Oligonucleotide bioanalysis in early drug development

EBF Spring Focus Workshop: Oligos & Peptides

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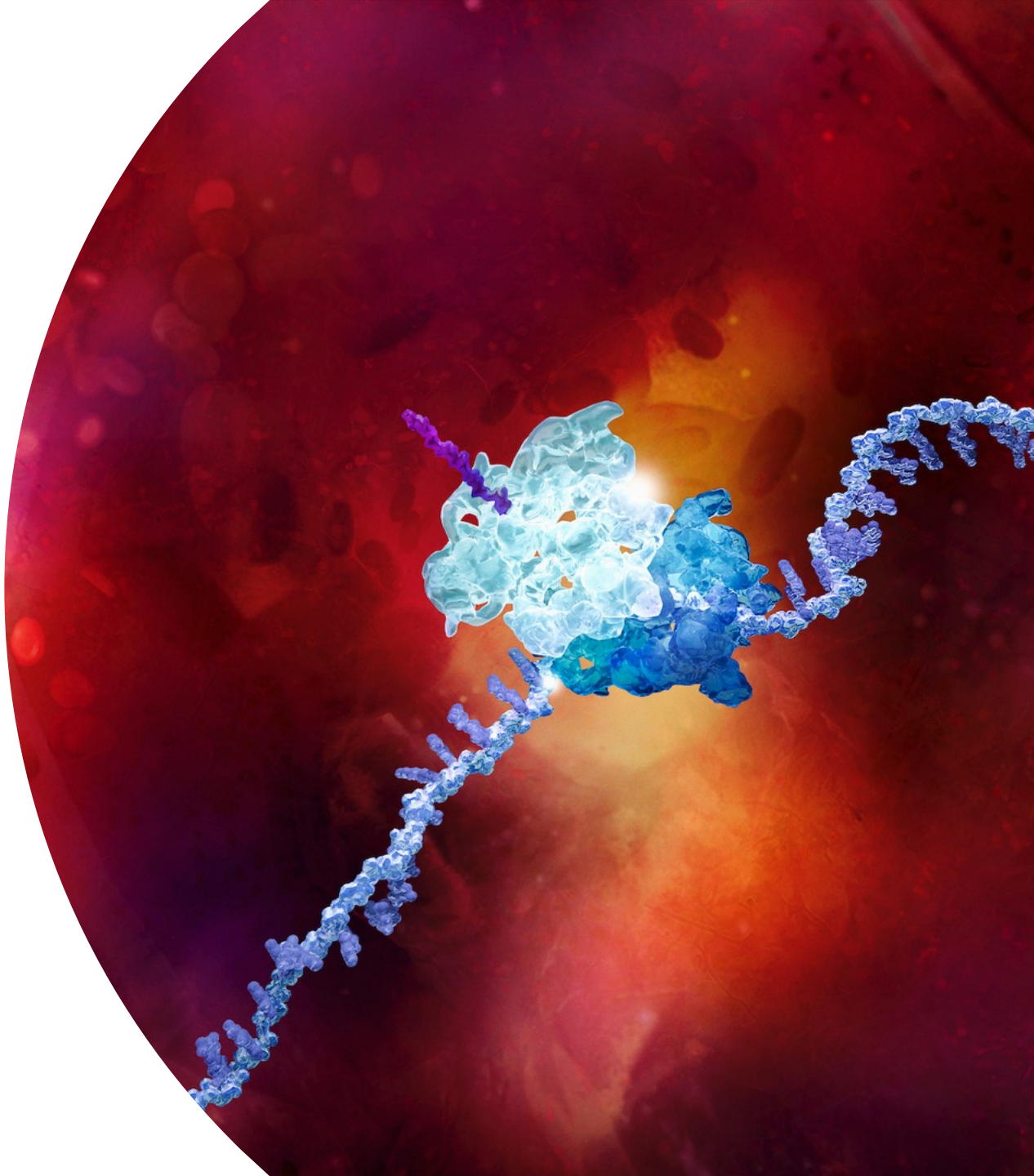


Oligonucleotide bioanalysis in early drug development

“Screening” of ASOs in-vivo

- Several sequences evaluated (10 not unusual)
- Exposure vs t½ vs knockdown

Small studies with tool or novel ASOs

- Matched IS (e.g. S34 labelled) not always available
- Planning and turn around times are short
- Limited time for method development

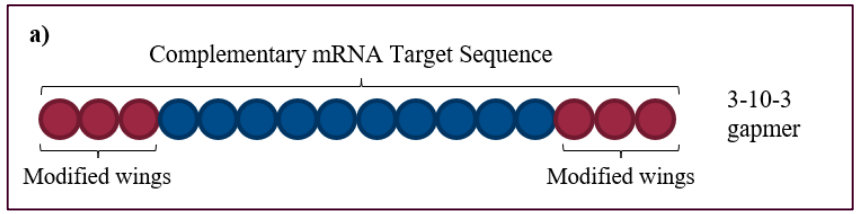
Novel chemistries

Novel conjugates

Oligonucleotides –type of analytes

Antisense oligonucleotides (ASO)

- Single strand
- Splice switching oligonucleotide (SSO)

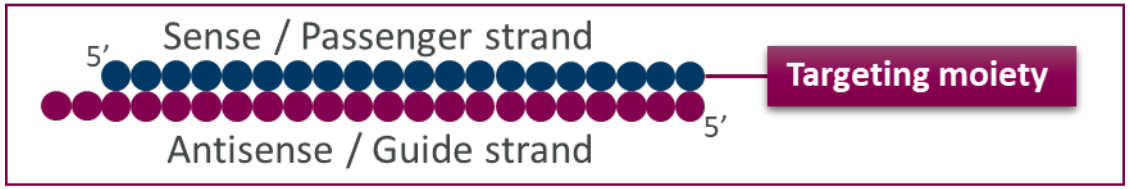


Antisense oligonucleotide

- 16-25 nucleotides
- Chemically modified nucleotides
- Phosphorothioate backbone

Small interfering RNA (siRNA / RNAi)

- Small interfering RNA
  - Double stranded
  - Guide and passenger strand
- Small activating RNA (saRNA)



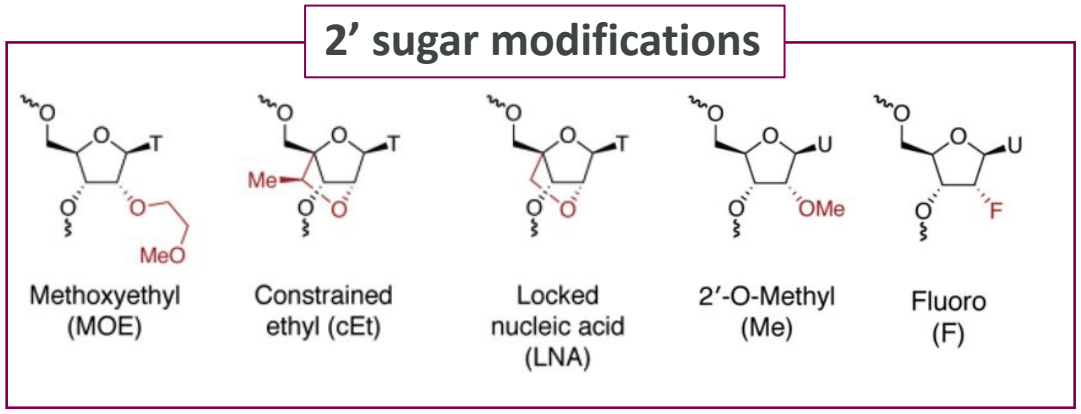
Small interfering RNA

- Antisense strand active
- Conjugated
- Bioanalytical endpoints: Guide / Passenger / RISC loaded
- Phoshporothioate only on 3' and 5' ends

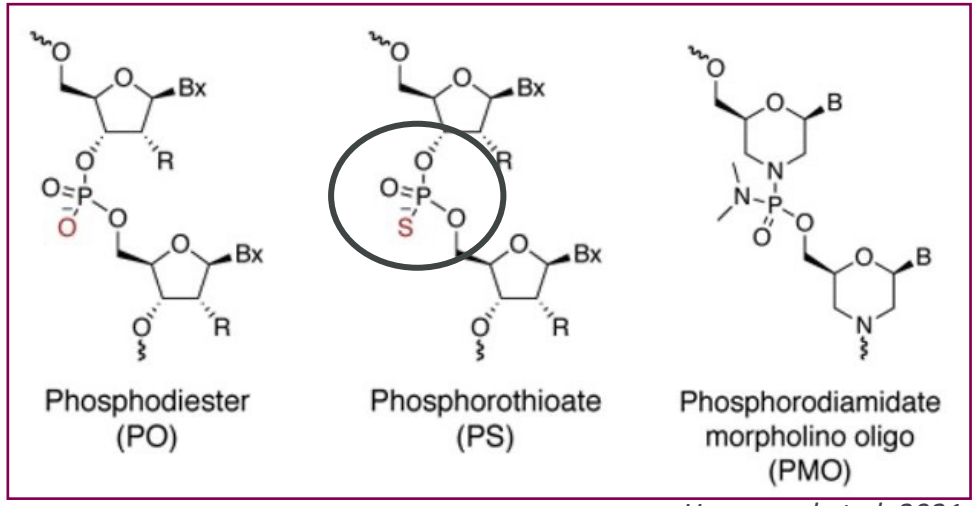
Antisense oligonucleotide chemistry

Phosphorothioate (PS) modified backbones

- Used in ASOs and siRNAs
- ASOs have more PS modifications than siRNAs
- Does not occur naturally -> can be used for specificity (m/z 95)
  - High collision energy to create as many PSO fragments as possible



Hammond et al.

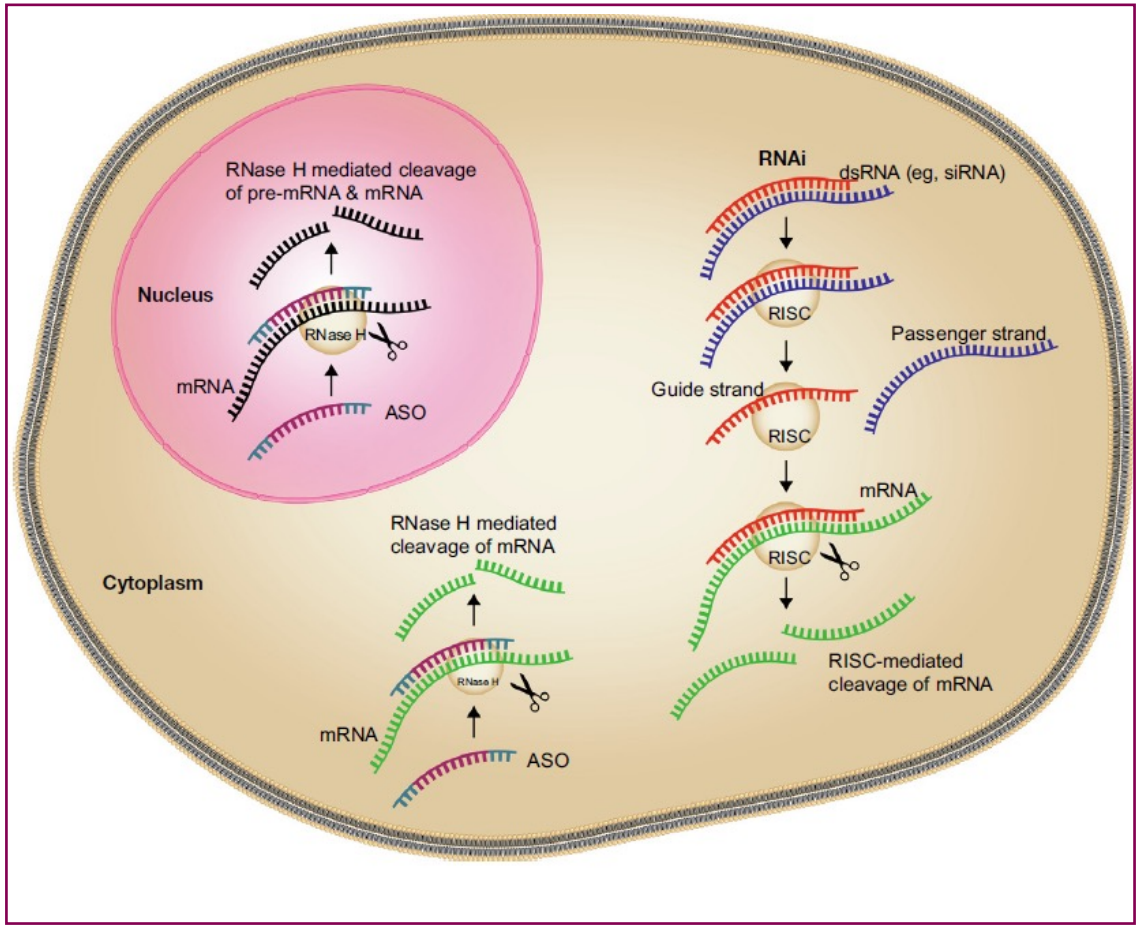


Hammond et al. 2021

Mechanism of action is different between ASOs and siRNA

ASOs are active in cytoplasm and nucleus
siRNA are active in the cytoplasm

- ASOs target pre-mRNA and mature mRNA
- siRNA target mature mRNA
- ASO-mRNA duplex recruits RNase H
- siRNA is taken up by Argonaut 2 and creates RISC[1] and binds mRNA



Weidolf et al 2021

1. RNA induced silencing complex

Analytical technologies for quantifying therapeutic oligonucleotides

- LC-MS – standard approach in early work
- Hybridisation assays
- HPLC-fluorescence
- PCR assays

TABLE 1

Considerations for selection of a suitable bioanalytical method for ON analysis.

Method	Sensitivity	Selective quantification of metabolites	Selective quantification of conjugates	Assay complexity (sample preparation/method development)	Comments	Refs
LCMS and LCMS/MS	Medium	Yes	Yes	High/Low	Needs dedicated LCMS for ONs	43
hELISA	Medium/ High	No	No	Low/Medium	Requires specific probe(s)	65
ECL	Very high	No	No	Low/High	Requires specific probe(s)	68
LC-Fl	High	Yes	Yes	Low/Medium	Requires specific probe(s)	71
LC-UV	Low	Yes	Yes	Low/Low		50

Weidolf et al 2021

LC-MS for oligonucleotide quantification

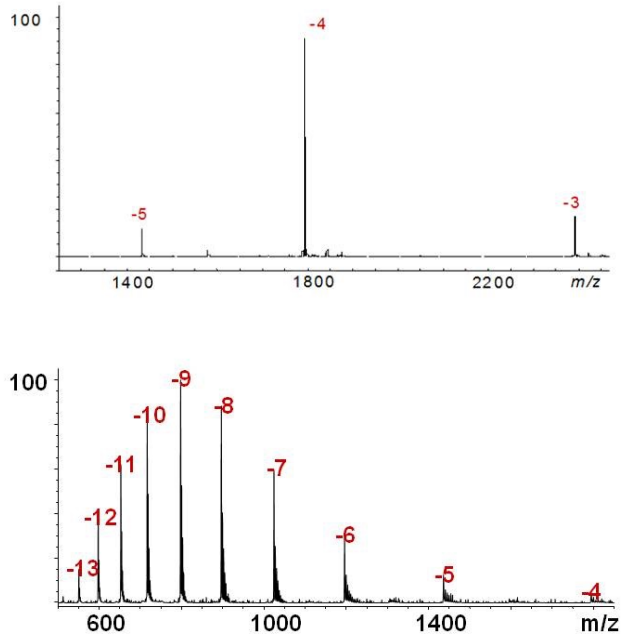
LCMS is the standard method for oligo quantification

- ASOs and siRNA have poor retention on C18 colums
- Use of ion pairing to increase retention
  - TBA[1]/HAc[2], TEA[3]/HFIP[4], DIPEA[5]/HFIP
  - Not suitable for analytes in positive mode later
- Alternatively use HILIC[6]

Sample extraction needed to break protein binding

- Extraction using liquid-liquid extraction

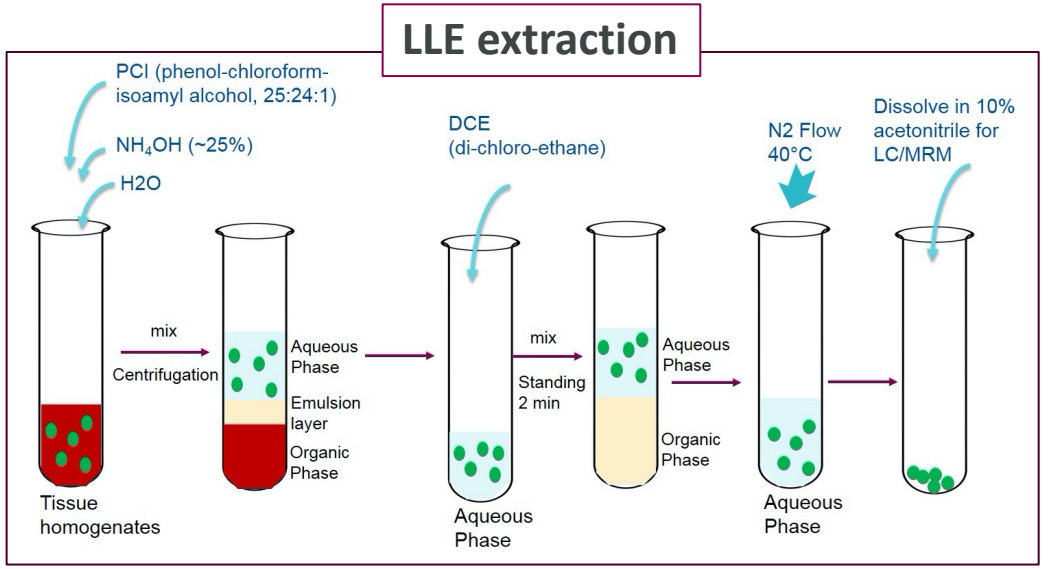
Charge state distributions



1. Tributylammonium acetate 2. Acetic acid 3. Triethylamine 4. Hexafluoroisopropanol 5. Diisopropylethylamine 6. Hydrophilic interaction chromatography

Liquid-liquid extraction is needed for efficient extration of oligonucleotides from tissue samples

- Use of tissue homogenate or plasma
- Internal Standard
  - Ideally labelled ASO/siRNA
  - Otherwise, surrogate oligonucleotide with similar chemistry but difference in size
- Liquid-liquid extraction (LLE) to extract oligos from tissues
  - Efficient extraction of ONs[1]
  - Dirty extract, needs SPE[2] or dilution



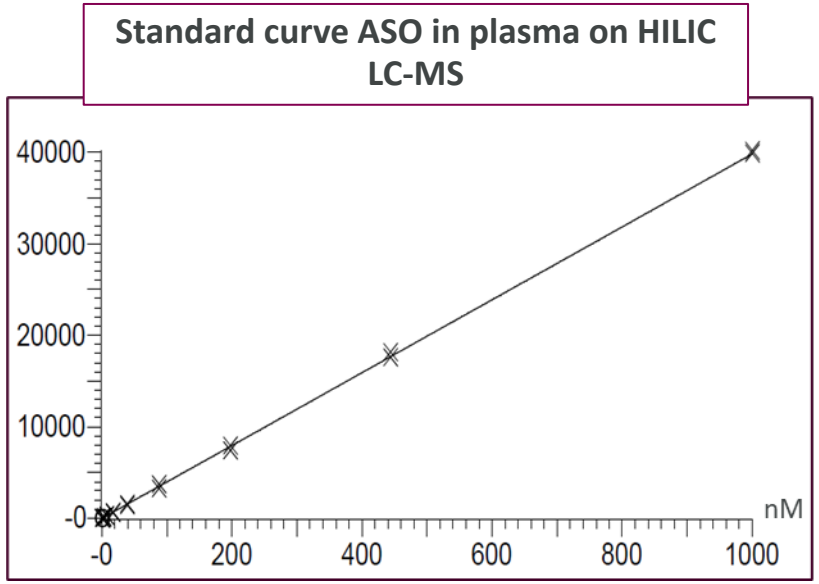
1. Oligonucleotide 2. Solid Phase Extraction

LC-MS – HILIC with sensitivity

Good retention of ASOs and siRNAs on HILIC[1] columns

Increases flexibility of instrument use

- HILIC on e.g. a Sciex 7500 matches sensitivity of ion-pairing on older systems
- HILIC mobile phases can be optimised for oligonucleotides
  - Possible to reach <0.1 nM robustly, and toward low/single digit pM for some ASOs
  - SPE gives cleaner background – increased sensitivity
    - Sample can be concentrated to increase sensitivity (most likely achieve single digit pM)



1. Hydrophilic interaction chromatography

LC-MS – usually good enough

- Limit of quantification (LOQ) usually sufficient
- LOQ achieved in tissue usually around 1-5 nM in homogenate (LLE only) – 5-30 nmol / kg
- Good enough for liver and kidney, exposure is high in these tissues
- For tissues such as heart and plasma, LOQ can be limiting
- In discovery there still needs to be ”high enough” exposure to give knockdown, usually this is followed by an oligonucleotide concentration that can be quantified

Availability of blank tissue can be a challenge

Blank tissue needed for matrix matchin can be a challenge to obtain

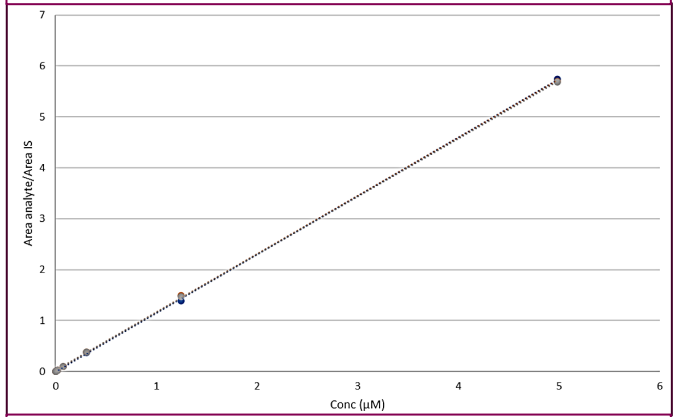
Use surrogate matrices for rare tissues

- Blank tissue for matrix matching can be challenging to obtain from smaller species in sufficient amounts
- Liver homogenate could be used as a surrogate for e.g. kidney and heart tissue

Mixed standards

- For screening studies, mixed standards can be an option
  - Check for sequence interactions – possibility of duplex formation

Overlap of standard curves in liver, kidney, and heart

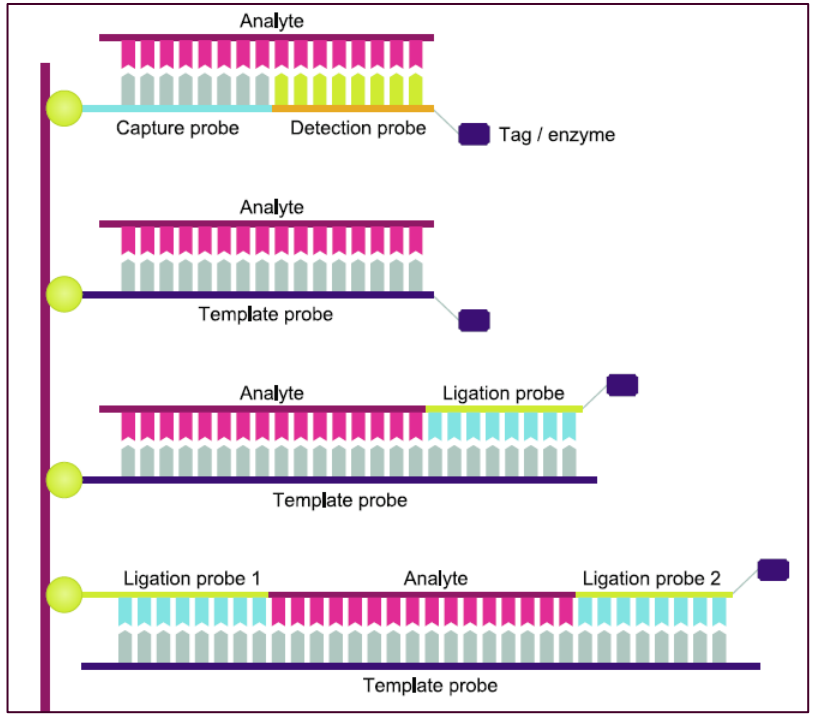


When using an internal standard the standard curves overlap. Can use liver standard curve for kidney and heart samples.

Hybridisation assays for oligos when sensitivity is needed

Hybridisation assays

- Hybridisation assays provides lower LOQs[1] than LC-MS
- Use complementary probes for capture/detection
  - Direct conjugates to reporter
  - Use antibody to detect tag
- Development time can substantial
  - Reactivity with conjugated and unconjugated needs to be the same
- Can be beneficial to use for conjugates in plasma for total concentration



Weidolf et al 2021

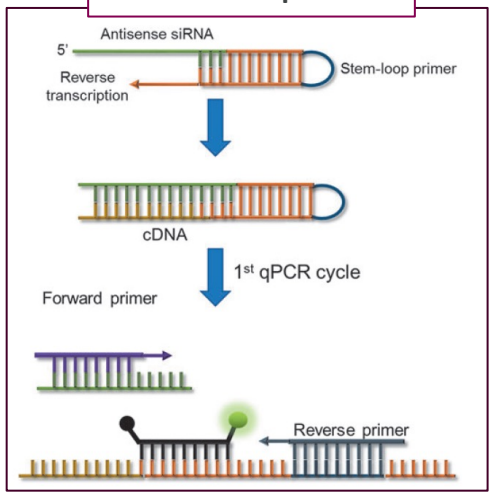
1. Limit of quantification

Stem loop qPCR assays for oligonucleotide quantification

Stem loop RT qPCR for ASO and siRNA quantification

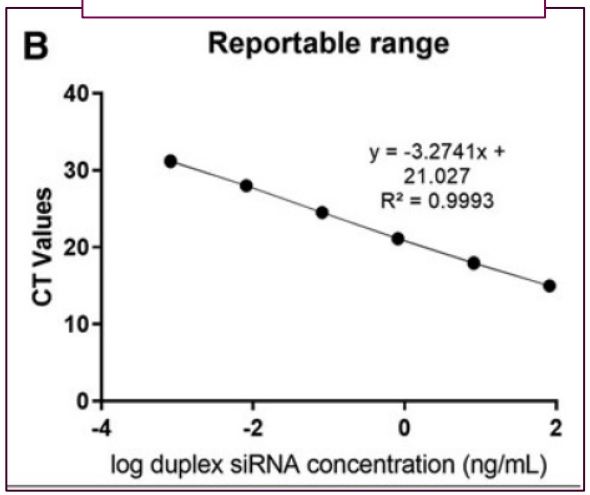
- Can be used for ASOs although challenging for short ASOs
- Shorter development time than for hybridisation assays
- Matrix effects, varying between tissues
- Not always 1:1 correlation with LC-MS

Stem loop PCR



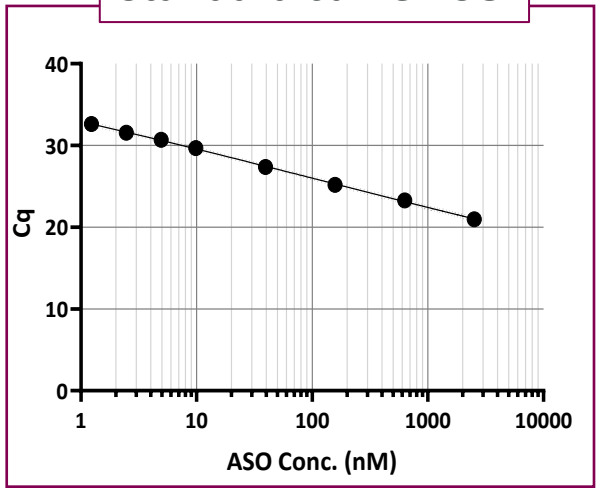
Castellanos Rizaldos et al 2020

Standard curve siRNA



Castellanos Rizaldos et al 2020

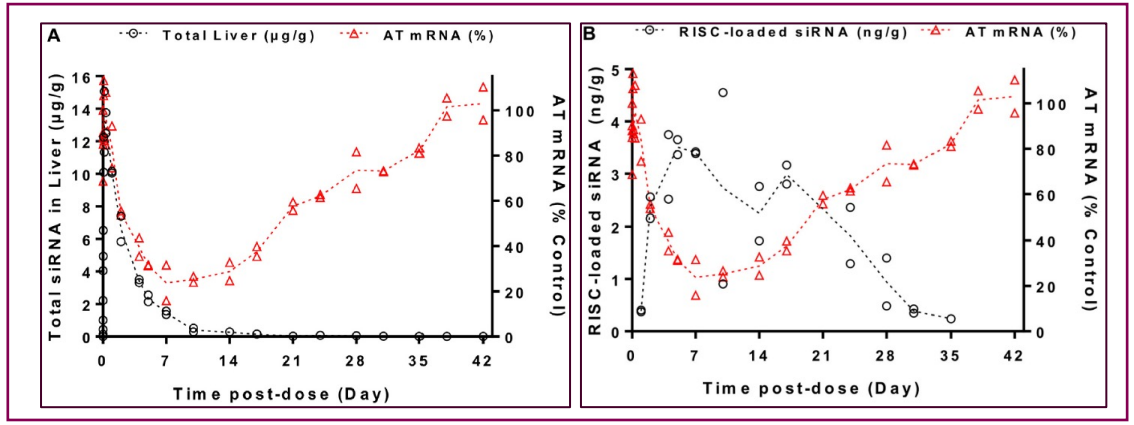
Standard curve ASO



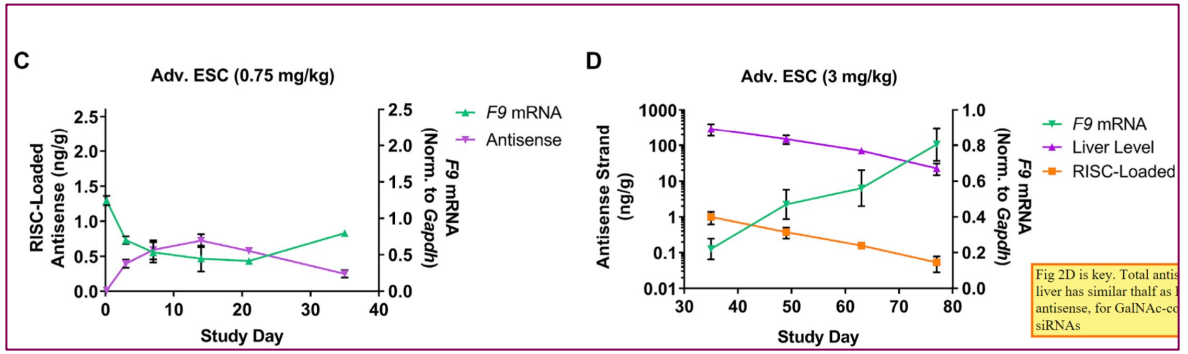
siRNA – RISC loading

PK of siRNA correlates with RISC loaded siRNA and total siRNA at different timepoints

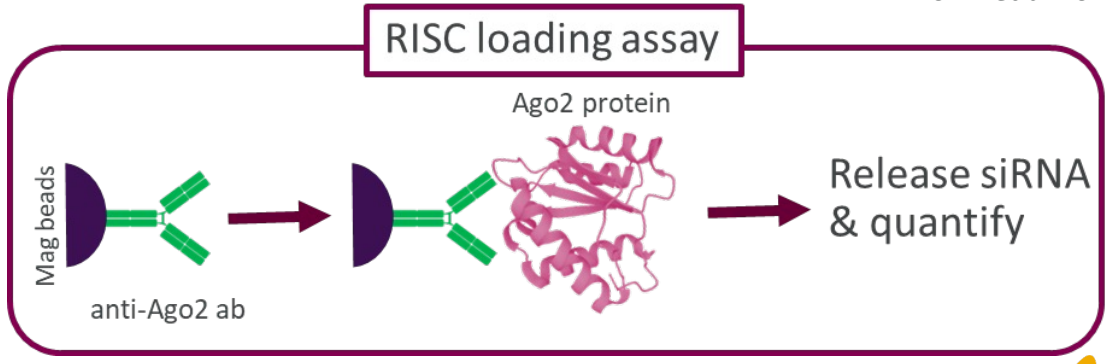
- **R**NA **I**nduced **S**ilencing **C**omplex
  - Argounaut 2 + siRNA = RISC
- siRNA needs to be taken up by Ago2 to create the catalytically active silencing complex
  - The sense/passenger strand is shedded after uptake
- Low amount of siRNA in RISC – sensitive method needed (e.g. qPCR method)
- RISC loaded siRNA correlates better with kd than total siRNA at early timepoints
  - Later parallel with total siRNA



Nair et al 2017



Brown et al 2021

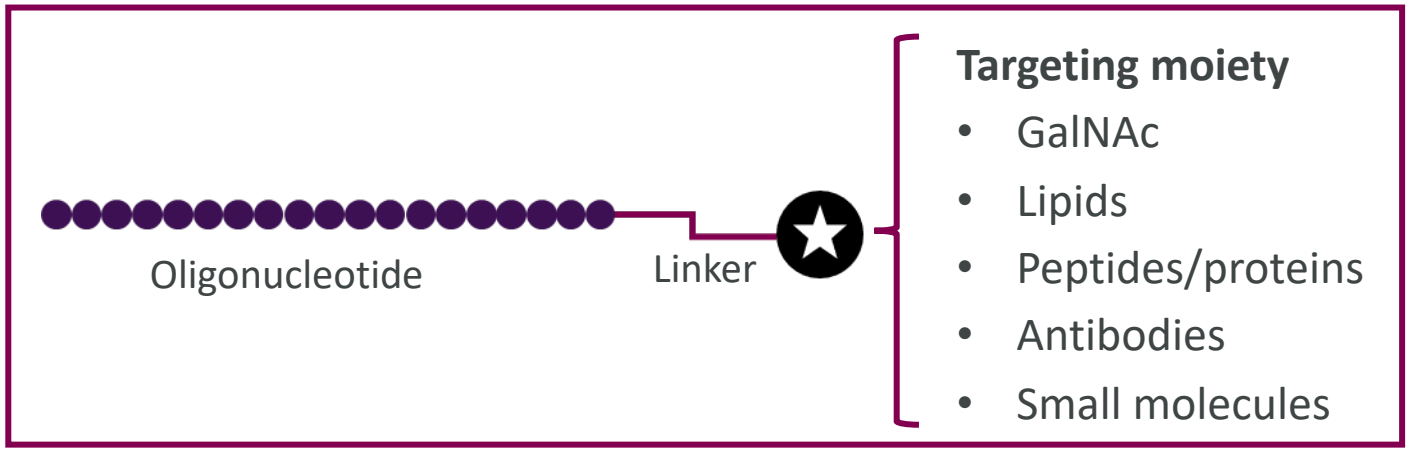


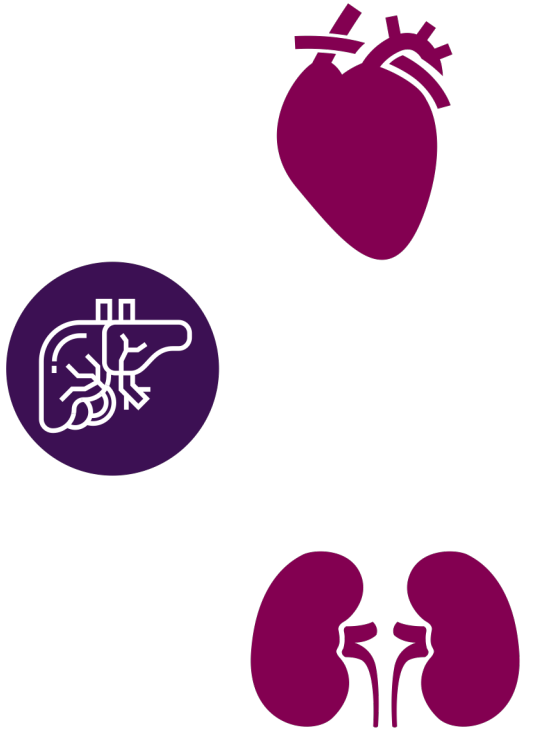
Conjugated oligonucleotides for tissue targeting

Reaching tissues outside of liver and kidney requires conjugation

- And for specific cell types

- Unconjugated siRNAs have poor tissue uptake
- ASO conjugates typically use cleavable linkers
- siRNA uses non-cleavable linkers on passenger strand





Olgionucleotide conjugates can be challenging to quantify

ASO conjugates

- Usually contain cleavable linkers
  - Targeting moiety is cleaved off in tissue
- Quantification on ”naked” ASO
- PK in plasma more challenging – contains conjugated and unconjugated forms
  - Can be beneficial to use hybridisation assay or similar to quantify ”total concentration”

siRNA conjugates

- Sense/passenger strand usually conjugated
- Active strand is antisense/guide strand
- Usually enough to quantify guide strand in early studies
- Non-cleavable linker for siRNA conjugates (conjugate strand can be a challenge)
  - Hybridisation or PCR assay

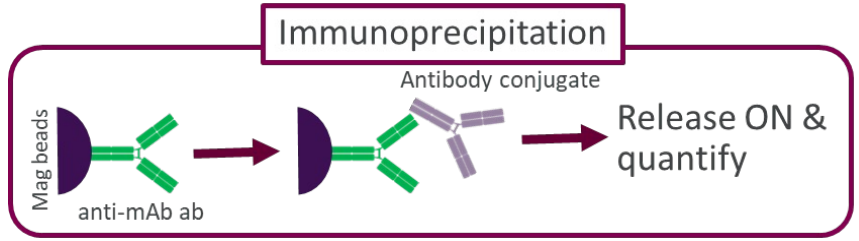
Conjugated oligonucleotides – sample preparation

Peptide & protein conjugates

- Oligonucleotides conjugated to peptides/proteins/antibodies will not ”survive” LLE extraction
  - If interested in intact conjugate. Need other extraction or analytical method
- Hybridisation catch and release assays
  - Use complementary probe for enrichment of oligonucleotide from sample

Antibody conjugates

- Immunoprecipitation to enrich for conjugate
  - Release oligonucleotide
- Use hybridisation based assay for total concentration



Selection of bioanalytical method

LC-MS is the go-to method

- High throughput / easy method development (mostly) / generic reagents

Hybridisation methods and PCR methods when extra sensitivity needed

- Usually plasma exposure (and in-vitro assays)
- Lead time for development can be long
- Ideally replaced with LCMS if sensitivity can be achieved

Method	LLOQ	Metabolites	Conjugates	Comment
LC-MS	1 nM	Can distinguish	Specific	µSPE for low pM LOQ
Hybridisation	0.01 – 0.1 nM	Captures metabolites	Measures 'total' conc	
RT-qPCR	1-10 pM	Captures metabolites	Measures 'total' conc	RISC loaded siRNA

Summary

- LC-MS is in most cases fit for purpouse
- HILIC on newer instruments provides good sensitivity
- Other assay formats available for more sensitivity
- siRNA have several bioanalytical endpoints & can require several analytical methods
- Tissue extraction requires strong chaotropic agents
- Conjugated oligonucleotides can be quantified as ‘total’ concentration or with assays targeting specific forms

Questions