Development and (Cross) Validation of a Triplex qPCR Assay for High Throughput Transgene Quantification, Normalization, and Monitoring Inhibition in Clinical Samples

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qPCR Key Applications for Transgene Detection in Cell Therapy

❑ Transgene Enumeration

- Quantification of the transgene in patient's (blood) samples before and after infusion at different time points

❑ Cellular Kinetics Monitoring

- Use the enumeration data to describe how the number of the transgene changes over time

❖ ***<u>The core requirements for accurate quantification</u>***

✓ Quantifying the copy number of the transgene (Gene of Interest (GOI))

✓ Quantifying the copy number of a reference gene (REF)

✓ Normalizing the GOI results with REF results

✓ Monitor potential PCR Inhibition using an inhibition control (IC) for results reliability

Investing in a Triplex: Benefits and Pitfalls

Benefits

- **Enhance Assay Performance**
  - ❖***Reduce Variability:*** Measure all target genes in the same well, eliminating inter-assay variation
  - ❖***Improved Accuracy:*** Simultaneous amplification under identical conditions enhances data reliability and consistency
- **Significant Efficiency Gains**
  - ❖***Higher Throughput and Streamlined Workflow*** with less processing time and fewer hands-on steps
  - ❖***Cost Reduction:*** Lower consumption of materials and reagents
- ***Reduce sample consumption***

Pitfalls

- **Complex Assay Design and Optimization**
  - ❖Designing of specific primers and probes without any cross reactivity, optimizing the annealing temperature and the concentrations of all primers and probes for three different targets simultaneously
  - ❖The presence of multiple primers and probes can affect the overall efficiency of the PCR reaction. A low concentration can lead to incomplete amplification, high concentration can cause inhibition
- **Potential for False Positives/Negatives**
  - ❖Inhibitory conditions or non-specific amplification can lead to inaccurate result

qPCR Evolution: Duplex to Triplex Transition

Current qPCR Bioanalysis Workflow

Extract genomic DNA from clinical samples, measure DNA concentration, dilute samples if needed

2X Duplex qPCR Assays (Parallel)

1. Gene of Interest /Inhibition Control Duplex qPCR

GOI IC

- Prepare Std and QCs in human genomic DNA
- Prepare Master Mix (primers and probes for GOI and IC, spike with IC plasmid to monitor inhibition)
- Add MM and samples to 96-well plate
- Run qPCR
- Calculate copy number based on GOI standard curve
- Assess inhibition to assure data reliability

2. Reference Gene /Inhibition Control Duplex qPCR

REF IC

- Prepare Std and QCs in rat genomic DNA
- Prepare Master Mix (primers and probes for REF and IC, spike with IC plasmid to monitor inhibition)
- Add MM and samples to 96-well plate
- Run qPCR
- Calculate copy number based on REF Std curve
- Assess inhibition to assure data reliability

Normalize results and report GOI as copy number/µg genomic DNA

qPCR Evolution: Duplex to Triplex Transition

Future qPCR Bioanalysis Workflow

Extract genomic DNA from clinical samples, measure DNA concentration, dilute samples if needed

1X Triplex qPCR Assay

Gene of Interest, Reference Gene and Inhibition Control Triplex qPCR

GOI **REF** **IC**

- Prepare standard curve and QCs contain both GOI and REF in rat genomic DNA
- Prepare one Master Mix (primers and probes for GOI, REF and IC, spike with IC plasmid to monitor inhibition)
- Add MM and samples to 96-well plate
- Run qPCR
- Calculate copy number based on Std curves
- Assess inhibition to assure data reliability

Normalize results and report GOI as copy number/µg genomic DNA

Assay Development

Development Strategy

1. In silico investigation

- identify hairpins, self- & heterodimers
- check the compatibility of the probe's fluorophores (no spectral overlap of dyes used)
- check for any cross reactivity with the matrix

✓ **No dimers**

✓ **No cross talk between probes**

✓ **No cross reactivity**

2. Wet Lab experimental optimization

- optimize Std curve and QCs preparation
- combine the two duplex methods as they are
- adjust and refine as needed

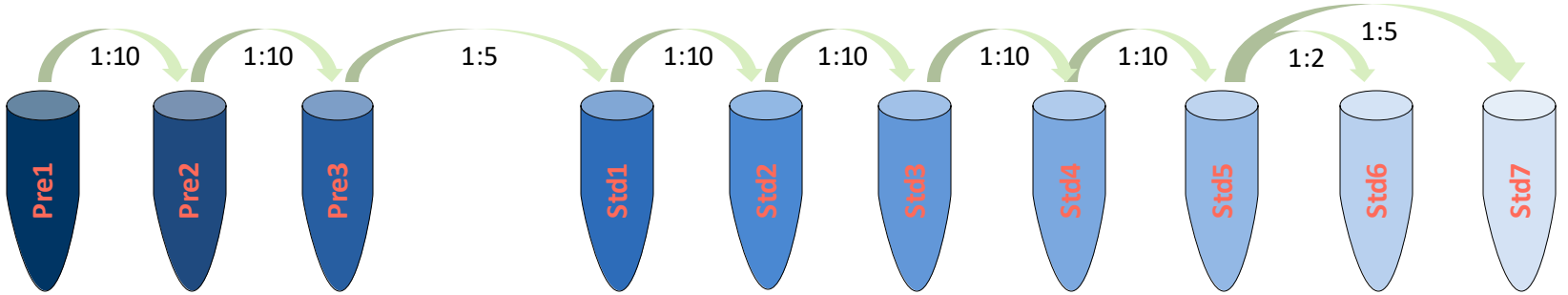
A journey in the wet Lab

Assay Development

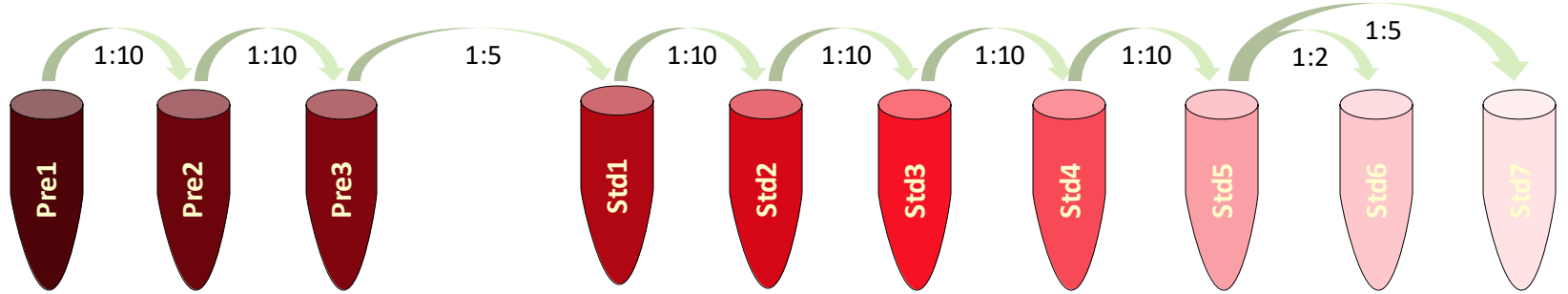
Standard Curve and QCs Preparation

Approach 1

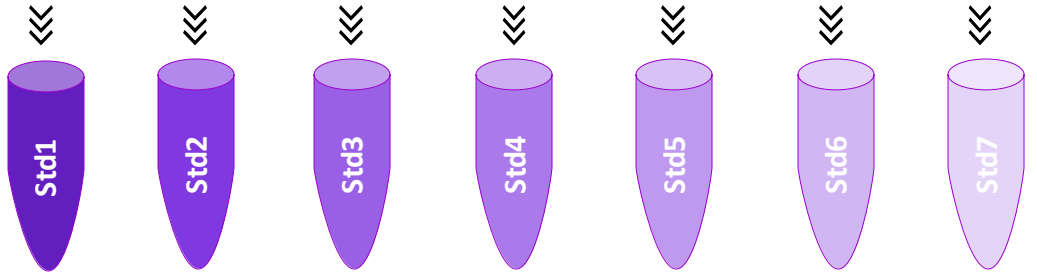
1. Prepare Std curve for REF



2. Prepare Std curve for GOI



3. Combine equal volume of opposite quantities



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Results

Standard Curve

GOI :

- passed for efficiency and R^2
- Same Ct and ΔRn as Duplex

REF:

- passed for efficiency and R^2 however higher efficiency
- higher Ct and same ΔRn as Duplex

QCs

GOI :

- all passed for %CV and %RE

REF:

- *partially passed*
- *most cases over recovery by ULOQ QC and LLOQ QC*

Acceptance Criteria:

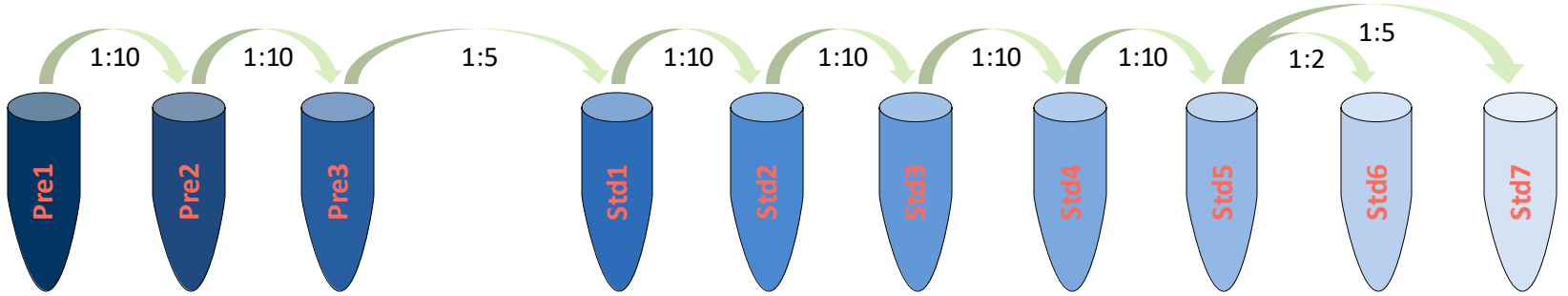
- *> 50 copies/ reaction: %CV and %RE ≤ 25.00%,*
- *≤ 50 copies/ reaction %CV and %RE ≤ 45.00%,*

Assay Development

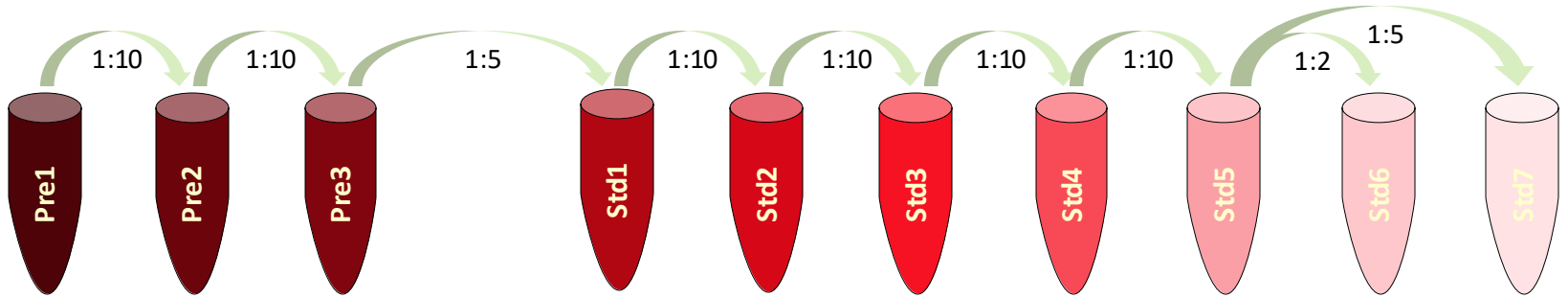
Standard Curve and QCs Preparation

Approach 2

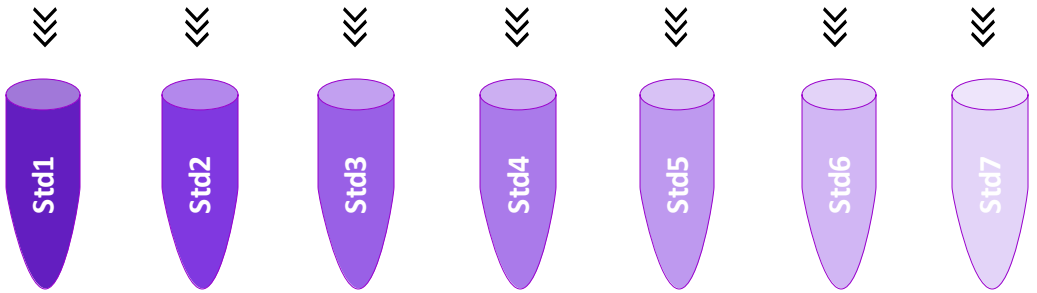
1. Prepare Std curve for REF



2. Prepare Std curve for GOI



3. Combine equal volume of equal quantities



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Results

Std curve results

- similar results to approach 1

QCs results

GOI :

- all passed for %CV and %RE

REF:

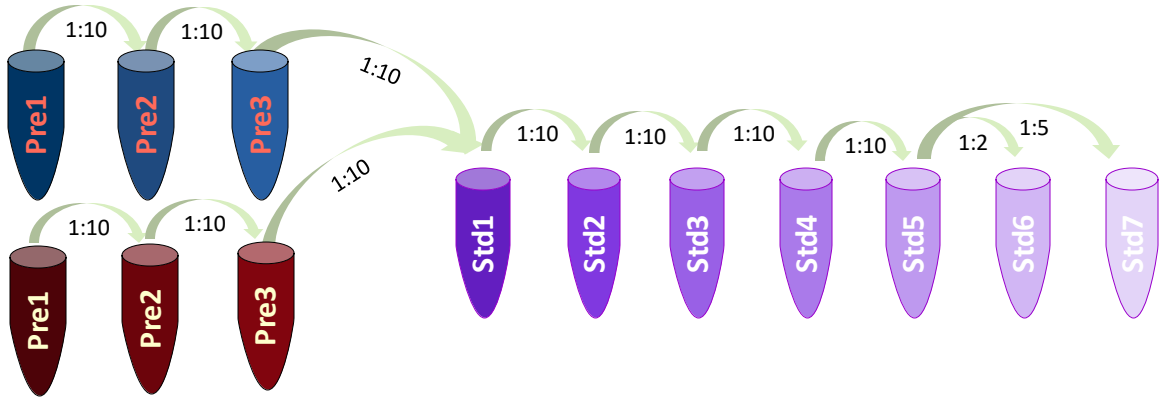
- *all passed for %CV and %RE*

Assay Development

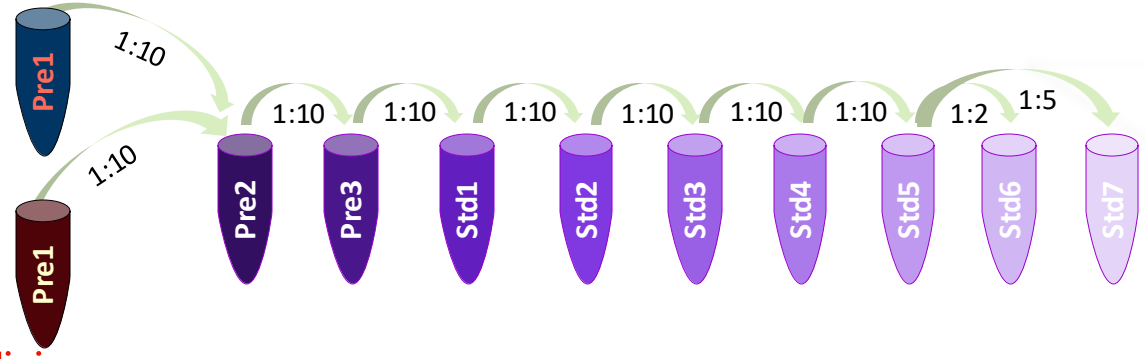
Standard Curve and QCs Preparation

Approach 3 and 4

- Prepare the pre dilutions of GOI and REF separately
- Prepare **ONE** Std curve for REF and GOI



- Prepare separate Pre1 for both the GOI and the REF
- Dilute the 2X Pre1 as 1:10 to prepare **ONE** Pre2 contains the GOI and the REF
- Dilute further to Pre3 till Std 7



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Results

- same results as approach 2
- more operative friendly
- less reagent consuming
- consider approach 4 as standard procedure

Assay Development

GOI LLOQ QC Sample Recovery Test

- **Procedure and results**

  Test the GOI LLOQ QC samples in the presence of different REF levels ULOQ -> LLOQ (REF +/- 200,000 copies/reaction) to mimic clinical samples with very low copy number

Sample name	Nominal Quantity of REF/reaction	Nominal Quantity of GOI/reaction	% Recovery of REF	% Recovery of GOI
Sample 1	1000000	20	Pass	Pass*
Sample 2	100000	20	Pass	Pass*
Sample 3	10000	20	Pass	Pass*
Sample 4	500	20	Pass	Pass
Sample 5	50	20	Pass	Pass
Sample 6	20	20	Pass	pass

- **Implemented solution**
  - ❑ Boost the GOI assay by increasing the primers concentration
  - ❑ Results were confirmed by different runs and different operators

Assay Development

Ct Values Comparison between Duplex and Triplex

	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7	
REF *(N Duplex= 113) (N Triplex= 35)*	-0.339	-0.325	-0.414	-0.324	-0.294	-0.16	-0.104	**< 1 Ct**
GOI *(N Duplex= 150) (N Triplex= 35)*	1.088	1.057	1.201	1.145	1.142	1.186	1.277	**< 1.5 Ct**

Ct mean difference (Duplex-Triplex)

Validation

Assay Validation

M10 as Inspiration for Partial and Cross Validation of Triplex qPCR

M10 BIOANALYTICAL METHOD VALIDATION AND STUDY SAMPLE ANALYSIS

Guidance for Industry

VI. PARTIAL AND CROSS VALIDATION (6)

A. Partial Validation (6.1)

Partial validations evaluate modifications to already fully validated bioanalytical methods. Partial validation can range from as little as one within-run accuracy and precision determination to a nearly full validation. If stability is established at one facility it does not necessarily need to be repeated at another facility.

For chromatographic methods, typical bioanalytical method modifications or changes that fall into this category include, but are not limited to, the following situations:

- Analytical site change using same method (i.e., bioanalytical method transfers between laboratories)
- A change in analytical method (e.g., change in detection systems, platform)

B. Cross Validation (6.2)

Cross validation is required to demonstrate how the reported data are related when multiple bioanalytical methods and/or multiple bioanalytical laboratories are involved.

Cross validation is required under the following situations:

- Data are obtained from different fully validated methods within a study.

Cross validation should be assessed by measuring the same set of QCs (low, medium, and high) at least in triplicate and study samples (if available) that span the study sample concentration range ($n \geq 30$) with both methods, or in both laboratories.

Bias can be assessed by Bland-Altman plots or Deming regression. Other methods appropriate for assessing agreement between two methods (e.g., concordance correlation coefficient) may be used too. Alternatively, the concentration vs. time curves for study samples could be plotted for samples analyzed by each method to assess bias.

Assay Validation

Partial Validation

Required Assay Parameters

❑ Std curve performance

- **Dynamic Range (Copies/ Reaction)**

	Duplex (N=113/150)	*Triplex (N=45)*
REF	10^6 - 20	10^6 - 20
GOI	10^6 - 20	10^6 - 20

Acceptance Criteria: %CV ≤ 2.00
Minimum of 6 points
2 out of 3 NTCs < LOD

- **Linearity (R^2)**

	Duplex (N=113/150)	*Triplex (N=45)*
REF	0.998 - 1.000	0.996 - 1.000
GOI	0.998 - 1.000	0.998 - 1.000

Acceptance_Criteria: ≥ 0.980

- **Efficiency (%)**

	Duplex (N=113/150)	*Triplex (N=45)*
REF	91.9-99.9	91.8-100.6
GOI	89.1-94.9	89.4-95.8

Acceptance Criteria: ≥ 85.0% and ≤ 110.0%

❑ Sensitivity

- **LLOQ (Copies/ µg genomic DNA)**

	Duplex (N=113/150)	*Triplex (N=45)*
REF	200	50
GOI	50	50

FDA recommendation: ≤ 50 copies for GOI

- **LOD (Copies/ Reaction)**

	Duplex N= 18 replicates /Dilution	*Triplex N= 18 replicates /Dilution*
REF	5	3
GOI	4	5

MIQE guidelines ≥ 3

Assay Validation

Partial Validation

Required Assay Parameters

❑ Accuracy and Precision (A&P) revealed as Total Error

	ULOQ QC	HQC	MQC	LQC	LLOQ QC
REF Triplex (N=18)	25.39	21.08	21.67	26.64	53.00
GOI Triplex (N=18)	17.06	15.45	10.24	16.10	40.83

Acceptance Criteria
LLOQ QC ≤ 70.00 %, All other QCs ≤ 40.00%

❑ Lowest gDNA revealed as %RE for back calculated results

	Dilution 1	*Dilution 2*
REF	-11.48	-18.81
GOI	-9.80	-9.84

Acceptance Criteria
%CV ≤ 25.00%
%RE within +/- 50.00%

❑ Stability

- **QC samples stability over time**
  (3, 6, 9, 12, 18, 24 months)
- **F/T Stability**
  3 cycles

❑ Robustness

- **Batch size**
  3 plates/day

Assay Validation

Cross Validation

Bland-Altman plot (% difference) & Deming regression (Triplex vs Duplex)

N=44 ***(24 Incurred Study Samples + 20 Spiked Matrix Control Samples)***

Bland Altman Percent Difference

The table of Bland-Altman percent differences is provided in Table 1.

Percent Difference	SD	Lower	Upper
-30.419	21.834	-73.212	12.375

Table 1: Percent Difference, Standard Deviation, Lower and Upper 95% Confidence intervals are presented.

The results in Figure 1 provide the Bland-Altman percent difference against average.

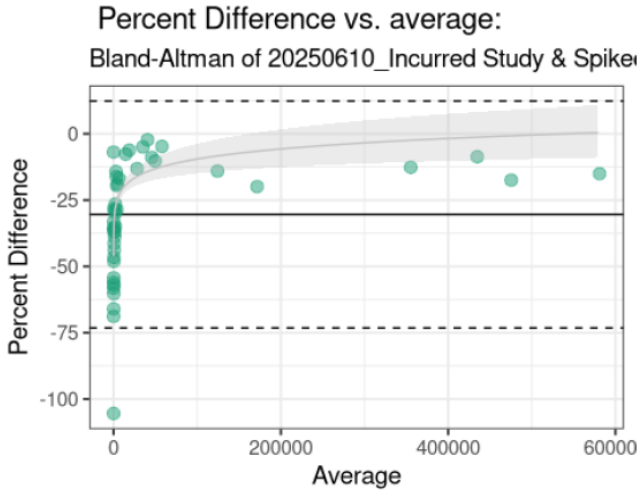
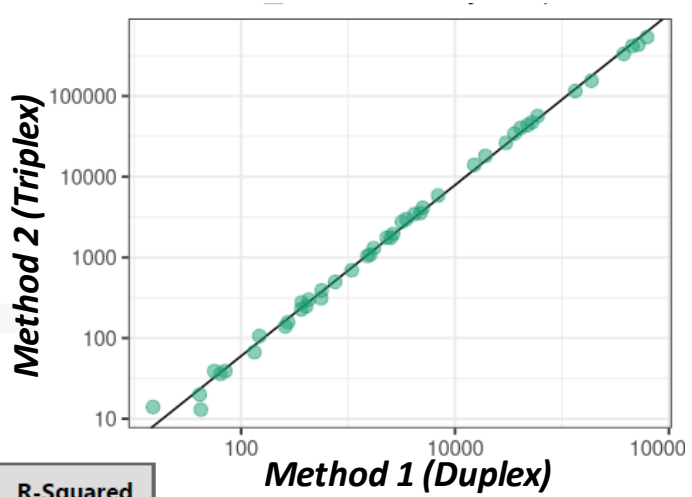


Figure 1: The figure provides the Bland-Altman percent difference. The solid line is the average ratio. The dashed lines from top to bottom are the respective upper 95% confidence limit, the unit line, and the lower 95% confidence limit.

Deming Regression Results on Log Scale

The Deming fit is given by:

```
[1] "log10(method 2) = -0.34 + 1.059 * log10(method 1)"
```



Deming Regression Log Scale Tables

The Deming model output is given in Table 2.

Intercept	Slope	X-Intercept	1/Slope	Concordance Cor.	R-Squared
-0.34	1.059	0.321	0.944	0.991	0.998

Table 2: Model output for Deming Regression is provided. The concordance correlation and the R squared statistic are also provided.

The coefficient bootstrapped confidence intervals are provided in Table 3.

Name	Estimate	Lower	Upper
Intercept	-0.340	-0.443	-0.238
Slope	1.059	1.035	1.086

Table 3: The 95% bootstrapped confidence intervals for the intercept and slope coefficients.

Conclusion and Future Perspectives

- **The triplex assay demonstrates strong performance without compromising quality**
- **Is user friendly, time and cost reducing**
  - ***3X plates (3x26=78 Results x 2 Assays= 158 results)/ Operator/Day***
- **Bland-Altman Plot Analysis and Deming Regression provide a clear visualization of agreement and potential bias between the two methods and can support confident decision-making**
- **Triplex design will be prioritized in the development of future qPCR assays**

Acknowledgement

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