Validation of immunoassay for protein biomarkers:

Bioanalytical study plan implementation and case study

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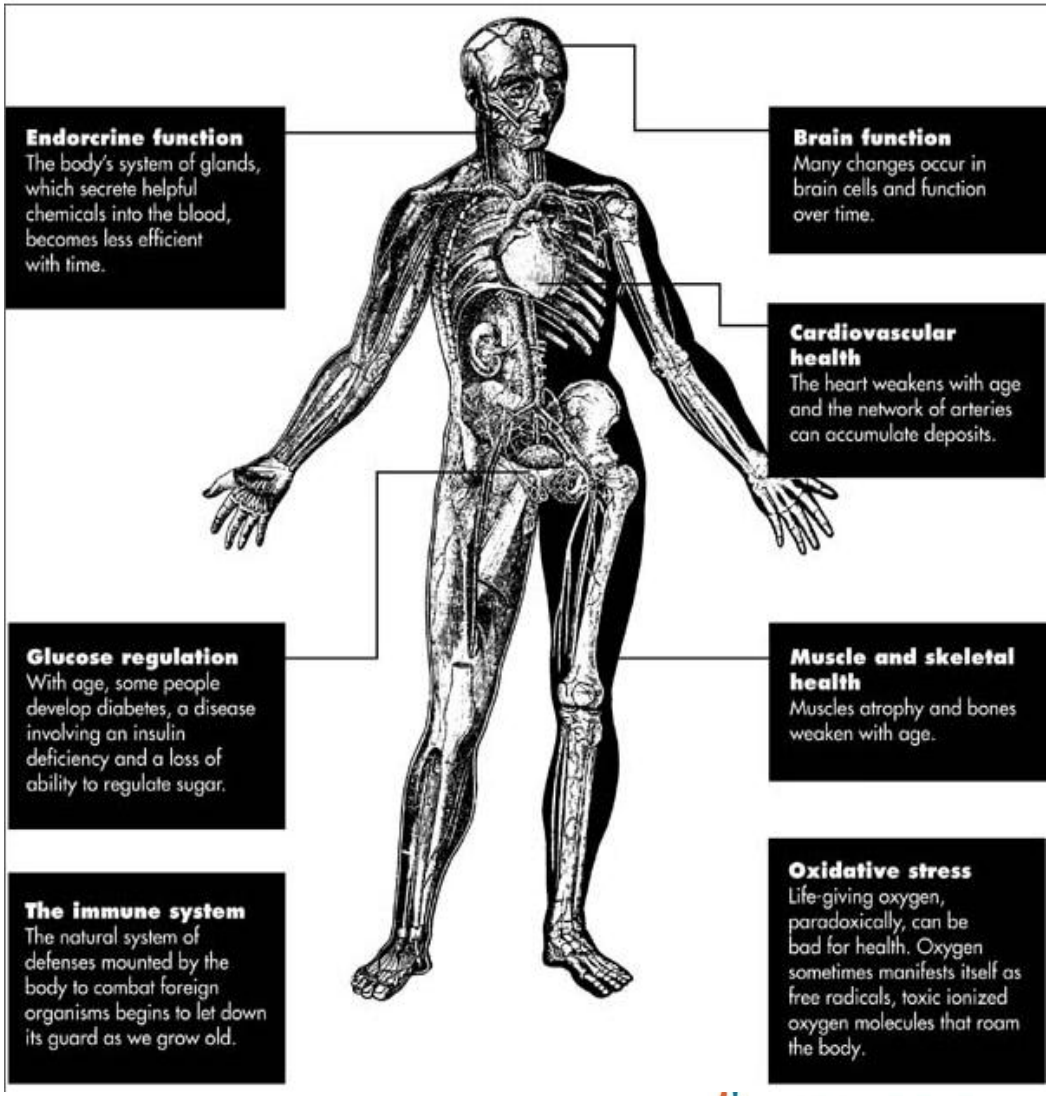
What is a biomarker?

A characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes, or a pharmacological response to a therapeutic intervention*

Examples include: blood pressure, β-Amyloid deposition (Alzheimer disease), and prostate specific antigen (PSA)



*J Clin Pharmacol Therpeut 2001;69:89-95

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What is the role of a biomarker?

Pharmacology markers for

- pharmacodynamic performance → **Compound selection**
- pharmacokinetic correlation → **Dose selection**

Disease biomarkers for

- diagnosis & prognosis
- predisposition assessment → **Dose monitoring**
- early detection of “toxicity”

Safety & efficacy biomarkers for

- clinical response monitoring → **Therapeutical performance**
- surrogate endpoints
- response prediction → **Patient selection**

➢ Utility of biomarkers to support decision making in clinical studies is directly related to quality of underlying bioanalytical data

How to measure a biomarker?

From assay development to clinical application

1. Establish biomarker assay

- Optimal assay condition settings (format, antibodies, detection system; critical assay reagent and stability; standard-curve model; matrix selection, sample preparation)
- Preliminary assessment of assay robustness

2. Validate biomarker assay

- Define assay sensitivity, parallelism and selectivity
- Assess assay robustness and variability

3. Analysis of clinical samples in house or by CRO

4. Biomarker data interpretation in the context of the clinical study

How to validate of a biomarker?

- ✓ Guidance documents published on validation of biotherapeutics (small- and macro-molecule drugs) measured by ligand-binding assay, mass-spectrometry or chromatographic-based system
- ✓ Small-molecule biomarkers measured by LC-MS fall into definitive quantitative assay category and will meet guidelines for PK assay
- ✓ No specific regulation on validation of methods to support biomarker study
- ✓ Immunoassays are relative quantitative assays which commonly experience the following issues:
  - ❑ lack of suitable reference material, use of unique analytical reagent / platform, presence of endogenous biomarker and disease-specific effects

➢ Implementation of bioanalytical study plan for the validation of immunoassays to support pre-clinical and clinical studies

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Milestone 1: Feasibility study

Parameters	Acceptance criteria	
	Biomarker selection	Decision-making biomarker
Calibration curve	≥ 6 non-zero STDs excluding blank and anchor point(s) Mean ACC: 80 – 120 % CV: ≤ 20 % ≥ 75 % of non-zero STDs should meet the acceptance criteria	
Parallelism	6 individual neat matrices	Mean ACC: 70 – 130 % compared to neat matrix undiluted or at the minimum required dilution CV ≤ 25 % For each dilution factor, at least 5/6 matrices should meet the acceptance criteria. If not, 6 additional matrices should be tested
Selectivity	6 individual matrices freshly spiked	Mean ACC: 70 – 130 % compared to freshly spiked matrix undiluted or at the minimum required dilution CV ≤ 25.0 % For each dilution factor, at least 5/6 matrices should meet the acceptance criteria. If not, 6 additional matrices should be tested
	DECISION POINT	

Milestone 1: Feasibility study: Case study: Angiopoietin-like protein biomarker in human serum

Parallelism

Serum	Dilution	Measured concentration (ng/mL)	Adjusted concentration (ng/mL)	CV (%)	Accuracy (%) based on dilution 1:5
Serum 1	1:5	6.57	32.9	0.0	/
	1:10	3.35	33.5	6.2	101.8
	1:20	1.57	31.4	3.5	95.4
	1:40	0.744	29.8	5.6	90.6
Serum 2	1:5	5.45	27.3	1.1	/
	1:10	2.67	26.7	0.0	97.8
	1:20	1.39	27.8	2.0	101.8
	1:40	0.740	29.6	11.7	108.4
Serum 3	1:5	4.75	23.8	0.5	/
	1:10	2.24	22.4	0.1	94.1
	1:20	0.989	19.8	1.1	83.2
	1:40	0.482	19.3	2.2	81.1
Serum 4	1:5	3.56	17.8	0.3	/
	1:10	1.78	17.8	2.7	100.0
	1:20	0.858	17.2	1.8	96.6
	1:40	0.586	23.4	36.0#	131.5#
Serum 5	1:5	5.65	28.3	0.2	/
	1:10	2.79	27.9	0.1	98.6
	1:20	1.36	27.1	0.6	95.8
	1:40	0.716	28.6	7.3	101.1
Serum 6	1:5	15.9	79.3	1.2	/
	1:10	6.96	69.6	0.3	87.8
	1:20	3.20	64.0	0.2	80.7
	1:40	1.59	63.7	2.6	80.3

- Accuracy and precision are met with a dilution 1:5
- Endogenous biomarker can be quantified in 6/6 matrices
- The overall range is 23.8 – 79.3 pg/mL in targeted population

Red: below the expected LLOQ (0.509 ng/mL)

Milestone 1: Feasibility study Feasibility study: Case study: Angiopoietin-like protein biomarker in human serum

Selectivity

		Neat serum	Serum spiked with 40 ng/mL of recombinant protein				
Serum	Dilution	Adjusted concentration (ng/mL)	Measured concentration (ng/mL)	Adjusted concentration (ng/mL)	Accuracy (%) based on dilution 1:5	Recovery (%)	CV(%
Serum 1	1:5	6.57	10.9	54.5	/	76.1	
	1:10	3.35	5.96	59.6	109.4		
	1:20	1.57	3.02	60.5	111.0		
	1:40	0.744	1.54	61.6			
Serum 2	1:5	5.45	10.3	51.3			
	1:10	2.67	5.60				1.9
	1:20	1.39	2.80				0.7
	1:40	0.740	1				0.6
Serum 3	1:5	4.75				78.3	1.0
	1:10	2.2				81.0	1.9
	1:20				103.5	81.0	1.3
	1:40				108.3	84.8	2.5
Serum				44.4	/	77.8	2.9
				50.9	114.6	89.2	3.7
			52	50.3	113.3	88.1	0.0
			1.26	50.6	114.0	88.6	0.2
Serum 5			9.84	49.2	/	73.2	0.8
		2.79	5.36	53.6	108.9	79.8	3.6
		1.36	2.69	53.9	109.6	80.2	1.8
	1:40	0.716	1.41	56.6	115.0	84.3	1.0
Serum 6	1:5	15.9	20.2	101	/	87.0	0.4
	1:10	6.96	8.99	89.9	89.0	77.4	3.1
	1:20	3.20	4.57	91.3	90.4	78.6	0.2
	1:40	1.59	2.26	90.2	89.3	77.7	0.8

- Accuracy and precision are met with a dilution 1:5
- Matrix does not interfere with quantification of biomarker
- No apparent effect of dilution observed (dilutions tested from 1:5 to 1:40)

Red: below the expected LLOQ (0.509 ng/mL)

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Milestone 2: Decision point

- If an assay is not satisfactory during feasibility study (sensitivity, parallelism...), decision may be taken to:
  - Evaluate new kit or develop a new assay (using new Ab‘s or reagents)
  - Proceed with validation if justified by a strong scientific and biological rationale (e.g. higher endogenous level of biomarker is expected in treated- or disease-sample)
- Goal is to avoid validating an immunoassay which is unlikely to meet the intended use
- Based on result of parallelism and selectivity, a minimum required dilution may be defined

Milestone 3: Definition of quality control nominal value

Parameters	Acceptance criteria	
	Biomarker selection	Decision-making biomarker
Calibration curve	≥ 6 non-zero STDs excluding blank and anchor point(s) Mean ACC: 80 – 120 % CV: ≤ 20 % ≥ 75 % of non-zero STDs should meet the acceptance criteria	
QC nominal value definition	3 QC levels: LLOQ, MID, ULOQ 3 independent runs 3 independent preparations per QC level	5 QC levels: LLOQ, LOW, MID, HIGH, ULOQ 3 independent runs 3 independent preparations per QC level

Milestone 3:Feasibility study: Case study: Angiopoietin-like protein biomarker in human serum Definition of quality control nominal value

Run	QC LLOQ (ng/mL) (Human serum neat and diluted 1:40)	QC Low (ng/mL) (Human serum neat and diluted 1:20)	QC Mid (ng/mL) (Human serum neat and diluted 1:5)	QC High (ng/mL) (Human serum neat and diluted 1:5)	QC ULOQ (ng/mL) (Human serum spiked with 40.0 ng/mL of protein and diluted 1:5)
1	0.415	0.930	7.10	17.8	22.2
	0.460	0.947	7.24	17.7	22.1
	0.497	1.01	7.30	18.9	22.5
2	0.531	0.997	7.16	18.0	21.3
	0.468	0.944	6.88	18.5	21.7
	0.547	0.969	7.13	18.3	21.4
3	0.648	1.07	7.80	19.5	23.0
	0.493	0.962	7.52	21.5	24.1
	0.526	0.997	7.65	20.8	23.5
Mean concentration (pg/mL)	0.509	0.981	7.31	19.0	22.4
CV (%)	13.0	4.4	4.0	7.1	4.3
n	9		9		9

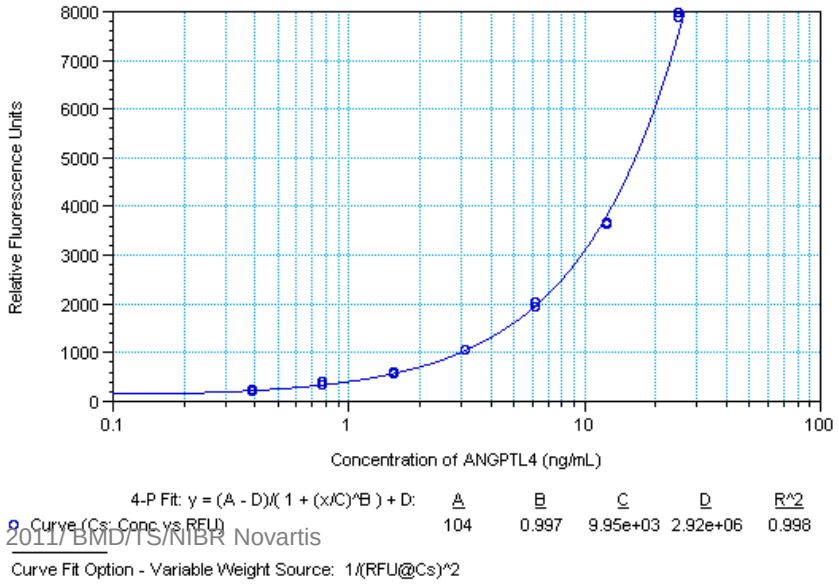
Milestone 4: Validation

	Biomarker selection		Decision-making biomarker	
	Parameters	Acceptance criteria	Parameters	Acceptance criteria
Calibration curve	≥ 6 non-zero STDs Mean ACC: 80 – 120 % CV: ≤ 20 % ≥ 75 % of non-zero STDs should meet the acceptance criteria STDs at LLOQ and ULOQ should meet the acceptance criteria			
Intra-, inter-run precision and accuracy	3 QC levels 3 independent runs 5 independent preparations per QC level	Mean ACC: 70 – 130 % CV ≤ 25 % 4/5 determination per QC level should meet the acceptance criteria	5 QC levels 6 independent runs 5 independent preparations per QC level	Mean ACC: 75 – 125 % (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % 4/5 determination per QC level should meet the acceptance criteria
Parallelism	No further assessment		6 additional individual neat matrices	Mean ACC: 75 – 125 % compared to undiluted neat matrix or matrix at MRD (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % For each dilution factor, at least 5/6 matrices should meet the acceptance criteria. If not, 6 additional matrices should be tested
Selectivity	No further assessment		6 additional individual matrices freshly spiked	Mean ACC: 75 – 125 % compared to undiluted spiked matrix or spiked matrix at MRD (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 %

Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum

Calibration curve: no anchoring point

	Nominal ANGPTL concentration (ng/mL)													
	STD1		STD2		STD3		STD4		STD5		STD6		STD7	
Date of analysis	25.0		12.50		6.25		3.13		1.56		0.781		0.391	
	Measured conc.	CV (%)	Measured conc.	CV (%)	Measured conc.	CV (%)	Measured conc.	CV (%)	Measured conc.	CV (%)	Measured conc.	CV (%)	Measured conc.	CV (%)
26-Jan-2011	25.9	0.5	12.0	0.1	6.17	0.8	3.18	1.6	1.59	1.5	0.789	2.7	0.383	1.1
27-Jan-2011	24.8	1.2	13.0	0.9	6.09	1.1	3.11	0.6	1.59	0.3	0.797	2.1	0.382	6.2
27-Jan-2011	24.7	1.2	13.0	0.1	6.06	0.7	3.12	2.3	1.55	0.6	0.839	0.0	0.366	3.3
01-Feb-2011	25.9	0.4	12.0	0.8	6.29	1.0	3.11	1.7	1.59	0.4	0.799	1.0	0.381	6.0
02-Feb-2011	25.0	1.9	12.8	0.0	5.92	2.2	3.33	0.4	1.54	1.1	0.778	6.9	0.394	3.6
02-Feb-2011	24.7	0.6	13.0	0.1	6.04	0.9	3.12	0.9	1.57	0.9	0.809	4.8	0.382	17.1
03-Feb-2011	24.8	0.7	12.9	0.9	6.06	0.8	3.13	0.5	1.59	0.5	0.790	2.1	0.387	14.4
03-Feb-2011	24.8	0.7	12.9	0.8	6.05	2.2	3.13	0.8	1.62	5.3	0.768	8.8	0.395	17.1
08-Feb-2011	25.8	0.0	11.9	1.0	6.32	0.1	3.25	0.2	1.54	7.0	0.783	8.3	0.398	19.6
08-Feb-2011	25.6	1.1	12.0	1.3	6.29	1.7	3.23	1.4	1.55	7.6	0.784	9.4	0.394	12.4
09-Feb-2011	26.2	0.8	11.7	1.7	6.34	1.9	3.20	0.4	1.56	1.6	0.792	7.2	0.385	1.4
15-Feb-2011	24.8	0.5	13.0	0.5	6.01	0.4	3.14	3.3	1.57	1.4	0.824	4.2	0.368	9.2
15-Feb-2011	25.1	0.5	12.4*	56.0*	6.26	2.3	3.07	0.9	1.57	3.2	0.839	5.7	0.361	8.8
01-Mar-2011	26.1	0.5	11.9	0.1	6.30	6.1	3.06	2.3	1.70	19.0	0.785	5.6	0.380	4.1
01-Mar-2011	25.6	0.0	11.9	1.2	6.42	3.8	3.12	2.7	1.76	4.0	0.649	13.2	0.450	6.3
05-May-2011	26.3	0.6	11.8	7.4	6.28	0.2	3.17	2.6	1.61	0.4	0.797	2.7	0.375	13.3
Mean concentration (ng/mL)	25.4		12.4		6.18		3.15		1.59		0.789		0.386	
Accuracy (%)	101.6		99.2		98.9		100.6		101.9		101.0		98.7	
CV (%)	2.3		4.4		2.4		2.2		3.7		5.4		5.2	
n	16		15		16		16		16		16		16	



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Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum

Intra- and inter-run precision and accuracy

QC level	Date of analysis		Run 6 26-Jan-2011 Measured concentration	CV (%)	Run 7 27-Jan-2011 Measured concentration	CV (%)	Run 8 27-Jan-2011 Measured concentration	CV (%)	Inter-run Mean concentration (ng/mL)	Mean Accuracy (%)	CV (%)
QC LLOQ	0.509	Preparation 1	0.528	4.3	0.491	3.3	0.459	3.1	0.489	**96.0**	**4.9**
		Preparation 2	0.477	4.7	0.420	3.2	0.505	5.7			
		Preparation 3	0.496	2.2	0.411	0.2	0.491	2.3			
		Preparation 4	0.558	4.2	0.506	3.3	0.495	2.4			
		Preparation 5	0.515	4.1	0.514	9.2	0.463	2.8			
	Intra-run	Mean concentration (ng/mL)	0.515		0.468		0.483				
		Accuracy (%)	**101.1**		**92.0**		**94.8**				
		CV (%)	**6.0**		**10.5**		**4.2**				
QC MID	7.31	Preparation 1	7.37	0.3	6.10	0.0	7.19	0.4	6.93	**94.8**	**7.8**
		Preparation 2	7.32	0.3	6.14	0.6	7.23	1.0			
		Preparation 3	7.27	1.1	6.14	2.3	6.89	1.1			
		Preparation 4	7.35	0.0	6.25	0.8	7.04	1.1			
		Preparation 5	7.32	0.0	6.91	2.5	7.39	0.4			
	Intra-run	Mean concentration (ng/mL)	7.33		6.32		7.15				
		Accuracy (%)	**100.2**		**86.4**		**97.8**				
		CV (%)	**0.5**		**5.3**		**2.7**				
ULOQ	22.4	Preparation 1	21.4	0.8	19.0	0.3	19.6	0.4	20.4	**91.1**	**6.8**
		Preparation 2	21.8	0.3	19.0	1.5	20.1	0.3			
		Preparation 3	22.9	1.0	19.4	2.0	20.2	1.0			
		Preparation 4	21.8	0.3	19.8	0.9	19.9	1.1			
		Preparation 5	22.0	0.7	19.7	0.0	19.5	0.9			
	Intra-run	Mean concentration (ng/mL)	22.0		19.4		19.9				
		Accuracy (%)	**98.1**		**86.5**		**88.7**				
		CV (%)	**2.5**		**2.0**		**1.5**				

Intra- and inter-run precision: 1.5 – 7.8 %

Intra- and inter-run accuracy: 86.4 – 101.1 %

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Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum *Working range / sensitivity*

- Defined by LLOQ and ULOQ
- Sensitivity based on LLOQ

➢ LLOQ = lowest concentration of biomarker in biological sample that can be quantitatively determined with acceptable precision and accuracy

➢ Example of working range:

  ➢ LLOQ = 0.509 ng/mL in calibration curve, corresponding to 2.55 ng/mL in human serum (taking into account the MRD 1:5)

  ➢ ULOQ = 22.4 ng/mL in calibration curve, corresponding to 112 ng/mL in human serum (taking into account the MRD 1:5)

Milestone 4: Validation

	Biomarker selection		Decision-making biomarker	
				For each dilution factor, at least 5/6 matrices should meet the acceptance criteria. If not, 6 additional matrices should be tested
Dilution linearity	Not assessed (unless biological evidence)		Minimum 3 individual matrices freshly spiked	Mean recovery: 75 – 125 % (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % 2/3 matrices should meet the acceptance criteria
Antibody specificity	Not assessed		Minimum 3 individual matrices	Mean recovery: 75 – 125 % (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % 2/3 matrices should meet the acceptance criteria
Short-term and bench top stability	2 individual neat matrices 3 independent preparations per matrix	Mean recovery: 70 – 130 % CV ≤ 25 % At least 4/6 matrices should meet the acceptance criteria	2 individual neat matrices 3 independent preparations per matrix	Mean recovery: 75 – 125 % (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % At least 4/6 matrices should meet the acceptance criteria
Long-term stability	Not assessed		2 individual matrices 3 independent preparations per matrix	Mean recovery: 75 – 125 % (70 – 130 % at LLOQ and ULOQ) CV ≤ 25 % At least 4/6 matrices should meet the acceptance criteria

Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum

Specificity

Serum	Dilution	Date of analysis: 03-Feb-2011							
		Neat		Spiked with 80.0 ng/mL ANGPTLx			Spiked with 5 000 ng/mL ANGPTLx		
		Adjusted concentration (ng/mL)	CV (%)	Adjusted concentration (ng/mL)	CV (%)	Accuracy (%)	Adjusted concentration (ng/mL)	CV (%)	Accuracy (%)
Serum 1	1:5	32.3	0.6	30.4	0.0	98.0	31.0	1.2	101.0
	1:10	31.8	1.9	30.0	1.0	98.3	30.6	0.2	101.3
	1:20	30.9	0.6	30.3	0.3	102.1	29.4	3.1	100.4
	1:40	30.1	0.7	29.0	0.3	100.4	28.1	3.9	98.3
Serum 2	1:5	28.7	1.5	27.4	1.2	99.4	27.6	1.5	101.2
	1:10	28.5	1.3	27.9	1.3	102.6	27.5	2.7	101.6
	1:20	28.3	0.2	26.7	3.4	98.3	27.2	1.2	101.2
	1:40	26.4	0.3	26.6	4.0	105.0	25.3	4.9	100.9
Serum 3	1:5	80.5	2.4	77.2	1.3	99.9	77.3	0.9	101.1
	1:10	68.7	1.0	65.8	1.5	99.8	67.2	1.3	103.0
	1:20	67.6	3.0	67.4	0.7	103.9	64.1	0.7	99.8
	1:40	65.7	6.0	65.5	2.3	103.8	61.6	2.5	98.7

The antibodies pair used in the assay does not cross-react with human analogue biomarker

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Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum

In some case: drug interference

Three different batches of human serum were spiked with 5.00 mg/mL of the drug and then diluted in sample diluent at the MRD 1:5. The accuracy of ANGPTL determination was assessed.

Accuracy (%): (adjusted concentration of spiked sample / neat adjusted concentration) x 100) . Volume of spiked solution is taken into account in the calculations

		Date of analysis: 05-May-2011				
		Neat		Spiked with 5.00 mg/mL of drug		
Serum	Dilution	Adjusted concentration (ng/mL)	CV (%)	Adjusted concentration (ng/mL)	CV (%)	Accuracy (%)
Serum 1	1:5	39.2	0.6	37.2	2.1	96.8
Serum 2	1:5	30.7	1.4	30.2	2.3	101.4
Serum 3	1:5	91.8	16.3	92.0	9.2	102.3

The drug doesn't interfere with the biomarker quantification

Milestone 4: Validation

Dilution linearity in another case study DKK-1 protein in serum

Human serum	Endogenous level (pg/mL)	Spiked level (pg/mL)	Dilution	Measured concentration (pg/mL)	Adjusted concentration (pg/mL)	Recovery (%)
Serum 1	101.3	25000.0	*1:25*	*>ULOQ*	*>U*	
			1:50	564.6		
			1:100	25		103.5
			1:500		00	109.6
Serum 2	91.5				*>ULOQ*	
				.1	29955	119.4
				247.2	24720	98.5
			.500	57.8	28900	115.2
Serum 3	16 .1	25000.0	*1:25*	*>ULOQ*	*>ULOQ*	
			1:50	555.8	27790	110.4
			1:100	230.8	23080	91.7
			1:500	55.4	27700	110.1

No apparent effect of the dilution was observed (dilutions tested from 1:50 up to 1:500)

Milestone 4: Validation Case study: Angiopoietin-like protein biomarker in human serum

Stability

Date of analysis	Conditions		Measured concentration (ng/mL)	Accuracy (%)	CV (%)
01-Feb-2011	T0	/	6.11 6.14 6.40	/ / /	0.3 0.7 0.2
		Mean (ng/mL) CV(%)	6.22 2.6		
08-Feb-2011	1 week	-80° C	5.88 5.90 5.81	94.6 94.9 93.5	1.9 0.9 0.6
15-Feb-2011	2 weeks	RT	5.61 5.37 5.38	90.2 86.4 86.5	3.5 2.1 1.8
		4° C	5.55 5.46 5.48	89.3 87.8 88.2	0.8 1.1 4.5
		-20° C	5.42 5.39 5.46	87.2 86.7 87.8	0.4 1.2 3.6
		-80° C	5.59 5.55 5.60	89.9 89.3 90.1	1.7 0.8 1.0
01-Mar-2011	1 month	4° C	5.82 5.62 5.40	93.6 90.4 86.9	1.3 0.9 2.4
		-20° C	6.27 6.22 6.10	100.9 100.1 98.1	1.6 2.5 1.5
		-80° C	6.28 6.41 6.33	101.0 103.1 101.8	0.8 0.7 0.7
05-May-2011	3 months	-20° C	6.65 6.38 6.22	107.0 102.6 100.1	2.3 0.3 0.8
		-80° C	6.72 6.61 6.67	108.1 106.3 107.3	1.4 2.0 0.2

Biomarker in human serum is stable at RT for 2 weeks, +4°C for 1 month, -20°C and -80°C for at least 3 months

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Conclusion

- Described implementation of bioanalytical study plan for the validation of immunoassays to support decision making biomarkers and biomarker selection
  - Establishes clear and complete operating procedure as well as parameters and their respective acceptance criteria
  - Proposes milestones and decision point to be followed during assay validation
- Application of such bioanalytical study plan should result in high quality data in a limited timeframe and with reduced costs
- Parallelism assessment is identified as a key milestone
- Bioanalytical study plan successfully applied to the validation of immunoassay based on classical monoplex ELISA, automated analyzer (Elecsys), multiplex planar array platform (MesoScale Discovery), multiplex suspension microbead platform (Luminex) and flow-based single molecule counting Erenna platform (Singulex)

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