Establishment of a baculovirus surface display library using the major envelope protein gp120 from human immuno deficiency virus 1 as a model.

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1. Abstract

Heterologous expression of functionally active protein requires correct posttranslational modifications and folding which can exclusively be accomplished by eukaryotic cells. During the last twenty years, the baculovirus expression system has become a competitive alternative to mammalian expression systems. Numerous heterologous proteins have been expressed successfully with the aid of baculoviruses taking advantage of the mammalian-like postranslational machinery of lepidopteran cell lines.

The aim of this work was to establish and to evaluate an authentic eukaryotic surface expression library on baculoviral particles and infected *Spodoptera frugiperda* (*Sf*9) insect cells for the purpose of epitope mapping. The complex envelope glycoprotein gp120 of human immunodeficiency virus (HIV-1) was chosen as challenging model protein. Different baculoviral expression vectors were tested for their surface display properties in order to select the optimal vehicle for construction of the library. Two different strategies for generation of the library were applied and evaluated. Several monoclonal antibodies were used for specific selection of binding partners from the libraries by means of fluorescence activated cell sorting (FACS).

A first library was generated by random fragmentation of the target protein gp120 by DNase digestion. Presentation of desired peptides on baculoviral particles and infected insect cells has been demonstrated, but no selection of binding partners could be achieved by means of FACS.

The second library which was generated by specific PCR amplification of gp120 fragments turned out to be very reliable and well-defined. Cytometric sorting with all three chosen antibodies led to specific amplification of binding partners and to verification of their epitopes. Moreover, FACS selection with the CD4-binding site antibody IAM 120-1B1, specific for a conformational epitope, resulted in the identification of several promising gp120 fragments with high binding affinity.

The presented work demonstrates the potential of the established libraries to serve as a tool for applications like epitope mapping and receptor binding studies and will be made available for other scientists.

2. Introduction

2.1 Expression systems

Expression of foreign genes in diverse host organisms has become a matter of particular interest in basic research as well as in terms of recombinant production of desired proteins. Apart from classic approaches with bacteria, the use of eukaryotic host cells has become more and more popular since they imply several decisive advantages: their ability to build disulfid-bonds and to perform essential modifications like glycosylation, phosphorylation, carboxylation, and acylation which are very crucial when it comes to correct folding and maintaining authentic functionality of the foreign protein. Furthermore, eukaryotic cells are capable of translocating proteins to the nucleus, the outer membranes as well as secreting them to the extracellular matrix, providing the basis for diverse sophisticated applications in modern biotechnology.

Insect cells (*Lepidoptera*) as hosts for recombinant protein expression possess several advantages over mammalian cell culture techniques. Apart from their high expression rates they are very easy to propagate and rather modest in their requirements. They possess specific transferases like glucosidases, mannosidases and N-acetylglucosamine-transferases which are essential for authentic expression of human proteins. Although it is not clear whether they are capable of performing sufficient and genuine sialyl- and galactosyl-transferase activity [Marchal *et al.* (2001)], diverse proteins of animal and human origin have been expressed functionally active with the aid of lepidopteran cell lines. Insect cells do not harbour retroviruses or promoters that are active in animal or human cells and, thus products derived from those systems can be included in appropriate applications without special safety concerns. In conjunction with the well explored baculovirus species this system represents a competitive alternative to mammalian cell culture expression techniques.

2.2 Overview on baculovirus

Baculoviruses [Blissard and Rohrmann (1990)] are dsDNA-viruses with a circular genome of about 88 to 220kB. They infect invertebrates, mainly insects such as different butterflies and moth species but also crustaceans, e.g. shrimps. Infection always leads to cell lysis and subsequent death of the host organisms. For this reason and because of their strict host specifity, baculo-viruses were considered as promising bio-insecticids in the early 1970s [Entwistle and Evans (1986)]. With the invention of baculoviral expression vectors during the early 1980s [Smith *et al.* (1983)] the baculovirus insect cell expression system has become a versatile tool for the expression of eukaryotic proteins. Expression vectors based on the genome of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) and *Bombyx mori* nuclear polyhedrosis virus (*Bm*NPV) have become the most important to date.

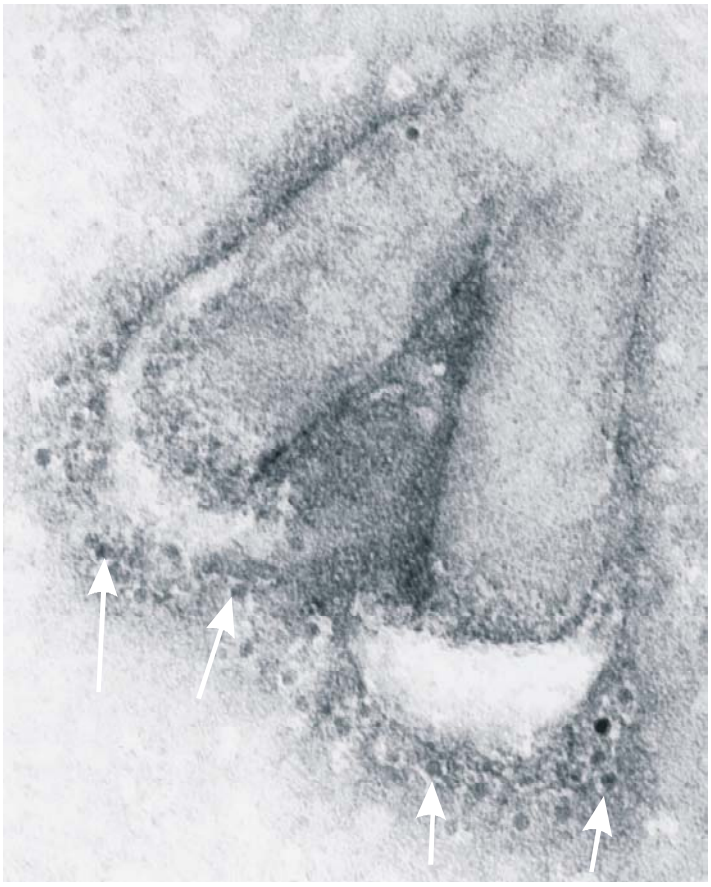

Fig.1: Electron micrograph of *Autographa californica* virus particles. Arrows indicating gold-conjugated antibodies binding to distal baculoviral envelope protein gp64. Electron micrograph was done in collaboration with Prof. Margit Sara (ZUF, BOKU Vienna).

The diameter of the rod-shaped baculoviral particles averages 40 to 50nm whereas their length varies from 200 to 400nm depending upon the size of their genome. This feature makes it possible to incorporate rather large pieces of foreign DNA into the virus. The baculoviral DNA is surrounded by a

nucleocapsid consisting mainly of a 39kD large protein. Expression occurs in the cytoplasm whereas assembly of these nucleocapsids takes place in the nucleus of the infected cell.

Baculoviral particles emerge in two distinct manifestations: the so called “budded viruses“ represent the primary and highly infectious form. They appear when viral nucleocapsids budd through the outer plasma membrane of their host cell, and thereby are surrounded by a lipid-bilayer. Viral budding and infection of new cells through adsorptive endocytosis are mainly triggered by the virus-coded glycoprotein gp64 (Fig.1) [Whitford *et al.* (1989)] which plays a key role in the baculoviral life cycle (Fig.2). This protein forms trimers and accumulates at the poles of the capsid giving it its characteristic morphology. The second manifestation of baculoviruses, the “occluded virus“, represents the resistant dormant body in which several nucleocapsid are incorporated in a special protein-matrix consisting of polyhedrin [Rohrmann (1986)]. This protein crystallises encasing the virions, and thereby provides protection against UV light and desiccation when being released from lysed host cells.

The family of *Baculoviridae* is divided into three different subgroups according to the nature of the matrix and the number of nucleocapsids being imbedded within the capsules. *Autographa californica* belongs to the subgroup A, where single (SNPV) or multiple (MNPV) nucleocapsids are embedded in a polyhedrin matrix. Subgroup B represents the so called granulosis viruses which produce a different protein, granulin, in which the nucleocapsids are incorporated. Finally, all remaining baculoviruses not expressing any characteristic structure like polyhedrin or granulin are pooled as “non-occluded“ viruses in subgroup C.

2.3 Replication of *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV)

Autographa californica nuclear polyhedrosis virus represents the best characterised and most widely used baculovirus. Occluded viral particles are uptaken by insect larvae with their nutrition and get released in the basic environment of their intestinal tract. After fusion of the viral lipoprotein

membrane with the membrane of the surrounding epithelial cells, baculoviral nucleocapsids are transported through the cytoplasm into the nucleus of the cell. This process of “uncoating“ takes about 15 to 240 minutes and is followed by the begin of virus replication starting at about six hours after infection. Progeny nucleocapsids accumulate after eight hours within the nucleus. They get transported through the cytoplasm and start budding across the outer membrane after 12 hours incorporating membrane - associated viral gp64 which was previously synthesised. Simultaneously, the production of polyhedrin inclusion bodies in the nucleus occurs which can be observed by light microscopy at about 24 hours after infection. Budded viral particles pass the insect larvae through the hemolymph infecting any accessible tissue and starting new generations of occluded and budded viruses. Within the next five to seven days, polyhedrin inclusion bodies extensively accumulate in the larvae leading to

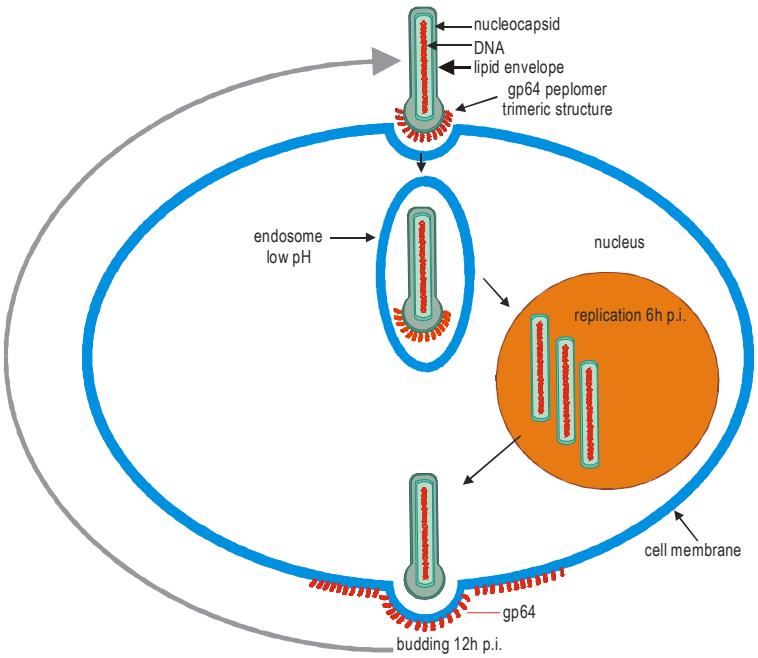

Fig.2: Life cycle of baculovirus. Budded viral particles enter the host cell by adsorptive endocytosis. Low pH triggers fusion of the viral membran with the endosome releasing nucleocapsids into the cytoplasm. The nucleocapsids are translocated into the nucleus where replication starts at about 6 hours post infection. Progeny Baculoviruses are transported through the cytoplasm and acquire their lipid-bilayer (containing gp64) while budding across the outer membrane of the host cell. Generation of occluded viruses is not shown. Taken from *Trends in Biotechnology* [Grabherr (2001)].

death of the host organism. After lysis of the larvae, the viruses are able to persist in the soil for years, representing potentially infective material for new generations of baculoviruses to come.

Gene expression of baculoviruses is strictly divided into four different stages: the immediate-early, the delayed-early, the late, and the very late phase. Virus replication separates the early from the late phases with the immediate-early phase being initiated only by transcription factors of the host cell. Characteristic promoters of the first stage are the immediate-early promotors IE-1 [Guarino and Summers (1986)] and IE-N [Carson *et al.* (1988)]. The production of budded viruses peaks in the late phase (12 to 20 hours post infection) with viral genes for DNA-polymerase and structural elements of the nucleocapsid and virus particles (e.g. gp64) reaching their maximum transcription levels. Finally, in the very late phase, starting at about 15 hours after infection, polyhedrin and other genes involved in the maturation of occlusion bodies get switched on leading to the clustering of crystalline embedded nucleocapsids.

2.4 Baculoviruses as expression vectors

Baculoviral expression vectors [O'Reilly *et al*. (1992)] usually lack the polyhedrin-gene because encapsulation of virus particles into the polyhedrin-matrix is dispensable and not desired. Subsequently, this region is replaced by homologous recombination or by insertion of foreign gene expression cassettes. Homologous recombination into the baculoviral genome [Smith *et al.* (1983); Pennock *et al.* (1984)] is mediated by first inserting the foreign gene into a transfer vector. This bacterial plasmid contains all necessary regulatory elements (promoter, terminator) as well as segments being homologous to viral sequences. The vector is co-transfected with wildtype baculoviral DNA and homologous recombination occurs with a frequency of about 0,1% to 1% [Kitts *et al.* (1990)]. Recombinant plaques lacking the feature to express the characteristic crystalline polyhedrin can be identified and isolated using a light-microscope. Because of the low recombination frequency and the involved effort in detecting positive clones new generations of transfer plasmids have

been established [Vlak *et al.* (1990)] using heterologous *E. coli* ß-galactosidase as a marker. Furthermore, linearised wildtype DNA, which is less infective than circular baculovirus-DNA was applied as acceptor molecule [Kitts *et al.* (1990)] increasing the portion of recombinant viruses to 30%. Eventually, a further development in this regard was the disruption of an essential gene (*ORF* 1629) on the baculoviral DNA (e.g. BaculoGold-DNA (BD Biosciences, Palo Alto, USA)) being complemented by the transfer vector [Kitts and Possee (1993)]. Thus, resulting baculoviruses feature a fraction of more than 99% recombinants.

A novel generation of baculoviral vectors was established by Ernst *et al.* [Ernst *et al.* (1994)]. The introduction of a unique *Sce*-I restriction site into the viral genome enables the direct insertion of foreign sequences into the genomic DNA. *Sce*-I is an intron-encoded restriction endonuclease from *Saccharomyces cerevisiae* that recognises a non-palindromic cleavage site of 18 basepairs allowing unidirectional ligation of suitable fragments [Colleaux *et al.* (1988)]. By this technique, recombination frequency was increased to $2*10^5$ recombinants per microgram viral DNA as compared to $2*10^3$ that can be achieved via homologous recombination [Ernst *et al.* (1994)].

Several hundred different genes of viral, human, animal and herbal origin have already been expressed successfully using the baculovirus expression system. Expression yields can reach as much as 500mg per litre culture volume taking advantage of the very strong polyhedrin and *p10* promoters. Moreover, the separation of virus production and foreign gene expression with these very late promoters facilitates the production of possibly cytotoxic proteins as shown by Baum *et al.* [Baum *et al.* (1987)].

Insect cells are capable of performing many of the posttranslational modifications necessary for the production of functionally active protein of eukaryotic origin. While O-glycosylation in lepidopteran cells is carried out authentically, the pattern of N-glycans may differ from those derived from other eukaryotic host cells [Jarvis and Summers (1989)]. Although insect cells mainly generate high-mannose N-glycosylated proteins instead of linking galactose or sialic acid, complex glycoproteins, like hemagluthinin A of influenza virus, have been expressed functionally active with the aid of baculoviruses [Van Wyke Coelingh *et al.* (1987); Kuroda *et al.* (1991)]. Correct phosphorylation and

acylation of fatty acids of SV 40 T-antigen produced in insect cells was demonstrated by O'Reilly and Miller [O'Reilly and Miller (1988)]. Moreover, endopeptidase and carboxypeptidase activity have been identified [Kuroda *et al.* (1990)] performing proteolytic cleavage of crude proteins. Signal peptides for redirection of proteins to the nucleus and to the endoplasmatic reticulum for secretion and membrane translocalisation have been used successfully [O'Reilly and Miller (1988); Jarvis and Summers (1989)]. Even several subdomains forming complex proteins have been expressed simultaneously [Kuroda *et al.* (1991)] as well as complete foreign viral capsids that were assembled correctly in insect cells [Hilditch *et al.* (1990)]. Human interleukin-5 monomers were dimerised building correct disulfid-bonds [Ingley *et al.* (1991)].

Altogether, posttranslational modifications in insect cells mainly result in native protein, although, like in most expression systems, authentic structure of the produced foreign polypeptides is not predictable.

Recombinant baculoviruses are considered safe, since they are unable to replicate or integrate their genes in non-vertebrate cells. Together with the possibility for scale-up as demonstrated by Maiorella *et al.* [Maiorella *et al.* (1988)] and Murhammer and Goochee [Murhammer and Goochee (1988)], the baculovirus expression system represents a feasible and competitive method for the production of complex proteins, especially for applications in biomolecular research and medicine.

2.5 Baculoviral surface display

The purpose of surface display is to present biomolecules authentically on viral particles or cells in order to make them accessible for applications like antigen and ligand display or for the establishment of epitope libraries (epitope mapping). Moreover, the host range of viruses can be altered by insertion of surface located proteins that specifically recognise cell receptors.

The general strategy of surface display is to fuse the protein of interest to a protein that is transported to the outer membrane of the host cell and is picked up by viral particles. Thus, the recombinant protein is made accessible for interaction studies in conjunction with its genetic information allowing easy

selection and amplification of desired constructs. Surface expression of murine and human proteins on non-lytic filamentous phages (phage display) has been achieved in numerous applications like the expression of F_{ab}-fragments [Hoogenboom *et al.* (1991)] and IgE receptors [Scarselli *et al.* (1993)]. Moreover, retroviral vectors have been used successfully to express surface-linked T-cell receptors [Pogulis and Pease (1998)] and single chain antibodies [Rode *et al.* (1996)].

Baculoviral vectors displaying foreign protein have first been published by Boublik *et al.* [Boublik *et al.* (1995)]. Fusion of eukaryotic proteins to a second copy of the envelope fusion protein gp64 resulted in surface presentation on viral particles and on infected insect cells. Improved vectors promoting the fusion of heterologous protein to the membrane anchor domain of gp64 have been invented by Grabherr *et al.* [Grabherr *et al.* (1997)]. Moreover, recombinant baculoviruses facilitating the insertion of peptides into the native gp64 have been constructed [Ernst *et al. (2*000)]. Chapple and Jones effectively enhanced the presentation of foreign proteins on baculoviral particles by applying the membrane anchor of vesicular stomatitis virus (VSV) [Chapple and Jones *(2*002)].

2.6 Surface libraries and vaccine development

Surface libraries facilitate high-throughput screening of large amounts of candidate peptides being presented on virus particles or cells. Small proteins that bind to a wide range of ligands such as receptors and carbohydrates have been successfully identified after screening random phage display peptide libraries [D'Mello and Howard *(2*001)]. Peptides like these could be the basis of a new diagnostic enzyme-linked immunosorbent assay, with sufficient specificity and sensitivity to replace expensive immunoblotting tests that are currently required for definitive serological diagnosis [Kouzmitcheva *et al.* (2001)]. Moreover, the elaborated sequences do not only provide data for basic research purposes, like general understanding of specific interactions and structural imaging, but could also serve as agents for therapeutic applications like immunisation and vaccination. The aim of vaccination is to evoke specific

immune responses, including potent cytotoxic T lymphocyte (CTL) and antibody responses, resulting in protective immunity against the administered vehicle. Thus, it is of great importance to determine exclusive sequences (epitopes) of high immunogenicity and antigenicity that raise specific cellular and neutralising humoral responses. Epitope surface libraries created by specific fragmentation of the candidate protein provide the neccessary tool for these purposes. In this respect, the mapping of linear non-glycosylated epitopes for monoclonal antibodies has already been accomplished by selection of phage-based surface libraries [Cwirla *et al.* (1990); Felici *et al.* (1991); McLafferty *et al.* (1993); Holzem *et al.* (2001)]. When it comes to more sophisticated challenges, e.g. epitope mapping of the extensively glycosylated and structurally complex HIV-1 gp120, the application of eukaryotic expression systems is inevitable. Libraries of randomly mutagenised single chain antibodies have already been expressed successfully on *S. cerevisiae* [Griffin *et al. (2*001)] but glycosylation patterns in yeast differ from those in human cells, limiting the range of possible applications for these systems. Retroviral surface libraries [Zannettino *et al.* (1996)] and human rhinovirus libraries [Smith *et al.* (1998)] have been established but must be regarded as suboptimal in respect of their expensive and demanding cultivation and genetic manipulation, their low frequency of recombinants as well as because of concerns in biosafety. Hence, the non-pathogenic baculovirus surface expression system with its well established and easy applicable methods of genetic engineering and propagation of viral particles offers a competitive technology for the surface display of human-like peptides and large proteins. Whole recombinant baculoviral particles have already been effectively used to directly immunise mice [Lindley *et al.* (2000)] demonstrating the potential of this system.

2.7 HIV-1 gp120 as a model for baculoviral surface display libraries

The human immunodeficiency virus (HIV-1) envelope glycoprotein gp120 being a complex glycoprotein of human origin was chosen as a model protein for the construction of baculoviral surface libraries. The unique properties of

gp120 and its importance in HIV transmission makes it an interesting candidate for antibody studies and vaccine development.

HIV-1 gp120 is synthesised as an envelope precursor molecule gp160 [Allan *et al.* (1985); Robey *et al.* (1985)], which oligomerises and is cleaved into gp120, the surface moiety and gp41, the transmembrane protein [Weiss and White (1993)]. The complex is arranged in a trimeric configuration of heterodimers, each consisting of a gp120 surface subunit non-covalently associated with a gp41 transmembrane subunit. By comparing the amino acid sequence of gp120 subunits of different HIV-1 isolates, five variable regions (V1-V5) and five conserved regions (C1-C5) have been identified [Starcich *et al.* (1986); Modrow *et al.* (1987)]. The aminoterminus of gp41 (residues 1-29) contains the hydrophobic, glycine-rich "fusion peptide" which plays a critical role in the fusion of viral and target cell membrane [Wyatt *et al.* (1997)].

The gp120 core is structurally organised into two major domains, the inner and outer domain, and a mini-domain termed the bridging sheet (Fig.3) [Kwong *et al.* (1998)]. The inner domain harbours both the N and C termini of gp120, which are involved in the interaction with gp41 [Wyatt *et al.* (1997)], and is the probable site of trimer packing [Kwong *et al.* (1998)].

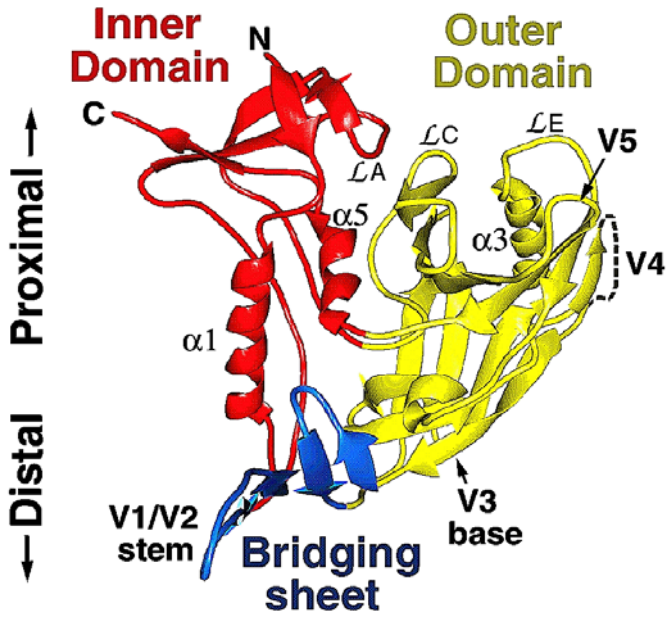

Fig.3: Backbone presentation of HIV-1 gp120 showing different domains [Kwong (1998)].

The HIV-1 mature envelope glycoprotein complex plays an essential role during the early events of virus attachment and entry into the target cell. The infection process is initiated by attachment of the virus to the target cell via the interaction between the gp120 subunit and the cellular receptor CD4. The binding of CD4 triggers conformational alterations in the HIV

envelope that promote recognition of the obligatory chemokine receptors CCR5 or CXCR4 and ultimately lead to membrane fusion [Choe *et al.* (1998)].

The CD4-binding site on gp120 is supposed to be conformational and of discontinuous structure [Kwong *et al.* (1998)]. Former assumptions of the CD4-domain being a discrete linear epitope (i.e. RIKQIINWQEVGKAMYAPPISGQIR) within the C4 region of gp120 are regarded as too simplistic because of the following reasons: mutation of almost all of the above residues has little or no effect on CD4 binding, with exception of Trp-427, whose mutations completely abolishes CD4 binding. Furthermore, mutations elsewhere in the gp120 are disruptive to CD4 binding as well (e.g. Asp-368, Glu-370, Asp-457, Asn-262) [Kwong *et al.* (1998)]. Finally, some antibodies to gp120 block CD4 without either binding to the above C4 peptide or, in some cases, are sensitive to amino acid substitutions in C4. Overall, the present view of the CD4-binding site is that it is a discontinuous structure formed by the juxtaposition of residues scattered among the gp120 sequence, but probably concentrated in the C-terminal half [Kwong *et al.* (1998)].

During the course of HIV-1 infection, neutralising antibodies are elicited against various elements of gp120. The dominant isotype in the anti-HIV-1 response is IgG1, in particular of the Env-specific antibodies [Klasse and Blomberg (1987); Mathiesen *et al.* (1989)]. Neutralising antibodies appear to be an important component of the host immune response as they do not only bind specifically to antigens but also recruit other molecules and cells in the immune system. Most clinical (primary) HIV-1 isolates are relatively resistant to neutralising antibodies, suggesting that these viruses are selected in the presence of neutralising antibodies in infected humans. In many HIV-1 infected individuals, two classes of neutralising antibodies are elicited: strain-restricted and broadly cross-reactive antibodies. The strain-restricted antibodies appear early after infection and are generally directed against linear determinants within the gp120 third variable region (V3 loop). These antibodies have been relatively easy to generate in both primate and non-primate animal systems but are not broadly protective. A subset of the broadly-neutralising antibodies recognise conformational-dependent, discontinuous epitopes that overlap with the discontinuous CD4-binding site on gp120 and are termed CD4-binding site antibodies. A second limited subset of broadly-neutralising antibodies recognise

discontinuous gp120 epitopes, overlapping with the chemokine receptor binding site, that are better exposed upon CD4 binding and thus are referred to as CD4-induced antibodies. In naive individuals, the presence of broadly cross-reactive neutralising antibodies and strain-restricted antibodies might help prevent or limit HIV-1 infection following exposure to the virus [Wyatt and Sodroski (1998)].

The development of an efficacious vaccine raising endogenous neutralising antibodies against HIV is of great urgency, since it is widely accepted that vaccination is the only means capable of controlling the AIDS (acquired immune deficiency syndrome) pandemic. There is obviously a lot of interest in the structure of gp120 because it has been a crucial target for some of the vaccines already in clinical trials. However, the broadly neutralising antibodies have been difficult to elicit in animals using wild-type gp120 glycoproteins as an immunogen. Having a better idea of what the antigenic surface on gp120 is and an advanced understanding of how antibodies bind to virus particles is central to these inquiries and would provide a far better chance of being able to design the appropriate gp120 presenting molecules for use as vaccines. The necessity and benefit of stimulating B cells that produce neutralising antibodies against the gp120-V3 loop has been clearly established in murine infection models with chimeric human rhinovirus [Smith *et al.* (1994)].

2.8 Screening of surface libraries with fluorescence activated cell sorting (FACS)

The analysis of surface display libraries and the selection of ligands requires a high-throughput system which allows to detect candidates specifically, e.g. by staining with monoclonal antibodies. Fluorescence activated cell sorting (FACS) [Radbruch 2000] is a technique to differentiate and count cells and microparticles by means of specific fluorescent labelling. Cells in suspension are passing a cuvette in a narrow stream and interfere with a focussed laser beam. Optical signals (scatter, fluorescence) are sequentially generated by each particle. While forward and side scatter preliminary correspond to the size and granularity of the particle, biochemical properties of the cells can by

visualised and analysed taking advantage of a wide range of specific and unspecific dyes and antibodies (e.g. anti-CD4 (lymphocyte surface marker), propidium iodide (DNA stain). Scattered light and emitted fluorescence are detected by a row of different photodetectors. The resulting signals are amplified and correlated by a fast data acquisition system.

Flow cytometers take advantage of laminar flow conditions in capillary tubes. Cell suspensions are injected into a flow channel where a carrier fluid (sheath fluid) surrounds the cells. Due to the Bernoulli effect the sheath fluid draws the cell suspension to the very middle of the fluid so that the particles are lined up in a narrow laminar stream. This so called hydrodynamic focussing allows the device to evaluate each single particle individually regarding its optical features.

The most important feature of flow cytometry is that large numbers (e.g. 10^4 or more) of cells can be analysed one by one in a timeframe of seconds. The detection limit is as low as 100 fluorescent molecules per cell. Statistical data like mean fluorescent intensity and its dependence on cell function are very reliable and can be generated reproducibly.

Separation of single cells or subgroups of cells (cell sorting) can be accomplished by electrostatic (e.g. BecktonDickinson FACSVantage) and mechanical (e.g. BecktonDickinson FACSCalibur) means: electrostatic sorting is achieved by ejecting the focussed stream of cells through a nozzle into air, resulting in single cells being separated in droplets. By vibrating the nozzle with a transducer, the droplets can be produced in a extremely stable and reproducible manner. The droplets are electrically charged and can subsequently be separated in an electrostatic field, evoked by plates held at high voltage (+/- 3000V). Mechanical sorting is facilitated in a modified flow chamber where a “catcher tube“ is moved into the stream to collect desired cells. One disadvantage of the latter system is the lower sorting rate (maximum of 300 per second compared to up to 5000/s using electrostatic sorting devices) and the diluted concentration of sorted material.

3. Aim of work

The purpose of this work was to establish and evaluate an eukaryotic display system for the screening of unknown epitopes binding to specific antibodies. HIV-1 gp120 was chosen as a promising target protein with future prospects of contributing to structural imaging of antibody interactions neccessary for vaccine development and basic research. Because of its unique properties the baculovirus surface display system was selected for easy and authentic expression and presentation of epitopes.

Several aspects in creating the library were regarded as crucial. Previously, a baculoviral surface library of HIV-1 gp120 has already been established by Spenger *et al.* [diploma thesis, IAM, BOKU, Vienna]. Fragments were produced by digesting the target protein with numerous sets of different restriction endonucleases resulting in only a limited number of peptides with different sizes. This approach led to the selection and identification of one out of three tested antibodies (the V3-loop binding monoclonal antibody ARP3025 aka 0.5ß). This low success rate might be explained by the insufficient diversity of the library and the varying fragment sizes leading to differences in growth rates and, thereby, inhomogeneous representation or even loss of viral clones.

The novel baculoviral HIV-1 gp120 library should be established with regard to the following features:

- the procedure of generating candidate fragments should be simplified
- the diversity of the library should be higher
- the library should be more homogenous
- the library should be well evaluated and lead to reproducible results
- the fluorescence activated cell sorter (FACS) should be chosen and established as available technology for screening and selection using several monoclonal antibodies

4. Materials and methods

4.1 Cloning procedures

Cloning of bacterial vectors was performed using standard methods according to Sambrook and Russell [Sambrook and Russell *(2*001)]. Baculoviral protocols were applied as described by O'Reilly *et al.* [O'Reilly *et al.* (1992)].

Sce-I was purchased from Boehringer-Mannheim (Mannheim, Germany). *DNase Shotgun Cleavage Kit* was obtained from Novagen (Madison, USA). All other restrictionendonucleases and DNA-modifying enzymes were purchased from MBI Fermentas (St.Leon-Rot, Germany).

All salts, alcohols, DMSO, H_2O_2, chloroform, ethidiumbromide and propidium iodide were purchased from Sigma (Munich, Germany) in analytical grade (*p.a.*). Sugars and ingredients for bacterial media were purchased from Merck (Darmstadt, Germany) in microbiology grade.

All buffers, solution and media were made with bidestilled water (AD) and stored at 4°C.

Small scale preparations of bacterial plasmid-DNA were performed with GFX-Kits (Amersham Pharmacia, Piscataway, USA), or, alternatively, by standard alkaline lysis [Sambrook and Russell (2001)]. Small fragments (<300bp) were purified with QIAEX (QIAGEN, Hilden, Germany). For mid-scale preparations (100µg DNA) QIAGEN-Kits (Qiagen) were applied. Baculoviral DNA was cleaned by PCI-purification and stored at 4°C.

Klenow treatment was accomplished with 1U/µg DNA and 0,1mM dNTPs at 37°C for 30 minutes on a Thermomixer compact 5350 (Eppendorf, Hamburg, Germany).

Phosphorylation was carried out using 10U of polynucleotide kinase per 1pmol/µl of 5'-termini in the presence of 1pmol/µl ATP (incubation for 30 minutes at 37°C).

Blunt ends were dephosphorylated with 1U calf intestine phosphatase per 1pmol/µl of 5'-termini at 37°C for 30 minutes.

Ligation was carried out at 16°C with 200ng of vector-DNA and a 5-fold molar excess of insert using 1U of T_4-ligase.

Polymerase chain reaction (PCR) with AmpliTaq-Gold (Applied Biosystems, Foster City, USA) was carried out applying a standard protocol (94°C, 3min; 92°C, 45s; 55°C, 45s; 72°C, 1,5min/1kb; 72°C, 10min) on a TGradient 96 Thermoblock (Biometra, Goettingen, Germany).

Electroporation of *E. coli* TG1 (Stratagene, Amsterdam, The Netherlands) was performed in disposable cuvettes (2mm gap) from BioRad (Munich, Germany) on a Gene-Pulser (BioRAD, Munich, Germany) at 2.5 kV (25 µF, 200Ω).

DNA samples and fragments were analysed and purified (GFX) by agarose gel electrophoresis. Size and quantity of samples was estimated by visual comparison with simultaneously analysed DNA standards (DNA-Standard III (MBI, St.Leon-Rot, Germany) for large fragments and plasmids; pBR322/MspI digested (NEB, Beverly, USA) and 100bp DNA ladder (NEB, Beverly, USA) for small fragments).

The vector envBH10 served as template for PCR amplification of the target protein HIV-1 (IIIB) gp120 [Allan 1985].

	5					10					15					20					25					30
1	M	R	V	K	E	K	Y	Q	H	L	W	R	W	G	W	R	W	G	T	M	L	L	G	M	L	M	I	C	S	A
31	T	E	K	L	W	V	T	V	Y	Y	G	V	P	V	W	K	E	A	T	T	T	L	F	C	A	S	D	A	K	A
61	Y	D	T	E	V	H	N	V	W	A	T	H	A	C	V	P	T	D	P	N	P	Q	E	V	V	L	V	N	V	T
91	E	N	F	N	M	W	K	N	D	M	V	E	Q	M	H	E	D	I	I	S	L	W	D	Q	S	L	K	P	C	V
121	K	L	T	P	L	C	V	S	L	K	C	T	D	L	K	N	D	T	N	T	N	S	S	S	G	R	M	I	M	E
151	K	G	E	I	K	N	C	S	F	N	I	S	T	S	I	R	G	K	V	Q	K	E	Y	A	F	F	Y	K	L	D
181	I	I	P	I	D	N	D	T	T	S	Y	T	L	T	S	C	N	T	S	V	I	T	Q	A	C	P	K	V	S	F
211	E	P	I	P	I	H	Y	C	A	P	A	G	F	A	I	L	K	C	N	N	K	T	F	N	G	T	G	P	C	T
241	N	V	S	T	V	Q	C	T	H	G	I	R	P	V	V	S	T	Q	L	L	L	N	G	S	L	A	E	E	E	V
271	V	I	R	S	A	N	F	T	D	N	A	K	T	I	I	V	Q	L	N	Q	S	V	E	I	N	C	T	R	P	N
301	N	N	T	R	K	S	I	R	I	Q	R	G	P	G	R	A	F	V	T	I	G	K	I	G	N	M	R	Q	A	H
331	C	N	I	S	R	A	K	W	N	N	T	L	K	Q	I	D	S	K	L	R	E	Q	F	G	N	N	K	T	I	I
361	F	K	Q	S	S	G	G	D	P	E	I	V	T	H	S	F	N	C	G	G	E	F	F	Y	C	N	S	T	Q	L
391	F	N	S	T	W	F	N	S	T	W	S	T	K	G	S	N	N	T	E	G	S	D	T	I	T	L	P	C	R	I
421	K	Q	I	I	N	M	W	Q	E	V	G	K	A	M	Y	A	P	P	I	S	G	Q	I	R	C	S	S	N	I	T
451	G	L	L	L	T	R	D	G	G	N	S	N	N	E	S	E	I	F	R	P	G	G	G	D	M	R	D	N	W	R
481	S	E	L	Y	K	Y	K	V	V	K	I	E	P	L	G	V	A	P	T	K	A	K	R	R	V	V	Q	R	E	K
511	R																													

Tab.1: Aminoacid sequence of HIV-1 (IIIB) gp120 [Allan 1985].

4.1.1 Construction of pCOPS and *Ac*COPS

The baculoviral transfer plasmid pCOPS for construction of baculoviral *Ac*COPS clones was generated by first inserting the unique cloning site *Sce*-I between the leader and the structural gene of gp64. The plasmid p64flank [Ernst *et al.* (2000)] which contains the *ORF* of baculoviral gp64 inserted in a pZIP1-backbone [Kerschbaumer *et al.* (1997)] served as template. Two separate PCR amplifications were carried out with the following primer pairs: gp64lea-PstI-back (5' GAT GAC CTG CAG AAT ATG GTA AGC GCT ATT GTT TTAT G 3') / gp64-117-Sce-COPS-for (5' GAT GAC GAT ATT ACC CTG TTA TCC CTA TCC GCC GCA AAG GCA GAA TGC G 3') and gp64-118-Sce-COPS-back (5' GAT GAC GAT TAG GGA TAA CAG GGT AAT GGA GCA CTG CAA CGC GCA AAT G 3') / gp64-MARS-Bsp120I-for (5' GAT GAC GGG CCC TTA ATA TTG TCT ATT ACG GTT TCT AAT C 3'). Both fragments were gel-purified and sequentially digested with *Pst*I and *Sce*-I or *Bsp*120I and *Sce*-I, respectively. After a standard ligation step with both fragments an additional PCR amplification with the terminal primers (gp64lea-PstI-back / gp64-MARS-Bsp120I-for) was performed. The resulting fragment was purified and ligated into a prepared the baculoviral transfer vector pBacPAK8 (BD Biosciences, Palo Alto, USA) which had been cut by *Pst*I and *Not*I. After transformation in electrocompetent TG1 and a PCR screening with –44back (5' TTT ACT GTT TTC GTA ACA GTT TTG 3') and +201for (5' CTT TTG CAG CGT TTC CTT GG 3') a positive clone was verified via DNA sequencing and designated as pCOPS. *Ac*COPS was generated by homologous recombination through co-transfection of *Sf*9 cells with 100ng BaculoGold-DNA (BD Biosciences) and 500ng of pCOPS. The supernatant was harvested five days post transfection and submitted to plaque assay. Single plaques were screened via PCR, amplified (4.3.5) and confirmed by DNA-sequencing.

4.1.2 Construction of pCOIN

The baculoviral transfer vector pCOIN was constructed in order to generate the viral clone *Ac*COIN-3D6. The *Sce*-I site was inserted into the

native gp64 of wt *Ac*NPV making use of the naturally occurring *Not*I site at position 278: A standard PCR was carried out with the primers gp64-Not-Sce-for (5‘ GAT GAC GAT TGC GGC CGC ATT ACC CTG TTA TCC CTA CCC CGC TTC TTG ACT CGG TGC TCG AC 3’) and gp64-684PstI-back (5’ GAT CTA GCT ATG CTG CAG CCA GTT ATC TGG CTC CAA AAT TGT ATC AGT GG 3’) using p64flank as template. The resulting fragment was purified, digested with *Not*I and ligated into prepared p64flank (*Not*I-cut, dephosphorylated). After transformation in TG1 several clones were screened: A PCR with the primers +781back (5’ CGC GAA CAC TGT TTG ATT GAC 3’) and +993for (5’ GTT TTC GTA CAT CAG CTC CTC 3’) was carried out and resulting fragments were displayed on 2% agarose gels. In addition to that, the PCR fragments were digested with *Sce*-I, verifying the existence and functionality of the inserted restriction site. Supposed positive clones were confirmed by DNA sequence analysis and designated as pCOIN.

4.1.3 Construction of *Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*COIN-3D6

The oligonucleotides 3*D6mutCOPSsenseOLIGO* (5‘ CGG TTG CTC TGG AAA GCT TAT TTG CAC CAC TGC TGT GCC TTG GAA TGC TAG TGA TAA 3’) and *3D6mutCOPSantisenseOLIGO* (5‘ CAC TAG CAT TCC AAG GCA CAG CAG TGG TGC AAA TAA GCT TTC CAG AGC AAC CGT TAT 3’) were diluted to 100µM each and annealed in a volume of 10µl: the solution was heated to 99°C for five minutes and then slowly chilled to RT. The DNA having *Sce*-I-compatible overhangs was concentrated by butanol-extraction and washed with 100% and 70% ethanol. After a standard ligation with *Sce*-I-cut and dephosphorylated pCOPS, pMARS, or pCOIN, respectively, the constructs were transformed into electrocompetent TG1. Bacterial clones were screened by PCR (w/ -44back / +201for for pCOPS-constructs; -44back / gp64-MARS-Bsp120I-for for pMARS constructs; gp64-Not-Sce-for / gp64-684PstI-back for pCOIN constructs) and positive clones were amplified. After a small scale DNA preparation, a co-transfection of the elaborated pCOPS-3D6, pMARS-3D6, and pCOIN-3D6 with BaculoGold DNA in *Sf*9 cells was performed. The supernatant of all three transfections was harvested five days post infection and submitted to

plaque assay. Single plaques were amplified in 24WP, infected cells were harvested five days post infection and, again, PCR-screened with the appropriate primers. Supposed positive clones were verified by DNA sequencing and corresponding viral clones were amplified on *Sf*9 in order to gain working stocks of high virus titre. The virus stocks were designated *Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*COIN-3D6, respectively.

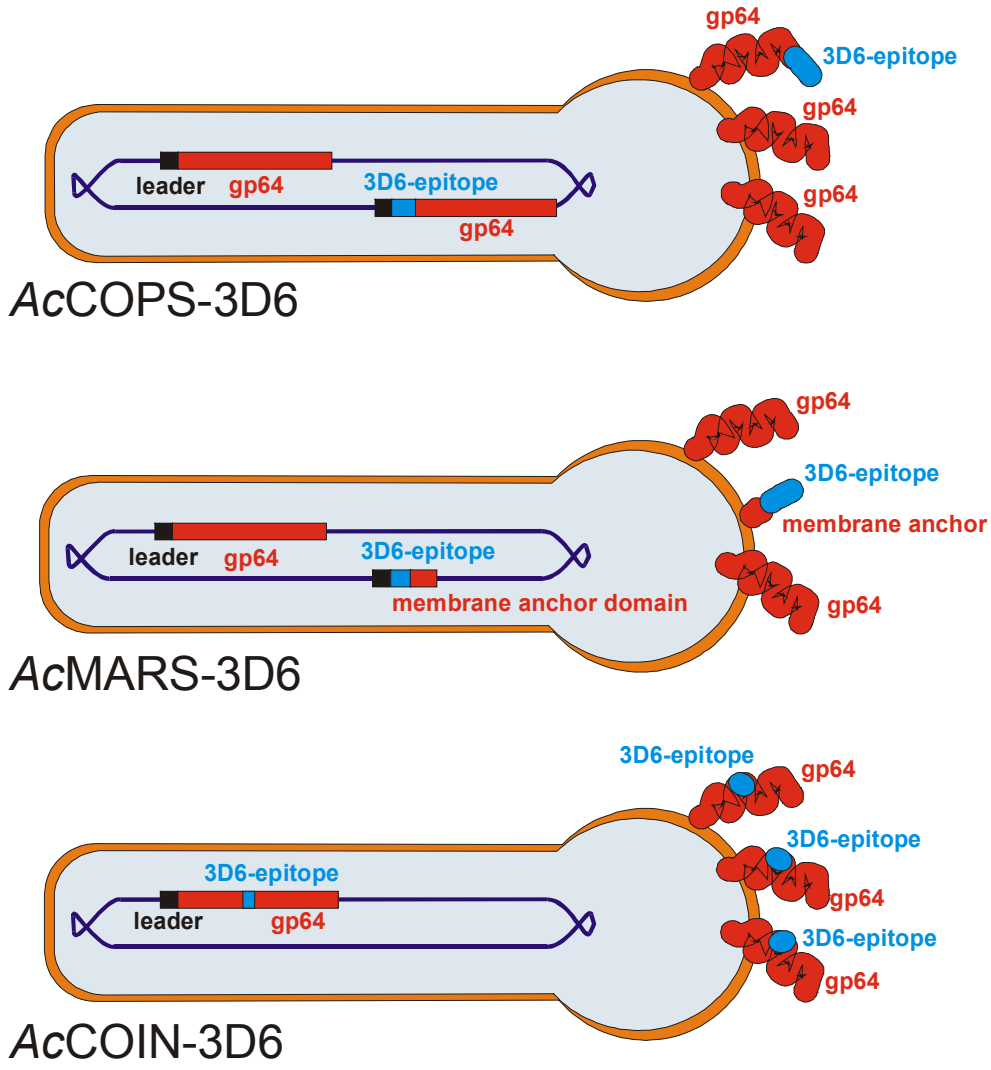

Fig.4: Genomic structure and surface properties of baculoviral constructs *Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*COIN-3D6. The vector *Ac*COPS-3D6 contains a second copy of the baculoviral gp64 with an N-terminal fusion of the epitope (SGKLICTTAVPWNAS) for the monoclonal antibody 3D6 [Buchacher *et al.* (1994)]. In the vector *Ac*MARS-3D6, the epitope for 3D6 is fused to the membrane anchor domain of gp64. Infection of *Sf*9 insect cells with these constructs leads to the expression of a mixture of wt gp64 and recombinant

protein fused to the membrane anchor domain on the surface of infected cells and progeny virus particles. Finally, in the vector *Ac*COIN-3D6 the epitope is inserted directly into the native gp64 at position 278 (naturally occurring *Not*I site) resulting in a high avidity display of heterologous gp64.

4.2 Construction and analysis of *Ac*COPS HIV-1 gp120 Libraries

4.2.1 Construction of DNase-digest-library *Ac*COPS-DNase-LIB

The *ORF* of HIV-1 (IIIB) gp120 was amplified by PCR amplification using AmpliTaq-Gold DNA polymerase and envBH10 as template. The PCR product was purified and digested with the *DNase Shotgun Cleavage Kit* as follows:

50µl of a solution containing 10µg gp120-DNA, 10µl of DNase-I (previously diluted 1/450 in 1fold reaction buffer) plus 5µl of 10fold reaction buffer and 5µl of supplied $MnCl_2$ solution were incubated at 10°C for 10 minutes. The reaction was stopped by adding 20µl EDTA (100mM). The originated DNA was analysed by agarose gel-electrophoresis and fragments within a range from 50 to 200bp were extracted using the QIAEX kit.

The gained fragments were treated with T_4-DNA Polymerase (10µg DNA, 50pMol dNTPs, 3U T_4-DNA Polymerase, 1fold reaction buffer in a total volume of 100µl were incubated for 1h at 37°C) in order to produce blunt ends and purified with QIAEX afterwards. Subsequently, the fragments were split in 9 aliquots and ligated to *Bst*XI-adapters.

Designation	Sequence
5-prime-BstXI-frame-1-sense	5’ TAC GAT CTG AGT CCA GAT AAC TGG 3’
5-prime-BstXI-frame-1-antisense	5’ CCA GTT ATC TGG ACT CAG ATC 3’
5-prime-BstXI-frame-2-sense	5’ TAC GAT CTG AGT CCA GAT AAC TGG C 3’
5-prime-BstXI-frame-2-antisense	5’ GCC AGT TAT CTG GAC TCA GAT 3’
5-prime-BstXI-frame-3sense	5’ TAC GAT CTG AGT CCA GAT AAC TGG CG 3’
5-prime-BstXI-frame-3antisense	5’ CGC CAG TTA TCT GGA CTC AG 3’
3-prime-BstXI-frame-1-sense	5’ TAC GAT CTG AGT CCA GTT ATC TGG 3’
3-prime-BstXI-frame-1-antisense	5’ CCA GAT AAC TGG ACT CAG ATC 3’
3-prime-BstXI-frame-2-sense	5’ TAC GAT CTG AGT CCA GTT ATC TGG C 3’
3-prime-BstXI-frame-2-antisense	5 ’GCC AGA TAA CTG GAC TCA GAT 3’
3-prime-BstXI-frame-3-sense	5’ TAC GAT CTG AGT CCA GTT ATC TGG CG 3’
3-prime-BstXI-frame-3-antisense	5’ CGC CAG ATA ACT GGA CTC AG 3’

Tab.2: DNA sequences of *Bst*XI-adapters for ligation of the DNase-fragments into pCOPS.

Antisense- and corresponding sense-*Bst*XI-adapter of the same reading frame were annealed (100pMol of both primers in a total of 50µl AD were heated to 99°C and slowly chilled down to RT) and ligated with one of the nine aliquots (1U T_4-DNA Ligase, 30pMol of each annealed adapter, 1fold reaction buffer in a total volume of 10µl were incubated at 16°C o/n) as follows:

Designation	annealed 5'-adapters	annealed 3'-adapters
#1 [5'(frame1)3'(frame1)]	5-prime-BstXI-frame-1-sense 5-prime-BstXI-frame-1-antisense	3-prime-BstXI-frame-1-sense 3-prime-BstXI-frame-1-antisense
#2 [5'(frame1)3'(frame2)]	5-prime-BstXI-frame-1-sense 5-prime-BstXI-frame-1-antisense	3-prime-BstXI-frame-2-sense 3-prime-BstXI-frame-2-antisense
#3 [5'(frame1)3'(frame3)]	5-prime-BstXI-frame-1-sense 5-prime-BstXI-frame-1-antisense	3-prime-BstXI-frame-3-sense 3-prime-BstXI-frame-3-antisense
#4 [5'(frame2)3'(frame1)]	5-prime-BstXI-frame-2-sense 5-prime-BstXI-frame-2-antisense	3-prime-BstXI-frame-1-sense 3-prime-BstXI-frame-1-antisense
#5 [5'(frame2)3'(frame2)]	5-prime-BstXI-frame-2-sense 5-prime-BstXI-frame-2-antisense	3-prime-BstXI-frame-2-sense 3-prime-BstXI-frame-2-antisense
#6 [5'(frame2)3'(frame3)]	5-prime-BstXI-frame-2-sense 5-prime-BstXI-frame-2-antisense	3-prime-BstXI-frame-3-sense 3-prime-BstXI-frame-3-antisense
#7 [5'(frame3)3'(frame1)]	5-prime-BstXI-frame-3-sense 5-prime-BstXI-frame-3-antisense	3-prime-BstXI-frame-1-sense 3-prime-BstXI-frame-1-antisense
#8 [5'(frame3)3'(frame2)]	5-prime-BstXI-frame-3-sense 5-prime-BstXI-frame-3-antisense	3-prime-BstXI-frame-2-sense 3-prime-BstXI-frame-2-antisense
#9 [5'(frame3)3'(frame3)]	5-prime-BstXI-frame-3-sense 5-prime-BstXI-frame-3-antisense	3-prime-BstXI-frame-3-sense 3-prime-BstXI-frame-3-antisense

Tab.3: Annealing scheme of *Bst*XI-adapters for construction of the *Ac*COPS-DNase-library

The resulting constructs were amplified by PCR: 2µl of the ligations were treated with AmpliTaq-Gold following the standard protocol for PCR-amplification and using 1pMol of the corresponding single-stranded BstXI-sense- and BstXI-antisense-oligos as primers.

Resulting fragments were pooled, purified with QIAEX and analysed by agarose gel-electrophoresis. Again, fragments ranging from 50 to 200bp were extracted and purified before being cut with *Bst*XI (approximately 10µg DNA,

50U *Bst*XI, 1fold reaction buffer in a total volume of 100µl were incubated at 55°C o/n). Following an additional QIAGEN-purification the fragments were directly ligated [Ernst *et al.* (1994)] to prepared *Ac*COPS (*Sce*-I-cut, dephosphorylated and PCI-purified): 250ng of vector were incubated with 10% total of the purified gp120-fragments in a standard ligation.

Finally, the complete preparation was transfected in *Sf*9 cells and amplified as described (4.3.4; 4.3.5). The working stock was designated *Ac*COPS-DNase-library.

The generated *Ac*COPS-DNase-library was analysed by PCR-screening of viral clones and by means of FACS with infected insect cells.

A working stock of the library was submitted to plaque assay and single clones were screened with –44back and +201for. Amplified fragments were analysed on a 2% agarose-gel. Additionally, a PCR amplification of an empty pCOPS vector (negative control) and of the complete library were assayed.

FACS analysis of *Sf*9 insect cell infected with *Ac*COPS-DNase-LIB with HIVIG, a polyclonal anti-HIV serum (NABI, Ft. Myers, Florida), were performed as described (4.4). Insect cells infected with *Ac*COPS (negative control) and *Ac*COPS-PCR-LIB (4.2.2) served as reference.

4.2.2 Construction of the PCR-library *Ac*COPS-PCR-LIB

60 pairs of primers were designed (Tab.4) covering the whole *ORF* of gp120 in overlapping fragments of 17aa each. Flanking *Bst*XI sites (5' CCA NNN NN^N TGG 3') were integrated into primers with *Sce*-I compatible restriction overhangs (5‘ ATA A 3‘).

Designation Fragment#_for/back_Base#	**Sequence (5'->3')**
1_gp120back_1	GATCTCCCAGATAACTGGATGAGAGTGAAGGAGAAATATCAG
1_gp120for_51	GATCTCCCAGTTATCTGGCCATCTCCACCCCCATCTC
2_gp120back_25	GATCTCCCAGATAACTGGCACTTGTGGAGATGGGGGTG
2_gp120for_75	GATCTCCCAGTTATCTGGCAACATCCCAAGGAGCATG
3_gp120back_52	GATCTCCCAGATAACTGGGGCACCATGCTCCTTGG
3_gp120for_102	GATCTCCCAGTTATCTGGCAATTTTTCTGTAGCACTACAGATC
4_gp120back_76	GATCTCCCAGATAACTGGATGATCTGTAGTGCTACAGAAAAAT
4_gp120back _91	GATCTGAGTCCAGATAACTGGACAGAAAAATTGTGGGTCACA
4_gp120for_126	GATCTCCCAGTTATCTGGTACCCCATAATAGACTGTGACC
5_gp120back_103	GATCTCCCAGATAACTGGTGGGTCACAGTCTATTATGGG
5_gp120for_153	GATCTCCCAGTTATCTGGAGTGGTGGTTGCTTCCTTCC
6_gp120back_127	GATCTCCCAGATAACTGGCCTGTGTGGAAGGAAGCAAC
6_gp120for_177	GATCTCCCAGTTATCTGGTTTAGCATCTGATGCACAAAATAG
7_gp120back_154	GATCTCCCAGATAACTGGCTATTTTGTGCATCAGATGCTAAAG
7_gp120for_204	GATCTCCCAGTTATCTGGAACATTATGTACCTCTGTATCATATG
8_gp120back_178	GATCTCCCAGATAACTGGGCATATGATACAGAGGTACATAATG
8_gp120for_228	GATCTCCCAGTTATCTGGGGGTACACAGGCATGTGTG
9_gp120back_205	GATCTCCCAGATAACTGGTGGGCCACACATGCCTG
9_gp120for_255	GATCTCCCAGTTATCTGGTACTACTTCTTGTGGGTTGGG
10_gp120back_229	GATCTCCCAGATAACTGGACAGACCCCAACCCACAAG
10_gp120for_279	GATCTCCCAGTTATCTGGAAAATTTTCTGTCACATTTACCAATAC
11_gp120back_256	GATCTCCCAGATAACTGGTTGGTAAATGTGACAGAAAATTTTAAC
11_gp120for_306	GATCTCCCAGTTATCTGGTTCTACCATGTCATTTTTCCAC
12_gp120back_280	GATCTCCCAGATAACTGGAACATGTGGAAAAATGACATGG
12_gp120for_330	GATCTCCCAGTTATCTGGACTGATTATATCCTCATGCATCTG
13_gp120back_307	GATCTCCCAGATAACTGGCAGATGCATGAGGATATAATCAG
13_gp120for_357	GATCTCCCAGTTATCTGGACATGGCTTTAGGCTTTGATC
14_gp120back_331	GATCTCCCAGATAACTGGTTATGGGATCAAAGCCTAAAG
14_gp120for_381	GATCTCCCAGTTATCTGGAACACAGAGTGGGGTTAATTTTAC

15_gp120back_358	GATCTCCCAGATAACTGGGTAAAATTAACCCCACTCTGTG
15_gp120for_408	GATCTCCCAGTTATCTGGATTCTTCAAATCAGTGCACTTTAAAC
16_gp120back_382	GATCTCCCAGATAACTGGAGTTTAAAGTGCACTGATTTGAAG
16_gp120for_432	GATCTCCCAGTTATCTGGGCTACTACTATTGGTATTAGTATCATTC
17_gp120back_409	GATCTCCCAGATAACTGGGATACTAATACCAATAGTAGTAGCGG
17_gp120for_459	GATCTCCCAGTTATCTGGCTCTCCTTTCTCCATTATCATTC
18_gp120back_433	GATCTCCCAGATAACTGGGGGAGAATGATAATGGAGAAAG
18_gp120for_483	GATCTCCCAGTTATCTGGGATATTGAAAGAGCAGTTTTTTATC
19_gp120back_460	GATCTCCCAGATAACTGGATAAAAAACTGCTCTTTCAATATC
19_gp120for_510	GATCTCCCAGTTATCTGGCTGCACCTTACCTCTTATGC
20_gp120back_484	GATCTCCCAGATAACTGGAGCACAAGCATAAGAGGTAAGG
20_gp120for_534	GATCTCCCAGTTATCTGGTTTATAAAAAAATGCATATTCTTTC
21_gp120back_511	GATCTCCCAGATAACTGGAAAGAATATGCATTTTTTTATAAAC
21_gp120for_561	GATCTCCCAGTTATCTGGATCATTATCTATTGGTATTATATCAAG
22_gp120back_535	GATCTCCCAGATAACTGGCTTGATATAATACCAATAGATAATG
22_gp120for_585	GATCTCCCAGTTATCTGGACTTGTCAACGTATAGCTGGTAG
23_gp120back_562	GATCTCCCAGATAACTGGACTACCAGCTATACGTTGACAAG
23_gp120for_612	GATCTCCCAGTTATCTGGGGCCTGTGTAATGACTGAGG
24_gp120back_586	GATCTCCCAGATAACTGGTGTAACACCTCAGTCATTACACAG
24_gp120for_636	GATCTCCCAGTTATCTGGTGGCTCAAAGGATACCTTTG
25_gp120back_613	GATCTCCCAGATAACTGGTGTCCAAAGGTATCCTTTGAG
25_gp120for_663	GATCTCCCAGTTATCTGGAGCCGGGGCACAATAATG
26_gp120back_637	GATCTCCCAGATAACTGGATTCCCATACATTATTGTGCC
26_gp120for_687	GATCTCCCAGTTATCTGGATTACATTTTAGAATCGCAAAAC
27_gp120back_664	GATCTCCCAGATAACTGGGGTTTTGCGATTCTAAAATG
27_gp120for_714	GATCTCCCAGTTATCTGGTGGTCCTGTTCCATTGAACG
28_gp120back_688	GATCTCCCAGATAACTGGAATAAGACGTTCAATGGAACAG
28_gp120for_738	GATCTCCCAGTTATCTGGTTGTACTGTGCTGACATTTGTAC
29_gp120back_715	GATCTCCCAGATAACTGGTGTACAAATGTCAGCACAGTACAATG
29_gp120for_765	GATCTCCCAGTTATCTGGTACTACTGGCCTAATTCCATGTG
30_gp120back_739	GATCTCCCAGATAACTGGTGTACACATGGAATTAGGCCAG
30_gp120for_789	GATCTCCCAGTTATCTGGGCCATTTAACAGCAGTTGAG
31_gp120back_766	GATCTCCCAGATAACTGGTCAACTCAACTGCTGTTAAATG
31_gp120for_816	GATCTCCCAGTTATCTGGAATTACTACCTCTTCTTCTGCCAG
32_gp120back_790	GATCTCCCAGATAACTGGAGTCTGGCAGAAGAAGAGGTAG
32_gp120for_840	GATCTCCCAGTTATCTGGATTGTCTGTGAAATTGGCAGATC
33_gp120back_817	GATCTCCCAGATAACTGGAGATCTGCCAATTTCACAGAC
33_gp120for_867	GATCTCCCAGTTATCTGGGTTCAGCTGTACTATTATGGTTTTAG

34_gp120back_841	GATCTCCCAGATAACTGGGCTAAAACCATAATAGTACAGCTG
34_gp120for_891	GATCTCCCAGTTATCTGGTGTACAATTAATTTCTACAGATTGG
35_gp120back_868	GATCTCCCAGATAACTGGCAATCTGTAGAAATTAATTGTACAAG
35_gp120for_918	GATCTCCCAGTTATCTGGACTTTTTCTTGTATTGTTGTTGG
36_gp120back_892	GATCTCCCAGATAACTGGAGACCCAACAACAATACAAGAAAAAG
36_gp120for_942	GATCTCCCAGTTATCTGGCCCTGGTCCTCTCTGGATAC
37_gp120back_919	GATCTCCCAGATAACTGGATCCGTATCCAGAGAGGACC
37_gp120for_969	GATCTCCCAGTTATCTGGTATTTTTCCTATTGTAACAAATGC
38_gp120back_943	GATCTCCCAGATAACTGGAGAGCATTTGTTACAATAGGAAAAATAG
38_gp120for_993	GATCTCCCAGTTATCTGGACAATGTGCTTGTCTCATATTTC
39_gp120back_970	GATCTCCCAGATAACTGGGGAAATATGAGACAAGCACATTG
39_gp120for_1020	GATCTCCCAGTTATCTGGGTTATTCCATTTTGCTCTACTAATG
40_gp120back_994	GATCTCCCAGATAACTGGAACATTAGTAGAGCAAAATGGAATAAC
40_gp120for_1044	GATCTCCCAGTTATCTGGTTTGCTATCTATCTGTTTTAAAGTG
41_gp120back_1021	GATCTCCCAGATAACTGGACTTTAAAACAGATAGATAGCAAAT
41_gp120for_1071	GATCTCCCAGTTATCTGGTTTATTATTTCCAAATTGTTCTC
42_gp120back_1045	GATCTCCCAGATAACTGGTTAAGAGAACAATTTGGAAATAATA
42_gp120for_1095	GATCTCCCAGTTATCTGGTGAGGACTGCTTAAAGATTATTG
43_gp120back_1072	GATCTCCCAGATAACTGGACAATAATCTTTAAGCAGTCCTC
43_gp120for_1122	GATCTCCCAGTTATCTGGGTGCGTTACAATTTCTGGGTC
44_gp120back_1096	GATCTCCCAGATAACTGGGGAGGGGACCCAGAAATTG
44_gp120for_1146	GATCTCCCAGTTATCTGGAAATTCCCCTCCACAATTAAAAC
45_gp120back_1123	GATCTCCCAGATAACTGGAGTTTTAATTGTGGAGGGG
45_gp120for_1173	GATCTCCCAGTTATCTGGAAACAGTTGTGTTGAATTACAGTAG
46_gp120back_1147	GATCTCCCAGATAACTGGTTCTACTGTAATTCAACACAACTG
46_gp120for_1197	GATCTCCCAGTTATCTGGAGTACTATTAAACCAAGTACTATTAAAC
47_gp120back_1174	GATCTCCCAGATAACTGGAATAGTACTTGGTTTAATAGTACTTGG
47_gp120for_1224	GATCTCCCAGTTATCTGGAGTGTTATTTGACCCTTTAGTACTC
48_gp120back_1198	GATCTCCCAGATAACTGGTGGAGTACTAAAGGGTCAAATAAC
48_gp120for_1248	GATCTCCCAGTTATCTGGGAGGGTGATTGTGTCACTTCC
49_gp120back_1225	GATCTCCCAGATAACTGGGAAGGAAGTGACACAATCACC
49_gp120for_1275	GATCTCCCAGTTATCTGGGTTTATAATTTGTTTTATTCTGCATG
50_gp120back_1249	GATCTCCCAGATAACTGGCCATGCAGAATAAAACAAATTATAAAC
50_gp120for_1299	GATCTCCCAGTTATCTGGTGCTTTTCCTACTTCCTGCC
51_gp120back_1276	GATCTCCCAGATAACTGGATGTGGCAGGAAGTAGGAAAAG
51_gp120for_1326	GATCTCCCAGTTATCTGGTTGTCCACTGATGGGAGGG
52_gp120back_1300	GATCTCCCAGATAACTGGATGTATGCCCCTCCCATCAG
52_gp120for_1350	GATCTCCCAGTTATCTGGTGTAATATTTGATGAACATCTAATTTG

53_gp120back_1327	GATCTCCCAGATAACTGGATTAGATGTTCATCAAATATTACAGG
53_gp120for_1377	GATCTCCCAGTTATCTGGACCACCATCTCTTGTTAATAGC
54_gp120back_1351	GATCTCCCAGATAACTGGGGGCTGCTATTAACAAGAGATG
54_gp120for_1401	GATCTCCCAGTTATCTGGGATCTCGGACTCATTGTTGC
55_gp120back_1378	GATCTCCCAGATAACTGGAATAGCAACAATGAGTCCGAG
55_gp120for_1428	GATCTCCCAGTTATCTGGCCTCATATCTCCTCCTCCAGG
56_gp120back_1402	GATCTCCCAGATAACTGGTTCAGACCTGGAGGAGGAG
56_gp120for_1452	GATCTCCCAGTTATCTGGATATAATTCACTTCTCCAATTGTC
57_gp120back_1429	GATCTCCCAGATAACTGGGACAATTGGAGAAGTGAATTATAT
57_gp120for_1479	GATCTCCCAGTTATCTGGTGGTTCAATTTTTACTACTTTATATTTA
58_gp120back_1453	GATCTCCCAGATAACTGGAAATATAAAGTAGTAAAAATTGAACC
58_gp120for_1503	GATCTCCCAGTTATCTGGTGCCTTGGTGGGTGCTAC
59_gp120back_1480	GATCTCCCAGATAACTGGTTAGGAGTAGCACCCACCAAG
59_gp120for_1533	GATCTCCCAGTTATCTGGTCTTTTTTCTCTCTGCACCAC

Tab.4: HIV-1 gp120 primers for construction of the *Ac*COPS-PCR-library. *Bst*XI sites with *Sce*-I compatible overhangs are underlined. Note: The primer 4_gp120back_91 starts from the very beginning of the core protein (w/o leader). Amplification with the for-primer 4_gp120for_126 results in a smaller fragment (12aa instead of 17aa) and led to the viral clone *Ac*COPS-PCR-LIB-Frg#4.1. The very last fragment (primers 59_gp120back_1480 and 59_gp120for_1533) implies 18aa and led to generation of the clone *Ac*COPS-PCR-LIB-Frg#59.

All fragments were PCR-amplified with envBH10 as template, purified and digested with *Bst*XI. After quantification on an agarose gel, ligations were performed with *Sce*-I-cut and dephosphorylated pCOPS. Following a transformation in TG1, colonies were PCR-screened for positive clones with –44back/+201for and checked on a 2% agarose gel. Three supposed positive clones of each ligation were grown o/n in 5ml LB-Amp and submitted to GFX-MiniPreps. Plasmid DNA was co-transfected with BaculoGold-DNA in *Sf*9 cells (24WP) and incubated for five days. Several clones of each transfection were screened (rapid protocol for PCR-screening (4.3.8) using primers –44back and +201for) and subsequently submitted to DNA-sequencing. Accurate clones were amplified twice, pooled and amplified once more. The gained virus stock was designated *Ac*COPS-PCR-library.

Designation of clone	Amino acid sequence
*Ac*COPS-PCR-LIB-Frg#1	M R V K E K Y Q H L W R W G W R W
*Ac*COPS-PCR-LIB-Frg#2	H L W R W G W R W G T M L L G M L
*Ac*COPS-PCR-LIB-Frg#3	G T M L L G M L M I C S A T E K L
*Ac*COPS-PCR-LIB-Frg#4	M I C S A T E K L W V T V Y Y G V
*Ac*COPS-PCR-LIB-Frg#4.1	T E K L W V T V Y Y G V
*Ac*COPS-PCR-LIB-Frg#5	W V T V Y Y G V P V W K E A T T T
*Ac*COPS-PCR-LIB-Frg#6	P V W K E A T T T L F C A S D A K
*Ac*COPS-PCR-LIB-Frg#7	L F C A S D A K A Y D T E V H N V
*Ac*COPS-PCR-LIB-Frg#8	A Y D T E V H N V W A T H A C V P
*Ac*COPS-PCR-LIB-Frg#9	W A T H A C V P T D P N P Q E V V
*Ac*COPS-PCR-LIB-Frg#10	T D P N P Q E V V L V N V T E N F
*Ac*COPS-PCR-LIB-Frg#11	L V N V T E N F N M W K N D M V E
*Ac*COPS-PCR-LIB-Frg#12	N M W K N D M V E Q M H E D I I S
*Ac*COPS-PCR-LIB-Frg#13	Q M H E D I I S L W D Q S L K P C
*Ac*COPS-PCR-LIB-Frg#14	L W D Q S L K P C V K L T P L C V
*Ac*COPS-PCR-LIB-Frg#15	V K L T P L C V S L K C T D L K N
*Ac*COPS-PCR-LIB-Frg#16	S L K C T D L K N D T N T N S S S
*Ac*COPS-PCR-LIB-Frg#17	D T N T N S S S G R M I M E K G E
*Ac*COPS-PCR-LIB-Frg#18	G R M I M E K G E I K N C S F N I
*Ac*COPS-PCR-LIB-Frg#19	I K N C S F N I S T S I R G K V Q
*Ac*COPS-PCR-LIB-Frg#20	S T S I R G K V Q K E Y A F F Y K
*Ac*COPS-PCR-LIB-Frg#21	K E Y A F F Y K L D I I P I D N D
*Ac*COPS-PCR-LIB-Frg#22	L D I I P I D N D T T S Y T L T S
*Ac*COPS-PCR-LIB-Frg#23	T T S Y T L T S C N T S V I T Q A
*Ac*COPS-PCR-LIB-Frg#24	C N T S V I T Q A C P K V S F E P
*Ac*COPS-PCR-LIB-Frg#25	C P K V S F E P I P I H Y C A P A
*Ac*COPS-PCR-LIB-Frg#26	I P I H Y C A P A G F A I L K C N
*Ac*COPS-PCR-LIB-Frg#27	G F A I L K C N N K T F N G T G P
*Ac*COPS-PCR-LIB-Frg#28	N K T F N G T G P C T N V S T V Q
*Ac*COPS-PCR-LIB-Frg#29	C T N V S T V Q C T H G I R P V V
*Ac*COPS-PCR-LIB-Frg#30	C T H G I R P V V S T Q L L L N G
*Ac*COPS-PCR-LIB-Frg#31	S T Q L L L N G S L A E E E V V I
*Ac*COPS-PCR-LIB-Frg#32	S L A E E E V V I R S A N F T D N
*Ac*COPS-PCR-LIB-Frg#33	R S A N F T D N A K T I I V Q L N
*Ac*COPS-PCR-LIB-Frg#34	A K T I I V Q L N Q S V E I N C T
*Ac*COPS-PCR-LIB-Frg#35	Q S V E I N C T R P N N N T R K S
*Ac*COPS-PCR-LIB-Frg#36	R P N N N T R K S I R I Q R G P G
*Ac*COPS-PCR-LIB-Frg#37	I R I Q R G P G R A F V T I G K I

*Ac*COPS-PCR-LIB-Frg#38	R A F V T I G K I G N M R Q A H C
*Ac*COPS-PCR-LIB-Frg#39	G N M R Q A H C N I S R A K W N N
*Ac*COPS-PCR-LIB-Frg#40	N I S R A K W N N T L K Q I D S K
*Ac*COPS-PCR-LIB-Frg#41	T L K Q I D S K L R E Q F G N N K
*Ac*COPS-PCR-LIB-Frg#42	L R E Q F G N N K T I I F K Q S S
*Ac*COPS-PCR-LIB-Frg#43	T I I F K Q S S G G D P E I V T H
*Ac*COPS-PCR-LIB-Frg#44	G G D P E I V T H S F N C G G E F
*Ac*COPS-PCR-LIB-Frg#45	S F N C G G E F F Y C N S T Q L F
*Ac*COPS-PCR-LIB-Frg#46	F Y C N S T Q L F N S T W F N S T
*Ac*COPS-PCR-LIB-Frg#47	N S T W F N S T W S T K G S N N T
*Ac*COPS-PCR-LIB-Frg#48	W S T K G S N N T E G S D T I T L
*Ac*COPS-PCR-LIB-Frg#49	E G S D T I T L P C R I K Q I I N
*Ac*COPS-PCR-LIB-Frg#50	P C R I K Q I I N M W Q E V G K A
*Ac*COPS-PCR-LIB-Frg#51	M W Q E V G K A M Y A P P I S G Q
*Ac*COPS-PCR-LIB-Frg#52	M Y A P P I S G Q I R C S S N I T
*Ac*COPS-PCR-LIB-Frg#53	I R C S S N I T G L L L T R D G G
*Ac*COPS-PCR-LIB-Frg#54	G L L L T R D G G N S N N E S E I
*Ac*COPS-PCR-LIB-Frg#55	N S N N E S E I F R P G G G D M R
*Ac*COPS-PCR-LIB-Frg#56	F R P G G G D M R D N W R S E L Y
*Ac*COPS-PCR-LIB-Frg#57	D N W R S E L Y K Y K V V K I E P
*Ac*COPS-PCR-LIB-Frg#58	K Y K V V K I E P L G V A P T K A
*Ac*COPS-PCR-LIB-Frg#59	L G V A P T K A K R R V V Q R E K R

Tab.5: Amino acid sequences displayed by baculoviral clones of the *Ac*COPS-PCR-library. All clones lead to surface presentation of gp120-fragments of 17 amino acids (exceptions: *Ac*COPS-PCR-LIB-Frg#4.1 (12aa) and *Ac*COPS-PCR-LIB-Frg#59 (18aa)).

Construction of the vector *Ac*MARS-gp120, which served as positive control for FACS experiments, was performed likewise: a PCR with the library primers 4_gp120back_ATG_91 and 59_gp120for_1533 using envBH10 as template was carried out. The amplified fragment was purified, cut and ligated into prepared pMARS. After transformation in TG1, clones were screened using –44back and 59_gp120for_1533 as primers. Positive clones were co-transfected with BaculoGold-DNA and submitted to plaque assay. A positive viral clone was verified by DNA-sequencing and designated as *Ac*MARS-gp120.

Analysis and sorting of the *Ac*COPS-PCR-library was performed as described (4.4). Several monoclonal antibodies were used for selection of specific binding partners:

Designation	Isotype	Host	Characteristics	Presentation	Reference
ARP360 (4A7C6)	IgG1	Mouse (BALB/c)	epitope sequence PQEVVLVNVT, binds native and denatured gp120	ascitic fluid, diluted 1/10 prior to use	[Moore *et al.* (1993)]
ARP3048 (IIIB-V3-21)	IgG1	Mouse (BALB/c)	binds to sequence INCTRPN of V3-loop	purified antibody, 50µg/ml	[Laman *et al.* (1992)]
ARP389 (ICR38.1a / ICR38.8f)	IgG2b	Rat/Y3	reacts with sequence EVGKAMYAPP, blocks CD4 binding	culture supernatant	[Cordell *et al.* (1991)]
1B1 (IAM 120-1B1)	IgG1	Human	? discontinuous, blocks CD4 binding	culture supernatant, 300µg/ml	[Buchacher *et al.* (1994)]

Tab.6: Monoclonal antibodies used for selection of the *Ac*COPS-PCR-library. ARP360, ARP3048 and ARP389 were ordered at *The Aids Reagent Project* (National Institute for Biological Standards and Control (NIBSC); http://www.nibsc.ac.uk/catalog/aids-reagent/Catalog2000/Mono.htm#HIV1ENV). The monoclonal antibody IAM 120-1B1 was kindly provided by H. Katinger (IAM, Vienna, Austria).

4.3 Insect cell culture methods

4.3.1 Cells and virus

All baculoviral constructs were derived from *Ac*NPV (*Autographa californica* nuclear polyhedrosis virus, VR-1345; ATCC, Rockville, USA) and were propagated in the insect cell line *Sf*9 (*Spodoptera frugiperda*, CRL-1711; ATCC, Rockville, USA). The baculoviral transfer vector pMARS was kindly provided by R. Grabherr (IAM, Vienna, Austria) [Grabherr *et al.* (1997)]. Genomic DNA of the viral clone Ac64$^-$ which lacks the gp64 structural gene was kindly provided by G. Blissard (Boyce Thompson Institute, Ithaca, USA).

4.3.2 Cultivation of *Sf*9 insect cells

*Sf*9 insect cells were grown in a IPL-41 medium (Sigma-Aldrich, Munich, Germany) containing yeast extract (DHW, Hamburg, Germany) and a lipid/sterol cocktail (Sigma). IPL-41 insect medium powder (w/ L-glutamine, w/o calcium chloride and sodium bicarbonate) was prepared pyrogenfree in 50l batches as follows:

- dissolve medium powder aliquot for 50l in 42,5l AD and stir for at least 2h
- add 742,5g Sucrose
  140g NaCl
  17,5g $NaHCO_3$
  375ml NaOH (2N)
  180g yeast extract (dissolved in 1250ml AD)
- stir for 30min and adjust pH to 6,2 with NaOH (2N)
- add lipid-cocktail: 50ml lipid-stock (Sigma, Munich, Germany) emulsified in 500ml Pluronic F68 (Sigma, Munich, Germany) solution (10% in AD)
- add 25g $CaCl_2$ (w/f)
- stir for 30min and sterile filter (Millipak-60 (Millipore, Bedford, USA)
- store aliquots at 4°C
- fetal calf serum (FCS) was added directly before use if desired

Insect cell culture was carried out in a biological safety cabinet class II (HeraSafe HS-P 12/2, Heraeus, Hanau, Germany). *Sf*9 insect cells were cultivated in cell culture flasks (Nunclon D SI, Nalge Nunc International, Rochester, USA) with IPL-41 containing 3% heat-inactivated FCS (HyClone, Logan, USA) at 27°C (incubator BK6160 (Heraeus). They were seeded at 10^5cells/cm^2 and passaged when reaching a confluent layer (usually after 3 to 4 days).

For long-term storage log-phase *Sf*9 cells were harvested, washed with IPL-41(0%FCS), diluted to 5*10^6cells/ml with IPL-41 (10% FCS, 10% DMSO) and stored in liquid nitrogen. Usually, *Sf*9 cells were substituted by freshly thawed cells after passage 100.

For mid scale production and infection of cells, *Sf*9 were grown in spinner flasks (Techne, Cambridge, UK) with volumes of 1000 to 5000ml (working volume was 25% of actual spinner capacity) in a waterbath (MWB-10L; tempunit TE-10D; stirrer MCS-104L (Techne)): cells were grown to a cell density of 3*10^6cells/ml and passaged to 10^5cells/ml. For infections, cells were used at a final density of 10^6cells/ml.

All cells used for infections, transfections and plaque assays were passaged 1:2 24h before the experiment. Before being subjected to virus particles or to complexed viral DNA in transfections, the cells were washed with serumfree IPL-41.

4.3.3 Harvest of insect cells

Adherently grown cells were detached from their surface by shaking or by repeated aspiring with a pipette. They were centrifuged (all centrifugation steps of insect cells were carried out at 1000rpm for five minutes in a centrifuge C312 (Juoan, Winchester, USA) or at 3000rpm for three minutes in a desktop-centrifuge 5415C (Eppendorf, Hamburg, Germany)), resuspended in sterile PBS and recentrifuged. The cell pellet was diluted in the appropriate volume of suitable buffer or medium.

4.3.4 Transfection of insect cells

Sf9 cells that were passaged 1:2 one day before transfection were harvested and washed twice with serumfree IPL-41. They were seeded in a Roux25 flask at a cell density of 10^5cells/cm^2 and supplied with 1ml IPL-41 (0%FCS).

In the meantime, the transfection-mixture was prepared as follows: a mixture of 20µl NovaFector (EquiBio, Ashford, U.K.) and 80µl AD was added to a tube containing the viral DNA (infective viral DNA: 20ng, for homologous recombination purposes: 100ng BaculoGold-DNA plus 500ng transfer plasmid) in 50µl AD. The mixture was shaken gently, applied to the arranged cells and incubated for 5h at 27°C.

Finally, IPL-41 containing an antibiotics-mix (100fold ATB = 6mg/ml penicillin G sodium (1662U/mg), 10mg/ml streptomycin sulfate (740µg/mg), 10mg/ml gentamycin sulfate (660U/mg) (Seromed, Berlin, Germany)) and FCS was added to give a final concentration of 1fold antibiotics-mix and 10%FCS.

Cells were incubated for at least three days and the supernatant containing the generated viral particles was harvested and amplified. Stock were stored at 4°C.

4.3.5 Infection of insect cells

Cells that have been passaged 1:2 one day before infection were harvested, resuspended in IPL-41 (0%FCS) and seeded in an appropriate flask at a cell density of 10^5cells/cm^2. After 15 minutes, the supernatant was removed, the adherent cells were washed with serumfree medium and supplied with a small amount of IPL-41 (1/3 of final volume) without FCS. The desired amount of plaque-forming units was applied and the flask was incubated for 1h on a rocking platform. Finally, IPL-41 containing antibiotics-mix and FCS was added to achieve a concentration of 1fold ATB and 10%FCS.

Infection of spinner cultures was performed likewise: cells and viruses were mixed in 1/10 of final volume in serumfree medium in a plastic tube before being expanded to the spinner flask.

In order to produce a high level of recombinant protein, cells were infected with an MOI of 20. Usually, cells were harvested 48h post infection.

Low MOI infections were used in order to generate virus stocks. Cells were infected with an MOI of 0,2 and were harvested 3 to 5 days p.i., according to the development of the infection visualised by microscopy. The cell suspension was centrifuged, the supernatant was harvested and stored at 4°C.

The originated stocks were designated as follows:

plaque pick (PP)	a single plaque-forming unit was transferred onto *Sf*9 cells in a 24WP and harvested 4 days post infection
seed stock (SS)	low MOI amplification of PP
intermediate stock (IS)	low MOI amplification of SS
working stock (WS)	low MOI amplification of IS

4.3.6 Harvest of baculovirus particles

In order to yield purified baculoviral particles, the supernatant of infected insect cells was harvested 3 to 5 days post infection and submitted to ultracentrifugation (ultracentrifuge L8-80M (Beckman, Fullerton, USA); cooling device ASW300 (Liebert, Columbus, USA)): the fluid was loaded on top of a small ($1/10^{th}$ of the SW28 tube volume) sucrose cushion (25% sucrose in AD) and centrifuged for 1h at 25000rpm/20°C. The pellet of one tube was dissolved in an appropriate volume of desired buffer (usually 100µl of PBS containing 0,1%NaN_3) and stored at 4°C.

4.3.7 Preparation of viral DNA for cloning and transfection purposes

Viral DNA for cloning and transfection purposes was extracted after ultracentrifugation of viral particles: the pellet was suspended in 4ml extraction-buffer [O'Reilly *et al.* (1992)] containing 0,5mg/ml proteinase K. The mix was incubated at 50°C for 2h and supplied with 2% (w/v) of N-lauroyl-sarcosine. After an additional incubation (50°C for 75 minutes), 20mg RNaseA were added and left at 50°C for 45 minutes. The aqueous solution was submitted to PCI-purified (2x PCI, 1x CI) and washed with 2 volumes of EtOH (96%). The originated DNA-precipitate was washed with 70% EtOH and left to air-dry for 15 minutes at RT before being dissolved in 100µl preheated (50°C) AD and stored at 4°C.

4.3.8 Rapid protocol for PCR screening of virus stocks

In order to PCR-screen numerous virus stocks very quickly the following protocol was established:

- take 1ml baculoviral supernatant and spin for 30 minutes at 15000g
- carefully remove liquid
- add 25µl AD containing 1mg/ml proteinase K and incubate for 20 minutes at 37°C
- heat for 10 minutes at 95°C
- spin down (1', 15000g)
- apply 5µl of supernatant as template for PCR-screen

Alternatively, standard alkaline lysis of infected insect cells was performed in order to extract viral DNA as template for PCR amplification.

4.3.9 Plaque Assay

Viral titres were determined by plaque assay and expressed as plaque-forming units (PFU/ml).

Insect cells were seeded at a cell density of $2*10^5$ cells/cm^2 on 60mm plates (Nunclon D SI, Nalge Nunc, Rochester, USA) and inoculated with 500µl of diluted virus (in serumfree IPL-41). Depending on the experiment, three relevant dilutions were plated out in two series.

The plates were incubated on a rocking platform for one hour in order to allow the viruses to enter the insect cells. Afterwards, the supernatant was aspirated and the cells were covered with 4ml of an seaplaque agarose (in IPL-41 / 10%FCS / 1xATB) (BMA, Rockland, USA) which was tempered at 39°C. After 15 minutes, the plates were subjected to the incubator in a humidified container.

The plates were incubated for five days and overlaid with 1mg/ml MTT solution (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide, Sigma) unless the translucent plaques could be clearly distinguished from the opaque surrounding cells.

The isolation of viral clones was performed likewise by means of plaque assay. After MTT-staining, the remaining fluid was removed and the plates were left to dry for approximately one hour. Subsequently, single plaques were transferred to freshly seeded *Sf*9 cells in 24WP using sterile pipettes.

4.4 Protocols for cytometric analysis and sorting of infected insect cells

4.4.1 Staining of infected insect cells

Cells infected with MOI=20 were harvested 48h p.i. and stained with 50µl/10^6cells of a mix of PBS containing 10% FCS and the appropriate amount of primary antibody as concluded from the titrations (Fig.11/12/13/14). The mixture was incubated for 1h on a rocking platform at RT. The cells were washed in PBS and mixed with secondary antibody (fluorescence conjugate) in 50µl PBS (2%FCS) per 10^6cells.

4.4.2 FACS analysis of infected insect cells

FACS analysis and sorting of infected insect cells was carried out on a FACSCalibur System (BD Biosciences, Palo Alto, USA). Threshold was set at 50. Detector setting were determined as follows:

Detector	Voltage	Amp Gain	Mode
FSC	E-1	6,27	Lin
SSC	321	1,00	Lin
FL1	367	-	Log
FL3	390	-	Log

Tab.7: Instrument setting for FACS analysis and sorting with the BD FACSCalibur system.

For analysis purposes, 10^3 events were surveyed. Infected cells were divided from non-infected cells and debris via their values in forward- and side-scatter (FSC/SSC) [Nordstrom *et al.* (1999)]. Only cells identified as being infected were included into analysis.

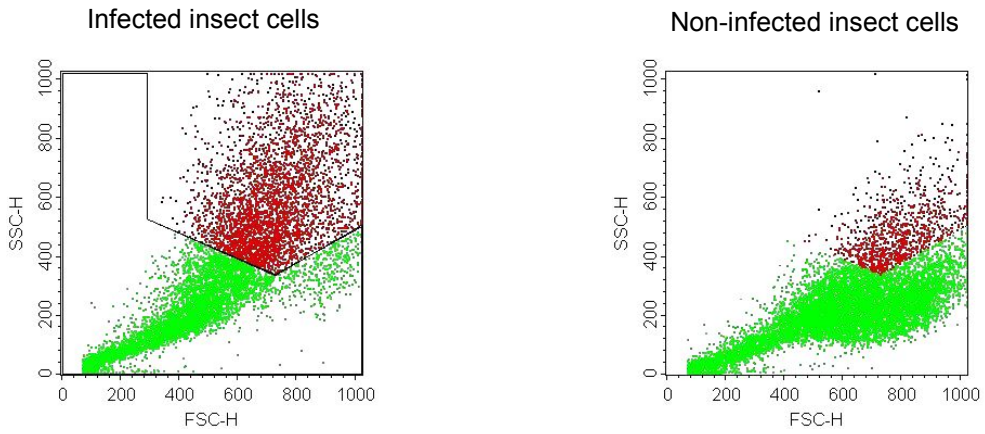

Fig.5.: Characteristical FSC/SSC-analysis of infected and non-infected insect cells. Supposed infected cells (red) were gated for further analysis.

In order to distinguish live and dead cells, 1/1000 v/v propidium iodide was added to the sample right before analysis. Dead cells assimilate PI and can be distinguished because of their high emission of PI-specific fluorescence (585nm, detected with FL-3). Because of their ability to bind primary and secondary antibodies unspecifically and due to their undetermined physiological state, dead cells were excluded from analysis.

Live cells (Fig.6) were defined by first gating out dead cells (red) from a positive control with high FL3-signal intensities (orange: live cells). A second gate (blue) was inserted containing approximately 99,5% of remaining live cells within the negative control with the lowest FL1-signal (FITC, detected at 530nm). All cells not included in these gates (green) were designated as live positive cells. The sample was analysed using the determined gates.

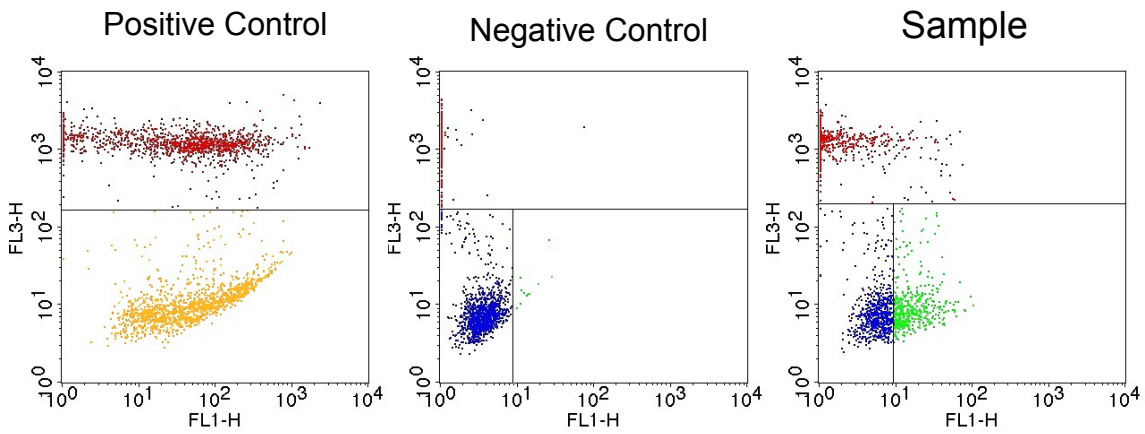

Fig.6: Representative FACS analysis of infected insect cells stained with an epitope-specific antibody and a suitable FITC-labelled conjugate plus propidium iodide. Different gates were set in order to determine live positive cells (green). FL1-H: relative fluorescence intensity (signal height of a single particle) of FITC-specific detector; FL3-H: propidium iodide-specific detector.

4.4.3 Fluorescence activated cell sorting of infected insect cells

Cytometric sorting of 10^6 infected insect cells was performed using sterile PBS as sheath fluid. Best sorting performance with the FACSCalibur was achieved by diluting the stained cells to a density of $3*10^6$cells/ml. Positive cells were sorted in 50ml polypropylene tubes that were saturated with sterile BSA (1% BSA in AD for 1h at 4°C). Subsequently, the selected infected cells were centrifuged at 1000g for 10 minutes and the cells were resuspended in the remaining liquid (approximately 100µl) and directly applied to freshly seeded *Sf*9 cells (in 24WP with IPL-41 plus 10%FCS and 1xATB). The next day, the supernatant was harvested and filtered through a 0,22µm sterile filter onto new cells in order to eliminate potential contaminations resulting from the sorting process. Three days after infection, the supernatant was designated intermediate stock and amplified in order to achieve a high-titre working stock.

For isolation of individual clones, these stocks were submitted to plaque assay and resulting plaques were used to infect *Sf*9 cells seeded in 24WP. After

4 days, infected cells and supernatant were harvested. Cells were stained with the appropriate antibody and submitted to FACS screening. In case of a positive signal, the recombinant insert of the viral DNA of the corresponding supernatant was amplified by means of PCR (rapid protocol for PCR screening of virus stocks (4.3.8)) with the relevant primers and submitted to DNA sequencing.

4.5 Protein analytics

4.5.1 Determination of total protein content of viral preparations

Protein content of viral preparations (4.3.6) was quantified with BioRad protein assay dye reagent concentrate (BioRad, Munich, Germany) applying the manufacturer‘s protocol for microassays. Samples were serially diluted in 96WP and analysed at 595nm on a Easy Reader EAR400AT (SLT-Labinstruments, Groedig, Austria) using ΔSoft, Version 1.80S (BioMetallics, Princeton, USA) for quantification. The mean of two independent experiments was taken as reference for enzyme linked immunosorbent assays (ELISA). Protein standard solution (Sigma) was used as standard.

4.5.2 Epitope surface localisation on viral particles

Comparison of the surface display properties of the three baculoviral constructs *Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*COIN-3D6 was done by means of ELISA of viral particles and by FACS analysis of infected insect cells.

Enzyme linked immunosorbent assays (ELISA) of recombinant viral particles were carried out as sandwich assays [Sambrook and Russell (2001)]: One of two antibodies (one specific for the recombinant epitope (human 3D6 [Buchacher 1994] (1,5mg/ml), kindly provided by H. Katinger (IAM, Vienna) and one antibody specific for viral gp64 (mouse B12D5 [Keddie *et al.* (1989)], kindly provided by L. Volkman (University of California Berkeley, USA) was coated on ELISA plates, whereas the other, together with a corresponding peroxidase conjugate (Sigma, Munich, Germany), served as detection antibody.

Coating was performed by incubating 10µg/ml antibody in 50µl coating-buffer o/n at 4°C on a rocking platform. Plates were washed with washing-buffer and loaded with purified viral particles (1µg/well viral particles (first row) in dilution-buffer were serially diluted). After one hour of incubation on a rocking platform (RT), the plates were washed again and supplied with the desired amount of detection antibody in dilution-buffer. Plates were washed and incubated with the matching conjugate (1/1000 in dilution-buffer) for one hour.

Staining of the ELISA-plates was performed adding 100µl/well staining-buffer (citrate-buffer; pH=5,0) supplied with 1mg/ml OPD (1,2-o-phenylenediaminedihydrochloride) and 1µl/ml H_2O_2 (35%). The reaction was stopped with 100µl H_2SO_4 (1,25M) and plates were analysed on an Easy Reader EAR400AT (extinction at 492nm).

5. Results

5.1 Comparison of the surface expression with different baculoviral vectors

The purpose of this work was to establish and to evaluate an authentic baculoviral surface expression library displaying fragments of the model protein HIV-1 gp120.

Several viral constructs were tested for their surface display properties in order to chose the suitable vehicle for the generation of the gp120 libraries. *Ac*COPS harbours a second copy of the envelope fusion protein gp64 under control of the polyhedrin promoter. Epitopes can be ligated to the N-terminus of the gp64 core sequence and get displayed as fusion proteins on viral particles and on infected insect cells (Fig.4) together with native gp64. Epitopes in the construct *Ac*MARS are linked to the membrane anchor sequence of gp64. In the vector *Ac*COIN, heterologous sequences are directly inserted into the native gp64 resulting in high avidity surface expression on viral particles (Fig.4). Choosing *Ac*COPS as the most suitable vector for the purpose of epitope display, two different libraries were generated. Fragments of the first library were produced by DNase-I digestion, the second library was elaborated by inserting well-defined PCR-fragments of gp120 into the baculoviral vectors.

5.1.1 Surface presentation of epitopes on viral particles

Surface presentation of heterologous peptides on viral particles was determined by sandwich-ELISA using the vectors *Ac*COPS, *Ac*MARS, and *Ac*COIN (Fig.4) harbouring the epitope for the monoclonal antibody 3D6. For each sample, two experiments with the same set of antibodies were carried out. In the first experiment (Fig.7; green columns), the plates were coated with 3D6 as specific catcher antibody and viral particles were detected with gp64-specific antibody B12D5. In the second set-up (Fig.7; red columns), which represents a verification of the first experiments, B12D5 was employed as catcher antibody and 3D6 served for detection of bound viruses harbouring the epitope.

ELISA data (Fig.7) revealed best surface presentation on recombinant *Ac*COIN viruses followed by *Ac*COPS. *Ac*MARS resulted in a very weak signal. The negative control *Ac*NPV showed no detectable binding of viral particles.

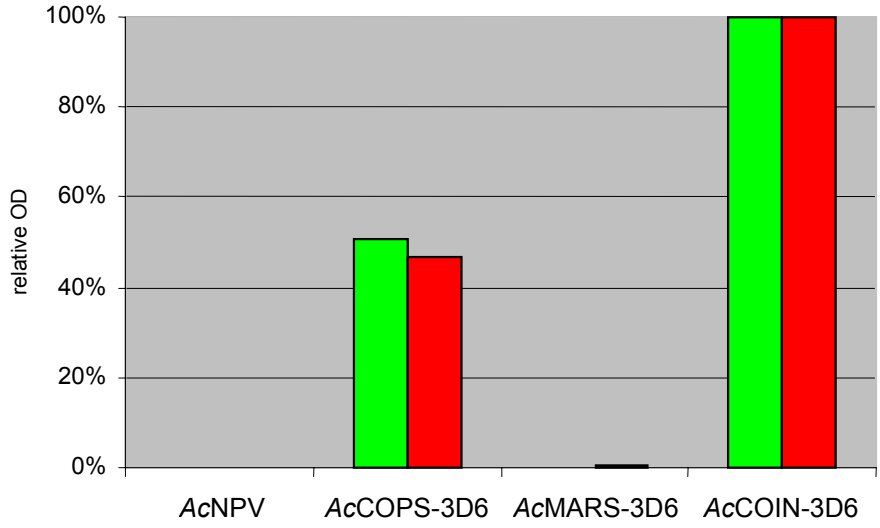

Fig.7: ELISA of different baculoviral particles. Recombinant viruses *Ac*COPS, *Ac*MARS and *Ac*COIN harbouring the epitope for the monoclonal gp41-antibody 3D6 (SGKLICTTAVPWNAS) were analysed in sandwich-ELISA. Green columns: 3D6 served as catcher antibody and viral particles were detected by B12D5. Red columns: B12D5 served as catcher antibody and 3D6 for detection. Wildtype *Ac*NPV was taken as negative control. Absorption (E) is indicated as relative OD in relation to protein content. Relative OD of *Ac*COIN-3D6 was set to 100%.

5.1.2 Surface presentation of epitopes on infected insect cells

Surface presentation on infected insect cells with the different vectors was evaluated by means of FACS analysis. Cells were infected with *Ac*COIN-3D6, *Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*NPV (negative control) and stained with the monoclonal antibody 3D6. Detection of bound 3D6 was done using anti-human IgG (γ-chain specific) FITC conjugate (Sigma).

Cells infected with *Ac*MARS-3D6 achieved highest fluorescence intensities, followed by *Ac*COPS-3D6. *Ac*COIN-3D6 displayed rather weak binding (Fig.8).

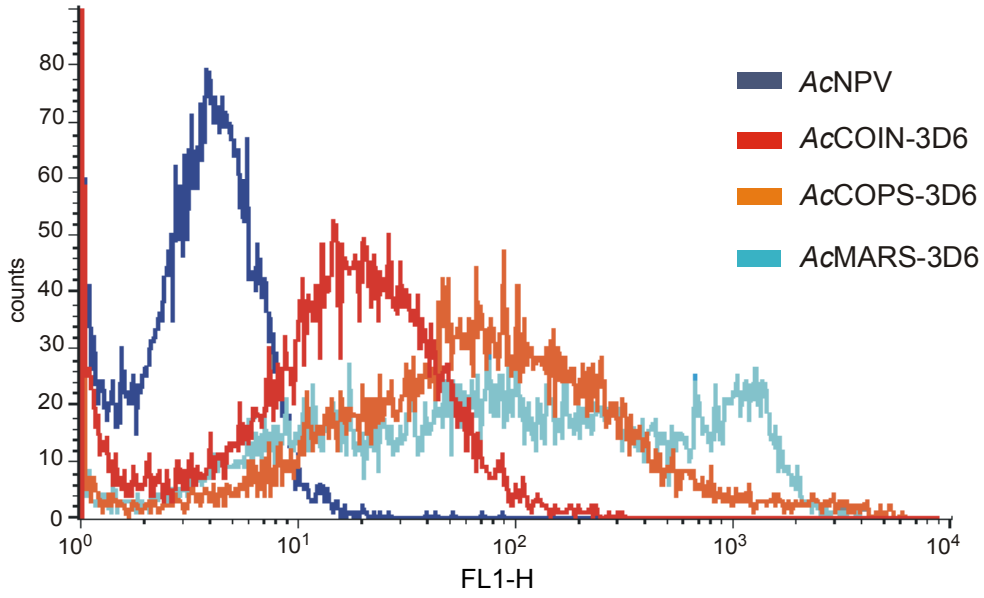

Fig.8: FACS analysis of *Sf9* cells infected with the recombinant baculoviruses *Ac*COPS-3D6, *Ac*MARS-3D6 and *Ac*COIN-3D6. Cells were harvested 48hp.i. and stained with the monoclonal antibody 3D6 (100µg/ml). Wildtype AcNPV was taken as negative control.

5.2 Construction of the *Ac*COPS-DNase-library

A baculoviral library presenting peptides on the surface of infected insect cells and viral particles was generated by random DNase digestion of the target protein gp120. The library was supposed to be highly divers and easy to create. The vector *Ac*COPS was chosen as suitable vehicle as surface expression is efficient on the surface of viral particles (Fig.7) as well as on infected insect cells (Fig.8). The baculoviral surface library was evaluated by PCR-screening of single virus clones and by means of FACS analysis of infected insect cells.

5.2.1 Characterisation of the *Ac*COPS-DNase-library

48 random clones of the library were isolated by plaque assay and analysed by PCR screening (-44back / +201for). 15 different clones are shown in Fig.9. Different insert sizes demonstrate the diversity of the library. 8 clones were non-recombinant background (not shown in Fig.9). The last lane (pool) represents PCR amplification of the complete library resulting in a smear of different insert sizes within the chosen size range of 50 to 200bp.

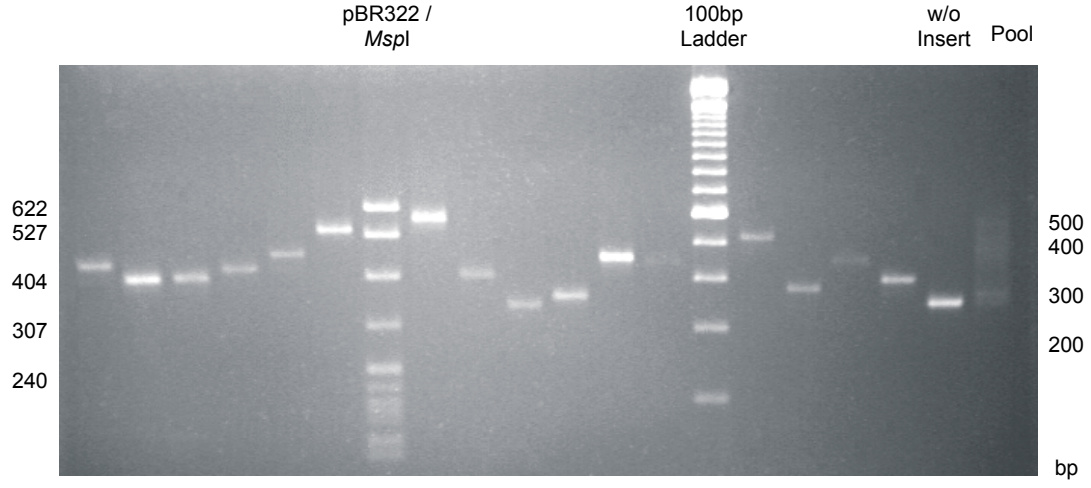

Fig.9: Agarose gel-electrophoresis of *Ac*COPS-DNase-library clones.

5.2.2 FACS analysis of insect cells infected with the *Ac*COPS-DNase-library

As the PCR-screening of single library clones confirmed proper insertion of target peptides into the baculoviral vector, FACS analysis of infected *Sf*9 cells with the polyclonal HIVIG serum was performed in order to evaluate specific binding of antibodies to the displayed peptides. The second library, generated by defined PCR amplification of gp120 fragments (5.3.2), was included in the experiment as reference. Libraries show a shift to higher FL1 signals (FITC-specific detector) indicating binding of the serum-antibodies and demonstrating the correct presentation of gp120 fragments.

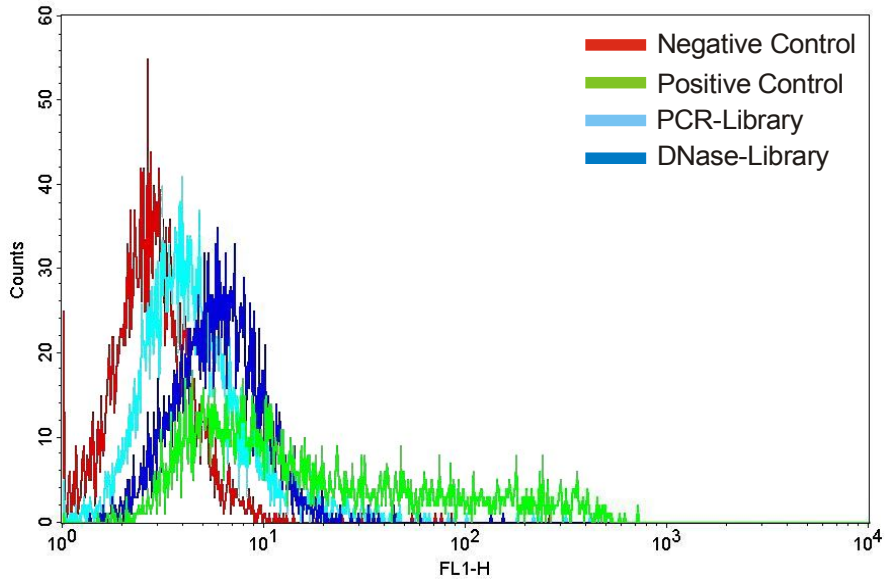

Fig.10: FACS analysis of different libraries: Comparison of *Ac*COPS-DNase-LIB and *Ac*COPS-PCR-LIB. Infected cells were harvested 48 hours after infection and stained with the polyclonal anti-HIV-gp120 serum HIVIG (500µg/ml) and detected with anti-human-FITC conjugate. Negative control: *Ac*COPS; positive control: *Ac*MARS-gp120.

5.2.3 FACS sorting of the DNase-digest library

The proper insertion of gp120 fragments and the surface presentation of peptides was demonstrated by PCR-screening and FACS analysis of the DNase-digest library. Cytometric sorting of the DNase-digest library with several monoclonal antibodies ARP360 [Moore *et al.* (1993)], ARP3048 [Laman *et al.* (1992)], ARP389 [Cordell *et al.* (1991)], 1B1 [Buchacher *et al.* (1994], ARP3025 [Matsushita *et al.* (1988)], ARP3076 [Shotton *et al.* (1995)], 1F7 [Buchacher *et al.* (1994)], 2G6 [Fouts *et al.* (1998)], and 2G12 [Buchacher *et al.* (1994)] was performed. FACS selection was performed using different sorting gates. But, as cytometric sorting did not lead to any amplification of specific clones a different strategy for the generation of a new library had to be pursued.

5.3 Characterisation of the *Ac*COPS-PCR-library

A second library with well-defined fragments of the model protein gp120 was generated by means of specific PCR amplification. PCR primers were defined to yield fragments of 17aa each, overlapping by 8 or 9aa. Fragments were ligated into the transfer vector pCOPS and integrated into the baculoviral genome by homologous recombination. All resulting baculovirus clones were confirmed by sequence analysis. A pool consisting of 60 individual clones was generated and used for further studies. Four different monoclonal antibodies against HIV-1 gp120 were chosen as candidates for FACS sorting of the *Ac*COPS-PCR-library. Three of the antibodies bind to known epitopes (ARP360, ARP389, ARP3048) whereas the binding site of the antibody 1B1 is supposed to be discontinuous and not elicited yet.

5.3.1 Titration of different monoclonal antibodies

Prior to usage all antibodies were submitted to titration experiments in order to specify their appropriate working concentrations. FACS analysis of infected insect cells (positive and negative control) was performed with varying concentrations of the mAb and the secondary conjugate in a 1/50 dilution. The FL1 signal of positive and negative control were taken into account and the antibody concentration with the best signal-to-noise ratio (S/N = geometric mean fluorescence intensity (positive control) / geometric mean fluorescence intensity (negative control)) was declared as working concentration. In an additional set of experiments, the concentration of the conjugated detection antibody was varied while the primary antibody was provided in the optimal dilution as concluded from the proceeding titration. Evaluation of the data was performed likewise.

The monoclonal mouse-antibody ARP360 (4A7C6) was chosen as appropriate candidate for FACS selection because of several circumstances: the epitope of ARP360 lies within a constant region (C1) of the gp120 rendering it relatively insusceptible for genetic variances between the different HIV-clades. Furthermore, ARP360 binds to a well known and rather short linear epitope of

10 aminoacids (PQEVVLVNVT). The antibody binds to native and denatured gp120 facilitating a possible implementation in western blots and other analyses. The library clone *Ac*COPS-PCR-LIB-Frg#10 was identified to contain the entire epitope of ARP360 and was taken as positive control for the experiments. Titration of ARP360 (Fig.11A/B) and its secondary antibody (Fig.11C/D) resulted in the signal-to-noise ratios presented in Fig.11E and Fig.11F, respectively. Titrations with the negative control (*Ac*COPS) revealed a very low background signal validating the strict specifity of ARP360. Only at very low dilutions (1/250 for primary and 1/25 for secondary antibody) the negative control generates an interfering signal. The optimal dilution for staining of infected insect cells with ARP360 was determined to be 1/25 for the primary antibody according to the run of the signal to noise-curve. The S/N-curve for the titration of the conjugate increases steadily depending on the concentration. A dilution of 1/50 was regarded as proper working concentration.

ARP3048 was chosen because of the distinct features of its challenging epitope. In contrast to ARP360, this antibody binds to a variable region of gp120, in particular the base of the V3-loop (INCTRPN). Like most V3-loop antibodies, ARP3048 is elicited very early after infection and does not neutralise HIV infection. Furthermore, it binds preferably to denatured gp120 and binding is probably obscured by local glycosylation. To sum up, this antibody was seen as a challenge not knowing whether the low binding affinity, which is certainly influenced by baculoviral (over-)glycosylation, would be sufficient enough in order to achieve the task. *Ac*COPS-PCR-LIB-Frg#35 was taken as positive control displaying the epitope for ARP3048. The titration curves (Fig.12) endorse these presumptions. The specific positive signal is extremely low with a wide spread and no real peak. The S/N-ratio of the primary antibody displays a rather flat line. Working concentration was chosen on the basis of the illustration of the histogram (Fig.12B) and set at 1/10. The secondary antibody performed likewise. Therefore, the standard dilution of 1/50 was applied for the experiments.

The rat-antibody ARP3089 (ICR38.1a/ICR38.8f) was chosen as it binds to a very promising epitope. As a CD4-binding site antibody, ARP389 blocks gp120/CD4 binding and neutralises HIV-1 strains IIIB, RF and MN at high concentrations (64mg/ml). The baculoviral clone *Ac*COPS-PCR-LIB-Frg#51

comprises the complete epitope (EVGKAMYAPP) for this antibody and was chosen as positive control. In addition to that, the tryptophane residue 427 being crucial for CD4 binding is also included in the fragment displayed by clone #51. Titration of ARP389 (Fig.13A/B/C/D) showed rather strong signal intensities. The signal to noise-curves (Fig.13E/F) of the titrations exhibit specific binding characteristics of both, primary and secondary antibody with a low background signal. The working concentration for ARP3048 was determined as 1/10 and 1/50 for the corresponding anti-rat IgG (whole molecule) FITC conjugate (Sigma).

The antibody IAM 120-1B1 [Buchacher 1994] was chosen as a promising candidate as well as a challenge for the system. 1B1 is a CD4-binding site antibody with a highly neutralising capacity showing strong synergistic effects with other HIV-antibodies [Burkly *et al.* (1995)]. The epitope of 1B1 is of conformational nature and not elicited to date. Consequently, *Ac*MARS-gp120 had to be taken as positive control. The FACS signal of 1B1 on insect cells infected with *Ac*MARS-gp120 displays a very expanded peak reaching high relative fluorescence levels of FL1>10^3 and, thus, demonstrating the strong binding affinity. The broad distribution of signals might be explained by the complexity of the epitope: as the baculoviral expression system is a system designed to express a heterologous gene at an extremely high rate, it may overwhelm the ability of the cell to modify the gene product [O'Reilly *et al.* (1992)]. When using the very late polyhedrin promoter, N-glycosylation and phosphorylation may fail to keep pace with the very high levels of expression occurring in the last phase of the infection process and may result in heterogeneous production of proteins [Jarvis and Summers (1989)] that are partially misfolded. Moreover, it is assumed that cells differ in multiplicity of infection as well as in individual features concerning their expression activity. Furthermore, it has been shown that some variable loops of gp120 (mainly V3 but also V1 and V2) do cover the cavity in which CD4 binds to the molecule [Kwong *et al.* (1998)]. Supposedly, these loops do also interfere with CD4-binding site antibodies. Titration of the secondary antibody (anti-human-FITC) displayed a negligible background, demonstration the high specifity of 1B1. With respect to the signal to noise-ratios, the standard dilutions of 1B1 and its secondary antibody where determined as 1/50 and 1/50, respectively.

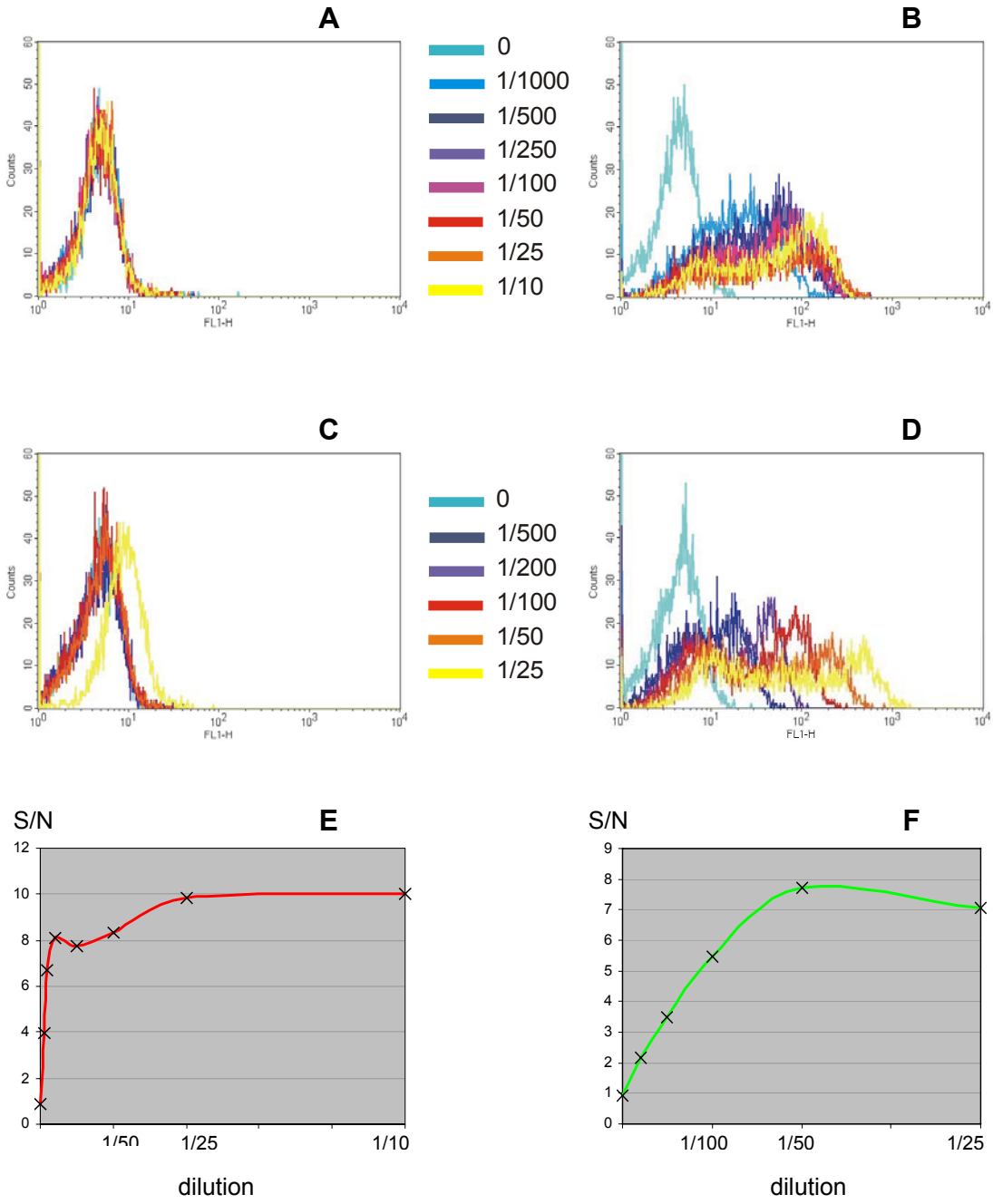

Fig.11: Titration of ARP360. A/B: FACS analysis of infected insect cells stained with different concentrations of primary antibody ARP360 (see legend). A: negative control (*Ac*COPS); B: positive control (*Ac*COPS-LIB-Frg#10); E: signal-to-noise (S/N) ratio for primary antibody.

C/D: corresponding analysis with different concentrations of secondary antibody (anti-mouse-FITC). Primary antibody was diluted 1/25 as concluded from E. C: negative control; D: positive control; F: S/N ratio for secondary antibody.

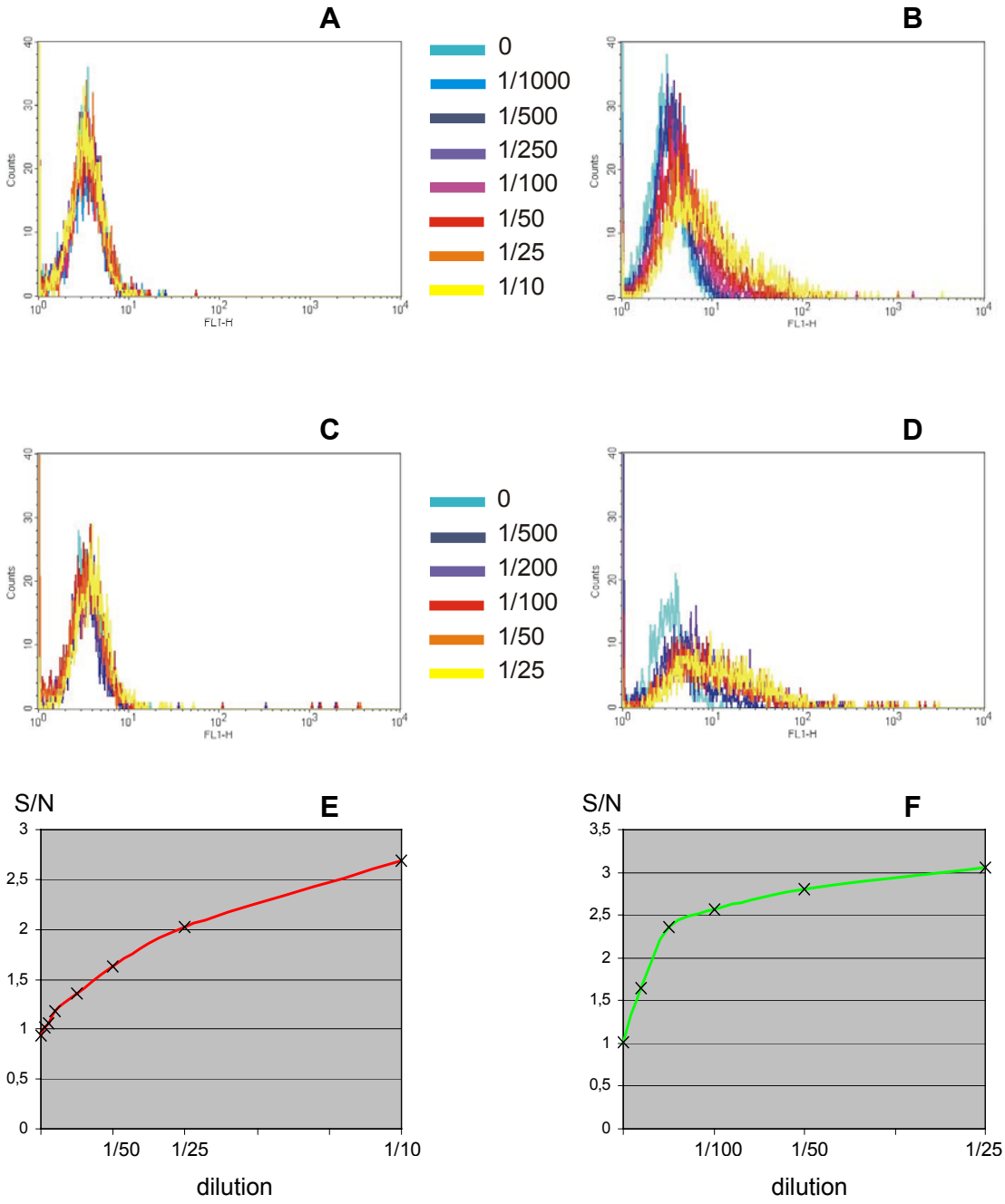

Fig.12: Titration of ARP3048. A/B: FACS analysis of infected insect cells stained with different concentrations of primary antibody ARP3048 (see legend). A: negative control (*Ac*COPS); B: positive control (*Ac*COPS-LIB-Frg#35); E: signal-to-noise ratio for primary antibody.

C/D: corresponding analysis with different concentrations of secondary antibody (anti-mouse-FITC). Primary antibody was diluted 1/10 as concluded from E. C: negative control; D: positive control; F: S/N ratio for secondary antibody.

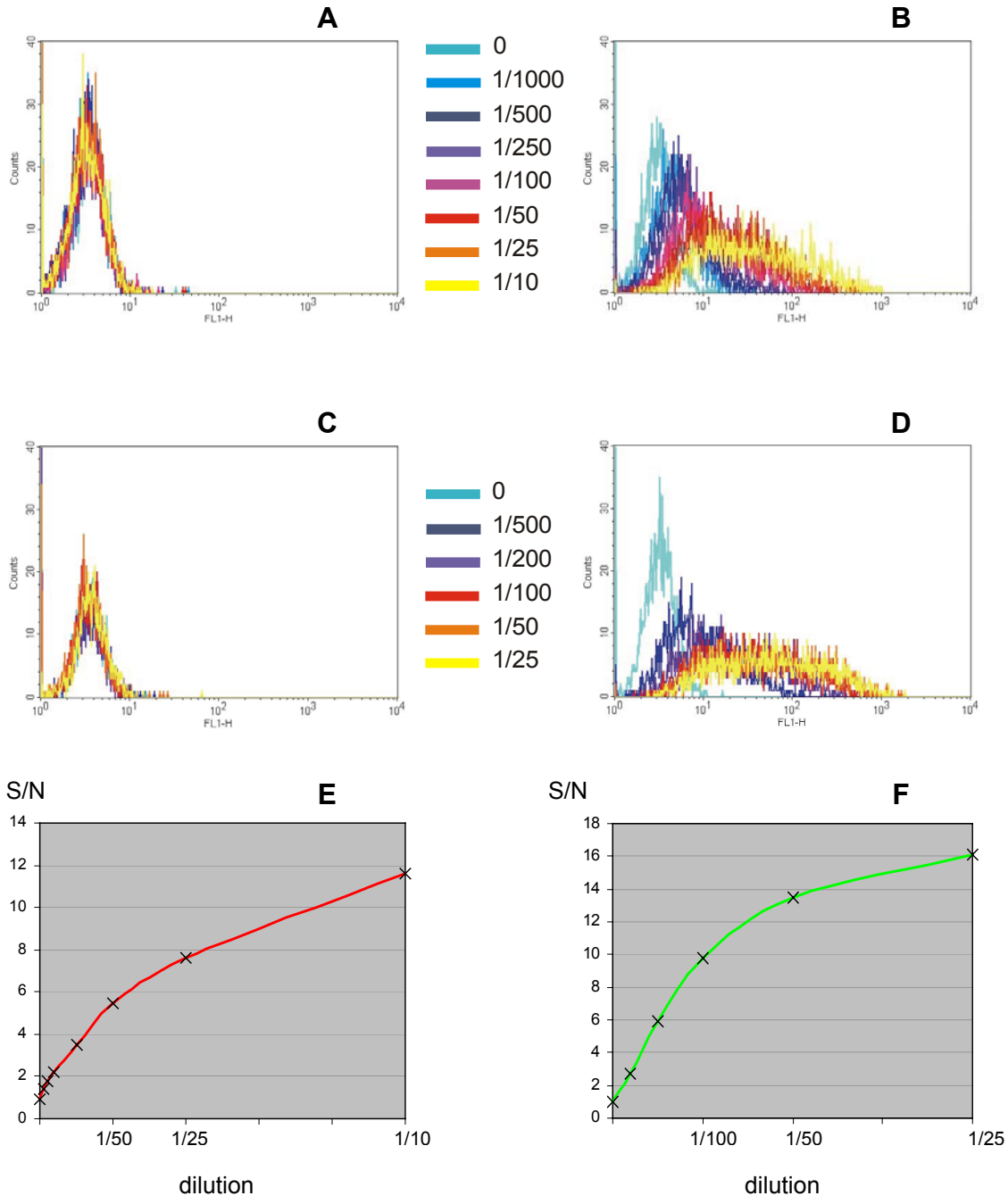

Fig.13: Titration of ARP389. A/B: FACS analysis of infected insect cells stained with different concentrations of primary antibody ARP389 (see legend). A: negative control (*Ac*COPS); B: positive control (*Ac*COPS-LIB-Frg#51); E: signal-to-noise ratio for primary antibody.

C/D: corresponding analysis with different concentrations of secondary antibody (anti-rat-FITC). Primary antibody was diluted 1/10 as concluded from E. C: negative control; D: positive control; F: S/N ratio for secondary antibody.

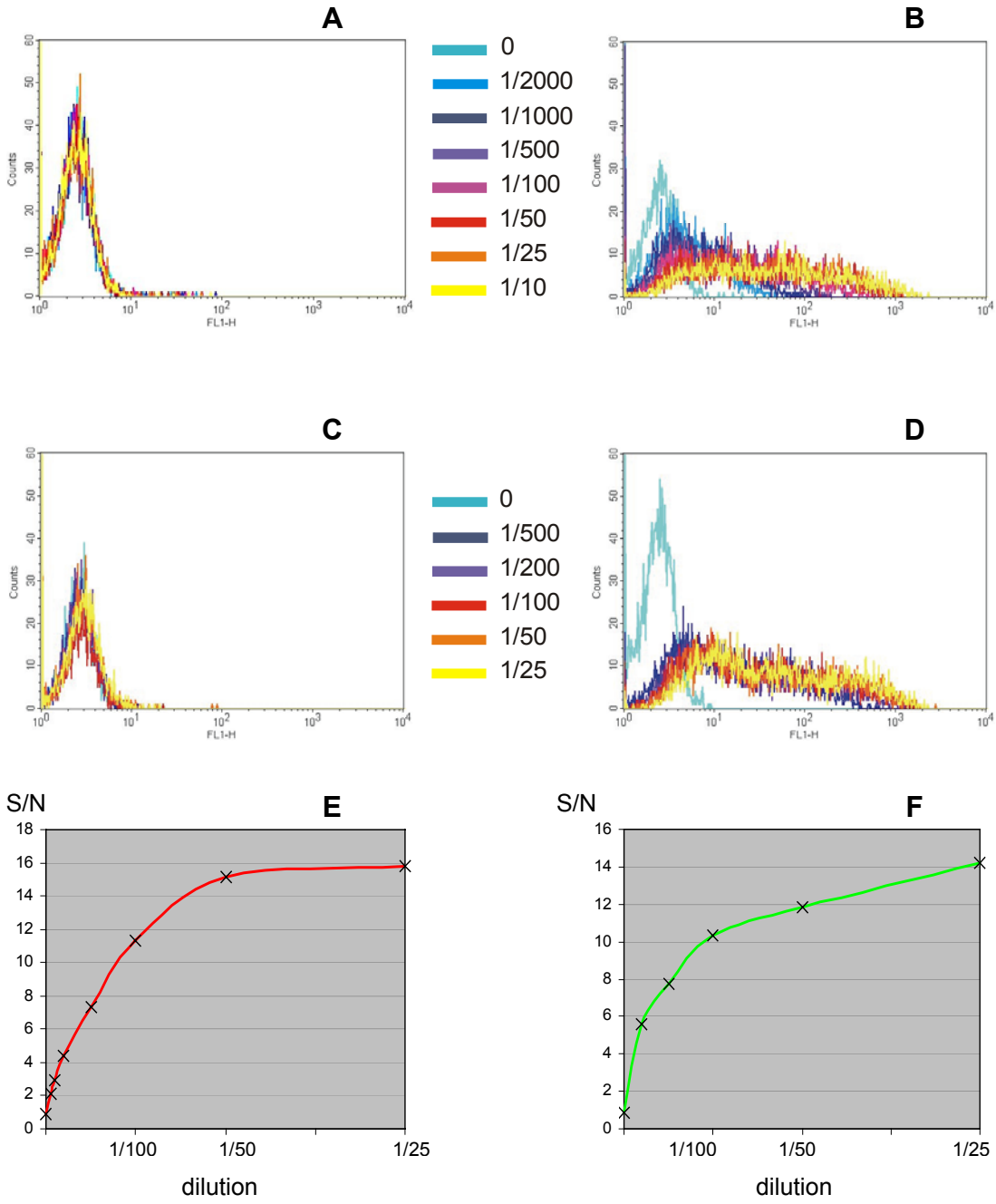

Fig.14: Titration of 1B1. A/B: FACS analysis of infected insect cells stained with different concentrations of primary antibody IAM 120-1B1 (see legend). A: negative control (*Ac*COPS); B: positive control (*Ac*MARS-gp120); E: signal-to-noise ratio for primary antibody.

C/D: corresponding analysis with different concentrations of secondary antibody (anti-human-FITC). Primary antibody was diluted 1/50 as concluded from E. C: negative control; D: positive control; F: S/N ratio for secondary antibody.

5.3.2 FACS sorting of the *Ac*COPS-PCR-library

As the proper working concentrations of the four monoclonal antibodies had been acquired by titration experiments, specific binding partners were selected from the *Ac*COPS-PCR-library by FACS sorting as described (4.4.3). *Sf*9 insect cells were infected by the baculoviral *Ac*COPS-PCR-LIB at MOI 20, harvested after 48 hours, labelled with specific antibodies and submitted to selection by fluorescence activated cell sorting. Sorting gate was set at FL1>3*10^1 in the first sorting step. Selected infected cells were incubated with fresh *Sf*9 insect cells for one day in order to amplify baculoviral particles. Subsequently, after two more amplification steps (three days each) a second round of FACS sorting was performed by infecting *Sf*9 cells with the selected and amplified baculoviral particles. The gate for the second round of sorting was set more stringent at FL1>10^2 in order to achieve selection of clones binding with high affinity to the applied monoclonal antibody. Again, the baculoviral particles from the sorted infected insect cells were amplified in order to generate high titres. FACS analysis of selected viral particles was performed using a negative control (*Ac*COPS), a positive control (*Ac*COPS-PCR-LIB-Frg#10, *Ac*COPS-PCR-LIB-Frg#35, *Ac*COPS-PCR-LIB-Frg#51, and *Ac*MARS-gp120, respectively) and the unsorted library as reference.

5.3.2.1 FACS-sorting of the *Ac*COPS-PCR-library with ARP360

The monoclonal antibody ARP360 was applied in a dilution of 1/25 (anti-mouse-FITC conjugate: 1/50) in order to select specific binding partner from the *Ac*COPS-PCR-library by means of FACS.

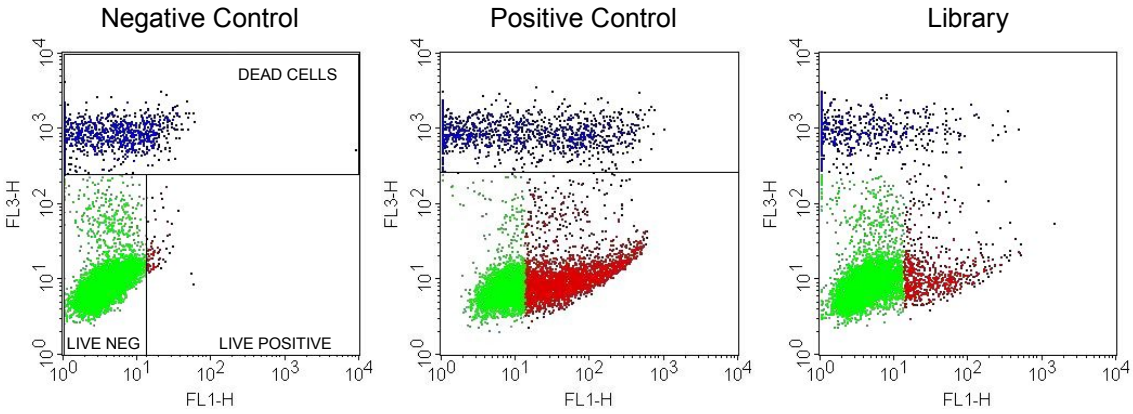

Fig.15: FACS analysis of insect cells infected with *Ac*COPS-PCR-LIB and stained with ARP360. FL1-signal (relative fluorescence at 530nm) corresponds to specifically bound antibody; FL3-signal distiguishes between dead (FL3>3*10^2) and live (FL3<3*10^2) cells. Negative control: *Ac*COPS; positive control: *Ac*COPS-PCR-LIB-Frg#10. First sorting gate was set at FL1>3*10^1.

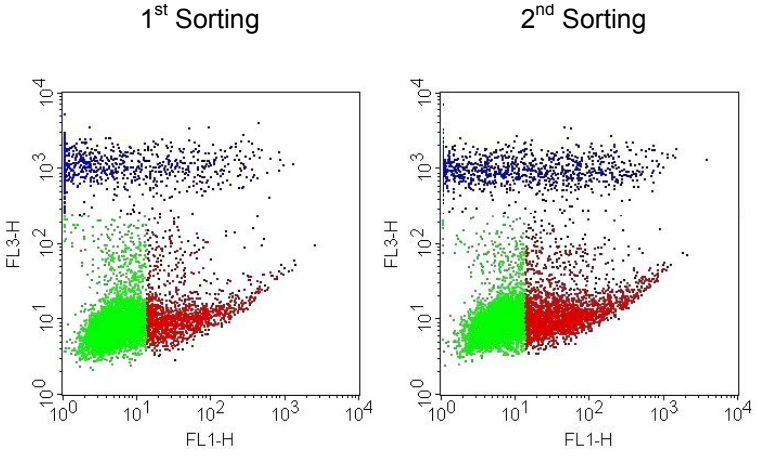

Fig.16: FACS analysis of infected insect cells. Left: Cells were infected with sorted material from 1st selection step. Right figure shows cells amplified after second round of sorting (2nd Gate FL1>10^2).

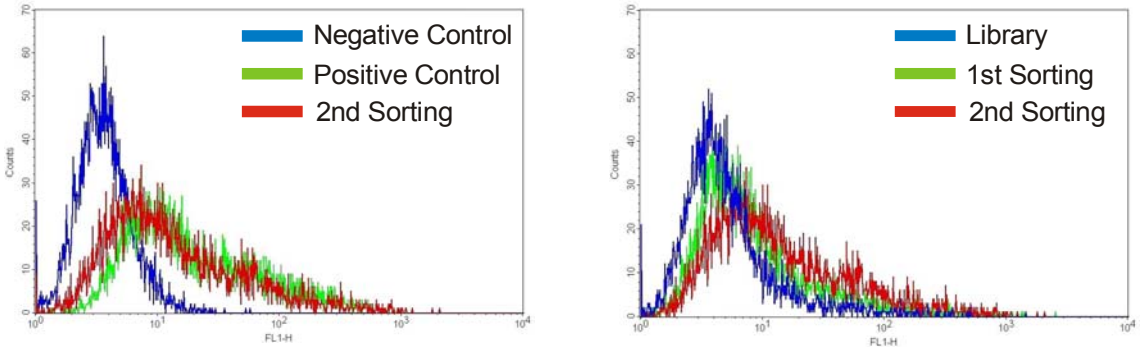

Fig.17: Histogram overlay demonstrating the FL1-signal enhancement achieved by means of FACS sorting with ARP360. Left: negative control *Ac*COPS (blue), positive control *Ac*COPS-PCR-LIB-Frg#10 (green), 2nd sorting of the library (red). The fluorescence signal of the 2nd sorting is almost identical to the curve of the positive control. Right: library (blue), 1st sorting (green), 2nd sorting (red). Sorting of the library led to a successive increase in signal magnitude.

Sample	Positive Cells [%]	Geo Mean	Positive Clones
Negative Control	0,98	3,50	-
Positive Control	41,93	17,55	-
Library	9,09	4,92	0 / 12
1st Sorting	19,75	7,87	2 / 12
2nd Sorting	34,22	12,77	3 / 12

Tab.8: Amount of positive cells and FACS signal values (geometric mean fluorescence of live cells) of different samples stained with ARP360. Last column: number of positive clones isolated by plaque assay. 2nd sorting of the library led to an approximately 3,8fold increase in positive cells.

Clone	1st Sort #1	1st Sort #2	2nd Sort #3	2nd Sort #4	2nd Sort #5
gp120-Fragment #	9	10	10	10	10

Tab.9: Identification of positive clones isolated by FACS sorting with ARP360. Single plaques were sequence analysed.

5.3.2.2 FACS-sorting of the *Ac*COPS-PCR-library with ARP3048

The monoclonal antibody ARP3048 was applied in a dilution of 1/10 (anti-mouse-FITC conjugate: 1/50) in order to select specific binding partner from the *Ac*COPS-PCR-library by means of FACS.

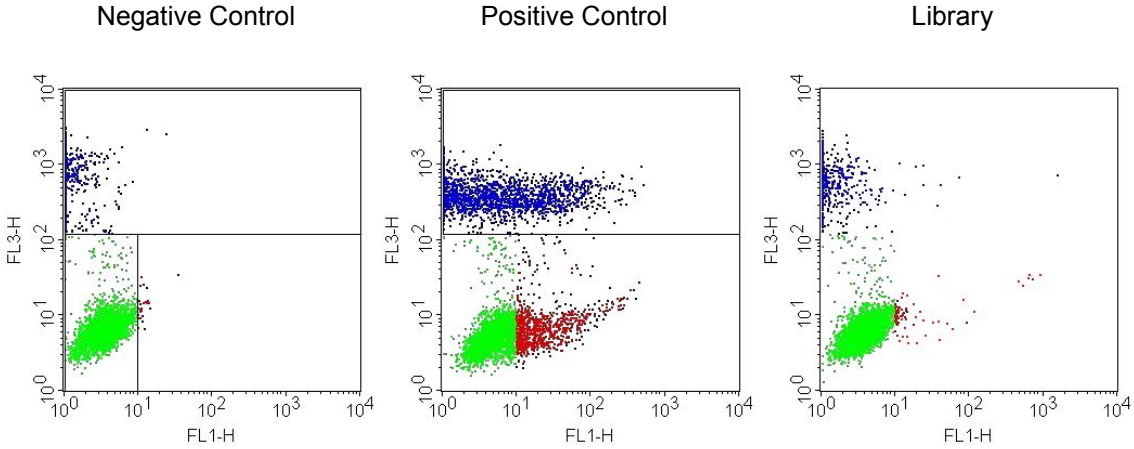

Fig.18: FACS analysis of insect cells infected with *Ac*COPS-PCR-LIB and stained with ARP3048. FL1-signal corresponds to specifically bound antibody; FL3-signal distinguishes between dead (FL3>3*10^2) and live (FL3<3*10^2) cells. *Ac*COPS served as negative control, *Ac*COPS-PCR-LIB-Frg#35 was taken as positive control. First sorting gate was set at FL1>3*10^1.

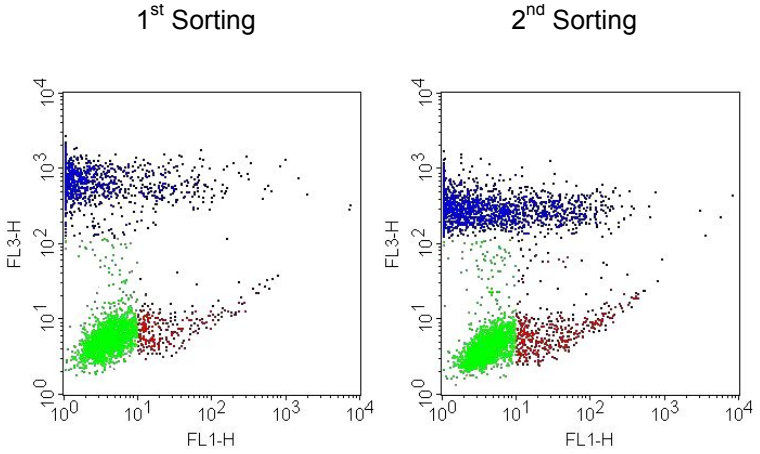

Fig.19: FACS of infected insect cells. Left: Cells were infected with sorted material from 1st selection step. Right figure shows cells amplified after second round of sorting (2nd Gate FL1>10^2).

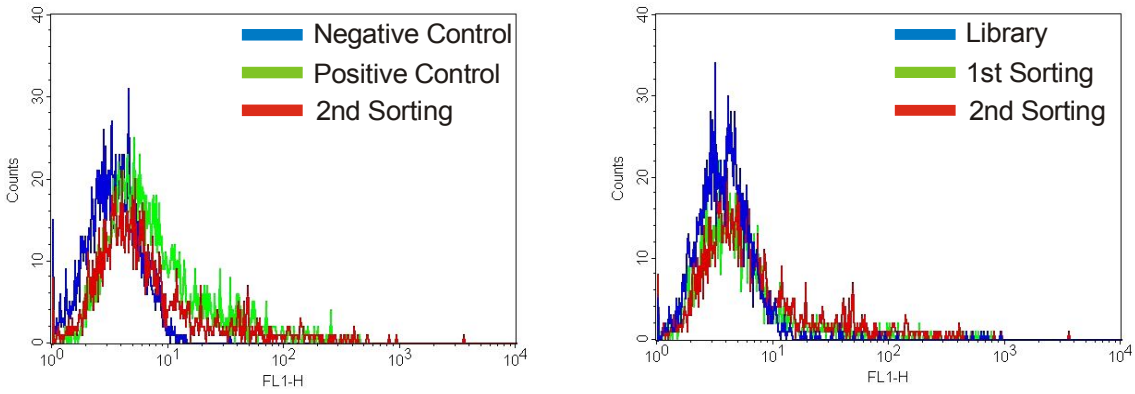

Fig.20: Histogram overlay demonstrating the FL1-signal enhancement achieved by means of FACS sorting with ARP3048. Left: negative control *Ac*COPS (blue), positive control *Ac*COPS-PCR-LIB-Frg#35 (green), 2nd sorting (red). Right: library (blue), 1st sorting (green), 2nd sorting (red). Signal amplification by FACS sorting was rather weak.

Sample	Positive Cells [%]	Geo Mean	Positive Clones
Negative Control	0,54	11,45	-
Positive Control	16,90	23,00	-
Library	1,67	19,07	0 / 12
1st Sorting	6,23	26,29	0 / 12
2nd Sorting	11,25	30,43	1 / 12

Tab.10: Amount of positive cells and FACS signal values (geometric mean fluorescence of live cells) of different samples stained with ARP3048. Last column: number of positive clones isolated by plaque assay.

Clone	1st Sort #1
gp120-Fragment #	35

Tab.11: Identification of positive clone isolated by FACS sorting with ARP3048. Single plaques were sequence analysed.

5.3.2.3 FACS-sorting of the *Ac*COPS-PCR-library with ARP389

The monoclonal antibody ARP389 was applied in a dilution of 1/10 (anti-rat-FITC conjugate: 1/50) in order to select specific binding partner from the *Ac*COPS-PCR-library by means of FACS.

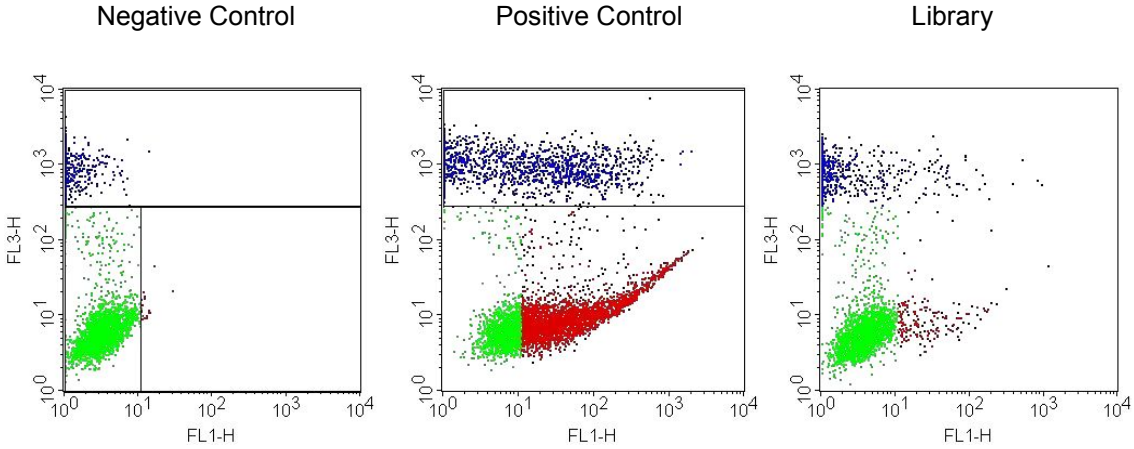

Fig.21: FACS analysis of insect cells infected with *Ac*COPS-PCR-LIB and stained with ARP389. FL1-signal corresponds to specifically bound antibody; FL3-signal distinguishes between dead (FL3>3*10^2) and live (FL3<3*10^2) cells. *Ac*COPS served as negative control, *Ac*COPS-PCR-LIB-Frg#51 was taken as positive control. First sorting gate was set at FL1>3*10^1.

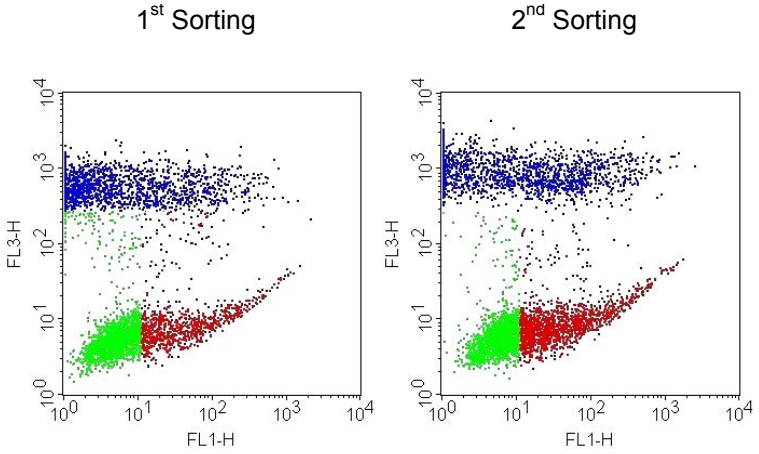

Fig.22: FACS analysis of infected insect cells. Left: Cells were infected with sorted material from 1st selection step. Right figure shows cells amplified after second round of sorting (2nd Gate FL1>10^2).

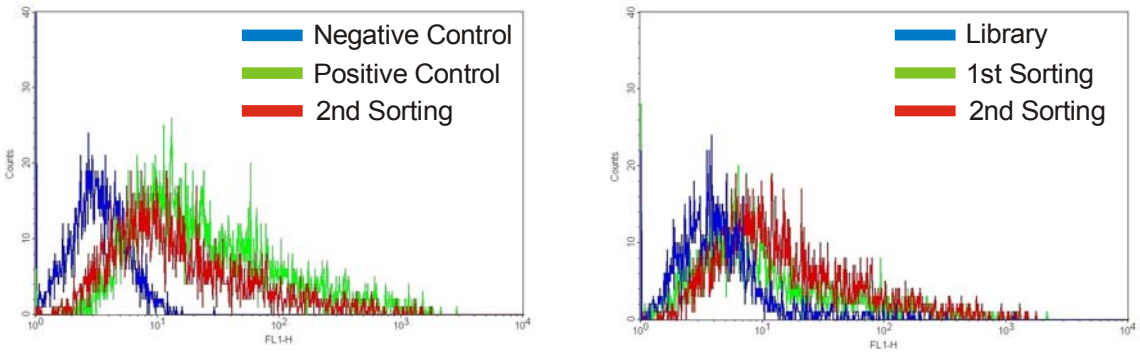

Fig.23: Histogram overlay demonstrating the FL1-signal enhancement achieved by means of FACS sorting with ARP389. Left: negative control *Ac*COPS (blue), positive control *Ac*COPS-LIB-Frg#51 (green), 2nd sorting (red). Signal curves of 2nd sorting and positive control are almost identical. Right histogram demonstrates the increasing signal magnitude achieved by FACS sorting: library (blue), 1st sorting (green), 2nd sorting (red).

Sample	Positive Cells [%]	Geo Mean	Positive Clones
Negative Control	0,39	3,06	-
Positive Control	50,63	23,71	-
Library	4,14	4,10	0 / 12
1st Sorting	21,16	8,83	3 / 12
2nd Sorting	32,54	14,92	1 / 12

Tab.12: Amount of positive cells and FACS signal values (geometric mean fluorescence of live cells) of different samples stained with ARP389. Last column: number of positive clones isolated by plaque assay. Enrichment factor of positive cells was approximately 7,9.

Clone	1st Sort #1	1st Sort #2	1st Sort #3	2nd Sort #4
gp120-Fragment #	51	51	51	51

Tab.13: Identification of positive clone isolated by FACS sorting with ARP389. Single plaques were sequence analysed.

5.3.2.4 FACS-sorting of the *Ac*COPS-PCR-library with IAM 120-1B1

The human monoclonal antibody ARP360 was applied in a dilution of 1/50 (anti-human-FITC conjugate: 1/50) in order to select specific binding partner from the *Ac*COPS-PCR-library by means of FACS.

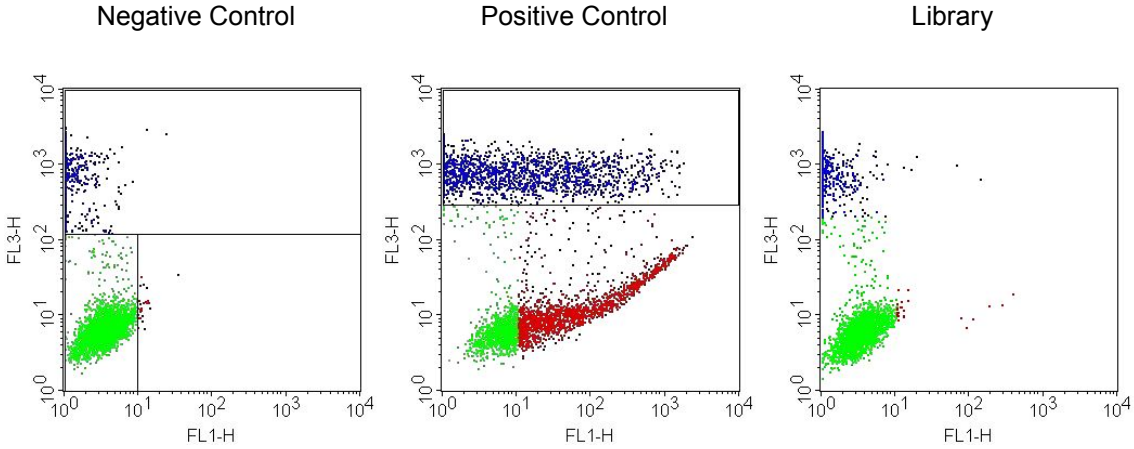

Fig.24: FACS analysis of insect cells infected with *Ac*COPS-PCR-LIB and stained with IAM 120-1B1. FL1-signal corresponds to specifically bound antibody; FL3-signal distinguishes between dead (FL3>3*10^2) and live (FL3<3*10^2) cells. *Ac*COPS served as negative control, *Ac*MARS-gp120 was taken as positive control. First sorting gate was set at FL1>3*10^1.

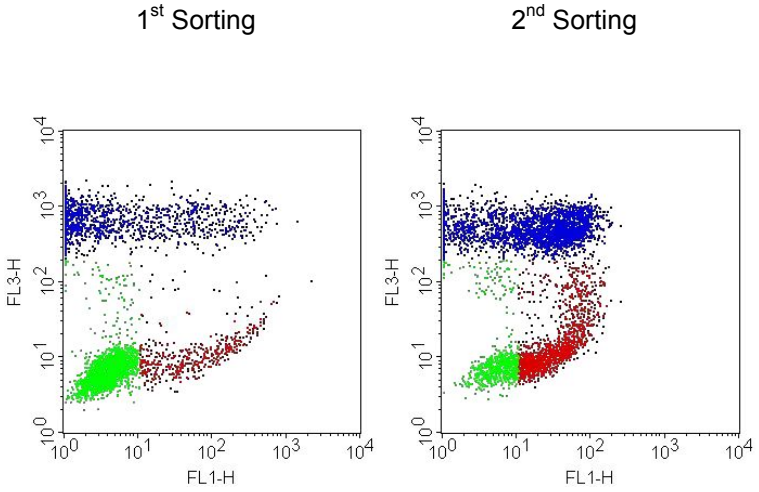

Fig.24: FACS analysis of infected insect cells. Left: Cells were infected with sorted material from 1st selection step. Right figure shows cells amplified after second round of sorting (2nd Gate FL1>10^2).

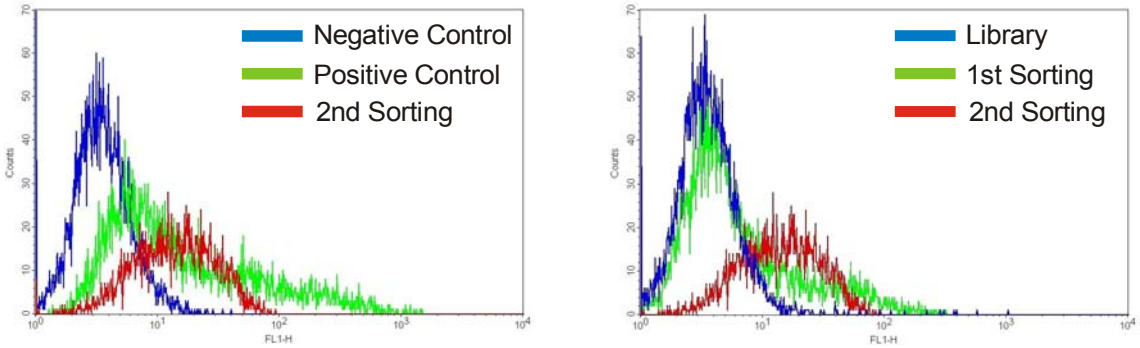

Fig.26: Histogram overlay demonstrating the FL1-signal enhancement achieved by means of FACS sorting with 1B1. Left: negative control *Ac*COPS (blue), positive control *Ac*MARS-gp120 (green), 2nd sorting (red). Second sorting step lead to a distinct signal peaking at approx. FL1=2*10^1. Right: library (blue), 1st sorting (green), 2nd sorting (red).

Sample	Positive Cells [%]	Geo Mean	Positive Clones
Negative Control	0,78	3,49	-
Positive Control	34,17	15,16	-
Library	1,09	3,51	0 / 12
1st Sorting	16,73	6,25	0 / 12
2nd Sorting	29,36	13,57	6 / 12

Tab.14: Amount of positive cells and FACS signal values (geometric mean fluorescence of live cells) of different samples stained with 1B1. Last column: number of positive clones isolated by plaque assay. Unsorted library contained almost no positive cells (1,09%) whereas 1st and 2nd sorting achieved high mean fluorescence levels (6,25 and 13,57) and large fractions of positive cells (16,73% and 29,36%).

Clone	2nd Sort #1	2nd Sort #2	2nd Sort #3	2nd Sort #4	2nd Sort #5	2nd Sort #6	2nd Sort #7	2nd Sort #8
gp120-Fragment #	17	30	40	48	52	55	32	48

Tab.15: Identififcation of positive clones isolated by FACS sorting with 1B1. Single plaques were sequence analysed. Clones #7 and #8 were identified in an additional experiment.

5.3.2.5 Enrichment of specific binding partners from the *Ac*COPS-PCR-library achieved by means of FACS sorting

Cytometric sorting of the *Ac*COPS-PCR-library with different monoclonal antibodies resulted in specific enrichment of positive cells. Enrichment factors were 3,8 for ARP360 (Tab.8), 6,7 for ARP3048 (Tab.10), 7,9 for ARP389 (Tab.12), and 26,9 for selection with IAM 120-1B1 (Tab.14).

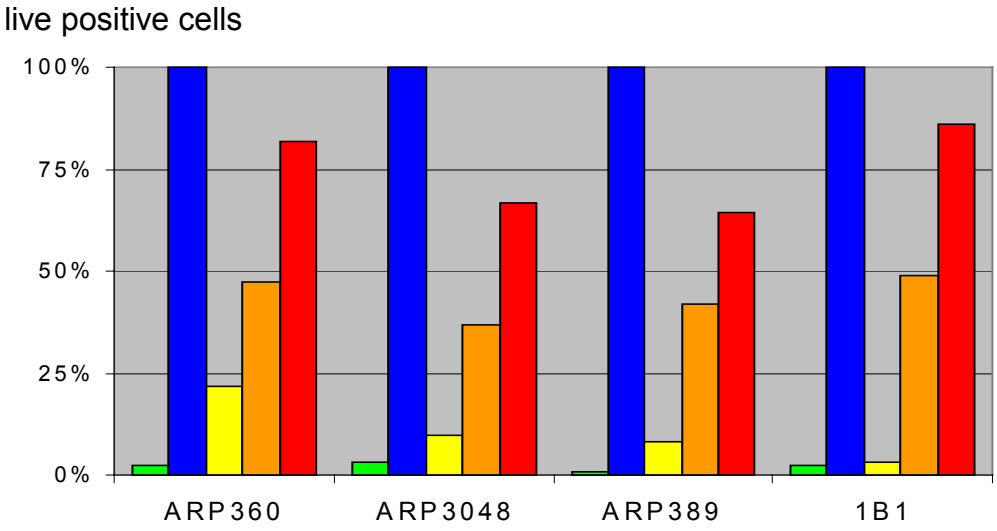

Fig.27: Overview on the enrichment of live positive cells achieved by means of FACS sorting of the *Ac*COPS-PCR-library with the monoclonal antibodies ARP360, ARP3048, ARP389, and 1B1. Green columns: negative control; blue columns: positive control (was set to 100%); yellow columns: unsorted *Ac*COPS-PCR-library; orange columns: 1st sorting of the library; red columns: 2nd sorting of the library.

Single positive clones from the sorting steps were analysed and identified by DNA sequencing. FACS selection of the *Ac*COPS-PCR-library with the monoclonal antibodies ARP360, ARP3048, and ARP389 led to the specific amplification and identification of their known epitopes (Tab.9; Tab.11; Tab.13). Moreover, sorting with ARP360 resulted in the amplification of the clone *Ac*COPS-PCR-LIB-Frg#9 which only harbours a 5 of the 10aa of the supposed epitope, demonstrating that this fragment is also able to bind ARP360 specifically. The geometric fluorescence intensity of cells infected with *Ac*COPS-PCR-LIB-Frg#9 was approximately 20fold lower (data not shown) as compared to those of the positive control *Ac*COPS-PCR-LIB-Frg#10. This indicates, that the binding affinity of ARP360 to the shorter peptide is noticeably lower. None the less, this weak binding partner could be selected successfully from the library.

FACS selection with the human monoclonal antibody IAM 120-1B1 led to the identification of several gp120 fragments binding specifically to the CD4-binding site antibody (Tab.15). Geometric mean fluorescence intensities of single clones displayed strong specific binding affinities (Fig.28; Fig.29). In this experimental set-up the selected epitopes bind far more specific to the antibody 1B1 than whole gp120 does and are supposed to present crucial interaction sites of the conformational epitope of IAM 120-1B1.

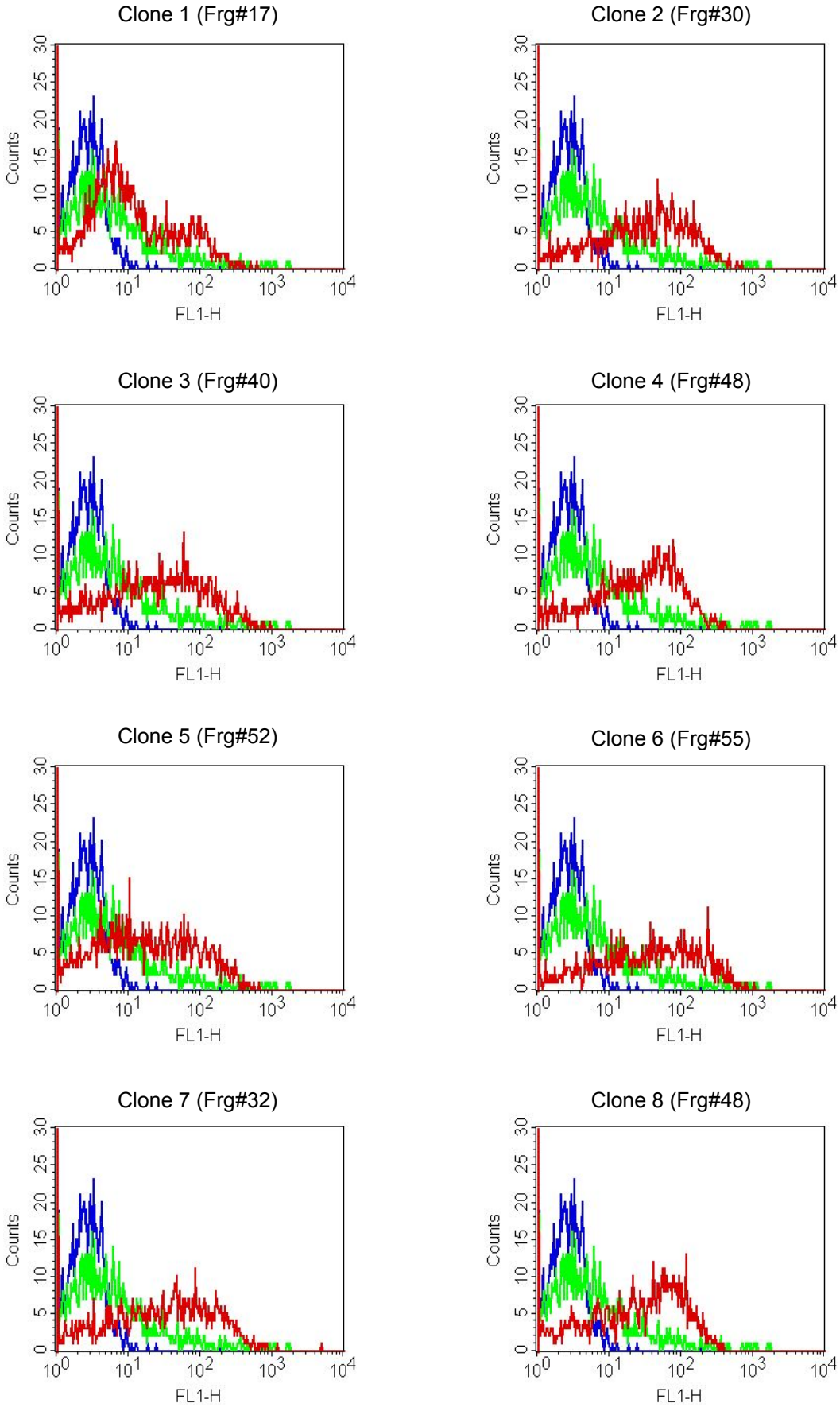

Fig.28: FACS analysis of insect cells infected with clones (red) selected from the *Ac*COPS-PCR-library by FACS sorting with IAM 120-1B1. *Ac*COPS (blue)

served as negative control and *Ac*MARS-gp120 (green) was taken as positive control. Selected clones achieve high relative fluorescence signals (FL1). In brackets: fragments of gp120 being displayed by the clones (identified by DNA sequence analysis).

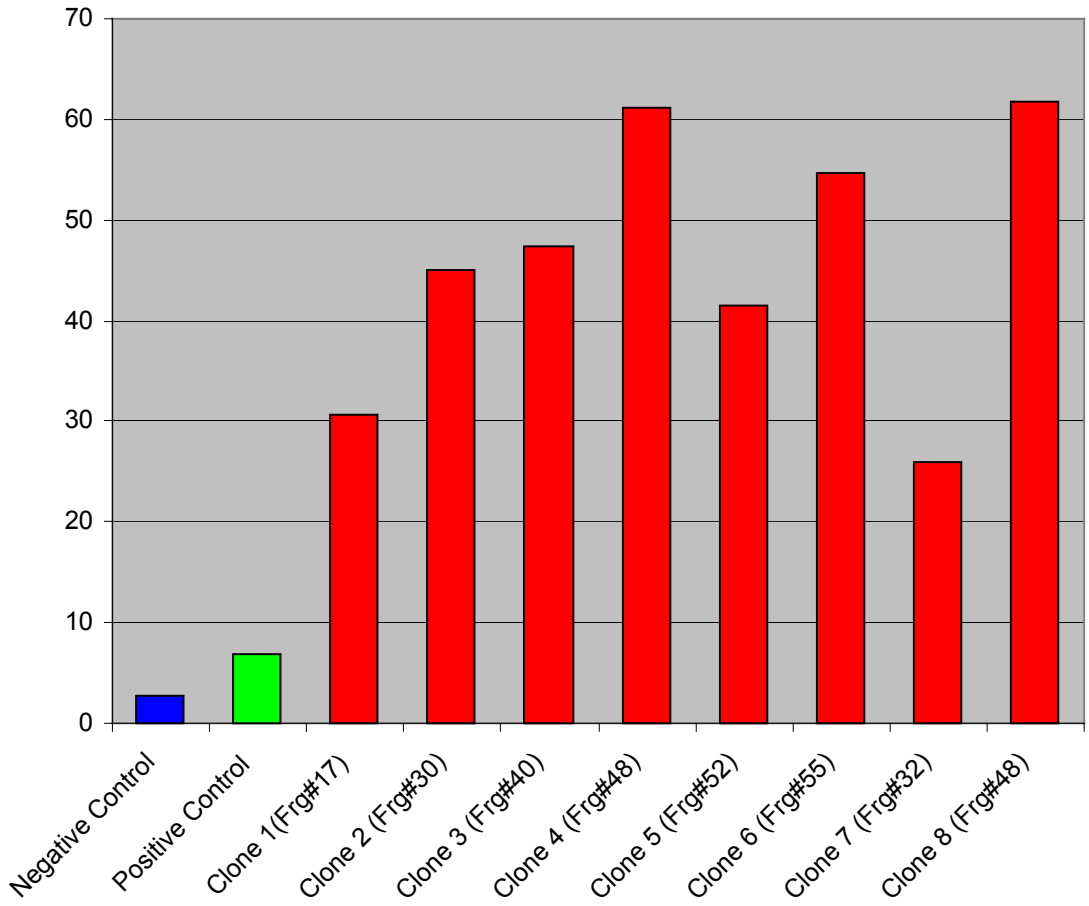

Fig.29: Geometric mean fluorescence intensities of insect cells infected with clones isolated from the *Ac*COPS-PCR-LIB by FACS sorting with 1B1. Red columns: insect cells infected with clones selected with IAM 120-1B1. Blue: negative control (*Ac*COPS), green: positive control (*Ac*MARS-gp120). Sorted clones displaying short peptides (17aa) of gp120 achieve higher geometric fluorescence intensities than the positive control (*Ac*MARS) which displays the complete gp120.

6. Discussion

The purpose of this work was to establish an authentic eukaryotic surface library of the model protein HIV-1 gp120, taking advantage of the baculovirus expression system. Epitope mapping using this library should be demonstrated by means of FACS selection of specific binding partners to several monoclonal antibodies. Different baculoviral surface expression vectors were tested for their suitability and two strategies for generation of the library were evaluated.

6.1 Comparison of the surface expression with different baculoviral vectors

In order to determine the optimal vector for surface presentation of the gp120 library, different baculoviral constructs were analysed. The 15aa epitope, specific for the monoclonal antibody 3D6 served as model. Three different constructs (*Ac*COPS-3D6, *Ac*MARS-3D6, and *Ac*COIN-3D6 (Fig.4)) were analysed for their efficiency in presenting the epitope on the surface of infected insect cells and on viral particles. An ELISA of viral particles was performed as well as a FACS analysis of infected insect cells.

In the *Ac*MARS vector, the recombinant core protein of gp64 with its oligomerisation domain is missing within the expression cassette. But, as gp64 is extremely important for viral entry into cells as well as for budding [Monsma and Blissard (1995)], progeny viruses presumably do mainly acquire the native gp64 which lacks the 3D6 epitope when passing across the outer cell membrane. In contrast to this, *Ac*COPS-3D6 with the epitope linked to the second copy of gp64 does harbour the oligomerisation domain within these heterologous fusion proteins. Consequently, progeny viruses accumulate native gp64 as well as heterologous gp64 when budding through the cell membrane resulting in a positive signal in the sandwich-ELISA. *Ac*COPS seemed to be far more potent in presenting peptides on the surface of baculoviral particles than *Ac*MARS.

In the vector *Ac*COIN-3D6 every single native gp64 is heterologous, presenting the binding site for the monoclonal antibody. Consequently, *Ac*COIN

viral particles display the highest avidity of epitopes on their surface. Thus, this construct reaches the highest signal in the ELISA. *Ac*COIN is supposed to be the best vehicle in respect to surface presentation of peptides on viral particles. However, as *Ac*COIN constructs are directly altered in their native gp64, problems and drawbacks in respect to their infectivity and propagation might occur. Fragments relevant for fusion to the native gp64 are limited in size. Small peptides do already decrease infectivity and prolong generation of high viral titres because they influence the complete structural conformation of gp64 [Ernst *et al.* (2000]]. Moreover, it has been shown that larger peptides usually abort infection as they lead to steric hindrance of viral adsorption and entry. Since the crystal structure of gp64 has not been elucidated yet, the site harbouring the recombinant peptide might be obscured by structural elements and loops of the gp64 glycoprotein. Novel *Ac*COIN vectors with different insertions sites have been constructed and evaluated [Spenger (IAM, Vienna) submitted for publication].

The comparison of surface display expression on infected insect cells by FACS led to a completely different view: the presentation on the cells is not depending on viral packaging of recombinant protein into progeny virus and, thus, is not contingent upon correct trimerisation of heterologous gp64. *Ac*MARS with its short expression cassette lacking the core gp64 consequently reaches highest levels of recombinant protein being translocated on the cell membrane. Insect cells infected with *Ac*COPS meet additional demands to their transcription- and translation-machinery when expressing the second copy of the gp64. Thus, the ratio of heterologous protein to native gp64 on the cell surface is supposed to be much smaller as compared to *Ac*MARS.

Surface presentation on infected cells with *Ac*COIN led to the lowest signal levels because expression of heterologous protein is driven by the weak native gp64 promoter.

*Ac*COPS was chosen to be the vector for surface presentation of the gp120-library. On one hand, this was due to the fact, that *Ac*COPS constructs yielded the second best presentation on infected insect cells (5.1.2) and, in respect to further studies, an acceptable surface expression on viral particles (5.1.1). On the other hand, studies have shown that simply fusing signal peptides to the recombinant protein often does not lead to adequate secretion

[Farrell *et al. (20*00)]. Subsequently, these results suggest that additional signals are required for passage of cytoplasmatic or nuclear polypeptides through the secretion pathway. Secreted and membrane-associated proteins (e.g. HIV-1 gp120) seem to harbour these structural elements *per se*. However, if a membrane-associated protein is cleaved into small pieces in order to establish a library, essential structures and signals within the sequence might be disrupted, aborting or decreasing surface presentation features. Thus, the *Ac*MARS construct containing only the leader and membrane-anchor sequence of baculoviral gp64 was regarded as not suitable for this purpose. A fusion of the library fragments to the whole membrane-associated gp64 seemed to be of basic necessity. Regarding the vector *Ac*COIN, an insertion of certain motifs in the native gp64 might decrease infectivity of these baculoviral vectors leading to unwanted shifts and loss of fragments in the library. Moreover, improved *Ac*COIN constructs are currently under investigation at the IAM achieving better surface presentation without influencing the native viral characteristics. For this reason, the *Ac*COPS constructs with the library fragments being fused to the second copy of the baculoviral gp64 were regarded as optimal for this purpose.

6.2 *Ac*COPS-DNase-Library

DNase-I was chosen for fragmentation of the candidate protein gp120 because it is supposed to cleave DNA randomly and mostly independent of sequence structure of the chosen gene. Thus, the established protocol could be used for other target proteins without changes. Actually, the additional ligation of reading-frame adapters was regarded as neccessary because an agarose gel-electrophoresis of DNase digested gp120 clearly visualised distinct bands appearing within the smear of digested DNA indicating that the enzyme does not generate stochastically independent fragments (data not shown). Thus, fragments might be represented in a wrong reading-frame decreasing the diversity of the library.

The ligation of the fragments is the limiting step in the protocol and monitoring of the cloning procedure is rather difficult. When working with a

complex pool of different fragments, one can never assure that ligation is performed properly and that all pieces are ligated efficiently.

The PCR amplification after ligation of the adapters may lead to a further disequilibrium between the fragments. Several inserts cloned into the transfer vectors have been analysed by DNA sequencing indicating that they indeed originated from gp120 (data not shown). However, some of these inserts were either in a wrong reading-frame, a false orientation, fused to some other fragment or a combination of all. Statistically, only one out of eighteen ligations of a specific fragment led to a proper surface presentation (three different reading frames for the epitope and three for the membrane anchor sequence plus two possible orientations of the insert (3*3*2=18). In addition to that, empty vectors appeared at approximately 15%.

Nevertheless, the FACS analysis of the DNase-library with polyclonal HIVIG serum confirmed proper binding of antibodies to the displayed epitopes. Signals were slightly shifted to higher fluorescence intensities as compared to the negative control (empty *Ac*COPS vector) indicating that some epitopes were presented correctly.

FACS sorting of the DNase-Library was performed with several monoclonal gp120-antibodies: apart from ARP360, ARP3048, ARP389, and 1B1, ARP3025, ARP3076, 1F7, 2G6, and 2G12 were applied for selection. Sorting gates were varied but no signal amplification could be achieved by means of cytometric sorting. Obviously, the correct presentation of specific epitopes was far too rare and interfering background signals were too plenty resulting in a poor signal-to-noise ratio. The difficult monitoring of the cloning procedures and the low probability of correct ligation (1 out of 18 fragments is presented correctly) of the library turned out to be disadvantageous.

6.3 Reduction of background signals generated by direct cloning

The direct cloning procedure used for establishment of the described DNase-library implies a non-recombinant viral background of about approximately 15% (8 out of 48 tested clones) resulting from uncleaved or religated wildtype DNA. In order to decrease background in the DNase-library,

an additional *Sce*-I digestion was integrated in the protocol right before transfection. Emersion of empty vectors decreased but transfection efficiency diminished due to the additional enzyme treatment and purification steps (data not shown).

Furthermore, a cell line constitutively expressing *Sce*-I was generated [Grabherr and Ernst (2001)]: the coding sequence of *Sce*-I under control of the immediate-early promoter IE-1 was cloned into a transfer vectors (pIE1-1 (Calbiochem, San Diego, USA)) and transfected in *Sf*9 cells. Different clones of the new cell line were tested for *Sce*-I activity and transfection efficiency over a period of six months. One clone of the cell line appeared to have the best features and was chosen for further analysis. Transfection efficiency reached as much as 73,0% compared to normal *Sf9* cells and the cell line was able to degrade viral DNA efficiently. Unfortunately, repeated transfection of the *Sce*-I cell line with ligated baculoviral DNA did not result in proper generation of progeny virus. Obviously, the amount of replication-competent DNA after ligation is not sufficient enough for proper transfection of this cell line. Scaling-up the ligation did lead to some infection but as side-effects and other disadvantages in reproducibility of the protocol appeared, this strategy was forsaken.

6.4 *Ac*COPS-PCR-Library

As FACS sorting of the DNase-Library did not lead to any reproducible result, a different strategy had to be applied. The drawbacks of having a far too high diversity and a perturbing share of misrepresented fragments was overcome by creating a well evaluated library by PCR amplification with the main focus directed towards quality control and evaluation of signal-to-noise ratios. Because all inserts were verified by DNA sequencing and all clones were generated separately, the library contains almost no background signals. Empty vectors were detected to a negligible extend when performing PCR screenings of single plaques (<1% (data not shown)) and mutations within the epitopes were kept low by limiting propagation steps of the library stocks. Furthermore, all overlapping fragments of the target protein gp120 are assuredly represented.

Insert sizes were set to 17 aminoacids in order to be in the range of common linear epitopes. Thus, all clones have similar replication rates and are believed to be represented at comparable virus titres within the library. Nevertheless, disparities in the amount of positive signals when staining the library with different antibodies indicate a certain inhomogeneity. This effect derives from varying growth rates and is superposed by different binding characteristics and signal magnitudes of the antibodies.

Cytometric sorting of the PCR-library turned out to be very effective and easy to perform. The epitopes of all chosen antibodies could be enriched. The fraction of positively stained cells could be raised up to 64,3 to 85,9% (compared to positive controls) after a second round of sorting (Fig.27). Isolated clones turned out to be rather inhomogenic with few positive clones (Tab.8/10/12/14) due to the high multiplicity of infection and inevitable cross-infections.

Sorting with ARP360 resulted in a 3,8fold increase in positive cells. Isolation of viruses by plaque assay and FACS screening of 12 clones each revealed two positive samples after first sorting and three after second sorting (Tab.8). None of the unsorted library clones turned out to be positive. Interestingly, one isolated positive clone from the first sorting turned out to be fragment #9, which shares only 5 (PQEVV) of the 10 amino acids (PQEVVLVNVT) representing the actual epitope of ARP360 [Moore *et al.* (1993)]. The FACS signal was markedly weaker (about 20fold lower, data not shown) when compared to library clone #10. Nevertheless, this has to be regarded as an additional refinement of the strategy and gives new prospects in regard to mapping the genuine epitope.

ARP3048 displayed rather low binding affinities to baculo-derived gp120: only 16,9% cells of the positive control were located in the desired gate (live positive cells) (Tab.10). This might be due to overglycosylation of two asparagine-residues located within the epitope (Asn-295, Asn-301). None the less, the amount of positive cells was raised to 11,3% in total which is equivalent to 66,6% when compared with the positive control. Only one out of 12 screened clones did indeed harbour the epitope, again affirming problem resulting from cross-infections.

FACS selection with the monoclonal antibody ARP389 led to an 7,9fold increase in positive signals. Already after the first round of sorting, 25% of the screened plaques (3/12) contained the correct fragment (Tab.12). By mischance, only one positive clone was identified after the second sorting. This clarifies that a screening of only 12 clones might by too less when working with such a high multiplicity of infection.

The antibody IAM 120-1B1 specific for a conformational epitope of unknown structure represented the biggest challenge for this system. Signals could be amplified drastically: 1,1% positive cells within the library could be raised up to 29,4% which is equal to an enrichment factor of more than 26. Screening of single clones revealed 50% positives (6/12) after second sorting. Surprisingly, inserts of different sequence were identified by DNA sequencing (fragments #17, 30, 32, 40, 48, 52, 55).

Kwong *et al.* [Kwong *et al.* (1998)] have shown that due to its conformation, different regions of gp120 lie in very close proximity forming the CD4-binding site. Several of the selected sequences (#48, 52, 55) are located near or even in the supposed main CD4-binding site at the carboxy terminus of gp120 (C4 region) [Lasky *et al.* (1987); Liu *et al.* (1998)]: fragments #48 and #52 directly join the crucial α4-helix whereas #55 comprises the important residues 439 and 442-444 and flanks 425-429 which are directly involved in CD4-gp120 interaction. Fragment #17 is situated in the region between the beta-strands β2 and β3 which is also supposed to contribute to this mechanism. Furthermore, the selected fragment #32 overlaps with the very important loop D (crucial sequence DNA(K)T) which represents an additional part of the CD4-contact site. Thus, it is most likely that the CD4-binding site antibody 1B1 interacts specifically with gp120 sequences being contiguous to these CD4 epitopes.

Specific binding to some of the sorted fragments resulted in a very high FACS signal (Fig.28/29) with almost all cells being positive. The cavity in gp120 for CD4 binding is partially occluded by hypervariable loops (mainly V1/V2) [Kwong *et al.* (1998)]. Thus, the absence of theses structures on the short library fragments might explain the improved binding characteristics. Still, competitive immuno-assays have to be performed in order to validate these

observations. As a matter of fact, these findings are currently under investigation in our laboratory.

6.5 Refinement of cytometric sorting

The major problem when sorting the library was the disproportion between positive cells and following analysis of the isolated viral clones. Since virus infection of cells is a random event that follows a Poisson distribution, synchronous infection can be achieved by infecting cultures with a high amount of virus, such that all cells within the culture become infected rapidly. To do this, one typically infects cells at a multiplicity of infection of 10 ore more. Granziero *et al.* [Granziero *et al.* (1997)] have established their baculoviral libraries with a MOI of 20 (titre was determined by plaque assay) in order to achieve a proper surface expression of foreign protein. Of course, this strategy goes along with a very high rate of cross-infections that are trailed through the whole experiment and generate background signals and lead to the isolation of false clones.

In order to overcome this drawback, cells infected with lower viral charge (MOI=2 and MOI=0,2) have been analysed by FACS (data not shown). Furthermore, samples have been analysed 24 hours post infection for the purpose of decreasing secondary infections that emerge already after 12 hours. Unfortunately, in both trials surface expression turned out to be rather low and FACS signals were to low to achieve proper sorting.

Moreover, as the geometric mean fluorescence of stained cells is supposed to correspond to the ratio of positive to negative surface linked epitopes (and thus, to the ratio of positive to negative virus particles per cell) the second sorting gate was restricted to high signal levels (relative fluorescence $FL1>3*10^2$). Granziero *et al.* [Granziero *et al.* (1997)] have already described that the first round of sorting should be rather less restrictive whereas the second gate should be set more stringent. Unfortunately, sorting experiments with ARP3048 and 1B1 did only led to an irrelevant increase in positive cells and viral clones (data not shown). The selection of fewer candidates (small gate) increases the amount of amplification steps neccessary for generating a proper virus stock and thus, amplifies unwanted background clones as well.

6.6 FACS analysis and sorting of viral particles

In order to evade problems with cross-infectivity, preliminary FACS experiments with virus particles were performed (data not shown). Moreover, baculoviral particles have the advantage of resulting in higher signal intensities because of the better gp64-to-background ratio as compared to infected cells.

Baculoviral particles were harvested and stained with monoclonal antibodies similar to the preparation of infected *Sf9* cells. FACS analysis of these samples, performed on the more powerful FACSVantage System (BD Biosciences), have shown that viral preparation often differ in purity. A simple ultracentrifugation leads to aggregation of particles and co-precipitation of heterologous gp64 being associated to cellular or viral membrane fractions. These contaminations do not only interfere with protein assays and staining procedures but do also create artificial signals when been FACS-analysed that cannot be distinguished properly from the very small virus particles. For this reason, different methods of sample purification were tested in order to set-up a standard procedure. Several ultra- and microfilter devices were applied for filtration and purification of virus stocks as well as a method for purifying baculoviral particles by ion-exchange chromatography [Barsoum (1999)], but none of the experiment led to satisfying results. Finally, viral particles were purified and concentrated using a tangential-flow system. The fact, that this method works with a tangential flow probably causing less aggregation and shearing of virus particles made it a promising alternative. In addition to that, separation of particles occurs only because of differences in diameter without being influenced by electrostatic interactions. Preliminary results indicate a reproducible and satisfactory concentration of viral particles (up to 100fold depending on the starting volume) without significant loss in total amount of infections particles due to aggregation or shearing. Total protein content could be decreased by approximately 30% and first FACS experiments point to an advanced quality of the samples compared with conventional ultracentrifugation.

The principle feasibility of cytometric sorting of baculoviral particles has already been proven: viruses stained with ARP389 could be enriched

specifically to approximately 20% positives (4 out of 24 tested clones (library: 0/18) (data not shown).

7. Conclusions and future prospects

An HIV-1 gp120 library has been established displaying fragments of the target protein authentically on the surface of infected insect cells and on viral particles. A first library was elaborated by DNase digestion in order to establish a highly divers system which is easy to generate. Analysis of this library by ELISA, by PCR screening and by staining with a polyclonal anti-HIV serum confirmed proper presentation of fragments on infected insect cells and on viral particles. Sorting of the DNase library did not result in amplification of desired clones, probably because this library was far too divers and background signals were too high. These bottlenecks have been overcome by generation of the second library which was done by specific PCR amplification and by separately cloning each single fragment. This second library turned out to be reliable and well defined. Epitope mapping of four different monoclonal antibodies has been performed successfully: specific signal intensities have been enriched by means of FACS sorting and binding partners for all tested antibodies have been selected. In one case, ARP360 (Tab.9), even a smaller part of the known binding site was amplified, thus, representing an additional refinement in epitope mapping. Moreover, several candidates obviously binding more specifically to the promising human antibody 1B1 selective for a conformational epitope [Kwong *et al.* (1998)] have been isolated. Further investigations on this result are currently under investigation.

Future trials are projected to investigate and to establish reproducible FACS selection of viral particles. Thus, the experimental protocol could be revised and efforts could be minimised. Preliminary experiments have already proven the general feasibility of this strategy. Moreover, purified baculoviral particles of this library could be used directly to immunise e.g. mice in order to induce specific humoral immune response of promising antibodies [Lindley *et al.* (2000)].

The presented data and the designed gp120 surface library is meant to serve as a tool for further advancement in vaccine development and drug design. Possible candidates for further studies could be soluble or associated CD4 and anti-HIV agents as well as receptors involved in HIV infection. The

established HIV-1 gp120 surface library will be made available for other scientists in order to contribute to further progress in HIV-research.

8. Abbreviations

1B1	monoclonal antibody IAM 120-1B1
24WP	24 well plate (Nunclon)
60mm plate	cell culture dish (Nunclon, ∅=60mm)
6WP	6 well plate (Nunclon)
96WP	96 well plate (Nunclon)
aa	amino acids
*Ac*NPV	*Autographa californica* nuclear polyhedrosis virus
ATB	antibiotics
bp	base pairs
E. coli	*Escherichia coli*
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Frg	fragment
IS	intermediate stock
LIB	library
MOI	multiplicity of infection
o/n	overnight
ORF	open reading-frame
PFU	plaque-forming units
p.i.	post infection
PI	propidium iodide
PP	plaque pick
Roux25/80/175	T-flask (Nunclon) with 25/80/175cm^2 area
RT	room temperature
Sce-I	I-*Sce* I restriction endonuclease
*Sf*9	*Spodoptera frugiperda*
S/N	signal-to-noise (ratio)
sort	sorting (step)
SS	seed stock
v/v	volume per volume
w/	with
w/o	without

w/v	weight per volume
WS	working stock

9. References

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