Multicenter evaluation of GraftAssureIQ™, a new generation dd-cfDNA assay

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Background:

Donor-derived cell-free DNA (dd-cfDNA) is playing a crucial role in post-transplant monitoring; however, the standard modality of testing currently limits its utility. Centralized laboratories offering dd-cfDNA testing have several time limitations that slow the time-to-diagnosis and usability of the results, such as sample transport time and the utilization of time-consuming NGS technologies.

GraftAssureIQ is a Research Use Only (RUO) test kit developed for use at any laboratory, decentralizing dd-cfDNA testing. The assay utilizes droplet-digital™ PCR to quantify dd-cfDNA across 45 highly curated preselected SNPs with a high probability of distinguishing donor from recipient (1). The assay workflow enables same day results within 6-8 hours from plasma separation and shows equivalency to the clinically available version, validated in large clinical studies (2-6). Through improved turnaround times and local availability, GraftAssureIQ fulfills numerous unmet needs for transplant researchers.

This report describes results from a multicenter global analytical validation of GraftAssureIQ utilizing contrived samples, providing robust evidence of assay performance across diverse laboratory settings.

Methods:

GraftAssureIQ Workflow

The assay, run on the QX600™ platform (Bio-Rad™, Hercules, CA), has a simple workflow, with minimal hands-on time. The workflow begins with a short pre-amplification reaction. The pre-amplified cfDNA is then added to the ddPCR™ master mix for 16 reactions. This mixture is distributed into 16 wells (2 strips) of 0.2mL PCR reaction cups, that include the lyophilized GraftAssureIQ specific primer/probe mixtures. A 96-well PCR plate can therefore host 6 samples (controls or target samples). It is recommended to have at least one non-template control per batch of pre-amplification. One positive control sample needs to be performed at the beginning of using GraftAssureIQ to calibrate the particular dd-cfDNA system used at the respective laboratory. It is recommended to repeat this control at regular intervals, but it is mandatory for each batch of GraftAssureIQ reagent. The kit contains all reagents needed to perform the test lyophilized, being stable at room temperature. The system consumables and reagents for the QX600™ are not part of the kit, but readily available from the manufacturer Bio-Rad™.

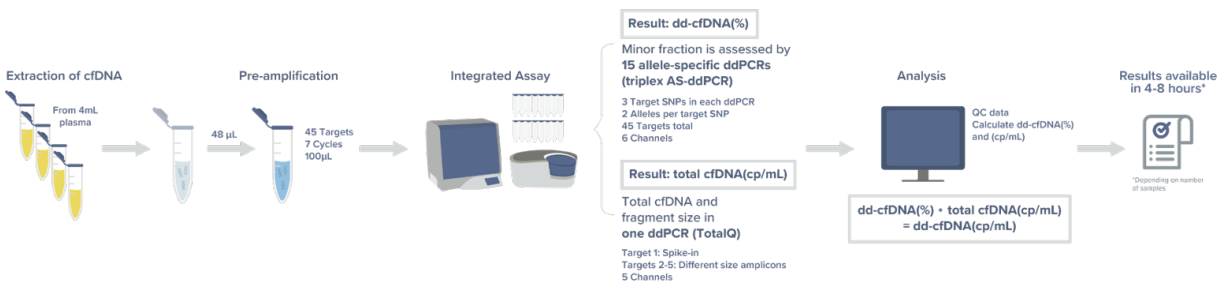

Figure 1 Overview of the GraftAssureIQ workflow.

Control Design

Eight different proficiency samples we prepared by the manufacturer (Insight Molecular Diagnostics Inc., Nashville, TN) and shipped to the participating laboratories located in North America, Europe, and Asia, reflecting the global scale of the validation effort. Each sample was measured 6 times over 3 days (335 measurements, 1 failed test, 99.7% success). The concentration of dd-cfDNA ranged from 3 to 570 copies per test in a background of approximately 38,000 copies. For the quantification of total cf-DNA, which is an integrative part of GraftAssureIQ, another eight proficiency samples with three different concentrations (equivalent to 3,000 to 47,000 cp/mL plasma), each with multiple fragmentation patterns, were generated and tested in 6 runs each (659 valid measurements, 13 fails, 98% success). All samples consisted of mixtures of synthetic gene fragments.

Results:

Figure 2 shows the measurement data of the dd-cfDNA proficiency samples stratified by center, yielding very homogenous results.

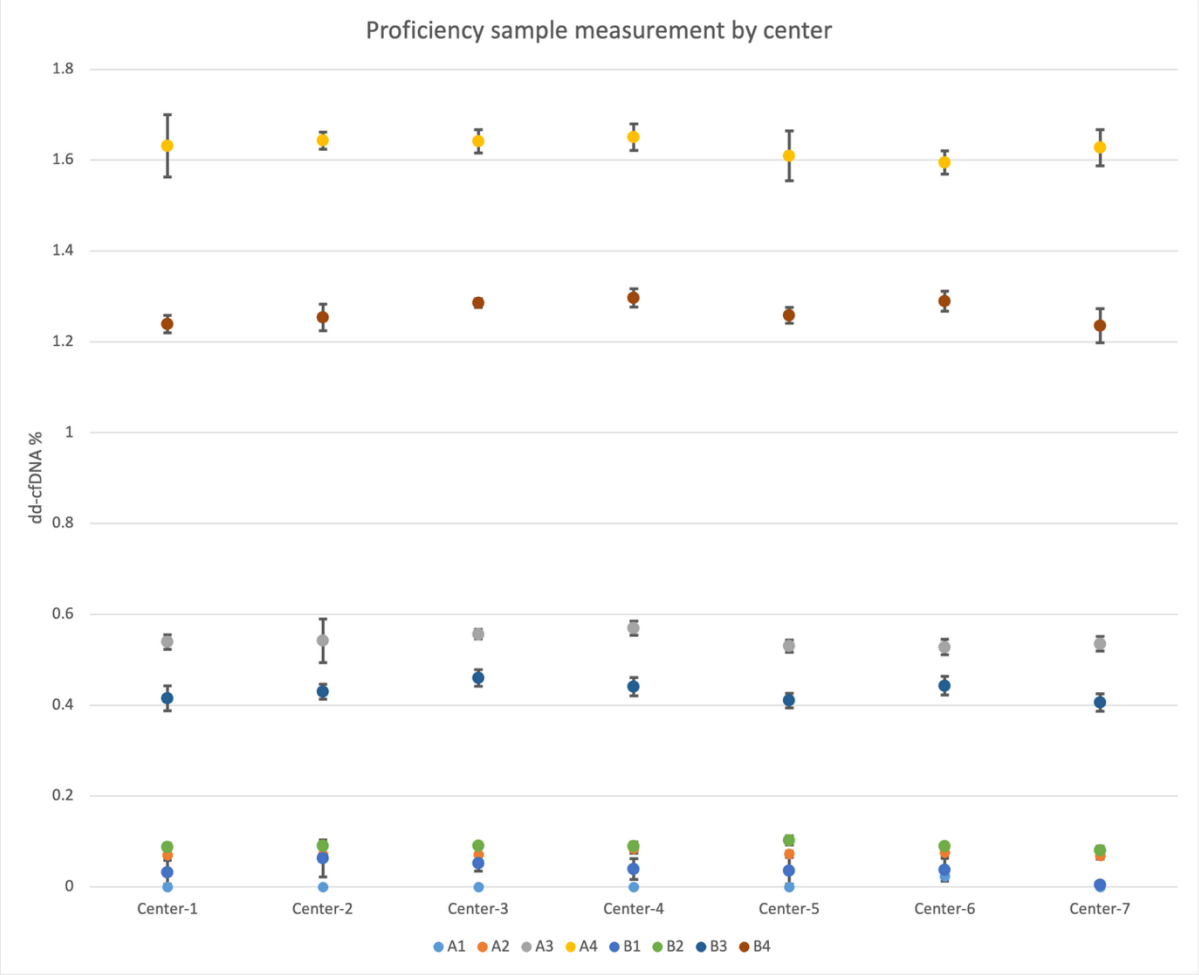

Figure 2. Percentage results of the proficiency samples stratified by center. Averages and standard deviations are given.

These data were used to evaluate the lower limit of quantification (LLoQ), which is given with 8.5 copies/test by the manufacturer assuming 13 informative assays. This number of assays represents the 5th percentile of the expected number in the U.S. population of kidney

transplanted patients. The data from Fig. 2, were: A) converted to 13 SNP assays using the gaussian error propagation (each calculated CV was multiplied by the square root of the number of total assays divided by 13) and B) ln/ln transformed and the upper 95th confidence limit of the regression was calculated (see Figure 3).

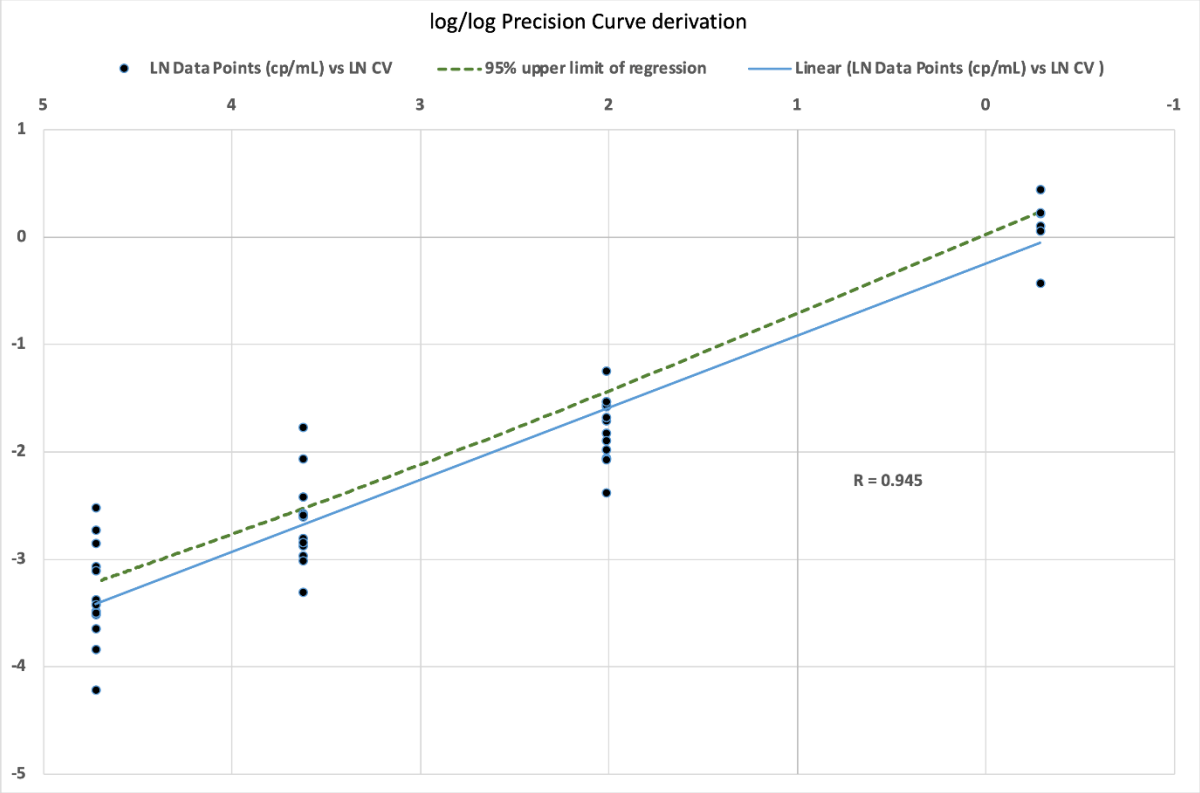

Figure 3. Derivation of the upper 95th percent confidence limit of the ln/ln transformed regression calculated for copy numbers using data given in Figure 2.

The precision curve was defined as the 95th CI from Figure 3 and the point where the CV was lower than 20% (standard definition of LLoQ) was derived is shown in Figure 4. **Note**: LLoQ was defined conservatively with an error of 5% (chance of having less than 13 SNPs) and 2.5% (upper 95th confidence limit of the regression), which is multiplied to assess the total a-error resulting in **a = 0.125%.** Therefore, **99.875%** of samples will have a **lower LLoQ** than the number presented in the following section.The precision curve was constructed using the 95th regression confidence given in Figure 4.

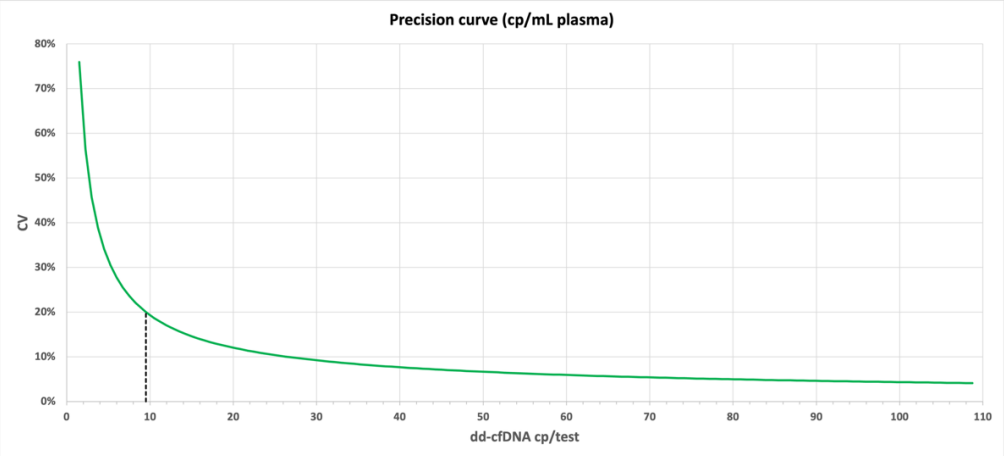

Figure 4. The LLoQ 95th confidence line from figure 3, yields a value of 9.5cp/test

From this curve, the LLoQ was computed to be **9.5 copies/test,** which closely resembles the specifications of the manufacturer.

The LLoQ of cp/mL can be converted to the corresponding percentage value, by using the total copies per mL of plasma cfDNA that is expected in a typical kidney transplanted patient. In the first year after transplantation, the median value of cfDNA is about 8,000 cp/mL plasma(2, 7). Since 4 mL of plasma is used, the value in a test shall be 32,000. To assess the efficiency of the cfDNA extraction using approximately 70%, the following calculations are shown below:

LLoQ:
cp/mL: 9.5cp/test / 4mL /0.7 = **4cp/mL**
Percent dd-cfDNA = 9.5/32,000/0.7 = **0.04%**

These values exactly resemble what the manufacturer showed for its internal assessment(8). Since the medial total cfDNA value of 50% was selected, the total a-error for percentage was 50% of the a for cp/mL.

Total cf-DNA

For the quantification of total cf-DNA, the results averaged for each concentration are shown by center in Figure 5. The concentrations were calculated based on the Instructions for Use, which recommends 4 mL of plasma as input to the entire assay, consisting of dd-cfDNA and total cfDNA measurements. Also 70% DNA extraction efficiency was assumed. The CVs were ≤9% over the entire tested range. Stability issues occurred during shipment of the proficiency samples to Asia, when censoring the results of that center from the analysis, the CVs were 2%, 6% and 6% respectively.

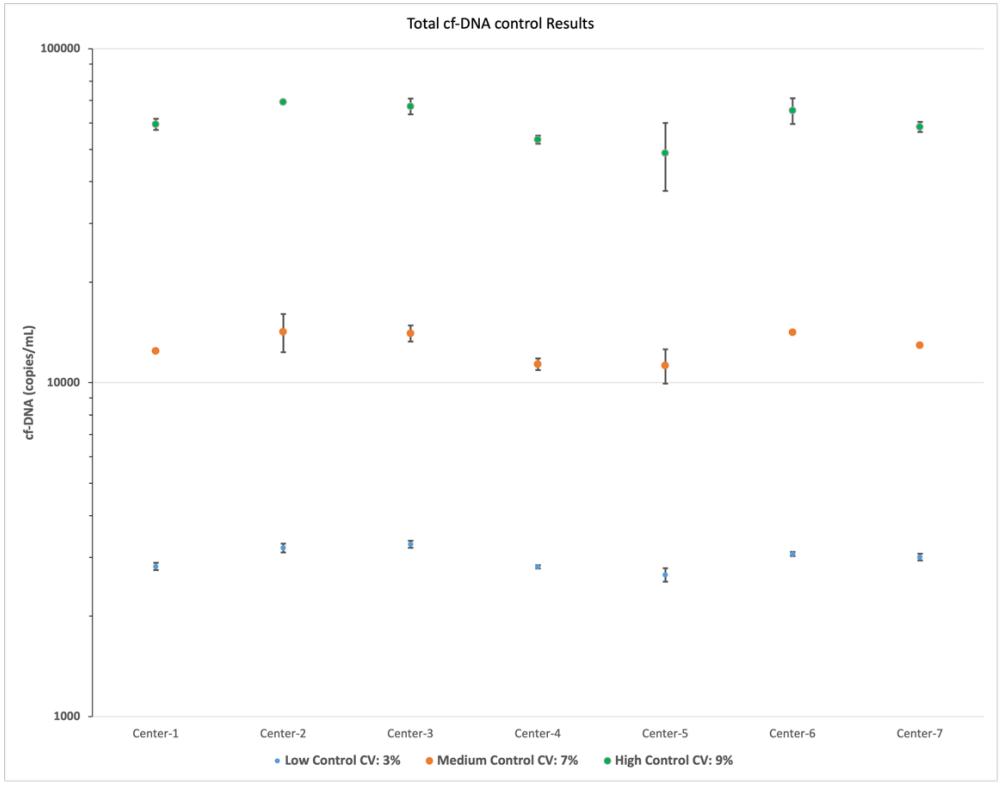

Figure 5. Total cf-DNA quantification for the three different amounts of copies/mL plasma used for proficiency testing

Conclusion:

This multicenter evaluation confirmed the manufacturer's reported performance of GraftAssureIQ, particularly its exceptionally low lower limit of quantification (LLoQ). The LLoQ is a critical determinant of test usability, and GraftAssureIQ outperforms first-generation dd-cfDNA assays, which typically rely on older NGS technologies. Published LLoQs for these assays range from 0.12% (9) to 0.23% (10, 11), three- to five-fold higher than GraftAssureIQ. The ability to reliably quantify dd-cfDNA below such thresholds provides important advantages for research applications, allowing investigators to explore biological variation and detect subtle changes that may be obscured by less sensitive methods. For instance, heart transplantation thresholds have been reported as low as 0.15%(11), which doesn't leave much room between the LLoQ and the diagnosis of rejection with the older high LLoQ methods. GraftAssureIQ therefore offers new opportunities in heart transplantation.

In addition, GraftAssureIQ demonstrated a lower reference change value of 41% at an average of 0.17%(8), compared with >60% (12, 13) reported for other approaches, demonstrating superior reproducibility and precision.

Demonstrated by this global multicenter evaluation, these results establish GraftAssureIQ as a sensitive, reproducible, and accessible assay. The technology outperforms older NGS methodologies, with its lower LLoQ and improved RCV. The droplet-digital PCR technology can provide same day results and is unincumbered by the risk of shipping delays since the testing is possible at transplant laboratory which is near to the patient.

Impact:

GraftAssureIQ has been validated in a multicenter evaluation to outperform older methods in terms of the lower limit of quantification by a factor of 3 or more and provides same-day dd-cfDNA results. Insight Molecular Diagnostics Inc. currently offers GraftAssureCore, a version of this assay available for clinical use, run at its CLIA-certified laboratory in Nashville, TN and covered by Medicare. This test is not approved or cleared by the FDA. Taken together, the data prove the GraftAssure technology to be also suitable in the longitudinal measurements of low dd-cfDNA amounts, where subtle upward trends below the clinical threshold for rejection may be seen and addressed early before full-blown rejection occurs.

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