ACUITAS THERAPEUTICS

Ionizable lipid impurities drive RNA-lipid adduct formation in LNP, impacting in vivo expression

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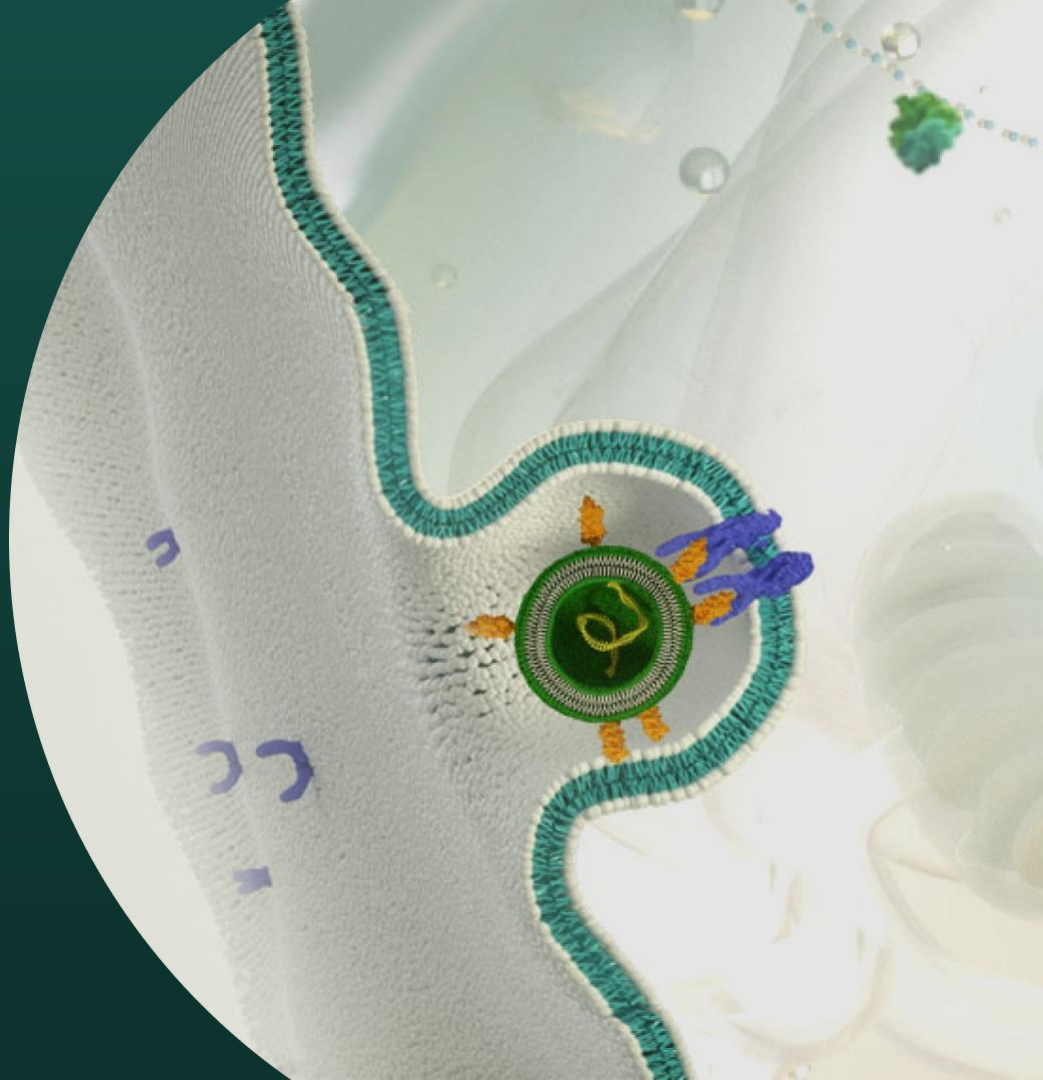


Who We Are

OUR LNP TECHNOLOGY

First Clinically Approved:

- **RNA interference-based Medicine (Onpattro®)**
- **mRNA Vaccine (Comirnaty®)**
- **Personalized CRISPR gene editing therapy (baby KJ)**

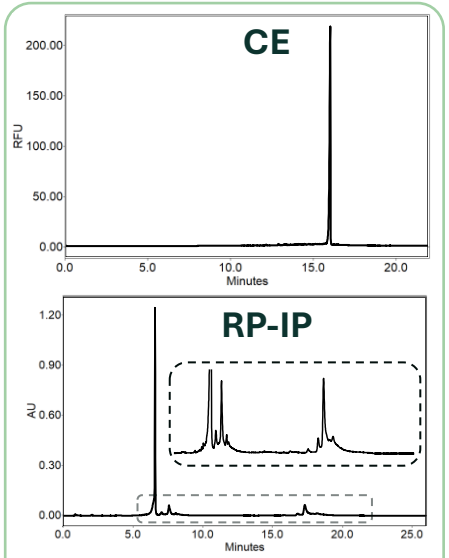
WHO WE WORK WITH

- Cutting edge **pharmaceutical & biotechnology** companies
- Leading **academics in universities & institutes**
- **Foundations** & **NGOs**

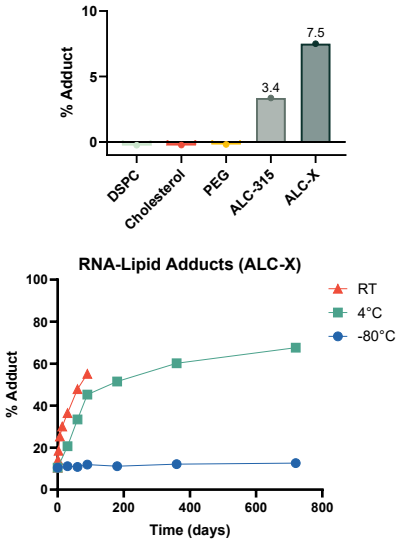
HOW WE WORK

We exclusively work in collaboration with partners and are **focused on supporting our partners to bring their drug products to patients**

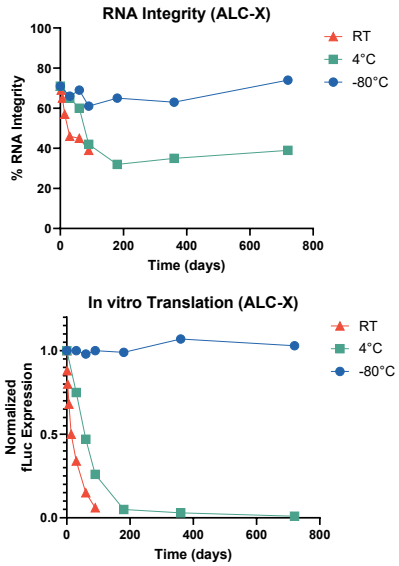
What are RNA-Lipid Adducts?



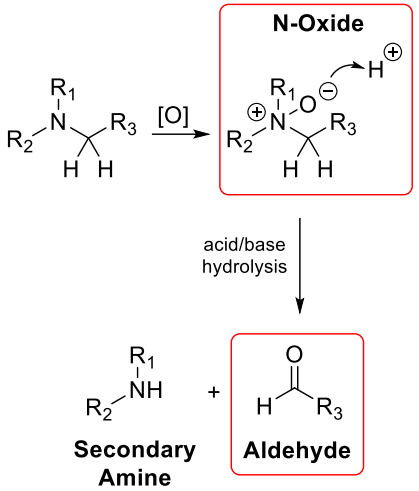
- RNA-lipid impurity undetected by typical mRNA purity techniques
- Detected as late-peaks in RP-IP



- Adduct formation linked to the ionizable lipid
- Formation is dependent on time and temperature



- RNA-lipid adducts, together with RNA integrity, **reduce *in vitro* protein expression**



- N-oxide forms through tertiary amine oxidation
- Acid/base-catalyzed hydrolysis generates aldehydes and secondary amines

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Investigative Goals

01

Develop Analytical Methods

Implement analytical methods to measure RNA-lipid adducts and adduct forming impurities

02

Impurity Testing

Screen multiple ionizable lipids and lipid lots for adduct formation and adduct forming impurities

03

Determine Root Cause

Identify the main impurities contributing to RNA-lipid adduct formation

04

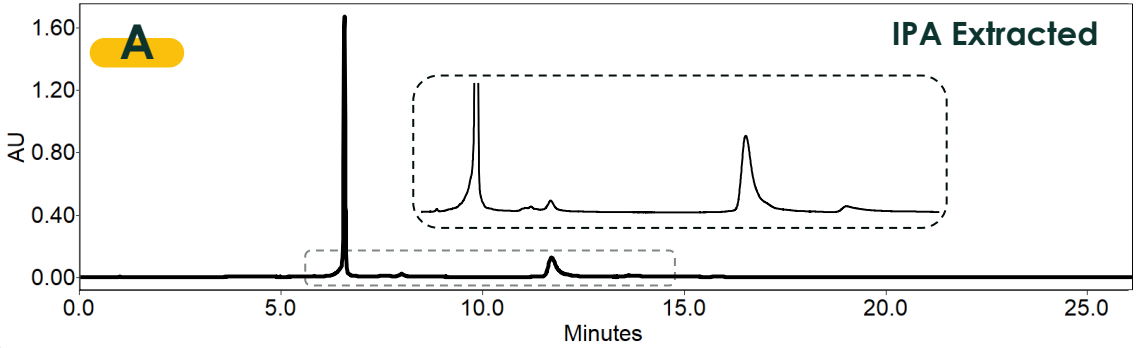
In vivo Expression and Safety

Assess impact to *in vivo* expression and safety in mice with high level of impurities

Measuring RNA-Lipid Adducts

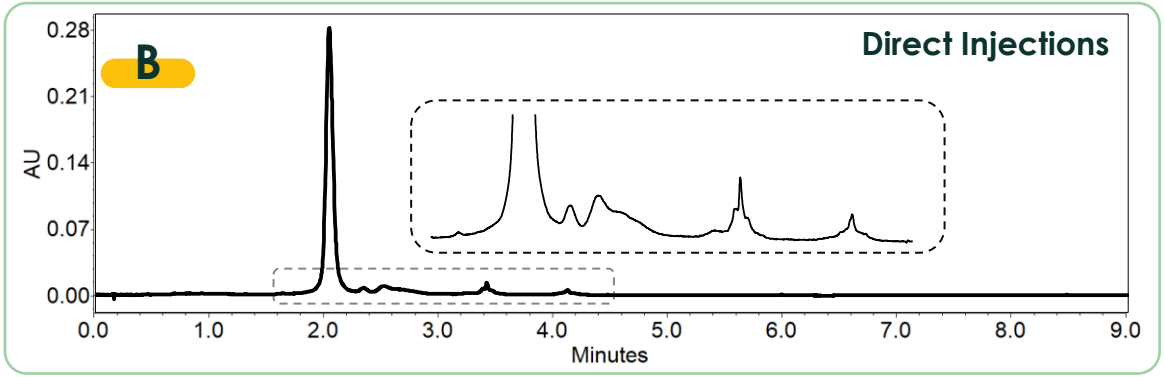
Binary Reactions

- Mixture of ionizable lipid (ethanol) and buffered mRNA (aqueous)
- Incubate at room temperature
- Extract RNA via IPA precipitation and analysis on **Method A** or direct analysis on **Method B**



LNP Analysis

- Extract RNA via IPA precipitation and analyze on **Method A**
- Disrupt LNP with surfactant and analyze directly on **Method B**



A. Packer, M., Gyawali, D., Yerabolu, R. *et al.* A novel mechanism for the loss of mRNA activity in lipid nanoparticle delivery systems. *Nat Commun* **12**, 6777 (2021).
B. Imiołek, M., Cojocaru, R., Fekete, S., Le Huray, J. & Lauber, M. High-Throughput Quantification and Characterization of Dual Payload mRNA/LNP Cargo via Deformulating Size Exclusion and Ion Pairing Reversed Phase Assays. *Anal. Chem.* **97**, 3091–3098 (2025).

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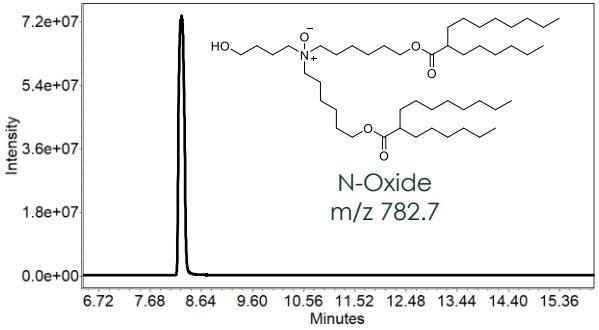
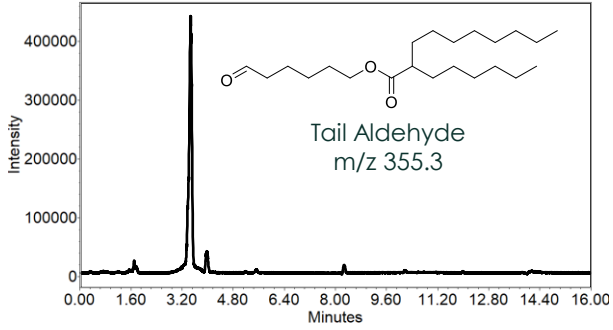
Measuring Lipid Impurities

Targeted Impurity Analysis

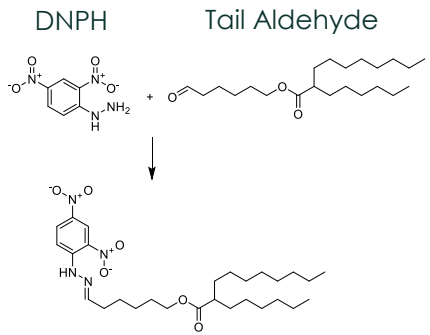
- Direct impurity detection in raw lipids or LNP by UPLC with MS detection
- Quantification using internally synthesized standards

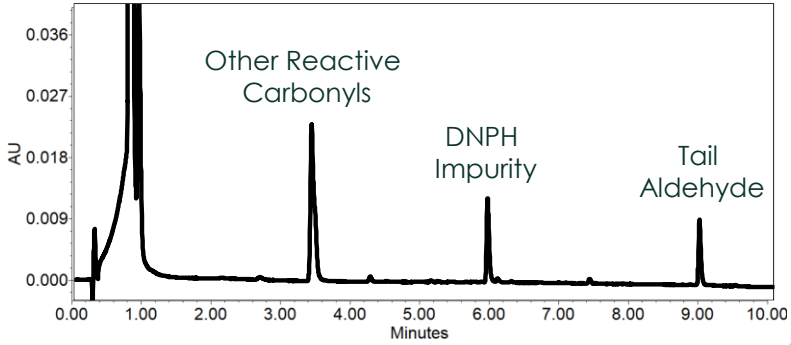




Derivatization with DNPH

- 2,4-Dinitrophenylhydrazine (DNPH) reacts specifically with carbonyls (ketones, aldehydes) to form derivatization products
- Inject into UPLC-PDA/MS monitoring for mass and A_{360}





Birdsall, R. E. et al. Monitoring stability indicating impurities and aldehyde content in lipid nanoparticle raw material and formulated drugs. *J. Chromatogr.* B **1234**, 124005 (2024).

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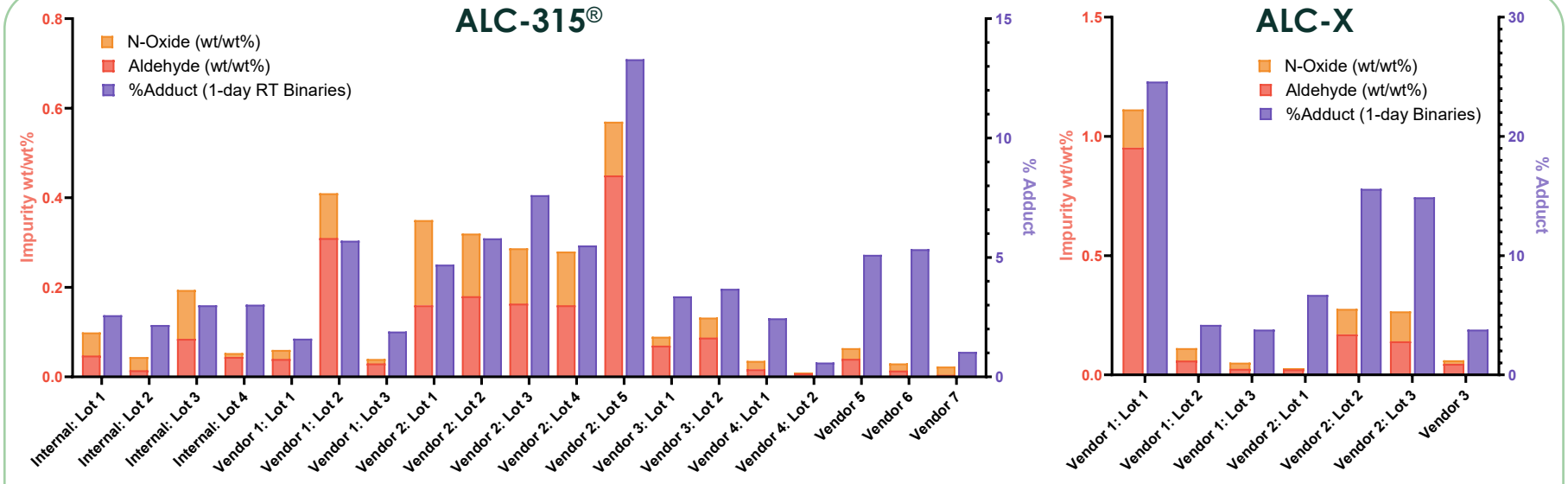
Impurity Screening of ALC-315® and ALC-X Lots



- Tail aldehyde and N-oxide content measured in 19 lots of ALC-315® and 7 lots of ALC-X, with RNA-lipid adducts measured in binary reactions
- Strong correlation between aldehyde content and RNA-lipid adduct formation

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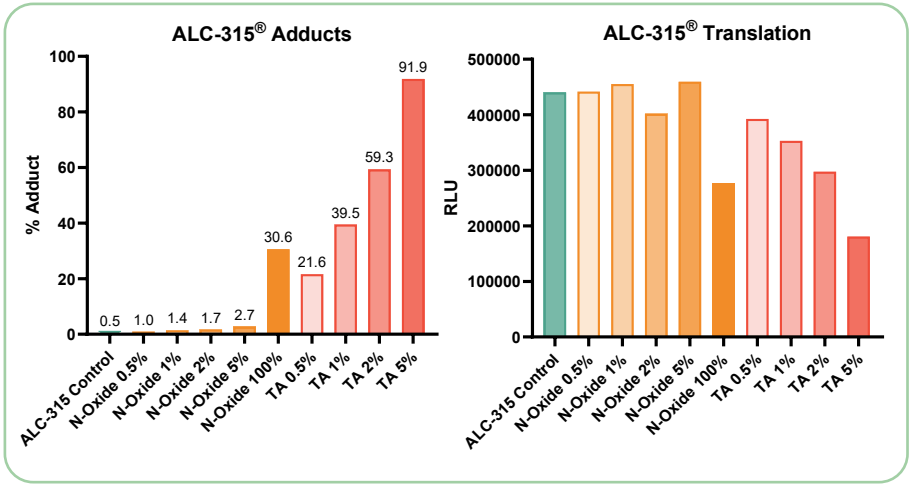
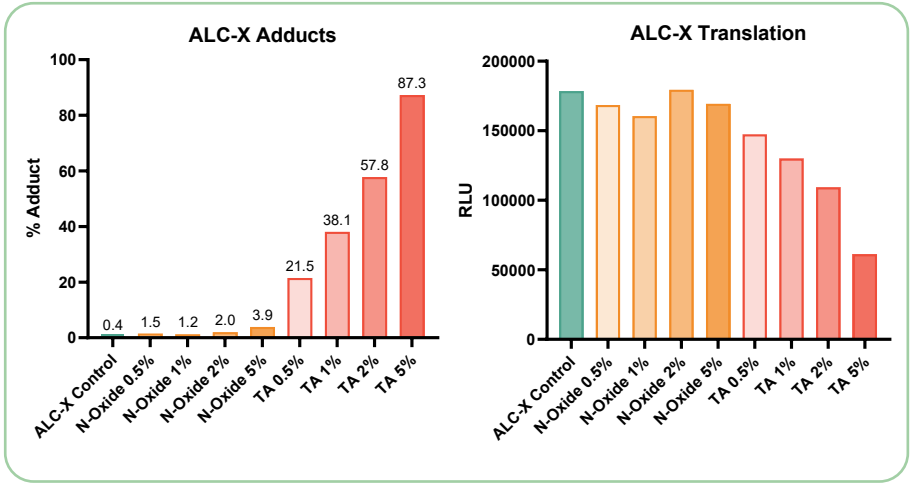
Validating Root Cause in Raw Material





- Aldehydes are the main contributing cause of RNA-lipid adduct formation
- N-Oxide is an indirect contributor to RNA-lipid adduct formation
- In vitro **translation is primarily affected by the aldehyde** derived RNA-lipid adducts

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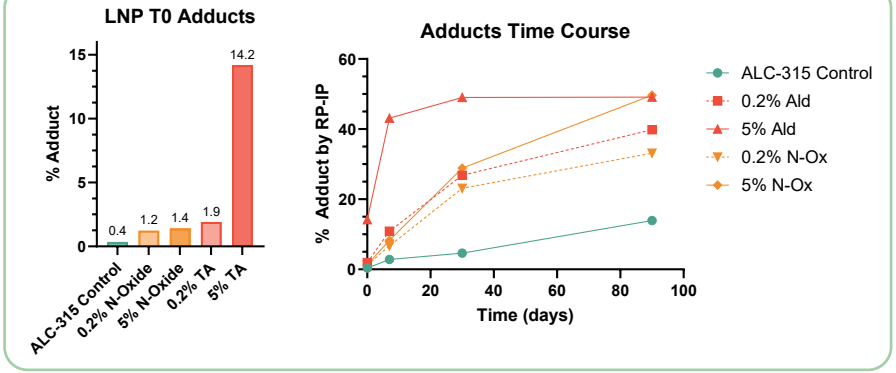
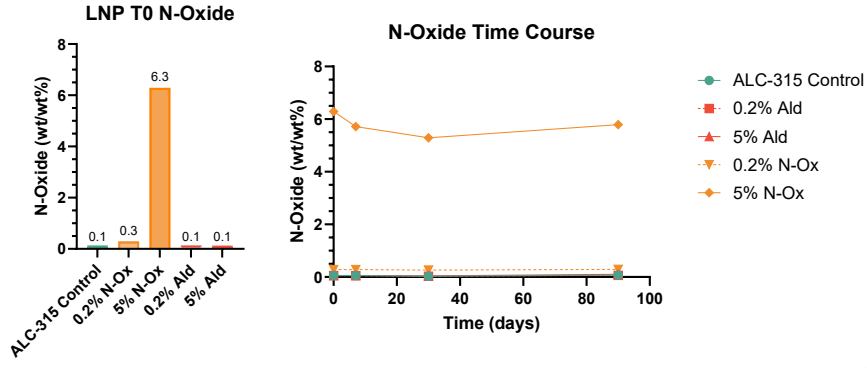
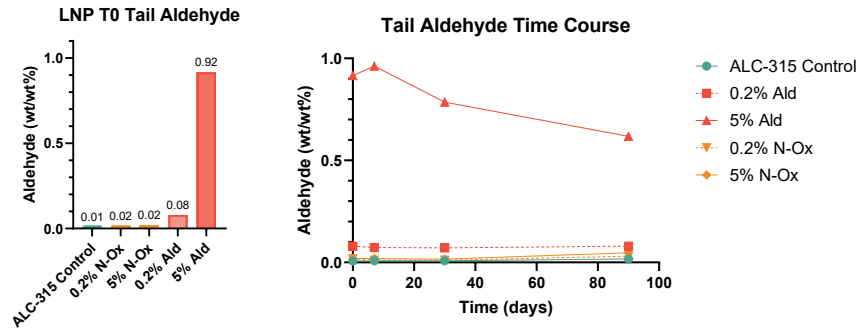
Validating Root Cause in LNP





- ALC-315® spiked with tail aldehyde (TA) or N-Oxide (0.2% or 5%)
- LNP formulated to monitor carryover of impurities into LNP
- LNP stored at room temperature for 3 months to monitor adduct formation
- Correlation between aldehyde content and RNA-lipid adduct formation is seen in LNP



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Impurity Impact on *in vivo* Translation (ALC-315®)

Group	Description	Storage	Measured Adducts (%)	Dose (mg/kg)	Animals
1	Control	T0 (-80°C)	0%	0.3	5F
2	Control	1wk (RT)	1%	0.3	5F
3	Spike Low Aldehyde (2%)	T0 (-80°C)	2%	0.3	5F
4	Spike Low Aldehyde (2%)	2d (RT)	5%	0.3	5F
5	Spike Low Aldehyde (2%)	1wk (RT)	19%	0.3	5F
6	Spike High Aldehyde (5%)	T0 (-80°C)	11%	0.3	5F
7	Spike High Aldehyde (5%)	1wk (RT)	37%	0.3	5F
8	Control	T0 (-80°C)	0%	5.0	5F
9	Spike High Aldehyde (5%)	1wk (RT)	37%	5.0	5F

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- **Study Parameters:**
  - **Pharmacodynamics**
    - IgG (50 µL serum) at 24h
  - **Safety/Tolerability**
    - Clin. Chem. (300 µL serum) at 24h (Gr.8-9 only)
    - Bodyweight at 24h
    - Clinical Observation scoring at 1h, EOD, 24h

- **mRNA:** CH65 IgG
- **Species:** Female CD-1, 5.5 weeks
- **Dose Administration**: i.v. bolus
- **Injection Site:** Tail vein
- **Frequency of Dosing:** Single dose
- **Monitoring Duration:** 24h
- **Biopsy/Necropsy:** 24h

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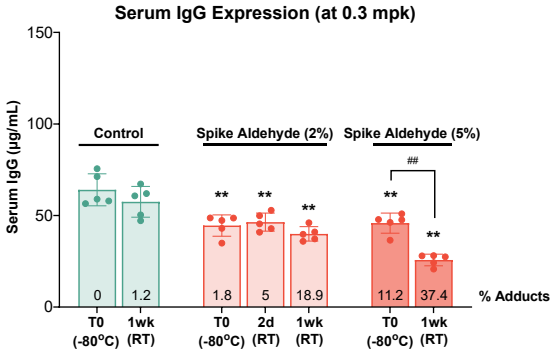
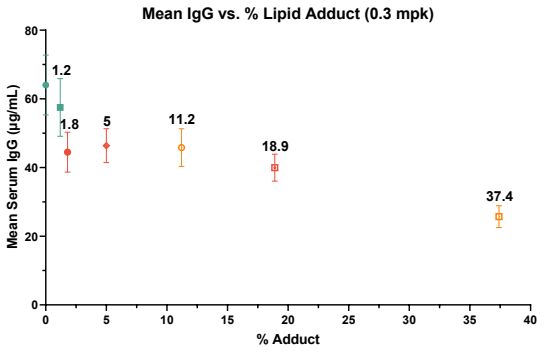
Impurity Impact on *in vivo* Translation (ALC-315®)

In vivo Protein Expression

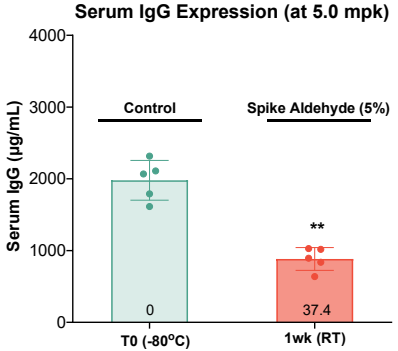
- Decreased serum IgG expression seen with increased % RNA-lipid adducts
- Good correlation between % RNA-lipid adducts and IgG expression (R^2 = 0.7824)

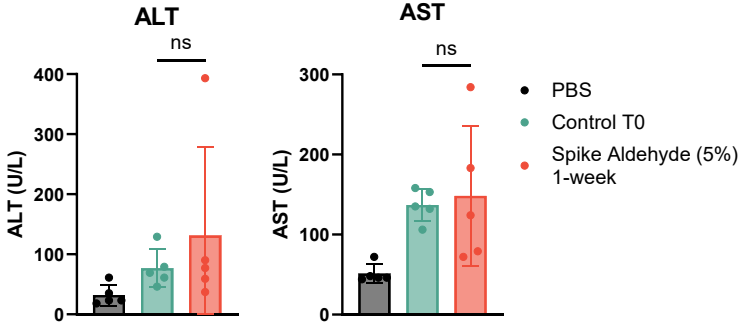




In vivo Safety

- A single animal in the 5% aldehyde group had higher ALT/AST than the rest of the group
- No significant difference between ALC-315® -80°C control and 5% aldehyde group in terms of liver tolerability





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Lot Impurity Impact on *in vivo* Translation (ALC-X)

Group	Description	Dose (mg/kg)	Animals
1	Vendor 3	0.3	5F
2	Vendor 2: Lot 1	0.3	5F
3	Vendor 2: Lot 2	0.3	5F
4	Vendor 2: Lot 3	0.3	5F
5	Vendor 2: Lot 4	0.3	5F

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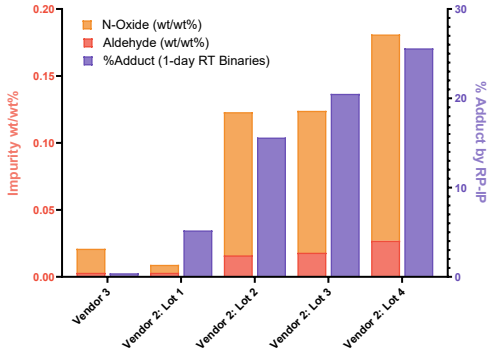
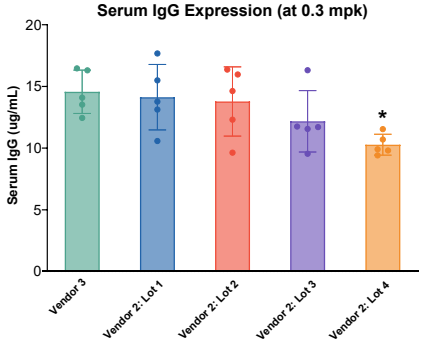
In vivo Expression

- ALC-X lots with higher level of impurities show higher RNA-lipid adducts
- ALC-X lots showed a decreasing trend in IgG expression levels as % adduct increased
- Vendor 2: Lot 4 had the lowest activity with statistical significance





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Key Takeaways

01

Analytical Methods

Robust analytical methods allow screening of raw materials and all LNP formulations

02

Raw Material Screening is Crucial

Thorough screening informs on LNP long term stability and protein expression

03

Contributing Impurities

Can guide lipid manufacturers towards directed impurity testing and new impurity acceptance criteria

04

RNA Expression

In vitro and *in vivo* expression are critical to determine biological functionality of adducted RNA

Acknowledgements

Waters™ ACUITAS THERAPEUTICS