Electronic Supplementary Material (ESI) for Analyst.
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A PCR-free technology to detect and quantify microRNAs directly from human plasma

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Electronic Supplementary Information (ESI)

Electronic Supplementary Information (ESI) Available: Information about materials and methods, instrumentations and sequences employed in this study.

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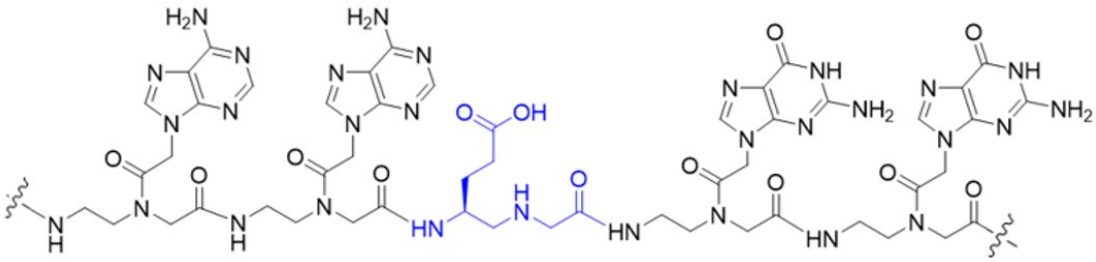
General – Reagents, buffers and instruments

Microspheres were purchased from ThermoFisher Scientific (Dynabeads® M-270 Carboxylic Acid). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was purchased from Sigma-Aldrich. Synthetic mimic miR-451a was purchased from Integrated DNA Technologies. TaqMan® MicroRNA Reverse Transcription Kit was purchased from Life Technologies. Stem-loop RT primer specific for miR-451 (Pub. No. 127AP11-01) was purchased from Applied Biosystems. FastStart TaqMan probe master mix was purchased from Roche. Hoechst 33342 (10 mg/mL in Water) was purchased from ThermoFisher Scientific. miRNeasy Serum/Plasma kit and reagents for RNA isolation were purchased from Qiagen.

Red Blood Cell (RBC) lysis buffer contains 150 mM ammonium chloride, 10 mM sodium bicarbonate and 10 mM disodium-EDTA in mili-Q water. Lysis buffer was prepared by mixing 21.4 g/L lithium chloride, 2.94 g/L ethylenediaminetetraacetic acid, 10.15 g/L lithium dodecyl sulphate, 0.79 g/L dithiothreitol and 0.4 g/L Proteinase K, in Tris-HCl 0.1M pH 7.5. Microsphere solution contains 10% PEG10K and 0.1% Tween in PBS 1X.

The thermoshaker (Biometra TS1 ThermoShaker), magnet rack (MagnaRackTM) and 96-black well plates (Nunc™ MicroWell™) were purchased from ThermoFisher Scientific. 7900 Fast Real-Time PCR System was purchased from Applied Biosystems. Microscopy chamber (Ref. No. 80826) was purchased from Ibidi. Confocal microscopy model *LSM 710 Confocal Laser Scanning Microscopy*, from Zeiss.



Region surrounding the abasic unit of the PNA probe complementary to target miR-451a. The abasic unit containing a propanoic acid side chain at the gamma position is highlighted in blue.

Figure S1 – Abasic 'blank' position contained in the abasic probe sequence

Table S1 - Sequences

Sequence ID	Name	Peptide with abasic position (N'-C')
1	Abasic probe	xx-AGTgluAATGGTgluAA*GL*GGTgluTT
		miRNA sequence (5'-3')
2	Target miR-451a	*AAACC**G**UUACCAUUACUGAGUU*
3	MiR-122	*UGGAGUGUGACAAUGGUGUUUG*
4	MiR-451a_T	*AAACC**T**UUACCAUUACUGAGUU*

ID 1: Abasic probe; ID 2: Synthetic mimic miR-451a; xx = amino-PEG-linker; Tglu = thymidine containing a propanoic acid side chain at the gamma position; *GL* = abasic "blank" monomer containing a propanoic acid side chain at the gamma position. The letters in **bold** represent the nucleobases under interrogation.

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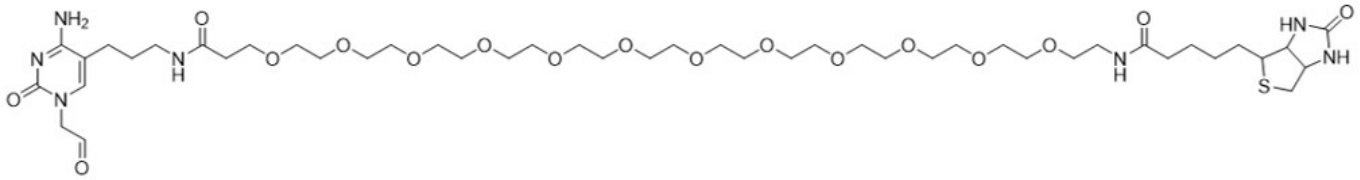
Section S1 - Microsphere coupling with abasic probe

Microspheres containing the abasic probe (Table S1 - 1) were synthesized as described previously [(1)] with some modification. The activation was carried out re-suspending 50 mg/mL of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in cold MES buffer pH 5 added with 0.1 % Tween®-20 at RT for 30 min with slow tilt rotation. After activation with EDC, the beads were washed two times with 200 µL cold MES buffer (50 mM, pH 5) and 0.1% Tween®-20 in milli-Q water. 100 µL of a solution containing 2100 pmoles of abasic probe in $NaHCO_3$ buffer (0.125M, pH 9.84) and 0.1% Tween®-20 in milli-Q water was added to the activated beads. Beads were resuspended on the solution containing the abasic probe and incubated at RT for 3 h with slow tilt rotation. After the incubation, beads were washed two times with 200 µL of MES buffer (50 mM, pH 5) and 0.1% Tween®-20 in milli-Q water. The amount of abasic probe loaded to the bead was measured as described previously.[(1)]



Chemical structure of SMART-C-Biotin

Figure S2 – SMART-C-Biotin structure

Section S2 - Calibration curve via Chem-NAT ELISA

Spike-in solutions were prepared by dissolving varying quantities of synthetic mimic miR-451a (96, 48, 24 and 12 fmoles) in 5 µL of water. Each solution was diluted with a ratio 1:20 in 95 µL of RBC lysis buffer 1X. As control, 5 µL of only water were diluted with 95 µL of RBC lysis buffer 1X.
The reaction was carried out in three steps:
1) miR-451a hybridization - 100 µL of each spike-in solution was diluted in an eppendorf of 1.5 mL with 198.75 µL of lysis buffer and 1.25 µL of microsphere solution containing 250000 beads (200,000 beads/µL). The eppendorf was placed in a thermoshaker, mixed at 1,200 rpm, RT for 1 h. The microspheres were pelleted by centrifugation at 6000 rpm for 10 sec and held on a magnet rack for 30 sec to discard the supernatant. The pellet was washed three times with 200 µL of 2X SSC and 0.1% SDS (pH 6).
2) The dynamic chemistry reaction was carried out by resuspending the pellet in 50 µL of solution containing 45.45 µL of 2X SSC and 0.1% SDS buffer (pH 6), 5 µM of SMART-C-Biotin (1.25 µL from a stock of 200 µM) and 1 mM of reducing agent (3.3 µL from a stock of 15 mM sodium cyanoborohydride). The eppendorf was incubated in a thermoshaker at 40ºC, 1,200 rpm for 1 h. 3) The microspheres were pelleted by centrifugation at 6000 rpm for 10 sec and held on a magnet rack for 30 sec to discard the supernatant. The pellet was washed three times with 200 µL of 2X SSC and 0.1% SDS (pH 6) to remove the excess of SMART-C-Biotin. After washings, the pellet was resuspended in 100 µL of SβG (250 pM), vortexed and incubated in a thermoshaker for 20 min at RT. Microphores were washed two times with 200 µL of 0.1%

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Tween®-20 in PBS (10 mM, pH 7.4) to remove the excess of SβG. Finally, microspheres were resuspended in 200 µL of 0.1% Tween®-20 in PBS (10 mM, pH 7.4) and transfer to a 96-black well plate. Supernatant was removed and 200 µL of resorufin-β-D-galactopyranoside (RGP) substrate was added. The fluorescence signal was measured by the FLUOstar OMEGA instrument. The values obtained were represented by the slope of the linear range of a plot of fluorescence measured.

Table S2 – Slope values obtained by Chem-NAT ELISA

Fmoles	Slope 1	Slope 2	Slope 3	Average slope	SD	CV
96	0.940	0.910	1.070	0.973	0.085	8.74%
48	0.550	0.570	0.580	0.567	0.015	2.70%
24	0.26	0.25	0.26	0.257	0.006	2.25%
12	0.170	0.160	0.190	0.173	0.015	8.81%
Water	0.060	0.090	0.060	0.070	0.017	24.74%

Using the formula based on the standard deviation of the blank (water) and the slope of the curve represented in Figure 2-A [(3xSD)/slope)], the limit of detection (LoD) is calculated about 6.89 fmoles (22.97 pM in a total hybridization volume of 300 µL).

Section S3 - Calibration curve via RT-qPCR

Spike-in solutions were prepared by dissolving varying quantities of synthetic mimic miR-451a (15, 1.5, 0.15, 0.015, 1.5 x 10-4, 1.5 x 10-5 and 1.5 x 10-6 fmoles) in 500 µL of water.

The reverse transcription was carried out using the TaqMan® MicroRNA Reverse Transcription Kit and a specific stem-loop RT primer specific for miR-451. The cDNA was generated according to manufacturer's protocol using 5 µL of each Spike-in solution. cDNA generated was amplified by quantitative real time PCR using a FastStart TaqMan probe master mix according to manufacturer's protocol. Briefly, PCR master mix was prepared, in 0,2 mL tube on ice, by mixing 10µL of master mix, 1µL of miR-451 specific miR451 TaqMan probe (Assay ID #001141; Life Technologies) and 6,5µL PCR-grade water. 2,5µL of total cDNA were added to the PCR master mix, in a final volume of 20µL. Quantitative PCR analysis was performed on a 7900 Fast Real-Time PCR System, with an initial activation step of 95ºC for 15 min followed by 40 cycles of 2-step cycling (denaturation: 15s at 95°C and Annealing/Extension: 1min at 60ºC).

Table S3 – Ct values obtained by qPCR

Fmoles	Ct 1	Ct 2	Ct 3	Average Ct	SD	CV
15	17.183	17.296	17.278	17.252	0.061	0.35%
1.5	21.476	21.287	20.998	21.253	0.241	1.13%
1.5×10^{-1}	26.440	26.770	26.561	26.590	0.180	0.68%
1.5×10^{-2}	30.181	29.714	29.825	29.907	0.244	0.82%
1.5×10^{-4}	36.379	36.875	35.388	36.627	0.351	0.96%
1.5×10^{-5}	37.701	38.215	n.d	37.958	0.363	0.96%
1.5×10^{-6}	n.d	n.d	n.d	n.d	n.d	n.d

PCR was performed in triplicate on three aliquots from this reaction solution. To estimate the LoD, the Ct values of the two lowest detectable quantities (1.5×10^{-5} and 1.5×10^{-4} fmoles) were averaged and the standard deviation determined. The Ct value at 3 s.d. below the average was 36.250, resulting in a LoD of 4.741×10^{-4} fmoles in a total volume of 15 µL (volume related to the generation of cDNA.), i.e. 31.60. n.d = not determined.

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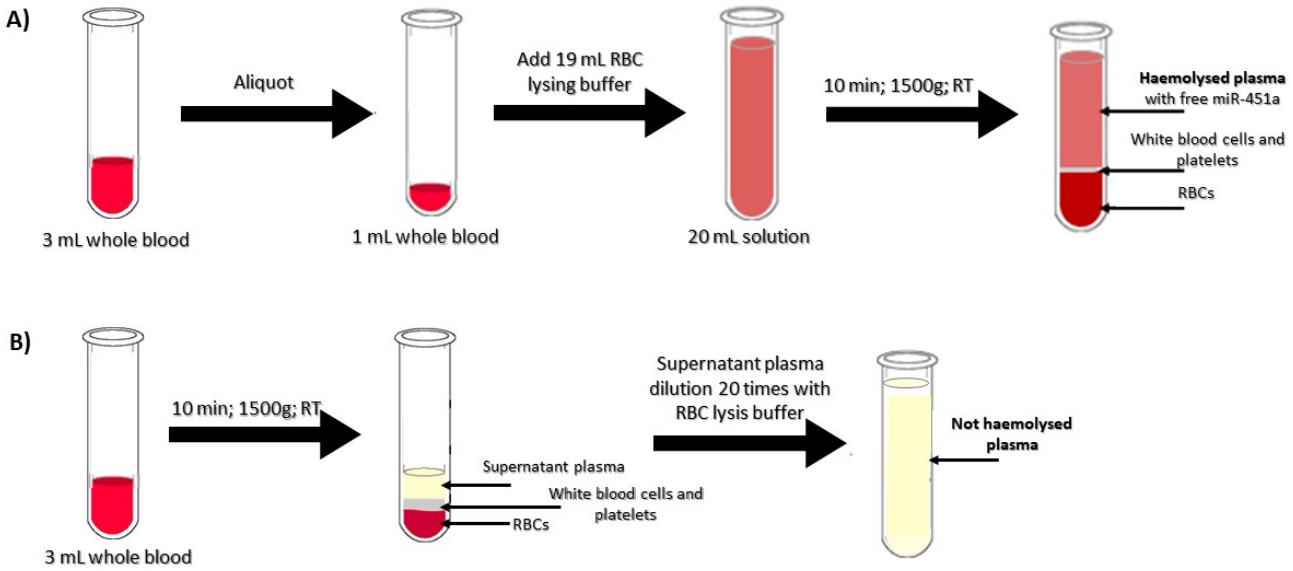

To obtain haemolysed plasma, 1 mL of whole blood is incubated 10 min with 19 mL of RBC lysis buffer, in a proportion sample: buffer of 1:20. The 20 mL solution is centrifuged for 10 min at RT to separate figurative elements of blood (pellet) from the supernatant plasma containing free miR-451a. The supernatant is aliquot into 0.2 mL Eppendorf 0.2 mL and storage at -80 ºC; B) To obtain not haemolysed plasma, 3 mL of whole blood is centrifuged at 1,500g for 10 min at RT to separate the supernatant plasma from the figurative elements of blood (pellet). The supernatant is collected and diluted 20 times with RBC lysis buffer. The diluted supernatant (not haemolysed plasma) is aliquoted into 0.2 mL Eppendorf and storage at -80 C

Figure S3 – Procedure for preparing haemolysed and not haemolysed plasmas

Section S4 – Haemolysis validation by microscopy

Haemolysed blood - 80 µL of Hoechst diluted in PBS 1X (1:1000). 80µL of the dilution were added to 20 µL of whole blood in a 0.5 mL eppendorf. The eppendorf was incubated for 15 min at RT. 1. 9 mL of RBC lysing buffer (1X) were added to the eppendorf and incubated for 10 min at RT.

Not haemolysed blood (control) - As control, 80µL of diluted Hoechst were added to 20 µL of whole blood in a 0.5 mL eppendorf. The eppendorf was incubated for 15 min at RT. 1. 9 mL of PBS (1X) were added to the eppendorf and incubated for 10 min at RT.

20 µL of both preparations were diluted 5 times with PBS 1X and loaded into a microscopy chamber for confocal microscopy analysis using a 40X objective (see Figure below).

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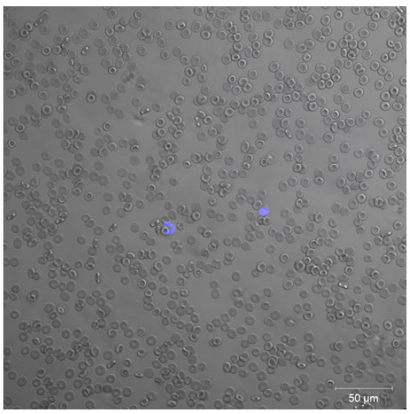
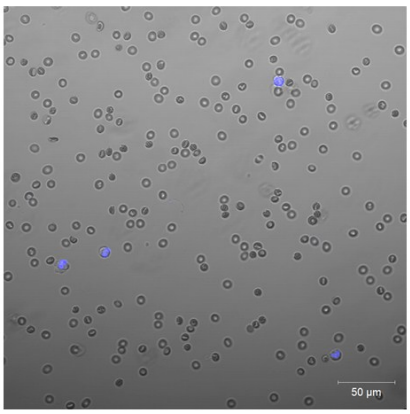
A) 

B) 

A) Not haemolysed blood - High number of erythrocytes are observed (not stained cells) as well as two leukocytes (stained cells); B) Haemolysed blood - A considerable low number of erythrocytes are observed (not staining cells). Some erythrocyte shows an unhealthy morphology based on the haemolysis treatment. Three leukocytes are observed (stained cells).

Section S5 – Healthy volunteer samples analysed via Chem-NAT ELISA

Analysis of healthy volunteer samples was carried out as described in Section S2. Instead of 100 µL of spike-in solution was used 100 µL of plasma solution prepared as described in Figure S3.

Table S4 – Slope values measured by Chem-NAT ELISA

Sample code	Measurement	Slope 1	Slope 2	Slope 3	Average slope	SD	CV
HV 1 – HP	Day 1	1.130	1.260	1.230	1.207	0.068	5.64%
	Day 2	1.110	1.380	1.290	1.260	0.137	10.91%
HV 2 - HP	Day 1	1.180	1.050	1.170	1.133	0.072	6.38%
	Day 2	1.330	1.070	1.160	1.187	0.132	11.13%
HV 3 - HP	Day 1	1.380	1.550	1.500	1.477	0.087	5.92%
	Day 2	1.640	1.620	1.380	1.547	0.145	9.35%
HV 1 – NHP	Day 1	0.080	0.120	0.040	0.080	0.040	50.00%
	Day 2	0.050	0.070	0.080	0.067	0.015	22.91%
HV 2 - NHP	Day 1	0.070	0.060	0.060	0.063	0.010	9.12%
	Day 2	0.080	0.070	0.070	0.073	0.006	7.87%
HV 3 - NHP	Day 1	0.130	0.140	0.080	0.117	0.030	27.55%
	Day 2	0.050	0.040	0.070	0.053	0.015	28.64%

HP: Haemolysed Plasma; NHP: Not haemolysed plasma.

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Section S6 - Isolation of RNA from HV plasma samples

RNA was isolated from haemolysed and not haemolysed plasma using the miRNeasy Serum/Plasma kit according to manufacturer's protocol. 100µL of each sample were mixed with 500µL of Qiazol, mixed and incubated at RT for 5 min. 100µL of chloroform ere added and mixed and incubated for 3 min. The mix was centrifuged at 12,000xg for 15 min at 4ºC and the upper aqueous phase was transferred to a new collection tube. 100% ethanol was added to the collection tube, mixed and transferred to the RNeasy MinElute column and centrifuged at 10,000 x g for 15 min at RT. Using the same centrifugation conditions, the column was washed with respectively RWT and RPE buffers followed by a further washing with 80% ethanol. The column was dried by centrifugation and small RNAs eluted with 14µL RNase-free water and stored at -80ºC.

Section S7 - Reverse transcription and amplification of HV plasma samples

RNA samples were isolated as described in section S6. Frozen RNA was thawed at day 1 and reverse transcripted and amplified as reported in Section S3. The aliquot was frozen and storage at -80ºC. For the inter-experimental repeatability study, the same aliquot of RNA was thawed after 24 hours and reverse transcripted and amplified as reported in Section S3.

Table S5 – Ct values measured by RT-qPCR

Sample code	Measurement	Ct 1	Ct 2	Ct 3	Average Ct	SD	CV
HV 1 – HP	Day 1	13.962	14.139	14.095	14.065	0.092	0.66%
	Day 2	13.518	14.128	15.108	14.251	0.803	5.63%
HV 2 - HP	Day 1	13.377	13.621	13.369	13.456	0.144	1.07%
	Day 2	14.392	14.237	14.307	14.312	0.077	0.54%
HV 3 - HP	Day 1	12.498	12.650	13.810	12.986	0.718	5.53%
	Day 2	15.321	15.180	15.811	15.438	0.332	2.15%
HV 1 – NHP	Day 1	23.452	23.658	23.448	23.520	0.120	0.51%
	Day 2	23.299	22.839	23.265	23.135	0.256	1.11%
HV 2 - NHP	Day 1	22.962	23.087	22.891	22.981	0.099	0.43%
	Day 2	22.656	22.716	22.838	22.737	0.093	0.41%
HV 3 - NHP	Day 1	24.581	23.491	23.366	23.429	0.668	2.85%
	Day 2	22.034	22.499	22.527	22.514	0.277	1.23%

Haemolysed Plasma; NHP: Not haemolysed plasma.

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Table S6 – Comparative analysis between the two methods

Method	Sample code	Concentration at day 1 (nM)	Concentration at day 2 (nM)	Average	SD	CV
Chem-NAT ELISA	HV 1 - HP	23.826	24.939	24.383	0.787	3.23%
	HV 2 - HP	22.295	23.408	22.852	0.787	3.45%
	HV 3 - HP	29.463	30.924	30.193	1.033	3.42%
RT-qPCR	HV 1 - HP	20.534	18.536	19.535	1.413	7.23%
	HV 2 - HP	28.713	17.925	23.319	7.628	32.71%
	HV 3 - HP	37.180	9.652	23.416	19.465	83.13%

Figure S4 – Correlation of concentration of miR-451a in haemolysed samples using the two technologies

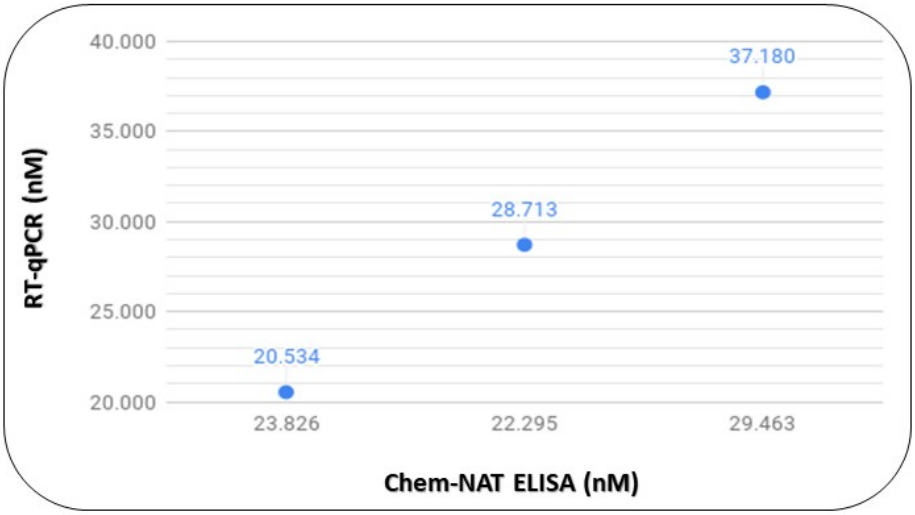

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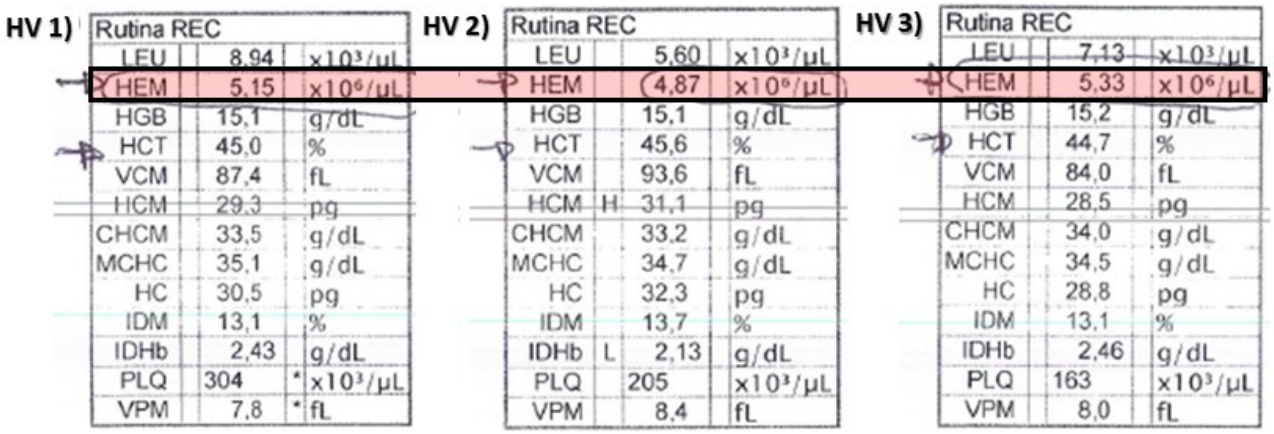
Figure S5 – Hemogram of HV samples



HV 1)

Rutina REC			
LEU	8,94		x10³/µL
HEM	5,15		x10⁶/µL
HGB	15,1		g/dL
HCT	45,0		%
VCM	87,4		fL
HCM	29,3		pg
CHCM	33,5		g/dL
MCHC	35,1		g/dL
HC	30,5		pg
IDM	13,1		%
IDHb	2,43		g/dL
PLQ	304	*	x10³/µL
VPM	7,8	*	fL

HV 2)

Rutina REC			
LEU	5,60		x10³/µL
HEM	4,87		x10⁶/µL
HGB	15,1		g/dL
HCT	45,6		%
VCM	93,6		fL
HCM	31,1	H	pg
CHCM	33,2		g/dL
MCHC	34,7		g/dL
HC	32,3		pg
IDM	13,7		%
IDHb	2,13	L	g/dL
PLQ	205		x10³/µL
VPM	8,4		fL

HV 3)

Rutina REC			
LEU	7,13		x10³/µL
HEM	5,33		x10⁶/µL
HGB	15,2		g/dL
HCT	44,7		%
VCM	84,0		fL
HCM	28,5		pg
CHCM	34,0		g/dL
MCHC	34,5		g/dL
HC	28,8		pg
IDM	13,1		%
IDHb	2,46		g/dL
PLQ	163		x10³/µL
VPM	8,0		fL

Hemogram was performed by the UGC laboratorios of Campus de la Salud (Granada, Spain) using an ADVIA 2120 Hematology System (Siemens Healthineers).

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