The Highs & Lows of Ultra-sensitive Immunoassays

Experiences from a CRO's perspective

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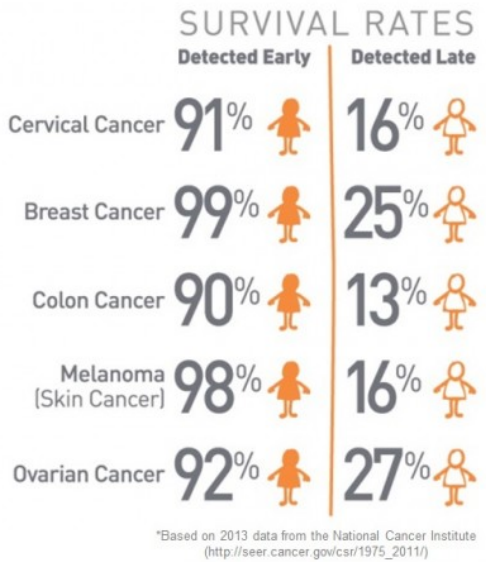
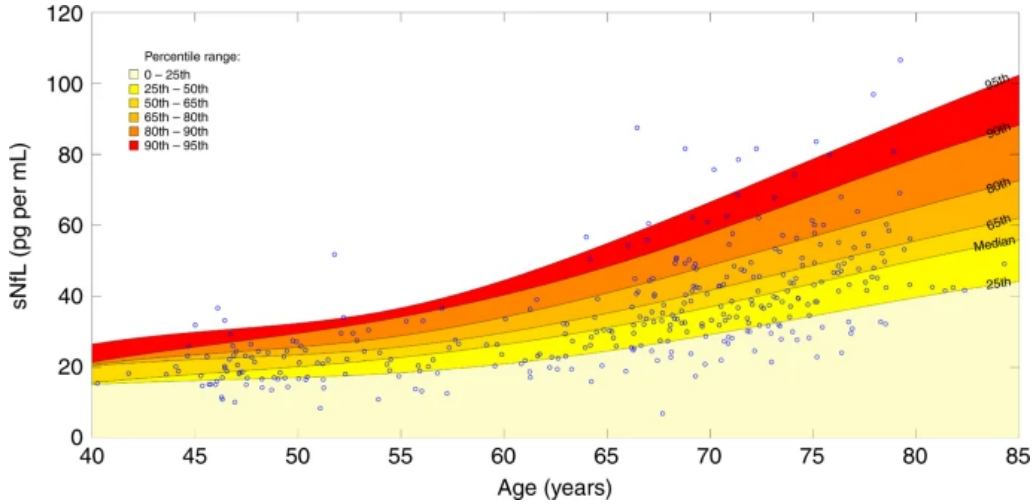
Topics to be Covered

- Why the need for ultra-sensitivity?
- Current platforms and technologies
- LGC's pros and cons from in-house evaluations of two platforms
- Case study 1: An overview of a validation of NF-L using the Quanterix HD-X
- Case study 2: A look at method development on the HD-X and both the benefits and potential pitfalls of this route for your assay, tips & tricks!
- Case Study 3: Cytokine 6-plex using Mitra VAMS

The growing demand for ultra-sensitive detection

- Early diagnosis key to improving patient survival rate
- Most prominent in cancers and neuro-degenerative diseases
- Early stage of disease = low concentration of key markers
- Target engagement assays: soluble target vs free fraction

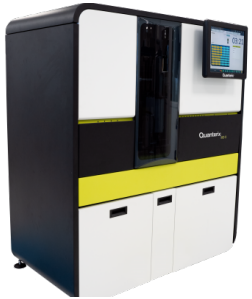




Khalil, M., Pirpamer, L., Hofer, E. *et al.* Serum neurofilament light levels in normal aging and their association with morphologic brain changes. *Nat Commun* **11,** 812 (2020). https://doi.org/10.1038/s41467-020-14612-6

Current Platforms & Technologies for a Regulated Environment

- Quanterix – primarily focused on ultra-sensitive platforms including: HD-X, SR-X & SP-X



- Merck – Platforms include Singulex Erenna and the SMCxPRO for ultra-sensitive detection

- MSD – S-plex technology allows ultra-sensitive detection using the current sector imaging instruments.

Implementation into the CRO

LGC evaluated two of these technologies with the aim to assess the robustness and suitability for implementation into the regulated workspace

	Quanterix HD-X	Merck SMCxPRO
Pros	Fully automated, 288 samples in ~5 hours	Plate based reading = no fluidics
	Multiplex capabilities	Reduced read time ~1 hour
	Homebrew capabilities for method development	Simple calibration ~10 minutes
	21CFR part 11 compliant	21CFR part 11 compliant
Cons	Requires large dedication from analysts to generate expertise on assay development	No change in workflow
	Calibration/qualification can be time consuming	No claim of improved sensitivity of the erenna
	Sample volumes needed can be large compared to other platforms	Assays not immediately transferable and may require re-development

LGC have since acquired and implemented the HD-X into our Fordham (UK) laboratory. The range of off the shelf kits available in both single and multiplex along with the ability to homebrew assays allows a CRO to offer multiple approaches to bespoke biomarker/PK assays.

Case Study 1: Validation of NF-L on the Quanterix HD-X

- Commercially available Quanterix NF-L kits used for validation
- Validation completed in nine days in both serum and plasma – Time saving, robust methods can prove to be invaluable for a busy CRO environment

Parameter Assessed	Outcome	Successful
Six P&A runs using three analysts	Inter-assay precision of ~5% across five QC levels	✓
Curve and weighting assessment	Statistical assessment of curve fit on six P&A runs concluded that a 5PL 1/Y2 weighting was optimal	✓
Suitability for singlicate assessment	Singlicate analysis can be completed on both serum and plasma	✓
Multiplate analysis	Instrument can run at full capacity (288 samples) across the validated range with acceptable precision and accuracy	✓
Kit lot to lot variation	Lot to lot assessment completed and minimal bridging will be required when changing kit lots during sample analysis studies	✓
6 X freeze/thaw and 2hr room temperature stability	Both assessment acceptable with minimal variation seen	✓
Matrix effects – Haemolysed and Lipaemic samples	No evidence of matrix effects from endogenous QCs at expected sample concentrations	✓
Parallelism of endogenous samples	Acceptable parallelism of 2-fold in both serum and plasma. This can be extended with incurred samples. All samples expected to come in with MRD (4-fold)	✓
LTS of up to one year pending	Up to 3 months completed successfully	**Pending**

Case Study 1: Validation of NF-L on the Quanterix HD-X

P&A in Serum using endogenous, spiked and kit controls							
	LLOQ	LQC	MQC	HQC	ULOQ	Kit control 1	Kit control 2
Mean Concentration Found (PG/ML)	2.34	5.34	12.8	38.9	123	3.93	163
Inter-run %CV	6.2	6.8	4.5	4.2	4.3	4.6	5.1
n	18	18	18	18	18	12	12

P&A data a good indicator of robustness in various QCs sources

Singlicate Vs Duplicate Assessment		
	LQC	MQC
Mean Concentration n=6 (pg/mL)	5.56	12.8
Inter-run %CV	4	2.5
%RE to Singlicate	4	0

Platform is capable of running singlicate assessments with reproducible results

Lot to Lot reproducibility using EQCs					
	LLOQ	LQC	MQC	HQC	ULOQ
Established QCs against new lot (pg/mL)	2.51	6.02	14	41.5	122
Inter-run %CV	7.6	5.1	4.7	2.8	3.1
Inter-run %RE to new curve	7.3	12.7	9.4	6.7	8.0
n	3	3	3	3	3

Lot to lot showed minimal variability which can save a significant amount of time and resource during a long study

Case Study 2: Method Development on the HD-X

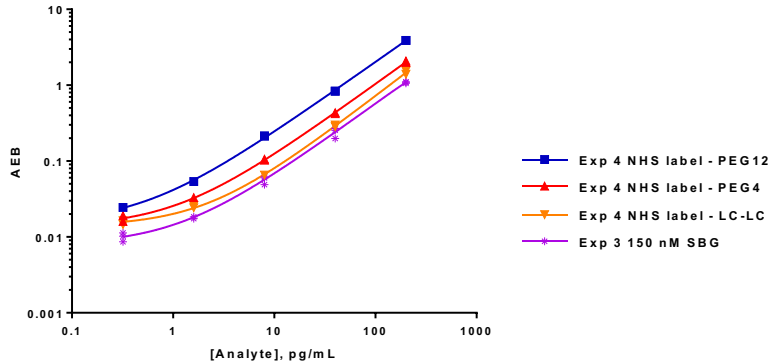
- Assay development using SIMOA technology can theoretically provide the user with a custom built assay for any biomarker needed with sensitivity in the sub pg/mL region

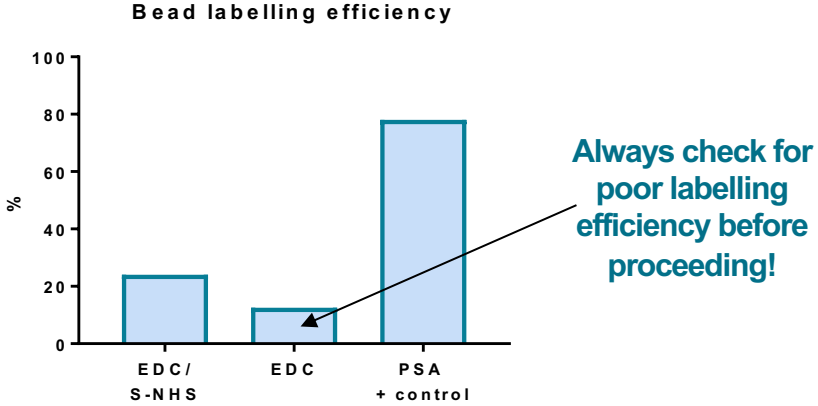
- To get the same performance as kit based assays however can take a lot of time, resource and knowledge of the platform

Case Study 2: Method Development on the HD-X

What to look out for during development:

1. Be aware of multiple label types/conjugation methods and establish reagent concentrations ASAP





- ✓ Multiple labelling approaches at this stage can prevent significant delays further down the line.
- ✓ Run parallel positive control if possible
- ✓ It is worth screening multiple Ab pairs and performing different labelling on each in order to establish the best combination via a chequerboard style assay format.

Case Study 2: Method Development on the HD-X

What to look out for during development:

2. Be aware of different buffer compositions and how they may affect your assay and when dealing with sensitivity keep an eye on the S/N of your raw data

	AEB			
	1	2	3	4
STD7 (1000pg/mL)	2.67	3.01	6.89	3.544
STD6 (500pg/mL)	2.55	2.22	4.21	2.567
STD5 (250pg/mL)	1.87	1.65	1.79	1.236
STD4 (100pg/mL)	0.860	0.760	0.659	0.563
STD3 (10pg/mL)	0.0776	0.0283	0.0552	0.0241
STD2 (1pg/mL)	0.0525	0.00888	0.0448	0.0178
STD1 (0.1pg/mL)	0.0511	0.00783	0.0200	0.0142
BLANK	0.0473	0.00746	0.0080	0.0071
	Diluent A	Diluent B	Diluent C	Diluent D

Average Curve bead number			
1	2	3	4
7757	17643	12473	15702

	S/N			
10pg/mL	1.6	3.8	6.9	3.4
1pg/mL	1.1	1.2	5.6	2.5
0.1pg/mL	1.1	1.1	2.5	2.0
	Diluent A	Diluent B	Diluent C	Diluent D

+ Top end of curve significantly improves with diluent C

+ S/N shows large improvement with Diluent C and D

- Not all developments will perform to the levels of commercial assays

Case Study 2: Method Development on the HD-X

What to look out for during development:

3. Add matrix into the assay ASAP, recombinants do not necessary mimic true endogenous behaviour

	AEB signal					
	1	2	3	4	5	6
1000	0.850	0.666	1.919	3.721	3.291	3.921
100	0.110	0.07550	0.192	0.387	0.310	0.411
10	0.0169	0.00899	0.0300	0.0625	0.0477	0.0641
1	0.00800	0.00385	0.0100	0.0249	0.0150	0.0275
0.1	0.00741	0.00355	0.00886	0.0231	0.0147	0.0202
Blank	0.00751	0.00368	0.0099	0.0228	0.0140	0.0213
Healthy IND	0.00551	0.00230	0.00452	0.0122	0.00655	0.0127
Disease IND	0.00760	0.00168	0.00262	0.0137	0.00572	0.0131
Det (ug/mL)	0.1	0.3	0.3	0.3	0.6	1.2
SBG (pM)	150	50	150	300	150	150

	S/N					
10pg/mL	2.250	2.441	3.030	2.741	3.407	3.009
1pg/mL	1.065	1.046	1.010	1.092	1.071	1.291
0.1pg/mL	0.987	0.964	0.894	1.013	1.050	0.948

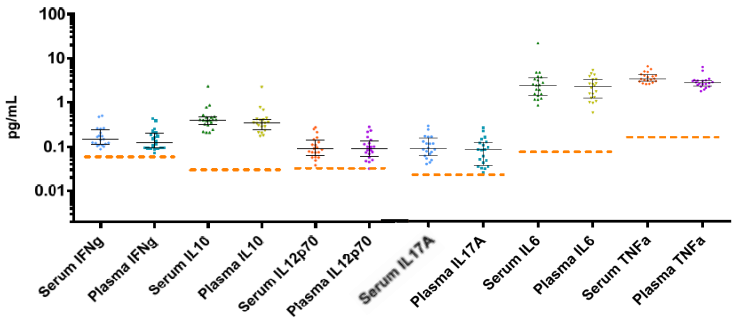
- General rule of thumb: >3.0 S/N is acceptable, >5.0 ideal. Reproducibility of blank is crucial
- Keep an eye on the whole curve, not just the bottom, see column 3, curve peaked much quicker than others but maintained good S/N.
- Although S/N is improving, when samples added to assay, no endogenous marker was recovered when expected from both healthy and disease state. Possible incorrect Ab pairing, only one pair tested for this assay.

Case Study 3: Cytokine 6-plex using Mitra VAMS

In collaboration with Quanterix, LGC has completed the assessment of samples using Neoteryx micro-sampling devices (VAMS) measuring six cytokines as part of a multiplex panel.

Experiments completed:

- General kit performance



Inter-assay precision

	IFN-γ	IL-10	IL-12p70	IL-17A	IL-6	TNF-α	
[Mean], pg/mL	1.73	0.568	1.03	0.530	2.64	0.682	Low Concentration QC
%CV	12.7	17.6	7.6	6.9	9.1	17.2	
n	19	19	19	19	19	19	
[Mean], pg/mL	43.5	9.43	20.5	14.6	60.5	22.6	High Concentration QC
%CV	11.7	19.9	7.0	9.3	7.5	12.2	
n	19	19	19	19	19	19	

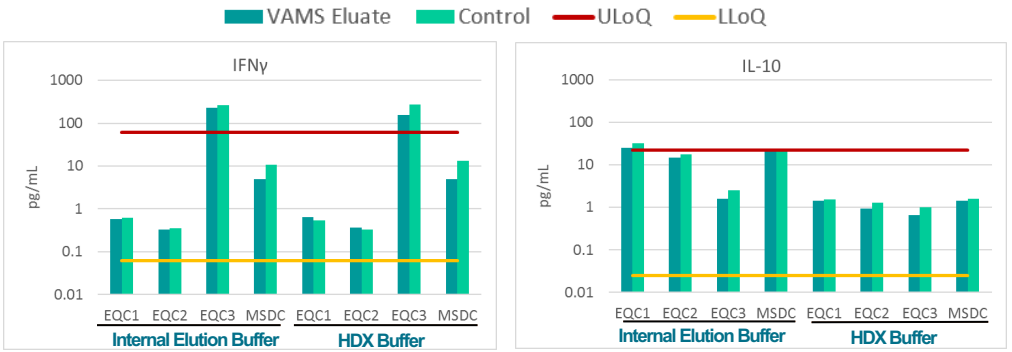
- Kits perform well from a robustness perspective
- Simple to run, bench to data = ~3 hrs

Case Study 3: Cytokine 6-plex using Mitra VAMS

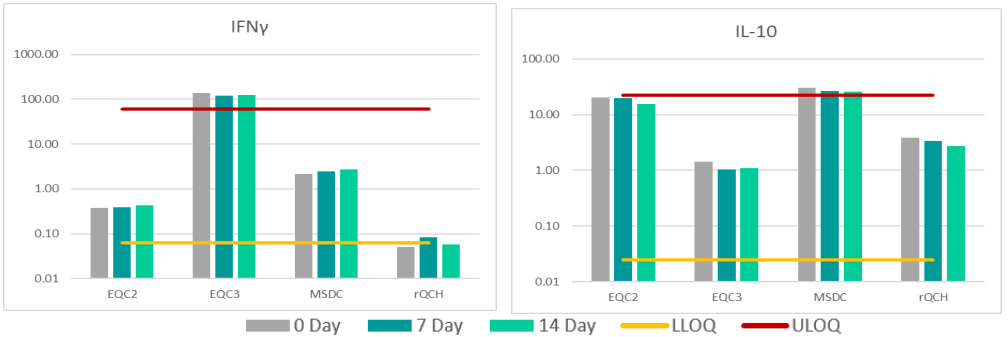
In collaboration with Quanterix, LGC has completed the assessment of samples using Neoteryx micro-sampling devices (VAMS) measuring six cytokines as part of a multiplex panel.

Experiments completed:

- General kit performance
- Elution buffer selection
- 2hr vs 24hr elution
- Fresh vs frozen QCs
- On-VAMS recovery up to 14 days
- VAMS to VAMS precision

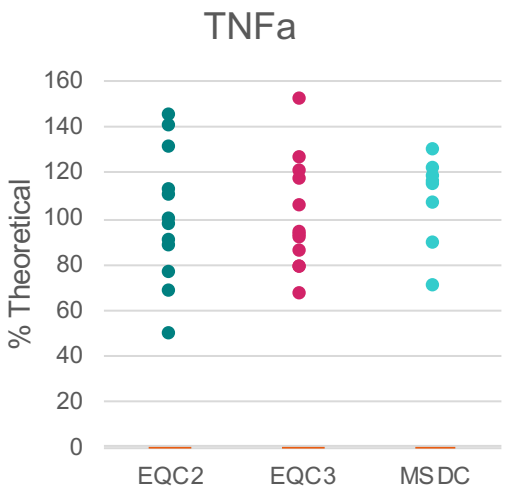


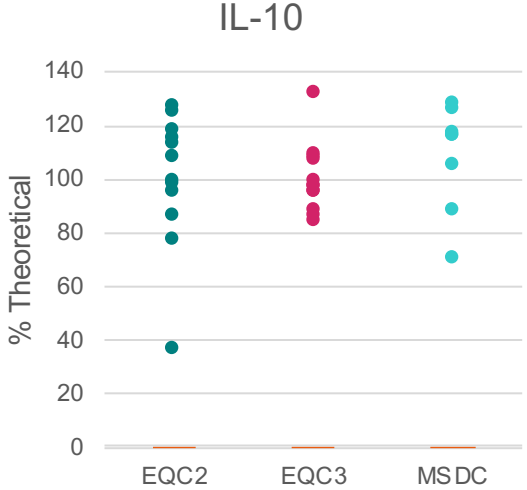
Evaluation of elution buffers

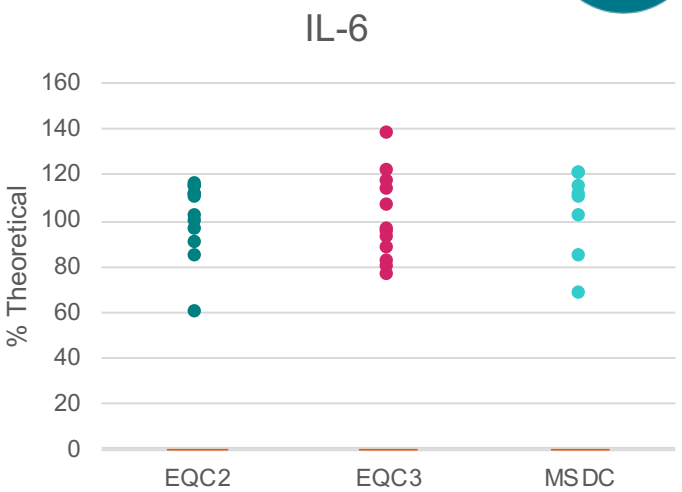


On-VAMS stability up to 14 days

Case Study 3: Cytokine 6-plex using Mitra VAMS VAMS to VAMS







	TNFa			IL-10			IL-6		
Conc (pg/mL)	24.4	116	21.2	33.9	1.58	43.6	645	277	181
CV%	29	24.3	17.9	24.9	12.86	18.2	16.2	18.8	17.8
Curve range (pg/mL)		0.037 – 40.7			0.025 – 22.6			0.084 – 67.5	

- Variation is slightly higher between the individual VAM devices than the pooled eluate from previous exps
- Experiment completed mimicking the “real life” collection technique. Variation could highlight collection issues
- With a level of tolerance, VAMS could be an efficient way for companies to conduct surveillance studies or monitor long term effects

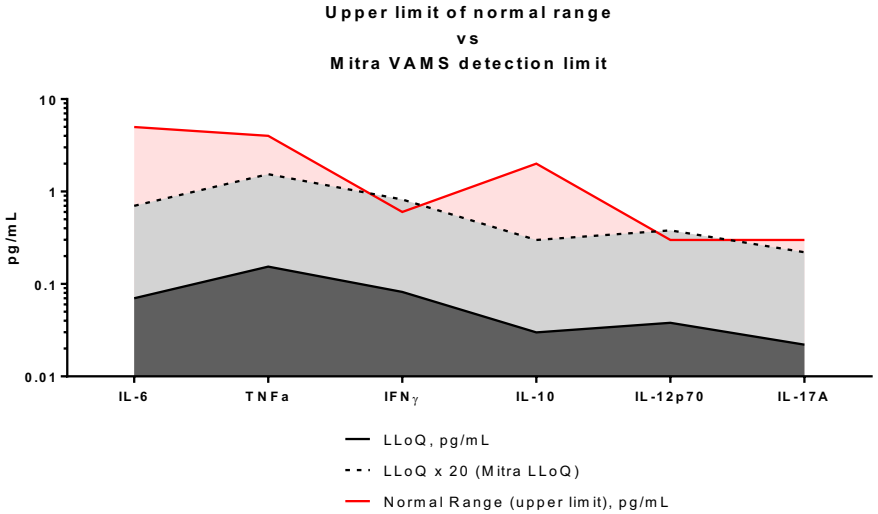
Case Study 3: Cytokine 6-plex using Mitra VAMS

Advantages

- Reproducible results from day to day and kit to kit
- Controls perform reliably although levels need to be established
- HDX sensitivity enables its use with VAMS devices (disease state)
- Clinically relevant cytokine levels post-VAMS elution in HDX range
- Analyte stability on the Mitra device confirmed

Limitations

- Highest calibrator failures due to too high signal
- Data lost from all analytes if one has very high (out of range) levels
- Not sensitive enough for healthy cytokine level post-VAMS dilution



Highs and Lows of Ultra-sensitive Assay Development and Validation on the HD-X

Highs	Lows
An open platform for commercially available kits and homebrew assays is a powerful tool for a CRO	Unless panels of tool antibodies are available, sensitivity of homebrew assays might not come close to commercial assays
Precision is reproducible in both kits and homebrews	
With the correct technical knowledge, development can be quick with multiple parameters being performed in one assay	Flexibility is required when developing with regards to Ab pairings, orientations, buffers and dynamic range
Several options and routes to test within development	
Fully automated assays mean minimal time is spent in the lab and turn around time increases	The instrument calibration can be problematic which can be very problematic for CROs. This is being addressed by Quanterix with a V2 calibration at the end of November 2020
Off the shelf kits perform very well and show high levels of robustness	CSV integration was lengthy and was met with unanticipated challenges.
Very strong technical support with Quanterix	CSV protocols changing regularly

Thankyou for listening!

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