EBF Spring Focus Workshop 2023
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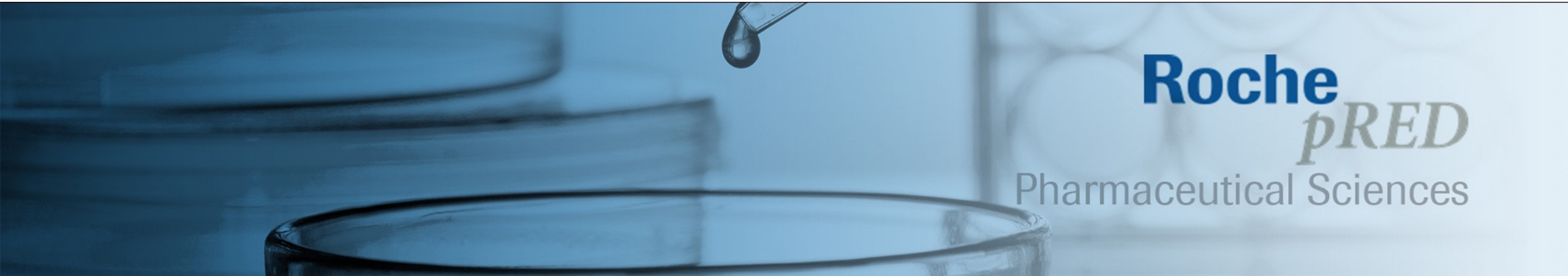
Selectivity and sensitivity challenges in the bioanalysis of oligonucleotides and their metabolites

Luca Ferrari
BioAM Bioanalytics and Biomarkers, pRED Pharmaceutical Sciences, Roche Innovation Center Basel – Switzerland



Outline

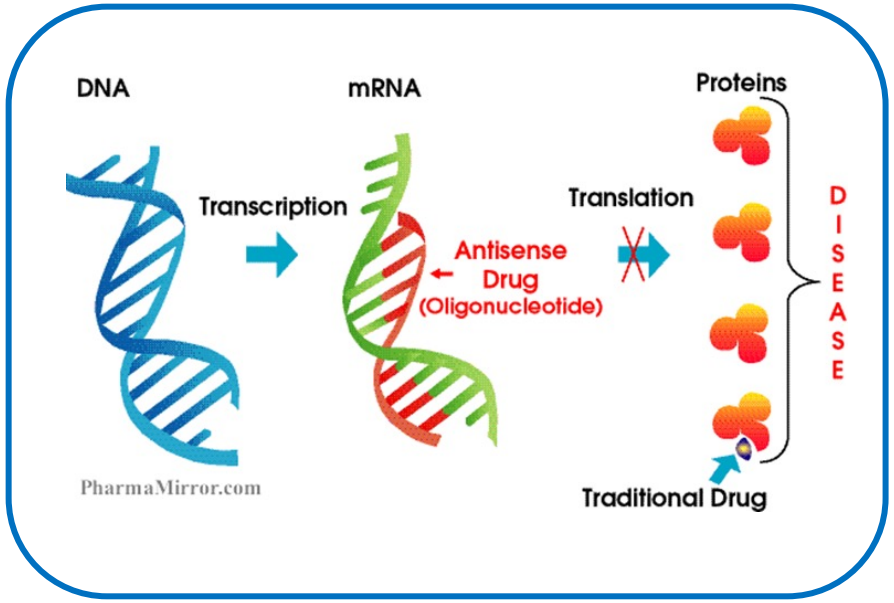
- State of the art technologies for quantitation of oligonucleotides
- Sensitivity/specificity challenges and possibility of switching technologies
- Quantification of metabolites
- A proposed bioanalytical strategy for technology selection
- Case studies

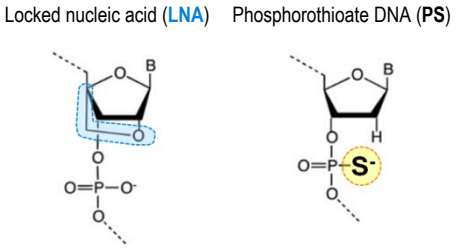
RNA-targeting oligonucleotide therapeutics

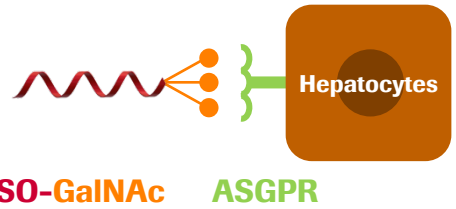
Single Stranded Oligonucleotides (SSOs)

Frequent chemistry modifications:

- **L**ocked **N**ucleic **A**cid (**LNA**)
- Phosphorothioate (**PS**) linkage
- Conjugation with GalNAc (N-Acetyl Galactosamine)

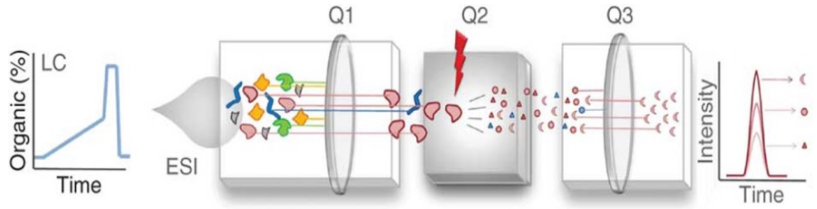




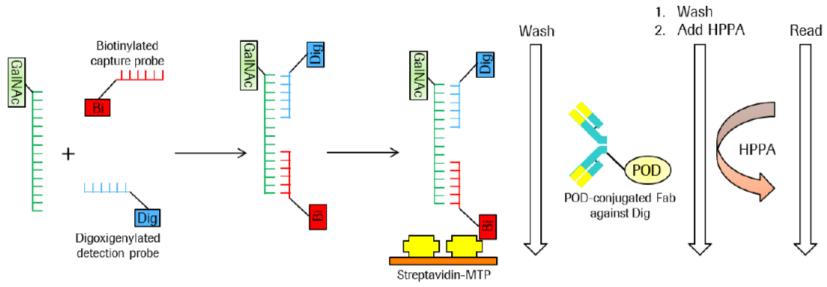


Bioanalysis of oligonucleotides – technologies

- **LC–MS**
  - Reagent free
  - Specific and selective
  - Sensitivity may be a limiting factor

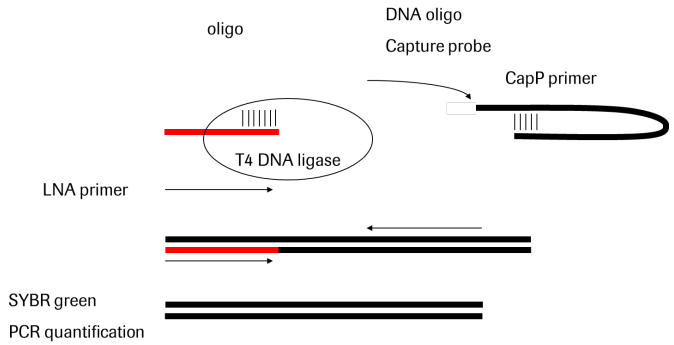


- **h-ELISA**
  - Requires specific reagents (capture, detection probes)
  - Highly sensitive
  - Narrow dynamic range

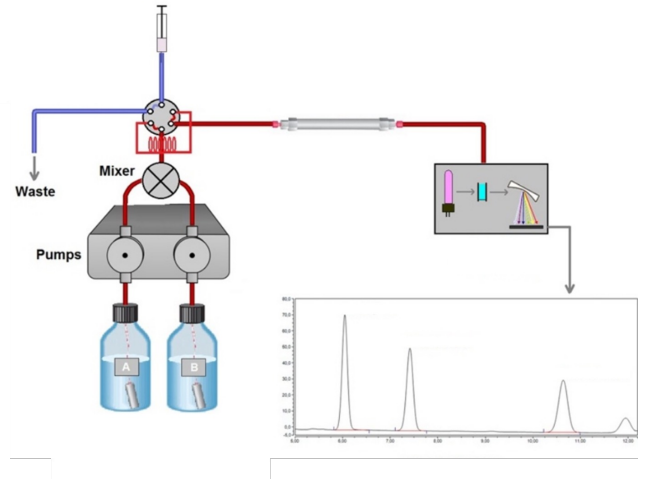


Bioanalysis of oligonucleotides – technologies

- **PCR, MOL-PCR**
  - Used for oligonucleotides, DNA, mRNA
  - High sensitivity
  - Very wide dynamic range (up to 6 orders of magnitude)
  - Limited experience in regulated settings

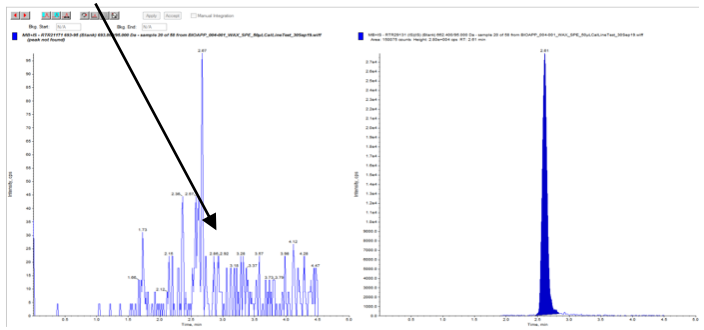


- **AEX LC-UV or AEX LC-FL**
  - Specific and selective
  - Sensitivity is a limiting factor
  - Allows quantification of metabolites in the lack of a reference standard

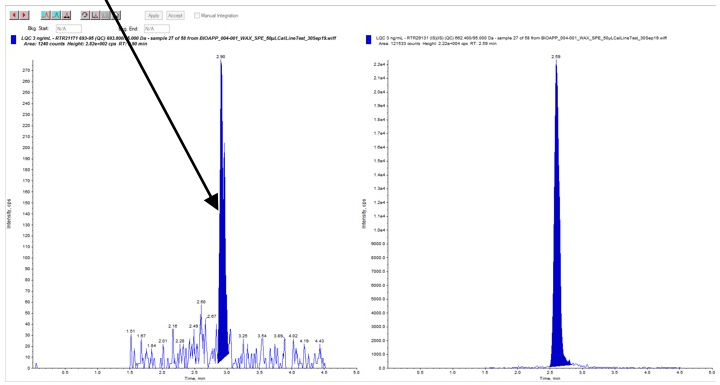


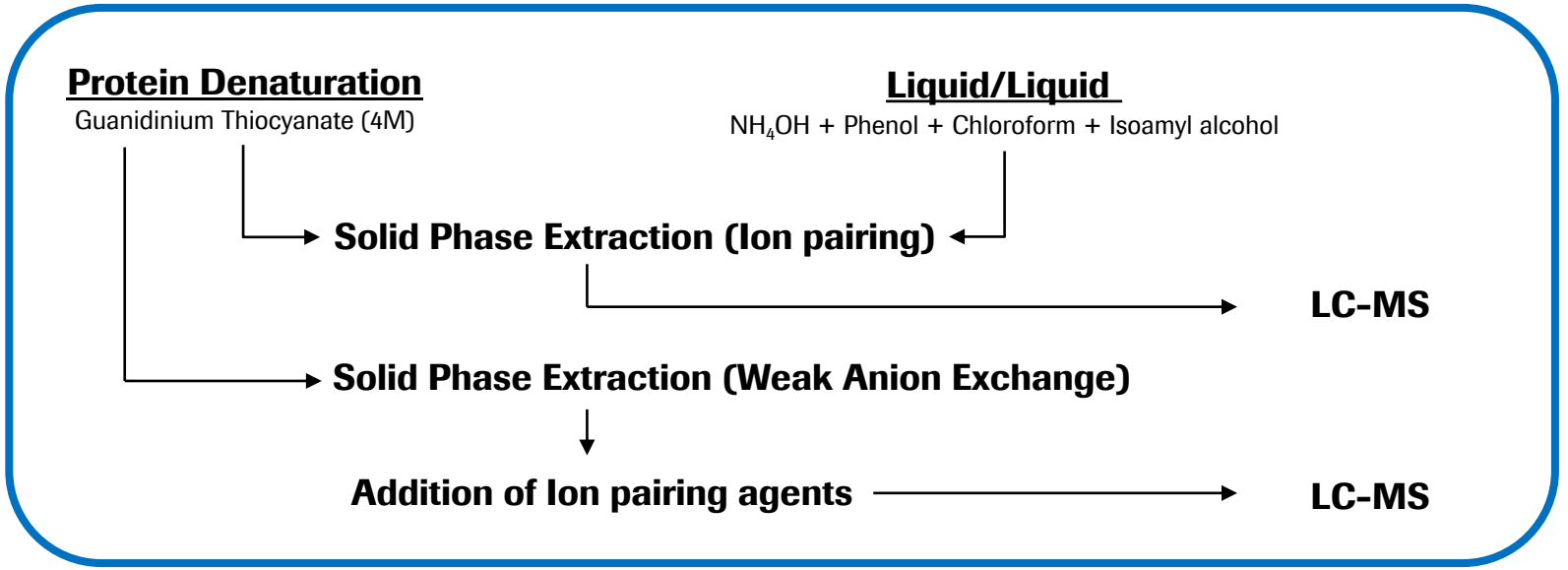
Example of a state of the art LC-MS method

Matrix Blank + IS



LLOQ: 0.250 ng/mL





Analytical Column (AC)	Xbridge Premier Oligo BEH C18 Column, 130A, 2.5 µm, 50 x 2.1mm (Waters)
Mobile Phase A	Water:Acetonitrile:HFIP:DIPEA 95:5:1:0.25 (v:v:v:v)
Mobile Phase B	Water:Acetonitrile:HFIP:DIPEA 60:40:1:0.25 (v:v:v:v)

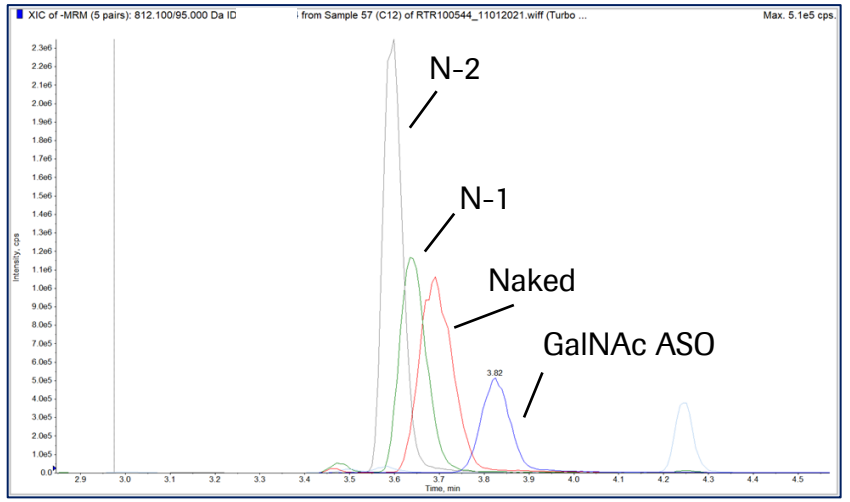
Precision and accuracy data

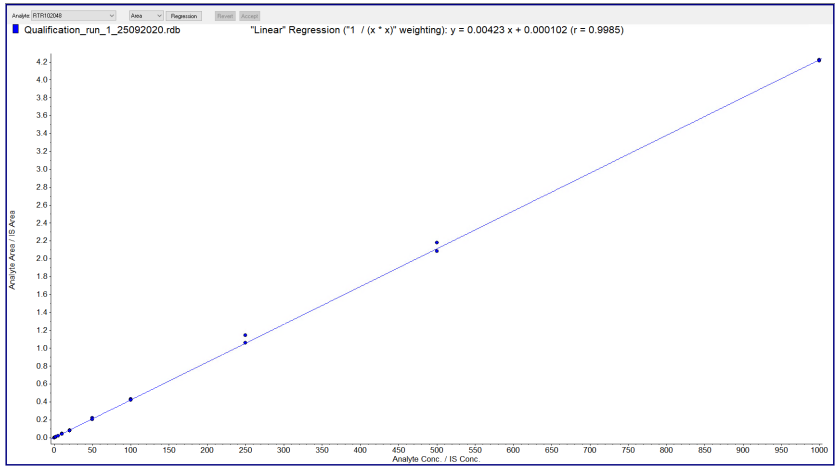
plasma

	LLOQ QC 0.250 ng/mL	QC 0.500 ng/mL	QC 1.00 ng/mL	QC 10.0 ng/mL	QC 100 ng/mL	QC 900 ng/mL
	0.273	0.468	1.01	9.63	99.6	919
	0.262	0.483	1.02	9.69	101	920
	0.221	0.454	0.957	9.83	105	968
	0.250	0.487	1.01	10.1	109	966
	0.264	0.477	1.01	10.1	99.9	985
	0.242	0.456	1.01	9.86	98.8	1000
Average	0.252	0.471	1.00	9.87	102.0	960
Within run precision	**7.4**	**2.9**	**2.3**	**2.0**	**3.9**	**3.5**
Within run accuracy	**100.8**	**94.2**	**100.3**	**98.7**	**102.0**	**106.6**

CSF

	LLOQ QC 0.250 ng/mL	QC 0.500 ng/mL	QC 1.00 ng/mL	QC 10.0 ng/mL	QC 100 ng/mL	QC 900 ng/mL
	0.256	0.472	0.892	8.75	96.5	864
	0.269	0.462	0.906	9.34	85.5	859
	0.251	0.460	0.905	9.15	92.7	835
	0.263	0.435	0.852	8.90	90.7	872
	0.225	0.426	0.901	9.25	92.4	915
	0.279	0.455	0.900	9.13	90.4	872
Average	0.257	0.452	0.89	9.09	91.4	870
Within run precision	**7.2**	**3.9**	**2.3**	**2.4**	**3.9**	**3.0**
Within run accuracy	**102.9**	**90.3**	**89.3**	**90.9**	**91.4**	**96.6**

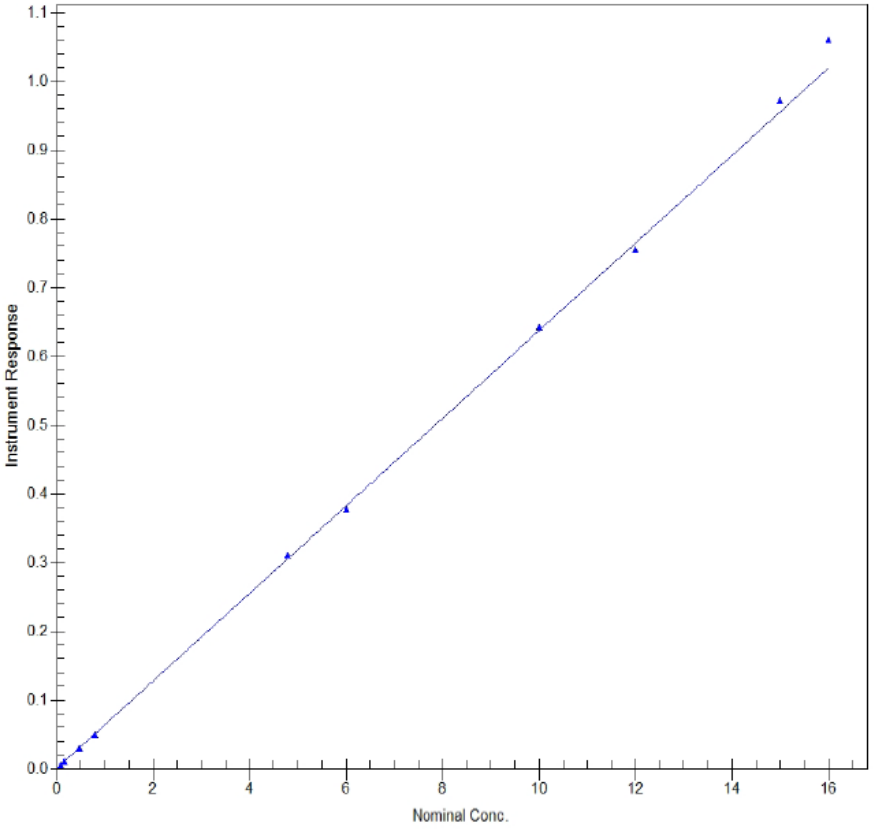




Method qualified in plasma, CSF and tissue homogenates (liver, kidney).
Shortmers also included.

Precision and accuracy data

	QC_LLOQ 0.0800 nM	QC_L 0.240 nM	QC_M 8.00 nM	QC_H 14.0 nM
	0.0782	0.24	7.82	13.7
	0.0805	0.22	7.64	13.4
	0.0776	0.23	7.73	13.7
	0.0807	0.23	7.73	13.7
	0.0725	0.23	7.87	13.4
	0.0749	0.23	7.81	13.5
Intrarun Mean	0.0774	0.23	7.77	13.6
Intrarun SD	0.0032	0.01	0.08	0.2
Intrarun % CV	**4.1**	**3.5**	**1.03**	**1.2**
Intrarun % Bias	**-3.2**	**-3.4**	**-2.9**	**-3.2**
n	6	6	6	6
Inter-run SD	0.0050	0.02	0.92	0.7
Inter-run % CV	**6.4**	**10.2**	**12.1**	**5.0**
Inter-run % Bias	**-2.2**	**-0.3**	**-4.9**	**-1.2**
n	18	18	18	18



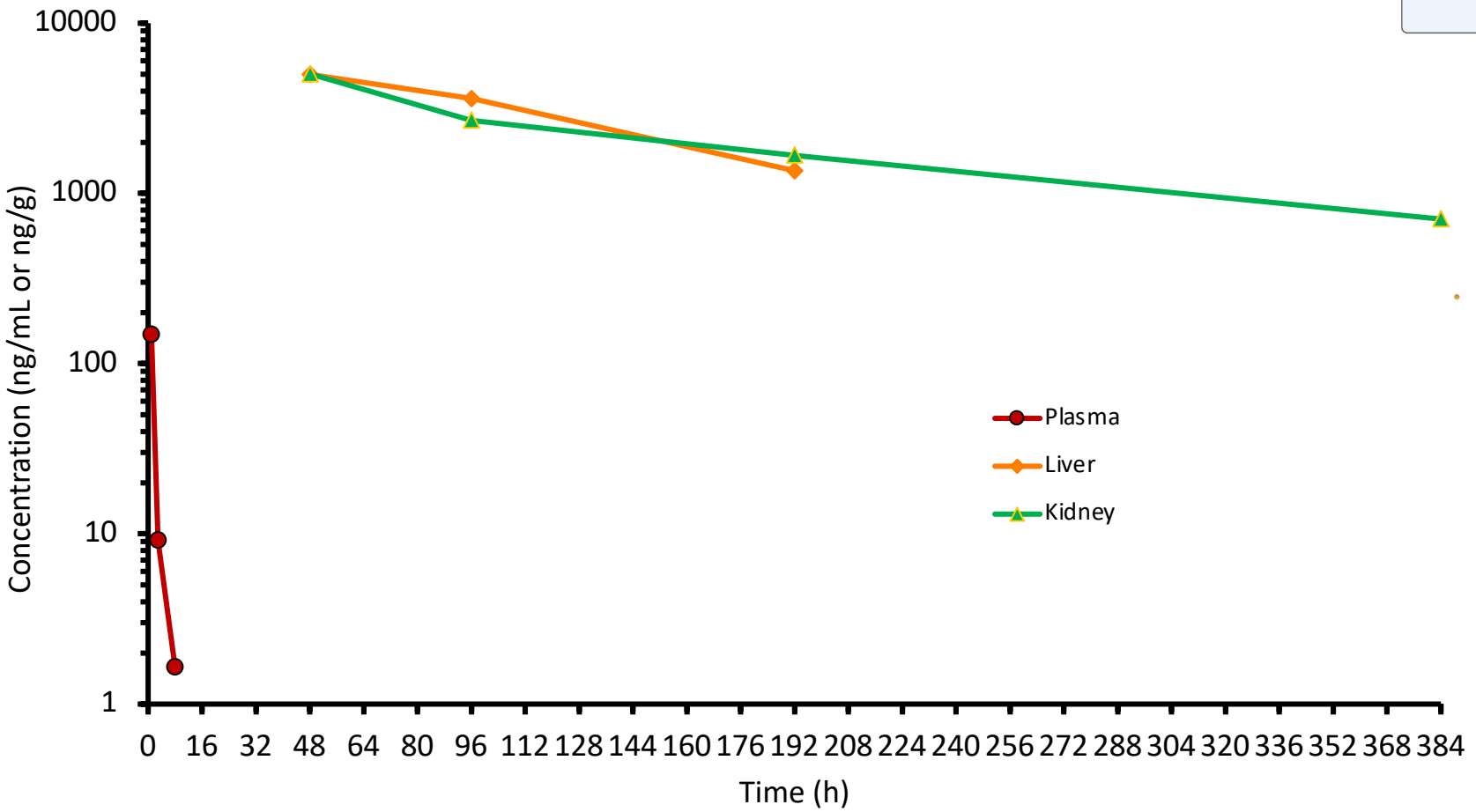
Method validated in cynomolgus monkey plasma and CSF
Method qualified in tissues (liver and kidney)

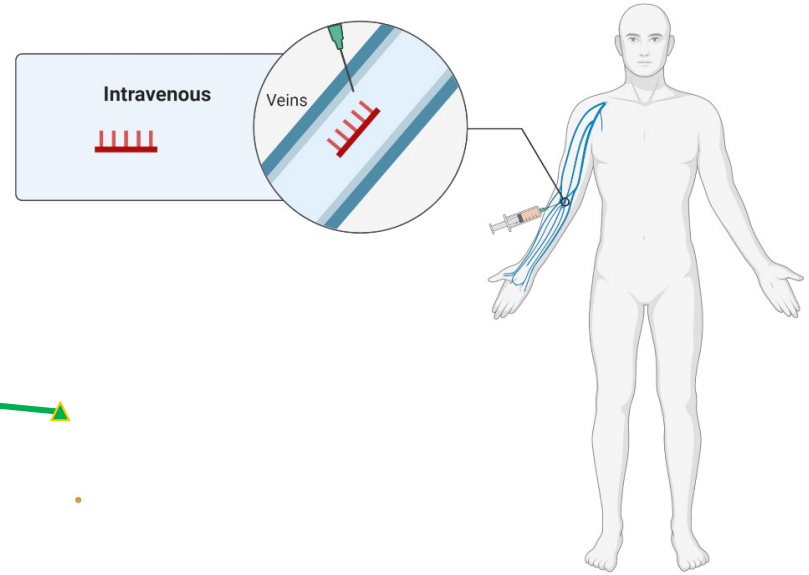
Sensitivity challenges

In order to link the concentration at the therapeutic site to an effect, **it is important to measure the concentration of ASOs in different matrices** (e.g. plasma and tissues).

- **Sensitivity can be challenging in certain matrices**, e.g. in plasma when the administration of the ASOs is not systemic.
- The LC-MS sensitivity is usually **sufficient to support preclinical studies**, particularly for analysis of **tissues** where the ASOs exhibit their efficacy and are present at high concentrations.
- The sensitivity might not be sufficient in the **clinical phase** when the administered doses and the observed exposures are expected to be lower.

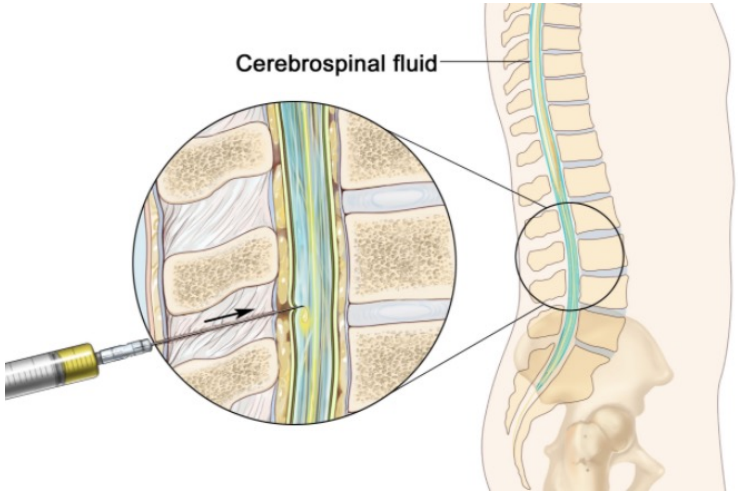
Sensitivity challenges – systemic administration

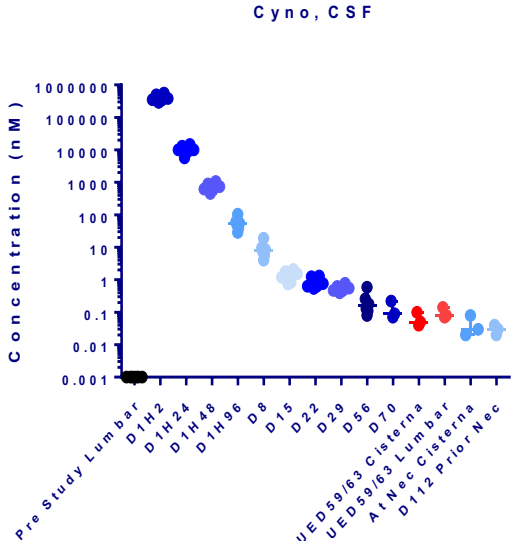


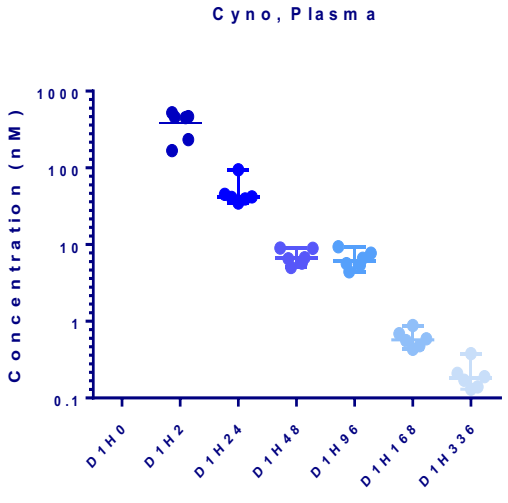


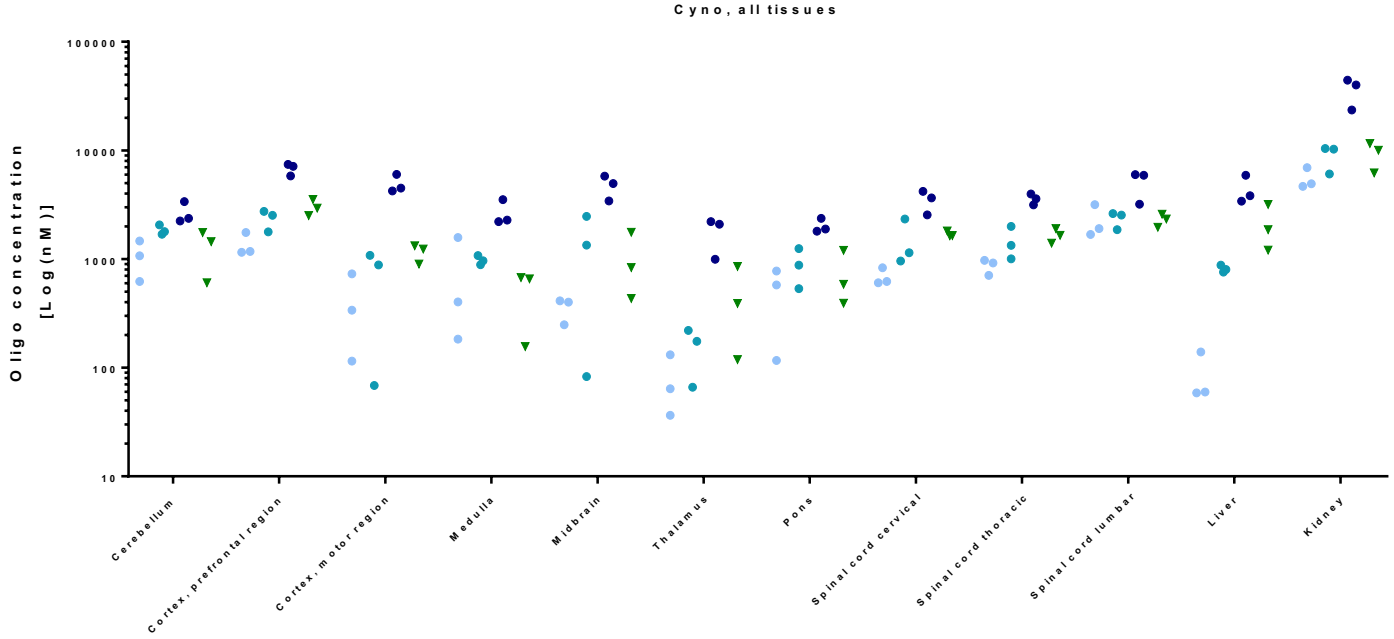
Sensitivity challenges: intrathecal administration

The question:
in case the LC-MS sensitivity in a given matrix is not sufficient, can another technology be used for quantitation?



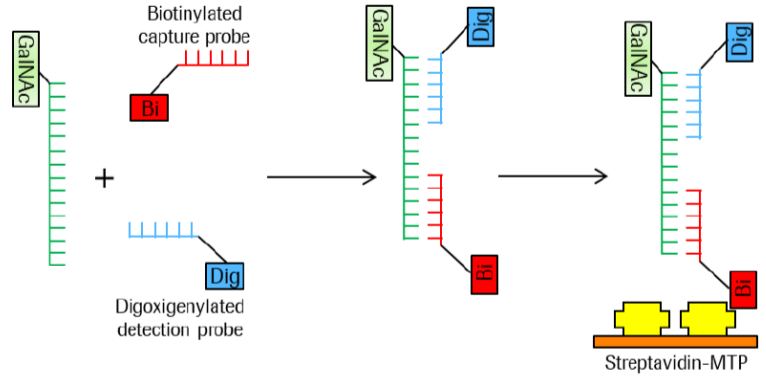


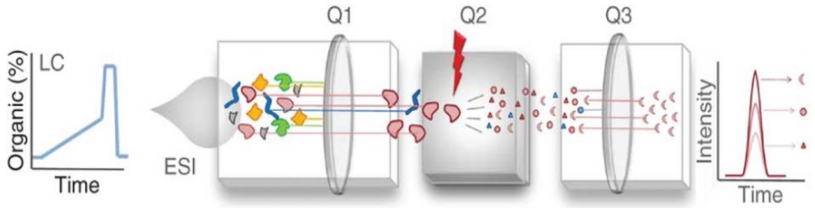




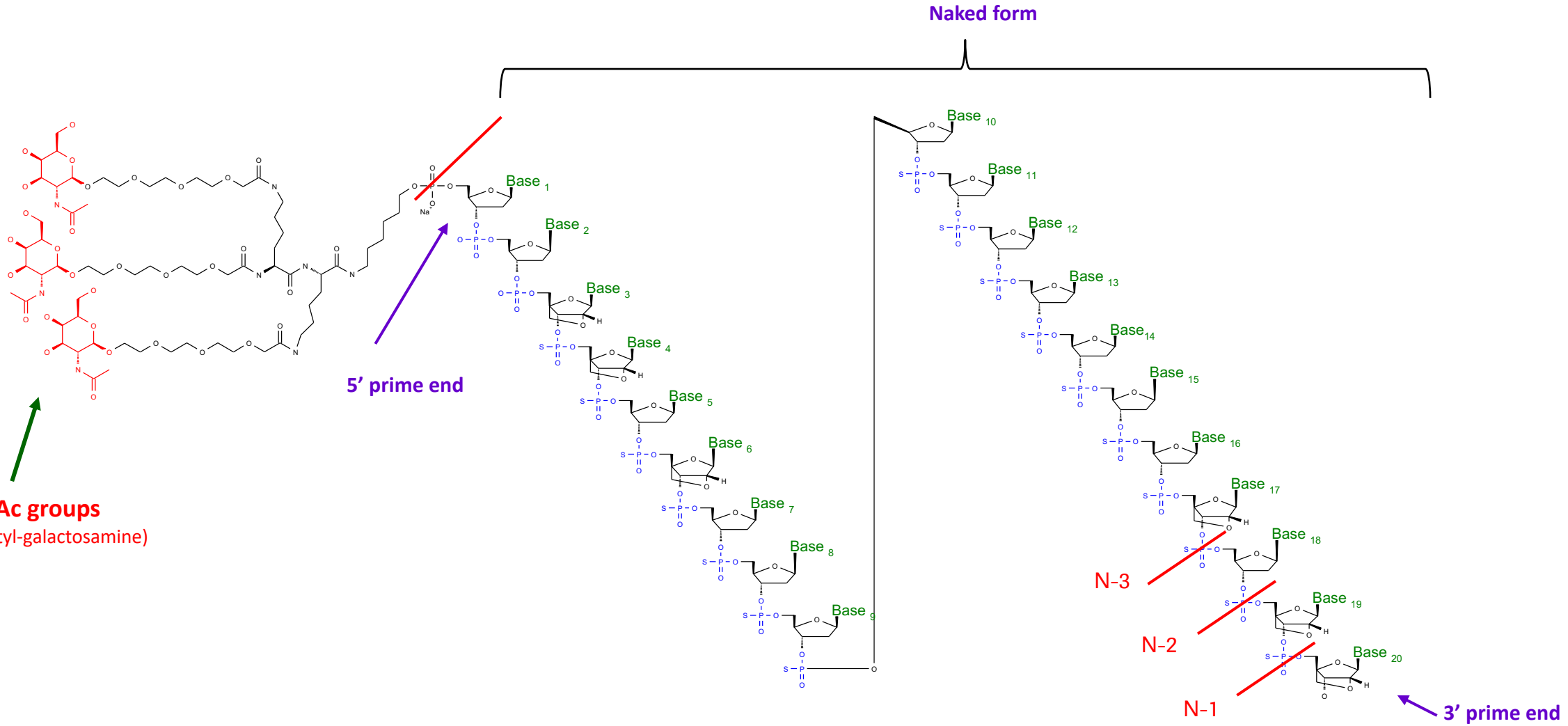
Potential specificity issues: h-ELISA vs. LC-MS

- LC-MS and h-ELISA are not equally selective
- h-ELISA might not be able to differentiate between intact and 3’ and/or 5’ truncated metabolites
- Quantitation using h-ELISA might result in an overestimation of the intact oligonucleotide

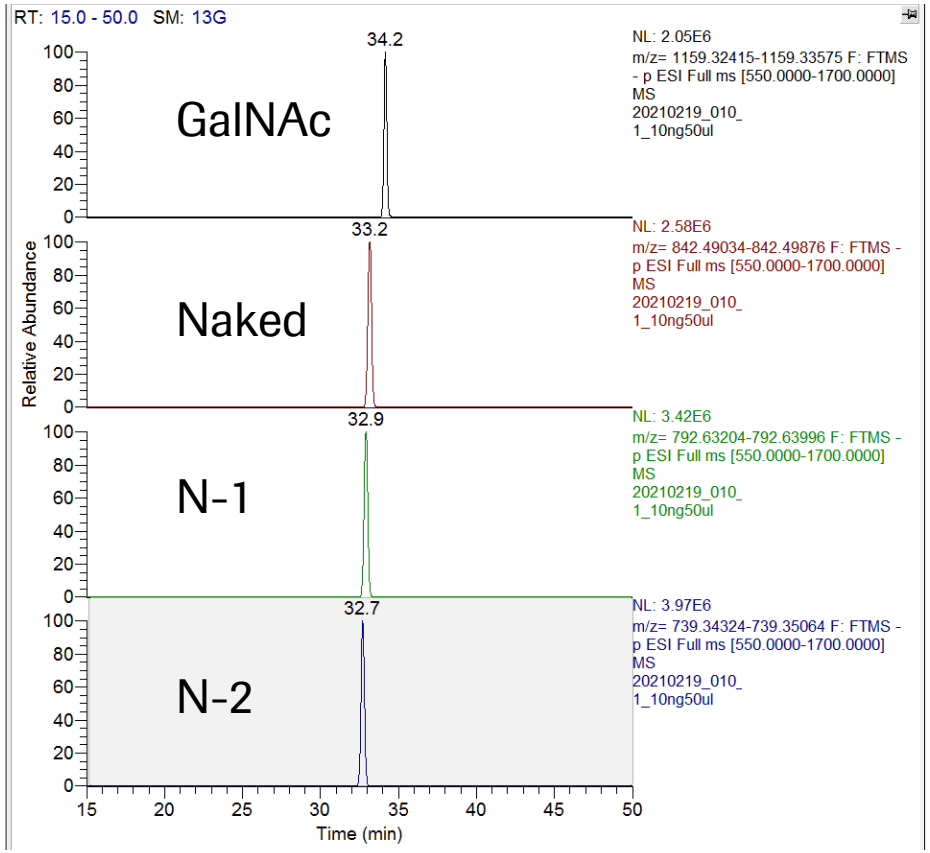




Potential specificity issues: h-ELISA vs. LC-MS

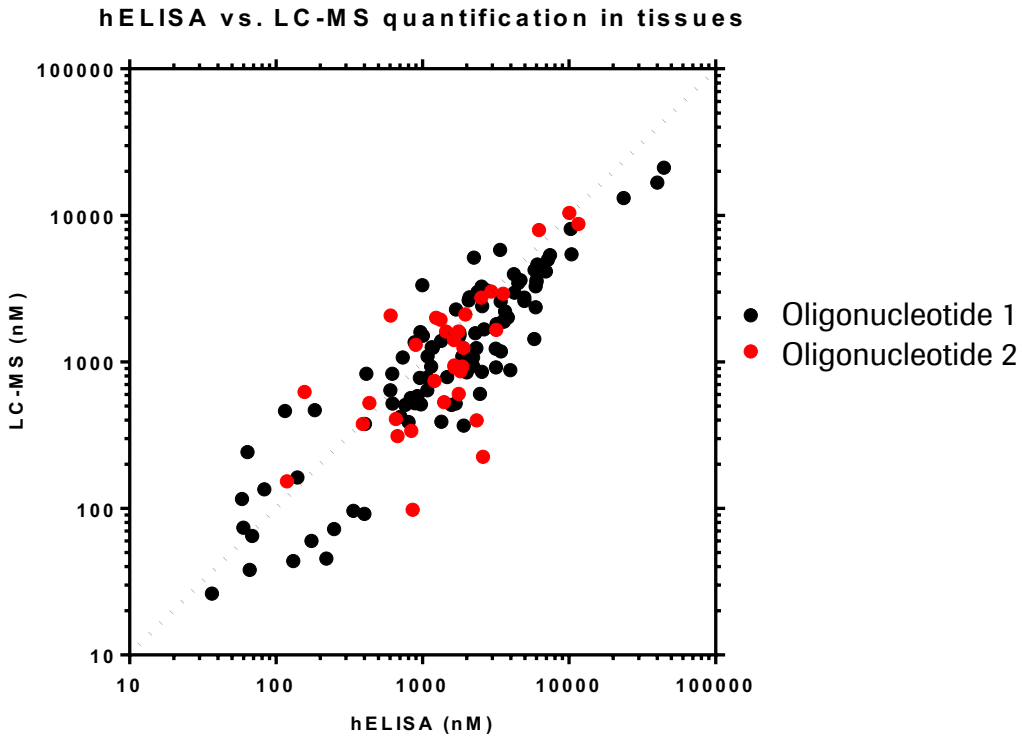


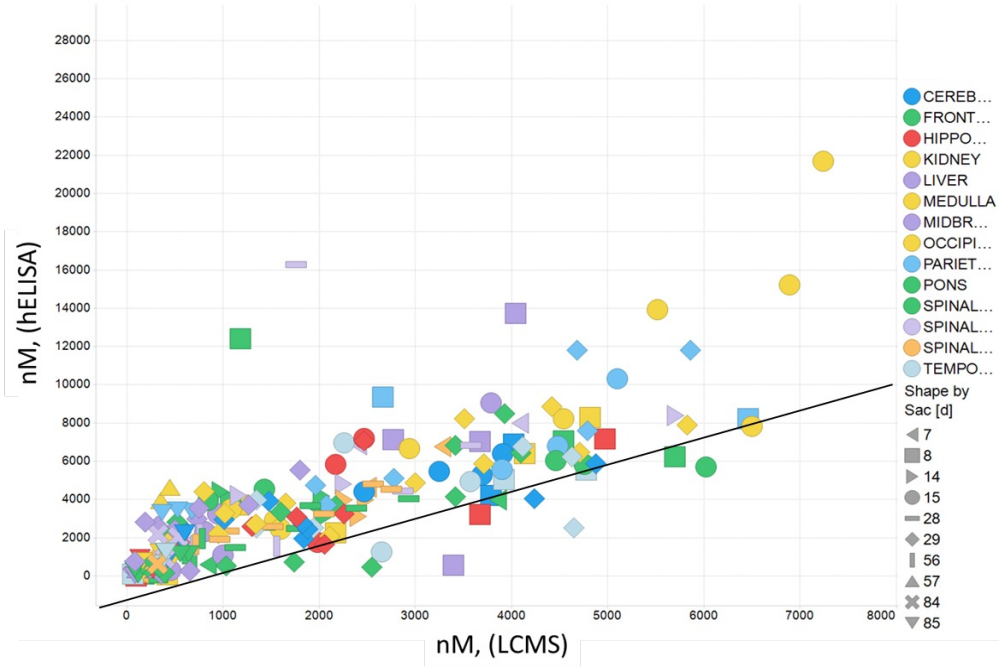
Potential specificity issues: h-ELISA vs. LC-MS



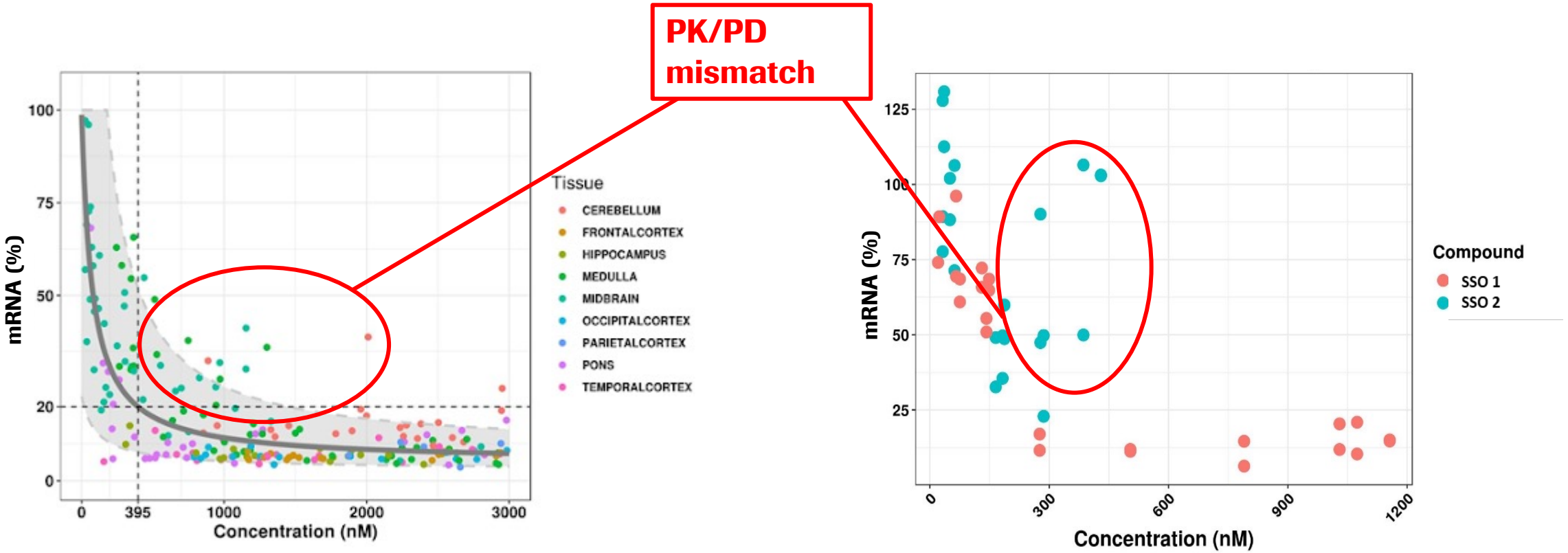
LC-MS/MS	h-ELISA	
✓	✓	
✓	✓	
✓	lower response factor?	sum
✓	lower response factor?	

Switching technologies for quantitation – what is the risk?





Switching technologies for quantitation – what is the risk?



mRNA vs. oligonucleotide conc. plot in different tissues

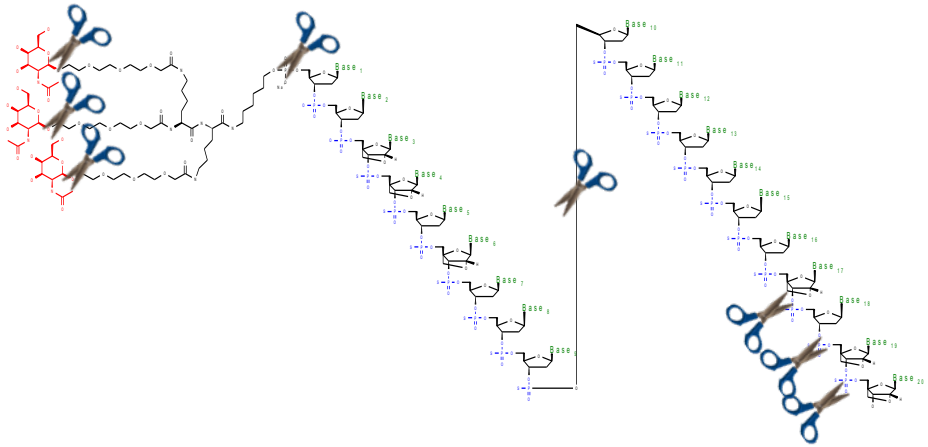
Switching technologies – how to avoid a potential PK/PD mismatch

A potential mismatch can be due to the following reasons:

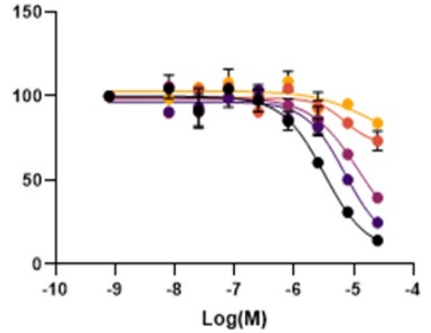
- Specificity issues.
- Different tissue portions collected for LNA and mRNA analysis.
- Different homogenization procedures adopted (with and w/o lysis buffer) for LC-MS and h-ELISA or PCR assays.
- Poor mRNA quality due to inappropriate sample treatment.

‘One tissue homogenate’ sample concept:
each tissue sample should be homogenized, the same homogenate split in two aliquots and analyzed for PK and PD assessment

Quantification of metabolites



- MIST Guideline
- The selection of the technology to use for their quantification should be very carefully evaluated because of potential specificity issues.
- The metabolic profiles of ASOs in plasma and in tissues are typically very different, resulting in different sensitivity requirements.



Metabolites: what to quantify?

Plasma **Day 8**

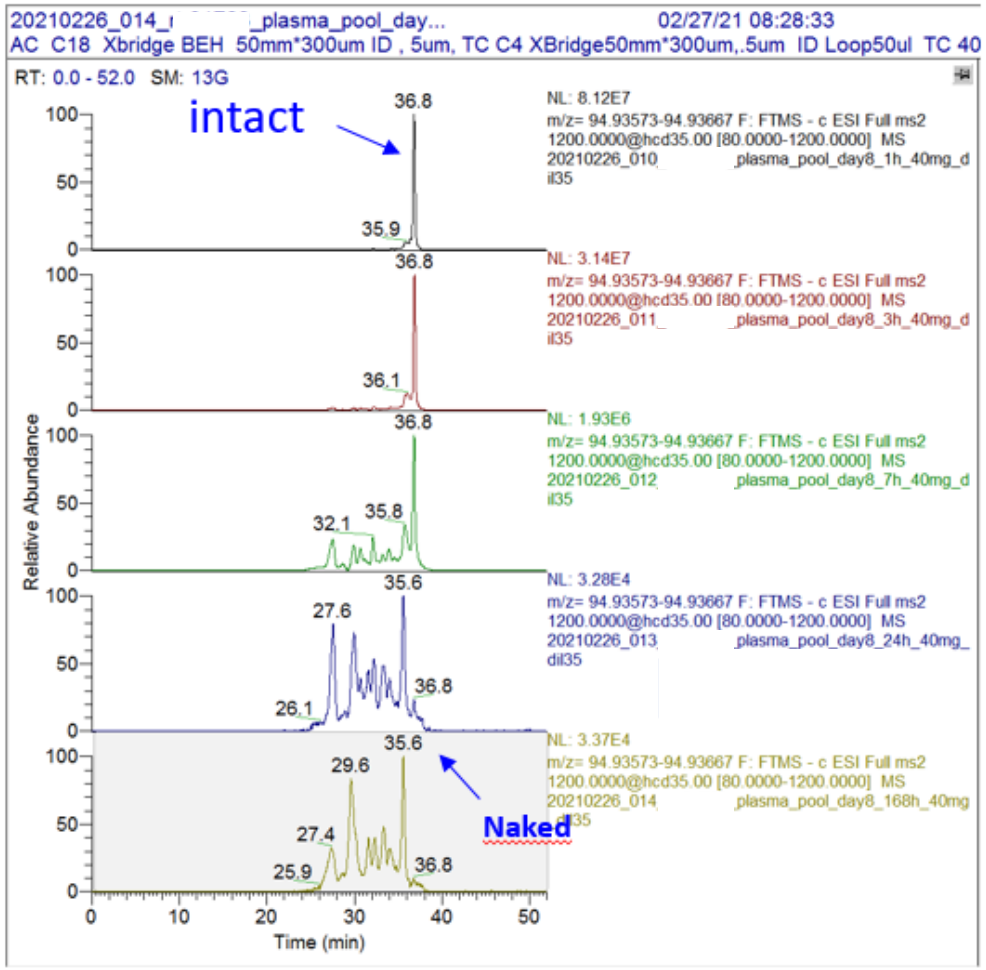
1h

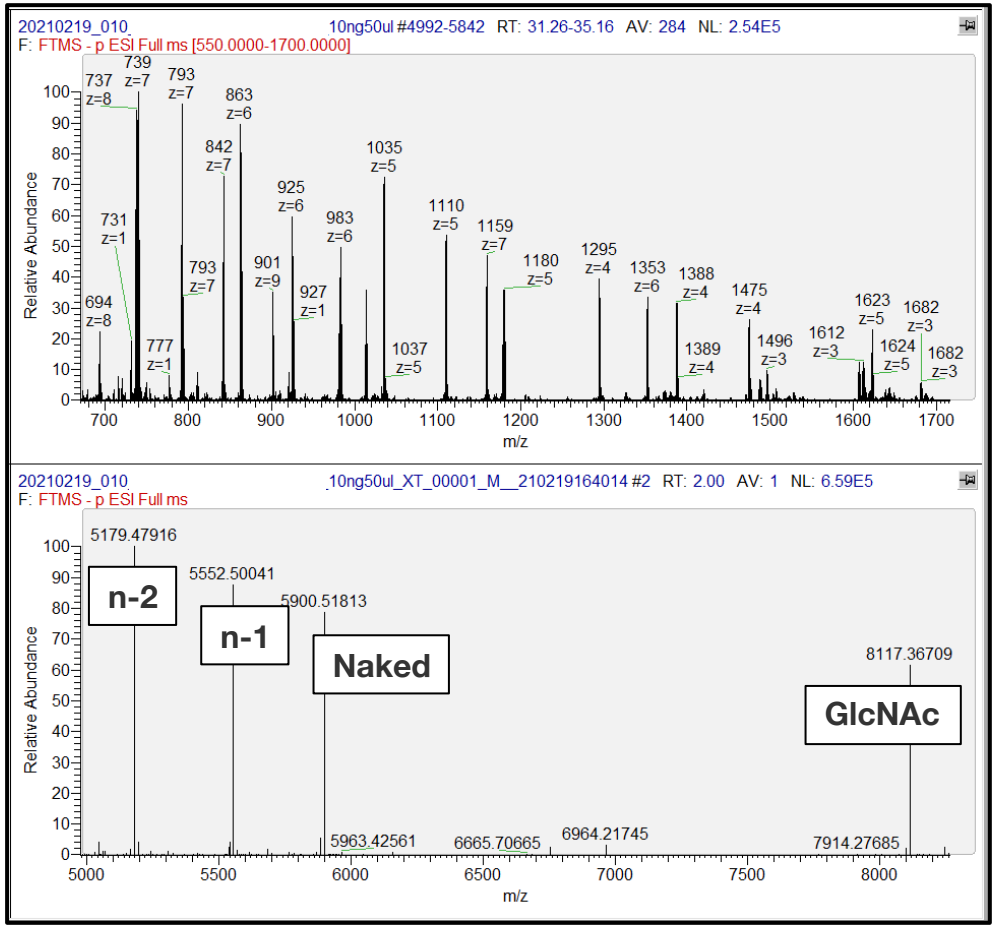
3h

7h

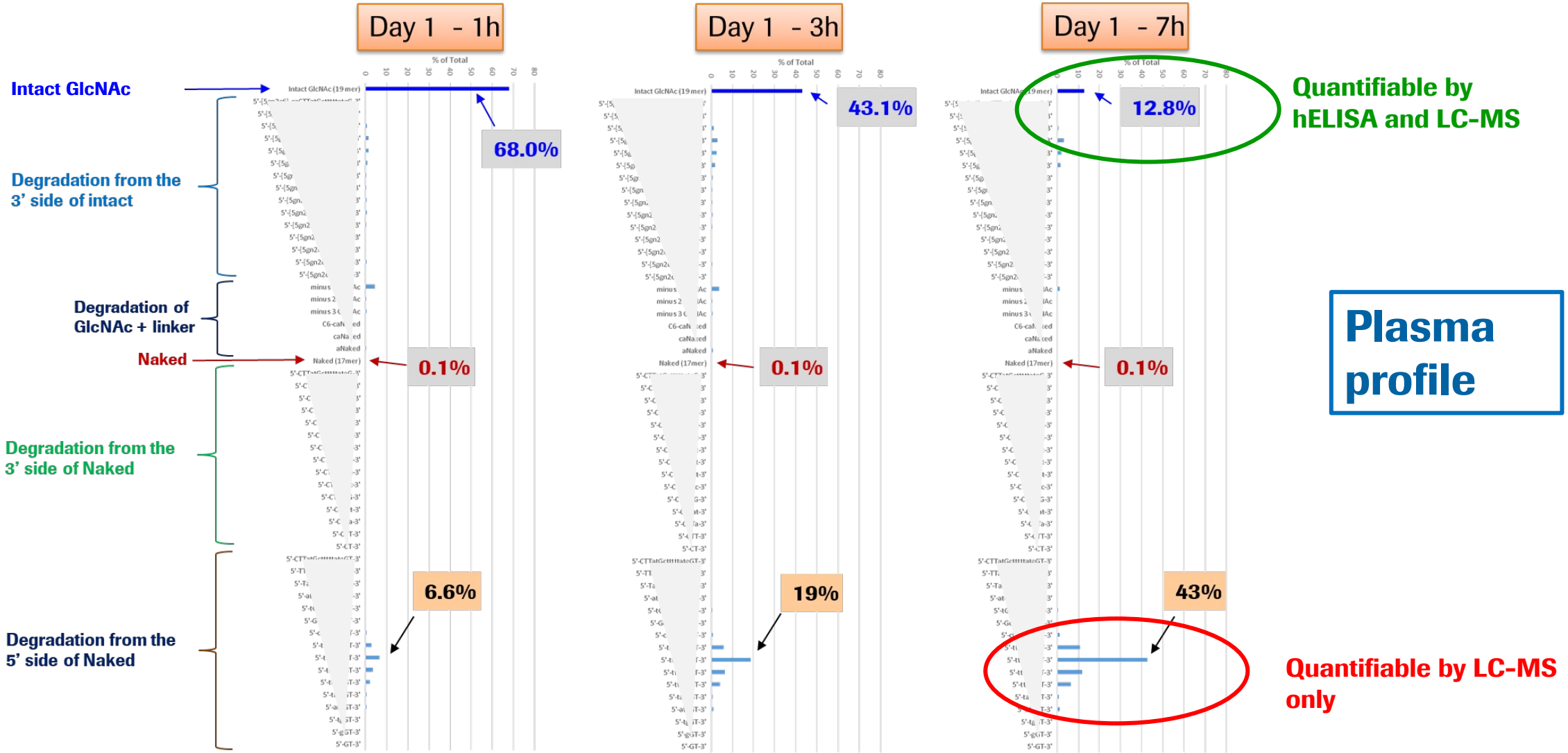
24h

168h

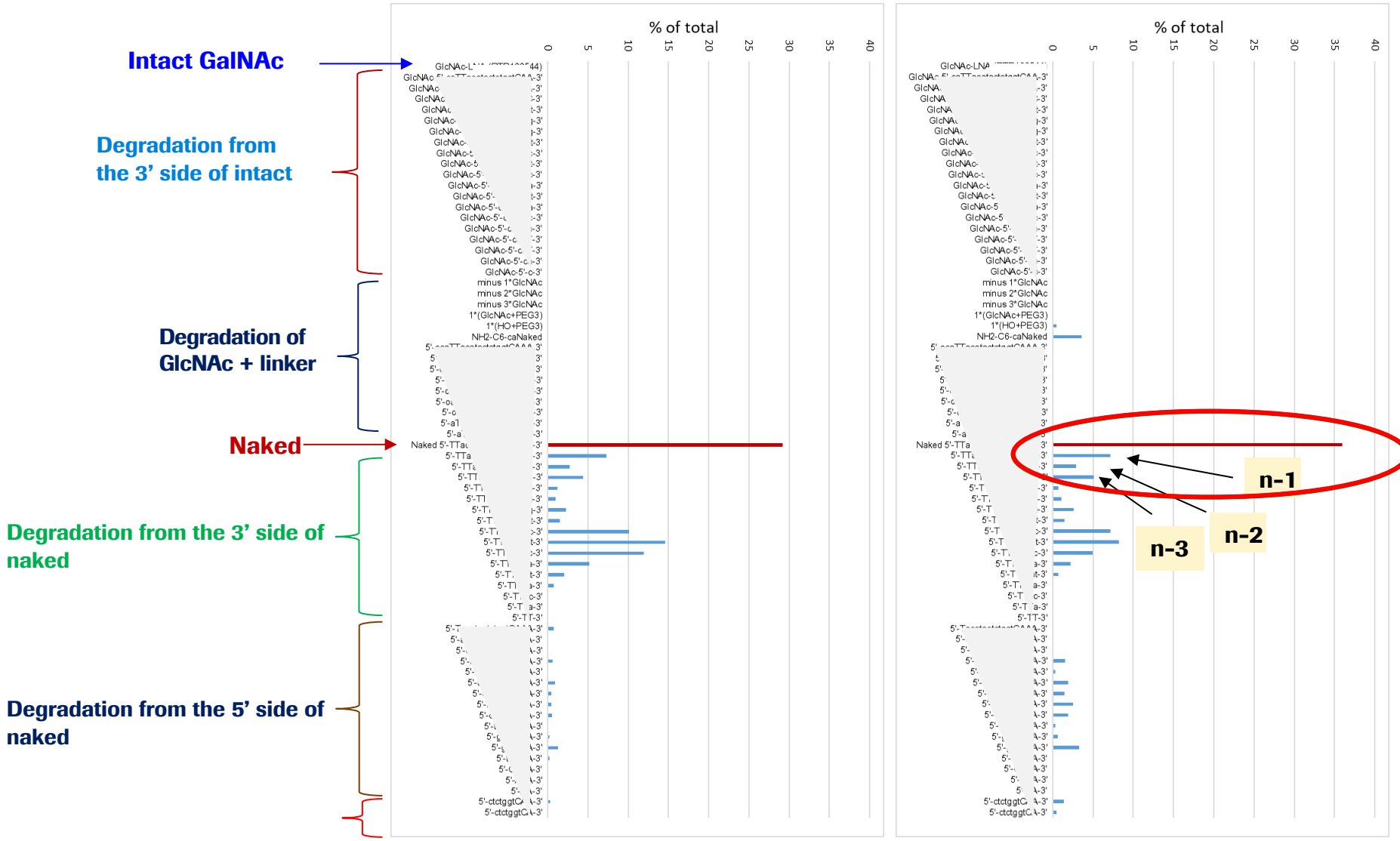




Metabolites: what to quantify in different matrices?



Metabolites: what to quantify in different matrices?



Selective quantification by LC-MS

Tissue profile

'Total active' quantification using hELISA

Specificity assessment of h-ELISA

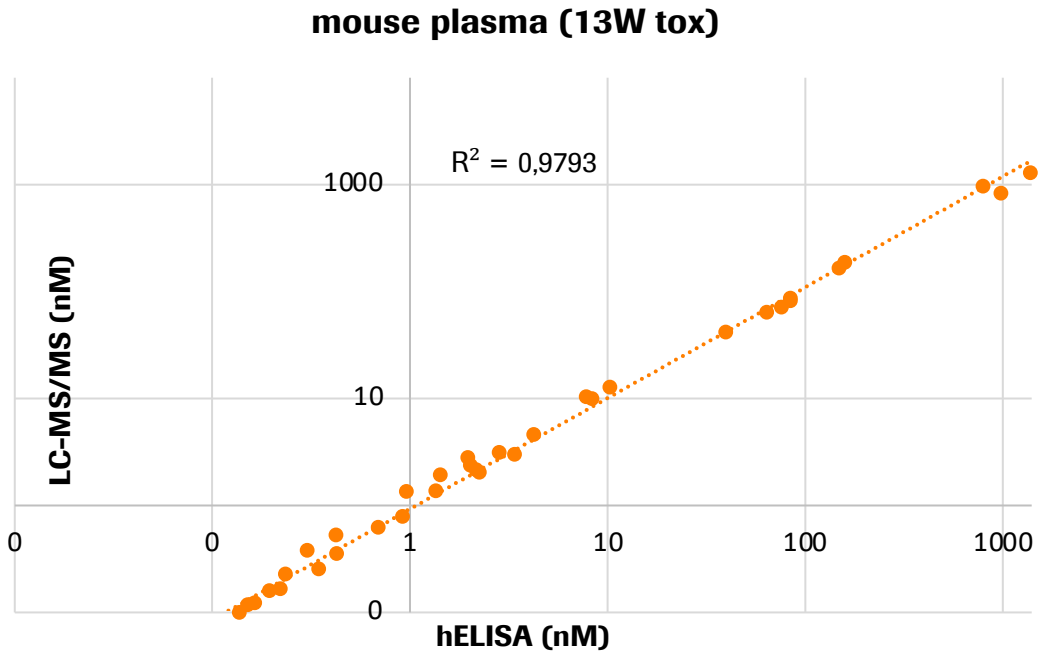
Description	ASO conc.	Metabolite conc.	h-ELISA response
ASO 1			
Full length	50nM	-	101%
Full length + 100% N-1	50nM	50nM	119%
Full length + 100% N-2	50nM	50nM	96%
Full length + 100% N-3	50nM	50nM	114%
Full length + 100% N-4	50nM	50nM	102%
ASO 2			
Full length	50nM	-	103%
Full length + 100% N-1	50nM	50nM	224%
Full length + 100% N-2	50nM	50nM	179%
Full length + 100% N-3	50nM	50nM	109%
Full length + 100% N-4	50nM	50nM	108%

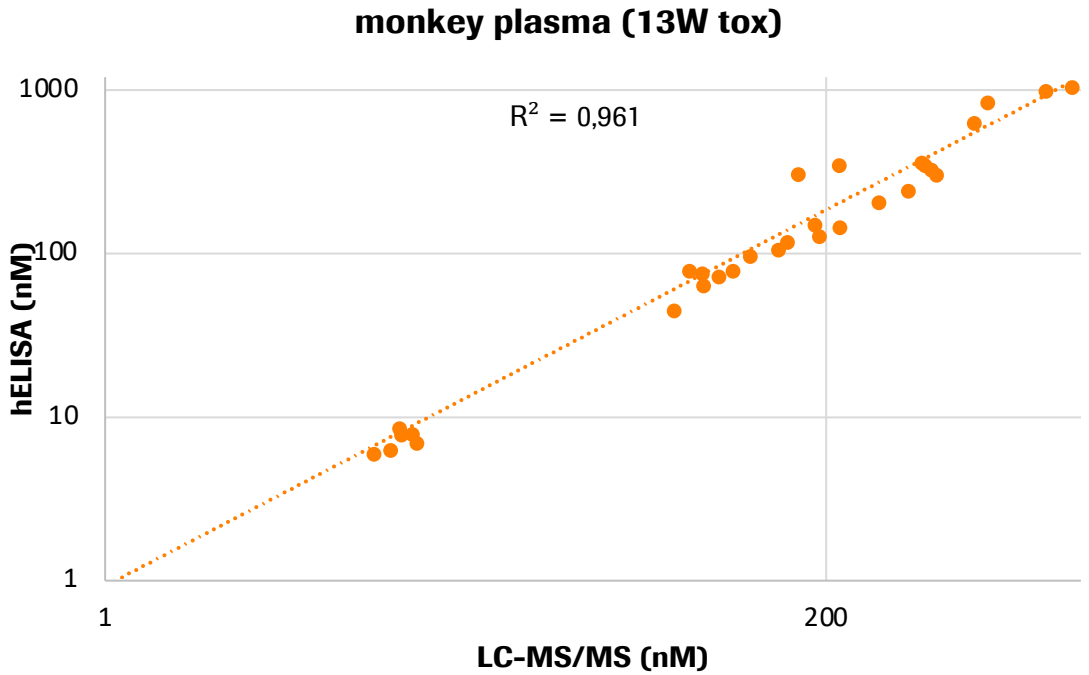
The specificity of LC-MS and hELISA assays vs. shortmers should always be assessed

Technology selection: recommendations

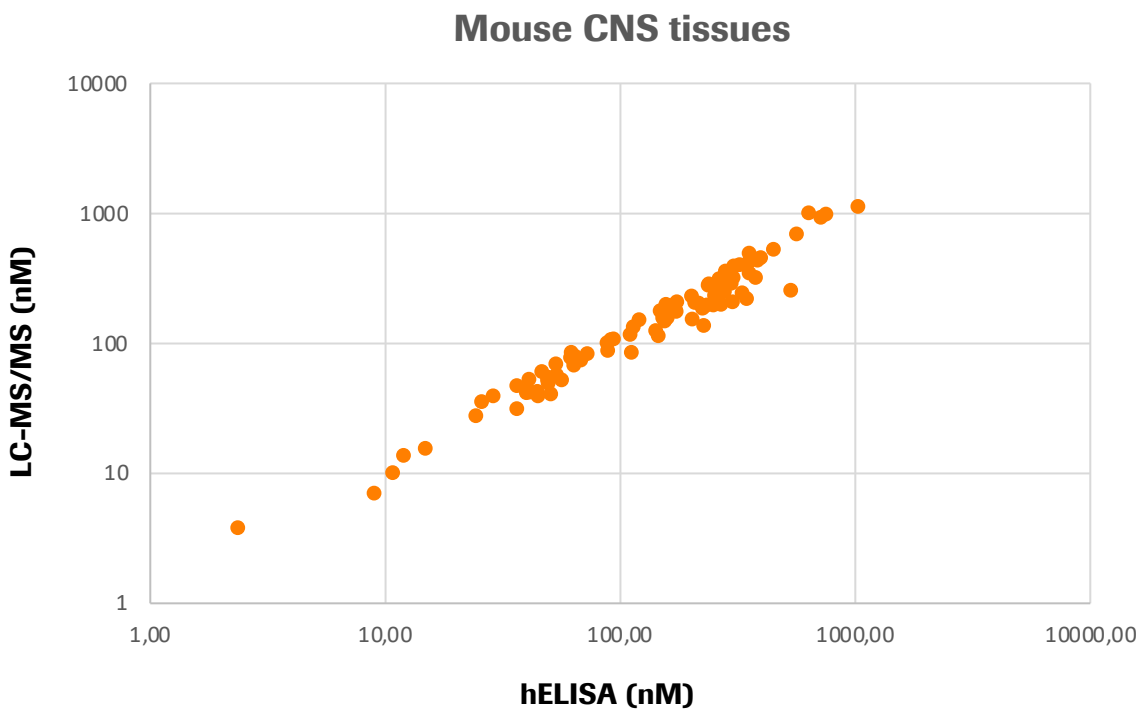
Criteria for selection of technology for ASO and metabolite quantification	**Sensitivity** **Specificity** **PK, PD and TK endpoints** (what needs to be measured) **Readiness for application in regulatory settings**
Biotransformation	**The biotransformation should be determined at early stage,** using i*n vitro* and *ex vivo* preparations
Shortmers - metabolites	**Shortmers** (typically N-1, N-2, N-3) should be **synthesized**, their **pharmacological activity** evaluated, and used to assess **specificity** of the methods used in regulated pre-clinical/clinical studies.
Possibility to perform a technology switch (e.g h-ELISA – LCMS – MOL PCR)	**In the lack of the above information, a technology switch is not recommended**, unless a comparison of the two techniques is performed using real samples, to demonstrate that comparable exposure data can be generated.

Comparison LC-MS vs. hELISA data





Comparison LC–MS vs. hELISA data



				hELISA quantification			LC-MS quantification	
Subject	Region	Time_days	Dose (µg)	Oligo concentration in homogenate (ng/l)	Oligo concentration in homogenate (ng/ml)	REPORTED DATA - hELISA nM	Conc. in homogenate (ng/mL)	%difference LC-MS/MS vs ELISA
101-17	A1	2 weeks	150	172860	172.86	550	176	1.8
102-18	A1	2 weeks	150	376463	376.46	942	323	-15.3
103-19	A1	2 weeks	150	148497	148.50	490	178	18.1
104-20	A1	2 weeks	150	249624	249.62	850	198	-23.1
105-21	A1	2 weeks	150	252658	252.66	963	236	-6.8
106-22	A1	2 weeks	150	111671	111.67	400	85.2	-26.9
101-17	CEREBELLUM	2 weeks	150	273138	273.14	1090	323	16.7
102-18	CEREBELLUM	2 weeks	150	322588	322.59	1058	406	22.9
103-19	CEREBELLUM	2 weeks	150	280933	280.93	1125	361	24.9
104-20	CEREBELLUM	2 weeks	150	238214	238.21	937	287	18.6
105-21	CEREBELLUM	2 weeks	150	201118	201.12	954	232	14.3
106-22	CEREBELLUM	2 weeks	150	164835	164.83	756	189	13.7
101-17	HIPPOCAMPUS	2 weeks	150	166361	166.36	1139	196	16.4
102-18	HIPPOCAMPUS	2 weeks	150	93474	93.47	995	109	15.3
103-19	HIPPOCAMPUS	2 weeks	150	163191	163.19	1342	182	10.9
104-20	HIPPOCAMPUS	2 weeks	150	91575	91.58	691	107	15.5
105-21	HIPPOCAMPUS	2 weeks	150	250883	250.88	1840	277	9.9
106-22	HIPPOCAMPUS	2 weeks	150	44387	44.39	419	42.7	-3.9
101-17	LUMBAR	2 weeks	150	72334	72.33	391	84.0	14.9
102-18	LUMBAR	2 weeks	150	46340	46.34	322	60.6	26.7
103-19	LUMBAR	2 weeks	150	28829	28.83	345	39.8	32.0
104-20	LUMBAR	2 weeks	150	36461	36.46	422	47.6	26.5
105-21	LUMBAR	2 weeks	150	25819	25.82	379.2	35.7	32.1
106-22	LUMBAR	2 weeks	150	110307	110.31	274.1	118	6.7
101-17	STRIATUM	2 weeks	150	49111	49.11	345	52.3	6.3
102-18	STRIATUM	2 weeks	150	49583	49.58	248	55.1	10.5
103-19	STRIATUM	2 weeks	150	39844	39.84	243.2	41.7	4.6
104-20	STRIATUM	2 weeks	150	68262	68.26	272	74.7	9.0
105-21	STRIATUM	2 weeks	150	53765	53.76	339.3	57.8	7.2
106-22	STRIATUM	2 weeks	150	36338	36.34	155	31.6	-13.9
101-17	MIDBRAIN	2 weeks	150	233671	233.67	1374	197	-17.0
102-18	MIDBRAIN	2 weeks	150	174529	174.53	983	210	18.4
104-20	MIDBRAIN	2 weeks	150	151574	151.57	1130	157	3.5
106-22	MIDBRAIN	2 weeks	150	50864	50.86	550	41.0	-21.5
102-18	LIVER	2 weeks	150	225821	225.82	352.8	189	-17.8
103-19	LIVER	2 weeks	150	202925	202.93	338.6	154	-27.4
104-20	LIVER	2 weeks	150	145670	145.67	199.2	115	-23.5
105-21	LIVER	2 weeks	150	268449	268.45	354.9	201	-28.7
106-22	LIVER	2 weeks	150	330225	330.22	469.2	245	-29.6
102-18	KIDNEY	2 weeks	150	563388	563.39	813.4	698	21.3
103-19	KIDNEY	2 weeks	150	1030984	1030.98	1568.8	1130	9.2
104-20	KIDNEY	2 weeks	150	715128	715.13	1051.2	941	27.3
105-21	KIDNEY	2 weeks	150	750402	750.40	957	991	27.6
106-22	KIDNEY	2 weeks	150	449550	449.55	643	529	16.2

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Doing now what patients need next