# 2 Material & Methods

## 2.1 Material

### 2.1.1 Technical equipment and disposable material

#### 2.1.1.1 Technical equipment

| Equipment | Type | Source |
|---|---|---|
| Autoclave | Automat 21/2 | Webeco, Bad Schwartau |
| | Varioklav® | H+P Labortechnik, Oberschleißheim |
| Balance | BP 310 p | Sartorius, Göttingen |
| | L 610 D | Sartorius, Göttingen |
| Biosafety cabinet | Antair BSK | Kendro, Langenselbold |
| | BioCard Hood | The Baker Company, Sanford, USA |
| Centrifuges | Biofuge Pico | Heraeus, Hanau |
| | Heraeus Megafuge 1.0 R | Heraeus, Hanau |
| | Sigma 2K15 Centrifuge | B. Braun, Melsungen |
| | Sigma 3K30C | B. Braun, Melsungen |
| | Centrifuge 5403 | Eppendorf, Hamburg |
| | Centrifuge 5415 C | Eppendorf, Hamburg |
| Developer (X-ray films) | Curix 60 | Agfa-Gevaert Group, Mortsel, Belgium |
| Dewar flask | KGW isotherm | Roth, Karlsruhe |
| Freezer | -20°C | Bosch, Stuttgart |
| | -20°C | Liebherr, Biberach a.d. Riß |
| | -70°C | Heraeus, Hanau |
| Freezer (liquid nitrogen) | | Union Carbide, Houston, USA |
| Gel documentation | CN-3000.WL/LC | Peqlab, Erlangen |
| Gel electrophoresis | ComPhor Mini/Midi | Biozym, Oldendorf |
| | Mini Protean® 3 | Bio-Rad, Munich |
| Glass ware | flasks, bottles, etc. | Schott Glas, Mainz |
| Heater/magnetic stirrer | IKA® RH basic 2 | IKA® Werke, Staufen |
| Heating block | Thermomixer comfort | Eppendorf, Hamburg |
| Incubators | | New Brunswick Scientific, Edison, USA |
| | | Heraeus, Hanau |
| Microscopes | Fluorescence microscope (Axioskop 20) | Zeiss, Jena |
| | Invert microscope (Axiovert 25) | Zeiss, Jena |
| | confocal laser scanning microscope (cLSM 510 Meta) | Zeiss, Jena |
| | Light microscope (Televal 3) | Zeiss, Jena |

| | | |
|---|---|---|
| Neubauer cell counting chamber | | Roth, Karlsruhe |
| Photometer | BioPhotometer | Eppendorf, Hamburg |
| Pipette assistance | Accujet® pro | Brand, Wertheim |
| Pipettes (bis 10 µL, 100 µL, 1,000 µL) | | Eppendorf, Hamburg<br>Finnpette<br>SLpette |
| Power pack | Standard Power Pack P25 | Biometra, Göttingen |
| | Model 200/2.0 | Bio-Rad, Munich |
| Refrigerator | | Bosch, Stuttgart |
| Rotation shaker | | Kisker, Steinfurt |
| Sequencer | ABI PRISM® 3100 Genetic Analyzer | Applied Biosystems, Foster City, USA |
| Sonifier | Branson Sonifier 450 | Branson, Danbury, USA |
| Spattle | | Roth, Karlsruhe |
| Thermocycler | Biometra T gradient cycler | Biometra, Göttingen |
| Thermal printer | DPU-414 | Seiko Instruments Inc., Japan |
| Tweezers | | Roth, Karlsruhe |
| UV-table | Transilluminator TC-312A | Spectroline, Westbury, USA |
| Vertical shaker | RS-PL 28-10 | Heto, Alerød, Denmark |
| | 3013 | GFL, Burgwedel |
| Vortexer | Vortex-Genie 2 | Scientific Industries, Bohemia, USA |
| Water heating bath | Type 1o13 | GFL, Burgwedel |
| Western blotting equipment | Fastblot B 34 | Biometra, Göttingen |

#### 2.1.1.2 Disposable material

| Type of article | Source |
|---|---|
| Cell culture bottles with filter cap (25 $cm^3$; 75 $cm^3$; 175 $cm^3$) | Nunc, Wiesbaden |
| Cell culture plates (6-, 12-, 24-, 48-, 96-well) | Nunc, Wiesbaden |
| Cell scraper (23 cm) | Nunc, Wiesbaden |
| CL-Xposure™ Film (18 x 24 cm) | Pierce, Rockford, USA |
| Cover glass slides (12 mm, round) | Roth, Karlsruhe |
| Cryotubes (1.8 mL) | Nunc, Wiesbaden |
| Cuvettes (disposable) | Eppendorf, Hamburg |
| ELISA Maxisorb 96-well | Nunc, Wiesbaden |
| Microcon® Centrifugal Filter Devices (5 ; 10 kDa) | Millipore, Billerica, USA |
| Nitrocellulose membranes (0.2 µm, 0.45 µm) | Schleicher & Schuell, Dassel |
| Parafilm | American National Can, Greenwich, USA |
| PCR reaction tubes 0.2 mL; 8-strips | Biozym, Oldenburg |
| Pipette filter tips (until 10 µL, 100 µL, 1,000 µL) | Biozym, Oldenburg |
| Pipette tips (until 10 µL, 100 µL, 1,000 µL) | Eppendorf, Hamburg |
| PVDF membranes/Westran® S (0.2 µm) | Schleicher & Schuell, Dassel |
| Reaction tubes 1.5 mL; 2.0 mL | Eppendorf, Hamburg |
| Reaction tubes 15 mL; 50 mL | Nunc, Wiesbaden |

| | |
|---|---|
| Scalpel | B. Braun Aesculap, Tuttlingen |
| Seropipettes (until 1 mL; 2 mL; 5 mL; 10 mL; 25 mL; 50 mL) | Nunc, Wiesbaden |
| Sterile filter (0.22 µm) | Millipore, Billerica, USA |
| Whatman blotting paper (102 mm x 133 mm) | Schleicher & Schuell, Dassel |
| X-ray film, BioMax MR (18 x 24 cm) | Kodak, Stuttgart |

### 2.1.2 Chemicals and enzymes

#### 2.1.2.1 Chemicals

| Chemicals | Source |
|---|---|
| Acetone | Roth, Karlsruhe |
| Acetic acid | Roth, Karlsruhe |
| Agar | Invitrogen, Karlsruhe |
| Agarose (NuSieve®3:1) | Biozym, Oldendorf |
| Ammoniumperoxodisulfate (APS) | Roth, Karlsruhe |
| Ampicillin | Sigma-Aldrich, Munich |
| Aqua dest. | Millipore, RKI |
| Aqua mol. (molecular grade) | Eppendorf, Hamburg |
| Bovine serum albumin (BSA) | New England Biolabs, Frankfurt a.M. |
| Bromphenol blue (Tetrabromophenol sulfonephthalein) | Sigma-Aldrich, Munich |
| Calcium chloride ($CaCl_2$) | Roth, Karlsruhe |
| Carbenicillin | Roth, Karlsruhe |
| Carboxymethyl-cellulose sodium salt (CMC) | BDH, Poole, UK |
| Chloramphenicol | Sigma-Aldrich, Munich |
| Chloric acid (HCl) | Merck, Darmstadt |
| Chloroform | Roth, Karlsruhe |
| Coomassie Brilliant Blue R-250 | Serva, Heidelberg |
| Dimethylsulfoxid (DMSO) | Roth, Karlsruhe |
| Disodiumhydrogenphosphate | Merck, Darmstadt |
| Dithiothreolin (DTT) | Sigma-Aldrich, Munich |
| dNTP mixture | Eppendorf, Hamburg |
| Ethanol (96 %) | Roth, Karlsruhe |
| Ethidium bromide | Roth, Karlsruhe |
| Ethylendiamine tetraaceticacid (EDTA) | Serva, Heidelberg |
| Fetal calf serum (FCS) | PAA Laboratories, Linz, Austria |
| Ficoll-paque Plus | Amersham, Freiburg |
| Formaldehyde | Roth, Karlsruhe |
| FuGENE® HD transfection reagent | Roche Diagnostics, Basel, Switzerland |
| Glucose | Sigma-Aldrich, Munich |
| Glycine | Merck, Darmstadt |
| Imidazole | Roth, Karlsruhe |
| Isoamyl alcohol | Roth, Karlsruhe |
| Isopropanol | Merck, Darmstadt |
| Isopropyl β-D-thiogalactosidase (IPTG) | Roth, Karlsruhe |
| Iodoacetamid | Sigma-Aldrich, Munich |
| Kanamycin | Roth, Karlsruhe |

| | |
|---|---|
| L-glutamine | PAA Laboratories, Linz, Austria |
| Lactose | Sigma-Aldrich, Munich |
| Lipofectamin™ 2000 | Invitrogen, Karlsruhe |
| Magnesium chloride ($MgCl_2$) | Invitrogen, Karlsruhe |
| Manganese chloride ($MnCl_2$) | Sigma-Aldrich, Munich |
| Methanol | Roth, Karlsruhe |
| Methionine [$^{35}$S] labelled | GE Healthcare, |
| Milk powder | Trade Service Int., Zeven, Netherlands |
| MOPS | Sigma-Aldrich, Munich |
| Naphthol blue black | Sigma-Aldrich, Munich |
| Nitrogen (liquid) | Linde, Berlin |
| Paraformaldehyde (PFA) | Roth, Karlsruhe |
| Penicillin/Streptomycin | PAA Laboratories, Linz, Austria |
| Phenol | Roth, Karlsruhe |
| Polyacrylamide (Rotiphorese® Gel 30; 37.5:1) | Roth, Karlsruhe |
| Poly-L-lysine | Sigma-Aldrich, Munich |
| Ponceau S | Roth, Karlsruhe |
| Potassium acetate (KAc) | Roth, Karlsruhe |
| Potassium chloride (KCl) | Roth, Karlsruhe |
| Propane-1,2,3,-triol (Glycerine) | Merck, Darmstadt |
| Protein A sepharose beads | Amersham, Freiburg |
| Protein G sepharose beads | Amersham, Freiburg |
| Protease-Inhibitor Cocktail Set I | Calbiochem, San Diego, USA |
| Protease-Inhibitor Cocktail Set II | Calbiochem, San Diego, USA |
| Protease-Inhibitor Cocktail Set III | Calbiochem, San Diego, USA |
| Rubidium chloride (RbCl) | Sigma-Aldrich, Munich |
| Sample buffer | EUROIMMUN, Lübeck |
| Silver nitrate | Merck, Darmstadt |
| Sodium carbonate anhydrous | Roth, Darmstadt |
| Sodium chloride (NaCl) | Merck, Darmstadt |
| Sodium dodecylsulfate (SDS) | Serva, Heidelberg |
| Sodium hydrogenphosphate | Merck, Darmstadt |
| Sodium hydroxid (NaOH) | Merck, Darmstadt |
| Sodium thiosulfate | Roth, Karlsruhe |
| Sucrose | Sigma-Aldrich, Munich |
| SuperSignal® West Dura Extended Chemiluminescent Substrate (Luminol) | Pierce, Rockford, USA |
| SuperSignal® West Femto Chemiluminescent Substrate (Luminol) | Pierce, Rockford, USA |
| SuperSignal® West Pico Chemiluminescent Substrate (Luminol) | Pierce, Rockford, USA |
| Tetramethylbenzidine (TMB) | Sigma-Aldrich, Munich |
| N,N,N´,N´-Tetramethyl ethylenediamin (TEMED) | Roth, Karlsruhe |
| Trichloric acetic acid | Sigma-Aldrich, Munich |
| Tris hydroxymethyl aminomethane (Tris) | Roth, Karlsruhe |
| Triton X-100 | Sigma-Aldrich, Munich |
| Trypane blue | Sigma-Aldrich, Munich |
| Trypsin | PAA Laboratories, Linz, Austria |
| Tween® 20 | Merck, Darmstadt |
| Urea | Sigma-Aldrich, Munich<br>Roth, Karlsruhe |

| | |
|---|---|
| β-cyclodextrin | Sigma-Aldrich, Munich |
| β-mercaptoethanol (ME) | Sigma-Aldrich, Munich |

#### 2.1.2.2 Enzymes

| Enzyme | Source |
|---|---|
| Benzonase® | Novagen, Madison, USA |
| BstXI | Fermentas, St. Leon-Rot |
| Calf intestine alkaline phosphatase (CIP) | Fermentas, St. Leon-Rot |
| DNase | Ambion, Huntingdon, UK |
| EcoRI | New England Biolabs, Frankfurt |
| Endoglycosidase H | New England Biolabs, Frankfurt |
| Lysozyme | Sigma-Aldrich, Munich |
| NotI | New England Biolabs, Frankfurt |
| Platinum® *Taq*-Polymerase | Invitrogen, Karlsruhe |
| Proteinase K | Sigma-Aldrich, Munich |
| RNase A | Roth, Karlsruhe |
| RNasin | Invitrogen, Karlsruhe |
| Superscript II and III reverse transcriptase | Invitrogen, Karlsruhe |
| SspI | Fermentas, St. Leon-Rot |
| T4-DNA-Ligase | Invitrogen, Karlsruhe |

### 2.1.3 Media

#### 2.1.3.1 Cell culture media

| Medium | Source |
|---|---|
| DMEM (Dulbecco´s Modified Eagles Medium) | GIBCO, Karlsruhe |
| RPMI 1640 (Roswell Park Memorial Institute 1640 Medium) | GIBCO, Karlsruhe |

DMEM and RPMI were supplemented with 10% FCS, 2 mM L-glutamine and 25 units/mL penicillin/streptomycin for passaging cell lines. When cells were transfected or infected no antibiotics were supplemented. Possible mycoplasma contamination was regularly controlled by PCR of supernatant as described elsewhere (van Kuppeveld et al., 1993).

#### 2.1.3.2 Others

| Medium | Ingredients | Source |
|---|---|---|
| Immu-Mount | | Thermo Shandon, Pittsburgh, USA |
| DakoCytomation | | DakoCytomation, Glostrup, Denmark |

| S.O.C. medium | | Invitrogen, Karlsruhe |
|---|---|---|
| LB medium* | 10 g/L bacto trypton,<br>5 g/L bacto yeast extract,<br>10 g/L sodium chloride,<br>pH 7.5 (with NaOH ) | - |

*LB medium (liquid) was autoclaved and supplemented with 100 µg/mL ampicillin, 50 µg/mL carbenicillin, 25 µg/mL kanamycin or 35 µg/mL chloramphenicol depending on the application. For solid LB medium 1.5% (w/v) agar was added.

### 2.1.4 Oligonucleotides, nucleic acid and protein standards, plasmids

#### 2.1.4.1 Oligonucleotides

| Name/ID | Sequence | +/- | Position/ Accession no. | nt | Tm | Order no. |
|---|---|---|---|---|---|---|
| Primers for sg mRNA PCR | | | | | | |
| NL63 F mis | TTT TgT ATg TAT AgA TAg AgA gTT TTC TTA TTT Ag | + | 11-45/ NC_005831 | 35 | 63.1 | 612659 |
| NL63 1a R | CTA ACC AgT TTA CAA ACT TgA Cg | - | 128-150 | 23 | 62.0 | 630851 |
| NL63 S R | AgC AAA ATC AAg AAA AgT TTC ATT T | - | 20470-20494 | 25 | 64.4 | 612660 |
| NL63 ORF3 R | CTT TTC AAA TTC gTA ATA Agg AAg TT | - | 24502-24527 | 26 | 63.4 | 612661 |
| NL63 E R | gAA ggA ACA TCT TCg TAT AgT TgA g | - | 25185-25209 | 25 | 62.3 | 612662 |
| NL63 M R | gAg gCA CAC TAC TAT TAg ACA TCg TT | - | 25438-25463 | 26 | 64.4 | 612663 |
| NL63 N R | CCA ATT TAC ACT AgC CAT TTT gT | - | 26128-26150 | 23 | 63.0 | 612664 |
| Primers for generation of ORF3 vector constructs | | | | | | |
| 5´Leader-NL | gAC TTT gTg TCT ACT CTT C | + | 45-63 | 19 | 49.5 | 542806 |
| 5´NL-O3-myc | ATg gAA CAA AAA CTT ATT TCT gAA gAA gAC TTg CCT TTT ggT ggC CTA TTT CAA C | + | 24543-24566 | 55 | 72.1 | 542809 |
| 5´NL-O3x | CAC CAT gCC TTT Tgg Tgg CCT ATT TCA AC | + | 24542-24566 | 29 | 72.1 | 542810 |
| 5´NL63-1 | CTT TTT ggT ATg TAT CTT gAC Ag | + | 24806-24828 | 23 | 58.6 | 552866 |
| 5´NL63-C | gTT ggC TAT TAT gCC TAT CTC | + | 24869-24889 | 21 | 58.0 | 557146 |
| 5´NL63-Cx | CAC CgT Tgg CTA TTA TgC CTA TCT C | + | 24869-24889 | 25 | 68.7 | 557147 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 5´NL63-Ctx | CAC CCT ATg CTT CgT TTC Agg CAA g | + | 24892-24912 | 25 | 72.1 | 578462 |
| 5´NL63-Ct | ATg CTA TgC TTCg TTT CAg gCA Ag | + | 24892-24912 | 24 | 70.8 | 578461 |
| 5´63-O3-HIND | CCC AAg CTT TCC TTT Tgg Tgg CCT ATT TC | + | 24545-24566 | 29 | 72.1 | 590314 |
| 5´Eco-FLAG_O3-63 | gC AgC AgA ATT CAT ggA CTA CAA ggA CgA CgA TgA CAA gCC TTT Tgg Tgg CCT ATT TCA ACT TAC | + | 24544-24570 | 65 | 60.0 | 761594 |
| 3´Not-O3-63 | CC TCC TgC ggC CgC TCA ATT AAT CgA Agg AAC ATC TTC gTA TAg | - | 25190-25219 | 44 | 60.0 | 761595 |
| 3´NL-O3s | ATT AAT CgA Agg AAC ATC | - | 25199-25216 | 18 | 51.2 | 542807 |
| 3´NL-O3 | ACA Agg AgC CAT AAA ATg | - | 25244-25261 | 18 | 56.2 | 542808 |
| 3´NL63-2 | CTg TCA AgA TAC ATA CCA AAA Ag | - | 24806-24828 | 23 | 58.6 | 552867 |
| Primers for generation of eukaryotic vector gene constructs | | | | | | |
| 5´NL63-E-GFP | TTC CTT CgA TTA ATT gAT gAC | + | 25203-25223 | 21 | 51.0 | 734877 |
| 5´NL63-M-GFP | TCT AAT AgT AgT gTg CCT C | + | 25445-25463 | 19 | 49.0 | 734878 |
| 5´NL63-N-GFP | gCT AgT gTA AAT Tgg gCC | + | 26136-26153 | 18 | 49.0 | 734879 |
| 5´NL63-O3-GFP | CCT TTT ggT ggC CTA TTT C | + | 24545-24563 | 19 | 51.0 | 734880 |
| 3´NL63-EeK | gAC ATT TAg TAC TTC AgC Tgg AAC | - | 25407-25430 | 24 | 61.7 | 630852 |
| 3´NL63-MeK | gAT TAA ATg AAg CAA CTT CTC TCT TTC | - | 26093-26119 | 27 | 64.1 | 630853 |
| 3´NL63-NeK | ATg CAA AAC CTC GTT gAC AAT | - | 27243-27263 | 21 | 65.0 | 630854 |
| Primers for generation of prokaryotic vector gene constructs | | | | | | |
| 5´NL63-EpK | CAC CTT CCT TCg ATT AAT TgA TgA C | + | 25203-25223 | 25 | 66.1 | 630855 |
| 5´NL63-MpK | CAC CTC TAA TAg TAg TgT gCC TC | + | 25445-25463 | 23 | 60.5 | 630856 |
| 5´NL63-NpK | CAC CgC TAg TgT AAA TTg ggC | + | 26136-26153 | 21 | 67.0 | 630857 |
| 3´NL63-EpK R | TTA gAC ATT TAg TAC TTC AgC Tgg | - | 25410-25433 | 24 | 60.3 | 642413 |
| 3´NL63-MpK R | TTA gAT TAA ATg AAg CAA CTT CTC | - | 26099-26122 | 24 | 58.9 | 642412 |
| 3´NL63-NpK R | TTA ATg CAA AAC CTC gTT gAC | - | 27246-27266 | 21 | 63.0 | 642414 |
| Vector primers | | | | | | |
| 5´pEGFP-forw | gTC CTg CTg gAg TTC gTg ACC | + | 1270-1290/ U57606 | 21 | 69.5 | 578463 |
| 5´pEGFP-rev | CAT TCA TTT TAT gTT TCA ggT TCA g | - | 1484-1508/ U57606 | 25 | 63.4 | 578464 |
| 5´T7 | TAA TAC gAC TCA CTA TAg gg | + | K4800-01* (Catalogue no.) | 20 | 52.1 | 592292 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 3´T7 reverse | TAg TTA TTg CTC AgC ggT gg | - | K100-01* (Catalogue no.) | 20 | 64.1 | 584141 |
| 3´Bgh rev | TAg AAg gCA CAg TCg Agg | - | K4800-01* (Catalogue no.) | 18 | 60.1 | 639174 |
| GFP forward | CgA CAC AAT CTg CCC TTT Cg | + | K4810-01* (Catalogue no.) | 20 | 68.7 | 687720 |
| pCAGGS-forw | CCT TCT TCT TTT TCC TAC Ag | + | 1655-1674 | 20 | 48.0 | n.a. |
| pCAGGS-rev | CCT TTA TTA gCC AgA AgT CAg | - | 1829-1849 | 21 | 50.0 | n.a. |
| Primers for detection of ACE2 | | | | | | |
| ACE2 F | gCC CAA CCC AAg TTC AAA g | + | 2-20/ NM_021804 | 19 | 51.0 | 503882 |
| ACE2 R | CCT CAA Tgg Tgg ACT gAg CA | - | 151-170/ NM_021804 | 20 | 54.0 | 503884 |
| Primers for detection of SARS-CoV | | | | | | |
| pp1a F | gCC gTA gTg TCA gTA TCA TCA CC | + | 4609-4631/ AY_274119 | 23 | 56.6 | n.a. |
| pp1a R | AAT Agg ACC AAT CTC TgT AAg AgC C | - | 4741-4717/ AY_274119 | 25 | 56.7 | n.a. |
| Probes | | | | | | |
| pp1a TM (SARS-CoV probe) | **F**-TCA CTT CgT CAT CAA AgA CAT C X**T** gAg gAg C p | + | 4661-4690/ AY_274119 | 30 | 66.2 | ABI |
| NL63 MGB (probe) | 6-**F**-CTT TgT gTC TAC TCT TCT-**MGB** | + | 47-65/ NC_005831 | 18 | 68.0 | ABI |

Primers were provided by TIBMolbiol, Berlin; *Vector primers were ordered according to sequences from the manual instructions of the Invitrogen kits (see catalogue no.).
Abbreviations: Tm: melting temperature; n.a.: not available; **F**: 6-carboxyfluorescein attached to 5´-terminus (FAM), **T**: 5-carboxytetramethylrhodamine (5-TAMRA) attached to 5-ethylamino-dThymidin. **MGB**: minor groove binder anchor; ABI: Applied Biosystems, Warrington, UK.

### 2.1.4.2 Nucleic acid- and protein standards

| Name | Source |
|---|---|
| 100-Bp Extended Range Ladder | Invitrogen, Karlsruhe |
| 1 kb PLUS Ladder | Invitrogen, Karlsruhe |
| MultiMark™ Multi-Colored Standard | Invitrogen, Karlsruhe |
| PageRuler™ Prestained Protein Ladder | Fermentas, St. Leon-Roth |
| TrackIt™ 100 bp DNA ladder | Invitrogen, Karlsruhe |
| TrackIt™ 1 Kb Plus DNA ladder | Invitrogen, Karlsruhe |

### 2.1.4.3 Plasmids

| Name | Type | Source |
|---|---|---|
| pcDNA3.1/V5-His-TOPO® | Eukaryotic vector | Invitrogen, Karlsruhe |
| pET100/D-TOPO® | Prokaryotic vector | Invitrogen, Karlsruhe |
| pCAGGS-MCS | Eukaryotic vector | Prof. Dr. S. Becker, RKI, Berlin |
| pcDNA3.1/GFP-NT-TOPO® | Eukaryotic vector | Invitrogen, Karlsruhe |
| pEGFP-C2 | Eukaryotic vector | BD Biosciences Clontech, Heidelberg |

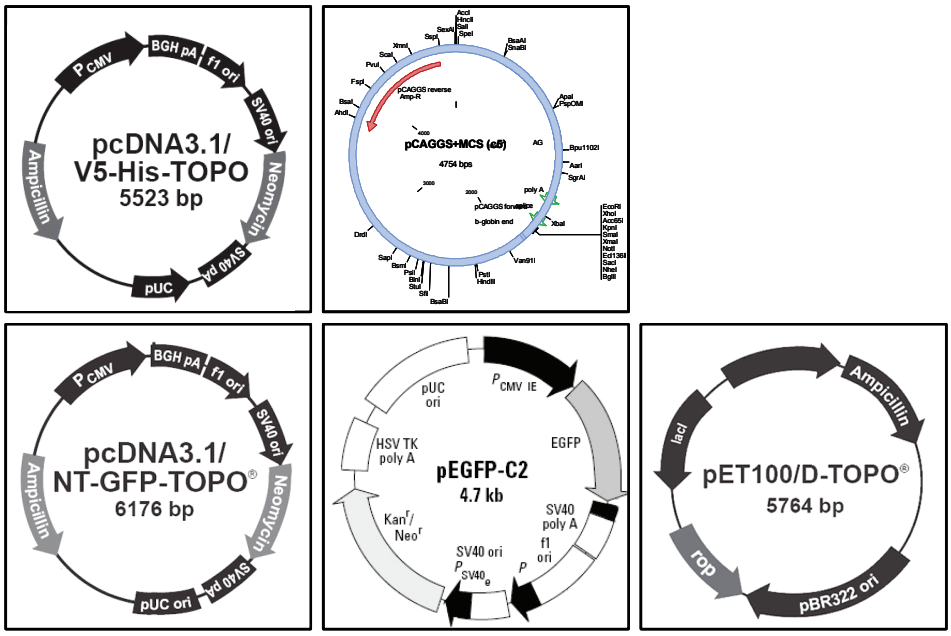


**FIG. 6. Maps of applied vectors for prokaryotic and eukaryotic expression of viral proteins.**
For expression of viral proteins different vectors providing proteins with different N-terminal or C-terminal tags were used. Expression in eukaryotes was either controlled by CMV promoters or in case of pCAGGS by β-actin chicken promoter. For expression of proteins in prokaryotes the pET100 expression vector using the lac operon for induction of expression was applied.

### 2.1.4.4 Vector gene constructs

| Designation | Vector | Insert | Abbreviation |
|---|---|---|---|
| **eukaryotic** | | | |
| K1-O3-250304 | pcDNA3.1/V5-His | SARS-ORF3-V5-His | SARS-ORF3-V5/H |
| K11-O8-250304 | pcDNA3.1/V5-His | SARS-ORF8-V5-His | SARS-ORF8-V5/H |
| K4-Ns-100604 | pcDNA3.1/V5-His | SARS-N-V5-His | SARS-N-V5/H |
| K3-NL63-080904 | pcDNA3.1/V5-His | ORF3-V5-His | ORF3-V5/H |
| K11-NL63-080904 | pcDNA3.1/V5-His | ORF3 | ORF3 |
| K22-NL63-080904 | pcDNA3.1/V5-His | myc-ORF3 | myc-ORF3 |
| K18-NL63-E-230806 | pcDNA3.1/V5-His | E-V5-His | E-V5/H |
| K9-NL63-M-230806 | pcDNA3.1/V5-His | M-V5-His | M-V5/H |
| K41-NL63-N-230806 | pcDNA3.1/V5-His | N-V5-His | N-V5/H |
| pEGFP-c2-011204 | pEGFP-c2 | EGFP | EGFP |
| pEGFP-K14-63-O3-160205 | pEGFP-c2 | EGFP-ORF3 | EGFP-ORF3 |
| K38-NL63-ORF3-C-040105 | pcDNA3.1/V5-His | ORF3ct-V5-His | ORF3ct-V5/H |
| GFP-NL63-E-300806 | pcDNA3.1/NT-GFP | GFP-E | GFP-E |
| GFP-NL63-M-300806 | pcDNA3.1/NT-GFP | GFP-M | GFP-M |
| GFP-NL63-N-160806 | pcDNA3.1/NT-GFP | GFP-N | GFP-N |
| GFP-NL63-O3-300806 | pcDNA3.1/NT-GFP | GFP-ORF3 | GFP-ORF3 |
| GFP-120906 control vector | pcDNA3.1/NT-GFP | GFP | GFP |
| K5-pCAGGS-NL63-O3-300806 | pCAGGS | FLAG-ORF3 | FLAG-ORF3 |

| | | | |
|---|---|---|---|
| **prokaryotic** | | | |
| pET100-E-K17-170805 | pET100D-His/Xpress | His/Xpress-E | H/X-E |
| pET100-M-K7-170805 | pET100D-His/Xpress | His/Xpress-M | H/X-M |
| pET100-N-K24-170805 | pET100D-His/Xpress | His/Xpress-N | H/X-N |
| pET100-O3-K24-220904 | pET100D-His/Xpress | His/Xpress-ORF3 | H/X-ORF3 |
| pET100-O3cT-K12-210105 | pET100D-His/Xpress | His/Xpress-ORF3ct | H/X-ORF3ct |
| **TaqMan PCR standards** | | | |
| TaqMan-63-1a-pK1-020805 | pcDNA3.1/V5-His | 1a gene fragment | Taq-1a |
| TaqMan-63-S-pK2-060705 | pcDNA3.1/V5-His | S gene fragment | Taq-S |
| TaqMan-63-ORF3-pK1-210705 | pcDNA3.1/V5-His | ORF3 gene fragment | Taq-ORF3 |
| TaqMan-63-E-pK58-080705 | pcDNA3.1/V5-His | E gene fragment | Taq-E |
| TaqMan-63-M-pK5-210705 | pcDNA3.1/V5-His | M gene fragment | Taq-M |
| TaqMan-63-N-pK48-060705 | pcDNA3.1/V5-His | N gene fragment | Taq-N |

### 2.1.5 Antibodies

**Primary antibodies**

| Name | Target (antigen) | Species | Order no. | Dilution (Application) | Source |
|---|---|---|---|---|---|
| Anti-Golgi 58K | Golgi protein 58K/ cytoplasmic part | mu | G2404 | 1:100 (IFA) | Sigma-Aldrich, Munich |
| Anti-ACE2 | ACE2 ectodomain (18-740 aa) | mu | ACE23-M | variable | Alpha Diagnostics, San Antonio, USA |
| Anti-ACE2 | ACE2 ectodomain (489-508 aa) | rb | ACE22-A | variable | Alpha Diagnostics, San Antonio, USA |
| Anti-alpha-Tubulin-FITC | C-terminus epitope α-tubulin | mu | F 2168 | 1:50 (IFA) | Sigma-Aldrich, Munich |
| Anti-Calreticulin (ab4) | Calreticulin of ER | rb | NB600-101 | 1:15 (IFA) | Abcam, Cambridge, UK |
| Anti-beta-Actin | N-terminus of beta-actin | mu | A 5316 | 1:2,000 (WB) 1:100 (IFA) | Sigma-Aldrich, Munich |
| Anti-V5 | V5 epitope | rb | A 190-120A | 1:5,000 (WB) 1:100 (IFA) | Bethyl, Montgomery, USA |
| Anti-His (C-term) | His epitope | mu | R930-25 | 1:1,000 (WB) 1:100 (IFA) | Invitrogen, Karlsruhe |
| Anti-GFP | GFP | rb | PA1-980A | 1:3,333 (WB) 1:100 (IFA) | Dianova, Hamburg |
| Anti-GFP | GFP | rb | ab290 | 1:10,000 (WB) 1:200 (IP) | Abcam, Cambridge, UK |
| Anti-GFP | GFP | mu | DLN-07227 | 1:5,000 (WB) | Dianova, Hamburg |
| Anti-Xpress | Xpress epitope | mu | R910-25 | 1:5,000 (WB) | Invitrogen, Karlsruhe |
| Anti-FLAG | FLAG-tag epitope | rb | F7425 | 1:5,000 (WB) 1:100 (IFA) | Sigma-Aldrich, Munich |

| | | | | | |
|---|---|---|---|---|---|
| Anti-FLAG (M2) | FLAG-tag epitope | mu | F1804 | 1:5,000 (WB)<br>1:400 (IP) | Sigma-Aldrich, Munich |
| Anti-ERGIC-53 | ERGIC protein 53 (LMAN1) | mu | ALX-804-602 | 1:100 (IFA) | Axxora, Grünberg |

Legend: mouse (mu); rabbit (rb); Western blot (WB); Immunofluorescence assay (IFA); Immunoprecipitation (IP).

**Secondary antibodies**

| Specificity | Label | Species | Order no. | Dilution (Application) | Source |
|---|---|---|---|---|---|
| Anti-Human | Rhodamine | go | 109-295-088 | 1:200 (IFA) | Dianova, Hamburg |
| Anti-Human | HRP | go | 31432 | 1:30,000 (WB) | Pierce, Rockford, USA |
| Anti-Mouse | FITC | go | A90-216F | 1:200 (IFA) | Bethyl, Montgomery, USA |
| Anti-Mouse | Cy5 | go | 115-175-146 | 1:100 (IFA) | Dianova, Hamburg |
| Anti-Mouse | Rhodamine | go | 115-295-146 | 1:200 (IFA) | Dianova, Hamburg |
| Anti-Mouse | HRP | go | 31450 | 1:30,000 (WB) | Pierce, Rockford, USA |
| Anti-Rabbit | Alexa594 | go | A11012 | 1:600 (IFA) | Invitrogen, Karlsruhe |
| Anti-Rabbit | FITC | go | 111-095-144 | 1:200 (IFA) | Dianova, Hamburg |
| Anti-Rabbit | HRP | go | 314060 | 1:30,000 (WB)<br>1:1,000 (ELISA) | Pierce, Rockford, USA |
| Anti-Rabbit | Cy3 | do | 711-165-152 | 1:200 (IFA) | Dianova, Hamburg |
| Anti-bat | - | go | A140-118A | 1:1,000 (IFA) | Bethyl, Montgomery, USA |
| Anti-bat | HRP | go | A140-118P | 1:10,000 (WB)<br>1:2,000 (ELISA) | Bethyl, Montgomery, USA |
| Anti-goat | FITC | do | | 1:100 (IFA) | Dianova, Hamburg |

Legend: goat (go); donkey (do); fluorescein isothiocyanate (FITC); horseradish peroxidase (HRP); Western blot (WB); Enzyme-linked immunosorbent assay (ELISA), Immunofluorescence assay (IFA); Immunoprecipitation (IP).

### 2.1.6 Buffer/Solutions

| Name | Ingredients | Source |
|---|---|---|
| 10 x Buffer O | | Fermentas, St.Leon-Rot |
| 10 x Buffer EcoRI | | Fermentas, St.Leon-Rot |
| 10 x *Taq* buffer | | Invitrogen, Karlsruhe |
| 5 x ABI sequencing buffer | | Applied Biosystems, Foster City, USA |
| 5 x Superscript RT buffer | | Invitrogen, Karlsruhe |
| 5 x T4 ligase buffer | | Invitrogen, Karlsruhe |
| Antibody buffer (for WB) | Washing buffer<br>+ 1% dried milk powder (w/v) | - |
| Blocking buffer (for IFA) | 10 mL PBS<br>3% FCS<br>0.1% Tween® 20 | - |

| | | |
|---|---|---|
| Blocking buffer (for WB) | Washing buffer<br>+ 10% dried milk powder (w/v) | - |
| BugBuster® | | Novagen, Madison, USA |
| Co-immunoprecipitation buffer (Co-IP buffer) | 20 mM Tris HCl, pH 7.5<br>100 mM sodium chloride<br>1% Nonidet P-40<br>5 mM EDTA | - |
| Coomassie Plus™ Protein Assay Reagent | | Pierce, Rockford, USA |
| Coomassie staining solution | Fixative solution<br>+ 0.1% Coomassie Brilliant Blue R-250 | - |
| Diluent solution | 8 g/L sodium chloride<br>0.4 g/L potassium chloride<br>0.06 g/L sodium hydrogenphosphate<br>0.06 g/L potassium hydrogenphosphate<br>1 g/L glucose<br>0.37 g/L sodium hydrogencarbonate<br>adjust to pH 7.0<br>add 0.2% EDTA | - |
| FACS-PBS | PBS<br>+2.5% FCS<br>+0.1% $NaN_3$<br>store at 4°C | - |
| FACS-antibody buffer | PBS<br>+0.2% BSA<br>+0.1% $NaN_3$ | - |
| Fixative solution | 40% methanol<br>10% ethanol<br>50% aqua dest. | - |
| DNA gel loading buffer (6 x) | 5 mL glycerine<br>40 mg bromphenolblue<br>5 mL TBE | - |
| Running buffer SDS-PAGE (5 x) | 15 g/L Tris<br>72 g/L glycine<br>5 g/L SDS | - |
| Buffer A* (5 x) | 6.9 g/L sodium hydrogenphosphate<br>17.54 g/L sodium chloride<br>500 µL/L Tween® 20<br>pH 8.0 (with NaOH) | - |
| Buffer B* (5 x) | 13.8 g/L sodium hydrogenphosphate<br>1.2 g/L Tris<br>500 µL/L Tween® 20<br>pH 8.0 (with NaOH) | - |
| Buffer C | 20 mM Tris<br>100 mM sodium chloride at pH 8.0 | - |
| M-PER lysis buffer | | Pierce, Rockford, USA |
| Naphthol black solution | 1 g naphthol blue black<br>13.6 g sodium acetate<br>60 mL glacial acetic acid<br>ad 1 L aqua dest. | - |

| | | |
|---|---|---|
| PBS (phosphate-buffered saline) buffer | 140 mM sodium chloride<br>2 mM potassium chloride<br>10 mM disodium hydrogenphosphate<br>2 mM potassium hydrogenphosphate | - |
| Ponceau red staining solution | 2% Ponceau S<br>3% trichloric acetic acid | - |
| Re-Blot Plus Strong Solution (for WB) | | Chemicon International, Temecula, USA |
| RIPA buffer | 150 mM sodium chloride<br>1% Igepal CA-630<br>0.5% sodium deoxycholat<br>0.1% SDS<br>50 mM Tris (pH 8.0) | - |
| Silver stain (SS)-developer solution | 0.38 M sodium carbonate<br>0.015% formaldehyde<br>20 µM sodium thiosulfate<br>In aqua dest. | - |
| SS-sensitivity solution | 80 µM sodium thiosulfate<br>In aqua dest. | - |
| SS-silvernitrate solution | 16.5 mM silvernitrate<br>0.026% formaldehyde<br>In aqua dest. | - |
| TBE (Tris-Borat-EDTA)-buffer | | Eppendorf, Hamburg |
| TFB I | 30 mM potassium acetate<br>50 mM mangenese chloride<br>100 mM rubidium chloride<br>10 mM calcium chloride<br>15% glycerine<br>pH 5.8 with acetic acid<br>(sterile filtrate before use) | - |
| TFB II | 10 mM MOPS<br>75 mM calcium chloride<br>10 mM rubidium chloride<br>15 % glycerine<br>pH 7.0 with KOH<br>(sterile filtrate before use) | - |
| Transfer buffer | 25 mM Tris<br>150 mM glycine<br>10% methanol | - |
| Washing buffer | 0.1% Tween® 20 in PBS | - |
| β-mercaptoethanol sample buffer (MESB) 4-fold | 50 mM Tris; pH 6.8<br>40% glycerin<br>8% β-mercaptoethanol<br>4 g/L bromphenolblue<br>80 g/L SDS | - |

*Buffer A and Buffer B were supplemented with 10 mM; 20 mM, 200 mM, 350 mM, 500 mM imidazole depending on use. Buffer B was also supplemented with 2 M, 4 M, 6 M or 8 M Urea. Generally percentage is understood as volume percentage.

### 2.1.7 Kits

| Name | Source |
|---|---|
| ABI PRISM® Big Dye® Terminator Cycle Sequencing Ready Reaction kit | Applied Biosystems, Foster City, USA |
| Endofree® Plasmid Maxi kit | Qiagen, Hilden |
| pcDNA3.1/V5-His TOPO® TA expression kit | Invitrogen, Karlsruhe |
| pcDNA3.1/GFP-NT-TOPO® TA expression kit | Invitrogen, Karlsruhe |
| pET100 Directional TOPO® TA expression kit | Invitrogen, Karlsruhe |
| QIAprep® Miniprep kit | Qiagen, Hilden |
| QIAquick® Gel Extraction kit | Qiagen, Hilden |
| QIAquick® PCR purification kit | Qiagen, Hilden |
| QIAamp® Viral RNA Mini kit | Qiagen, Hilden |
| RNeasy® Protect Mini kit | Qiagen, Hilden |
| TNT T7 quick coupled reticulocyte lysate system | Promega, Mannheim |

### 2.1.8 Animals, cell lines, viruses and bacteria

#### 2.1.8.1 Animals

For immunization chinchilla rabbits were used. Animal facilities were located at the Robert Koch-Institut, Berlin, Germany. Animal experiments were authorized and supervised by an institutional animal protection officer.

#### 2.1.8.2 Cell lines

| Designation | Tissue | Source | Organism |
|---|---|---|---|
| Huh-7D12 | Liver | ECACC 01042712 | Human |
| Huh-7 | Liver | AMC[a] | Human |
| Hep2 | Liver | ATCC HB-8065 | Human |
| 293 | Fetal kidney | ATCC CRL-1573 | Human |
| HEK293T | Fetal kidney | RKI[b] | Human |
| RH | Kidney | RKI | Human |
| MRC-5 | Lung fibroblast | RKI | Human |
| Chang Liver | Hela contaminant | ATCC CCl-13 | Human |
| RD | Mouth carcinoma | RKI | Human |
| Wil2.NS.6TG | Spleen | ECACC 93031001 | Human |
| C8166 | T-lymphocyte | ECACC 88051601 | Human |
| U937 | Monocyte | ATCC CRL-1593.2 | Human |
| H9 | T-lymphocyte | ATCC HTB-176 | Human |
| Vero E6 | Kidney | ATCC CRL-1586 | Monkey |
| LLC-MK2 | Kidney | ATTC CCL-7 | Monkey |
| PBMC | | Charité[c] | Porcine |
| POEK | Fetal kidney | RKI | Porcine |
| PS | Kidney | RKI | Porcine |
| PK | Kidney | ATCC CCL-33 | Porcine |
| MDBK | Kidney | ATCC CCL-22 | Bovine |

| | | | |
|---|---|---|---|
| PG-4 | Fibroblasts | ATCC CRL-2032 | Feline |
| AK-D | Lung | ATCC CCL-150 | Feline |
| FeT-J | T-lymphocyte | ATCC CRL-11967 | Feline |
| CTL-6 | Fibroblasts | RKI | Murine |
| RAT-2 | Fibroblasts | RKI | Murine |
| Embryo Fibroblasts | 11 day old embryo | RKI | Chicken |

Legend: [a]AMC (Academic Medical Center, Amsterdam); [b]RKI (Robert Koch-Institut, Berlin); [c]Charité (Charité University Hospital, Berlin)

#### 2.1.8.3 Viruses

| Designation | Strain/accession no. | Source |
|---|---|---|
| HCoV-NL63 | Amsterdam I; 8$^{th}$ passage/ NC_005831 | Dr. Lia van der Hoek, Academic Medical Center (AMC), Amsterdam, The Netherlands |
| SARS-CoV Hong Kong | Hong Kong 6109/ AY_278491 | Dr. Wilina Lim, University of Hong Kong, China |

#### 2.1.8.4 Bacteria

| Designation | Genotype | Source |
|---|---|---|
| *Escherichia coli* Top10 | F$^-$*mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lacZ*ΔM15 Δ*lac*X74 *rec*A1 *deo*R *ara*D139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str$^R$) *end*A1 *nup*G | Invitrogen, Karlsruhe |
| *Escherichia coli* BL21 StarTM (DE3) | F$^-$ *omp*T *hsd*SB (*r*B$^-$, *m*B$^-$) *gal dcm* (DE3) | Invitrogen, Karlsruhe |
| *Escherichia coli* Rosetta™ (DE3) | F$^-$ *omp*T *hsd*SB (*r*B$^-$, *m*B$^-$) *gal dcm* (DE3) pRARE² (Cam$^R$) | Novagen, Madison, USA |
| *Escherichia coli* Rosetta2™ (DE3) | F$^-$ *omp*T *hsd*SB (*r*B$^-$, *m*B$^-$) *gal dcm* (DE3) pRARE² (Cam$^R$) | Novagen, Madison, USA |

### 2.1.9 Software

| Software | Source |
|---|---|
| AxioVSLE V 4.4.0.0 | Zeiss, Jena |
| BCM Search Launcher | (Baylor College of Medicine HGSC; www.searchlauncher.bcm.tmc.edu/) |
| BestKeeper analysis tool | See reference (Pfaffl et al., 2004) |
| BLAST 2.2.9 | NCBI, Bethesda, USA |
| cLSM 510 META Software, Version 3.0, SP3 | Zeiss, Jena |
| EditSeq | DNAStar Inc. Madison, USA |
| GCUA (Graphical Codon Usage Analyzer) | See reference (Fuhrmann et al., 2004) |
| GeNorm analysis tool | See reference (Vandesompele et al., 2002) |

| | |
|---|---|
| HMMTOP Version 2.0 | Hungarian Academy of Science, Budapest, Hungary |
| OligoAnalyzer 3.0 | Integrated DNA Technologies, Inc., Coralville, USA |
| Primer3 Release 1.0 | Whitehead Institute for Biomedical Research, Cambridge, UK |
| Protean | DNAStar Inc. Madison, USA |
| SeqMan® II | DNAStar Inc. Madison, USA |
| SOSUI (Classification an Secondary Structure Prediction of Membrane Proteins) | Tokyo University of Agriculture and Technology, Tokyo, Japan |
| Topology analysis | www.cbs.dtu.dk/services/; www.ncbi.nlm.nih.gov/, www.npsa-pbil.ibcp.fr |

## 2.2 Methods

### 2.2.1 Cell culture and virus propagation

#### 2.2.1.1 Cell culture

**Cell culture conditions**

Most cell lines (2.1.8.2) were grown at 37°C and 5% $CO_2$ in Dulbecco´s Modified Eagles Medium (DMEM) containing 10% fetal calf serum (FCS), 2 mM L-glutamine and 25 units of penicillin/mL as well as 25 units streptomycin/mL. Porcine peripheral blood mononuclear cells (PBMC) were isolated from a healthy pig (see below) and grown in Roswell Park Memorial Institute 1640 Medium (RPMI 1640) supplemented with FCS and antibiotics as described above. When cells were frozen for long-time storage cell pellets were resuspended in a freeze mix medium consisting of 90% FCS and 10% DMSO. After one week at -80°C cells were transferred to liquid nitrogen.
All cells were tested negative for mycoplasma contamination by polymerase chain reaction (PCR) as described elsewhere (van Kuppeveld et al., 1993).

**Cell harvesting, counting and seeding**

In case of adherent cells medium was immersed, the monolayer rinsed with diluent solution (2-5 mL), followed by trypsin solution (1 mL) and incubated cells for 5 to 10 min at 37°C. Afterwards cells were resuspended in adequate cell culture medium containing FCS for trypsin inactivation and centrifuged for 5 min at 800 x g. Pellets were resuspended 20 times in appropriate medium and cell suspension was split 1:3 to 1:10 depending on the cell line. When cells were used for infection or transfection experiments antibiotics were omitted. Cell counting was done with the help of a Neubauer cell counting chamber. Therefore cell suspension was diluted 1:10 in PBS and counted 4 x 16 squares. With the formula: ([total amount of cells/4] x dilution x $10^4$) the number of cells/mL were calculated.
Usually cells were seeded at densities of 4 to 6 x $10^5$ cells per mL to reach 90% confluency after 24 h incubation.

**Isolation of PBMC by Ficoll-paque Plus centrifugation**

Isolation of PBMCs was done using 15 mL Ficoll-paque Plus (Amersham) in a 50 mL centrifugation tube. After carefully adding full heparin pig blood on top of the Ficoll-paque Plus layer, tubes were centrifuged at 1,800 rpm (Megafuge, Heraeus) and 20°C for 40 min. The PBMC ring was transferred to 40 mL sterile PBS and centrifuged again at 1,600 rpm for 10 min. After discarding the supernatant the pellet was washed twice with PBS and centrifuged at 1,200 rpm and 1,100 rpm for 10 min, respectively. Finally, cells were resuspended in 20 mL RPMI medium and cultured at 37°C and 5% $CO_2$.

### 2.2.1.2 Virus propagation

**Virus stock production of SARS-CoV strain Hong Kong 6109**

For stock production, SARS-CoV (strain 6109) isolated from a Hong Kong patient (2.1.8.3; kindly provided by Dr. Wilina Lim, University of Hong Kong, China) was added to Vero E6 cells (2.1.8.2) under biosafety level 3 conditions, usually at a multiplicity of infection (MOI) of 0.1 to 1. After 8 to 24 h of incubation the supernatant and the infected cells were harvested, stringently centrifuged (10 min at 6,000 x g) and the supernatant was aliquoted. Afterwards the virus titer was determined by plaque assay (2.2.1.3).

**Virus stock production of HCoV-NL63 strain Amsterdam I**

For virus stock production LLC-MK2 cells (2.1.8.2) were inoculated with HCoV-NL63 (2.1.8.3; 8th passage Amsterdam strain I; accession no. NC_005831) at a MOI of 0.01 and infected cells were cultured at 37°C and 5% $CO_2$ for five to seven days before harvested. After centrifugation at 6,000 x g for 10 min supernatant was aliquoted and stored at –80°C. Virus titers were determined by plaque assay (2.2.1.3).

### 2.2.1.3 Plaque assay for detection of viral particles

Plaque assays are useful tools for determination of infectious viral particles in a sample. Briefly, a cell monolayer is inoculated with a virus sample and overlaid with a viscous medium that inhibits the spreading of viral particles via the supernatant. Only neighbouring cells are infected resulting in plaque formation. Generally, plaque assays were performed as described previously with minor modifications (Niedrig et al., 1999).

**Plaque assay for detection of viral particles of HCoV-NL63**

For preparation, carboxymethyl-cellulose (CMC) powder (1.6% w/v) including a magnetic stirrer was autoclaved and DMEM was added. After stirring for several hours at room temperature the CMC solution was ready to use. Cells were harvested, counted and adjusted to 5 x $10^5$ cells/mL. Virus dilutions in medium were prepared at least in quadruplicates/dilution starting at dilution 1:100; 1:500; 1:1,000; 1:2,000; 1:4,000; 1:8,000. A negative cell control in which the virus was replaced by medium was included. Per well 200 µL cell suspension and 200 µL virus dilutions were added and incubated at 37°C for 4 h. Afterwards 400 µL of 1.6% CMC in DMEM was put carefully in each well and plates were incubated at 37°C with 5% $CO_2$. After five days the medium was removed without disturbing the cell layers and 2 mL formaldehyde (4 %) was added to each well. After incubation for 30 min (fixation and inactivation) formaldehyde was discarded and 1 mL naphthol black staining solution was added to the fixed cells. Staining was performed for 30 min, naphthol black solution was removed and plates were washed with aqua dest. For determination of plaque forming units (PFU)/mL the following formula was used: (total number of plaques/total volume of positive virus dilutions) x dilution of lowest dilution = PFU/mL.

**Plaque assay for detection of viral particles of SARS-CoV**

Plaque assays were performed as described above with the following modifications. Vero E6 cells cultured in DMEM and SARS-CoV Hong Kong isolate 6109 were used. Dilutions in quadruplicates usually ranged from 1:1,000 to 1:32,000.

#### 2.2.1.4 Virus neutralization test (SARS-CoV) for screening of bat sera

Virus neutralization tests are used to analyze the ability of antibodies to neutralize virus particles in a way that infection of cells is inhibited. Procedures are similar to the above described plaque assay with the difference that virus dilutions are pre-incubated with antisera for 1 h at 37°C before added to the cells.

Bat serum dilutions (quadruplicates) ranged from 1:10 to 1:320. SARS-CoV Hong Kong was applied at a dilution of 1:5,000 (Titer: 3.25 x $10^7$ PFU/mL). To exclude the possibility that complement factors could influence neutralizing ability, 10% guinea pig serum was added to 9 selected sera. After three days incubation the cells were fixed with 8% formaldehyde for 10

min, then plates were immersed in 4% formaldehyde for 30 min to ensure virus inactivation, cells were stained and results were evaluated as described previously (Niedrig et al., 1999).

### 2.2.2 Sampling of bat sera

Bat serum samples (n= 705) which had been collected for unrelated purposes from 1986 to 99 in the Republic of South Africa (RSA) and the Democratic Republic of the Congo (DRC) were tested. The initial 248 sera were collected from 1986-89 in Limpopo and Mpumalanga Provinces of the RSA (Fig. 7C and D) for studies on rabies-related viruses, with the approval of the Directorates of Nature Conservation of each Province and the Animal Ethics Committee of the University of the Witwatersrand, to which the National Institute for Communicable Diseases (NICD), RSA, is affiliated. The remaining 457 sera were collected in 1995-99 in Oriental and Bandundu Provinces of the DRC (Fig. 7A and B) for studies on Ebola and Marburg viruses, under the auspices of International Committees for the Control of Ebola hemorrhagic fever in Kikwit, and Marburg hemorrhagic fever in Durba-Watsa, coordinated by the World Health Organization (WHO) on behalf of the government of DRC. Bats were caught in mist nets, anesthetized, and exsanguinated by cardiac puncture. Serum samples which could be acquired for this study were stored at -70ºC until analyzed.


**FIG. 7. Map of sampling sites for collecting African bat species.**
Indicated are sampling sites for African bat species. Oriental Province (A) and Bandundu Province (B) of the Democratic Republic of the Congo (DRC) as well as Limpopo Province (C) and Mpumalanga Province (D) of the Republic of South Africa. Picture is adapted from Microsoft Corp. 2006.

### 2.2.3 Molecularbiological methods

Working with nucleic acids in particular with RNA presupposes good laboratory practice. Therefore plastic material was autoclaved (120°C and 1 bar pressure) and equipments like pipettes were pretreated with 0.1 M HCl and UV light radiation to avoid nucleic acid contaminations and RNase or DNase activity. Additionally, only commercially available molecular grade water was applied. Generally commercial kits were used to guarantee contamination free working. All below mentioned buffers were included in the kits and are named as described in the manual instructions.

#### 2.2.3.1 Isolation of RNA

**Isolation of viral RNA**

For isolation of viral RNA the QIAamp® Viral RNA Mini kit (Qiagen) was used. Basically, the sample is lysed under denaturing conditions to inactivate RNases and supplemented with a so-called carrier RNA to reduce unspecific binding of viral RNA to plastic surfaces. With the help of a silica gel-based membrane placed in a microspin column RNA is extracted from the sample. Before lysis buffer AVL was added supernatant from infected cells was centrifuged at 1,000 rpm in a Heraeus Varifuge to remove cells and RNA was extracted from 140 µL supernatant according to the manual description. Briefly, after binding of RNA to the membrane two washing steps were performed with buffers AW1 and AW2 and eluted the RNA with 60 µL pre-warmed (80°C) elution buffer AVE.

**Isolation of total RNA**

Total RNA was isolated using RNeasy Protect Mini kit (Qiagen). Similarly to the above mentioned method, spin columns with a silica gel membrane were used for extraction of total RNA from cells. Inactivation of RNases was ensured by using a highly denaturing guanidine isothiocyanate lysis buffer (RLT buffer). Before lysates were applied to the membrane, samples were homogenized with the help of QIAshredder® spin columns. Afterwards samples were loaded onto the spin columns and washed with two different washing buffers i.e. RW1 and RPE. Elution of RNA was performed by adding 50 µL RNase-free water. In cases where contaminating genomic DNA had to be removed the total RNA was twice digested with

DNase I (AMBION) as described in the manual instructions. Nucleic acid concentrations were measured by photometrical determination (2.2.3.3).

### 2.2.3.2 Isolation of plasmid DNA

**Plasmid DNA preparation (Mini plasmid preparation)**

After identification of positive *E. coli*-clones by colony-PCR (2.2.3.7.1), overnight cultures were produced in selective LB medium. Bacterial cell suspension was pelleted (10 min at 6,000 rpm; Heraeus Megafuge) and plasmid DNA was purified by means of QIAprep® Spin Miniprep kit (Qiagen). Briefly, the process followed a simple bind–wash–elute procedure. First, bacterial cells were lysed by adding buffers P1 and P2, neutralized by adding buffer P3 and the lysates were cleared by centrifugation. Lysates were then applied to the QIAprep® spin column where plasmid DNA adsorbs to the silica-gel membrane. Impurities were washed away by applying buffer PB and PE and purified DNA was eluted in 50 µL of elution buffer (EB). When low copy plasmids were used elution was done with 30 µL EB buffer. Finally nucleic acid concentrations were measured by photometrical determination (2.2.3.3) and plasmid DNA was sequenced to control correct cloning (2.2.3.4).

**Plasmid DNA preparation (Maxi plasmid preparation)**

For transfection experiments of eukaryotic cell lines (2.1.8.2) large amounts of ultra pure plasmid DNA were needed. To ensure that no bacterial endotoxins influence transfection efficiency the EndoFree Plasmid Maxi kit (Qiagen) was used. A 100 mL overnight culture was prepared by inoculation of selective LB medium with transformed *E. coli*-cells from a glycerol stock that had been produced after sequencing of the corresponding plasmid DNA. After pelleting (10 min at 6,000 rpm; Megafuge, Heraeus) bacterial cells were lysed under alkaline conditions with P1 and P2 buffer and neutralized by adding P3. With the help of a QIAfilter® Maxi Cartridge the lysates were cleared. At this stage, the endotoxin removal buffer (ER buffer) was added to the filtered lysate and was then loaded onto the anion-exchange column where plasmid DNA selectively binds under appropriate low-salt and pH conditions. RNA, proteins, metabolites, and other low molecular weight impurities were removed by a medium-salt wash (QC buffer). In high-salt buffer (QN buffer) pure plasmid DNA was eluted. The DNA was concentrated and desalted by isopropanol (0.7 volumes) precipitation, washed with 70% ethanol and collected by centrifugation (15,000 x g for 10

min). After air-drying, pellets were re-dissolved in 300 to 700 µL endotoxin-free buffer TE. Concentrations were determined photometrically (2.2.3.3) and correct cloning was confirmed by sequencing (2.2.3.4).

#### 2.2.3.3 Photometrical determination of nucleic acid concentration

Nucleic acids are able to absorb monochromatic light and concentrations can thus be determined photometrically. Disposable plastic cuvettes were applied with a thickness of 1 cm in a Biophotometer (Eppendorf). As reference, a minimum of 50 µL water or the corresponding solving liquid was used. Samples were measured at wavelength 260 nm and at 320 nm to consider impurities. For DNA a result of 1 in comparison to the reference means a concentration of 50 µg/mL and for RNA 40 µg/mL, respectively.

Formula for calculation: $C = (E_{260} - E_{320}) \times d \times f$ [µg/mL]
C = concentration
E = extinction
d = dilution factor
f = factor for DNA (50 µg/mL) and RNA (40 µg/mL) when using a 1 cm cuvette

#### 2.2.3.4 Sequencing of DNA

Sequencing was done with the help of the ABI PRISM® Big Dye® Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems) which is based on Sanger´s method (Sanger et al., 1977). All plasmids (vector gene constructs from 2.1.4.4) were sequenced with a forward and reverse primer except for the N gene. Due to its size it needed two additional internal primers (5´NL63-N-GFP, 3´NL-NpK R). In a total volume of 10 µL the DNA template (300 ng) was amplified with the help of the following protocol: 96°C for 2 min and 10 s, followed by 25 cycles of 55°C for 5 s and 60°C for 4 min, with a final cooling at 4°C. DNA sequencing was performed at an internal facility of the RKI.

#### 2.2.3.5 Ethanol precipitation of nucleic acids (DNA)

In order to purify or concentrate plasmid DNA an ethanol precipitation was used. Therefore ethanol is added for dehydration (reduces the ability for ions to move freely) and salts are

added to neutralize the charge on the sugar-phosphate backbone of the DNA. Thus ion pairs between the polyanion (DNA) and the cation (usually sodium or ammonium) can be built.
DNA solutions were supplemented with 1/10 volume 3 M sodium acetate (pH 5.5) and 2.5 volumes ethanol (96%). After incubation at -80°C for 5 min samples were centrifuged for 15 min at 15,000 x g and 4°C. The DNA pellet was washed once with 70% ethanol and centrifuged again for 5 min. After air-drying of the pellet at 37°C for 10 min, DNA was re-dissolved in an appropriate volume aqua mol.

#### 2.2.3.6 Reverse transcription

Reverse transcription (RT) was used to produce stable complementary DNA (cDNA) from isolated total or viral RNA. One can either use oligo(dT) primers that are able to anneal to the poly(A)-tail of mRNA or one can apply random hexamer primers that anneal randomly. After annealing the mRNAs are transcribed in 5´end direction with the help of a reverse transcriptase.

**General RT for cDNA synthesis using Superscript II**

Synthesis of cDNA using Superscript II (Invitrogen) was done as described by the manual descriptions. Briefly, 250 ng of total RNA was reverse transcribed in 20 µL volume with 5 ng oligo(dT) primer or random hexamer primer, 20 nmol dNTPs, 40 units RNasin, 4 µL 5 x buffer, 100 nmol $MgCl_2$, 200 nmol dithiothreolin (DTT) and 200 units SuperScript II RT (Invitrogen). Before adding the enzymes the reaction mixture was heated to 65°C for 5 min and placed on ice for 1 min. The programme for the RT was 42°C for 50 min and 70°C for 15 min.

**RT using Superscript III**

Complementary DNA was produced using the Superscript III RT-PCR System (Invitrogen) according to the manufacturer´s recommendations for oligo(dT)20 primed cDNA-synthesis. cDNA synthesis was performed using 1 µg of RNA, at 50°C. Finally, cDNA was diluted 1:5 before use in quantitative PCR (2.2.3.7.6; 2.2.3.7.7).

### 2.2.3.7 Polymerase chain reaction

The polymerase chain reaction (PCR) is used for the amplification of specific DNA fragments. Principally one uses the ability of DNA to denature at 94°C and the special feature of the heat-stable DNA polymerase isolated from *Thermus aquaticus* which has an optimal working temperature at 72°C. Typically, the PCR process can be divided into three phases: denaturing, annealing and elongation:

1. Denaturing of double-stranded into single-stranded DNA is caused by destruction of hydrogen bonds between the opposite base pairs.
2. Annealing of two specific oligonucleotides (primers) occurs after cooling down to a primer specific temperature. Thereby primers bind complementary to the single-stranded DNA and flank the amplified area. The annealing temperature (Tm) can be calculated by the formula: Tm = (G + C) x 4 + (A + T) x 2
3. During elongation primers are elongated in 3´end direction by the thermo stable *Taq*-DNA-polymerase at a speed of approximately 1,000 bp per min.

The three phases are repeated 25 to 40 times whereby after each cycle the amplified DNA can be used as a new matrix. Generally, the following protocol was used:

| | |
|---|---|
| 5 µL | 10 x DNA-polymerase buffer |
| 75 to 125 nmol | $MgCl_2$ |
| 1.5 units | Platinum® *Taq*-DNA-polymerase |
| 10 nmol | per dNTP |
| 25 pmol | 5´- or 3´-primer |
| 5 to 10 ng | cDNA |
| ad 50 µL | Aqua mol. |

PCR programme was performed as follows:

| | | |
|---|---|---|
| Initial Denaturing: | 94°C | 2 min |
| 30-35 cycles: | | |
| Denaturing: | 94°C | 30 s |
| Annealing: | Primer specific | 20 to 30 s |
| Elongation: | 72°C | 60 s per 1,000 bp |

#### 2.2.3.7.1 Colony-PCR

With the help of a colony-PCR one is able to evaluate if transformed *E. coli*-cells incorporated plasmids with the correct DNA inserts. By the freeze and thaw method plasmids were isolated from bacterial cells and supernatants could be tested by PCR.

Therefore, 50 µL aqua mol. was inoculated with a bacterial clone picked from an agar plate. From this solution 5 µL were used to inoculate 50 µL LB medium containing the appropriate antibiotic providing a rescue for subsequent processing (2.2.3.2). The aqueous bacterial solution was vortexed, frozen at -70°C, thawed, vortexed again and boiled at 100°C. After centrifugation 2.5 µL supernatant was used in a conventional PCR (2.2.3.7.1).

#### 2.2.3.7.2 Cloning-PCR

In order to express HCoV-NL63 proteins E, M, N, ORF3 and ORF3 C-terminus (ORF3ct, comprising the 96 amino acids of the C-terminus) recombinantly in prokaryotes and eukaryotes the different genes were cloned into a variety of expression vectors.
Generally cloning-PCR was performed with Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen), and conditions were as follows: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, primer specific temperature for 30 s, and 72°C for 90 s, with a final extension at 72°C for 10 min.
For the isolation of viral genes total RNA from infected LLC-MK2 cells was reverse transcribed with oligo(dT) primers as described above (2.2.3.6).
For the generation of GFP-constructs PCR was performed with the following specific primers (2.1.4.1); for E: 5´NL63-E-GFP and 3´NL63-EpK R; for M: 5´NL63-M-GFP and 3´NL63-MpK R; for N: 5´NL63-N-GFP and 3´NL63-NpK R; for ORF3: 5´NL63-O3-GFP and 3´NL63-O3.
Prokaryotic pET-vector constructs were generated with primers for E: 5´NL63-EpK and 3´NL63-EpK R; M: 5´NL63-MpK and 3´NL63-MpK R; N: 5´NL63-NpK and 3´NL63-NpK R; ORF3: 5´NL-O3x and 3´NL-O3; O3cT: 5´NL63-Ctx and 3´NL-O3.
For PCR amplification of FLAG-ORF3 and subsequent cloning into a pCAGGS vector we used 5´Eco-FLAG_O3-63 and 3´Not-O3-63. In this case PCR products were digested with restriction endonucleases EcoR1 and Not1 (2.2.3.11) before cloning into the pCAGGS which had also been digested and additionally dephosphorylated before use (2.2.3.12).

#### 2.2.3.7.3 RT-PCR for detection of sg mRNA

To be able to detect viral genome equivalents and differentiate between specific sg transcripts a PCR was designed with a single 5´end primer named 5´NL63 F mis positioned within the leader sequence and different 3´end primers in proximity to the transcription regulating

sequence i.e. NL63 1a R; NL63 ORF3 R; NL63 M R; NL63 N R. RT of the same amounts of total RNA (250 ng) was done with random hexamer primers as described above (2.2.3.6) using random primers. By using 1 µL cDNA (i.e. approximately 12.5 ng reverse transcribed total RNA) per 25 µL PCR sample volume in a stringent PCR protocol with a combined short annealing/elongation step (initial denaturation for 2 min at 94°C followed by 38 cycles of 15 sec at 94°C and 20 sec at 60°C) we managed to specifically amplify each subgenomic transcript without cross-amplifying longer transcripts. To generate standards for semi-quantification we cloned PCR amplicons of the different subgenomic transcripts into the pcDNA3.1/V5-His TOPO® vector (2.2.3.12). For analyzing correct cloning plasmids were sequenced (2.2.3.4). Plasmid dilutions were used in the same PCR at copy numbers $10^6$ to $10^1$. Optimization experiments confirmed that the PCR is still in the range of exponential growth which is demanded for semi-quantification.

#### 2.2.3.7.4 RT-PCR for detection of ACE2 and GAPDH mRNA

For the detection of ACE2 mRNA, cDNA was produced from total RNA of all cell lines susceptible for SARS-CoV and from some non-susceptible cell lines using the above mentioned protocol applying oligo(dT) primers (2.2.3.6). To control if RT was successful and transcribed RNA was free of PCR inhibitors all human and simian cDNA samples were tested in a control PCR using primers detecting the glycerylaldehyde 3-phosphate dehydrogenase (GAPDH) gene (Radonic et al., 2004). Amplification was performed in a 25 µL reaction volume using 1 µL of cDNA, 2.5 µL 10 x buffer, 5 nmol dNTPs, 75 nmol $MgCl_2$, and 1 unit Platinum® *Taq* Polymerase [94°C, 2 min; 35 x ( 94°C, 30 s; 59°C, 30 s; 72°C, 1 min) 72°C, 3 min]. The resulting PCR product had a length of 225 bp. Same PCR conditions were used for the amplification of ACE2 cDNA with primers ACE2 F and ACE2 R. The resultant PCR product of 168 bp was sequenced and analyzed in BLAST.

#### 2.2.3.7.5 Quantitative RT-PCR for coronavirus nucleic acids in bat sera

Viral RNA was extracted from serum with QIAamp® viral RNA extraction kits as described above (2.2.3.1). Quantitative RT-PCR was performed as described elsewhere (de Souza Luna et al., 2007). Briefly, reactions (25 µL mixtures) were carried out using the Qiagen one-step RT-PCR kit, with 200 nM of primer PC2S2 (equimolar mixture of TTATGGGTTGGGATTATC and TGATGGGATGGGACTATC), 900 nM of primer

PC2As1 (equimolar mixture of TCATCACTCAGAATCATCA, TCATCAGAAA GAATCATCA, and TCGTCGGACAA GATCATCA), 1 µL one-step RT-PCR kit enzyme mix, and 5 µL RNA extract. The amplification procedure comprised 30 min at 50°C; 15 min at 95°C; 10 cycles of 20 s at 94°C, 30 s starting at 62°C with a decrease of 1°C per cycle, and 40 s at 72°C; and 30 cycles of 20 s at 95°C, 30 s at 52°C, and 40 s at 72°C. For nested PCR 1 µL of first-round PCR product was used together with 1 x Platinum *Taq* buffer (Invitrogen), 200 µM deoxynucleoside triphosphate, 2.5 mM $MgCl_2$, 80 nM of primer PCS (equimolar solutions of CTTATGGGTTGGGATTATCCTAAGTGTGA and CTTATGG GTTGGGATTATCCCAAATGTGA), 400 nM primer PCNAs (CACACAACACCTTCA TCAGATAGAATCATCA), and 1 unit Platinum *Taq* polymerase. The amplification procedure comprised 3 min at 94°C and 30 cycles of 20 s at 94°C, 30 s at 60°C, and 30 s at 72°C. Detection limits were approximately 45 copies of RNA per reaction.

#### 2.2.3.7.6 Quantitative real-time PCR (TaqMan) for detection of SARS-CoV nucleic acids in infected cells

The amount of SARS-CoV RNA was determined in triplicate by quantitative real-time PCR (TaqMan) as described elsewhere (Nitsche et al., 2004). For the real-time PCR performed on the Applied Biosystems platforms, each 25 µL reaction contained 12.5 µL 2xQuantiTect Probe real-time PCR Master Mix (Qiagen, Hilden, Germany), 10 pmol of each primer i.e. pp1a F and pp1a R, 3 pmol 5´-nuclease probe (pp1a TM), 0.25 µL QuantiTect Probe real-time Mix. RNAse-free water was added up to 23 µL, and 2 µL of RNA preparation were used. Cycling conditions were 30 min at 50°C for reverse transcriptase reaction, 15 min at 95°C for inactivation of reverse transcriptase, activation of the *Taq* DNA polymerase and cDNA denaturation, followed by 45 cycles of 15 s at 95°C and 30 s at 60°C.

#### 2.2.3.7.7 Quantitative real-time PCR (TaqMan) for reference genes

Primers, TaqMan probes and quantitative PCR conditions for reference gene analysis were used as previously described (Radonic et al., 2004). PCR was performed in a Perkin Elmer 7700 Sequence Detection System in 96-well microtiter plates using a final volume of 25 µL.

#### 2.2.3.8 Agarose gel electrophoresis of nucleic acids

As nucleic acids are charged negatively due to their phosphate residues they can be separated by agarose gel electrophoresis using an electric field. The detection is performed with the help of ethidium bromide which interacts with nucleic acids and can be visualized by UV light. Generally a 1-2% agarose gel was used which was supplemented with 0.5 µg/mL ethidium bromide. Samples were diluted in 6-fold DNA sample buffer before applied to the gel. Running of gels in TBE buffer was done at 80 to 100 V.

#### 2.2.3.9 Gel extraction of nucleic acid fragments

In order to extract DNA fragments from agarose gels the QIAquick® gel extraction kit from Qiagen was apllied. After excision of DNA fragments from the agarose gel with a scalpel the gel slice was dissolved in 3 volumes buffer QG. One gel slice-volume of isopropanol was added and the solution was applied to the QIAquick® spin column. Washing was done with buffers QG and PE. Elution was performed by adding 30 to 50 µL buffer EB. In some cases pre-heated aqua mol. was used for elution. The concentration of DNA was measured photometrically (2.2.3.3).

#### 2.2.3.10 Purification of PCR products and nucleic acid fragments

The purification of PCR products or of digested nucleic acids was done as described by the manufacturer of QIAquick® PCR purification kit (Qiagen). Briefly 5 volumes PB buffer was added to the sample which was applied to a QIAquick® spin column. After centrifugation bound DNA was washed once with buffer PE and eluted in 30 to 50 µL buffer EB.

#### 2.2.3.11 Restriction endonuclease digestion and dephosphorylation of DNA

Restriction endonucleases are able to recognize palindromic sequences and cut specifically particular nt motifs. The digestion of DNA results in sticky or blunt DNA ends depending on the endonuclease. Generally, one unit of enzymes digests 1 µg DNA in 1 h at an enzyme-specific temperature usually between 30°C and 37°C.
Restriction digestion was used for cloning of FLAG-ORF3 into a pCAGGS vector (2.2.3.11; 2.2.3.12). Amplification was done by PCR using primers 5´Eco-FLAG_O3-63 and 3´Not-O3-

63 which had restriction sites included. PCR products and pCAGGS vector were digested with restriction endonucleases EcoRI and NotI before cloning. In a volume of 40 µL either 3 µg vector or eluated PCR product were digested by adding 100 units EcoRI, 10 units NotI and the corresponding EcoRI buffer including BSA. After a 2 h or overnight incubation at 37°C endonucleases were inactivated by incubation at 65°C for 20 min.

For dephosphorylation of the digested pCAGGS vector 5 units calf intestine alkaline phosphatase were added and the sample was incubated for 1 h at 37°C. Afterwards the digested PCR product and the vector were separated by agarose gel electrophoreses, DNA fragments were excised and purified with the QIAquick® gel extraction kit (2.2.3.9).

#### 2.2.3.12 Ligation of DNA

The different genes E, M, N, ORF3 and ORF3ct were cloned into pET100/D-TOPO® vector (prokaryotic expression), pcDNA3.1/V5-His-TOPO® and pcDNA3.1/NT-GFP-TOPO® (eukaryotic expression) with the help of TOPO® expression kits (Invitrogen) according to the manual instructions.

Generally, the *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxyadenosine at the 3´ends of PCR products. As the supplied linearized vector has a single, overhanging 3´deoxythymidine residue PCR inserts are efficiently ligated. Basically, 2 µL PCR product of amplified genes, 0.5 µL salt solution and 0.5 µL TOPO® vector were incubated for 10 min. The complete reaction was used for transformation of One Shot TOP10 chemically competent *E. coli*-cells (2.2.3.13). When using the pET100/D-TOPO® cloning kit PCR amplicons needed a 5´end CACC overhanging residue in order to perform directional cloning. When tags were added to the N-terminus the start codon was left out but a stop codon included whereas for C-terminal tags the start codon was included and the stop codon omitted.

Construction of pCAGGS-FLAG-ORF3 vector was done by conventional cloning. Thus, approximately 10 ng digested and dephosphorylated pCAGGS vector was ligated with 200 ng digested and purified PCR product (FLAG-ORF3) in a volume of 10 µL. After adding T4-ligase (Invitrogen) and a corresponding buffer the reaction was incubated at 14°C over night and subsequently used for transformation of *E. coli*-cells (2.2.3.14).

#### 2.2.3.13 Generation of chemically competent *E. coli*-cells

The generation of chemically competent *E. coli*-cells was based on methods described elsewhere (Hanahan, 1983). Briefly, 5 mL LB medium (in case of Rosetta™ strains supplemented with selective chloramphenicol [35 mg/L]) were inoculated with a single colony of a particular strain (2.1.8.4). After incubation at 37°C and 200 rpm over night a larger volume (250 mL) LB medium was inoculated with 1/100 volume of the grown culture and shaken at 37°C until the $OD_{600}$ reached 0.5 (log phase of bacterial growth). Then the cells were cooled down to 4°C and pelleted by centrifugation at 4,000 rpm and 4°C (Megafuge, Heraeus). The cell pellet was resuspended in 40 mL ice-cold TFB I buffer and after incubation for 10 min on ice, the cells were pelleted as described above. After discarding the supernatant the pellet was resuspended in TFB II buffer and the bacterial suspension was transferred (80 µL aliquots) into pre-cooled reaction tubes. Shock-freezing of aliquots was done with the help of liquid nitrogen. Chemically competent cells were stored at -80°C and thawed on ice before use. Test transformations were done with a pUC19 plasmid provided by the manufacturer (Invitrogen).

#### 2.2.3.14 Transformation of *E. coli*-cells

The transformation of *E. coli*-strains TOP10, BL21 (Invitrogen) and Rosetta2™ (Novagen) was performed according to the manual instructions of the TOPO® TA expression kit (Invitrogen) with slight modifications. The complete ligation reaction (2.2.3.12) was added to chemically competent *E. coli*-cells and incubated for 15 min on ice. After heat-shock at 42°C for 30 s the vials were put on ice and supplemented with 250 µL S.O.C medium. The samples were shaken horizontally (200 rpm) at 37°C for 30 min. Finally, 25 µL to 200 µL suspension was spread on pre-warmed selective plates and incubated at 37°C over night. The clones were analyzed by colony-PCR (2.2.3.7.1).

### 2.2.4 General proteinbiochemical methods

#### 2.2.4.1 Detection and protein analysis by SDS-PAGE

**Determination of protein concentration (Bradford assay)**

The determination of protein concentrations was done based on the principle of a Bradford assay (Compton and Jones, 1985) using Coomassie Plus™ protein assay reagent (Pierce) according to the manual instructions. Briefly, BSA standard dilutions ranging from 1 mg/mL to 31.25 µg/mL were generated and 6.25 µL of BSA solution was diluted with 187.5 µL Coomassie Plus™ protein assay reagent. This reagent interacts unspecifically with cationic, non-polar and hydrophobic aa side chains of proteins and thereby changes its absorption maximum from 465 nm to 595 nm. In parallel we diluted protein samples and a blank probe using the protein solving buffer. After mixing and incubation at room temperature for 10 min absorption at $OD_{595}$ was measured with the help of the Bradford programme of the Biophotometer (Eppendorf).

**Concentration and buffer change of proteins by Microcon® centrifugal devices**

In cases when proteins needed to be concentrated or buffer conditions had to be altered Microcon® Centrifugal Filter Devices (Millipore) were used according to the suppliers´ description. Depending on the volumes devices were applied with volumes ranging from 500 µL (1.5 mL reaction tube) to 4 mL (15 mL reaction tube) and filter cut-offs 5 to 10 kDa. Small vials (1.5 mL) were centrifuged for 30 to 40 min at 14,000 x g whereas 15 mL tubes were centrifuged for 40 min at 4500 rpm (Megafuge, Heraeus). Afterwards concentrated proteins on the upper filter side were resuspended in an appropriate buffer.

**SDS-PAGE (Sodium dodecylsulfate-polyacrylamide gelelectrophoresis)**

SDS-PAGE is a method for separating denatured proteins according to their molecular weight. In the process one uses the negatively charged detergent sodium dodecylsulfate (SDS) which is able to mask the different charges of the aa residues. All proteins are thus stretched, negatively charged polypeptides that can be separated by an electric field in a polyacrylamide matrix. As proteins have a constant negative charge per mass unit (1.4 g SDS/1 g protein in 1% SDS solution) separation occurs exclusively by their molecular weight. Moreover, disulfide bridge formation is inhibited by adding either β-mercapthoethanol (ME) or DTT.

The samples are commonly boiled at 95°C for 5 min to enhance the denaturing effect of the SDS but as some proteins are sensitive to heat the boiling step was skipped in some experiments.

A discontinuous SDS-PAGE was used with a stacking and a separating gel. This has the advantage that proteins of different sizes are accumulated within the stacking gel and have therefore the same starting point when entering the separating gel.

In Table 3 the different components are shown for a discontinuous 15% SDS-PAGE gel. The proteins were mixed with β-mercaptoethanol sample buffer (MESB) and applied to the slots of the gel. Running conditions were 14 V/cm gel i.e. 200 V (running time approximately 45 min). Afterwards the gels were either stained (coomassie or silver stain) or used for Western blot (WB) analysis (2.2.5.4).

**TABLE 3. Components of a 15% SDS-PAGE gel**

| **Separating gel** | | **Stacking gel** | |
|---|---|---|---|
| Aqua dest. | 1.38 mL | Aqua dest. | 1.75 mL |
| 2 M Tris (pH 8.8) | 1.05 mL | 0.5 M Tris (pH 6.8) | 0.5 mL |
| 20% SDS | 25 µL | 20% SDS | 18.75 µL |
| PAA | 2.475 mL | PAA | 0.625 mL |
| | | 60% sucrose | 0.875 mL |
| APS | 60 µL | APS | 87.5 µL |
| TEMED | 7.5 µL | TEMED | 8.75 µL |

**Silver staining**

For the detection of separated proteins a silver staining (SS) procedure was applied which is based on a complex formation of silver ions with glutamine, asparagine and cysteine residues of proteins. In this process alkaline formaldehyde is reducing ions to elementary silver, staining proteins dark brown. Sensitivity of the procedure is high so that 5-100 ng protein/lane could be detected.

SDS-PAGE gels were washed once with aqua dest. before put into fixative solution (2.1.6; 25 mL/gel) supplemented with 12.5 µL formaldehyde (37%) for 20 min. The gels were washed three times for 5 min with 50% ethanol solution and rinsed with SS-sensitivity solution for 1 min. Then the gels were washed three times with aqua dest. and incubated in SS-silvernitrate solution for 25 min. After washing with aqua dest. for 1 min the gels were incubated in SS-developer solution until proteins could be seen (approximately 1 to 10 min). The reaction was stopped by putting the gels into fixative solution.

**Coomassie blue staining**

Coomassie staining was done when higher protein concentrations >200 ng/lane should be detected. Principally, coomassie brilliant blue interacts with alkaline aa residues e.g. arginine, lysine, histidine of proteins. After SDS-PAGE gels were washed once with aqua dest. and subsequently incubated in coomassie staining solution for 1 h. Destaining was performed with fixative solution for 1 to 2 h or with aqua dest. over night.

### 2.2.4.2 Heterologous expression and purification of proteins in prokaryotes

**Prokaryotic expression of recombinant viral proteins**

The transformation of *Escherichia coli* (*E. coli*) strains BL21 Star (DE3) One Shot and Rosetta2™ (Novagen) was done as described above (2.2.3.14). Optimisations of expression conditions by varying temperature and isopropyl β-D-thiogalactoside (IPTG) concentrations were done as described in the bachelor thesis of Axel Schulz (http://tu-dresden.de/die_tu_dresden/fakultaeten/fakultaet_mathematik_und_naturwissenschaften/fachrichtung_biologie/biotechnologen/Wirt-Parasit-Interaktion/Bachelorarbeit.Axel.pdf).
Optimally, heterologous protein expression was performed by inoculating 1,000 mL LB medium supplemented with carbenicillin [50 mg/L], chloramphenicol [35 mg/L] (in case of Rosetta2™) and 1% glucose with 50 mL of an overnight culture. Incubation took place at 200 rpm, 37°C for approximately 1.5 h until the $OD_{600}$ reached 0.6 and cultures were then induced with 0.5 mM IPTG. After 5 h at 200 rpm at 37°C *E. coli*-cells were centrifuged at 6,000 rpm (Heraeus Megafuge) for 10 min and stored at -80°C.

**Preparation of crude protein extracts from *E. coli*-cells**

In order to decide whether recombinant proteins are in the supernatant fraction (soluble proteins) or in the pellet fraction (insoluble inclusion bodies) of lysed *E. coli*-cells a protein preparation was performed with subsequent analysis by SDS-PAGE and WB (2.2.5.4). Therefore the pelleted cells were resuspended in buffer A (5 mL/g wet cell pellet) supplemented with Benzonase® [25 units/mL], lysozyme [1 mg/mL], Protease Inhibitor Cocktail III and 10 mM imidazole and the cells were disrupted by the freeze and thaw method. After 5 cycles of freezing in liquid nitrogen and thawing at 42°C the samples were centrifuged at 15,000 x g for 10 min. The supernatant containing the soluble proteins was stored at -80°C until analyzed by SDS-PAGE and WB (2.2.5.4). The pellet fraction was

washed once with buffer A and centrifuged again for 10 min at 15,000 x g. After discarding the supernatant the pellet was resuspended in buffer B (containing 8 M urea). After vortexing and incubation of 1 h at room temperature the pellet should have been dissolved. Otherwise samples were sonicated additionally for 2 min. After centrifugation at 15,000 x g for 10 min the supernatant of the pellet fraction was frozen at -80°C until analyzed.

**Purification of recombinant proteins by affinity chromatography**

For the purification of His tagged proteins an affinity chromatography was performed. In the course of this immobilized nickel-agarose interacts with the His tags of recombinant proteins. The bound proteins can subsequently be washed and eluted by either decreasing the binding affinity by lowering the pH or by adding a competitor such as imidazole.
Approximately 3 g *E. coli*-cells were lysed in BugBuster® (5 mL/g wet cell pellet) supplemented with Benzonase® [25 units/mL], lysozyme [1 mg/mL], Protease Inhibitor Cocktail III and 10 mM imidazole. After centrifugation (20,000 x g at 4°C for 10 min) the supernatant was discarded and the pellet fraction was washed twice in buffer A, centrifuged at 6,000 x g at 4°C for 5 min and the pellet (inclusion bodies) was dissolved in buffer B (supplemented with 8 M urea) as described above. The cleared lysate was applied to a nickel-agarose column (Novagen), washed twice with buffer B and afterwards with buffer C. For the refolding of the protein on column the urea was substituted by buffer C supplemented with 0.1% Triton X-100 which was thereafter replaced by the stabiliser β-cyclodextrin (5 mM in buffer C) and incubated at 4°C over night. After extensive washing for 1 h twice with buffer C the recombinant protein was eluted in buffer C with increased imidazole concentrations (200, 350, 500 mM).

### 2.2.4.3 Heterologous expression of proteins in eukaryotes

**Cell lysis and protein extraction**

In order to be able to analyze the expression of heterologous expressed proteins cells had to be disrupted to produce protein lysates. First, transfected or mock transfected cells were washed three times with ice-cold PBS. This ensures that cell metabolism and especially protease activity is stopped and that FCS containing medium is removed. The cells were scraped off the bottom of the 6-well plate or flask, resuspended in PBS and centrifuged at 4°C for 5 min at 800 x g. The cells (approximately 2-4 x $10^7$ cells/mL buffer) were lysed with

different buffers (RIPA, M-PER, Co-IP buffer) supplemented with Protease Inhibitor cocktail III (1:100), Benzonase® (25 units/mL) and 1 mM DTT, incubated for 20 min on ice and pulse-sonicated twice for 30 s. After centrifugation for 1 to 10 min at 15,000 x g supernatants were either diluted in 4-fold MESB for WB analysis (2.2.5.4) or used for immunoprecipitation experiments (2.2.5.6).

**Transient transfection in 24-well plate format**

Transfections of HEK293T and Huh-7 cells with eukaryotic expression vectors containing the fusion genes GFP-E, GFP-M, GFP-N and FLAG-ORF3 were performed with the help of FuGENE® HD transfection reagent (Roche) according to the manufacturer´s instructions. Typically, 2 to 4 x $10^5$ cells were plated on a 24-well plate containing glass slides (in case of HEK293T cells slides were coated with poly-L-lysine) and the cells were allowed to attach over night to reach 90% confluency. Optimally, 1.5 µg DNA and 3 µL FuGENE® HD Reagent were diluted in 25 µL DMEM without supplements. For complex formation the samples were incubated for 15 min at room temperature and applied to the cells. After a 24 h incubation at 37°C and 5% $CO_2$ the transfected cells were washed with PBS and fixed with paraformaldehyde (PFA; 4%) for IFA (2.2.5.2).

**Transient transfection of HEK293T cells in 6-well plate format**

Transfections of HEK293T cells with eukaryotic expression vectors containing the genes GFP-E, GFP-M, GFP-N, GFP as control and FLAG-ORF3 were performed with the help of FuGENE® HD transfection reagent (Roche) according to the manufacturer´s instructions. To achieve a 90% confluent cell monolayer 1.6 x $10^6$ cells/well were plated on 6-well plates and incubated over night. The transfection was performed with 6 or 8 µg DNA and 12 or 16 µL FuGENE® HD in 100 µL DMEM whereas in the cases of co-transfection 4 µg of each plasmid were applied. After incubation for 15 min at room temperature complexes were applied to the cells. After incubation of 26 to 48 h at 37°C and 5% $CO_2$ the transfected cells were washed three times with ice cold PBS, harvested and lysed as described above for immunoprecipitation (2.2.5.6) or WB analysis (2.2.5.4).

### 2.2.4.4 Recombinant expression by *in vitro* translation

*In vitro* translation of ORF3 and M protein was done for the analysis of N-glycosylation. Plasmids pcDNA3.1-ORF3-V5/His and pcDNA3.1-M-V5/His were employed in the TNT T7

quick coupled reticulocyte lysate system (Promega) according to the suppliers´ description. The proteins were metabolically labelled with [$^{35}$S]methionine (GE Healthcare) and translated in the presence of canine pancreatic microsomal membranes (Promega). Membrane-bound proteins were pelleted at 13,000 x g for 15 min and resuspended in PBS. The samples were split in half and incubated for 1 h at 37°C with endoglycosidase H (Endo H) or, as control, without additives. Afterwards the samples were subjected to SDS-PAGE. Radioactive signals were visualized by exposing dried gels to BioImage plates, which were scanned by using a bioimager analyzer (BAS-1000; Fuji).

### 2.2.5 Immunodetection assays

#### 2.2.5.1 Generating polyclonal antisera against ORF3, M and N.

The generation of polyclonal antisera against HCoV-NL63 proteins M, N was done by Eurogentec (Seraing, Belgium) with the help of keyhole limpet hemocyanin (KLH) coupled peptides. In the case of M, N and ORF3 protein, 2 peptides for each protein were synthesized (aa positions 180-195 and 212-226 for M, aa positions 1-16 and 113-127 for N and aa positions 182-197 and 211-225 for ORF3; Eurogentec). For M and N immunization of rabbits was performed by Eurogentec protocol I using each peptide separately, and for ORF3 the immunization was performed in-house. Briefly, in case of ORF3 a chinchilla rabbit was immunized four times with 200 µg of a mixture of the two KLH coupled peptides and sera were tested as suggested by the manufacturer by enzyme-linked immunosorbent assay (ELISA) using the corresponding uncoupled peptides (2.2.5.3).

Moreover, we tested all sera with immunofluorescence assay (IFA) using infected LLC-MK2 cells (2.2.5.2) as well as with prokaryotic recombinant proteins with the help of WB and Dot blot analysis (2.2.5.4; 2.2.5.5). The bleedings for the applied anti-ORF3 (No. 87-3, mixed peptides), anti-M (No. 21; peptide aa position 180-195) and anti-N sera (No. 23; peptide aa position 1-16) were carried out 20 days (anti-ORF3) or 32 days (anti-M, anti-N) after the 4th injection and the sera were used directly without further purification. For long-time storage the sera were kept at -20°C.

#### 2.2.5.2 Immunofluorescence assay

For the indirect immunofluorescence assay (IFA) monolayer of cells are fixed to glass slides by using different fixatives. Whereas PFA maintains cell structures and protein epitopes by building protein networks, fixation with methanol and acetone results in relatively strong defragmentation of membrane systems. Nonetheless the dehydrating and precipitating features of methanol and acetone lead to optimal access to epitopes. After fixation the cells are incubated with primary and secondary antibodies whereas the detection of secondary antibodies is performed by fluorescence microscopy. This is possible as secondary antibodies are labelled with different fluorescent dyes that can adsorb certain wavelength and excitate different wavelengths.

**Analysis of expression and subcellular localization of HCoV-NL63 proteins by IFA**

Typically, 8 x $10^4$ LLC-MK2 cells were seeded on a 24-well plate and infected with HCoV-NL63 (2.2.1.2). Four days after the infection the cells were fixed with PFA (4%) for 15 min, permeabilized with 0.1% Triton X-100 for 10 min. Afterwards the cells were washed with PBS again and then incubated with the primary antibody, diluted 1:100 in sample buffer at 37°C for 1 h. For the detection of the different viral proteins (E, M, N, ORF3) peptide generated rabbit antisera (2.2.5.1) were utilized. The ER-Golgi intermediate compartment (ERGIC) was stained with the help of mouse-anti-ERGIC53 (2.1.5). Secondary detection was done with FITC-conjugated goat-anti-rabbit or rhodamine-conjugated goat-anti-mouse antibody (2.1.5) at 37°C in a wet chamber for 30 min. Slides were mounted with DakoCytomation Fluorescent Mounting Medium and analyzed by cLSM 510 META laser confocal microscope (Zeiss).

**Indirect IFA for co-localization studies in transiently transfected cells**

Transfections of HEK293T and Huh-7 cells with eukaryotic expression vectors containing the fusion genes GFP-E, GFP-M, GFP-N and FLAG-ORF3 were performed with the help of FuGENE® HD transfection reagent (2.2.4.3). After a 24 h incubation at 37°C and 5% $CO_2$ the transfected cells were washed with PBS and fixed with PFA (4%), permeabilized with Triton X-100 and incubated with rabbit-anti-FLAG (2.1.5) and mouse-anti-ERGIC53 (2.1.5) primary antibodies, both diluted 1:100 with sample buffer at 37°C in a wet chamber for 1 h. Secondary detection was performed with the help of Cy3-conjugated goat-anti-rabbit (1:200) and Cy5

labelled goat-anti-mouse (1:100) antibodies (2.1.5) at 37°C in a wet chamber for 30 min. Slides were mounted and analyzed by confocal laser microscopy (Zeiss).

**Indirect IFA for detection of SARS-CoV related antibodies**

To analyze if African bat sera have antibodies reactive with SARS-CoV antigen a SARS-CoV-IIFT kit (EUROIMMUN) was used as described by the manufacturer with few modifications. As a fluorescently labelled secondary antibody was not available a goat anti-bat immunoglobulin (2.1.5) was applied as second antibody which was stained by a third fluorescein isothiocyanate labelled donkey anti-goat immunoglobulin (2.1.5). To eliminate the possibility that second or third antibody reacts unspecifically with SARS-CoV antigen the dilution that produced no background signal (i.e. 1:1,000 and 1:100, respectively) was determined. Bat sera were diluted 1:100 in sample buffer, which was found to be the optimal dilution, and slides were incubated at room temperature for 2 h. Specific reactions were detected with goat anti-bat immunoglobulin (2.1.5) at a dilution of 1:1,000 (incubation time 1 h at room temperature), followed by incubation for 30 min with FITC labelled donkey anti-goat immunoglobulin (2.1.5) at a dilution of 1:100. Additionally, to exclude false positive results, a control sera panel was tested (3.1.1.1).

**IFA for detection of SARS-CoV antigen in infected cells**

For susceptibility studies of eukaryotic cell lines infected with SARS-CoV, infection rates were determined by IFA. Experimental procedures were performed as described elsewhere (Hattermann et al., 2005). Briefly, cells were fixed and permeabilized similarly as described above. For detection of SARS-CoV antigen slides were incubated with a SARS-CoV patient serum (diluted 1:100). Secondary detection was done with the help of a FITC labelled anti-human-antibody, mounted and analyzed by confocal laser scanning microscopy (Zeiss).

#### 2.2.5.3 Enzyme-linked immunosorbent assay

Generally, a direct enzyme-linked immunosorbent assays (ELISA) was used in our studies. Therefore an antigen (purified SARS-CoV antigen or peptides) was coated on to a fixed phase (96-well plate) and incubated with sera. Antibodies recognizing the antigen will bind specifically and can be detected by a secondary antibody which binds to the conserved part (Fc) of the primary antibody and is moreover labelled with the enzyme horseradish peroxidase

(HRP). By adding a chromogenic substrate, that is metabolized by the enzyme and subsequently changes its colour, one can measure the amount of bound antibody with the help of an ELISA reader. Absorption was measured at a wavelength of 450 nm and a reference wave length of 605-650 nm.

**ELISA for detection of SARS-CoV antibodies**

For the screening of the 705 bat sera the SARS-CoV ELISA kit (EUROIMMUN) was used with minor modifications. The bat sera were tested at dilutions of 1:50, and horseradish peroxidase (HRP) labelled goat anti-bat immunoglobulin conjugate (2.1.5) was used as secondary antibody at a dilution 1:2,000. Both antibody incubations took place at 37°C for 1 h. Negative bat serum was obtained from a captive bred bat (*Rousettus aegytiacus*) at NICD, Johannesburg (RSA), and the cut-off OD value used for interpretation of results was fixed as 3x mean $OD_{450/605}$ value determined for negative control samples, an approach deliberately intended to be conservative. Tests on positive sera were repeated and titers were determined. To evaluate test specificity and to exclude possible cross-reactivity to other viruses, especially to human pathogenic coronaviruses which have a seroprevalence in humans > 90% (Hofmann et al., 2005), 662 human sera were screened including 90 patients with other acute respiratory infections, 70 HCoV-229E positive sera and 4 HCoV-NL63 positive sera (kindly provided by Lia van der Hoek, AMC, Amsterdam, The Netherlands). To exclude background signals from putative unspecific binding of secondary antibodies goat-anti-human HRP and goat-anti-bat were also tested alone.

**ELISA for evaluation of antisera against viral HCoV-NL63 proteins**

The sera were tested as suggested by the manufacturer (Eurogentec) by ELISA using the corresponding uncoupled peptides. Briefly, peptides (100 ng/well) were coated on an ELISA plate using coating buffer (0.1 M $NaHCO_3$) and incubated at 4°C over night. After blocking (coating buffer supplemented with 1 mg/mL BSA) and washing, the sera, diluted in conjugate buffer (PBS supplemented with 0.1% Tween and 2% BSA), were applied to the wells and incubated at room temperature for 2 h. Secondary detection was performed with goat-anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (2.1.5) at room temperature for 2 h using tetramethylbenzidine solution as substrate. Absorption was measured at $OD_{450/620}$ nm.

#### 2.2.5.4 Western blot analysis

Western blot (WB) analysis is based on the principle that proteins bind to nitrocellulose or polyvinylidenfluorid (PVDF) membranes and that those immobilized and stabilized proteins can subsequently be incubated with antibody solutions. Secondary detection is performed with the help of HRP labelled antibodies and detection is done by autoradiography of chemiluminescence signals. Performed WB analysis was based on the manual instructions of Biometra Fastblot.

After SDS-PAGE (2.2.4.1) the gel, a membrane and 6 Whatman blotting paper were equilibrated in transfer buffer for 5 min. Beforehand PVDF membranes were activated by incubation in 100% methanol for 1 min. On top of three blotting papers the membrane, the gel and again three blotting papers were placed and occurring air bubbles removed. Per blot (10 x 7 cm) we used 150 to 200 mA with a maximum of 25 V applying a run time of 1 to 2 h. Afterwards membranes were washed with PBS-Tween washing buffer and blocked with 10% non-fat dry milk for 1 h, followed by an overnight incubation at 4°C or 2 h at room temperature with a primary antibody (2.1.5). Then the membranes were washed with washing buffer three times for 5 min followed by incubation with an appropriate HRP-conjugated secondary antibody (Pierce) for 1 h at room temperature. After extensive washing (6 x 5 min), detection was performed by using SuperSignal® West Dura Extended, Pico or Femto Chemiluminescent Substrate (Pierce).

**WB analysis of bat sera with SARS-CoV cell lysate**

WB was done using protein lysates, prepared from Vero E6 cell cultures infected with SARS-CoV Hong Kong isolate 6109 and from uninfected cultures (2.2.1.2). Proteins (5 µg per lane) were separated by 12% SDS-PAGE and blotted onto a 0.45 µm Protran nitrocellulose membrane. Bat sera diluted in antibody buffer were applied at dilutions 1:500 and 1:2,000. Secondary detection was performed by incubating blots with goat-anti-bat HRP (1:10,000) for 1 h and applying the SuperSignal® West Dura Extended Chemiluminescent Substrate detection assay. Films were equally exposed for 1 min. The signal intensity of the 150 kDa S and 50 kDa N proteins was evaluated independently by two operators.

**WB analysis of bat sera with recombinant SARS-CoV proteins S and N**

For a second confirmatory WB recombinant SARS-CoV proteins were utilized. For the prokaryotic expression of recombinant SARS-CoV N protein and a fragment of the S protein

(aa positions 318-510, accession no. AAP50485) the instructions of the Champion pET Directional TOPO® Expression kit (2.2.3.14) were followed using plasmids pET101-N and pET102-Saa318-510 (kindly provided by Klaus Grywna, Bernhard Nocht-Institute, Hamburg, Germany).

The purified recombinant protein (15 µg) was resolved on a discontinuous 12% SDS-PAGE gel (2.2.4.1). After blotting using 0.45 µm Protran nitrocellulose the membrane was cut in strips, which were incubated with bat sera diluted 1:2,500 and 1:5,000. A selected bat serum (No. 17) was used as a reference to exclude variations in the experimental procedure for the comparison of signal intensities in different blots. Sera which produced signals at a dilution 1:5,000 were considered as positive. To evaluate specificity a selection of 19 control sera was included comprising 12 ELISA negative bat sera, 2 SARS-CoV positive human sera and 5 SARS-CoV negative human sera including 4 HCoV-NL63 patients. Secondary detection was performed as described above.

**WB analysis of the expression of HCoV-NL63 viral proteins**

A WB analysis of viral proteins was done as described above. For the titration of the different antisera (2.2.5.1) HCoV-NL63 cell lysate generated from LLC-MK2 infected cells seven days post infection (~1 x $10^7$ cells/blot) was used for WB and the produced nitrocellulose strips were incubated with the different rabbit antisera (pre-immune sera as negative control) at dilutions ranging from 1:500 up to 1:256,000. Moreover generated specific antisera were used to analyze the expression pattern of HCoV-NL63 proteins during the course of infection. In that case LLC-MK2 cells (3.2 x $10^5$) were inoculated with HCoV-NL63 (MOI 0.01) and the infected cells were harvested at different time points i.e. zero until seven days post infection (dpi). The cells were lysed in RIPA buffer (2.2.4.3) and separated on a 12% SDS-PAGE gel (2.2.4.1). WB was performed by using anti-ORF3, anti-M, anti-N at dilutions 1:4,000, 1:250,000 and 1:24,000 respectively. Secondary detection was done with the help of SuperSignal® West Dura Extended Chemiluminescent Substrate.

**WB analysis of recombinant HCoV-NL63 viral proteins (prokaryotes)**

For WB analysis a maximum of 10 µg purified recombinant viral proteins (2.2.4.2) were separated on a 12% SDS-PAGE gel and transferred to 0.2 µm PVDF membranes. As primary antibodies mouse-anti-Xpress (2.1.5) diluted 1:5,000 or the specific rabbit anisera anti-M, anti-N and anti-ORF3 were used at dilutions 1:64,000, 1:16,000 and 1:4,000, respectively

(2.2.5.1). Detection was performed by using SuperSignal® West Dura Extended Chemiluminescent Substrate.

**WB analysis of recombinant HCoV-NL63 viral proteins (eukaryotes)**

Transfections of HEK293T cells with eukaryotic expression vectors containing the genes GFP-E, GFP-M, GFP-N, GFP as control and FLAG-ORF3 were performed with the help of FuGENE® HD transfection reagent (2.2.4.3). The cell lysis (2.2.4.3) was performed with RIPA lysis buffer (~4 x $10^7$ cells/mL). For the detection of the different proteins mouse-anti-GFP (1:5,000), mouse-anti-FLAG (1:5,000) or mouse-anti-β-actin (1:2,000) was used. As secondary antibody a goat-anti-mouse horseradish peroxidase (HRP)-conjugated antibody (1:30,000) was applied for 1 h at room temperature. Detection was performed by using SuperSignal® West Femto Chemiluminescent Substrate.

**Re-probing of PVDF membranes**

When it was necessary to reprobe PVDF membranes with another antibody, stripping was performed with the help of Re-Blot Plus Strong Solution (Chemicon). After incubation of 20-25 min in 10 mL/blot Re-Blot solution, membranes were washed with washing buffer and blocked again before use.

### 2.2.5.5 Dot blot analysis

For Dot blot analysis recombinant proteins (approximately 2 µg in 20 µL volume supplemented with 0.2% SDS) were spotted onto a 0.45 µm nitrocellulose membrane. After air-drying at room temperature, the membranes were blocked and treated the same way as a WB. Specific antisera (anti-M, N, ORF3) as well as the corresponding pre-immune sera as negative control were used at a dilution 1:1,000.

### 2.2.5.6 Immunoprecipitation

Immunoprecipitation is performed with either protein A or G sepharose. Protein A or G isolated from *Staphylococcus aureus* binds to the conserved part of antibodies (Fc). When a primary antibody forms a complex with a protein, it can thus be immunoprecipitated by adding protein A/G sepharose and subsequent centrifugation. On the one hand, antibody

specificity can be shown by adding a known protein. On the other hand, proteins can be isolated and concentrated from a protein mixture.

**Immunoprecipitation of recombinant proteins**

HEK293T cells were transfected with various GFP-tagged constructs, GFP as control or with FLAG-ORF3 as described above (2.2.4.3). Cells were lysed in RIPA buffer (2.2.4.3) 30 to 48 h after transfection. The lysates were precleared with 25 µL protein A sepharose beads (Amersham) for 1 h at 4°C. After a 2 h incubation at 4°C with rabbit-anti-GFP antibody (ab290) or mouse-anti-FLAG antibody (2.1.5), antibody-antigen complexes were adsorbed with 25 µL protein A sepharose beads. The collected beads were washed four times with cold RIPA buffer, mixed with MESB, incubated for 5 min at 37°C and subjected to WB analysis. The proteins were detected with the help of mouse-anti-GFP (diluted 1:5,000), rabbit-anti-FLAG (diluted 1:5,000) and the corresponding secondary antibodies using SuperSignal® West Femto or Dura Extended Chemiluminescent Substrate.

**Co-immunoprecipitation**

HEK293T cells were transfected with various GFP-tagged constructs, GFP alone as control, or with FLAG-ORF3 as described above (2.2.4.3). Cells transfected with GFP-tagged constructs were lysed in RIPA buffer (2.2.4.3). FLAG-ORF3 transfected cells were directly lysed in co-immunoprecipitation (Co-IP) buffer (2.1.6). As transfection efficiency was higher for FLAG-ORF3 GFP-protein lysates were mixed 12:1 with FLAG-ORF3 lysates. In a volume of 150 µL (Co-IP buffer) the lysates were precleared with 25 µL protein A sepharose beads (Amersham). After 2 h or over night incubation at 4°C with mouse-anti-FLAG (1:400) antibody-antigen complexes were adsorbed with 25 µL protein A sepharose beads. The collected beads were washed four times with cold Co-IP buffer, mixed with MESB, incubated for 5 min at 37°C and subjected to WB analysis. The different proteins were detected with the help of rabbit-anti-GFP (ab290; diluted 1:5,000), rabbit-anti-FLAG (diluted 1:5,000) or mouse-anti-β-actin (diluted 1:2,000) and the corresponding secondary antibodies using SuperSignal® West Femto or Dura Extended Chemiluminescent Substrate.

#### 2.2.5.7 Fluorescent activated cell sorting

The quick and precise measurement of large numbers of stained cells is realized by flow cytometry. The so-called fluorescent activated cell sorting (FACS) allows to study the

properties of individual cells using antibodies directed against their cell-surface proteins. In the presented case that was the announced receptor of SARS-CoV i.e. the angiotensin converting enzyme 2 protein.

Principally, a mixture of labelled cells in saline solution is forced through a nozzle creating a fine stream of liquid containing single cells at intervals. As each cell passes through a laser beam it scatters the laser light, and any dye molecules bound to the cell is detected by fluorescence signals. Sensitive photomultiplier tubes detect both the scattered light, which gives information on the size and granularity of the cell, and the fluorescence emissions, which give information on the binding of labelled antibodies, and hence on the expression of cell-surface proteins by each cell analyzed by computer.

The cells were harvested and counted as described above (2.2.1.1) and distributed at density 5 x $10^5$ cells per reaction tube. After pelleting the cells at 400 x g, 4°C for 5 min indirect immuno staining was performed with unlabelled primary antibodies rabbit-anti-ACE2 (dilutions 1:50 to 1:1600) and mouse-anti-ACE2 (concentrations: 40 µg/mL to 1.25 µg/mL) in 50 µL FACS-PBS. The cells were resuspended thoroughly by pipetting carefully up and down. After incubation on ice for 20 min, 1,000 µL ice-cold FACS-PBS was added to each vial and the samples were centrifuged at 400 x g, 4°C for 5 min. Afterwards 50 µL fluorophore-conjugated anti-immunoglobulin antibody (secondary reagent) in FACS-PBS was added (FITC labelled goat-anti-mouse or rabbit antibody) and the samples were incubated on ice for 20 min. The cells were washed with 1,000 µL FACS-PBS and centrifuged as described above. Before the samples were subjected to analysis on a flow cytometer, the cells were resuspended in 200 µL FACS-PBS supplemented with 100 µL PI solution immediately before analysis (0.33 µg/mL final concentration). Propidium iodide (PI) stains the dead cells which can be excluded during FACS analysis by gating on the PI negative (live) cell population. Samples of at least 10,000 cells were measured by applying an optimal flow rate of 500-1500 events/s.