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## 2 Methods

Relevant chemicals, buffers, and solutions are listed in chapter 5 ("Materials").

### 2.1 Cell culture

#### 2.1.1 Standard cell culture

All cell lines were cultivated according to the guidelines given by the DSMZ (German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany). Standard conditions for incubation were 37°C, 5% CO<sub>2</sub> in a humidified incubator.

#### 2.1.2 T-cell isolation from human buffy coats

"Buffy coats" are concentrated suspensions of lymphocytes obtained during procession of total blood. Peripheral blood mononuclear cells (PBMCs) can be isolated from buffy coats by Ficoll density-gradient centrifugation. Lymphocytes and monocytes will according to their specific density concentrate at the interphase between the upper phase (plasma, thrombocytes) and the lower phase (Ficoll). Erythrocytes and granulocytes of higher density will form a cell pellet.

Briefly, the buffy coat cell suspension was diluted 1:2 with PBS. 30ml cell suspension was layered on top of 15ml Ficoll solution (specific density 1.077g/ml, Biochrom) and was separated by centrifugation at 870x g, for 30min at RT, w/o break. The interphase, containing the PBMC, was carefully transferred into a fresh tube and washed twice in 50ml PBS. The cells were then resuspended in RPMI 1640 complete medium and depletion of monocytes and macrophages was carried out o/n in 150cm<sup>2</sup> cell culture flasks at standard cell culture conditions (monocytes and macrophages will adhere to the flask). Subsequently, depletion of CD19<sup>+</sup> B-cells was performed with anti-CD19 coated paramagnetic beads (Dynal). Briefly, ~4x10<sup>7</sup> non-adherent cells were washed once in 50ml PBS, then resuspended in 4ml cold PBS + 2% FCS (i.e., 1-2x10<sup>7</sup> cells/ml). Anti-CD19 Dynabeads® were washed and prepared as described by the manufacturer, then 2x10<sup>7</sup> beads were added to the cells. Assuming a CD19<sup>+</sup> B-cell population of 10% of the total PBMC, this would equal five beads/B-cell. The cells were incubated for 30min on ice, carefully inverting the tube every 5min. Finally, PBS + 2% FCS was added to 10ml final volume and the anti-CD19 beads-covered B-cells were captured

at the tube wall using a MPC® magnet (Magnetic Particle Concentrator, Dynal). The supernatant containing the T-cells was transferred into a fresh tube and washed once with PBS. Cells were counted, adjusted to  $1 \times 10^6$  cells/ml in RPMI 1640 complete medium, and cultured at standard cell culture conditions for up to 4 days. Purity of the T-cell fraction (usually > 90%) was determined by FACS analysis with anti-CD3 antibody.

## **2.2 Preparative protein techniques**

### **2.2.1 Production and purification of anti-Wue-1 antibody**

#### **2.2.1.1 Production and isotyping**

Anti-Wue-1 ascites solution was produced by Eurogentec, Belgium. The antibody-containing ascites fluid was generated in mice, which were immunized with anti-Wue-1 secreting Balb-c/NSO-D mouse hybridoma cells. The antibody isotype was determined using the "IsoStrip Antibody Isotyping Kit" (Roche) as described in the manual. Anti-Wue-1 was determined to be IgG<sub>1</sub> isotype.

#### **2.2.1.2 Purification of anti-Wue-1 from ascites fluid**

##### **2.2.1.2.1 Removal of oil and fat**

Synthetic oil and tissue fat were removed from the ascites fluid by filtration through silanized glasswool. In a beaker the glasswool was shortly covered with silane solution (Sigmacote®, Sigma), and then submerged in water several times to wash. The silanized glasswool was spread out on paper towels and left to dry o/n under a fume hood. A 5ml syringe was plugged (~1cm) with the coated glasswool, and the ascites fluid was filtered through the plug.

##### **2.2.1.2.2 Affinity chromatography**

The antibody was purified from the ascites fluid via affinity chromatography using a protein A sepharose column. The agarose slurry was prepared by adding approximately ¼ volume protein A agarose (Pharmacia) to 1ml H<sub>2</sub>O bi-dest and was left to swell at RT for 30min. The slurry was transferred into a disposable mini-column (Pierce), to form a gel bed of ~0.5ml volume. The column was washed twice with 10ml H<sub>2</sub>O bi-dest and was then equilibrated with

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10ml Binding buffer pH8 (Pierce). The Wue-1 ascites fluid was diluted to equal parts with Binding buffer to 10ml final volume and applied to the column. This was followed by five washes with 5ml Binding buffer each. The bound antibody was eluted from the column with 2ml Mild Elution buffer pH6.0 (Pierce) first, followed by a more stringent elution using 8ml IgG<sub>1</sub> Elution buffer pH2.8 (Pierce). Fractions of ~500µl were collected and each fraction was neutralized by addition of 25µl 1M Tris-HCl pH7.5. The fractions were analyzed on a NuPAGE 4-12% Bis-Tris gradient gel (Novex, Invitrogen) and the antibody concentration was determined by ELISA as described in section 2.2.1.3.

### **2.2.1.2.3 Dialysis**

Where needed the antibody solution was further purified by dialysis in order to remove sodium azide, glycerol, or primary amines. Dialysis was carried out against dialysis buffer (usually PBS), using 10kDa molecular weight cut-off (10K MWCO) "Slide-A-Lyzer" cassettes (Pierce). Dialysis was carried out for 2 hours at RT or 4°C, then the dialysis buffer was changed and dialysis was performed for another 2 hours. Finally, the dialysis buffer was changed once more and the sample was dialyzed o/n at 4°C. Dialysis was carried out in 200-500 times the volume of the sample.

### **2.2.1.3 Determination of antibody concentration by ELISA**

Concentration of the purified antibody was determined by capture ELISA. MaxiSorp ELISA plates (Nunc) were coated with anti-mouse IgG capture antibody (Sigma) at a concentration of 5µg/ml in PBS-T (section 5.7.1) for 60min at RT. The wells were washed three times with 100µl PBS-T, then blocked for 60min at RT with 200µl PBS-B<sup>+</sup> (section 5.7.6). The plate was dried upside down on a paper towel, wrapped in cling film, and stored upside down at 4°C for up to 4 weeks.

Using IgG<sub>1</sub> isotype control (Dako), a standard dilution series was prepared ranging from 50ng/ml to 0.79ng/ml (i.e. 6 steps of 1:2 dilutions). Samples to be tested were diluted in steps of 1:10 ranging from 10<sup>-1</sup> to 10<sup>-8</sup>. All samples were set up in duplicates, and diluted to a final volume of 50µl in PBS-B (section 5.7.6). The samples were incubated for 60min at RT, followed by three washes with 100µl PBS-T each. The anti-mouse IgG F(ab')<sub>2</sub>-Biotin detection antibody (Dako) was diluted 1:5000 in PBS-B, allowing 50µl/well. The plate was incubated and washed as before. 50µl of streptavidin-HRP (Amersham) diluted 1:5000 in

PBS-B was added to each well. The plate was incubated at RT for 45min then it was washed three times with 100 $\mu$ l PBS-T per well. Substrate solution was prepared from two "Sigma Fast" Tablets (Sigma) in 20ml H<sub>2</sub>O bi-dist according to the manufacturer's instructions. 50 $\mu$ l of the substrate solution was added to each well and the plate was incubated at RT as required. The reaction was stopped by addition of 25 $\mu$ l/well stop solution (2.5M H<sub>2</sub>SO<sub>4</sub>; section 5.7.6). The plate was measured in a "Benchmark" plate reader (Biorad) reader at  $\lambda_{415\text{nm}}$  and analyzed with the associated software.

### **2.2.1.4 Biotinylation of purified anti-Wue-1 antibody**

The procedure was carried out using NHS-Biotin (Pierce), mainly according to the supplier's manual. The antibody solution was dialysed against PBS using a 10K MWCO "Slide-A-Lyzer" cassette (Pierce). Antibody was labelled using a 30-fold molar excess of NHS-biotin. The molar weight of the anti-Wue-1 antibody was estimated to ~150.000g/mol (~150kDa). The antibody was concentrated [1mg/ml], equalling 6.5x10<sup>-6</sup> mmol/ml. A 30-fold molar excess equals 2x10<sup>-4</sup> mmol. Thus, a 10mM NHS-Biotin solution was prepared and 20 $\mu$ l of this solution were added to 1mg anti-Wue-1 antibody in PBS. Labelling was performed o/n slowly rotating at 4°C. The antibody was then dialysed again against sodium carbonate (NaCO<sub>3</sub>) buffer pH7 (section 5.7.6), in order to remove unbound biotin from the solution. Biotinylation of the antibody was then monitored by Western blotting and FACS analysis.

### **2.2.2 Generation of bispecific single-chain (bssc) anti-Wue-1 x anti-CD3**

The construct was cloned, expressed and purified as described elsewhere<sup>73, 76, 82</sup>. Briefly, the V<sub>H</sub>- and V<sub>L</sub>-sequences of anti-Wue-1 and the 15 aminoacid glycine-serine linker were amplified by means of RT-PCR, and the cDNAs were subsequently cloned into a pBluescript KS cloning vector, which already contained the V<sub>H</sub>- and V<sub>L</sub>-sequences of the anti-CD3 arm. The resulting construct, coding for bssc-anti-Wue-1xCD3 (with the domain structure V<sub>L</sub> Wue-V<sub>H</sub> Wue-V<sub>H</sub> CD3-V<sub>L</sub> CD3) was then cloned into the eukaryotic expression plasmid pEF-DHFR. The plasmid was transfected into mammalian CHO (chinese hamster ovarian cells) cells, and the secreted recombinant protein was purified from the cell culture supernatant in a three step process, i.e. ion exchange chromatography, immobilized metal affinity chromatography via the C-terminal 6x histidine tag and finally gel filtration.

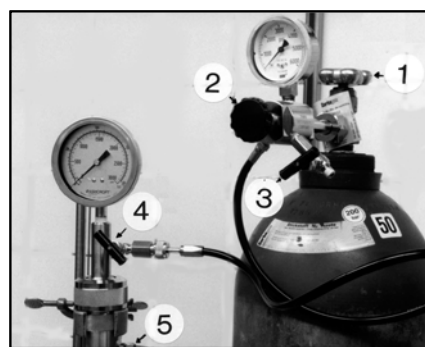
## 2.2.3 Protein preparations

### 2.2.3.1 Preparation of membrane proteins

For preparation of membrane proteins the cells were disrupted using a nitrogen (N<sub>2</sub>)-cavitation bomb as described elsewhere<sup>95</sup>. This method allows isolation of proteins in their native plasma membrane environment. Briefly, a cavitation bomb is a pressure-safe, hollow metal cylinder with a pressure gauge mounted on top. It comprises two valves, one for allowing N<sub>2</sub> influx, the other to discharge the cell suspension (figure 2.1). When N<sub>2</sub> is discharged into a cell suspension under high pressure the gas will dissolve in the buffer and in the cells. When the pressure is released after equilibration of the sample the solubilized gas will spontaneously fumigate, expand, and thereby disrupt the cells.

Membrane preparations were usually prepared from 1x10<sup>8</sup> cells in total, either freshly harvested or from frozen pellets. The cells were resuspended in 3ml buffer H (section 5.7.2), containing protease inhibitors (section 5.7.3). Disruption of the cells was carried out in a pre-cooled cavitation cylinder. The lysate was centrifuged at 1000x g, 4°C in order to remove large debris, nuclei, etc.

The supernatant was then transferred into ultracentrifuge tubes (No. 355645, Beckman) and spun at 230.000x g for 30min, 4°C in a Optima L60 ultracentrifuge (Rotor Ti 70.1, Beckman). The supernatant was discarded and the pellets were thoroughly broken up in 500µl



**Figure 2.1: Example for a nitrogen cavitation setup** (cavitation bomb model 4639, Parr Instruments). Valves are depicted as (1) nitrogen tank valve, (2) filling connection control valve, (3) dump valve, and the pressure bomb (4) inlet and (5) outlet valves. From: Harder & Kuhn, 2001<sup>95</sup>

Urea/CHAPS buffer + 5mM DTT (section 5.7.2), if necessary samples were carefully heated to 50°C. The samples were spun for 60sec at 20.000x g, 4°C in a tabletop centrifuge. The supernatants containing the "soluble membrane proteins" were transferred into fresh tubes. The remaining pellets, i.e. the "non-soluble membrane proteins" were resuspended in 500µl Urea/CHAPS buffer + 5mM DTT.

#### 2.2.3.1.1 Acetone precipitation

In order to remove lipids from the protein lysates, which may disturb gelelectrophoresis and/or MALDI/MS some protein samples were acetone precipitated. After ultracentrifugation the membrane protein pellets were resuspended in 100µl H<sub>2</sub>O. Five volumes of iccold

acetone were added and the samples were incubated at  $-20^{\circ}\text{C}$  for 30min. This was followed by centrifugation for 5min at  $9000\times g$ ,  $4^{\circ}\text{C}$  in a tabletop centrifuge. The pellets were air-dried and subsequently resuspended in  $500\mu\text{l}$  Urea/CHAPS buffer + 5mM DTT (section 5.7.2), if necessary samples were carefully heated to  $50^{\circ}\text{C}$ . The samples were spun for 60sec at  $20.000\times g$ ,  $4^{\circ}\text{C}$  in a tabletop centrifuge. The supernatants containing the "soluble membrane proteins" were transferred into fresh tubes. The remaining pellets i.e. the "non-soluble membrane proteins" were resuspended in  $500\mu\text{l}$  Urea/CHAPS buffer + 5mM DTT.

### **2.2.3.2 Preparation of total cell proteins**

#### **2.2.3.2.1 NP40 lysis**

$1\times 10^6$ – $1\times 10^8$  freshly harvested cells or frozen cell pellets were resuspended in 50-200 $\mu\text{l}$  freshly prepared 1x NP-40 lysis buffer (section 5.7.2). The cells were left on ice for 15min, followed by centrifugation of the lysate at  $20.000\times g$  for 20min,  $4^{\circ}\text{C}$  in a tabletop centrifuge. The supernatant was used as whole cell extract.

#### **2.2.3.2.2 SDS lysis**

The volumes of the freshly harvested cells or frozen cell pellets were estimated. Subsequently an equal volume of 2x SDS lysis buffer (section 5.7.2) was added to the cells. The suspensions were incubated at  $95^{\circ}\text{C}$  for 15min, followed by centrifugation of the lysate at  $20.000\times g$  for 15min, RT in a tabletop centrifuge. The supernatant was used as whole cell extract.

#### **2.2.3.2.3 Hypotonic lysis**

Freshly harvested cells or frozen cell pellets were taken up in hypotonic lysis buffer (section 5.7.2), allowing 5ml for  $1\times 10^7$ – $1\times 10^8$  cells. The resuspended cells were placed on ice for 30min and were then transferred into a pre-cooled glass homogenisator (Sartorius). Cell disruption was done by sheer force with 10-20 strokes, cooling the lysate every 5 strokes. The suspension was adjusted to 15ml with hypotonic lysis buffer and 15ml 2x Triton X-100 buffer (section 5.7.2) were added. The lysate was placed on ice for 30min and was then cleared by centrifugation ( $1000\times g$ , 10min,  $4^{\circ}\text{C}$ ). The supernatant was used as whole cell extract.

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### **2.2.3.3 Determination of protein concentration**

#### **2.2.3.3.1 "BCA Protein Assay Kit" (Pierce)**

The protein concentrations of lysates were determined according to the manufacturer's manual. 1µl of a protein sample was used in the assay (considering the concentration limitations set by the different detergents).

#### **2.2.3.3.2 Bradford assay (BioRad)**

The protein concentrations of lysates were determined according to the manufacturer's manual. 10µl of a protein sample were used in the assay (considering the concentration limitations set by the different detergents).

## **2.3 Analytical protein techniques**

### **2.3.1 FACS analysis (flow cytometry)**

Cells were harvested and were washed 1-2x in cold PBS. For each FACS reaction 0.5-1x 10<sup>6</sup> cells were resuspended in 500µl cold AKV buffer (section 5.7.5), and blocked on ice for 30min. The cells were pelleted for 5min. at 1100x g in a tabletop centrifuge and resuspended in 50µl AKV buffer containing the primary antibody at the required concentration. The samples were incubated on ice for 30min, then 500µl cold PBS was added. The cells were pelleted as above and were washed again 1-2x in 500µl cold PBS. Then the secondary antibody (directly conjugated with either FITC or R-PE) was diluted 1:100 in 50µl AKV buffer and was added. Isotype controls and background controls (i.e. cells w/o antibody and cells incubated with secondary antibody only) were usually set up alongside. Incubation and washing steps were carried out as above. Finally, the cells were resuspended in 200µl cold PBS, transferred into 0.6ml FACS tubes (Greiner) and analysed.

Where necessary, the cells were fixed prior to the FACS preparation. Approximately 3x10<sup>6</sup> cells were taken up in 1.5ml PBS in a 15ml Falcon tube. While vortexing gently 6ml icecold EtOH were added dropwise to the cells and the tube was incubated on ice for 30min. This was followed by centrifugation for 10min at 400x g, 4°C. The pellet was washed with PBS-T (section 5.7.1) and the cells were prepared for FACS analysis as described above

The samples were measured in a FACS-Calibur flow cytometer (BD Biosciences). Usually FITC labelled samples were measured in channel FL1 and R-PE samples in channel FL2 and for each sample  $1-2 \times 10^4$  events were counted. Results were analyzed using the associated CellQuest Pro software (BD Biosciences) and visualized in a histogram plot (counts over fluorescence) or a dot plot (FL2 over FL1).

### **2.3.2 SDS PAGE and Western blotting**

#### **2.3.2.1 Conventional one-dimensional gels**

The procedure was followed mainly as described by Sambrook *et al.*<sup>96</sup>. Usually SDS-PAGE was performed on 9 x 6cm or 12 x 6cm gels, comprising a 5% stacking gel (pH6.8) and a 10% or 12% separation gel (pH8.8). Electrophoresis of 50 $\mu$ g protein/lane was carried out at 80V through the stacking gel, followed by separation at 140V for 1h.

Transfer of the separated proteins onto Immobilon-P PVDF membrane (Millipore) was performed by semi-dry blotting with PVDF transfer buffer (section 5.7.1) at 20V for 45min. The blots were subsequently blocked in TBS-T (section 5.7.1) + 5% non-fat dry milk for 1h at RT or o/n at 4°C and subjected to WB analysis.

#### **2.3.2.2 Precast one-dimensional gradient gels (Novex)**

12- or 17-well NuPAGE 4-12% Bis-Tris Zoom gradient gels (Novex, Invitrogen) were used for separation of up to 150 $\mu$ g protein/lane as described in the supplier's manual. Electrophoresis was carried out in MES running buffer (Novex, Invitrogen) usually under reducing conditions for 40min at 200V using the NuPAGE "X-Cell Sure Lock Mini Cell". Transfer of the separated proteins onto Immobilon-P PVDF membrane (Millipore) was performed by semi-dry blotting with PVDF transfer buffer (section 5.7.1) at 20V for 45min. The blots were subsequently blocked in TBS-T (section 5.7.1) + 5% non-fat dry milk for 1h at RT or o/n at 4°C and subjected to WB analysis.



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### 2.3.2.3 Precast two-dimensional gels (Novex)

2-D-gelelectrophoresis was performed using the "Zoom IPG Runner System" (Invitrogen) according to the manufacturer's instructions.

After rehydration each IEF Zoom Strip (pH3-10; Novex, Invitrogen) was loaded with 50µg of membrane protein and incubated o/n at RT. The IEF the strips were then stored at -80°C in 15ml Flacon tubes until needed. After thawing the strips were incubated for 15min at RT in LDS sample buffer (Novex, Invitrogen) and then isoelectric focussing (IEF) was performed. For second dimension electrophoresis the IEF strips were assembled with NuPAGE 4-12% Bis-Tris Zoom gradient gels (Novex, Invitrogen) as described in the supplier's manual. Electrophoresis was carried out in MES running buffer (Novex, Invitrogen) for 40min at 200V.

Transfer of the separated proteins onto Immobilon-P PVDF membrane (Millipore) was performed using the NuPAGE "X-Cell Sure Lock Mini Cell" and "X-Cell II Blot Module" (Novex, Invitrogen) as described in the manual. The tank-blot was run for 60min at 30V (230mA). The blots were subsequently blocked in TBS-T (section 5.7.1) + 5% non-fat dry milk o/n at 4°C and subjected to WB analysis. When further analysis of the proteins by MALDI/MS was desired the gels were fixed and stained with colloidal coomassie solution (Sigma) as described in section 2.3.2.5.2. The stained gels were compared to the radiographies of the WBs and the appropriate spots were excised from the gel.

### 2.3.2.4 Western blot analysis

Usually 50µg proteins, in 1x Laemmli sample buffer (section 5.7.1) were separated by SDS-PAGE. Transfer to Immobilon-P PVDF membrane (Millipore) was done by semi-dry blotting with PVDF transfer buffer (section 5.7.1) at 20V for 45min. Membranes were blocked with 5% non-fat dry milk in TBS-T (section 5.7.1) for 30min, gently rocking at RT. Incubation with the first antibody was performed in TBS-T + 3% non-fat dry milk for 1h at RT, followed by four washes with TBS-T for 15min each. HRP-conjugated secondary antibody was diluted 1:10.000 in TBS-T + 3% non-fat dry milk, the blots were incubated for 1h at RT and washed with TBS-T as stated before. The blots were then developed with "Enhanced Chemi-Luminescence Kit" (ECL, Amersham) as recommended by the manufacturer, and exposed on X-omat LS films (Kodak). Finally, the membranes were stained with coomassie solution

(section 5.7.1) for 1-3min and destained as required to visualize the total protein as a loading control.

### **2.3.2.5 Staining of SDS-PAGE gels**

#### ***2.3.2.5.1 Conventional Coomassie***

Gels were generally stained in standard Coomassie solution when simple examination of protein loading was required. Besides, PVDF membranes were stained in Coomassie solution after Western blot analysis. Solutions were set up and the procedure was performed as described by Sambrook *et al.*<sup>96</sup>.

#### ***2.3.2.5.2 Colloidal Coomassie***

Brilliant Blue G Colloidal Coomassie (Sigma) was used to stain gels when bands were excised for further analysis by MALDI/MS. The staining procedure is more sensitive than conventional coomassie staining, thus also revealing weaker bands. Gels were first fixed for 1h. Colloidal coomassie working solution was prepared according to the suppliers instructions and the gels were stained for 2h at RT gently rocking. The gels were then destained and finally stored in 25% methanol (solutions see section 5.7.1).

#### ***2.3.2.5.3 Silver nitrate***

Silver nitrate staining is a sensitive staining procedure, therefore suitable to visualize weak protein bands. However, proteins excised from silver nitrate stained gels are only limited suitable for analysis by MALDI/MS. Staining of the gels was performed using the "Plus One" protein silver nitrate staining kit (Pharmacia) according to the manufacturer's manual.

### **2.3.3 Immunoprecipitation**

#### **2.3.3.1 Paramagnetic beads (Dynabeads®)**

##### ***2.3.3.1.1 Protein A Dynabeads®***

Dynabeads® Protein A (Dyna) are uniform, magnetizable polystyrene beads covalently coupled with recombinant protein A. Protein A is a bacterial cell wall protein with four Fc

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binding sites, thus immunoglobulins will bind to protein A through their Fc regions. For direct use in immunoprecipitation of a target antigen the IgG coupled Dynabeads® were directly added to the protein sample and incubated to allow the antibody/antigen complex to form. The paramagnetic beads were collected at the tube wall using a MPC® magnet (Magnetic Particle Concentrator, Dynal). The beads were then resuspended or the target protein was eluted directly from the beads using glycine buffer pH2.0 (section 5.7.1), or by boiling in a small volume of reducing Laemmli buffer (section 5.7.1).

The IP of WUE-1 was carried out using a modification of the protocol suggested in the Dynabead manual and the method described by Harder and Kuhn<sup>95</sup>. First, the paramagnetic beads were coated with anti-Wue-1 antibody. Briefly, for each sample of  $\sim 3 \times 10^7$  cells 100 $\mu$ l Dynabeads® were washed as described in the manual. After the final wash the beads were resuspended in 70 $\mu$ l 0.1M sodium phosphate buffer (section 5.7.1), and then incubated with 25 $\mu$ l anti-Wue-1 ascites fluid + 5 $\mu$ l 0.5M sodium phosphate buffer for 60min, slowly rotating at RT. This was followed by three washing steps with 0.1M sodium phosphate buffer, using a MPC® magnet to capture the antibody-coated beads. After the last wash, all liquid was removed and the cells were added to the beads in a final volume of 100 $\mu$ l 0.1M sodium phosphate buffer. In order to allow the antibody to bind to the WUE-1 antigen the suspension was incubated for 60min slowly rotating at 4°C. The cells were recovered by centrifugation, for 60sec at 400x g in a tabletop centrifuge. A sample of the supernatant was kept as control (F<sub>0</sub>). The coated cells were washed three times with 1ml PBS, then resuspended in 1ml Buffer H (section 5.7.2) and transferred into the pre-cooled N<sub>2</sub>-cavitation chamber. Cells were disrupted as described above (section 2.2.3.1), and a control sample was kept (F<sub>1</sub>). Using a MPC® magnet the bead-antibody-coated cell debris was retrieved for 5min slowly rotating at 4°C. A sample of the supernatant was kept as control (F<sub>2</sub>). The beads were washed three times in 1ml PBS using a MPC® magnet. After the last wash, all liquid was removed and 2x Laemmli buffer (section 5.7.1) was added to 100 $\mu$ l final volume. The samples were heated to 95°C for 10min and then subjected to SDS PAGE. Gels were fixed and stained with colloidal coomassie or silver nitrate (section 2.3.2.5).

### **2.3.3.1.2 Pan Mouse IgG Dynabeads®**

Dynabeads® Pan Mouse IgG (Dynal) are uniform, magnetizable polystyrene beads coated with a monoclonal human anti-mouse IgG antibody, which recognizes all mouse IgG subclasses and is Fc specific. In the assay, the primary mouse IgG antibody is either added to the

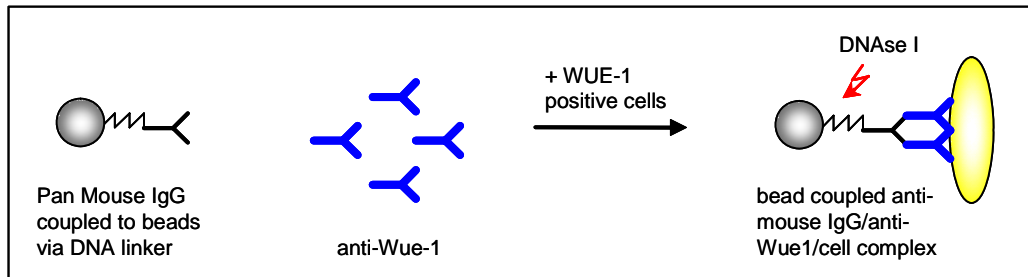
cell sample (indirect technique) or pre-coated onto the beads (direct technique) prior to cell isolation. The beads are then added to the cell sample, and will bind to the target cells. The cells which are attached to the beads via the antibody, can be retrieved with a MPC® magnet (Magnetic Particle Concentrator, Dynal)

The IP was carried out as described in the manufacturer's manual, using the indirect technique. For each sample  $2-5 \times 10^7$  cells were pre-incubated with 100µl anti-Wue-1 ascites, rotating at 4°C for 2hrs. In the negative control the AB was omitted. For IP five beads/cell were used, i.e.  $1-2.5 \times 10^8$  beads/sample. One set of experiments was set up where disruption of the cells was carried out after incubation of the anti-Wue-1 coated cells with the Pan Mouse beads, in a second set the beads were added after disruption of the AB coated cells. Cells were disrupted using the N<sub>2</sub>-cavitation chamber as described above (section 2.2.3.1). The bound proteins were eluted from the beads using 100µl glycine buffer pH2.0 (section 5.7.1). The samples were neutralized by addition of 50µl 1M Tris-HCl buffer, pH8.3. Then 50µl 4x Laemmli buffer (section 5.7.1) was added. The samples were heated to 95°C for 10min and then subjected to SDS PAGE. Gels were fixed and stained with colloidal coomassie (Sigma) according to the manufacturer's instructions (section 2.3.2.5.2).

### **2.3.3.1.3 "CELLlection™ Pan Mouse IgG Kit" (Dynal)**

CELLlection™ Pan Mouse IgG beads (Dynal) differ from the beads described in section 2.3.3.1.2 only in the DNA linker by which the monoclonal human anti-mouse IgG antibody is attached to the beads. The linker provides a cleavable site for cell release. Cells can be directly isolated from any biological sample. The bead-bound cells are retrieved with a MPC® magnet (Magnetic Particle Concentrator, Dynal), washed, and released from the beads by cleavage of the DNA linker with DNase I (figure 2.2). The enriched cells are pure and viable and can subsequently be used in any downstream application.

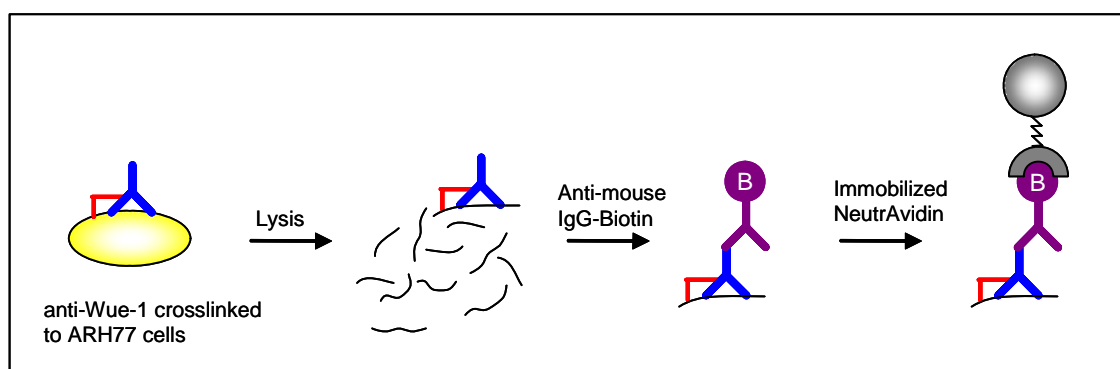
Preparation of the CELLlection™ beads and incubation of the cells was carried out as described in the manufacturer's manual, using the indirect method. The cells were released from the beads according to the supplier's protocol. Cells were prepared for FACS analysis as described in section 2.3.1. When the cells were intended for IP experiments the protocol described in section 2.3.3.1.1 was followed, and when taken into culture the isolated cells were treated according to the DSMZ recommendations for the respective cell line.



**Figure 2.2: Principle of CELlection™ Pan Mouse IgG Kit.** See text for explanation

### 2.3.3.2 Immobilized NeutrAvidin™

NeutrAvidin™ is a chemically modified version of avidin. Unlike avidin, NeutrAvidin™ has no carbohydrate portion and a neutral isoelectric point (pI 6.3), resulting in minimal non-specific binding. NeutrAvidin™ is immobilized on beaded agarose and therefore can be used to separate biotinylated proteins or to affinity purify antigens when used with biotinylated antibodies (figure 2.3). The biotinylated proteins can be eluted with 8M guanidine-HCl pH1.5 (Pierce), or the beads can be boiled directly in SDS-PAGE sample buffer. Crosslinking of anti-Wue-1 antibody to the cell surface was performed using the water soluble, thiol-cleavable NHS-ester DTSSP (Pierce).  $2 \times 10^8$  ARH77 cells were incubated with 100µg anti-Wue-1 antibody (controls: IgG<sub>1</sub> isotype control, anti-CD54) in 10ml PBS on ice for 60min. The cells were then washed twice in PBS and resuspended in 9ml PBS. A 20mM DTSSP stock solution was prepared in H<sub>2</sub>O just prior to use. The stock solution was added dropwise to each sample to 2mM final concentration. The samples were incubated at RT for 30min and the reaction was stopped by addition of 1M Tris-HCl, pH7.5 (20mM final concentration). Subsequently the samples were washed three times in 1ml PBS and processed further as required.



**Figure 2.3: Immunoprecipitation with immobilized NeutrAvidin™.** See text for explanation.

The cells crosslinked with antibody were pelleted and disrupted by hypotonic lysis as described in section 2.2.3.2.3. Large debris was removed by centrifugation (1000x g, 10min, 4°C). Protein concentration of the supernatant was determined by BCA assay (Pierce). Biotinylated anti mouse F(ab')<sub>2</sub> antibody (Dako) was added at a ratio of 1:500 [antibody:total protein]. The reactions were incubated o/n at 4°C slowly rotating. NeutrAvidin™ beads were then added at 1µl beads/3µg biotinylated protein (i.e. F(ab')<sub>2</sub>) and the samples were incubated for 3hrs at RT slowly rotating. The beads were collected by centrifugation at 2500x g for 2min. The beads were transferred into 1.5ml Eppendorf tubes and were washed 4x with 1ml PBS. Finally, elution of the antibody-immune complex was performed with 100µl Elution buffer pH2.8 (Pierce). The beads were collected by centrifugation at 2500x g for 2min, the supernatant was transferred into fresh tubes and was neutralized with 5µl 1M Tris-HCl, pH8.3. The remaining beads were resuspended in 100µl 1M Tris-HCl, pH7.5. Eluates and beads were subsequently analyzed on a NuPAGE 4-12% Bis-Tris gradient gel (Novex, Invitrogen) in 50µl total volume (i.e. 32.5µl eluate, 12.5µl 4x LDS sample buffer (Invitrogen), 5µl DTT). Detection was performed by silver nitrate staining of the gel (section 2.3.2.5.3)

### **2.3.3.3 Metabolic labelling with L-[<sup>35</sup>S]-methionine**

Metabolic [<sup>35</sup>S]-labelling of cell proteins was mainly performed as described in the "Current Protocols in Immunology", Unit 8.12<sup>97</sup>. The cells were incubated with antibody, then treated with a cleavable crosslinker (DTSSP, Pierce) in order to conserve the antibody/antigen complex prior to lysis. The crosslinker was cleaved by treatment with 5% β-mercaptoethanol (100°C, 5min), contained in the SDS sample buffer.

For each labelling experiment 3x10<sup>7</sup> cells were used. The cells were washed twice in 10ml labelling medium (PAA Laboratories) each, collecting them carefully by centrifugation at 300x g for 5min. The pellets were resuspended in 20ml labelling medium, incubated at 37°C for 30min and collected as above. The pellets were then taken up in 20ml labelling medium and 0.1mCi/ml [<sup>35</sup>S] labelling mix (NEN) was added, containing the L-[<sup>35</sup>S]-methionine. The cells were transferred into 25cm<sup>2</sup> cell culture flasks and incubated at standard conditions for 15hrs. The cells were harvested by centrifugation at 300x g for 10min and washed twice in 10ml icecold PBS. The cells were aliquoted into Eppendorf tubes, allowing 1x10<sup>7</sup> cells for each IP reaction. Each sample was then incubated with 1mg of an appropriate antibody in a

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final volume of 500µl AKV buffer (section 5.7.5) for 120min slowly rotating at 4°C, then washed twice and finally resuspended 500µl PBS. The antibody was crosslinked to the cells using DTSSP as described in section 2.3.3.2. Cell lysis was performed in 1ml/1x10<sup>7</sup> cells Triton X-100 lysis buffer (section 5.7.2) for 30min on ice, inverting the tubes periodically. Cell debris was removed from the lysates by centrifugation at 20.000x g for 10min, 4°C. Pre-clearing of the supernatants, containing the soluble proteins was performed using 50µl/sample of a protein A slurry (Pharmacia), letting the tubes rotate slowly at 4°C for 30min. The protein A agarose was removed by centrifugation at 9000x g for 60sec, then 20µl of a protein G slurry (Santa Cruz) were added to each supernatant. Immunoprecipitation was carried out for 2hrs at 4°C, rotating the tubes gently. IP was followed by two washes in 500µl Triton X-100 lysis buffer, two washes in 500µl High Salt buffer (section 5.7.1), and one wash in 1ml 50mM Tris-HCl, pH6.8, collecting the agarose beads at 400x g for 60sec. After the final wash the beads were taken up in 25µl Tris-HCl, pH6.8 and 25µl 2x Laemmli buffer (section 5.7.1) were added. The samples were heated to 95°C for 10min and the subjected to gradient gelelectrophoresis. The results were analyzed by autoradiography of the dried gels.

#### **2.3.3.4 Biotin labelling**

The labelling procedure was mainly performed as described by Altin *et al.*, 1995<sup>98</sup> using NHS-sulfo-biotin (Pierce).

##### **2.3.3.4.1 FACS analysis of biotinylated cells**

FACS analysis for WUE-1 on biotin labelled cells was performed in order to ensure that antibody binding to the antigen was not disturbed by biotinylation.

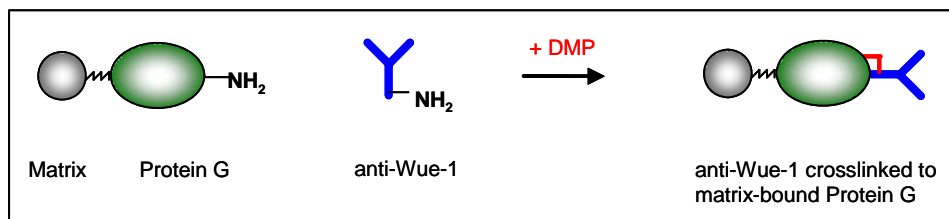
Two sets of 6x10<sup>6</sup> cells each were harvested by centrifugation, washed twice with PBS. Cells not treated with biotin were aliquoted at 1x10<sup>6</sup> cells/sample in AKV buffer (section 5.7.5), and were kept on ice until needed. The other 6x10<sup>6</sup> cells were resuspended in 240µl PBS (2.5x10<sup>7</sup> cells/ml). The biotin stock solution was prepared just prior to use at a concentration of 5mg/ml. 24µl of the stock were added to the cell suspension (final concentration 0.5mg/ml). The cells were left at RT for 30min, followed by three washes with PBS. The cells were then taken up in AKV buffer and aliquoted at 1x10<sup>6</sup> cells/sample. All samples, biotinylated and non-biotinylated, were prepared for FACS analysis as described in section 2.3.1. The samples were tested for CD54 (positive control) and WUE-1. Controls were set up

w/o antibody, with secondary antibody only and with IgG<sub>1</sub> isotype control. Biotinylation was monitored using a Streptavidin-FITC conjugate (Dianova). Double staining was performed by pre-incubation with Streptavidin-FITC conjugate, followed by anti-Wue-1 incubation and finally goat anti-mouse (GaM) PE conjugate incubation, and *vice versa* (anti-Wue-1, GaM-PE, Streptavidin-FITC).

### 2.3.3.4.2 Affinity chromatography

#### Preparation of the affinity column

Using the "ImmunoPure® Protein G IgG Plus Orientation Kit" (Pierce) a anti-Wue-1 affinity column was prepared. Protein G binds to most mammalian IgGs through their Fc regions, thus the antibody is immobilized and orientated in a way that the antigen-binding site remains available for antigen purification (figure 2.4).



**Figure 2.4: Principle of the ImmunoPure® Protein G IgG Orientation Kit.** See text for explanation.

The antibody is covalently coupled to the immobilized protein G using the NHS-ester cross-linker DSS (Pierce). Once the antibody is crosslinked to the protein G matrix the antigen samples can be purified by adding the crude sample to the column to form the immune complex. The column is washed to remove non-bound proteins and the remaining antigen is eluted from the antibody at pH2.8. There should be no contamination with primary antibody in the eluate.

Protein G was coupled with affinity chromatography purified anti-Wue-1 antibody (section 2.2.1.2.2). Prior to application to the protein G matrix the antibody solution was dialysed against PBS in order to remove any traces of primary amines (\*). 2ml of the dialyzed solution (containing approximately 10mg immunoglobulin) was mixed with 2ml of antibody binding buffer pH8 (\*) supplied with the kit (Pierce) and added to 2ml protein G gel matrix in a mini-column (Pierce). The column was incubated at RT slowly rotating for 1hr. It was then drained (\*) and washed with Binding buffer (pH8). The antibody was crosslinked to the protein G matrix using DSS and the active sites were blocked as described in the supplier's manual.



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Finally the column was pre-eluted once after addition of 5ml Elution buffer pH2.8 (Pierce), slowly rotating for 5min at RT (\*). Then the column was washed twice with 5ml Binding buffer (pH8), storage buffer (section 5.7.1) was added, and the column was stored at 4°C in the dark. The samples were analyzed by ELISA, as described in section 2.2.1.3, and the binding efficiency was calculated.

Note: The asterisk (\*) indicates at which steps samples were taken to use for determination of the binding efficiency.

#### Biotinylation of the samples

$7 \times 10^8$  ARH77 cells were harvested, washed three times in 50ml icecold PBS and, were resuspended in 25ml PBS (i.e.  $2.8 \times 10^7$  cells/ml). The biotin stock solution was prepared just prior to use at a concentration of 5mg/ml. 3ml of the stock were added dropwise to the cell suspension while vortexing carefully (final concentration 0.5mg/ml). The cells were left at RT for 40min, collected by centrifugation at 300x g for 5min, and washed twice with 50ml icecold PBS. After the final wash the pellet was resuspended in 5ml hypotonic lysis buffer (section 5.7.2), and the cell lysate was prepared as described in section 2.2.3.2.3. The lysate, containing the soluble, biotinylated proteins was then applied to the column.

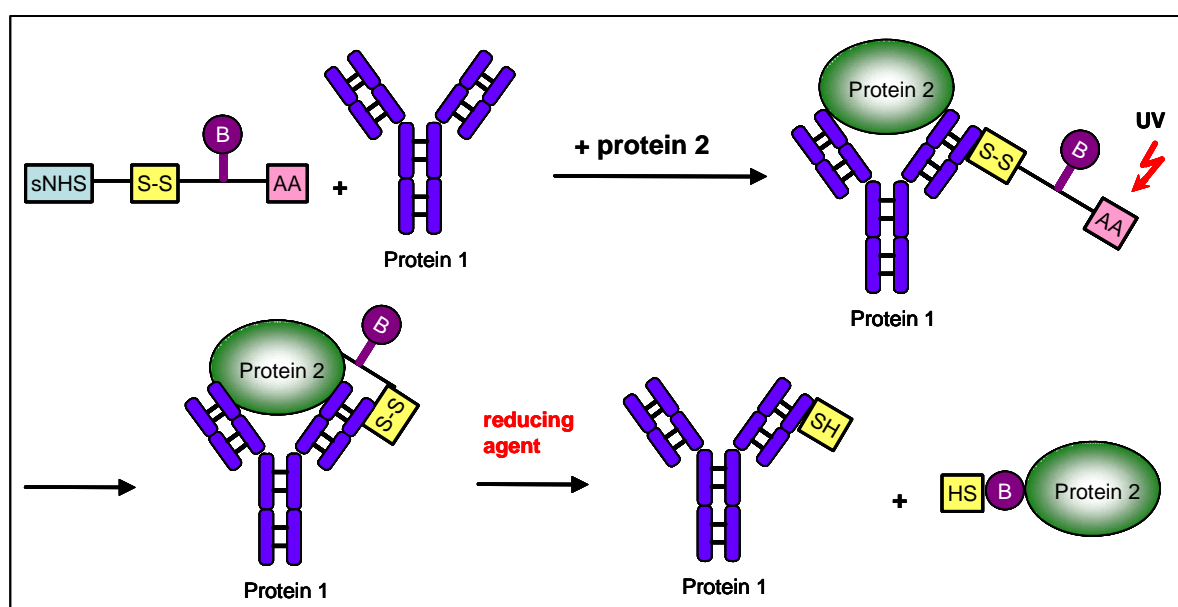
#### Loading of the column, elution and analysis of the samples

The column was equilibrated with 20ml Binding buffer pH8.0 (Pierce). The lysate was mixed to equal parts with Binding buffer and applied to the column in a steady flow circuit, using an aquarium pump. The column was loaded for 5hrs at 4°C and was then eluted with 5ml stringent Elution buffer pH2.8 (Pierce). During elution, fractions of ~0.5ml each were collected. The column was washed with 30ml Binding buffer pH8 and stored in storage buffer (section 5.7.1) at 4°C in the dark.

The fractions were adjusted to neutral pH by adding 40µl/ml 1M Tris-HCl, pH8.3, and the protein concentration of the samples was measured by BCA protein assay (Pierce). When the protein concentration was low, the fractions were pooled and protein was recovered by acetone precipitation as described in section 2.2.3.1.1. The pellet was resuspended in 500µl H<sub>2</sub>O and dialysed in PBS using a 10K MWCO "Slide-A-Lyzer" cassette (Pierce). The sample was reduced to 30µl in a Speed Vac SC 110A (Savant) and applied to a NuPAGE 4-12% Bis-Tris gradient gel (Novex, Invitrogen) in 50µl total volume (i.e. 32.5µl eluate, 12.5µl 4x LDS sample buffer (Invitrogen), 5µl DTT). Detection was performed by silver staining of the gel.

### 2.3.3.5 ProFound™ sulfo-SBED label transfer

Sulfo-SBED (Pierce) is a trifunctional crosslinking reagent comprising a Sulfo-NHS active ester, biotin, and a photoactivatable aryl azide. NHS esters react with primary amines at pH 7-9 to form covalent amide bonds. Upon exposure to UV light photolysis occurs and aryl azides form short-lived nitrenes. Nitrenes react non-specifically or undergo ring expansion and react with nucleophiles, especially amines. Using the label transfer method an antigen interacting with an antibody that has been biotinylated using sulfo-SBED can be captured by means of the photoreactive aryl azide moiety. The interacting complex can be isolated and the disulfide bond can subsequently be reduced with DTT or  $\beta$ -mercaptoethanol. Upon reduction of the disulfide bond, the biotin is transferred from the antibody to the antigen (figure 2.5).



**Figure 2.5: Protein biotinylation with ProFound™ Label Transfer Sulfo-SBED Protein:Protein Interaction Reagent (Pierce).** In the first step protein 1 (i.e. the antibody) is biotinylated with Sulfo-NHS-biotin. In the presence of UV light an interacting protein 2 (i.e. the antigen) is captured by the photoreactive aryl azide (AA) moiety. The interacting complex is isolated and the disulfide bond is reduced resulting in the biotin label being “transferred” to the interacting protein.

sNHS: sulfonated-NHS ester, S-S: disulfide bond, B: biotin, AA: aryl azide

The biotin labelled protein can then be detected by Western blot using streptavidin-HRP and an appropriate substrate. For proteins > 150kDa in size a molar excess of S-SBED of up to 20-fold is recommended. In order to avoid precipitation of the antibody by the labelling procedure a molar ratio of 1:8 [antibody:sulfo-SBED] was found most suitable. The MW of a 150kDa protein equals  $1.5 \times 10^5$  g/mol, the MW of sulfo-SBED is 880g/mol. Thus 3mg (~20nmol) anti-Wue-1 and IgG<sub>1</sub> isotype control, respectively were incubated with 141 $\mu$ g

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(~160nmol) sulfo-SBED as described in the supplier's manual. The labelled antibodies were dialysed against PBS using 10K MWCO "Slide-A-Lyzer" cassettes (Pierce), and the protein content of the samples was determined by BCA protein assay (Pierce). Subsequently  $2 \times 10^7$  ARH77 cells were incubated with 0.2 $\mu$ g sulfo-SBED labelled antibody (anti-Wue-1 and IgG<sub>1</sub> isotype control, each). Photolysis and thus label transfer was performed for 3min under UV light (340-380nm). The cells were harvested, washed once in PBS, total protein was isolated by SDS lysis (section 2.2.3.2.2), the protein content was determined by BCA protein assay (Pierce), and subsequently 15 $\mu$ g of total protein were separated on a NuPAGE 4-12% Bis-Tris gradient gel (Novex, Invitrogen) under reducing conditions, alongside 2 $\mu$ g of each labelled and non-labelled antibody. Successful labelling was monitored by detection of the biotin-tag with streptavidin-HRP conjugate (Amersham, 1:5000).

### **2.3.4 Biochemical characterization of anti-Wue-1 mAB**

#### **2.3.4.1 Depletion**

Anti-Wue-1 ascites fluid was diluted 1:500 in 0.5ml PBS. 50 $\mu$ l protein A/G-agarose (Santa Cruz) was added and the sample was incubated on an overhead shaker at 4°C o/n. The agarose was then pelleted by careful centrifugation at 800x g for 5min. A 100 $\mu$ l sample of the supernatant was kept, to the remainder 100 $\mu$ l (~ $4 \times 10^7$ ) Pan Mouse Dynabeads® (Dynal) were added, and the sample was incubated on an overhead shaker at 4°C for 2hrs. The beads were removed using a MPC® magnet (Magnetic Particle Concentrator, Dynal), then 100 $\mu$ l fresh Pan Mouse Dynabeads® were added, and the sample was incubated as before. Analysis of the samples was carried out by means of SDS-PAGE and Western blotting. In addition, FACS analysis was performed with the depleted antibody solution on ARH77, NCI-H929, and BL60-2 cells as described in section 2.3.1.

#### **2.3.4.2 Zenon™ dye**

With the "Zenon™ Mouse IgG Labelling Kit" (Molecular Probes) antibody conjugates can be generated which will carry a fluorophore of choice. An unlabelled IgG is incubated with a fluorophore-labelled F(ab')-fragment, which binds to the Fc portion of the IgG antibody to form the labelling complex. The labelled antibody can subsequently be used in downstream applications like FACS analysis just like a directly conjugated construct.

Approximately 1 µg of each antibody (i.e. anti-Wue-1, IgG<sub>1</sub> isotype control (Dako), and anti-CD54 (Becton Dickinson)) were adjusted to 10 µl final volume using AKV buffer (section 5.7.5). 5 µl each of reagent A and B were added and the labelling procedure was performed as described in the supplier's manual. For each reaction 1x10<sup>6</sup> cells of ARH77 and NCI-H929, respectively were incubated with 5 µl of the labelled antibodies in a final volume of 50 µl. The cell samples were further processed for FACS analysis as described in section 2.3.1.

### **2.3.5 Biochemical characterization of the WUE-1 antigen**

#### **2.3.5.1 Protease digestion**

Pronase (Roche) is a preparation comprising broad specificity proteases, such as various types of endopeptidases (serine and metalloproteases) and exopeptidases (carboxypeptidases and aminopeptidases). Typically, neutral protease, chymotrypsin, trypsin, carboxypeptidase, and aminopeptidase are present, together with neutral and alkaline phosphatases. Pronase is therefore able to breaking down virtually all proteins into their individual amino acids.

Prior to FACS analysis 1x10<sup>6</sup> ARH77 cells/reaction were incubated with 100 µg/ml Pronase in PBS for 30min at 37°C. Control reactions w/o Pronase were incubated likewise. For inhibition experiments 1mM EDTA-Na<sub>2</sub>, 0.2mM PMSF, 1 µM Pepstatin A or 100 µl/ml "Complete mini" (Roche) stock solution were added to 100 µg/ml Pronase and pre-incubated for 10min at RT. Subsequently the cells were added to the mixtures and incubated as above. All samples were then prepared for FACS analysis as described in section 2.3.1, with IgG<sub>1</sub> isotype control, anti-Wue-1, anti-CD19, anti-CD48, and anti-CD54 primary antibodies, respectively.

#### **2.3.5.2 Phospholipase C treatment**

Phospholipase C treatment will release glycosyl-phosphatidylinositol (GPI)-anchored proteins from membranes. The protocol used here was modified from "Current Protocols in Molecular Biology", Unit 17.8<sup>99</sup>. Briefly, two sets of 7x10<sup>6</sup> cells each were harvested by centrifugation, washed twice with PBS and were then resuspended in 1.2ml PBS. To one tube 1U (10 µl) PI-PCL (Sigma) was added. Both tubes were incubated at 37°C for 60min. After the incubation the cells were aliquoted into 6 tubes each. Each sample was prepared for FACS analysis as

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described in section 2.3.1. The samples were tested for CD19 (negative control), CD48 (positive control) and WUE-1. Controls were set up w/o antibody, with secondary antibody only and with IgG<sub>1</sub> isotype control.

### 2.3.5.3 Carbohydrate ELISA

Specific binding of anti-Wue-1 to carbohydrate antigens was investigated using a protocol modified from the publication by Butschak and Karsten, 2002<sup>100</sup>. In this method microtiter plates are coated with polyacrylamid (PAA) conjugated saccharides (i.e. the "antigens") and ELISA is performed with anti-Wue-1 in order to detect reactivity. The polyacrylamid (PAA) conjugated saccharides were a generous gift from Dr. Uwe Karsten, MDC, Berlin, Germany.

Briefly, the PAA conjugates were diluted to 10µg/ml in 50mM carbonate buffer, pH9.6 (section 5.7.6). MaxiSorp microtiter plates (Nunc) were coated with and 50µl/well of each conjugate and then incubated o/n at RT in a humid chamber. The coating solution was aspirated, followed by three washing steps with PBS-T (section 5.7.1). The remaining binding sites were blocked with 100µl/well PBS-B (section 5.7.6) for 2h at RT and then washed once as above. The anti-Wue-1 antibody was diluted 1:100 and the IgG<sub>1</sub> isotype control (Dako) was diluted 1:10, both in PBS-B and 50µl/well were applied. After 2h at RT and three further washing steps, the wells were incubated with 50µl each of anti mouse IgG-HRP conjugate (Promega), diluted 1/5000 in PBS for 90 min at RT. Substrate solution was prepared from two Sigma Fast Tablets (Sigma) in 20ml H<sub>2</sub>O bi-dist according to the manufacturer's instructions. The wells were washed again three times, 50µl of the substrate solution was added to each well, and the plate was incubated at RT as required. The reaction was stopped with 25µl/well stop solution (2.5M H<sub>2</sub>SO<sub>4</sub>; section 5.7.6). Absorbance was measured in a "Benchmark" plate reader (Biorad) at  $\lambda_{415\text{nm}}$  and analyzed with the associated software.

### 2.3.5.4 Periodate oxidation

The treatment with periodate destroys carbohydrates containing vicinal OH groups and thus prevents or reduces the binding of carbohydrate-specific antibodies. The procedure was carried out according to the method of Woodward *et al.*<sup>101</sup>. MaxiSorp microtiter plates were coated with the PAA-conjugates as described above (section 2.3.5.3). After washing three times with PBS-T (section 5.7.1) and a further wash step with 50mM sodium acetate buffer,

pH4.5 (section 5.7.6) the wells were incubated with 10mM sodium periodate solution (section 5.7.6) for 60 min at RT in the dark. In the controls, Na-periodate was replaced by acetate buffer. The plates were washed once with acetate buffer and incubated with 50mM sodium borohydride solution (section 5.7.6) for 30 min at RT in the dark. The further steps were carried out as described for the carbohydrate ELISA (section 2.3.5.3).

### **2.3.5.5 Blocking with lectins**

Lectins are non-immunoglobulin like proteins or glycoprotein structures, which are capable of reversibly forming ligand/receptor-like complexes with glycoconjugates (mono- or oligosaccharide structures) such as polysaccharides, glycoproteins, glycolipids, etc. The strength of the binding depends on the number of molecular interactions and increases from monovalent interactions to multivalent complexes. In general, lectin/carbohydrate interactions are weaker than antibody/antigen complexes. Due to the ability to bind to blood group antigens most lectins are capable to agglutinate erythrocytes. Several lectins display blood group specificity.

Here, a panel consisting of 21 different lectins ("Biotinylated Lectin Kit" I-III, Vector) with varying binding specificities was used to further characterize the nature of the WUE-1 antigen. Briefly, the cells were harvested by centrifugation and washed twice in PBS and were aliquoted at  $1 \times 10^6$  cells/sample in AKV buffer (section 5.7.5). The control samples not treated with lectins were kept on ice until needed. Each lectin was diluted to  $0.5 \mu\text{g}/\mu\text{l}$  and  $1 \mu\text{l}$  of a dilution was added to  $1 \times 10^6$  cells in  $50 \mu\text{l}$  AKV buffer (final concentration  $10 \mu\text{g}/\text{ml}$ ). The cells were left on ice for 30min, followed by two washes with PBS. For each lectin five samples were prepared: w/o antibody, secondary antibody (GaM-PE) only, IgG<sub>1</sub> isotype, anti-Wue-1, and streptavidin-PE conjugate (control for successful binding of lectin). The controls not treated with lectin were set up accordingly. All samples were then prepared for FACS analysis as described in section 2.3.1.

### **2.3.5.6 Cellular ELISA**

For use with suspension cells a 96-well cell culture plate (Cotec) was coated with sterile 0.01% poly-L-Lysine solution (Sigma) for 10min at RT. The solution was aspirated and the plate was dried. ARH77 and NCI-H929 cells were adjusted to  $4 \times 10^5$  cells/ml in culture

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medium.  $4 \times 10^4$  cells (i.e. 100 $\mu$ l) were added to each well and the plate was incubated o/n at standard cell culture conditions. With a microscope attachment of the cells to the plate was controlled. The wells containing the attached cells were carefully washed twice with 200 $\mu$ l PBS each. To each well 125 $\mu$ l 10% buffered formalin solution, pH7.0 (section 5.7.6) were added and the cells were fixed for 15min at RT. This was followed by three wash steps with 200 $\mu$ l/well H<sub>2</sub>O bi-dest. The plate was dried, wrapped in cling film, and stored upside down at 4°C until needed.

The plate was washed once with 200 $\mu$ l/well H<sub>2</sub>O bi-dest, and was then blocked 60min at 37°C with 250 $\mu$ l/well PBS-B (section 5.7.6). This was followed by three wash steps with 200 $\mu$ l/well H<sub>2</sub>O bi-dest. Anti-Wue-1 was diluted 1:10 and 1:100 in PBS-B and 50 $\mu$ l were aliquoted into the appropriate wells. Control antibody reactions were set up likewise. Incubation with the antibody was performed for 2h at 37°C. The plate was washed 5 times with 200 $\mu$ l/well H<sub>2</sub>O bi-dest. The secondary anti mouse IgG-HRP antibody (Pharmacia) was diluted 1:2500 in PBS-B allowing 50 $\mu$ l/well. The plate was incubated 60min at 37°C, then washed 5 times with 200 $\mu$ l/well H<sub>2</sub>O bi-dest and once with 200 $\mu$ l/well carbonate buffer, pH9.6 (section 5.7.6). Substrate solution was prepared from two "Sigma Fast" tablets (Sigma) in 20ml H<sub>2</sub>O bi-dest according to the manufacturer's instructions. 50 $\mu$ l of the substrate solution was added to each well and the plate was incubated at RT as required. The reaction was stopped by addition of 25 $\mu$ l/well stop solution (2.5M H<sub>2</sub>SO<sub>4</sub>; section 5.7.6). Absorbance was measured in a "Benchmark" plate reader (Biorad) at  $\lambda_{415\text{nm}}$  and analyzed with the associated software.

## **2.4 RNA techniques**

### **2.4.1 Preparation of RNA**

#### **2.4.1.1 Small scale preparations of total RNA**

Total RNA was prepared from cell pellets using the "RNeasy Mini Kit" (Qiagen), according to the manufacturer's manual. RNA concentration and purity was determined by spectrophotometry using a 1:100 diluted RNA sample. Pure RNA has an  $A_{260\text{nm}}/A_{280\text{nm}}$  absorbance ratio of  $\geq 2.0$ , i.e. a solution containing 40 $\mu$ g/ml RNA will have an absorbance  $A_{260\text{nm}}$  of  $\sim 1$ . RNA quality was controlled on a 1.2% agarose/1.1% formaldehyde gel (section 2.4.2.1).

#### **2.4.1.2 Large-scale preparations of total RNA**

Large-scale RNA preparations were carried out using the caesium chloride (CsCl<sub>2</sub>) gradient centrifugation method.  $1 \times 10^7$ - $1 \times 10^9$  cells per preparation were harvested and washed once with ice-cold PBS. Pellets were resuspended in 9ml 4M guanidinium solution (section 5.7.4). The cells were disrupted by passing the suspension through a needle and syringe several times. Large cell debris was removed by centrifugation for 2min at 900x g. For each preparation 2.6ml 5.7M CsCl<sub>2</sub> (section 5.7.4) were aliquoted into ultracentrifuge tubes (Beckman) and carefully overlaid with the cell lysates. Centrifugation was carried out at 31.000x g, 18°C, 22hrs in an Optima L60 ultracentrifuge (Rotor Ti 70.1, Beckman).

The supernatant was carefully aspirated and residual liquid was removed by drying the tube upside down on filter paper for 30min at RT. The pellet was resuspended in 360µl 10mM TE<sub>10/5</sub> (section 5.7.4) then 1ml 100% EtOH was added. The liquid was transferred into 1.5ml screw-cap tubes, 40µl 3M sodium acetate pH5.2 (NaAc; section 5.7.4) were added and mixed by carefully inverting and tapping the tube. Precipitation of the RNA was carried out for 30min at -80°C. The tubes were subsequently centrifuged in a tabletop centrifuge (30min, maximum speed, 4°C). The supernatant was carefully aspirated and the pellet was resuspended in 100µl DEPC-H<sub>2</sub>O (section 5.7.4). Following the addition of 12µl 3M NaAc and 280µl 100% EtOH the RNA was precipitated a second time as described above. The RNA pellet was then washed once in 70% EtOH and centrifuged for 15min at 4°C, maximum speed. The supernatant was aspirated, the pellet was dried in a Speed Vac SC 110A (Savant) for 5-10min and finally resuspended in 100µl DEPC-H<sub>2</sub>O. If necessary a precipitation step using 7.5M LiCl<sub>2</sub> (Ambion) was carried out according to the supplier's instructions in order to remove residual contaminating DNA and proteins. RNA quantity and quality were determined as described above (section 2.4.1.1).

#### **2.4.1.3 Isolation of polyA<sup>+</sup> mRNA**

It was assumed that approximately 1-5% of total RNA is mRNA. The mRNA was isolated from total RNA samples using the "PolyAtract® mRNA Isolation Kit" for large-scale isolations (Promega) as described in the manufacturer's manual. RNA quantity was determined as described in section 2.4.1.1.



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## 2.4.2 Northern blot analysis

Northern-blot analysis was performed essentially as described by Kroczeck *et al.*<sup>102</sup>. In brief, total RNA was isolated, separated by denaturing agarose gelelectrophoresis, transferred onto nitrocellulose membrane, cross-linked by high dose UV radiation, and hybridized with a specific radioactively labelled riboprobe, as described below. Hybridization was then detected by autoradiography.

### 2.4.2.1 RNA agarose gelelectrophoresis

Total RNA was separated by denaturing agarose gelelectrophoresis in order to eliminate secondary structures. 8µg RNA (in 5.5µl H<sub>2</sub>O) were mixed with 19.5µl of denaturing premix, 5µl RNA loading-buffer (both section 5.7.4), and 1µl ethidium bromide (0.5mg/ml). Samples were denatured at 55°C for 15min prior to loading. Electrophoresis was performed at RT in 1x MOPS buffer (section 5.7.4) at 80V under constant buffer circulation for about 4 h. Separation of the RNA was visualized on a UV-screen by detection of the 28S (4.7kb), 18S (1.87kb) and 5S (0.16kb) rRNA bands.

### 2.4.2.2 RNA transfer

The RNA-gel was equilibrated in 20x SSC (section 5.7.4) for 10min and RNA was then transferred onto a Hybond-N membrane (Amersham) overnight by capillary forces. Fixation of the RNA to the membrane was performed by crosslinking with 120mJ/cm<sup>2</sup> UV light (Stratalinker). Transfer was controlled on a UV-screen by detection of rRNA bands. The membranes were stored at -20°C until needed.

### 2.4.2.3 Generation of RNA probes

Northern blot analysis was mainly carried out in order to examine the clones obtained from the ARH77 and MM library panning. For generation of the riboprobes the library plasmids were linearized within the multiple cloning site (MCS) by restriction digestion with *EcoRI*. The DNA was purified by phenol-chloroform extraction (section 2.5.1) and resuspended at a concentration of [1µg/µl] in TE buffer (section 5.7.4). The run-off antisense transcripts for hybridization were obtained from 1µg plasmid DNA, using the "Riboprobe *in vitro*

Transcription Kit" (Promega) according to the manufacturer's instructions. Unincorporated [<sup>32</sup>P]-labelled nucleotides were removed by cleaning up the RNA probes using RNeasy Mini columns (Qiagen) as recommended by the supplier. The probes were eluted with 2x 50µl RNase free water and specific activity was measured in a "Tri Carb 1900 TR" liquid scintillation analyzer (Packard).

### **2.4.2.4 Generation of DNA probes**

In some cases, DNA probes were generated for Northern blot analysis. Compared to riboprobes DNA probes usually show less background but on the other hand are also less sensitive. For generation of DNA probes the library plasmids were digested with *EcoRI* and *XhoI* to release the insert. The inserts were separated from the vector by agarose gelelectrophoresis, excised from the gel, and purified using the "QiaEx II" kit (Qiagen) according to the manufacturer's instructions. Concentration of the fragments was estimated semi-quantitatively by running out DNA samples alongside 5µl Smartladder (Eurogentec). Approximately 50ng of the fragments were used for the generation of radioactively labelled probes. Incorporation of [ $\alpha$ -<sup>32</sup>P] dCTP into complementary strands generated by Klenow polymerase was carried out with the "HexaLabel DNA Labelling Kit" (Fermentas) according to the manufacturer's instructions. Unincorporated [<sup>32</sup>P]-labelled nucleotides were removed by separation on a "Quick Spin" size exclusion column (Roche) and specific activity was measured in a "Tri Carb 1900 TR" liquid scintillation analyzer (Packard).

### **2.4.2.5 Northern blot hybridization**

The Hybond-N membranes (Amersham) were pre-hybridized in 10ml ExpressHyb solution (Clontech) for 1h at 68°C, constantly rotating. The radioactively labelled probes were denatured for 5min at 95°C and then added at a concentration of 1-3x10<sup>6</sup> cpm/ml to the pre-incubated membrane. Hybridisation was carried out o/n at 68°C, constantly rotating. Subsequently, the membranes were washed in wash buffer I (section 5.7.4) for 2x 15min at RT, followed by 2x 10min at 68°C. Final washes were performed in wash buffer II (section 5.7.4) for 3-4x 15min at 68°C. The blots were then exposed to Xomat LS films (Kodak) for autoradiography, either at RT or at -80°C using intensifying screens.

## 2.5 DNA techniques

### 2.5.1 General DNA protocols

All standard methods like plasmid-preparation, restriction digestion, cloning methods, electrophoresis on agarose gels in TBE etc. were performed according to Sambrook *et al.*<sup>96</sup> or according to the manufacturer's instructions. Modifications of any of these protocols are indicated where necessary.

### 2.5.2 Conventional PCR

When the nucleic acid concentration of the sample was known usually 1-10ng template DNA were used per PCR reaction. From unknown samples 0.25-0.5µl were used. All PCR reactions were carried out in 20µl final volume as follows:

#### PCR reactions:

stock	final concentration	volume
template	1-10ng	0.25-0.5µl
10 x buffer (+ MgCl <sub>2</sub> )	1x	2µl
dNTP mix (20mM each)	0.2mM	0.2µl
primer 1 (10µM)	0.2µM	0.4µl
primer 2 (10µM)	0.2µM	0.4µl
<i>Taq</i> polymerase (5U/µl)	1.25U	0.25µl
H <sub>2</sub> O		ad 20µl

#### PCR program:

1)	5min	94°C
2)	30sec	94°C
3)	40sec	60°C
4)	40sec	72°C
5)	5min	72°C
Repeat steps 2)-4) 24 times		

### 2.5.3 PCR screen of bacteria

The PCR screen is an adapted protocol of the conventional PCR. This method allows for quick control of a plasmid or insert present in large numbers of bacteria without the need of doing minipreps. However, this method is only a rough screen for putative positive clones. The risk of obtaining false positives or of missing truly positive clones has to be considered. The need for plasmid miniprep and proper analysis of the plasmid DNA by PCR and/or restriction digestion persists.

The PCR screen was usually carried out in 0.25µl PCR tubes or 96-well PCR-plates depending on the number of colonies to be screened. With a sterile toothpick a tiny amount of

cells is transferred from a single colony into the tube or plate, by lightly streaking the cells off on the bottom wall of the tube. With the same toothpick a LB-agar masterplate is dotted. The masterplate is necessary for subsequent identification and amplification of the positive colonies. Depending on the number of reactions a PCR mastermix is set up, including the no template control (ntc). To each tube or well 20 $\mu$ l mastermix are added. The ntc-reaction should be the last well to be pipetted in order to ensure that a cross contamination with bacteria can be detected. *Taq* polymerase and buffer were used from (Invitek). The same PCR program as for a conventional PCR was used (section 2.5.2).

### Mastermix (for one reaction)

stock	final concentration	volume
10 x buffer (+ MgCl <sub>2</sub> )	1x	2 $\mu$ l
dNTP mix (20mM each)	0.2mM	0.2 $\mu$ l
primer 1 (100 $\mu$ M)	0.2 $\mu$ M	0.04 $\mu$ l
primer 2 (100 $\mu$ M)	0.2 $\mu$ M	0.04 $\mu$ l
<i>Taq</i> polymerase (5U/ $\mu$ l)	0.5U	0.1 $\mu$ l
H <sub>2</sub> O		17.62 $\mu$ l

### **2.5.4 Sequencing**

Sequence analysis of miniprep DNA samples was performed by AGOWA GmbH, Berlin. Sequence files were obtained online (ABI format), and were subsequently analyzed and aligned using "4Peaks" (non-commercial) and Lasergene (DNA Star) software.

### **2.6 Expression library construction**

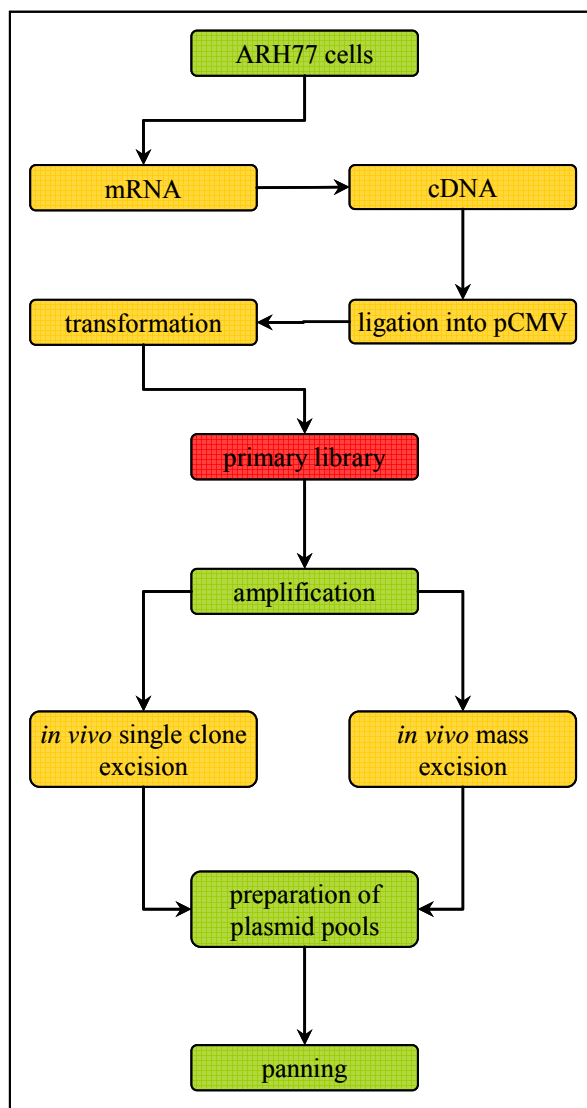
The use of cDNA libraries is an elegant method for examination of new genes and/or assigning gene function. cDNA libraries represent the information encoded in the mRNA of a particular sample (tissue, cell, or organism). Generally, RNA molecules are not very stable and therefore difficult to amplify in their natural form. Thus, the information encoded by the RNA is converted into a stable DNA duplex (cDNA) and is then inserted into a self-replicating plasmid vector. Once the genetic information is available in the form of a cDNA library, individual questions can be assessed.

### 2.6.1 ARH77 "Lambda ZAP®-CMV XR" library

The library was generated from ARH77 mRNA, using the "Lambda ZAP®-CMV XR Library Construction Kit" (Stratagene), following the instructions given by the supplier.

Briefly, after transcription of the mRNA the newly synthesized cDNA is size-fractionated, precipitated, and ligated directionally into the "Lambda ( $\lambda$ ) ZAP®-CMV XR" vector. The recombinant lambda library is then packaged in a high-efficiency system and is plated on *E. coli* to produce bacteriophage-containing plaques and the Lambda phage can subsequently be harvested. The phage-containing supernatant is the primary library. The titer of the primary library can be determined as described below (section 2.6.2.1). Since primary libraries can be unstable one round of amplification is recommended, in order to produce a large, stable quantity of a high-titer library stock. The  $\lambda$ -ZAP® library is converted into a plasmid (i.e. phagemid) library by performing *in vivo* excision. The cDNA inserts cloned into the

$\lambda$ -ZAP®-CMV XR vector are released from the phage in the form of a pCMV-Script® EX phagemid vector, thus converting the bacteriophage library into a plasmid system which allows expression in mammalian cells and easy insert characterization. Alternatively, the entire library can be mass excised for functional screening in mammalian cells. The pCMV-Script® phagemid does not contain an ATG initiation codon, thus ensuring that only clones



**Figure 2.6: Overview of the “ $\lambda$ -ZAP®-CMV XR” library construction.** The primary bacteriophage library is amplified prior to single clone- or mass excision. Excision of the pCMV-Script Ex phagemid from the original vector converts the phage library into a plasmid library, which can be used for transfection of COS7 cells (panning).

containing their own ATG within the cDNA insert will be expressed. Figure 2.6 summarizes the experimental procedure of the library construction in a brief flowchart.

## 2.6.2 Multiple Myeloma "Lambda ZAP®-CMV XR" library

The Multiple Myeloma cDNA library was kindly provided by Dr. Sun-Jin Choi, University of Pittsburgh Medical Centre, USA (see also reference [103]).

The library was constructed in the "Lambda ZAP® Express™" vector (Stratagene). For cDNA synthesis mRNA from four MM patient bone marrow samples was used. The patients were phase III stage and never treated with drugs. The cDNA fragments were unidirectionally cloned into the vector (5'-*EcoRI*--*XhoI*-3'). The insert size cut off was 700bp, 11 out of 12 clones contained inserts  $\geq 1.6$ kb. The initial titer of the library was  $3 \times 10^5$  pfu/ml. The primary library has been amplified once and the independent number was  $2.5 \times 10^5$  pfu per library, the titer of the amplified library was  $> 10^8$  pfu/ml. Upon receipt *in vivo* mass excision of the MM library was carried out in order to convert the lambda phage library into a phagemid library as described in (section 2.6.2.2).

### 2.6.2.1 Titration and amplification of the library

Titration and amplification of the ARH77  $\lambda$ -ZAP® primary library was performed according to the manual. Briefly, the entire cDNA recovered after size fractioning was ligated into the " $\lambda$ -ZAP®-CMV XR" vector, the ligations were packaged, and the titer of the primary library was determined. Since primary libraries can be unstable, the ARH77  $\lambda$ -ZAP® library was amplified. A good representational library consists of  $\sim 1 \times 10^6$  clones and to create this amount of clones 20 aliquots of the primary library were amplified, containing  $\sim 5 \times 10^4$  pfu (plaque forming units) each. The titer of the amplified library was calculated using the equation below. The amplified library was aliquoted and stored at  $-80^\circ\text{C}$  for further use.

$$\text{Titer [pfu/}\mu\text{g]}^5 = \left[ \frac{\text{No. of plaques} \times \text{DF}^1 \times \text{total packaging volume}^2 [\mu\text{l}]}{\text{total DNA packaged}^3 [\mu\text{g}] \times \text{volume plated}^4 [\mu\text{l}]} \right]$$

<sup>1)</sup> dilution factor (pre-dilution of packaging reaction); <sup>2)</sup> volume of packaging reaction mix; <sup>3)</sup> total insert DNA used from ligation mix for packaging; <sup>4)</sup> volume of packaging mix plated; <sup>5)</sup> plaques obtained per  $\mu\text{g}$  insert DNA

### 2.6.2.2 Conversion of the ARH77 "Lambda ZAP®-CMX XR" primary library

By means of *in vivo* excision and re-circularization a pCMV®-phagemid is generated comprising the lambda vector, which contains the cloned cDNA insert. *E. coli* cells are simultaneously infected with the target lambda phage and a M13 helper phage. Inside *E. coli*, the M13 helper proteins recognize the initiator DNA within the lambda vector. One of the two DNA strands is nicked and new DNA synthesis begins downstream of the nicking site. Synthesis of a new ssDNA molecule continues through the cloned insert up to a termination signal, positioned 3' of the initiator signal. Subsequently the single-strand (ss) DNA molecule is circularized, packaged into phagemid particles, and secreted from *E. coli*. When *E. coli* is re-infected with the phagemid the bacteria can be plated on selective media to form colonies.

First titration of the mass-excised phagemid was performed on Ø 10cm plates. The phagemid supernatant was diluted up to  $10^{-4}$  and dilutions  $10^0$ – $10^{-4}$  were subsequently plated out. The titer of the excised phagemid was calculated using the formula below.

$$\text{Titer} = \frac{(\text{No. of colonies}) \times \text{dilution factor}}{\text{volume plated} [\mu\text{l}]}$$

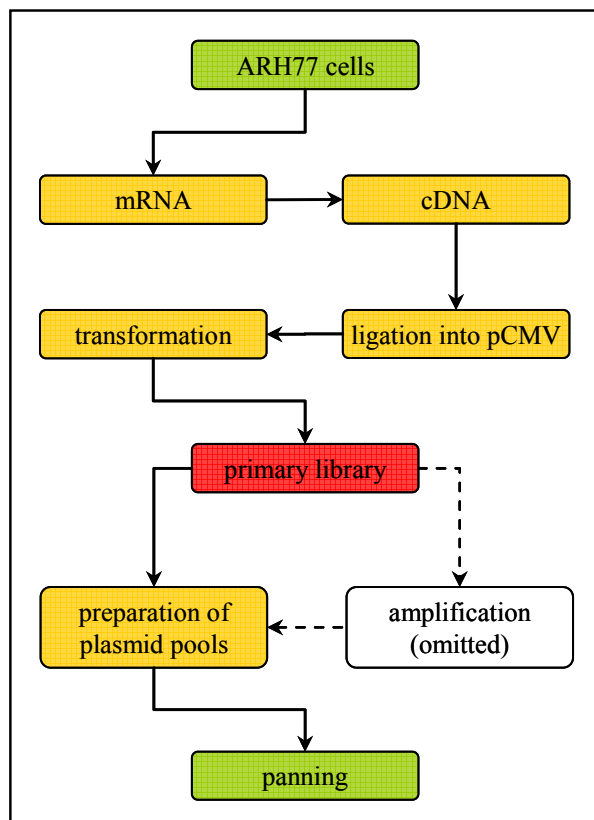
Following the manufacturer's guidelines *in vivo* mass excision of the ARH77 λ-ZAP® library was performed directly from the primary library in order to maintain the original clonal representation. 25 clonal pools were prepared as follows: *E. coli* cells were transformed with the required amount of phagemid to represent a pool of  $\sim 2.5 \times 10^5$  cfu (colony forming units) each, and plated onto selective LB agar (section 5.9.3) in large 530cm<sup>2</sup> square dishes. *In vivo* excision was also carried out from the MM library. 28 clonal pools were prepared from  $\sim 2.5 \times 10^5$  cfu each. Using 2x 20ml and 1x 10ml LB medium (section 5.9.3) for each large dish the *E. coli* cells were washed off the agar and pooled in falcon tubes. Additionally six clonal pools from the MM library were set up in shakeflasks. 200ml LB medium were inoculated with  $\sim 5 \times 10^9$  cfu +  $4 \times 10^{10}$  *E. coli* cells (i.e. a ratio 1:8 phagemid:cells), and the flasks were incubated at 37°C, shaking for 9hrs. The bacteria were harvested by centrifugation at 9000x g for 15min, the approximate pellet sizes were determined for each sample by weighing and the plasmid DNA was recovered from each pool by carrying out maxiprep (Qiagen). The recovered plasmid DNA was subsequently used for panning. For control of incorporated inserts and size distribution single plaques from the ARH77 λ-ZAP® library were picked and subjected to single clone excision. The plaques were first transferred into 500µl SM buffer +

20 $\mu$ l CHCl<sub>3</sub> (section 5.7.7) to release the phage particles. Then samples from these solutions were subjected to PCR analysis with T3<sub>fw</sub> and T7<sub>rev</sub> primers (section 5.6.1). The phage supernatants (mass- and single clone excision) were stored at 4°C.

### 2.6.3 ARH77 "pCMV-Script® XR" plasmid library

Plasmid-based functional screening is largely facilitated by cDNA libraries constructed directly into plasmid vectors. The "pCMV-Script® XR cDNA Library Construction Kit" (Stratagene) is designed for generating directional cDNA libraries in the pCMV-Script® mammalian expression vector, which is suitable for functional assays in mammalian cells. pCMV-Script® is derived from a high copy number pUC-based plasmid and is designed to allow efficient protein expression in mammalian systems, driven by the human cytomegalovirus (CMV) immediate early promoter. The vector does not contain an ATG initiation codon, thus only clones containing their own ATG initiation codon within the cDNA insert will be expressed. Individual colonies can be examined to determine the percentage of vectors containing an insert and the average insert size. This can be achieved either by PCR directly from the colony with T3 and T7 primers or by restriction analysis of individually prepared plasmid DNA. Figure 2.7 summarizes the experimental procedure of the library construction in a brief flowchart.

The ARH77 plasmid library was generated by means of the "pCMV-Script® XR cDNA Library Construction Kit" (Stratagene) following the instructions stated in the manual. The entire cDNA recovered after size fractioning was ligated into the pCMV-Script® vector. Each individual ligation was transfected into ultracompetent *E. coli* cells. The efficiency of transformation was calculated using the equation below.



**Figure 2.7: Overview of the "pCMV-Script® XR" cDNA library construction.** Amplification of the primary library was omitted in order to maintain the original clonal complexity. Plasmid DNA from bacterial pools of the primary library were directly used for transfection of COS7 cells (panning).



$$\text{Titer [cfu/}\mu\text{g]}^5 = \left[ \frac{\text{No. of colonies} \times \text{DF}^1 \times \text{total volume}^2 [\mu\text{l}]}{\text{total DNA}^3 [\mu\text{g}] \times \text{volume plated}^4 [\mu\text{l}]} \right]$$

<sup>1)</sup> DNA dilution factor (DNA contained in volume ligation mix used for transformation, in relation to DNA contained in total volume of ligation mix; <sup>2)</sup> volume of transformation mix; <sup>3)</sup> total insert DNA used in ligation; <sup>4)</sup> volume of transformation mix plated; <sup>5)</sup> colony forming units obtained per  $\mu\text{g}$  insert DNA

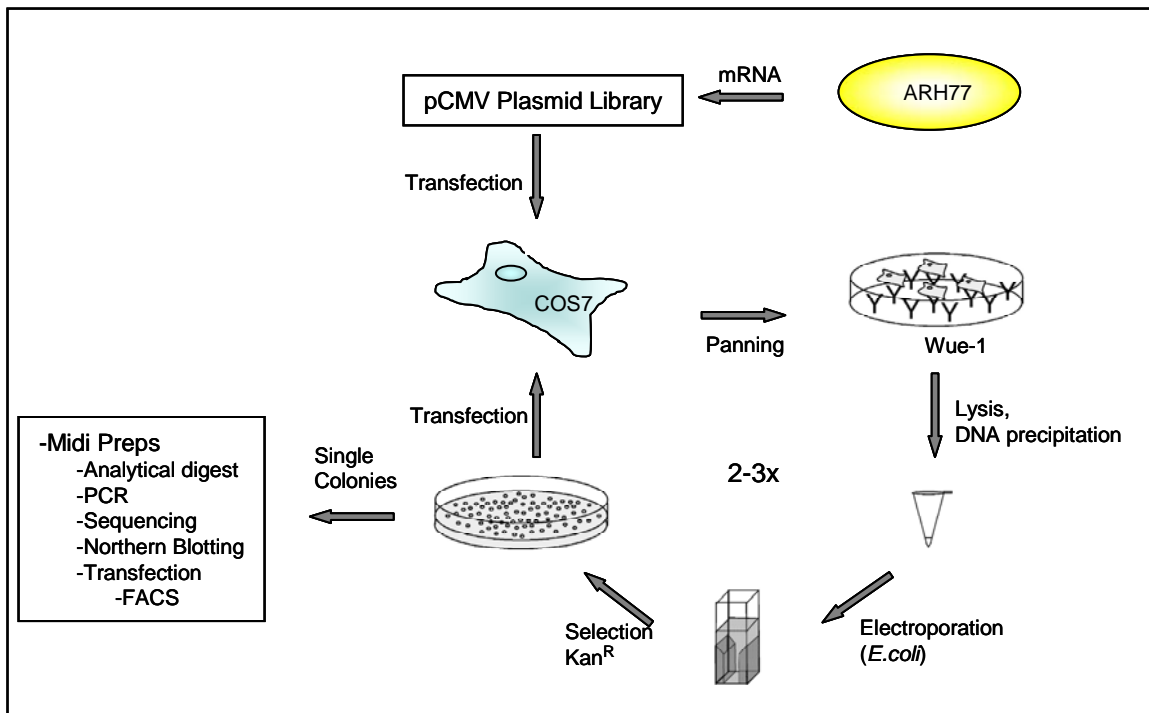
To control for presence of incorporated inserts and size distribution single colonies were picked, miniprep DNA was prepared and subsequently digested with *EcoRI* and *XhoI*, in order to release the insert. The entire plasmid-ligated cDNA was finally transformed and plated on 530cm<sup>2</sup> LB-agar plates. Amplification of the primary plasmid library was omitted in order to maintain the original clonal representation. Using 2x 20ml and 1x 10ml LB medium (section 5.9.3) for each large dish the *E. coli* cells were washed off the agar and pooled in falcon tubes. The bacteria were harvested by centrifugation at 9000x g for 15min, the approximate pellet sizes were determined for each sample by weighing and the plasmid DNA was recovered from each pool by means of midi preps (Qiagen). The recovered plasmid DNA was subsequently used for panning.

## 2.7 Expression cloning (immunoselection)

### 2.7.1 Principle of "panning" using mAB

In this project the panning procedure was carried out using a modification of the method described in "Current Methods in Molecular Biology", Section VI, Units 6.11<sup>104</sup> and Section III, Units 16.12<sup>105</sup> – 16.13<sup>106</sup>. Briefly, this method is designed to isolate cDNAs coding for cell surface proteins by screening cDNA libraries, which are transiently expressed in COS7 cells with monoclonal antibodies (mABs). The whole screening procedure requires multiple rounds of transfection and immunoselection in which the number of cells carrying the plasmid, which contains the sequence of interest, will be enriched (figure 2.8). When the cDNA library is first introduced into mammalian cells it is important to ensure that a complete representation of the library is transferred into the host cells. This is to guarantee that the protein of interest is expressed by any of the transfectants. In the first round of enrichment transfection of the cDNA library into COS7 cells is therefore carried out using a highly efficient method, such as the DEAE-dextran method. Cells expressing the target

protein are isolated from the bulk of transfected cells by means of protein-protein interaction. Transfected cells are incubated with an antibody against the protein of interest, then the cells are panned on plastic petridishes which have previously been coated with an antibody directed against the target-recognizing antibody<sup>107</sup>. Plasmid DNA is recovered from the cells attached to the plates by the method of Hirt<sup>108</sup> ("Hirt supernatant"). Following amplification in *E. coli*, the plasmids can then be reintroduced into COS7. At this point transfection is performed via spheroplast fusion, a rather inefficient procedure, resulting only in up to 2% transfected cells. This method is meant to ensure that only a single plasmid type is delivered into each transfectant, in order to maximize the level of enrichment obtained in the immunoselection steps. After four rounds of transfection and immunoselection the plasmid of interest should be enriched sufficiently. Subsequently plasmid DNA can be prepared from single *E. coli* colonies for further analysis like PCR, restriction digestion, sequencing, and database sequence comparison. Moreover cells not expressing the target protein can be transfected with the single plasmid DNAs and can then be subjected to flow cytometry and stable transfectants can be selected for use in functional analysis of the target protein.



**Figure 2.8: Expression cloning and immunoselection of WUE-1.** ARH77 and MM cDNA libraries, respectively are transfected into COS7 cells. The transfectants are incubated with anti-Wue-1 antibody and subsequent screening (“panning”) was performed on anti-mouse IgG coated petridishes. Plasmid DNA is isolated from the attached cells, amplified in *E.coli* and finally re-introduced into COS7 cells to perform a new round of panning. This is repeated three times and the resulting clones are further analysed by FACS and Northern blotting. Modified method from Seed and Aruffo<sup>103-105</sup>

### **2.7.1.1 Coating of the plates with anti-Wue-1 antibody**

Anti-mouse IgG was diluted to 10µg/ml in 50mM Tris-HCl, pH9.6. Standard bacterial culture dishes were coated with 10ml of this solution for 2hrs at RT or at 4°C o/n (the anti-mouse IgG coating solution was used up to three times). The plates were washed 3x with 0.15M NaCl at RT, then they were blocked o/n at RT with 3ml of BSA solution (1mg/ml). The blocking solution was aspirated and the plates were stored wrapped in cling-film at -20°C until needed.

### **2.7.1.2 Transfection of COS7 cells (DEAE-dextran/chloroquine method)**

#### Properties of COS7 cells

COS cells are African Green Monkey kidney cells (CV-1) that have been transformed with an origin-defective SV40 virus, which has integrated into the cell chromosomal DNA<sup>109</sup>. The cells produce high levels of wild-type SV40 large tumour (T) antigen but no other viral particles. SV40 large T antigen is necessary to initiate virus DNA replication at the SV40 origin without the need of being located on the DNA molecule on which it acts. Approximately 48-72hrs after transfection plasmids containing a SV40 origin replicate in COS cells to a large copy number ( $10^4$ - $10^5$  copies/cell). The transfectants will express the foreign protein at high levels over a short period of time, and will therefore allow rapid characterization of cDNA clones. Protein production starts in a burst approximately 24hrs post-transfection and can last for up to a week, after which the transfected cells usually die or lose the plasmid.

#### DEAE-dextran/chloroquine transfection

DEAE-dextran/chloroquine transfection is an efficient procedure to introduce a cDNA library into COS cells. The efficiency can be up to 70% transfected cells and allows for delivery of up to 200 different plasmids into each transfected cell. By means of this method the host cells will ideally receive a complete representation of the library. The mechanism of the DNA transfer is yet poorly understood. It is believed that the positive charge of the DEAE-dextran polymer neutralizes the negative charge of the DNA polymer, resulting in formation of a fine precipitate, which comes in contact with the plasma membrane of the host cell. The DEAE-dextran/DNA complex is then internalized by pinocytosis. Chloroquine is added to the transfection mix to prevent the acidification of the endosomes, by means of which the DEAE-

dextran/DNA complex is presumable transported into the cells. Acidification would result in hydrolysis of the DNA and in consequence in lower transfection efficiency.

DEAE-dextran transfection of COS7 cells with the ARH77- and MM- cDNA libraries was carried out as described in "Current Methods in Molecular Biology", Section VI, Unit 9.2<sup>110</sup>. In preliminary tests the transfection conditions had already been optimized. Exponentially growing COS7 cells were seeded into Ø10cm cell culture dishes at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup>. After 24h the DEAE-dextran transfection was performed using 10µg plasmid DNA/plate. The cells were incubated with the DEAE-dextran/DNA transfection mix (section 5.7.7) for 2hrs at standard cell culture conditions. The medium was aspirated and this was followed by a DMSO shock (10%) for 2min at RT. DMSO was aspirated, the cells were washed once with PBS, 10ml cell culture medium was added and cells were incubated at standard cell culture conditions for 18hrs. Subsequently the cells of each plate were detached with Trypsin-EDTA (Biochrom), re-seeded in two plates each and incubated o/n.

### **2.7.1.3 Panning**

The DEAE-dextran transfected cells were detached with EA buffer (section 5.7.7), blocked in EAV buffer (section 5.7.7) on ice for 30min, and subsequently incubated with purified anti-Wue-1 ascites at a dilution of 1:50 for 60min on ice. Excess or unbound anti-Wue-1 antibody was removed by Ficoll gradient centrifugation. The cells were carefully layered on top of EAF buffer (section 5.7.7) and centrifuged at 200x g for 4min. The cell pellets were resuspended in EA and transferred into two petridishes each, which were previously coated with anti-mouse IgG capture antibody (section 2.7.1.1). The plates were then left standing at RT for 3hrs to allow the immune-complex to form. After incubation, the plates were carefully washed with PBS. Using a light microscope the number of cells still attached to the plates after the wash (i.e. potentially positive clones) was estimated.

### **2.7.1.4 Isolation of the plasmid DNA ("Hirt" method)**

Lysis of the attached cells was performed with "Hirt" lysis buffer (section 5.7.7) for 20min at RT. The lysate ("Hirt" supernatant) was transferred into 2ml Eppendorf tubes, 5M NaCl was added, mixed very carefully, and incubated o/n at 4°C. The tubes were spun for 4min, 4°C, at max. speed. This was followed by standard phenol-chloroform extraction. After the final

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CHCl<sub>3</sub> wash 10µg co-precipitant (Gylcobblue, Ambion) were added to the aqueous phase and standard EtOH DNA-precipitation was carried out. The DNA pellets were resuspended in 10µl sterile H<sub>2</sub>O bi-dest.

#### **2.7.1.5 Electroporation of *E. coli***

Electrocompetent *E. coli* were prepared from a fresh culture of a DH5α strain. 1000ml bacterial culture was grown up to OD<sub>600nm</sub> = 0.5-0.8 and harvested by centrifugation at 4000x g, 15min, 4°C. This was followed by steps of resuspending and centrifugation in 1000ml icecold H<sub>2</sub>O, 500ml icecold H<sub>2</sub>O, 20ml icecold sterile 20% glycerol, and finally 3ml icecold sterile 10% glycerol. The bacteria were aliquoted on dry ice (50µl/tube, cell density approximately 1-3x10<sup>10</sup> cells/ml), shock frozen in liquid nitrogen, and stored at -80°C.

Prior to electroporation the cuvettes (for bacteria, 0.2cm gap width, Invitrogen) were pre-cooled on ice and SOC medium was pre-warmed to 37°C. Gene Pulser and Controller (BioRad) were set to 25µF, 2.5 kV, 200Ω. The required amount of electrocompetent *E. coli* aliquots were thawed on ice, ¼ of the precipitated DNA was added to each tube and mixed thoroughly. The bacteria were incubated with the DNA on ice for 60sec, transferred into the pre-cooled electroporation cuvettes and electroporated with one pulse (time constant 4-5, field strength 12.5V). Immediately after the pulse 1ml pre-warmed SOC medium (section 5.9.3) was added, the cell suspension was mixed thoroughly, then transferred into Falcon 2063 polypropylene tubes and finally incubated on a orbital shaker for 60min at 37°C, 200rpm. The cells were subsequently plated onto LB-agar plates + 50µg/ml kanamycin (section 5.9.3) and were incubated o/n at 37°C.

#### **2.7.1.6 Transfection of COS7 cells using spheroplast fusion**

Spheroplast fusion is a rather inefficient method, resulting in only up to 2% transfected cells. On the other hand this method is meant to ensure that ideally only a single plasmid type is delivered into each transfectant. This in consequence will result in greater enrichment of a target plasmid in subsequent rounds of transfection and immunoselection. Bacteria containing the plasmids, which were recovered by the "Hirt" method (section 2.7.1.4) are treated with lysozyme in order to remove their cell walls. The resulting spheroplasts are then fused with mammalian host cells using polyethylene glycol (PEG). This allows the introduction of the

foreign DNA directly into the host cell cytoplasm. Because of the inefficiency of the method each host cell statistically fuses only with one spheroplast, on average, thereby introducing only a single plasmid type into each transfected cell.

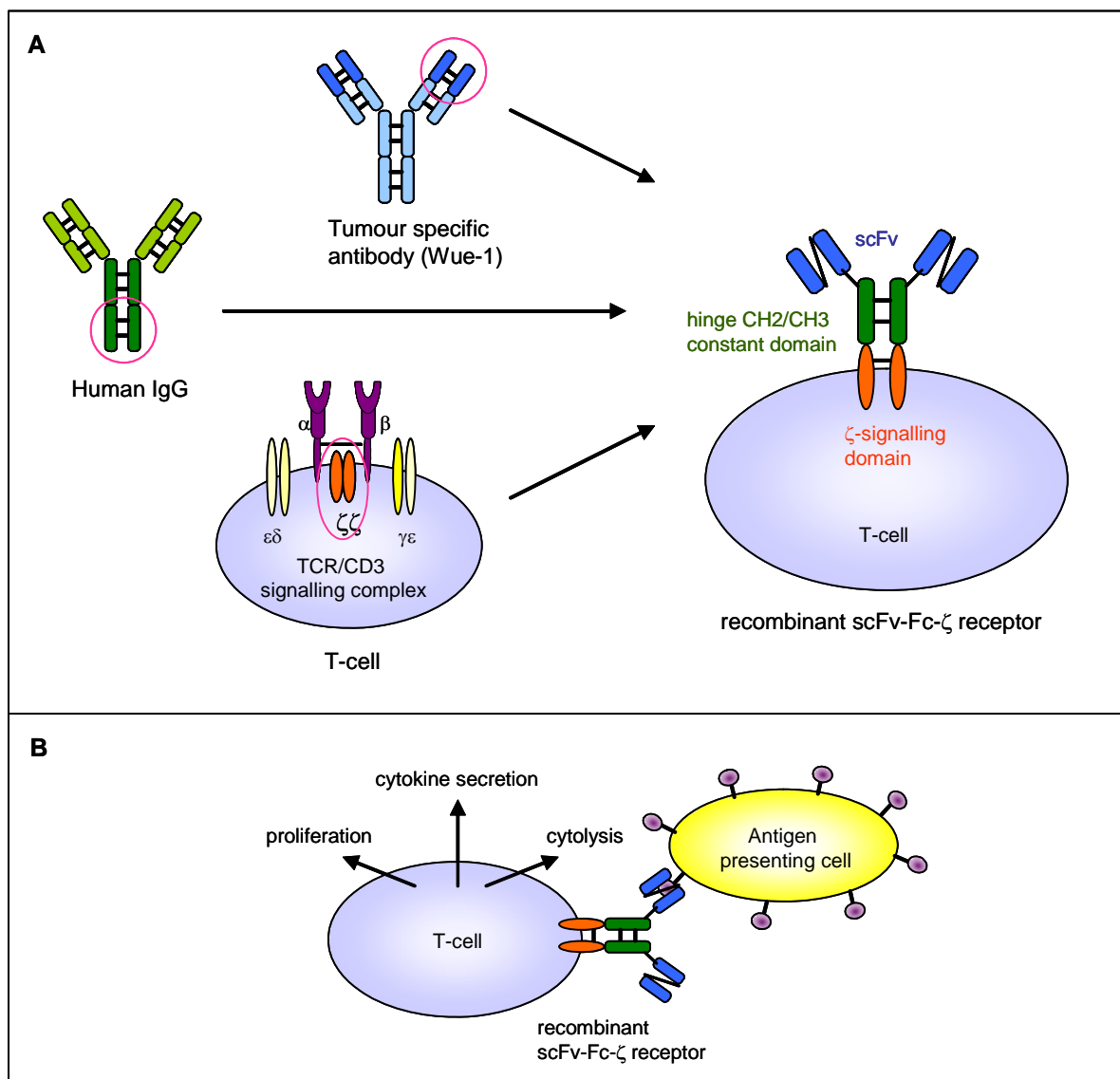
The bacterial colonies were washed off the selective agar plates (section 2.7.1.5) with LB medium, carefully scraping the cells off when necessary. Each plate represents one pool of plasmids. 200ml LB medium + 50µg/ml kanamycin (section 5.9.3) were each inoculated with 1/10 of each pool and the suspensions were grown up to an  $OD_{600nm} = 0.5$ . Spectinomycin (Sigma) was added to a final concentration of 100µg/ml and the cultures were grown on an orbital shaker at 37°C, 200rpm for up to 16hrs. 100ml of each culture were harvested by centrifugation for 5min, 4000x g, 4°C, the pellets were resuspended in icecold sucrose/Tris-HCl solution (section 5.7.7), lysozyme (Sigma) was added to a final concentration of 1mg/ml and the cells were incubated on ice for 5min. Icecold 250mM EDTA was added and the tubes were incubated on ice for 5min. After addition of 50mM Tris-HCl and incubation for 5min at 37°C the cells were placed on ice and the generation of spheroplasts was validated using a light microscope. When >80% spheroplasts were generated icecold sucrose/MgCl<sub>2</sub> solution (section 5.7.7) was added slowly and carefully. This spheroplast suspension was then aliquoted onto Ø 6cm cell culture plates (TPP), containing exponentially growing COS7 cells which have been seeded out the previous day at a density of  $1.5 \times 10^4$  cells/cm<sup>2</sup>. The Ø 6cm cell culture plates were then spun for 10min, 100x g, RT in order to pellet the spheroplasts onto the COS7 cells. A 50% PEG 1450 solution (ATCC) was added and incubated for 2min at RT. The plates were washed twice with serum-free DMEM, 3ml cell culture medium + 15µg/ml gentamycin (Biochrom) were added, and the cells were incubated at standard cell culture conditions for 5hrs. Then the medium was exchanged once and the plates were incubated for another 48-72hrs.

## **2.8 Functional assays**

### **2.8.1 T-cell reporter assay**

The immunoreceptor strategy for adoptive immunotherapy combines the advantages of MHC-independent binding to an antigen with efficient T cell activation through the T-cell receptor (TCR) ligand. For this purpose T cells are grafted with recombinant TCRs, which comprise an antibody-derived single-chain domain to bind to the antigen of interest, and an intracellular

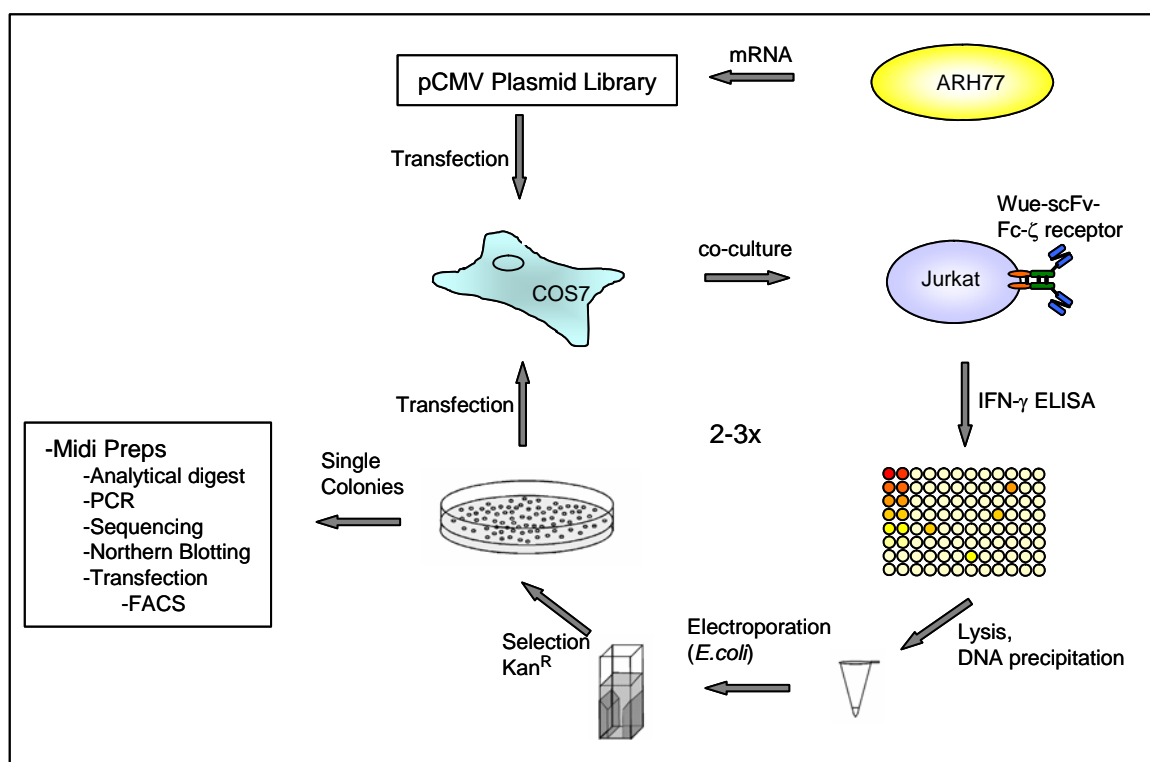
signalling domain which is obtained from the cytoplasmatic part of a membrane bound receptor e.g., the Fc $\epsilon$ RI receptor  $\gamma$ -chain or the CD3 $\zeta$  chain<sup>88, 111</sup>. Such constructs are also referred to as "T-bodies", which provide the grafted T-cells with antibody like specificity and, upon antigen-mediated receptor cross-linking, render the cells capable of inducing an antigen-specific, MHC-independent immune response. A schematic outline of the generation of a T-body and its activation is depicted in figure 2.9.



**Figure 2.9: Generation and activation of a recombinant chimeric T-cell receptor ("T-body").** **A)** The single chain variable region of anti-Wue-1 was fused to a IgG Fc hinge region and the  $\zeta$ -domain of the CD3 signalling complex. The resulting recombinant immunoreceptor bears antibody-like, MHC molecule independent specificity. **B)** Effector T-cells are transfected with the plasmid, which codes for the chimeric TCR. Upon T-body expression specific receptor crosslinking (antibody/antigen) results in MHC-independent effector cell activation, which can be monitored by proliferation- or kill- assays, or by cytokine ELISA.

Modified from Hombach *et al.*, 2002<sup>85</sup>

The approach used here is a modification of the panning procedure described in section 2.7.1. As depicted in figure 2.10 the whole experiment differs only in the way a putative positive clone is detected. In this assay, a chimeric T-cell receptor was generated, comprising the anti-Wue-1 single-chain variable domain (scWue1-Fv) fused to a chimeric CD28/CD3 $\zeta$ -domain of the T-cell signalling complex. COS7 cells were transfected with the library and co-cultured with Jurkat cells expressing the chimeric TCR. Upon binding of the antigen to sc-Wue1-Fc, the TCR mediated signalling cascade is initiated, resulting in secretion of IFN- $\gamma$ , which will subsequently be detected by ELISA.



**Figure 2.10: Functional library screen with a WUE-1 specific chimeric T-cell receptor (TCR).** The ARH7 plasmid library is transfected into COS7 cells. Screening is performed by means of co-culture with effector T-cells expressing a chimeric TCR, which contains the anti-Wue-1 single-chain variable region. Identification of putative positive clones is done by an IFN- $\gamma$  ELISA of cell culture supernatants. The experimental set-up was scaled down to 96-well format.

### 2.8.1.1 WUE-1 specific "T-bodies"

The vector backbone for cloning of the chimeric single-chain (sc) Wue-1 TCRs, namely pBullet-607 is based on the Moloney murine leukaemia virus-derived retroviral expression vector pSTITCH<sup>112</sup>, and was kindly provided by Prof. H. Abken, University of Cologne.



pBullet-607 comprises the anti-CEA (carcinoembryonic antigen) chimeric receptor BW431/26-scFv-Fc-CD28/CD3  $\zeta$ . The extracellular moiety of the receptor comprises a humanized single-chain antibody fragment (scFv) derived from the anti-CEA mAB BW431/26, and a human IgG constant domain (Fc). The transmembrane/intracellular domain is composed of the signalling moieties of CD28 and CD3 $\zeta$ <sup>88</sup> (plasmid map see figure 7.1, sequences see section 7.1).

### 2.8.1.1.1 Generation of $\kappa$ -scWue-1 and $\kappa$ -HA-scWue-1

Two recombinant chimeric scWue1-Fc-CD28/CD3 $\zeta$  receptor constructs were created, one of which was designed with a HA-tag at the N-terminus of the scWue-1 binding domain, in order to facilitate identification of positive transfectants. Amplification of the single-chain V<sub>H</sub> and V<sub>L</sub> Wue-1 moiety was carried out with 20ng template (pBluescript KS-FSS-LSP-scWue-1, provided by Dr. K. Bommert, MDC Berlin) and the cloning primers *XbaI*-wue<sub>fw</sub>, *XbaI*-HA-wue<sub>fw</sub>, and *BamHI*-wue<sub>rev</sub> (section 5.6.2), thus introducing 5'-*XbaI* and 3'-*BamHI* restriction sites and the HA-tag. For each construct (i.e. +/- HA-tag) 2x50 $\mu$ l PCR reactions were set up. PCR was set up as a two-step reaction for 10 cycles using the following program:

#### PCR reactions:

stock	final concentration	volume
template	20ng	X $\mu$ l
10 x buffer	1x	5 $\mu$ l
MgSO <sub>4</sub> (50mM)	0.4mM	0.4 $\mu$ l
dNTP mix (20mM each)	0.3mM	1.5 $\mu$ l
primer 1 (10 $\mu$ M)	0.3 $\mu$ M	1.5 $\mu$ l
primer 2 (10 $\mu$ M)	0.3 $\mu$ M	1.5 $\mu$ l
<i>Pfu</i> polymerase (5U/ $\mu$ l)	2.5U	0.5 $\mu$ l
H <sub>2</sub> O		ad 50 $\mu$ l

#### PCR program:

1)	5min	94°C
2)	15sec	94°C
3)	60sec	68°C
4)	5min	68°C
Repeat steps 2)–3) 9 times		

The PCR products were analyzed on a 1% agarose gel, the correct bands were excised, and the DNA was extracted with the "QiaEx II Agarose Gel Extraction Kit" (Qiagen). PCR was performed with the proofreading *Pfu* DNA polymerase, which possesses 3'→5' exonuclease activity that removes the 3'-A overhangs. Since 3'-A overhangs are necessary for TA-cloning 3'-A tailing was performed after the PCR amplification. 3'-A tailing and TA cloning was

performed as described in the pGEM®-T Easy manual (Promega). Positive clones were identified by PCR screening as described in section 2.5.3, using T7 and SP6 primers.

In order to ensure correct transportation of the recombinant chimeric molecule to the cell surface a  $\kappa$ -leader sequence was introduced at the N-terminus of scWue-1. The  $\kappa$ -leader sequence was removed from pBullet-607 by restriction digestion of 10 $\mu$ g plasmid DNA for 3hrs with 50U *Xba*I in 50 $\mu$ l final volume. The 60bp  $\kappa$ -leader fragment was separated on a 2% agarose gel, excised, and the DNA was recovered from the gel using the "QiaEx II" kit (Qiagen) as recommended by the supplier.

Subsequently four clones of each pGEM®-T Easy vector containing the scWue-1 inserts (+/- HA-tag) were linearized with *Xba*I, using 2 $\mu$ g miniprep DNA/digestion. Each digest was treated with 2U SAP (shrimp alkaline phosphatase, Promega) for 30min at 37°C in order to dephosphorylate the 5'-ends. The DNA concentrations of pGEM®-T Easy vector (containing the scWue-1 inserts) and  $\kappa$ -leader were estimated. Ligations were set up at a molar ratio of 1:3 (vector:insert) i.e. ~100ng digested plasmid DNA and ~5ng gel-purified  $\kappa$ -leader, in a final volume of 10 $\mu$ l. The reactions were incubated o/n at 16°C and 5 $\mu$ l of each ligation was then used for transformation of competent *E. coli* DH5 $\alpha$  cells. Clones containing an insert of the correct size (820bp) were identified by PCR screen (section 2.5.3) using T7 and SP6 primers. Since the  $\kappa$ -leader was not inserted by directional cloning, an analytical restriction digest was carried out with *Nco*I in order to confirm the correct orientation of the sequence (table 2.1 and figure 2.11). The orientation of the  $\kappa$ -(HA)-scWue-1 insert as a whole was monitored by double digestion with *Aat*II and *Bam*HI. Clones containing a correct insert were then sequenced and analyzed, using the Lasergene software (DNA Star).

**Table 2.1:** Analytical digest of the pGEM®-T Easy- $\kappa$ -(HA)-scWue-1 clones (possible fragments)

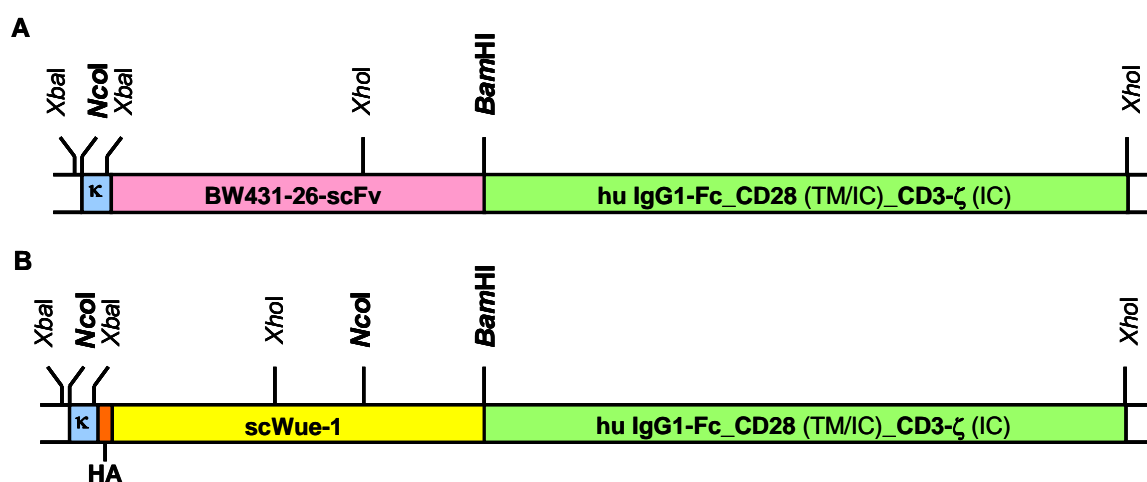
	<b>A</b> ( $\kappa$ -leader in correct orientation )	<b>B</b> ( $\kappa$ -leader in correct orientation )	C	D
<i>Aat</i> II/ <i>Bam</i> HI	873, 2983 bp	48, 3808 bp	873, 2983 bp	48, 3808 bp
<i>Nco</i> I	36, 586, 3234 bp	261, 586, 3009 bp	86, 536, 3234 bp	261, 536, 3059 bp



**Figure 2.11: Restriction map of the  $\kappa$ -HA-scWue-1 cassette in pGEM®-T Easy.** The correct orientation of the whole insert was confirmed by double digestion with *AatII* and *BamHI* (fragment sizes for correct orientation: 873bp and 2983bp). The orientation of the  $\kappa$ -leader within the insert was controlled by restriction digestion with *NcoI* (fragment sizes for correct orientation: 36bp, 586bp and 3234bp). Total size of the plasmid: 3856bp; size of  $\kappa$ -HA-scWue-1 cassette: ~820bp.

### 2.8.1.1.2 Construction of the $\kappa$ -(HA)-scWue-1 expression vector

The expression cassette for the scWue-1 chimeric TCR was created by replacing the anti-CEA portion (BW431-26-scFv) of pBullet-607 by the  $\kappa$ -scWue-1 or the  $\kappa$ -HA-scWue-1 inserts, respectively (figure 2.12). 5 $\mu$ g pBullet-607 were digested with 50U *NcoI* and 50U *BamHI* in a final volume of 50 $\mu$ l at 37°C for 2hrs to release the anti-CEA scFv fragment. Subsequently 3U SAP (Promega) were added, and the digests were incubated for another 30min in order to dephosphorylate the 5'-ends. The digest were separated on a 1% agarose gel and the vector backbone (~6.8kb) was excised and purified. The  $\kappa$ -(HA)-scWue-1 inserts were cloned into the pBullet backbone directionally, i.e. as *NcoI* - *BamHI* fragments. Since an additional *NcoI* restriction site is present within the scWue-1 sequence it was necessary to carry out a partial *NcoI* digest.



**Figure 2.12: Expression cassettes of recombinant chimeric T-cell receptors (TCRs).** **A)** The parental vector pBullet-607 contains a CEA-specific binding sequence (BW431-26-scFv), which was removed by double digestion with *NcoI*/*BamHI*. **B)** The BW431-26-scFv fragment was replaced with the  $\kappa$ -HA-scWue-1 sequence to create an expression cassette for a WUE-1 specific TCR.

TM: transmembrane; IC: intracellular; CEA: carcinoembryonic antigen

For the partial digest 50µg of each plasmid containing  $\kappa$ -scWue-1 (+/- HA-tag) were linearized for 2hrs with 100U *Bam*HI in a final volume of 100µl. Complete digestion was monitored on an agarose gel, and five 1.5ml tubes were prepared for each plasmid, containing 30µl H<sub>2</sub>O and 50µl phenol:chloroform. The digested DNA was ethanol precipitated and resuspended in 90µl H<sub>2</sub>O. To the resuspended, linearized DNA 10µl 10x restriction buffer and 2U *Nco*I were added and the digests were incubated at 37°C. 20µl samples were removed from the reactions each after 5min, 8min, 12min, 15min, and 18min and immediately transferred to phenol:chloroform. All samples were phenol:chloroform extracted and ethanol precipitated as described elsewhere<sup>96</sup>. The precipitated samples were resuspended and run out on a 1% agarose gel. The 820bp bands were excised, pooled for each construct, and the DNA was recovered using the "QiaEx II" kit (Qiagen) as described in the manual.

The concentrations of the pBullet backbone and the  $\kappa$ -(HA)-scWue-1 inserts were determined semi-quantitatively on a 1% agarose gel alongside 5µl Smartladder (Eurogentec). Ligations were set up at a molar ratio of 1:3 (vector:insert), i.e. 100ng vector (6800bp) + 35ng insert (820bp). Ligations were performed o/n at 16°C. 3µl of each ligation mix were transformed into competent *E. coli* DH5 $\alpha$ . From each construct five colonies were picked, minipreps were prepared and analyzed by restriction digestion with *Nco*I/*Bam*HI. Clones containing an insert of the right size were further analyzed by sequencing of the entire  $\kappa$ -(HA)-scWue1-Fc-CD28/CD3 $\zeta$  cassette. The sequences were confirmed by DNA sequence alignments, using BLAST and the Lasergene software (DNASar). The constructs were renamed pBullet- $\kappa$ -Wue1 and pBullet- $\kappa$ -HA-Wue1 and were subsequently used for the functional assay.

### **2.8.1.2 Transfection, transformation**

COS7 cells were transfected using the DEAE-dextran/chloroquine-method as described in section 2.7.1.2. Jurkat cells (T cells) were transiently transfected with the expression vectors pBullet-607, pBullet- $\kappa$ -Wue1, and pBullet- $\kappa$ -HA-Wue1, respectively. The cells were adjusted to 5x10<sup>5</sup> cells/ml one day prior to transfection and were then transfected by electroporation, using 10µg DNA with 1x10<sup>7</sup> cells per electroporation at 250V, 960µF.

Transformation of electrocompetent *E. coli* was performed to amplify the plasmid DNA, which was recovered from the 96-well plates with the Hirt method (section 2.7.1.4). Half the volume of each recovered plasmid solution and 40µl aliquots of electrocompetent *E. coli* were

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used for each electroporation at 2.5kV, 200Ω, 25μF. Subsequently the entire volume of the transformations was plated on selective agar.

### **2.8.1.3 Co-culture**

In the preliminary tests "triple" co-culture was performed with non-transfected COS7 cells, pBullet-607 or pBullet-κ-(HA)-Wue1 transfected Jurkat (T-) cells and WUE-1 positive cells. Usually,  $1.5 \times 10^4$  COS7 cells/cm<sup>2</sup> were seeded into each well of a 96-well cell culture plate (i.e.  $5 \times 10^3$  cells/well). The cells were left to attach o/n. Then  $2.5 \times 10^4$  transfected Jurkat cells and  $2.5 \times 10^4$  MM cells were added to each well. Control reactions were set up with mock transfected (i.e. w/o DNA) Jurkat cells, WUE-1<sup>-</sup> control cells, or anti-hu IgG Fc (Dianova, 1:100) was added instead of WUE-1<sup>+</sup> cells. The cultures were incubated for 24h at standard cell culture conditions.

### **2.8.1.4 IFN-γ ELISA**

The IFN-γ ELISA was performed using a commercially available kit (Immunotools), according to the manual. Wash steps were done with 100μl IFN-γ wash buffer/well (section 5.7.6). The wells were blocked with 250μl IFN-γ blocking buffer/well (section 5.7.6) for 2hrs at RT, gently rocking. Sample volume was 100μl cell culture supernatant. Special care was taken to not transfer any cells to the ELISA plates. When necessary the samples were centrifuged for 1min max. speed to pellet the cells. For detection the wells were incubated with 100μl NeutrAvidin™-HRP (Pierce, 1:1000) followed by addition of 100μl OPD/H<sub>2</sub>O<sub>2</sub> substrate solution (section 5.7.6). The reactions were stopped by addition of 50μl/well stop solution (2.5M H<sub>2</sub>SO<sub>4</sub>; section 5.7.6). The plates were then measured at  $\lambda_{492\text{nm}}$  in a "Benchmark" plate reader (Biorad).

## **2.8.2 Cytotoxicity assay**

Tests for induction of specific T-cell mediated lysis by the bispecific single-chain antibodies bssc-anti-Wue-1xCD3 and bssc-anti-CD19xCD3 were performed with NCI-H929 (WUE-1<sup>+</sup>, CD19<sup>-</sup>) and Nalm-6 (WUE-1<sup>-</sup>, CD19<sup>+</sup>) target cells, respectively. The bssc-anti-EpCAMxCD3

antibody served as negative control. T-cells (effector cells) were isolated from human buffy coats as described in section 2.1.2. The target cells were labelled with the cell-membrane dye PKH-26 (Sigma) as recommended by the manufacturer. Labelled target cells and unlabelled effector cells were mixed at a ratio of 10:1 (effector:target) in their appropriate medium in 96-well flat bottom culture plates (Nunc). Antibodies were added to the following final concentrations: bssc-anti-Wue-1xCD3 (2µg/ml), bssc-anti-CD19xCD3 (1µg/ml), and bssc-anti-EpCAMxCD3 (2µg/ml). The plates were incubated at standard cell culture conditions for 48h. Subsequently the cells were harvested, washed twice with PBS, stained with propidium iodide (PI; section 5.7.5) at a final concentration of 2.5µg/ml, and analyzed by FACS. Specific cytotoxicity was calculated by comparing the relative number of PI/PKH<sup>+</sup> cells (viable target cells) in the bssc-anti-Wue-1xCD3 and bssc-anti-CD19xCD3 samples, respectively with the viable target cell fraction in the bssc-anti-EpCAMxCD3 control samples using the equation below:

$$\text{Specific lysis} = \left[ 1 - \frac{\text{No. viable target cells (bssc - anti - Wue - 1xCD3)}}{\text{No. viable target cells (bssc - anti - EpCAMxCD3)}} \right] \times 100$$

In order to normalize the decrease in the viable target cell fractions against possible increase of the T-cell fraction (due to proliferation) the absolute number of cells in culture was determined for each sample by adding a known number of CaliBRITE beads (BD Biosciences). CaliBRITE beads and cells can be distinguished by flow cytometry based on their forward and side scatter properties. The ratio of live cells to beads was then used to calculate the total number of cells in each sample<sup>113</sup>.