Tiered validation of LC+AMS assays: Recommendations for best practices

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Overview

- Tiered validation and validation options
- LC-MS vs. LC+AMS, similarities and differences
- LC+AMS assay
- Full validation
  - Calibration line
  - QCs
  - LLOQ / carryover
- Selectivity and matrix effects
- Assay performance

Tiered validation approach

- Dave Higton and Mark Seymour, “Application of a tiered approach to the validation of accelerator MS assays.” Bioanalysis Vol 6 No. 5, 665-672 (2014).
- Three methods: screening method, qualified method, and validated method

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Tiered validation approach

	Screening	Qualified method	Validated method
Type of study	^{14}C-metabolite profiling	LC+AMS (parent quantification) = investigative studies e.g. phase 0	LC+AMS (parent quantification) = regulated studies
Use of ^{14}C std	No	Optional	Yes
Use of calibration line / QCs	No	Optional	Yes
ISTD	No	Optional	Yes
Selectivity	Yes	Yes	Yes
Carryover	Yes	Yes	Yes

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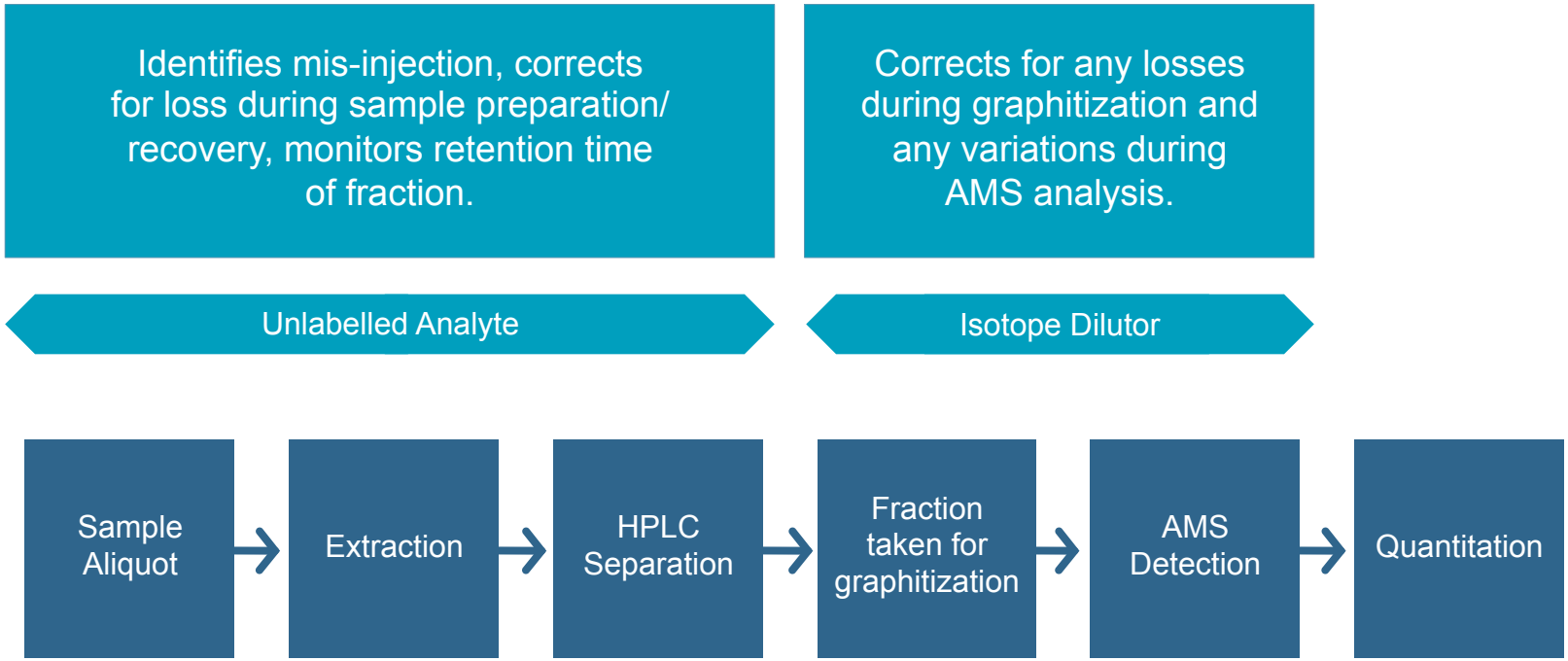
LC-MS and LC+AMS: Key similarities and Differences

	LC-MS	LC+AMS
Sensitivity	Dependent upon structure	Structure-independent
Matrix effects	Yes	No
Structural information	Yes	No, lost during graphitization process
Complexity	On-line process	Off-line process (HPLC fractionation + AMS)
Speed	1-2 days per batch	7-10 days per batch

LC+AMS assay steps

- Sample processing
  - Addition of cold internal standard
  - Extraction (protein precipitation)
- HPLC separation
- Transfer of HPLC fraction of parent peak, observed by UV, to quartz insert with isotope dilutor (sodium benzoate)
- AMS analysis
- Quantitation

Fully validated LC+AMS assay



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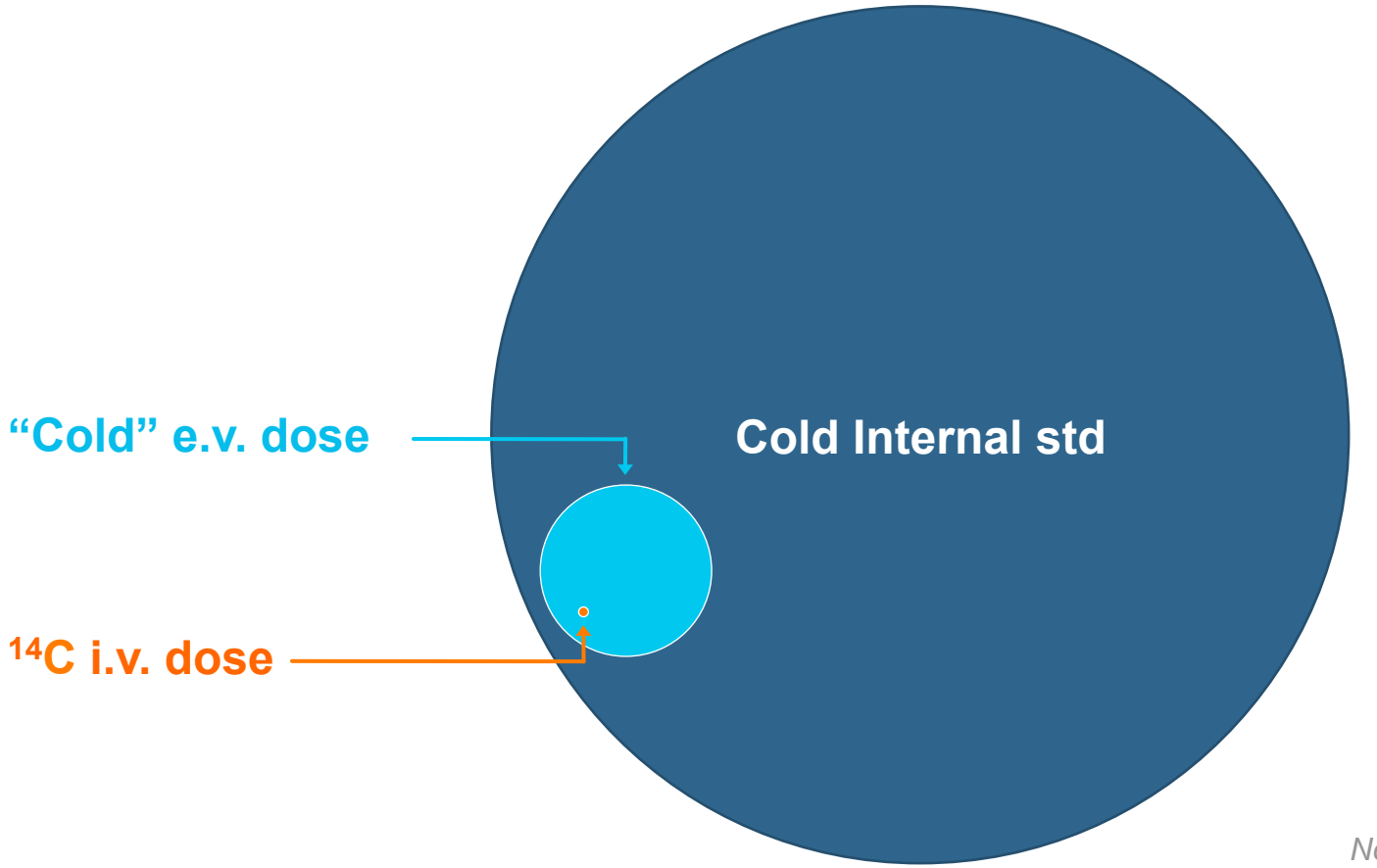
Unlabelled analyte in samples



Not exact scale

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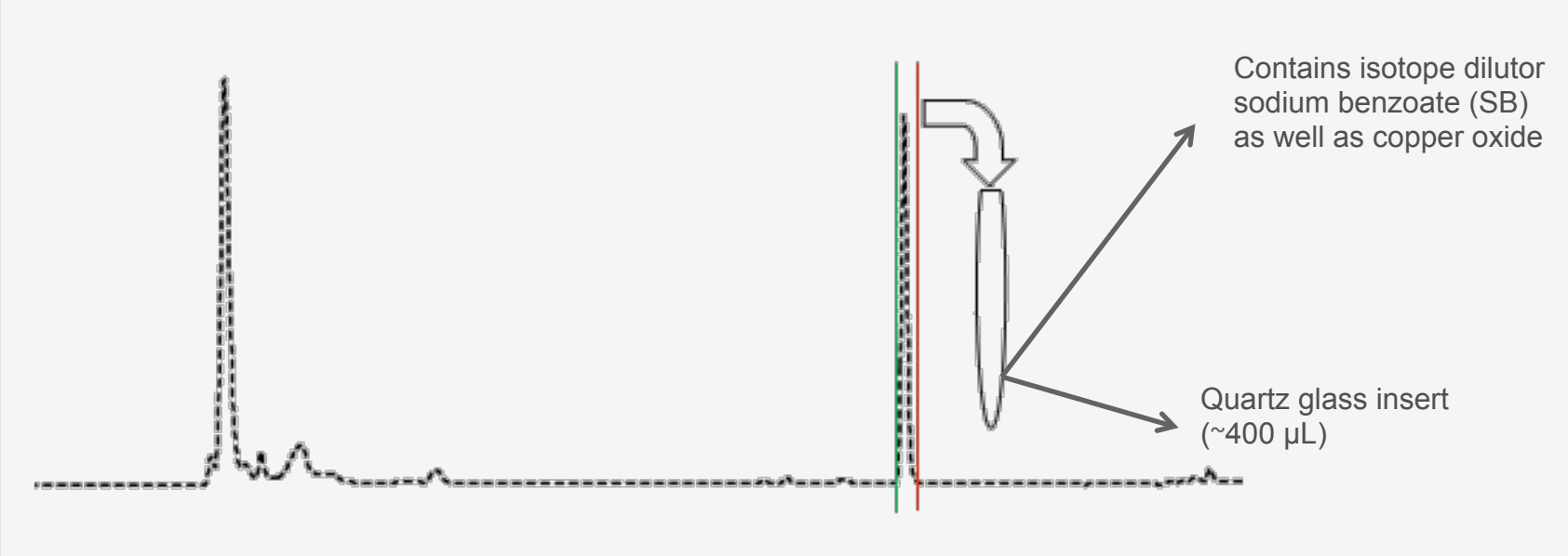
HPLC Separation



- Carbon content of fraction is negligible
- SB adds 1.7 mg of carbon
- Example weight of IS collected in fraction ~0.003 mg with a fraction of that being carbon

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Fully validated LC+AMS assay

- 3 batches
- Calibration line
  - 6-8 concentrations (*n*=2)
- QCs
  - Four concentrations (*n*=6)
    - Low / Mid / High /LLOQ
  - Dilution QC – one occasion
- Carryover
  - Assessed in blank matrix and aqueous wash solution

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Calibration line and QCs

- Calibration line
  - 75% must pass (incl. LLOQ & ULOQ), minimum of 6 points
  - Accuracy: ± 20% (± 25% @ LLOQ)
- QCs
  - High, Mid, Low, Dilution
    - LLOQ (Accuracy ± 25%; Precision ≤ 25%)
    - Low, Mid, High (Accuracy ± 20%; Precision ≤ 20%)
    - Dilution QC on one occasion (Accuracy ± 20%; Precision ≤ 20%)
    - Dilution QC for other dilution levels (10 fold dilution typical)

LLOQ & Carryover

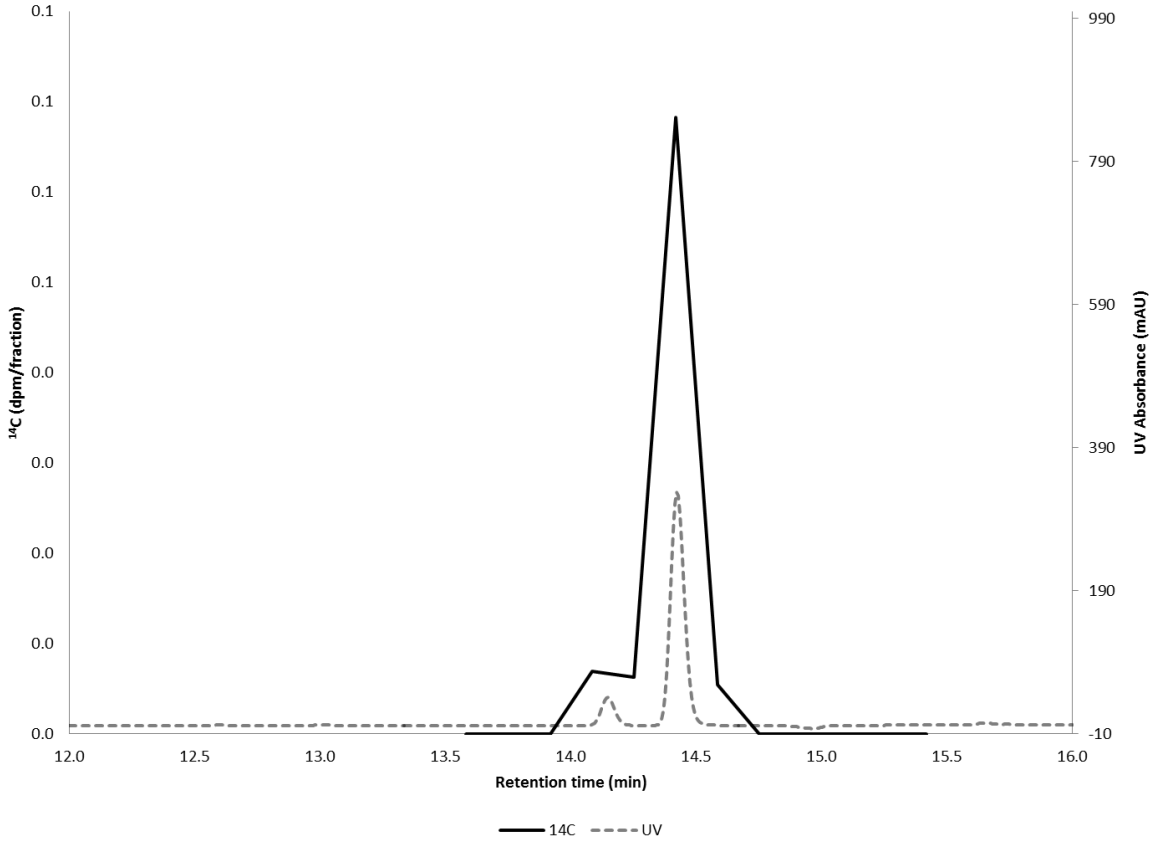
- LLOQ
  - At least 5 times mean b/g
    ~10 percent Modern carbon (pMC)
- Carryover
  - Blank (matrix only)
    - Peak area (mAU) ≤5% of mean peak area for standards
  - Zero (matrix plus IS) / Wash (solvent blank)
    - ≤ 3 times the mean b/g
    - Net pMC ≤ 20% of the lowest calibration standard (LLOQ)

Note: b/g = sodium benzoate used in graphitization process as carbon carrier (typically 2-3 pMC)

Matrix effects / selectivity

- Matrix effects
  - AMS response matrix-independent
  - Matrix may affect chromatography
  - HPLC mobile phase devoid of non-volatile ^{12}C
- Selectivity
  - Control plasma – UV signal
  - Radio-profile of *in vitro* or pre-clinical samples if available
  - Definitive experiment requires clinical samples - run during/before first clinical sample batch

Non-selectivity in plasma (16 h post dose)



- 12.5 second fractions

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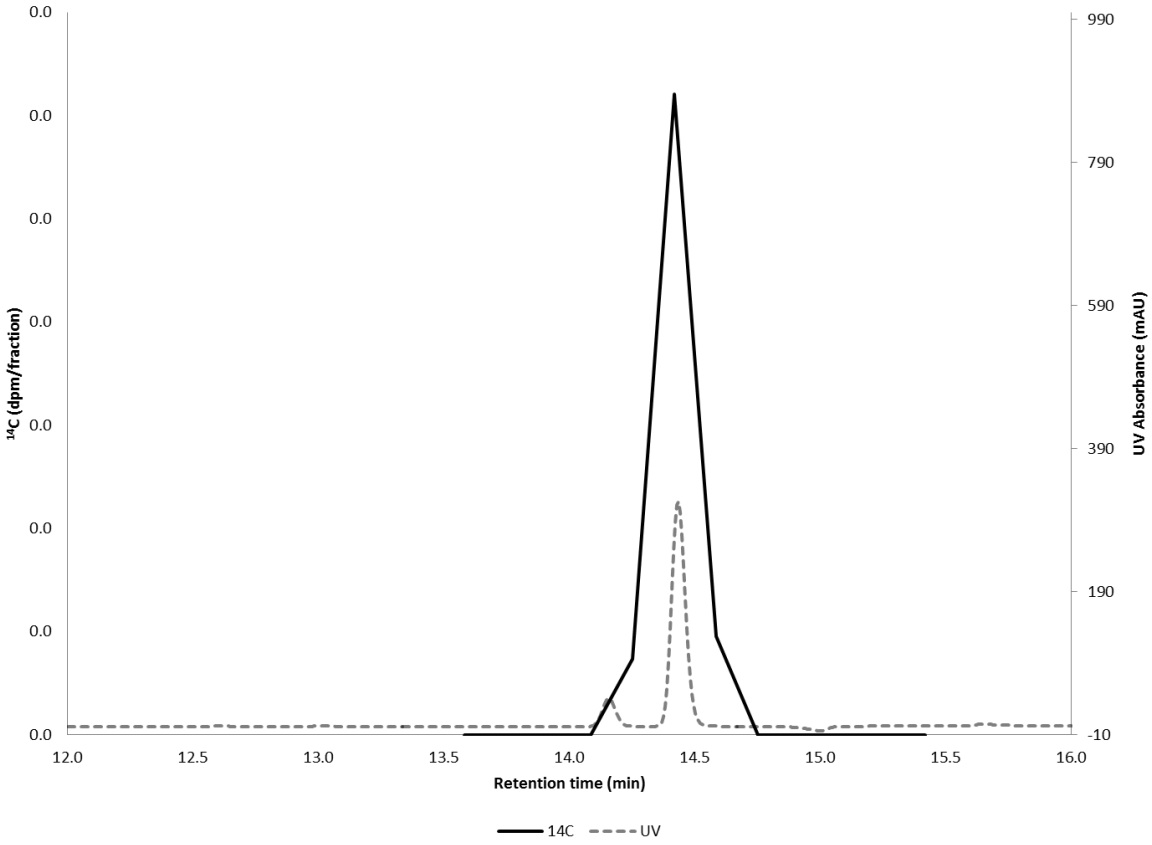
Non-selectivity in plasma (76 h post dose)



- 12.5 second fractions

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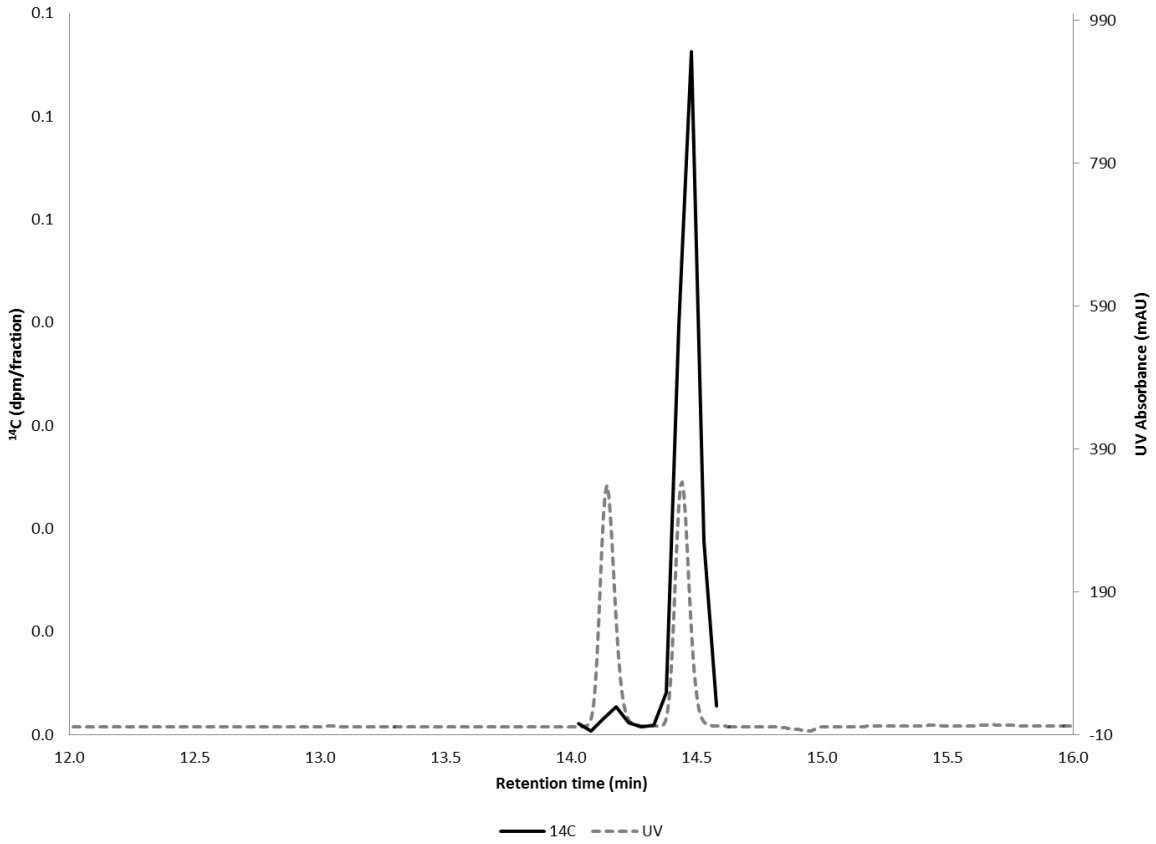
Selectivity in plasma (16 h post dose)



- 3 second fractions
- Spike with known metabolite for UV response

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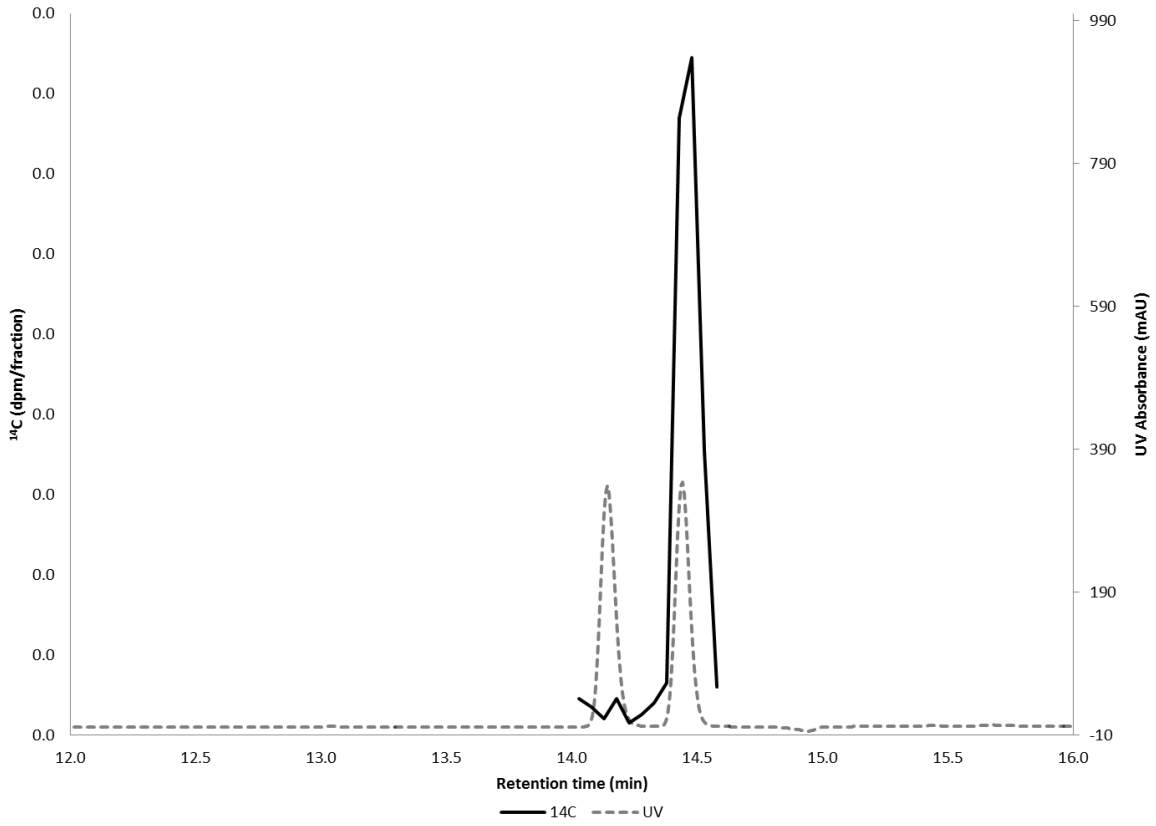
Selectivity in plasma (76 h post dose)



- 3 second fractions
- Spike with known metabolite for UV response

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Selectivity summary

- Small sample volume necessary for AMS allowed for smaller fraction size
- Availability of metabolite reference standard
- Fraction collection settings

LC+AMS Assay Performance – Interday

Assay ID	LLOQ (pg/mL)	Cal Stds	QCs	Notes
37/009VA	29.7	**A: 96-107%**	**A: 93-109%** **P: 4-9%**	Two analyte assay
37/009VB	29.7	**A: 99-102%**	**A: 93-112%** **P: 5-6%**	
137/002V	13.1	**A: 93-105%**	**A: 96-107%** **P: 3-21%**	Feces Homogenate*
137/002V	13.1	**A: 97-105%**	**A: 105-118%** **P: 5-12%**	Plasma
128/004V	5.0	**A: 97-103%**	**A: 103-113%** **P: 0.4-5%**	
139/002V	3.2	**A: 95-104%**	**A: 85-103%** **P: 3-9%**	
31/013V	3.1	**A: 90-104%**	**A: 89-105%** **P: 3-19%**	

LC-MS:	Accuracy (±15%, 20% at LLOQ)	Precision (≤15%, 20% at LLOQ)
LC+AMS:	Accuracy (±20%, 25% at LLOQ)	Precision (≤15%, 20% at LLOQ)

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LC+AMS Assay Performance – Interday (cont)

Assay ID	LLOQ (pg/mL)	Cal Stds	QCs	Notes
31/022V	2.1	**A: 96-105%**	**A: 90-**117% **P: 1-10%**	
144/004V	1.8	**A: 91-106%**	**A: 85-110%** **P: 2-15%**	
18/009V	0.87	**A: 96-103%**	**A: 100-109%** **P: 5-11%**	
144/007V	0.69	**A: 96-106%**	**A: 94-108%** **P: 1.5-10%**	
82/004V	0.50	**A: 92-107%**	**A: 88-107%** **P: 4-8%**	
146/002V	0.36	**A: 96-104%**	**A: 101-115%** **P: 5-9%**	
135/002V	0.1	**A: 96-109%**	**A:** 80-**100%** **P: 5-**19%	2D Chiral HPLC Method

LC-MS:	Accuracy (±15%, 20% at LLOQ)	Precision (≤15%, 20% at LLOQ)
LC+AMS:	Accuracy (±20%, 25% at LLOQ)	Precision (≤15%, 20% at LLOQ)

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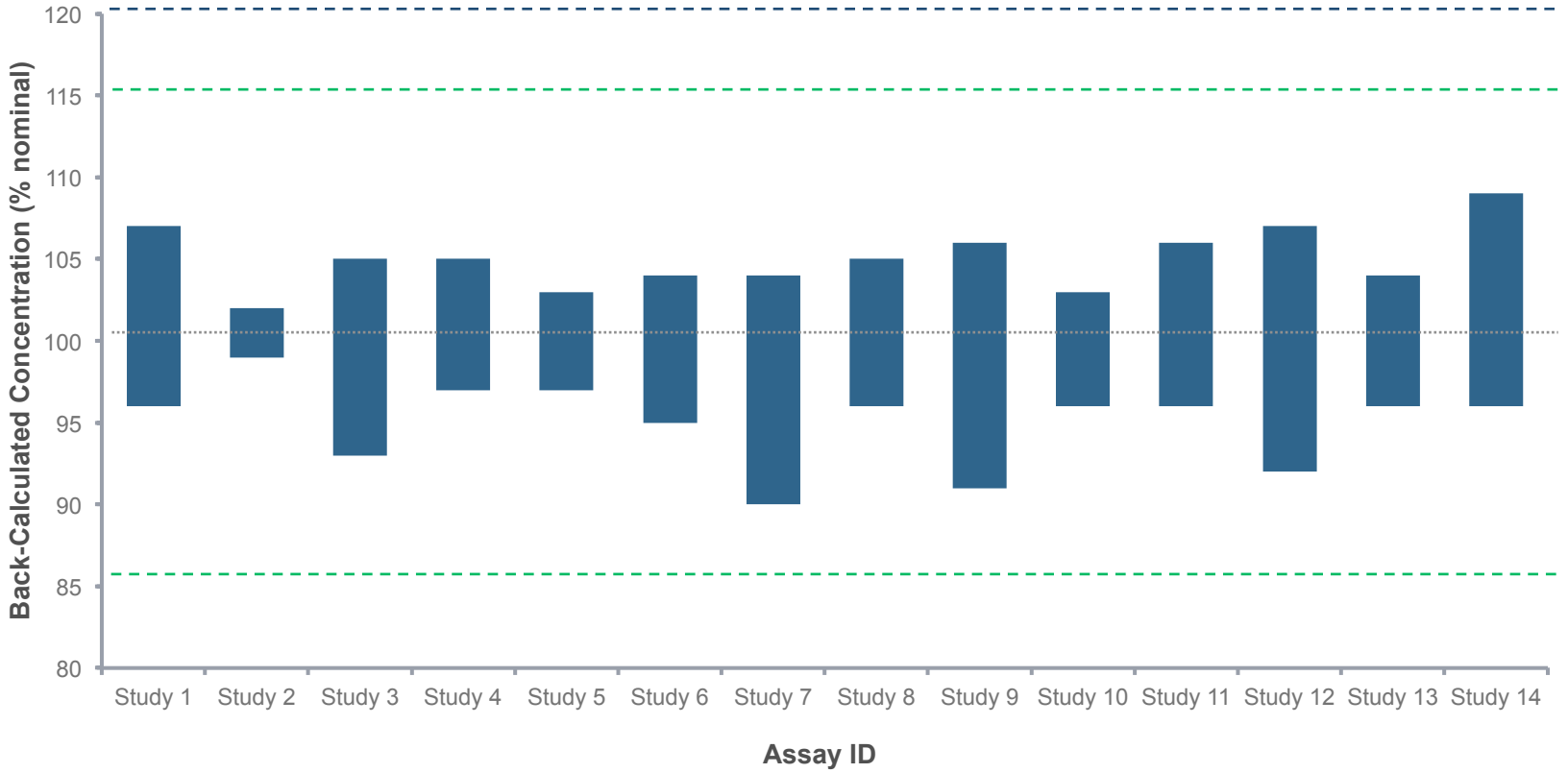
LC+AMS assay performance: interday calibration standard accuracy



120: LC+AMS acceptable range
85-115: LC-MS/MS acceptable range

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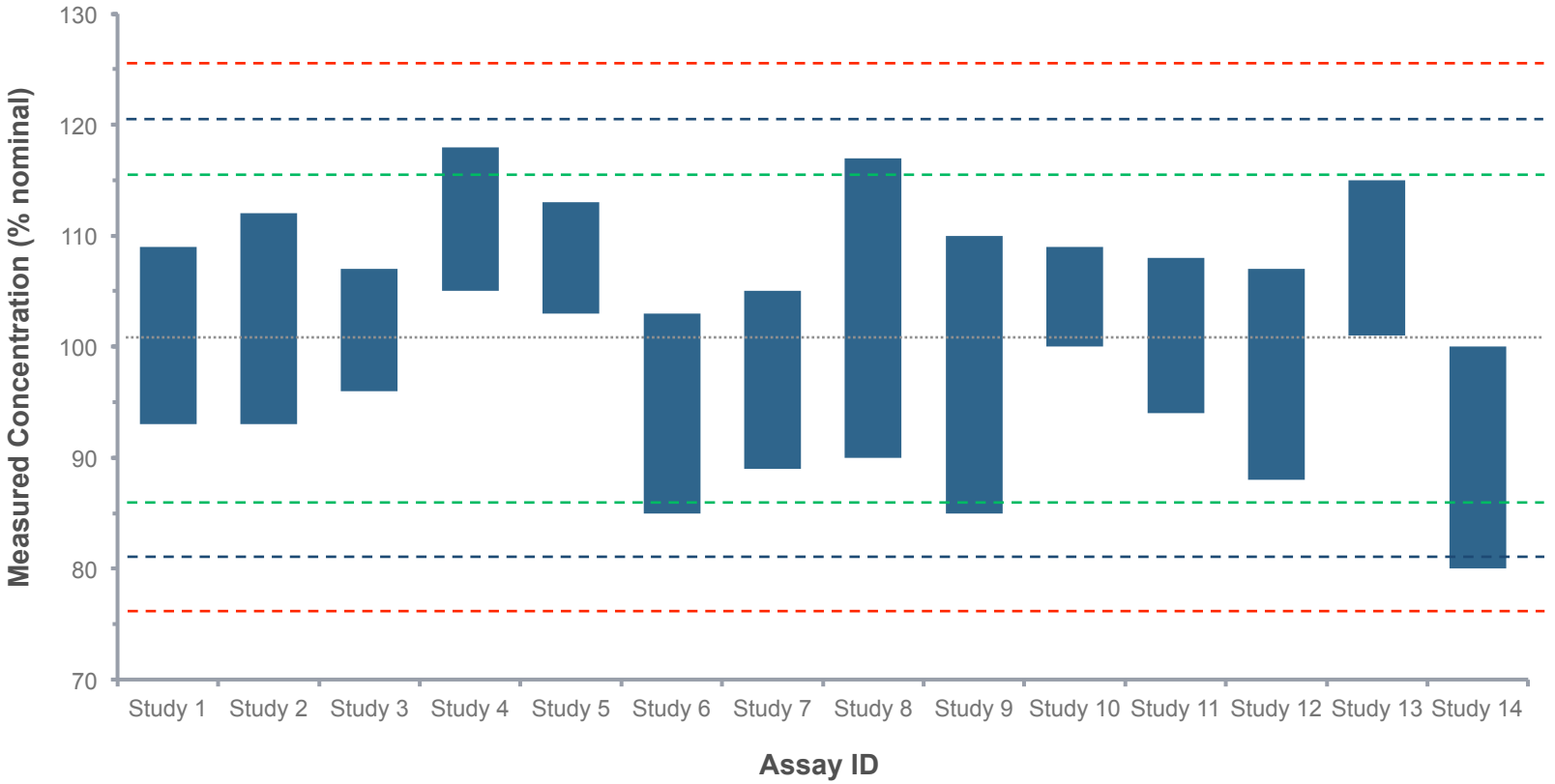
LC+AMS assay performance: QC accuracy (interday)



- - - - - - 75-125: LC+AMS acceptable range for LLOQ QC level
- - - - - - 80-120: LC+AMS acceptable range
- - - - - - 85-115: LC-MS/MS acceptable range

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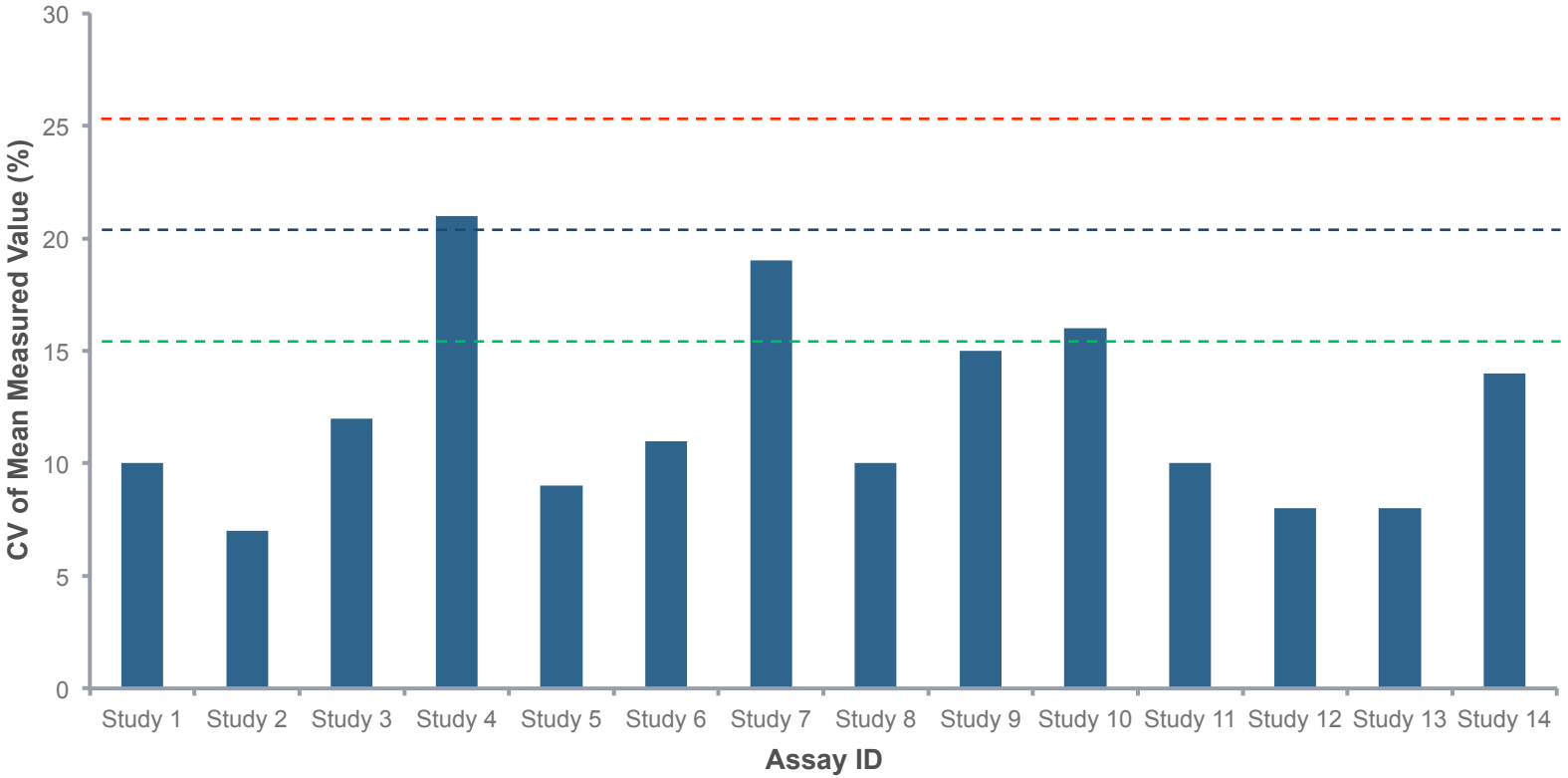
LC+AMS assay performance: QC max precision (interday)



25: LC+AMS acceptable range for LLOQ QC
20: LC+AMS acceptable range
15: LC-MS/MS acceptable range

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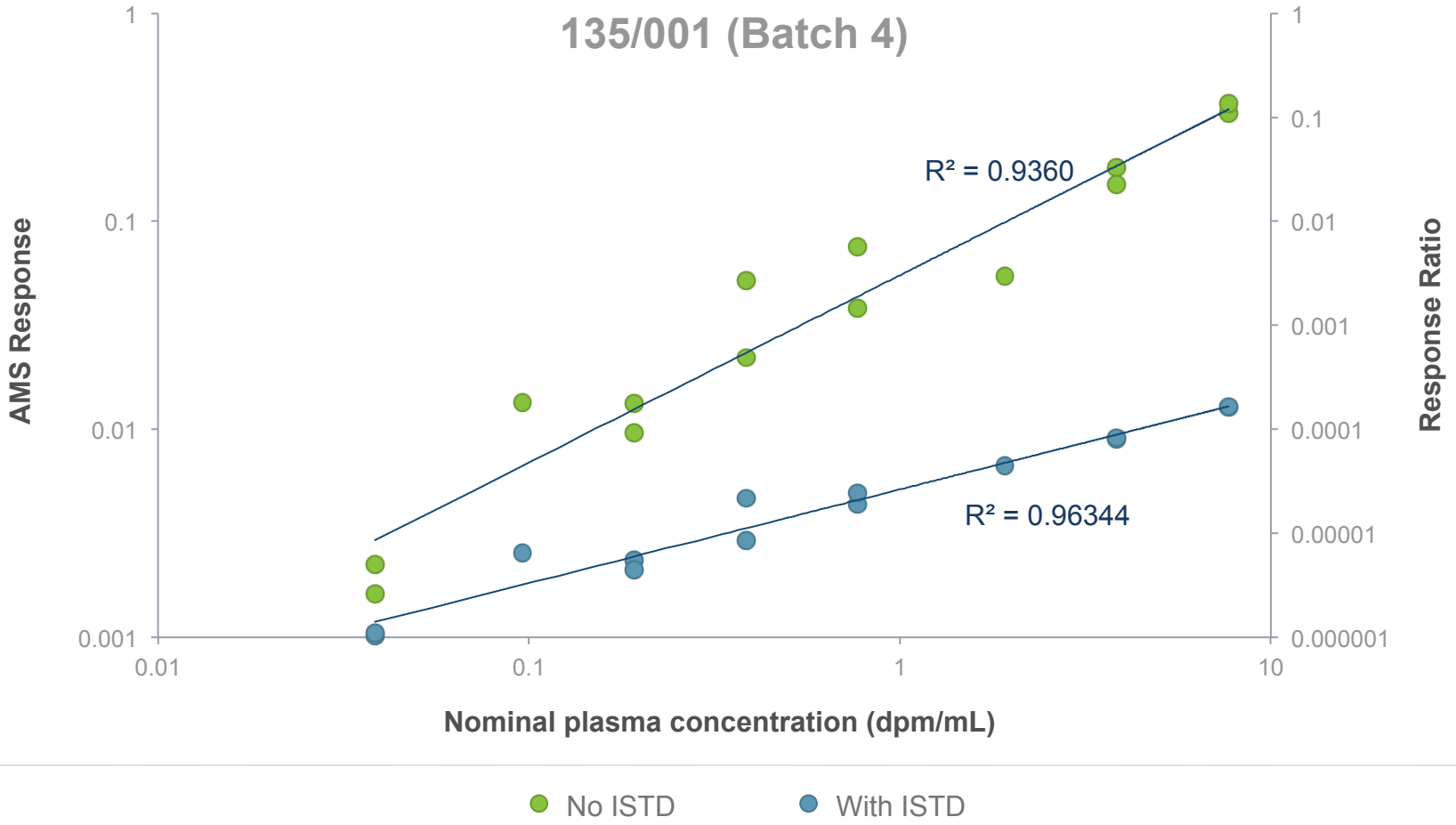
Response ratio with and without the use of internal standard



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Response ratio with and without the use of internal standard

QC Level (nominal conc)		Results (% nominal) No ISTD	With ISTD
HQC (5.77 dpm/mL)		133.6	102.7
		103.6	96.8
		91.1	92.3
	Min	**91.1**	**92.3**
	Max	**133.6**	**102.7**
	Mean	**109.4**	**97.3**
MQC (0.576 dpm/mL)		115.0	106.6
		NR	NR
		98.5	95.7
	Min	**98.5**	**95.7**
	Max	**115.0**	**106.6**
	Mean	**106.8**	**101.2**
LQC (0.115 dpm/mL)		98.5	87.3
		125.0	104.9
		135.9	119.7
	Min	**98.5**	**87.3**
	Max	**135.9**	**119.7**
	Mean	**119.8**	**104.0**

Results outside acceptance criteria shown in orange.
NR: no results (AMS results invalid).

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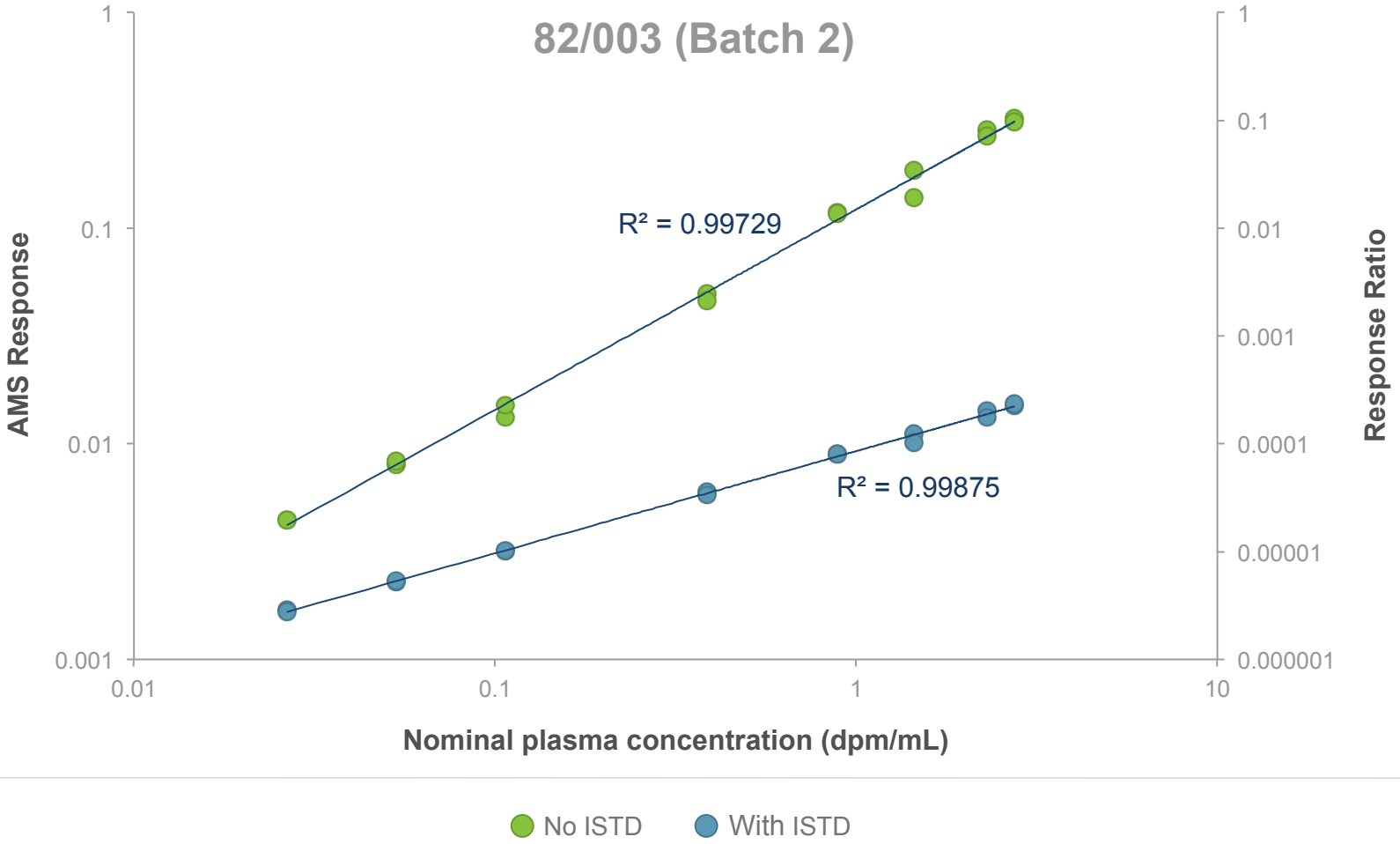
Response ratio with and without the use of internal standard



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Response ratio with and without the use of internal standard

QC Level (nominal conc)		Results (% nominal) No ISTD	With ISTD
HQC (2.13 dpm/mL)		96.8	106.8
		85.9	95.5
		101.1	103.3
	Min	**85.9**	**95.5**
	Max	**101.1**	**106.8**
	Mean	**94.6**	**101.9**
MQC (1.22 dpm/mL)		102.8	104.9
		104.9	107.4
		102.1	98.9
	Min	**102.8**	**98.9**
	Max	**104.9**	**107.4**
	Mean	**103.3**	**103.7**
LQC (0.115 dpm/mL)		95.8	96.1
		126.2	144.8
		89.7	91.9
	Min	**89.7**	**91.9**
	Max	**126.2**	**144.8**
	Mean	**103.9**	**110.9**

Results outside acceptance criteria shown in orange.

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Internal standard summary

- Approximately 80% of the assay batches considered do not rely on the internal standard for acceptance criteria
- 2D chiral method more greatly affected – assay specific
- Right-first time and the preference of utilizing the internal standard approach to keep up with the natural flow of the laboratory

Recommendations for full validation

- Full validation
  - 3 batches
    - Two calibration lines: Accuracy ± 20% (± 25% @ LLOQ)
    - QCs at four concentrations, incl. LLOQ, plus dilution QCLLOQ (Accuracy ± 20% (25% at LLOQ); Precision ≤ 20% (≤ 25% at LLOQ)
  - Carryover
  - Selectivity
- Acceptance criteria
  - Appropriate for off-line process

Acknowledgements

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Thank you

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