Electronic Supplementary Information

Protamine-adsorbed magnetic nanoparticles for efficient isolation and concentration of Hepatits-C-Virus from human plasma samples

Abdallah A. Yassin,[*a,b] Ahmed M. Elwaseef,[c] Magdy M. Elnashar,[b] Johannes Oldenburg,[a] Günter Mayer,[d] Bernd Pötzsch,[a] and Jens Müller[*a]

[a] *Institute for Exp. Haematology and Transfusion Medicine, University of Bonn Medical Centre, Bonn, Germany.*

Fax: +49 228 287 14762

Tel: +49 228 287 16735

E-mail: jens.mueller@ukb.uni-bonn.de; a.yassin@daad-alumni.de

[b] *Encapsulation & Nanobiotechnology Group, Center of Excelence; Polymers Dept., National Research Center, Cairo, Egypt. E-mail: a_yassin2009@hotmail.com*

[c] *Division of Biochemistry, Mansoura University, Mansoura, Egypt.*

[d] *Life and Medical Sciences Institute, University of Bonn, Bonn, Germany.*

Experimental Section

Chemicals and reagents

All chemicals including Iron (III) hexahydrate ($FeCl_3.6H_2O$), Iron (II) chloride tetrahydrate ($FeCl_2.4H_2O$), tri-Sodium Citrate ($Na_3C_6H_5O_7$), Sodium hydroxide, Sodium di-hydrogen phosphate (NaH_2PO_4), Di-Sodium hydrogen phosphate (Na_2HPO_4), NaCl, in addition to Bovine Serum Albumin (BSA) were purchased from Sigma Aldrich, Taufkirchen, Germany. Human alpha-thrombin was purchased from Cellsystems (Troisdorf, Germany). The fluorogenic thrombin peptide substrate (# I-1560) was purchased from Bachem (Bubendorf, Switzerland). Protamine hydrochloride (5,000 U/ml [50 mg/ml], medical grade) was purchased from MEDA Pharma (Bad Homburg, Germany). The 5'-FAM-labelled ssDNA-oligonucleotide (FAM-SA; Sequence: 5'-TCA GGC AGT ACC ACA AGG C-3') was synthesized and purified by HPLC by Microsynth (Balgach, Switzerland).

Preparation of citrated nanoparticles (cNPs)

The co-precipitation method was employed for the production of super-paramagnetic magnetite (Fe_3O_4) Nanoparticles (SPIONS) as previously described by Mehta et al.[1] with some modifications. The method depended on mixing ferric and ferrous ions in a 1:2 molar ratio in highly basic solution at elevated temperature.

In brief, Iron (III) chloride hexahydrate (0.0551mol) was dissolved in 150 ml of ammoniated water while 0.0275mol of iron (II) chloride tetrahydrate was dissolved in 150 ml of ammoniated water. Then, the two solutions were mixed in a 500 ml conical flask and placed in a temperature controlled water bath equipped with a magnetic stirrer. A sodium hydroxide aqueous solution (12.8 g in 120 ml of distilled water) was then added with a flow rate of 10 ml/min while continuously stirring at 80° C and the reaction was continued for 60 min under the same conditions. The resulting Fe_3O_4 particles were washed 3 times repeatedly with 500 ml of distilled water until neutral pH using magnetic field separation and finally kept in 150 ml distilled water in the fridge for further treatment.

For coating of particles with citrate ions, 10 ml of Magnetite solution were re-dispersed in a 100ml backer using ultrasonic for 5 min. Then, 1.5 mol of tri-sodium-citrate (5 gm in 10ml dist. water) was added to the magnetite under vigorous stirring at 85°C for 90 min. At the high temperature introduced, citrate molecules easily dissociated, leading to more ions formed in the solution. These negatively charged citrate ions induced the hydrogen bonding between

the structural (-OH) groups that fully cover the magnetite surfaces and the carboxyl (-COONa) group from citrate ions, yielding citrate-coated nanoparticles (cNPs).

Finally, nanoparticles were washed 2 times repeatedly with 100 ml distilled water using magnetic field separation and re-dispersed in 150 ml of distilled water. Prepared particles were stored in the fridge until used.

Determination of NP-concentration and yield

To measure the concentration of SPIONs yielded, one ml of the nanoparticles stock solution was dried and weighted. The weight of 1 ml of nanoparticles was equivalent to 0.062 g. Thus the total output of SPIONs in 150 ml was calculated to be 9.3 g. From this solution, 10 ml (0.62 g) were treated with citrate. The total output of citrate-coated nanoparticles preserved in 150 ml was calculated as shown above, yielding 0.525 g at a concentration of 3.5 mg/ml.

Nanoparticle characterisation

Chemical, structural and physical characterization of the two prepared forms of nanoparticles was done using Fourier transform infrared spectroscopy (FTIR) and Transmission electron microscopy (TEM). For this purpose, aliquots of the prepared nanoparticles were prepared as 1:10 dilution of its original concentration in distilled water.

Fourier transform infrared spectroscopy (FTIR)

From each sample, 5 ml were dried in a furnace at 70°C for 2 hours. The dried powders were collected, grind with potassium bromide (KBr) and compressed as disks. After compression, each disk was put into a Fourier transform infrared spectroscope (JASCO FTIR [6000 series]), with digital band analysis (JASCO spectra manager TM II cross-platform software, Mary's Court Easton, USA) and analyzed.

Transmission electron microscopy (TEM):

From each sample, a droplet was placed on parafilm for 10 min. A formavar covered 200 mesh grid coated with carbon (Plano GmbH, Wetzlar, Germany) was placed on the droplet for 5 min. The remaining solution was soaked with a filter paper. After drying, the grid was put into a transmission electron microscope (Philips CM 10) with digital imaging analysis from SIS (Olympus Europa GmbH, Hamburg, Germany) and images taken.

Adsorption of protamine to cNPs

Citrated nanoparticles (3.5 mg/ml stock solution) were transferred into 1.5 ml reaction tubes, separated by magnetic field and supernatants removed. Subsequently, cNPs were taken up in

500 µl of 10 mM phosphate buffer (pH 7, +/- 0.1% BSA) (PB) to reach a final concentration of 2 mg/ml. Then, 500 µl of protamine hydrochloride (pre-diluted to 2-fold the desired final concentration [2,000 U/ml used under optimized conditions] in the same buffer) was added and the tubes incubated under shaking for 2h at RT. After incubation, yielded PaNPs were washed 2x (by magnetic separation using a strong neodymium magnet) using PB and finally taken up in PB to yield a final concentration of 15 mg/ml. During control experiments in the absence of protamine, cNPs at the same final concentrations were incubated with either PB w/o BSA and PB in the presence of 0.1% or 1.0% BSA (final concentrations).

Thrombin-binding assay

Samples (cNPs or prepared PaNPs) were dispersed in PBS-buffer (pH 7.4, 0.1% BSA) at a final concentration of 1 mg/ml and purified human alpha thrombin (10 ng/ml [270 pM] final concentration) added to reach a final sample volume of 100µl. After incubation at RT for 30 min under shaking, particles were separated by magnetic field and 60 µl of the supernatants mixed with 60 µl of 400 µM fluorogenic thrombin peptide substrate (Boc-Asp(OBzl)-Pro-Arg-AMC · HCl) in PBS. Subsequently, 2 x 50 µl of the solution were transferred to the wells of black microtiter-plates and change in fluorescence over time (measured at 360/460 nm [ex/em]) using a Synergy 2 microplate reader (Biotek, Bad Friedrichshall, Germany). For calculation of residual thrombin activity in the samples, corresponding controls were run in parallel.

ssDNA-binding assay

Samples (cNPs or prepared PaNPs) were dispersed in PB-buffer (pH 7.4, 0.1% BSA) at a final concentration of 0.3 mg/ml and the FAM-labelled ssDNA-oligonucleotide (FAM-SA) added (10 nM final concentration) to reach a final sample volume of 300 µl. After incubation at RT for 2 min, particles were separated by magnetic field. Subsequently, replicates of 50 µl of the supernatants were transferred to the wells of black microtiter-plates and fluorescence intensity measured (485/528 nm [ex/em]). Buffer only and FAM-SA controls were measured in parallel.

HCV-positive plasma material

HCV RNA-positive plasma (EDTA) was prepared from whole blood donated by a HCV-positive haemophilic and determination of the HCV viral load in the plasma (1.37 x 10^6 IU/ml) performed by parallel testing with WHO standard 98/576 as previously described (one

unit of HCV-RNA corresponds to ~ 3 to 5 genome equivalents).[2] The HCV-positive plasma was diluted using confirmed HCV-RNA negative plasma to reach the desired concentrations stated in the manuscript. All samples were stored at -80°C until use.

Viral RNA isolation and detection of HCV-RNA by real-time RT-PCR

In general, isolation of viral RNA was done using the Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Accordingly, 140 µl of (plasma) sample volume were introduced to the assay. Purified total plasma RNA was tested for HCV RNA using an in-house real-time PCR assay. The overall system has been validated according to regulatory guidelines and is described in methodological details elsewhere.[2] The assay shows a limit of detection with respect to 95% and 50% cut-offs of 114 IU/ml and 28 IU/ml of plasma HCV-RNA, respectively.

Capturing of HCV from plasma samples by PaNPs and viral RNA isolation

Plasma samples of volumes up to 560 µl were added to 1.5 ml or 2.0 ml reaction tubes (Eppendorf, Hamburg, Germany) and diluted up to 1 in 3 using phosphate buffer (0.1% BSA, pH 6 [or pH-values as indicated in the manuscript]). Then, either cNPs or PaNPs were added from stock solutions to reach the final concentrations stated in the manuscript. Subsequently, mixtures were incubated at RT under shaking for 20 min. After incubation, particles were collected by magnetic field, all plasma remains removed, and particles dispersed in 140 µl of 0.9% NaCl-solution. Then, 560 µl of viral lysis buffer (buffer AVL, content of used Viral RNA Mini Kit) were added to the samples and incubated for 10 min at RT under shaking. Subsequently, particles were again separated by magnetic field and supernatants transferred to a new 1.5 ml reaction tube. Then, 560 µl of 100% ethanol were added to removed supernatants and samples introduced to the silica-based spin-column procedure as specified by manufacturer. Plasma control samples (140) were run in parallel according to the manufacturer's instructions. Here, 560 µl of buffer AVL were added to samples and incubated for 10 min. Then, 560 µl of 100% ethanol were added and samples handled as described above. Finally, plasma total RNA was eluted in 50 µl of pure water and analyzed by HCV real-time RT-PCR (at least in triplicates) as previously described.[2]

Supplementary results

Fig. S1. Appearance of unmodified (NPs, yellow label) and citrate-stabilized magnetite nanoparticles (cNPs, green label) at concentrations of 3.5 mg/ml in water after 1 h of settling at RT under normal gravity. While magnetite nanoparticles showed strong precipitation, the negative surface charge of the citrate-coated nanoparticles (cNPs) ensure the presence of a colloidal solution.

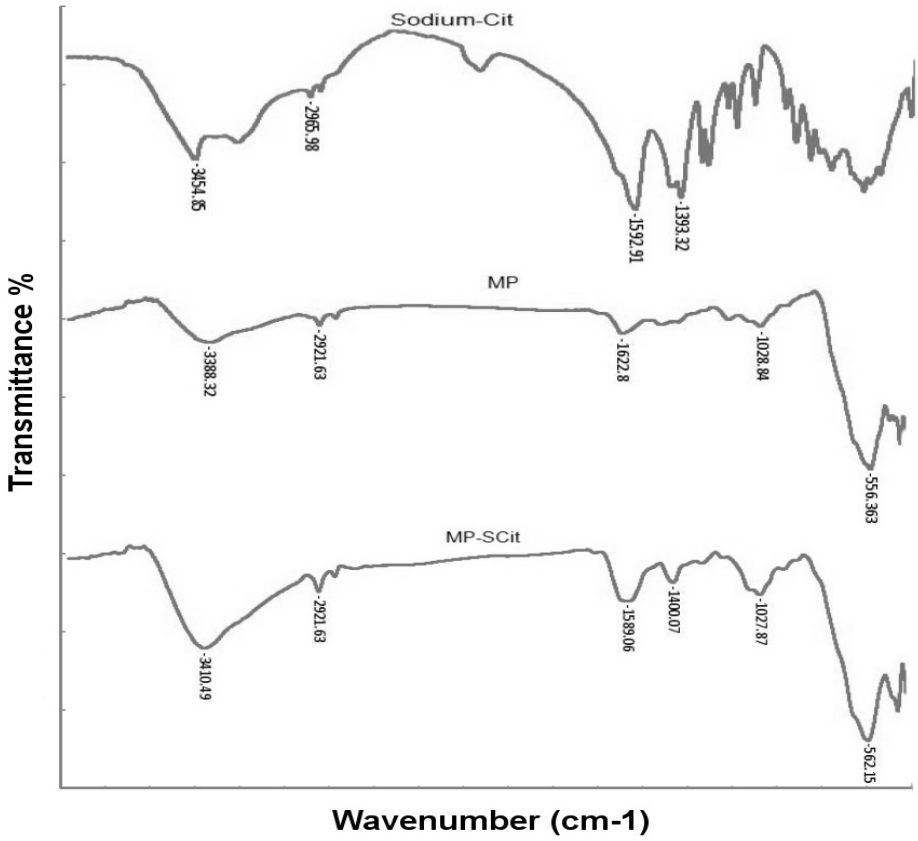

Fig. S2. Infrared spectra for pure sodium citrate (Sodium-Cit), magnetite nanoparticles (MP, SPIONS) and citrate-coated magnetite nanoparticles (MP-SCit, cNPs).

The IR spectrum (4000 cm^{-1} – 400 cm^{-1}) of the citrate-coated SPIONs showed collective bands from the Magnetite / and pure citrate spectrums: i) the broad and intense band of the hydroxyl group (-OH) at 3410 cm^{-1} indicated a combination between the sharp band of citrate ions' hydroxyl (-OH) groups at 3454 cm^{-1} and the broad band of the structural (-OH) groups located on the magnetite surface. ii) A weak and splitted band of the CH_2 stretching could be seen at 2921 cm^{-1}. iii) The sharp band of C=O vibration (symmetric stretching) at 1592 cm^{-1} from the COONa group of sodium citrate (sodium-Cit) has shifted to an intense band at 1589 cm^{-1}, indicating the adsorption of sodium-Cit radicals to the magnetite surface; iv) An adjacent band at 1400 cm^{-1} in cNP-spectra indicated the asymmetric stretching of CO from the COONa group; v) The very sharp and intense bands between 556 cm^{-1} and 562 cm^{-1} indicated the stretching and bending vibration of tetrahedral structure of the magnetite in SPION.[3]

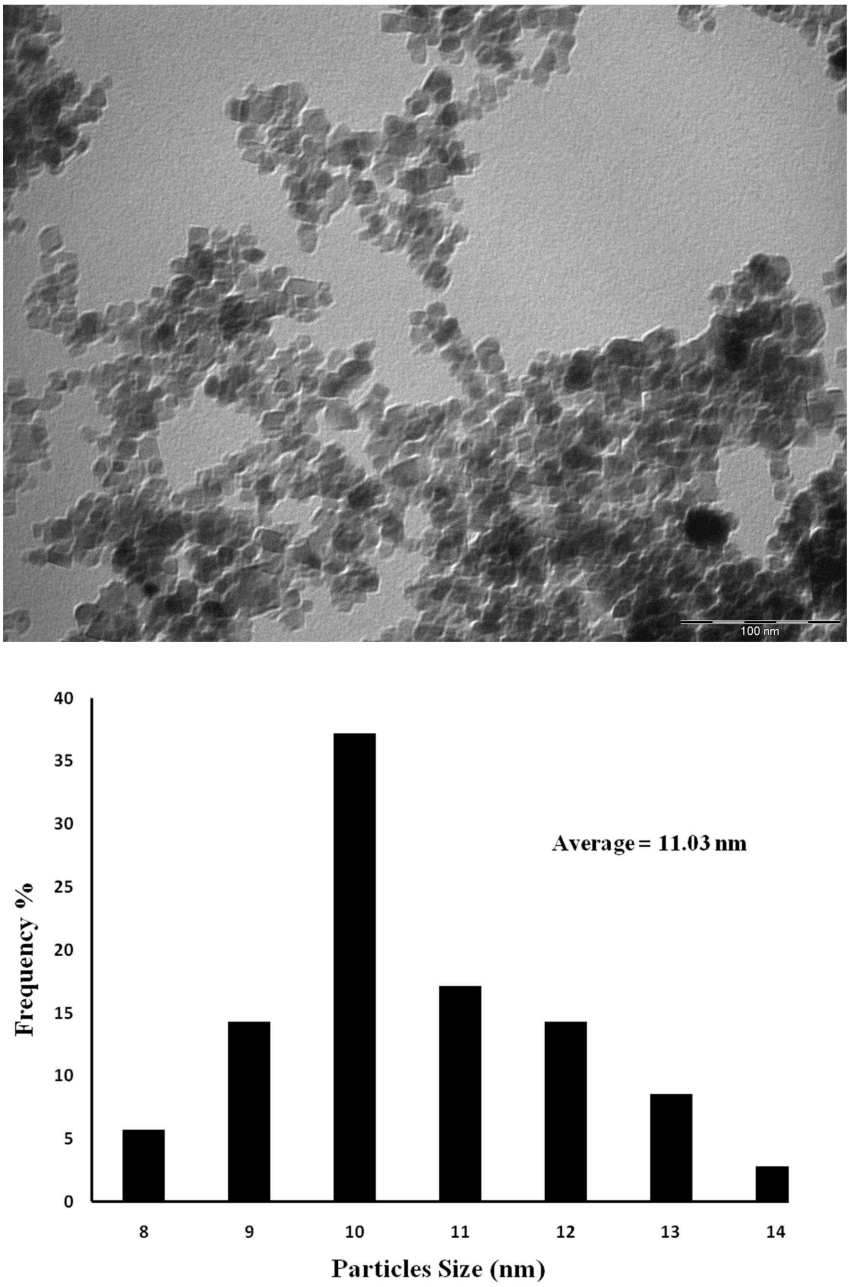

Fig. S3. TEM image (scale bar represents 100 nm) and histogram analysis of citrated nanoparticles (cNPs).

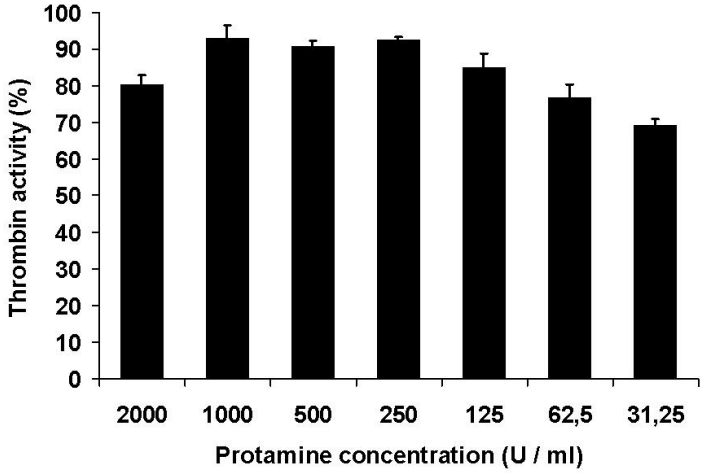

Fig. S4. Effect of applied final concentration of protamine on adsorption efficiency to cNPs as determined by the thrombin-binding assay. Although a plateau between 250 U/ml (2.5 mg/ml) and 1000 U/ml (10 mg/ml) was observed, a final concentration of 1000 U/ml was used during all further experiments.

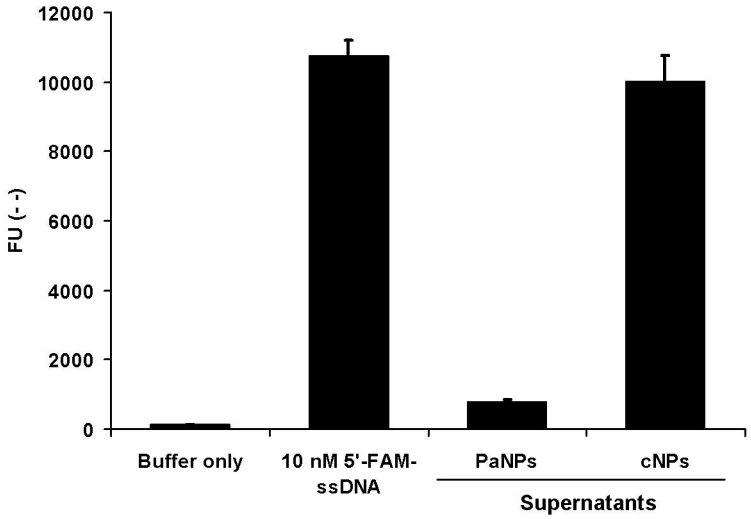

Fig. S5. Binding of 5'-FAM-labelled ssDNA-oligonucleotide to PaNPs and cNPs. After 2 min of incubation, particles (0.3 mg/ml final concentration) were removed from solutions and remaining fluorescence measured in the supernatants. Results are shown in comparison to buffer background- and oligo-only controls.

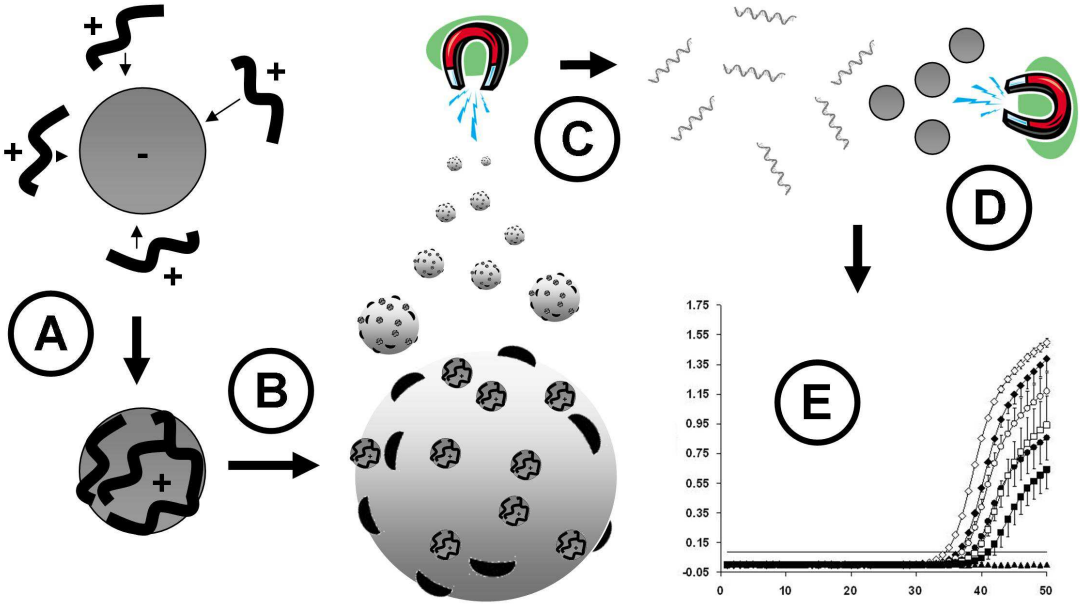

Fig. S6. Schematic presentation of assay principle. Protamine is added to cNPs yielding stable PaNPs (**A**). Then, PaNPs are added to plasma, binding to HCV present in the samples (**B**). After magnetic separation of PaNPs-covered HCV, supernatants are removed, the collected fraction taken up in NaCl-solution and (viral) lysis buffer added (**C**). After lysis, magnetic particles are removed from the solution (**D**) and viral RNA is further purified by a standard silica-based spin column procedure for subsequent real-time RT-PCR analysis (**E**).

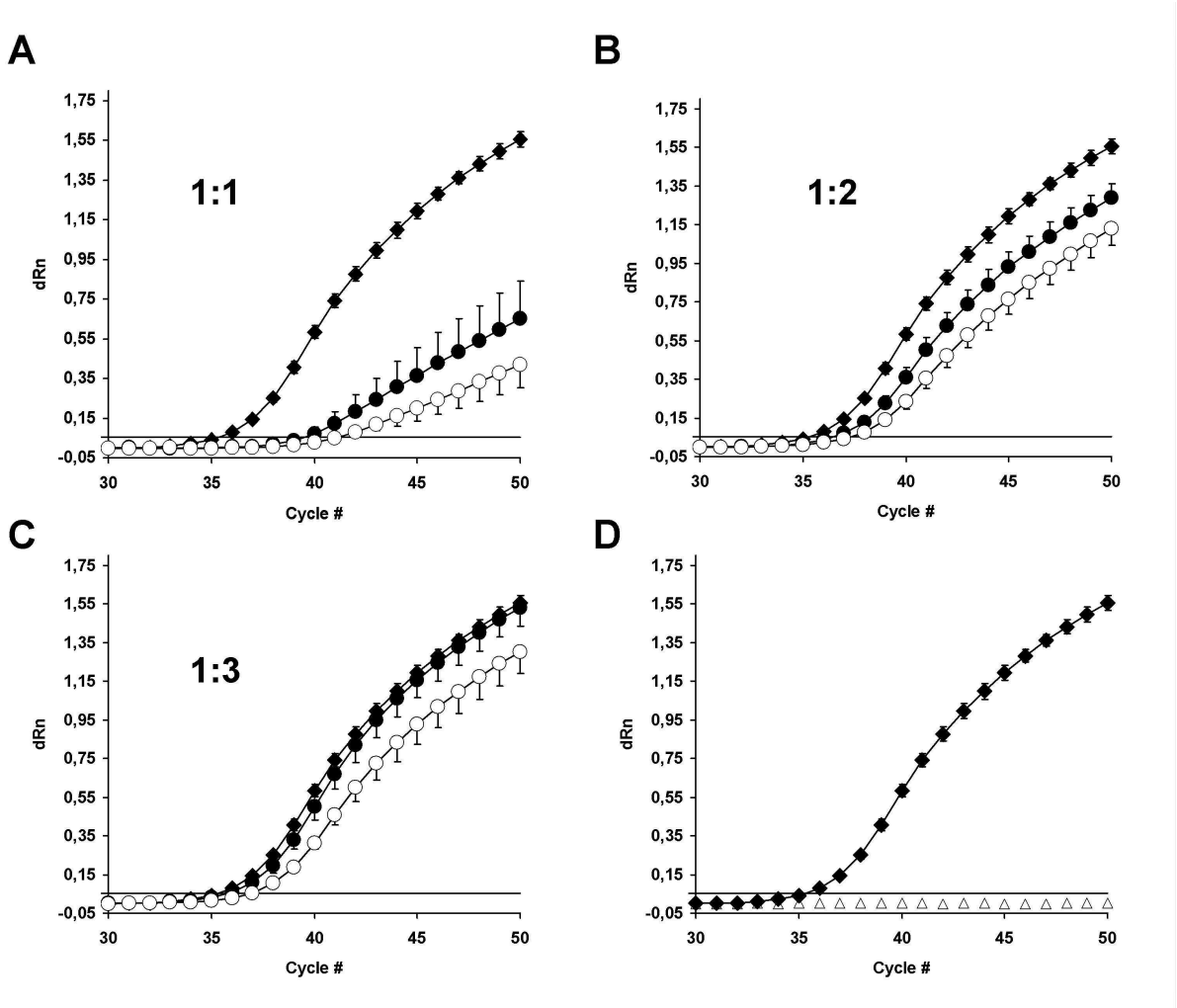

Fig. S7. Single view on mean fluorescence signals over amplification cycles as yielded by PaNP- (closed circles) and cNP-based (open circles) HCV-isolation at different plasma dilutions (phosphate buffer, pH 7) in comparison to the positive control sample (filled diamonds). **A**, undiluted plasma; **B**, 1:2 diluted plasma; **C**, 1:3 diluted plasma; **D**, positive control sample in comparison to applied negative control sample (HCV-RNA negative plasma [open triangles]).

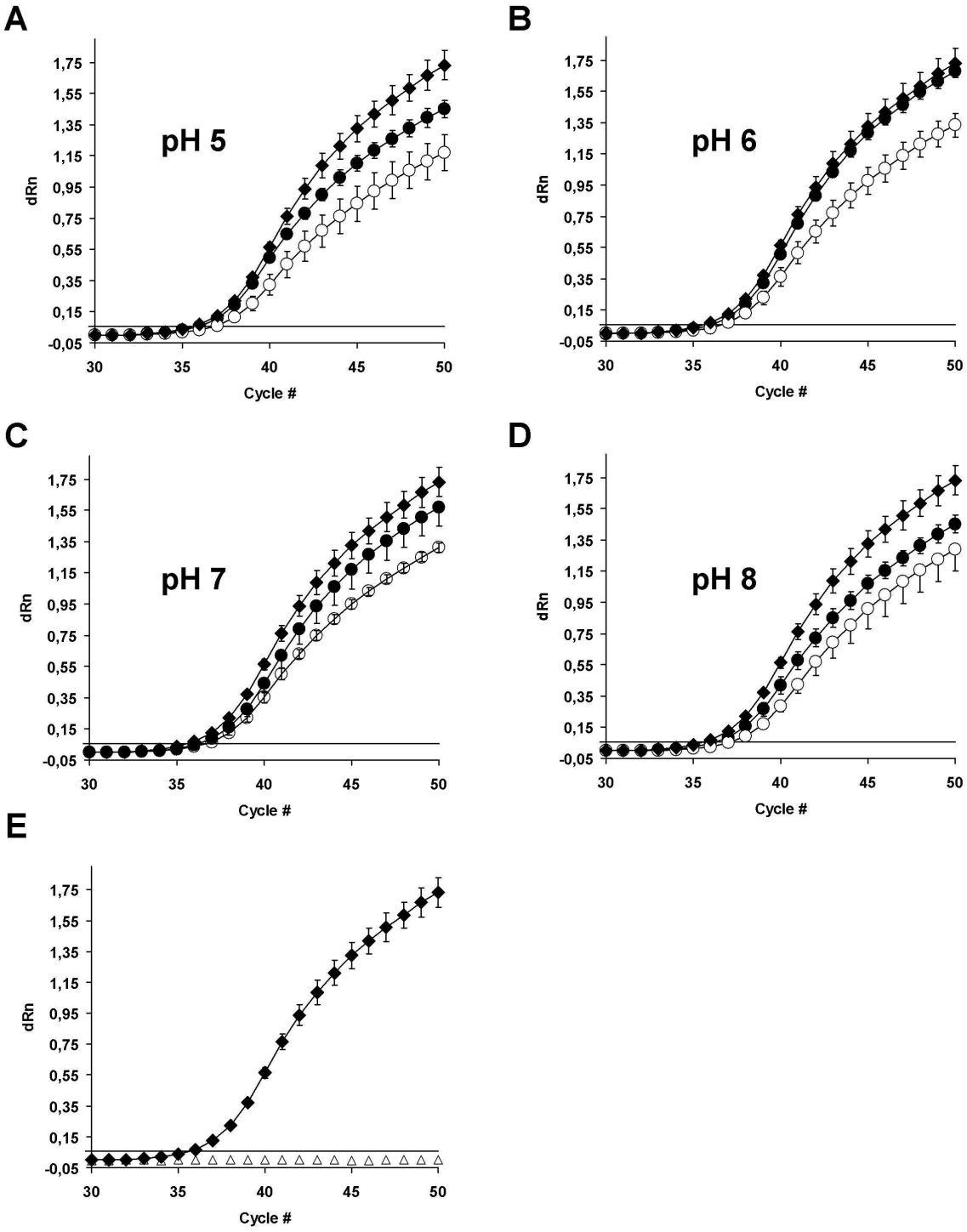

Fig. S8. Single view on mean fluorescence signals over amplification cycles as yielded by PaNP- (closed circles) and cNP-based (open circles) plasma HCV-isolation at different pH-values of the dilution buffer (at a final PaNP/cNP-concentration of 0.35 mg/ml) in comparison to the positive control sample (filled diamonds). **A**, pH 5; **B**, pH 6; **C**, pH 7; **D**, pH 8; **E**, positive control sample in comparison to applied negative control sample (HCV-RNA negative plasma [open triangles]).

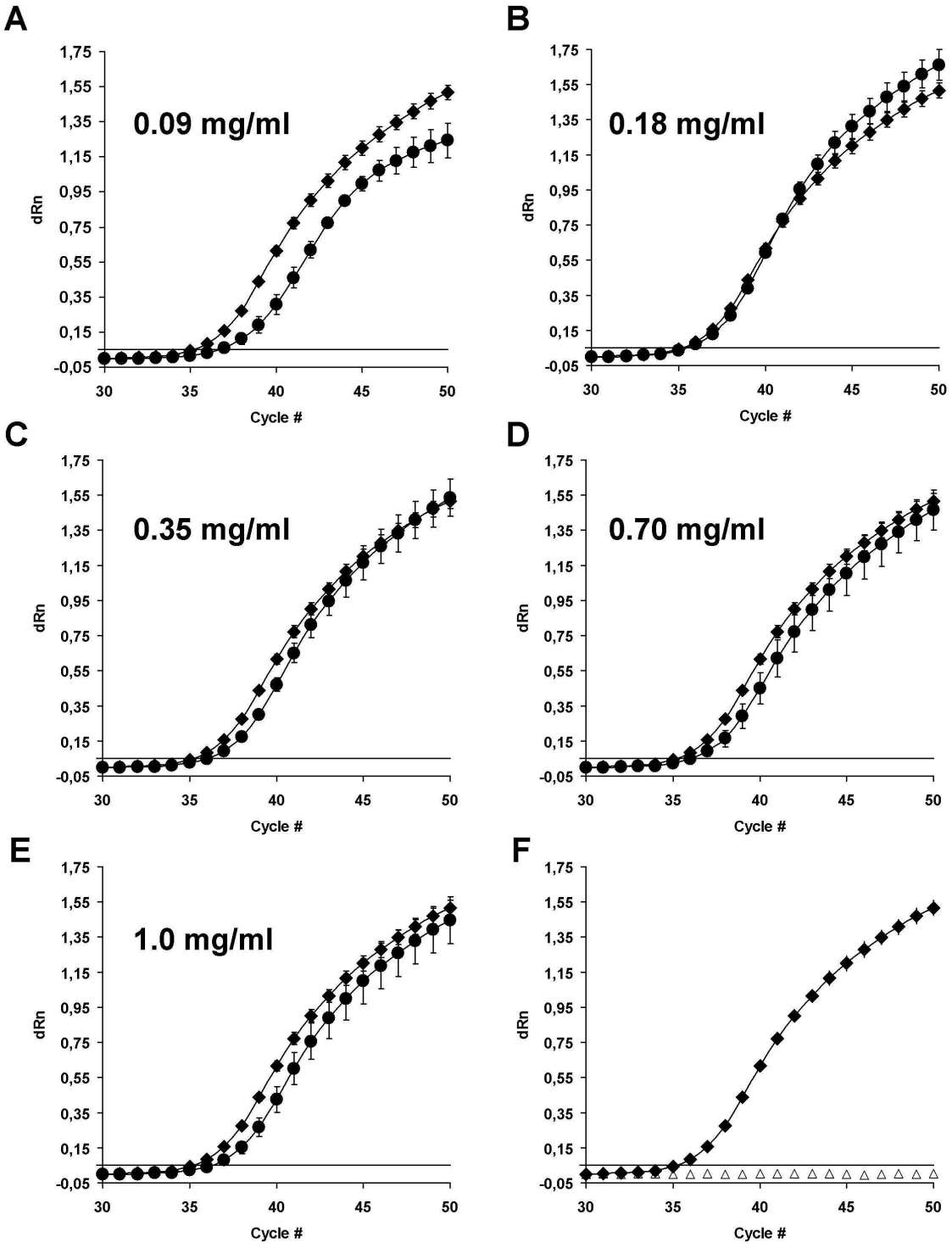

Fig. S9. Single view on mean fluorescence signals over amplification cycles as yielded by PaNP-based (closed circles) plasma HCV-isolation using different final PaNP-concentrations (at pH 7 of dilution buffer and a plasma dilution of 1 in 3) in comparison to positive control sample (filled diamonds). **A**, 0.09 mg/ml; **B**, 0.18 mg/ml; **C**, 0.35 mg/ml; **D**, 0.7 mg/ml; **E**, 1.0 mg/ml; **F**, positive control sample in comparison to applied negative control sample (HCV-RNA negative plasma [open triangles]).

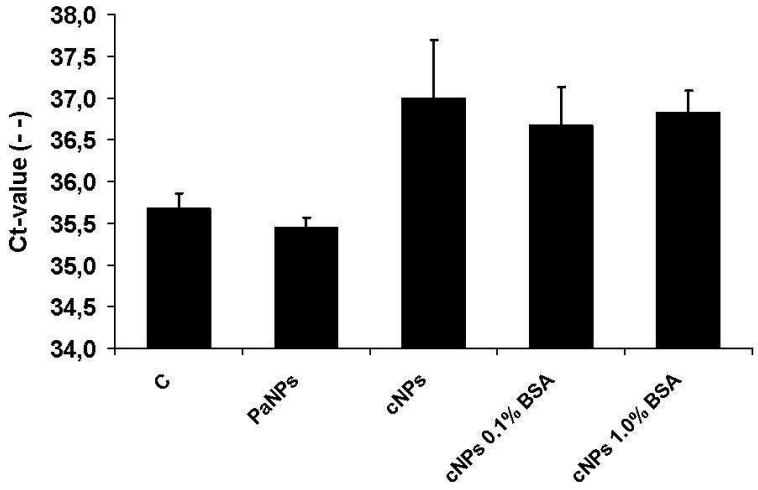

Fig. S10. Performance of PaNPs and cNPs under optimized conditions (plasma dilution 1 in 3; dilution buffer of pH 6) in comparison to positive control sample (C). To further examine the influence of protein-adsorption, cNPs were either tested without prior incubation with protein (cNPs) or after incubation in phosphate buffer (pH 7) in the presence of 0.1% or 1% of BSA. PaNPs were prepared in parallel, applying a final concentration of 1,000 U/ml protamine in the presence of 0.1% BSA. At this, no effect of BSA on assay performance was observed. The slightly better performance of PaNPs-based capturing of HCV in comparison to the positive control sample might be attributed to a lower amount of (RT)-PCR inhibiting substances due to removal of plasma supernatants after binding and magnetic separation of the viruses.

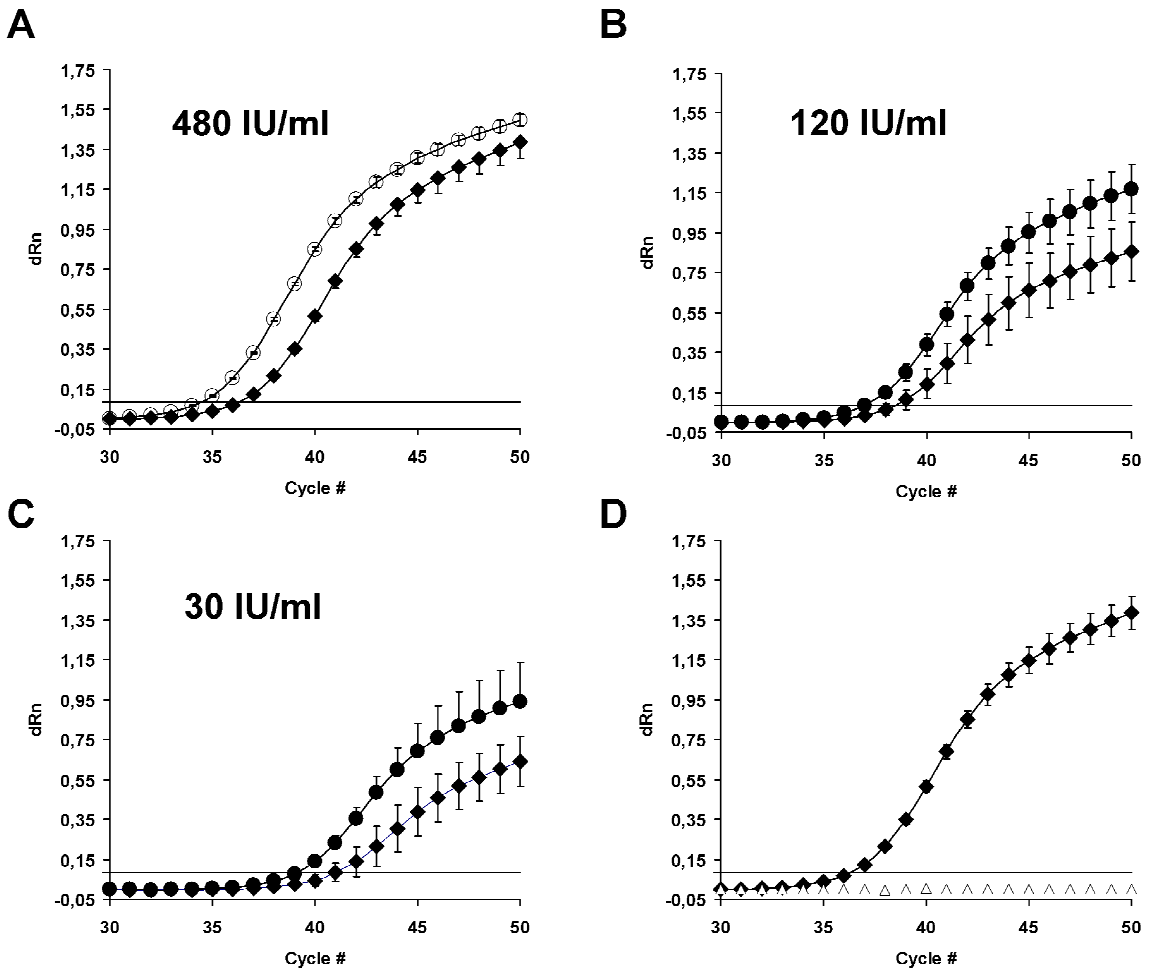

Fig. S11. Single view on mean fluorescence signals over amplification cycles as yielded by PaNP-based isolation of HCV from 4-fold plasma volumes (560 µl, circles) in comparison to controls (140 µl, diamonds). **A**, 480 IU/ml HCV-RNA; **B**, 120 IU/ml; **C**, 30 IU/ml; **D**, positive control sample (480 IU/ml, 140 µl) in comparison to applied negative control sample (HCV-RNA negative plasma [open triangles]).

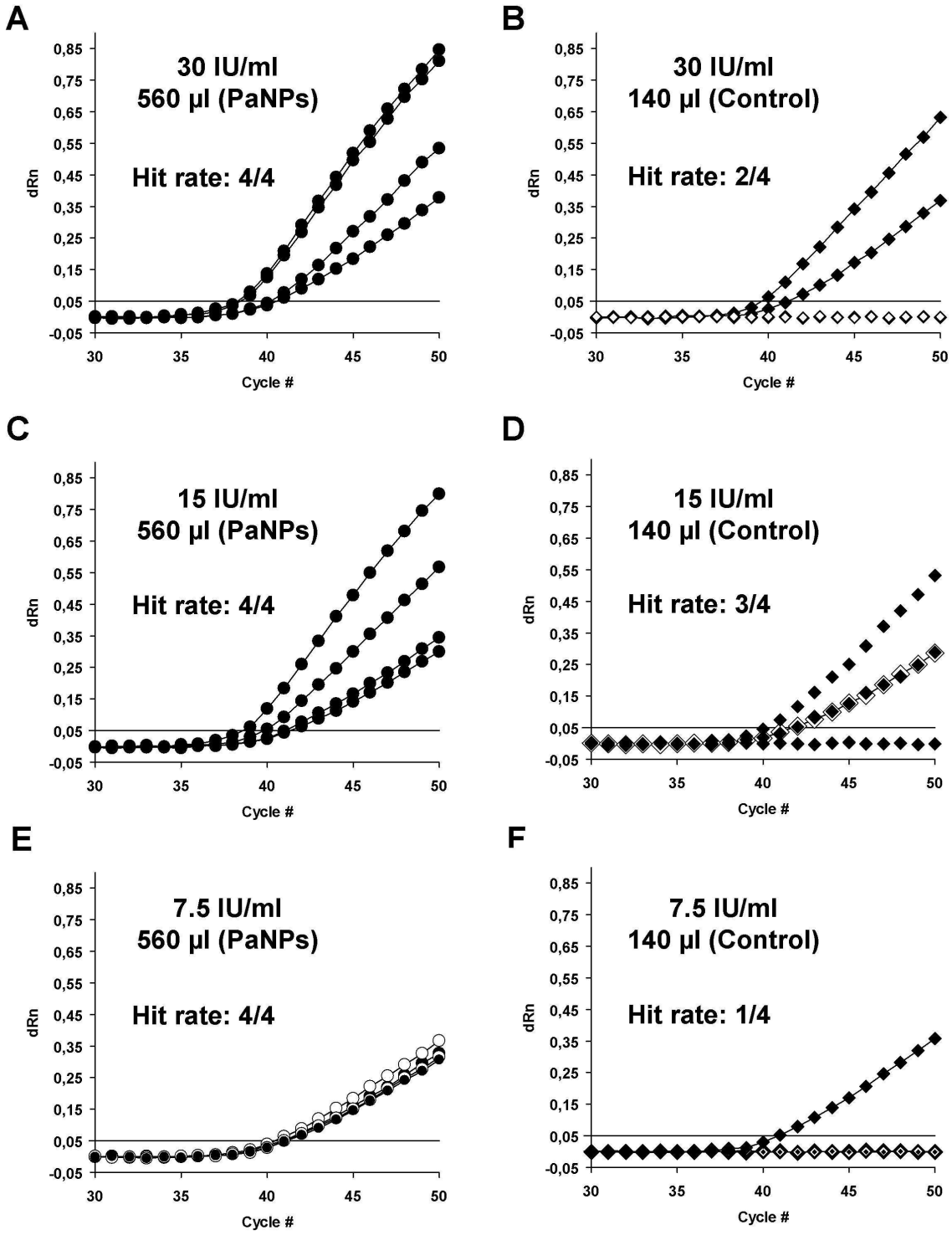

Fig. S12. Single view on mean fluorescence signals over amplification cycles as yielded by PaNP-based plasma HCV-isolation and concentration from 4-fold up-scaled sample volumes (560 µl, circles, left column) in comparison to non-enriched controls (diamonds, right column). Different concentrations of HCV-RNA below the validated LOD of the assay (114 IU/ml) were used. **A/B**, 30 IU/ml; **C/D**, 15 IU/ml; **E/F**, 7.5 IU/ml. The number of positive results is given by the hit rate. Open and closed symbols were used to also illustrate overlaid curves.

References

1 R.V. Mehta, R. Desai, P. Bhat and R.V. Upadhyay, *Indian journal of pure & applied physics*, *2006*, **44**, 537.

2 J. Mueller, M. Gessner, A. Remberg, J. Hoch, G. Zerlauth and P. Hanfland, *Clin. Chem. Lab. Med.*, 2005, **43**, 827.

3 J.T. Keiser, C.W. Brown and R.H. Heidersbach, *Journal of The Electrochemical Society*, *1982*, **129**, 2686.