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Vorstand: Prof.Dr.Dr.h.c. Ulrich Koszinowski

Proteome-wide production of monoclonal antibodies and study of intracellular localisation for Varicella-zoster virus (VZV)

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Venkata Raveendra Pothineni

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Berichterstatter:	Prof.Dr. Jürgen Haas
Mitberichterstatter:	Priv.Doz.Dr. Jürgen Schauber
	Prof.Dr. Hans-Walter Pfister
Dekan:	Prof.Dr.med.Dr.h.c M. Reiser, FACR, FRCR
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With love and gratitude,

Dedicated to my parents

1. Zusammenfassung

Das Varicellazoster-Virus (VZV) gehört zur Unterfamilie der Alphaherpesviren und ist mit einem Genom, das für nur 70 Proteine kodiert, das kleinste aller humanpathogenen Herpesviren. Die primäre Infektion etabliert sich meist bei Kindern als Varizellen bzw. Windpocken. Infolge dessen erreicht das Virus Ganglien sensorischer Nerven, wo es latent verbleibt. Bei Reaktivierung kommt es vor allem bei Erwachsenen zu einer Sekundärerkrankung, dem sogenannten Herpes Zoster. Mangels zellfreien Virus in Zellkultur, Virus-spezifischer Werkzeuge und eines effektiven Tiermodells gehört VZV zu den am wenigsten untersuchten humanen Herpesviren. Zahlreiche Aspekte des VZV-Replikationszyklus, der Latenz und der Reaktivierung sind deshalb wenig charakterisiert. Ausserdem konnte die Funktion vieler VZV-spezifischer Proteine bisher nicht identifiziert werden.

Das Ziel dieses Ansatzes war es, Hybridoma-Zelllinien als permanente Quelle von VZV-spezifischen Antikörpern zu etablieren und mit den gewonnenen Antikörpern die Lokalisierung von VZV-Proteinen im Viruskontext proteomweit zu untersuchen. Dazu wurde mittels der Gateway® Rekombinationstechnologie eine ORFeom-weite Entry-Bibliothek aller VZV-Gene hergestellt. Zur Proteinexpression in *E. coli* wurde die Entry-Bibliothek in vier verschiedene Expressionsvektoren der pET-Reihe umkloniert, die entweder für ein N-terminales His6-, ein C-terminales His6-, ein N-terminales MBP- oder ein N-terminales GST-Tag kodieren. Mäuse wurden mit 64 erfolgreich gereinigten VZV-Proteinen immunisiert und anschliessend zur Hybridomaherstellung verwendet. Die Klonkollektion besteht derzeit aus 218 Mutterklonen, die Antikörper gegen 61 (87%) VZV-Proteine produzieren. Dabei waren 190 Klone, die insgesamt 57 VZV-Proteine erkennen, im Westernblot reaktiv, während 123 gegen 52 VZV-Proteine gerichtete Antikörper in der Immunfluoreszenz positiv waren.

Mit Hilfe dieser neuen Antikörper-Kollektion konnte die Lokalisierung von 52 (74%) Proteinen, 22 davon zum ersten Mal, im Kontext der VZV-Infektion untersucht werden. Insgesamt waren 20 ORFs im Zellnukleus, 16 ORFs im Cytoplasma und 16 ORFs in beiden Kompartimenten lokalisiert. Der Vergleich von 41 Core-Orthologen, die sowohl in HSV-1, VZV, CMV, EBV als auch KSHV vorkommen, hat eine ausgezeichnete Űbereinstimmung in der Lokalisierung von konservierten Glykoproteinen, Capsid- und Tegumentproteinen gezeigt. Mittels der Pepscan-Methode konnten auf den viralen Glykoproteinen gK, gB, gL, gI, gE und dem

membran-assoziierten Phosphoprotein ORF24 einige immundominante Regionen identifiziert werden. Diese kostbare Antikörper-Kollektion kann in mehreren methodischen Ansätzen genutzt werden und wird es ermöglichen, biologischen Rätseln im Bereich der Herpesvirologie auf die Spur zu kommen.

1. Summary

Varicella zoster virus (VZV) is a member of the alphaherpesvirus subfamily and with a genome encoding 70 proteins the smallest of all human herpesviruses. Upon primary infection it causes varicella also called chickenpox in children. As a consequence, it reaches sensory nerve ganglia where latency is established. Upon reactivation it causes a secondary disease called Herpes zoster mostly in adults. Todate, VZV is the least studied human herpesvirus due to the lack of cell-free virus in culture, of virus-specific tools and an effective animal model. Therefore, many aspects of the VZV infection cycle, of latency and reactivation are poorly characterized. Moreover, the function of many proteins specific to VZV has not been identified.

The goal of this research was to generate hybridoma clones as a permanent source of VZV specific antibodies and to use the antibodies produced to study the localisation of VZV proteins in the viral context on a proteome-wide level. To this end, a VZV ORFeome entry library was constructed using the Gateway® recombinational cloning technology. For VZV protein expression in E. coli, the entry library was subcloned into four different pET derived expression vectors providing either an N-terminal His6, a C-terminal His6, an N-terminal MBP, or an N-terminal GST tag. Following purification of 64 VZV proteins, mice were immunised and subsequently used to generate antibody producing hybridoma clones. So far, our clone collection contains 218 mother clones producing antibodies to 61 (87%) VZV proteins. In this clone collection 190 clones were identified as positive in Western blotting covering 57 VZV ORFs while 123 antibodies were tested positive in immunofluorescence covering 52 VZV ORFs.

Using this novel antibody collection, the localisation of 52 (74%) proteins could be determined in the context of VZV infection 22 of which were analysed for the first time. In total, 20 ORFs were localised in the nucleus, 16 ORFs were present in the cytoplasm and 16 ORFs were found in both the nucleus and cytoplasm. Comparison of 41 core proteins present in HSV-1, VZV, CMV, EBV as well as KSHV showed excellent agreement in localisation of conserved glycoproteins, capsid and tegument proteins. Several immunodominant regions on the viral glycoproteins gK, gB, gL, gI, gE and the membrane associated phosphoprotein ORF24 were identified using the pepscan technique. This precious antibody collection gives access to various

experimental approaches and will allow to unveil biological secrets in the field of Herpesvirology.

2. Introduction

2.1 Herpesviridae

Herpesviruses are linear double-stranded (ds) DNA viruses that cause diseases in animals, including humans. The family name is derived from the greek word herpein ("to creep"), referring to latency, which is the characteristic of the herpes family [(1)]. To date, nearly 130 herpesviruses were identified. The genome organization and sequence as well as the architecture of the virion determines the membership of the *Herpesviridae* family. The size of the herpesvirions varies from 120 to 300 nm depending on the variability of tegument thickness and immune modulation. The structural components of the virions comprise the core, the capsid, the tegument and the envelope. The capsid measures 100 to 110 nm in diameter with an icosahedral symmetry and contains 162 capsomers. It surrounds the core which contains the dsDNA in the form of a torus (Figure 1). The envelope is a host-derived lipid membrane containing viral glycoproteins with a trilaminar appearance. It contains numerous protrusions of spikes made up of glycoproteins. The tegument is the structure between capsid and envelope. It consists of structural and transcriptional regulatory proteins.

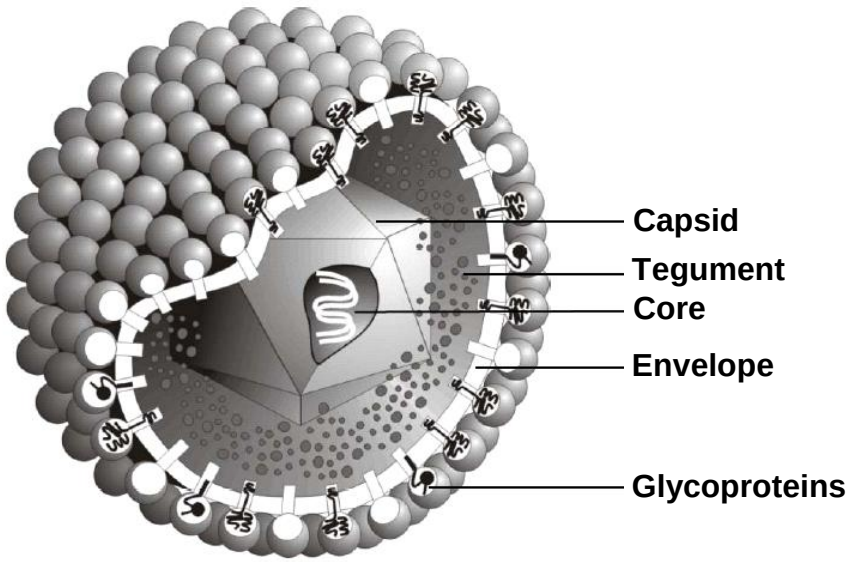


Figure 1: The herpesvirus particle consists of an outer envelope, tegument, capsid and core. (http://www.virology.net/Big_Virology/BVDNAherpes.html)

Based on their biological properties and genome sequencing the *Herpesviridae* family was classified into three subfamilies (Table 1): *Alphaherpesvirinae, Betaherpesvirinae*, and the *Gammaherpesvirinae* [(1)]. *Alphaherpesvirinae* are characterized by a variable host range, a relatively short reproductive cycle, the efficient destruction of infected cells, rapid spreading in culture and the capacity to establish latency primarily but not exclusively in sensory ganglia. *Betaherpesvirinae* are classified based on a restricted host range, a long reproductive cycle and a slow progression of infection in culture. Latency is maintained in secretory glands, lymphoreticular cells, kidneys, and other tissues. This subfamily is classified into three genera, the Cytomegaloviruses, Muromegaloviruses, and Roseoloviruses. The *Gammaherpesvirinae* show a host range restricted to the family or order to which the natural host belongs. *In vitro* they replicate in lymphoblastoid cells. *Gammaherpesvirinae* exhibit specificity for either T or B lymphocytes, and latency is maintained in lymphoid tissue. This subfamily is divided into two genera, the Lymphocryptoviruses and Rhadinoviruses [(2)].

Table 1: Members and classification of Human Herpesviruses

Species	Sub-family	Site of latency	Pathophysiology
HHV-1 Herpes simplex virus-1	α	Neurons	Gingivostomatitis, conjunctivitis, keratitis, encephalitis
HHV-2 Herpes simplex virus-2	α	Neurons	Herpes genitalis, Herpes neonatorum, a.o.
HHV-3 / VZV Varicella zoster virus	α	Neurons	Varicella, Herpes zoster
HHV-4 Epstein-Barr virus	γ	B cells	Congenital infection, infection in immuno-suppressed individuals
HHV-5 Cytomegalovirus	ß	Monocytes, Lymphocytes	Roseola infantum
HHV-6 Roseolovirus	ß	T cell	no clear evidence for a direct involvement of HHV-7 in any human disease
HHV-7 Roseolovirus	ß	T cell	infectious mononucleosis, Burkitt's lymphoma, nasopharyngeal carcinoma and others
HHV-8 Kaposi's sarcoma-associated herpesvirus	γ	B cell	Kaposi's sarcoma, primary effusion lymphoma (PEL), multicentric Castleman's diseases (MCD)

2.2 Varicella-zoster virus (VZV)

2.2.1 Disease association

Varicella-zoster virus is a dsDNA virus, member of the alphaherpesvirus subfamily and the only varicellovirus that can infect humans. It causes a primary disease called varicella (chickenpox). Upon reactivation from latency it causes a secondary disease called zoster (shingles) [3]. Weller and colleagues proved from *in vitro* studies that chickenpox and shingles were both caused by VZV. In the course of infection, VZV exhibits cell tropism to three major cell types, peripheral blood mononuclear cells (PMBC), skin cells, and sensory nerve ganglions [3].

2.2.2 Varicella (chickenpox)

The term "chickenpox" might originate from the old English word "gicin" which means itching. The other version of chickenpox might be the anglicization of the old French word "chiche-pois" for chickpea, due to the similarity in size of seeds to the chickenpox lesions [4]. In 1767, William Heberden, an English physician was the first to differentiate chickenpox from smallpox [5]. By isolating virus from cell cultures, Thomas H. Weller proved that chickenpox and shingles were both caused by VZV. In temperate regions >90% of individuals were infected with VZV by 18 years of age whereas in tropical regions less than 60% of adults are immune. Varicella illness begins with a rash, low-grade fever, and malaise in young children (Figure 2). In older children and adults prodromal symptoms like headache, myalgia, anorexia, nausea and vomiting are followed by the eruption of a rash (exanthema). The rash first starts on the trunk of the face, develops in crops, and spreads to the skin surface [6,7].

Complicatians of varicella more often occur in immunocompromised people, pregnant women, and also increases with age. The most common complications of varicella were secondary bacterial infections with *S. aureus*, or GroupA Streptococci, which cause skin and soft tissue infections, osteomyelitis, septicaemia or toxic shock syndrome. Central nervous system complications which precede varicella were cerebellar ataxia, encephalitis, aseptic meningitis and transverse myelitis [8]. Mostly in adults varicella causes pneumonia (only in 30% of patients).

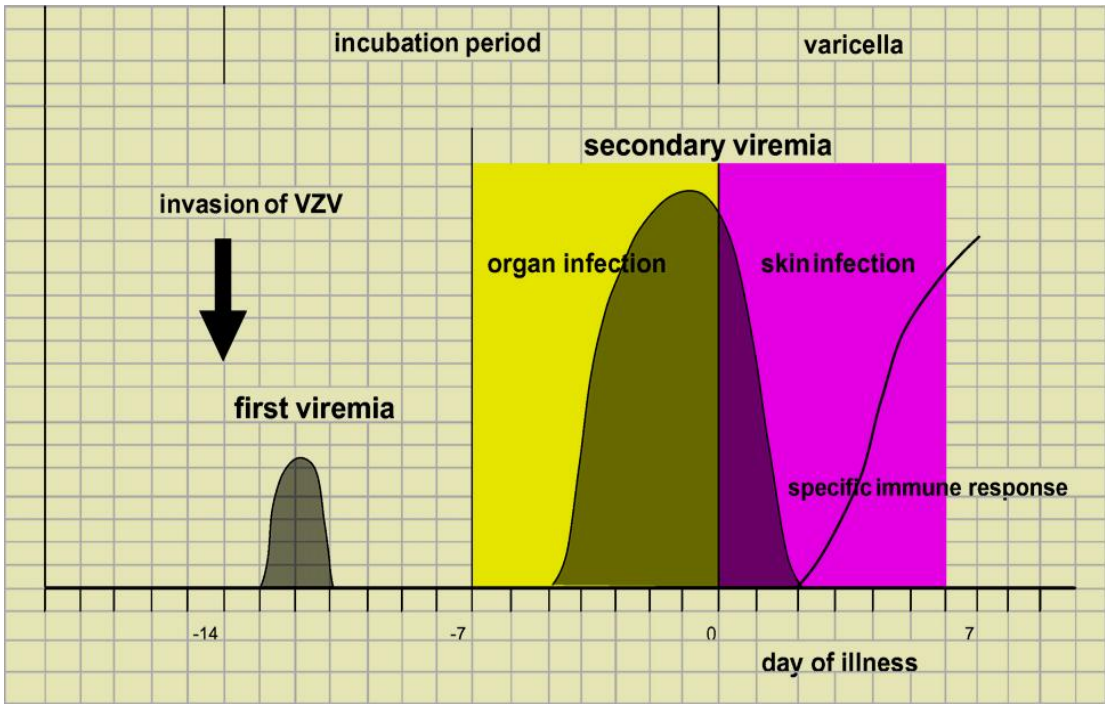


Figure 2: Varicella replication between invasion of VZV and clinical signs of varicella [9].

2.2.2.1 Congenital Varicella

Transfection of varicella to fetuses causes congenital varicella syndrome (CVS) and occurs mostly before week 20 of the gestation period. The risk of CVS is lower between 0 to 12 weeks (0.55%) and it is higher between weeks 13 to 20 (1.4%) [10,11]. It is characterised by scarring skin lesions, hypoplasia, low birth weight, damage to the eyes, neurological disorders and retarded psychomotor development [12].

2.2.2.2 Neonatal Varicella

In 1978, Hubbard for the first time reported on congenital varicella. It can be expected if a mother contracts chickenpox during the last 3 weeks of pregnancy [13]. If the mother develops a varicella rash between 5 days before birth to 2 days after birth then the risk for congenital varicella might be 20 to 50% and the mortality rate is 20%. The fatality is due to the lack of acquired protective antibodies and insufficient cell-mediated immunity. Neonatal varicella is not fatal for fetuses exposed between 20 and 6 days before delivery [14].

2.2.3 Herpes Zoster (shingles)

The Herpes zoster (HZ) occurs by reactivation of latent VZV existing in the dorsal root ganglia (Figure 2). HZ most frequently occurs in the thoracic or lumbar nerve

segments (T3-L2) and the distribution area of the trigeminal nerve (V1-3) [12]. The risk of HZ increases with advancing age at least partly caused by immunosenescence, the gradual deterioration of the immune system due to natural aging and also decline of cell mediated immunity. In addition, immunocompromised patients who have undergone organ transplantation, chemotherapy for cancer, individuals with HIV / AIDS are at greater risk for developing HZ [15].

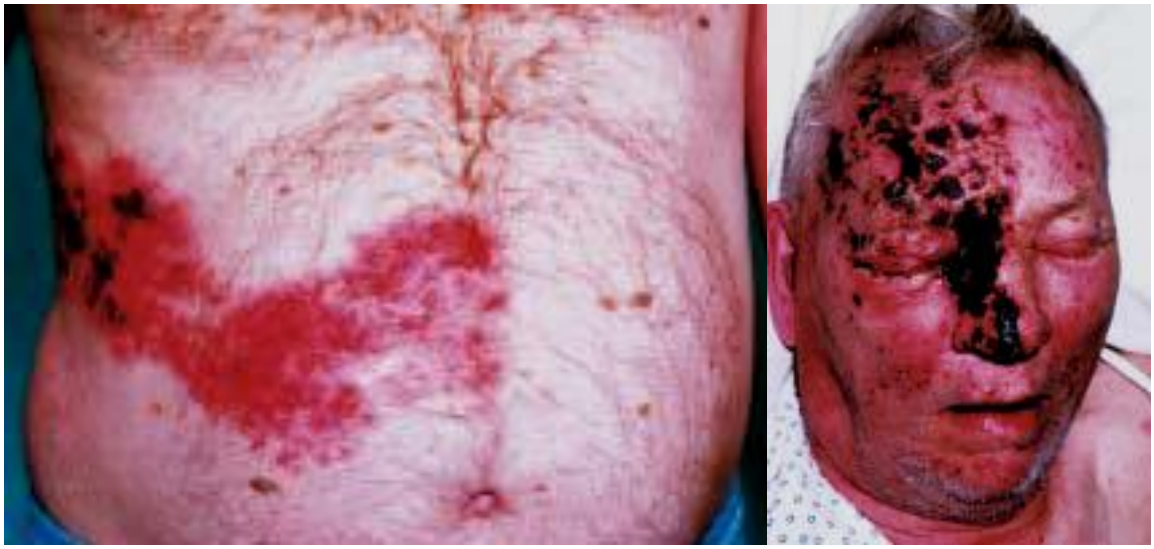
In Hope-Simpson's paper on HZ epidemiology the overall annual incidence of HZ was 3.4 per 1000 person / year. It increases with age and the incidence of HZ is greater in females compared to males. The incidence of HZ increased from 2.4 per 1000 persons / year in people of age 40 to 49 years to 5.6 per 1000 persons / year in those of age 50 to 59, 6.8 per 1000 persons / year in 60 to 69 year olds, 7.2 per 1000 persons / year in 70 to 79 year olds and 11.0 per 1000 persons / year in people above 80 years of age. The estimation of the current incidence rate of HZ shows that approximately 1 million cases of HZ occur every year in the USA (for 300 million population), 1.7 million in the European Union (for 460 million population), 100,000 in Canada (for 32 million population), 80,000 in Australia and New Zealand (for 24 million population) [16].

The clinical manifestation of HZ starts with a prodromal phase. Patients were reported with symptoms of headache, radicular pain, malaise, and acute photophobia before rash appears. An acute phase follows the prodromal phase characterised by a unilateral dermatomal rash not crossing the midline of the body. The rash is followed by unbearable itching and allodynia (pain provoked by innocuous stimuli). Some patients experience allodynia, pain, itching and burning without rash, the condition is known as zoster sine herpete. The rash appears as erythematous and maculopapular initially, turns to coalescing clusters of clear vesicles containing high concentration of VZV. The rash lasts 7 to 10 days and fully heals within 2 to 4 weeks (Figure 3) [17,18,19]. The common complications during HZ are postherpetic neuralgia (PHN), zoster ophthalmicus, vasculopathy, myelitis, retinal necrosis, meningitis and encephalitis.

2.2.3.1 Postherpetic neuralgia

PHN occurs in 10 to 20% of HZ patients and the risk of PHN is 50% if the patients are above 50 years of age [12]. PHN is an intractable pain occurring in a dermatomal distribution which can persist for months to several years. It is defined as pain

persisting more than 4 to 6 weeks after the rash resolves, and is the most frequent neurologic complication of zoster. PHN patients have a central area of cutaneous scarring and sensory loss surrounded by an area of hypersensitivity and allodynia. Allodynia is present in nearly 70% of PHN patients and patients with allodynia experience severe pain after a light, normally innocuous, touch of the affected skin by things as trivial as cold wind or a piece of cloth [16,12]. Oakleander and Rowbotham along with their colleagues have shown a greater loss of small cutaneous nerves from the skin biopsy of zoster patients experiencing PHN [20]. Neuronal loss, scarring in the region of sensory ganglions and the spinal dorsal horn corresponding to the area of the affected skin are observed in individuals with prolonged PHN. Many of the sensory abnormalities that characterise PHN are due to death of primary neurons involved in sensory ganglions and secondary neurons in the corresponding dorsal horn of the spinal cord.



a) **b)**

Figure 3: Herpes zoster. a) The classic skin findings of herpes zoster are grouped vesicles on a red base in a unilateral, dermatomal distribution. b) The progression of herpes zoster lesions through stages, beginning as red macules and papules, finally evolve into vesicles and form pustules and crusts. A common site for the distribution of ophthalmic division is the trigeminal nerve (taken from [21]).

2.2.4 Latency

After primany infection, VZV becomes latent in the trigeminal and dorsal root ganglia (DRG). In 1965, Hope-Simpson originally proposed that during varicella infection a viraemia disseminates VZV to the skin. The virus then reaches to sensory nerve ganglia and establishes latency which may subsequently reactivate to cause zoster [22]. Although the molecular mechanism for reactivation from latency is unknown, VZV

reactivation can occur spontaneously or by triggering factors like infection, immunosuppression, trauma, x-irradiation and malignancy. Reactivation is more common in elderly people and immunosuppressed individuals such as those with HIV infection [23,22].

In 1995, Dueland and collegues combined VZV *in situ* amplification with *in situ hybridization* (ISH) to report the presence of latent VZV DNA exclusively in the cytoplasm of neurons in the trigeminal ganglia (TG) [24]. By using ISH, Lungu *et al.* were the first to report the presence of latent VZV in both neuronal and non-neuronal cells like satellite cells [25]. This was further clarified by Kennedy *et al.* combining molecular techniques like PCR, FISH, and *in situ* DNA amplification by PCR on 30 human TG. This showed that latent VZV was located predominantly in the nuclei of 2 to 5% of neurons and less than 0.1% of non-neuronal satellite cells [22]. VZV does not contain any homologues of the HSV-1 LAT genes. During latency in sensory nerve ganglia, VZV gene expression is restricted to expression of a limited number of genes. So far transcripts and translates of ORF4, ORF21, ORF29, ORF62, ORF63 were identified in latently infected human trigeminal ganglia. The proteins of the mentioned ORFs were localised in the cytoplasm of latently infected neurons of dorsal root ganglia which is contrasting with the localisation of these proteins during the lytic cycle. In the presence of ORF62, a transactivator protein, some of the VZV latently expressed proteins may restrict regulatory proteins from entering the nucleus [26]. Although many of these VZV genes were expressed during latency, only transcripts mapping to gene 63 were consistently detected in latently infected TG, a suggested hallmark of VZV latency [27].

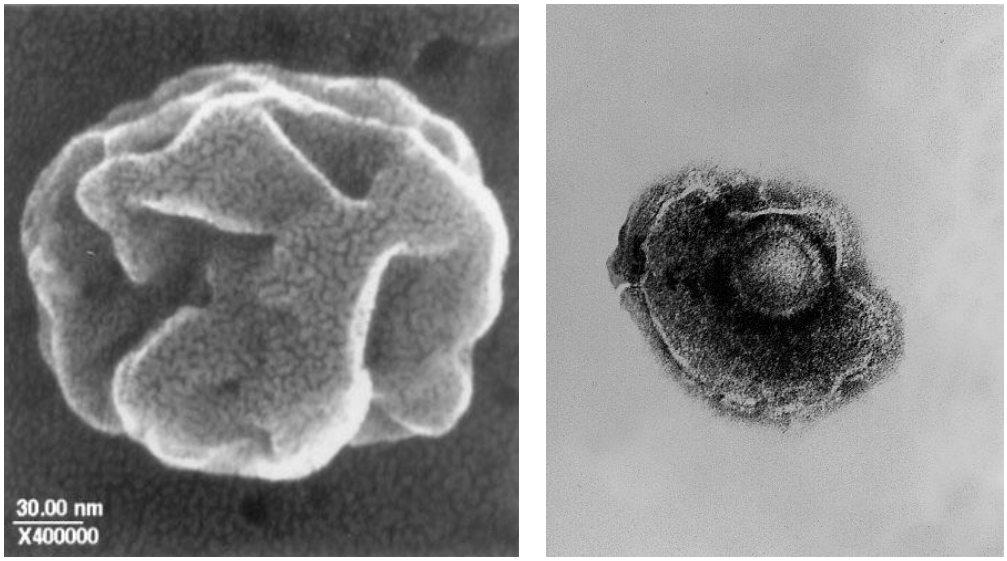
2.2.5 Virus Particle

The VZV virion is pleomorphic to spherical in shape and measures approximately 180 to 200 nm in diameter [1]. The electron-dense core contains one copy of the linear double-stranded DNA genome. The genome of VZV is wound tightly as a toroid around a dense protein rod which is similar to that observed in HSV-1. But this has been questioned by recent findings since virion particles fixed for electron microscopy with non-dehydrating agents show a more loose fibrillar cage of strands surrounding a dense cylindrical core of DNA fibres [28].

The VZV nucleocapsid is indistinguishable in appearance with respect to other herpesviruses. Electron microscopy studies revealed that the VZV nucleocapsid

contains 162 capsomers arranged like an icosahedron with a 5:3:2 axial symmetry and measuring 80 to 120 nm. The pentameric proteins are located at each of the 12 vertices and hexameric elements comprise its facets [29]. The predicted ORFs that form the structural components of nucleocapsids are ORFs 20, 23, 33, 33.5, 40, and 41 which are the orthologues of the HSV-1 capsid proteins VP19C (UL38), VP26 (UL35), VP24 (UL26), VP22 (UL26.5), VP5 (UL19), and VP23 (UL18), respectively (Figure 4).

The tegument of VZV contains immediate-early proteins like ORF4, 62, 63 and also ORFs 10, 47, and 66. While transported along various membranes, the nucleocapsid assembles at host cellular membranes thus modified to display virally encoded glycoproteins. Nucleocapsids acquire a primary envelope in patches from nuclear membrane, and a secondary one from the Golgi apparatus or other cytoplasmic membranes, so most likely no singular source of the VZV envelope exists [30].



a) **b)**

Figure 4: Varicella zoster virions. a**)** High resolution image of VZV at 400,000x magnification. The VZV envelopes were covered by small protrusions [31]. b) Electronmicrograph of enveloped particle. (http://en.wikipedia.org/wiki/File:Varicella_%28Chickenpox%29_Virus_PHIL_1878_lores.jpg)

2.3 Genome Organisation of VZV

In 1986, Davidson and Scott determined the complete nucleotide sequence of the VZV genome (strain Dumas) [32]. It is a linear double-standard DNA molecule of approximately 125 kbp encoding 71 unique viral proteins. It consists of two coding

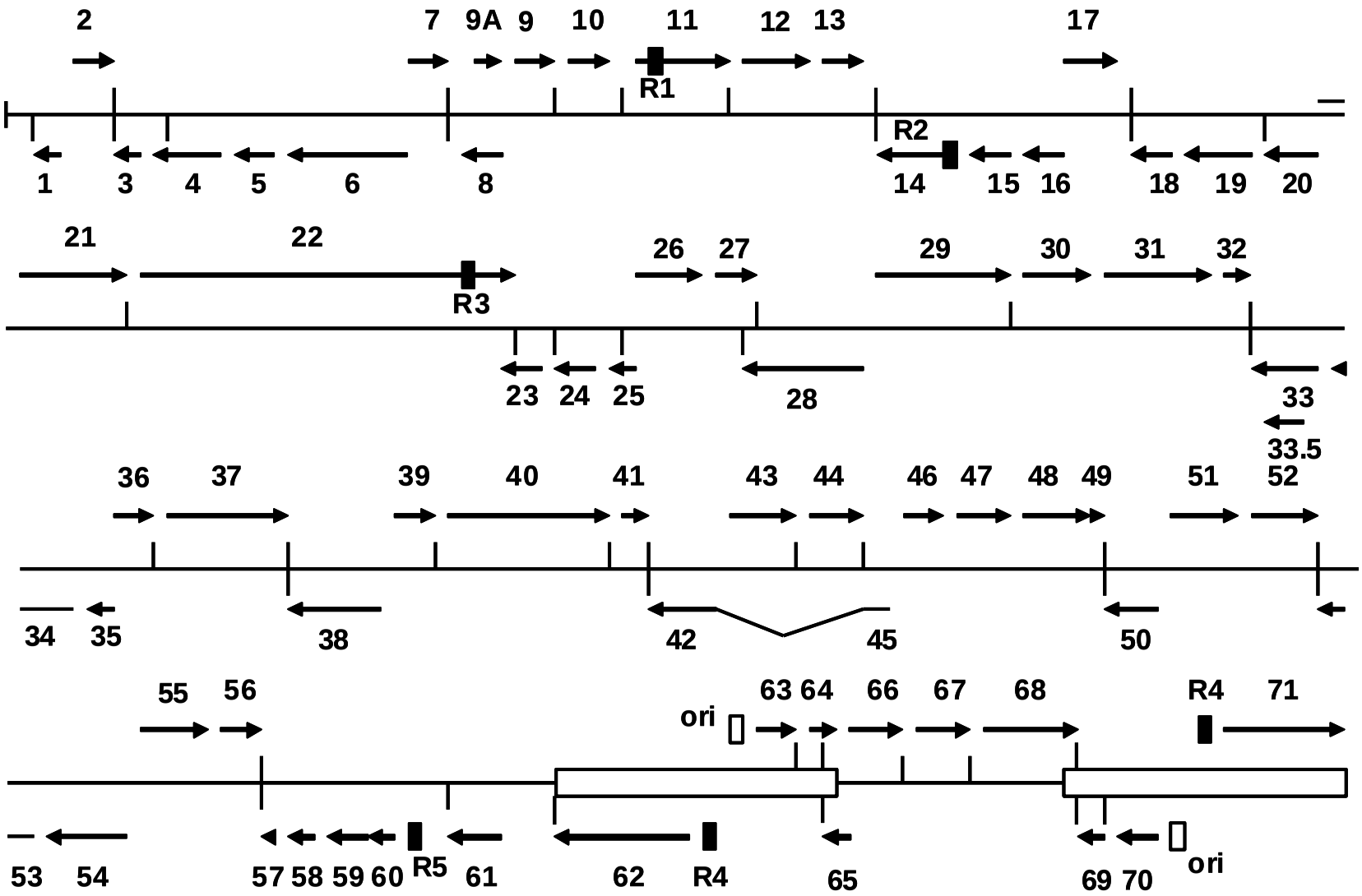


Figure 5: Organisation of the VZV genome. The VZV genome contains 70 unique ORFs not counting ORFs 69 through 71, which are duplications of ORF62, ORF63 and ORF64. The VZV genome contains unique regions (horizontal lines), terminal and internal repeats flanking the unique short region (open boxes). ORF transcripts are numbered and depicted by arrows and potential polyadenylation sites were shown by vertical lines. Repeats R1 to R5 are shown in filled rectangles. The origins of replication (ori) are indicated (unfilled rectangles). ORF S/L is not shown. [(1)]

regions: the unique long region (UL, about 105,000 bp) and unique short region (US, 5,232 bp), both flanked by the internal repeat regions (IRL, IRS) and terminal repeat regions (TRL, TRS). Three ORFs (ORF63/70, ORF62/71, ORF64/69) are located within the IRS and TRS, and are therefore duplicated within the viral genome (Figure 5).

By using electron microscopy the inverted repeat structure of a VZV genome was verified. In total, 46% of the VZV genome is composed of Guanine and Cytosine (GC). The UL and US regions contain 43 to 44% of GC, whereas the GC content of the TRS and IRS regions is 59% and that of the TRL and IRL regions is 68%[(1)]. Usually, the VZV genome is present as a linear molecule in virions but in rare cases it may also exist as a circular, supercoiled form. The genome of VZV can exist in four different isomeric forms while two forms predominate [(33)]. Both ends of the VZV genome contain a single unpaired nucleotide, Cytosine (C) at the left end and Guanine (G) at the right end. Due to complementarity they make basepairs to form a circular molecule [(34)]. The genome consists of five regions with direct sequence repeats, termed R1 to R5. Due to the difference in lengths, R1 to R5 is being used to distinguish different VZV strains by restriction endonuclease analysis. So, due to the variations in number of repeats, the genome size of different VZV strains has different lengths [(35)].

Though some of the genes have putative polyadenylation signals shortly after the end of their ORF, many are arranged in unidirectional families of up to four genes with single putative polyadenylation signals at the 3' end of the family. For 11 VZV genes the reading frames overlap. ORF42 and 45 are splice products of the same gene and ORFS/L splicing occurs in noncoding regions [(36)]. Most of the VZV genome is co-linear with the HSV-1 genome. The comparison of the HSV-1 and VZV predicted aminoacid sequences indicates that homologous genes are organised similarly in both genomes. In the UL region, 56 out of 62 and in the US region all four genes of VZV genes are homologous to the HSV-1 genome [(37)]. Six of the VZV genes (ORF1, ORF2, ORF13, ORF32, ORF57, ORFS/L) do not have homologs in HSV-1 [(36)].

VZV shares 41 conserved genes in comparison with seven other herpesviruses and all are located in the UL region of the VZV genome. These conserved genes are located within a given block and co-linear between viruses in a given block. The genes that are mostly conserved are the IE gene ORF4, viral enzymes, as well as

structural proteins (major capsid protein, glycoproteins gB, gH, gL). The three regions of the VZV genome that do not contain ORFs were 3' to the UL region between ORF60 and 61, between ORF62 and 63 and at the origin of replication. The phylogenetic tree of known human herpesviruses as well as several other (non-human) herpesviruses was derived by comparing the aminoacid sequences of the major capsid protein gene [38] (Figure 6).

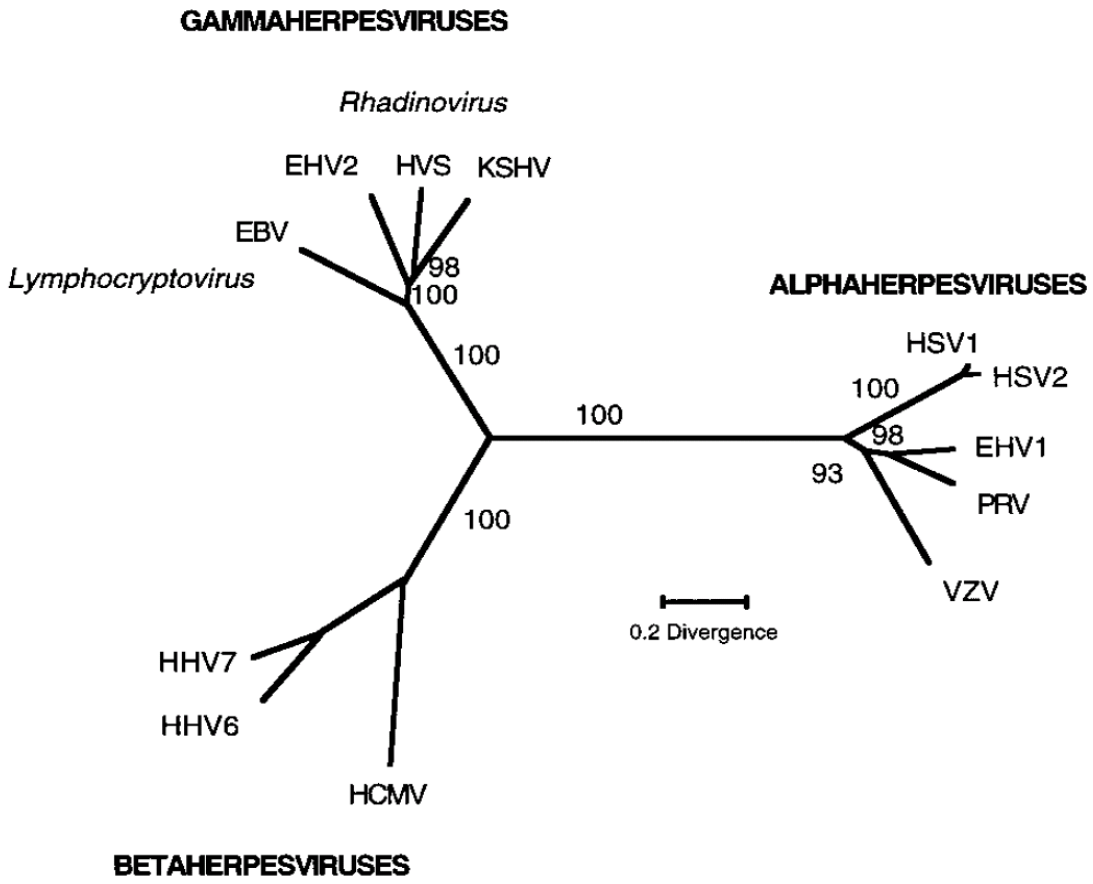


Figure 6: Phylogenetic tree of herpesviruses. The phylogenetic tree was constructed by comparing the amino acid sequences of the major capsid protein gene. EBV denotes Epstein-Barr virus, EHV-2 Equine herpesvirus type 2, HVS Herpesvirus saimiri, HSV-1 Herpes simplex virus type 1, HSV-2 Herpes simplex virus type 2, EHV-1 Equine herpesvirus type 1, PRV Pseudorabies virus, VZV Varicella-zoster virus, HCMV Human Cytomegalovirus, HHV-6 Human herpesvirus 6, and HHV-7 Human herpesvirus 7 (according to [38]).

2.4 Replication cycle of VZV

VZV attaches to the host cell surface through interactions of the mannose-6-phosphate (Man-6-P) groups present on gB, gH, gL molecules in the outer envelope with heparin sulphate proteoglycans, and also with the insulin-degrading enzyme (IDE) through gE. The viral envelope fuses with the host cell plasma membrane to

enter the cell [3,39]. Then tegument proteins are partially removed from the capsid that is transported through the host cell cytosol to the nuclear pore where the viral DNA genome is injected into the nucleus [40].

Following infection into the nucleus, the linear VZV genome circularises controlled by several tegument proteins such as ORF4, ORF10, ORF62, ORF63. In the nucleus the viral genome is expressed in a cascade manner in three stages, each of which is dependent on the host cell RNA polymerase. In the first stage, immediate early (IE) genes are expressed, which are proteins involved in transcriptional regulation. Upon synthesis in the host cytoplasm the IE proteins enter the nucleus where they further downregulate IE gene transcription and initiate synthesis of early (E) proteins. The E proteins consist of the DNA helicase / primase complex, the DNA polymerase and single stranded DNA binding proteins, together forming the machinery that replicates viral DNA. In the third stage of gene expression, late (L) proteins which encode structural components like capsomers and glycoproteins are synthesized. VZV DNA circularises by ligation of single nucleotide extensions at the 3' termini of the L and S segment. The replication starts after circularisation of the VZV genome. The head to tail concatemers that are cleaved into unit-length genomes are packed into newly assembled capsids [3].

The immature nucleocapsids are assembled inside of the host nucleus, packed with one viral genome each and equipped with several tegument proteins. Subsequently, these nucleocapsids bud through the inner nuclear membrane into the perinuclear cisternae thereby acquiring a primary envelope (Figure 7). Upon fusion of the primary envelope with the outer nuclear membrane, the naked nucleocapsid is released into the cytoplasm [41].

Secondary envelopment occurs at the trans-Golgi network (TGN). As a prerequisite, viral glycoproteins have to be synthesized at the endoplasmic reticulum (ER), processed posttranslationally and transported to the site of envelopment (Figure 7). The final envelopment and tegumentation of the VZV virions occur at specialised TGN cisternae. The glycoproteins with adhered tegument proteins are concentrated in the concave membrane wrapping TGN cisternae [3]. The viral nucleocapsids converge with the glycoproteins and tegument and the TGN sacs wrap around the nucleocapsid and fuse, giving rise to mature virions. A range of glycoproteins are exposed on the virion surface required for its docking at and fusion with the plasmamembrane to enter a new host cell for further amplification.

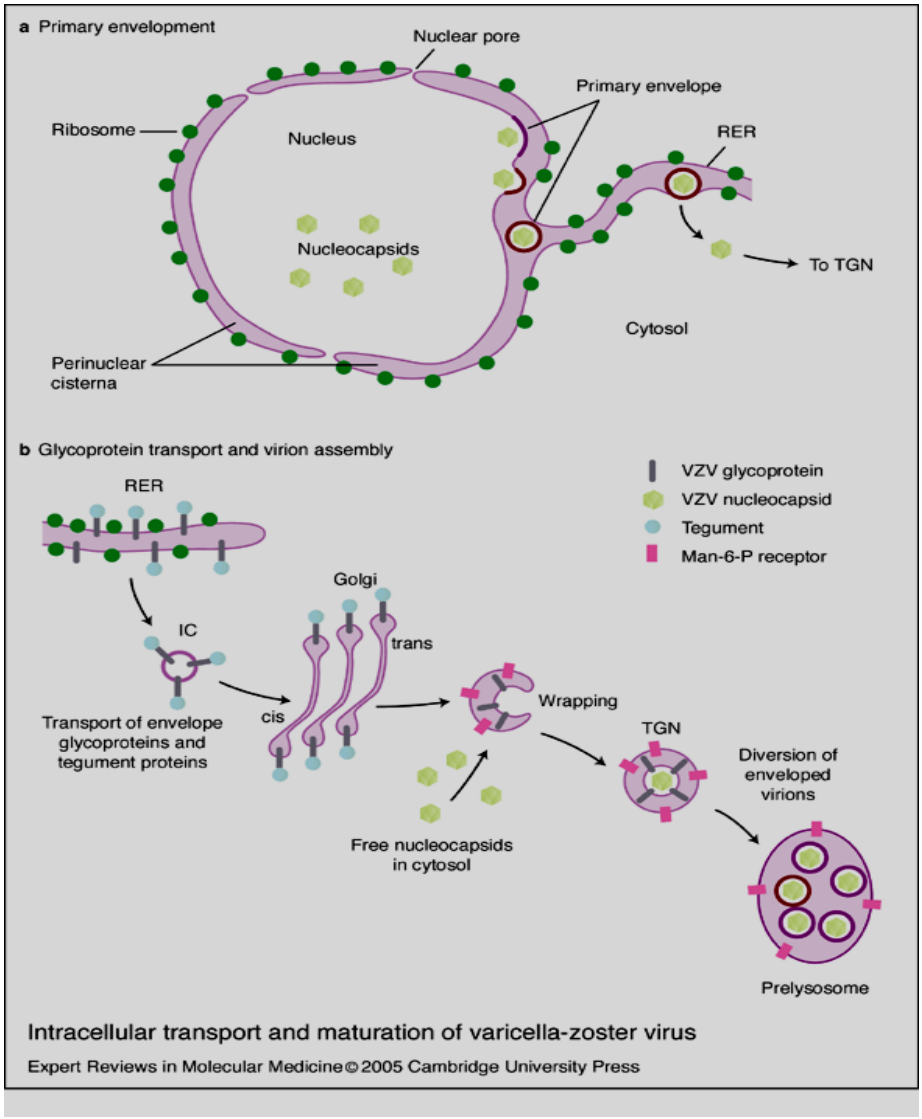


Figure 7: Intracellular transport and maturation of varicella-zoster virus [41].
(http://journals.cambridge.org/fulltext_content/ERM/ERM7_15/S146239940500966Xsup009.htm)

2.5 Limitations within the VZV research

Research on VZV is restricted for several reasons. First, it is impossible to prepare reproducible high titers of cell-free virus since VZV is highly cell-associated [42,43]. Furthermore, VZV exhibits a highly restricted host specificity, so there is no appropriate small animal model available. This is in contrast to HSV-1, which has a broad host specificity, and is able to replicate within mice. To overcome these limitations in the VZV field, new technologies have been developed. For example, a chimeric mouse model based on the implantation of small pieces of human fetal tissue, e.g. skin, thymus or liver, into SCID mice was developed to analyze the viral organotropism *in vivo* [44]. After engraftment the xenografts form a small human

“microenvironment” that enables the analysis of viral replication within these different human tissues.

2.6 Gateway® Technology

Recombinatorial cloning (Invitrogen Gateway® system) is a procedure based on the bacteriophage lambda site-specific recombination system. This recombination mechanism is responsible for the site-specific integration of this phage into the *E.coli* chromosome [45]. The lambda recombination is site-specific and occurs between attB sites on the *E.coli* chromosome and attP sites on the Lambda DNA and gives rise to attL and attR sites [46].

Figure 8 explains the Gateway® technology. An ORF is first cloned into a so-called donor vector (BP-reaction) and in a second, consecutive step into one or several different destination vectors (LR-reaction). The BP reaction requires attB sites on the gene of interest (in our case a PCR-amplified viral ORF) and attP sites on the donor vector. The recombination reaction is catalysed by the BP-clonase enzyme mix. After recombination, the resulting vectors comprise the subcloned viral ORF with attL sites. The LR reaction requires attL sites on the gene of interest and attR sites on the destination vectors. This recombination reaction is catalysed by the LR Clonase enzyme mix. After the recombination process, the resulting subcloned viral ORFs are flanked by attB sites.

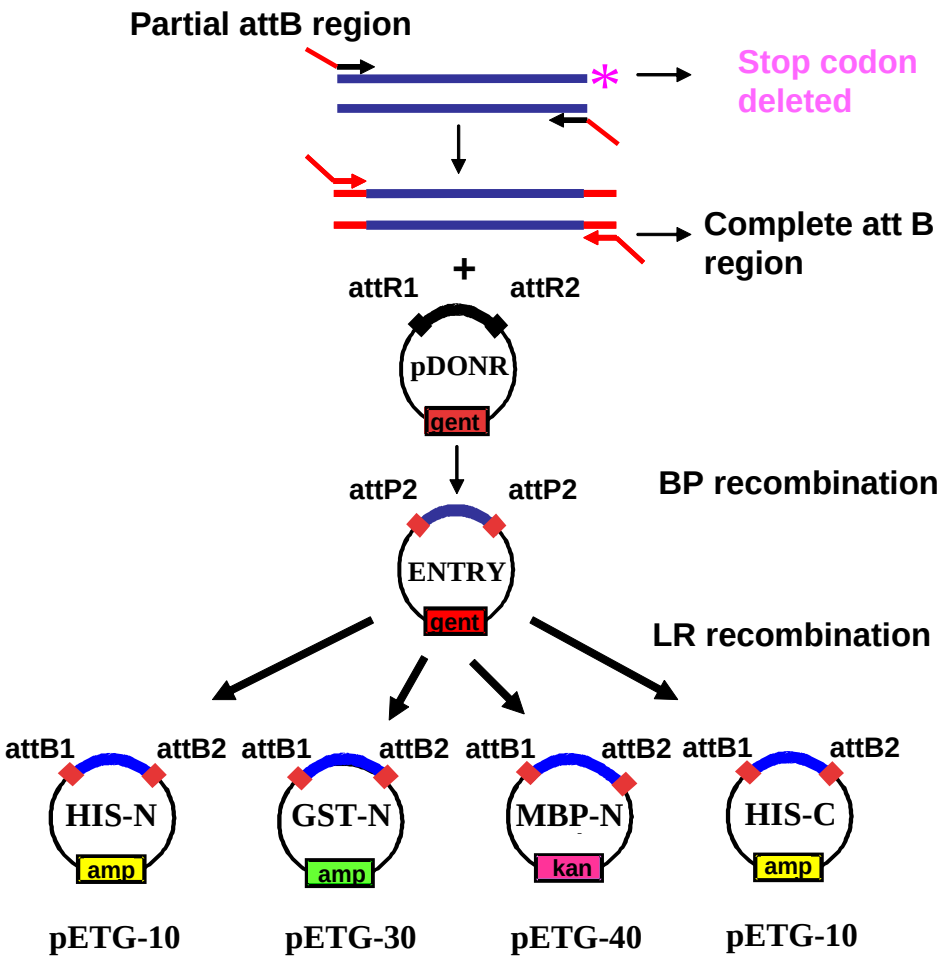


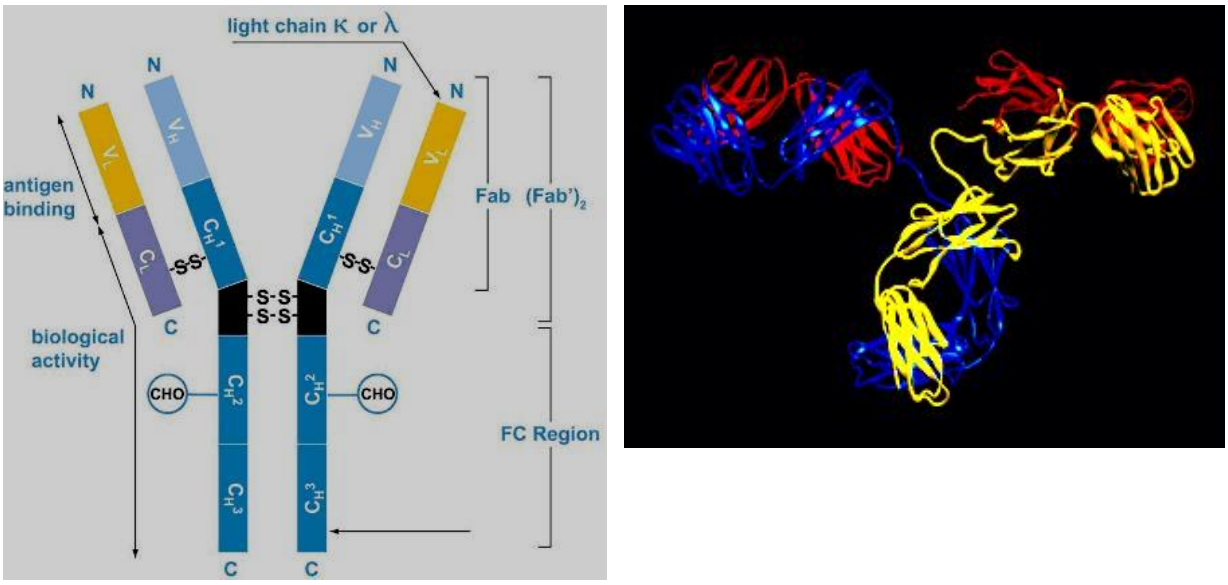
Figure 8: Schematic diagram of recombinational cloning (Invitrogen Gateway®) procedure.

2.7 Antibodies

Antibodies are proteins present on B-cell membranes and secreted by plasma cells and able to bind to specific sites of an antigen. In principle, antibodies of the IgG type are composed of four peptide chains (Figure 9), two identical heavy (H) chains with a molecular weight (MW) of 50,000 and two identical light (L) chains of MW of 25,000 daltons. Each light chain is joined to a heavy chain by a single intermolecular disulfide bond and by non-covalent interactions. Similarly, both heavy chains are joined by inter-chain disulphide bonds and noncovalent interactions. Both light and heavy chains constitute the antigen-binding site of the antibody molecule. The first 110 aminoacids of the amino-terminal region of a heavy or light chain possess a high degree of variability in aminoacid sequence called V region: V_L in light chain and V_H in heavy chain. The specific nature of an antibody is defined by this V region. Within the V regions most of the differences fall into the so-called *complementarity-determining region (CDRs).* The regions beyond the variable regions are relatively

constant, termed C regions, C_L in light chain and C_H in heavy chain. Based on structural and functional patterns antibodies are classified into five types IgA, IgD, IgE, IgG, IgM. Glycosylation in antibodies is restricted to the constant region.

Chemical and proteolytic cleavage of IgGs provided the first clue of their three-dimensional structure. Proteolytic cleavage of IgG with papain produces three fragments, two of which are identical while the third is different. Due to their activity to bind the antigen, two identical fragments are called antigen-binding fragments (Fab). The Fab fragments contain both variable and constant regions of heavy and light chains. The non-identical fragment designated Fc fragment (fragment crystallizable) is composed by the constant regions of two heavy chains [47].



a) **b)**

Figure 9: Graphical depiction of immunoglobulins. a) Schematic diagram of immunoglobulin structure derived from amino acid sequencing studies. H, heavy chain; L, light chain; V, variable region; C, constant region; CHO, carbohydrate attachment site; S-S, disulfide bond; Fab, antigen-binding fragment; Fc, crystallizable fragment. b) Ribbon presentation of an intact monoclonal antibody [47].

http://www.abcam.com/index.html?pageconfig=resource&rid=11258&pid=11287
http://en.wikipedia.org/wiki/Antibody

2.8 Aim of the PhD thesis

VZV is a neurotropic alphaherpesvirus that contains a 125 kb dsDNA genome encoding 70 viral proteins. Research on VZV is restricted due to various reasons, like difficulty in isolating virus from cells, or lack of animal model. Because of this very little is known about its morphogenesis, replication, reactivation from latency and the

functional and biochemical characteristics of many VZV specific genes were not studied so far.

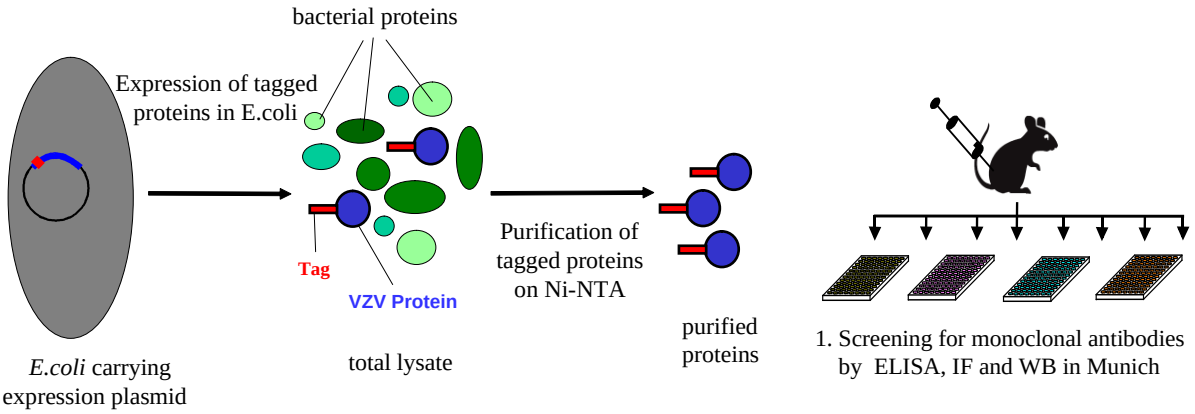


Figure 10: Schematic diagram depicting the pipeline for high-throughput antibody screening.

The aims of this study and the experimental approach of which is schematically depicted in Figure 10 were (i) to construct a VZV ORFeome by recombinational cloning, (ii) to construct expression libraries by subcloning a VZV ORFeome library into pET derived expression vectors containing four different tags like N-His6, C-His6, N-MBP, and N-GST, (iii) to express and purify all VZV encoded proteins and immunize them to mice, (iv) to screen hybridoma clones for secretion of VZV-specific antibodies using ELISA, Western Blotting and indirect immunofluorescence analysis, (v) to study the localization of the VZV proteome in the viral context.

3. Materials and Methods

3.1 Materials

3.1.1 Equipment

Bacterial Shaker	Kühner, Bürsfelden, Switzerland
Balances	Sartorius, Göttingen, Germany
Centrifuge GP	Beckman, Palo Alto, USA
Centrifuge J2-21	Beckman, Palo Alto, USA
Centrifuge Varifuge 3.0R	Heraeus, Hanau, Germany
Centrifuge Minifuge RF	Heraeus, Hanau, Germany
Centrifuge Labofuge T	Heraeus, Hanau, Germany
Centrifuge, refrigerated / non-refrigerated	Heraeus, Hanau, Germany
Confocal microsope	Leica, Mannheim, Germany
Film Developing Machine	Optimax Typ TR MS Laborgeräte, Heidelberg, Germany
Fluorescence Microscope	Leica, Mannheim, Germany
Fridge (4°C)	Liebherr, Ochsenhausen, Germany
Freezer (-20°C)	Liebherr, Ochsenhausen, Germany
Freezer (-80°C)	Forma Scientific, Inc., Marietta, Ohio, USA
Cryo 1°C Freezing Container	Nalgene Nunc, Wiesbaden, Germany
Gel Dryer	Bio-Rad, Munich, Germany
GelAir Drying System	Bio-Rad, Munich, Germany
Incubators for Cell Culture (37°C)	Forma Scientific, Inc., Marietta, Ohio, USA
Laminar Flow Hood Steril Gard II A/B3	The Baker Company, Sanford, Maine, USA
Magnetic Stirrer with heating block	Janke & Kunkel, Staufen, Germany
Microwave	AEG, Berlin, Germany
PCR Thermal Cycler GeneAmp 2400	Perkin Elmer, Weiterstadt, Germany
pH-Meter	WTW, Weilheim, Germany
Pipettes	Gilson, Villies Le Bel, France; Eppendorf, Hamburg, Germany
Pipetting Aid	Technomara, Zürich, Switzerland

Electrophoresis Power supply EPS200	Amersham-Pharmacia, Freiburg, Germany
Overhead Mixer	Heidolph, Schwabach, Germany
384-Pin Replicator	Nalge Nunc International
Sonifier 450	Branson Ultrasonics Corp., Danbury, USA
Thermomixer	Eppendorf, Hamburg, Germany
UV-Transilluminator (366 nm)	Vetter, Wiesloch, Germany
(254 nm)	Konrad Benda, Wiesloch, Germany
Vortex Mixer	IKA Works, Inc, Wirmington, USA
Water Bath	Julabo, Seelbach, Germany
	GFL, Burgwedel, Germany

3.1.2 Chemicals

Acetic Acid	Roth, Karlsruhe, Germany
Acrylamide/Bisacrylamide 37,5/1 (Rotiphorese Gel 30)	Roth, Karlsruhe, Germany
Agar for plates	Gibco BRL, Karlsruhe, Germany
Agarose Electrophoresis Grade	Invitrogen, Karlsruhe, Germany
Ammonium Persulfate (APS)	Sigma, Munich, Germany
Ampicillin	Roche Diagnostics, Mannheim, Germany
Bacto Peptone	BD Biosciences Clontech, Heidelberg, Germany
Bacto Tryptone	BD Biosciences Clontech, Heidelberg, Germany
Bacto Yeast Extract	BD Biosciences Clontech, Heidelberg, Germany
Bromophenol Blue	Serva, Heidelberg, Germany
BSA (Bovine Serum Albumin)	Sigma, Munich, Germany
Calcium Chloride	Merck, Darmstadt, Germany
Chloramphenicol	Sigma, Munich, Germany
Coomassie Brilliant Blue R-250	Bio-Rad, Munich, Germany
Dextrose	BD Biosciences Clontech, Heidelberg,

	Germany
Dimethylsulfoxid (DMSO)	Merck, Darmstadt, Germany
Dithiothreitol (DTT)	Roth, Karlsruhe, Germany
dNTPs	RocheDiagnostics,Mannheim, Germany
Dulbecco's modified Eagle's medium (DMEM)	Gibco BRL, Karlsruhe, Germany
Ethanol (EtOH)	Riedel-de Haën, Seelze, Germany
Ethidium Bromide	Sigma, Munich, Germany
Ethylendiamintetraacetate Disodium Salt	Roth, Karlsruhe, Germany (EDTA)
Ethylene Glycol	Sigma, Munich, Germany
Fetal Calf Serum (FCS)	Gibco BRL, Karlsruhe, Germany
Gentamycin	Serva, Heidelberg, Germany
Glucose	Merck, Darmstadt, Germany
Glycerol	Roth, Karlsruhe, Germany
Glycine	Serva, Heidelberg, Germany
Hydrochloric Acid (HCl)	Merck, Darmstadt, Germany
Imidazole	Fluka, Seelze, Germany
Isopropanol	Riedel-de Haën, Seelze, Germany
Isopropylthio-b-D-galactosid (IPTG)	Roth, Karlsruhe, Germany
Kanamycin	Serva, Heidelberg, Germany
Magnesium Chloride	Merck, Darmstadt, Germany
Magnesium Sulfate	Merck, Darmstadt, Germany
2-Mercaptoethanol	Merck, Darmstadt, Germany
Methanol	Merck, Darmstadt, Germany
Polyethylene Glycol (PEG 1000)	Sigma, Munich, Germany
Phosphate Buffered Saline (PBS)	Dulbecco's Gibco BRL, Karlsruhe, Germany
Ponceau S	Sigma, Munich, Germany
Potassium Acetate	Riedel-de Haën, Seelze, Germany
Potassium Chloride	Merck, Darmstadt, Germany
Potassium Phosphate Salts	Merck, Darmstadt, Germany
Protein G Sepharose Fast Flow	Amersham-Pharmacia, Freiburg, Germany
RPMI (Rosswell Park Memorial Institute)	1640 Gibco BRL, Karlsruhe, Germany

Skim Milk Powder	Merck, Darmstadt, Germany
Sodium Acetate	Riedel-de Haën, Seelze, Germany
Sodium Azide	Serva, Heidelberg, Germany
Sodium Chloride	Riedel-de Haën, Seelze, Germany
Sodium Carbonate	Merck, Darmstadt, Germany
Sodium Hydroxid	J.T.Baker B.V., Deventer, Holland
Sodium Phospate salts	Merck, Darmstadt, Germany
Tetramethylethylendiamin (TEMED)	Amersham-Pharmacia, Freiburg, Germany
Tris (hydroxymethyl) aminomethan (Tris)	Roth, Karlsruhe, Germany
Triton X-100	Serva, Heidelberg, Germany
Trypsin	Gibco BRL, Karlsruhe, Germany
Tween 20	Merck, Darmstadt, Germany
Urea	Roth, Karlsruhe, Germany

3.1.3 Additional materials

Autoradiography Films BIOMAX-MR	Eastman-Kodak, Rochester, USA
Cell Culture Plastic Ware	Greiner, Nürtingen, Germany Nunc, Wiesbaden, Germany Falcon/Becton Dickinson, Heidelberg, Germany
Filter Paper (3 mm)	Whatman Ltd., Maidstone, England
Glass Slides for IF	Marienfeld, Bad Mergentheim, Germany
Protran Nitrocellulose Transfer Membranes	Schleicher & Schuell, Dassel, Germany
Sterile Filter Units	Millipore
Single-well Microtiter Plates	Omnitray; Nalge Nunc International

3.1.4 Cell lines

HEK 293	human embryonal kidney cell line (ATCC: CRL-1573)
MeWo	human melanoma cell line ((ATCC: HTB-65)

3.1.5 Bacterial strains

DH5α	Invitrogen, Karlsruhe, Germany Genotype: F¯ϕ80*dlacZ*ΔM15 *endA1 recA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1* λ¯ *gyrA96 relA1* Δ (*lacIZYA-argF*)*U169*
DB3.1	Invitrogen, Karlsruhe, Germany Genotype: F¯*gyr*A462 *en*dA *(sr*1-*rec*A) *mcr*B *mrr hs*dS20 (r B - m B -) *su*pE44 *ara*14 *ga*lK2 *la*cY1 *pro*A2 *rps*L20(Str R) *xyl*5 ë - *leu mt*l1
DH10B	Invitrogen, Karlsruhe, Germany Genotype: F¯*mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80d*lacZ*ΔM15 Δ*lacX74 deoR recA1 araD139* Δ(*ara leu*)7697 *galU galK rpsL endA1 nupG*

3.1.6 Plasmids

pDONR207 (Gm^r)	Invitrogen, Karlsruhe, Germany
pETG based vectors	N-His, C-His, N-GST, N-MBP.

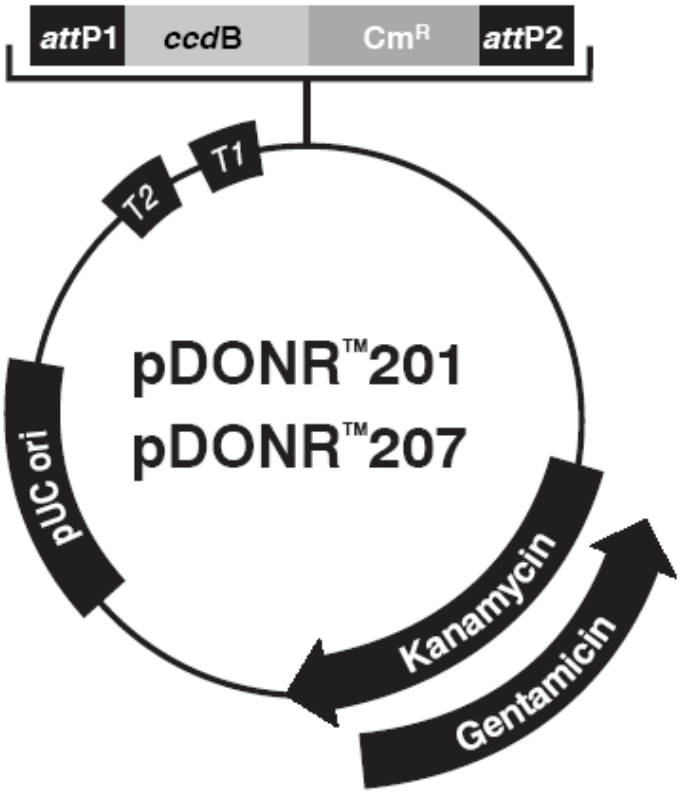


Figure 11: The pDONR207 vector contains attP sites at both sides of the ccdB cassette.

3.1.7 Oligonucleotides

Table 2: Forward Primers

ORF No.	**Forward Primer**
ORF1 for	AAAAAGCAGGCTCCGCCatgTCCAGGGTATCGGAGTATGG
ORF2 for	AAAAAGCAGGCTCCGCCatgCATGTAATTTCTGAGACACTTGC
ORF3 for	AAAAAGCAGGCTCCGCCatgGATACAACGGGAGCTTCCGAAAG
ORF4 for	AAAAAGCAGGCTCCGCCatgGCCTCTGCTTCAATTCCAACC
ORF5 for	AAAAAGCAGGCTCCGCCatgCAGGCTTTAGGAATCAAGACAGAAC
ORF5F for	AAAAAGCAGGCTCCGCCatgTATTTACAATTTCGACGAATACGTCG
ORF6 for	AAAAAGCAGGCTCCGCCatgGATAAATCCTCCAAACCGACGATTCG
ORF7 for	AAAAAGCAGGCTCCGCCatgCAGACGGTGTGTGCCAGCTTATGTG
ORF8 for	AAAAAGCAGGCTCCGCCatgAACGAAGCGGTAATTGATCCCATCTTG
ORF9 for	AAAAAGCAGGCTCCGCCatgGCATCTTCCGACGGTGACAGACTTTG
ORF9a for	AAAAAGCAGGCTCCGCCatgGGATCAATTACCGCTTCGTTCATATTAATAAC
ORF10 for	AAAAAGCAGGCTCCGCCatgGAGTGTAATTTAGGAACCGAACATCCTAG
ORF11 for	AAAAAGCAGGCTCCGCCatgCAGTCGGGTCATTATAACCGGAGGC
ORF12 for	AAAAAGCAGGCTCCGCCatgTTTTCTCGGTTTGCGCGTTCCTTTTCCAGCG

ORF No.	Forward Primer
ORF13 for	AAAAAGCAGGCTCCGCCatgGGAGACTTGTCATGTTGGACAAAGGTGC
ORF14/14N for	AAAAAGCAGGCTCCGCCatgAAGCGGATACAAATAAATTTAATTTTAACGATCG
ORF15 for	AAAAAGCAGGCTCCGCCatgGCCGTGAATGGTGAAAGAGCTGTCCATG
ORF15F for	AAAAAGCAGGCTCCGCCatgGTCGATGCCGCCACCGATTTGCAAGATACG
ORF16 for	AAAAAGCAGGCTCCGCCatgGATTTGAGGTCGCGTACAGACGATGCTTTGG
ORF17 for	AAAAAGCAGGCTCCGCCatgGGGCTCTTTGGACTGACACGCTTTATCC
ORF18 for	AAAAAGCAGGCTCCGCCatgGATCAGAAAGATTGCAGTCATTTTTTTTACAGGC
ORF19 for	AAAAAGCAGGCTCCGCCatgGAGTTCAAAAGAATTTTTAATACGGTTCATGAC
ORF20 for	AAAAAGCAGGCTCCGCCatgGGGAGTCAACCAACCAACTCGCATTTTAC
ORF21 for	AAAAAGCAGGCTCCGCCatgGAAGAACCAATTTGTTATGATACAC
ORF22N for	AAAAAGCAGGCTCCGCCatgGATATAATTCCGCCTATAGCTGTCACTGTTGC
ORF22C for	AAAAAGCAGGCTCCGCCatgATATTACGAACCTCGGGGGATCTATTAC
ORF23 for	AAAAAGCAGGCTCCGCCatgACACAACCCGCATCGTCTCGTGTAGTC
ORF24/24N for	AAAAAGCAGGCTCCGCCatgTCACGGAGAACGTATGTACGGAGTGAAC
ORF25 for	AAAAAGCAGGCTCCGCCatgTACGAATCGGAAAATGCGTCGGAAC
ORF26 for	AAAAAGCAGGCTCCGCCatgGATCGGGTAGAATCAGAAGAACCCATGG
ORF27 for	AAAAAGCAGGCTCCGCCatgCATTTAAAGCCTACCAGATTTTTCCACGC
ORF28 for	AAAAAGCAGGCTCCGCCatgGCGATCAGAACGGGGTTTTGTAATCCC
ORF29 for	AAAAAGCAGGCTCCGCCatgGAAAATACTCAGAAGACTGTGACAGTGC
ORF30 for	AAAAAGCAGGCTCCGCCatgGAATTGGATATTAATCGAACATTGTTGG
ORF31/31N for	AAAAAGCAGGCTCCGCCatgTTTGTTACGGCGGTTGTGTCGGTCTCTCC
ORF31C for	AAAAAGCAGGCTCCGCCatgCTTAAAACAAGCCCGATGAAGGC
ORF32 for	AAAAAGCAGGCTCCGCCatgGAATCGTCTAACATTAACGCGCTAC
ORF33 for	AAAAAGCAGGCTCCGCCatgGCTGCTGAAGCTGACGAAGAGAAC
ORF34 for	AAAAAGCAGGCTCCGCCatgACGGCGAGATATGGGTTCGGATC
ORF35 for	AAAAAGCAGGCTCCGCCatgTCCGCTAGTCGAATTCGGGCCAAG
ORF36 for	AAAAAGCAGGCTCCGCCatgTCAACGGATAAAACCGATGTAAAAATGGGCG
ORF37/37N for	AAAAAGCAGGCTCCGCCatgTTTGCGCTAGTTTTAGCGGTGGTAATTCTTCC
ORF38 for	AAAAAGCAGGCTCCGCCatgGAATTTCCATATCATTCAACCGTATCTTATAAC
ORF39/39N for	AAAAAGCAGGCTCCGCCatgAACCCACCCCAAGCCCGCG
ORF40 for	AAAAAGCAGGCTCCGCCatgACAACGGTTTCATGTCCCGCTAAC
ORF41 for	AAAAAGCAGGCTCCGCCatgGCTATGCCATTTGAGATAGAGGTATTG
ORF42 for	AAAAAGCAGGCTCCGCCatgCCGCGTGTTTTAGCACATAGCG
ORF43 for	AAAAAGCAGGCTCCGCCatgGAAGCCCATTTGGCAAATGAAACCAAAC
ORF44 for	AAAAAGCAGGCTCCGCCatgGAATTACAACGCATATTTCCGCTGTACACCG
ORF45 for	AAAAAGCAGGCTCCGCCatgTCATTGATAATGTTTGGTCGTACGCTTGGTG
ORF46 for	AAAAAGCAGGCTCCGCCatgTCAGGCCACACTCCAACCTAC
ORF47 for	AAAAAGCAGGCTCCGCCatgGATGCTGACGACACACCCCCC
ORF48 for	AAAAAGCAGGCTCCGCCatgGCACGATCGGGATTGGATAGG
ORF49 for	AAAAAGCAGGCTCCGCCatgGGACAATCTTCATCCAGCGGTC

ORF No.	Forward Primer
ORF50 for	AAAAAGCAGGCTCCGCCatgGGAACTCAAAAGAAGGGGCCGC
ORF50C for	AAAAAGCAGGCTCCGCCatgCGTGCATATGTGTATCATCGACAGAAACGC
ORF51 for	AAAAAGCAGGCTCCGCCatgTCTCCCAACACCGGGGAGAG
ORF52 for	AAAAAGCAGGCTCCGCCatgGACGCAACGCAGATTACCTTGGTTAG
ORF53 for	AAAAAGCAGGCTCCGCCatgCAGCGGATTCGACCTTACTGG
ORF54 for	AAAAAGCAGGCTCCGCCatgGCCGAAATAACGTCTCTTTTTAATAACAGTTCC
ORF55 for	AAAAAGCAGGCTCCGCCatgAAAAGATCAATTTCTGTAGACAGTTCTTCACCC
ORF56 for	AAAAAGCAGGCTCCGCCatgAAAAATCCGCAGAAATTAGCGATCACATTCTTG
ORF57 for	AAAAAGCAGGCTCCGCCatgGACGTACGAGAACGTAATGTGTTTGG
ORF58 for	AAAAAGCAGGCTCCGCCatgTTTTCGGAGTTGCCTCCTTCCGTACC
ORF59 for	AAAAAGCAGGCTCCGCCatgGATGTGTCTGGGGAGCCGACCG
ORF60 for	AAAAAGCAGGCTCCGCCatgGCATCACATAAATGGTTACTGCAG
ORF60C for	AAAAAGCAGGCTCCGCCatgCCGCTATCGGATGTGAGTTTG
ORF61 for	AAAAAGCAGGCTCCGCCatgGATACCATATTAGCGGGCGGTAG
ORF62(71) for	AAAAAGCAGGCTCCGCCatgGATACGCCGCCGATGCAGCGCTC
ORF63(70) for	AAAAAGCAGGCTCCGCCatgTTTTGCACCTCACCGGCTACGCG
ORF64(69) for	AAAAAGCAGGCTCCGCCatgAATCTCTGCGGATCCCGCGGTG
ORF65 for	AAAAAGCAGGCTCCGCCatgGCCGGACAAAACACCATGGAGG
ORF66 for	AAAAAGCAGGCTCCGCCatgAACGACGTTGATGCAACAGACACC
ORF67/67N for	AAAAAGCAGGCTCCGCCatgTTTTTAATCCAATGTTTGATATCGGCCG
ORF68 for	AAAAAGCAGGCTCCGCCatgGGGACAGTTAATAAACCTGTGGTGGGGG
ORF68F for	AAAAAGCAGGCTCCGCCatgACGAATCCGGTCAGAGCATCCGTC

Table 3: Reverse Primers

ORF No	Reverse primers
ORF1 rev w/o stop	AGAAAGCTGGGTC TTCTCGCTTGCAGCTTGTCG
ORF1N rev w/o stop	AGAAAGCTGGGTC CCTGTCCATTTGCATTTTCA
ORF2 rev w/o stop	AGAAAGCTGGGTC CATCAATACGCCCTCCGTAG
ORF3 rev w/o stop	AGAAAGCTGGGTC TAGTCCGCCGACAGCCGCTC
ORF4 rev w/o stop	AGAAAGCTGGGTC GCAGTTAAAGGTACTACACT
ORF5 rev w/o stop	AGAAAGCTGGGTC ATGCTTCTGGGAGTTTTCAC
ORF5F rev w/o stop	AGAAAGCTGGGTC CATCAATAACGTAACACCTT
ORF6 rev w/o stop	AGAAAGCTGGGTC ACTCGAAGTTAAATTTGGAT
ORF7 rev w/o stop	AGAAAGCTGGGTC TACAAGCATAACATGGGATT
ORF8 rev w/o stop	AGAAAGCTGGGTC ATGTTTTAGTAGAAAATCGA
ORF9a rev w/o stop	AGAAAGCTGGGTC CCACGTGCTGCGTAATACAG
ORF9aN rev w/o stop	AGAAAGCTGGGTC AAGGGTGGTGATCATTGATC
ORF9 rev w/o stop	AGAAAGCTGGGTC TTTTCGCGTATCAGTTCTTG
ORF10 rev w/o stop	AGAAAGCTGGGTC ACGCGTTAAAAACCCACACG

ORF No	Reverse primers
ORF11 rev w/o stop	AGAAAGCTGGGTC ATATTTTCGTAGTAAATGCA
ORF12 rev w/o stop	AGAAAGCTGGGTC ATGATGACTCTTAGGCGTAT
ORF12N rev w/o stop	AGAAAGCTGGGTC TGAGACCGCAGGACCCGTTT
ORF13 rev w/o stop	AGAAAGCTGGGTC AAGAGCCATTTCCATTTTTA
ORF14 rev w/o stop	AGAAAGCTGGGTC TGAACAGCAACGGATGCATAA
ORF14N rev w/o stop	AGAAAGCTGGGTC GTAGGTAGATGCATCGTAGG
ORF15 rev w/o stop	AGAAAGCTGGGTC CGATACATATGTACCACATA
ORF15N rev w/o stop	AGAAAGCTGGGTC CCGCGATGCTGAGGTTATTG
ORF15F rev w/o stop	AGAAAGCTGGGTC ACGAATTCCTTTAAACACGC
ORF16 rev w/o stop	AGAAAGCTGGGTC TTTAACTGTACATATTACGT
ORF17 rev w/o stop	AGAAAGCTGGGTC ATTCCAATATTTTGTTAATA
ORF18 rev w/o stop	AGAAAGCTGGGTC TAAATCGTTTATCACTGTGC
ORF18N rev w/o stop	AGAAAGCTGGGTC TTCTGCAACGGATGGGTTGT
ORF19 rev w/o stop	AGAAAGCTGGGTC TAAAGCACAACTGGTACAGG
ORF20 rev w/o stop	AGAAAGCTGGGTC ATAATAACATTCGTTCCATG
ORF21 rev w/o stop	AGAAAGCTGGGTC AGGGTCACTCCCACTTGTAT
ORF22 rev w/o stop	AGAAAGCTGGGTC TATATATGTTCCATCTAATA
ORF22N rev w/o stop	AGAAAGCTGGGTC ATCTCGGTAGTTAGGTATTC
ORF23 rev w/o stop	AGAAAGCTGGGTC CACCCTACGACTTCTTGAAG
ORF24 rev w/o stop	AGAAAGCTGGGTC TTTCCAGAAAAGCACCGCCC
ORF24N rev w/o stop	AGAAAGCTGGGTC TACGGGTAGAGCAAGTTTCC
ORF25 rev w/o stop	AGAAAGCTGGGTC AGCATCCTTCAATATTTCAT
ORF26 rev w/o stop	AGAAAGCTGGGTC GACATACTTCGATAGGGTGT
ORF27 rev w/o stop	AGAAAGCTGGGTC CCGAGGAGGAACAAAGTCAT
ORF28 rev w/o stop	AGAAAGCTGGGTC ACTTTGATGGAGAATTGCTT
ORF29 rev w/o stop	AGAAAGCTGGGTC AATCATTTCCATTGTAATGT
ORF30 rev w/o stop	AGAAAGCTGGGTC TGAAAACGCCGGGTCCGTTG
ORF31 rev w/o stop	AGAAAGCTGGGTC CACCCCCGTTACATTCTCGG
ORF31N rev w/o stop	AGAAAGCTGGGTC AAACGTGGTAAATCCGTGTA
ORF32 rev w/o stop	AGAAAGCTGGGTC ATCGGTGTCAGAATCTTCAT
ORF33 rev w/o stop	AGAAAGCTGGGTC ACACCGCCCCACCATCATCT
ORF34 rev w/o stop	AGAAAGCTGGGTC CGGTGTGGAGGCAAACTGAG
ORF35 rev w/o stop	AGAAAGCTGGGTC CCCATGGGAAAACATCCCGG
ORF36 rev w/o stop	AGAAAGCTGGGTC GGAAGTGTTGTCCTGAACGG
ORF37 rev w/o stop	AGAAAGCTGGGTC TGTCAGAGGTATTTTATTAT
ORF37N rev w/o stop	AGAAAGCTGGGTC CATTCGTATGGCTTGTCGTC
ORF38 rev w/o stop	AGAAAGCTGGGTC CCTTTGGGTTTTTTTCCCGT
ORF39 rev w/o stop	AGAAAGCTGGGTC AAACGAAATAGATGTTTTTA
ORF39N rev w/o stop	AGAAAGCTGGGTC ATAAAACACGGAGTGTTGCG
ORF40 rev w/o stop	AGAAAGCTGGGTC CGCGGAAGAGGAAGACATC

ORF No	Reverse primers
ORF41 rev w/o stop	AGAAAGCTGGGTC CACTTGAATCACGGCCGTGC
ORF42 rev w/o stop	AGAAAGCTGGGTC TTTAATAGGCATAAACACGG
ORF43 rev w/o stop	AGAAAGCTGGGTC TTTATGGGGGTTGGGAATAG
ORF44 rev w/o stop	AGAAAGCTGGGTC GGTGGTTGTAGGTTCCGGTT
ORF45 rev w/o stop	AGAAAGCTGGGTC TAAAACACTCACGTTTGTGT
ORF46 rev w/o stop	AGAAAGCTGGGTC CACATCCGTGTGTGGGGTTG
ORF47 rev w/o stop	AGAAAGCTGGGTC TGTCGATCCTATCCAATCCC
ORF48 rev w/o stop	AGAAAGCTGGGTC AAGCAACGGTTTCTCCGTTG
ORF49 rev w/o stop	AGAAAGCTGGGTC ACATTTTGCGCATTTGGAAT
ORF50 rev w/o stop	AGAAAGCTGGGTC CTCCCACCCACTGTTTGATC
ORF51 rev w/o stop	AGAAAGCTGGGTC TAAACTTTCAAAATTTACCG
ORF52 rev w/o stop	AGAAAGCTGGGTC TAAAAACAAGAAGTTATATG
ORF53 rev w/o stop	AGAAAGCTGGGTC CTTTACAACCCGTGGTGAAT
ORF54 rev w/o stop	AGAAAGCTGGGTC AGATCTTCGATCACGTCGCT
ORF55 rev w/o stop	AGAAAGCTGGGTC ATACACAACGTGTACGTTGG
ORF56 rev w/o stop	AGAAAGCTGGGTC CGCGTTTGCGGCGTCCCGTA
ORF57 rev w/o stop	AGAAAGCTGGGTC ACGTTGATGAGCCTTGCAGG
ORF58 rev w/o stop	AGAAAGCTGGGTC CGTTCTCGTACGTCCATGAC
ORF59 rev w/o stop	AGAAAGCTGGGTC TATAACACTCCAATCGATCT
ORF60 rev w/o stop	AGAAAGCTGGGTC TTGGCATACGCGTTGGAACA
ORF61 rev w/o stop	AGAAAGCTGGGTC GGACTTCTTCATCTTGTTTG
ORF62 rev w/o stop	AGAAAGCTGGGTC CCCCCGACTCTGCGGGGGGC
ORF63 rev w/o stop	AGAAAGCTGGGTC CACGCCATGGGGGGGCGGTA
ORF64 rev w/o stop	AGAAAGCTGGGTC GGATCTCTCGTAGGTTCTTG
ORF65 rev w/o stop	AGAAAGCTGGGTC TCCAACAAATTGTGACGTTA
ORF65N rev w/o stop	AGAAAGCTGGGTC TTTTTTACGATGATATATTT
ORF66 rev w/o stop	AGAAAGCTGGGTC ATCTCCAACTTCCATTGGAT
ORF67 rev w/o stop	AGAAAGCTGGGTC TTTAACAAACGGGTTTACAA
ORF67N rev w/o stop	AGAAAGCTGGGTC ATTTTCTGGAGGATCATTAA
ORF68 rev w/o stop	AGAAAGCTGGGTC CCGGGTCTTATCTATATACA
ORF68F rev w/o stop	AGAAAGCTGGGTC TCGTAGAAGTGGTGACGTTC
ORFS/L rev w/o stop	AGAAAGCTGGGTC TGTAGTTGAGTTGGGAGGTT

All oligonucleotides were obtained from Invitrogen, Karlsruhe, Germany.

3.1.8 Molecular weight markers

Gene Ruler 100 bp DNA ladder	MBI Fermentas, St.Leon-Rot, Germany
Gene Ruler DNA 1 kb ladder	MBI Fermentas, St. Leon-Rot, Germany
See Blue Plus 2 Prestained Protein	Invitrogen, Karlsruhe, Germany

3.1.9 Kits

PCR DNA Gel Purification	Amersham-Pharmacia, Freiburg, Germany
Qiafilter Plasmid Maxi Kit	Qiagen, Hilden, Germany
Qiagen Plasmid Mini Kit	Qiagen, Hilden, Germany

3.1.10 Secondary antibodies

Alexa 488	Invitrogen, Karlsruhe, Germany
Peroxidase-conjugated goat anti-Mouse IgG	Invitrogen, Karlsruhe, Germany
Alkaline-phosphatase-conjugated goat anti-Mouse IgG	Invitrogen, Karlsruhe, Germany

3.1.11 Enzymes

BP Clonase	Invitrogen, Karlsruhe, Germany
BanII	New England Biolabs, USA
EcoR1	New England Biolabs, USA
HindIII	New England Biolabs, USA
LR Clonase	Invitrogen, Karlsruhe, Germany
T4 DNA Polymerase	New England Biolabs, USA
XbaI	New England Biolabs, USA

3.1.12 Viral Stains

VZV-pOKA	Takahashi et al 1974 [48]

3.2 Methods

3.2.1 Bacterial cultures

3.2.1.1 Cultivating Bacteria

E. coli bacteria were grown in LB medium or on LB agar plates with specific selective antibiotics. Incubation was performed at 37°C with constant shaking.

LB Medium (1l)

Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g

LB Agar (1l)

Agar	15 g
Bacto Tryptone	10 g
Bacto Yeast Extract	5 g
NaCl	5 g

LB Medium for selection: containing either 100 µg/ml Ampicillin, 34 µg/ml Chloramphenicol, 15 µg/ml Gentamycin, or 50 µg/ml Kanamycin.

3.2.1.2 Preparation of electrocompetent *E. coli* DH10 B cells

The day before the experiment, a preculture of *E. coli* DH10B was inoculated in 2YT-medium. The next day, 10ml of the preculture was transferred to a flask containing 1 litre of 2YT medium and grown at 37°C to obtain an OD_{600} of 0.6. The cells were incubated for 20 min on ice and then centrifuged for 10 min at 4000 rpm at 4°C. Note: all further steps were performed on ice or in pre-cooled centrifuges (4°C). The resulting bacterial pellet was resuspended in 250 ml ice-cold water and centrifuged for 10 min at 4000 rpm. This washing step was repeated two more times with ice-cold water and two more times in 150 ml of ice-cold 10% glycerol. After the final washing step, cells were resuspended in 5 ml 10% glycerol, aliquoted, shock-frozen in liquid nitrogen, and stored at –80°C.

2YT Medium (1l)

Bacto Tryptone	16 g
Bacto Yeast Extract	10 g
NaCl	5 g

3.2.1.3 Preparation of chemically competent *E. coli* DH5α cells

For preparation of chemically competent cells, a pre-culture of *E.coli* DH5α was grown overnight in 50 ml of 2YT medium. To 1 litre of 2YT medium, 20 ml of preculture was added and incubated at 37°C until an OD_{600} of 0.6 was reached. At that point the culture was rapidly chilled on ice for 20 min. From here, all further steps were performed on ice and also in pre-cooled centrifuges (4°C). The bacterial culture was centrifuged at 4000 rpm for 10 min and the pellet was resuspended in 40ml (1/25 volume of culture) of icecold TFB I buffer. After 15min incubation on ice, the resuspended bacterial culture was centrifuged at 4000 rpm for 10min. The supernatant was discarded and the pellet was resuspended in 10 ml of icecold TFB II buffer. Finally, aliquots of 200 µl were made, shock-frozen in liquid nitrogen, and stored at –80°C.

TFB I (200ml)

30mM KAc	0.59 g
50mM $MnCl_2$	1.98 g
100 mM RbCl	2.42 g
10mM $CaCl_2$	0.29 g
15% (v/v) Glycerol	30 ml

TFB II (200ml)

10 mM MOPS pH 7.0	0.46 g
75 mM $CaCl_2$	2.21 g
10mM RbCl	0.24 g
15% (v/v) Glycerol	30 ml

Both buffers were sterilized by filtration (Ø0.2 µm) and stored at 4°C.

3.2.1.4 Bacterial transformation

To transform entry vectors into electrocompetent bacteria DH10B were made electrocompetent. About 2 µl of the BP reaction was transformed into 50 µl of

eletrocompetent DH10B cells. The parameters for electroporation were 2500 V, 25 µF, 200 Ω. After electroporation 500µl of SOC medium was added to the cells and incubated in the shaker at 900 rpm for 2 hours at 37°C. After this incubation, the transformed cells were plated on LB media containing Gentamycin (15 µg/ml) to select for pDONR207 and incubated over night at 42°.

Destination vectors were transformed into chemically competent bacteria. About 2 µl of the LR reaction was transformed into 50 µl of DH5α cells by heat shock. 500µl of SOC medium was added to the cells and incubated in the shaker at 900 rpm for 2 hours at 37°C. After this incubation, the transformed cells were plated on LB media containing the respective antibiotic: Ampicillin (100 µg/ml) for pHis tag, Kanamycin (50 µg/ml) for pMBP tag, and incubated over night at 37°

3.2.2 DNA techniques

3.2.2.1 Purification of plasmid DNA

Plasmid DNA was purified in small scale (minipreps) with the QIAGEN plasmid extraction kit (QIAGEN, Hilden, Germany) and in large scale (maxiprep) with the (Promega Germany) according to the manufacturer's protocol.

3.2.2.2 Estimation of DNA concentration

The purified DNA concentration and purity was determined by measuring the UV absorbance at 260 and 280 nm. The concentration of the DNA was calculated using the formula 1 OD_{260} = 50 µg/ml dsDNA or 33 µg/ml ssDNA. The purity of the DNA was determined using the OD_{260}/OD_{280} ratio, with a ratio of ca. 1.8 indicating a low degree of protein contamination.

3.2.2.3 Primer design

While the forward primers were provided by Armin Baiker, the reverse primers were designed by using pDRAW32 software. The internal forward primer used comprises 12bp of the attB1, a translational consensus sequence (KOZAK) and 20bp of the respective 5' specific ORF sequence. The internal reverse primer contains 12bp of the attB2 sites, the 3' end of the respective ORF with no stop codon and 20bp sequence complementary to the coding region.

3.2.2.4 Nested Polymerase Chain Reaction

All the single ORFs in the VZV were amplified by two rounds of PCR (nested PCR). The primer design for the first round of PCR were described in 3.2.2.3. The nested PCR consists of two steps. In the first step the individual genes were amplified by a pair of specific forward and reverse primers. For the second PCR, the amplicon of the first PCR was used as a template. In the second round of PCR the full length attB site was added to the respective PCR fragment. All PCR amplifications were performed using the EXPAND LONG Polymerase (Roche, Mannheim, Germany). The typical reaction was performed in a 50 µl volume as shown in Figure 12.

Reaction mixture of the first PCR:

10X buffer	5 µl
10mM dNTPs	1 µl
1unit expand long polymerase	1 µl
gene specific For Primer (10pmol/U)	2 µl
gene specific Rev Primer (10pmol/U)	2 µl
VZV template DNA(100ng)	8 µl
Sterile water	31 µl

Typical reaction conditions:

1 - 95°C for 2 min (initial denaturation)
2 - 95°C for 30 sec (denaturation)
3 - 55°C for 30 sec (annealing)
4 - 68°C for [1000bp x = 1 min] (extension)
5 - 15 – 20 cycles
6 - 68°C final extension

For some of the PCR fragments, the annealing temperature had to be varied (decreased 2 to 5°C) in order to get better amplification products.

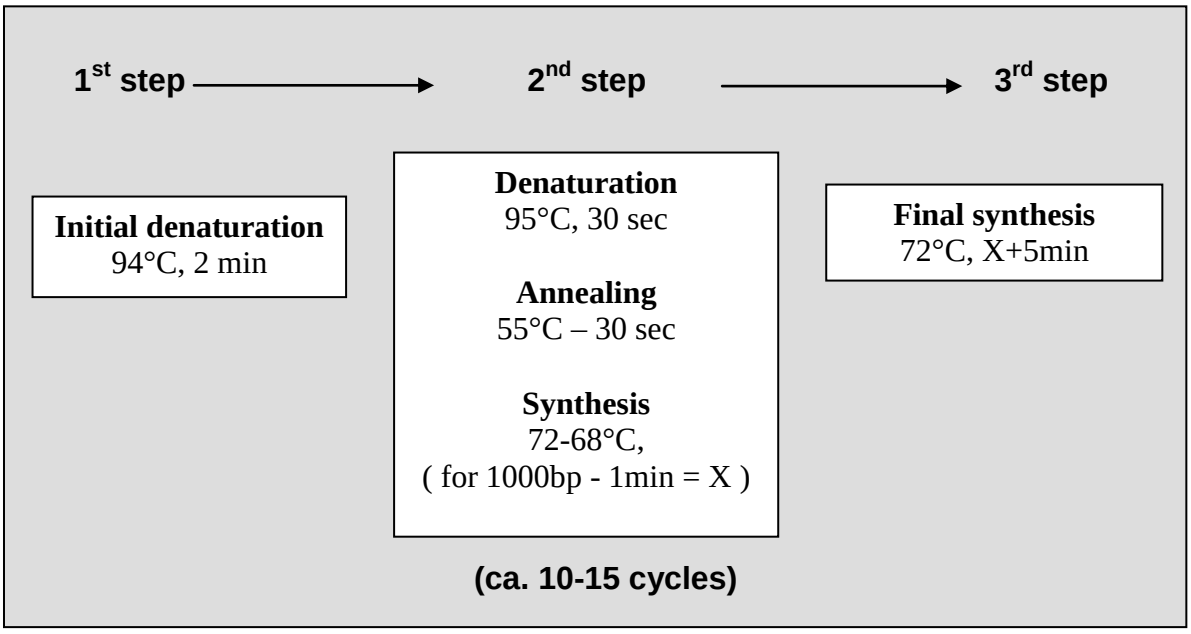


Figure 12: The schematic diagram representing the 1st and 2nd PCR rounds

Reaction mixture of the second PCR:

10X buffer	5 µl
10mM dNTPs	1 µl
1unit expand long polymerase	1 µl
attB1 Primer (10pmol/U)	2 µl
attB2 Primer (10pmol/U)	2 µl
first PCR product DNA(100ng)	8 µl
Sterile water	31 µl

The amplification conditions used for the second PCR were identical to the first PCR (see above). After PCR amplification, the resulting products were separated on an 0.8% agarose gel by electrophoresis.

3.2.2.5 Agarose gel electrophoresis

PCR products, restricted fragments and plasmids were analysed by agarose gel electrophoresis in 1x TAE. An agarose concentration between 0.8 to 1.5% was used for analysis depending on the molecular weight of the DNA to be analysed. The agarose was solubilised in 1X TAE by heating in a microwave oven and 0.25 µg/ml (2.5 µl stock to 100 ml) of ethidium bromide was added before casting the gels. Gels of different sizes were cast based on the number of DNA samples. The DNA samples

to be analysed were mixed with 6x loading dye. Gels were run from anode to cathode at 120 to 160 V based on the length of the gel. DNA was detected using UV light, λ=254 nm or λ=366 nm to cut out specific fragments.

Loading Buffer (6x in water) MBI Fermentas, St. Leon-Rot, Germany

50x TAE (1l)

50 x (pH 8.4)	1 x (pH 8.0)	
2 M Tris	40 mM Tris	242 g
Acetic acid (glacial)	20 mM acetic acid	57.1 ml
0.05 M EDTA	1 mM EDTA	100 ml 0.5M EDTA

Ethidium Bromide (stock): 10 mg/ml

3.2.2.6 Isolation of PCR fragments from agarose gels

The DNA fragments obtained by PCR and separated on 0.8% agarose gels and determined to be of the correct size were cut out from the gel by using a sharp razor blade. The PCR fragments were purified by using the QIAGEN Gel extraction kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol.

3.2.2.7 Recombinational cloning of the PCR fragments

To clone the resulting PCR product into the pDONR207, a BP reaction was performed. A typical BP reaction was set up in a reaction volume of 5 µl.

purified PCR product	2-3 µl
pDONR 207	1 µl
BP Clonase	1 µl
Ampuwa water	1 µl

The reaction mixture was incubated at room temperature for 4 to 5 hours and then transformed into *E. coli* DH10B by electroporation. Bacteria were plated on selective media. Positive clones were analysed by plasmid purification and restriction analysis. Note: the pDONR207 vector containing a subcloned viral ORF was renamed in pENTR207 (Gateway® nomenclature). The original pDONR207 comprised attB sites, whereas the recombination product comprised attL sites.

To subclone the viral ORFs from the pDONR207 into a destination vector, an LR reaction is performed. The LR reaction was set up in a reaction volume of 5 µl.

purified entry vector	1 µl
Destination vector	1 µl
LR Clonase	1 µl
Ampuwa water	2 µl

The reaction mixture was incubated at 37°C for 2 hours and then transformed into *E. coli* DH10B by electroporation. Positive clones were analysed by plasmid purification and restriction analysis.

3.2.2.8 Isolation of plasmid DNA in 96 well format

Bacterial colonies were picked from LB-plates and an overnight culture was grown at 37°C in a 96-well block containing LB supplemented with gentamycin. The next day, the 96-well block was centrifuged for 10 min at 4000 rpm to harvest the cells. After discarding the supernatant, cells were resuspended thoroughly in 300 µl of resuspension buffer. After that, lysis buffer was added to the cell lysate and mixed by inverting the 96-well block several times. After incubation of the lysates for 5 min at room temperature, 300 µl of neutralization buffer was added. The lysate was mixed again by inversion, and incubated on ice for 10 min. After this step, the cells in the 96 well block were centrifuged for 30 min at 4000 rpm. Then, 800 µl of supernatant was transferred into a fresh 96-well block. After addition of 580 µl of isopropanol, the cells were kept at –20°C for one hour and then centrifuged for 45 min at 4000 rpm at 4°C to precipitate the DNA. The pelleted DNA was washed briefly with 500 µl of 75% ethanol and then air-dried for 20 min. Finally 50µl of sterile water was added to resuspend the plasmid DNA.

3.2.2.9 Restriction analysis of pENTR207 (entry vector) clones

The restriction digest of the pENTR207 entry clones was set up in a reaction volume of 30 µl.

Reaction mixture:

pENTR207 miniprep DNA	5 µl
buffer 4	3 µl
RNase (1 mg/ml)	0.5 µl
BSA	0.3 µl
BanII	0.3 µl
sterile water	20.9 µl

The restriction analysis was performed using the BanII enzyme, since BanII restriction releases the subcloned ORF fragment out of the pENTR207 vector backbone. The restriction mixture was placed in 96 well ELISA plates, sealed with plastic tape, and incubated for 3 hours at 37°C. After restriction, the plasmid DNAs were separated on a 0.8% agarose gel, visualized and documented.

3.2.2.10 Restriction analysis of destination vector clones

The restriction digest of the pENTR207 entry clones was set up in a reaction volume of 30 µl.

Reaction mixture:

miniprep DNA	5 µl
buffer	3 µl
RNase (1 mg/ml)	0.5 µl
BSA	0.3 µl
HindIII	0.3 µl
XbaI	0.3 µl
sterile water	20.9 µl

The restriction analysis was performed by HindIII and XbaI since these enzymes release the subcloned ORF fragment out of the pENTR207 vector backbone. The restriction mixture was placed in 96 well ELISA plates, sealed with plastic tape, and

incubated for 3 hours at 37°C. After restriction, the plasmid DNAs were separated on a 0.8% agarose gel, visualized and documented.

3.2.3 Tissue culture

3.2.3.1 MeWo cell culture and infection with p-Oka strain

MeWo cells were cultured on MEM medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and µg/ml fungitone at 37°C and 5% CO_2. The MeWo cell cultures were propagated by detaching them with trypsin and seeding the cells in 1:4 ratios. By adding p-Oka infected MeWo cells to uninfected MeWo cells at a ratio of 1:50 the virus was propagated.

3.2.3.2 Immunofluorescence microscopy

Uninfected MeWo and infected MeWo cells were cultured in the ratio of 1:50 on glass coverslips for 48h. At 48h post infection coverslips were washed once with phosphate buffered saline (PBS) and fixed for 15min using 2% formaldehyde (FA). Cells were washed twice with PBS. Permeabilization was done by incubation with icecold PBS containing 0.5% TritonX-100 for 5min followed by a wash step with PBS. After this, the cells were blocked with PBS containing 10% goat serum and 0.3% TritonX-100 for 1h. Then the cells were incubated with primary antibody (monoclonal antibody supernatant obtained from positive B cell fusions) supplemented with 3% goat serum and 0.3% TritonX-100 for 3h at RT. The cells were washed thrice for 15 min and subsequently incubated with secondary antibody (goat anti-mouse immunoglobulin G [IgG]-Alexa Fluor 488-conjugated antibody) diluted 1:400 in PBS containing 3% goat serum and 0.3% TritonX-100. Nuclei were counterstained with DAPI (4-6 diamidino-2-phenylindole) and the cells were washed thrice for 15min using PBS. Finally, coverslips were mounted onto glass slides with mounting medium (vectra shield) and analysed using a fluorescence microscope at a magnification of 40X.

3.2.4 Protein Techniques

3.2.4.1 Purification of recombinant His-tagged VZV proteins

20 ml of overnight culture of BL21 RIL cells containing VZV ORFs in expression vectors were inoculated into 1l of selective LB medium (50 µg/ml of ampicillin, 50 µg/ml of chloramphenicol) were grown at 37°C until an OD_{600} of 0.6 was reached. 10 ml of 1mM IPTG was added and cells were cultured for 4 to 6 hours at 37°C.

Bacterial cells were harvested by centrifugation for 30 min at 4000 rpm and the pellet was resuspended in 40 ml of lysis buffer (50mM Tris-HCl pH 6.8, 0.3M NaCl, 6M guanidinium hydrochloride, 5mM imidazole). Then the bacterial suspension was sonicated 3 times for 2min (output control level 6) on ice and shaken at room temperature for 1h. The bacterial suspension was centrifuged for 30 min at 16,000g and the supernatant was collected by centrifugation. Simultaneously 1ml of Ni-NTA agarose beads were equilibrated by washing with Urea buffer w/o imidazole. Equilibrated Ni-NTA agarose beads were incubated with supernatant for 30min by shaking and the suspension was loaded onto the column. Then the flowthrough was discarded and the column was washed 4x using 10ml of Urea buffer w/o imidazole, 3x with 10ml of Urea buffer containing 10mM imidazole (50mM Tris-Hcl pH 6.8, 0.3M NaCl, 8M urea, 10mM imidazole) and 2x with 10ml of Urea buffer containing 50mM imidazole (50mM Tris-Hcl pH 6.8, 0.3M NaCl, 8M urea, 50mM imidazole). Finally the protein was eluted 3x with 500 µl of elution buffer (50mM Tris-Hcl pH 6.8, 0.3M NaCl, 4M urea, 450mM imidazole). Eluted fractions and samples of solutions were analysed by SDS-PAGE.

Urea Buffer w/o imidazole (1l)

50 mM Tris-Hcl pH 6.8	6.05 g
0.3 M NaCl	18 g
8 M urea	180 g

3.2.4.2 Sample preparation for SDS-PAGE

Bacterial cell lysates containing expressed proteins, purified proteins, MeWo cell lysates either uninfected or VZV infected were resuspended in 2xSDS protein sample buffer and heated for 5 min at 95°C. After cooling the samples were centrifuged for 1 min at 14,000 rpm and stored at -20°C.

3.2.4.3 SDS-PAGE

Gel electrophoresis was performed with minigels using the Protean II system (Bio-Rad) with 6 to 15% gels (80 x 50 x 1 mm). The separating gel was mixed and poured between the glass plates and covered with distilled water. After polymerisation the distilled water was removed. Then the stacking gel was poured and the comb was

inserted. The samples were loaded on the gel along with the protein marker. The separation was done for 1 hour at 120 V.

Separation gel:

components	6%	8%	12%	15%
Acrylamide/ Bisacrylamide (37.5:1)	2.68 ml	2.35 ml	1.67 ml	1.17 ml
1.5 M Tris pH 8.8	1.25 ml	1.25 ml	1.25 ml	1.25 ml
10 % SDS	50 µl	50 µl	50 µl	50 µl
Distilled water	1 ml	1.32 ml	2.0 ml	2.5 ml
10 % APS	25 µl	25 µl	25 µl	25 µl
TEMED	2.5 µl	2.5 µl	2.5 µl	2.5 µl

Stacking gel:

Components	5%
Acrylamide/ Bisacrylamide (37.5:1)	1.35 ml
1.5 M Tris pH 8.8	0.625 ml
10 % SDS	25 µl
Distilled water	1.53 ml
10 % APS	12.5 µl
TEMED	2.5 µl

Electrophoresis buffer (10x) (1l):

50 mM Tris	60.6 g
384 mM Glycine	288.0 g
0.1% SDS	20.0 g

3.2.4.4 Western Blot

The proteins separated on the SDS-Gel were transferred to nitrocellulose membranes using the Trans-Blot wet Transfer Cell (Bio-Rad). The SDS-gel was directly placed on the nitrocelluose membrane and two pieces of Whatman filter paper were placed on either side of them. The internal air bubbles were removed by rolling the smooth glass rod on the top of the sandwich. The proteins on the gel were transferred to the membrane at 120 V for one hour. Proteins transferred were confirmed by incubating the nitrocelluose membrane in ponceau solution. The

nitrocelluose membrane was blocked in 5% milk solution for one hour. Then the membrane was incubated with primary antibody for one hour. The membrane was washed thrice with PBS for 10 min. After that, the membrane was incubated with horse radish peroxidase conjugated goat anti-mouse secondary antibody (1:2500 in PBS) and washed three times with PBS for 30min. The blotted proteins were visualized by ECL according to the manufacturer's instructions. The membrane was exposed to BIOMAX-MR autoradiography films for different time periods and films were developed using an automatic film developing machine.

Transfer buffer (1l)

Tris base	5.8 g
Glycine	2.9 g
SDS	0.37 g
Methanol	200 g
H_2O	ad 1l

Ponceau solution (100 ml)

Ponceau S	0.5 g
Glacial acetic acid	1 ml
H_2O	98.5 ml

3.2.5 Generation of monoclonal antibodies

3.2.5.1 Immunization

50 micrograms of protein or peptide were dissolved in PBS and mixed with the complete Freund´s adjuvant (CFA) in a ratio of 1:1 (e.g. 400 µl of PBS / protein + 400 ul CFA). Mouse were immunized subcutaneously. After 15 days the same amount of protein mixed with incomplete Freund´s adjuvant was mixed and used for immunization of the mouse by injecting 2/3 of the volume subcutaneously and 1/3 intraperitoneally. After 10 days blood was drawn from the tail vein and the titer of antibodies in the sera was determined by ELISA. In the case of a low titer, a 3rd immunization was applied). 3 to 5 days before the spleen was removed for hybridoma fusion mice were injected with 50µg of protein dissolved in PBS ("boost").

3.2.5.2 Fusion

SP2/o cells were expanded 5 days before the scheduled fusion. The spleen was isolated under sterile conditions and homogenized in cold RPMI. Then the cell suspension was centrifuged at 1500 rpm for 5 min at RT. The supernatant was removed and the cells were resuspended in 1 ml of RPMI. In the same time SP2/o cells were harvested, centrifuged, resuspended in 10 ml of warm RPMI w/o HEPES and counted. SP2/o cells and splenocytes were then mixed in a 1:1 ratio, centrifuged and the supernatant was removed. Blue cap containing cells were placed into the glass, prefilled with warm water in the 37°C water bath. In a dropwise manner 1 ml of pre-warmed PEG was added throughout 1 minute, mixing it continuously. After the addition of PEG, mixing was continued for one more minute. In a dropwise manner 1 ml of pre-warmed RPMI was added throughout 1 minute, continuously mixing. Then again 1 ml of RPMI (w/o HEPES) was added. Add 7 ml of warm RPMI (w/o HEPES) in a dropwise manner throughout 2-3 min. Then the cells were centrifuged at 800 rpm for 5 min. Supernatant was removed and 10ml of 20% warm RPMI with HAT was added and care is taken not to resuspend the cells. The suspension is aliquoted into 96-well plates (200 ul/well) and the cells were grown in the incubator (37°C, 5% CO_2). The wells were observed to follow the growth of hybridoma cells by microscopy. Then the cells were feeded for every few days by replacing the old media with new 20% RPMI containing HAT. The supernatant of growing hybridomas were tested after 12 days post fusion by ELISA. Finally, motherwells that recognised the protein of interest were expanded.

3.2.5.3 Cloning of Mother wells

For cloning of mother well clones, the cells were taken from 1 well of a 24-well plate and counted. The cell number was adjusted by serial dilution so that a) 30 cells/well, b) 3 cells/well, c) 0.3 cells/well were plated per well (volume per well is 200 ul). Each well in the 96-well plate was filled with these suspensions. One half of the 96-well plate was filled with suspension a) while the other half was filled with suspension b), suspension c) was again plated on a half of a 96-well plate. The cells were placed in the incubator, their growth was observed under the microscope and finally, the supernatant produced by hybridomas singled out on 0.3 cells / plate were tested by ELISA.

3.2.5.4 ELISA

ELISA is performed on plates coated with the antigen of interest. Supernatant of the growing hybridoma is added to the coated plates in duplicates and incubated for 1h at RT. Excess of antibodies is washed off and secondary antibody is added (goat anti-mouse IgG conjugated to peroxidase) and incubated for 1h at RT. After washing, the ELISA plates were incubated with substrate (OPD pill is dissolved in 50 ml of citrate buffer and 50 ul of 6% H_2O_2) for 5 to 10 min in the dark. The reaction is stopped by the addition of 50 ul of 1M H_2SO_4 (per well). ELISA plates were read on the spectrophotometer (492 nm/630 nm).

3.2.6 Epitope mapping

Peptides consisting of 15 residues were spotted on the cellulose membrane with each peptide shifted in sequence by 3 residues. Subsequently, the membrane was first incubated with methanol for 10min, then washed thrice with TBS-T for 10min. Unspecific binding was blocked for 3h with 2% milkpowder in 0.2% TBS-T. Followed by this, the membrane was washed for 10min with TBS-T (0.05% Tween) and incubated with primary antibody for 3h (monoclonal antibody supernatant obtained from positive B cell fusions raised against VZV ORFs). Then the membrane was washed thrice for 10min using TBS-T (0.05% Tween). After this, incubation with secondary antibody was performed for 1.5h (goat anti-mouse antibodies conjugated to alkaline phosphatase at a dilution of 1:3000) and the membrane was washed thrice for 10min using PBS-T. The immunodominant region (epitope) was detected using the ECL detection system (Amersham-Pharmacia/GE Healthcare) according to the manufacturer's instructions. Finally, the membrane was exposed to BIOMAX-MR autoradiography films for different periods of time and films were developed using an automatic film developing machine.

3.2.7 Computer-assisted prediction of nuclear transport signals

To predict the nuclear localisation of all VZV proteins, the presence of putative nuclear localisation signals (NLS) was determined using http://cubic.bioc.columbia.edu/predictNLS/ (NLS) and http://www.expasy.org/cgi-bin/nicedoc.pl?PDOC00015 (bipartite NLS). The presence of putative nuclear export sequences (NES) was determined using http://www.cbs.dtu.dk/services/NetNES/.

4. Results

4.1 Generation of VZV expression libraries

To generate antibodies for all ORFs of VZV, expression libraries of VZV were constructed where the encoded proteins were equipped with an N-terminal His, N-terminal MBP, N-terminal GST, or C-terminal His tag.

4.1.1 Amplification of VZV ORFs by nested PCR

For the construction of the VZV ORFeome, primers were designed for amplification of all 70 ORFs in full length. In addition, primers were also designed to amplify fragments (29) of some of the ORFs. ORFs with cytoplasmic or luminal domains of transmembrane proteins or subfragments of very long genes (>3kb) were selected for fragmented amplification.

First, each ORF was amplified by nested PCR in two steps. In the first PCR, the ORF was amplified by using gene-specific forward and reverse primers containing partial attB regions. A characteristic primer pair is shown in Figure 13. The second PCR was performed by using complete attB (common to all ORFs) primers in order to attach full length attB sites to the viral genes pre-amplified in the first PCR. Then the amplified PCR products were separated on 0.8% agarose gels and the pre-determined size of the amplicons was verified. Finally, the amplified and verified PCR products containing complete attB sites were compatible for Gateway® cloning. All the 70 ORFs along with 29 partial ORFs (some encoding N and C terminal domains) comprising 99 amplicons in total, were amplified successfully [32].

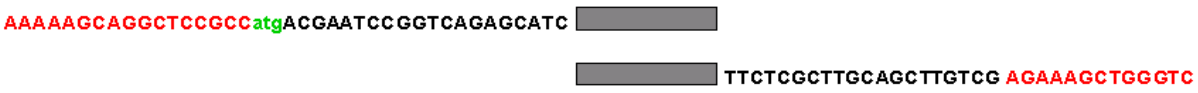


Figure 13: A PCR-product with VZV specific forward and reverse primers and partial attB sites.

The sequence on the left hand side represents the forward primer, the sequence in black represents the complementary sequence to the respective ORF, indicated in green is the start codon while the sequence in red represents the partial attB1 region. The sequence on the right hand side is the reverse primer. In black the sequence complementary to the respective ORF is indicated while the partial attB2 region is

shown in red. By adding primers containing complete attB regions in the second PCR round the respective ORFs were amplified.

4.1.2 Construction of the Gateway® compatible ORFeome in the pDONR207 vector

After PCR amplification of all VZV genes they were cloned into the Gateway® compatible pDONR207 vector by BP recombinational cloning. The BP reaction mixture was transformed into DH10B cells and selected on LB plates containing gentamycin. The positive clones were screened by isolating plasmids from DH10B cells and analysing them by restriction digest using the BanII enzyme (Figure 14). Finally, 99 clones encoding all VZV ORFs either in full length or fragments were cloned into the vector pDONR207. The VZV ORFeome cloned in pDONR207 (entry vector) is also called the VZV entry library. The positive clones were verified by sequencing.

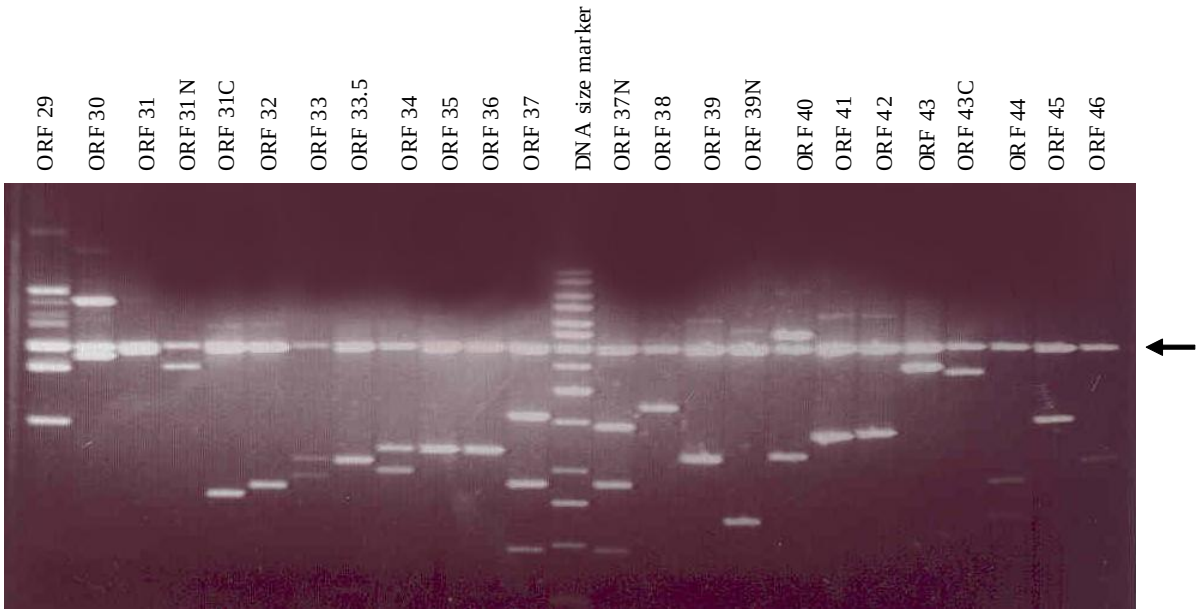


Figure 14: Restriction analysis of the VZV entry library. The restriction analysis of selected pENTR207 plasmids containing the subcloned viral ORFs or ORF fragments is displayed. The restriction analysis was done by BanII digestion, and analysed on a 1% agarose gel. The arrow represents the vector backbone and DNA ladder is as follows from the bottom (0.25, 0.5, 0.75, 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8 and 10 kbp).

4.1.3 Sequence analysis of the VZV ORFeome in pDONR207 vector

The entry clone sequences were analysed using the pDRAW32 software. The respective individual ORF sequences were translated into protein sequences, and

compared to the “original” viral protein sequences available in the NCBI database. All the 99 entry clones were found to be correct by sequencing (100 % alignment to the “original” DNA sequence).

4.1.4 Subcloning of entry clones into expression vectors

The entry clones of the VZV ORFeome can be cloned into any Gateway® compatible destination vector. For protein expression of VZV proteins in *E. coli* cells, an LR reaction was performed to subclone the VZV ORFeome into the Gateway® compatible pETG expression vectors providing N-terminal His, N-terminal MBP, N-terminal GST, or C-terminal His tags to the protein of interest. The positive clones were screened by isolating plasmids from DH5α cells and analysing them by restriction digest using HindIII and XbaI enzymes. Thus, three complete expression libraries encoding Orfs with N-terminal His, N-terminal MBP, N-terminal GST and a partial library encoding C-terminal His tagged proteins were constructed. As the expression libraries contain different resistance genes, the subcloning could be performed in a one-step reaction. The status of the VZV ORFs that were cloned from the VZV ORFeome to four different expression vectors (N-His, N-MBP, N-GST and C-His) is listed in Table 4.

Table 4: Status of clones in expression libraries

VZV (ORF)	position (nt)	pDONR207	N-His	N-MBP	N-GST	C-His
Orf1	1-327	**XX**	**XX**	**XX**	**XX**	**XX**
Orf1N	1-222	**XX**	**XX**	**XX**	**XX**	**XX**
Orf2	1-717	**XX**	**XX**	**XX**	**XX**	**----**
Orf3	1-540	**XX**	**XX**	**XX**	**XX**	**XX**
Orf4	1-1359	**XX**	**XX**	**XX**	**XX**	**No**
Orf5	1-1023	**XX**	**XX**	**XX**	**XX**	**XX**
Orf5 F	396-642	**XX**	**XX**	**XX**	**XX**	**No**
Orf6	1-3252	**XX**	**XX**	**XX**	**XX**	**XX**
Orf7	1-780	**XX**	**XX**	**XX**	**XX**	**XX**
Orf8	1-1191	**XX**	**XX**	**XX**	**XX**	**XX**
Orf9	1-909	**XX**	**XX**	**----**	**XX**	----
Orf9a	1-264	**XX**	**XX**	**XX**	**XX**	**XX**
Orf9a N	1-159	**XX**	**XX**	**XX**	**XX**	**XX**
Orf10	1-1233	**XX**	**XX**	**XX**	**XX**	**XX**
Orf11	1-2460	**XX**	**XX**	**XX**	**XX**	**XX**
Orf12	1-1986	**XX**	**XX**	**XX**	**XX**	**XX**
Orf12 N	1-894	**XX**	**XX**	**XX**	**XX**	**XX**
Orf12 C	1065-1986	**XX**	**XX**	**XX**	**XX**	**XX**

Orf13	1-906	XX	XX	XX	XX	XX
Orf14	1-1683	XX	XX	XX	XX	XX
Orf14 N	1-1593	XX	XX	XX	XX	----
Orf15	1-1221	XX	----	----	----	----
Orf15 N	1-141	XX	XX	XX	XX	----
Orf15 F	609-810	XX	XX	XX	XX	XX
Orf16	1-1227	XX	XX	XX	XX	XX
Orf17	1-1368	XX	XX	XX	XX	XX
Orf18	1-921	XX	XX	XX	XX	XX
Orf18 N	1-450	XX	XX	XX	XX	XX
Orf18 C	507-921	XX	XX	XX	XX	XX
Orf19	1-2328	XX	XX	XX	XX	XX
Orf20	1-1452	XX	XX	XX	XX	XX
Orf21	1-3	XX	----	----	XX	----
Orf21.1	1-1000	XX	XX	XX	XX	----
Orf22 N	1-4119	XX	XX	XX	XX	----
Orf23	1-708	XX	XX	XX	XX	----
Orf24	1-810	XX	XX	XX	XX	----
Orf24 N	1-735	XX	XX	XX	XX	----
Orf25	1-471	XX	XX	XX	XX	----
Orf26	1-1758	XX	XX	XX	XX	----
Orf27	1-1002	XX	XX	XX	XX	XX
Orf28	1-3585	XX	XX	XX	XX	----
Orf29.1	1-3615	XX	XX	XX	XX	----
Orf29.3		XX	XX	XX	----	----
Orf30	1-2313	XX	XX	XX	XX	----
Orf31	1-2607	XX	XX	XX	XX	----
Orf31 N	1-2175	XX	XX	XX	XX	----
Orf31 C	2250-2607	XX	XX	XX	XX	----
Orf32	1-432	XX	XX	XX	XX	----
Orf33	1-1818	XX	XX	XX	XX	----
Orf33.5	909-1818	XX	XX	XX	XX	----
Orf34	1-1740	XX	XX	XX	XX	----
Orf35	1-777	XX	XX	XX	XX	----
Orf36	1-126	XX	XX	XX	XX	XX
Orf37	1-2526	XX	XX	XX	XX	----
Orf37 N	1-2409	XX	XX	XX	XX	XX
Orf38	1-1626	XX	XX	XX	XX	XX
Orf39	1-723	XX	XX	XX	XX	----
Orf39 N	1-237	XX	XX	XX	XX	----
Orf40	1-4191	XX	XX	XX	XX	----
Orf41	1-951	XX	XX	XX	XX	----
Orf42	1-966	XX	XX	XX	XX	XX
Orf43	1-2031	XX	XX	XX	XX	----
Orf43 C	141-2031	XX	XX	XX	XX	----
Orf44	1-1092	XX	XX	XX	XX	----
Orf45	1-1071	XX	XX	XX	XX	XX
Orf46	1-600	XX	XX	XX	XX	XX
Orf47	1-1533	XX	XX	XX	XX	XX
Orf48	1-1656	XX	XX	XX	XX	XX
Orf49	1-246	XX	XX	XX	XX	XX
Orf50	1-1308	XX	XX	XX	XX	----
Orf50 C	1074-1308	XX	XX	XX	XX	XX

Orf51	1-2508	**XX**	**XX**	**XX**	----	----
Orf52	1-2316	**XX**	**XX**	**XX**	----	----
Orf53	1-996	**XX**	**XX**	**XX**	**XX**	**XX**
Orf54	1-2310	**XX**	**XX**	**XX**	**XX**	----
Orf55	1-2646	**XX**	**XX**	**XX**	**XX**	----
Orf56	1-735	**XX**	**XX**	**XX**	**XX**	----
Orf56 C	183-735	**XX**	**XX**	**XX**	**XX**	**XX**
Orf57	1-216	**XX**	**XX**	**XX**	**XX**	**XX**
Orf58	1-666	**XX**	**XX**	**XX**	**XX**	**XX**
Orf59	1-918	**XX**	**XX**	**XX**	**XX**	----
Orf60	1-480	**XX**	**XX**	**XX**	**XX**	----
Orf60 C	87-480	**XX**	**XX**	**XX**	**XX**	----
Orf61	1-1404	**XX**	**XX**	**XX**	**XX**	----
Orf62	1-3933	**XX**	**XX**	**XX**	**XX**	----
Orf63	1-837	**XX**	**XX**	**XX**	**XX**	----
Orf64	1-543	**XX**	**XX**	**XX**	**XX**	----
Orf65	1-309	**XX**	**XX**	**XX**	**XX**	----
Orf65 N	1-222	**XX**	**XX**	**XX**	**XX**	----
Orf66	1-1182	**XX**	**XX**	**XX**	**XX**	----
Orf67	1-1065	**XX**	**XX**	**XX**	**XX**	----
Orf67 N	1-816	**XX**	**XX**	**XX**	**XX**	----
Orf67 C	882-1065	**XX**	**XX**	**XX**	**XX**	----
Orf68	1-1872	**XX**	**XX**	**XX**	**XX**	----
Orf68 F	72-1611	**XX**	**XX**	**XX**	**XX**	----
Orf68 C	1680-1872	**XX**	**XX**	**XX**	**XX**	----
S/L		**XX**	**XX**	**XX**	**XX**	----
S/L-C	1-954	**XX**	**XX**	**XX**	**XX**	----
S/L-MKKVSV	234-954	**XX**	**XX**	**XX**	**XX**	----

4.2 Expression and purification of VZV proteins

Compared to other human herpes viruses, VZV research is progressing slowly. In order to study VZV proteins both biochemically and functionally, it is necessary to generate monoclonal antibodies against all VZV proteins. These monoclonal antibodies are indispensable molecular detection tools used in a vast number of techniques. To raise mabs, first of all, the expression of all VZV proteins cloned in N-His, C-His and N-MBP expression vectors was verified. To test for expression, the vectors containing VZV ORFs with N-His, C-His and N-MBP tags were transformed into bacterial BL21RIL cells. Single bacterial colonies were grown in liquid LB media supplemented with antibiotics and at an OD_{600} of 0.5 induced with 1 mM IPTG. Expression of the VZV proteins was verified by Western blotting using α-His, α-MBP, or anti-GST antibodies. Once the expression was confirmed, the positively tested bacterial strains were cultured in large scale (1 liter). Bacterial pellets were collected, taken up in lysis buffer and sonified. After centrifugation of the debris, His- and MBP-tagged proteins were purified from the supernatant via affinity chromatography using

Ni-NTA-agarose or amylose beads. The purified proteins were analysed once again with α-His and α-MBP antibodies. All proteins of the VZV genome could be expressed while only 65 VZV proteins could be purified efficiently. Of the 65 VZV purified proteins, 36 VZV proteins were purified by using an N-terminal His-tag, 14 VZV proteins were purified based on their C-terminal His-tag, and 15 VZV proteins were purified by N-terminal MBP tags (Table 5).

Table 5: Status of purified proteins with different tags

Type of tag	No of proteins
N-His	36
C-His	14
N-MBP	15

4.3 Generation of monoclonal antibodies against the VZV proteome

4.3.1 Immunisation of mice with purified proteins and peptides

To generate monoclonal antibodies, purified VZV proteins were used for immunisation of Balb/c mice. Per immunization 50 micrograms of protein were injected. Normally two doses were given but if the antibody titer remained below the titration level a third booster dose was applied. This was done in collaboration with Stipan Jonjic, University of Rijeka, Croatia. Mice were immunised with 65 VZV proteins equipped with different tags (Table 6). In addition, for 6 ORFs peptides were generated and used for immunization. Out of 65 VZV proteins, 36 VZV proteins contained N-terminal His tags, 14 VZV proteins contained C-terminal His tags and 15 VZV proteins contained MBP tags. For ORF37 and ORFS/L (ORF0), immunisation was done using N-terminally His-tagged proteins as well as peptides. The hybridoma supernatants that were tested positive in ELISA (against specific antigens used for immunisation) were screened finally both by Western blotting and indirect immunofluorescence.

Table 6: Status of immunisation

Material used for immunisation	No of proteins
N-His	36
C-His	14

N-MBP	15
peptides	6

4.3.2 Screening of hybridoma supernatants by Western blotting

The hybridoma supernatants of mother wells (MW) and subclones that were tested positive on ELISA were screened again by Western blotting to confirm and finalise that the subclones are producing antibodies specific to VZV ORFs. Infected and uninfected MeWo cell lysates were generated, run in parallel on SDS gels and blotted onto nitrocellulose membrane. Subsequently, the nitrocellulose membranes were cut into small stripes so that each stripe contained both infected and uninfected MEWO lysates adjacently. These stripes were first incubated with hybridoma supernatants tested positively on ELISA and subsequently with horse radish peroxidase conjugated goat α-mouse secondary antibodies. The Western blots were processed by ECL and detected using a BIOMAX-MR film.

Antibodies in most of the tested hybridoma supernatants decorated bands that correlated with the predicted size of the respective VZV ORF. The predicted molecular weight (MW) of the proteins is summarized in Table 8. By Western blot screening we identified positive hybridoma clones for 57 VZV ORFs, the corresponding pictures are shown in Figure 15 with the exception of ORF60. In total, 47 of the 57 VZV ORFs (83%) migrated at the predicted MW, and the remaining 10 (17%) migrated aberrantly. This aberrant migration could be due to protein proteolysis, to alternative splicing, to posttranslational modification, or simply mixup of samples. Sofar, we have identified 190 antibodies produced by hybridoma clones which specifically detect VZV proteins in Western blotting (Table 9).

Figure 15: Testing of hybridoma supernatants with cell lysates of infected and uninfected MeWo cells by Western blot. Hybridoma supernatants were tested before and after subcloning to identify specific positive clones for all VZV ORFs. Lysates of MeWo cells infected with pOKA (1:50 uninfected to infected cells for 48hrs) and uninfected MeWo cells were generated and applied to SDS-PAGE adjacent to each other. Proteins were separated electrophoretically and subsequently transferred to nitrocellulose membranes. The membranes were cut into small stripes, each stripe containing both infected (as indicated by +) and uninfected protein lysates (as indicated by -). Each stripe was incubated with a supernatant containing primary antibodies originating from various hybridoma clones and then incubated with secondary peroxidize-conjugated goat anti-mouse antibodies. Finally, the stripes were processed by ECL and exposed to BIOMAX-MR films. Specific bands were indicated by stars. The molecular weight marker in kilodalton (MW in kDa) is indicated on the left. Depending on the predicted size of the VZV ORF, infected and uninfected MeWo cell lysates were run on different concentrations of gels as indicated in colored ORFs (blue: 6%, pink: 8%, green: 12%, black15%).

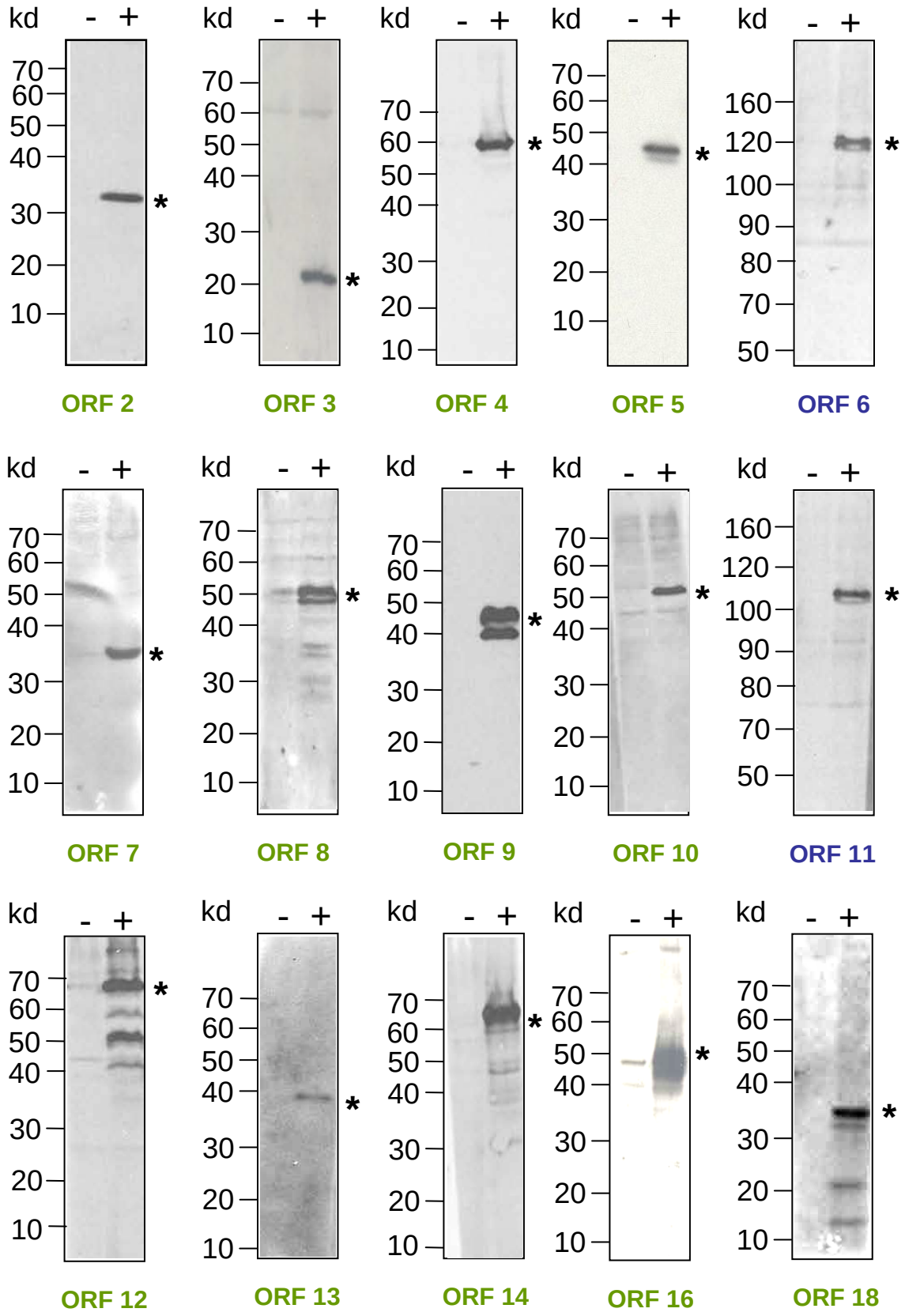


Figure 15 (for legend see above)

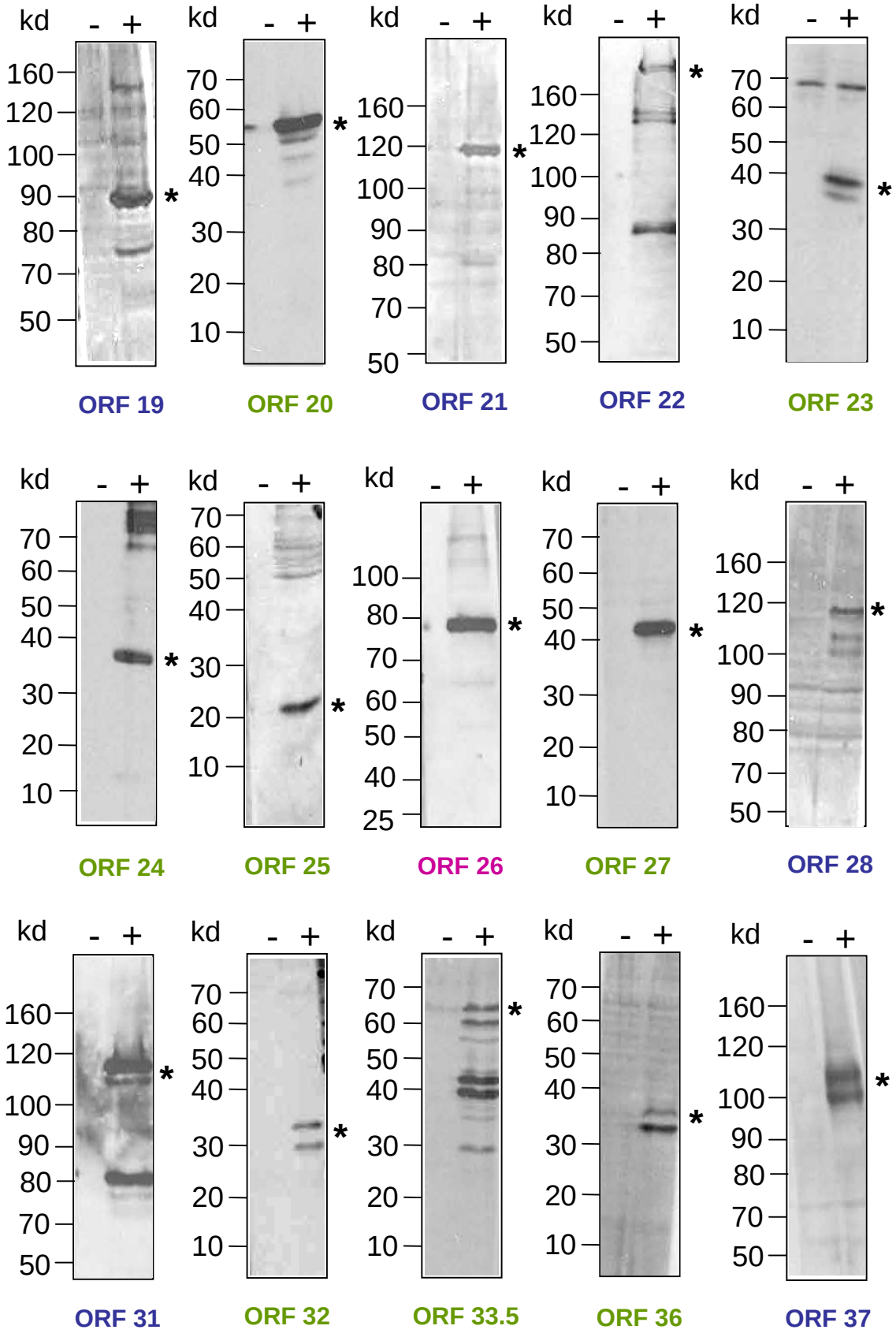


Figure 15 cont. (for legend see above)

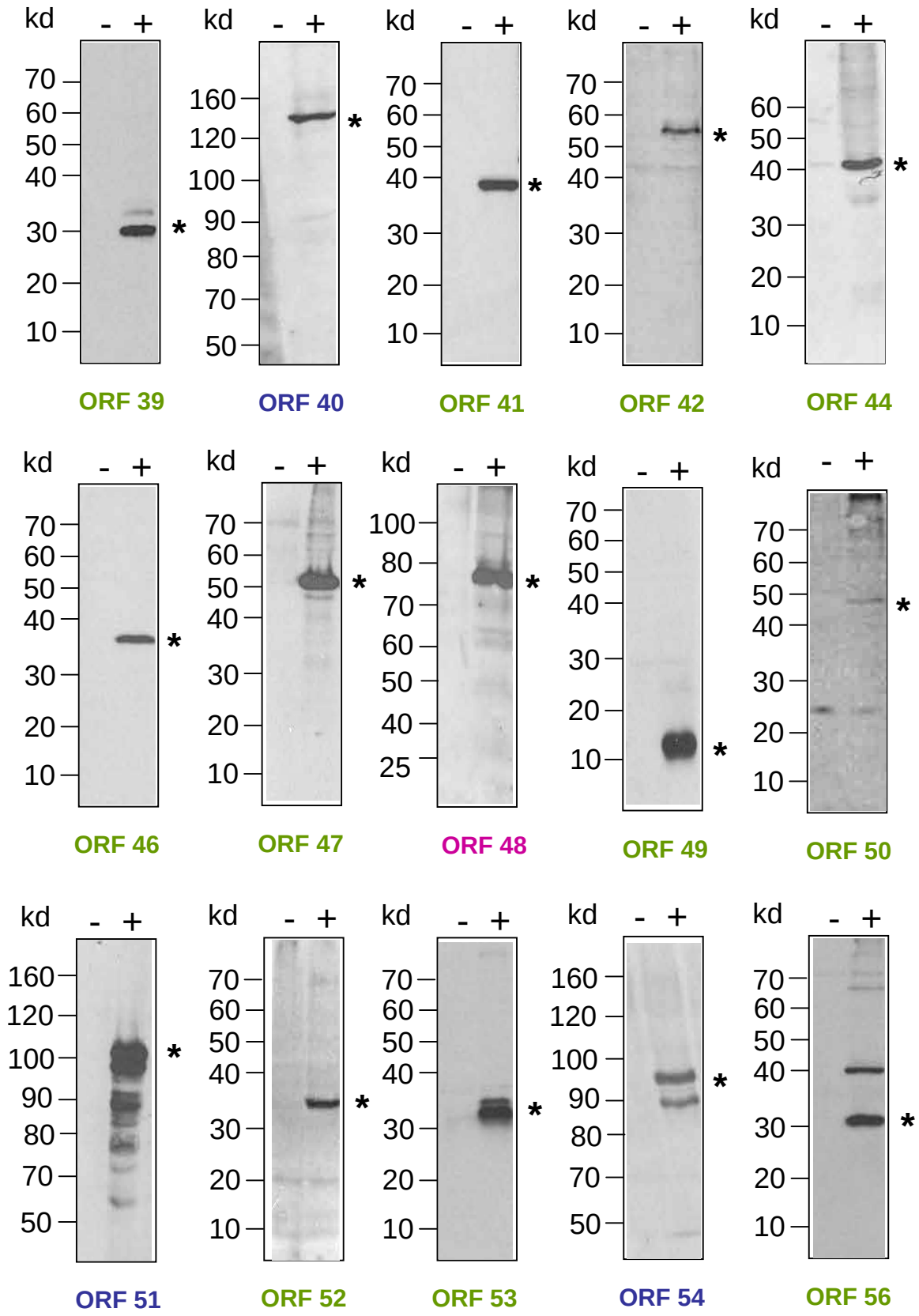


Figure 15 cont. (for legend see above)

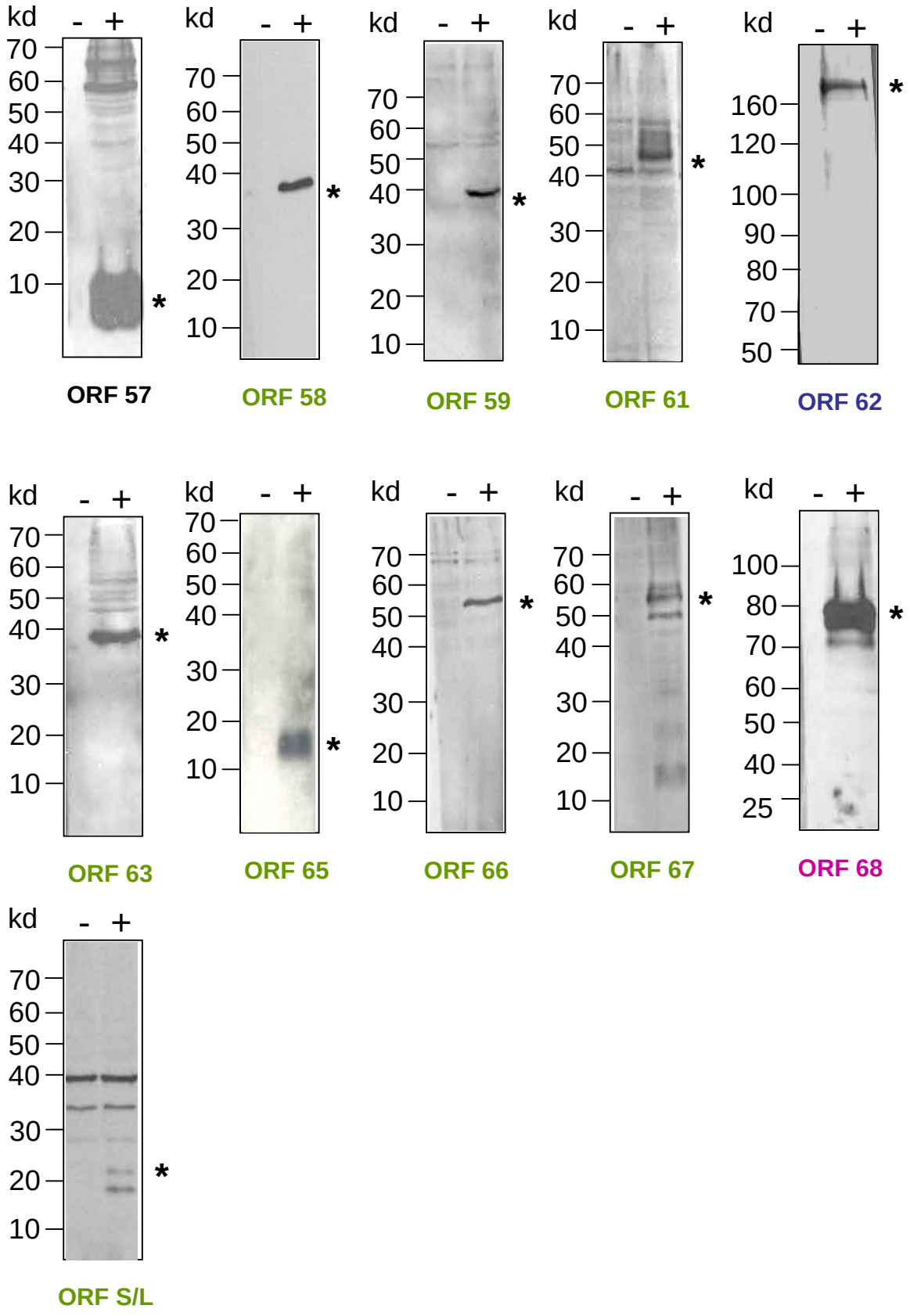


Figure 15 cont. (for legend see above)

4.3.3 Screening of hybridoma supernatants by indirect immunofluorescence

The hybridoma supernatants of mother wells (MW) and subclones that were tested positive on ELISA were screened again by indirect immunofluorescence in parallel to Western blotting to confirm and finalise the subclones that are producing antibodies specific to VZV ORFs. MeWo cells were used as a cell system for these studies because they can be infected *in vitro* and form syncytia that allow easy detection of infection-associated antigens. A syncytium is formed by fusion of a group of cells resulting in a shared and continuous cytoplasm and plasmamembrane and a ring of nuclei surrounding a concentration of Golgi bodies in the center of the syncytium [49],[50].

MeWo cells were seeded in the ratio of 1 infected cell per 50 uninfected cells and infection was allowed to proceed for 48 hours. Then the cells were fixed by 2% formaldehyde and permeabilized using 0.5% Triton-X 100 in PBS. Subsequently, the cells were incubated with ORF specific primary antibodies. The primary antibodies were detected by incubation with secondary Alexa 488-conjugating goat anti-mouse antibodies. Epifluorescence microscopy was used to detect whether the antibodies tested gave a specific signal and if yes, where in the infected cell the respective VZV protein was detected. With this approach antibody producing clones against VZV proteins were screened, detected and studied in VZV infected MeWo cells. In total, 123 mother wells which were producing VZV antibodies were screened positive covering 52 VZV ORFs (Table 9). In average every VZV ORF was covered by 3 to 4 different hybridoma clones.

4.3.4 Comparison of influence of acetone and formaldehyde on fixation

In order to determine whether the fixation / permeabilization agents affect the reactivity of the positive antibodies, we compared acetone and formaldehyde fixation. The affects of acetone and formaldehyde was tested on five different ORF specific antibodies (ORF4, ORF16, ORF62, ORF63, ORF66). Although an immunofluorescence signal was detected for all five antibodies using both fixation methods, a weaker signal was observed for the ORF62 and ORF63 antibodies upon use of acetone (Figure 16). So, for the subsequent immunofluorescence analysis, formaldehyde was generally used.

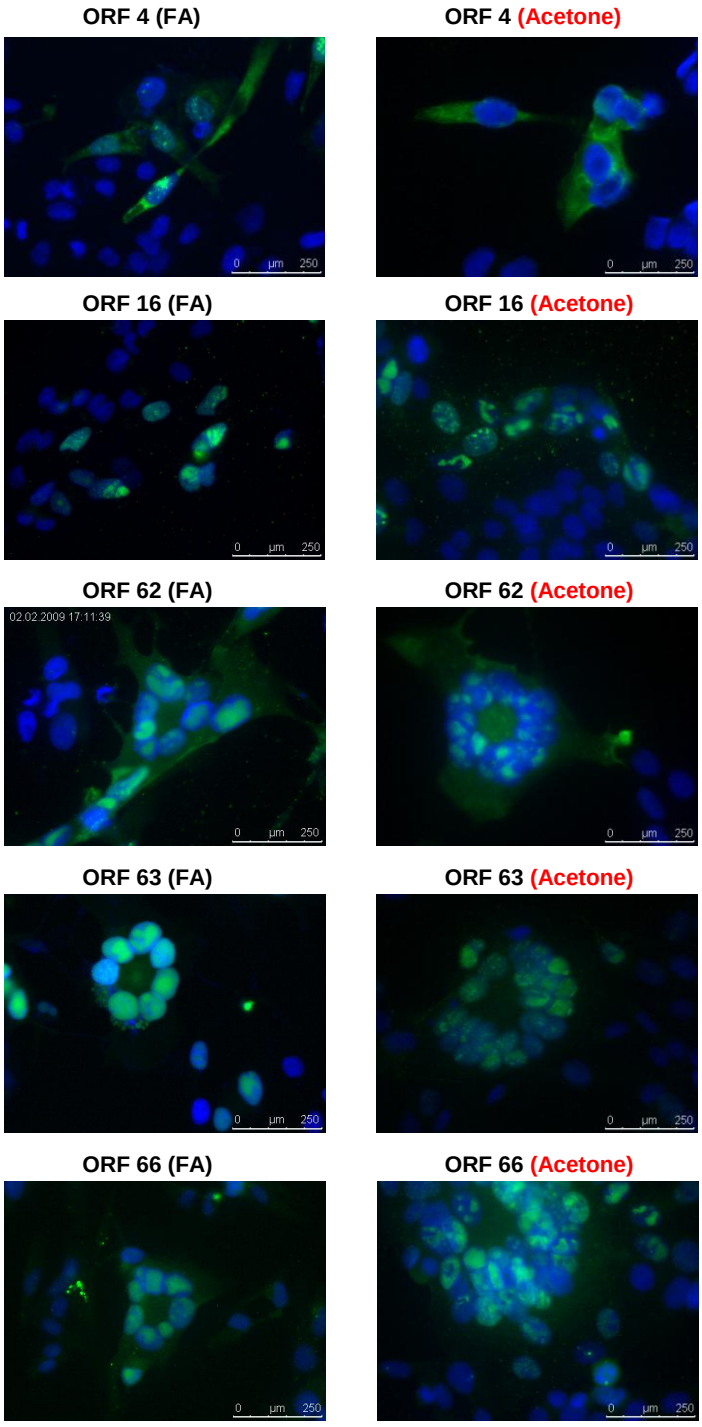


Figure 16: Comparison of influence of fixation reagents in immunofluorescence analysis. Monolayers of uninfected MeWo cells mixed with pOKA infected MeWo cells at a ratio of 50:1 were seeded on glass slides. Two days post infection, cells were fixed with 3% formaldehyde and permeabilized by 0.5 % TritonX-100 (left side). Alternatively, cells were fixed with icecold acetone (right side). Indirect immunofluorescence was performed using ORF specific mouse monoclonal antibodies followed by Alexa 488-conjugated secondary goat-anti mouse antibodies. DNA was visualised by DAPI staining. Pictures were obtained using an epifluorescence microscope (Leica). The bar shown on the bottom right hand side of each picture corresponds to 250µm and the same magnification was maintained in all panels. The DAPI (blue) and fluorescence (green) pictures were merged using Leica software.

4.3.5 Cellular localisation of all VZV proteins

In parallel to screening of positive hybridoma clones, subcellular localisation of the VZV proteins was studied using the same cell system. In order to obtain a general overview of the subcellular localisation of each protein, epifluorescence microscopy was applied. With this approach all VZV proteins could be clearly detected in numerous VZV infected single cells or syncytia. We have chosen to study the localisation separately in single cells and syncytia since changes in localisation of VZV proteins are expected to occur upon syncytial cell fusion and ongoing infection associated with formation of syncytia. Such a high-throughput localisation study in the context of VZV infection with ORF specific antibodies has not been attempted by any investigator sofar.

According to the localisation observed, the proteins could be classified into three groups: 1) cytoplasmic localisation, 2) nuclear localisation or 3) both cytoplasmic and nuclear localization. In total, we have obtained localisation data for 52 ORFs (74%) in syncytia and for 50 ORFs (71%) in single cells. In VZV infected syncytial cells, 20 ORFs were localised in the nucleus (38%), 16 ORFs were localised in the cytoplasm (31%) and 16 ORFs localised to both the nucleus and cytoplasm (31%). In VZV infected single cells, 18 ORFs were found in the nucleus (36%), 20 ORFs were located in the cytoplasm (40%) and 12 ORFs localised to both the nucleus and cytoplasm (24%). The summary of these results is presented in Figure 17, Figure 19 and Table 7. The subcellular localisation of each individual VZV ORF is shown in detail in Table 8. The immunofluorescence pictures of three ORFs (ORF2, ORF39, and ORF53) were not included in the data. We conclude from our observation that the subcellular localisation of 9 VZV proteins (ORF3, ORF8, ORF9, ORF12, ORF21, ORF27, ORF32, ORF57, ORF58) were altered between syncytia and single cells. An exceptional localization was observed for ORF24 and its partner protein ORF27 which both localised to the nuclear membrane in accordance with the localization of their orthologous proteins in HSV, CMV, EBV and KSHV. A picture obtained by confocal microscopy that shows the confinement of ORF24 to the nuclear periphery of syncytia is shown in Figure 18.

Of the 70 VZV proteins we could visualise, subcellular localisation of 30 VZV ORFs was published previously, and there is an excellent correlation between our data and previous reports (Table 8). In contrast, the subcellular localisation of 3 VZV ORFs (ORF37, ORF60, ORFS/L) was not in agreement with published data as is shown in

Table 8. The transmembrane glycoproteins ORF37 (gH) and ORF60 (gL) were published to localize to the ER, Golgi or to the plasmamembrane reflecting their biochemical properties and their association with the secretory pathway. Quite in contrast, the antibodies generated reacted with an antigen located to the nucleus. ORF10 and ORFS/L were previously reported to reside in the cytoplasm while we observed a nuclear signal. The reason for this discrepancy could be a mixup of samples, cross-reactivity of antibodies with an unrelated protein or less likely, sofar undetected properties of these proteins. Apart from these some minor differences were observed for ORF20 and ORF25. Both of these proteins were detected as cellular in our observation but other investigators have only found them in the cytoplasm.

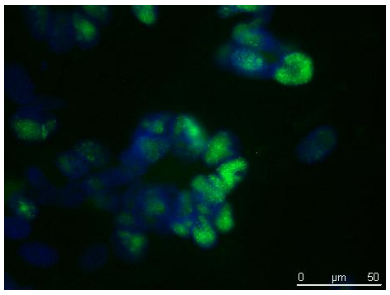
The localisation was also classified on the basis of different functions of proteins like structural and non-structural proteins. The structural proteins comprise glycoproteins, tegument and capsid proteins. The localisation of all VZV glycoproteins that were observed, were in excellent agreement with published data except for gH and gL as mentioned above. Six out of 8 tegument proteins could be localised. The subcellular localisation of 3 tegument proteins (ORF9, ORF21 and ORF65) coincided with studies of other researchers. ORF21 was found in the nucleus of single cells whereas in syncytia it was located to both the nucleus and cytoplasm. For 3 tegument proteins (ORF11, ORF12, and ORF22) no localisation was reported sofar and the localisation newly determined in this study is shown in Table 8. ORF22 is the largest tegument protein of the VZV proteome and found to localise to the cytoplasm. The subcellular localisation of 2 additional tegument proteins (ORF43 and ORF46) could not be observed by us and has also not been reported by anyone else. Two out of 4 capsid proteins (ORF20, ORF23, but not ORF40 and ORF41) could be localized by us. Both were found in the nucleus consistent with their function and previous reports. The scaffolding protein ORF33 localized similar to the capsid proteins ORF20 and ORF23. All transactivator proteins (ORF4, ORF61, ORF62, ORF63) except for ORF10 showed a localization consistent with previous data.

Table 7: Subcellular localization of VZV proteins in syncytia and single cells

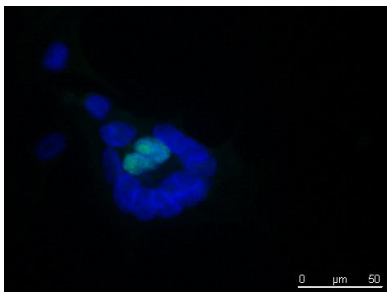
Cell type	Nucleus and cytoplasm	Cytoplasm	Nucleus
syncytia	16 ORFs	16 ORFs	20 ORFs
single cells	12 ORFs	20 ORFs	18 ORFs

a) Nuclear localization (Figure 17 cont.)

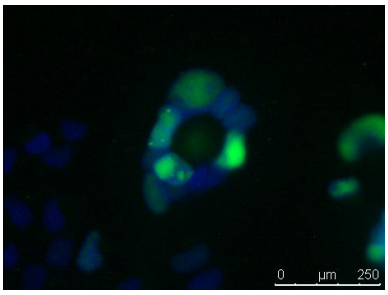
ORF6



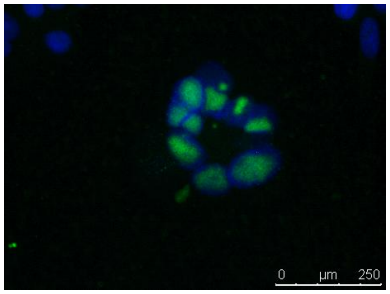
ORF10



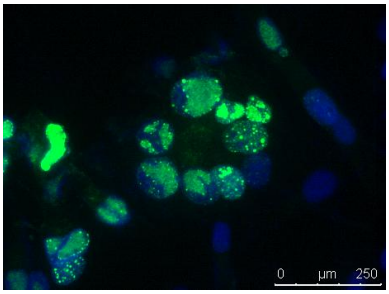
ORF16



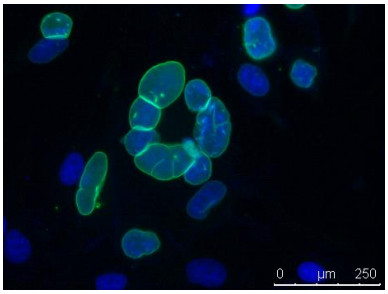
ORF17



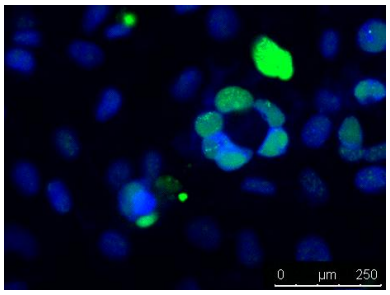
ORF23



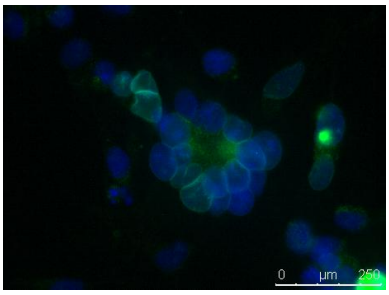
ORF24



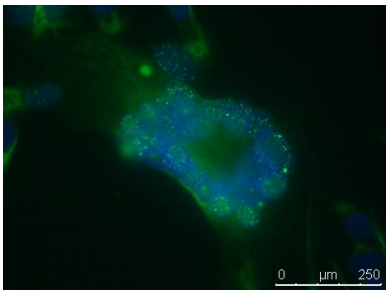
ORF26



ORF27



ORF29



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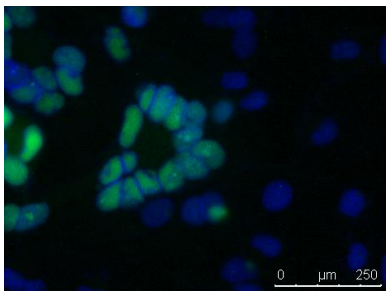

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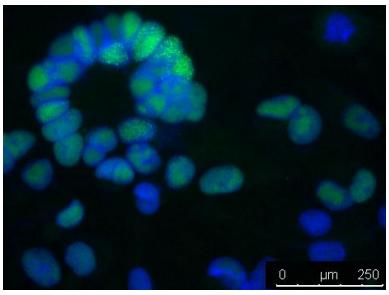

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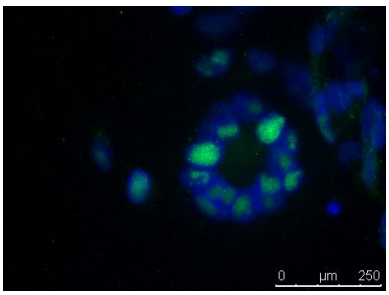

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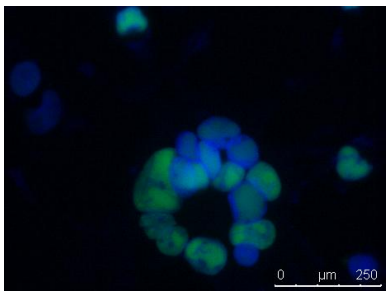

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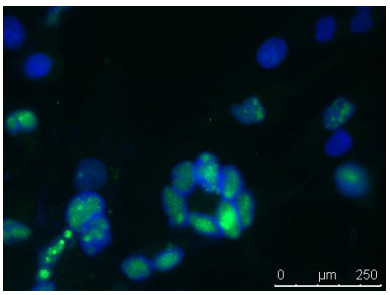

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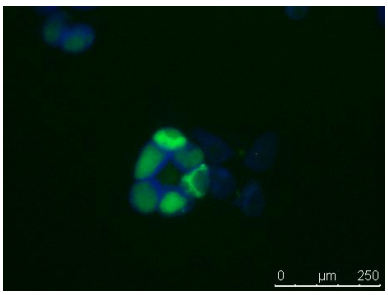

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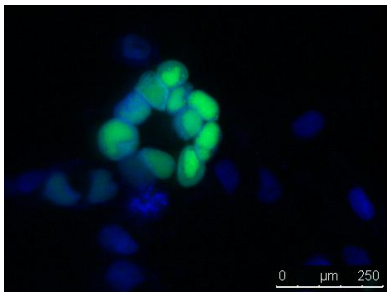

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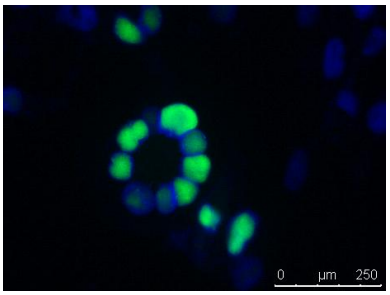

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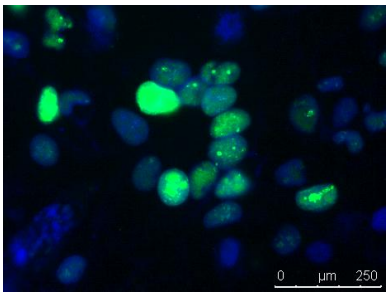

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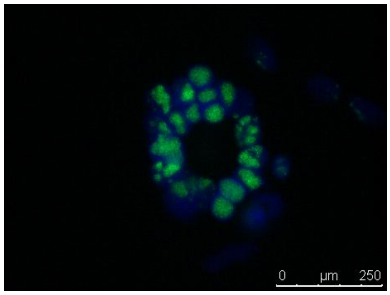
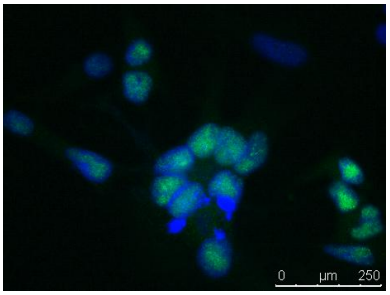
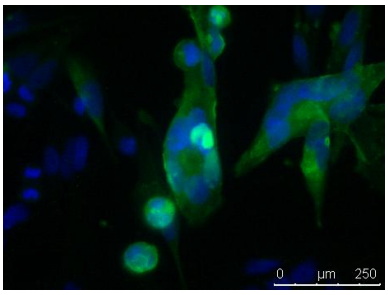


ORFS/L



b) Cytoplasmic localization (Figure 17 cont.)

ORF5



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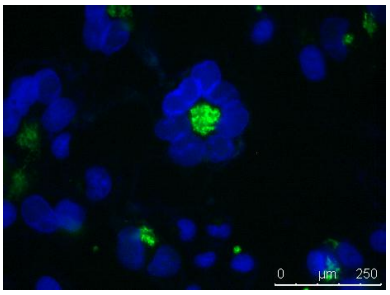

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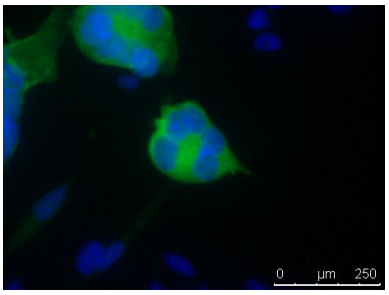

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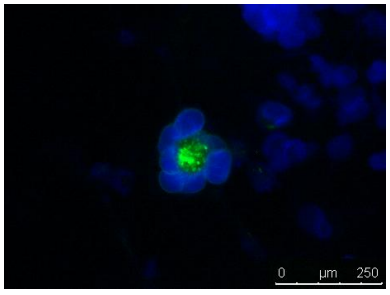

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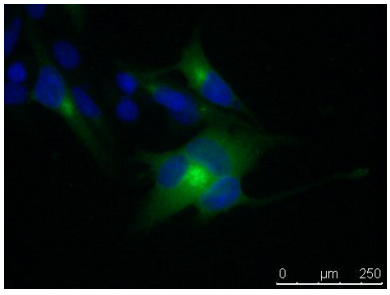

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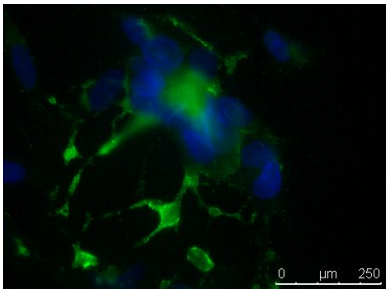

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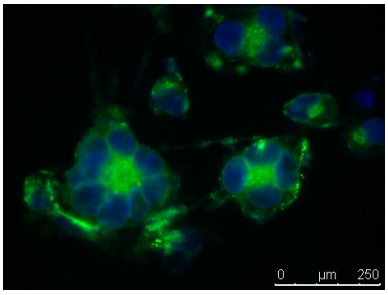

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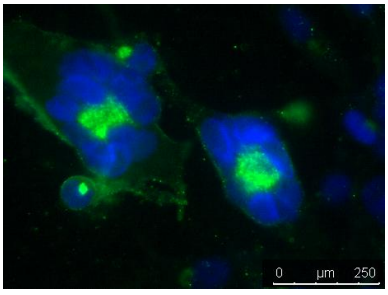

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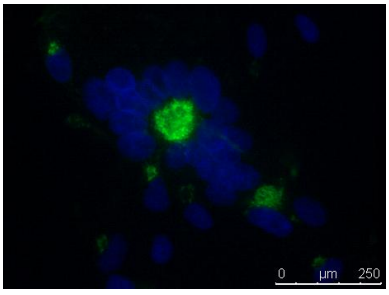

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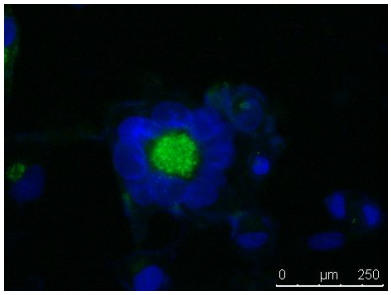

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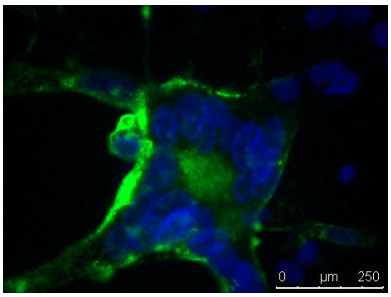

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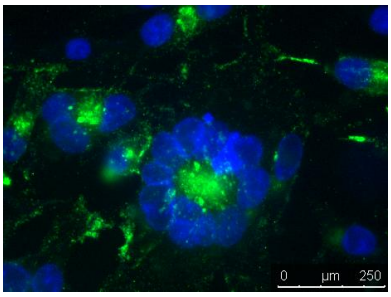
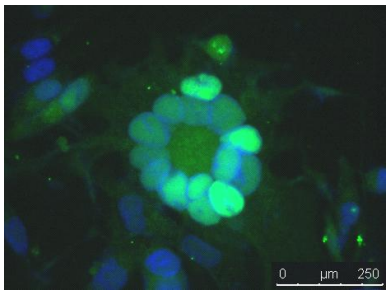


c) Nuclear and cytoplasmic localization (Figure 17 cont.)

ORF3



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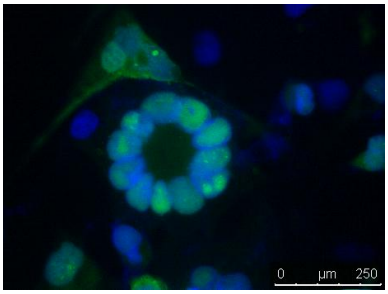

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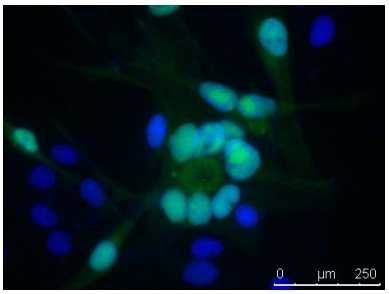

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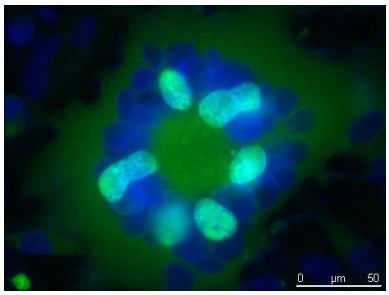

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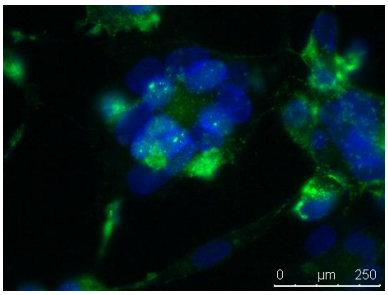

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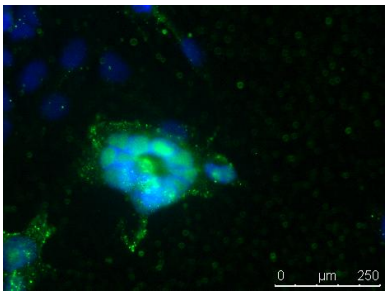

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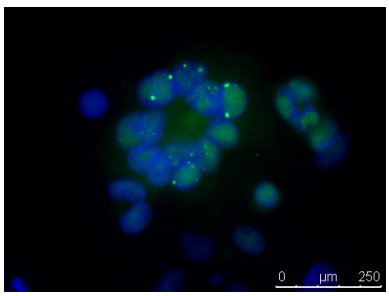

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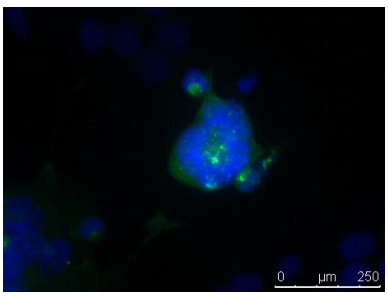

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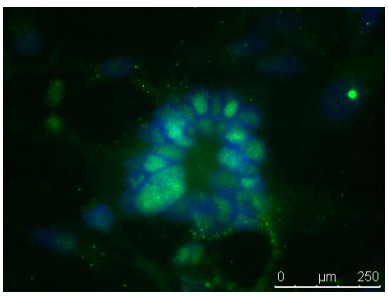

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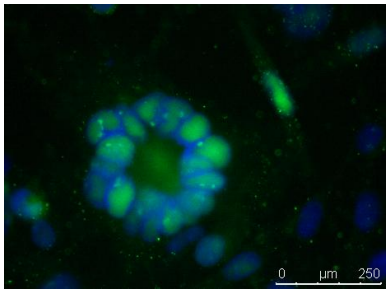

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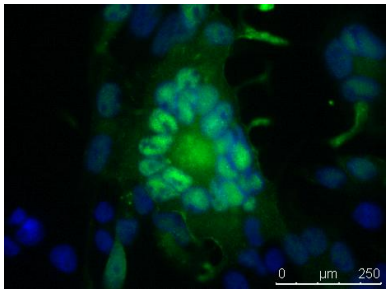

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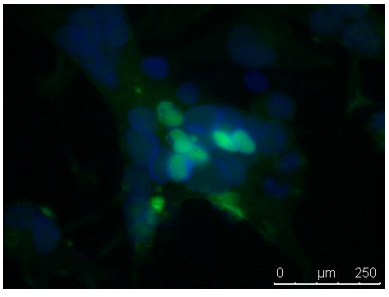


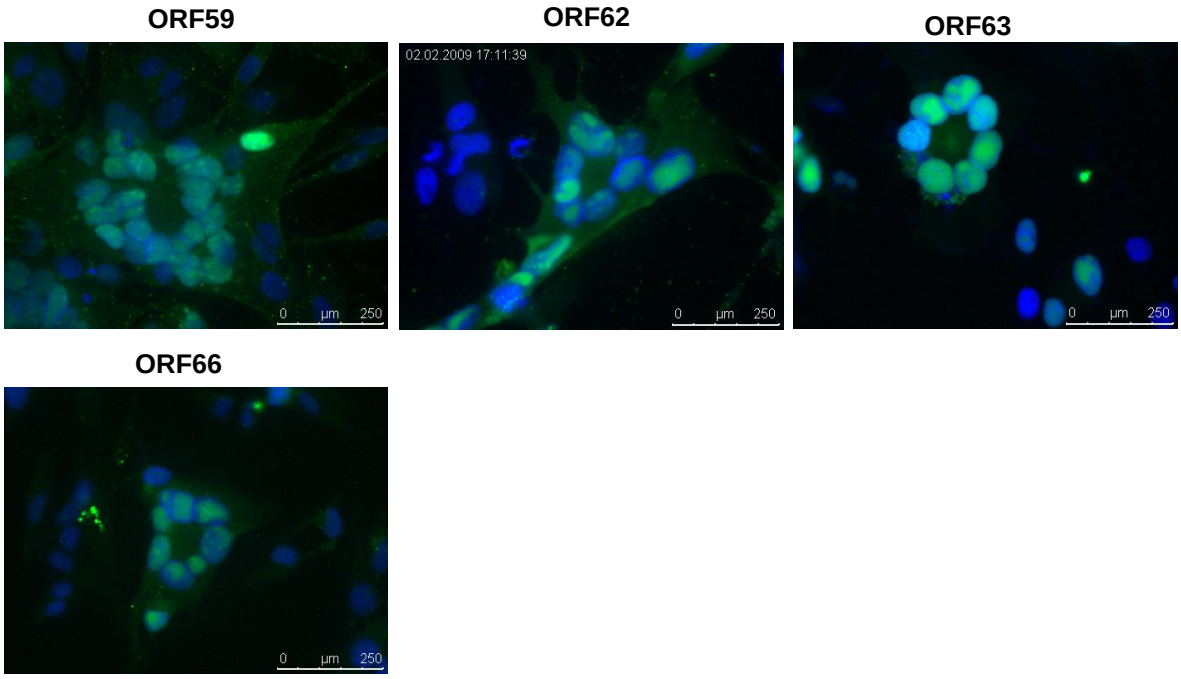


Figure 17: Subcellular localisation of VZV encoded proteins in syncytia. Localisation observed for ORF proteins in syncytia was categorized as nuclear (a), cytoplasmic (b), and both nuclear and cytoplasmic (c). Monolayers of uninfected MEWO cells mixed with pOKA infected MEWO cells at a ratio of 50:1 were seeded on glass slides. Two days post infection, cells were fixed with 3% formaldehyde and permeabilized using 0.5 % TritonX-100. Indirect immunofluorescence was performed using ORF specific mouse monoclonal antibodies followed Alexa 488-conjugated secondary goat-anti mouse antibodies. DNA was visualised by DAPI staining. Pictures were obtained using an epifluorescence microscope (Leica). The bar shown on the bottom right hand side of all pictures corresponds to 250µm and the same magnification was maintained in all panels. The DAPI (blue) and fluorescence (green) pictures were merged using Leica software.

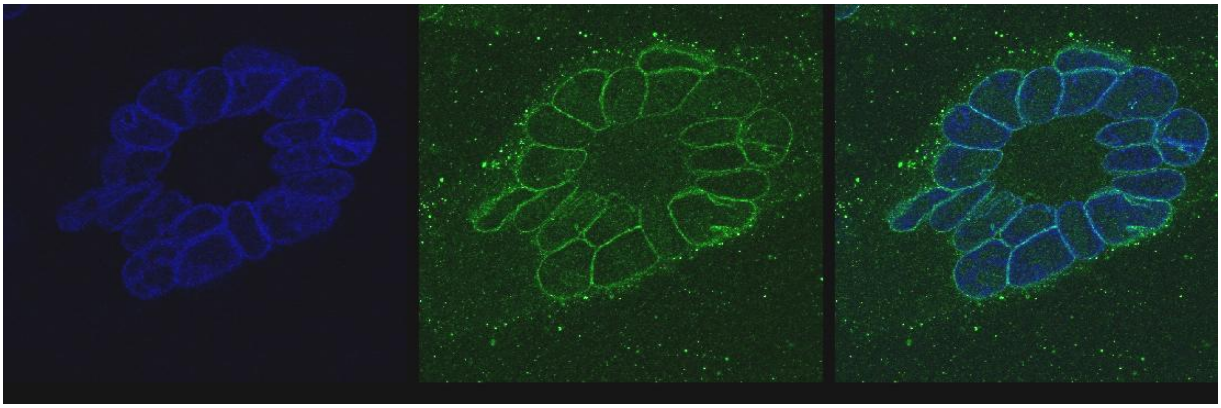
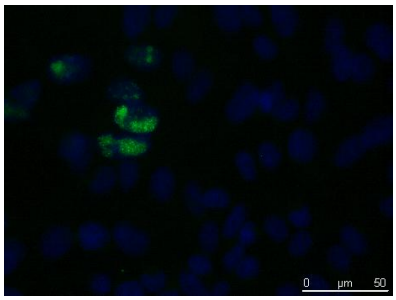


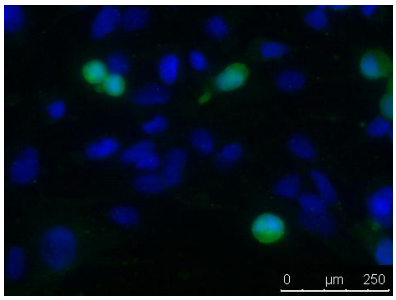
Figure 18: Subcellular localisation of VZV protein ORF24.
Monolayers of uninfected MeWo cells mixed with pOKA infected MeWo cells at a ratio of 50:1 were seeded on glass slides. Two days post infection, cells were fixed with 3% formaldehyde and permeabilized by 0.5 % TritonX-100. Indirect immunofluorescence was performed using ORF24 specific mouse monoclonal antibodies followed by Alexa 488-conjugated secondary goat-anti mouse antibodies. DNA was visualised by DAPI staining. Pictures were obtained using an confocal microscope (Leica). The DAPI (blue) and fluorescence (green) pictures were merged using Leica software.

a) Nuclear localisation (Figure 19 cont.)

ORF6



ORF8



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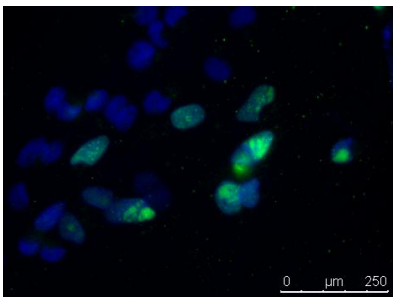

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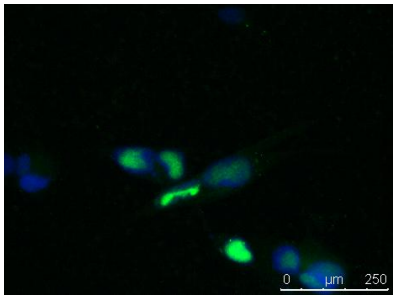

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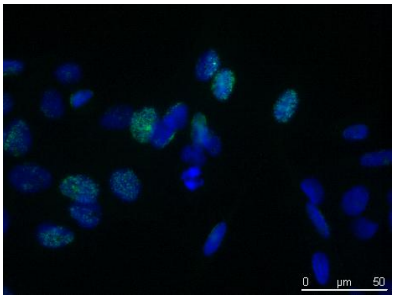

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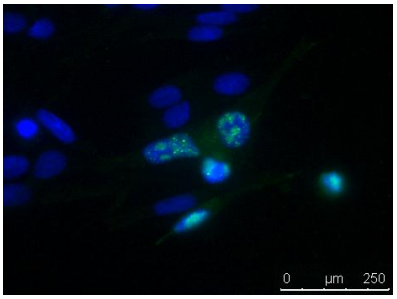
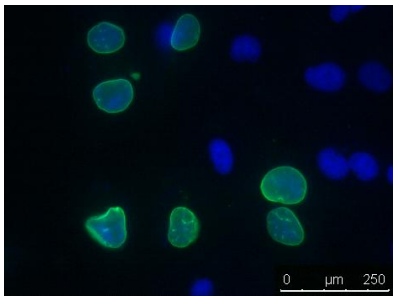


ORF24



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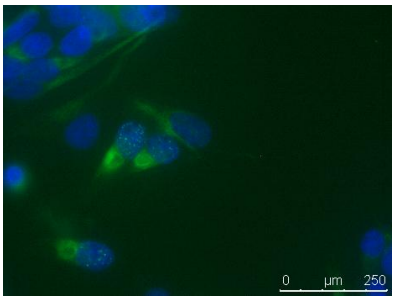

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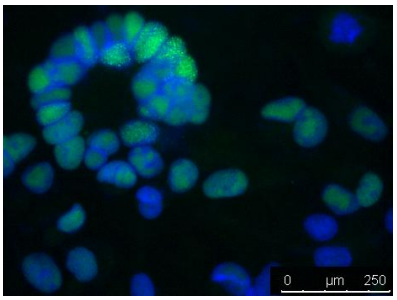

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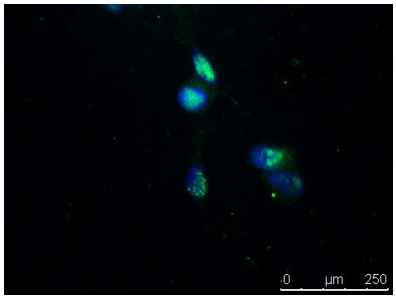

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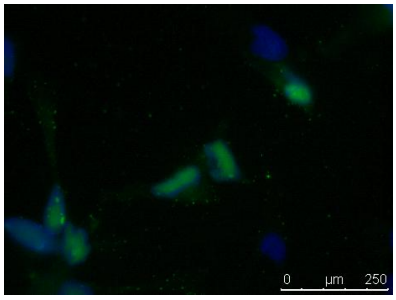
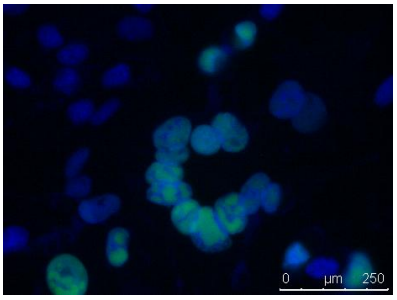


ORF48



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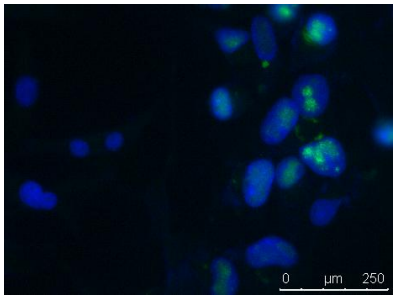

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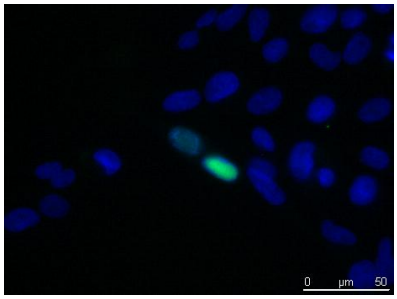

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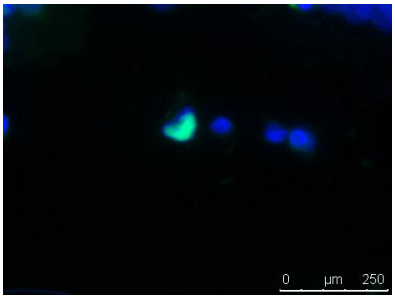
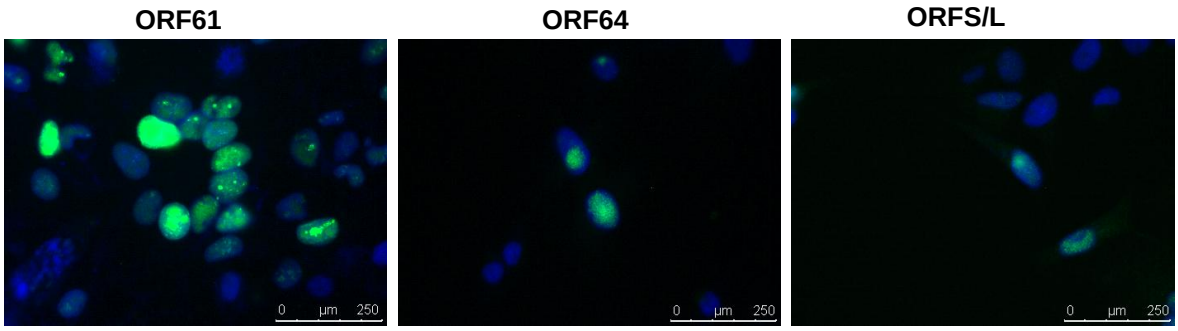
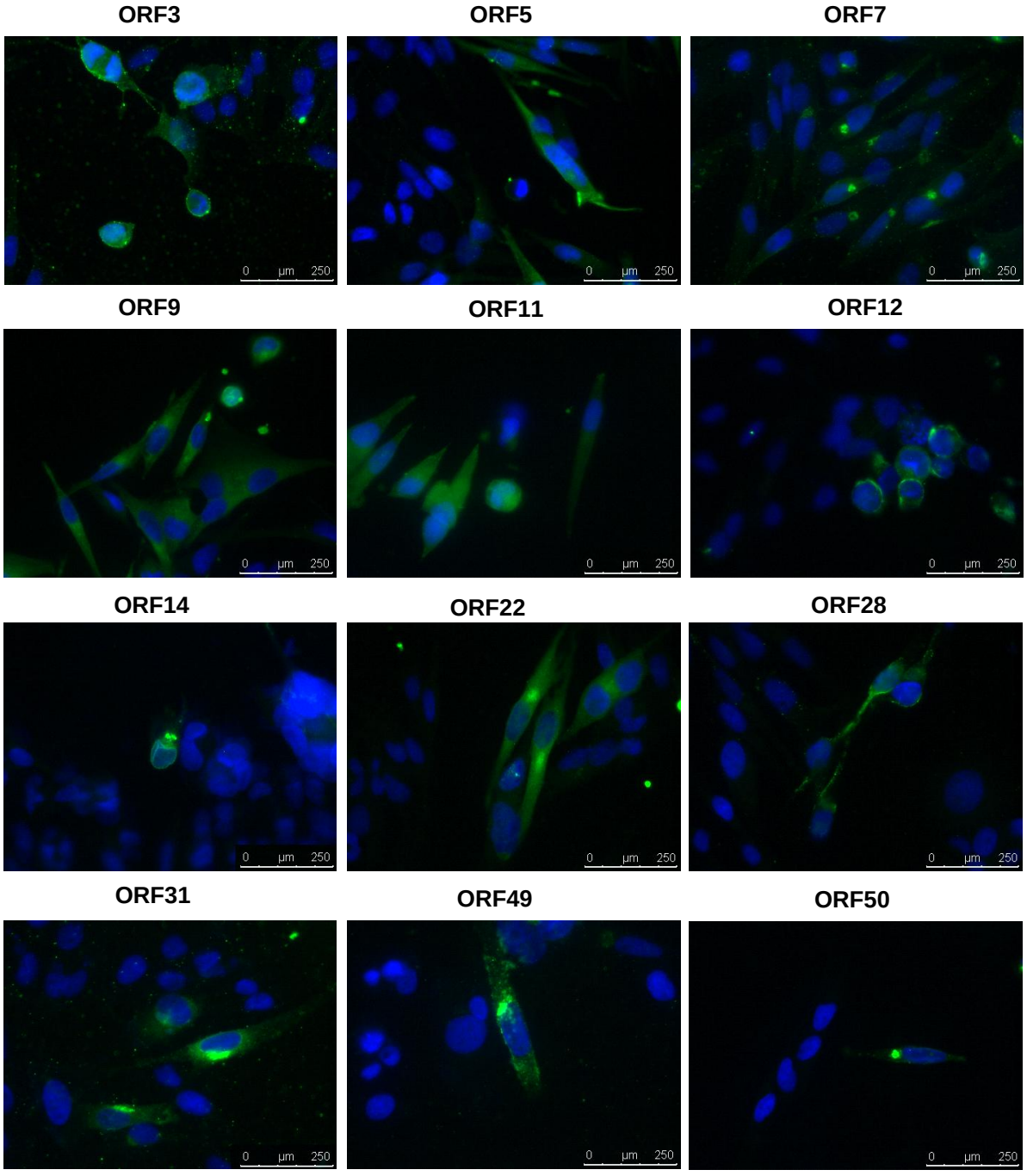




b) Cytoplasmic localisation (Figure 19 cont.)



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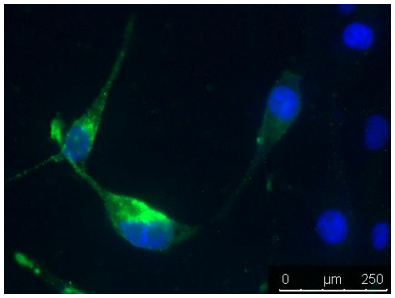
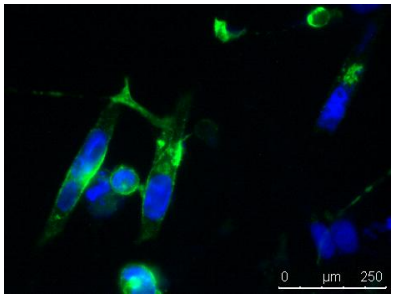
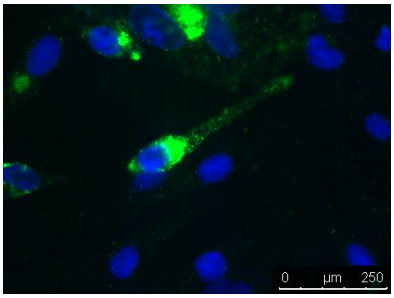


ORF67

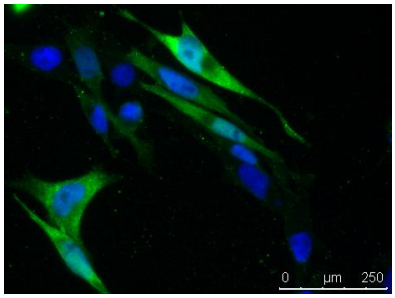


ORF68

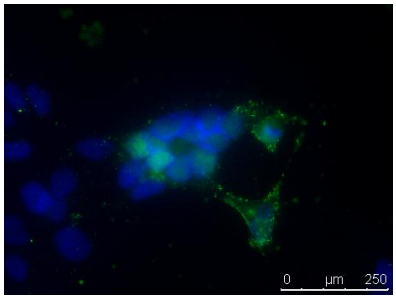


c) Nuclear and cytoplasmic localisation (Figure 19 cont.)

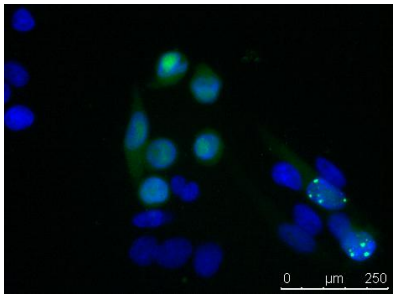
ORF4



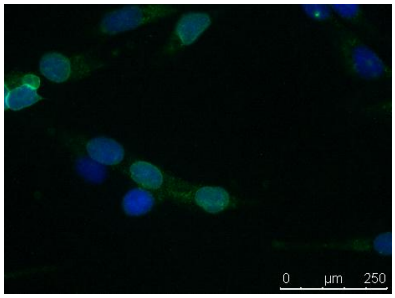
ORF13



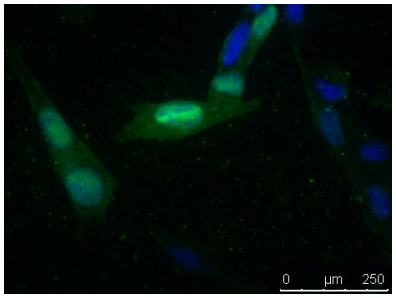
ORF20



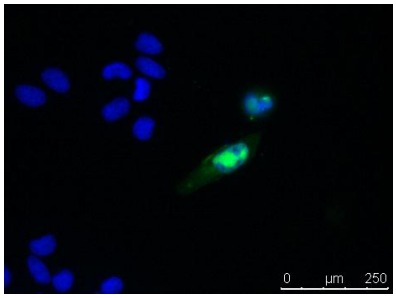
ORF27



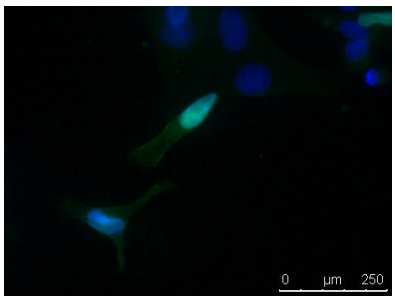
ORF32



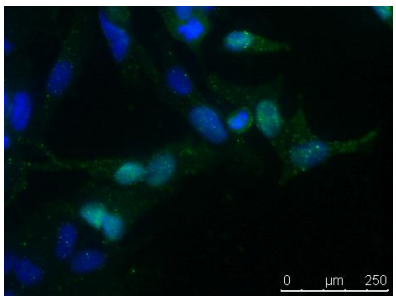
ORF47



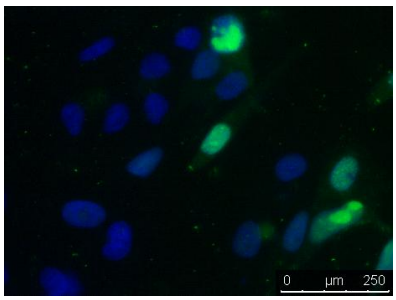
ORF58



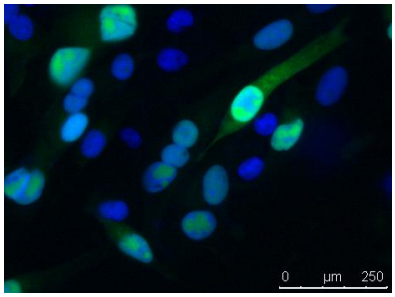
ORF59



ORF62



ORF63



ORF66

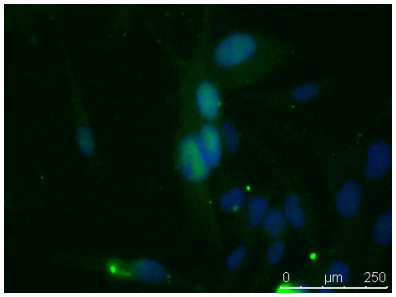


Figure 19: Subcellular localisation of VZV encoded proteins in single cells. Localisation of individual ORF proteins was categorized as nuclear (a), cytoplasmic (b), and both nuclear and cytoplasmic (c). Monolayers of uninfected MeWo cells mixed with pOKA infected MeWo cells at a ratio of 50:1 were seeded on glass slides. Two days post infection, cells were fixed with 3% formaldehyde and permeabilized using 0.5 % TritonX-100. Indirect immunofluorescence was performed using ORF specific mouse monoclonal antibodies followed by Alexa 488-conjugated secondary goat-anti mouse antibodies. DNA was visualised by DAPI staining. Pictures were obtained using an epifluorescence microscope (Leica). The bar shown on the bottom right hand side of all pictures corresponds to 250 µm and the same magnification was maintained in all panels. The DAPI (blue) and fluorescence (green) pictures were merged using Leica software.

Table 8: Subcellular localisation of VZV proteins in single cells or syncytia

ORF No	MW (kDa) calc	MW This work	Localization (Ref)	Localization (this work)		function	HSV orthol locali Ref.
				single	sync.		
ORF1	14.3	10	C [51]			Membrane protein	
ORF2	31.4	32		C	C	Immediate early	
ORF3	23.6	24		C	N&C	hypothetical	
ORF4	59.5	60	**N&C [52]**	**N&C**	**N&C**	Transactivator	N [53]
ORF5	44.8	45	**C [54,55]**	**C**	**C**	Glycoprotein K	
ORF6	142.3	120		N	N	DNA-helicase-Primase compleax	C [53]
ORF7	34.1	34		C	C	Virion phosphoprotein	N&C [53]
ORF8	52.1	52		N	N&C	dUTPase	N&C [53]
ORF9	39.8	45/40	C [56]	C	N&C	Tegument protein	C [53]
ORF9a	11.6					Hypothetical (MP)	C [53]
ORF10	54.0	54			N	Transactivator	N&C [53]
ORF11	107.7	108		C	C	Tegument protein / Transactivator	
ORF12	87.0	70		C	N&C	Tegument protein	
ORF13	39.7	40			N&C	Thymidilate syntethase	
ORF14	73.7	65	**C [57]**	**C**	**C**	Glycoprotein C	
ORF15	53.4					Membrane protein	N&C [53]
ORF16	53.7	50		N	N	DNA-polymerase-associated protein	N [53]
ORF17	59.9			N	N	Host''shutoff'' protein	
ORF18	40.3	35				Ribonucleotide reductase (small SU)	C [53]
ORF19	101.8	90		C	C	Ribonucleotide reductase (large SU)	
ORF20	63.5	55	C [58]	N&C	N&C	Capsid protein	N [53]
ORF21	136.4	120	**N&C [59,60,61]**	N	**N&C**	Tegument protein	C [53]
ORF22	360.3	360		C	C	Tegument protein	C
ORF23	31.0	38	**N [58,62]**	**N**	**N**	Capsid protein	N&C [53]
ORF24	35.5	36		N	N	Membrane associated phosphoprotein	
ORF25	20.6	21	C [63]		N&C	DNA packaging protein	N&C [53]
ORF26	76.9	77			N	Virion protein	C [53]
ORF27	43.9	44		N&C	N	Hypothetical protein	N&C [53]
ORF28	156.9	120		C	C	DNA polymerase	N&C [53]
ORF29	158.2	158	**N [59,62,64]**	**N**	**N**	SS-DNA binding protein	N&C [53]
ORF30	101.2		N&C [65]			Virion protein	

ORF31	114.1	114	**C** [66,57,67]	**C**	**C**	Glycoprotein B	C
ORF32	18.9	30		N&C	N	Hypothetical protein	
ORF33.5	79.6	80	**N** [58]	**N**	**N**	Protease	**N** [53]
ORF34	76.2					Virion protein	N&C [53]
ORF35	34.0					Virion protein	N [53]
ORF36	44.9	35				TK	
ORF37	110.6	111	**C** [66,57]	**N**	**N**	Glycoprotein H	C
ORF38	71.2					Virion protein	N&C [53]
ORF39	31.6	32	**C** [54]	**C**	**C**	Membrane protein	
ORF40	183.4	140	C [58]			Major capsid protein	C [53]
ORF41	41.6	40	C [58]			Capsid protein	N&C [53]
ORF42	42.3	55				Not known	
ORF43	88.9					Tegument protein	
ORF44	47.8	45		N	N&C	Virion protein	N&C [53]
ORF45	46.9		C [65]			DNA packaging protein	
ORF46	26.3	35				Minor tegument protein	N&C [53]
ORF47	67.1	55	**C** [68,69]	N&C	N&C	S/T kinase	**C** [53]
ORF48	72.5	74		N	N	Deoxyribonuclease	C [53]
ORF49	10.8	10	**C** [70]	**C**	**C**	Myristylated virion protein	
ORF50	57.2	52	**C** [71]	**C**	**C**	Glycoprotein M	**C** [72]
ORF51	109.8	110				Origin binding protein	
ORF52	101.4	35				DNA helicase-primase-complex	
ORF53	43.6	44		C	C	Hypothetical	
ORF54	101.1	98				Virion protein	
ORF55	115.8			N	N	DNA helicase-primase-complex	
ORF56	32.2	32		N	N	Virion protein	C [53]
ORF57	9.5	10		C	N&C	Hypothetical	
ORF58	29.2	35		N&C	N	Nuclear phosphoprotein	N [53]
ORF59	40.2	40		N&C	N&C	Uracil DNA glycosylase	
ORF60	21.0	18	**C** [57,67]	**N**	**N**	Glycoprotein L	C [73,74]
ORF61	61.4	60	**N** [62,61]	N	N	Transactivator	
ORF62	172.1	172	**N&C** [68,56,52,62]	**N&C**	**N&C**	Major transactivator	
ORF63	36.6	37	**N&C** [75,62,61]	**N&C**	**N&C**	Co-Transactivator	N [53]
ORF64	23.8			N	N	Virion protein	N&C [53]
ORF65	13.5	14	**C** [66,76]	**C**	**C**	Tegument Phosphoprotein	**C** [53]
ORF66	51.7	52	**N&C** [77]	**N&C**	**N&C**	S/T kinase	**N&C** [53]
ORF67	46.6	50	**C** [78,79]	**C**	**C**	Glycoprotein I	N&C [53]
ORF68	81.9	82	**C** [66,62,79]	**C**	**C**	Glycoprotein E	N&C [53]
ORFS/L	21.0	18/20	**C** [80]	**N**	**N**	Cytoplasmic protein	

4.3.6 Subcloning of positively screened hybridoma clones in WB and IF

Approximately 1500 mother clones were initially tested positive by ELISA and of these, 218 were found reactive with VZV specific proteins. A greater number of VZV specific antibodies secreted by mother clones were positive in Western blotting than in indirect immunofluorescence. In total, an average of 3 to 4 positive clones was

identified specific for an individual VZV ORFs. For some ORFs the screening is still incomplete because either only Western blotting or indirect immunofluorescence was performed, the status of the screening is described in Table 9.

The positively screened antibody secreting mother clones had to be subcloned. The purpose of subcloning is to isolate a specific hybridoma cell line that is clonally expanded and produces only a single kind of epitope specific antibody. For subcloning, positively screened mother wells were seeded in serial dilutions into 96 well plates at the ratio of 0.3 cells / well. After cultivation, the subclones that were producing only one ORF specific VZV antibody were screened again by Western blotting and indirect immunofluorescence. So far, clones derived of 218 mother clones (14%) covering 61 VZV proteins were re-isolated. Of these 218 clones, 190 clones were found to be positive in Western blotting while 123 clones were identified to be positive in immunofluorescence (Table 9).

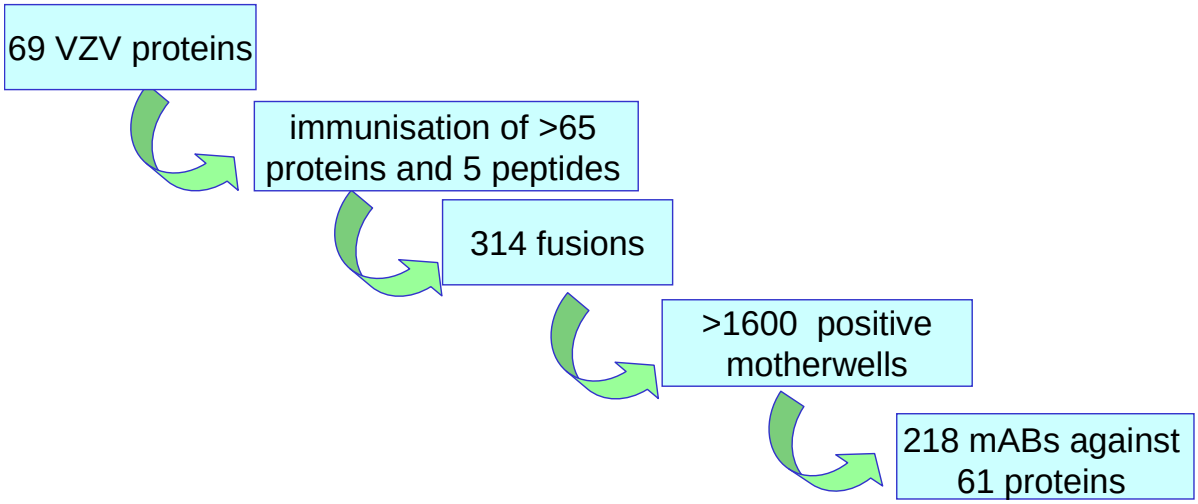


Figure 20: Summary of screening VZV-specific antibodies produced by hybridoma clones.
The figure shows status of the current project at different steps. It explains nearly 65 proteins were immunised and 314 fusions were made successfully. So far, 1600 positive motherwells were screened by ELISA and from this 218 hybridoma clones producing antibodies to 61 VZV ORF's were tested positive.

Table 9: List of all mother wells screened positively in WB and IF

ORF	MW	subclones	WB	IF
Orf2	1G12		**pos**	**pos**
	4G11	2_1	**pos**	**pos**
		2_2	**pos**	**NT**
	7H2		**pos**	**pos**
Orf3	3B10	3_1	**pos**	**pos**
	3B10	3_2	**pos**	**pos**

Orf4	3B6	4_2	**pos**	**pos**
	-//-	4_5	**pos**	**pos**
	-//-	4_4	**pos**	**pos**
	2F7		**pos**	neg
Orf5	6A6		neg	**pos**
	7E5		**pos**	**pos**
	1C12		**pos**	neg
	5A6		**pos**	**pos**
	5B6		nt	**pos**
	6B11		**pos**	**pos**
	7F4		**pos**	**pos**
	-//-	5_12	**pos**	neg
	-//-	5_13	**pos**	neg
	8E5		**pos**	**NT**
	8C4		**pos**	**NT**
	6A9		**pos**	**NT**
	1H2		**pos**	**NT**
	3H7		**pos**	**NT**
	2H11		**pos**	**NT**
Orf6N	-//-	6N_01	**pos**	neg
	-//-	6N_02	**pos**	neg
	7B9		neg	**pos**
Orf7	1H11	7_1	weakly pos	**pos**
	-//-	7_2	weakly pos	**pos**
	2F2	7_3	**pos**	**pos**
	2F2	7_4	**pos**	**pos**
	3D6	7_5	**pos**	**pos**
	-//-	7_6	**pos**	**pos**
	4F9	7_7	**pos**	**pos**
	-//-	7_8	neg	**pos**
Orf8	6A10	8_1	**pos**	**pos**
	-//-	8_2	**pos**	**pos**
	-//-	8_3	**pos**	**pos**
Orf9C	7B5		neg	**pos**
	3H4		neg	**pos**
	4A3		**pos**	**pos**
	1C7		**pos**	neg
	-//-	9C_11	**pos**	**NT**
	-//-	9C_12	**pos**	**NT**
	6H3		neg	**pos**
	-//-	9C_09	**pos**	**NT**
	-//-	9C_10	**pos**	**NT**
Orf10	1A4		**pos**	**NT**
	-//-	10_9	**pos**	**pos**
	1B9		weakly pos	**NT**
	2D4		**pos**	**NT**
	2H8		**pos**	**NT**
	-//-	10_12	**pos**	**pos**

	-//-	10_13	**pos**	**NT**
	3E2		weakly pos	**pos**
	3F4		**pos**	**NT**
	3F5		weakly pos	**NT**
	3A12		**pos**	**pos**
	5F2		**pos**	neg
	5H3		**pos**	**NT**
	5H6		**pos**	neg
	5A8		**pos**	neg
	-//-	10_10	**pos**	**pos**
	-//-	10_11	**pos**	**pos**
	5D11		weakly pos	**NT**
	4G3		weakly pos	**NT**
	4A7		weakly pos	**NT**
Orf11N	1C3		**pos**	**NT**
	1E11		**pos**	neg
	-//-	11_04	**pos**	**NT**
	-//-	11_05	**pos**	**NT**
	3E2		**pos**	**NT**
	-//-	11_06	**pos**	**NT**
	-//-	11_07	**pos**	**NT**
	6A10		**pos**	**NT**
	6B11		**pos**	**NT**
	8F5		**pos**	**NT**
	-//-	11_08	**pos**	**NT**
	1B6		**pos**	**NT**
	1D9		**pos**	**NT**
	-//-	11_03	**pos**	**NT**
	5E9		**pos**	**pos**
	7A2		**pos**	weakly pos
	7C11		**pos**	**pos**
	8F8		**pos**	**NT**
	8B11		**pos**	**pos**
	4B11		**pos**	
Orf 12	1E10	12_3	**pos**	**pos**
	-//-	12_4	**pos**	**pos**
Orf 13	3G4	13_8	neg	**pos**
	2B9	13_10	**pos**	neg
	-//-	13_11	**pos**	neg
Orf 14	4A12	14_1	**pos**	**pos**
	-//-	14_2	**pos**	**pos**
	-//-	14_3	**pos**	**pos**
	-//-	14_4	**pos**	**pos**
	-//-	14_5	**pos**	weakly pos
	2E7	14_6	**pos**	**pos**
	-//-	14_7	**pos**	neg
	-//-	14_9	**pos**	weakly pos
Orf 16	9C6	16_6 asc	**pos**	**pos**
Orf17C	2G10		neg	**pos**

	-//-	17C_7	**NT**	**pos**
	-//-	17C_8	**NT**	**pos**
Orf18C	3H6		**pos**	neg
	-//-	18C_7	**pos**	xreact
	-//-	18C_8	**pos**	neg
	4A12		weakly pos	neg
	-//-	18C_9	**pos**	xreact
	-//-	18C_10	**pos**	neg
	5F1		**pos**	neg
	-//-	18C_11	**pos**	neg
	6H2		**pos**	**NT**
	-//-	18C_12	**pos**	xreact
	-//-	18C_13	**pos**	**pos**
	7F10		**pos**	**NT**
Orf19N	4A11		**pos**	neg
	-//-	19N_1	**pos**	**pos**
	-//-	19N_2	**pos**	neg
	-//-	19N_3	**pos**	**pos**
Orf20	1E2		**pos**	neg
	-//-	20_ 7	**pos**	neg
	-//-	20_ 8	**pos**	neg
	4D4		**pos**	**pos**
	7H5		**pos**	**pos**
	8F5		**pos**	**pos**
	-//-	20_09	**pos**	**pos**
	-//-	20_10	**pos**	**pos**
	1G7		**pos**	pos/xreact
	2H10		**pos**	**pos**
		20_13	**pos**	**pos**
		20_14	**pos**	**pos**
	8E_10		**pos**	neg
	-//-	20_11	**pos**	neg
	-//-	20_12	**pos**	neg
	7B4		**pos**	**pos**
	4A10		**pos**	**NT**
	6C5		**NT**	**pos**
Orf21_3	1C1	21_3_1	**pos**	**pos**
	-//-	21_3_2	**pos**	neg
Orf22_3	4C8	22_3_5	**pos**	neg
	-//-	22_3_6	**pos**	neg
	5A11	22_3_7	**pos**	**pos**
Orf23	7C12	23_1	neg	**pos**
	-//-	23_5	**pos**	**NT**
	6H8	23_2	**pos**	**pos**
	-//-	23_3	**pos**	**pos**
	8D7	23_6	**pos**	**NT**
Orf24	1E1	24_1	weakly pos	**pos**
	-//-	24_2	weakly pos	**pos**

	-//-	24_3	weakly pos	**pos**
	1G11	24_4	**pos**	**pos**
	-//-	24_5	**pos**	**pos**
	-//-	24_6	**pos**	**pos**
	3B7	24_7	**pos**	**pos**
	-//-	24_8	**pos**	**pos**
	-//-	24_9	weakly pos	**pos**
	3G10	24_10	weakly pos	**pos**
	-//-	24_11	weakly pos	weakly pos
Orf25	1B12		**pos**	**pos**
	-//-	25_10	**pos**	neg
	-//-	25_11	**pos**	neg
	5B11		**pos**	neg
Orf26	1E6	26_1	neg	**pos**
	-//-	rekl.26_5	weakly pos	neg
	-//-	rekl.26_6	weakly pos	neg
	-//-	26_4	neg	**pos**
	1H12	26_2	**pos**	neg
Orf27	7A10		**pos**	**pos**
		27_1	**pos**	**pos**
		27_2	**pos**	**pos**
	4G3		**pos**	**pos**
		27_3	**pos**	**pos**
		27_4	**pos**	**pos**
	7H9		**pos**	neg
Orf28.1	1-E9		**pos**	**pos**
Orf29.1	8C1		neg	pos-xreaxt
	5B4		neg	pos-xreaxt
Orf31	1C1			
	-//-	31C_1	**pos**	**pos**
	-//-	31C_2	**pos**	**pos**
	-//-	31C_5	**pos**	**pos**
	-//-	31C_6	**pos**	**pos**
	2A7	31C_7	**pos**	**pos**
	-//-	31C_8	**pos**	**pos**
Orf32	6E1	32_1	**pos**	**pos**
	-//-	32_2	**pos**	**pos**
	5E3	32_4	**pos**	neg
Orf33, 33.5	4F1	33.5_1	**pos**	**pos**
	-//-	33.5_2	**pos**	**pos**
	-//-	33.5_3	**pos**	**pos**
Orf36	8B11		**pos**	**pos**
	-//-	36_15	**pos**	**NT**
	-//-	36_16	**pos**	neg
	5C2		**pos**	neg
	-//-	36_13	**pos**	neg

	-//-	36_14	**pos**	neg
Orf37	3A11		**pos**	**pos**
		37_14	**pos**	neg
	2D12		**pos**	neg
	1H6		**pos**	neg
	1C6		**pos**	**pos**
		37_12	**pos**	**pos**
		37_13	**pos**	**pos**
	6A7		**NT**	**pos**
	3C10		**NT**	**pos**
	4F7		**NT**	**pos**
	1A4		**NT**	**pos**
	2E6		**NT**	**pos**
	7D3		neg	**pos**
	-//-	37N_9	neg	**pos**
	-//-	37N_10	neg	**pos**
	-//-	37N_11	neg	**pos**
Orf39N	3B3	39N_2	weakly pos	**pos**
	-//-	39N_7	weakly pos	**pos**
	3A8	39N_3	neg	weakly pos
	-//-	39N_4	**pos**	neg
	-//-	39N_5	**pos**	weakly pos
	-//-	39N_6	**pos**	weakly pos
	-//-	39N_8	weakly pos	weakly pos
Orf40.1	6H2		**pos**	neg
	7F6		**pos**	neg
Orf41	5F6	41_1	**pos**	neg
	-//-	41_2	**pos**	neg
	-//-	41_4	**pos**	neg
	3G8		**pos**	neg
	5F5		**pos**	neg
	3F8		**pos**	neg
	5E4		**pos**	neg
	5F6		**pos**	neg
	3A4		**pos**	neg
	1F10		**pos**	neg
	4B9		**pos**	neg
Orf42	3E9		**pos**	neg
	2F11		**pos**	neg
Orf44N	1E6		**pos**	**pos**
	1C8		**pos**	**pos**
		44N_3	**pos**	neg
		44N_4	**pos**	**NT**
	2E9		**pos**	neg
	4G2		**pos**	neg
	4G11		**pos**	neg
	6G3(6B3)		**pos**	**NT**
	8D6		**pos**	**NT**
	3F1		**pos**	**NT**

	4F7		**pos**	**NT**
	5B10		**pos**	**NT**
	2F4		**pos**	**NT**
		44N_1	**pos**	neg
		44N_2	**pos**	**pos**
	8C10		**pos**	**NT**
	6D3		**pos**	**NT**
Orf46	2C11		**pos**	neg
		46_9	**pos**	neg
		46_10	**pos**	neg
Orf47	6D12		**pos**	**pos**
	-//-	47_9	**pos**	**NT**
	-//-	47_10	**pos**	**NT**
	4E4		**pos**	neg
	7E11		**pos**	neg
	6A4		weakly pos	**NT**
	7B9		**pos**	pos/xreact
	6H8		**pos**	pos/xreact
	6B8		**pos**	**pos**
	-//-	47_1	**pos**	**NT**
	-//-	47_2	**pos**	**NT**
	2H8		**pos**	**pos**
	-//-	47_5	**pos**	**NT**
	-//-	47_6	**pos**	**NT**
	8C12		**pos**	**NT**
	7D6		**pos**	**pos**
	-//-	47_3	**pos**	**NT**
	-//-	47_4	**pos**	**NT**
	4C3		**pos**	**NT**
	-//-	47_7	**pos**	**NT**
	-//-	47_8	**pos**	**NT**
	8D8		**pos**	**NT**
Orf48	6H3	48_1	**pos**	**pos**
	-//-	48_2	**pos**	**pos**
	-//-	48_3	**pos**	**pos**
	-//-	48_4	**pos**	**pos**
	-//-	48_5	neg	**pos**
	-//-	48_7	neg	**pos**
	3E12	48_6	**pos**	**pos**
	-//-	48_8	**pos**	**pos**
	6G9	48_9	weakly pos	**pos**
	-//-	48_10	weakly pos	**pos**
	-//-	48_11	weakly pos	**pos**
	-//-	48_12	neg	**pos**
	-//-	48_15	neg	**pos**
	6G6	48_16	**pos**	**pos**
	-//-	48_17	**NT**	**pos**
Orf49	6B2	49_7	**pos**	**pos**
	-//-	49_8	**pos**	**pos**
	4G9		**pos**	**pos**
	3E9		**pos**	**pos**

	6A4		**pos**	neg
	2E3		weakly pos	neg
	4G10		**pos**	neg
Orf50	3D7		**pos**	**pos**
Orf51	3G5		**pos**	neg
	6A11		**pos**	**NT**
Orf52N	2H12		**pos**	neg
	4F4		**pos**	neg
	1C2		**pos**	neg
	2G5		**pos**	neg
	5B5		**pos**	neg
	6H11		**pos**	neg
	5A9		**pos**	neg
	5D6		**pos**	neg
	3C3		**pos**	**NT**
Orf53	2C3		neg	**pos**
	-//-	53_10	**pos**	neg
	4F2		**pos**	neg
	-//-	53_11	**pos**	neg
	-//-	53_12	**pos**	neg
Orf54	1B3	54_1	**pos**	neg
	-//-	54_2	**pos**	neg
	5G11		**pos**	**NT**
	-//-	54_8	**pos**	**NT**
	-//-	54_9	**pos**	**NT**
Orf55	2F11	55_1	neg	**pos**
	-//-	55_2	neg	**pos**
	4B6	55_3	neg	**pos**
	-//-	55_4	neg	**pos**
Orf56	1B3	56_1	neg	**pos**
	-//-	56_2	neg	**pos**
	7D1	56_4	**pos**	neg
	9D7	56_5	neg	**pos**
	-//-	56_6	**pos**	**pos**
	10C2	56_7	**pos**	**pos**
	-//-	56_8	**pos**	**pos**
	10E7	56_9	neg	**pos**
	-//-	56_10	neg	**pos**
Orf57	6A2	57_3	neg	**pos**
	-//-	57_4	neg	**pos**
	8C4	57_7	**pos**	**pos**
	-//-	57_10	**pos**	**pos**
Orf58	9A2	58_1	**pos**	**pos**
	-//-	58_3	**pos**	**pos**
	-//-	58_4	neg	**pos**

Orf59	10F11	59_1	**pos**	**pos**
	-//-	59_3	**pos**	**pos**
Orf60C	1H4	60C_1	neg	**pos**
	-//-	60C_2	neg	**pos**
	5E5	60C_5	**pos**	neg
	-//-	60C_6	**pos**	neg
Orf61	1G6	61_1	neg	**pos**
	-//-	61_2	**pos**	**pos**
	2H4	61_3	**pos**	**pos**
	-//-	61_4	neg	**pos**
Orf62	4D1	62_1	weakly pos	**pos**
	-//-	62_2	weakly pos	**pos**
	3A8	62_3	weakly pos	**pos**
	-//-	62_4	weakly pos	**pos**
	2D3	62_5	**pos**	**pos**
	-//-	62_8	**pos**	neg
	3B3	62_6	**pos**	**pos**
	-//-	62_7	**pos**	**pos**
Orf63	4B11	63_1	**pos**	**pos**
	-//-	63_2	**pos**	**pos**
	-//-	63_3	**pos**	**pos**
	-//-	63_4	neg	**pos**
	4C11	63_5	neg	**pos**
	4B11	63_6	neg	**pos**
	4C11	63_7	neg	**pos**
	-//-	63_8	**pos**	**pos**
	-//-	63_9	**pos**	**pos**
Orf64	5G10		neg	**pos**
	1D4		neg	**pos**
Orf65N	7B12		**pos**	**NT**
	7F12		**pos**	**pos**
Orf66	10H10	66_7	**pos**	neg
	2D7	66_8	**pos**	**pos**
	-//-	66_9	**pos**	neg
	-//-	66_10	neg	**pos**
Orf67	3D6	67C_1	**pos**	**pos**
	-//-	67C_2	**pos**	**pos**
	8A4	67C_3	neg	**pos**
	-//-	67C_4	neg	**pos**
	-//-	67C_5	neg	**pos**
	3D6	67C_6	**pos**	**pos**
Orf68	6B12	68_2	**pos**	**pos**
	-//-	68_3	**pos**	**pos**
	-//-	68_4	**pos**	**pos**
	3E2	68_5	**pos**	neg
	-//-	68_6	**pos**	neg

S/L	4E3	SL_C_1	**pos**	**pos**
	-//-	SL_C_2	**pos**	**pos**

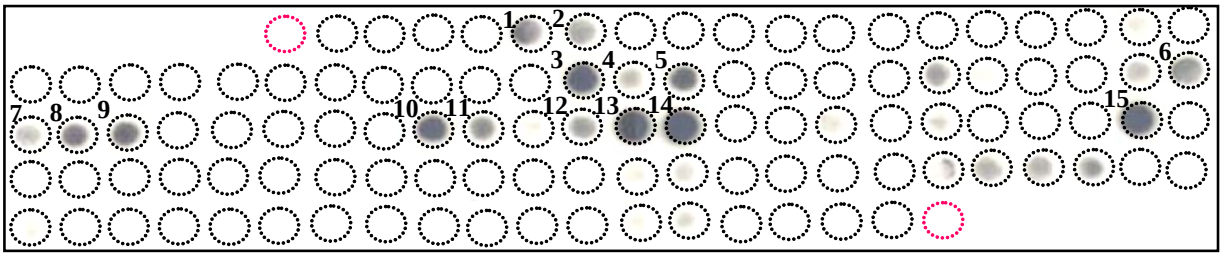
4.4 Epitope Mapping by Pepscan

The synthetic peptides for the VZV antigens used in the Pepscan analysis were deduced from nucleotide sequence data of pOKA VZV strain. To map the epitopes the respective antigen peptides (15-mers, overlapping by 12 aa spanning the entire or partial antigen sequences) were synthesized and coupled to continuous cellulose membranes. Subsequently, the cellulose membranes spotted with respective antigenic peptides were blocked and then incubated with specific hybridoma supernatants. After washing, the cellulose membranes were incubated with alkaline phosphatase-conjugated goat-anti mouse secondary antibodies. The immunodominant regions (epitope) were detected using the ECL detection system (Amersham-Pharmacia / GE Healthcare) and visualized using BIOMAX-MR autoradiography.

4.4.1 Mapping of epitopes recognized on gK

Two anti-gK mAbs from clone 6B11 and 5A6 were tested for identification of immunodominant regions. The anti-gK mAbs from clone 6B11 generated against glycoprotein K identified five discrete stretches of reactive peptides (Figure 21 a).

a) ORF5 / gK – clone 6B11



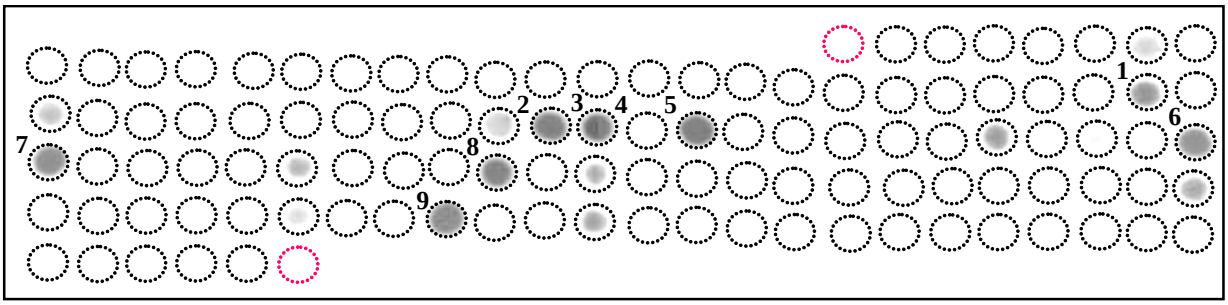
1. LLS**GHAVFTLWYTAR**
2. **GHAVFTLWYTAR**VKF
3. VKTAIS**TPLHDKIRI**
4. AIS**TPLHDKIRI**VLG
5. **TPLHDKIRI**VLGTRN
6. AWFVYGMYL**QFRRIR**
7. VYGMYL**QFRRIR**RMF
8. MYL**QFRRIR**RMFGPF
9. **QFRRIR**RMFGPFRSS
10. ISP**TSYSLNYVTRVI**
11. **TSYSLNYVTRVI**SNI
12. YVTRVI**SNILLGYPY**
13. RVI**SNILLGYPY**TKL
14. **SNILLGYPY**TKLARL
15. **KVFNADPISFLYMHK**

MQALGIKTEHFIIMCLLS$_{18}$**GHAVFTLWYTAR**$_{31}$VKFEHECVYATTVINGGPVVWGS
YNNSLIYVTFVNHSTFLDGLSGYDYSCRENLLSGDTMVKTAIS$_{96}$**TPLHDKIRI**$_{106}$VL
GTRNCHAYFWCVQLKMIFFAWFVYGMYL$_{135}$**QFRRIR**$_{142}$RMFGPFRSSCELISP$_{156}$**T**
SYSLNYVTRVISNILLGYPY$_{178}$TKLARLLCDVSMRRDGMS$_{195}$**KVFNADPISFLYMH**
K$_{210}$GVTLLMLLEVIAHISSGCIVLLTLGVAYTPCALLYPTYIRILAWVVVCTLAIVELIS
YVRPKPTKDNHLNHINTGGIRGICTTCCATVMSGLAIKCFYIVIFAIAVVIFMHYEQR
VQVSLFGESENSQKH

Figure 21 (for legend see below)

The residues in common between the different peptides were delimited by residues $_{18}$GHAVFTLWYTAR$_{31}$, $_{96}$TPLHDKIRI$_{106}$, $_{135}$QFRRIR$_{142}$, $_{156}$TSYSLNYVTRVISNILLGYPY$_{178}$, and $_{195}$KVFNADPISFLYMHK$_{210}$. All of these five immunodominant peptides contain either arginine or isoleucine. MAbs derived of clone 5A6 also identified five different discrete stretches (Figure 21 b). The identified residues are $_{90}$VKTAISTPLHDKIRI$_{106}$, $_{135}$QFRRIR$_{142}$, $_{168}$SNILLGYPYTKL$_{181}$, $_{195}$KVFNADPISFLYMHK$_{210}$, and $_{264}$LISYVRPKPTKDNHL$_{279}$. All of these five immunodominant residues contain isoleucine. The immunodominant regions from $_{135}$QFRRIR$_{142}$ and $_{195}$KVFNADPISFLYMHK$_{210}$ were reactive to two clones are very similar.

b) ORF5 / gK – clone 5A6



1. **VKTAISTPLHDKIRI**
2. AWFVYGMYL**QFRRIR**
3. VYGMYL**QFRRIR**RMF
4. MYL**QFRRIR**RMFGPF
5. **QFRRIR**RMFGPFRSS
6. RVI**SNILLGYPYTKL**
7. **SNILLGYPYTKL**ARL
8. **KVFNADPISFLYMHK**
9. **LISYVRPKPTKDNHL**

MQALGIKTEHFIIMCLLSGHAVFTLWYTARVKFEHECVYATTVINGGPVVWGSYN
NSLIYVTFVNHSTFLDGLSGYDYSCRENLLSGDTM$_{90}$**VKTAISTPLHDKIRI**$_{106}$VLGT
RNCHAYFWCVQLKMIFFAWFVYGMYL$_{135}$**QFRRIR**$_{142}$RMFGPFRSSCELISPTSYSLN
YVTRVI$_{168}$**SNILLGYPYTKL**$_{181}$ARLLCDVSMRRDGMS$_{195}$**KVFNADPISFLYMHK**$_{210}$
GVTLLMLLEVIAHISSGCIVLLTLGVAYTPCALLYPTYIRILAWVVVCTLAIVE$_{264}$**LIS**
YVRPKPTKDNHL$_{279}$NHINTGGIRGICTTCCATVMSGLAIKCFYIVIFAIAVVIFMHYE
QRVQVSLFGESENSQKH

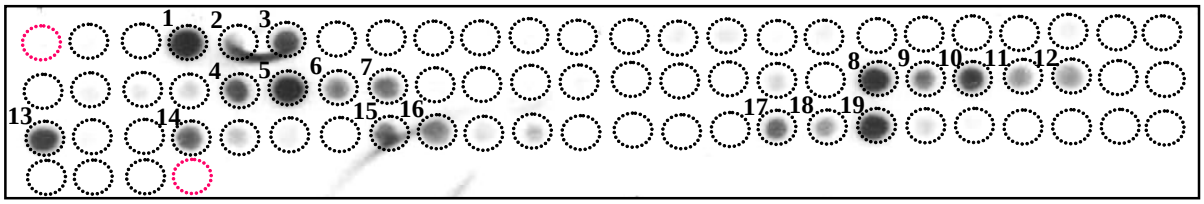
Figure 21: Epitope mapping of monoclonal antibodies to gK. Synthetic peptides covering the complete sequence of gK were coupled to cellulose membranes as 15 mers overlapping by 12 aa. The blocked membrane was incubated with the mAbs (a) clone 6B11 or (b) clone 5A6 of gK, followed by addition of secondary goat anti-mouse antibodies conjugated to alkaline phosphatase. The membrane was detected using ECL and developed by BIOMAX-MR autoradiography. Five discrete peptide stretches were recognised by mAb clones 6B11 and 5A6, and residues that were important for binding within the reactive peptide sequences are marked in bold. The localisation of the reactive peptide streches is shown on the bottom in the primary sequence of gK.

4.4.2 Mapping of epitopes recognized on ORF24

The clones 1G11 and 3B7 were raised against the N-terminal ORF24 fusion protein comprising residues 1 to 245. So, for screening purposes ORF24 peptides (15-mers, overlapping by 12 aa spanning residues 1 - 245) coupled to cellulose membrane were selected to map the epitopes. The mAb 1G11 detected seven discrete stretches of reactive peptides (Figure 22 a). The five residues in common between the different peptides were delimited by the residues $_{15}$GDNLLQRIR$_{25}$, $_{93}$VLFQGF$_{100}$, $_{135}$IKR$_{139}$, $_{168}$MGPEDPSRTIKL$_{180}$, and $_{195}$NLDEYIRWR$_{205}$. Two stretches $_{144}$RPLQALMWVNCF$_{156}$ and $_{157}$VRMPYVQLSFRF$_{168}$ represented single reactive peptides. The mAb 3B7 identified three spots and these spots correspond to peptides $_{9}$ERRRGCGDNLLQRIR$_{25}$, $_{153}$NCFVRMPYVQLSFRF$_{169}$, and $_{189}$YKETGNNLDEYIRWR$_{205}$ (Figure 22 b). Three epitopes recognized by antibodies

produced by clone 3B7 ($_{153}$NCFVRMPYVQLSFRF$_{169}$, $_{15}$GDNLLQRIR$_{25}$, and $_{195}$NLDEYIRWR$_{205}$) showed also reactivity with clone 1G11 suggesting they are immunologically active.

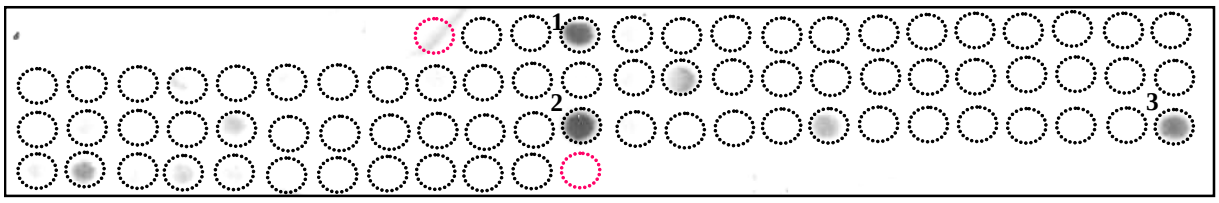
a) ORF24 – clone 1G11



1. ERRRGC**GDNLLQRIR**
2. RGC**GDNLLQRIR**LVV
3. **GDNLLQRIR**LVVPSA
4. IKIQNTGVS**VLFQGF**
5. QNTGVS**VLFQGF**FFR
6. GVS**VLFQGF**FFRPTN
7. **VLFQGF**FFRPTNAPV
8. LSTGINLSALES**IKR**
9. GINLSALES**IKR**GGG
10. LSALES**IKR**GGGIDR
11. LES**IKR**GGGIDRRPL
12. **IKR**GGIDRRPLQAL
13. **RPLQALMWVNCFVRM**
14. **NCFVRMPYVQLSFRF**
15. FRF**MGPEDPSRTIKL**
16. **MGPEDPSRTIKL**MAR
17. YKETGN**NLDEYIRWR**
18. TGN**NLDEYIRWR**PSF
20. **NLDEYIRWR**PSFRSP

MSRRTYVRSERRRGC$_{15}$**GDNLLQRIR**$_{25}$LVVPSALQCCDGDLPIFDPQRPPARCV
FQFNGEDNVSEAFPVEYIMRLMANWAQVDCDPYIKIQNTGVS$_{93}$**VLFQGF**$_{100}$FF
RPTNAPVAEVSIDSNNVILSSTLSTGINLSALES$_{135}$**IKR**$_{139}$GGGIDR$_{144}$**RPLQALM**
WVNCF$_{156}$**VRM**PYVQLSFRF$_{168}$**MGPEDPSRTIKL**$_{180}$MARATDAYMYKETGN$_{195}$
NLDEYIRWR$_{205}$PSFRSPPENGSPNTSVQMQSDIKPALPDTQTTRV

b) ORF24 – clone 3B7



1. $_{9}$**ERRRGCGDNLLQRIR**$_{25}$
2. $_{153}$**NCFVRMPYVQLSFRF**$_{169}$
3. **189YKETGNNLDEYIRWR205**

MSRRTYVRS$_{9}$**ERRRGCGDNLLQRIR**$_{25}$LVVPSALQCCDGDLPIFDPQRPPARCVF
QFNGEDNVSEAFPVEYIMRLMANWAQVDCDPYIKIQNTGVSVLFQGFFFRPTN
APVAEVSIDSNNVILSSTLSTGINLSALESIKRGGGIDRRPLQALMWV$_{153}$**NCFVR**
MPYVQLSFRF$_{169}$MGPEDPSRTIKLMARATDAYM$_{189}$**YKETGNNLDEYIRWR**$_{205}$P
SFRSPPENGSPNTSVQMQSDIKPALPDTQTTRV

Figure 22: Epitope mapping of monoclonal antibodies to ORF24. The overall reactivity of two anti-ORF24 antibodies from two different clones 1G11 (a) and 3B7 (b) with peptides spanning the N-terminal ORF24 sequence from residues 1 to 245 (15-mers, overlapping by 12aa) was tested. Cellulose membranes with spotted peptides were blocked and subsequently incubated with the mAbs 1G11 and 3B7 reactive with ORF24, followed by addition of goat anti-mouse secondary antibodies conjugated to alkaline phosphatase and developed as described in Figure 19. The sequence corresponding to the numbers adjacent to the reactive spots are highlighted in the primary aminoacid sequence of ORF24 shown on the bottom.

4.4.3 Mapping of epitopes recognized on gB

To determine the epitopes clone 2A7 recognized on gB, a cellulose membrane with 15-mer peptides with 12 aminoacid overlap spanning gB residues 750 to 869 was prepared. A single linear stretch of 30 residues within the carboxy-terminal part of gB was shown to bind clone 2A7 antibodies and is depicted in Figure 23 a.

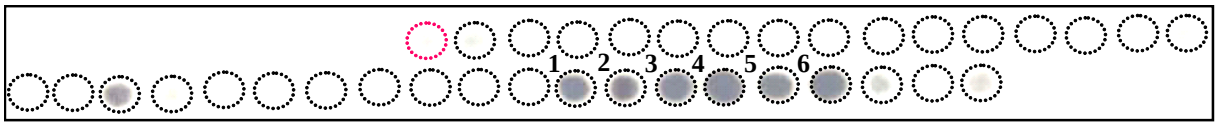
4.4.4 Mapping of epitopes recognized on gL

To identify epitopes on gL recognized by clone 5E5 a cellulose membrane with 15-mer peptides with 12 amino acids overlaps spanning aminoacids 29 to 160 was generated. A single linear stretch of 33 residues within the carboxy-terminal part of gL reacted with clone 5E5 antibodies (Figure 23 b).

4.4.5 Mapping of epitopes recognized on gI

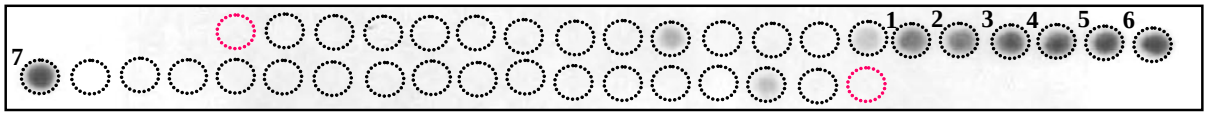
The mAbs 3D6 were raised against a C-terminal gI fusion protein comprising 294 to 355 aa. For epitope mapping, gI peptides (15-mers, overlapping by 12 aa spanning residues 294 to 355) were coupled to cellulose membranes and tested for antibody binding. Three discrete stretches were recognised by clone 3D6 antibodies as shown in Figure 23 c. IYR is a common region shared by all peptides.

a) ORF31 / gB – clone 2A7



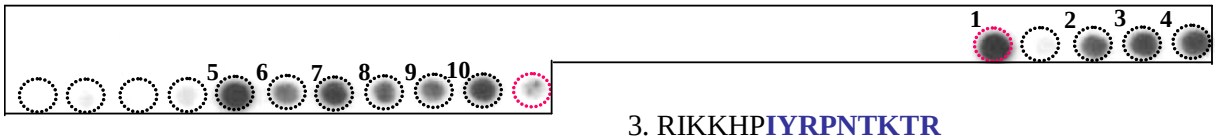
KTSPMKALYPLTTKGLKQLPEGMDPFAEKPNATDTPIEEIGDSQNTEPSVNSGFDPD
KFREAQEMIKYMTLVSAAERQESK$_{831}$**ARKKNKTSALLTSRLTGLALRNRRGYSRV**
R$_{862}$TENVTGV

b) ORF60 / gL – clone 5E5



PLSDVSLIITEPCVSSVYEAWDYAAPPVSNLSEALSGIVVKT$_{77}$**KCPVPEVILWFKDKQ**
MAYWTNPYVTLKGLAQSV$_{111}$GEEHKSGDIRDALLDALSGVWVDSTPSSTNIPENGCV
WGADRLFQRVCQ

c) ORF67 / gI – clone 3D6



1. ISVKRRRIKKHPIYR

3. RIKKHP**IYRPNTKTR**
4. KHP**IYRPNTKTR**RGI
5. **IYRPNTKTR**RGIQNA

6.TPESDVMLEAAIAQLATIREESPPHSVVNP

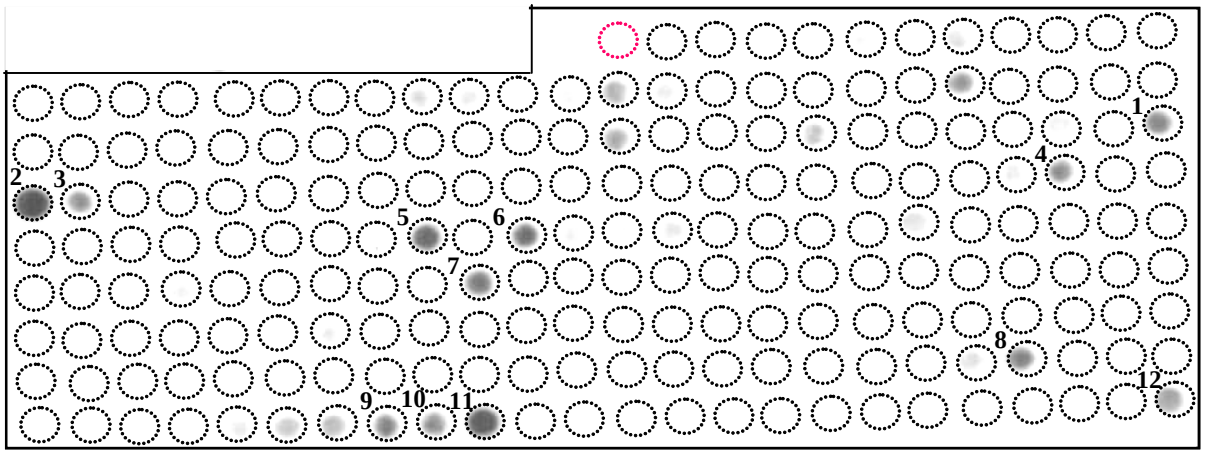
$_{294}$**ISVKRRRIKKHPIYRPNTKTR**$_{316}$RGIQNA$_{321}$**TPESDVMLEAAIAQLATIREESPP**
HSVVNP$_{352}$FVK

Figure 23: Epitope mapping of monoclonal antibodies to ORFs 31, 60, and 67. For epitope mapping, pepscan cellulose membranes were spotted with peptides (13-mers, 12 overlapping amino acids) covering the C-terminal sequence of gB (residues 750 to 869), gL (residues 29 to 160) and gI (residues 294 to 355). The membranes were incubated with antibodies of clone 2A7 produced to gB (a), clone 5E5 produced to gL (b) and clone 3D6 produced to gI (c), followed by addition of goat anti-mouse secondary antibodies conjugated with alkaline phosphatase. The membrane was developed as described in Figure 19. The epitopes recognized within gB, gL or gI are marked schematically.

4.4.6 Mapping of epitopes recognized on gE

For epitope mapping, antibodies produced by clone 6B12 were tested for reactivity on cellulose membranes spotted with 15-mer peptides harbouring a 12 amino acid overlap spanning gE residues 560 to 624. Eight peptide stretches were recognised by the specific antibodies (Figure 24). Peptide $_{282}$PGVLKVLRTEKQYLG$_{298}$ is the common epitope region recognised by reactive spots 5 and 6.

ORF68 / gE – clone 6B12



1. ENHPFT**LRAPIQRIY**
2. PFT**LRAPIQRIY**GVR
3. **LRAPIQRIY**GVRYTE
4. **QLAEISYRFQGKKEA**
5. **DPPEIEPGVLKVLRT**
6. **PGVLKVLRTEKQYLG**
7. **SVGDTFSLAMHLQYK**
8. **NPGTSPLLRYAAWTG**
9. KRMRVK**AYRVDKSPY**
10. RVK**AYRVDKSPY**NQS
11. **AYRVDKSPY**NQSMYY
12. **SHGGSSYTVYIDKTR**

MGTVNKPVVGVLMGFGIITGTLRITNPVRASVLRYDDFHIDEDKLDTNSVYEPYY
HSDHAESSWVNRGESSRKAYDHNSPYIWPRNDYDGFLENAHEHHGVYNQGRGI
DSGERLMQPTQMSAQEDLGDDTGIHVIPTLNGDDRHKIVNVDQRQYGDVFKGDL
NPKPQGQRLIEVSVEENHPFT$_{183}$**LRAPIQRIY**$_{193}$GVRYTETWSFLPSLTCTGDAAP
AIQHICLKHTTCFQDVVVDVDCAENTKED$_{243}$**QLAEISYRFQGKKEA**$_{259}$DQPWIV
VNTSTLFDELEL$_{276}$**DPPEIEPGVLKVLRTEKQYLG**$_{298}$VYIWNMRGSDGTSTYATF
LVTWKGDEKTRNPTPAVTPQPRGAEFHMWNYHSHVF$_{351}$**SVGDTFSLAMHLQY**
K$_{367}$IHEAPFDLLLEWLYVPIDPTCQPMRLYSTCLYHPNAPQCLSHMNSGCTFTSPH
LAQRVASTVYQNCEHADNYTAYCLGISHMEPSFGLILHDGGTTLKFVDTPESLSG
LYVFVVYFNGHVEAVAYTVVSTVDHFVNAIEERGFPPTAGQPPATTKPKEITPV$_{528}$
NPGTSPLLRYAAWTG$_{544}$GLAAVVLLCLVIFLICTAKRMRVK$_{567}$**AYRVDKSPY**$_{577}$
NQSMYYAGLPVDDFEDSESTDTEEEFGNAIGG$_{608}$**SHGGSSYTVYIDKTR**$_{624}$

Figure 24: Epitope mapping of monoclonal antibodies to ORF68. The complete sequence of gE was coupled to cellulose membrane as synthetic peptides of 15mers overlapping by 12 residues. The membrane was incubated with the antibodies produced by clone 6B12 followed by addition of goat anti-mouse secondary antibodies conjugated with alkaline phosphatase. The membrane was developed as described in Figure 19. Five discrete peptide stretches were recognised by clone 6B12, and residues that were important for binding within the reactive peptide sequences are shown in bold. The localisation of the reactive peptide streche is shown on the bottom in the primary sequence of gE.

5. Discussion

Varicella zoster virus causes chicken pox as primary infection which upon reactivation results in herpes zoster. VZV is of high clinical relevance due to the high prevalence of Varicella zoster in humans, its highly contagious nature and a high morbidity rate associated with reactivation. Despite of this, many aspects of the infection cycle, of latency and reactivation are not well understood. Moreover, the function of many individual proteins specific to VZV is poorly characterized. Until today, research is hampered by the lack of cell-free virus and effective animal models restricting analysis to *in vitro* cell culture using cell-associated non-synchronized virus.

Like all herpesviruses, VZV are large viruses that exhibit complex biological mechanisms to proliferate efficiently. This is simply exemplified by the association of a large number of viral proteins to form new viral particles. In the course of infection, herpes virus proteins however do not only interact with each other but also with a large number of host proteins. To analyse and understand these complex mechanisms, systems biology approaches represent a powerful tool. So far, genome wide interactions of five different herpesviruses, comprising all subfamilies of human herpesviruses (HSV, VZV, EBV, CMV, KSHV) were studied using yeast-two-hybrid (Y2H) ([81,82]). However the Y2H does not provide a means to understand and study fundamental aspects of viral morphogenesis in the viral or cellular context. To systematically detect and characterize all VZV expressed proteins in the course of infection we generated a comprehensive antibody collection. To our knowledge this is the first approach to generate an ORFeome-wide antibody collection. With certainty, this collection will reveal many so far uncovered biological aspects of VZV infection and will be highly welcomed by the VZV research community.

5.1 Pipeline for high-throughput generation of antibodies against the VZV proteome

As a permanent source of antibodies for future studies, we chose to produce monoclonal antibodies in mice using the conventional hybridoma technology. To generate VZV proteins for immunization we implemented some strategies to increase the efficiency in a limited time. For cloning a VZV entry library and subcloning it into different expression vectors we have chosen the Gateway® technology from

Invitrogen because it is rapid, robust, cost-efficient and highly amenable to high-throughput systems. By using the recombinational Gateway® technology we have cloned all 70 open reading frames of VZV without stop codon, and thus constructed a VZV entry library ([32]). The Gateway® technology gives flexibility to subclone the entry clones into different Gateway® compatible vectors, for eg. into a variety of expression vectors with different tags for protein expression and purification in bacteria. So, to maximise our chance of protein expression and purification we have cloned the entry library into four different pET derived expression vectors containing four different tags like N-terminal His6, C-terminal His6, N-terminal MBP, or N-terminal GST tag. Far beyond, this entry library can be transferred into any compatible vector allowing expression in various cellular systems like yeast, insect or mammalian cells.

The vast majority of VZV proteins used for immunization was produced by bacterial expression and subsequent protein purification as this system is economical, fast and requires minimal technical expertise to establish a laboratory protein production system [83]. Proteins that could not be obtained using the bacterial expression system were produced using the baculovirus-driven expression in infected insect cells. Moreover, synthetic peptides were generated for several proteins that were not obtained by either system.

Purified proteins and synthetic peptides were immunised to mice and screened initially in ELISA for positive mABs. To screen a large number of clones a high-throughput 96 well ELISA platform was established. To this end, ELISA plates were coated with bacterially expressed and purified VZV proteins to screen hybridoma mother clones for production of VZV specific antibodies. Antibodies giving positive signals on the immunogen using ELISA were subsequently tested by Western blotting (WB) on VZV infected cell material. This enabled us to determine whether a specific antibody recognized a protein of the correct molecular weight in lysates of VZV infected but not of uninfected MeWo cells. The same antibodies were then tested by indirect immunofluorescence to determine the subcellular localization of the VZV encoded protein in infected cells again in comparison to uninfected cells. Altogether the second part of screening in the virus context resulted in a collection of faithfully tested antibodies that can be used for various studies of specific VZV encoded proteins.

5.2 Limitations of the pipeline

In the process of generating antibodies we have encountered several limitations at every step while implementing our strategies. Problems occurred at various steps like PCR amplication of large DNA fragments and cloning into the entry vector. Large DNA sequences of more than 4000kb were difficult to amplify due to premature chain termination, or frame shifts and stop codons were introduced randomly because of high mutation rates. So, ORF21, ORF22, ORF28 and ORF29 were amplified as smaller fragments. To facilitate purification of certain VZV ORFs, proteins predicted to contain transmembrane regions were cloned as soluble domains lacking the transmembrane domains. We have also generated small fragments for ORFs where a full length protein showed poor expression in bacteria.

Several VZV proteins gave difficulties during expression / purification using the bacterial system. If fragmentation did not solve the problem, the baculovirus expression system was applied. This system facilitates high levels of protein expression, posttranslational modifications, such as protein N-glycosylation (similar to mammalian cells) and easy to grow insect cells in large amounts ([84,85,86]). If all expression systems were exhausted, synthetic peptides were ordered.

Due to poor expression of VZV proteins, the concentration of protein used for immunization was low resulting in few positive hybridoma clones. A second problem arose during ELISA testing. Low amounts of specific protein available for coating of ELISA plates led to problems in discriminating positive antibody producing clones from negative clones. Moreover, we observed cross-reactivity. Many clones that were screened positive by ELISA were detected negative in Western blotting and / or IF. Many false positive clones were also obtained because of low specificity and high background in ELISA. Cross-reactive clones were produced by the addition of a 50 amino acid sequence which was encoded by the linker region up to a late occurring Stop codon in the Gateway® vector resulting in a general C-terminal extension of the protein of interest. Due to the generation of antibodies against this C-terminally encoded Gateway® vector sequence many clones initially tested positive by ELISA turned out negative when tested on VZV infected cells. To overcome this problem a control protein carrying this same 50 aminoacid extension was used for ELISA screening. Moreover, for some of the VZV proteins we could not generate antibody producing positive clones. These proteins might be poorly immunogenic. Finally, we

have observed that several clones producing monoclonal antibodies specific to VZV proteins also exhibit cross-reactivity with host proteins.

5.3 Potentiality of the Antibody Collection

To our knowledge this is the first time, antibodies were generated against the whole genome of such a complex virus. This will enable the comprehensive analysis of all VZV encoded proteins in the virus context. So far, our antibody clone collection contains 218 mother clones producing antibodies to 61 VZV proteins (87%). In average, each VZV ORF is covered by 3 to 4 positive clones, however depending on the ORF varying between one to 10 different clones. In total, 190 clones were identified as positive in Western blotting covering 57 ORFs (81%). In immunofluorescence 123 antibodies were tested positive covering 52 VZV ORFs 74%). To our surprise 50 VZV ORFs (71%) could be detected both by WB and IF, two methods that vary in conserving protein conformation (Figure 25). While screening the antibodies with IF gives a specific subcellular signal WB allows detection of the molecular weight of the protein in question, providing an intrinsic control due to detection of the molecular weight. Thus, the fact that 50 ORFs (71%) are detected by specific antibodies using both of these methods underscores the validity and high fidelity of our collection. Moreover, a selection of these antibodies tested under altered IF conditions indicate that many antibodies are tolerant to sample preparation and thus should be widely applicable in using various methods including immunocytochemistry, immunoelectronmicroscopy or immunoprecipitation.

More than 83% (47 of 57) of the antibodies that were screened positive in WB recognised a VZV protein of the predicted molecular weight. Nearly 17% (10 proteins) of the VZV proteins migrated aberrantly in SDS-PAGE possibly due to protein truncation, proteolytic processing, or to a highly basic or acidic nature. In IF all the proteins showed the expected localisation in comparison to their herpesviral conserved and predicted function. Future studies aim at detailed analysis of those antibodies where detection of the 10 remaining ORFs by WB was unsatisfactory. Sofar, antibodies for 9 VZV ORFs were not generated and the process of production of antibodies recognizing these ORFs is ongoing.

In summary, our collection of antibodies allows detection of VZV proteins in absence of a protein tag either *in situ*, under native or denatured conditions, in the viral context but also under ectopic expression in any cell system. Moreover, since many ORFs

are covered by several different monoclonal antibodies we expect that this collection also enables the discrimination of splice and modification variants thus potentially giving many novel insights into VZV replication.

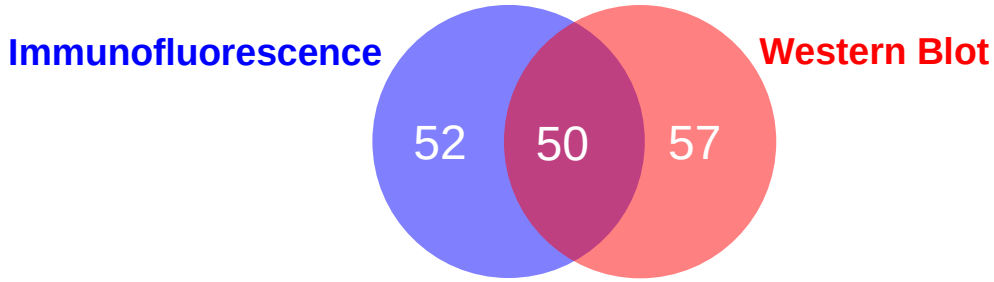


Figure 25: Status of hybridoma clones reactive in WB and IF for VZV ORFs. The picture indicates how many VZV ORFs are positively recognized by hybridoma secreted antibodies in WB and IF. In total, 52 VZV ORFs were recognized in IF, 57 in WB and 50 in both WB and IF.

5.4 Intracellular localisation of VZV proteins

Localization of VZV proteins in the viral context was part of the screening procedure. At the same time a nearly comprehensive analysis of the subcellular localization of 52 (74%) VZV proteins - 22 of them for the first time - was achieved. To our knowledge, the intracellular localisation of only 30 VZV encoded proteins - however not always in the viral context - was previously investigated and published. Localisation was studied both in syncytia and single cells to understand the complexity and dynamics of VZV infection. So far, we have obtained localisation for 52 (74%) proteins in syncytia and for 50 (71%) proteins in single cells. In VZV infected syncytial cells 20 ORFs were localised to the nucleus (38%), 16 ORFs were localised in the cytoplasm (31%) and localisation of 16 ORFs was observed in both nucleus and cytoplasm (31%). For proteins observed in VZV infected single cells 18 were nuclear (36%), 20 in the cytoplasm (40%) and 12 ORFs were distributed throughout the cell (24%). The subcellular localisation of four ORFs (ORF2, ORF19, ORF39, and ORF53) was not included in the thesis but observed by us. For nine VZV encoded proteins, ORF3, ORF8, ORF9, ORF12, ORF21, ORF27, ORF32, ORF57, ORF58 (17%), subcellular localisation was altered when comparing single cells and syncytia. This difference in subcellular localisation could indicate that during infection VZV exhibits different dynamics in syncytia and single cells.

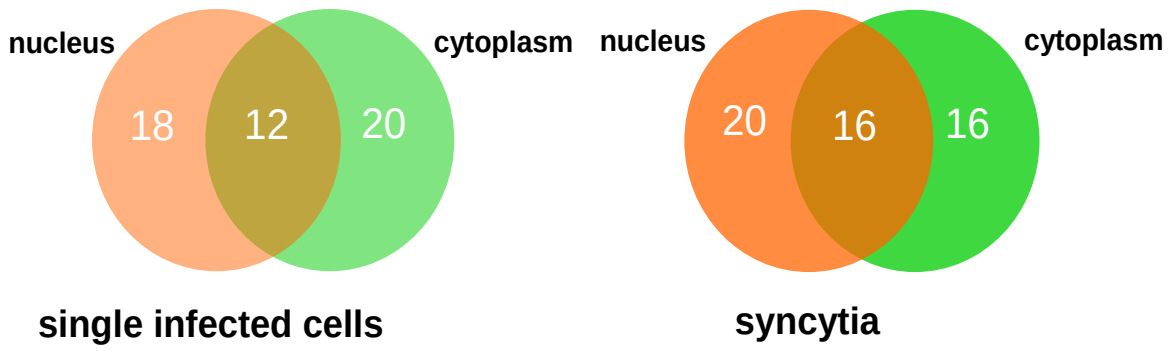


Figure 26: Intracellular localisation status of VZV proteins in single cells and syncytia. The figure indicates how many VZV proteins were found in the nucleus, in the cytoplasm, both nucleus and cytoplasm in syncytia and single cells using hybridoma produced antibodies.

The nuclear preponderance of VZV encoded proteins is in good agreement with the viral lifecycle, which is preferentially associated with the nucleus. In syncytial cells, 18 VZV proteins (35%) localised to the nucleus. To gain insight into their mode of subcellular trafficking, we analysed whether nuclear VZV proteins exhibit an NLS using the prediction algorithms *PredictNLS* and *Bipartite NLS* (Table 10). In this study an NLS was detected in ORF4, ORF9, and ORF62 in addition to proteins ORF29 and ORF63, which were previously reported to contain an NLS [87,88]. Except for ORF9 all of these proteins localise in the same compartment both in single cells and syncytia. The VZV encoded ORF9 protein localises in the cytoplasm in single cells while in syncytia it is found both in the nucleus and cytoplasm. Thus, assuming that single cells represent an earlier infection compared to syncytia, the ORF9 protein might be translocating from the cytoplasm to the nucleus at a later stage of infection. Proteins that carry an NLS have the potential to be actively imported into the nucleus using the importin alpha driven classical pathway [89]. So far, for VZV ORF29 and ORF63, investigators have identified an NLS that is responsible for nuclear localisation (for references see Table 10). Stallings and Silverstein have found that an NLS within ORF29 is responsible for its transport into the nucleus in a Ran-, Karyopherin alpha- and beta-dependent mechanism [70]. In ORF63 a predicted NLS localised between residues 260 and 263 may be responsible for transport of the protein to the nucleus [88]. While for ORF4 and ORF62 bipartite NLSs were predicted (Table 10), researchers have shown that translocation of these proteins into the nucleus may not depend on these NLSs but also be supported by viral interaction partners [52,77].

Table 10: Nuclear localisation sequences of VZV encoded proteins

Protein	NLS	Amino acid position	Type of prediction/Ref.
ORF4	RRSSRSYNTQSSRKHRD RKHRDRSLSNRRRRP	130 - 144 118 - 134	bipartiteNLS predictNLS
ORF9	RRKTTPSYSGQYRTARR	16 – 32	bipartiteNLS
ORF29		9 - 154	[176]
ORF62	RRFGPPSGVEALRRRCA	395 - 412	bipartiteNLS
ORF63	KRRR	778 – 789	[187]

http://cubic.bioc.columbia.edu/predictNLS/ (NLS)
http://www.expasy.org/cgi-bin/nicedoc.pl?PDOC00015 (bipartite NLS)

Many proteins that enter the nucleus are equipped with sequences that drive their active nuclear export. One of the best characterized signals is the leucine-rich nuclear export sequence (NES) that is predicted for a number of VZV proteins (Table 11). A NES was identified in 14 proteins, ORFs 4, 6, 10, 21, 27, 29, 32, 33, 47, 48, 55, 62, 64, 66. Of those, the VZV proteins ORF6, 10, and 33 showed a steady state localization in the nucleus while all other ORFs are distributed throughout the cell. Subcellular localisation of ORF21, ORF27 and ORF32 varies in single cells and syncytia. These findings are consistent with shuttling of these proteins between both compartments and their behaviour might be regulated. These observations provide starting points for a detailed analysis of nucleocytoplasmic trafficking of VZV proteins in the course of infection.

Table 11: Nuclear export sequences of VZV encoded proteins

Protein	NES	Reference
ORF4	LGPFVRCLLL	[90]
ORF6	LQAIKDLFL	[90]
ORF10	LARLLYLHLYL	[176]
ORF21	LPGLLFWRLDL	www.cbs.dtu.dk
ORF27	LSGMGYHLGL	www.cbs.dtu.dk
ORF29	LAVVQDLAL	www.cbs.dtu.dk
ORF32	ISLALEI	www.cbs.dtu.dk
ORF33	LSPLERALYL	www.cbs.dtu.dk
ORF47	LLGRLPGQLPI	www.cbs.dtu.dk
ORF48	LSGYFPALKL	www.cbs.dtu.dk
ORF55	LCWFKQLLEL	www.cbs.dtu.dk
ORF62	LAALSNRLCL	www.cbs.dtu.dk
ORF64	LELSERLIL	www.cbs.dtu.dk
ORF66	LWTNLYELPI	www.cbs.dtu.dk

http://www.cbs.dtu.dk/services/NetNES/

5.5 Comparative analysis

The available published results on localisation of VZV proteins were in clear agreement with our studies except for three VZV encoded proteins (ORF 37, ORF60, ORFS/L). In our observation all four VZV encoded proteins were detected in the nucleus instead of the cytoplasm. The ORF37 and ORF60 encode glycoproteins gH and gL which based on their function are expected to be localised at the ER, Golgi or in the plasmamembrane. Many groups have studied gH and gL thoroughly and reported their localisation to cytoplasmic membranes which is in complete contrast to our observation [66,57]. ORFS/L was previously shown to be a cytoplasmic protein while we observed it in the nucleus [80]. These discrepancies might be caused by mishandling of hybridoma clones, antibodies or false detection in ELISA due to cross reactivity.

We have also observed minor differences for two other proteins encoded by ORF20 and ORF25. Specifically, ORF20 and ORF25 were found only in the cytoplasm by other investigators, whereas in our study these two proteins were detected in both the nucleus and the cytoplasm [58,63]. Interestingly, in the reported analysis transfection expression of the specific ORFs was done while in our case VZV infected cells were analysed. Thus, the difference could be due to altered expression in these studies or due to the lack of viral interaction partners. It is well understood that ORF20 encodes for a capsid protein which can be found in both the cytoplasm and nucleus. Yeast-2 hybrid analysis showed that VZV ORF25 interacts with 15 other VZV encoded proteins and may form functional complexes. Indeed, Visalli and colleagues showed that ORF25 forms a complex with ORF30, ORF42 and ORF45 [63]. These studies give clear evidence that ORF25 brings viral proteins together and forms a functional network. So, these published observations are consistent with the pan-cellular distribution of ORF20 and 25 we observed in our study. Taken together, our study provides new localisation data for 22 ORFs which accounts to 32% of the VZV genome. By combining the published data with our localisation data the intracellular localisation for 57 VZV (81%) encoded proteins could be determined (Figure 27).

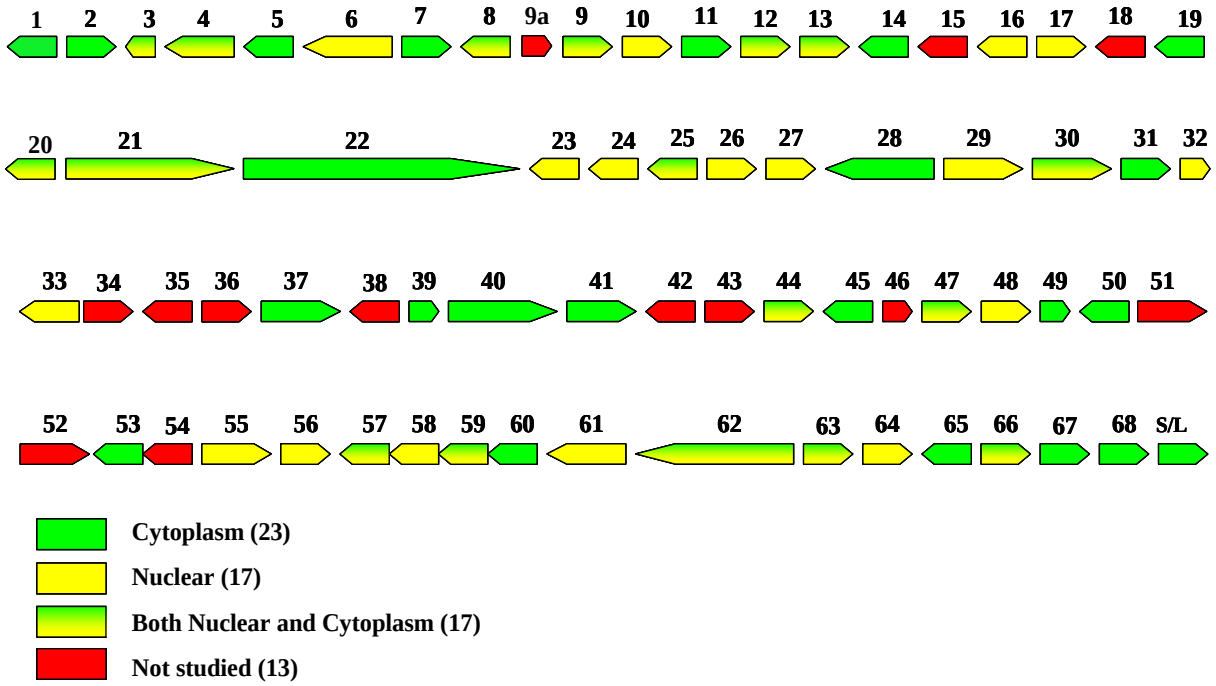


Figure 27: Schematic diagram of intracellular localisation in respect of the viral genome. All VZV encoded proteins are shown. The arrows indicate the transcriptional orientation of ORFs in the genome. Genes are colour coded as indicated in the figure.

5.6 Comparison of subcellular localisation of herpesviral proteins

Herpesviruses are subdivided into three different subfamilies based on the genomic composition and biology [91,32]. They possess dsDNA genomes ranging from the smallest 120kbp of the VZV to 240kbp of CMV[32]. In all three subfamilies, 41 core orthologs are thought to be conserved comprising half of the genome of HSV-1, VZV, EBV, and KSHV but less than 25% of CMV. They are further subdivided into a group of 31 orthologs with relatively high sequence similarity (approximately 30 to 60% sequence similarity), and a group of 10 orthologs with little sequence similarity (approximately 16 to 30%) [81]. These herpesviral core orthologs are generally involved in fundamental aspects of viral replication like entry, DNA replication and packaging, structure and primary and secondary envelopment [92,93].

Nowadays, systems biology approaches are used as a powerful tool to analyse and understand complex biological processes of different pathogens [93,94,95]. By using the yeast-2 hybrid system, Uetz *et al.* have studied protein-protein interactions in VZV and HHV-8 in a proteome-wide fashion [82]. Fossum *et al.* have compared protein interaction networks of core orthologs for 5 different human herpesviruses (HSV-1, VZV, CMV, EBV, KSHV) [81]. The usefulness and validity of such systems biology

approaches was supported by subcellular co-localization of HHV-8 proteins K10 and its interaction partners previously identified using the yeast-2 hybrid system [82],[96]. Sofar, the subcellular localisation of proteins derived of 4 herpesviruses (HSV-1, CMV, EBV and KSHV) covering 3 herpesviral subfamilies was analysed. In this study, we compared the subcellular localisation of 41 core proteins of 5 different herpesviruses (HSV-1, VZV, CMV, EBV, KSHV). Our comparative results of 41 core proteins conserved in 5 different herpesviruses are shown in Table 12. We have classified the subcellular localisation as nuclear, cytoplasmic or both nuclear and cytoplasmic. If the interaction between core proteins is conserved they are likely to be found in the same compartment. The data received here on subcellular localization should in near future be compared to the interaction data received by yeast-2 hybrid analysis for VZV. Since the subcellular localisation of a protein is closely associated with its function, the subcellular localisation screening is an appropriate starting point to characterise the function of so far poorly analysed VZV proteins as well as other herpesviral proteins during viral infection.

For all transmembrane glycoproteins conserved throughout the herpesviral family, we localised them in the cytoplasm and to cytoplasmic membranes consistent with their biosynthesis at the endoplasmatic reticulum and maturation in and transport along the secretory pathway. The interaction between gH:gL and gM:gN is conserved in all 3 subfamiles. For VZV tegument proteins supposed to function in either nucleus or cytoplasm depending on the envelopment step a cytoplasmic and/or nuclear localization is expected. A good agreement is seen for VZV ORFs 40 and 41 and all orthologues compared in Table 10 since all of them localized to the nucleus and / or the cytoplasm, compartments that are in continuous exchange. The same is true for the subcellular localisation of capsid proteins compared between all 5 herpesviruses. Although their localization is not identical when comparing a set of five orthologs, it shows a great amount of similarity between all herpesvirus subfamilies. In conclusion, all over there is excellent agreement in localization when core orthologs are compared. Of the 32 VZV core orthologs studied, all showed a good match in localization with their functional relatives in other herpesviral subfamilies.

Table 12: Subcellular localisation of 41 herpesvirus core proteins

The colors in the table represents different types of proteins. The blue color represents glycoproteins, red represents for capsid proteins and pink for tegument proteins.

VZV (ORF)	Localisation	HSV-1	Localisation	CMV	Localisation	KSHV	Localisation	EBV	localisation
ORF4	**N&C**	UL54	**N**	UL69		57	**N**	BMLF1	**N**
ORF6	N	UL52	C	UL70		56	C	BSLF1	C
ORF7	**C**	UL51	**N&C**	M71		55		BSRF1	
ORF8	**N&C**	UL50	**N&C**	UL72		54	**N**	BLLF3	**N**
ORF9a		UL49.5	**C**	M73	**C** (97)	53	**C**	BLRF1	
ORF 16	**N&C**	UL42	**N**	UL82		59		BMRF1	
ORF 19	**C**	UL39		UL45	XX	61	**C**	BORF2	
ORF 20	**N&C**	UL38	**N**	M46		62		BORF1	
ORF 21	N&C	UL37	C	US29	N	63		BOLF1	
ORF 22	**C**	UL36	C(98)	UL48		64	**C**	BPLF1	
ORF 23	**N&C**	UL35	**N&C**	M48.2		65		BFRF3	
ORF 24	N	UL34	N&C	UL50	C	67	C	BFRF1	C
ORF 25	**N&C**	UL33	**N&C**	UL51	**N&C**	67.5	C	BFRF4	
ORF 26	N	UL32	C	UL52		68	C	BFLF1	
ORF 27	C	UL31	N&C	UL53	N	69	N	BFLF2	N
ORF 28	**C**	UL30	**N&C**	UL54		9	**C**	BALF5	
ORF 29	**N**	UL29	**N&C**	UL57		6	**N**	BALF2	**N**
ORF 30	**N&C**	UL28		UL56	**N&C**	7	**N&C**	BALF3	
ORF 31	**C**	UL27	**C** (99)	UL55	**C** (100,101)	8	**C**	BALF4	**C** (102,103)
ORF 33	**N**	UL26	**N**	UL80	**N**	17	**N**	BVRF2	
ORF 33.5	**N**	UL26.5		M80.5		17.5		BdRF1	
ORF 34		UL25	**N&C**	UL77	**C**	19	**N&C**	BVRF1	
ORF 35		UL24	**N**	UL76		20	**N**	BXRF1	
ORF 37	**C**	UL22	**C** (104,73)	UL75	**C**	22	**C** (105)	BXLF2	**C** (106)
ORF 38		UL21	N&C	M88		23		BTRF1	
ORF 40	**C**	UL19	**C**	UL86	**C**	25	**C**	BCLF1	
ORF 41	**C**	UL18	**N&C**	UL85	**N&C**	26	**N&C**	BDLF1	**N&C**
ORF 42		UL15		UL89		29b	N&C	BDRF1	N&C
ORF 43		UL17		UL93	**N&C**	32	**N&C**	BGLF1	**C**
ORF 44	**N&C**	UL16	**N&C**	UL94	**N&C**	33	**N**	BGLF2	**N&C**
ORF 46		UL14	N&C (107)	M95		34		BGLF3	
ORF 47	N&C	UL13	C	UL97		36	N	BGLF4	N&C
ORF 48	N	UL12	C	UL98	nuc	37	N	BGLF5	
ORF 49	C	UL11		M99		38		BBLF1	
ORF 50	**C**	UL10	**C** (72)	UL100	**C** (97)	39	**C**	BBRF3	
ORF 52		UL8	(108)	M102		40		BBLF2	
ORF 53	**C**	UL7	(108)	UL103	**C**	42	**N&C**	BBRF2	**N&C**
ORF 54		UL6		UL104		43	**C**	BBRF1	**C**
ORF 55	N	UL5		UL105		44	C	BBLF4	N&C
ORF 59	**N&C**	UL2		UL114	**N&C**	46	**N&C**	BKRF3	**N&C**
ORF 60	**C**	UL1	**C** (74,73)	M115	**C** (109,110)	47	**C** (111,105)	BKRF2	

VZV and HSV-1 share 65 orthologs and for 39 proteins information on their subcellular localization is available either from the literature or our own results (Figure 28). Our data show that 35 out of 39 orthologs (90%) show a similar localization. Out of these, 21 orthologs show complete identity in subcellular localisation while 14 orthologs localize similarly. So, comparing the subcellular localisation of orthologs of

herpes viruses will provide good information to study functions of unstudied orthologs and their interaction partners.

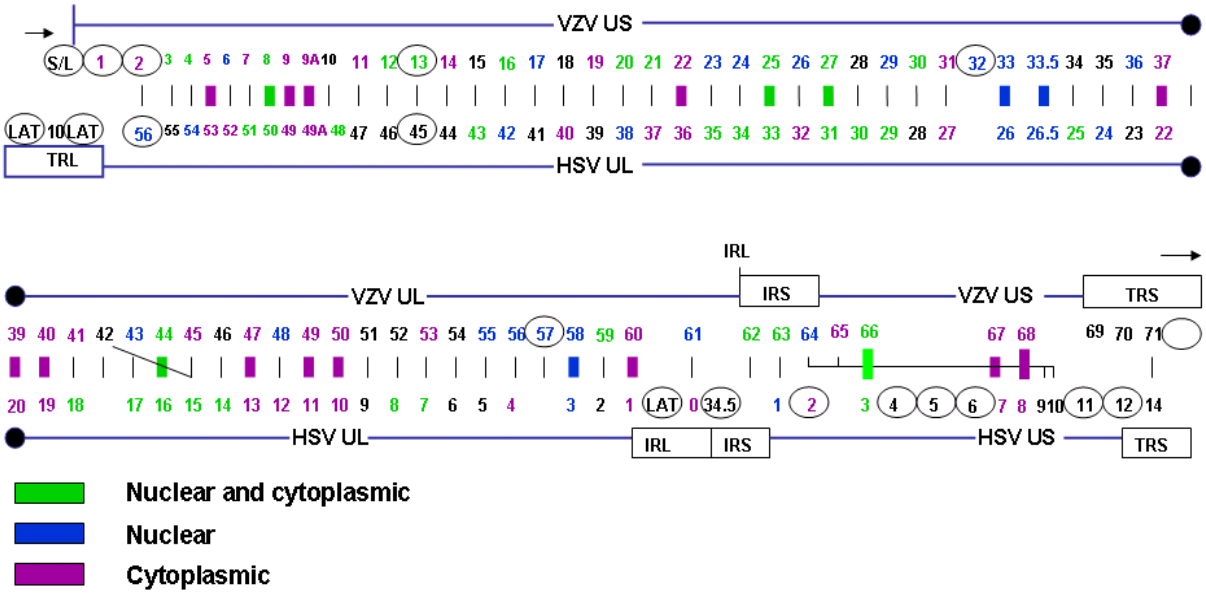


Figure 28: Comparison of the subcellular localisation of VZV and HSV-1 orthologs. The subcellular localisation was represented by specific colors shown in the figure. Identical subcellular localisation of VZV and HSV-1 orthologs was highlighted by the boxes present between ORF numbers (modified from [112]).

5.7 Epitope mapping

Herpesvirus glycoproteins are part of the virus outer envelope that is responsible for inducing B-cell based immune response and are widely used as serological agents. The antibodies against viral glycoproteins play a major role in neutralisation and immune defence of the host. Previously, for many herpesviral glycoproteins epitope mapping was done using the pepscan technique to identify their antigenic determinants [113]. In our study the same method was applied to identify several immunodominant regions for the viral glycoproteins gK, gB, gL, gI, gE and the membrane-associated phosphoprotein ORF24.

The positive clones 6B11 and 5A6 specific for gK identified five immunodominant regions. Antibodies produced by clone 6B11 detected five discrete stretches of aminoacids, $_{18}$GHAVFTLWYTAR$_{31}$, $_{96}$TPLHDKIRI$_{106}$, $_{135}$QFRRIR$_{142}$, $_{156}$TSYSLNYVTRVI**SNILLGYPY**$_{178}$ and $_{195}$KVFNADPISFLYMHK$_{210}$. MAbs from clone 5A6 also identified five different discrete stretches. The identified residues are $_{90}$VKTAIS**TPLHDKIRI**$_{106}$, $_{135}$QFRRIR$_{142}$, $_{168}$SNILLGYPYTKL$_{181}$, $_{195}$KVFNADPISFL-YMHK$_{210}$ and $_{264}$LISYVRPKPTKDNHL$_{279}$. Two very similar immunodominant regions

are recognized by both monoclonal antibodies, $_{135}$QFRRIR$_{142}$ and $_{195}$KVFNADPISFLYMHK$_{210}$, an overlap between two other sequences in residues $_{96}$**TPLHDKIRI**$_{106}$ and $_{168}$**SNILLGYPY**$_{178}$ was observed. These observations suggest that these regions in gK are highly immunogenic.

The clones 2A7 generated against gB and 5E5 generated against gL detect a long continuous stretch at the C-terminal end of each immunogen, $_{831}$ARKKNKTSALLTSRLTGLALRNRRGYSRVR$_{862}$ in gB and $_{77}$KCPVPEVILWFK-DKQMAYWTNPYVTLKGLAQSV$_{111}$ in gL. The mAbs 3D6 made against gI identified three discrete stretches at the C-terminal end of gI and IYR is the common epitope region recognised by the first two stretches. The mAbs 6B12 recognised eight discrete stretches from the complete antigenic region and $_{282}$PGVLKVLRTEKQYLG$_{298}$ is the common epitopic region recognised by reactive spots 5 and 6.

The mAbs 1G11 and 3B7 raised against the membrane phosphoprotein ORF24 recognise seven and three discrete stretches in the N-terminal region. Both mAbs identify one common immunodominant region, $_{153}$NCFVRMPYVQLSFRF$_{169}$ and two regions with a partial overlap, $_{15}$GDNLLQRIR$_{25}$ and $_{195}$NLDEYIRWR$_{205}$. The detection of common immunodominant regions in both clones shows that these regions are antigenic and elicit B cells to produce antibodies.

From these observations it can be concluded that the antibodies to gB and gI recognize linear epitopes while antibodies to gK, gL, gE and ORF24 have complex, conformational epitopes. It should be noted however, that the pepscan technique is biased towards detection of short linear stretches of aminoacids [114]. So, the antibodies we have tested could recognize additional more complex epitopes in the protein that cannot be identified by the pepscan technique.

Monoclonal antibodies could potentially be used for neutralization of VZV infection. Researchers have indeed identified antibodies that are capable to neutralise VZV virions by binding to the surface proteins gH and gE [115,116]. The identification of immunodominant regions by epitope mapping is an initial step to characterise our mAb clone collection. This characterisation will help to perform further analysis to identify additional neutralising monoclonal antibodies and the regions within VZV proteins targeted by these antibodies.

6. References

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7. Abbreviations

μ	micro (10^{-6})
°C	degrees Celsius
Ap^r	ampicillin resistence
ATP	adenosine triphoshate
bp	base pair
BSA	bovine serum albumin
C. elegans	Caenorhabditis elegans
cDNA	complementary DNA
CDR	complementary determining region
Cm^r	chloramphenicol resistence
CMV	Cytomegalovirus
CNS	Central Nervous System
CVS	congenital varicella syndrome
C	centigrade
C	cytoplasm
DAPI	4-6 diamidino-2-phenylindole
DHFR	dihydrofolate reductase
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleoside triphosphate
DRG	dorsal root ganglia
ds	double stranded
DTT	dithiothreitol
E	early
E. coli	Escherichia coli
e.g.	exempli gratia (Lat. = for instance)
EBV	Epstein-Barr-Virus
EDTA	ethylene-diamine-tetraacidic acid
EHV	equine herpesvirus type
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
et al.	et alii (Lat. = and others)
EtOH	ethanol
FA	formaldehyde
Fab	fragment antibody binding
FC	fragment crystallizable
FCS	fetal calf serum
FISH	fluorescent in situ hybridization
FL	full length
g	gram
GC	Guanine and Cytosine
Gm^r	gentamycin resistence
h	hour(s)
H	heavy
HA	hemagglutinin
HAT	Hypoxanthine Aminopterin Thymidine
HCl	hydrochloric acid
HCV	hepatitis C virus
HHV	human herpesvirus
HIS	histidine
HIV	human immundeficiency Virus
HSV	Herpes simplex virus

HVS	herpesvirus saimiri
HZ	Herpes zoster
IE	immediate-early
ISH	in situ hybridization
kbp	kilobasepairs
kDa	kilo Dalton
Km^r	kanamycin resistence
KSHV	Kaposi's sarcoma-associated herpesvirus
L	late
L	light
l	litre
IDE	insulin-degrading enzyme
IF	immunofluorescence
LB	Luria Bertani medium
LEU	leucin
m	milli (10^{-3})
Mab	monoclonal antibody
Man-6-P	mannose-6-phosphate
MBP	Maltose-binding protein
MCD	multicentric Castleman's diseases
MCP	major capsid protein
MHC	major histocompatibility complex
min	minute(s)
mRNA	messenger RNA
MW	molecular weight
Mw	mother well
n	nano (10^{-9})
N	nuclear
NLS	nuclear localization signal
Neg	negative
NES	nuclear export signal
NT	not tested
OD	optical density
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEL	primary effusion lymphoma
PHN	Postherpetic neuralgia
PMBC	peripheral blood mononuclear cells
p-OKA	parental OKA
pos	positive
PRV	pseudorabies virus
RC	recombinatorial cloning
Ref	reference
RNA	ribonucleic acid
RNAi	RNA-mediated interference
rpm	revolutions per minute
RT	room temperature
s	second(s)
S. cerevisiae	Saccharomyces cerevisiae
SCID	Severe combined immunodeficiency
S. aureus	Staphylococcus aureus
SDS	sodium dodecylsulfat
SOC	Super optimal broth

ssDNA	single-stranded DNA
TEMED	tetramethylethylendiamin
TG	trigeminal ganglia
TGN	trans-Golgi network
TK	thymidine kinase
TPA	tissue plasminogen activator
TRP	tryptophan
TS	thymidylate synthase
UL	unique long
US	unique short
UV	ultraviolet
VZV	varizella zoster virus
WB	western blot
w/o	without
Y2H	yeast two-hybrid

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9. Publications

1) Stellberger, T., Haeuser, R., Baiker, A., **Pothineni, V. R.,** Haas, J., Uetz, P. (2010) Improving the yeast two-hybrid system with permutated fusion proteins: the Varicella Zoster Virus interactome. *Proteome Science*, **8**:8

2) Ceroni, A., Sibani, S., Baiker, A., **Pothineni, V. R.,** Bailer, S., M., LaBaer, J., Haas, J., Campbell, C. (2010) Systematic analysis of the IgG antibody immune response against Varicella Zoster virus (VZV) using a self-assembled protein microarray (NAPPA) (*Molecular Biosystems*, accepted)

3) **Pothineni, V. R.,** Bailer, S., Babic, M., Lenac, T., Baiker, A., Rose, D., Endesfelder, M., Simic, H., Jonjic, S., Haas, J. Genome-scale generation of antibodies and intracellular localisation map for Varicella zoster virus (Manuscript in preparation).

Abstracts

1) Babic, M., **Pothineni**, **R.,** Lenac, T., A. Baiker, Markovic, F., Miklic, K., Endesfelder, M., Rose, D., Simic, H., Bailer, S., Haas, J., Jonjic, S. Proteome-wide Production of Antibodies against Varicella Zoster Virus. *Proteomics Forum ,Berlin, Germany, April 2009.*

2) **Pothineni**, **R.,** Babic, M., Lenac, T., Baiker, A., Endesfelder, M., Rose, D., Simic, H., Jonjic, S., Haas, J. S. Proteome-wide Production of Antibodies against Varicella Zoster Virus. *19th Annual Meeting of the Society for Virology, Leipzig, Germany, Mar 2009.* (Oral presentation)

3) Baiker, A., Lueking, A., Pinto. M. V., **Pothineni**, **R.,** Arvin, A., Jaeger, G. High-throughput expression of the Varicella-Zoster Virus (VZV) proteome for serum profiling experiments. *9th Annual VZV Satellite Workshop, Estirol, Portugal, Jy 2008.*

4) Simic, H.; Baiker, A., Babic, M., **Pothineni, R.,** Miklic, K., Bazdaric, D., Endesfelder, M., Malic, S., Polic, B., Jonjic, S., Haas, J. High-throughput monoclonal

antibody production against the varicella zoster virus (VZV) proteome. *Annual Meeting of the Croatian Immunological Society , Oct 2007.*

5) Baiker, A., **Pothineni, R.**, Endesfelder, M., Babic, M., Simic, H., Jonjic, S., Arvin, A. Haas, J. High-throughput expression of the Varicella-Zoster Virus (VZV) proteome for serum profiling experiments and the production of monoclonal antibodies. *8th Annual VZV Satellite Workshop, Asheville, NC, USA, July 2007.*

10. Curriculum vitae

Personal Information

Name	Venkata Raveendra Pothineni
Nationality	Indian
Date of birth	30.01.1978
Marital status	Married
Address	Max von Pettenkofer-Institut, Pettenkofer Strasse 9a 80336 München
E-mail	Ravindra179@yahoo.com

Education

Since October 2006	Pursuing Ph.D. at Max von Pettenkofer-Institut Supervisor: Prof. Dr. Dr. Jürgen Haas Deputy Director, Division of pathway Medicine University of Edinburgh Medical School, Chancellor`s Building Little France Crescent Edinburgh EH 16 4SB,UK
January 2004 – 2006 September	International Master of Science Program Major in Molecular Biology at University of Skovde, Sweden
October 1999 – June 2001	Master of Science in Biotechnology at Bharathidasan University, India
1996 – 1999	Bachelors of Science in Microbiology, Biochemistry and Aquaculture at Nagarjuna University, India
1996 - 1994	Intermediate College (Gymnasium), India