Development and Validation of an Enzyme-linked Immunosorbent Assay (ELISA) for the quantification of Trastuzumab in human serum to support Pharmacokinetics in Clinical Trial.

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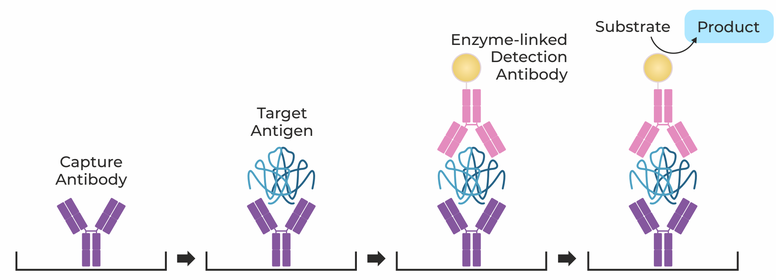
INTRODUCTION

- Trastuzumab (brand name, Herceptin) is a humanized monoclonal antibody used as immune therapeutics for early and metastatic breast cancer and gastric/gastroesophageal cancer.
- Trastuzumab selectively targets the extracellular domain of the human epidermal growth factor receptor 2 protein (HER2) and prevents the growth of cancer cells.
- Trastuzumab is composed of 1,328 amino acids and has a molecular weight of ~148 kDa with human framework constant regions and complementarily-determining regions of a murine anti-p185 HER2 antibody that binds to HER2.
- An ELISA method was developed for quantitation of Trastuzumab in normal human serum to support the Pharmacokinetics study of Phase-I clinical trials.

ASSAY SUMMARY

Trastuzumab ELISA is based on the quantitative sandwich enzyme immunoassay technique. A commercially available anti idiotypic antibody specific for Trastuzumab are coated onto a microplate. Standards and samples are pipetted into the wells and Trastuzumab present is bound by the immobilized antibody. After washing away any unbound substances, polyclonal HRP-conjugated antibody is added to the wells as a detection antibody. Following a washing step to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells and the color developed is proportional to the amount of Trastuzumab bound to the immobilized antibody. The color development is stopped and the intensity of the color is measured.



METHOD DEVELOPMENT

- Selection of Coating and detection reagent, coating buffer, diluents and calculating required sensitivity and design of the assay
- Capture and Detection concentration optimization
- MRD optimization
- Optimization of diluent compositions and washing steps
- Calibration Curve Range
- Quality Control Samples
- Regression Type of the Calibration Curve and Weighting
- Optimization of incubation time and temperature

PRE-VALIDATION EVALUATIONS

- Selectivity in Haemolysed, Lipemic and different lots of Drug Naïve Serum at HQC and LLOQ levels
- Specificity in presence of anticipated concomitant and coadministered drug at ULOQ and LLOQ level
- Precision Accuracy at ULOQ, HQC, MQC LQC LLOQ levels for 03 batches
- Bench Top Stability at Room Temperature for 20 hours
- Freeze Thaw Stability for 03 cycles
- Blood Stability for 02 Hours
- Dilution Linearity and Prozone Hook effect

METHOD DEVELOPMENT SUMMARY

Assay Format	:	Quantitative Sandwich Immunoassay
Capture Antibody	:	Human Anti-Trastuzumab (Idiotypic)
Detection Antibody	:	Peroxidase conjugated anti Trastuzumab
Matrix	:	Human Serum
Sensitivity	:	312.5 ng/mL
Regression type	:	5 Parameter Logistic (1/X2 weighing)

METHOD VALIDATION

PRECISION & ACCURACY

Precision: Precision is the closeness of agreement (i.e., degree of scatter) among a series of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions.
Accuracy: Accuracy is the degree of closeness of the determined value to the nominal or known true value under prescribed conditions. Accuracy is also sometimes termed trueness.

Precision Accuracy experiment was performed by two different analyst on several days. Interrun and Intrarun %CV and %Bias evaluated from QC Results.

CALIBRATION GRAPH

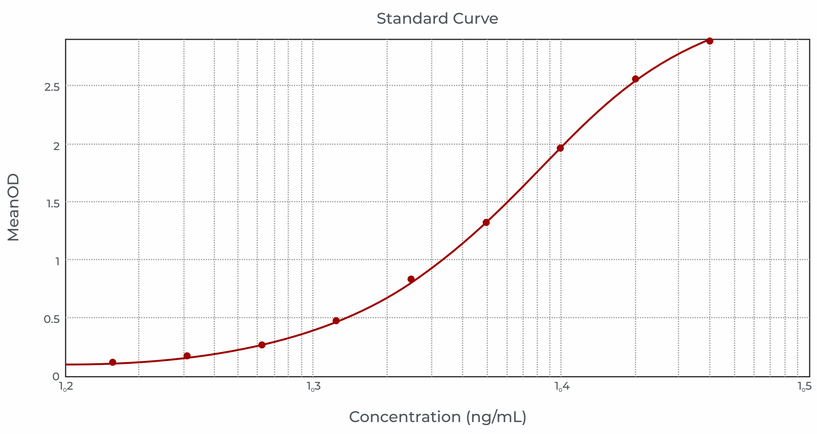


Fig 1: Representative 5 Parameter Logistic Fit calibration curve of Trastuzumab [Concentration Vs Mean OD].

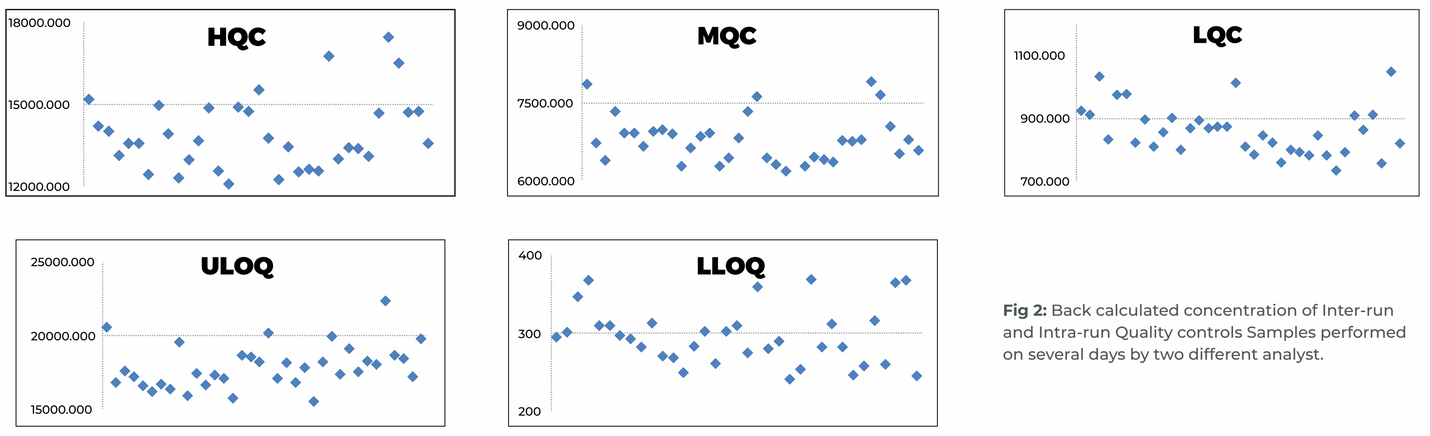


Fig 2: Back calculated concentration of Inter-run and Intra-run Quality controls Samples performed on several days by two different analyst.

Intra-run Quality Control Samples for %CV are within 3.1 to 15.2 and % Bias are within -14.5 to 4.7.
Inter-run Quality Control Samples for %CV are within 6.9 to 13.8 and %Bias are within -10.6 to -4.3.

SELECTIVITY

Selectivity experiment was performed to measure the analyte of interest in the presence of unrelated compounds in the matrix in which it interfere with the analyte of interest.
Evaluated Selectivity using 10 different lots of Human Serum including Lipemic and Haemolysed Serum at HQC and LLOQ level.

Serum	S-1	S-2	S-3	S-4	S-5	S-6	S-7	S-8	S-9	S-10
HQC %Bias	-7.1	-5.7	-14.8	-9.7	-6.7	-8.3	-15.1	-7.3	4.6	-14.0
LLOQ %Bias	-16.2	-11.1	-15.2	1.2	-1.9	-14.2	-17.2	-8.0	5.3	0.1

HQC Samples for %CV are within 0.5 to 7.0 and % Bias are within -15.1 to 4.6.
LLOQ Samples for %CV are within 0.1 to 14.3 and %Bias are within -17.2 to 5.3.

SPECIFICITY

Specificity experiment was performed to measure analyte of interest in the presence of anticipated concomitant, coadministerd and physio-chemically similar drugs.
Evaluating Specificity at ULOQ and LLOQ level in the presence of physio-chemically similar compounds and anticipated concomitant medication.

Drugs	Acetaminophen, Diphenhydramide Adalimumab, HER2 (ERbB2)
ULOQ (ng/mL) (%Bias)	-0.5
LLOQ (ng/mL) (%Bias)	-6.9

There is no interference of anticipated concomitant medications and physico chemically similar compounds.

DILUTION LINEARITY

Dilution Linearity experiment was performed to measure serially diluted Spiked Concentration Samples higher than the Calibration Range.
Evaluating Dilution Linearity by Diluting DQC Sample with Drug naive Serum for Dilution factor 25, 50, 100, 200 and 400 fold.

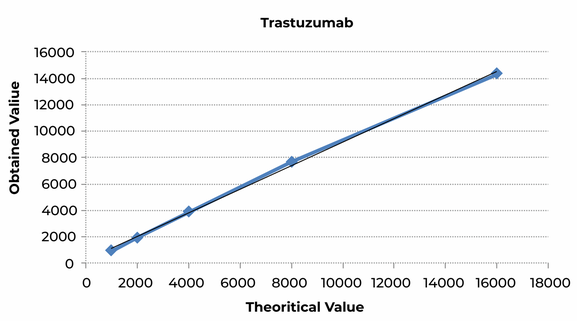


Fig 3: Dilution Linearity established up to 400 fold dilution.

PROZONE HOOK EFFECT (PHE)

Prozone Hook Effect is identified as increasing analyte concentration results in no change or decreased signals compared to preceding concentration.
Evaluating Prozone Hook Effect for two different high concentration (PHE2 & PHE1) sample above the calibration standards.

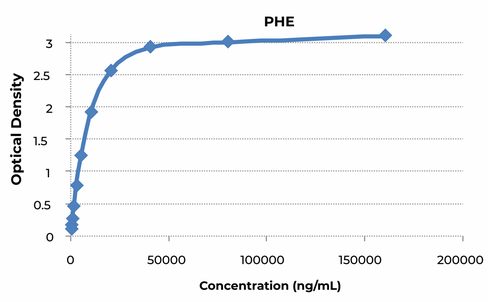


Fig 4: OD Value of PHE2 Sample ≥ OD Value of PHE1 Sample ≥ OD value of Highest Calibration Standards.

No prozone Hook Effect Observed.

STABILITY

Stability experiment was performed to measure of the intactness an analyte (lack of degradation) in a matrix under specific storage conditions.

Evaluating Stability at HQC and LQC Evaluating stability at ULOQ and LLOQ level in the presence of anticipated for reconstituted reference drug and concomitant medication at expected working solution.
high concentration.

QC ID	Blood Stability	Bench Top	Freeze Thaw (-20±7°C)	Freeze Thaw (-70±10°C)	Long Term (-20±7°C)	Long Term (-70±10°C)
HQC (%Bias)	-6.2	3.8	-5.3	-8.8	8.6	4.9
LQC (%Bias)	-2.3	-1.3	-5.4	-4.7	-2.5	-10.0

QC ID	Working Solution Stability (Room Temperature)	Reconstituted Reference Drug stability (2-8°C)
ULOQ (%Bias)	10.1	-13.6
LLOQ (%Bias)	6.9	NA

- **Blood Stability – 02 Hours**
- **Bench Top Stability at Room Temperature – 20 Hours**
- **Freeze and Thaw Stability (-20±7°C & -70±10°C) – Cycle 6**
- **Long Term Freezer Stability (-20±7°C & -70±10°C) – 124 Days**
- **Working Solution Stability at Room Temperature – 18 Hours**
- **Reconstituted Reference Drug Stability – 14 days**

COMPARABILITY

Comparability experiment was performed to measure both the innovator and the biosimilar drug similarity against Bioanalytical method.

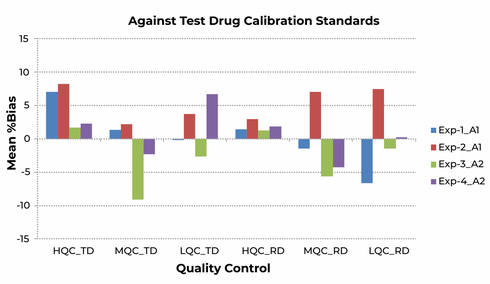


Fig 5: Comparison of Mean %Bias of two different analyst using Test Drug Calibration Standards on several days

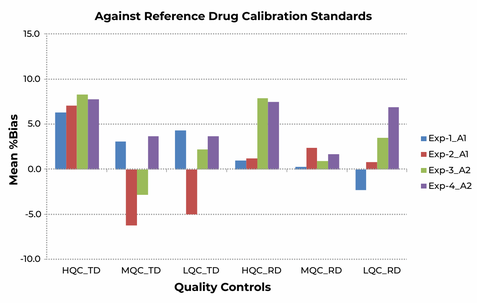


Fig 6: Comparison of Mean %Bias of two different analyst using Reference Drug Calibration Standards on several days

Test Drug and Reference Drug are comparable.

ROBUSTNESS

Robustness Experiment was performed to measure consistency of the method when certain changes occurred
Evaluating Robustness at HQC, MQC and LQC levels by changing condition of the assay.

QC ID (ng/ml)	Blocking incubation	Sample incubation	Peroxidase stability	Without plate shaker	Manual washing	Incubation temperature
HQC (%Bias)	-14.1	3.1	-8.8	6.6	-8.0	-17.4
MQC (%Bias)	-12.3	3.8	-0.6	-11.1	-15.0	-10.3
LQC (%Bias)	-7.4	8.5	-16.2	-12.0	-8.8	-3.4

- **Extended Blocking Incubation and Sample Incubation Time**
- **Manual washing instead of Automated Washer**
- **Assay at static conditions**
- **Different in Incubation temperature**
- **Stability of working HRP at room temperature**

CONCLUSION: A robust in house ELISA method has been developed in normal human serum. This method is validated as accurate, precise, stable, selective and robust.