GLP Validation of a Gyrolab® Assay for the Determination of Interleukin 8 (IL-8) Levels in Cynomolgus Monkey Serum

Theodore Baginski, Mark Abrams, Caroline Green, and John Kamerud
Eurofins Pharma Bioanalytical Services US Inc, St Charles, MO
*corresponding author: johnkamerud@eurofins.com

Introduction

Inflammatory cells are thought to be instrumental in the pathophysiology of diseases and the control of their recruitment and activation appears to be an attractive strategy for therapeutic intervention. Chemokines are a family of small molecular weight (7–15 kDa) proteins that in conjunction with adhesion molecules play a crucial role in leukocyte recruitment, cellular activation and proliferation at sites of inflammation. Chemokines are produced by a variety of cell types, including leukocytic and non-leukocytic cells, usually in response to antigens, irritants and other cytokines (White *et al.,* 2007).

A diverse variety of biological effects are attributed to CXCL8 chemokines, including several involving inflammatory cell activation and chemotaxis, production of reactive oxygen species, increased expression of the integrin CD11b-CD18, enhancement of cell adhesion to endothelial cells, promotion of angiogenesis, modulation of histamine and lipid mediator release as well as azurophil granule release.

Interleukin-8 (IL-8) is a molecule produced by many cell types. IL-8 has a potent neutrophilic chemoattractant activity, having other functions as well, including enhancement of angiogenesis and modulation of the expression of adhesion and MHC molecules. IL-8 also acts as a chemoattractant for T lymphocytes, basophiles, NK cells, and melanocytes (Alvarez *et al.,* 1996).

Circulating cytokine levels can provide valuable information about immune status, but often require high-sensitivity assays. Here we describe a GLP validation of a Gyrolab® microfluidics method for the determination of IL8 in Cynomolgus monkey serum.

The Gyrolab microfluidics platform is idealy suited for the determination of a variety of chemokines including IL-8, see **figure 1**. The standard biotin- streptavidin surface chemistry of the microstructures within each Bioaffy® CD enables the interogation of a variety of antibody pairs in a rapid and efficient manner, see **figure 3a and 3b**. The chemistry required for Alexa® - 647 labeling of the detection antibody is well established and available to use as a commercial kit.

Materials, Methods

Capture and Detection Antibody Conjugations

The capture antibody is prepared by biotinylating purified mouse IgG1 anti-IL8 antibody, (R&D Systems cat# MAB208) using the EZ-Link Sulfo-NHS-LC-Biotin labeling kit (Thermo Scientific cat#21327). The labeling reaction is designed to accommodate a 12 -1 challenge ratio. Resultant conjugated product is quantitated on a Nanodrop® system using the IgG instrument configuration. Final product is diluted 1:1 with AB-P assay buffer, aliquots were made, and stored at -70˚ C.

The detection antibody is supplied by R&D Systems as a purified unlabeled goat anti-IL8 IgG polyclonal antibody (cat. #AF-208-NA) and is labeled with Alexa Fluor® -647 using a commercially available kit from Molecular Probes (Alexa Fluor® 647 Monoclonal Antibody Labeling Kit, Catalog # A-20186). The product concentration is determined using the Nanodrop® system and the degree of Alexa Fluor® 647 labeling is calculated using kit-provided equation (a dye incorporation ratio of 2-8 is considered within normal range).

Determination of IL-8 in Cynomolgus Monkey Serum

Following the conjugations of the capture and detection antibodies optimal working concentration of the detection antibody is determined by "checkerboarding" detection antibody concentrations against a 100 µg/mL capture antibody (recombinant IL8 , R&D Systems #208-IL, standard curves). A Gyrolab Bioaffy® 1000 CD (analyte determinations in the picomolar range) was selected based upon the anticipated levels of analyte in serum , see **figure 2**. Experimental parameters were inputted into the Gyrolab® "Client" software and a Gyrolab® loader list containing plate positions and volumes of capture antibody, detect antibody, IL8 standards, and unknowns was generated. A PCR micro-plate was loaded with the required components according to the generated "Loading List". Primary (1% PBST) and secondary (20% EtOH+0.5% SDS) wash stations were connected/confirmed and the assay run initiated.

GLP Validation Parameters

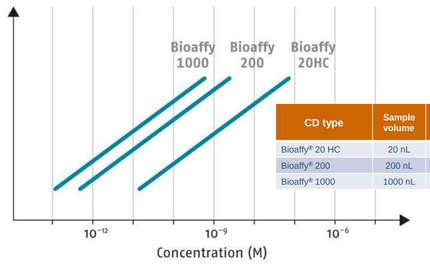
The assay validation parameters examined were primarily defined following a "Fit for Purpose" Method Validation (Lee *et al.,* 2006) see **figure 4**.

Figure 1: Gyrolab® Reader Instrument

- Nanoliter-scale, CD-based flow-through technology
  - High accuracy and precision
  - Broad dynamic range
  - nL sample / low reagent use
- Automated assay workflow
  - Includes pipeting, incubation, washing, detection & analysis
- High sensitivity laser induced fluorescence (LIF) detection
- Speed and throughput
  - < 1 hour per CD
  - Up to 5 CDs/batch (in our hands -3 CDs/batch)
- Control and analysis software

Images courtesy of Gyros AB

Figure 2:Gyrolab Bioaffy® CD selection

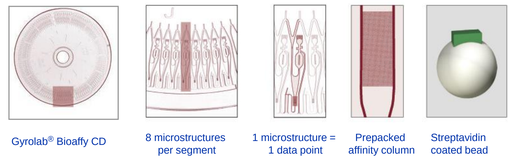


CD type	Sample volume	Typical LLOD	Number of Data points
Bioaffy® 20 HC	20 nL	µM range	112
Bioaffy® 200	200 nL	nM range	112
Bioaffy® 1000	1000 nL	pM range	96

Images courtesy of Gyros AB

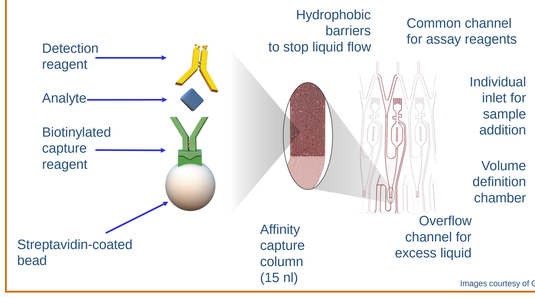
Figure 3a: Anatomy of a Bioaffy® 1000 CD, Integrated assay workflow using microfluidics and optimized surface chemistry

- Nanoliter-scale precision
- Fluidics facilitated by centrifugal force and capillary action



Gyrolab® Bioaffy CD | 8 microstructures per segment | 1 microstructure = 1 data point | Prepacked affinity column | Streptavidin coated bead

Figure 3b: Gyrolab Bioaffy® CD microstucture surface chemistry:



Images courtesy of Gyros AB

Figure 4: Performance guidelines for a GLP validated assay

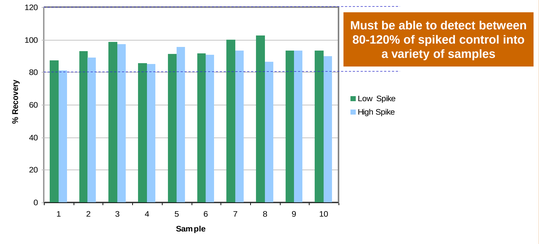
- Curve-fitting / Calibration model assessment
- Sensitivity (LOD / LLOQ)
- Selectivity / Specificity
- Accuracy / Precision
- Dilutional Linearity / Parallelism
- Range of BM concentrations in target population
- Robustness
- Stability
- Study specific factors:
  - interference from study drug, metabolites, & co-administered medications
  - matrix compatibility
  - disease state interference

Bioanal. 2009, 1, 1461-1474

Figure 5: Summary of Gyrolab® accuracy and precision validation assays

Characteristic	Statistic	Acceptance Criteria	78.1 pg/mL	156 pg/mL	250 pg/mL	750 pg/mL	2,500 pg/mL	5,000 pg/mL	Pass/Fail
Total N	N_obs	NA	15	18	17	18	18	17	Pass
MEAN	AVERAGE(Results)	NA	61.5	130	216	697	2,470	5,130	Pass
Precision (%CV)	Inter-batch	±20% CV ±25% CV of Nominal at LLOQ	15	17	15	10	10	8	Pass
Accuracy (%RE)	Inter-batch	±25% RE of Nominal ±30% RE of Nominal at LLOQ	-21	-17	-14	-7	-1	3	Pass
Total Error |Bias| + Precision	|Mean| + Inter-batch	≤40% all concentrations except ≤50% at LLOQ	36	34	29	17	11	10	Pass

Figure 6: Spike and recovery (selectivity) of recombinant IL-8 in Cynomolgus monkey serum



Must be able to detect between 80-120% of spiked control into a variety of samples

Figure 7: Parallelism & selectivity determined using endogenous IL-8

Sample ID	Endogenous Level IL-8 pg/mL
High Cynomolgus: CYN99124	4160.0
Low Cynomolgus: CYN99127	745.0

High : Low	% of Expected
100% / 0%	100.0
75% / 25%	101.6
50% / 50%	105.2
25% / 75%	108.2
0% / 100%	100.0

Sample ID	Endogenous Level IL-8 pg/mL
High Cynomolgus: CYN99132	2970.0
Low Cynomolgus: CYN99129	506.0

High : Low	% of Expected
100% / 0%	100.0
75% / 25%	95.6
50% / 50%	96.1
25% / 75%	91.8
0% / 100%	100.0

Figure 8: Dilutional linearity for quantitation of IL-8. Signal generation is proportional to the degree of dilution

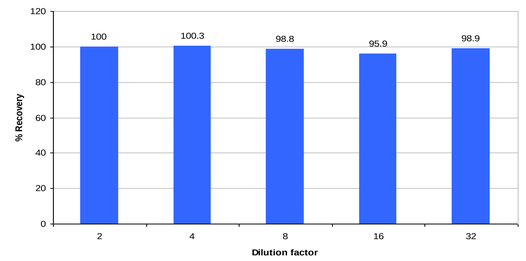
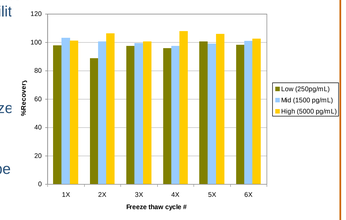


Figure 9: Stability

- IL-8 remained stable over short term (4 hr and 24 hour) stabilit tested in matrix at 15-30˚ C and 2-8˚ C
- IL-8 samples were also subjected to 6 cycles of freeze thaw and found to be stable
- Long term stability will also be evaluated for up to 1 year



Summary

- Custom biotinylated mouse IgG1 anti-IL-8 and Alexa® -647 labeled goat anti-IL8 IgG polyclonal antibody perform in an efficient and efficacious manner as antibody detection pairs for the determination of IL-8 levels in Cynomolgus monkey serum.
- The Gyrolab® platform used in conjunction with vendor supplied Bioaffy® 1000 CDs provide sufficient sensitivity to detect IL-8 (pg/mL range) in Cynomolgus monkey serum.
- A 1:2 MRD was determined to produce optimal assay sensitivity with acceptable levels of matrix interference.
- A full GLP method validation including: accuracy & precision, selectivity, dilutional linearity, endogenous dilutional linearity, parallelism, and stability were performed. Validation assays all passed previously established "*a priori*" acceptance criteria (Accuracy ±25% RE, Precision ±30% RE, Selectivity 70-130% recovery, Parallelism 70-130% Recovery, Dilutional Linearity ±25%RE/≤20%CV, Endogenous Dilutional Linearity ±25% RE/≤20% CV, and Stability ± 30% of reference.
- A Gyrolab® platform assay for the determination of IL-8 in Cynomolgus serum was validated. The validated range of the method was 78.1 to 5,000 pg/mL. IL-8 was found to exhibit dilutional linearity up to 1:32 dilution (1:16 initial + 1:2 MRD). IL-8 was found to be stable for up to 24 hours at both ambient temperature (15-30˚ C) and refrigerated temperature (2-8˚ C).

REFERENCES

• White, J., Sarau, H., IL-8 receptor antagonist: basic research and clinical utility. Chemokine Biology Vol II: pp. 89 – 102 (2007).

• Alvarez, V., Coto, E., Setien, F., Characterization of IL-8 receptors in non-human primates. Immunogenetics 43: pp 261-267 (1995).

•Lee, J., Devanarayan, V., Barret, Y., Weiner, R., Allinson, J., Fountain, S., Keller, S., Weinryb, I., Green, M., Duan, L., Rogers, J., Millham, R., O'Brien, P., Sailstad, J., Khan, M., Ray, C., Wagner, J., Fit-for Purpose method development and validation for successful biomarker measurement, Pharmaceutical Research 2: pp 312 – 328 (2006).

•Gyrolab User Guide, Gyros AB, pp 1 – 312 (2010).