.

It should be noted that blank serves to check the non-specific binding of conjugate to the solid phase upon antigen immobilization and blocking. To remove loosely bound materials, we used Tween-20, a detergent that can also act as blocking agent (Esser, 2010), in both dilution buffer and wash buffer. Tween-20 may (i) replace loosely bound antigen and blocking agent after coating and blocking steps, respectively; and (ii) bind to the hydrophobic moieties of blocking agent, increasing the surface polarity of a solid phase. Both mechanisms may suppress nonspecific binding and improve the selectivity of indirect ELISA.

After shaking incubation of diluted plasma for 2 h at room temperature, the microplate was washed four times with wash buffer. Goat anti-human IgG, HRP conjugate (KPL, USA) at 1:20,000 was added to the microplates, 50 µl per well, followed by shaking incubation for 1 h at room temperature. The four wash steps were repeated.

SureBlue Reserve TMB (3,3',5,5'-tetramethylbenzidine) substrate (KPL, USA) was added, 100 µl per well, followed by static incubation for 10 min at room temperature in the dark. TMB BlueStop solution (KPL, USA) was then added immediately, 100 µl per well. $A_{630}$ was measured. To normalize the absorbance values between microplates, percentage positivity (PP) was used to express the ELISA data (Wright et al., 1993):

$$PP = (A_{630} \text{ of a sample} / A_{630} \text{ of C++ from the same microplate}) \times 100 \quad (1)$$

In the present study, we wanted to classify the study subjects correctly into disease (NPC) and non-disease (normal) using the EBV serologic tests. To assess the diagnostic accuracy of EBV serologic tests, receiver operating characteristics (ROC) analyses of the serologic tests were done using ROCR package 1.0-4 (Sing et al., 2005) for R 2.13.0 (R Development Core Team, 2011). The optimal cutoff value−a cutoff

value with the highest Youden's score (Bewick et al. (2004); see Equation (2))−for each EBV serologic test was obtained from the ROC analyses, in which one is predicted to have NPC (test positive) if his or her PP value (normalized anti-EBV IgG level) is higher than the optimal cutoff value. The results of each EBV serologic test can be presented in a confusion matrix (e.g., Table 3.11).

**Table 3.11.** Confusion matrix for the outcome of each EBV serologic test.

| | NPC | |
|---|---|---|
| Test | Present | Absent |
| Positive | True positive | False positive |
| Negative | False negative | True negative |

Youden's index = sensitivity + specificity – 1 (2)

Sensitivity = true positive / (true positive + false negative) (3)

Specificity = true negative / (false positive + true negative) (4)

Accuracy = (true positive + true negative) / (true positive + true negative + false positive + false negative) (5)

Performance characteristics such as sensitivity, specificity and accuracy of each EBV serologic test can be calculated based on the confusion matrix. Sensitivity is the proportion of those who are correctly classified as NPC cases by the EBV serologic test in total NPC cases (see Equation (3)). Specificity is the proportion of those who are correctly classified as normal subjects by the EBV serologic test in total normal subjects (see Equation (4)). On the other hand, accuracy is the proportion of those who are correctly classified as NPC and normal subjects by the EBV serologic test in total study subjects (see Equation (5)).

Because the ELISA data are continuous variables, a range of cutoff values can be obtained from the ROC analyses, in which sensitivity and specificity can be calculated based on the outcome of each cutoff value. To construct the ROC curve for each EBV serologic test, sensitivity (y-axis) was plotted in function of 1 – specificity (x-axis), illustrating the trade-off between sensitivity and specificity across varying cutoff values in the binary classification process.

Areas under the ROC curves (AUCs), a performance characteristic that is independent of the cutoff value, were calculated by trapezoidal rule. The guidelines to interpret AUC (Swets, 1988; Zweig et al., 1992; Zweig & Campbell, 1993; Levinson, 2010) are: (i) maximum and minimum AUC scores are 1.0 (perfect classification) and 0.5 (random classification), respectively. (ii) AUC scores of 0.5–0.7, 0.7–0.9, and above 0.9 represent low, intermediate, and high classification accuracies, respectively. (iii) AUC score of below 0.7 shows weak association of a marker with the disease.

To compare the performance between the EBV serologic tests, Mann-Whitney U-statistic implemented in StAR (statistical analysis of ROC curves) web server version (Vergara et al., 2008; http://protein.bio.puc.cl/star/roc_analysis.php) was used for pairwise comparison of the AUC scores. *p*-value greater than 0.05 indicates statistical significance.

### 3.3.6. Assay reproducibility

The reproducibility of ELISA serology, i.e., inter-assay and intra-assay precisions were calculated from the quadruplicates of full assay controls across routine runs ($n$ = 10) on different days (see Section 3.3.5).

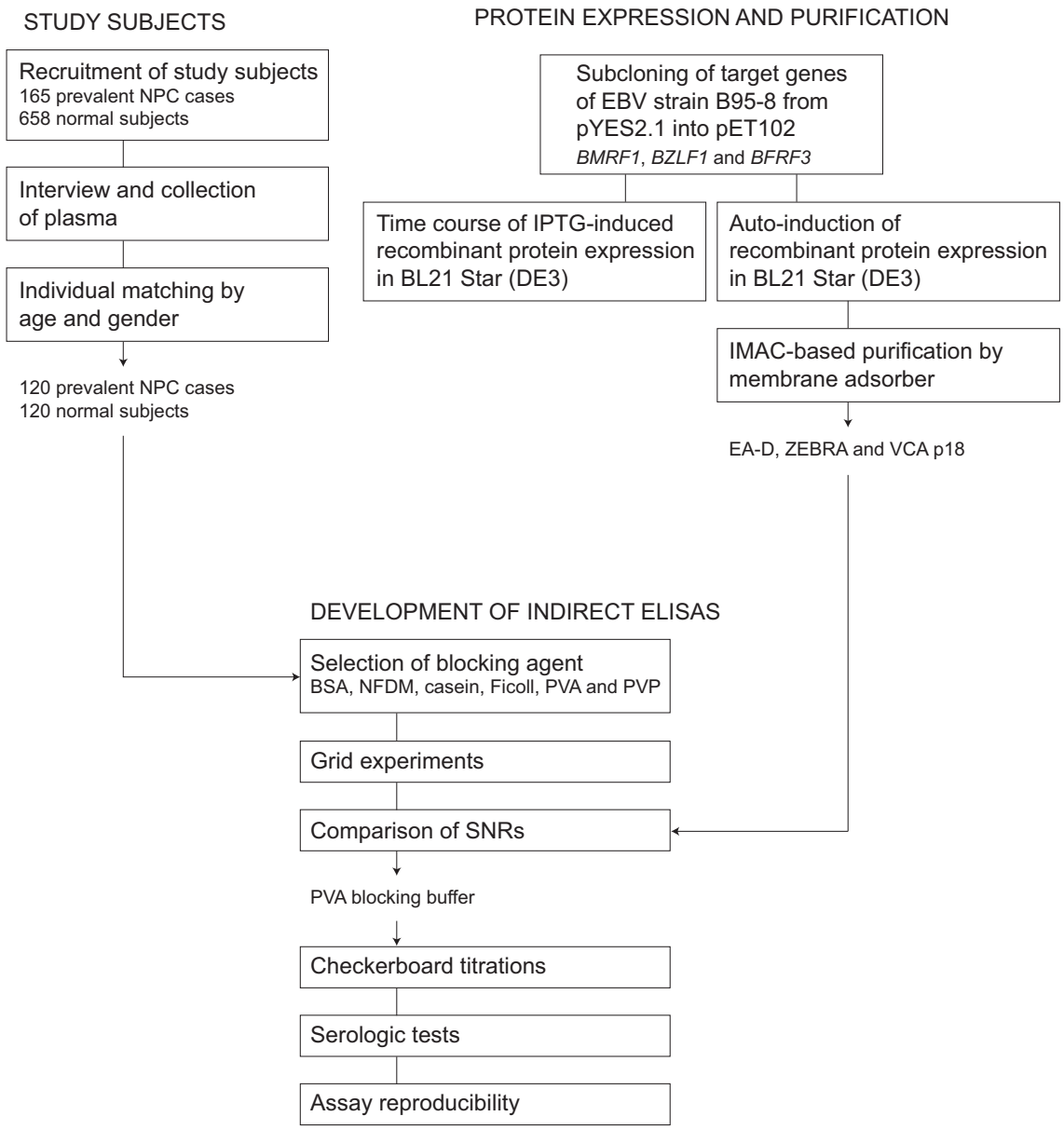


**Figure 3.3.** Workflow of experimental procedures in the present study.

# CHAPTER 4. RESULTS

## 4.1. STUDY SUBJECTS

Over a 3.5-year period in sample collection, a total of 165 prevalent cases of NPC were enrolled. For Chinese patients, the highest frequencies of both male and female cases were in the 50–59 age group (Table 4.1), which was in agreement with age-specific incidence curves (Figure 2.2). Also, we observed familial clustering of NPC, in which 23 NPC patients (all Chinese) had family history of NPC: five of them had two NPC-affected first-degree relatives, 16 of them had a NPC-affected first-degree relative, and two of them had a NPC-affected second-degree relative. NPC was most prevalent in Chinese ($n$ = 148), which comprised of 89.7% (148/165) total cases. In particular, 14.2% (21/148) Chinese cases were familial NPC (had at least one NPC-affected first-degree relative). Chinese males ($n$ = 111) were 3-fold more prevalent than females ($n$ = 37).

In our pilot survey of prevalence, two of 334 Chinese normal subjects had a first-degree family member affected by NPC. The prevalence of family history of NPC among the Chinese controls was 0.6% (2/334). None of the controls of other ethnicity ($n$ = 324) had family history of NPC.

**Table 4.1.** Prevalent cases of NPC for Chinese by age and gender.

| Age | 10–19 | 20–29 | 30–39 | 40–49 | 50–59 | 60–69 | ≥70 |
|---|---|---|---|---|---|---|---|
| Male ($n$) | 1 | 0 | 6 | 28 | 37 | 27 | 12 |
| Female ($n$) | 0 | 1 | 4 | 10 | 17 | 3 | 2 |

Abbreviation: NPC, nasopharyngeal carcinoma.

## 4.2. PROTEIN EXPRESSION AND PURIFICATION

### 4.2.1. Construction of prokaryotic expression vectors

The target DNA fragments of EBV were subcloned from the pYES2.1 yeast expression vectors (Figure 4.1) into pET102 prokaryotic expression vector. *E. coli* TOP10 cells were transformed with the freshly ligated pET102 vectors. Colony PCR results showed that the pET102 plasmids with positive inserts were harbored by the *E. coli* transformants (Figure 4.2). The inserts were verified by DNA sequencing.

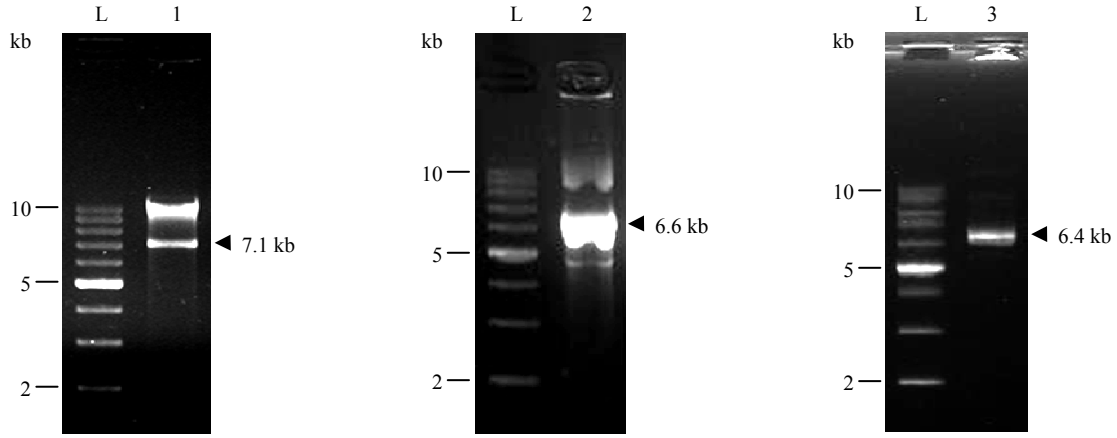


**Figure 4.1.** Electrophoretic patterns of pYES2.1 plasmids with inserts encoding EA-D (1), ZEBRA (2) and VCA p18 (3) in 0.8% Tris-borate-EDTA (TBE) agarose gel. Arrows indicate the bands of plasmids. L denotes supercoiled DNA ladder (Promega, USA).

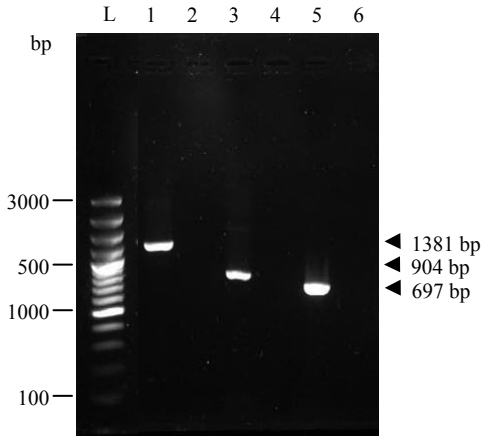


**Figure 4.2.** Electrophoretic patterns of colony PCR products in 1% TBE agarose gel. The DNA templates used were the lysates of TOP10 transformants harboring pET102 with inserts encoding EA-D (1), ZEBRA (3) and VCA p18 (5). Even-numbered lanes represent the negative controls for PCR reactions. Arrows indicate the bands of PCR products. L denotes GeneRuler 100 bp Plus DNA ladder (Fermentas, Canada).

### 4.2.2. Time course of IPTG-induced protein expression

The target proteins produced by this prokaryotic expression system were tagged with His-patch thioredoxin at N-terminus, V5 and 6×His at C-terminus.

To express the target proteins, the recombinant PET102 plasmids were transformed into prokaryotic expression host, BL21 Star (DE3). IPTG was used as artificial inducer for the T7 *lac* promoter containing plasmid for protein expression.

To harvest the target proteins at optimum levels, we monitored the expression cultures at varying time points by SDS-PAGE (Figure 4.3). Both ZEBRA (Figure 4.3B) and VCA p18 (Figure 4.3C) were expressed at high levels starting from two-hour IPTG induction. However, the expression level of EA-D was low across the time points (Figure 4.3A).

Western blotting was performed using the whole cell extracts of *E. coli* from the IPTG-induced cultures at four-hour time point. Anti-V5 antibody was used to probe the fusion proteins. The blots showed that the V5-tagged proteins are present at the expected molecular weights as suggested by SDS-PAGE (EA-D, 65 kDa; ZEBRA, 55 kDa; VCA p18, 40 kDa) (Figure 4.4), indicating that the EBV proteins were expressed as fusion proteins in full lengths.

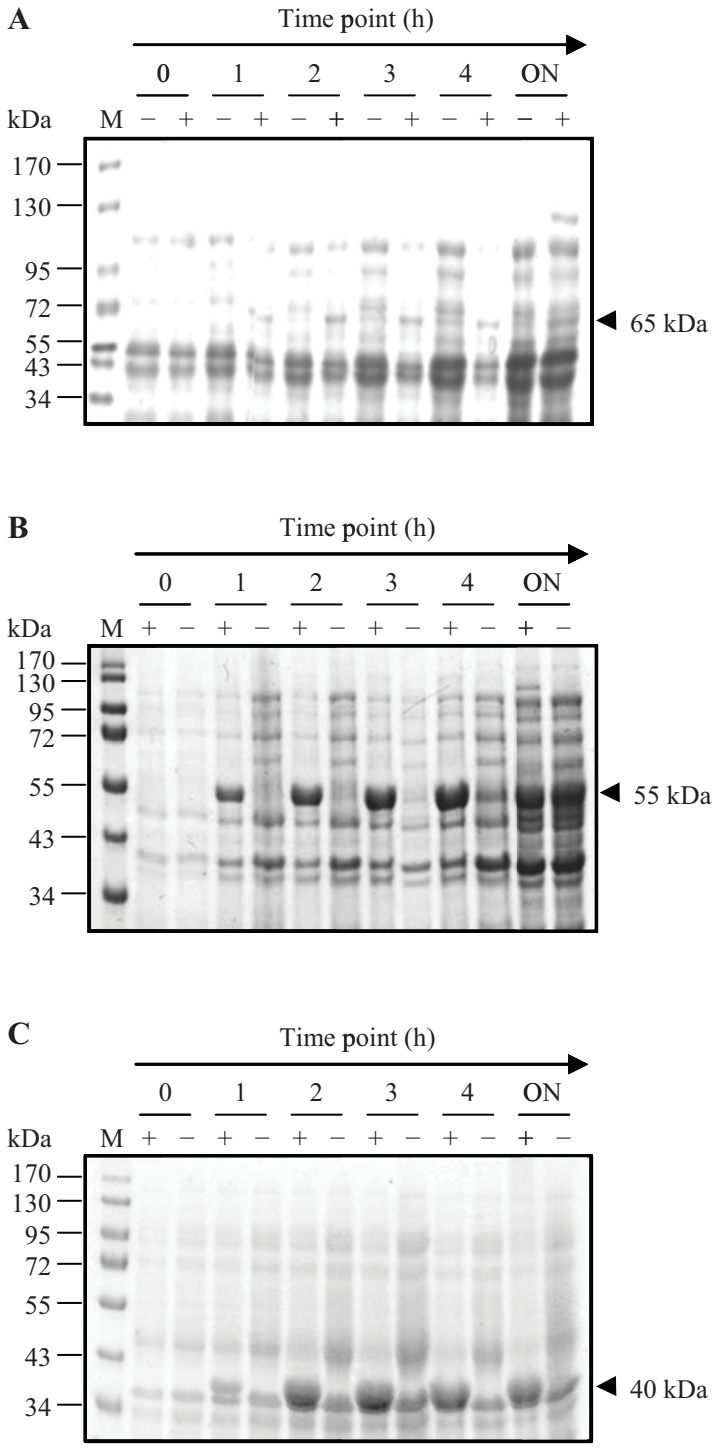


**Figure 4.3.** Time course of IPTG-induced expression of recombinant EBV proteins in BL21Star (DE3). SDS-PAGE gels show the migration patterns of whole cell extracts of IPTG-induced (+) and uninduced (−) *E. coli* samples expressing (A) EA-D (65 kDa), (B) ZEBRA (55 kDa), and (C) VCA p18 (40 kDa). M denotes PageRuler prestained protein ladder (Fermentas, Canada). ON denotes overnight.

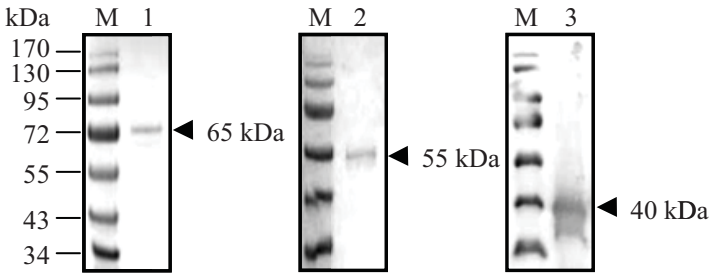


**Figure 4.4.** Western blots show the protein bands of V5-tagged EA-D (65 kDa) (1), ZEBRA (55 kDa) (2) and VCA p18 (40 kDa) (3), which were detected from *E. coli* samples after a four-hour IPTG induction, using anti-V5 antibody. M denotes PageRuler prestained protein ladder (Fermentas, Canada).

### 4.2.3. Auto-induction of recombinant protein expression

It is costly to purify EA-D with low expression level because its culture work needs to be several-fold more than the work for ZEBRA and VCA p18. We therefore adopted an alternative expression strategy, i.e., auto-induction (see Section 3.2.3) (Studier, 2005). MDG non-inducing media were used to prepare starter cultures for the expression hosts. Auto-induction was performed by seeding ZYM-5052 auto-induction media with the MDG starter cultures.

The recombinant EA-D protein was expressed at a higher level compared with that of IPTG induction (Figure 4.5A, left panel versus Figure 4.3A). The expression levels of ZEBRA and VCA p18 by both induction methods were comparable (Figure 4.3B and 4.3C versus Figure 4.5A, left panel). Therefore, auto-induction was used to express the recombinant EBV proteins in the subsequent scale-up cultures (in 100 ml volumes) for protein purification.

### 4.2.4. IMAC-based purification by membrane adsorber

Cobalt-charged membrane adsorbers were employed to purify the His-tagged proteins. Under native conditions, these target proteins were eluted by imidazole from

the IMAC-based device. SDS-PAGE analysis showed a single protein band for each pooled, purified fraction (Figure 4.5A, right panel). The protein bands were then identified by both anti-V5 antibody, and specific monoclonal or polyclonal antibodies in Western blotting (Figure 4.5B), suggesting that all three target proteins have been expressed and purified from *E. coli*.

The yields of purified EA-D, ZEBRA and VCA p18 from 100 ml cultures were 2.03, 1.96 and 2.87 mg, respectively.

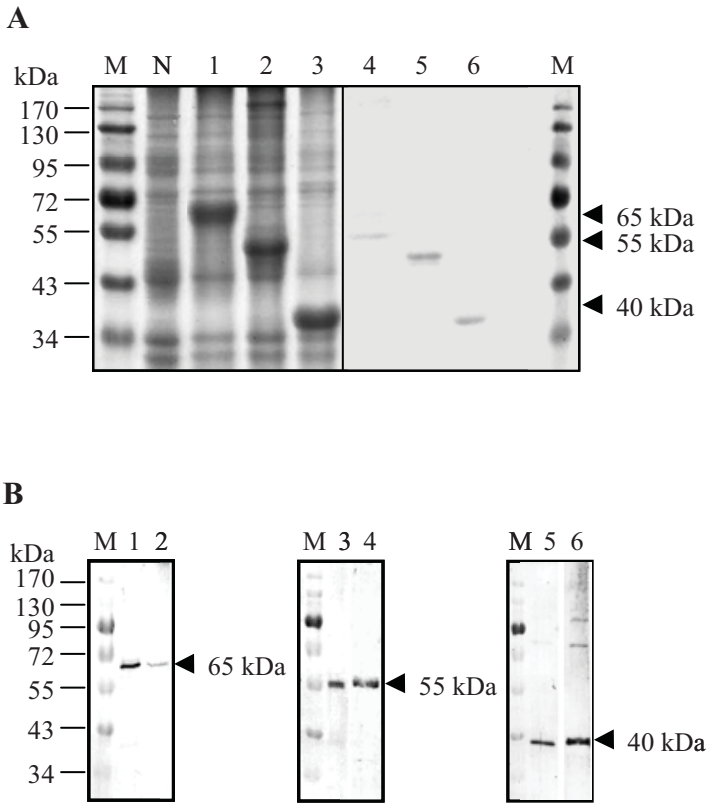


**Figure 4.5.** Auto-induced expression of recombinant EBV proteins in BL21 Star (DE3) and IMAC-based purification of target proteins. (A) SDS-PAGE gel (left) shows the migration patterns of the whole cell extracts of *E. coli* samples expressing EA-D (65 kDa) (1), ZEBRA (55 kDa) (2) and VCA p18 (40 kDa) (3). SDS-PAGE gel (right) shows the migration patterns of the pooled fractions of EA-D (4), ZEBRA (5) and VCA p18 (6) after purification. (B) Western blots show the protein bands of recombinant EA-D (1, 2), ZEBRA (3, 4) and VCA p18 (5, 6), which were detected from the pooled fractions, using anti-V5 antibody (odd-numbered lanes), and EBV antigen-specific monoclonal or polyclonal antibodies (even-numbered lanes). N denotes the negative control for protein expression, i.e., the mock-transformed BL21Star (DE3). M denotes PageRuler prestained protein ladder (Fermentas, Canada).

## 4.3. DEVELOPMENT OF INDIRECT ELISAS

### 4.3.1. Selection of blocking agent

The purified proteins served as antigens in indirect ELISAs. Because indirect ELISA is very sensitive, antigen is often coated at low concentration. The remaining active sites, which account for the majority of the binding surface, are then blocked with a blocking buffer. Therefore, the blocking agent used should prevent nonspecific binding of materials from primary (diluted plasma) and secondary antibodies. Only non-immunogenic blocking agent that adsorbed tightly on the solid phase can provide low background.

To choose a suitable blocking agent, we determined the effectiveness of commonly used (BSA, NFDM and casein) and synthetic polymer (Ficoll, PVA and PVP) blocking agents (see Section 3.3.1). Backgrounds for 12 pairs of case-control samples were estimated from the microplates that blocked with different blocking buffers in the absence of immobilized antigen, because the lower the sample backgrounds, the more effective the blocking, and hence the higher the assay reliability.

Here, the sample backgrounds provided by commonly used blocking agents were relatively higher than those provided by synthetic polymer blocking agents (Table 4.2). Exceptionally high sample backgrounds were observed when NFDM was used as blocking agent. Only Ficoll (1×PBS and coating buffer) and PVA (coating buffer) were able to provide relatively low sample backgrounds with small variations. Also, sample backgrounds that correspond to synthetic polymers at 4% (w/v) in coating buffer were relatively lower than those in 1×PBS.

**Table 4.2.** Sample backgrounds (12 NPC and 12 normal subjects) provided by different blocking buffers.

| Blocking buffer | $A_{630}$ | | | | |
|---|---|---|---|---|---|
| | Maximum | Minimum | Range | Mean ± *SD* | *CV* (%) |
| BSA | 0.56 | 0.16 | 0.40 | 0.23 ± 0.09 | 41.6 |
| NFDM | 2.83 | 0.18 | 2.66 | 0.86 ± 0.82 | 95.4 |
| Casein | 0.64 | 0.18 | 0.47 | 0.27 ± 0.10 | 38.6 |
| Ficoll in 1×PBS | 0.26 | 0.17 | 0.10 | 0.19 ± 0.02 | 11.2 |
| Ficoll in coating buffer | 0.24 | 0.17 | 0.08 | 0.19 ± 0.02 | 9.0 |
| PVA in 1×PBS | 0.44 | 0.15 | 0.29 | 0.25 ± 0.07 | 29.2 |
| PVA in coating buffer | 0.32 | 0.17 | 0.15 | 0.22 ± 0.04 | 17.7 |
| PVP in 1×PBS | 0.57 | 0.16 | 0.40 | 0.28 ± 0.09 | 31.8 |
| PVP in coating buffer | 0.46 | 0.17 | 0.29 | 0.24 ± 0.06 | 25.5 |

Abbreviations: $A_{630}$, absorbance at 630 nm; *SD*, standard deviation; *CV*, coefficient of variation; BSA, bovine serum albumin; NFDM, nonfat dry milk; PBS, phosphate-buffered saline; PVA, poly(vinyl alcohol); PVP, poly(vinyl pyrrolidone).

### 4.3.2. Grid experiments

Because PVA was effective for blocking and the cheapest among the tested blocking agents, we further examined the optimum blocking conditions of PVA using grid experiments (see Section 3.3.2). At different polymer concentrations, compositions of kosmotrope(s), temperatures, and pHs, we estimated the backgrounds for plasma pools of NPC ($n$ = 4) and normal ($n$ = 4) subjects at different dilutions, because sample backgrounds should be considerably low and close to each other, regardless of case-control status and dilution factor.

In Figure 4.6: (i) decreasing backgrounds were observed with increasing PVA concentration, in which 4% (w/v) PVA provides the lowest backgrounds. The concentrations of PVA at 0.5% and 1% (w/v), which were shown to be effective in

previous studies (Huber et al., 2009; Rodda & Yamazaki, 1994; Studentsov et al., 2002), were insufficient for blocking in our study. (ii) 1×PBS was a better diluent than coating buffer for PVA below 2% (w/v), which is in agreement with a recent finding (Huber et al., 2009). However, the effects of diluents were not obvious for 4% (w/v) PVA.

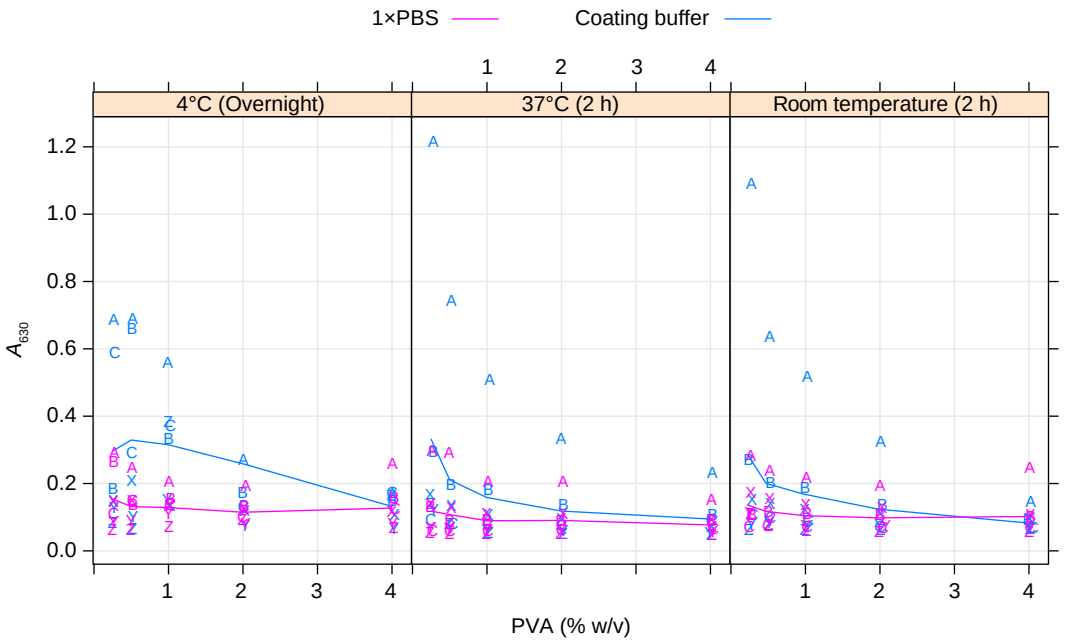


**Figure 4.6.** Trellis plot shows the blocking effectiveness of varying PVA concentrations at different compositions of kosmotrope(s), incubation temperatures and pHs, obtained from the indirect ELISA results for plasma pools of NPC ($n$ = 4) and normal ($n$ = 4) subjects at different dilutions. The grid experiments were performed in the absence of immobilized antigen. Alphabets on the figure annotate the plasma dilutions as follows: A, B and C correspond to NPC plasma pool at 1:100, 1: 400 and 1: 1,600, respectively, whereas X, Y and Z correspond to normal plasma pool at 1:100, 1: 400 and 1: 1,600, respectively. Pink and blue alphabets represent backgrounds ($A_{630}$ values) for the diluted plasma, provided by the PVA blocking layers assembled in 1×PBS and coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6), respectively.

### 4.3.3. Comparison of SNRs

Additional 24 NPC samples were included to seek the best diluent for PVA (see Section 3.3.3). Signals and backgrounds for the samples were obtained from the assays in the presence and absence of ZEBRA, respectively. We chose ZEBRA as antigen for this experiment because circulating anti-ZEBRA IgG is one of the most promising anti-EBV IgG biomarkers for NPC (Dardari et al., 2001; Mathew et al., 1994), and therefore the 24 NPC samples should give strong signals. The blocking effectiveness of (i) NFDM, and 4% (w/v) PVA in (ii) 1×PBS and (iii) coating buffer was compared in terms of backgrounds and SNRs. The SNRs of individual samples were estimated by dividing their signals by backgrounds.

When using NFDM as blocking agent, the distribution for signals obtained from 24 NPC samples were completely overlapped with the distribution for their backgrounds (Figure 4.7) (Wilcoxon signed rank test: $z = 1.97$, $p = 0.049$, $r = 0.40$; in which $z$ corresponds to $z$-score, $p$ corresponds to $p$-value, $r$ corresponds to Pearson's correlation coefficient, as an effect size measure). By contrast, when using 4% (w/v) PVA as blocking agent, the distribution for signals did not overlap with the distribution for backgrounds (Wilcoxon signed rank test: $z = 4.29$, $p = 1.19\times10^{-7}$, $r = 0.87$). Here, sample backgrounds that correspond to 4% (w/v) PVA in coating buffer ($A_{630}$, 0.22 ± 0.06) were relatively lower than those in 1×PBS ($A_{630}$, 0.28 ± 0.07), suggesting that the data from Table 4.2 and Figure 4.7 are in agreement.

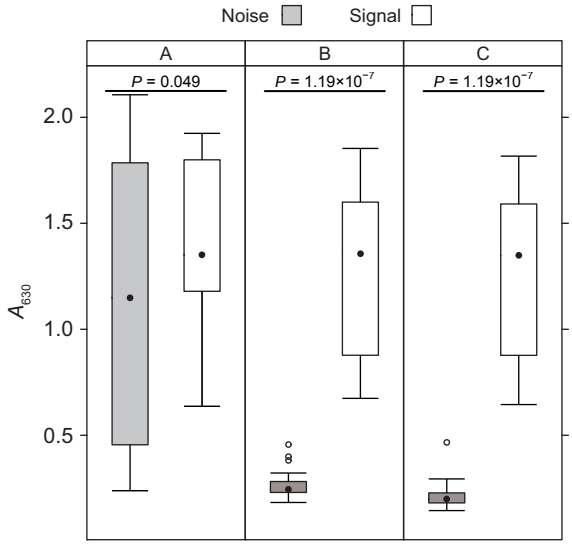


**Figure 4.7.** Box plots shows the signals (□) and noises (■) of 24 NPC samples in ELISAs using NFDM (A), and PVA in 1×PBS (B) and coating buffer (15 mM $Na_2CO_3$, 35 mM $NaHCO_3$, pH 9.6) (C) as blocking buffers. The signals of assayed samples were recorded as routine indirect ELISA, from solid phases that coated with antigen (ZEBRA) and blocked with blocking buffers. The noises of assayed samples (sample backgrounds) were estimated from solid phases blocked with blocking buffers, but not coated with antigen. The box plots should show distinct gaps between signals and noises. *p*-values were obtained from Wilcoxon signed rank test on the paired data, i.e., signals and noises for 24 NPC samples.

For SNRs, blocking with 4% (w/v) PVA in coating buffer was 4.26-fold, on average, higher than blocking with NFDM. Also, it was 1.25-fold, on average, higher than blocking with 4% (w/v) PVA in 1×PBS. Therefore, 4% (w/v) PVA in coating buffer was chosen for the subsequent experiments.

### 4.3.4. Checkerboard titrations

To optimize reagents in ELISA serology, checkerboard titrations were performed (see Section 3.3.4). Four-parameter logistic function was used to fit the endpoint titration curves for C++, C+ and C− at optimum reagents (Figure 4.8). Here, coefficients of determination, $R^2$ above 0.99 were obtained.

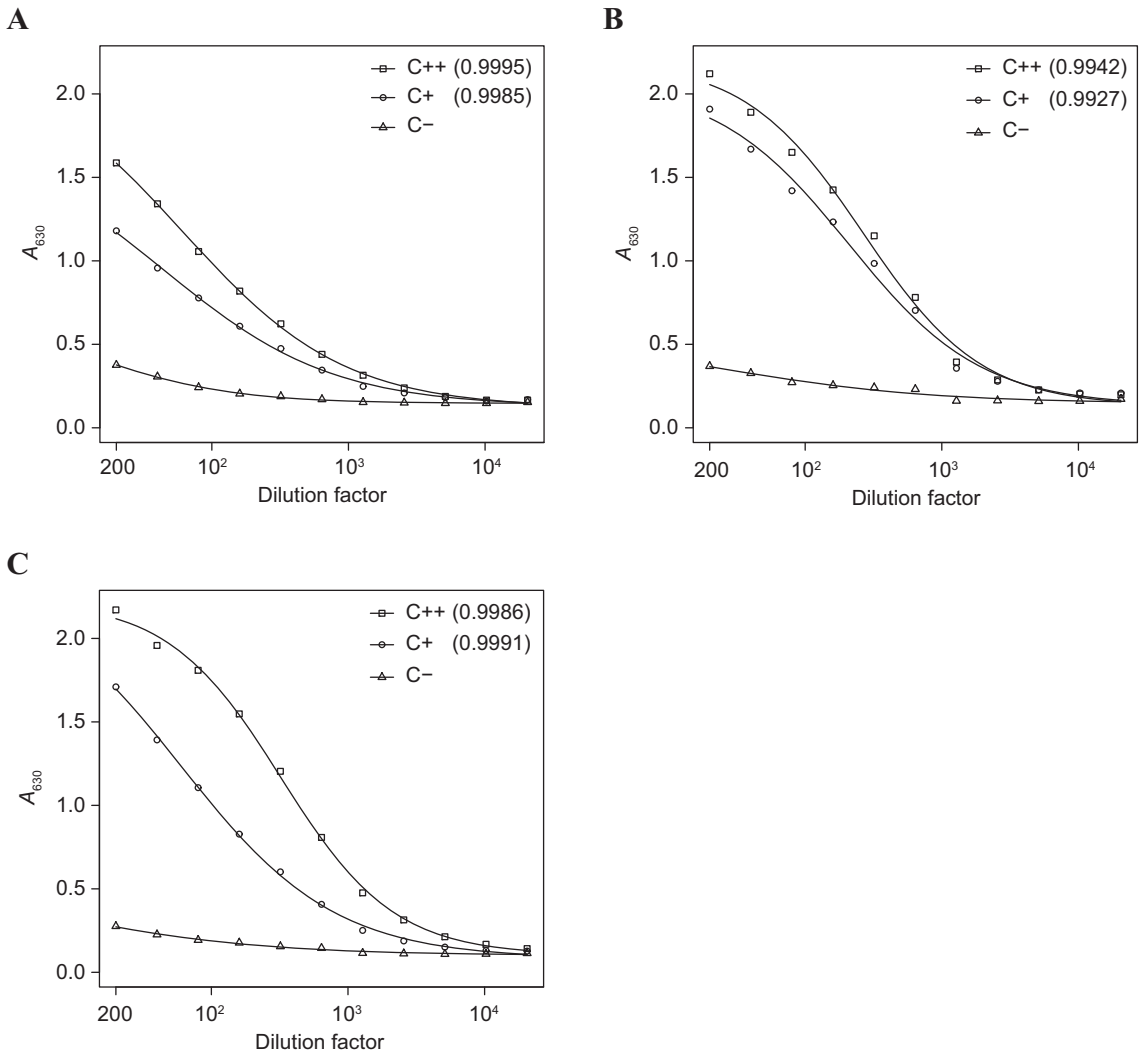


**Figure 4.8.** Endpoint titration curves for C++ ( ), C+ ( ) and C− ( ), obtained from the checkerboard titrations of (A) EA-D/IgG, (B) ZEBRA/IgG and (C) VCA p18/IgG indirect ELISAs. C++, C+ and C− denote strong positive, moderate positive and negative assay controls, respectively. The $R^2$ of the four-parameter logistic curves are shown in parentheses.

### 4.3.5. Serologic tests

Based on the optimized setup, samples from 120 age- and gender-matched, case-control pairs were subjected to EA-D/IgG, ZEBRA/IgG and VCA p18/IgG indirect ELISAs. The ELISA data were analyzed by ROC. Appendix A, B and C show the outputs of the ROC analyses, including (i) lists of cutoff values for the EBV serologic tests, in which each cutoff value shows the number of study subjects with test positive, and (ii) sensitivity, specificity and Youden's index calculated for each cutoff value (see Equation (3), (4) and (5), respectively). Sensitivity was plotted against 1 − specificity, generating ROC curves for the EBV serologic tests (Figure 4.9). The cutoff values with the highest Youden's score were chosen, and their corresponding sensitivity, specificity and accuracy for the EBV serologic tests are reported in Table 4.3.

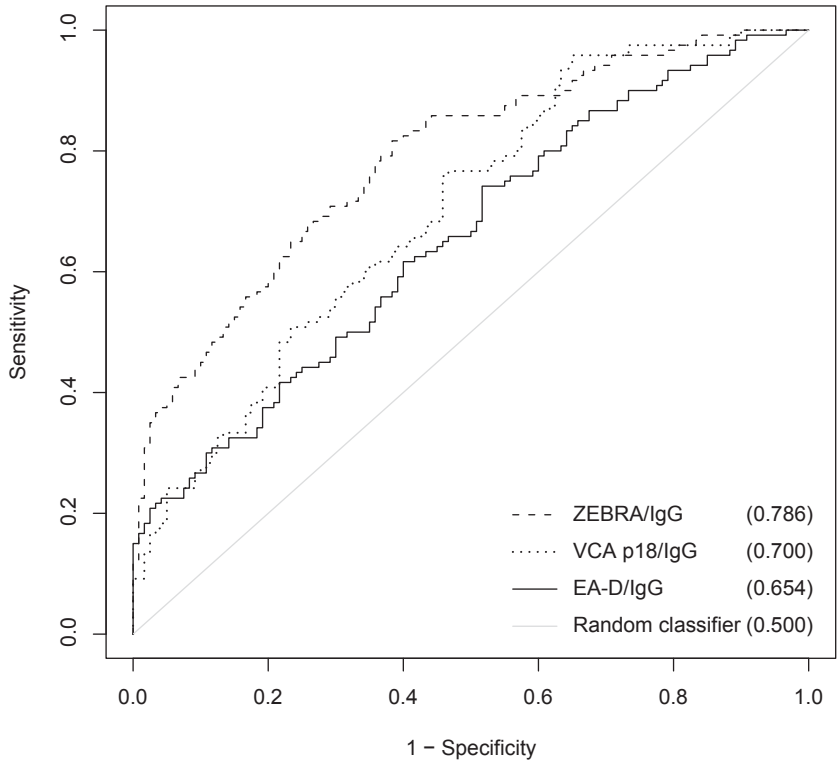


**Figure 4.9.** ROC curves for the EBV serologic tests in the classification of NPC ($n$ = 120) and normal ($n$ = 120) subjects. AUCs are shown in parentheses.

**Table 4.3.** Performance characteristics for the EBV serologic tests in the classification of NPC cases ($n$ = 120) and controls ($n$ = 120).

| | EA-D/IgG | ZEBRA/IgG | VCA p18/IgG |
|---|---|---|---|
| Cutoff (PP; $A_{630}$) | >41.64; >0.66 | >55.08; >1.18 | >21.53; >0.43 |
| AUC (95% CI) | 0.654 (0.590–0.714) | **0.786 (**0.729–0.836) | 0.700 (0.638–0.757) |
| True positive ($n$) | 89 | 98 | 115 |
| True negative ($n$) | 58 | 74 | 42 |
| False positive ($n$) | 62 | 46 | 78 |
| False negative ($n$) | 31 | 22 | 5 |
| Sensitivity (%) | 74.2 | 81.7 | **95.8** |
| Specificity (%) | 48.3 | **61.7** | 35.0 |
| Accuracy (%) | 61.3 | **71.7** | 65.4 |

Numbers in boldface denote the highest performance estimates.
Abbreviations: EBV, Epstein-Barr virus; NPC, nasopharyngeal carcinoma; EA-D, early antigen-diffuse; IgG, immunoglobulin G; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen; PP, percentage positivity; $A_{630}$, absorbance at 630 nm; AUC, area under the ROC curve; ROC, receiver operating characteristics; CI, confidence interval.

In EA-D/IgG test, 89 of 120 NPC patients (74.2% sensitivity) were EA-D/IgG positive, whereas 58 of 120 normal subjects were EA-D/IgG negative (48.3% specificity). The remaining subjects could not be classified correctly. The test misclassified 31 of 120 NPC patients (25.8%), and 62 of 120 normal subjects (51.7%).

In ZEBRA/IgG test, 98 of 120 NPC patients (81.7% sensitivity) were ZEBRA/IgG positive, while 74 of 120 normal subjects were ZEBRA/IgG negative (61.7% specificity). The test misclassified 22 of 120 NPC patients (18.3%), and 46 of 120 normal subjects (38.3%).

In VCA p18/IgG test, 115 of 120 NPC patients (95.8% sensitivity) were VCA p18/IgG positive, whereas 42 of 120 normal subjects were VCA p18/IgG negative (35.0%

specificity). The test misclassified 5 of 120 NPC patients (4.2%), and 78 of 120 normal subjects (65.0%).

Further, AUC scores for the EBV serologic tests were compared using Mann-Whitney U-statistic (Table 4.4). ZEBRA/IgG was the best performed test in classification of NPC (AUC differences between ZEBRA/IgG and VCA p18/IgG, and ZEBRA/IgG and EA-D/IgG were statistically significance). ZEBRA/IgG offered the highest scores of AUC (0.786), thereby yielding the best trade-off between sensitivity (81.7%) and specificity (61.7%). The best sensitivity (95.8%) was provided VCA p18/IgG, but its specificity was low (35.0%). With AUC scores between 0.7–0.9, ZEBRA/IgG and VCA p18/IgG had intermediate classification accuracies. Among the three serologic tests, EA-D/IgG was the poorest in classifying the case-control status (but the AUC difference between VCA p18/IgG and EA-D/IgG was not statistically significance). With an AUC score below 0.7, EA-D/IgG had low classification accuracy, and a weak association with NPC. Nevertheless, EA-D/IgG was better than a random classifier.

**Table 4.4.** Comparison of AUC scores for the EBV serologic tests.[a]

| EBV serologic test | EA-D/IgG | ZEBRA/IgG | VCA p18/IgG |
|---|---|---|---|
| EA-D/IgG | — | 0.132 | 0.046 |
| ZEBRA/IgG | 0.00001 | — | 0.086 |
| VCA p18/IgG | 0.15807 | 0.00231 | — |

[a] Pairwise comparison by Mann-Whitney U-statistic implemented in StAR. Upper triangle: AUC differences; lower triangle: *p*-values.
Abbreviations: AUC, area under the ROC curve; ROC, receiver operating characteristics; EBV, Epstein-Barr virus; EA-D, early antigen-diffuse; IgG, immunoglobulin G; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen; StAR; statistical analysis of ROC curves.

### 4.3.6. Assay reproducibility

For each indirect ELISA, intra-assay precisions ($n$ = 10) were determined from quadruplicate readings (well-to-well) of assay controls, whereas inter-assay precision was determined from average quadruplicate readings (plate-to-plate, also day-to-day here) of assay controls across 10 runs. Overall, the intra-assay and inter-assay precisions were less than or equal to 7% and 16%, respectively (Table 4.4).

**Table 4.5.** Reproducibility of the indirect ELISAs.

| | Assay control | | |
|---|---|---|---|
| | C++ | C+ | C− |
| Intra-assay precision (*CV*%) ($n$ = 10)[a] | | | |
| EA-D/IgG | 0.4–1.7 | 0.9–**7.0** | 0.5–5.8 |
| VCA p18/IgG | 0.8–2.0 | *0.1*–5.6 | 1.0–5.2 |
| ZEBRA/IgG | 1.1–3.9 | 0.3–1.5 | 1.2–3. 6 |
| Inter-assay precision (*CV*%) ($n$ = 10)[b] | | | |
| EA-D/IgG | 8.1 | **16.0** | 12.9 |
| VCA p18/IgG | 3.9 | 4.9 | 13.3 |
| ZEBRA/IgG | *2.9* | 3.8 | 6.4 |

Numbers in italic and boldface denote the lowest and highest *CV*s, respectively, among the immunoassays.
[a] Determined from quadruplicate readings (well-to-well) of assay controls in the same runs ($n$=10).
[b] Determined from average quadruplicate readings (plate-to-plate, also day-to-day here) of assay controls across 10 runs.
Abbreviations: ELISA, enzyme-linked immunosorbent assay; C++, strong positive control; C+, moderately positive control; C−, negative control; *CV*, coefficient of variation; EA-D, early antigen-diffuse; IgG, immunoglobulin G; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen.

# CHAPTER 5. DISCUSSION

We attempted to develop in-house ELISAs to investigate the diagnostic values of anti-EBV IgG levels in a high-risk NPC population, i.e., Malaysian Chinese. Therefore, we adopted recombinant DNA approaches to express and purify the target proteins of EBV from *E. coli*. We then used the recombinant EBV proteins as antigen for our in-house ELISAs.

## 5.1. PROTEIN EXPRESSION AND PURIFICATION

### 5.1.1. Expression of recombinant proteins

Because of simplicity, efficiency and cost, we chose *E. coli* to produce the target proteins. Despite recombinant proteins produced by *E. coli* are possibly misfolded and lack of post-translational modification, many ELISA studies have shown the feasibility of EBV proteins produced by *E. coli* (Dardari et al., 2001; Fachiroh et al., 2010; Mathew et al., 1994).

However, when using IPTG induction, the expression of EA-D in expression host, BL21 Star (DE3) was low (Figure 4.3A). It was undesirable for protein purification because the yield and purity of the recombinant protein might be low. To increase the expression level of recombinant EA-D, we tried MDG non-inducing media to prepare the starter culture, and ZYM-5052 media to grow and to auto-induce BL21 Star (DE3) for protein expression (Table 2.4 and 2.5) (Studier, 2005). MDG media prevent the leakage of T7 *lac* promoter of the harbored vector, while ZYM-5052 media provide rich nutrients that are necessary for the expression of certain recombinant proteins in *E. coli* (Studier, 2005). Under autoinduction, EA-D was expressed at a higher level (Figure 4.5A, left panel versus Figure 4.3A).

Unlike LB media, certain extra nutrients supplied by ZYM-5052 media might be essential for the expression of EA-D in BL21 Star (DE3). However, it is not clear that

which nutrient(s) had stimulated the cells to express EA-D at a higher level. For future work, its nutrient requirement can be investigated by factorial design experiment. We can also explore the physiological states of *E. coli* samples that have different expression levels of the recombinant protein. Besides, the role of MDG starter culture in preventing the leakage of the protein expression system should be taken into account.

Based on the successful expression of EA-D at a higher level, auto-induction method was adopted to express other target proteins in BL21 Star (DE3). This approach allows easy handling of scale-up cultures in one-liter shake flasks because we do not need to monitor the cell growths in order to add IPTG timely. As expected, the expression levels of ZEBRA and VCA p18 using auto-induction method were comparable with conventional IPTG induction method (Figure 4.3B, C versus Figure 4.5A, left panel). Taken together, our data suggested that auto-induction was the preferred approach for high level expression of the EBV proteins in *E. coli*. Our protein expression strategy was therefore changed to auto-induction method.

### 5.1.2. Purification of recombinant proteins

To purify the His-tagged proteins of EBV, we employed membrane adsorbers, i.e., scale-down units that have 15 layers in each unit to carry metal-chelating groups of iminodiacetic acid. Membrane adsorber can be easily customized for IMAC-based purification because (i) its capacity can be multiplied by connecting to additional unit(s), (ii) it can be charged with the metal ion of choice, (iii) it is reusable by stripping and recharging of metal ion, and (iv) its flow rate can be controlled either by the plunger of syringe or peristaltic pump.

For our application, two connecting membrane adsorbers and a syringe were sufficient. Cobalt ion was used to charge the purification device because it provides the

greatest selectivity toward His-tagged protein among the commonly used metal ions, such as copper, nickel and zinc ions (Sulkowski, 1989).

For indirect ELISA, the native state of antigen is required to exhibit its conformational epitopes (Crowther, 2009). Hence, all recombinant proteins were purified under native conditions by competitive elution with imidazole for this study.

The eluted fractions of individual proteins were pooled (Figure 4.5A, right panel), analyzed by SDS-PAGE, detected by Western blotting (Figure 4.5B), and quantified by Bradford assay. As a preliminary study, we wanted to evaluate the potential use of the recombinant EBV proteins produced by *E. coli* as antigens before further refinements, i.e., removing fusion tags and performing further purification.

## 5.2. DEVELOPMENT OF INDIRECT ELISAS

### 5.2.1. Selection of blocking agent

We employed Maxisorp poly(styrene) microplate (Nunc, Denmark) as the solid phase for indirect ELISA because it is suitable for general use, i.e., coating of both hydrophobic and hydrophilic antigens. For indirect ELISA, the remaining binding surface after antigen immobilization must be shielded from primary and secondary antibodies to avoid background or noise. Because blocking agent occupies most binding surface of a solid phase (microplate), its ability to prevent nonspecific binding determines the SNR and reliability of an ELISA.

To choose a compatible blocking agent, commonly used (BSA, NFDM and casein) and synthetic polymer (Ficoll, PVA and PVP) blocking agents (Huber et al., 2009; Rodda & Yamazaki, 1994; Studentsov et al., 2002) were evaluated. BSA, NFDM and casein are conventional, widely used blocking agents. Their blocking effectiveness was therefore concerned in this experiment. On the other hand, we selected the synthetic polymers based on their high affinity toward poly(styrene) surfaces but lack of affinity

toward biomolecules (Barrett et al., 2001; Denhardt, 1966; Robinson & Williams, 2002). In addition, we chose low molecular weight PVA (15,000) and PVP (10,000) to achieve good surface coverage (Barrett et al., 2001), meanwhile avoiding steric hindrance in the subsequent immunodetection steps. With different building blocks from proteins—hence no antigenic determinant—these synthetic polymers should not interfere with the immunoassay.

The fundamental difference between the assay of this experiment with a normal run of indirect ELISA is that empty microplates were directly blocked with blocking buffer instead of coated with antigen. Therefore, the detection of sample background was solely based on nonspecific binding of materials from a diluted plasma sample to an immobilized layer of blocking agent.

In this experiment, full assay controls for each microplate comprised of SC1, SC2, CC, C++, C+ and C− (Table 2.8). The positivity for C++, C+ and C− assay controls was determined by checkerboard titrations (Figure 4.8).

From the raw absorbance values, we noticed that the variations of sample backgrounds were attributed to the blocking buffers used (Table 4.2). It should be emphasized that a blocking agent is suitable for ELISA serology only if sample backgrounds from both cases and controls are homogeneous (close to each other) and sufficiently low to avoid false positive signals. However, high backgrounds were observed when using NFDM as blocking agent. Unlike commonly used blocking agents, Ficoll (in both 1×PBS and coating buffers) and PVA (in coating buffer) provided relatively low and narrow range of sample backgrounds.

### 5.2.2. Grid experiments

The blocking ability of PVA was further investigated because it is clinically proven to be non-immunogenic (Nair, 1998), and 30-fold cheaper than Ficoll (Table

5.1). Because the assembly of PVA chains on a solid phase may be influenced by polymer concentration, composition of kosmotrope(s), temperature (Barrett et al., 2001; Kozlov & McCarthy, 2004) and pH, we optimized the blocking steps in grid experiments (Figure 2.1).

**Table 5.1.** Price of blocking agents by November 2011.

| Blocking agent | Company | Product code | Quantity | Unit price (MYR) | Cost per plate[a] (MYR) |
|---|---|---|---|---|---|
| BSA | Sigma | A6003 | 100 g | 4,525.22 | 18.10 |
| NFDM | KPL | 50-82-01 | 200 ml | 683.00 | 1.71 |
| Casein | Merck | 218682 | 500 g | 624.00 | 0.50 |
| Ficoll | Sigma | F4375 | 500 g | 4,715.22 | 3.77 |
| PVA | Merck | 843865 | 1 kg | 323.00 | 0.13 |
| PVP | Sigma | PVP10 | 1 kg | 937.46 | 0.37 |

[a] A total of 30 microplates are needed in the present study (three assays in 10 routine runs).
Abbreviations: MYR, Malaysian ringgits; BSA, bovine serum albumin; NFDM, nonfat dry milk; PVA, poly(vinyl alcohol); PVP, poly(vinyl pyrrolidone).

From the grid experiments, 4% (w/v) PVA provided the most effective blocking, viz., the lowest backgrounds across different dilutions of plasma pools from both NPC ($n$ = 4) and normal ($n$ = 4) subjects (Figure 4.6). For polymer concentrations below 2% (w/v), backgrounds that correspond to PVA layers formed in 1×PBS were relatively lower than those formed in coating buffer. It may be due to higher concentrations of kosmotropes (especially NaCl) in 1×PBS, which could promote the adsorption of PVA (Kozlov & McCarthy, 2004). The kosmotropes could stabilize water structure, and exhibit "salting out" effect by strengthening the hydrophobic interactions between PVA and poly(styrene) surface. Therefore, available surface on the solid phase could be occupied more readily at lower polymer concentrations. This mechanism clarifies why

Huber et al. (2009) demonstrated the effective blocking of 1% (w/v) PVA (no data for other concentrations) in 1×PBS rather than coating buffer. By contrast, the effects of kosmotropes were not obvious above 2% (w/v) PVA (Figure 4.6). Moreover, Table 4.2 shows that the sample backgrounds that correspond to all 4% (w/v) synthetic polymers in coating buffer are relatively lower than those in 1×PBS.

In order to select a diluent for 4% (w/v) PVA, we included additional 12 NPC samples to study their sample backgrounds and SNRs. For sample backgrounds, our results (Figure 4.7) agree well with the observation from Table 4.2, suggesting that the assembly of PVA chains at 4% (w/v) and high pH (coating buffer, pH 9.6) is more resistant to nonspecific binding. However, the underlying mechanism is still not clear to us. Overnight incubation was not further examined because a stable PVA layer could be formed in 2 h at room temperature (Barrett et al., 2001).

### 5.2.3. Comparison of SNRs

The commonly used blocking agents are able to occupy available active sites of a solid phase well (no anomalous background for CC, i.e., $A_{630}$ values lower than 0.20), but the immunogenicity of these animal proteins in human (Dise & Brunell, 1987) may adversely affect the SNRs of serological assays. To demonstrate this point, we compared the SNRs for this 24 NPC samples in assays using NFDM and PVA as blocking agents. The SNR for individual sample was estimated as the ratio of corresponding $A_{630}$ values from assays with to without immobilized antigen (ZEBRA).

The raw absorbance values of signals and noises (sample backgrounds) that correspond to NFDM were indistinguishable (Wilcoxon signed rank test: $z = 1.97$, $p = 0.049$, $r = 0.40$) (Figure 4.7). By contrast, a clear-cut between signals and noises were shown when PVA was used as blocking agent (Wilcoxon signed rank test: $z = 4.29$, $p =$

$1.19\times10^{-7}$, $r = 0.87$). Here, the SNRs that correspond to blocking using 4% (w/v) PVA in coating buffer were 4.26-fold, on average, higher than those blocking using NFDM.

With equivalent signal intensities but relatively lower sample backgrounds (Figure 4.7), the SNRs that correspond to blocking using 4% (w/v) PVA in coating buffer were 1.25-fold, on average, higher than those blocking using 4% (w/v) PVA in 1×PBS. These results again suggested that 4% (w/v) PVA in coating buffer is optimum. Thus, the optimum blocking step was used in the subsequent experiments.

Besides blocking agent, other possibilities for high sample backgrounds are incomplete washing and poor quality of conjugate. From our observations, however, both possibilities can be ruled out because: (i) compared with NFDM, sample backgrounds that correspond to other blocking agents were much lower. Moreover, 4% (w/v) synthetic polymers that prepared in coating buffer were more effective than those prepared in 1×PBS although all microplates underwent the same wash steps by an automated washer. Both observations cannot be explained if the former possibility (incomplete washing) is true. (ii) The latter possibility (poor quality of conjugate) may not valid because wells for CC did not produce anomalous background ($A_{630}$ values lower than 0.20).

### 5.2.4. Checkerboard titrations

Using C++, C+ and C− assay controls, checkerboard titrations were performed to titrate the key reagents for indirect ELISAs (antigen, plasma, and conjugate) (see Section 3.3.4). With optimized reagents, decreasing $A_{630}$ values were observed with increasing dilutions of the assay controls until their endpoints (Figure 4.8). Four-parameter logistic function was used to fit the endpoint titration curves for C++ and C+. The $R^2$ of the fitted curves were above 0.99, suggesting that backgrounds across the titrations are minimal (if the blocking agent contributes high backgrounds, endpoint

titrations of C++ and C+ could not be obtained, therefore $R^2$ values of the non-sigmoidal titration curves will be lower than 0.99). On the other hand, the data also suggested that the PVA blocking layer does not interfere with the signal formations upon the titrations of reagents.

### 5.2.5. Serologic tests

With optimized reagents, ELISA serology was performed on 120 case-control pairs. Because age, gender and ethnicity are confounding factors on NPC risk, only age- and gender-matched, case-control pairs of Malaysian Chinese were assayed (see Section 3.1.2 and 4.1).

The ELISA results for NPC cases and controls were expressed as PP values (see equation (1) in Section 4.2.5). NPC cases were labeled as true positive while normal subjects were labeled as true negative. On this basis, ELISA results from EA-D/IgG, ZEBRA/IgG and VCA p18/IgG were used to classify NPC cases and controls. The performance for each serologic test was characterized by ROC (Figure 4.9).

Table 4.3 summarizes the cutoff value, sensitivity, specificity and AUC for each serologic test. Certainly, a good serologic test has high sensitivity and specificity. In real world situations, however, ROC curves for serologic tests often display the trade-off between sensitivity and specificity at different cutoff values, as shown in Figure 4.8. From the ROC curves, ZEBRA/IgG is the best performed test, followed by VCA p18/IgG and EA-D/IgG, because (i) the ROC curve for ZEBRA/IgG lies nearer to upper left corner of the ROC space, scoring the highest AUC (0.786), and (ii) the AUC differences between ZEBRA/IgG and the other two EBV serologic tests were statistically significance (Table 4.4). Therefore, ZEBRA/IgG had the highest sensitivities and specificities at most of the cutoff values.

Despite no consensus in experimental setup and data analysis, ZEBRA/IgG by ELISA had been suggested as a potential test for Malaysian NPC patients by a previous study (Mathew et al., 1994). Mathew et al. (1994) developed their in-house ELISA and IFA for ZEBRA/IgG (using recombinant ZEBRA protein produced by *E. coli*) and VCA/IgA, respectively. From 100 NPC patients with VCA/IgA (IFA) positive, they found that 75 were ZEBRA/IgG positive (75%). Although the VCA/IgA (IFA) positivity of our NPC cases were unknown, the sensitivity (81.7%) of our assay was higher. On the other hand, out of 83 normal subjects, they found that three were ZEBRA/IgG positive (3.6%), indicating a specificity of 96.4%, which was much higher than the specificity (61.7%) of our assay. However, this difference might be due to spectrum bias because their normal subjects ($n$ = 83) did not match with NPC patients ($n$ = 100).

Besides ZEBRA/IgG, the trade-offs between sensitivity and specificity for both VCA p18/IgG and EA-D/IgG were not satisfactory.

A few limitations in the present study should be noted. (i) The EBV proteins are based on a single EBV strain B95-8 (GenBank V01555) originated from a North American case of infectious mononucleosis (Miller & Lipman, 1973), which may not be as effective as the EBV proteins based on GD1 originated from a Guangdong Chinese case of nasopharyngeal carcinoma (Zeng et al., 2005). We have done multiple sequence alignments of EA-D, ZEBRA and VCA p18 for the only available EBV strains, B95-8, AG876 (originated from a West African case of Burkitt's lymphoma (Pizzo et al., 1978)) and GD1 using Clustal Omega (Sievers et al., 2011) at UniProt (http://www.uniprot.org) (see Appendix D). We found several variations between the amino acid sequences, suggesting comparisons of immunogenicity between the EBV proteins of different strains are necessary for the development of the ELISAs. (ii) During the development of the ELISAs, we chose PVA instead of Ficoll as blocking agent, which was based on

cost considerations (Table 5.1) and some published data showing the effective blocking of PVA (Rodda & Yamazaki, 1994; Studentsov et al., 2002; Huber et al., 2009). To the best of our knowledge, however, only Huber et al. (2009) reported Ficoll as an effective blocking agent. The results in Table 4.2 suggested that Ficoll contributed lower backgrounds with tighter range than PVA, ELISAs using Ficoll as blocking agent may be more reliable than that of PVA. (iii) Our NPC samples were obtained from prevalent cases, which were in remission. After cancer therapy, their anti-EBV antibody levels (IgG against EA-D, and synthetic peptides of EBNA-1, and gp78; IgA against EA-D, gp125, and synthetic peptides of EBNA-1, gp78, VCA p18) might be altered (Gu et al., 2009b). Therefore, the overall diagnostic performances of ZEBRA/IgG, VCA p18/IgG, and EA-D/IgG for detection of NPC in other geographical areas (Table 5.1) are better than our results. (iv) In addition, the ELISA results may be bias, representing only a portion of the prevalent NPC cases who survive longer (long enough to attend the follow-up appointments) because we might not be able to collect blood samples from NPC patients who die quickly. (v) Because of the discrepancies in EBV infection status and health condition, anti-EBV IgG levels in cases and controls may have temporal variations. (vi) Because of limited number of study subjects, we did not randomly split the study subjects into two sets, in which one set is for training (to obtain cutoff values of the EBV serologic tests by ROC analysis) and another independent set is for testing (to evaluate the sensitivities and specificities of the EBV serologic tests). Therefore, the cutoff values may not robust and the diagnostic performances of the serologic tests may not reproducible. (vii) We did not assess the EBV serologic tests with positive predictive values and negative predictive values because these parameters depend on the prevalence of NPC in Malaysian Chinese population that is still undetermined.

For future work, the serological assays should be repeated using EBV proteins with higher purity and without fusion tags in order to improve the diagnostic

performances of the in-house ELISAs. Also, the diagnostic performances of ELISA using PVA as blocking agent should be compared with that of Ficoll. More samples are required to obtain EBV serologic data in full spectrum of NPC subtypes and staging. In addition, all samples should be screened for long term, including the use of other serologic markers such as EBNA-1/IgG, and IgA-based indirect ELISA. Notably, the prognostic value of EBNA-1/IgA by ELISA was recently found to be higher than the most popular EBV marker for NPC, VCA/IgA by IFA (Yu et al., 2011). Besides, the performances of our in-house assays should be compared with commercial kits.

**Table 5.2.** Diagnostic performances of ELISAs (EA-D/IgG, VCA/IgG, and ZEBRA/IgG) for detection of NPC in different geographical areas (selected communications since 2000).

| Author and year | Area (population) | Incident cases and controls | ELISA approach | Sensitivity and specificity (%) | | |
|---|---|---|---|---|---|---|
| Abdulamir et al., 2010 | Iraq and Jordan (native Middle Eastern people of Arabic ethnicity) | 42 NPC cases;<br>66 carcinoma of larynx;<br>14 hypopharyngeal carcinoma;<br>300 healthy controls;<br>cases were matched with controls by age and gender. | VCA/IgG (in-house ELISA using VCA purchased from Wellcome, England) | 76.2; 86 | | |
| Dardari et al., 2008 | Rabat and Casablanca, Morocco | 255 NPC cases: 25 children (≤15 years), 59 young (16–30 years), 171 adults (>30 years);<br>266 healthy controls;<br>cases were matched with controls by age and gender. | *In-house ELISA* | *Children (≤15 years)* | *Young (16–30 years)* | *Adults (>30 years)* |
| | | | ZEBRA/IgG (using recombinant ZEBRA protein produced by *E. coli*) | 100; 100 | 93; 100 | 96; 96 |
| | | | Zp125/IgG (using synthetic peptide) | 80; 88 | 69; 94 | 73; 83 |
| | | | Zp130/IgG (using synthetic peptide) | 67; 100 | 41; 100 | 48; 100 |
| Gu et al., 2008b | Guangzhou, China | 135 NPC cases;<br>130 heathy controls. | EA-D/IgG (ELISA kit purchased from IBL, Germany, using recombinant EA-D protein produced by *E. coli*) | 85.2; 82.3 | | |
| Paramita et al., 2007 | Yogyakarta, Indonesia | 151 NPC cases;<br>259 healthy controls. | EA-D/IgG (in-house ELISA using EA-D extract from HH514.c16 cell, which was induced by 3mM sodium butyrate, 20 ng/ml 12-O-tetradecanoylphorbol-13-acetate and 0.5 mM phosphonoacetic acid) | 90.4; 95.5 | | |

**Table 5.2,** continued.

| Author and year | Area (population) | Incident cases and controls | ELISA approach | Sensitivity and specificity (%) |
|---|---|---|---|---|
| Tedeschi et al., 2007 | Italy (native and resident of Italy) | 77 NPC cases;<br>54 healthy controls;<br>cases were matched with controls by age and gender. | ZEBRA/IgG (in-house ELISA using synthetic peptide) | 61; 94.4 |
| Chan et al., 2003 | Hong Kong | 218 suspected NPC subjects:<br>cases (51 developed NPC, 4 developed lymphoepithelioma like cancer of the lung);<br>controls (23 developed other cancer, 140 did not have tumor). | ZEBRA/IgG (ELISA kit purchased from Sinoclone, Hong Kong, using a recombinant polypeptide produced by *E. coli*) | 74.5, 82.8 |
| Cheng W.-M. et al., 2002 | Hong Kong and Zhongshan, China (residents of Pearl River estuary) | 121 NPC cases;<br>332 healthy controls. | ZEBRA/IgG (ELISA kit purchased from Sinoclone, Hong Kong, using a recombinant polypeptide produced by *E. coli*) | 79; 80 |

Abbreviations: ELISA, enzyme-linked immunosorbent assay; EA-D, early antigen-diffuse; IgG, immunoglobulin G; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen; NPC, nasopharyngeal carcinoma.

### 5.2.6. Assay reproducibility

In our routine ELISA study, all microplates were incorporated with full assay controls, namely SC1, CC, blank, C++, C+ and C− in quadruplicates (see Section 3.3.6). The assay reproducibility was determined from the measurements of C++, C+ and C−. The intra- and inter-assay precisions obtained were less than 7% and 16%, respectively (Table 4.4), suggesting that the assay reproducibility is acceptable.

The measurements of SC1, CC and blank were consistently lower than 0.05, 0.15 and 0.20, respectively. For SC1, the low readings suggested that the substrate system is in good condition. For CC and blank, the low backgrounds suggested the lack of nonspecific binding of conjugate to the solid phase upon PVA blocking, and upon antigen coating and PVA blocking, respectively. Taken together, these observations also suggested that PVA blocking agent may consistently form an inert layer, which favors the assembly of specific immune complex.

For future work, we would like to examine: (i) the effects of interfering substances in plasma or serum, (ii) the cross-reactivity, (iii) the limit of detection and limit of quantification, and (iv) the linearity of the assays.

### 5.2.7. Suggested use of serologic test

Because there are high-risk and low-risk populations for NPC (Figure 2.1), a good population screening strategy would be adopting a highly sensitive test and a highly specific test to the high-risk and low-risk populations, respectively. Using the NPC incidence rate of 1 per 100,000 as a benchmark, individuals above age 25 from the high-risk populations should participate in the screening program adopting a highly sensitive test in order to minimize the number of those with disease undetected. On the other hand, individuals above age 45 from the low-risk populations should participate in the screening program adopting a highly specific test in order to minimize the number

of those without disease subjected for further monitoring. For Malaysian Chinese, one of the high-risk populations for NPC (Figure 2.2), ZEBRA/IgG and VCA p18/IgG tests with higher sensitivities may be better choices than EA-D/IgG test (Table 4.3).

However, the sensitivity and specificity of a serologic test should not be used to estimate the probability of a disease in individuals because they are merely the quantitative estimates of the diagnostic ability of the test (Deeks, 2004).

By contrast, likelihood ratio (LR), which derived from the sensitivity and specificity of a test, is more clinically useful than other statistics in calculating the probability of a disease (Deeks, 2004).

Positive LR, $LR^+$ = sensitivity / (1 − specificity) (6)

Negative LR, $LR^-$ = (1 – sensitivity) / specificity (7)

In the context of evidence-based laboratory medicine, LR greater than 10 or less than 0.1 has a strong impact on clinical decision (Deeks, 2004). Based on Bayes' theorem and above statistics, here we illustrate a fictional case study to suggest the practical application of our EBV serologic test.

A 45-year-old Malaysian Chinese male and his first-degree family members participated in a serologic screening program that organized by our research group. We assume that the pretest probability for the Chinese male to have NPC is 0.6% (see Section 4.1). To the best of our knowledge, the population-based data for family history of NPC in Malaysian Chinese is not publicly available.

His ZEBRA/IgG test had a PP value of 92.70. $LR_1^+$ at this level is 0.367 / (1 – 0.967) = 11.12 (see Appendix B). This means that he was about 11 times more likely to have NPC than a person with the same PP value without NPC. He was therefore referred to an otolaryngologist for clinical examinations. Endoscopy and MRI were done. To the best of our knowledge, the diagnostic performances of Endoscopy and MRI for

Malaysian Chinese population are not publicly available. Therefore, we assume that the data from King et al. (2011) are applicable to Malaysian Chinese population.

Situation A: what about if he has positive results in both endoscopy and MRI examinations? For endoscopy, sensitivity and specificity are 90% and 93%, respectively (King et al., 2011); $LR_2^+$ is 0.90 / (1 − 0.93) = 12.86. For MRI, sensitivity and specificity are 100% and 93%, respectively (King et al., 2011); $LR_3^+$ is 1 / (1 – 0.93) = 14.29.

Because this is a sequential testing, LR should be calculated as $LR_1^+ \times LR_2^+ \times LR_3^+ = 11.12 \times 12.86 \times 14.29 = 2043.52$. One can use Fagan's nomogram to project a pretest probability to a post-test probability (probability of a subject to have a disease after a test) via LR (Deeks, 2004). However, hand-charting is not suitable here because the LR is beyond the scale of a standard Fagan's nomogram (see gray dashed line in Figure 5.1). Therefore, we calculate his post-test probability by the following equations (Deeks, 2004):

Let Pretest probability = $p_1$, Pretest odds = $p_1 / (1 - p_1)$ (8)

Post-test odds, $o_2$ = Pretest odds × LR (9)

Post-test probability = $o_2 / (1 + o_2)$ (10)

Pretest odds = 0.006 / (1 − 0.006) = 0.006. Post-test odds = 0.006 × 2043.52 = 12.261. Post-test probability = 12.261 / (1 + 12.261) = 0.925. Here, the probability of this man to have NPC increases from 0.6% to 92.5%, suggesting that endoscopic biopsy is needed for definitive histological diagnosis.

Situation B: what about if he has a negative result in endoscopy inspection, but a positive result in MRI examination? This may happen when MRI reveals the tumor site that missed during endoscopic inspection. For endoscopy, $LR_2^-$ is (1 – 0.9) / 0.93 = 0.11. For MRI, $LR_3^+$ is the same, 14.29. LR can be calculated as $LR_1^+ \times LR_2^- \times LR_3^+ = 11.12$

× 0.11 × 14.29 = 17.48. Post-test probability can be obtained from Fagan's nomogram (see gray solid line in Figure 5.1), or calculation in the following paragraph.

Post-test odds = 0.006 × 17.48 = 0.105. Post-test probability is 0.105 / (1 + 0.105) = 0.095. The probability of this man to have NPC increases from 0.6% to 9.5%, suggesting that endoscopic biopsy is needed for definitive histological diagnosis. If he is having NPC but do not undergo the serologic test at first, he may be ruled out from having NPC, because of no further clinical examination when he is negative in endoscopic inspection.

In both possible outcomes, the result of EBV serologic test can exert a strong influence on clinical management. However, we suggest validating such approach on an independent set of samples before applying it to patient management.

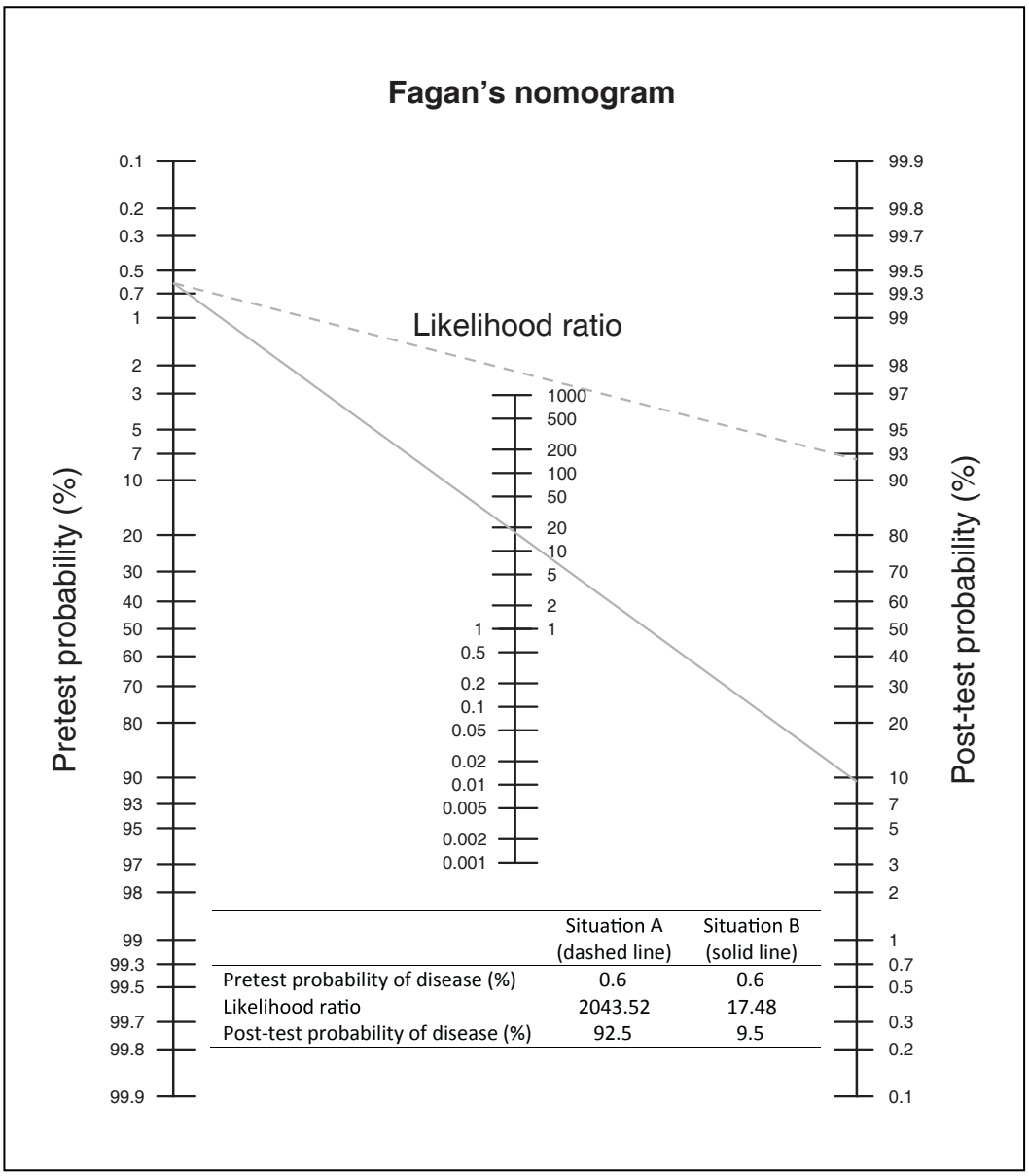


**Figure 5.1.** Fagan's nomograms show the leverages of pretest into post-test probabilities by LRs in a fictional NPC case study (see gray lines). Given that his has a PP value of 92.70 in ZEBRA/IgG test ($LR_1^+$ = 11.12), in situation A, positive in both endoscopy ($LR_2^+$ = 12.86) and MRI ($LR_3^+$ = 14.29) examinations could yield a post-test probability of 92.5% (see dashed line); in situation B, negative in endoscopic inspection ($LR_2^-$ = 0.11) but positive in MRI examination ($LR_3^+$ = 14.29) could yield a post-test probability of 9.5% (see solid line).

# CHAPTER 6. CONCLUSION

NPC is a prevalent cancer in Malaysian Chinese, especially in male population. The ratio of enrolled Chinese male to female patients was 3:1. Also, we noted the familial clustering of NPC, in which 14.19% Chinese patients were familial NPC cases. In our pilot survey of prevalence, three of 334 normal subjects of Chinese descent had a first-degree relative ($n$ = 2) or spouse ($n$ = 1) affected by NPC. Notably, these three NPC cases are males. By contrast, 324 normal subjects of other ethnicity did not have family history of NPC.

Using recombinant EBV proteins (EA-D, ZEBRA, and VCA p18) purified from *E. coli*, we developed indirect ELISAs (EA-D/IgG, ZEBRA/IgG, and VCA p18/IgG) for detection of NPC.

Among the 120 age- and gender-matched case-control pairs (all Chinese), NPC patients showed elevated levels of anti-EBV antibodies. Our study also demonstrated that IgG against ZEBRA is a better clinical indicator for NPC (81.7% sensitivity, 61.7% specificity) compared with IgG against EA-D (74.2% sensitivity, 48.3% specificity) and VCA p18 (95.8% sensitivity, 35.0% specificity). The diagnostic accuracies of in-house ELISAs must be improved before selective screening of NPC in high-risk population in Malaysia can be proposed.

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**Appendix A.** Counting of study subjects according to EA-D/IgG positivity (cutoff values), Youden's index and coordinates of the ROC curve.[a]

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| ≥11.80 | 120 | 120 | 1.000 | 0.000 | 0.000 | 1.000 |
| >11.80 | 120 | 119 | 1.000 | 0.008 | 0.008 | 0.992 |
| >14.35 | 120 | 118 | 1.000 | 0.017 | 0.017 | 0.983 |
| >14.81 | 120 | 117 | 1.000 | 0.025 | 0.025 | 0.975 |
| >14.94 | 120 | 116 | 1.000 | 0.033 | 0.033 | 0.967 |
| >16.48 | 119 | 116 | 0.992 | 0.033 | 0.025 | 0.967 |
| >16.94 | 119 | 115 | 0.992 | 0.042 | 0.033 | 0.958 |
| >17.24 | 119 | 114 | 0.992 | 0.050 | 0.042 | 0.950 |
| >19.10 | 119 | 113 | 0.992 | 0.058 | 0.050 | 0.942 |
| >20.25 | 119 | 112 | 0.992 | 0.067 | 0.058 | 0.933 |
| >20.45 | 119 | 111 | 0.992 | 0.075 | 0.067 | 0.925 |
| >20.61 | 119 | 110 | 0.992 | 0.083 | 0.075 | 0.917 |
| >20.83 | 119 | 109 | 0.992 | 0.092 | 0.083 | 0.908 |
| >21.00 | 118 | 109 | 0.983 | 0.092 | 0.075 | 0.908 |
| >21.31 | 118 | 108 | 0.983 | 0.100 | 0.083 | 0.900 |
| >21.50 | 118 | 107 | 0.983 | 0.108 | 0.092 | 0.892 |
| >22.94 | 117 | 107 | 0.975 | 0.108 | 0.083 | 0.892 |
| >23.84 | 116 | 107 | 0.967 | 0.108 | 0.075 | 0.892 |
| >24.48 | 116 | 106 | 0.967 | 0.117 | 0.083 | 0.883 |
| >24.49 | 115 | 106 | 0.958 | 0.117 | 0.075 | 0.883 |
| >24.94 | 115 | 105 | 0.958 | 0.125 | 0.083 | 0.875 |
| >25.18 | 115 | 104 | 0.958 | 0.133 | 0.092 | 0.867 |
| >25.53 | 115 | 103 | 0.958 | 0.142 | 0.100 | 0.858 |
| >25.65 | 115 | 102 | 0.958 | 0.150 | 0.108 | 0.850 |
| >25.70 | 114 | 102 | 0.950 | 0.150 | 0.100 | 0.850 |
| >25.83 | 113 | 102 | 0.942 | 0.150 | 0.092 | 0.850 |
| >26.06 | 113 | 101 | 0.942 | 0.158 | 0.100 | 0.842 |
| >26.16 | 113 | 100 | 0.942 | 0.167 | 0.108 | 0.833 |
| >26.52 | 113 | 99 | 0.942 | 0.175 | 0.117 | 0.825 |
| >27.12 | 112 | 99 | 0.933 | 0.175 | 0.108 | 0.825 |
| >27.75 | 112 | 98 | 0.933 | 0.183 | 0.117 | 0.817 |
| >28.33 | 112 | 97 | 0.933 | 0.192 | 0.125 | 0.808 |
| >28.42 | 112 | 96 | 0.933 | 0.200 | 0.133 | 0.800 |
| >29.15 | 112 | 95 | 0.933 | 0.208 | 0.142 | 0.792 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >29.34 | 111 | 95 | 0.925 | 0.208 | 0.133 | 0.792 |
| >29.41 | 110 | 95 | 0.917 | 0.208 | 0.125 | 0.792 |
| >29.48 | 110 | 94 | 0.917 | 0.217 | 0.133 | 0.783 |
| >30.04 | 109 | 94 | 0.908 | 0.217 | 0.125 | 0.783 |
| >30.39 | 109 | 93 | 0.908 | 0.225 | 0.133 | 0.775 |
| >30.74 | 108 | 93 | 0.900 | 0.225 | 0.125 | 0.775 |
| >30.75 | 108 | 92 | 0.900 | 0.233 | 0.133 | 0.767 |
| >30.92 | 108 | 91 | 0.900 | 0.242 | 0.142 | 0.758 |
| >31.21 | 108 | 90 | 0.900 | 0.250 | 0.150 | 0.750 |
| >31.38 | 108 | 89 | 0.900 | 0.258 | 0.158 | 0.742 |
| >31.53 | 108 | 88 | 0.900 | 0.267 | 0.167 | 0.733 |
| >31.65 | 107 | 88 | 0.892 | 0.267 | 0.158 | 0.733 |
| >31.67 | 106 | 88 | 0.883 | 0.267 | 0.150 | 0.733 |
| >31.88 | 106 | 87 | 0.883 | 0.275 | 0.158 | 0.725 |
| >31.93 | 106 | 86 | 0.883 | 0.283 | 0.167 | 0.717 |
| >32.39 | 105 | 86 | 0.875 | 0.283 | 0.158 | 0.717 |
| >32.48 | 104 | 86 | 0.867 | 0.283 | 0.150 | 0.717 |
| >32.82 | 104 | 85 | 0.867 | 0.292 | 0.158 | 0.708 |
| >32.91 | 104 | 84 | 0.867 | 0.300 | 0.167 | 0.700 |
| >33.19 | 104 | 83 | 0.867 | 0.308 | 0.175 | 0.692 |
| >33.42 | 104 | 82 | 0.867 | 0.317 | 0.183 | 0.683 |
| >33.60 | 104 | 81 | 0.867 | 0.325 | 0.192 | 0.675 |
| >33.84 | 103 | 81 | 0.858 | 0.325 | 0.183 | 0.675 |
| >34.17 | 102 | 81 | 0.850 | 0.325 | 0.175 | 0.675 |
| >34.27 | 102 | 80 | 0.850 | 0.333 | 0.183 | 0.667 |
| >34.33 | 102 | 79 | 0.850 | 0.342 | 0.192 | 0.658 |
| >34.38 | 101 | 79 | 0.842 | 0.342 | 0.183 | 0.658 |
| >34.52 | 101 | 78 | 0.842 | 0.350 | 0.192 | 0.650 |
| >34.69 | 100 | 78 | 0.833 | 0.350 | 0.183 | 0.650 |
| >34.73 | 100 | 77 | 0.833 | 0.358 | 0.192 | 0.642 |
| >35.11 | 99 | 77 | 0.825 | 0.358 | 0.183 | 0.642 |
| >35.34 | 98 | 77 | 0.817 | 0.358 | 0.175 | 0.642 |
| >35.40 | 97 | 77 | 0.808 | 0.358 | 0.167 | 0.642 |
| >35.63 | 97 | 76 | 0.808 | 0.367 | 0.175 | 0.633 |
| >36.12 | 96 | 76 | 0.800 | 0.367 | 0.167 | 0.633 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >36.13 | 96 | 75 | 0.800 | 0.375 | 0.175 | 0.625 |
| >36.42 | 96 | 74 | 0.800 | 0.383 | 0.183 | 0.617 |
| >36.44 | 96 | 73 | 0.800 | 0.392 | 0.192 | 0.608 |
| >36.54 | 95 | 73 | 0.792 | 0.392 | 0.183 | 0.608 |
| >36.63 | 95 | 72 | 0.792 | 0.400 | 0.192 | 0.600 |
| >36.67 | 94 | 72 | 0.783 | 0.400 | 0.183 | 0.600 |
| >36.88 | 93 | 72 | 0.775 | 0.400 | 0.175 | 0.600 |
| >37.01 | 92 | 72 | 0.767 | 0.400 | 0.167 | 0.600 |
| >37.16 | 92 | 71 | 0.767 | 0.408 | 0.175 | 0.592 |
| >37.51 | 91 | 71 | 0.758 | 0.408 | 0.167 | 0.592 |
| >38.61 | 91 | 70 | 0.758 | 0.417 | 0.175 | 0.583 |
| >38.81 | 91 | 69 | 0.758 | 0.425 | 0.183 | 0.575 |
| >39.01 | 91 | 68 | 0.758 | 0.433 | 0.192 | 0.567 |
| >40.05 | 91 | 67 | 0.758 | 0.442 | 0.200 | 0.558 |
| >40.52 | 90 | 67 | 0.750 | 0.442 | 0.192 | 0.558 |
| >40.69 | 90 | 66 | 0.750 | 0.450 | 0.200 | 0.550 |
| >41.05 | 89 | 66 | 0.742 | 0.450 | 0.192 | 0.550 |
| >41.14 | 89 | 65 | 0.742 | 0.458 | 0.200 | 0.542 |
| >41.18 | 89 | 64 | 0.742 | 0.467 | 0.208 | 0.533 |
| >41.33 | 89 | 63 | 0.742 | 0.475 | 0.217 | 0.525 |
| >41.64 | 89 | 62 | 0.742 | 0.483 | 0.225 | 0.517 |
| >41.86 | 88 | 62 | 0.733 | 0.483 | 0.217 | 0.517 |
| >41.98 | 87 | 62 | 0.725 | 0.483 | 0.208 | 0.517 |
| >42.06 | 86 | 62 | 0.717 | 0.483 | 0.200 | 0.517 |
| >42.22 | 85 | 62 | 0.708 | 0.483 | 0.192 | 0.517 |
| >42.51 | 84 | 62 | 0.700 | 0.483 | 0.183 | 0.517 |
| >42.67 | 83 | 62 | 0.692 | 0.483 | 0.175 | 0.517 |
| >42.76 | 82 | 62 | 0.683 | 0.483 | 0.167 | 0.517 |
| >42.94 | 82 | 61 | 0.683 | 0.492 | 0.175 | 0.508 |
| >43.64 | 81 | 61 | 0.675 | 0.492 | 0.167 | 0.508 |
| >43.64 | 80 | 61 | 0.667 | 0.492 | 0.158 | 0.508 |
| >43.80 | 80 | 60 | 0.667 | 0.500 | 0.167 | 0.500 |
| >43.91 | 79 | 60 | 0.658 | 0.500 | 0.158 | 0.500 |
| >44.47 | 79 | 59 | 0.658 | 0.508 | 0.167 | 0.492 |
| >45.06 | 79 | 58 | 0.658 | 0.517 | 0.175 | 0.483 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >45.35 | 79 | 57 | 0.658 | 0.525 | 0.183 | 0.475 |
| >45.44 | 79 | 56 | 0.658 | 0.533 | 0.192 | 0.467 |
| >45.82 | 78 | 56 | 0.650 | 0.533 | 0.183 | 0.467 |
| >45.86 | 78 | 55 | 0.650 | 0.542 | 0.192 | 0.458 |
| >45.90 | 77 | 55 | 0.642 | 0.542 | 0.183 | 0.458 |
| >45.91 | 77 | 54 | 0.642 | 0.550 | 0.192 | 0.450 |
| >46.04 | 76 | 54 | 0.633 | 0.550 | 0.183 | 0.450 |
| >46.52 | 76 | 53 | 0.633 | 0.558 | 0.192 | 0.442 |
| >47.46 | 76 | 52 | 0.633 | 0.567 | 0.200 | 0.433 |
| >47.49 | 75 | 52 | 0.625 | 0.567 | 0.192 | 0.433 |
| >48.28 | 75 | 51 | 0.625 | 0.575 | 0.200 | 0.425 |
| >48.46 | 75 | 50 | 0.625 | 0.583 | 0.208 | 0.417 |
| >48.70 | 74 | 50 | 0.617 | 0.583 | 0.200 | 0.417 |
| >48.74 | 74 | 49 | 0.617 | 0.592 | 0.208 | 0.408 |
| >48.78 | 74 | 48 | 0.617 | 0.600 | 0.217 | 0.400 |
| >48.87 | 73 | 48 | 0.608 | 0.600 | 0.208 | 0.400 |
| >49.22 | 72 | 48 | 0.600 | 0.600 | 0.200 | 0.400 |
| >49.27 | 71 | 48 | 0.592 | 0.600 | 0.192 | 0.400 |
| >49.39 | 71 | 47 | 0.592 | 0.608 | 0.200 | 0.392 |
| >49.98 | 70 | 47 | 0.583 | 0.608 | 0.192 | 0.392 |
| >50.06 | 69 | 47 | 0.575 | 0.608 | 0.183 | 0.392 |
| >50.12 | 68 | 47 | 0.567 | 0.608 | 0.175 | 0.392 |
| >50.21 | 68 | 46 | 0.567 | 0.617 | 0.183 | 0.383 |
| >50.52 | 67 | 46 | 0.558 | 0.617 | 0.175 | 0.383 |
| >50.68 | 67 | 45 | 0.558 | 0.625 | 0.183 | 0.375 |
| >50.88 | 67 | 44 | 0.558 | 0.633 | 0.192 | 0.367 |
| >51.58 | 66 | 44 | 0.550 | 0.633 | 0.183 | 0.367 |
| >51.95 | 65 | 44 | 0.542 | 0.633 | 0.175 | 0.367 |
| >51.98 | 65 | 43 | 0.542 | 0.642 | 0.183 | 0.358 |
| >52.11 | 64 | 43 | 0.533 | 0.642 | 0.175 | 0.358 |
| >52.19 | 63 | 43 | 0.525 | 0.642 | 0.167 | 0.358 |
| >52.32 | 62 | 43 | 0.517 | 0.642 | 0.158 | 0.358 |
| >52.40 | 62 | 42 | 0.517 | 0.650 | 0.167 | 0.350 |
| >52.53 | 61 | 42 | 0.508 | 0.650 | 0.158 | 0.350 |
| >52.97 | 60 | 42 | 0.500 | 0.650 | 0.150 | 0.350 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >53.07 | 60 | 41 | 0.500 | 0.658 | 0.158 | 0.342 |
| >53.26 | 60 | 40 | 0.500 | 0.667 | 0.167 | 0.333 |
| >53.61 | 60 | 39 | 0.500 | 0.675 | 0.175 | 0.325 |
| >53.93 | 60 | 38 | 0.500 | 0.683 | 0.183 | 0.317 |
| >53.99 | 59 | 38 | 0.492 | 0.683 | 0.175 | 0.317 |
| >54.60 | 59 | 37 | 0.492 | 0.692 | 0.183 | 0.308 |
| >54.62 | 59 | 36 | 0.492 | 0.700 | 0.192 | 0.300 |
| >54.76 | 58 | 36 | 0.483 | 0.700 | 0.183 | 0.300 |
| >55.03 | 57 | 36 | 0.475 | 0.700 | 0.175 | 0.300 |
| >55.14 | 56 | 36 | 0.467 | 0.700 | 0.167 | 0.300 |
| >55.22 | 55 | 36 | 0.458 | 0.700 | 0.158 | 0.300 |
| >55.69 | 55 | 35 | 0.458 | 0.708 | 0.167 | 0.292 |
| >56.12 | 54 | 35 | 0.450 | 0.708 | 0.158 | 0.292 |
| >56.60 | 54 | 34 | 0.450 | 0.717 | 0.167 | 0.283 |
| >56.80 | 54 | 33 | 0.450 | 0.725 | 0.175 | 0.275 |
| >56.96 | 53 | 33 | 0.442 | 0.725 | 0.167 | 0.275 |
| >57.21 | 53 | 32 | 0.442 | 0.733 | 0.175 | 0.267 |
| >57.68 | 53 | 31 | 0.442 | 0.742 | 0.183 | 0.258 |
| >58.16 | 53 | 30 | 0.442 | 0.750 | 0.192 | 0.250 |
| >58.68 | 52 | 30 | 0.433 | 0.750 | 0.183 | 0.250 |
| >58.94 | 52 | 29 | 0.433 | 0.758 | 0.192 | 0.242 |
| >59.73 | 51 | 29 | 0.425 | 0.758 | 0.183 | 0.242 |
| >59.86 | 51 | 28 | 0.425 | 0.767 | 0.192 | 0.233 |
| >60.19 | 50 | 28 | 0.417 | 0.767 | 0.183 | 0.233 |
| >60.21 | 50 | 27 | 0.417 | 0.775 | 0.192 | 0.225 |
| >60.76 | 50 | 26 | 0.417 | 0.783 | 0.200 | 0.217 |
| >61.41 | 49 | 26 | 0.408 | 0.783 | 0.192 | 0.217 |
| >62.14 | 48 | 26 | 0.400 | 0.783 | 0.183 | 0.217 |
| >62.47 | 47 | 26 | 0.392 | 0.783 | 0.175 | 0.217 |
| >62.62 | 46 | 26 | 0.383 | 0.783 | 0.167 | 0.217 |
| >63.13 | 46 | 25 | 0.383 | 0.792 | 0.175 | 0.208 |
| >63.29 | 45 | 25 | 0.375 | 0.792 | 0.167 | 0.208 |
| >63.42 | 45 | 24 | 0.375 | 0.800 | 0.175 | 0.200 |
| >63.56 | 45 | 23 | 0.375 | 0.808 | 0.183 | 0.192 |
| >63.63 | 44 | 23 | 0.367 | 0.808 | 0.175 | 0.192 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >64.04 | 43 | 23 | 0.358 | 0.808 | 0.167 | 0.192 |
| >64.09 | 42 | 23 | 0.350 | 0.808 | 0.158 | 0.192 |
| >64.32 | 41 | 23 | 0.342 | 0.808 | 0.150 | 0.192 |
| >64.68 | 41 | 22 | 0.342 | 0.817 | 0.158 | 0.183 |
| >64.96 | 40 | 22 | 0.333 | 0.817 | 0.150 | 0.183 |
| >65.60 | 39 | 22 | 0.325 | 0.817 | 0.142 | 0.183 |
| >65.91 | 39 | 21 | 0.325 | 0.825 | 0.150 | 0.175 |
| >66.28 | 39 | 20 | 0.325 | 0.833 | 0.158 | 0.167 |
| >67.17 | 39 | 19 | 0.325 | 0.842 | 0.167 | 0.158 |
| >67.59 | 39 | 18 | 0.325 | 0.850 | 0.175 | 0.150 |
| >68.66 | 39 | 17 | 0.325 | 0.858 | 0.183 | 0.142 |
| >68.95 | 38 | 17 | 0.317 | 0.858 | 0.175 | 0.142 |
| >70.41 | 37 | 17 | 0.308 | 0.858 | 0.167 | 0.142 |
| >70.74 | 37 | 16 | 0.308 | 0.867 | 0.175 | 0.133 |
| >71.56 | 37 | 15 | 0.308 | 0.875 | 0.183 | 0.125 |
| >72.23 | 37 | 14 | 0.308 | 0.883 | 0.192 | 0.117 |
| >72.35 | 36 | 14 | 0.300 | 0.883 | 0.183 | 0.117 |
| >73.38 | 36 | 13 | 0.300 | 0.892 | 0.192 | 0.108 |
| >73.73 | 35 | 13 | 0.292 | 0.892 | 0.183 | 0.108 |
| >74.48 | 34 | 13 | 0.283 | 0.892 | 0.175 | 0.108 |
| >74.79 | 33 | 13 | 0.275 | 0.892 | 0.167 | 0.108 |
| >75.75 | 32 | 13 | 0.267 | 0.892 | 0.158 | 0.108 |
| >76.05 | 32 | 12 | 0.267 | 0.900 | 0.167 | 0.100 |
| >76.47 | 32 | 11 | 0.267 | 0.908 | 0.175 | 0.092 |
| >76.54 | 31 | 11 | 0.258 | 0.908 | 0.167 | 0.092 |
| >77.49 | 31 | 10 | 0.258 | 0.917 | 0.175 | 0.083 |
| >78.30 | 30 | 10 | 0.250 | 0.917 | 0.167 | 0.083 |
| >78.82 | 29 | 10 | 0.242 | 0.917 | 0.158 | 0.083 |
| >78.83 | 29 | 9 | 0.242 | 0.925 | 0.167 | 0.075 |
| >79.02 | 28 | 9 | 0.233 | 0.925 | 0.158 | 0.075 |
| >79.79 | 27 | 9 | 0.225 | 0.925 | 0.150 | 0.075 |
| >79.92 | 27 | 8 | 0.225 | 0.933 | 0.158 | 0.067 |
| >80.30 | 27 | 7 | 0.225 | 0.942 | 0.167 | 0.058 |
| >80.60 | 27 | 6 | 0.225 | 0.950 | 0.175 | 0.050 |
| >81.96 | 27 | 5 | 0.225 | 0.958 | 0.183 | 0.042 |

**Appendix A,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >82.63 | 26 | 5 | 0.217 | 0.958 | 0.175 | 0.042 |
| >82.82 | 26 | 4 | 0.217 | 0.967 | 0.183 | 0.033 |
| >83.04 | 25 | 4 | 0.208 | 0.967 | 0.175 | 0.033 |
| >83.49 | 25 | 3 | 0.208 | 0.975 | 0.183 | 0.025 |
| >83.92 | 24 | 3 | 0.200 | 0.975 | 0.175 | 0.025 |
| >84.86 | 23 | 3 | 0.192 | 0.975 | 0.167 | 0.025 |
| >88.17 | 22 | 3 | 0.183 | 0.975 | 0.158 | 0.025 |
| >88.24 | 22 | 2 | 0.183 | 0.983 | 0.167 | 0.017 |
| >89.07 | 21 | 2 | 0.175 | 0.983 | 0.158 | 0.017 |
| >89.49 | 20 | 2 | 0.167 | 0.983 | 0.150 | 0.017 |
| >91.60 | 20 | 1 | 0.167 | 0.992 | 0.158 | 0.008 |
| >92.91 | 19 | 1 | 0.158 | 0.992 | 0.150 | 0.008 |
| >93.30 | 18 | 1 | 0.150 | 0.992 | 0.142 | 0.008 |
| >94.29 | 18 | 0 | 0.150 | 1.000 | 0.150 | 0.000 |
| >94.41 | 17 | 0 | 0.142 | 1.000 | 0.142 | 0.000 |
| >97.80 | 16 | 0 | 0.133 | 1.000 | 0.133 | 0.000 |
| >98.92 | 15 | 0 | 0.125 | 1.000 | 0.125 | 0.000 |
| >99.05 | 14 | 0 | 0.117 | 1.000 | 0.117 | 0.000 |
| >101.63 | 13 | 0 | 0.108 | 1.000 | 0.108 | 0.000 |
| >105.34 | 12 | 0 | 0.100 | 1.000 | 0.100 | 0.000 |
| >106.76 | 11 | 0 | 0.092 | 1.000 | 0.092 | 0.000 |
| >106.97 | 10 | 0 | 0.083 | 1.000 | 0.083 | 0.000 |
| >107.63 | 9 | 0 | 0.075 | 1.000 | 0.075 | 0.000 |
| >108.82 | 8 | 0 | 0.067 | 1.000 | 0.067 | 0.000 |
| >110.15 | 7 | 0 | 0.058 | 1.000 | 0.058 | 0.000 |
| >111.50 | 6 | 0 | 0.050 | 1.000 | 0.050 | 0.000 |
| >112.35 | 5 | 0 | 0.042 | 1.000 | 0.042 | 0.000 |
| >112.67 | 4 | 0 | 0.033 | 1.000 | 0.033 | 0.000 |
| >131.23 | 3 | 0 | 0.025 | 1.000 | 0.025 | 0.000 |
| >132.00 | 2 | 0 | 0.017 | 1.000 | 0.017 | 0.000 |
| >134.35 | 1 | 0 | 0.008 | 1.000 | 0.008 | 0.000 |
| >140.94 | 1 | 0 | 0.000 | 1.000 | 0.000 | 0.000 |

[a] Gray shading denotes the cutoff value, sensitivity and specificity at the highest Youden's score.

Abbreviations: EA-D, early antigen-diffuse; IgG, immunoglobulin G; ROC, receiver operating characteristics; PP, percentage positivity; NPC, nasopharyngeal carcinoma.

**Appendix B.** Counting of study subjects according to ZEBRA/IgG positivity (cutoff values), Youden's index and coordinates of the ROC curve.[a]

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| ≥9.51 | 120 | 120 | 1.000 | 0.000 | 0.000 | 1.000 |
| >9.51 | 120 | 119 | 1.000 | 0.008 | 0.008 | 0.992 |
| >11.84 | 120 | 118 | 1.000 | 0.017 | 0.017 | 0.983 |
| >14.09 | 120 | 117 | 1.000 | 0.025 | 0.025 | 0.975 |
| >19.12 | 120 | 116 | 1.000 | 0.033 | 0.033 | 0.967 |
| >21.73 | 120 | 115 | 1.000 | 0.042 | 0.042 | 0.958 |
| >21.83 | 120 | 114 | 1.000 | 0.050 | 0.050 | 0.950 |
| >21.86 | 120 | 113 | 1.000 | 0.058 | 0.058 | 0.942 |
| >21.92 | 120 | 112 | 1.000 | 0.067 | 0.067 | 0.933 |
| >22.11 | 120 | 111 | 1.000 | 0.075 | 0.075 | 0.925 |
| >22.63 | 120 | 110 | 1.000 | 0.083 | 0.083 | 0.917 |
| >23.20 | 120 | 109 | 1.000 | 0.092 | 0.092 | 0.908 |
| >24.63 | 120 | 108 | 1.000 | 0.100 | 0.100 | 0.900 |
| >24.90 | 119 | 108 | 0.992 | 0.100 | 0.092 | 0.900 |
| >25.00 | 119 | 107 | 0.992 | 0.108 | 0.100 | 0.892 |
| >25.65 | 119 | 106 | 0.992 | 0.117 | 0.108 | 0.883 |
| >26.19 | 119 | 105 | 0.992 | 0.125 | 0.117 | 0.875 |
| >26.32 | 119 | 104 | 0.992 | 0.133 | 0.125 | 0.867 |
| >26.48 | 119 | 103 | 0.992 | 0.142 | 0.133 | 0.858 |
| >26.70 | 119 | 102 | 0.992 | 0.150 | 0.142 | 0.850 |
| >26.90 | 119 | 101 | 0.992 | 0.158 | 0.150 | 0.842 |
| >27.01 | 118 | 101 | 0.983 | 0.158 | 0.142 | 0.842 |
| >28.24 | 118 | 100 | 0.983 | 0.167 | 0.150 | 0.833 |
| >28.38 | 117 | 100 | 0.975 | 0.167 | 0.142 | 0.833 |
| >29.16 | 117 | 99 | 0.975 | 0.175 | 0.150 | 0.825 |
| >29.17 | 117 | 98 | 0.975 | 0.183 | 0.158 | 0.817 |
| >29.50 | 117 | 97 | 0.975 | 0.192 | 0.167 | 0.808 |
| >30.37 | 116 | 97 | 0.967 | 0.192 | 0.158 | 0.808 |
| >30.49 | 116 | 96 | 0.967 | 0.200 | 0.167 | 0.800 |
| >31.97 | 116 | 95 | 0.967 | 0.208 | 0.175 | 0.792 |
| >32.51 | 115 | 95 | 0.958 | 0.208 | 0.167 | 0.792 |
| >33.39 | 115 | 94 | 0.958 | 0.217 | 0.175 | 0.783 |
| >33.93 | 115 | 93 | 0.958 | 0.225 | 0.183 | 0.775 |
| >34.27 | 115 | 92 | 0.958 | 0.233 | 0.192 | 0.767 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >34.36 | 115 | 91 | 0.958 | 0.242 | 0.200 | 0.758 |
| >34.37 | 115 | 90 | 0.958 | 0.250 | 0.208 | 0.750 |
| >34.75 | 115 | 89 | 0.958 | 0.258 | 0.217 | 0.742 |
| >35.32 | 115 | 88 | 0.958 | 0.267 | 0.225 | 0.733 |
| >35.39 | 115 | 87 | 0.958 | 0.275 | 0.233 | 0.725 |
| >35.56 | 115 | 86 | 0.958 | 0.283 | 0.242 | 0.717 |
| >35.76 | 115 | 85 | 0.958 | 0.292 | 0.250 | 0.708 |
| >36.15 | 114 | 85 | 0.950 | 0.292 | 0.242 | 0.708 |
| >36.41 | 113 | 85 | 0.942 | 0.292 | 0.233 | 0.708 |
| >36.49 | 113 | 84 | 0.942 | 0.300 | 0.242 | 0.700 |
| >36.79 | 113 | 83 | 0.942 | 0.308 | 0.250 | 0.692 |
| >36.85 | 113 | 82 | 0.942 | 0.317 | 0.258 | 0.683 |
| >37.15 | 112 | 82 | 0.933 | 0.317 | 0.250 | 0.683 |
| >37.81 | 112 | 81 | 0.933 | 0.325 | 0.258 | 0.675 |
| >38.78 | 112 | 80 | 0.933 | 0.333 | 0.267 | 0.667 |
| >39.14 | 111 | 80 | 0.925 | 0.333 | 0.258 | 0.667 |
| >39.72 | 111 | 79 | 0.925 | 0.342 | 0.267 | 0.658 |
| >39.94 | 110 | 79 | 0.917 | 0.342 | 0.258 | 0.658 |
| >40.02 | 110 | 78 | 0.917 | 0.350 | 0.267 | 0.650 |
| >40.32 | 109 | 78 | 0.908 | 0.350 | 0.258 | 0.650 |
| >41.45 | 108 | 78 | 0.900 | 0.350 | 0.250 | 0.650 |
| >41.48 | 108 | 77 | 0.900 | 0.358 | 0.258 | 0.642 |
| >42.04 | 108 | 76 | 0.900 | 0.367 | 0.267 | 0.633 |
| >42.09 | 107 | 76 | 0.892 | 0.367 | 0.258 | 0.633 |
| >42.11 | 107 | 75 | 0.892 | 0.375 | 0.267 | 0.625 |
| >42.12 | 107 | 74 | 0.892 | 0.383 | 0.275 | 0.617 |
| >42.29 | 107 | 73 | 0.892 | 0.392 | 0.283 | 0.608 |
| >42.38 | 107 | 72 | 0.892 | 0.400 | 0.292 | 0.600 |
| >42.44 | 107 | 71 | 0.892 | 0.408 | 0.300 | 0.592 |
| >42.49 | 107 | 70 | 0.892 | 0.417 | 0.308 | 0.583 |
| >42.88 | 107 | 69 | 0.892 | 0.425 | 0.317 | 0.575 |
| >43.05 | 107 | 68 | 0.892 | 0.433 | 0.325 | 0.567 |
| >43.07 | 106 | 68 | 0.883 | 0.433 | 0.317 | 0.567 |
| >43.52 | 105 | 68 | 0.875 | 0.433 | 0.308 | 0.567 |
| >43.54 | 105 | 67 | 0.875 | 0.442 | 0.317 | 0.558 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >43.70 | 105 | 66 | 0.875 | 0.450 | 0.325 | 0.550 |
| >44.19 | 104 | 66 | 0.867 | 0.450 | 0.317 | 0.550 |
| >44.35 | 103 | 66 | 0.858 | 0.450 | 0.308 | 0.550 |
| >44.40 | 103 | 65 | 0.858 | 0.458 | 0.317 | 0.542 |
| >44.71 | 103 | 64 | 0.858 | 0.467 | 0.325 | 0.533 |
| >44.83 | 103 | 63 | 0.858 | 0.475 | 0.333 | 0.525 |
| >45.50 | 103 | 62 | 0.858 | 0.483 | 0.342 | 0.517 |
| >45.57 | 103 | 61 | 0.858 | 0.492 | 0.350 | 0.508 |
| >45.72 | 103 | 60 | 0.858 | 0.500 | 0.358 | 0.500 |
| >45.80 | 103 | 59 | 0.858 | 0.508 | 0.367 | 0.492 |
| >46.25 | 103 | 58 | 0.858 | 0.517 | 0.375 | 0.483 |
| >46.62 | 103 | 57 | 0.858 | 0.525 | 0.383 | 0.475 |
| >46.87 | 103 | 56 | 0.858 | 0.533 | 0.392 | 0.467 |
| >47.02 | 103 | 55 | 0.858 | 0.542 | 0.400 | 0.458 |
| >47.04 | 103 | 54 | 0.858 | 0.550 | 0.408 | 0.450 |
| >48.48 | 103 | 53 | 0.858 | 0.558 | 0.417 | 0.442 |
| >49.39 | 102 | 53 | 0.850 | 0.558 | 0.408 | 0.442 |
| >50.69 | 102 | 52 | 0.850 | 0.567 | 0.417 | 0.433 |
| >51.18 | 101 | 52 | 0.842 | 0.567 | 0.408 | 0.433 |
| >51.24 | 100 | 52 | 0.833 | 0.567 | 0.400 | 0.433 |
| >51.61 | 100 | 51 | 0.833 | 0.575 | 0.408 | 0.425 |
| >51.90 | 100 | 50 | 0.833 | 0.583 | 0.417 | 0.417 |
| >52.21 | 100 | 49 | 0.833 | 0.592 | 0.425 | 0.408 |
| >53.75 | 99 | 49 | 0.825 | 0.592 | 0.417 | 0.408 |
| >53.82 | 99 | 48 | 0.825 | 0.600 | 0.425 | 0.400 |
| >54.09 | 98 | 48 | 0.817 | 0.600 | 0.417 | 0.400 |
| >54.44 | 98 | 47 | 0.817 | 0.608 | 0.425 | 0.392 |
| >55.08 | 98 | 46 | 0.817 | 0.617 | 0.433 | 0.383 |
| >55.09 | 97 | 46 | 0.808 | 0.617 | 0.425 | 0.383 |
| >55.48 | 96 | 46 | 0.800 | 0.617 | 0.417 | 0.383 |
| >55.70 | 95 | 46 | 0.792 | 0.617 | 0.408 | 0.383 |
| >55.85 | 95 | 45 | 0.792 | 0.625 | 0.417 | 0.375 |
| >56.18 | 95 | 44 | 0.792 | 0.633 | 0.425 | 0.367 |
| >56.69 | 94 | 44 | 0.783 | 0.633 | 0.417 | 0.367 |
| >56.89 | 94 | 43 | 0.783 | 0.642 | 0.425 | 0.358 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >56.90 | 93 | 43 | 0.775 | 0.642 | 0.417 | 0.358 |
| >57.26 | 92 | 43 | 0.767 | 0.642 | 0.408 | 0.358 |
| >58.12 | 91 | 43 | 0.758 | 0.642 | 0.400 | 0.358 |
| >58.30 | 91 | 42 | 0.758 | 0.650 | 0.408 | 0.350 |
| >58.41 | 90 | 42 | 0.750 | 0.650 | 0.400 | 0.350 |
| >58.68 | 89 | 42 | 0.742 | 0.650 | 0.392 | 0.350 |
| >58.98 | 89 | 41 | 0.742 | 0.658 | 0.400 | 0.342 |
| >59.11 | 88 | 41 | 0.733 | 0.658 | 0.392 | 0.342 |
| >59.88 | 87 | 41 | 0.725 | 0.658 | 0.383 | 0.342 |
| >60.13 | 87 | 40 | 0.725 | 0.667 | 0.392 | 0.333 |
| >60.51 | 86 | 40 | 0.717 | 0.667 | 0.383 | 0.333 |
| >60.63 | 86 | 39 | 0.717 | 0.675 | 0.392 | 0.325 |
| >60.75 | 86 | 38 | 0.717 | 0.683 | 0.400 | 0.317 |
| >60.81 | 85 | 38 | 0.708 | 0.683 | 0.392 | 0.317 |
| >61.67 | 85 | 37 | 0.708 | 0.692 | 0.400 | 0.308 |
| >61.88 | 85 | 36 | 0.708 | 0.700 | 0.408 | 0.300 |
| >62.14 | 85 | 35 | 0.708 | 0.708 | 0.417 | 0.292 |
| >63.11 | 84 | 35 | 0.700 | 0.708 | 0.408 | 0.292 |
| >63.48 | 83 | 35 | 0.692 | 0.708 | 0.400 | 0.292 |
| >64.37 | 83 | 34 | 0.692 | 0.717 | 0.408 | 0.283 |
| >64.86 | 83 | 33 | 0.692 | 0.725 | 0.417 | 0.275 |
| >65.97 | 82 | 33 | 0.683 | 0.725 | 0.408 | 0.275 |
| >66.37 | 82 | 32 | 0.683 | 0.733 | 0.417 | 0.267 |
| >67.17 | 81 | 32 | 0.675 | 0.733 | 0.408 | 0.267 |
| >67.38 | 81 | 31 | 0.675 | 0.742 | 0.417 | 0.258 |
| >67.94 | 80 | 31 | 0.667 | 0.742 | 0.408 | 0.258 |
| >68.39 | 80 | 30 | 0.667 | 0.750 | 0.417 | 0.250 |
| >69.59 | 79 | 30 | 0.658 | 0.750 | 0.408 | 0.250 |
| >69.92 | 78 | 30 | 0.650 | 0.750 | 0.400 | 0.250 |
| >70.77 | 78 | 29 | 0.650 | 0.758 | 0.408 | 0.242 |
| >71.06 | 78 | 28 | 0.650 | 0.767 | 0.417 | 0.233 |
| >71.14 | 77 | 28 | 0.642 | 0.767 | 0.408 | 0.233 |
| >71.54 | 76 | 28 | 0.633 | 0.767 | 0.400 | 0.233 |
| >71.69 | 75 | 28 | 0.625 | 0.767 | 0.392 | 0.233 |
| >71.74 | 75 | 27 | 0.625 | 0.775 | 0.400 | 0.225 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >72.39 | 75 | 26 | 0.625 | 0.783 | 0.408 | 0.217 |
| >72.82 | 74 | 26 | 0.617 | 0.783 | 0.400 | 0.217 |
| >72.96 | 73 | 26 | 0.608 | 0.783 | 0.392 | 0.217 |
| >73.19 | 72 | 26 | 0.600 | 0.783 | 0.383 | 0.217 |
| >73.55 | 72 | 25 | 0.600 | 0.792 | 0.392 | 0.208 |
| >73.56 | 71 | 25 | 0.592 | 0.792 | 0.383 | 0.208 |
| >73.71 | 70 | 25 | 0.583 | 0.792 | 0.375 | 0.208 |
| >73.95 | 70 | 24 | 0.583 | 0.800 | 0.383 | 0.200 |
| >74.40 | 69 | 24 | 0.575 | 0.800 | 0.375 | 0.200 |
| >75.72 | 69 | 23 | 0.575 | 0.808 | 0.383 | 0.192 |
| >76.22 | 68 | 23 | 0.567 | 0.808 | 0.375 | 0.192 |
| >76.23 | 68 | 22 | 0.567 | 0.817 | 0.383 | 0.183 |
| >76.47 | 67 | 22 | 0.558 | 0.817 | 0.375 | 0.183 |
| >77.24 | 67 | 21 | 0.558 | 0.825 | 0.383 | 0.175 |
| >77.25 | 67 | 20 | 0.558 | 0.833 | 0.392 | 0.167 |
| >77.32 | 66 | 20 | 0.550 | 0.833 | 0.383 | 0.167 |
| >77.61 | 65 | 20 | 0.542 | 0.833 | 0.375 | 0.167 |
| >78.19 | 65 | 19 | 0.542 | 0.842 | 0.383 | 0.158 |
| >78.30 | 64 | 19 | 0.533 | 0.842 | 0.375 | 0.158 |
| >78.39 | 63 | 19 | 0.525 | 0.842 | 0.367 | 0.158 |
| >78.52 | 63 | 18 | 0.525 | 0.850 | 0.375 | 0.150 |
| >78.60 | 62 | 18 | 0.517 | 0.850 | 0.367 | 0.150 |
| >78.77 | 62 | 17 | 0.517 | 0.858 | 0.375 | 0.142 |
| >79.02 | 61 | 17 | 0.508 | 0.858 | 0.367 | 0.142 |
| >79.10 | 61 | 16 | 0.508 | 0.867 | 0.375 | 0.133 |
| >79.64 | 60 | 16 | 0.500 | 0.867 | 0.367 | 0.133 |
| >79.83 | 59 | 16 | 0.492 | 0.867 | 0.358 | 0.133 |
| >79.85 | 59 | 15 | 0.492 | 0.875 | 0.367 | 0.125 |
| >80.66 | 58 | 15 | 0.483 | 0.875 | 0.358 | 0.125 |
| >81.72 | 58 | 14 | 0.483 | 0.883 | 0.367 | 0.117 |
| >82.52 | 57 | 14 | 0.475 | 0.883 | 0.358 | 0.117 |
| >83.02 | 56 | 14 | 0.467 | 0.883 | 0.350 | 0.117 |
| >83.34 | 56 | 13 | 0.467 | 0.892 | 0.358 | 0.108 |
| >83.61 | 55 | 13 | 0.458 | 0.892 | 0.350 | 0.108 |
| >84.30 | 54 | 13 | 0.450 | 0.892 | 0.342 | 0.108 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >84.70 | 54 | 12 | 0.450 | 0.900 | 0.350 | 0.100 |
| >86.16 | 53 | 12 | 0.442 | 0.900 | 0.342 | 0.100 |
| >86.60 | 53 | 11 | 0.442 | 0.908 | 0.350 | 0.092 |
| >86.84 | 52 | 11 | 0.433 | 0.908 | 0.342 | 0.092 |
| >87.03 | 51 | 11 | 0.425 | 0.908 | 0.333 | 0.092 |
| >87.13 | 51 | 10 | 0.425 | 0.917 | 0.342 | 0.083 |
| >87.33 | 51 | 9 | 0.425 | 0.925 | 0.350 | 0.075 |
| >88.52 | 51 | 8 | 0.425 | 0.933 | 0.358 | 0.067 |
| >88.59 | 50 | 8 | 0.417 | 0.933 | 0.350 | 0.067 |
| >89.10 | 49 | 8 | 0.408 | 0.933 | 0.342 | 0.067 |
| >89.36 | 49 | 7 | 0.408 | 0.942 | 0.350 | 0.058 |
| >90.05 | 48 | 7 | 0.400 | 0.942 | 0.342 | 0.058 |
| >90.26 | 47 | 7 | 0.392 | 0.942 | 0.333 | 0.058 |
| >90.80 | 46 | 7 | 0.383 | 0.942 | 0.325 | 0.058 |
| >90.86 | 46 | 6 | 0.383 | 0.950 | 0.333 | 0.050 |
| >91.60 | 45 | 6 | 0.375 | 0.950 | 0.325 | 0.050 |
| >91.69 | 45 | 5 | 0.375 | 0.958 | 0.333 | 0.042 |
| >92.28 | 44 | 5 | 0.367 | 0.958 | 0.325 | 0.042 |
| >92.66 | 44 | 4 | 0.367 | 0.967 | 0.333 | 0.033 |
| >92.71 | 43 | 4 | 0.358 | 0.967 | 0.325 | 0.033 |
| >94.38 | 42 | 4 | 0.350 | 0.967 | 0.317 | 0.033 |
| >94.58 | 42 | 3 | 0.350 | 0.975 | 0.325 | 0.025 |
| >94.77 | 41 | 3 | 0.342 | 0.975 | 0.317 | 0.025 |
| >94.86 | 40 | 3 | 0.333 | 0.975 | 0.308 | 0.025 |
| >94.92 | 39 | 3 | 0.325 | 0.975 | 0.300 | 0.025 |
| >95.02 | 38 | 3 | 0.317 | 0.975 | 0.292 | 0.025 |
| >96.02 | 37 | 3 | 0.308 | 0.975 | 0.283 | 0.025 |
| >97.38 | 37 | 2 | 0.308 | 0.983 | 0.292 | 0.017 |
| >97.66 | 36 | 2 | 0.300 | 0.983 | 0.283 | 0.017 |
| >98.45 | 35 | 2 | 0.292 | 0.983 | 0.275 | 0.017 |
| >99.09 | 34 | 2 | 0.283 | 0.983 | 0.267 | 0.017 |
| >99.23 | 33 | 2 | 0.275 | 0.983 | 0.258 | 0.017 |
| >100.65 | 32 | 2 | 0.267 | 0.983 | 0.250 | 0.017 |
| >100.72 | 31 | 2 | 0.258 | 0.983 | 0.242 | 0.017 |
| >101.06 | 30 | 2 | 0.250 | 0.983 | 0.233 | 0.017 |

**Appendix B,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >101.37 | 29 | 2 | 0.242 | 0.983 | 0.225 | 0.017 |
| >103.11 | 28 | 2 | 0.233 | 0.983 | 0.217 | 0.017 |
| >103.23 | 27 | 2 | 0.225 | 0.983 | 0.208 | 0.017 |
| >103.62 | 27 | 1 | 0.225 | 0.992 | 0.217 | 0.008 |
| >103.70 | 26 | 1 | 0.217 | 0.992 | 0.208 | 0.008 |
| >104.39 | 25 | 1 | 0.208 | 0.992 | 0.200 | 0.008 |
| >104.63 | 24 | 1 | 0.200 | 0.992 | 0.192 | 0.008 |
| >104.72 | 23 | 1 | 0.192 | 0.992 | 0.183 | 0.008 |
| >104.93 | 22 | 1 | 0.183 | 0.992 | 0.175 | 0.008 |
| >105.59 | 21 | 1 | 0.175 | 0.992 | 0.167 | 0.008 |
| >105.94 | 20 | 1 | 0.167 | 0.992 | 0.158 | 0.008 |
| >106.57 | 19 | 1 | 0.158 | 0.992 | 0.150 | 0.008 |
| >107.13 | 18 | 1 | 0.150 | 0.992 | 0.142 | 0.008 |
| >107.21 | 17 | 1 | 0.142 | 0.992 | 0.133 | 0.008 |
| >107.77 | 16 | 1 | 0.133 | 0.992 | 0.125 | 0.008 |
| >108.04 | 15 | 1 | 0.125 | 0.992 | 0.117 | 0.008 |
| >108.06 | 14 | 1 | 0.117 | 0.992 | 0.108 | 0.008 |
| >109.01 | 14 | 0 | 0.117 | 1.000 | 0.117 | 0.000 |
| >109.58 | 13 | 0 | 0.108 | 1.000 | 0.108 | 0.000 |
| >110.20 | 12 | 0 | 0.100 | 1.000 | 0.100 | 0.000 |
| >110.53 | 11 | 0 | 0.092 | 1.000 | 0.092 | 0.000 |
| >110.60 | 10 | 0 | 0.083 | 1.000 | 0.083 | 0.000 |
| >111.80 | 9 | 0 | 0.075 | 1.000 | 0.075 | 0.000 |
| >111.83 | 8 | 0 | 0.067 | 1.000 | 0.067 | 0.000 |
| >112.15 | 7 | 0 | 0.058 | 1.000 | 0.058 | 0.000 |
| >112.78 | 6 | 0 | 0.050 | 1.000 | 0.050 | 0.000 |
| >113.29 | 5 | 0 | 0.042 | 1.000 | 0.042 | 0.000 |
| >116.26 | 4 | 0 | 0.033 | 1.000 | 0.033 | 0.000 |
| >116.27 | 3 | 0 | 0.025 | 1.000 | 0.025 | 0.000 |
| >116.55 | 2 | 0 | 0.017 | 1.000 | 0.017 | 0.000 |
| >119.61 | 1 | 0 | 0.008 | 1.000 | 0.008 | 0.000 |
| >123.16 | 0 | 0 | 0.000 | 1.000 | 0.000 | 0.000 |

[a] Gray shading denotes the cutoff value, sensitivity and specificity at the highest Youden's score.

Abbreviations: ZEBRA, Z-encoded broadly reactive activator; IgG, immunoglobulin G; ROC, receiver operating characteristics; PP, percentage positivity; NPC, nasopharyngeal carcinoma.

**Appendix C.** Counting of study subjects according to VCA p18/IgG positivity (cutoff values), Youden's index and coordinates of the ROC curve.[a]

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| ≥6.41 | 120 | 120 | 1.000 | 0.000 | 0.000 | 1.000 |
| >6.41 | 120 | 119 | 1.000 | 0.008 | 0.008 | 0.992 |
| >7.87 | 120 | 118 | 1.000 | 0.017 | 0.017 | 0.983 |
| >8.03 | 120 | 117 | 1.000 | 0.025 | 0.025 | 0.975 |
| >8.13 | 120 | 116 | 1.000 | 0.033 | 0.033 | 0.967 |
| >8.84 | 120 | 115 | 1.000 | 0.042 | 0.042 | 0.958 |
| >9.05 | 120 | 114 | 1.000 | 0.050 | 0.050 | 0.950 |
| >9.37 | 120 | 113 | 1.000 | 0.058 | 0.058 | 0.942 |
| >9.83 | 120 | 112 | 1.000 | 0.067 | 0.067 | 0.933 |
| >11.36 | 120 | 111 | 1.000 | 0.075 | 0.075 | 0.925 |
| >11.80 | 120 | 110 | 1.000 | 0.083 | 0.083 | 0.917 |
| >12.38 | 120 | 109 | 1.000 | 0.092 | 0.092 | 0.908 |
| >12.46 | 120 | 108 | 1.000 | 0.100 | 0.100 | 0.900 |
| >12.62 | 119 | 108 | 0.992 | 0.100 | 0.092 | 0.900 |
| >12.76 | 119 | 107 | 0.992 | 0.108 | 0.100 | 0.892 |
| >13.18 | 119 | 106 | 0.992 | 0.117 | 0.108 | 0.883 |
| >13.57 | 118 | 106 | 0.983 | 0.117 | 0.100 | 0.883 |
| >13.67 | 117 | 106 | 0.975 | 0.117 | 0.092 | 0.883 |
| >13.89 | 117 | 105 | 0.975 | 0.125 | 0.100 | 0.875 |
| >13.95 | 117 | 104 | 0.975 | 0.133 | 0.108 | 0.867 |
| >14.67 | 117 | 103 | 0.975 | 0.142 | 0.117 | 0.858 |
| >15.42 | 117 | 102 | 0.975 | 0.150 | 0.125 | 0.850 |
| >15.55 | 117 | 101 | 0.975 | 0.158 | 0.133 | 0.842 |
| >15.99 | 117 | 100 | 0.975 | 0.167 | 0.142 | 0.833 |
| >16.21 | 117 | 99 | 0.975 | 0.175 | 0.150 | 0.825 |
| >16.26 | 117 | 98 | 0.975 | 0.183 | 0.158 | 0.817 |
| >16.30 | 117 | 97 | 0.975 | 0.192 | 0.167 | 0.808 |
| >16.70 | 117 | 96 | 0.975 | 0.200 | 0.175 | 0.800 |
| >16.93 | 117 | 95 | 0.975 | 0.208 | 0.183 | 0.792 |
| >17.48 | 117 | 94 | 0.975 | 0.217 | 0.192 | 0.783 |
| >17.76 | 117 | 93 | 0.975 | 0.225 | 0.200 | 0.775 |
| >17.81 | 117 | 92 | 0.975 | 0.233 | 0.208 | 0.767 |
| >18.04 | 117 | 91 | 0.975 | 0.242 | 0.217 | 0.758 |
| >18.05 | 117 | 90 | 0.975 | 0.250 | 0.225 | 0.750 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >18.41 | 117 | 89 | 0.975 | 0.258 | 0.233 | 0.742 |
| >19.22 | 117 | 88 | 0.975 | 0.267 | 0.242 | 0.733 |
| >19.63 | 116 | 88 | 0.967 | 0.267 | 0.233 | 0.733 |
| >19.95 | 115 | 88 | 0.958 | 0.267 | 0.225 | 0.733 |
| >20.06 | 115 | 87 | 0.958 | 0.275 | 0.233 | 0.725 |
| >20.18 | 115 | 86 | 0.958 | 0.283 | 0.242 | 0.717 |
| >20.26 | 115 | 85 | 0.958 | 0.292 | 0.250 | 0.708 |
| >20.95 | 115 | 84 | 0.958 | 0.300 | 0.258 | 0.700 |
| >21.03 | 115 | 83 | 0.958 | 0.308 | 0.267 | 0.692 |
| >21.15 | 115 | 82 | 0.958 | 0.317 | 0.275 | 0.683 |
| >21.16 | 115 | 81 | 0.958 | 0.325 | 0.283 | 0.675 |
| >21.34 | 115 | 80 | 0.958 | 0.333 | 0.292 | 0.667 |
| >21.43 | 115 | 79 | 0.958 | 0.342 | 0.300 | 0.658 |
| >21.53 | 115 | 78 | 0.958 | 0.350 | 0.308 | 0.650 |
| >21.61 | 114 | 78 | 0.950 | 0.350 | 0.300 | 0.650 |
| >22.17 | 113 | 78 | 0.942 | 0.350 | 0.292 | 0.650 |
| >22.29 | 112 | 77 | 0.933 | 0.358 | 0.292 | 0.642 |
| >23.01 | 112 | 76 | 0.933 | 0.367 | 0.300 | 0.633 |
| >23.67 | 111 | 76 | 0.925 | 0.367 | 0.292 | 0.633 |
| >24.32 | 110 | 76 | 0.917 | 0.367 | 0.283 | 0.633 |
| >25.45 | 109 | 76 | 0.908 | 0.367 | 0.275 | 0.633 |
| >25.85 | 109 | 75 | 0.908 | 0.375 | 0.283 | 0.625 |
| >26.88 | 108 | 75 | 0.900 | 0.375 | 0.275 | 0.625 |
| >27.06 | 107 | 75 | 0.892 | 0.375 | 0.267 | 0.625 |
| >27.21 | 106 | 75 | 0.883 | 0.375 | 0.258 | 0.625 |
| >27.40 | 105 | 75 | 0.875 | 0.375 | 0.250 | 0.625 |
| >27.43 | 105 | 74 | 0.875 | 0.383 | 0.258 | 0.617 |
| >27.65 | 104 | 74 | 0.867 | 0.383 | 0.250 | 0.617 |
| >28.31 | 104 | 73 | 0.867 | 0.392 | 0.258 | 0.608 |
| >28.36 | 103 | 73 | 0.858 | 0.392 | 0.250 | 0.608 |
| >28.63 | 103 | 72 | 0.858 | 0.400 | 0.258 | 0.600 |
| >29.65 | 102 | 72 | 0.850 | 0.400 | 0.250 | 0.600 |
| >30.07 | 102 | 71 | 0.850 | 0.408 | 0.258 | 0.592 |
| >30.09 | 101 | 71 | 0.842 | 0.408 | 0.250 | 0.592 |
| >30.32 | 101 | 70 | 0.842 | 0.417 | 0.258 | 0.583 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >30.43 | 100 | 70 | 0.833 | 0.417 | 0.250 | 0.583 |
| >30.44 | 100 | 69 | 0.833 | 0.425 | 0.258 | 0.575 |
| >30.79 | 99 | 69 | 0.825 | 0.425 | 0.250 | 0.575 |
| >31.29 | 98 | 69 | 0.817 | 0.425 | 0.242 | 0.575 |
| >31.29 | 97 | 69 | 0.808 | 0.425 | 0.233 | 0.575 |
| >31.77 | 96 | 69 | 0.800 | 0.425 | 0.225 | 0.575 |
| >32.19 | 96 | 68 | 0.800 | 0.433 | 0.233 | 0.567 |
| >32.23 | 95 | 68 | 0.792 | 0.433 | 0.225 | 0.567 |
| >32.34 | 95 | 67 | 0.792 | 0.442 | 0.233 | 0.558 |
| >32.41 | 95 | 66 | 0.792 | 0.450 | 0.242 | 0.550 |
| >32.49 | 94 | 66 | 0.783 | 0.450 | 0.233 | 0.550 |
| >32.70 | 94 | 65 | 0.783 | 0.458 | 0.242 | 0.542 |
| >33.12 | 94 | 64 | 0.783 | 0.467 | 0.250 | 0.533 |
| >33.33 | 93 | 64 | 0.775 | 0.467 | 0.242 | 0.533 |
| >33.37 | 93 | 63 | 0.775 | 0.475 | 0.250 | 0.525 |
| >33.43 | 92 | 63 | 0.767 | 0.475 | 0.242 | 0.525 |
| >33.54 | 92 | 62 | 0.767 | 0.483 | 0.250 | 0.517 |
| >33.97 | 92 | 61 | 0.767 | 0.492 | 0.258 | 0.508 |
| >34.08 | 92 | 60 | 0.767 | 0.500 | 0.267 | 0.500 |
| >34.37 | 92 | 59 | 0.767 | 0.508 | 0.275 | 0.492 |
| >34.37 | 92 | 58 | 0.767 | 0.517 | 0.283 | 0.483 |
| >34.56 | 92 | 57 | 0.767 | 0.525 | 0.292 | 0.475 |
| >34.67 | 92 | 56 | 0.767 | 0.533 | 0.300 | 0.467 |
| >34.71 | 91 | 56 | 0.758 | 0.533 | 0.292 | 0.467 |
| >34.76 | 91 | 55 | 0.758 | 0.542 | 0.300 | 0.458 |
| >34.90 | 90 | 55 | 0.750 | 0.542 | 0.292 | 0.458 |
| >35.01 | 89 | 55 | 0.742 | 0.542 | 0.283 | 0.458 |
| >35.84 | 88 | 55 | 0.733 | 0.542 | 0.275 | 0.458 |
| >35.98 | 87 | 55 | 0.725 | 0.542 | 0.267 | 0.458 |
| >36.45 | 86 | 55 | 0.717 | 0.542 | 0.258 | 0.458 |
| >36.46 | 85 | 55 | 0.708 | 0.542 | 0.250 | 0.458 |
| >36.82 | 84 | 55 | 0.700 | 0.542 | 0.242 | 0.458 |
| >36.98 | 83 | 55 | 0.692 | 0.542 | 0.233 | 0.458 |
| >37.08 | 82 | 55 | 0.683 | 0.542 | 0.225 | 0.458 |
| >37.12 | 82 | 54 | 0.683 | 0.550 | 0.233 | 0.450 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >37.12 | 82 | 53 | 0.683 | 0.558 | 0.242 | 0.442 |
| >37.32 | 81 | 53 | 0.675 | 0.558 | 0.233 | 0.442 |
| >37.38 | 81 | 52 | 0.675 | 0.567 | 0.242 | 0.433 |
| >37.78 | 80 | 52 | 0.667 | 0.567 | 0.233 | 0.433 |
| >38.35 | 79 | 52 | 0.658 | 0.567 | 0.225 | 0.433 |
| >39.18 | 79 | 51 | 0.658 | 0.575 | 0.233 | 0.425 |
| >39.54 | 79 | 50 | 0.658 | 0.583 | 0.242 | 0.417 |
| >39.55 | 78 | 50 | 0.650 | 0.583 | 0.233 | 0.417 |
| >39.67 | 78 | 49 | 0.650 | 0.592 | 0.242 | 0.408 |
| >40.11 | 77 | 49 | 0.642 | 0.592 | 0.233 | 0.408 |
| >40.30 | 77 | 48 | 0.642 | 0.600 | 0.242 | 0.400 |
| >40.79 | 77 | 47 | 0.642 | 0.608 | 0.250 | 0.392 |
| >40.92 | 76 | 47 | 0.633 | 0.608 | 0.242 | 0.392 |
| >40.99 | 76 | 46 | 0.633 | 0.617 | 0.250 | 0.383 |
| >41.15 | 75 | 46 | 0.625 | 0.617 | 0.242 | 0.383 |
| >41.63 | 74 | 46 | 0.617 | 0.617 | 0.233 | 0.383 |
| >41.70 | 74 | 45 | 0.617 | 0.625 | 0.242 | 0.375 |
| >41.71 | 74 | 44 | 0.617 | 0.633 | 0.250 | 0.367 |
| >42.38 | 74 | 43 | 0.617 | 0.642 | 0.258 | 0.358 |
| >43.04 | 73 | 43 | 0.608 | 0.642 | 0.250 | 0.358 |
| >43.48 | 73 | 42 | 0.608 | 0.650 | 0.258 | 0.350 |
| >43.72 | 72 | 42 | 0.600 | 0.650 | 0.250 | 0.350 |
| >44.31 | 72 | 41 | 0.600 | 0.658 | 0.258 | 0.342 |
| >45.08 | 71 | 41 | 0.592 | 0.658 | 0.250 | 0.342 |
| >45.38 | 70 | 41 | 0.583 | 0.658 | 0.242 | 0.342 |
| >45.78 | 70 | 40 | 0.583 | 0.667 | 0.250 | 0.333 |
| >45.86 | 70 | 39 | 0.583 | 0.675 | 0.258 | 0.325 |
| >45.94 | 69 | 39 | 0.575 | 0.675 | 0.250 | 0.325 |
| >46.91 | 69 | 38 | 0.575 | 0.683 | 0.258 | 0.317 |
| >47.37 | 68 | 38 | 0.567 | 0.683 | 0.250 | 0.317 |
| >47.53 | 68 | 37 | 0.567 | 0.692 | 0.258 | 0.308 |
| >48.24 | 67 | 37 | 0.558 | 0.692 | 0.250 | 0.308 |
| >48.37 | 67 | 36 | 0.558 | 0.700 | 0.258 | 0.300 |
| >49.43 | 66 | 36 | 0.550 | 0.700 | 0.250 | 0.300 |
| >49.84 | 65 | 36 | 0.542 | 0.700 | 0.242 | 0.300 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >50.83 | 65 | 35 | 0.542 | 0.708 | 0.250 | 0.292 |
| >51.18 | 64 | 35 | 0.533 | 0.708 | 0.242 | 0.292 |
| >51.33 | 63 | 35 | 0.525 | 0.708 | 0.233 | 0.292 |
| >52.09 | 63 | 34 | 0.525 | 0.717 | 0.242 | 0.283 |
| >52.29 | 63 | 33 | 0.525 | 0.725 | 0.250 | 0.275 |
| >52.47 | 62 | 33 | 0.517 | 0.725 | 0.242 | 0.275 |
| >52.57 | 62 | 32 | 0.517 | 0.733 | 0.250 | 0.267 |
| >52.73 | 62 | 31 | 0.517 | 0.742 | 0.258 | 0.258 |
| >52.79 | 61 | 31 | 0.508 | 0.742 | 0.250 | 0.258 |
| >53.19 | 61 | 30 | 0.508 | 0.750 | 0.258 | 0.250 |
| >54.22 | 61 | 29 | 0.508 | 0.758 | 0.267 | 0.242 |
| >54.49 | 61 | 28 | 0.508 | 0.767 | 0.275 | 0.233 |
| >55.03 | 60 | 28 | 0.500 | 0.767 | 0.267 | 0.233 |
| >55.12 | 59 | 28 | 0.492 | 0.767 | 0.258 | 0.233 |
| >56.30 | 58 | 28 | 0.483 | 0.767 | 0.250 | 0.233 |
| >56.39 | 58 | 27 | 0.483 | 0.775 | 0.258 | 0.225 |
| >57.85 | 58 | 26 | 0.483 | 0.783 | 0.267 | 0.217 |
| >57.92 | 57 | 26 | 0.475 | 0.783 | 0.258 | 0.217 |
| >58.02 | 56 | 26 | 0.467 | 0.783 | 0.250 | 0.217 |
| >58.37 | 55 | 26 | 0.458 | 0.783 | 0.242 | 0.217 |
| >59.02 | 54 | 26 | 0.450 | 0.783 | 0.233 | 0.217 |
| >59.41 | 53 | 26 | 0.442 | 0.783 | 0.225 | 0.217 |
| >59.44 | 52 | 26 | 0.433 | 0.783 | 0.217 | 0.217 |
| >60.04 | 51 | 26 | 0.425 | 0.783 | 0.208 | 0.217 |
| >60.34 | 50 | 26 | 0.417 | 0.783 | 0.200 | 0.217 |
| >60.81 | 49 | 26 | 0.408 | 0.783 | 0.192 | 0.217 |
| >61.16 | 49 | 25 | 0.408 | 0.792 | 0.200 | 0.208 |
| >61.44 | 49 | 24 | 0.408 | 0.800 | 0.208 | 0.200 |
| >61.58 | 49 | 23 | 0.408 | 0.808 | 0.217 | 0.192 |
| >61.91 | 48 | 23 | 0.400 | 0.808 | 0.208 | 0.192 |
| >63.07 | 47 | 23 | 0.392 | 0.808 | 0.200 | 0.192 |
| >63.11 | 46 | 23 | 0.383 | 0.808 | 0.192 | 0.192 |
| >63.28 | 46 | 22 | 0.383 | 0.817 | 0.200 | 0.183 |
| >63.51 | 46 | 21 | 0.383 | 0.825 | 0.208 | 0.175 |
| >63.77 | 45 | 21 | 0.375 | 0.825 | 0.200 | 0.175 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >63.99 | 44 | 21 | 0.367 | 0.825 | 0.192 | 0.175 |
| >64.86 | 44 | 20 | 0.367 | 0.833 | 0.200 | 0.167 |
| >65.41 | 43 | 20 | 0.358 | 0.833 | 0.192 | 0.167 |
| >65.49 | 42 | 20 | 0.350 | 0.833 | 0.183 | 0.167 |
| >66.07 | 41 | 20 | 0.342 | 0.833 | 0.175 | 0.167 |
| >66.61 | 40 | 20 | 0.333 | 0.833 | 0.167 | 0.167 |
| >67.23 | 40 | 19 | 0.333 | 0.842 | 0.175 | 0.158 |
| >67.47 | 40 | 18 | 0.333 | 0.850 | 0.183 | 0.150 |
| >67.68 | 40 | 17 | 0.333 | 0.858 | 0.192 | 0.142 |
| >68.70 | 40 | 16 | 0.333 | 0.867 | 0.200 | 0.133 |
| >69.03 | 39 | 16 | 0.325 | 0.867 | 0.192 | 0.133 |
| >69.82 | 39 | 15 | 0.325 | 0.875 | 0.200 | 0.125 |
| >69.86 | 38 | 15 | 0.317 | 0.875 | 0.192 | 0.125 |
| >70.10 | 37 | 15 | 0.308 | 0.875 | 0.183 | 0.125 |
| >70.91 | 36 | 15 | 0.300 | 0.875 | 0.175 | 0.125 |
| >70.98 | 36 | 14 | 0.300 | 0.883 | 0.183 | 0.117 |
| >71.76 | 35 | 14 | 0.292 | 0.883 | 0.175 | 0.117 |
| >73.50 | 34 | 14 | 0.283 | 0.883 | 0.167 | 0.117 |
| >73.58 | 34 | 13 | 0.283 | 0.892 | 0.175 | 0.108 |
| >73.62 | 33 | 13 | 0.275 | 0.892 | 0.167 | 0.108 |
| >74.95 | 33 | 12 | 0.275 | 0.900 | 0.175 | 0.100 |
| >75.05 | 32 | 12 | 0.267 | 0.900 | 0.167 | 0.100 |
| >75.71 | 32 | 11 | 0.267 | 0.908 | 0.175 | 0.092 |
| >75.71 | 31 | 11 | 0.258 | 0.908 | 0.167 | 0.092 |
| >75.94 | 30 | 11 | 0.250 | 0.908 | 0.158 | 0.092 |
| >76.51 | 29 | 11 | 0.242 | 0.908 | 0.150 | 0.092 |
| >76.70 | 29 | 10 | 0.242 | 0.917 | 0.158 | 0.083 |
| >76.83 | 29 | 9 | 0.242 | 0.925 | 0.167 | 0.075 |
| >77.23 | 29 | 8 | 0.242 | 0.933 | 0.175 | 0.067 |
| >78.28 | 29 | 7 | 0.242 | 0.942 | 0.183 | 0.058 |
| >78.88 | 29 | 6 | 0.242 | 0.950 | 0.192 | 0.050 |
| >78.98 | 28 | 6 | 0.233 | 0.950 | 0.183 | 0.050 |
| >79.20 | 27 | 6 | 0.225 | 0.950 | 0.175 | 0.050 |
| >80.40 | 26 | 6 | 0.217 | 0.950 | 0.167 | 0.050 |
| >80.48 | 25 | 6 | 0.208 | 0.950 | 0.158 | 0.050 |

**Appendix C,** continued.

| Cutoff value (PP) | NPC (*n*) | Normal (*n*) | Sensitivity | Specificity | Youden's index | 1 − Specificity |
|---|---|---|---|---|---|---|
| >82.71 | 24 | 6 | 0.200 | 0.950 | 0.150 | 0.050 |
| >83.55 | 23 | 6 | 0.192 | 0.950 | 0.142 | 0.050 |
| >83.94 | 22 | 6 | 0.183 | 0.950 | 0.133 | 0.050 |
| >84.27 | 22 | 5 | 0.183 | 0.958 | 0.142 | 0.042 |
| >84.83 | 21 | 5 | 0.175 | 0.958 | 0.133 | 0.042 |
| >84.98 | 21 | 4 | 0.175 | 0.967 | 0.142 | 0.033 |
| >86.18 | 20 | 4 | 0.167 | 0.967 | 0.133 | 0.033 |
| >86.51 | 20 | 3 | 0.167 | 0.975 | 0.142 | 0.025 |
| >87.25 | 19 | 3 | 0.158 | 0.975 | 0.133 | 0.025 |
| >87.38 | 18 | 3 | 0.150 | 0.975 | 0.125 | 0.025 |
| >87.68 | 17 | 3 | 0.142 | 0.975 | 0.117 | 0.025 |
| >88.14 | 16 | 3 | 0.133 | 0.975 | 0.108 | 0.025 |
| >91.35 | 16 | 2 | 0.133 | 0.983 | 0.117 | 0.017 |
| >92.03 | 15 | 2 | 0.125 | 0.983 | 0.108 | 0.017 |
| >93.15 | 14 | 2 | 0.117 | 0.983 | 0.100 | 0.017 |
| >94.16 | 13 | 2 | 0.108 | 0.983 | 0.092 | 0.017 |
| >94.72 | 12 | 2 | 0.100 | 0.983 | 0.083 | 0.017 |
| >96.24 | 11 | 2 | 0.092 | 0.983 | 0.075 | 0.017 |
| >98.00 | 11 | 1 | 0.092 | 0.992 | 0.083 | 0.008 |
| >99.42 | 11 | 0 | 0.092 | 1.000 | 0.092 | 0.000 |
| >100.66 | 10 | 0 | 0.083 | 1.000 | 0.083 | 0.000 |
| >100.69 | 9 | 0 | 0.075 | 1.000 | 0.075 | 0.000 |
| >101.00 | 8 | 0 | 0.067 | 1.000 | 0.067 | 0.000 |
| >101.03 | 7 | 0 | 0.058 | 1.000 | 0.058 | 0.000 |
| >102.71 | 6 | 0 | 0.050 | 1.000 | 0.050 | 0.000 |
| >103.43 | 5 | 0 | 0.042 | 1.000 | 0.042 | 0.000 |
| >105.23 | 4 | 0 | 0.033 | 1.000 | 0.033 | 0.000 |
| >105.29 | 3 | 0 | 0.025 | 1.000 | 0.025 | 0.000 |
| >108.07 | 2 | 0 | 0.017 | 1.000 | 0.017 | 0.000 |
| >109.30 | 1 | 0 | 0.008 | 1.000 | 0.008 | 0.000 |
| >120.47 | 0 | 0 | 0.000 | 1.000 | 0.000 | 0.000 |

[a] Gray shading denotes the cutoff value, sensitivity and specificity at the highest Youden's score.

Abbreviations: VCA, viral capsid antigen; IgG, immunoglobulin G; ROC, receiver operating characteristics; PP, percentage positivity; NPC, nasopharyngeal carcinoma.

**Appendix D.** Protein multiple sequence alignments (EA-D, ZEBRA and VCA p18) for EBV strains (B95-8, AG876 and GD1).

| EBV protein | Multiple sequence alignment[a] | | | UniProt entry | EBV strain |
|---|---|---|---|---|---|
| EA-D | 1 | METTQTLRFKTKALAVLSKCYDHAQTHLKGGVLQVNLLSVNYGGPRLAAVANAGTAGLIS | 60 | P03191 | B95-5 |
| | 1 | METTQTLRFKTKALAVLSKCYDHAQTHLKGGVLQVNLLSVNYGGPRLAAVANAGTAGLIS | 60 | P0C6Z0 | AG876 |
| | 1 | METTQTLRFKTKALAVLSKCYDHAQTHLKGGVLQVNLLSVNYGGPRLAAVANAGTAGLIS | 60 | Q3KSU3 | GD1 |
| | | ************************************************************ | | | |
| | 61 | FEVSPDAVAEWQNHQSPEEAPAAVSFRNLAYGRTCVLGKELFGSAVEQASLQFYKRPQGG | 120 | P03191 | B95-5 |
| | 61 | FEVSPDAVAEWQNHQSPEEAPAAVSFRNLAYGRTCVLGKELFGSAVEQASLQFYKRPQGG | 120 | P0C6Z0 | AG876 |
| | 61 | FEVSPDAVAEWQNHQSPEEAPAAVSFRNLAYGRTCVLGKELFGSAVEQASLQFYKRPQGG | 120 | Q3KSU3 | GD1 |
| | | ************************************************************ | | | |
| | 121 | SRPEFVKLTMEYDDKVSKSHHTCALMPYMPPASDRLRNEQMIGQVLLMPKTASSLQKWAR | 180 | P03191 | B95-5 |
| | 121 | SRPEFVKLTMEYDDKVSKSHHTCALMPYMPPASDRLRNEQMIGQVLLMPKTASSLQKWAR | 180 | P0C6Z0 | AG876 |
| | 121 | SRPEFVKLTMEYDDKVSKSHHTCALMPYMPPASDRLRNEQMIGQVLLMPKTASSLQKWAR | 180 | Q3KSU3 | GD1 |
| | | ************************************************************ | | | |
| | 181 | QQGSGGVKVTLNPDLYVTTYTSGEACLTLDYKPLSVGPYEAFTGPVAKAQDVGAVEAHVV | 240 | P03191 | B95-5 |
| | 181 | QQGSGGVKVTLNPDLYVTTYTSGEACLTLDYKPLSVGPYEAFTGPVAKAQDVGAVEAHVV | 240 | P0C6Z0 | AG876 |
| | 181 | QQGSGGVKVTLNPDLYVTTYTSGEACLTLDYKPLSVGPYEAFTGPVAKAQDSGAVEAHVV | 240 | Q3KSU3 | GD1 |
| | | ************************************************** ******** | | | |
| | 241 | CSVAADSLAAALSLCRIPAVSVPILRFYRSGIIAVVAGLLTSAGDLPLDLSVILFNHASE | 300 | P03191 | B95-5 |
| | 241 | CSVAADSLAAALSLCRIPAVSVPILRFYRSGIIAVVAGLLTSAGDLPLDLSVILFNHASE | 300 | P0C6Z0 | AG876 |
| | 241 | CSVAADSLAAALSLCRIPAVSVPILRFYRSGIIAVVAGLLTSAGDLPLDLSVILFNHASE | 300 | Q3KSU3 | GD1 |
| | | ************************************************************ | | | |
| | 301 | EAAASTASEPEDKSPRVQPLGTGLQQRPRHTVSPSPSPPPPPRTPTWESPARPETPSPAI | 360 | P03191 | B95-5 |
| | 301 | EAAASTASEPEDKSPRVQPLGTGLQQRPRHTVSPSPSPPPPPRTPTWESPARPETPSPAI | 360 | P0C6Z0 | AG876 |
| | 301 | EAAASTASEPEDKSPRVQPLGTGLQQRPRHTVSPSPSPPPPPRTPTWESPARPETPSPAI | 360 | Q3KSU3 | GD1 |
| | | ************************************************************ | | | |
| | 361 | PSHSSNTALERPLAVQLARKRTSSEARQKQKHPKKVKQAFNPLI 404 | | P03191 | B95-5 |
| | 361 | PSHSSNTALERPLAVQLARKRTSSEARQKQKHPKKVKQAFNPLI 404 | | P0C6Z0 | AG876 |
| | 361 | PSHSSNTALERPLAVQLARKRTSSEARQKQKHPKKVKQAFNPLI 404 | | Q3KSU3 | GD1 |
| | | ******************************************** | | | |

**Appendix D,** continued.

| EBV protein | Multiple sequence alignment[a] | UniProt entry | EBV strain |
|---|---|---|---|
| ZEBRA | 1 MMDPNSTSEDVKFTPDPYQVPFVQAFDQATRVYQDLGGPSQAPLPCVLWPVLPEPLPQGQ 60<br>1 MMDPNSTSEDVKFTPDPYQVPFVQAFDQATRVYQDLGGPSQAPLPCVLWPVLPEPLPQGQ 60<br>1 MMDPNSTSEDVKFTPDPYQVPFVQAFDQATRVYQDLGGPSQAPLPCVLWPVLPEPLPQGQ 60<br>********** | P03206<br>Q1HVG1<br>Q3KSS8 | B95-5<br>AG876<br>GD1 |
| | 61 LTAYHVSTAPTGSWFSAPQPAPENAYQAYAAPQLFPVSDITQNQQTNQAGGEAPQPGDNS 120<br>61 LTAYHVSAAPTGSWFPAPQPAPENAYQAYAAPQLFPVSDITQNQQTNQAGGEAPQPGDNS 120<br>61 LTAYHVSAAPTGSWFPAPQPAPENAYQAYAAPQLFPVSDITQNQLTNQAGGEAPQPGDNS 120<br>*******:******* ********** ********** | P03206<br>Q1HVG1<br>Q3KSS8 | B95-5<br>AG876<br>GD1 |
| | 121 TVQTAAAVVFACPGANQGQQLADIGVPQPAPVAAPARRTRKPQQPESLEECDSELEIKRY 180<br>121 TVQPAAAVVFACPGANQGQQLADIGAPQPAPAAAPARRTRKPLQPESLEECDSELDIKRY 180<br>121 TVQPAAAVVLACPGANQEQQLADIGAPQPAPAAAPARRTRKPLQPESLEECDSELEIKRY 180<br>*** *****:******* *******.*****.********** ***********:**** | P03206<br>Q1HVG1<br>Q3KSS8 | B95-5<br>AG876<br>GD1 |
| | 181 KNRVASRKCRAKFKQLLQHYREVAAAKSSENDRLRLLLKQMCPSLDVDSIIPRTPDVLHE 240<br>181 KNRVASRKCRAKFKHLLQHYREVASAKSSENDRLRLLLKQMCPSLDVDSIIPRTPDVLHE 240<br>181 KNRVASRKCRAKFKHLLQHYREVASAKSSENDRLRLLLKQMCPSLDVDSIIPRTPDVLHE 240<br>**********:*********:********** | P03206<br>Q1HVG1<br>Q3KSS8 | B95-5<br>AG876<br>GD1 |
| | 241 DLLNF 245<br>241 DLLNF 245<br>241 DLLNF 245<br>***** | P03206<br>Q1HVG1<br>Q3KSS8 | B95-5<br>AG876<br>GD1 |

**Appendix D,** continued.

| EBV protein | Multiple sequence alignment[a] | | | | UniProt entry | EBV strain |
|---|---|---|---|---|---|---|
| VCA p18 | 1 | MARRLPKPTLQGRLEADFPDSPLLPKFQELNQNNLPNDVFREAQRSYLVFLTSQFCYEEY | 60 | | P14348 | B95-5 |
| | 1 | MARRLPKPTLQGRLEADFPDSPLLPKFQELNQNNLPNDVFREAQRSYLVFLTSQFCYEEY | 60 | | Q1HVI0 | AG876 |
| | 1 | MARRLPKPTLQGRLEADFPDSPLLPKFQELNQNNLPNDVFREAQRSYLVFLTSQFCYEEY | 60 | | Q3KSU9 | GD1 |
| | | ************************************************************ | | | | |
| | 61 | VQRTFGVPRRQRAIDKRQRASVAGAGAHAHLGGSSATPVQQAQAAASAGTGALASSAPST | 120 | | P14348 | B95-5 |
| | 61 | VQRTFGVPRRQRAIDKRQRASVAGAGAHAHLGGSSATPVQQAQAAASAGTGALASSAPST | 120 | | Q1HVI0 | AG876 |
| | 61 | VQRTFGVPRRQRAIDKRQRASVAGAGAHAHLGGSSATPVQQAQAAASAGTGALASSAPST | 120 | | Q3KSU9 | GD1 |
| | | ************************************************************ | | | | |
| | 121 | AVAQSATPSVSSSISSLRAATSGATAAASAAAAVDTGSGGGGQPHDTAPRGARKKQ | 176 | | P14348 | B95-5 |
| | 121 | AVAQSATPSVSSSISSLRAATSGATAAASAAAAVDTGSGGGGQPQDTAPRGARKKQ | 176 | | Q1HVI0 | AG876 |
| | 121 | AVAQSATPSVSSSISSLRAATSGATAAASAAAAVDTGSGGGGQPQDTAPRGARKKQ | 176 | | Q3KSU9 | GD1 |
| | | ******************************************:*********** | | | | |

[a] Clustal Omega (Sievers et al., 2011) at UniProt (http://www.uniprot.org) was used. Gray shading and asterisk (*), fully conserved residue; colon (:), highly similar residue; period (.), weakly similar residue.

Abbreviations: EBV, Epstein-Barr virus; EA-D, early antigen-diffuse; ZEBRA, Z-encoded broadly reactive activator; VCA, viral capsid antigen.