2 Material and methods

2.1 Material

2.1.1 Expendable items

Material	Specification	Manufacturer
96 well plates	Real-time PCR	ABgene, Rapidozym GmbH, Luckenwalde, Germany
Blood collection set	BD vacutainer ® Safety lok™ Blood collection set	BD Biosciences, Heidelberg, Germany
Cell culture material	Flasks 25 $cm^{2/}$60 mL 75 $cm^{2/}$270 mL 150 $cm^{2/}$690 mL	TPP Techno Plastic Products, Trasadingen, Switzerland and Nunc, Wiesbaden, Germany and Greiner, Frickenhausen, Germany
Chromatography paper	3 mm Whatman paper	Whatman Ltd., Maidstone, England
Cryotubes	1.5 mL and 2.0 mL	Nunc, Roskilde, Denmark
Cuvettes	2.0 mL	Sarstedt AG & Co, Nümbrecht, Germany
Ethidium bromide adsorber	Columns	Merck KGaA, Darmstadt, Germany
Micro test tube racks		Brand GmbH & Co KG, Wertheim, Germany Nalgene, Hamburg, Germany
Needles	BD vacutainer®Passive shielding Blood collection needle Valu Set 621 3/4 “	BD Biosciences, Heidelberg, Germany
Parafilm	2”x2 Ft	American National Can, Greenwich, USA
PCR tubes	0.2 mL	ABgene house, Epsom, Surrey, UK
pH indicator	Indicator paper 0-14, 0-6, and 4.5-10	Carl Roth GmbH & Co, Karlsruhe, Germany
Pipette tips	With aerosol protection	Nerbe Plus GmbH, Winsen-Luhe, Germany
	Without aerosol protection	ART, Sigma Aldrich, München, Germany
Qiashredder spin columns	Mini columns	Qiagen GmbH, Hilden, Germany
Quick seal tubes	1 × 3½”	Beckman Instruments GmbH, München, Germany

Material	Specification	Manufacturer
Reaction tubes	0.5 mL	Sarstedt AG & Co, Nümbrecht, Germany
	1.5 mL and 2.0 mL	Eppendorf, Hamburg, Germany Brand GmbH & Co KG, Wertheim, Germany
	15 mL and 50 mL	Falcon, Oxnard, USA Greiner, Nürtingen, Germany
Seals (real-time PCR)	Clear seal strong	ABgene, Epsom, UK
Sterile filter	0.22 µm and 0.45 µm	Nalgene, Hamburg, Germany
Syringes and needles	1 mL to 50 mL	Braun Melsungen, Melsungen, Germany
Ultracentrifugation polyallomer tubes	½x2" and 9/16x3¾"	Beckman Instruments GmbH, München, Germany
X-ray film	BioMax MR-1; 14x17"	Kodak, Integra, Fernwald, Germany

2.1.2 Chemicals

Chemical	Supplier
Agarose, peq Gold Universal Agarose	peQ Lab GmbH, Erlangen, Germany
Ampicillin	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Bidistilled water (DNase and RNase free)	Fluka Chemie, Ulm, Germany
Bromophenol blue	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Chloramin T	Carl Roth GmbH & Co, Karlsruhe, Germany
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Dimethyl formamide (DMF)	Merck KGaA, Darmstadt, Germany
Di-Sodium Hydro Phosphate (Na_2HPO_4)	Merck KGaA, Darmstadt, Germany
Dithiothreitol (DTT)	Invitrogen, Karlsruhe, Germany
Ethanol	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany
Ethidium bromide (3,8-Diamino-5-ethyl-6-phenyl-phenanthridinium-bromid)	Carl Roth GmbH & Co, Karlsruhe, Germany
EDTA (Ethylene diamine tetraacetic acid)	Merck KGaA, Darmstadt, Germany
Glycerol	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany
Glycine	Merck KGaA, Darmstadt, Germany
Hydrochloric acid (HCl)	Merck KGaA, Darmstadt, Germany
Igepal CA 630	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany

Chemical	Supplier
Isopropyl-thio-galactosid (IPTG)	BTS Biotech Trade & Service GmbH, St. Leon-Rot, Germany
Isopropanol	Merck KGaA, Darmstadt, Germany
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Manganese chloride ($MnCl_2$)	Merck KGaA, Darmstadt, Germany
Polyethylene glycol 8000, (PEG 8000)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Potassium chloride (KCl)	Merck KGaA, Darmstadt, Germany
Potassium di-hydro phosphate (KH_2PO_4)	Merck KGaA, Darmstadt, Germany
Sodium butyrate ($CH_3(CH_2)_2COONa$)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Sodium chloride, (NaCl)	Merck KGaA, Darmstadt, Germany
Sodium hydroxide, (NaOH)	Merck KGaA, Darmstadt, Germany
Sodium dodecylsulfate (SDS)	Merck KGaA, Darmstadt, Germany
β-Mercapto ethanol	Merck KGaA, Darmstadt, Germany
Sucrose	Merck KGaA, Darmstadt, Germany
TEMED (N,N,N',N'-Tetramethylendiamin)	Merck KGaA, Darmstadt, Germany
Tris (Tris-(hydroxyl-methyl)-amino-methan, powder)	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Tris (1M solution)	Ambion, Huntingdon, Cambridgeshire, UK
Triton X-100	Merck KGaA, Darmstadt, Germany
Tween	Fluka Chemie, Ulm, Germany
X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase)	Biomol Feinchemikalien GmbH, Hamburg, Germany

2.1.3 Kits

Kit	Supplier
ABI PRISM BigDye Terminators v2.0	Applied Biosystems, Darmstadt, Germany
QIAamp DNA Mini Kit	Qiagen GmbH, Hilden, Germany
QIAquick gel extraction kit	Qiagen GmbH, Hilden, Germany
RNase-free DNase Set	Qiagen GmbH, Hilden, Germany
RNeasy Mini Kit	Qiagen GmbH, Hilden, Germany
Oligotex mRNA	Qiagen GmbH, Hilden, Germany
PAXgene Blood RNA Kit	Qiagen GmbH, Hilden, Germany
PAXgene RNA tubes	Qiagen GmbH, Hilden, Germany
TOPO TA cloning kit	Invitrogen, Karlsruhe, Germany
Nucleospin Plasmid Kit	Rapidozym, Luckenwalde, Germany

Kit	Supplier
Jetquick Gel	Genomed, Löhne, Germany
NucleoSpin kit	Machery-Nagel, Düren, Germany
Z Competent *E.coli* Transformation Kit	Zymo Research, Orange, CA, USA

2.1.4 Nucleic acids, nucleotides, and markers

Material	Supplier
λ DNA/Hind III	MBI Fermentas GmbH, St. Leon-Rot, Germany
^{33}P-labelled dATP	DuPont NEN, DuPont de Nemours Deutschland GmbH, Bad Homburg, Germany
Calf-thymus DNA, Type XV, activated	Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany
Carrier RNA	Qiagen GmbH, Hilden, Germany
Gene ruler 100 bp ladder +	MBI Fermentas GmbH, St. Leon-Rot, Germany
MS2 RNA	Roche Diagnostics GmbH, Mannheim, Germany
Oligonucleotides*	TIB Molbiol GmbH, Berlin, Germany
pEMBL	Heinz Ellerbrok, RKI
Ultrapure dNTP set	Pharmacia Biotech Europe GmbH, Freiburg, Germany

*list of used oligonucleotides see addendum 5.12.

2.1.5 Enzymes and inhibitors

Enzyme	Supplier
DNase, RNase free	Qiagen GmbH, Hilden, Germany
Protease inhibitor (Complete mini)	Boehringer Mannheim, Germany
RNase A, DNase and protease free	MBI Fermentas GmbH, St. Leon-Rot, Germany
RNasin	Promega, Mannheim, Germany
Superscript I	Invitrogen, Karlsruhe, Germany
Taq polymerase	Invitek GmbH, Berlin, Germany
Uracil DNA Glycosylase UDG	Invitrogen, Karlsruhe, Germany

2.1.6 Buffers and solutions

2.1.6.1.1 Bacterial and cell culture media

All powdered media were dissolved according to the manufacturer's instructions and filtrated (0.22 µm, Nalgene) or autoclaved (121°C, 1.2 bar, 30 minutes).

2.1.6.1.1.1 Bacterial culture

Media/solution	Concentration	Compound
LB medium	10 g/L	Tryptone
	5 g/L	Yeast extract
	10 g	NaCl

Media/solution	Concentration	Compound
SOC	20 g/L	Tryptone
	5 g/L	Yeast extract
	0.5 g/L	NaCl
	20 mL/L	1M glucose
IPTG solution	0.1 M	In H_2O
Ampicillin	100 µg/ml	Final concentration
X-Gal solution,	200 mg/mL	In DMF

2.1.6.1.1.2 Cell culture media

Media/Solution	Concentration	Supplier/compound
D-MEM	Ready to use powder	Life Technologies, Paisley, UK
RPMI 1640	Ready to use powder	Life Technologies, Paisley, UK
PBS (pH 7.2)	137 mM	NaCl
	2.7 mM	KCl
	10 mM	Na_2HPO_4
	2 mM	KH_2PO_4
L-glutamine	200 mM	Biochrom AG, Berlin, Germany
Trypsin EDTA	0.25%	Trypsin
	3 mM	EDTA in PBS, pH 7.2
Fetal calf serum (FCS)	5-10%	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany

2.1.6.2 Solutions for cDNA synthesis and PCR

Solution	Supplier
DTT (0.1 M)	Invitrogen, Karlsruhe, Germany
First strand buffer, 5x	Invitrogen, Karlsruhe, Germany
$MgCl_2$ (1 M)	Invitek, Berlin, Germany
Nucleotide mix (10 mM)	Amersham Biosciences, Pisctaway, NJ, USA
PCR-Puffer NH4 (10x)	Invitek, Berlin, Germany
TaqMan® Universal PCR Master Mix	Applied Biosystems, Weiterstadt, Germany

2.1.6.3 Solutions for gel electrophoresis

Solution/buffer	Concentration/volume	Compound
Loading buffer (6x)	10 mM	Tris HCl, pH 7.5
	2 mM	EDTA
	15% (v/v)	Glycerol
	0.1 % (w/v)	Bromophenol blue

Solution/buffer	Concentration/volume	Compound
TAE (pH 8.0)	40 mM	Tris-acetate
	1 mM	EDTA
TBE (pH 8.0)	45 mM	Tris-borate
	1 mM	EDTA
Formamide loading buffer	97.5% (w/v)	Deionized formamide
	10 mM	EDTA
	0.3 % (w/v)	Xylene cyanol
	0.3 % (w/v)	Bromophenol blue
Polyacrylamide gel (6%)	9 mL	40% acrylamid/bisacrylamid (19:1)
	6 mL	10x TBE
	30 g	Urea
	23.5 mL	H_2O
	24 µL	TEMED
	180 µL	APS
Sequencing gel (5%)	7.5 mL	40% acrylamid/bisacrylamid (19:1)
	30 g	Urea
	6.0 mL	10x TBE
	25 mL	H_2O

2.1.6.4 Other solutions and buffers

Solution	Supplier
Bubble Free Solution™	Zymo Research, Orange, CA, USA
Buraton	Schülke & Mayr, Norderstedt, Germany
LTK 008	Biodelta, Bad Oeynhausen, Germany
Lysoform	Lysoform-Dr. Hans Rosemann AG, Berlin, Germany
Sterilium Virugard	Bode Chemie, Hamburg, Germany

2.1.7 Laboratory apparatus

Apparatus	Specification	Supplier
Balance	L610D	Sartorius AG, Göttingen, Germany
Cell counter	Casy 1, model TT	Schärfe System GmbH, Reutlingen, Germany
Centrifuges:		
Desktop	5415	Eppendorf, Hamburg, Germany
Desktop	5402	Eppendorf, Hamburg, Germany
Sorvall	RC 28 S, GSA rotor	DuPont, Bad Homburg, Germany
Ultracentrifuge	XL-90 Rotors: SW40,70Ti TST54	Beckman Instruments GmbH, München, Germany Kontron ltd. Zurich, Switzerland
Developer	Curix 60	AGFA, Köln, Germany

Apparatus	Specification	Supplier
Electrophoresis chambers		
Agarose gels	Horizon™ 58	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany
PAGE	S2	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany
Geiger counter	LB122	Berthold Technologies GmbH & Co KG, Bad Wildbad, Germany
Gel dryer	Model 583	Bio-Rad Laboratories GmbH, München
Glassware	Flasks, pipettes	Brand GmbH & Co KG, Wertheim, Germany
Freezers	+ 4°C	Bosch, Berlin, Germany
	- 20°C	Siemens, Berlin, Germany
	- 70°C	Bosch, Berlin, Germany
Incubators		
Cell culture	BB 6220	Heraeus, Osterode, Germany
Bacteria culture	Type B 6030	Heraeus, Osterode, Germany
Magnetic stirrer	Variomag® Telemodul 20P	H+P Labortechnik GmbH, Oberschleissheim, Germany
Microscope	ID03	Carl Zeiss Jena GmbH, Jena, Germany
PCR machines		
Gradient PCR machine	Mastercycler Gradient	Eppendorf, Hamburg, Germany
PCR machine	AMP PCR System 7600	ABI, Weiterstadt, Germany
PCR machine	MJ research PTC 2000	M.J.Research inc. via Biozym Diagnostic, Hameln, Germany
Real-time PCR machine	TaqMan® 7700	ABI, Weiterstadt, Germany
Photometer	Ultraspec III	Pharmacia Amersham Biotech Europe GmbH, Freiburg, Germany
pH-meter	CG 837	Schott AG, Mainz, Germany
Pipettes	Different volumes (1-1000 µL)	Eppendorf, Hamburg, Germany and Gilson, Abimed Analysen Technik, Langenfeld, Germany
Pipetman	Pipetboy acu	Tecnomara Biosciences, Wallisellen, Switzerland
Power supply	ST 304, ST 150D	GIBCO/BRL, Life Technologies GmbH, Eggenstein, Germany
Safety cabinet	Laminar biogard	The Baker Company Inc., Sandford, Maine, USA
Shaker	Lab-Shaker	Adolf Kühner AG, Birsfelden, Switzerland

Apparatus	Specification	Supplier
Sequencer	ABI 3100 Genetic Analyzer	Applied Biosystems, Weiterstadt, Germany
Speed vac		Savant, Hicksville, USA
Videodocumentation	UVT-20 M/W	Herolab GmbH, Wiesloch, Germany
Vortex	Reax 2000	Heidolph Instruments GmbH &Co KG, Schwabach, Germany
	Vortex	Bender & Hobein AG, Zürich, Switzerland
Water bath		Julabo Labortechnik GmbH, Seelbach, Germany
X-Ray cassettes	35 × 43 cm	Siemens, Berlin, Germany

2.1.8 Software

Software	
ABI PRISM DNA Sequencing Analysis	Applied Biosystems, Weiterstadt, Germany
AutoAssembler 2.1	Applied Biosystems, Weiterstadt, Germany
Corel draw	Corel Corporation limited
Free cell	Microsoft Corp.
Microsoft Office	Microsoft Corp.
MS Internet Explorer	Microsoft Corp.
Netscape Navigator 4.5	Netscape Communications Corp. Mountain View, USA
Photoshop 5.0	Adobe Systems Inc., San Jose, USA
Prism 3.02	GraphPad Software, Inc
Reference Manager	ISI Research Soft.
Sequence Detector 1.6.3	Applied Biosystems, Weiterstadt, Germany
Sequencing analysis	ABI, Weiterstadt, Germany

2.2 Methods

2.2.1 Cell culture

2.2.1.1 General safety measures

Working with infected cell cultures and viruses requires safe waste disposal. Solid waste and used glass ware were collected in metal buckets with lids and autoclaved. Liquid waste was inactivated with 2.5% Chloramin T (final concentration). Surface decontamination was carried out with Lysoform. Contaminated material was inactivated in Buraton. Not contaminated surface and working material was cleaned with 70% (v/v) isopropanol. Retroviruses can be inactivated by treatment with 70% isopropanol (v/v), or 80% ethanol (v/v), with detergent (such as Tween, Triton X-100, or Nonidet), 3.7% (w/v) formaldehyde or 0.05% glutardialdehyde, or NaOCl-solution.

2.2.1.2 Cultivation of cell lines

Sterilization of cell culture media, other solutions and materials was achieved by autoclaving (121°C, 1.2 bar, 30 minutes) or sterile filtration (0.45 µm). FCS was heat-inactivated for one hour at 56°C. Cells were cultivated in one-way plastic cell culture material of appropriate sizes. All cell lines were cultivated at 37°C and 5% CO_2 in a humid atmosphere.

2.2.1.2.1 Adherent cell lines

Adherently growing cell lines were cultivated in D-MEM with 2 mM L-glutamine. FCS was added, either 5% (PK15, PS, Vero) or 10% (293T, 293 Graham, G-RM, MelJuso, Mewo, G361, HEpG2, GH). Medium of confluent cells was removed and cells were washed with PBS. 500 µL Trypsin·EDTA solution was added per 75cm^2 flask and cells were incubated at room temperature or 37°C for 5 to 10 minutes. The detached cells were suspended in fresh cell culture medium and diluted 1:5 to 1:20, depending on cell growth rates.

2.2.1.2.2 Suspension cell lines

Suspension cell lines were cultivated in RPMI 1640 supplemented with 10% FCS and 2 mM L-glutamin. Cells were cultivated up to a maximum density of 1×10^6 cells per mL, then resuspended and isolated by pipetting. Subsequently they were diluted 5- to 10-fold every 3 to 7 days depending on cell growth rate.

2.2.1.2.3 Employed cell lines

Cells	Cell type	Growth type	Provenance
293 Graham	Human epithelial kidney cell line	Adherent	RKI
293T	Human epithelial kidney cell line	Adherent	ATCC No: CRL-11268
CEM	Human T-lymphoblast cells	Suspension	ATCC No: CRL-2264
G361	Human Caucasian malignant melanoma	Adherent	ECACC No: 88030401
GH	Human teratocarcinoma	Adherent	RKI, A.Mahini
G-RM	Human Caucasian melanoma	Adherent	ECACC No: 95032301
HEpG2	Human hepatocellular carcinoma	Adherent	ATCC No: CRL-11997
Kasumi-1	PBMC from an acute myeloid leukemia patient	Suspension	DSMZ No: ACC 220
MelJuso	Human melanoma	Adherent	DSMZ No: ACC 74
Mewo	Human malignant melanoma cells	Adherent	ECACC No: 93082609

Cells	Cell type	Growth type	Provenance
Molt 4/8	Human lymphoblasts (CD4+)	Suspension	ATCC No: CRL-1582
MS1533	Long term cell culture of PBL from MS patient	Suspension	PEI, Langen
MS1845	Long-term cell culture of PBL from MS patient	Suspension	PEI, Langen
MT2	Human T cells, HTLV- transformed	Suspension	RKI
PK15	Porcine embryonic kidney cells	Adherent	ATCC No: CCL-33
PS	Porcine kidney cells	Adherent	RKI, C. Schmitt
Vero	African green monkey kidney cells	Adherent	ATCC No: CCL-81

2.2.2 Isolation of nucleic acids

2.2.2.1 Quantification

Nucleic acid concentrations were determined by spectrophotomectric measurement of the amount of ultraviolet irradiation absorbed by the bases. The readings were taken at 260 nm and 280 nm. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. An OD of 1 corresponds to approximately 50 µg/mL for double-stranded DNA and 40 µg/mL for single-stranded DNA and RNA. The ratio between the readings at 260 nm and 280 nm (OD_{260}/OD_{280}) provides an estimate of the purity of the nucleic acid. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 and 2.0, respectively. Purified mRNA was not quantified because of low nucleic acid content.

2.2.2.2 DNA isolation

2.2.2.2.1 DNA isolation from whole blood

Whole blood EDTA samples were centrifuged at 1500 × g for 10 minutes. The leukocytes (buffy coat) which were enriched between plasma and erythrocytes were collected. 200 µL buffy coat were used for DNA extraction, the rest was stored at -70°C. DNA preparation was performed using the 'DNA Blood and Body Fluid' (DNA Blood Mini Kit) protocol according to the manufacturer's instructions. DNA was eluted in 200 µL H_2O and stored at -70°C.

2.2.2.2.2 DNA isolation from PBMC

PBMCs were isolated from whole blood and stored at -70°C. After thawing 5×10^6 to 1×10^7 cells were pelleted at 800 × g for 10 minutes. DNA extraction was performed using the 'Blood and Body Fluid' protocol according to the manufacturer's instructions. DNA was eluted in 200 µL H_2O and stored at -70°C.

2.2.2.3 RNA isolation

2.2.2.3.1 Specific aspects for RNA

All work involving RNA was performed either using sterile RNase-free single use plastic ware or DEPC-treated glass ware. Only purchased RNase free or DEPC-treated H_2O was used. RNA extraction was performed spatially distinct from the PCR set-up or product analysis.

2.2.2.3.2 RNase-inhibitor

RNase inhibitor (RNasin) was used according to the manufacturer's instruction.

2.2.2.3.3 Total RNA isolation from cell culture

5×10^6 to 1×10^7 cells were pelleted at $800 \times g$ for 10 minutes. The pellet was re-suspended in the appropriate volume RLT buffer (Qiagen) containing 1% (v/v) β-mercapto ethanol; total RNA extraction was performed according to the manufacturer's instructions using the RNeasy Mini kit. DNase digestion was performed on minispin columns according to the manufacturer's instructions using RNase free DNase. RNA was eluted in H_2O and stored at -70°C.

2.2.2.3.4 Total RNA isolation from virus pellets

Cell-free cell culture supernatant was either centrifuged at 14,000 × g for 90 minutes using a desktop centrifuge or ultracentrifuged at 100,000 × g for 1 hour (SW40 rotor). The pellet was resuspended in 100 µL H_2O and 350 µL RLT buffer containing 1% (v/v) β-mercapto ethanol and 1 µg carrier RNA. RNA extraction was performed according to the Qiamp RNA Mini Protocol for RNA Cleanup. RNA was eluted in 50 µL H_2O and stored at -70°C.

2.2.2.3.5 Total RNA isolation from whole blood (EDTA/buffy coat)

Whole blood (EDTA) samples were centrifuged at $1500 \times g$ for 10 minutes. The subsequently enriched leukocytes (buffy coat) between plasma and erythrocytes were collected. 200 µL were used for RNA extraction following the 'Isolation of Total RNA from Animal Cells' protocol (RNeasy Mini Kit), the remaining buffy coat and extracted RNA was stored at -70°C.

2.2.2.3.6 Total RNA isolation from whole blood (PAX-system)

All blood samples for quantitative expression analysis were collected in PAX system tubes (Qiagen). An RNA protection buffer is contained in the vacutainer system stabilizing the RNA composition of a given sample at the time of sampling. RNA extraction was performed according to the manufacturer's instructions. Initial centrifugation was performed at $5000 \times g$. The RNA preparation included an RNase-free DNase digestion (Qiagen). RNA was eluted twice in 40 µL BR5 and stored at -70°C. For photometrical quantification of PAX-buffer derived RNA in Tris the relationship $A_{260}=1 \Rightarrow 44$ µg/mL was used according to the manufacturer's directions for samples eluted in buffer BR5.

2.2.2.3.7 Preparation of mRNA

The Oligotex kit (Qiagen) was used according to the manufacturer's instructions for mRNA preparation from total RNA. Samples were eluted in 40 µL buffer OEB twice and stored at -70°C.

2.2.3 Reverse transcriptase PCR (RT-PCR)

Reverse transcriptase PCR (RT-PCR) can be used to detect RNA, e.g. viral genomic RNA or RNA transcripts and involves first the synthesis of a single stranded complimentary DNA (cDNA) using reverse transcriptase. Depending on the purpose of the experiment, the primers for first-strand synthesis can be specifically designed to (1) hybridize to a particular target sequence, (2) random hexanucleotides can be used, which are capable of priming cDNA synthesis at many points along the RNA templates, or (3) oligo(dT) primer binding generally to all mRNAs can be used. The cDNA synthesis is followed by amplification using a thermo stable DNA polymerase.
The cDNA synthesis was performed using random hexamer oligonucleotides and mRNA in a final volume of 20 µL.

RT-PCR: cDNA synthesis

Reagent/step	**Concentration**	**(µL)**
Random Hexamer primer	100 ng/µL	1.0 µL
Calf thymus DNA	500 ng/µL	1.0 µL
dNTP (each)	25 mM	0.4 µL
mRNA	-	10.6 µL
Incubation at 65°C for 5 minutes, cooling to 4°C		
DTT	0.1 M	2.0 µL
1st strand buffer	5x	4.0 µL
Superscript I	200U/µL	1.0 µL
Incubation at 37°C for 50 minutes, denaturation at 70°C for15 minutes.		

2.2.4 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) is used to amplify small quantities of DNA and cDNA which can then be detected by gel electrophoresis or real-time PCR. The use of thermo stable DNA polymerases allows automated cycles (Mullis and Faloona, 1987). Amplification by PCR requires the presence of two oligonucleotide primers 15 to 30 bases long (each complementary to sequences on one of the two strands of DNA), the four deoxynucleotide triphosphates (dNTPs), a thermo stable DNA polymerase (e.g. *Taq* DNA polymerase from *Thermus Aquaticus*), template and appropriate buffer conditions. Three distinct events occur during a PCR cycle: (1) Denaturation of the extracted DNA or cDNA

(template) at 92°C-96°C, (2) annealing of the oligonucleotide primers to complementary sequences on the template at temperatures between 37°C and 65°C, and (3) DNA synthesis by a thermo stable polymerase through extension of the primers at 72°C-74°C.

2.2.4.1 Specific PCR

PCR using specific primers was performed in order to amplify DNA or cDNA, a list of used primers is provided in the addendum (5.12).

PCR mix:

Reagent	Final concentration
10xNH4 buffer	1x
$MgCl_2$	1-4 mM
dNTP	200-800 µM
Sense primer	100-800 nM
Reverse primer	100-800 nM
Taq DNA polymerase	50 mU/µL
Template	Up to 500 ng
H_2O to final volume	

PCR profile:

Step	Time	Temperature
Denaturation	2-5 minutes	94°C
35-45 cycles		
denaturation	15-30 seconds	94°C
annealing	30 seconds	48°C-60°C
elongation	30 seconds-2 minutes	72°C
Final elongation step	10 minutes	72°C

Annealing temperatures were chosen according to the optimal primer conditions.

2.2.4.2 Detection of PCR products – gel electrophoresis

DNA has a negative charge in solution therefore it migrates to the anode in an electric field. In agarose gel electrophoresis the DNA is forced to move through a sieve of molecular proportions that is made of agarose. The rate of diffusion is reciprocally proportional to the $\log_{10}$ of the number of base pairs. The separated nucleic acids can be visualized by staining with ethidium bromide which contains a tricylcic planar group that intercalates between the stacked bases of DNA. UV radiation at 254 nm is absorbed by the DNA and transmitted of the dye. Radiation at 302 nm and 366 nm is absorbed by the bound dye itself. In both cases, the energy is re-emitted at 590 nm in the red-orange region

of the visible spectrum. The detection limit is ~10 ng DNA (Sambrook and Russell, 2001a). Depending on the size of DNA fragments different agarose concentrations from 0.8% (1-15 kb) up to 2% (0.1-2 kb) were used. Agarose was dissolved in 1x TAE by boiling and ethidium bromide was added (0.25 µg/mL). The liquid agarose was poured into a horizontal mold of a gel electrophoresis apparatus and an adequately formed comb was positioned in the liquid. After cooling the comb was removed, 1x TAE buffer was added to cover the gel. DNA samples were mixed with 6x loading buffer and loaded into the slots. A voltage of 1-5 V/cm (distance between the anode and cathode) was applied for 30 minutes to 1 hour.

2.2.4.3 Quantitative PCR – real-time PCR

The TaqMan® method of real-time PCR (Holland et al., 1991, Livak et al., 1995) uses an oligonucleotide (probe) that anneals to an internal sequence within the amplified DNA fragment. The oligonucleotide, usually 20-24 bases in length, is labeled at one end with a fluorescent group (e.g. Fam) at its 5'end and a quenching group (usually Tamra) at its 3'end, which is blocked with PO_4, NH_2 or a blocked base, thus it cannot act as a primer itself. The labeled oligonucleotide is added to the PCR together with primers required to drive amplification of the target sequence. When both the fluorescent and quenching groups are present in close apposition on the intact hybridization probe, any emission from the reporter dye during the course of real-time PCR is absorbed by the quenching dye, and the fluorescent emission is low. As the reaction progresses, and the amount of target DNA increases, progressively greater quantities of oligonucleotide probe hybridize to denatured target DNA. However, during the extension phase of the PCR cycle, the 5'→3'exonuclease activity of the thermo stable polymerase cleaves the fluorophore from the probe. Because the fluorescence is no longer in close proximity to the quencher, it begins to fluoresce. Because of the high sensitivity of fluorometric detection, real-time PCR is capable of measuring the initial concentration of target DNA over a range of five or six orders of magnitude.

2.2.4.3.1 TaqMan® protocol

Real-time PCR was either performed using the Universal Master Mix or an in-house PCR mix.

2.2.4.3.1.1 Universal Master Mix

The Universal Master Mix (UMM) contains dNuTPs, $MgCl_2$, thermo stable DNA polymerase, uracil *N*-glycosylase (UDG), and provides the necessary buffer requirements. Thus, only primers, probe and template have to be added:

Real-time PCR with Universal Master mix:

Reagent	Concentration
UMM	0.5x
Sense primer	100-200 nM
Reverse primer	100-200 nM
Probe	100 nM
Template DNA/cDNA	Up to 500 ng
H_2O to final volume	

2.2.4.3.1.2 In house real-time mix

Reagent	Concentration
10x NH4 buffer/Rox	1x
$MgCl_2$	1.5-3.5 mM
dNuTP	100 nM each
Sense primer	100-200 nM
Reverse primer	100-200 nM
Probe	100 nM
UDG	20 mU/µL
Taq DNA polymerase	50 mU/µL
Template DNA/cDNA	Up to 500 ng
H_2O to final volume	

2.2.4.3.1.3 PCR profiles for real-time PCR

PCRs were carried out as two-step or three-step PCRs depending on the required conditions for optimal performance. Annealing temperatures were adapted to the primers.

Real-time PCR profiles:

Step	Three-step PCR	Two-step PCR	Temperature
UDG digestion	2 minutes	2 minutes	50°C
Denaturation	10 minutes	10 minutes	94°C
45 cycles:			
denaturation	20 seconds	20 seconds	94°C
annealing	30 seconds	-	60°C-64°C
elongation	30 seconds-2 minutes	15 seconds -1 minute	72°C

2.2.4.4 Analysis of TaqMan® data

Because the target sequences are amplified and detected in the same instrument, there is no need to withdraw aliquots during the reaction or to process them. Instead, the instrument plots the rate of accumulation of amplified DNA over the course of an entire PCR. The greater the initial concentration of target sequences in the reaction mixture, the fewer the number of cycles required to achieve a particular yield of amplified product. The initial concentration of target sequences can therefore be expressed as the fractional cycle number (C_T, ct), required to achieve a present threshold of amplification. A plot of C_T against the $\log_{10}$ of the initial copy number of a set of standard DNAs yields a straight line. The target sequences in an unknown sample may easily be quantified by interpolation into this standard curve.

2.2.4.5 Preventing contamination

Several preventive measures were taken in order to avoid PCR contamination with exogenous DNA sequences. The rooms for nucleic acid extraction, cDNA synthesis, PCR set-up and PCR product analysis were spatially distinct. Basic reagents such as dNTP mix, water, and $MgCl_2$ aliquots were also prepared in a separate room. Only pipettes, glass, and plastic ware which were not exposed to DNAs in the laboratory were used to make and store solutions. Buffers and enzymes were stored in small aliquots and discarded after use. PCR mixes were prepared in no-DNA boxes, DNA or cDNA were added in separate DNA-boxes. Safety cabinets used for nucleic acid extraction and PCR boxes were cleaned after each usage and contaminating DNA was inactivated by UV irradiation at 254 nm wavelength for at least 10 minutes. Potential contaminations of cabinets with nucleic acid were eliminated with LTK 800. Uracil *N*-glycosylase (uracil DNA glycosylase, UDG) was used to destroy amplified DNAs that are unintentionally carried from one PCR to another (Hartley and Rashtchian, 1993). This enzyme cleaves uracil-glycosidic bonds in DNA that contains dU residues incorporated in place of dT residues but will not cleave RNA or double stranded DNA that contains rU or dT residues, respectively. The contamination protocol was initiated by routinely substituting dUTP for dTTP in PCR. This substitution has little effect on the specificity of the PCR or the analysis of PCR products. When subsequent sets of PCRs are briefly treated with uracil *N*-glycosylase, contaminating DNA containing uracil residues is destroyed. The UDG digestion was performed routinely for all real-time PCRs.

2.2.5 Differential Display Reverse Transcriptase PCR (DDRT-PCR)

In 1992 Liang and Pardee, and independently Welsh et al., described a subtractive method for using PCR to amplify and display many cDNAs derived from mRNAs of a given cell or tissue type (Liang and Pardee, 1992, Welsh et al., 1992). The method relies on two different types of synthetic oligonucleotides: anchored antisense primers and arbitrary

sense primers. A typical anchored primer is complementary to ~12 nucleotides of the poly(A) tail of mRNA and the adjacent one or two nucleotides of the transcribed sequence. Anchored primers therefore anneal to the junction between poly(A) tail and the 3'-untranslated region of mRNA templates, from where they can prime synthesis of first-stand cDNA. A second primer, an arbitrary sequence of ~10 nucleotides, is then added to the reaction mixture and double stranded cDNAs are produced by conventional, radioactively labeled PCR, carried out at low stringency. Usually eight different arbitrary primers are used with each cDNA in parallel. The products of the amplification reaction are separated by a denaturing polyacrylamide gel electrophoresis (PAGE) and visualized by autoradiography. By comparing the banding patterns of cDNA products derived from two different cell types or from the same cell type grown under different conditions, it is possible to identify the products of differentially expressed genes. Bands of interest can then be recovered from the gel, amplified, cloned, and sequenced.

2.2.5.1 DDRT-PCR: cDNA-synthesis

The cDNA mix including RNA was incubated at 65°C for 5 minutes in order to disintegrate secondary structures of the RNA template. The temperature was reduced to 37°C, after 10 minutes 1 µL of M-MLV reverse transcriptase was added. Following incubation for 50 minutes at 37°C, the samples were heated to 75°C for 5 minutes. Aliquots of the cDNA were subsequently used as template in radioactively labeled PCRs. Instead of oligo(dT) primer PBS-specific primers were used (results, 3.1.1, addendum 5.13, oligonucleotide sequences see addendum 5.12.4).

DDRT-PCR (cDNA synthesis):

Reagent/step	**Final concentration**	**(µL)**
RNA 100 ng/µL	200 ng/µL	2,0 µL
Reverse primer (2 µM) *	0.1 µM	2,0 µL
dNTP (250 µM each)	20 µM	1.6 µL
H_2O	Ad 11.4 µL	7.4 µL
Incubation at 70°C for 10 minutes, cooling to 4°C		
DTT (0.1 M)	0.01 M	2.0 µL
1st strand buffer (5x)	1x	4.0 µL
Superscript I (200 U/µL)	10 U/µL	1.0 µL
Incubation at 40°C for 60 minutes		

* oligo(dTA, dTC, dTG), PBS-specific primer

2.2.5.2 Radio-labeling of PCR products

Double stranded radio-labeled DNA was produced by conventional PCR, using the cDNA synthesized with an outer PBS-specific primer now in combination with the respective

inner PBS-specific primer and an arbitrary primer (AP) in sense direction. Up to eight different AP were used in parallel. The PCR was always performed using the same PCR machine in order to rule out apparatus dependent variations. PCR was carried out using the following conditions: (94°C, 30 sec (40x: 94°C, 15 sec; 40°C, 2 min; 72°C, 30 sec) 4°C ∞)

DDRT-PCR (PCR setup):

Reagent	Final concentration	(µL)
10x PCR buffer (NH4)	1x	1.0
$MgCl_2$ (50 mM)	2 mM	0.4
dNTP (25 µM each)	2 µM	0.8
AP (arbitrary primer, 2 µM)	0.2 µM	1.0
PBS (inner PBS, 2 µM)	0.2 µM	1.0
cDNA	-	1.0
α[^{33}P]-dATP (2000 Ci/mMol)	20 Ci/mMol	0.1
Taq polymerase (5 U/µL)	0.05 U/µL	0.1
H_2O to final volume		5.0
Final volume		10.0

2.2.5.3 Denaturing polyacrylamide gel electrophoresis

The radioactively labeled products of the amplification reaction were separated by a denaturing polyacrylamide gel electrophoresis (PAGE). Acrylamide, 10x TBE, and H_2O were mixed and urea was dissolved by stirring and heating. The mixture was filtrated and degassed for at least 10 minutes. TEMED and APS were added and the mixture was poured between prepared glass plates (34 cm × 25 cm). The plates were rinsed, impregnated with Bubble Free Solution™, separated by spacers, and fixated by large bulldog clips. A comb was inserted to provide slots. Polymerization time was at least 2 hours. The plates holding the gel were attached to the electrophoresis tank. The reservoirs of the electrophoresis tank were filled with 1x TBE buffer prepared from the same batch used to cast the gel. The wells formed by the comb were flushed with buffer using a syringe. 3.5 µL of radioactively labeled PCR product was mixed with 2.0 µL denaturing loading buffer and heated to 85°C for 2 minutes in order to separate the DNA strands. Samples were placed on ice immediately after heating until they were applied to the gel. Samples were pipetted into the slots in appropriate order. The gel was run for 2.5 to 3 hours at 40 W to 60 W, until the marker dyes had migrated to the desired segment.

2.2.5.4 Autoradiography

Following PAGE the glass plates were detached carefully. The gel was blotted to a filter paper and dried for 2 hours at 80°C under vacuum. After drying and cooling the gel was

attached to X-ray film and the exact position on the film was marked with pinpricks. Exposition time was 12 to 48 hours at room temperature using appropriate cassettes.

2.2.5.5 Selection of candidate bands and DNA elution

Banding patterns of non-infected and infected samples were compared. All bands of the same lengths which were present in the infected samples and not detectable in non-infected samples were analyzed as follows. These bands were marked with pinpricks and the dried gel was removed from the autoradiography. The marked bands including the blot paper were cut out and transferred to a reaction tube containing 50 µL H_2O. The samples were kept at 4°C for 10 minutes, then heated to 95°C for 15 minutes and cooled to 4°C again. The eluted DNA was subjected to re-amplification by PCR in order to yield enough material for cloning and sequencing.

2.2.5.6 Re-amplification of PCR products

The same primers and PCR conditions as for the radioactive-labeling PCR were used.

DDRT-PCR (re-amplification):

Reagent	Final concentration	(µL)
10x PCR buffer (NH4)	1x	4.0
$MgCl_2$ (50 mM)	2 mM	1.6
dNTP (2.5 mM each)	0.2 mM	3.2
Arbitrary primer (2 µM)	0.2 mM	4.0
PBS (inner primer, 2 µM)	0.2 mM	4.0
Eluted DNA		4.0
Taq polymerase (5 U/µL)	0.05 U	0.4
H_2O ad 40.0 µL		18.8
Final volume		40.0

2.2.5.7 Purification of PCR products

The products of the re-amplification PCR were analyzed by agarose gel electrophoresis. Amplified products were purified using the Qiagen PCR purification kit according to the manufacturer's instructions. If necessary, PCR products were separated by agarose gel electrophoresis. Gels were analyzed under UV light and candidate bands cut out. DNA was recovered using the Genomed Gel Extraction kit according to the manufacturer's instructions. DNA concentration was determined photometrically.

2.2.6 Cloning

2.2.6.1 Cloning of PCR fragments

PCR products were cloned using the TOPO TA Cloning™ kit according to the manufacturer's instructions. The pCR®II vector contains antibiotic resistance and β-galactosidase genes for selection and identification of recombinant plasmids. The ligation was performed for 5 minutes at room temperature.

Cloning mix:

Reagent	(µL)
TOPO™ vector pCR®II	0.5 µL
PCR product	1.0 µL
Salt solution	1.0 µL
H_2O	2.5 µL

2.2.6.2 Transformation of competent cells

The ligation probe (5 µL) was added to 50 to 100 µL of competent bacteria (*E.coli*, XL-1 MRF). The mixture was incubated on ice for 15 to 45 minutes. The bacteria were then stroked out on LB plates containing ampicillin, X-Gal (4 µL), and IPTG (4 µL). Incubation was performed at 37°C overnight. White colonies represented transformed bacteria. An appropriate number of white colonies were picked and the transformation was controlled by colony PCR.

2.2.6.2.1 Preparation of competent bacteria

Transformation competent bacteria were prepared using the Z Competent *E.coli* Transformation Kit according to the manufacturer's instructions. Freshly prepared *E. coli* (XL-1 MRF) were used. Divergent from the instructions the bacteria were cooled to -70°C immediately following preparation. They were stored at -70°C up to several months. The transformation competence was determined as follows: 1 µL of a control plasmid (2 ng/µL, pEMBL) were mixed gently with 100 µL of freshly thawed (on ice) competent bacteria and incubated on ice for 20 minutes. 10 µL were stroked out on LB-ampicillin plates and incubated overnight at 37°C. Colonies were counted. The transformation rate was determined as transformed bacteria per µg DNA, only bacteria with transformation rates $>10^6$ of transformed bacteria per µg DNA were used.

2.2.6.3 Colony PCR

White colonies were picked and dipped in 50 µL PCR mix. PCR products were analyzed by agarose gel electrophoresis, the length of the detected products showed whether PCR products were inserted into the plasmids.

PCR mix for colony PCR:

Reagent	Final concentration
10xNH4 buffer	1x
$MgCl_2$	3.5 mM
dNTP (each)	200 nM
Primer M13	200 nM
Primer T7	140 nM
Taq DNA polymerase	30 mU/µL
Template	dipped
H_2O to the final volume 50 µL	

PCR profile for colony PCR

Step	Time	Temperature
Denaturation	4 minutes	94°C
30 cycles:		
denaturation	30 seconds	94°C
annealing	30 seconds	55°C
elongation	30 seconds	72°C
Final elongation	10 minutes	72°C

2.2.6.4 Plasmid preparation

3 mL LB medium containing 100 µg/mL ampicillin were inoculated with successfully transformed bacteria as determined by colony PCR. Incubation was carried out in a shaker at 37°C overnight. 1.5 mL of the overnight culture was pelleted at 8000 × g for 10 minutes at room temperature. Plasmids were prepared using the NucleoSpin-Kit™ according to the manufacturer's instructions.

2.2.6.5 Plasmid standards for quantitative PCR

Plasmid standards for real-time PCRs were cloned as described above. The sequences were controlled by sequencing. Plasmid preparations were performed as described (2.2.6.4) and DNA concentration was determined in triplicate for three separate dilutions by spectrophotomectric analysis. The number of plasmids was calculated based on the following data: 1 base pair (dsDNA) has an average molecular weight of 660 g/mol, the number of base pairs of the respective plasmid, and the length of the inserted PCR fragment is known. For example, a 12 kb plasmid with a concentration of 60 ng/µL yields approximately 4.5×10^9 plasmids per µL. Plasmid standard were diluted in DNase and RNase free water containing1 ng/µL λDNA.

2.2.7 DNA Sequencing

DNA sequencing was performed according to Sanger (Sanger et al., 1977). The double-stranded DNA is denatured and hybridized with an oligonucleotide. Starting from this sequencing primer a complementary DNA strand is synthesized. In addition to dNTPs dideoxynucleoside triphosphates (ddNTPs) are added to the reaction mix. Whenever a ddNTP instead of a dNTP is attached to the nascent DNA, the strand synthesis stops since the 3'-hydroxygroup which is necessary for elongation is lacking. Each of the used ddNTP is coupled to a different fluorescent dye and can thus be traced separately.

2.2.7.1 Sequence reactions

20 ng to 30 ng of PCR product was used for each sequencing reaction using the ABI PRISM BigDye Terminators v2.0 kit according to the manufacturer's instructions. For cell culture supernatant derived samples derived by DD, the inner PBS-specific primer was used as sequencing primer. PCR products originating from patients blood samples had to be cloned prior to sequencing because often overlapping PCR fragments with the same length were obtained.

Sequencing reaction:

Reagent	(µL)
PCR product	20-30 ng
Primer (2 µM)	1.0 µL
Big dye stock solution	2.0 µL
H_2O	ad 10 µL

PCR profile for sequencing reactions:

Step	Time	Temperature
Denaturation	2 minutes	94°C
25 cycles of		
denaturation	15 seconds	94°C
annealing	15 seconds	55°C
elongation	2 minutes	60°C

2.2.7.2 Purification of sequencing reactions

In order to remove excess of fluorescently labeled ddNTP the sequencing reactions were added to a mix of 2 µl of 3 M sodium acetate pH 4.6 and 50 µL ethanol. The samples were then centrifuged for 20 minutes at 14,000 × g. The supernatant was removed, the pellets washed with 500 µL 70 % ethanol and re-centrifuged. The supernatant was removed. The pellets were dried and stored at -20°C until they were analyzed.

2.2.7.3 Sequencing gel

The gel was casted like the PAGE gel (see 2.2.5.3). DNA samples were dissolved in a denaturing buffer without additional dye and denatured by heating to 95°C for 5 minutes. Electrophoresis was carried out in an ABI sequencer. The fluorescent signals of separated DNA bands were detected during electrophoresis by laser excitation. The sequencing pattern was analyzed using the 'Sequence Analysis' software (ABI).

2.2.7.4 Analysis of sequence data

BLAST analyses (Altschul et al., 1997) were performed with the NCBI databank (National Center for Biotechnology Information). Sequences were identified by comparison to known sequences available in the public databank.

2.2.8 Reverse Transcriptase Activity Assay (RTA)

The reverse transcriptase assay uses a defined RNA template and appropriate conditions in combination with a retrovirus-containing sample (Pyra et al., 1994, Boni et al., 1996). This sample might act as exogenous source for reverse transcriptase activity. If no other exogenous RT activity is added, the detection of cDNA serves as proof of retroviral reverse transcriptase activity. The presence of cDNA is detected and quantified with a real-time PCR assay. This assay can be performed using cell culture supernatant or plasma samples, however, different sample preparations have to be considered.

2.2.8.1 Sample preparation

2.2.8.1.1 Preparation of cell culture supernatant

Cell culture supernatant from confluent adherent cells or densely grown suspension cells was harvested and stored at -70°C. Cell free cell culture supernatant was used. 2 mol of a 20% sucrose solution was overlaid with 2 mol 10 mM Tris (in DEPC·H_2O, pH 8.0) in a ½ × 2" Beckman tube. 1 mol supernatant was put on top. The sample was centrifuged for 1 h 30 minutes at 70,000 × g at 4°C using the TST54 rotor. The supernatant was removed completely.

2.2.8.1.2 Preparation of plasma

Plasma, either derived from EDTA whole blood by centrifugation at 1500 × g for 10 minutes or cell free citrate plasma, was diluted 1:10 with 10 mM Tris (in DEPC·H_2O, pH 8.0) and cleared by centrifugation at 5000 × g for 10 minutes. 2 mol of a 20 % sucrose solution was overlaid with 1.5 mol 10 mM Tris (in DEPC·H_2O, pH 8.0) in a ½ × 2" Beckman tube. 1.5 mol of the diluted and cleared plasma was put on top. The sample was centrifuged for 1 h 30 minutes at 70,000 × g, 4°C using a TST54 rotor. The supernatant was completely removed.

2.2.8.2 Resupension and lysis of the virus pellet, cDNA-synthesis

Following ultracentrifugation and removal of supernatant, 200 µL of 2-fold cDNA buffer were added to the pellet and incubated for 10 minutes at room temperature. The pellet was resuspended by pipetting. The buffer was composed as cDNA buffer which at the same time lyses virus particles.

RTA cDNA/lysis buffer:

Reagent	Concentration (2x buffer)
Tris HCl, pH 8.0	112 mM
KCl	112 mM
DTT	11.2 mM
Igepal	1.8%

A pre-mix containing the RNA template, calf thymus DNA, bivalent cation, reverse primer as well as dNTPs was incubated for 30 minutes at 37°C.

Pre-mixes:

Reagent	Mg^{2+} buffer	Mn^{2+} buffer (0.8 mM)	Mn^{2+} buffer (0.4 mM)
MS2 RNA	300 ng/reaction	300 ng/reaction	300 ng/reaction
Calf thymus DNA	500 ng/reaction	500 ng/reaction	500 ng/reaction
MS2 reverse primer	500 nM	500 nM	500 nM
$MgCl_2$	5 mM	/	/
$MnCl_2$	/	0.8 mM	0.4 mM
dNTP	500 µM each	1 mM each	1 mM each

Following the pre-incubation, 10 µL of the lysed virus particle preparation (2.2.8.2) and H_2O to a final volume of 20 µL was added to the pre-mix. In parallel to the lysate at least two dilutions (1:10 and 1:100, in case of high virus titers up to 1:10000) were analyzed. As internal cDNA standard a serial dilution of commercially available M-MLV reverse transcriptase was used (0.06 µU to 60,000 µU). All samples were tested in three dilutions and each dilution in triplicate. As negative control water instead of RT or virus preparation was added to cDNA mix. The cDNA synthesis was carried out for 5 hours at 37°C, followed by a denaturing step of 70°C for 15 minutes. Samples were kept at 4°C until PCR was performed.

2.2.8.3 Quantification of reverse transcriptase activity

The quantification of cDNA and thus RT activity was conducted by real-time PCR. An RNase digestion of the target RNA was performed in the 96-well PCR plate prior to real-time PCR according to the manufacturer's instructions.

Real-time PCR buffers for RTA:

Reagent	Mg^{2+} buffered cDNA	Mn^{2+} buffered cDNA
10x buffer NH4/Rox	1x	1x
$MgCl_2$	5 mM	8 mM
dNTP	100 nM each	100 nM each
Sense primer	300 nM	300 nM
Reverse primer	300 nM	300 nM
Probe	100 nM	100 nM
UDG	40 mU/µL	40 mU/µL
RNase A	1 U/µL	1 U/µL
Taq DNA polymerase	10 mU/µL	10 mU/µL
cDNA mix	10 µL	10 µL
H_2O	Ad 25 µL	Ad 25 µL

PCR profile for reverse transcriptase activity assay:

Step	Time	Temperature
RNase digestion	30 minutes	37°C
UDG digestion	2 minutes	50°C
Denaturation	10 minutes	94°C
45 cycles of		
denaturation	20 seconds	94°C
annealing	20 seconds	64°C
elongation	30 seconds	72°C

2.2.8.4 Antibody-stripping

HIV-positive plasma was mixed with an equal volume of a (2x) antibody-stripping buffer. The mixture was immediately put onto a sucrose step gradient (20%, 25% and 30% sucrose) and subjected to ultracentrifugation. The obtained virus pellet was lysed and employed in the RTA.

Reagent	Final concentration (2x)
Glycine	0.2 M
Magnesium acetate	0.04 M
Potassium chloride	0.1 M
pH 2.2	

2.2.8.5 Determination of viral load

The units of reverse transcriptase activity were determined in relation to a serial dilution of Superscript I. The enzyme activity is defined as follows: one unit incorporates 1 nmol of dTTP into acid-precipitable material in 10 minutes at 37°C using poly(A)*oligo$(dT)_{25}$ as template-primer (according to the manufacturer). Standard dilutions of 0.006 µU to 60,000 µU were used in triplicate in parallel in each assay. It was assumed that approximately 100 RT molecules are incorporated within one virion (Telesnitsky and Goff, 1997). Other publications give similar ratios: approximately 20 RT molecules per virion show 1 nU of RT activity (Andrews et al., 2000); 20 to 100 RT molecules are found within one virion (Maudru and Peden, 1997); and 10^{-5} U are equivalent to 4.25×10^4 virions, this corresponds to 1 nU and 4.3 virions, respectively (Sears et al., 1999). For the calculation of the virus load, it was assumed that 10 pU of RT activity are the equivalent of one RT molecule (Sears and Khan, 2003). Thus, one nU is approximately equivalent to one retrovirus particle.

2.2.9 Polyethylene glycol precipitation of virus particles

Cell free supernatant of retrovirus infected cell culture was filtrated (0.45 µm). Solid NaCl and polyethylene glycol were added to final concentrations of 0.5 M and 10 % (w/v), respectively. The solution was stored at 4°C overnight. The solution was centrifuged at 7000 rpm using a GSA rotor for 10 minutes at 4°C. The supernatant was removed and the pellet resuspended in an appropriate volume of PBS. A 20 % sucrose·PBS solution was filled into $^9/_{16} \times 3^3/_4$“ centrifuge tubes. The resuspension was layered on top the sucrose cushion. Ultracentrifugation was performed using a SW40 rotor at 35,000 rpm for 1.5 hours at 4°C. The supernatant was removed and the virus pellet was resuspended in an appropriate volume PBS (usually a 1:1000 concentration compared to the starting volume; according to Yamamoto et al., 1970, Sambrook and Russell, 2001b).

Alternative procedure:

This procedure is faster but yields 1-2 $\log_{10}$ less particles (comparison was performed with SMRV infected cell culture supernatant, quantification by RT-real-time PCR). Cell free supernatant of virus infected cell culture was filtrated (0.45 µm) and filled into 1 × 3½” Quick seal tubes. Ultracentrifugation was performed using a 70Ti rotor at 60,000 rpm (250,000 × g) for 25 minutes at 4°C. Supernatant was removed completely and the virus pellet was resuspended in an appropriate volume PBS. The suspension was transferred to new tubes and centrifuged again with 250,000 × g. Supernatant was removed completely and the pellet was resuspended in an appropriate volume PBS (usually a 1:1000 concentration compared to the starting volume).