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Supplemental Information

Regulatory-Compliant Validation of a Highly Sensitive qPCR for Biodistribution Assessment of Hemophilia A Patient Cells

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Supplemental Information

Supplemental Data Items

Table S1 Method development and robustness evaluation. The initial qPCR protocol including the mastermix composition and the qPCR program comprising parameters for time and temperature for preincubation, initial denaturation, denaturation, annealing/amplification, and cooling. During method development and optimization, several values for the parameters annealing/amplification temperature, primer concentration, probe concentration and annealing/amplification time (elongation time) were investigated.

qPCR program	Time	Temperature	Mastermix	Concentration
Preincubation	2 min	50°C	Primer	250 – 700 nM
Initial denaturation	5 min	95°C	Probe	50 – 150 nM
Denaturation	10 sec	95°C	Volume DNA	100 ng solved in
Annealing/	10 - 30 sec	60 – 64 °C		1 µl TE-buffer
amplification				
Cooling	10 sec	40°C		
qPCR-cycles	45			

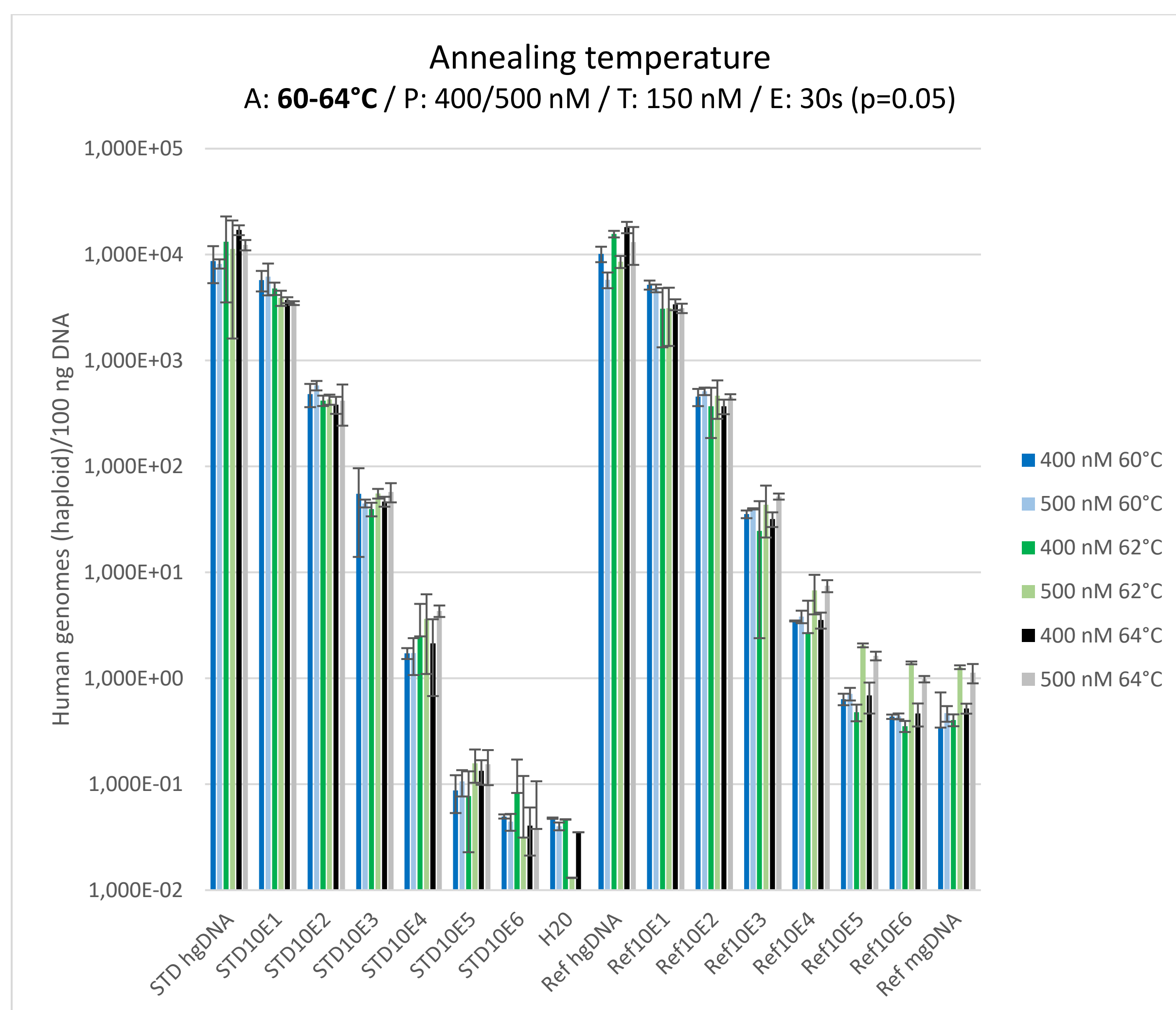

Figure S1 Method development and robustness evaluation. Concentration of haploid human genomes for STD samples and reference standards applying the initial qPCR protocol investigating two different primer

concentrations of 400 nM and 500 nM and annealing temperatures ranging from 60°C – 64°C. The data represent the mean haploid genome concentration of three replicates (n=3) for different samples. For the experiment 500 nM 62°C the data for H2O represent the mean haploid genome concentration of two replicates (n=2), the third replicate was negative. For the experiment 400 nM 62°C the data for H2O represent the haploid genome concentration of one replicate (n=1), the other two replicates were negative. For the experiment 400 nM 64°C the data for H2O represent the mean haploid genome concentration of two replicates (n=2), the third replicate was negative. For the experiment 500 nM 64°C for all H2O control samples no signal could be detected. The error bars represent the significance level values for p=0.05. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, H2O = negative control sample.

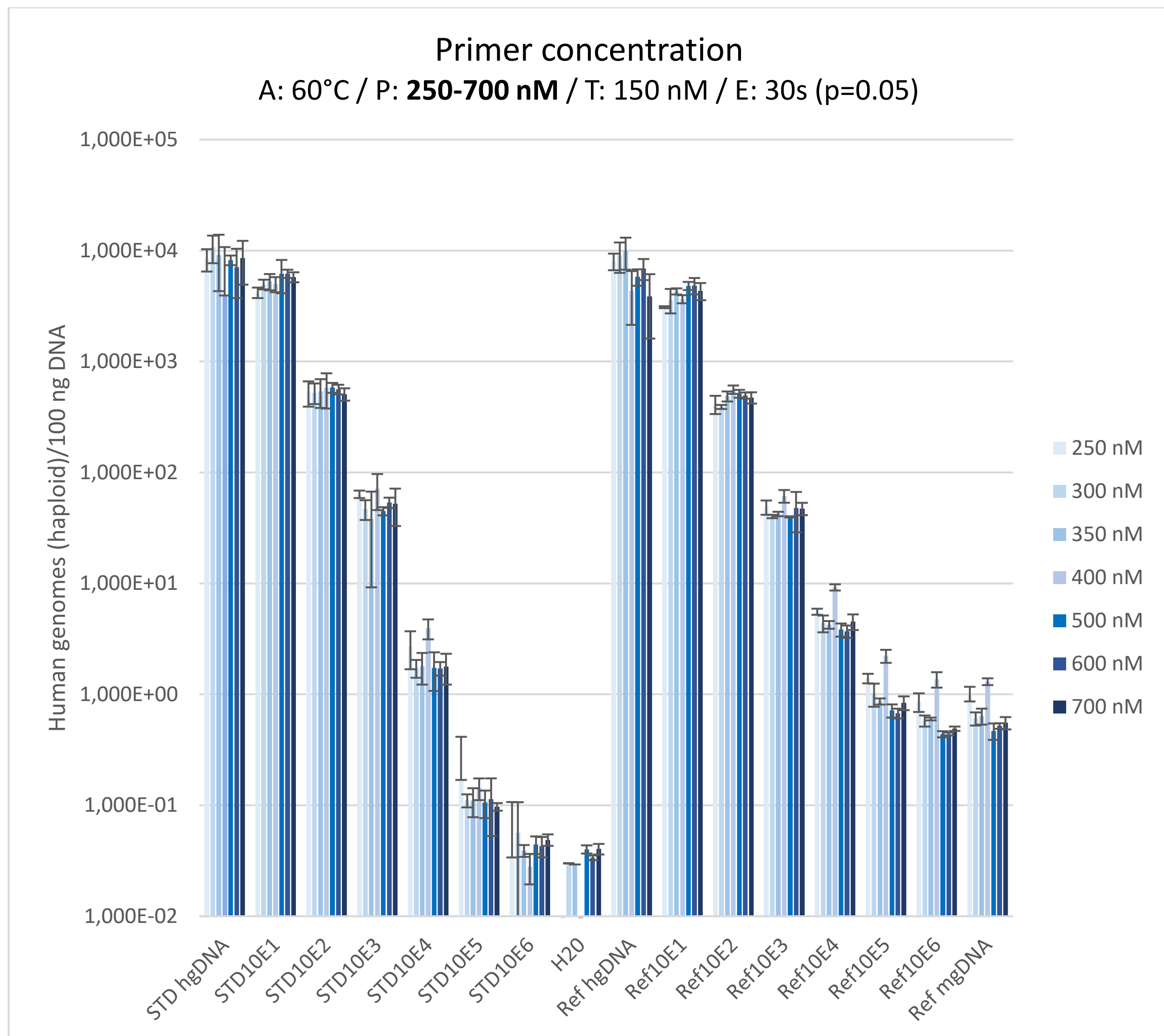

Figure S2 Method development and robustness evaluation. Concentration of haploid human genomes for STD samples and reference standards applying the initial qPCR protocol investigating seven different primer concentrations ranging from 250 nM to 700 nM. The data represent the mean haploid genome concentration of three replicates (n=3) for different samples. The error bars represent the significance level values for p=0.05. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, H2O = negative control sample.

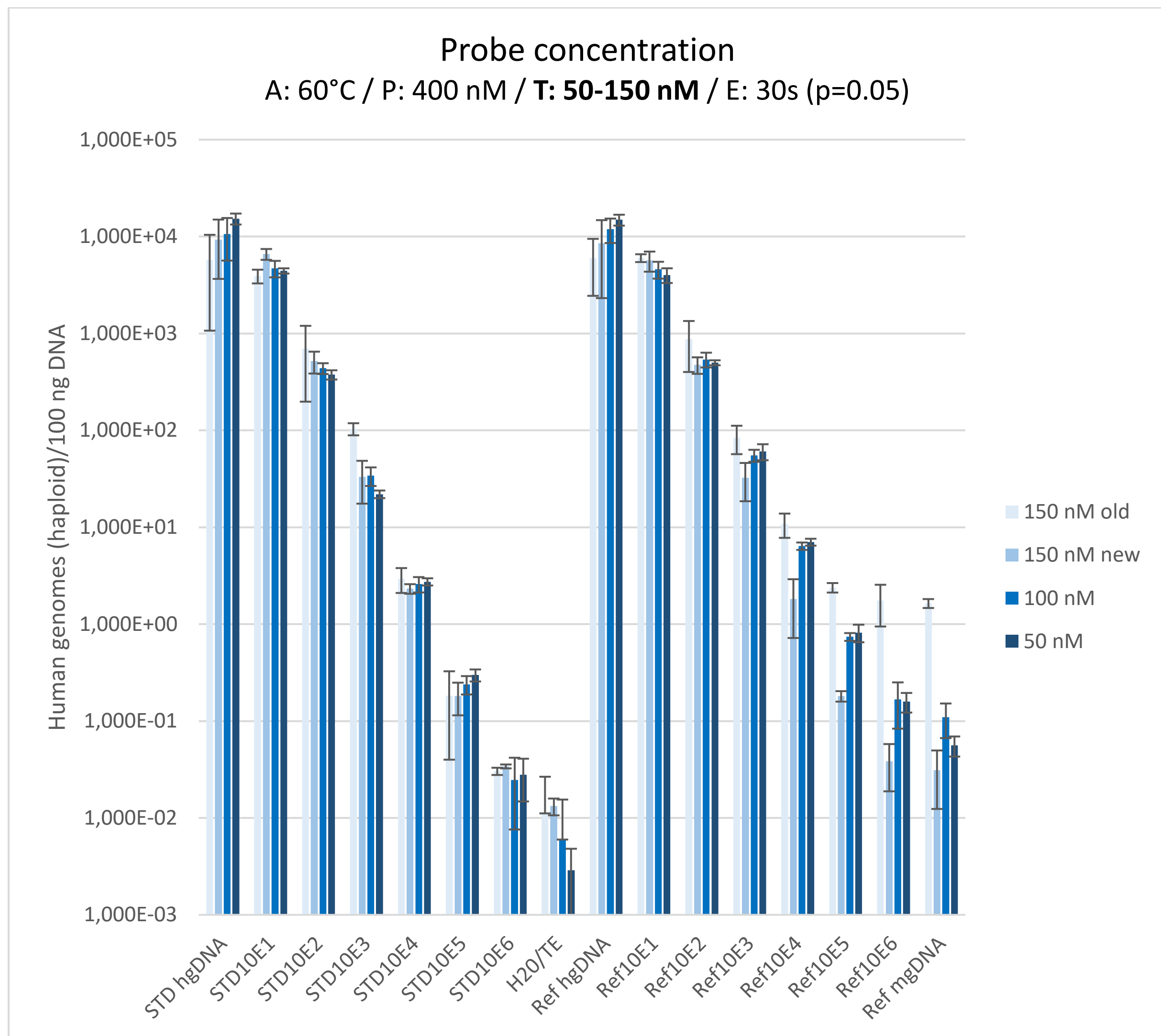

Figure S3 Method development and robustness evaluation. Concentration of haploid human genomes for STD samples and reference standards applying the initial qPCR protocol investigating three different probe concentrations ranging from 50 nM to 150 nM. The primer concentration was 400 nM and the annealing/amplification temperature was 60°C. The data represent the mean haploid genome concentration of three replicates (n=3) for experiments 150 nM old and new and five replicates (n=5) for experiments with 100 nM and 50 nM probe concentrations for different samples. For the experiment 150 nM new the data for H2O represent the mean haploid genome concentration of two replicates (n=2), the third replicate was negative. The terms old and new describe different STD sample and reference standard batches. The error bars represent the significance level values for p=0.05. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, H2O/TE = negative control sample.

Table S2 Method development and robustness evaluation. Parameters for the evaluation of the calibration curves, error, efficiency, slope and *y*-Intercept for experiment 1 and repeats 1 to 5. The parameters that have been varied in repeats 1 to 5 are the applied STD sample and reference standard batches and the operator performing the experiment.

Parameter (Acceptance criterion)	Experiment 1	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Repeat 5
Error (≤ 0.2)	0.0507	0.0675	0.0559	0.0379	0.0365	0.0496

Efficiency (%) (90 – 110%)	1.828 (91.4%)	1.825 (91.3%)	1.976 (98.8%)	2.006 (100.3%)	1.998 (99.9%)	2.032 (101.6%)
Slope (-3.100 – 3.580)	**-3.817**[a]	**-3.829**[a]	-3.381	-3.307	-3.327	-3.246
y-Intercept (only reported)	30.10	30.04	27.89	27.40	27.61	27.52
STD sample and reference standard batch	2018-07-12	2018-07-12	2018-07-12	2018-07-27	2018-07-27	2018-07-27
Operator (OP)	OP1	OP2	OP2	OP3	OP2	OP1

[a]The values in bold do not match the acceptance criteria in comparison to the validation runs.

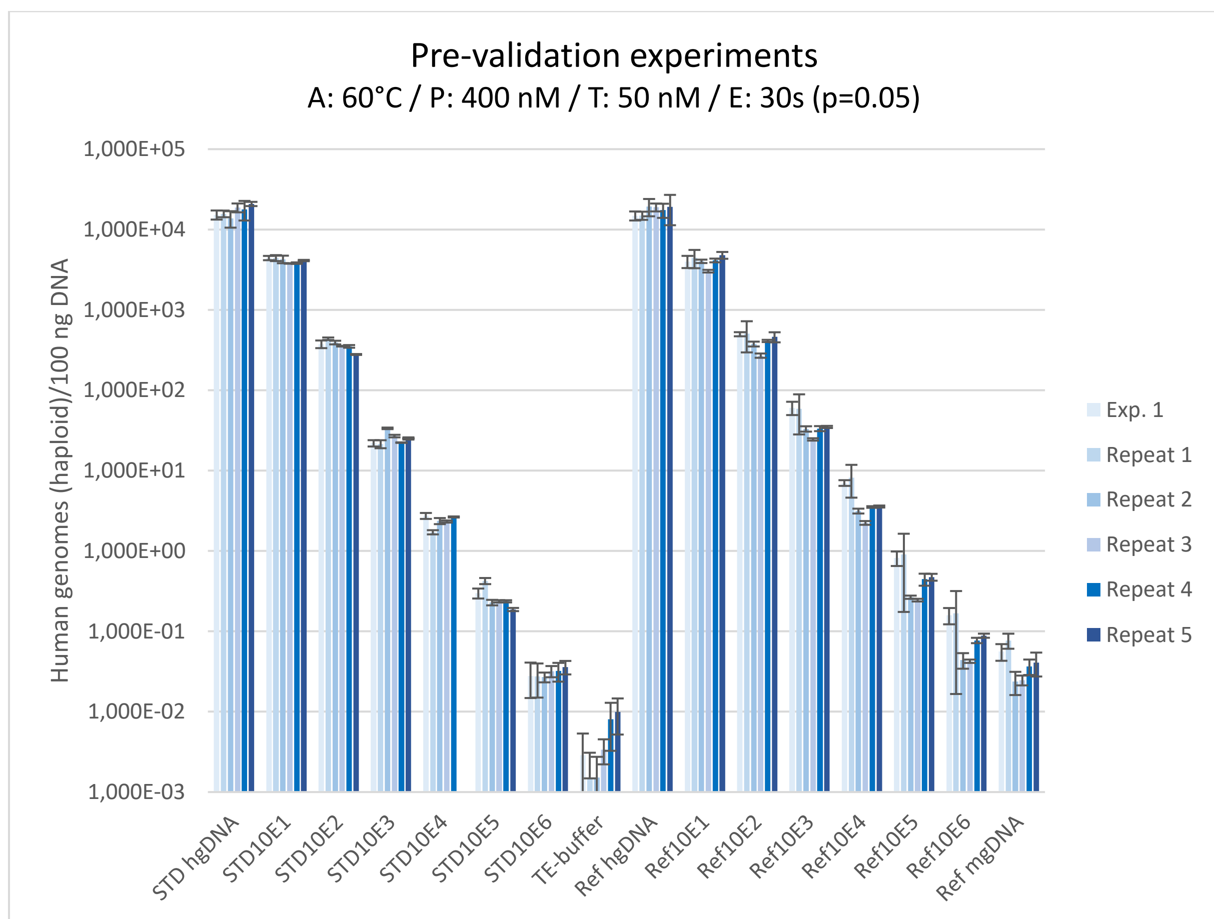

Figure S4 Method development and robustness evaluation. Concentration of haploid human genomes for STD samples and reference standards applying the optimized qPCR protocol with 50 nM probe concentration, 400 nM primer concentration and 60°C annealing/amplification temperature for experiment 1 and repeats 1 to 5. The data represent the mean haploid genome concentration of five replicates (n=5) for different samples. For repeat 3 the data for TE-buffer represent the mean haploid genome concentration of three replicates (n=3), two replicates were negative. The error bars represent the significance level values for p=0.05. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, TE-buffer = negative control sample, Exp. 1 = Experiment 1

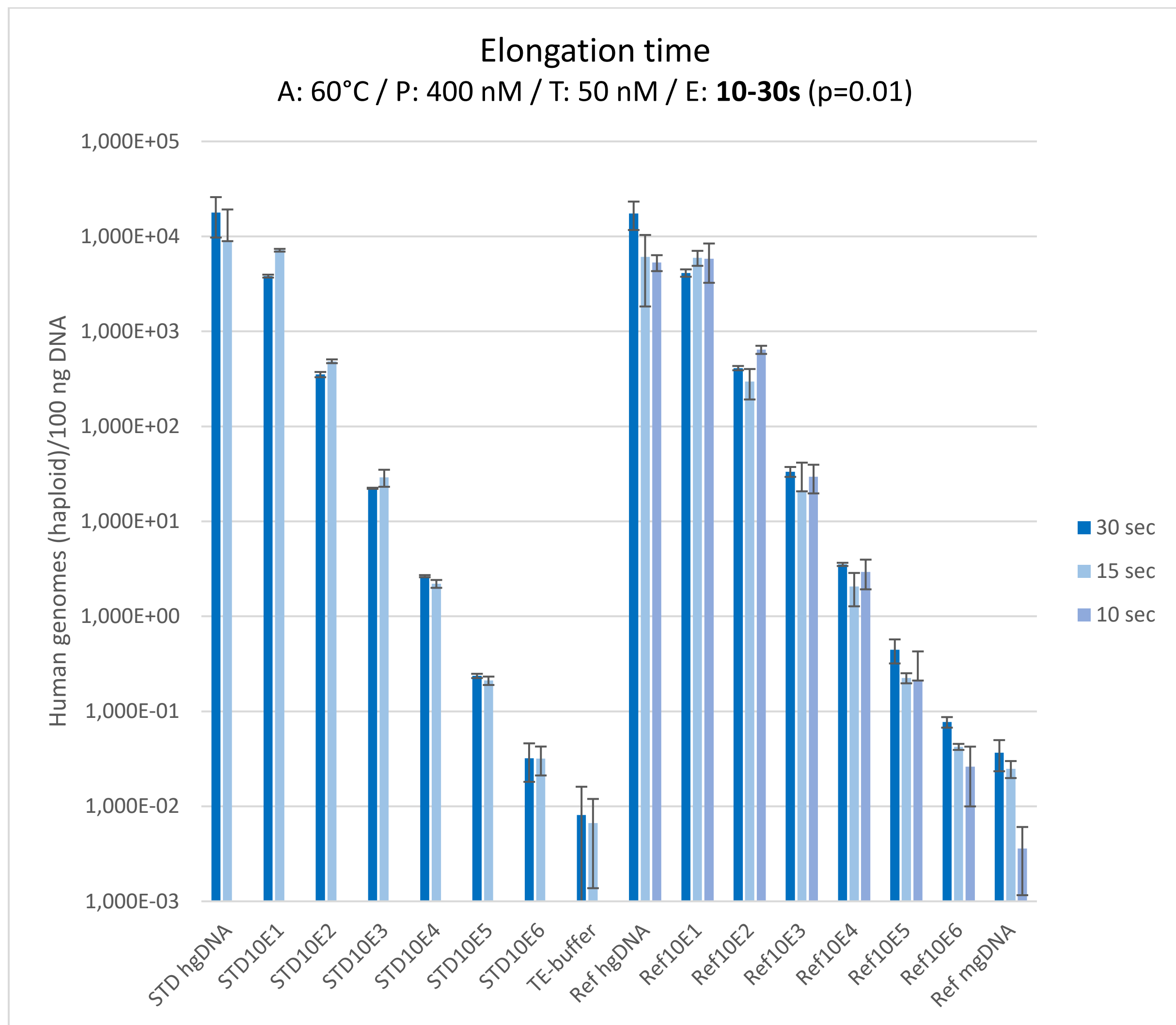

Figure S5 Method development and robustness evaluation. Concentration of haploid human genomes for STD samples and reference standards applying the optimized qPCR protocol with 50 nM probe concentration, 400 nM primer concentration and 60°C annealing/amplification temperature investigating three different elongation times ranging from 10 seconds to 30 seconds. The data represent the mean haploid genome concentration of five replicates (n=5) for different samples. For the experiment with 10 sec elongation time, all TE-buffer control samples were negative. The experiment with 10 seconds elongation time has been performed exclusively with reference standards. The error bars represent the significance level values for p=0.01. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, TE-buffer = negative control sample.

Table S3 Method development and robustness evaluation. Parameters for the evaluation of the calibration curves, error, efficiency, slope and *y*-Intercept for experiment with three different elongation times, 30, 15 and 10 seconds.

Parameter (Acceptance criterion)	Elongation time 30 seconds	Elongation time 15 seconds	Elongation time 10 seconds
Error (≤ 0.2)	0.0365	0.111	0.1270
Efficiency (%) (90 – 110%)	1.998 (99.9%)	2.153 (107.7%)	2.142 (107.1%)
Slope (-3.100 – 3.580)	-3.327	**-3.003**[a]	**-3.022**[a]
y-Intercept (only reported)	27.61	28.77	30.77

[a]The values in bold do not match the acceptance criteria in comparison to the validation runs.

Table S4 Method development and robustness evaluation. The final qPCR protocol including the mastermix composition and the qPCR program parameters for time and temperature for preincubation, initial denaturation, denaturation, annealing/amplification and cooling. The experiments in the following are performed applying this qPCR protocol.

qPCR program	Time	Temperature	Mastermix	Concentration
Preincubation	2 min	50°C	Primer	400 nM
Initial denaturation	5 min	95°C	Probe	50 nM
Denaturation	10 sec	95°C	Volume DNA	100 ng solved in
Amplification/Annealing	30 sec	60°C		1-2 µl TE-buffer
Cooling	10 sec	40°C		
qPCR-cycles	45			

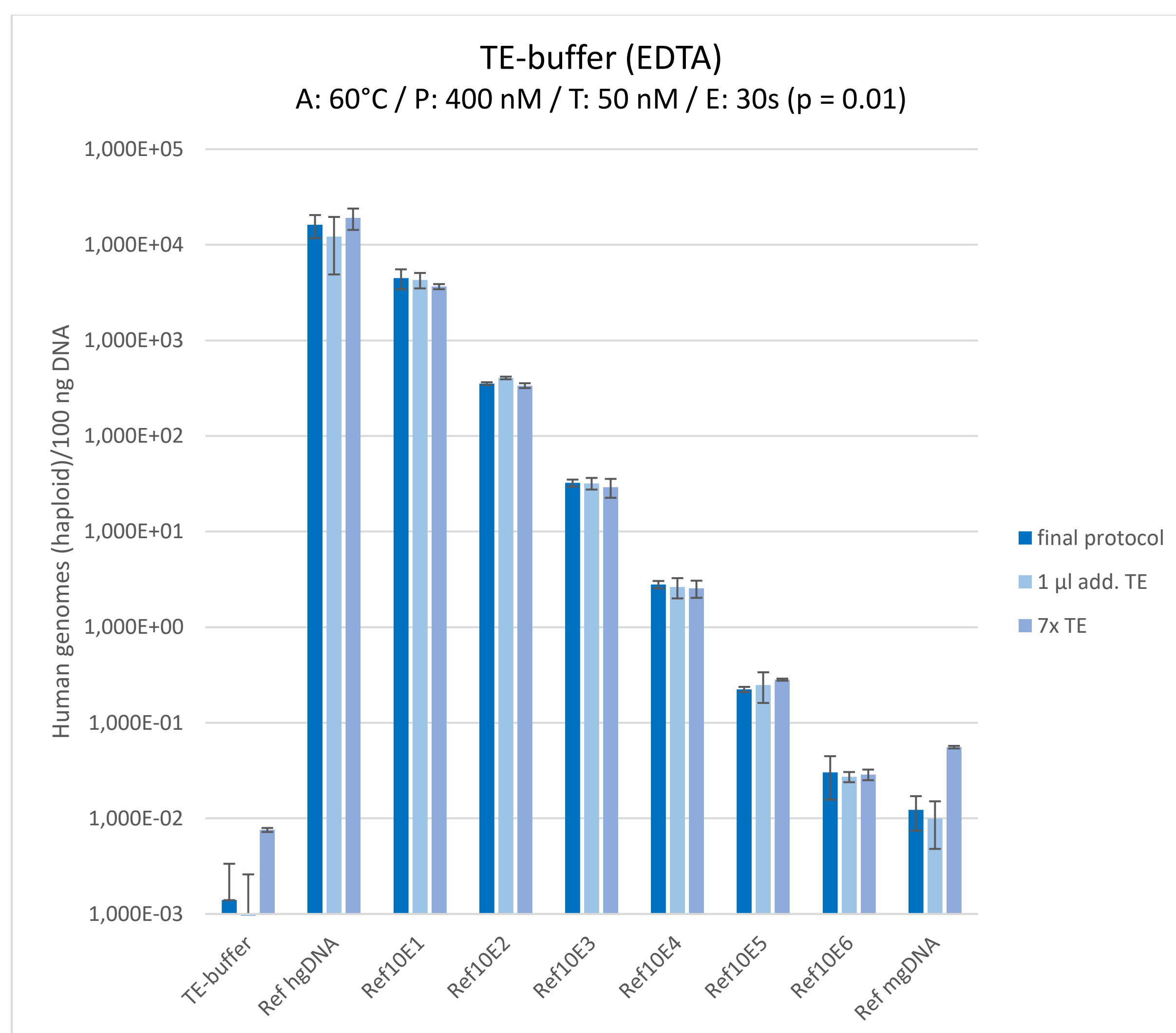

Figure S6 Method development and robustness evaluation. Concentration of haploid human genomes for reference standards applying the final qPCR protocol investigating different amounts of TE-buffer (EDTA) in the qPCR mastermix. The mastermix composition of the final protocol was compared to a mastermix containing 1 µl additional TE-buffer (1 µl add. TE) and a mastermix containing a seven times higher TE-buffer concentration (7x TE), meaning that the whole qPCR-water was replaced by TE-buffer. The data represent the

mean haploid genome concentration of five replicates (n=5) for different samples. The error bars represent the significance level values for p=0.01. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, TE-buffer = negative control sample.

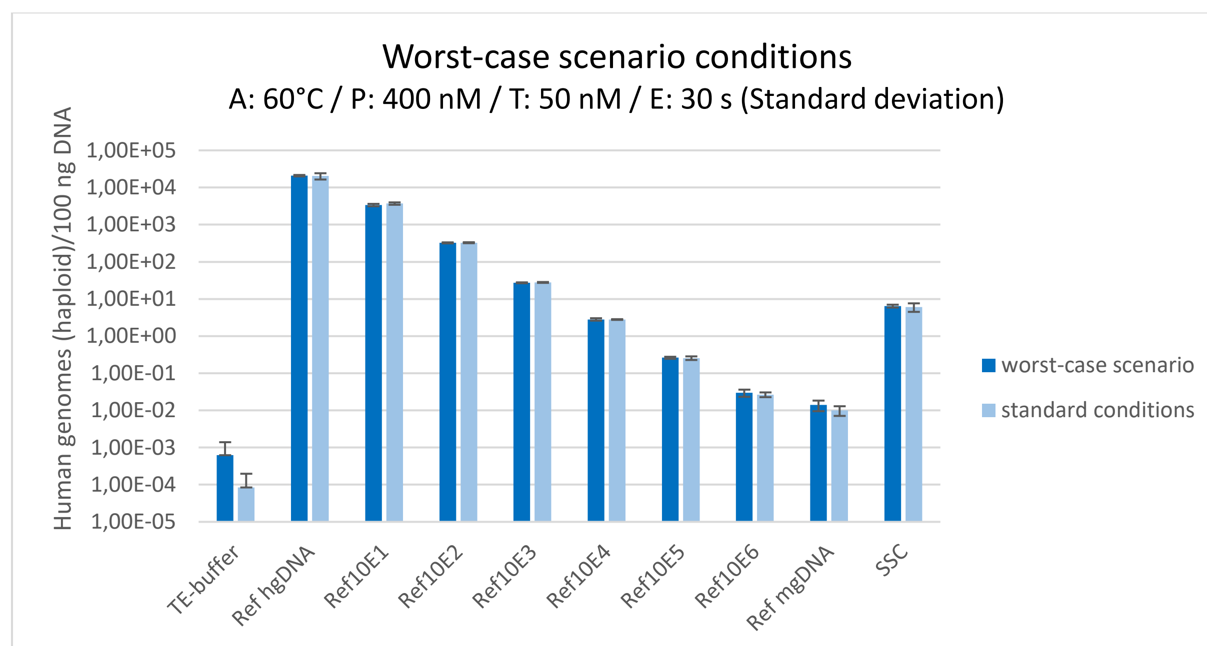

Figure S7 Method development and robustness evaluation. Concentration of haploid human genomes for reference standards and SSC applying the final qPCR protocol under worst-case scenario conditions meaning maximum light exposure and no cooling of mastermix samples containing the probe for two hours and under standard conditions meaning normal light exposure and without cooling of samples for exactly one hour. The data represent the mean haploid genome concentration of five replicates (n=5) for different samples. For the experiment under worst-case scenario conditions the data for Ref mgDNA represent the mean haploid genome concentration of four replicates (n=4) and for TE-buffer the mean haploid genome concentration of three replicates (n=3), two replicates were negative. For the experiment under standard conditions the data for Ref mgDNA and SSC represent the mean haploid genome concentration of four replicates (n=4), for Ref hgDNA the mean haploid genome concentration of three replicates (n=3) and for TE-buffer the mean haploid genome concentration of two replicates (n=2), three replicates were negative. The error bars represent the standard deviation. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, SSC = system-suitability control (as described in the main article), TE-buffer = negative control sample.

Table S5 Method development and robustness evaluation. Intra-assay accuracy and precision values and acceptance criteria for experiments performed under worst-case scenario and standard conditions for different reference standards.

Intra-assay accuracy and precision[a]								
Acceptance criteria		Ref hgDNA	Ref10E1	Ref10E2	Ref10E3	Ref10E4	Ref10E5	Ref10E6
% of nominal value	Accuracy	±15%	±15%	±15%	±15%	±15%	±15%	±20%
CV[b]	Precision	≤15%	≤15%	≤15%	≤15%	≤15%	≤15%	≤20%
Worst-case scenario	Accuracy	75.37%	122.25%	116.97%	98.45%	101.92%	94.61%	107.49%
conditions	Precision	4.46%	7.41%	3.17%	2.72%	7.46%	6.35%	**21.93%**[d]
Standard conditions	Accuracy	73.42%[c]	133.89%	118.20%	100.69%	101.05%	92.59%	96.21%

	Precision	19.27%[c]	7.29%	3.01%	2.67%	2.21%	11.13%	14.63%

[a]The intra-assay accuracy and precision values are calculated with five replicates for each experiment (n=5). [b]Precision is expressed as the coefficient of variation (CV) in percentage. [c]The intra-assay accuracy and precision values are calculated with three replicates for this sample. [d]The value in bold does not match the acceptance criteria in comparison to the validation runs.

Table S6 Complete calculation and manufacturing of reference standards.

Reference standard	µl hgDNA[a] solution (100 ng/µL) [µL]	Dilution factor (with TE-buffer)	Human genomes/µL	µl mgDNA[b] solution 97.64 ng/µL) [µL]	Human genomes/µL in the reference standard	Total human genomes in the reference standard	Total murine genomes	Factor
hgDNA	20	undiluted	2.76E+04	0	2.76E+04	5.53E+05	0	/
Ref10E1	2	undiluted	2.76E+04	18	2.76E+03	5.53E+04	5.53E+05	0.100003
Ref10E2	2	1 to 10	2.76E+03	18	2.76E+02	5.53E+03	5.53E+05	0.010000
Ref10E3	2	1 to 100	2.76E+02	18	2.76E+01	5.53E+02	5.53E+05	0.001000
Ref10E4	2	1 to 1000	2.76E+01	18	2.76E+00	5.53E+01	5.53E+05	0.000100
Ref10E5	2	1 to 10000	2.76E+00	18	2.76E-01	5.53E+00	5.53E+05	0.000010
Ref10E6	2	1 to 100000	2.76E-01	18	2.76E-02	5.53E-01	5.53E+05	0.000001
mgDNA	0			18			5.53E+05	

[a]For all calculations the value for the haploid human genome size was 3.30E+09 base pairs and the weight of on base pair was 1.10E-15 µg. [b]The value for the haploid murine genome size was 2.90E+09 base pairs.

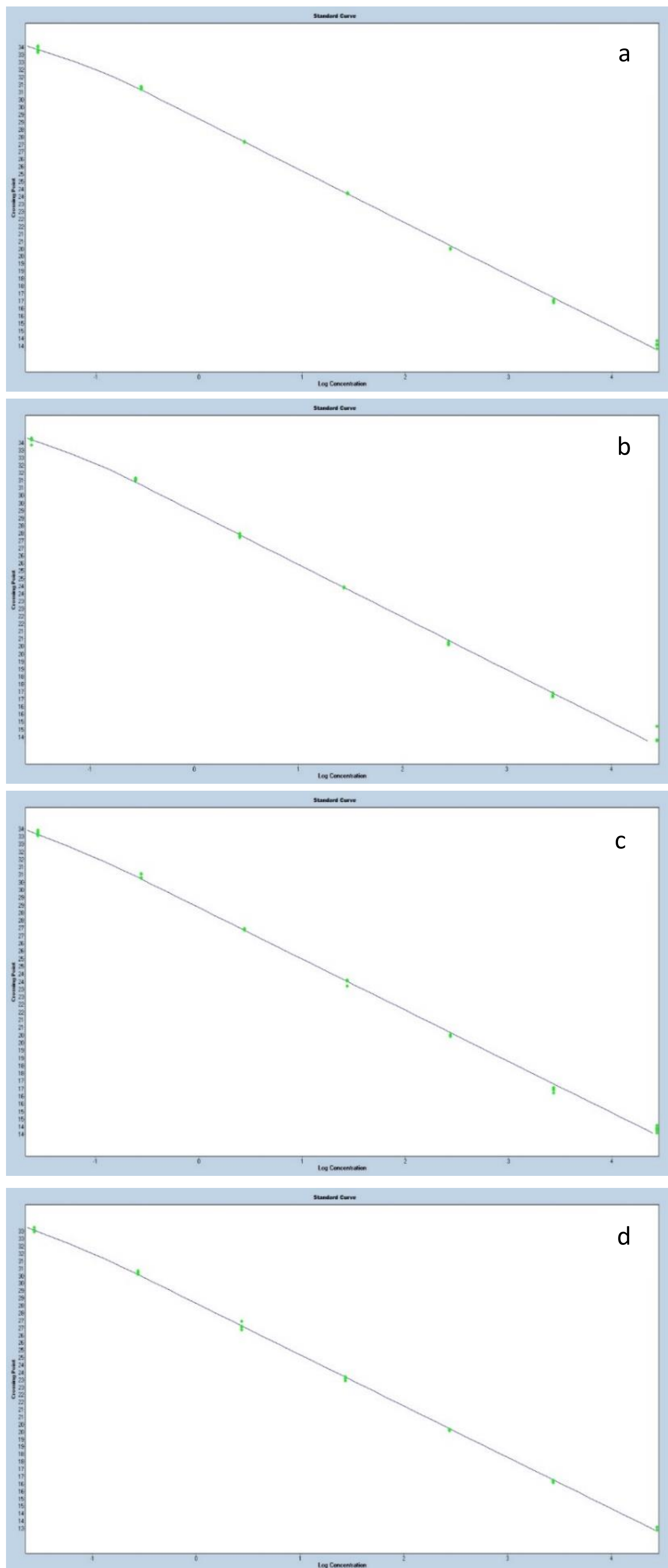

Figure S8 Plots of calibration curves for validation runs. The plots of validation run 1 (a), 2 (b), 3 (c) and 4 (d) are presented. The x-axis represents the log concentration of human haploid genomes and the y-axis the Cq-value (=Crossing Point).

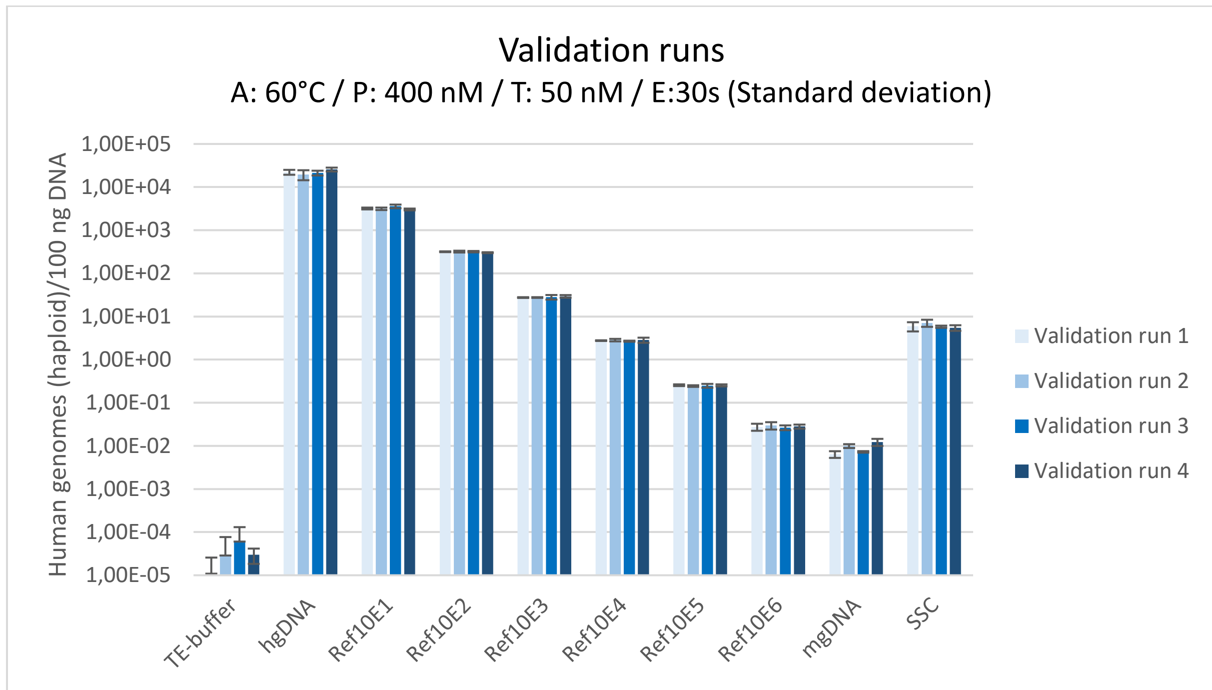

Figure S9 Concentrations and standard deviations for all samples of all validation runs. The concentration of haploid human genomes and standard deviations for reference standards, TE-buffer, hgDNA, mgDNA and SSC applying the final qPCR protocol for validation runs 1-4 are reported. The data represent the mean haploid genome concentration of five replicates (n=5) for different samples. For validation run 1 the data for mgDNA represent the mean haploid genome concentration of four replicates (n=4) and for TE-buffer the mean haploid genome concentration of three replicates (n=3), two replicates were negative. For validation run 2 the data for hgDNA and mgDNA represent the mean haploid genome concentration of four replicates (n=4) and also for TE-buffer the mean haploid genome concentration of four replicates (n=4), one replicate was negative. For validation run 3 the data for mgDNA represent the mean haploid genome concentration of four replicates (n=4) and for TE-buffer the mean haploid genome concentration of two replicates (n=2), three replicates were negative. For validation run 4 the data for mgDNA, Ref10E1 and SSC represent the mean haploid genome concentration of four replicates (n=4) and for hgDNA the mean haploid genome concentration of two replicates (n=2). The data for TE-buffer represent the mean haploid genome concentration of four replicates (n=4), one replicate was negative. The error bars represent the standard deviation. A = annealing/amplification temperature, P = primer concentration, T = TaqMan© probe concentration, E = elongation time, SSC = system-suitability control (as described in the main article), TE-buffer = negative control sample.

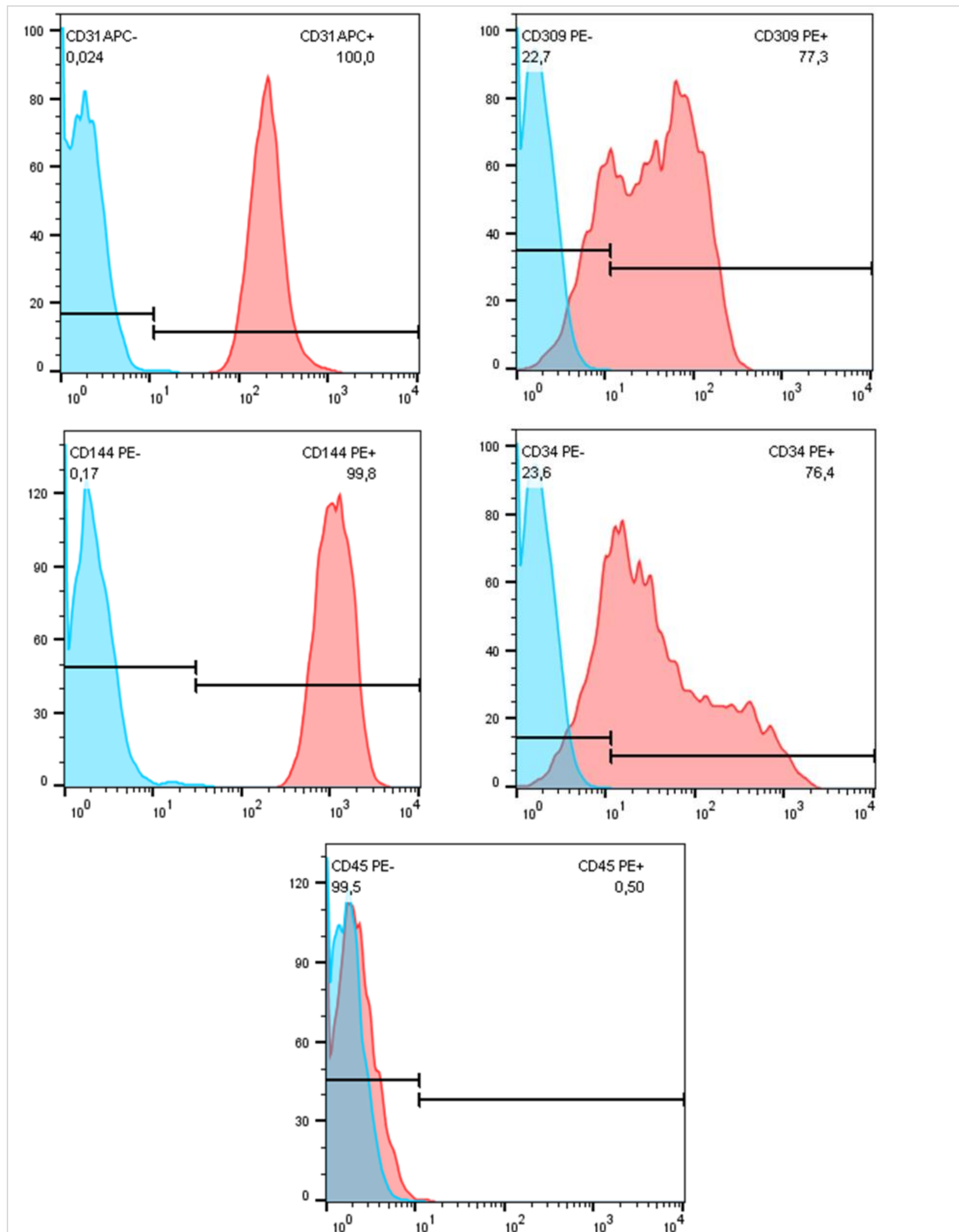

Figure S10 Characterization of genetically modified HA-BOECs by flow cytometry. BOECs were trypsinized and stained with antibodies directed against specific surface markers (red line and filled peak) and isotype controls (blue line and filled peak). Surface markers for cytometric characterization of genetically modified HA-BOECs included the endothelial surface markers CD31 and VEGFR2 (CD309), VE-Cadherin (CD144), the progenitor and activation marker CD34 and the leukocyte marker CD45.

Supplemental Methods

During method development samples containing hgDNA diluted in TE-buffer (STD samples: STD hgDNA, STD10E1-STD10E6) were analyzed and compared to samples containing hgDNA diluted in sample matrix mgDNA (Reference standard: Ref hgDNA, Ref10E1-Ref10E6, Ref mgDNA). Sample preparation was performed following Table S6 with the exception that mgDNA was replaced with TE-buffer for STD10E1-STD10E6. The reason for that was to investigate if and in case how the dilution with mgDNA influences the qPCR performance. Several different batches of reagents, e.g. primers, probe, reference standards, hgDNA reference material, TE-buffer, etc. and consumables were applied during method development and robustness evaluation. In addition, three different operators prepared critical reagents requiring preparation steps before use, e.g. dilution to a defined concentration.

Flow cytometry analysis

BOECs were characterized by flow cytometric analysis. Cells were detached with TrypLE Select 1x no phenol red, re-suspended in staining buffer containing DPBS-, FCS 0,5% (Bio&SELL, Nuremberg, Germany) and NaN_3 0,1% and incubated with the respective antibody for 30 min on ice. Antibodies used are listed below. For each sample, $1.5x10^5$ live events were acquired on the BD FACSCalibur (BD Biosciences, San Jose, CA, USA). Data were analyzed using the BD FACStation software Version 6.0.

Antibody	Manufacturer	Format	Clone
CD144	Miltenyi Biotech	PE	REA199
CD309	Miltenyi Biotech	PE	ES8-20E6
CD45	Miltenyi Biotech	PE	5B1
CD31	ImmunoTools	APC	MEM-05
CD34	ImmunoTool	PE	4H11