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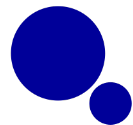
Comparison of QuantiGene and LC-MS/MS technologies for quantification of antisense oligonucleotides in tissue and plasma matrices

Davy Guignard, Evotec



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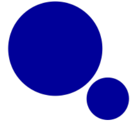
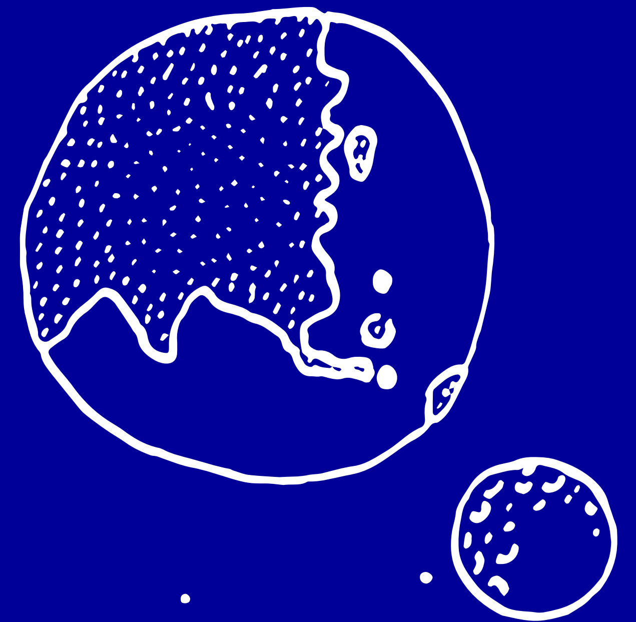


Agenda

1. Quantification of ASOs in biological matrices
2. Objectives & Methods
3. Development of the QuantiGene methods
4. Sample analysis
5. Method comparison: QuantiGene vs LC-MS/MS
6. Conclusion & Perspectives



Context - Quantification of ASOs in biological matrices

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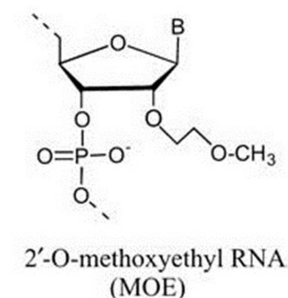
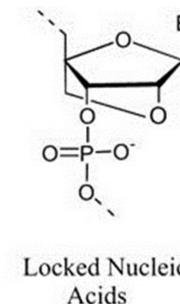
Pharmacokinetics of antisense oligonucleotides

Peculiarities of ASOs compared to small molecules

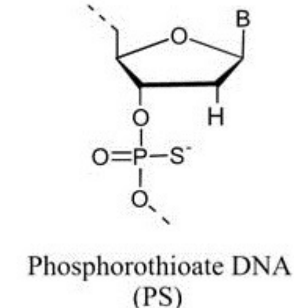
- **ASO = antisense oligonucleotide**
  - Synthetic short sequence (15-25 nt) of RNA, DNA or a mix
  - Single strand
  - Can modulate the expression of a gene through different mechanisms of action
  - Back bone chemistries that increase stability and potency (↓ dose)



2′-O-methoxyethyl RNA (MOE)

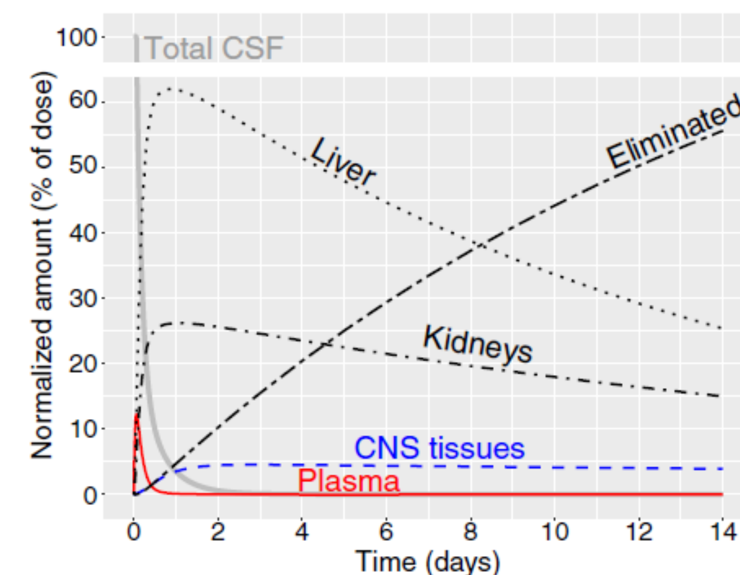


Locked Nucleic Acids



Phosphorothioate DNA (PS)

- **PK evaluation**
  - High concentration in tissues (kidneys and liver)
  - Fast elimination from systemic circulation → very low concentration in plasma
  - Need a very senstive bioanalytical method to quantify ASOs in plasma (accessible matrix for repeated samplings)



Shadid et al., 2021 – Expert Opinion on Drug Metabolism & Toxicology – doi: 10.1080/17425255.2021.1992382
Monine et al., 2021 - Journal of Pharmacokinetics and Pharmacodynamics – doi: 10.1007/s10928-021-09761-0

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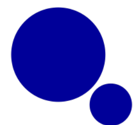


Quantification of oligonucleotides

Overview of the current technologies

Chromatography-based methods	Hybridization-based methods
• LC-UV • LC-MS/MS	• Single-probe hybridization ELISA • Dual-probe ECL (MSD technology) • RT-qPCR
• Hybridization–LC-MS/MS • Hybridization–LC-UV	

Limitation of some technologies: lack of sensitivity

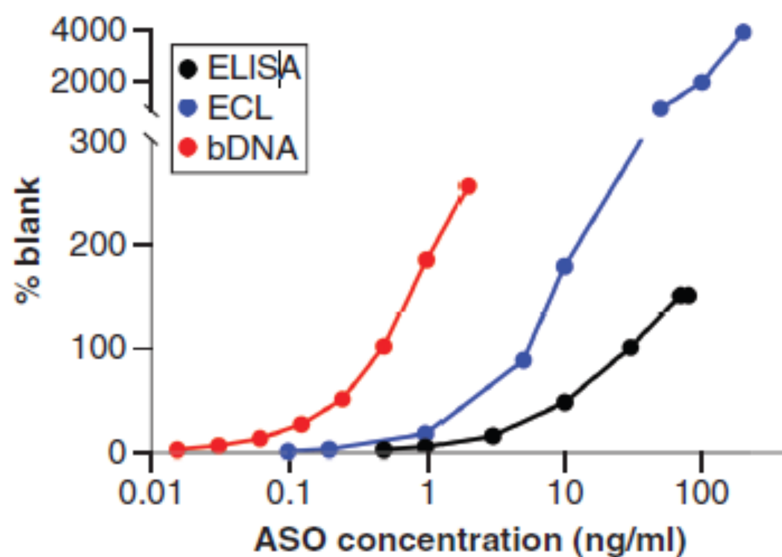
(especially when low-dose and non-intravenous ASOs are administered)

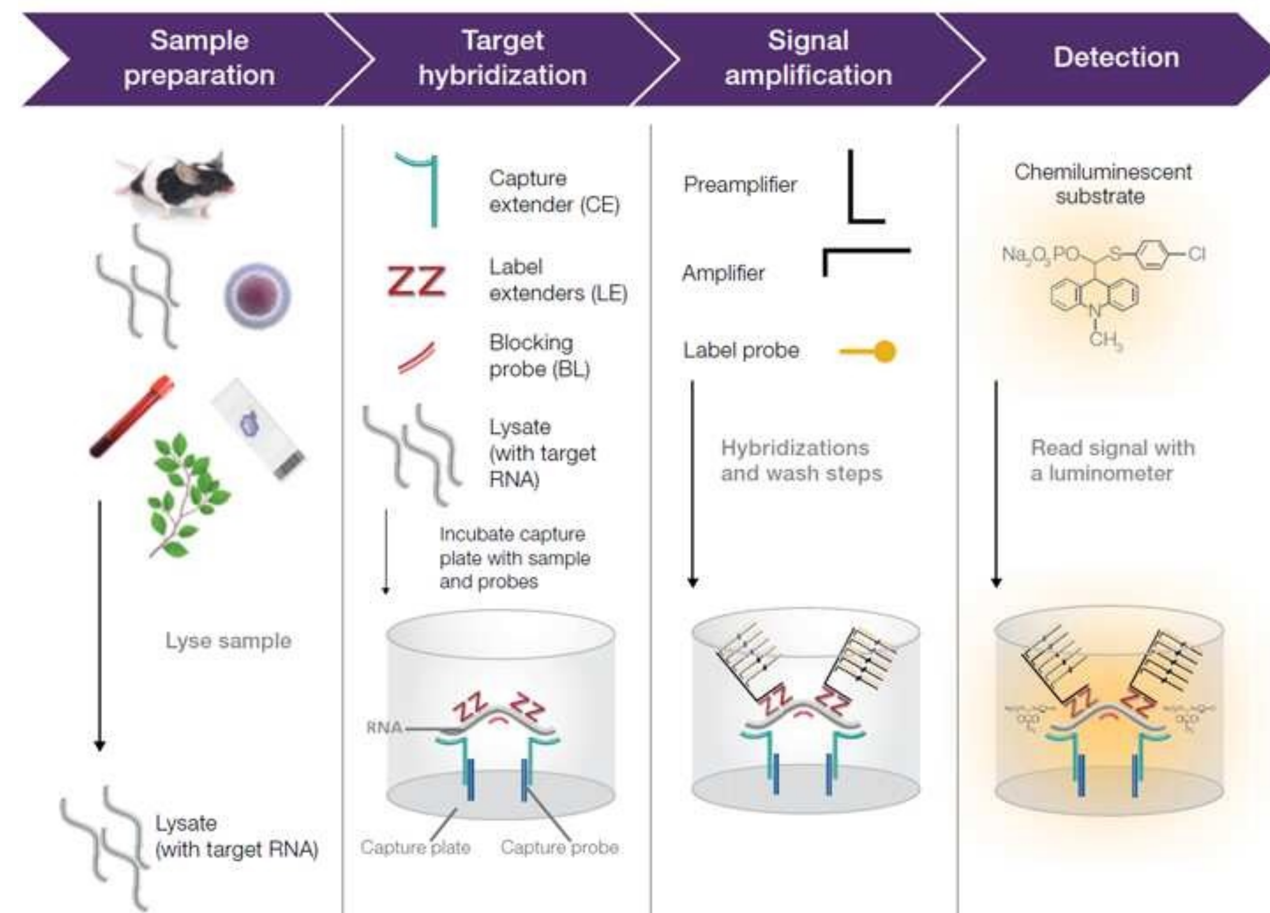
Shadid et al., 2021 – Expert Opinion on Drug Metabolism & Toxicology – doi: 10.1080/17425255.2021.1992382
Mahajan et al., 2022 – Bioanalysis - doi: 10.4155/bio-2022-0035
Haegele et al., 2022 – Nucleic Acid Therapeutics – doi: 10.1089/nat.2021.0100

Quantification of oligonucleotides

QuantiGene technology

- Hybridization-based gene expression assay that uses branched DNA for signal amplification
- The luminescent signal is proportional to the number of RNA molecules present in the sample
- Originally designed for detection of mRNA molecules; adapted for ASO quantification
- Directly applicable to a biological matrix (no sample purification)
- Highly sensitive





Mahajan et al., 2022 – Bioanalysis - doi: 10.4155/bio-2022-0035

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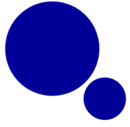
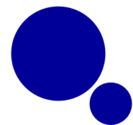


Objectives & Methods



Questions & Objectives

Is the QuantiGene technology

- Applicable for the quantification of ASOs in solid tissues?
- Applicable for the quantification of short sequence ASOs (>16 nt)?
- Applicable for the quantification of ASOs with chemical modifications (PS; LNA)?
- Comparable with the LC-MS/MS technology?

Objectives

- Compare the QuantiGene and the LC-MS/MS technologies (reliability)
- Characterize the PK profiles of different anti-Malat1 antisense oligonucleotides (tool ASOs) after a SC administration in mouse

QuantiGene LC-MS/MS

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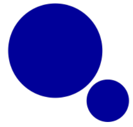


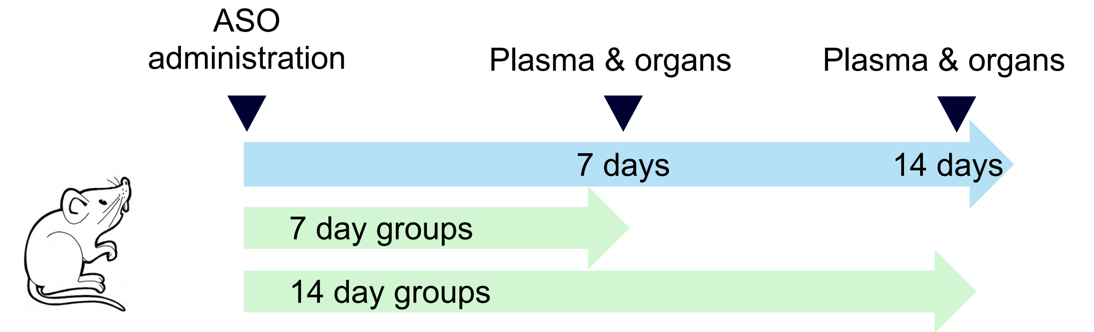
Material & Methods

In vivo study design

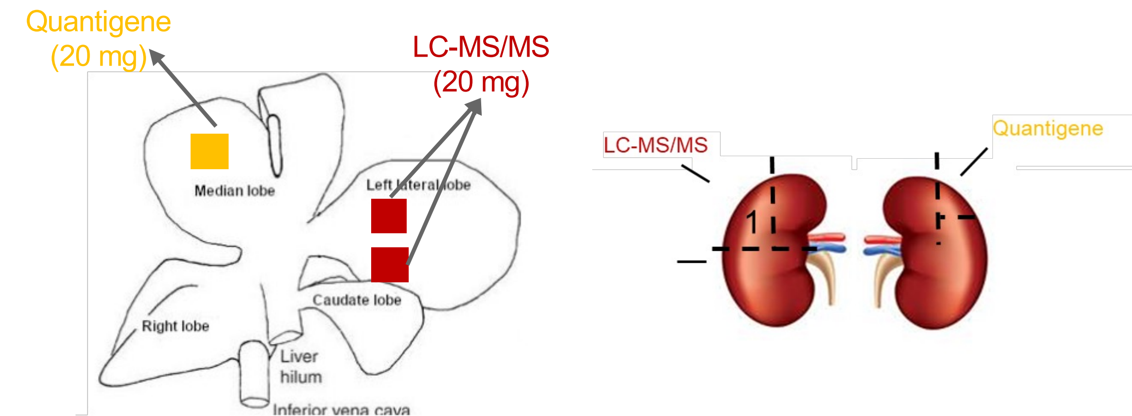
- **Model**
  - C57Bl/6JRJ female mice (6-8 weeks old)
  - N=3 /group
- **Administration**
  - single SC administration
  - 3 ASOs anti-Malat1: MM1 – MM2 or MM5
  - 3 dose levels: 8.3 – 25 or 75 mg/kg
- **Samplings**
  - Timepoints: 7 and 14 days post-administration
  - Blood sampling
  - Organ collection: : liver, kidneys

Quantification of the ASOs by:

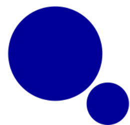
- **QuantiGene**
- **LC-MS/MS**



ASO	Length	Chemistry
MM1	16 nt	LNA-Gapmers (3 LNA at each end) and full PS backbone
MM2	20 nt	
MM5	16 nt	

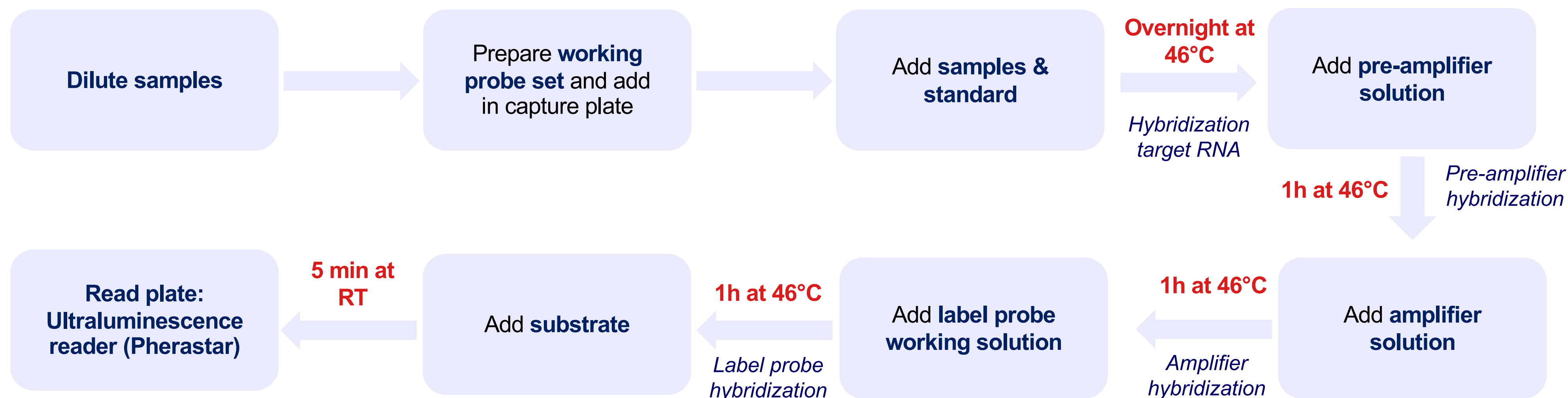


Development of the QuantiGene methods

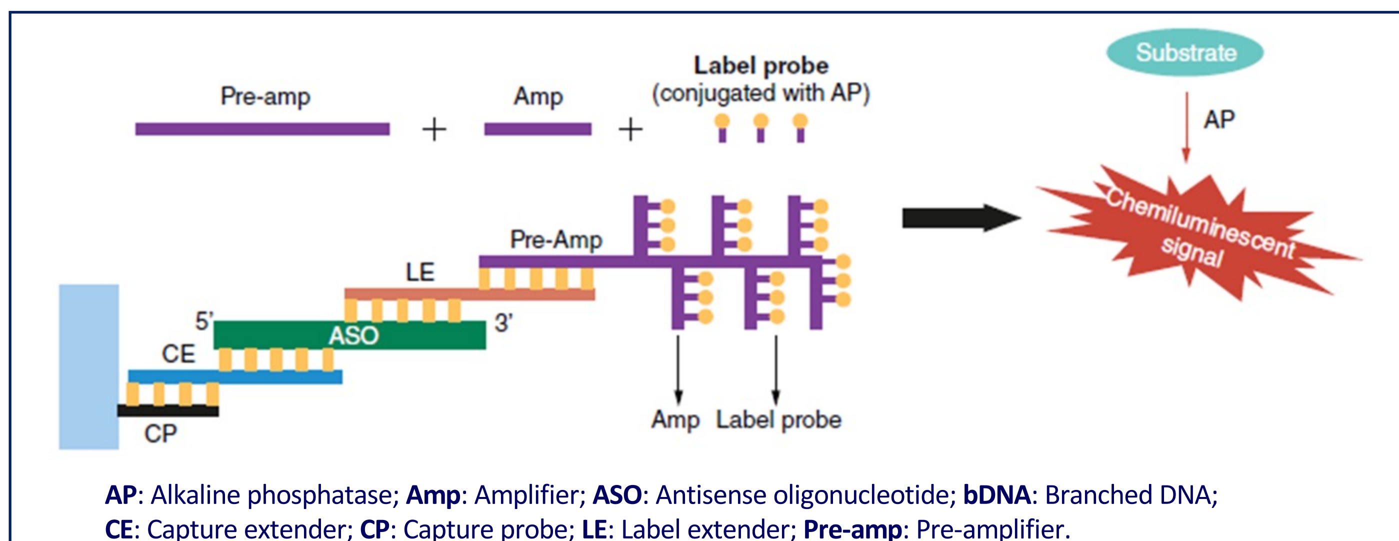


QuantiGene Kit

Optimized protocol - Assay

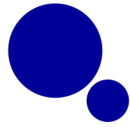


Use of "QuantiGene™ Singleplex assay kit", but protocol from "QuantiGene® 2.0 miRNA Assay"



AP: Alkaline phosphatase; **Amp**: Amplifier; **ASO**: Antisense oligonucleotide; **bDNA**: Branched DNA; **CE**: Capture extender; **CP**: Capture probe; **LE**: Label extender; **Pre-amp**: Pre-amplifier.

Mahajan et al., 2022 – Bioanalysis - doi: 10.4155/bio-2022-0035

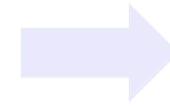


Development of the QuantiGene methods

Matrix effect and dilution linearity

1) Definition of the MRD

- Standard curves in buffer (PBS), plasma, liver homogenate and kidney homogenate
- Comparison of the blank signal and the SNR
- Test of few samples at different dilutions



For the 3 ASOs, preparation of a standard curve:
- in mouse plasma 1/10
- in tissue homogenate 1/100

2) Possibility to use a pool (50/50) of « liver – kidney homogenate »

Comparison of the concentration obtained with a calibration curve prepared in liver homogenate or in kidney homogenate

MM1 - Analysis of liver samples with calibration curve in kidney				
Sample ID	Dilution	Concentration with the calibration curve in **liver** (ng/mL)	Concentration with the calibration curve in **kidney** (ng/mL)	%Difference
#14-D7	100	164	142	-14
#12-D14	100	16	13	-19

MM1 - Analysis of kidney samples with calibration curve in liver				
Sample ID	Dilution	Concentration with the calibration curve in **kidney** (ng/mL)	Concentration with the calibration curve in **liver** (ng/mL)	%Difference
#14-D7	100	173	199	14
#12-D14	100	214	245	14

3) Dilution linearity

- Up to 1/10 000 for plasma samples
- Up to 1/250 000 for liver and kidney samples

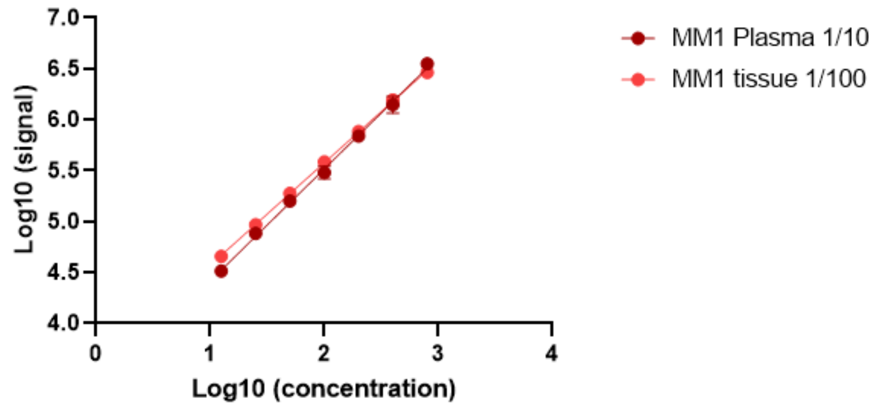
MM5 - Kidney samples			
Sample ID	**Dilution**	**Final concentration (µg/mL)**	**Accuracy (%RE) versus smallest dilution**
#55 – D7	25 000	4.44	N/A
	50 000	4.67	5
	100 000	4.76	7
	250 000	5.00	13
#51 – D7	50 000	7.87	N/A
	100 000	8.55	9
	250 000	8.31	6

Development of the QuantiGene methods

Qualification of the methods

For discovery purpose	• Based on the criteria descibed in the EMA and FDA bioanalytical guidelines but criteria may be slightly adapted • Standard curve and QCs (LLOQ, low QC, mid QD, high QC, ULOQ) • Evaluation of the precision and the accuracy

MM1

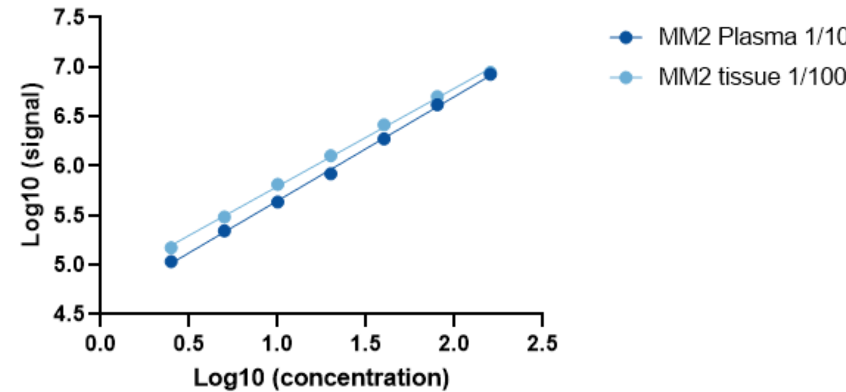


→ Calibration range for MM1 ASO in both matrices:

Range: ~ 25 – 800 pg/mL

→ QCs slightly more variable in plasma for lower concentrations

MM2

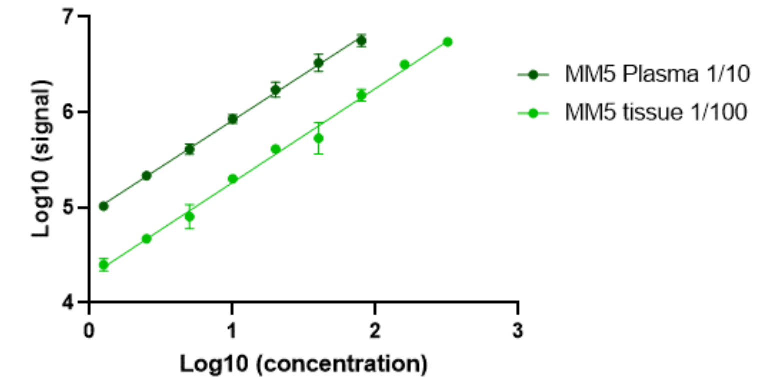


→ Calibration range for MM2 ASO in both matrices:

Range: 2.50 - 160 pg/mL

→ QCs slightly more variable in plasma for lower concentrations

MM5

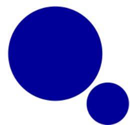


→ Calibration range for MM5 in both matrices:

- **Plasma 1/10: ~ 1.25 - 80 pg/mL**
- **Tissue homogenate 1/100: ~ 1.25 - 320 pg/mL**

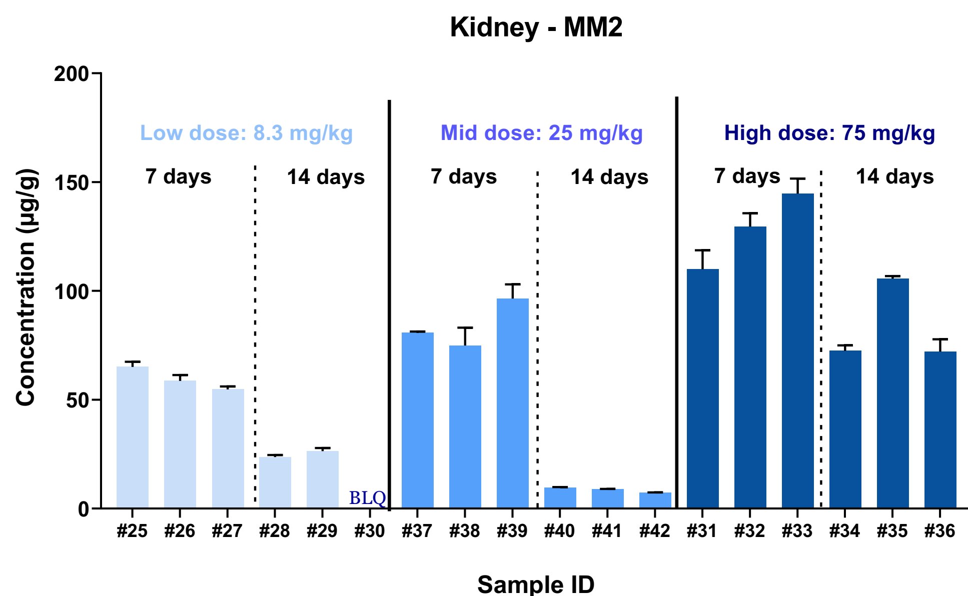
→ Inter-run variability with this ASO (high background signal with this probe)

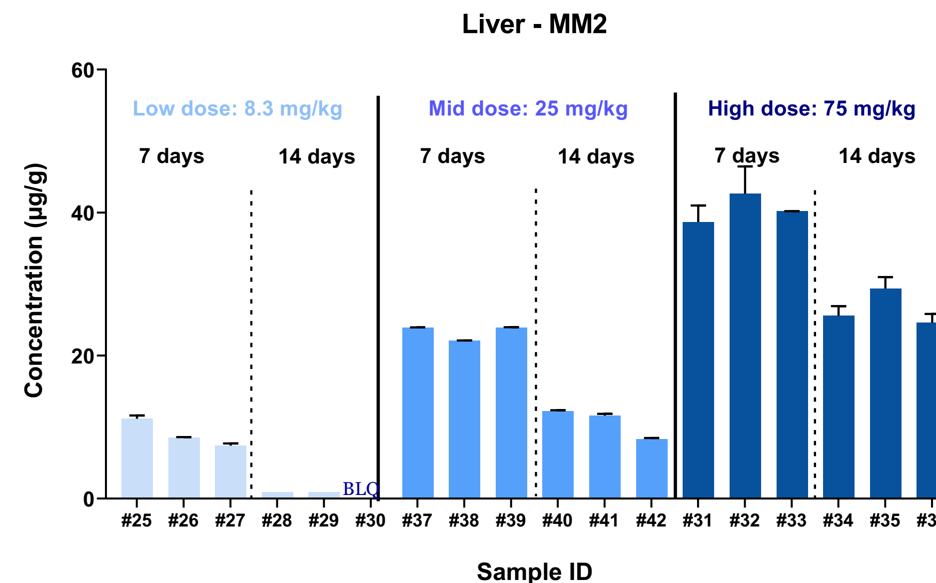
Sample analysis

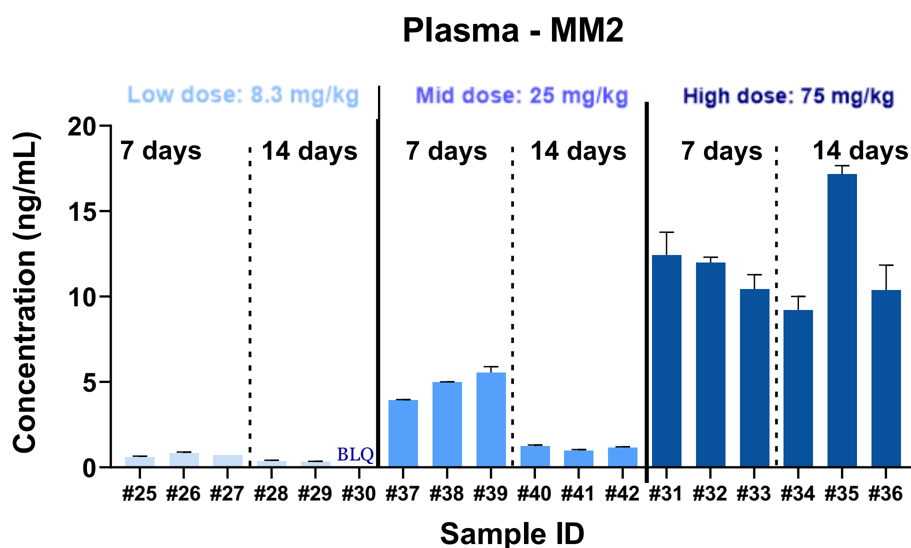


Sample analysis with the QuantiGene technology

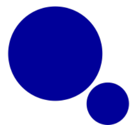
Concentrations of the ASO MM2





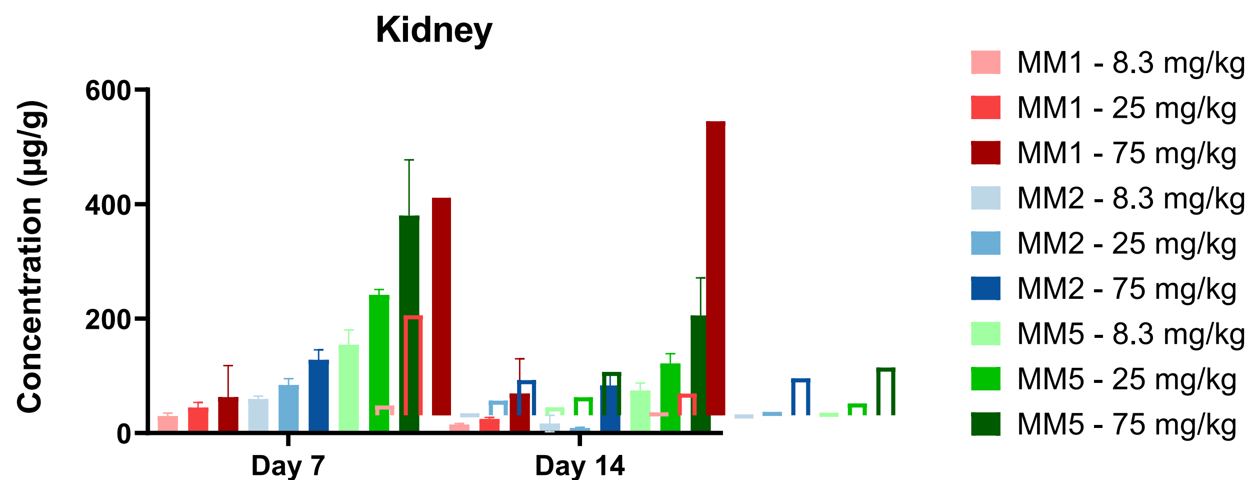


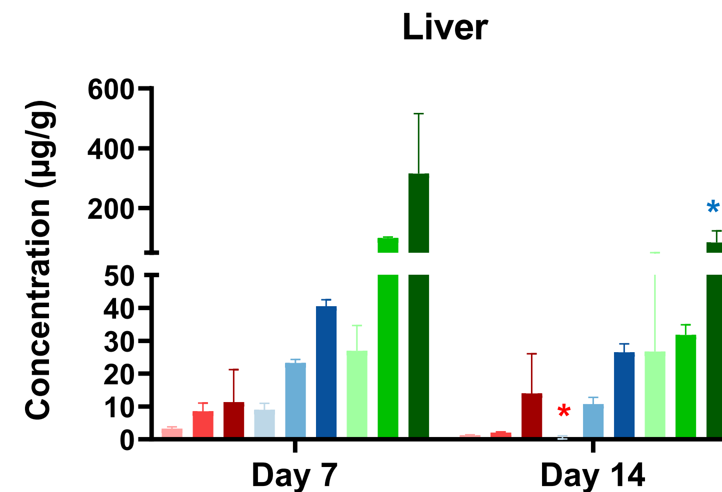
- All samples could be quantified except for the mouse #30
- Very low variability between technical replicates
- Kidney and liver: concentrations in the range of µg/g of tissue
  →high dilution needed (up to 1/250 000)
- Plasma: concentration in the range of ng/mL
- Dose-dependent concentrations
- Time-dependent concentrations
- Low inter-animal variability



Sample analysis with the QuantiGene technology

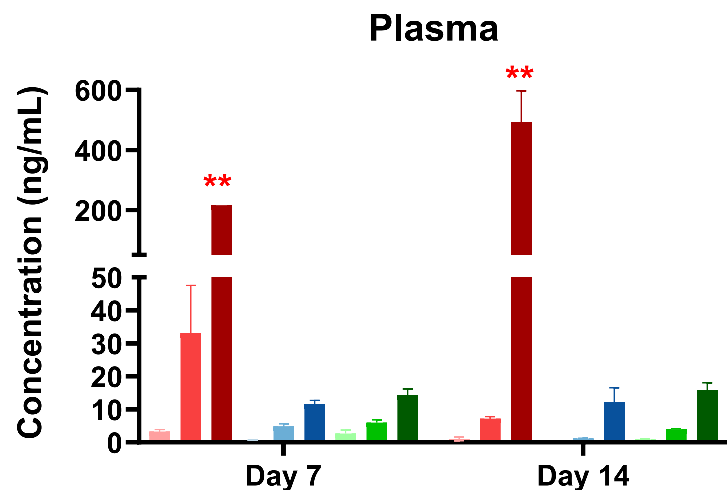
Summary of the 3 ASOs





*Data above ULOQ after dilution 1/100 for two samples (> 867 ng/mL)

*Extrapolated data for one sample (<LLOQ of the run)



**Data above ULOQ after dilution 1/250 (> 200 ng/mL)

- Dose-response observed for all ASOs in all matrices
- High exposure in kidneys and liver for the 3 ASOs
- Higher exposure for MM5 ASO in solid tissues
- High MM1 level in plasma observed, especially at higher dose:
  → Hepatic cell lysis? Clear colored spotted liver for samples from this group; ↑ ALT & AST

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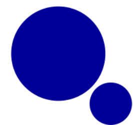
Method comparison: QuantiGene vs LC-MS/MS



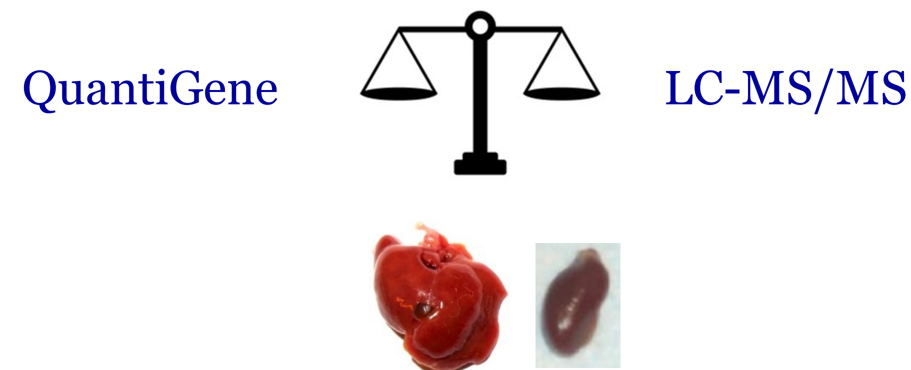
Method comparison: QuantiGene vs LC-MS/MS

LC-MS/MS conditions – Generic method

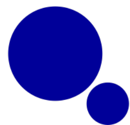
UPLC Conditions	
Instrument	Agilent 1290 Bio
Phase A:	water 0.02%DPA, 0.1% HIFP
Phase B:	acetonitrile/MeOH (75/25)
Column:	Acquity ™ Premier Oligonucleotide BEH C18 2.1X50; 130A, 1.7um
Column Temperature:	50 °C
Flow rate	0.7 mL/min
Injection Volume	5 µL

Gradient profile	
Time (min)	**%B**
0	1
1.1	30
1.2	80
1.4	80
1.6	1
2.2	1

Mass Spec Conditions	
Instrument	Sciex API 6500+
Ionization	TIS
Temperature	550 °C
Mrm for MM1	658.5>95.00
Mrm for MM2	733.2>95.00
Mrm for MM5	660.5>95.00
MM2 and MM5 used as internal standard for MM1/MM5 and MM2, respectively	

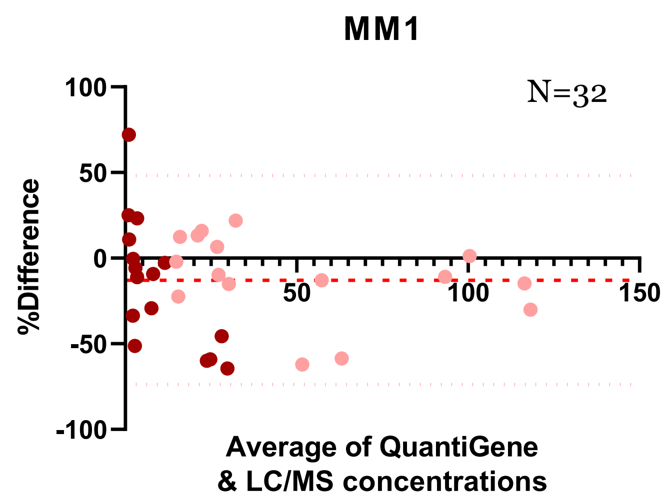


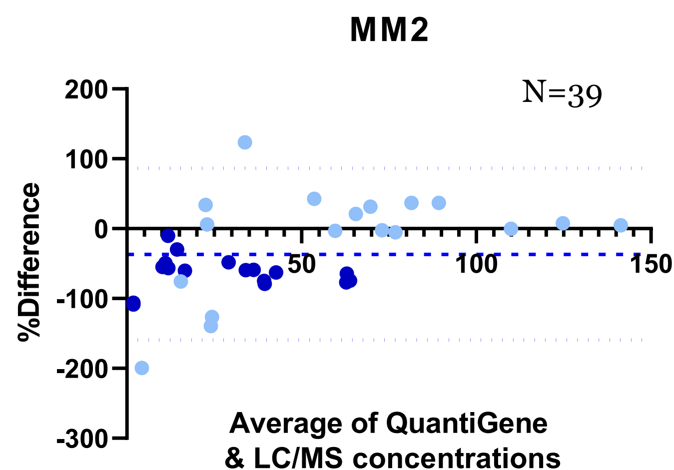
DPA: dipropylamine
Hexaisofluoropropil alcool

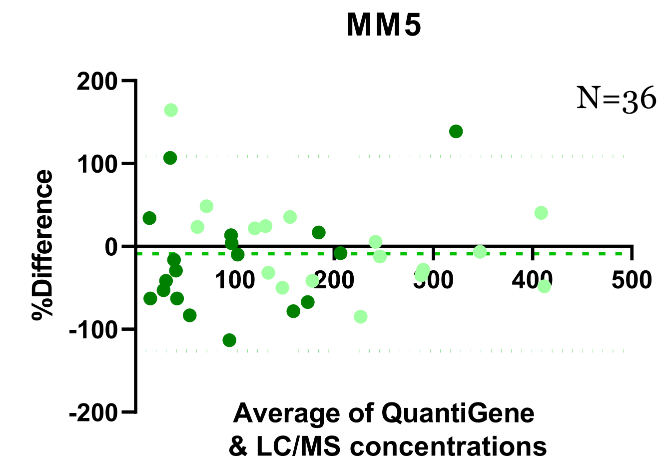


Method comparison: QuantiGene vs LC-MS/MS

Bland Altman: % difference vs average





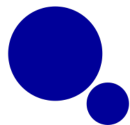


MM1: QuantiGene – LC-MS/MS	
Average of bias	-13 %
SD of bias	31 %
95% Limits of Agreement	
From	-74 %
To	48 %

MM2: QuantiGene – LC-MS/MS	
Average of bias	-37 %
SD of bias	63 %
95% Limits of Agreement	
From	-160 %
To	86 %

MM5: QuantiGene – LC-MS/MS	
Average of bias	-9 %
SD of bias	60 %
95% Limits of Agreement	
From	-126 %
To	108 %

- Fold change < 2 for the majority of samples → **acceptable for discovery purpose** (especially regarding the very high dilutions applied)
- Comparable at high concentrations; QuantiGene slightly lower than LC-MS/MS especially for liver samples
- Note: samples were not exactly collected from the same area (liver: different lobs)

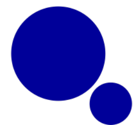


Method comparison: QuantiGene vs LC-MS/MS

Pros & Cons

Parameter	QuantiGene	LC-MS/MS
Sensitivity	Probe-dependent and matrix dependent About 50 pg/mL in plasma About 500 - 1000 pg/g in solid tissue	Matrix dependent About 150 ng/g in tissue
Specificity	Dependent on the design of the probes, may recognize the parent and some metabolites	High selectivity: possibility to distinguish parent and metabolites
Dynamic range	Medium; 2 orders of magnitude (Hook effect at high concentration)	Wide (3 orders of magnitude)
Applicability	ASOs shorter than 16 nt → potential difficulties for optimal binding	Better with ASOs < 40 nt
Time of development	3 weeks per ASO + delay due to probe design	1 week per ASO
Price	High (about 1200 € per 96-well plate)	Low
General comment	Method is **highly dependent on the design of the probe** by ThermoFisher	**Generic method** with minor fine tuning applicable

Conclusion & Perspectives



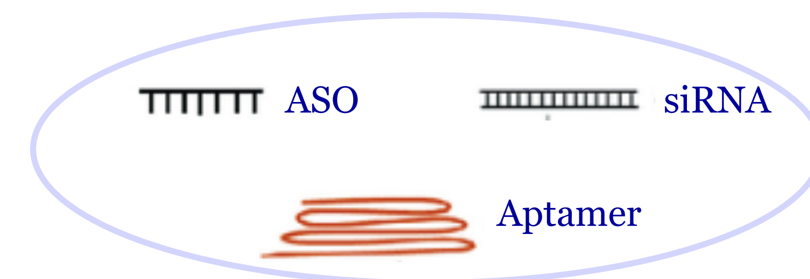
Conclusion & Perspectives

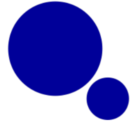
- The QuantiGene technology can be used to quantify ASOs in different matrices
- It can be used for LNA-PS –ASOs (>16nt)
- Results obtained in the current study showed comparable data between the QuantiGene and the LC-MS/MS technologies (for discovery purpose)
- The QuantiGene technology is highly sensitive (may be useful to quantify very low concentrations) but not specific (parent and truncated metabolites)
- Development of method with the QuantiGene technology is more expensive and time-consuming than with the LC-MS/MS technology

QuantiGene technology may be used as an alternative of LC-MS/MS in case of technical issues with LC-MS/MS (administration of a low dose; use of a non-intravenous route)

Continue to explore the capabilities of the QuantiGene technology

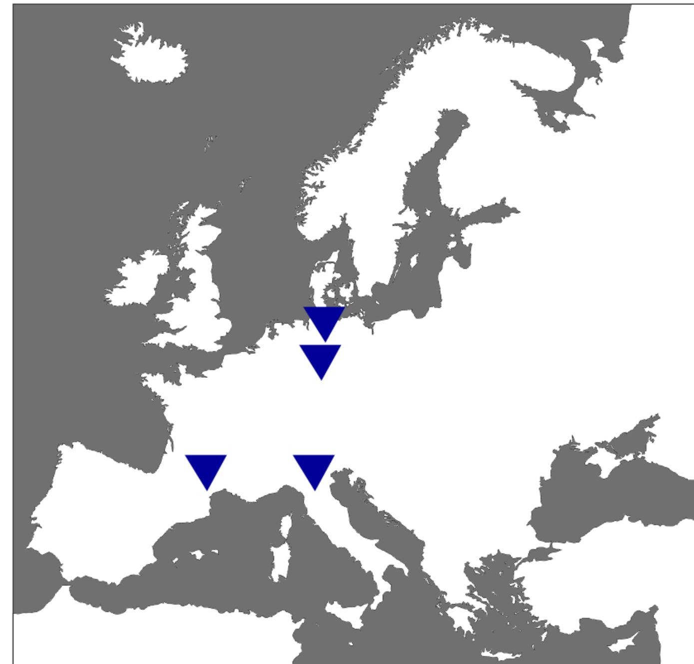
- Test other biological matrices (brain tissue)
- Test ASOs with different chemical modifications (MOEs)
- Test other oligonucleotide-based modalities (siRNA, aptamers…)





Acknowledgements

A collaborative effort



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QUESTIONS AND ANSWERS