

# Method Development and Validation of an Alternative Immunoassay Platform for Pharmacokinetic Studies in a Regulated Environment

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## ABSTRACT

**Purpose:** The need for a sensitive immunoassay to support clinical pharmacokinetic studies presented an opportunity to integrate the Erenna® platform in a regulated environment. Here, we describe how we addressed reagent availability, consistency and storage of reagents, performance specifications, sample preparation, automation of liquid handling steps, through-put, data analysis and assay validation.

**Methods:** A sensitive ligand binding assay for the quantification of a therapeutic protein in human plasma was developed at Singulex then transferred and validated at Pfizer. During method development, requirements related to implementation of a new technology in a regulated environment were addressed.

### Results:

- 1) Screening experiments for selection of the optimal capture and detection reagents were performed. To ensure consistent lot-to-lot performance of the kits, Pfizer in-house reagents were selected as commercial reagents were not consistent from lot-to-lot. Acceptance specifications including LLoQ, ULoQ, intra and inter-assay precision were met. Stability of the bulk labeled kit reagents were also evaluated by storage at -80°C and 4°C.
  - 2) An alternative sample preparation step was required when filtration of quality control (QC) samples prior to testing in the assay indicated poor (50%) recovery. Unfiltered QC samples had acceptable recovery (80-120%). In addition, a minimum required dilution in clarified pooled plasma was performed for all samples to minimize matrix effects observed in individual donors.
  - 3) Assay throughput was increased by running samples in duplicate, instead of triplicate, and automating several liquid handling steps which included washing of microparticles, sample transfer and addition of buffers.
  - 4) A custom tool to interface Erenna system output was developed and validated by Pfizer. This tool helped with aspects of Part 11 compliance and integration with Watson LIMS for data analysis, reporting and storage.
- Conclusion:** Several activities related to running assays in a regulated environment were addressed when this technology was implemented. A strong partnership between the vendor and Pfizer was essential for successful implementation. A sensitive clinical assay with a lower limit of quantification (LLOQ) of 32 pg/mL was successfully validated and is being used currently in Pfizer non-clinical and clinical programs.

## Method Development

Initial feasibility studies including reagent selection and assay optimization were conducted by Singulex (Alameda, CA). Several reagents were evaluated for their ability to quantify the therapeutic protein in human plasma. Reagents were labeled and screened as either capture or detection reagents. To ensure consistent lot-to-lot performance of the kits, Pfizer in-house reagents were selected. The assay format is illustrated in Figure 1 below. Assay acceptance results are summarized in Table 1. Stability of kit reagents is summarized in Graph 1.

Figure 1: Schematic of the Ligand Binding Assay and Critical Reagents

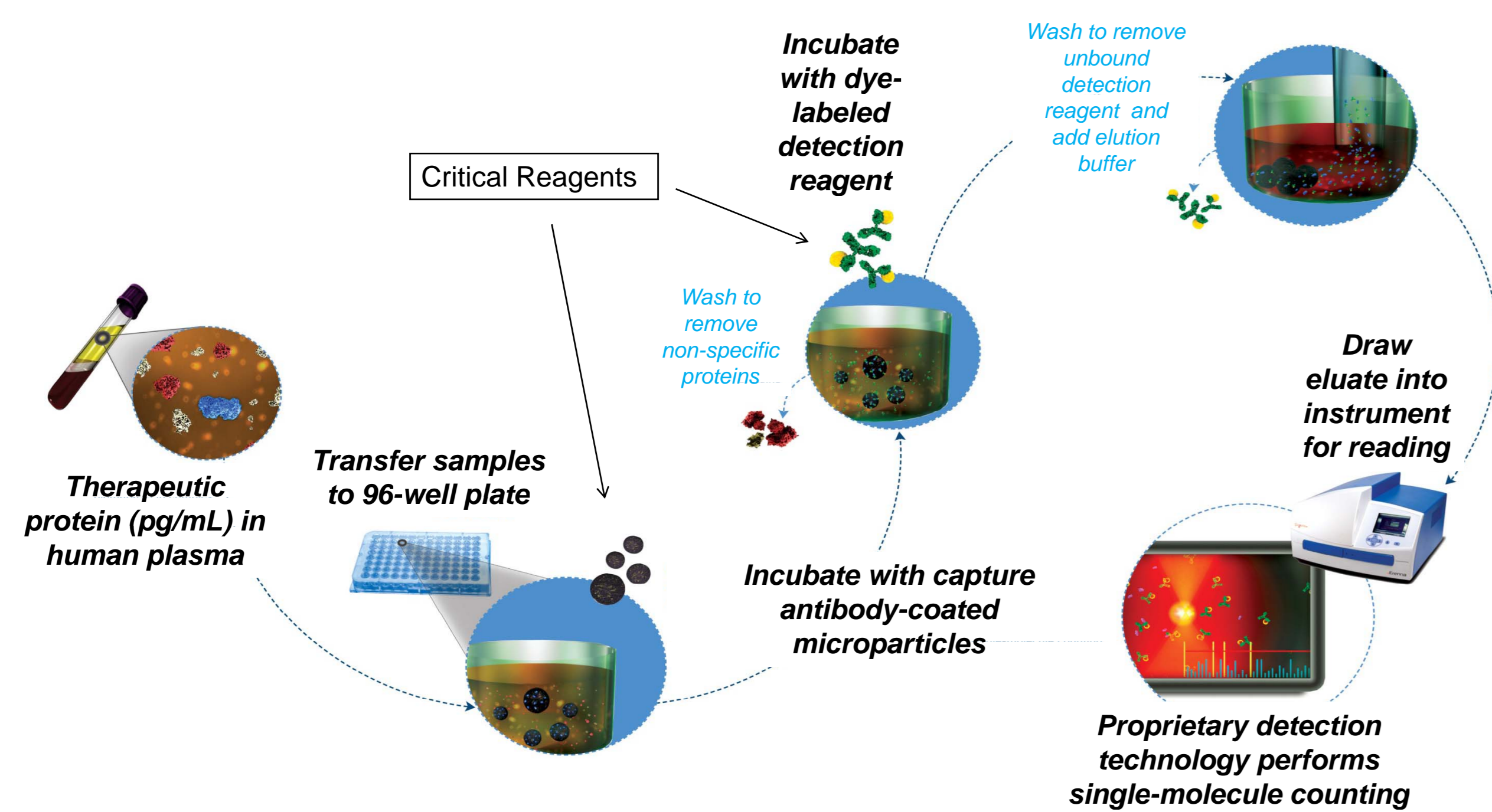


Table 1: Singulex Assay Feasibility and Acceptance Results

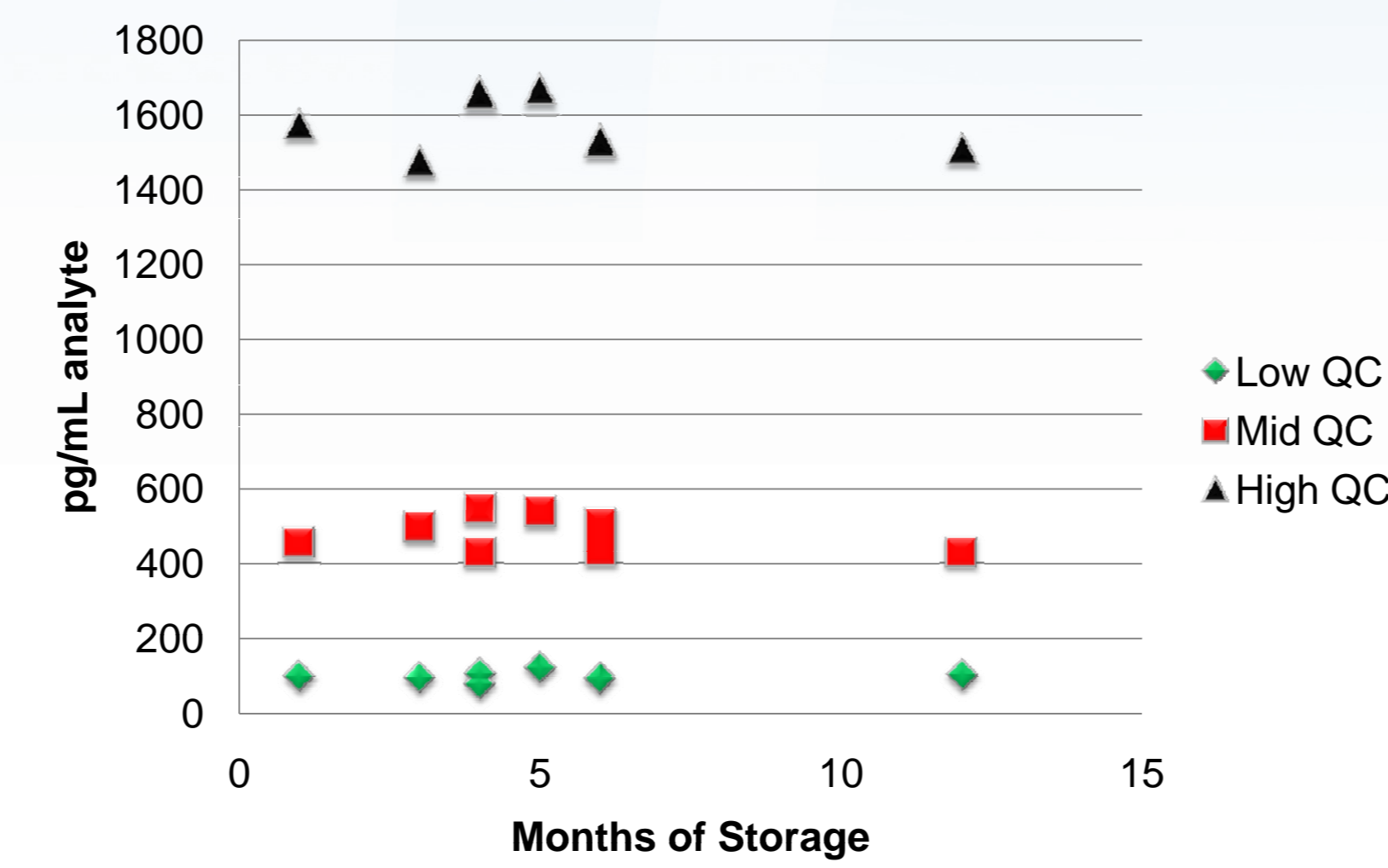
Parameter Evaluated	Results	Notes
Lower Limit of Quantification	< 20 pg/mL*	Estimated LLoQ was 7.8 pg/mL based on lowest point on the standard that back interpolates with a bias and CV < 20%
Upper Limit of Quantification	> 400 pg/mL	Estimated ULoQ was 1000 pg/mL based on lowest point on the standard that back interpolates with a bias and CV < 20%
Inter-assay precision	< 20% CV	Coefficient of Variation (CV) for replicate spikes on one plate over 3 days
Intra-assay precision	< 20% CV	Coefficient of Variation (CV) for replicate spikes on 3 plates over 1 day

\* Additional experiments were performed at Pfizer to determine the actual LLoQ (32 pg/mL) by spiking the therapeutic protein in individual donor plasma.



Graph 1: Long Term Stability Data for Assay Kit Lots.

Long term storage of kits stored at 4°C and detector reagent stored at -80°C was assessed by periodically analyzing low, mid and high QC samples. The long term stability data up to 12 months is displayed in Graph 1. The biases of the QCs were in the acceptable range for all lots of kits tested.



## Optimization

The method was optimized by Singulex and Pfizer. An alternative sample preparation step was required when filtration of quality control (QC) samples prior to testing in the assay indicated poor recovery (Table 2). On day 1, pooled plasma was spun and filtered prior to spiking with analyte. On day 2, pooled plasma was neither spun nor filtered prior to spiking with analyte. On each day, after spiking, samples were either filtered or not filtered prior to diluting 1:2 in pooled plasma and testing in the assay.

Table 2: Sample Preparation Determined by Spike and Recovery Experiments

Analyte Conc. (pg/mL)	Recovery of Analyte in Pooled Plasma			
	Day 1		Day 2	
	Not filtered	Filtered	Not filtered	Filtered
1000	89.4 %	48.4 %	114 %	68.9 %
500	101 %	51.0 %	113 %	69.6 %
62.5	79.7 %	48.1 %	87.6 %	ND
32.3	124 %	42.0 %	81.7 %	61.9 %

ND= Not Determined

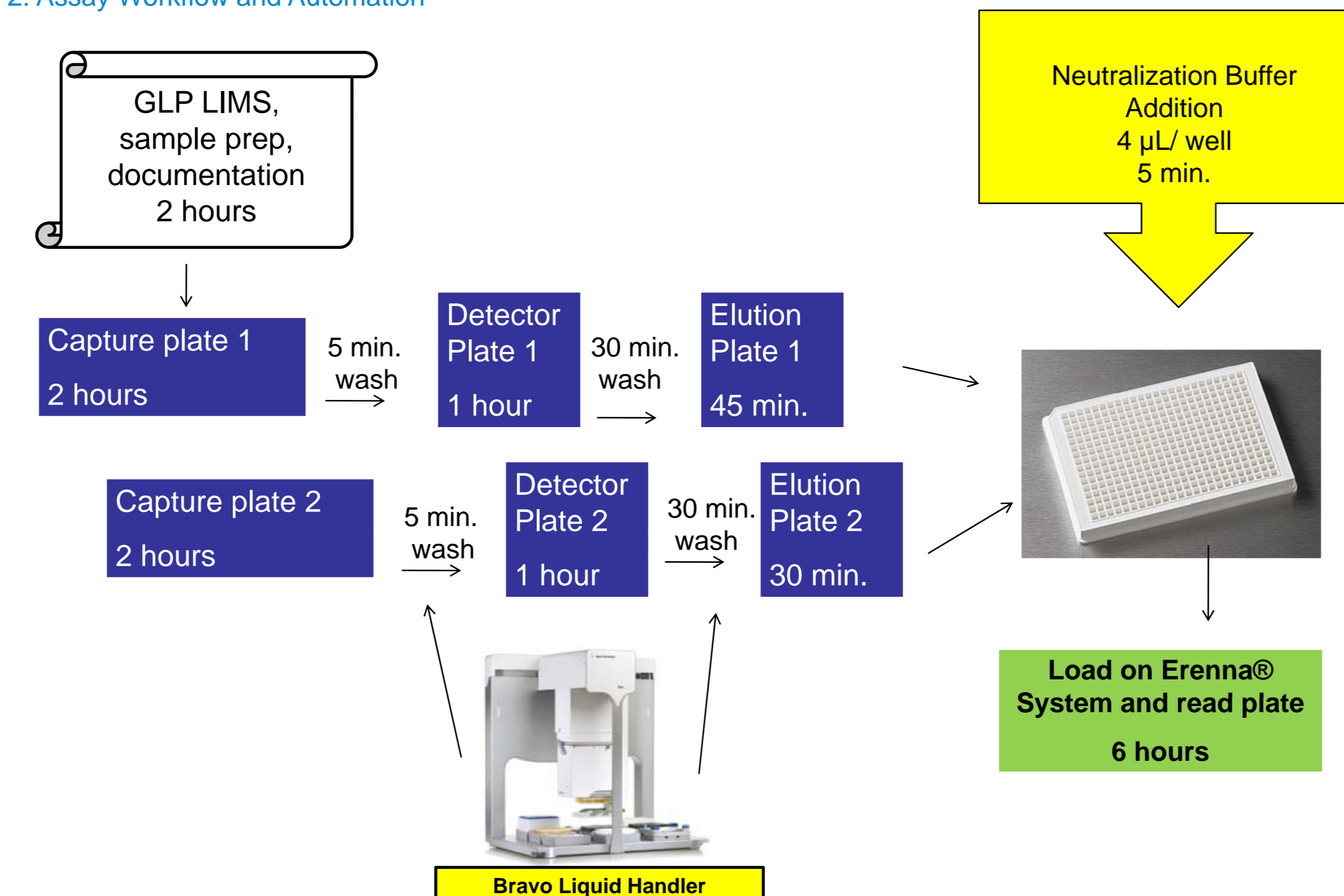
% Recovery was acceptable when spiked QC samples were not filtered prior to testing. Additional spike and recovery experiments were performed in individual donors. A 1:4 minimum required dilution in clarified plasma (supernatant from centrifuged pooled plasma) was performed on all samples to minimize matrix interference.

## Assay Throughput

Several liquid handling steps were automated with Agilent Bravo Liquid Handler as illustrated in Figure 2. These steps include sample transfer, addition of buffers, and washing of microparticles. With automation of these steps, time is now available in the workflow for real-time documentation of experiments which is required in a regulated environment.

To improve assay precision and throughput, an additional liquid handling step, the addition of 4 µL/well of buffer to each quadrant of a 384 well plate, was automated at Pfizer. Prior to implementing the automated step, samples were tested in triplicate and CVs were <30%. With automation, samples could be run in duplicate with CVs <20%. The liquid handling step is highlighted in yellow in Figure 2.

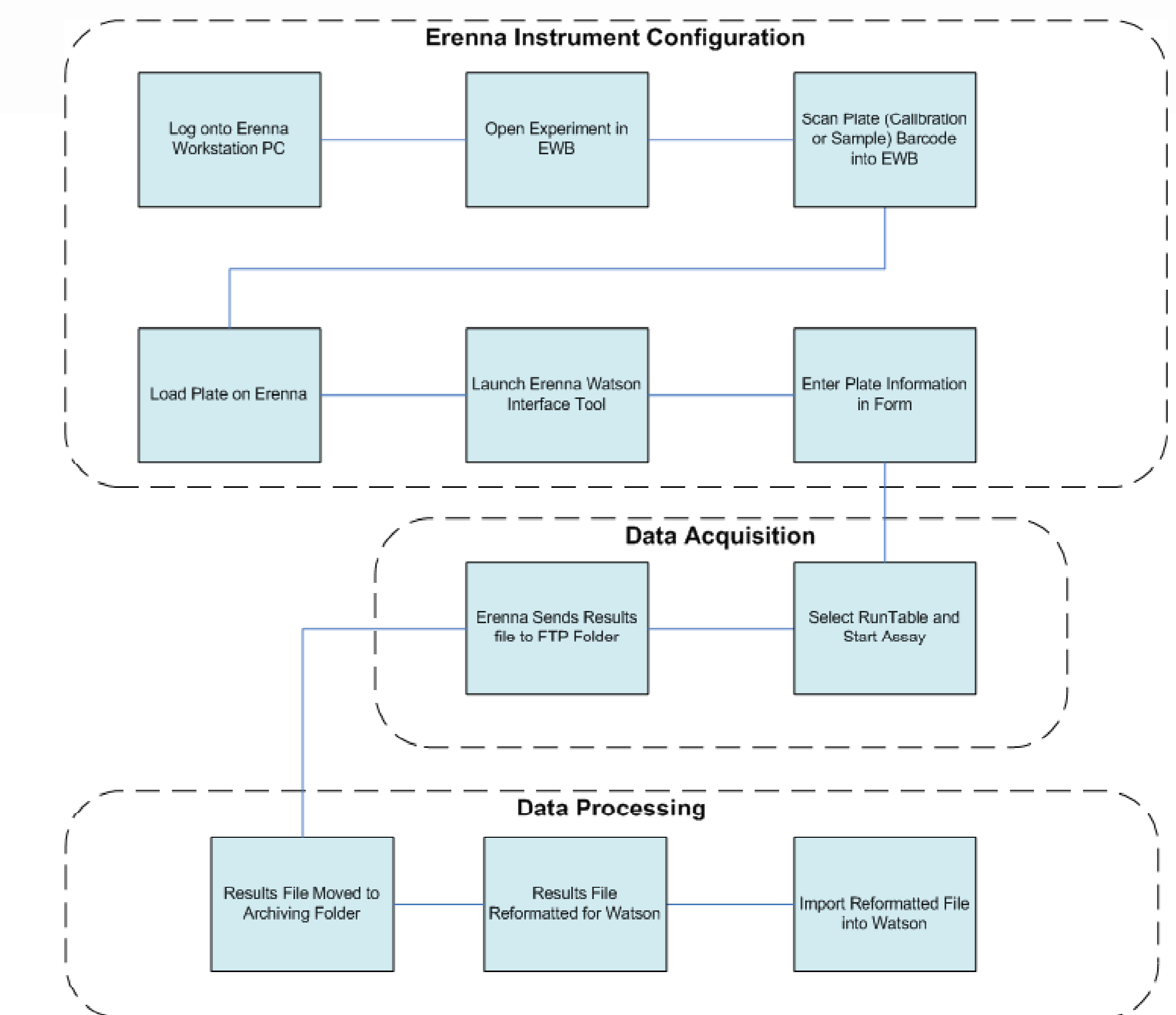
Figure 2: Assay Workflow and Automation



## Custom Interface for Data Analysis

A custom tool to interface Erenna system output was developed and validated by Pfizer. This tool was written in Visual Basic .NET (VB.NET) and was deployed on the dedicated instrument PC. It provides Part 11 compliance, integration with Watson LIMS for data analysis, reporting and storage, and facilitates archival of the raw data. The workflow is illustrated in Figure 3.

Figure 3: Erenna Immunoassay System Use Workflow Process Diagram



## Assay and System Validation

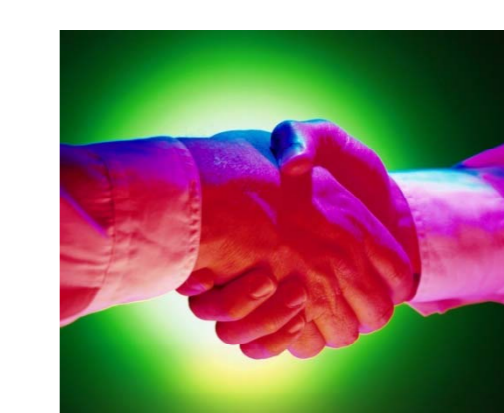
The assay is currently used to support Pfizer non-clinical and clinical studies. The assay was validated at Pfizer and has a range of quantification from 32 pg/mL to 2000 pg/mL in 100% plasma. Accuracy and inter-assay precision data is summarized in Table 3. Each concentration was tested in replicates of 5 over 3 days. The custom interface tool for data analysis was validated and approved for regulated use.

Table 3: Accuracy and Inter-assay Precision

Analyte Concentration	32 pg/mL (LLOQ)	98.0 pg/mL	480 pg/mL	1500 pg/mL	2000 pg/mL (ULoQ)
Mean Concentration (pg/mL)	27.7	108	504	1592	1963
SD	2.19	3.61	11.1	102	105
%CV	7.9	3.3	2.2	6.4	5.3
%Bias	-15.3	-10.0	4.9	6.2	-1.8

## Conclusions

A strong partnership between Singulex and Pfizer was essential for successful implementation of a new technology in a regulated environment. To meet the challenges of validating the Erenna in a GLP environment, issues regarding throughput, sample preparation, reagent stability, precision, data analysis and part 11 compliance were addressed. Innovative solutions were successfully employed by the joint Pfizer/Singulex team to accomplish the goal of validating this instrument to support regulated clinical and non-clinical studies.



## ACKNOWLEDGEMENTS

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