EBF Raise the Anchor

To sensitivity or too sensitivity

Case studies on challenges and trends in bioanalytical assay sensitivity

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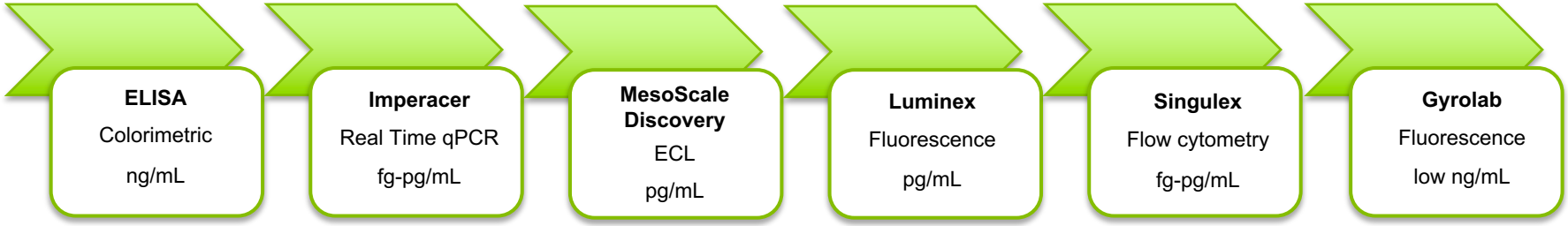
Advances in immunoassay technologies

- Ligand binding assays (LBAs) have been used routinely for more than 40 years to quantify biologicals and biomarkers.
- New platforms are now competing with the traditional ELISA and offer various advantages in terms of sensitivity, dynamic range, sample volume and throughput.



- Development of more sensitive assays allowing the discovery of new biomarkers

Assay requirements for sensitivity

- Required sensitivity is one of the first questions asked before developing an assay at Envigo
- No guidance from the regulating authorities detailing how sensitive an assay should be
- Knowing the required sensitivity of the assay is important to develop a fit for purpose assay:
  - PK/TK: Will the assay be used to support PK studies with low doses or to support TK studies where large dilutions are expected?
  - ADA: the sensitivity requirements from the FDA have been reduced due to evidence that even low concentrations of ADAs (around 100 ng/mL) are a safety issue. Should we be developing the most sensitive assay (problems around the choice of the positive controls)?
  - **Do we know what the normal range of the biomarker is? Are the concentrations expected to increase or decrease?**

Case study 1: Validation of a sensitive assay to measure cardiac Troponin (ADVIA Centaur XP)

- **Troponin as a biomarker**
  - Protein complex which regulates the contraction of striated muscle
  - **Cardiac troponin** is released into the bloodstream within hours of the onset of cardiac damage
  - Known biomarker for 50 years, now used as a prognostic biomarker as assays have become more sensitive

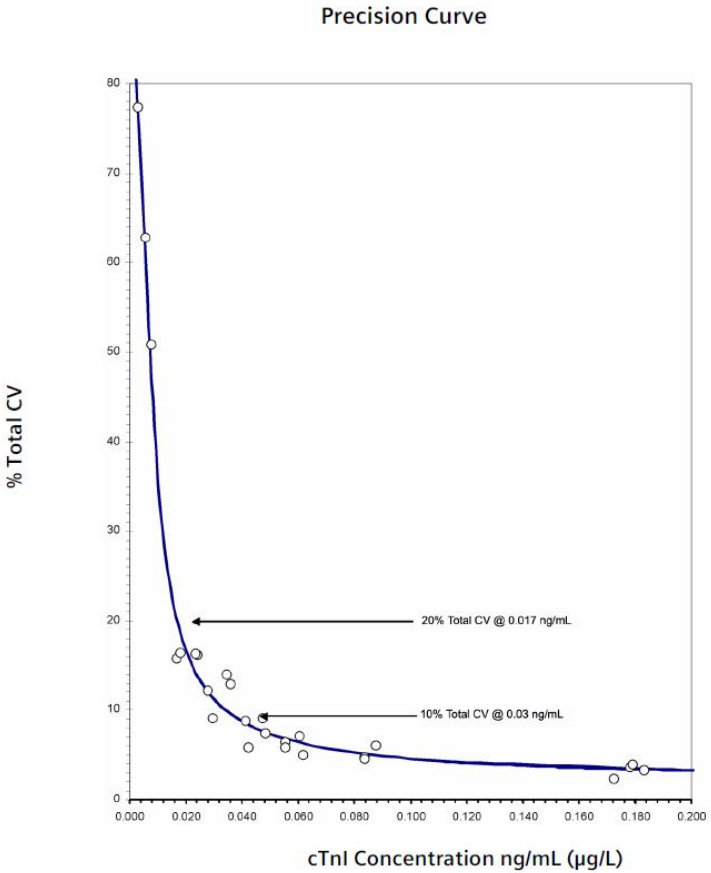
- **ADVIA Centaur XP system**
  - Clinical Immunoassay analyser
  - Chemiluminometric technology
  - Mid volume (10 µL to 200 µL assay dependent)
  - High-throughput (240 tests/hour)
  - Automatic dilutions
  - More than 100 assays available

- **TnI-Ultra assay**
  - 3-site sandwich immunoassay (2 biotinylated mouse monoclonal anti-troponin I antibodies + polyclonal goat anti-troponin I antibody labelled with acridinium ester)
  - Solid phase: magnetic latex particles conjugated with streptavidin

Case study 1: cTnI assay validation and sensitivity

- **Manufacturer's data**
  - Sensitivity: 0.017 ng/mL (17 pg/mL) – 20% precision
  - Low end precision assessed by manufacturer in triplicate in 20 runs over a 20-day period
  - Assay calibrated once a month using the provided kit (no standard curve in run)

- **Envigo validation**
  - Assay to be validated in dog, rat, minipig and cynomolgus monkey plasma
  - Yes/No answer required with a cut off at 20 pg/mL
  - Matrix QCs prepared across the assay range by diluting positive sample obtained by cardiac puncture/homogenised cardiac tissue (20 pg/mL to 50,000 pg/mL)
  - Parameters being assessed: precision and accuracy, sensitivity, selectivity, stability



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Case study 1: cTnI assay results

+ Achieved sensitivities:

Species	Rat		Dog		Minipig		Monkey	
matrix QCs	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2	QC 1	QC 2
concentration (pg/mL)	21	547	27	77	20	156	31	79
precision (%CV)	21.7%	3.3%	66.3%	16.6%	7.5%	1.0%	4.3%	0.5%

+ Better precision compared to the start due to kit production issues at the start

+ Assay performance monitored using a large batch of controls in all runs to identify potential batch to batch variations

+ Assay successfully validated in all four species and stability demonstrated up to 1 year

Case study 2: Assessment of GLP-1 kits using multiple reference materials (Gyrolab and MSD)

- Glucagon-like peptide-1 (GLP-1) is a 3.5 kD protein with 2 biologically active forms: GLP-1 (7-36)amide and GLP-1 (7-37)

- MSD Kit:
  - Mouse/Rat Total Active GLP-1, Insulin, Glucagon Kit (Cat. No. K15171C)
  - Calibrator: GLP-1 (7-36)amide
  - Dynamic range: 14 to 10,000 pg/mL

- Millipore Kit on Gyrolab platform (discontinued)
  - GyroMark™ HT Total GLP-1 Assay (Cat No. GYGLP1T-36K)
  - Calibrator: Total GLP-1 Standard
  - Dynamic range: 1.6 to 3500 pg/mL

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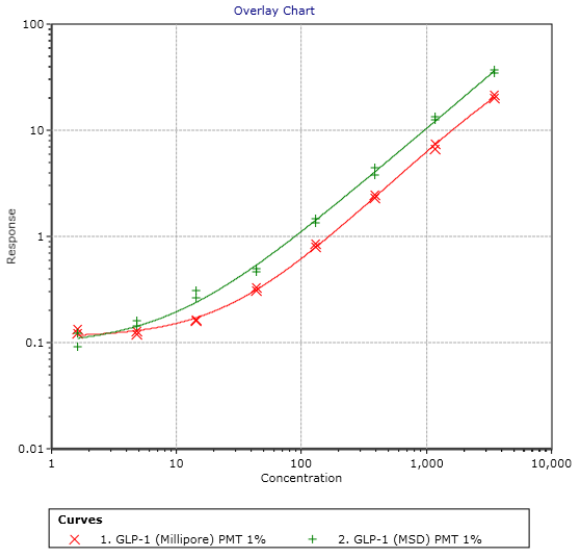
Case study 2:
Comparison of reference materials and rat plasma

- Run content:
  - Millipore and MSD reference materials assessed in both assays
  - 5 rat plasma individuals spiked with 400 pg/mL of Millipore material and analysed with unspiked samples



GLP-1 concentrations (pg/mL) measured in Gyrolab assay using Millipore and MSD reference materials

Rat plasma individual	Millipore reference	MSD reference
1	14.3	9.04
2	36.2	18.7
3	18.2	10.7
4	40.2	20.6
5	31.4	16.6

- Results and discussions:
  - Difference between the two reference materials observed in both assays
  - Endogenous levels of GLP-1 measurable in all individuals in the Gyrolab assay (>14 pg/mL) but not in the MSD assay
  - None of the kits found suitable due to the variability of the Gyrolab assay and the lack of sensitivity of the MSD assay

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Case study 3: Sample analysis using a non-validated multiplex assay kit (Luminex)

- Context for the analysis:
  - Client requested to measure FGF23, PTH and ACTH in rat EDTA plasma
  - Multiplex kit was available from Luminex (MILLIPLEX MAP Rat Bone Magnetic Bead Panel 1, Cat No RBN1MAG-31K) but had never been assessed at Envigo
  - Plan was to assess 90 samples preceded by a parallelism experiment to establish MRD and linearity (using 3 rat plasma individuals)
  - Parameters evaluated during manufacturer's validation process:
    - Sensitivity (minimum detectable concentrations)
    - Precision and accuracy
    - Cross reactivity between analytes
    - Dilution linearity
    - Kit stability
    - Sample behaviour (detectability and stability)
- Initial results:
  - No linearity observed, MRD set at 1 in 10

Samples	Dilution	Nominal Concentration (pg/mL)	Observed Concentration (pg/mL)	Recovery %
Ind 1	1	3680	1790	48.6
	10	368	368	100.0
	20	184	156	84.8
	40	92.0	93.1	101.2
	80	46.0	43.7	95.0
	160	23.0	BLQ	NA
			Mean	95.2
			%CV	7.8
Ind 2	1	4180	1450	34.7
	10	418	418	100.0
	20	209	198	94.7
	40	104.5	88.9	85.1
	80	52.3	47.8	91.5
	160	0.29	BLQ	NA
			Mean	92.8
			%CV	6.7
Ind 3	1	1660	857	51.6
	10	166	166	100.0
	20	83.0	91.0	109.6
	40	41.5	47.4	114.2
	80	20.8	BLQ	NA
	160	10.38	BLQ	NA
			Mean	108.0
			%CV	6.7

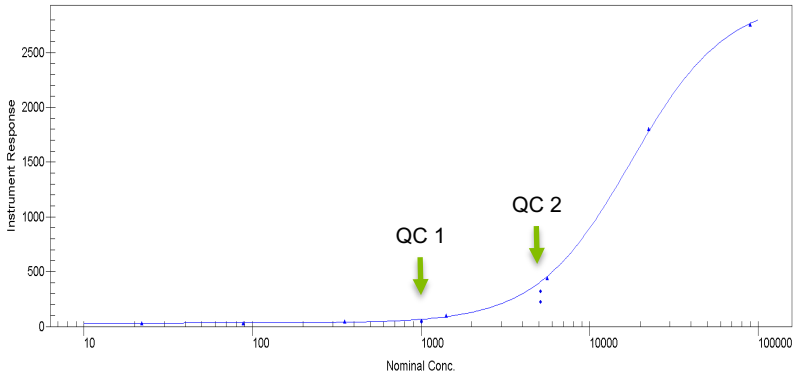
BLQ: Below limit of quantification (39.0 pg/mL)
Recovery calculated against the 1 in 10 result

Case study 3: Performance of the Low QC

+ Assay run out of the box and low QCs failed in initial runs

	ACTH	FGF23	PTH
Standard concentrations (pg/mL)			
Std 1	9.77	22.0	0.366
Std 2	39.1	87.9	1.46
Std 3	156	352	5.86
Std 4	625	1406	23.4
Std 5	2500	5625	93.8
Std 6	10000	22500	375
Std 7	40000	90000	1500
Kit controls concentrations (pg/mL)			
control 1 range	322 - 668	657 - 1364	11 - 23
control 1 mean	495	1010	17
control 2 range	1462 - 3036	3335 - 6927	49 - 101
control 2 mean	2250	5130	75

	ACTH	FGF23	PTH
Signal to background ratio			
Std 1	1.1	1.1	1.0
Std 2	1.6	1.1	1.3
Std 3	**6.4**	1.7	2.3
Std 4	**41.1**	3.7	**5.2**
Std 5	**191.6**	**16.5**	**20.2**
Std 6	**447.5**	**67.4**	**80.6**
Std 7	**576.8**	**102.9**	**162.7**



+ Solutions:
  + Matrix QC prepared to control the assay instead of using the low kit QC
  + Dilution scheme modified to include more standards points with an acceptable signal to background ratio

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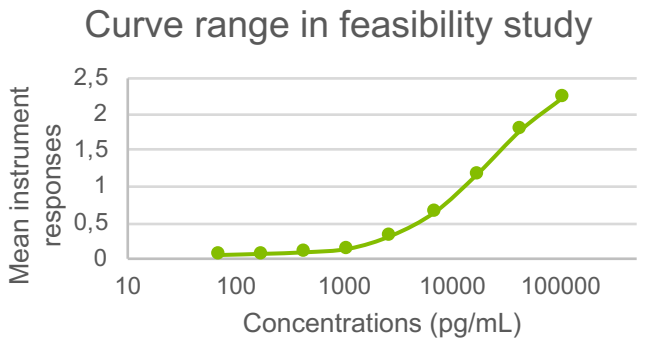
Case study 4: Development of a PK assay using a biomarker kit (ELISA)

- Project details:
  - Recombinant human protein used to prevent viral infection
  - Human Quantikine ELISA kit available from R&D Systems
  - Kit initially assessed to support a pharmacokinetic study in sheep using topical application
- Feasibility study
  - MRD assessed using standards curves prepared in 1%, 5%, 10% and 25% serum and compared to buffer curve (MRD set at 1 in 10)
  - Curve range: 70.5 to 108,000 pg/mL (serum concentrations)
  - Precision and accuracy assessed over 3 runs using 3 levels of serum QCs (430, 2690 and 43,000 pg/mL)
  - Assay considered fit for purpose to measure drug in serum and tissue homogenates for preliminary studies although the lowest QC didn't perform well
  - Sample analysis carried out over 12 acceptable runs (6 runs rejected due to low QC outside acceptance criteria)
- Follow up study:
  - New pharmacokinetic study performed 3 years later (new route of administration)
  - Better performance observed during sample analysis

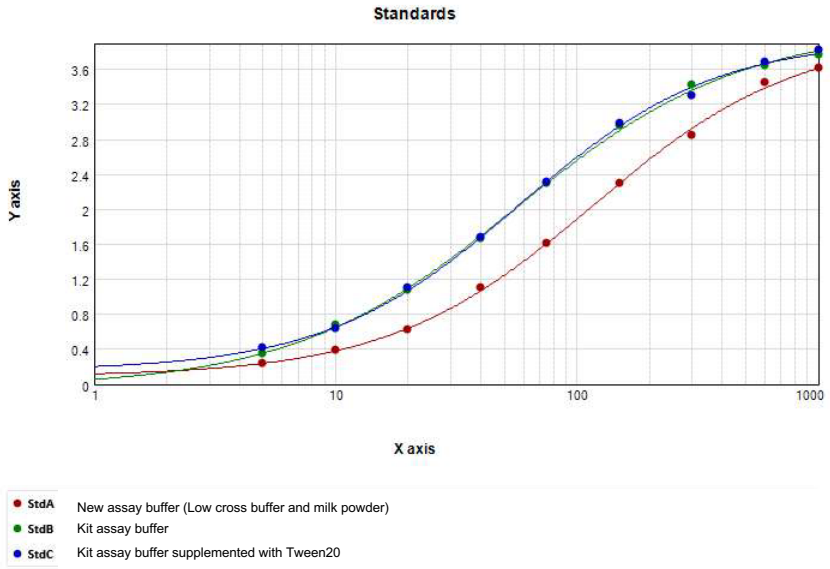
Case study 4:
Assay validation in rat and rabbit serum

- Validation:
  - Validation initiated after increasing the LLOQ of the assay (282 pg/mL) but terminated due to the selectivity assessment not meeting the acceptance criteria



concentration (pg/mL)	70.5	176	440	1100	2750	6880	17200	43000	108000
S/B	1.2	1.6	2.1	3.3	**7.6**	**15.2**	**27.7**	**41.9**	**52.7**

- Development of a new assay
  - Changes made to the method which had been used in another laboratory (assay buffer and volumes)
  - LLOQ of the assay increased to 10.0 ng/mL
  - More variability observed with the rat assay



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Conclusions

- Understanding the assay requirements is critical
- Choosing the assay platform will meet most of the assay requirements
- Setting LLOQ:
  - Good signal to background (ideally 5)
  - Multiple LLOQs assessed during development if required
  - Push for sensitivity only if required
- Reference material
  - Sensitivity dependant on reference material
  - Important to screen individual samples early on rather than trusting the manufacturer range
- Non-validated commercial kits
  - Review kit performance before analysing samples
  - Increase the number of standards to improve assay performance

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Together, we make the world a
safer and healthier place to live.

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