Implementation of SplintR ligation qPCR as novel bioanalytical method for tissues quantification of oligonucleotide therapeutics for the expansion of the Evotec oligonucleotide drug discovery platform

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Abstract

Evotec possesses a comprehensive oligo drug discovery platform to support oligo-based drug discovery from *in silico* design to clinical translation. Recently, we presented a "toolbox" *in vivo* study with a MALAT1-targeting oligonucleotide (MM5) systemically delivered to mice, performed to help the generation of PK/PD models. Here, we show our ongoing effort to further expand bioanalytical methods for oligos by employing the recently developed splintR-qPCR for quantification of oligonucleotides in tissues within the MALAT1 toolbox study and comparing it to LC/MS and branched DNA (bDNA) in use in Evotec. SplintR-qPCR method was investigated for quantification of LNA/PS MALAT1 gapmer. A pool of in-house designed probes were tested, and the selected pair was utilized to quantify MM5-treated PK liver and kidney samples. The results indicate that splintR qPCR is a promising bioanalytical method for oligonucleotide quantification in tissues. However, as novel analytical method, further optimization is required to establish the optimal analytical conditions for each different tissue matrix as highlighted by the differences in kidney and liver samples. The high sensitivity, throughput and low costs compared to LC/MS and bDNA assay place splintR-qPCR as pivotal method that will strengthen Evotec oligonucleotides expertise on PK/PD modelling.

Method workflow and probes design

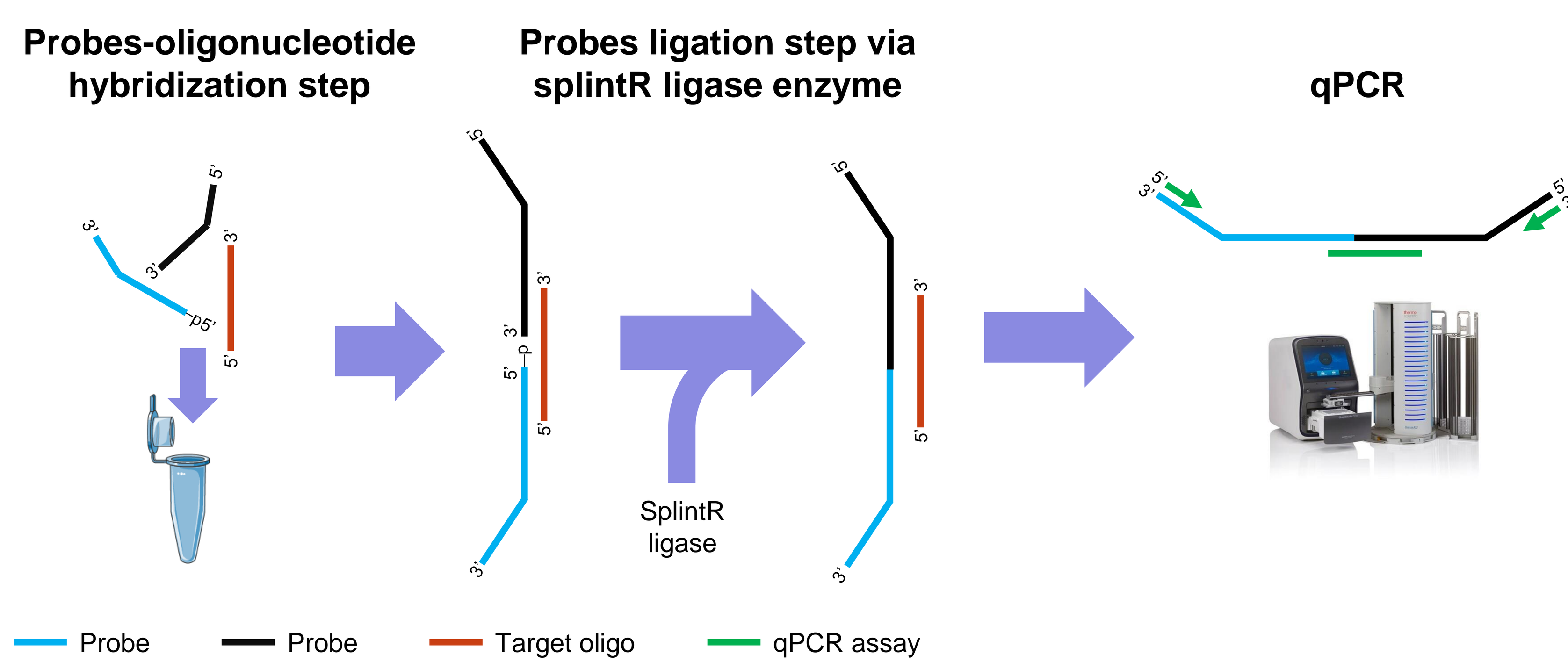


Figure 1. Method workflow: In-house designed probes are added to the reaction mix containing the processed tissue to be tested or a spiked MM5 (hybridization step between probe set and the target oligonucleotide). Then, SplintR ligase enzyme is added to the reaction mix to ligate only the probes that hybridize with the target. The ligated probes become the template for the qPCR and the amount of signal will be proportional to the amount of oligonucleotide originally present in the tissue.

ASO ID	ASO structure
MM5	[+C]*[+T]*[+A]*G*T*T*C*A*C*T*G*A*A*[+T]*[+G]*[+C]

Probes ID	Probe structure
P1	CTCGACCTCTCTATGGGCAGTCACGACAGGCATT pCAGTGAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P2	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTC pAGTGAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P3	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCA pGTGAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P4	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCAG pTGAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P5	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCAGT pGAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P6	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCAGTG pAACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P7	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCAGTGA pACTAGCGCTGAGTCGGAGACACGCAGGGCTTAA
P8	CTCGACCTCTCTATGGGCAGTCACGACAGGCATTCAGTGAA pCTAGCGCTGAGTCGGAGACACGCAGGGCTTAA

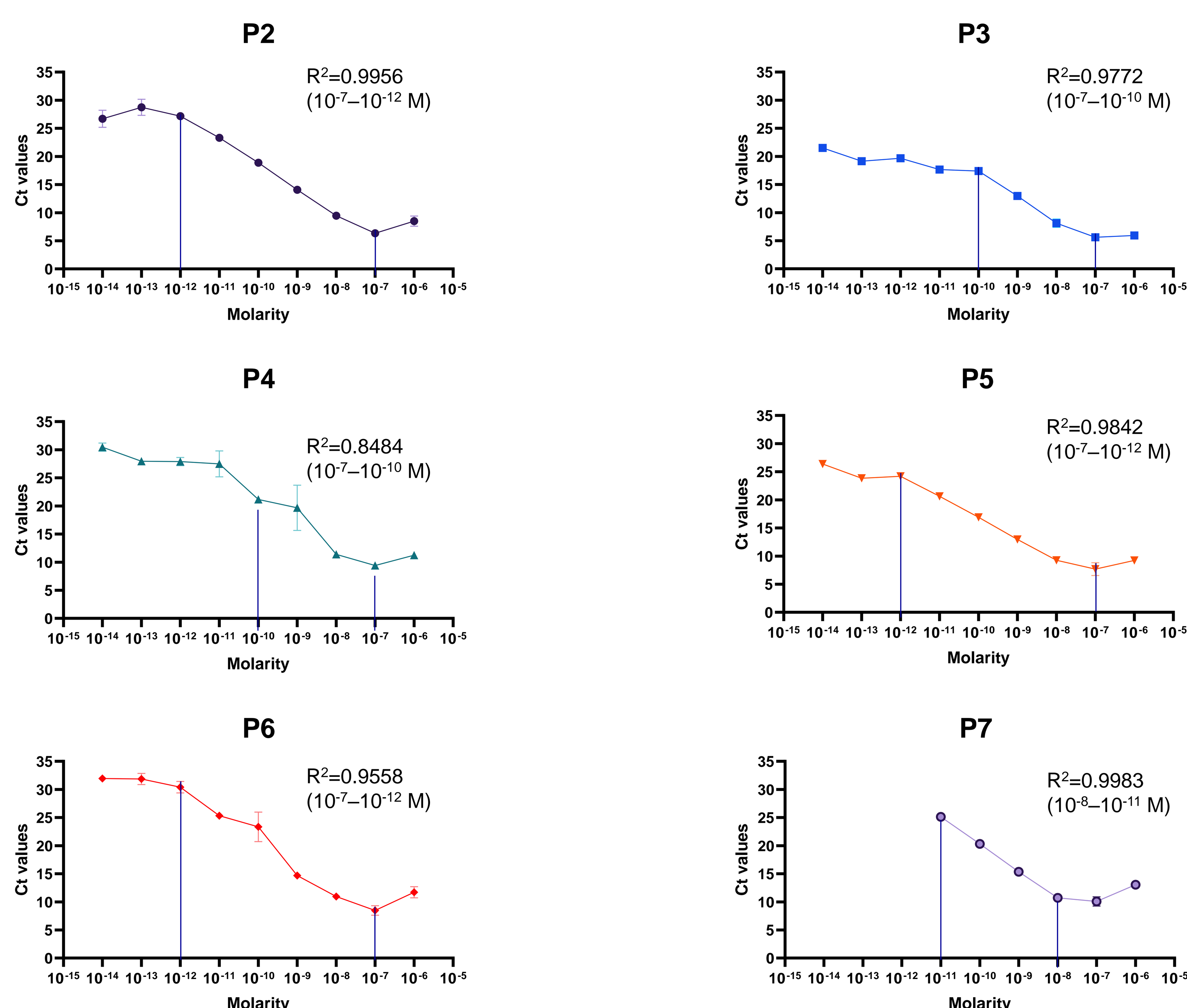


Figure 2. Eight probe sets were designed comprising an oligonucleotide binding part (in red and blue in Figure 1) and qPCR primer binding part (as shown in Figure 1). Each probe set was tested in a 9-point dilution series standard curve ranging between 10^{-6}–10^{-13} M in water. Probes P1 and P8 were not tested due to failed QC during probes design (GCs content, hairpin, duplexes formation). The graphs show that all six tested probes did amplify the target over a variable range of concentrations (samples n=3 technical replicate qPCR). Probes P2 and P5 detected all points of the curve and showed the widest linear range (10^{-7}–10^{-12} M) with R^2 >0.98. Probe P3, P4 and P6 did detect all points, but high variability was shown. P7 probe did not detect the target oligo below 10^{-11} M, with a linear range 10^{-8}–10^{-11} M. Probe set P5 was selected for the study. MM5 chemical modifications: *=PS, [+N]=LNA

Matrix effect evaluation

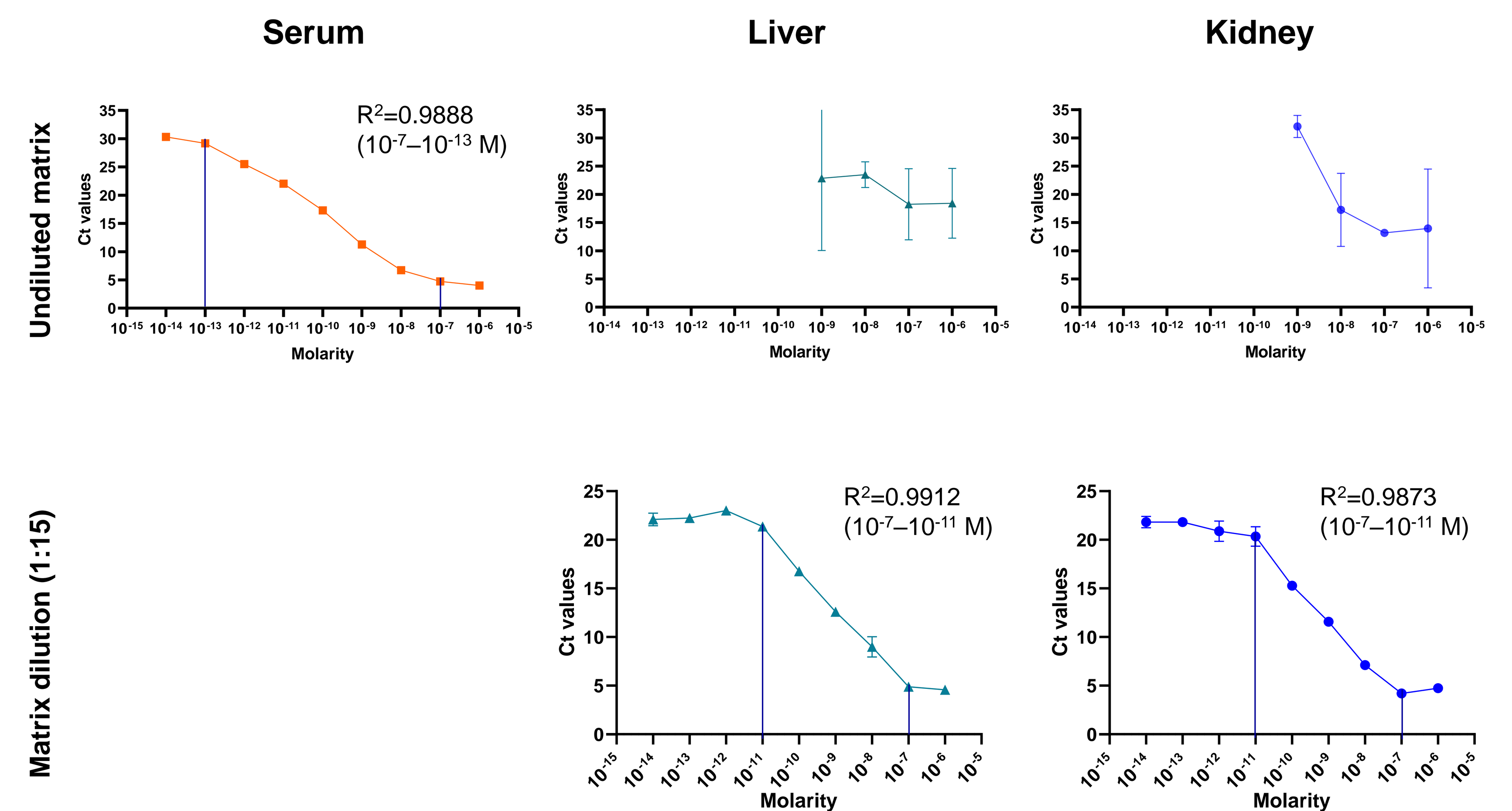
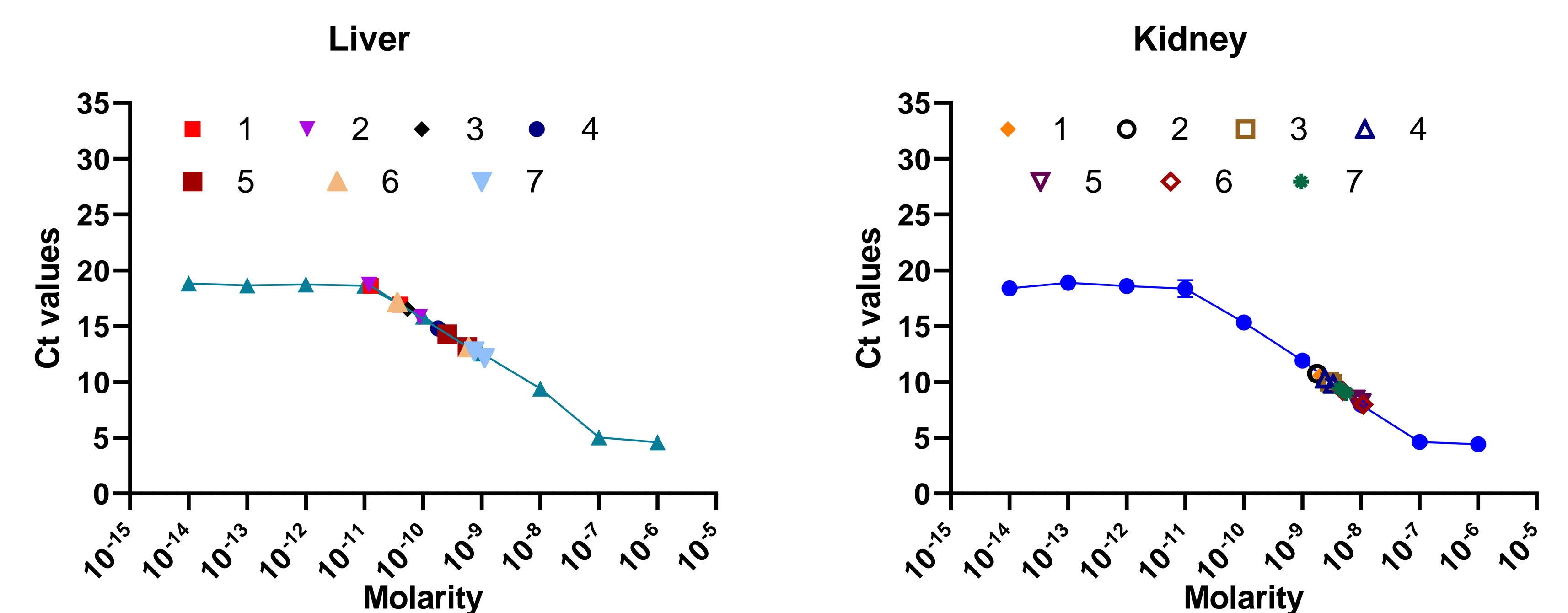


Figure 3. After the selection of the P5 probe set, standard curves with MM5 spiked in serum, liver and kidney matrixes were performed to assess possible matrix effect on the oligonucleotide detection. Tissues of untreated mice were processed using Tissuelyser II in 30 µL/mg RIPA buffer, followed by 1h incubation on ice and centrifugation at 16,000 g, at 4°C for 30 min. The supernatant was collected into a new Eppendorf and stored at -80°C. To collect serum, blood of untreated mice was incubated 1h at RT, centrifuged at 10,000 g for 5 min at RT, and the serum supernatant was collected. Undiluted liver and kidney matrices exhibited qPCR inhibition while serum did not show interference resulting in a 6-log linear range (10^{-7}–10^{-13} M, R^2>0.98). Further optimisation for liver and kidney matrixes was performed. A series of dilutions of matrix solutions (5, 7.5, 10, 15, 20, 25 – data not shown) were tested. Dilutions ≥15 allowed to obtain 5-log linear range (10^{-7}–10^{-11} M, R^2>0.98) MM5 standard curves. A dilution factor 1:15 was selected for following experiments using liver and kidney matrixes.

MALAT1 toolbox PK/PD study samples quantification and method comparison to LC/MS and bDNA



Tissue	Dose (mg/kg)	Animal ID	ng ASO/ g tissue	Ratio bDNA/Splint	Ratio LC/MS/Splint	Ratio LC/MS/bDNA
Liver	8.3	1	644	83.30	25.33	0.30
		2	1168	8.67	16.62	1.92
	25	3	3023	9.47	18.16	1.92
		4	3917	8.12	19.68	2.42
	75	5	9934	11.60	23.35	2.01
		6	7616	12.73	29.02	2.28
		7	22375	1.83	6.61	3.61
Kidney	8.3	1	49105	1.81	1.11	0.61
		2	37880	1.71	0.17	0.10
	25	3	73215	1.52	2.53	1.67
		4	62857	2.25	3.42	1.52
	75	5	217295	1.15	1.52	1.33
		6	190361	1.25	1.78	1.42
		7	118986	1.10	2.71	2.48

Figure 4. Liver and kidney samples of MM5 PK/PD study were quantified using splintR qPCR and then compared to previous MM5 quantification run using LC/MS and bDNA methods. Tissue of three groups of treated mice (8.3, 25 and 75mg/kg, 14d, n=3/group) were processed as described in Figure 3. Quantification of kidney samples showed correlation with the 3 treatment dosages, and the comparison to LC/MS and bDNA confirmed the validity of splintR-qPCR showing 1.5 bDNA/SplintR and 1.9 LC/MS/SplintR average ratios. In liver samples, a dose-relation was exhibited although higher discrepancy was observed between bioanalysis methods, suggesting that further optimization is needed and that a specifically developed protocol for each tissue may be required.

Conclusions

Overall, the splintR-qPCR was proven as valid and comparable method to bDNA and gold-standard LC/MS. As novel analytical method, further optimization is required to establish the optimal analytical conditions for each different tissue matrix as highlighted by the differences in kidney and liver samples. Interestingly, the highest sensitivity was shown for serum, which will allow to generate more accurate PK/PD models. A complete panel of tissues will be required to estimate the method sensitivity for each tissue and the range of applications the SplintR qPCR can be employed for. The combination of in-house probes design, high throughput performance of 384w-format qPCR brings a reduction of costs and timelines compared to bDNA and LC/MS assay as well as the high sensitivity place splintR-qPCR as pivotal method that will strengthen Evotec oligonucleotides expertise on PK/PD studies.