Section S1.

Table S1. Detailed Experimental Design.

Performance Metric	Sample	Sample Mixtures	Related/ Unrelated	Input Mass (ng)	dd-cfDNA Fraction (%)	Number of Replicates	Number of Measurements	Total Measurements
LoB	Reference (n = 4, blank)	N/A	N/A	15	N/A	3	12	128
	Reference (n = 1, blank)	N/A	N/A	15	N/A	11	11	
	Reference (n = 5 blanks)	N/A	N/A	30	N/A	3	15	
	Reference (n = 5 blanks)	N/A	N/A	45	N/A	6	30	
	Plasma (n = 10)	N/A	N/A	Variable	N/A	3	30	
	Plasma (n = 5)	N/A	N/A	Variable	N/A	6	30	
LoD	Reference	1	Related	15,45	0.1, 0.3, 0.6	6	36	389
		1	Related	30	0.1, 0.3, 0.6	22	66	
		1	Related	30	0.6	64	64	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6	6	108	
	Plasma mixtures	3	Unrelated	15	0.1	8	24	
		3	Unrelated	15	0.3,0.6	6	36	
		3	Related	Variable	0.1,0.3,0.6	3	27	
		2	Related	Variable	0.3,0.6	4	16	
		1	Related	Variable	0.3,0.6	6	12	
LoQ, linearity	Reference	1	Related	15,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	96	798

		1	Related	30	0.1, 0.3, 0.6, 1.2	22	88	
		1	Related	30	2.4	20	20	
		1	Related	30	5.0, 10.0, 15.0	6	18	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	288	
		1	Related	30	0.6,2.4	64	128	
	Plasma mixtures	3	Unrelated	15	0.1	8	24	
		3	Unrelated	15	0.3,0.6	6	36	
		3	Unrelated	15	1.2,2.4,5.0,10.0	3	36	
		3	Related	Variable	0.1,1.2	3	18	
		2	Related	Variable	0.3,0.6	4	16	
		1	Related	Variable	0.3,0.6	6	12	
		3	Related	Variable	0.3,0.6	3	18	
Accuracy	Reference	1	Related	15,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	96	638
		1	Related	30	0.1, 0.3, 0.6, 1.2	22	88	
		1	Related	30	2.4	20	20	
		1	Related	30	5.0, 10.0, 15.0	6	18	
		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0, 15.0	6	288	
		1	Related	30	0.6,2.4	64	128	
Reproducibility, per input	Reference	1	Related	15	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	12	84	504

		1	Related	30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	84	
		2	Unrelated	15	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	12	168	
		2	Unrelated	30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	168	
Reproducibility, Per Lot	Reference	1	Related	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	126	378
		2	Unrelated	15,30,45	0.1, 0.3, 0.6, 1.2, 2.4, 5.0, 10.0	6	252	
Linearity, Reproducibility	Transplant Patient Samples (n = 6)	4	Related	Variable	Variable	2	8	12
		2	Unrelated	Variable	Variable	2	4	
Repeatability	Reference	1	Related	30	0.6, 2.4	64	128	128

N/A, Not applicable.

Section S2.

Table S2. Mean, Median, and Standard Deviation Values of Measured dd-cfDNA for Related and Unrelated Cases for Lots 1 and 2.

LoB – Statistics, Estimation Methods	Lot 1	Lot 2
Mean, related, %	0.03	0.06
Mean unrelated, %	0.02	0.03
Median, related, %	0.01	0.03
Median, unrelated, %	0.01	0.01
Standard deviation, related, %	0.05	0.1
Standard deviation, unrelated, %	0.02	0.05

To demonstrate the performance of the test for reference and plasma samples separately, LoB was computed for each case by using 60 and 68 measurements obtained from plasma and reference samples, respectively. To increase the sample size, lots were not distinguished. **Figure S1** and **Table S3** depict the histograms and LoB values for each sample type and estimation method, respectively.

Figure S1. Histograms of measured dd-cfDNA for LoB analysis. (a) Related method, reference samples. (b) Unrelated method, reference samples. (c) Related method, plasma samples. (d) Unrelated method, plasma samples.
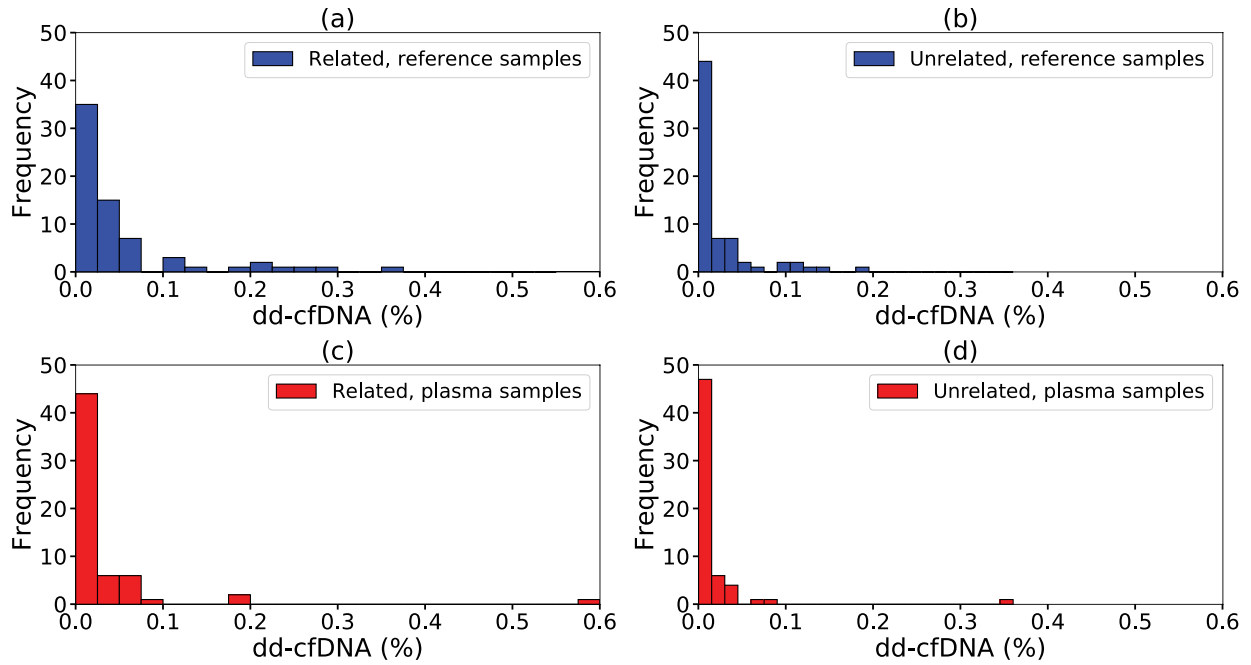
Table S3. LoB Values for Related and Unrelated Estimation Methods for Reference and Plasma Samples.

LoB, Estimation Methods	Reference Samples	Plasma Samples
Related, %	0.23	0.08
Unrelated, %	0.11	0.04

Section S3.

LoD Analysis: The parametric LoD computation method necessitates that: (1) The measurements from low-level samples, approximately follows a Gaussian distribution, (2) the empirical standard deviations of the described samples, approximately remain constant as a function of empirical mean. **Figure S2** depicts the histograms of centered, measured DFs for each lot and each test mode. **Figure S3** shows empirical standard deviation as a function of empirical mean for both lots and test modes. **Figures S2** and **S3** demonstrate that these two conditions are satisfied for both related and unrelated low-level samples.

Figure S2. Histograms of centered, measured dd-cfDNA for LoD analysis. (a) Related samples from Lot 1. (b) Related samples from Lot 2. (c) Unrelated samples from Lot 1. (d) Unrelated samples from Lot 2.
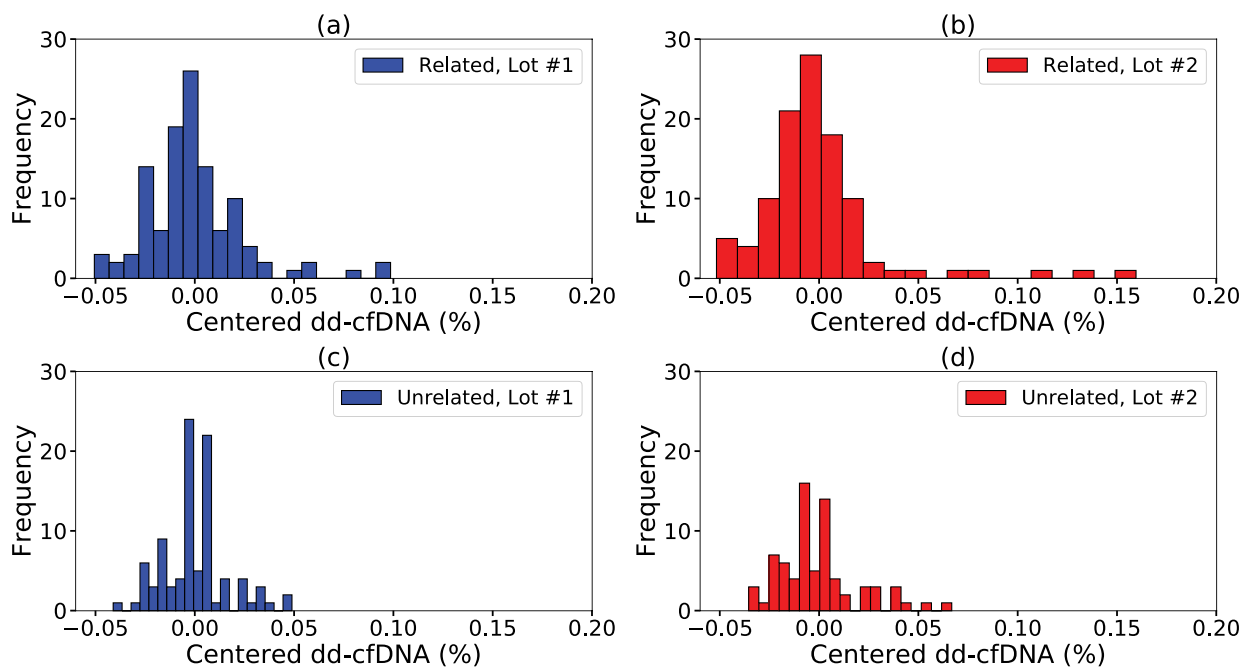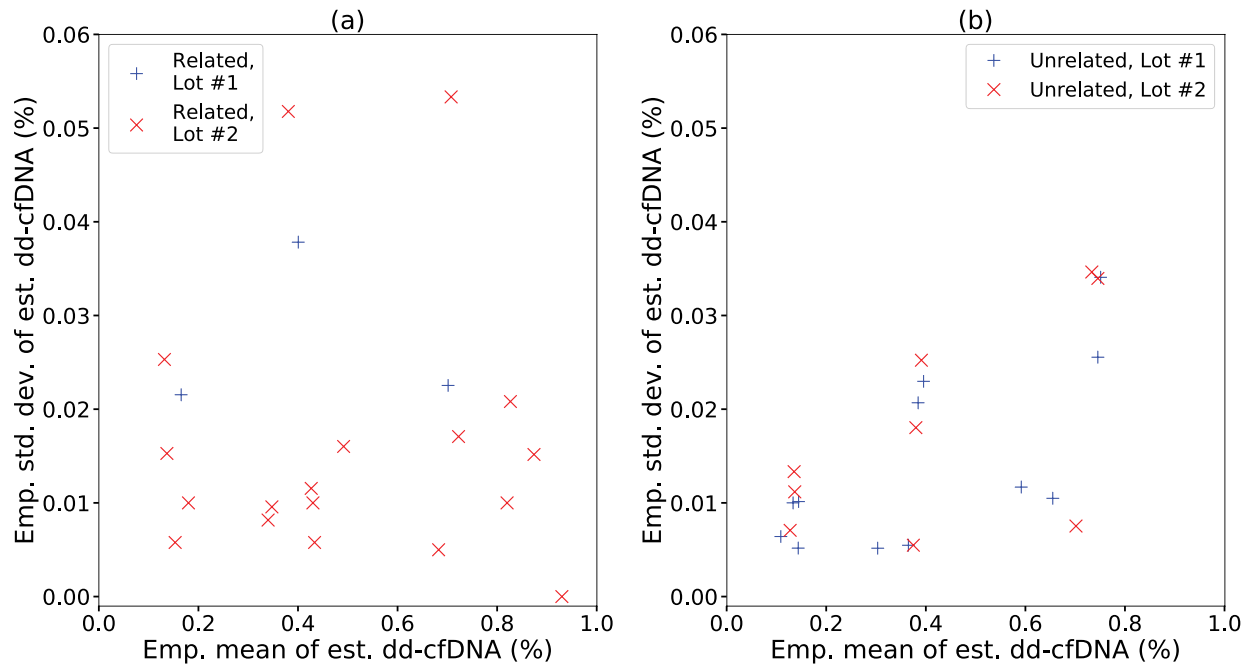
Figure S3. Empirical standard deviations as a function of the corresponding empirical means for LoD analysis. (a) Related samples from Lot 1 and Lot 2. (b) Unrelated samples from Lot 1 and Lot 2.

To demonstrate LoD for reference and plasma mixture samples separately, and to observe the effect of input amount on reference samples, LoD analysis for these sets of samples was carried out separately, by using their corresponding LoB values. Furthermore, for reference samples, 18 related and 36 unrelated measurements were used at 15 ng and 45 ng inputs; 130 related and 36 unrelated measurements were used at 30 ng input. **Tables S4** and **S5** provides a breakdown of the computed LoD values with respect to their estimation method and input amount for reference samples and for plasma mixture samples, respectively.

Table S4. LoD Values for Related and Unrelated Estimation Methods, for Reference Samples at 15, 30, and 45 ng Inputs.

| Reference samples, Estimation Methods	LoD		
	15 ng	30 ng	45 ng
Related, %	0.28	0.26	0.25
Unrelated, %	0.13	0.13	0.12

Table S5. LoD Values for Related and Unrelated Estimation Methods, for Plasma Mixture Samples.

| Plasma Mixture Samples, Estimation Methods	LoD
Related, %	0.11
Unrelated, %	0.05

Section S4.

LoQ Analysis: Similar to LoD analysis, LoQ was evaluated for reference samples, which were further partitioned with respect to their input amounts. As depicted in **Figure S4**, all the measured CV values for all the spike levels tested were below 20% cutoff for related samples at all input levels, as well as related samples at 15 and 45 ng input levels. Thus, for all the cases, lower LoQ was equal to LoD, by definition. For related samples with 30 ng input level, fitted curve intersects 20% CV level at approximately 0.174%, which was lower than the corresponding LoD, ie, 0.26%, for this case. Thus, the lower LoQ is again equal to LoD, by definition.

Figure S4. Measured percent CV values as a function of the corresponding percent empirical means, particularized with respect to input amount, for LoQ analysis of reference samples. (a) Related samples. (b) Unrelated samples.
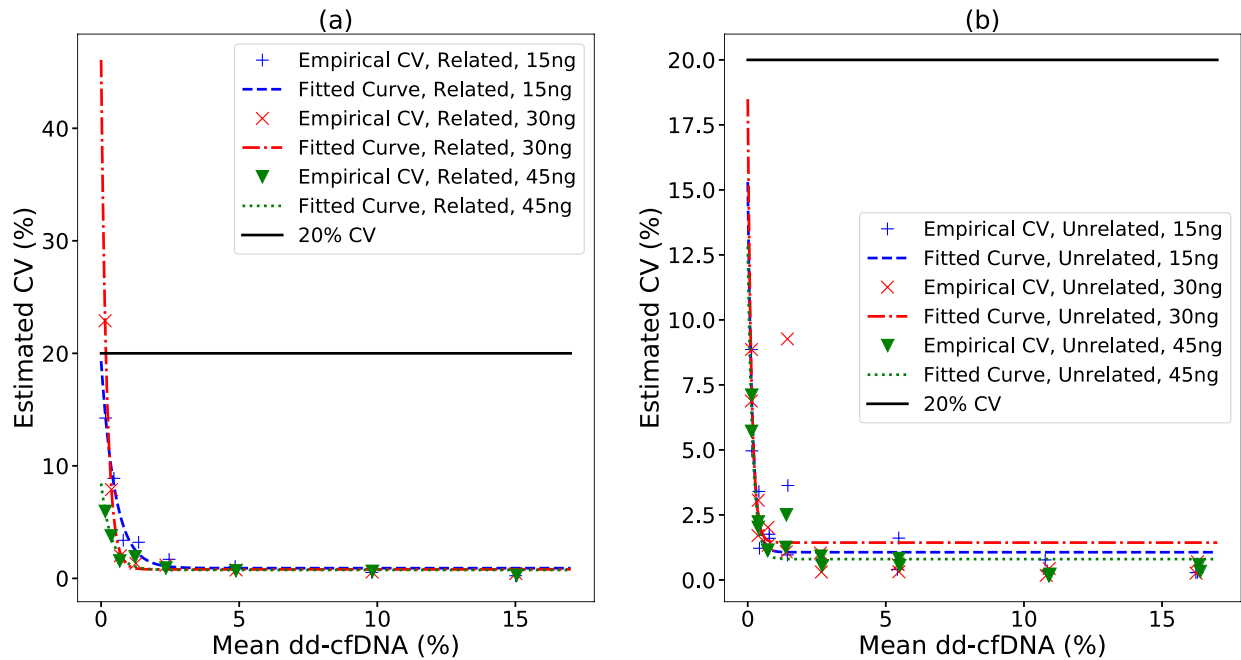
Furthermore, LoQ values for plasma mixture samples were also computed (**Figure S5**).

For both cases, a lower LoQ was observed equal to the corresponding LoD. It should be

noted that the exponential fit for **Figure S5a** was not accurate, which limited readability

of the graph, and hence omitted. The main reason for the inaccuracy of the fit was the

lack of higher DF level samples, compared to all other scenarios. This exclusion,

however, did not affect our inference about the lower LoQ, since all the estimated CV

values were well below 20% cutoff.

Figure S5. Measured percent CV values as a function of the corresponding percent

empirical means for LoQ analysis of plasma mixture samples. (a) Related. (b)

Unrelated.
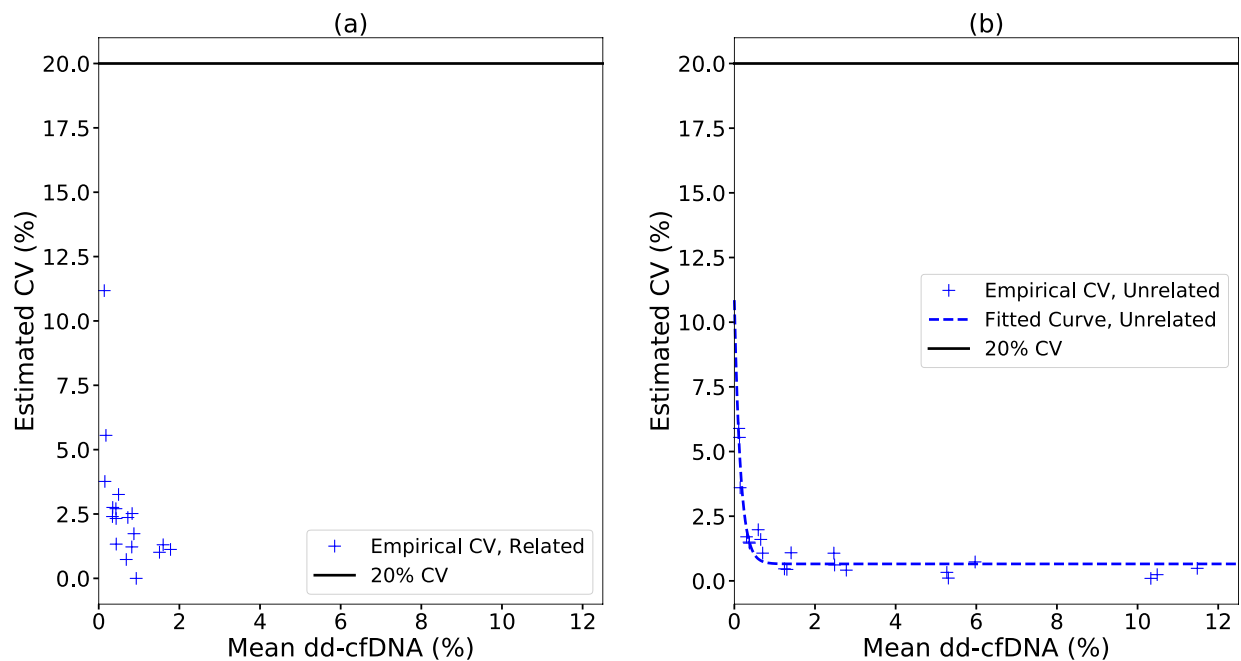
Finally, **Table S6** summarizes the estimated parameters of the nonlinear fit for CV in all

different scenarios:

Table S6. Estimated Parameters of the Exponentially Decaying Model of the CV for Every Scenario—LoQ.

LoQ, Data Set, Estimation Method	Estimated Parameters		
	a	b	c
Combined, lot 1, related	0.950216	16.4685	1.88562
Combined, lot 2, related	1.82651	24.2948	6.82745
Combined, lot 1, unrelated,	0.557873	6.16417	1.53284
Combined, lot 2, unrelated,	0.715364	7.00144	1.00344
Reference samples, 15 ng, related,	0.907757	18.3994	1.97114
Reference samples, 30 ng, related,	0.798892	45.2805	4.943
Reference samples, 45 ng, related,	0.746606	7.69009	2.62489
Reference samples, 15 ng, unrelated,	1.06598	14.2357	6.04647
Reference samples, 30 ng, unrelated,	1.4362	17.0526	7.3715
Reference samples, 45 ng, unrelated,	0.801393	12.0185	5.69333
Plasma mixture samples, related	1.88546	13275.4	53.5112
Plasma mixture samples, unrelated	0.654995	10.1971	6.67823

Section S5.

Linearity Analysis: Similar to previous performance metrics, linearity analyses for reference and plasma mixture samples are reported separately. Specifically, analysis for reference samples used 349 related and 285 unrelated measurements, whereas analysis for plasma mixture samples used 63 related and 96 unrelated measurements. **Figure S6** depicts the individual measurements and linear regression lines for reference samples. Similarly, **Figure S7** depicts the individual measurements and linear regression lines for plasma mixture samples. **Table S7** lists corresponding linear regression results for reference and plasma mixture samples, respectively.

Figure S6. Measured dd-cfDNA as a function of the corresponding attempted (targeted) spike levels, along with the calculated linear fit, for linearity analysis of reference samples. (a) Related only. (b) Unrelated only. (c) Related and unrelated cases together.
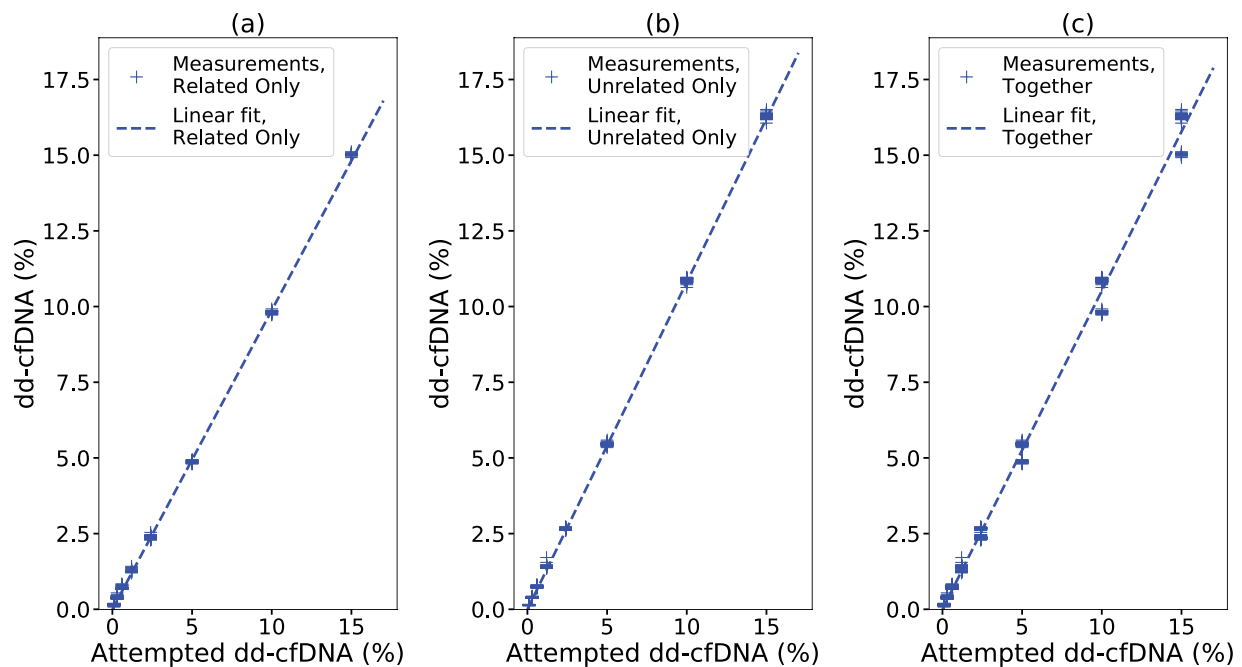
Figure S7. Measured dd-cfDNA as a function of the corresponding attempted (targeted) spike levels, along with the calculated linear fit, for linearity analysis of plasma mixture samples. (a) Related only. (b) Unrelated only. (c) Related and unrelated cases together.
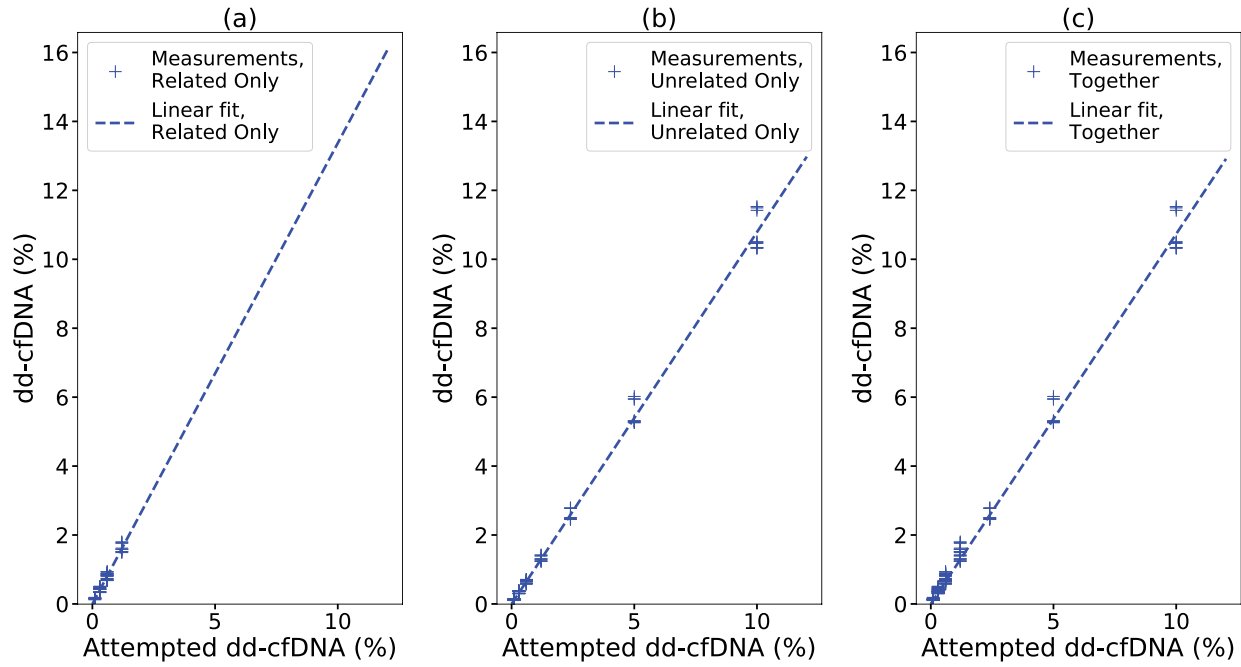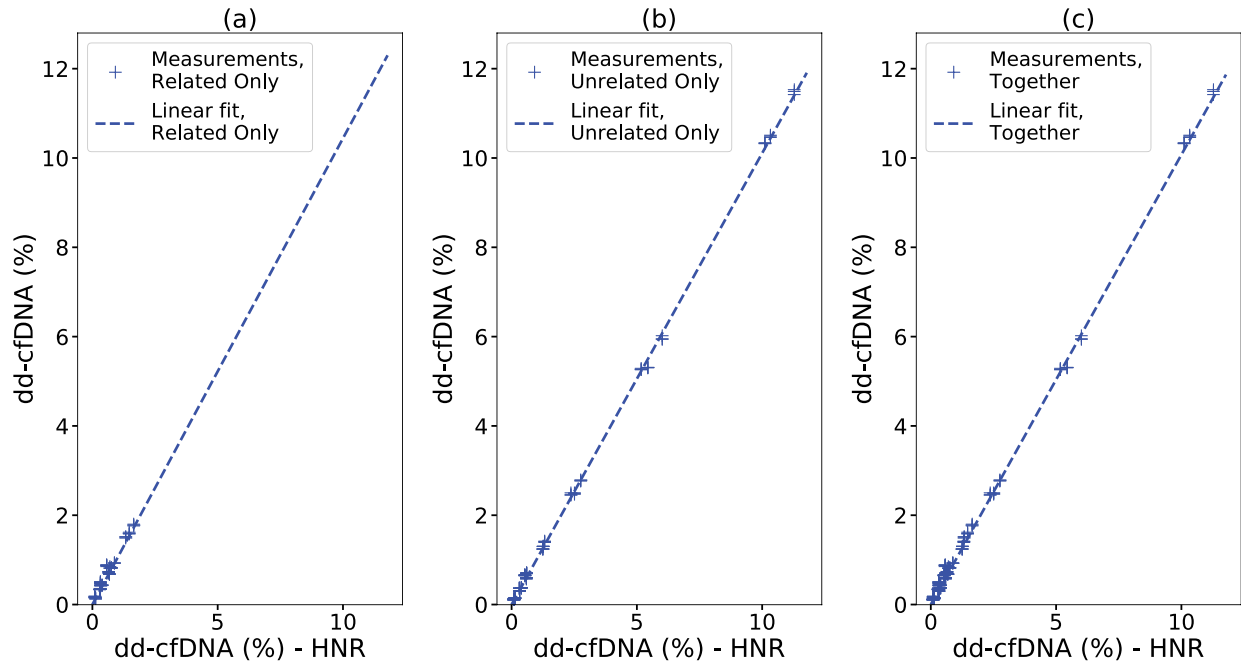
Table S7. Linear Regression Results for Linearity of Reference Samples and Plasma Mixture Samples, Including 95% CI.

Sample Type	Linearity Parameters		
Reference Samples	Slope	Intercept	R^2
Unrelated	1.0804 (0.9540, 1.2069)	0.0007 (−0.0077, 0.0091)	0.99989 (0.99986,0.99992)
Related	0.9876 (0.8833, 1.0920)	0.0005 (−0.0041, 0.0052)	0.9994 (0.9974, 0.9995)
Related and unrelated	1.0515 (0.9693, 1.1338)	0.0003 (−0.0043, 0.0049)	0.9969 (0.9964, 0.9974)
Plasma mixture samples	Slope	Intercept	R^2
Unrelated	1.0787 (0.8574, 1.300)	0.0002 (−0.0076, 0.0080)	0.9962 (0.9943, 0.9975)
Related	1.3368 (0.9895, 1.6841)	0.0001 (−0.0020, 0.0022)	0.9713 (0.9528, 0.9965)
Related and unrelated	1.0734 (0.9038, 1.2430)	0.0008 (−0.0039, 0.0055)	0.9953 (0.9935, 0.9965)

Section S6.

Accuracy Analysis: In order to demonstrate the accuracy for plasma mixture samples, a DF estimated by using SNP's from homologous non-recombining region in lieu of ddPCR for reference samples. The rationale of using this method as a more precise alternative to the conventional DF estimate is as follows: the non-recombining nature ensures that the targets in this region have the property of X chromosome always provides the reference allele and the Y chromosome always provides the mutant allele. Thus, the allele ratio reflects the ratio of X and Y chromosomes in the sample. This observation, coupled with the fact that plasma mixture samples are designed to have a female background with a male spike-in, which implies that the allele ratio is directly proportional to the half of DF. Hence, DF can be estimated without the background interference. The analysis was carried out using 63 related and 96 unrelated plasma mixture sample measurements, which excludes one sample that failed QC. The individual measurements and linear regression lines are shown in **Figure S8**, and the corresponding linear regression results are shown in **Table S8**. It is anticipated that the relative wider confidence intervals for plasma mixture sample estimates compared to their reference sample counterparts might be due to the relatively smaller sample size of the former compared to the latter.

Figure S8. Measured dd-cfDNA (y-axis) as a function of the corresponding dd-cfDNA values measured using homologous non-recombining region (x-axis), along with the calculated linear fit, for accuracy analysis of plasma mixture samples: (a) Related only. (b) Unrelated only. (c) Related and unrelated cases together.

HNR, Homologous non-recombining region

Table S8. Linear Regression Results for Accuracy of Plasma Mixture Samples, Including 95% CI.

Plasma Mixture Samples	Accuracy		
	Slope	Intercept	R^2
Unrelated	1.0108 (0.8038, 1.2179)	0.0002 (−0.0076, 0.0080)	0.9996 (0.9994, 0.9997)
Related	1.0440 (0.7727, 1.3153)	0.0007 (−0.0012, 0.0027)	0.9706 (0.9517, 0.9993)

Plasma Mixture Samples	Accuracy		
	Slope	Intercept	R^2
Combined	1.0073 (0.8484, 1.1662)	0.0005 (−0.0042, 0.0053)	0.9991 (0.9987, 0.9993)

Section S7.

Precision Analysis: In order to compute the confidence intervals on the estimated CV's for repeatability analysis, the classical bounds of McKay was used based on a chi-squared approximation.[1] The derivation of these bounds assumes that the underlying measurements from which CV is estimated are realizations from a Gaussian distribution. **Figure S9** illustrates that the said assumption was justified in our case.

Figure S9. Histograms of measured dd-cfDNA for repeatability analysis. (a) 0.6% targeted dd-cfDNA. (b) 2.4% targeted dd-cfDNA.
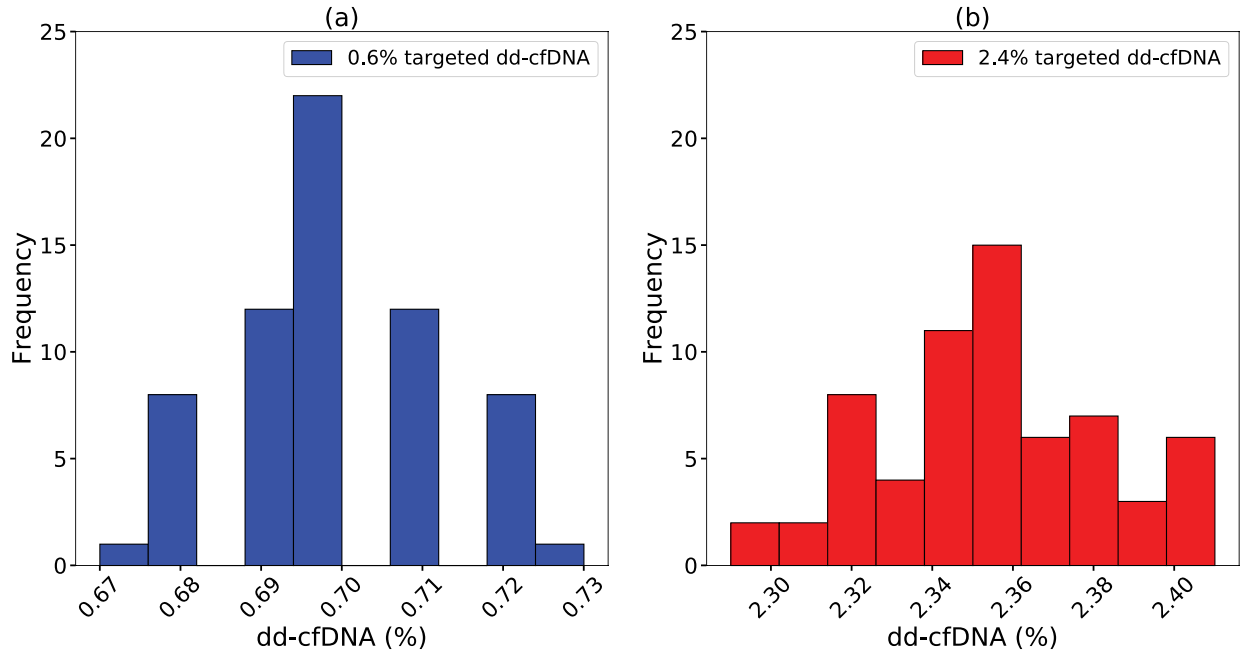
It should be noted that chi-squared approximation-based bounds used in repeatability analysis is not suitable to compute the confidence intervals of the estimated CV's for reproducibility analysis. The reason is that the underlying measurements from which CV value is estimated do not follow a Gaussian distribution, because of the broad range of underlying DFs. Thus, confidence intervals by a standard bootstrapping technique was computed. Because of the inherent stochasticity of the approach, the values may slightly vary for each trial of the method.

Confidence intervals of the estimated concordance between clinical samples was computed via Clopper-Pearson method for binomial proportions. Specifically, the closed-form expression of the said method for 100% observed success rate was used.

Reference

1.   McKay AT. Distribution of the coefficient of variation and the extended "t" distribution. *J R Stat Soc.* 1932;95(4):695–698.