Tricky analyte, challenging matrix and a new high sensitivity analytical platform:

How to overcome major challenges for a successful biomarker assay validation on the SMCxPRO® platform

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Roche pRED

Challenging the Quantification Limit of LBAs: Case Study

Introduction

Tricky analyte, challenging matrix

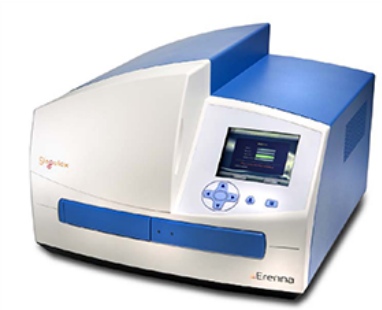
- Biomarker for neurodegenerative disease in human cerebrospinal fluid (CSF)
- Analyte heterogenous in length among patients & prone to aggregation
- Low pg/mL concentration (low fM range)
- Exploratory assay available on the Erenna® platform (Merck) in a research laboratory

Context of use: core surrogate biomarker in late stage clinical development:

- Assay transfer to a regulated laboratory mandatory
- Full assay validation required
- Highest possible sensitivity to be achieved

Assay Transfer Strategy

Research lab (CRO)
Assay qualification



Erenna®:
end of support
announced for 2021

Q1 - Q2 2019

failed

Q3 2019 - Q2 2020

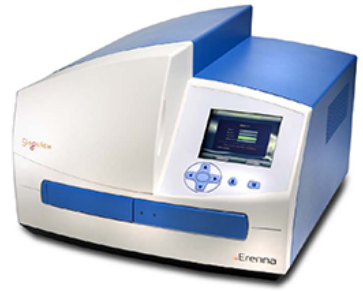
Roche internal Reg BA lab
Establishment of platform
Full assay validation

Regulated lab (CRO)
Full assay validation
Clinical sample analysis

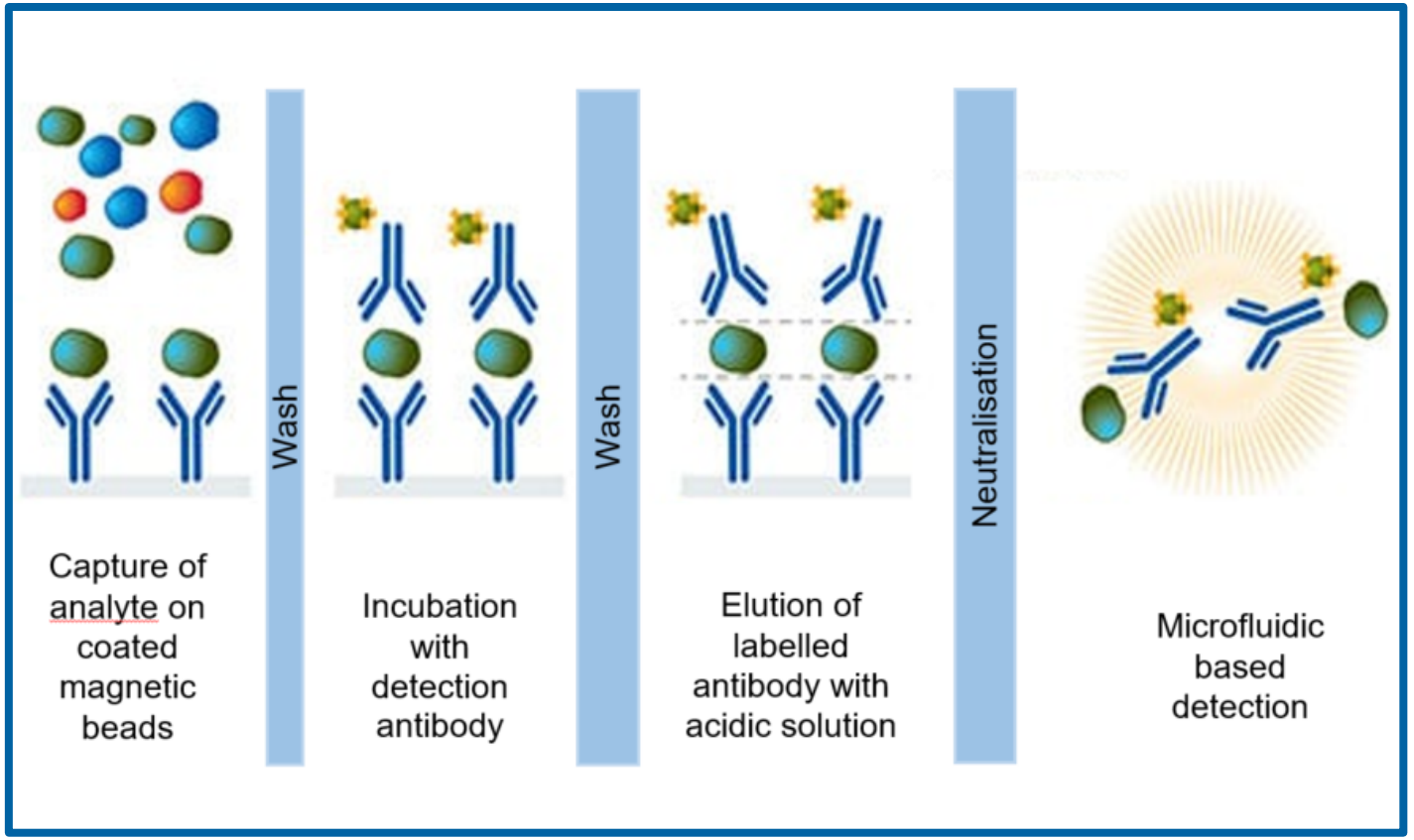
SMCxPro®
introduced in 2018

Single Molecule Counting Technology

Erenna® Platform



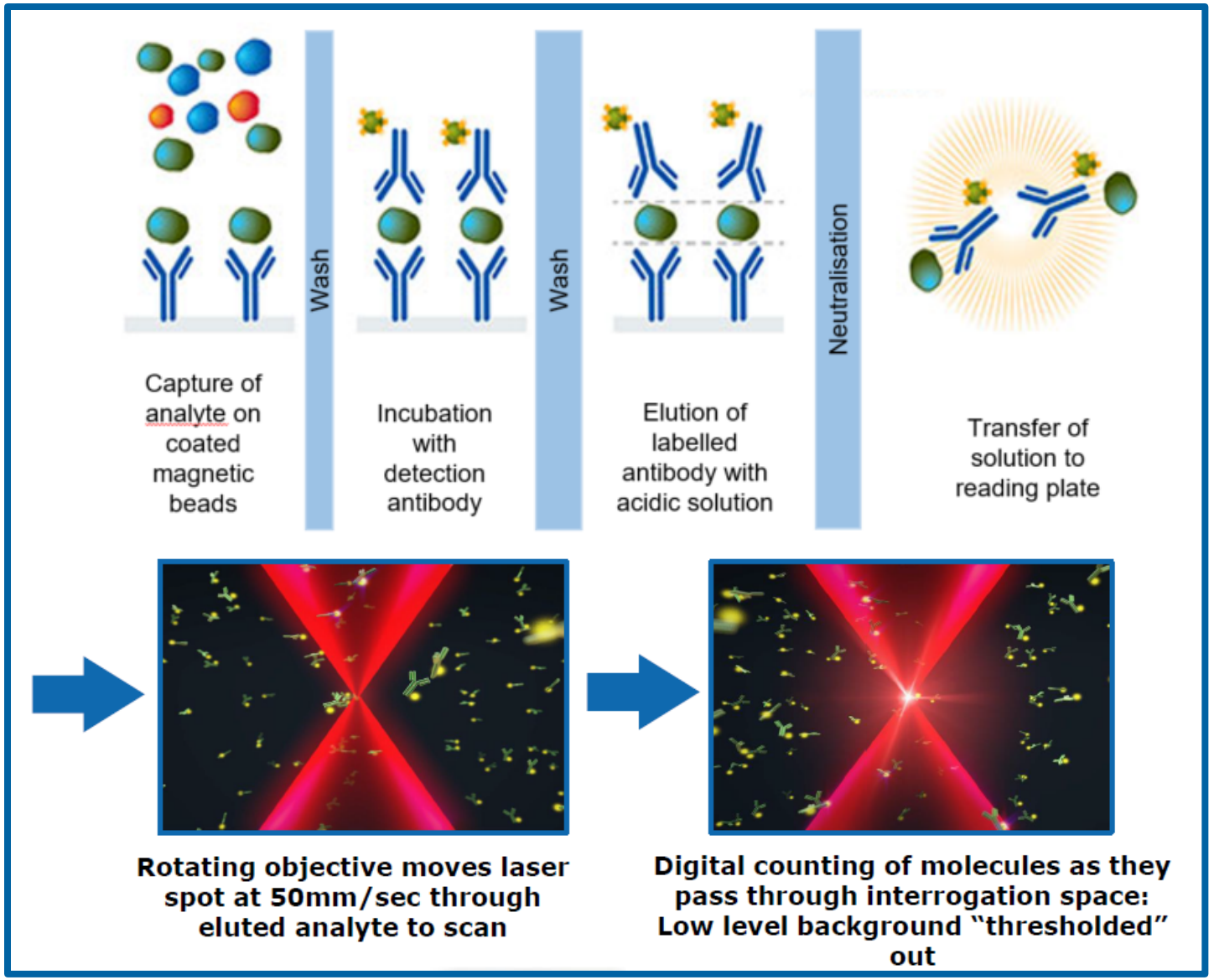
- Ultra high sensitivity platform originally developed by Singulex®
- Manual bead-based sandwich immunoassay in 96-well plate format
- Elution of detection antibodies from immune complexes
- Quantification via capillary flow fluorescence detection



Single Molecule Counting Technology

SMCxPro® Platform

- Same bead-based immunoassay as Erenna® (identical kits)
- Readout in 384-well plate with a rotating laser allowing for individual photon counting
- Sophisticated laser optic: daily self-calibration and weekly external calibration of instrument

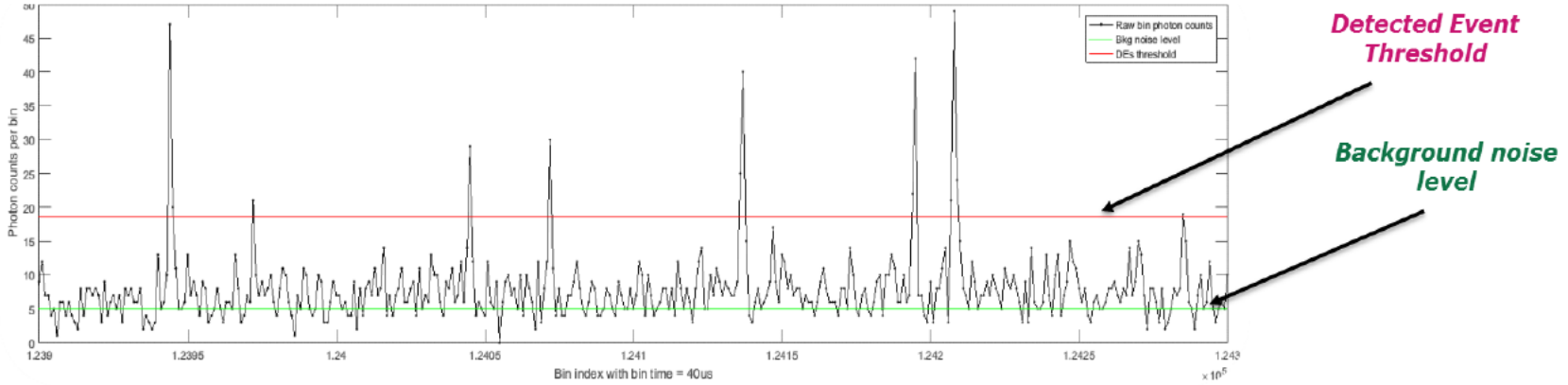


Robustness of the SMCxPRO® Platform

Device Installation Jun – Oct 2019

Technical challenges

- No proper assay signals obtained during on site analyst training using IL6 assay kit
- Red flags popped up at calibration: several visits of engineer required for laser & software adjustment
- The fixed, automatic distinction between background noise and event (event threshold) lead to artifacts at very low signal levels. Demo software update offered.



→ Intense information exchange with Merck team to solve issues

Robustness of the SMCxPRO® Platform

Device Performance Monitoring

Systematic checks implemented to discriminate between assay problems and device related issues

- Reading plate (96-well quadrant) loaded with unique concentration of detection antibody providing a low assay signal and stored refrigerated for 1 month
- Daily analysis and signal precision across plate calculated
- Plate precision ranged between 6-11%; higher compared to ELISA

Detection Antibody diluted 1: 200'000'000

37.81	40.23	50.59	47.68	44.26	49.21	41.53	49.94	50.61	42.23	45.18	47.84
45.05	44.53	44.58	44.73	48.25	47.58	45.53	46.07	43.72	39.18	45.01	44.82
44.42	37.55	46.35	46.83	49.08	48.98	42.52	45.07	49.02	46.27	48.14	48.06
45.78	44.32	50.12	42.92	48.86	41.89	48.81	53.93	43.96	49.23	48.56	51.22
49.41	46.10	47.12	45.10	49.65	48.62	43.06	53.10	51.91	43.28	49.76	51.21
43.30	40.19	44.97	46.72	46.34	47.31	45.92	54.79	48.04	44.22	42.11	48.01
48.77	48.22	41.16	48.04	48.21	50.58	43.42	48.51	49.04	45.12	45.66	46.00
47.00	48.66	40.30	42.27	47.25	47.73	44.71	40.42	42.61	42.25	41.71	45.50

Mitigation: Samples analyzed in triplicates, possibility to remove one outlier, replicate precision to be ≤20%

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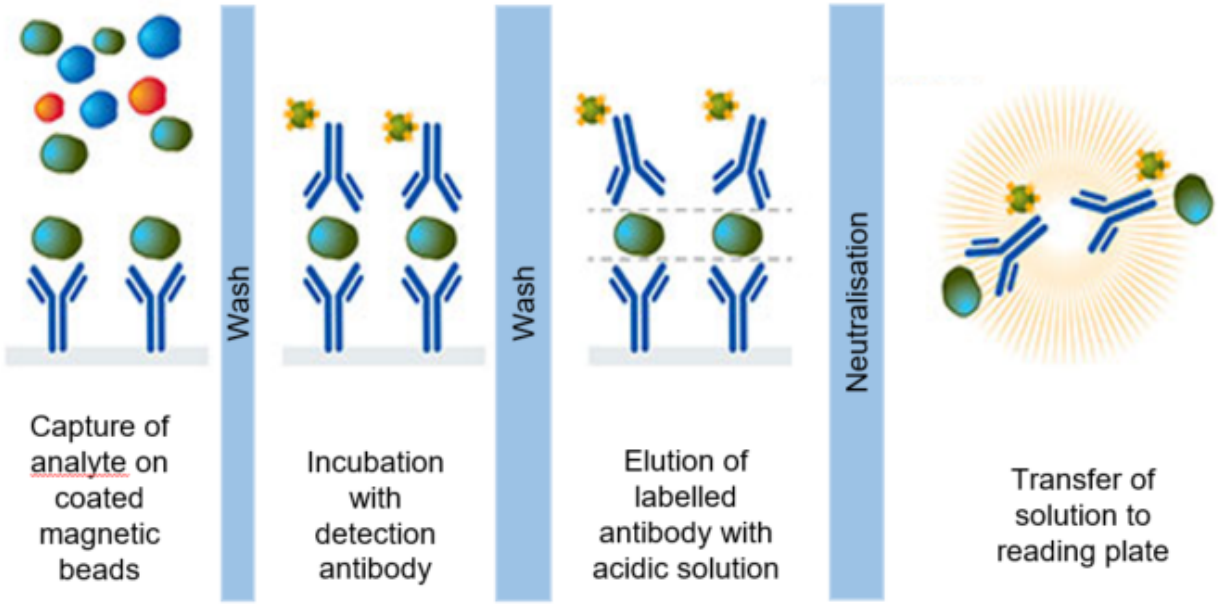
Robustness of the SMCxPRO® Platform

Pipetting & Bead Handling

Non-automated high sensitivity assay

- Each assay step crucial
- Visual check of proper bead peletting & resuspension is essential
- Pipetting out of biosafety cabinet to avoid air flows (except biosample preparation)



Proper resuspension of bead stock (volume of aliquots)

Pipetting of bead solution on 96 well plate with manual pipette instead of electronic pipettes (multi-stepper) to avoid plate inhomogeneity

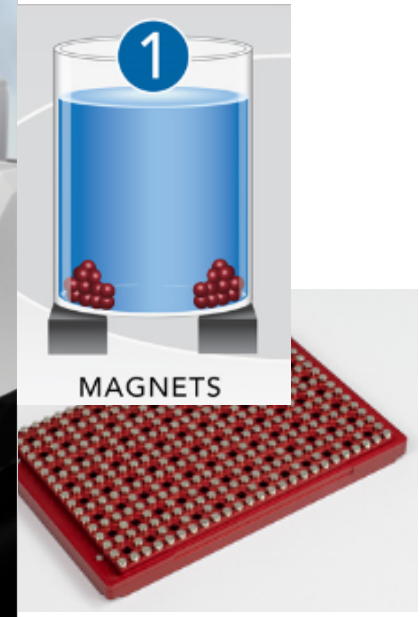
Homogenization of bead solutions during incubation steps: homogenous shaking over the plate to be ensured (selection of shaker crucial)

Accuracy of plate transfer (10 µL out of 12 µL!)

Robustness of the SMCxPRO® Platform

Microplate Washer Technology

- SMCxPro delivered with BioTek 405 TS Plate Washer: aspiration washer used with a magnetic carrier plate
- Washer settings pre-adjusted at manufacturing but not re-adjusted/checked at installation (Merck engineers now trained)
- Device adjusted by BioTek in Nov 2019: performance improved but still inhomogeneity throughout plate



24.86	15.42	20.93	27.73	18.27	22.31	26.37	21.25	23.09	20.21	22.66	20.33
24.55	25.00	20.80	25.43	25.49	24.37	21.71	23.51	23.95	27.90	28.28	27.06
24.36	21.81	25.29	25.18	26.69	23.45	20.88	25.14	20.68	28.01	23.24	30.79
28.76	26.72	31.52	31.14	29.19	28.37	25.91	28.52	25.37	36.19	31.64	32.49
29.13	21.38	27.04	28.16	26.94	25.01	20.34	23.18	22.25	29.76	30.09	25.42
28.59	30.48	35.78	28.12	34.63	32.71	32.87	34.18	32.88	31.50	34.17	32.14
21.31	19.52	31.92	20.89	30.60	26.23	21.66	26.11	20.78	21.82	21.87	22.39
25.74	28.14	37.14	31.77	27.61	34.34	32.37	38.81	29.91	33.00	31.36	31.30

Assay signal, unique concentration of analyte loaded all over the plate

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Robustness of the SMCxPRO® platform

Microplate Washer Technology

- Centrifugal Blue®Washer designed for cell- and bead-based assays
- Wash step example:
  - Plate 2 min on magnetic carrier
  - Buffer spinned out at 800 rpm
  - 2 cycles of buffer dispense & centrifugation
- Optimization of wash programm required
- Better signal homogeneity obtained: Blue®Washer used from there

Robustness of the SMCxPRO® Platform

Accuracy and Precision Data After Optimization – Research Grade Reagents

	1	2	3	4	5	6	7	8	9	10	11	12
A	STD1	STD1	STD1							HQC	HQC	HQC
B	STD2	STD2	STD2							MQC	MQC	MQC
C	STD3	STD3	STD3							LQC	LQC	LQC
D	STD4	STD4	STD4									
E	STD5	STD5	STD5									
F	STD6	STD6	STD6							HQC	HQC	HQC
G	STD7	STD7	STD7							MQC	MQC	MQC
H	STD8	STD8	STD8							LQC	LQC	LQC

Calibration samples

Run Date	Nom Conc [pg/mL]	0.00	1.63	4.08	10.2	25.6	64.0	160	400	S/N at LLOQ	S/N at ULOQ
19-Dec-2019	Signal [RU]	1.60	5.47	10.6	23.1	56.2	114	260	407	3.4	254
	Conc [pg/mL]		1.63	4.09	10.1	26.7	59.6	174	386		
07-Jan-20	Signal [RU]	1.84	5.59	10.5	27.6	69.1	165	437	828	3.0	450
	Conc [pg/mL]		1.67	3.88	10.70	26.0	60.4	169	394		
07-Jan-20	Signal [RU]	NV	7.80	15.7	35.2	91.6	186.0	550.0	1477	NA	NA
	Conc [pg/mL]		1.59	4.18	10.4	27.8	56.2	162	423		
09-Jan-20	Signal [RU]	2.83	6.39	12.7	29.3	72.1	169	409	1184	2.3	418
	Conc [pg/mL]		1.62	4.1	10.4	26.5	62	150	428		
10-Jan-20	Signal [RU]	2.02	4.37	7.7	16.7	37.7	85.9	230	601	2.2	298
	Conc [pg/mL]		1.67	4.0	10.3	25.3	59.7	163	431		
17-Jan-20	Signal [RU]	2.02	8.12	16.6	35.5	82.3	188	566	1272	4.0	630
	Conc [pg/mL]		1.57	4.3	10.4	25.2	58.6	176	397		
20-Jan-20	Signal [RU]	3.09	8.22	16.9	32.6	90.6	220	488	1343	2.7	435
	Conc [pg/mL]		1.61	4.4	9.3	27.3	67.4	150	411		
21-Jan-20	Signal [RU]	3.54	11.1	18.8	45.7	102.0	300	749	1730	3.1	489
	Conc [pg/mL]		1.64	4.0	10.9	24.1	66.1	160	399		
21-Jan-20	Signal [RU]	6.19	11.5	22.3	67.2	164.0	388	954	2320	1.9	375
	Conc [pg/mL]		1.72	3.60	11.2	27.1	63.5	156	396		
Mean Conc [pg/mL]			**1.64**	**4.05**	**10.4**	**26.2**	**61.5**	**162**	**407**		
Interbatch Accuracy [%]			**100.4**	**99.3**	**102.1**	**102.4**	**96.1**	**101.4**	**101.8**		
Interbatch Precision [%]			**2.8**	**5.8**	**5.2**	**4.5**	**5.9**	**5.8**	**4.0**		
Total error [%]			**3.2**	**6.5**	**7.2**	**6.9**	**9.8**	**7.2**	**5.9**		

QC samples

	Back-calculated concentrations at QC level [pg/mL]		
Run Date	**LQC**	**MQC**	**HQC**
	4.50	**40.00**	**300**
19-Dec-19	4.56	38.8	290
	4.16	45.0	258
07-Jan-20	5.16	46.5	308
	4.95	46.1	304
7-Jan-20	4.74	40.3	312
	6.21	47.2	301
9-Jan-20	6.37	32.1	321
	4.39	40.8	299
10-Jan-20	5.06	40.0	295
	4.88	44.1	336
20-Jan-20	5.69	43.5	364
	5.28	48.4	337
21-Jan-20	4.65	40.0	352
	4.64	45.5	325
21-Jan-20	4.09	37.1	297
	4.86	37.5	284
Mean Conc [pg/mL]	**4.98**	**42.1**	**311**
Interbatch Accuracy [%]	**110.7**	**105.1**	**103.8**
Interbatch Precision [%]	**13.1**	**10.6**	**8.6**
Total error [%]	**23.8**	**15.7**	**12.4**

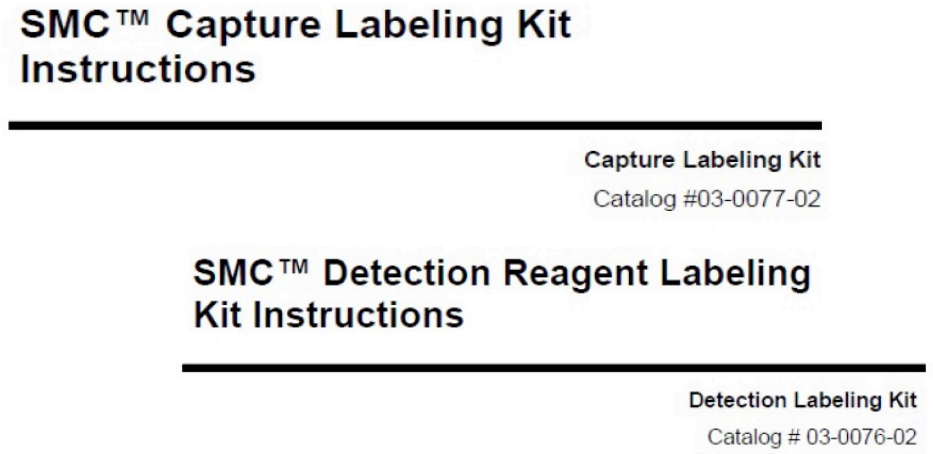
QC sample data acknowlege for signal homogeneity over the plate

Towards Assay Validation

Labeling of Critical Reagents

Initial procedure: use of SMC labeling kits

- Black box approach, no quality control for product available
- Batch-to-batch variability observed



Optimization at Roche Diagnostics

- Analytical characterization before and after labeling (purity, incorporation rate, fluorescence emission, functional testing)
- Buffer exchange/desalting optimized
- Variation of labeling ratio to enhance S/N ratio in assay

Performance of labeled antibodies

- S/N ratio at LLOQ
  - Labeling with kits: 2 to 4
  - Optimized reagents: 5 to 7
- Long term stability assessed
- CoA generated

Towards Assay Validation

Assay Matrix

Sample collection

- Sample analysis in triplicates, 135 µL sample per well: 500 µL aliquots required
- Samples shock frozen directly after preparation to ensure analyte integrity

Surrogate assay matrix

- Large volume of rare matrix: surrogate assay matrix for preparation of calibration and QC samples
- Ready to use artificial CSF

Human CSF

- Commercially available human CSF issued from leftover samples: uncontrolled chain of custody
- Samples gave high background → selectivity demonstrated via parallelism assessement

Towards Assay Validation

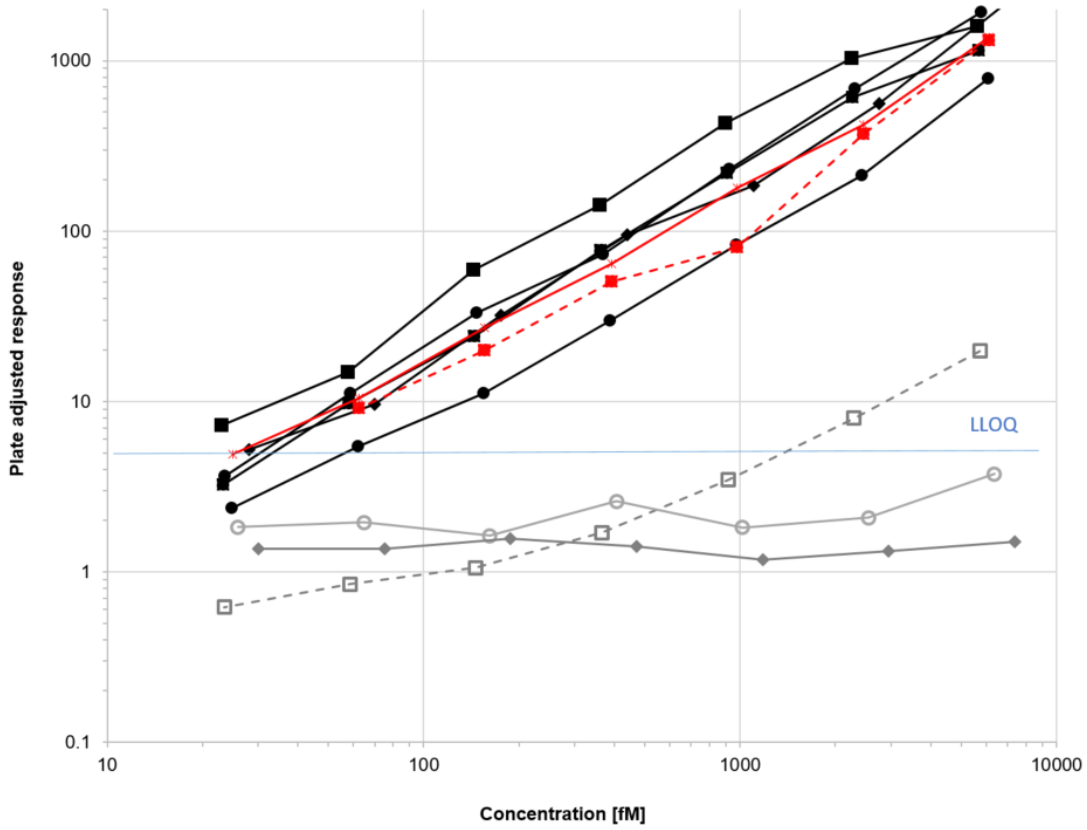
Reference Standard

- **Analyte heterogenous in length among patients**: surrogate reference standard of defined length

- **Suitability of surrogate reference standard** demonstrated on
  - recombinant fragments: response of fragments of different lengths parallel to reference standard curve

    → **relative quantitative assay**

  - patient samples via parallelism experiment



Red: Surrogate reference standard (different lots)

Black: Representative recombinant forms of analyte

Grey: Wild type fragments (not detected at relevant concentrations)

Towards Assay Validation

Validation Strategy

Mitigation of assay variability

- Samples analyzed in triplicates, possibility to remove one outlier, precision to be ≤20%
- Acceptance criteria extended from ±20%/±25% A&P to ±30% based on pre-validation data

Validation parameters

- Inter- and intra-assay accuracy and precision on QC samples (surrogate matrix)
- Inter-assay precision on patient samples
- Parallelism on patient samples
- Determination of LOD
- Plate homogeneity
- Hook effect
- Interferences
- Stability in surrogate matrix
- Incurred sample stability

Validation results

- In house validation completed within one month. All pre-set criteria met. Target LLOQ validated

Challenging the Quantification Limit of LBAs: Case Study Conclusion

- Discrimination between technical and analytical challenges allowed for successful assay optimization & validation
- SMCxPro platform requires lab excellence:
  - extended control of devices
  - experienced & trained analysts
- Communication between manufacturers and labs key factor for implementation of new analytical platforms
- Deep in-house assay understanding allowed for efficient trouble shooting at CRO: external assay validation successfully completed

GxP lab (CRO)
Full assay validation ✓

Q1-Q3 2020

Acknowledgement

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