Materials and methods, supporting online information for protocols

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DNA sequences homologous to hepatitis C virus (HCV) in the extrachromosomal circular DNA in peripheral blood mononuclear cells of HCV-negative subjects

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1. **Subjects and Isolation of peripheral blood mononuclear cells (PBMCs) from human plasma**

   From healthy subjects (blood donors), both negative and below the limit of the detection of the tests used: anti-HCV, HCV-RNA, anti-HBc, HBV-DNA, anti-HIV, 5 mL venous blood (+EDTA-Na, final concentration 5 mmol/L) was drawn. Written informed consent was given.

   The white blood cell fraction (PBMC) was isolated by Lympholyte®-H Cell Separation Media, Cedarlane/www.cedarlanelabs.com (Isolation of lymphocytes from Human peripheral blood). Access: "*CL5020-Lympholyte®-H.pdf*". The manufacture's protocol was followed throughout.

2. I**solation of the whole DNA from PBMCs**

   The QIAamp® DNA Mini and Blood Mini Kit was used to extract total DNA from PBMC; Access via: HB-0329-002, 1072897_HB_QIAamp_DNA_Mini_Blood_Mini_0612_20120709.pdf. The extracted nucleic acids were processed with RNase accordingly.

3. **Preparation of eccDNA from the whole DNA extracted from PBMCs**

   The Plasmid Safe™ ATP-Dependent DNase kit was used (PSAD digest). 5 to 10 μg PBMC DNAs were treated with Plasmid-Safe™ ATP-Dependent DNase as follows: 210 μL sterile water, 10 μL 25 mmol/L ATP, 25 μL 10× Reaction Buffer, 5 μL Plasmid-Safe DNase (50 U), 250 μL total volume. Incubation at 37 °C overnight (more than 12 h). 3 U of Plasmid-Safe DNase will digest 1 μg of DNA in 30 min at 37 °C. Access: "Plasmid-safe-atp-dependent-dnase.pdf", epicenter, an Illumina® company; Access: http://www.epibio.com/enzymes/nucleases-glycosylases-dna-binding-proteins/dna-exonucleases/plasmid-safe-atp-dependent-dnase?details. The instructions given in the manufacture's protocol were followed.

4. **Isolation of gel purified PSAD-resistant DNA/ecc DNA:**

   The PSAD-digested PBMC DNA was separated in a 0.6% agarose gel in TAE (Tris-Acetate/EDTA) buffer. After the electrophoresis, the DNA bands around (35±15) kb were excised from the agarose gel, e.g., shown in Fig. 1, from each lane shown. The recovered 35-kb gel segments were submitted to the QIAEX II Gel Extraction Kit. Access: 20021-QIAEX-II-Handbook--April-2012-EN.pdf. The procedure followed the manufacturer's instructions. "The QIAEX II Gel Extraction Kit is designed to extract and purify DNA from any agarose gel in either TAE (Tris-Acetate/EDTA) or TBE (Tris-Borate/EDTA) buffer, without phenol extraction or ethanol precipitation. QIAEX II silica particles have been optimized to enhance recovery of

very small and very large DNA fragments. DNA molecules of 40 bp to 50 kb are adsorbed to QIAEX II particles in the presence of high salt."

5. **Protocol to check for patterns of methylation in the HCV-DNA sequences of the HCV 5'-NCR targeted here: the pre-PCR digestion protocol with restriction endonucleases prior to the PCR with gene-specific primers**

This protocol was established (1) to exclude possible contaminations in PCRs by amplicons which are not methylated from prior PCRs, i.e., they are sensitive to the restriction endonucleases (RE) applied, and (2) to check for individual pattern of methylation within the original subject's DNA sequence section of the 5'-non-coding region (5'-NCR) of HCV, i.e., the target sequence in this study. We made consistent use of four restriction endonucleases selected for certain features: *Sma*I, *Xma*CI, *Msp*I, and *Hpa*II are inhibited if methylation(s) are present at certain cytosines within their cutting sequences. The protocol used here followed the same as described before (Dennin and Wo, 2003). As a control for the activity of the restriction endonucleases, we used the vector plasmid pCR 2.1 (Invitrogene) with a cloned fragment of the 5'-NCR sequence from a HCV-positive patient covering nucleotides 66 to 321 that contained the CCGG, CCCGGG sequences that in turn were confirmed by sequencing.

6. **PCR assays to test for the PSAD-resistant DNA/eccDNA with HCV gene-specific primers**

We applied nested resp. semi-nested Polymerase chain reaction (PCR without a reverse transcriptase (RT) step). We used AmpliTaq Gold® 360 Master Mix (AmpliTaq Gold® 360 DNA Polymerase include) from Applied Biosystems according to the manufacturer's instructions. Available: "AmpliTaq Gold® 360 Master Mix protocal-detail.pdf"; "AmpliTaq Gold® 360 Master Mix-Quick Reference Card.pdf" and " AmpliTaq-Gold-360-DNA-polymerase-and-master-mix-product-bulletin.pdf".

7. Details of the PCR protocols

For the PCR experiments presented here, we have selected published HCV gene-specific primers (GSPs) of its 5'-NCR, since this region is commonly used for routine diagnostics. HCV GSPs were used which span the nucleotide section nt 57 to nt 342: "E" stands for external, "I" for internal primer location; "1" stands for downstream: 5'>3', "2" for upstream: 3'>5' direction in the PCR;

PTNC-E1, nt 89–108; PTNC-E2, nt 152–170; PTNC-I1, nt 99–118; PTNC-I2, nt 139–158 (Garson et al., 1991);

AE1/2 and AI1/2 spanning nt 57–328 (Bukh et al., 1992); AK3 nt: 252–275 (Zemer et al., 2008).100 ng DNA per tube (50 µl) were used for PCR. For sequences see Table 1.

1. PCR: outer primers	
AE1(57-86):	actgtcttcacgca gaaagcgtct agccat
AE2(299-329):	ggtgcttgcga gtgccccggg aggtctcg
2. PCR: inner (nested) primers	
PTNC E1(89-108):	gttagtatgag tgtcgtgc
AK3(251-275):	agccgagta gtgttgggtc gcgaa
3. PTNC-P (122–141), Digoxigenin-labeled probe:	atggctctcc ccggaggggg

Table 1: Sequences of the primers and the probe used for PCR and hybridization.

For hybridization tests, the probe PTNC-P was used throughout; it is located on the sequence between the inner primers PTNC-E1 and AK3.

8. **Cloning of the amplicons after PCR for sequencings**

We used the TOPO TA Cloning Kit, Invitrogen, TOPO TA Cloning®, pCR®2.1 TOPO® vector.

9. **Sequencing**

Cloned plasmids were sequenced by Sanger's sequencing machine (ABI 3730xl). The sequences are available upon request.

10. **Alignments by BLASTn**

We used the Basic Local Alignment Search Tool (BLASTn) to align selected reference sequences of the Hepatitis C virus GT1b with and without query subranges against the human genome sequences provided by BLASTn. We followed the presets given. (Figure 1)

11. Protocol used to demonstrate the relative amount of the PSAD-resistant eccDNA fraction

The protocol used to demonstrate the relative amounts of the PSAD resistant "leftover" eccDNA (Fig. 1, right hand part, b) compared to the whole DNA extracted from PBMCs (Fig. 1, left hand part, a) depending on the subjects:

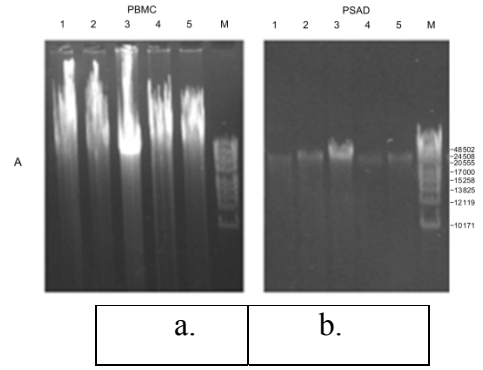

Fig. 1 Agarose gel-electrophoresis of (a.) the whole PBMC-DNA (left part) and (b.) the PSAD-resistant-DNA (right part).[1] It was used to measure the integrated optical density (OD) for comparison—see below Table 2:

	Sample No.:	23	24	25	26	28
	Lane in Fig. 1:	1	2	3	4	5
a.	PBMC, whole DNA:	144.80	621.85	205.08	235.76	196.95
b.	PSAD, eccDNA:	31.136	30.240	42.636	22.836	22.583
	PSAD/PBMC (%):	21.50	4.86	20.79	9.69	11.45

Table 2 Results of the integrated OD (IOD) value detected with *Gel-Pro Analyzer* software of the whole lanes; IOD comparison between *whole DNA of PBMCs* and *PSAD-resistant DNA* after gel-electrophoresis. The Gel-Pro Analyzer check was performed to obtain a semi-quantitative appraisal of the amount of eccDNA to be expected: a rough estimation of the size of the eccDNA leftover fraction after PSAD digestion of PBMC DNA relative to the whole PBMC DNA. We arranged the complete protocol to show the remaining leftover eccDNA±the range of bp after PSAD digest of the entire PBMC DNA in the middle of the gel.

References

Bukh J, Purcell RH, Miller RH, 1992. Importance of primer selection for the detection of hepatitis C virus RNA with the polymerase chain reaction assay. *Proc Natl Acad Sci USA*, 89:187-191.

Garson JA, Ring CJA, Tuke PW, 1991. Improvement of HCV genome detection with 'short' PCR products. *Lancet*, 338:1466-1467.

Gel-Pro Analyzer; https://meyerinst.com/imaging-Software/image-pro/gel/index.htm

Zemer R, Cohen YK, Naftaly T, et al., 2008. Presence of hepatitis C virus DNA sequences in the DNA of infected patients. *Eur J Clin Invest*, 38(11):845-848. https://doi.org/10.1111/j.1365-2362.2008.02029.x

[1] This is the same figure as shown in Fig. 2, part A, of the manuscript;