Case studies for testing stabilities in biomarker assays

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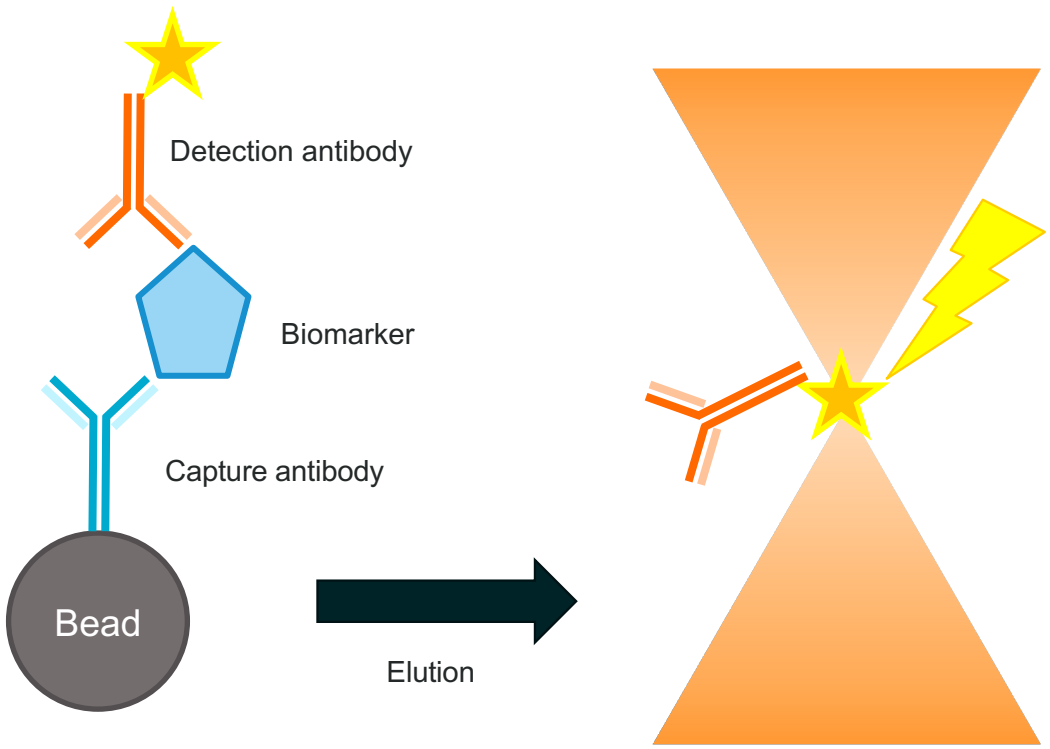
How to ideally test stabilities in biomarker assays

- Matrices needed for endogenous QC samples that cover the range of the assay
- Fresh matrix, collected identically to the clinical trial, screened and aliquoted
- Establish nominal concentration of endo QCs in 6 runs immediately after collection
- Store at desired temperatures and time periods for stability experiments
- Analyze and compare to established nominal concentration

What issues have we encountered at ICON?

- Difficult to obtain really fresh matrices to establish concentration at t = 0
- Not enough volume for endogenous QCs and stability experiments
- (Healthy) endogenous samples do not cover the range of the assay
- Biomarkers unstable or standard stability experiments not suitable

Case 1: Testing stabilities using rare matrix



Context of use Biomarker

Biomarker measured in cerebrospinal fluid (CSF) of patients

Low concentrations expected, so ultrasensitive assay required: low picogram range

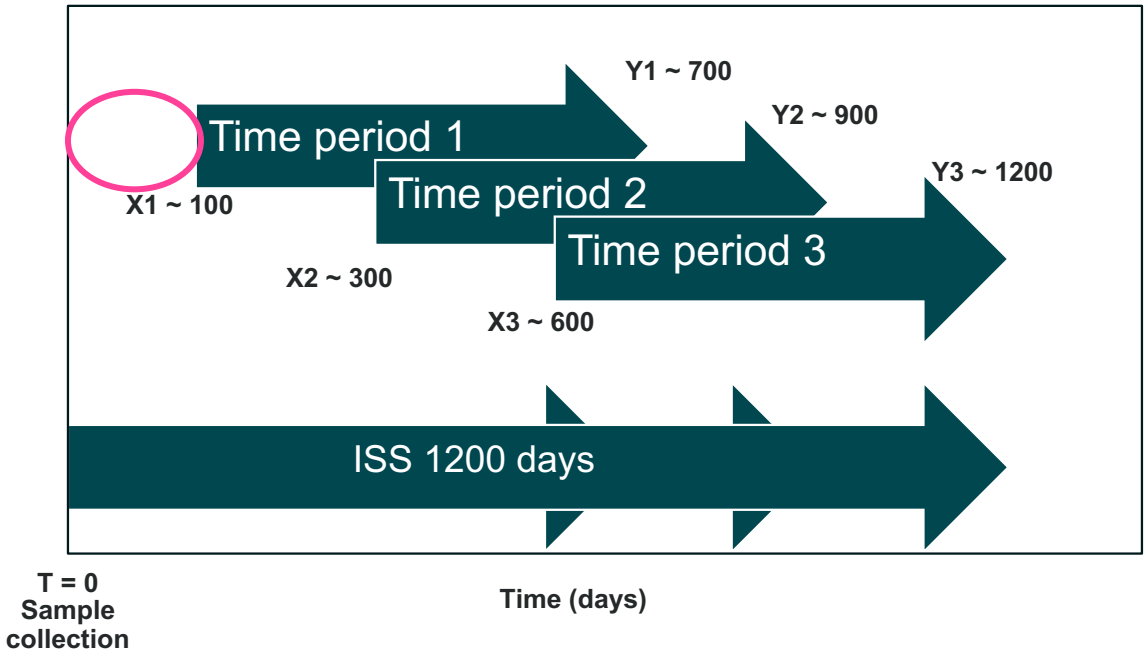
No patient CSF available for endogenous QCs, so not possible to test stabilities with QC samples

Freeze/thaw and bench top stability only determined in buffer

Stability of patient samples determined by incurred sample stability (ISS)

Case 1: Design incurred sample stability

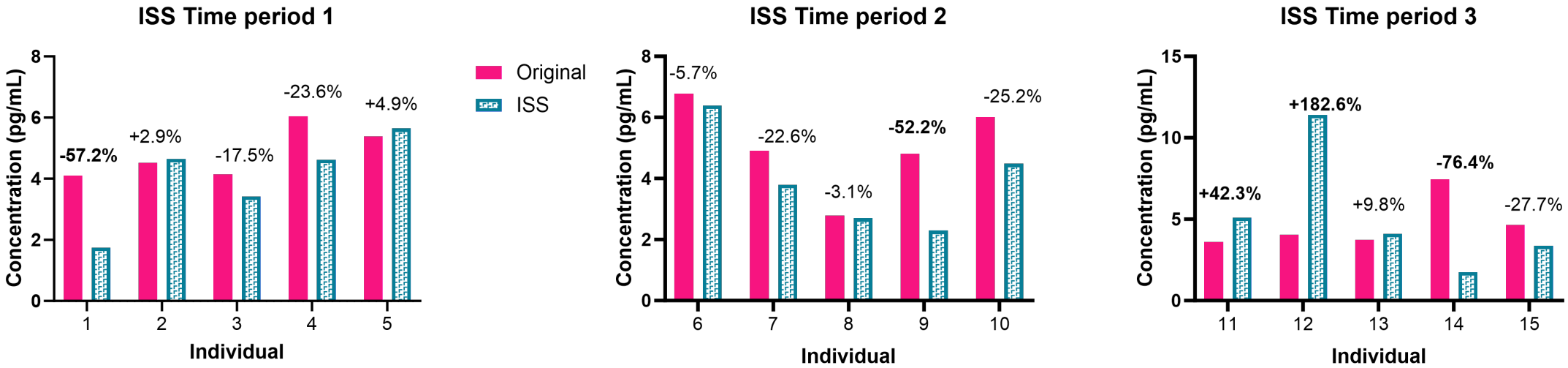
- Measure samples ASAP after collection
- ISS performed ~600 days after initial analysis at 3 time periods
  - 5 samples per time period, 3/5 samples bias <30.0% at each time period
- X is time point first analysis and Y is analysis after storage
- Samples 100 days old at first analysis, unknown stability for first 100 days



Vauleon, S., Schutz, K., Massonnet, B. *et al.* Quantifying mutant huntingtin protein in human cerebrospinal fluid to support the development of huntingtin-lowering therapies. *Sci Rep* **13**, 5332 (2023). https://doi.org/10.1038/s41598-023-32630-4

Case 1: Incurred sample stability in practice

3/5 individuals should have bias <30.0% at each time period



Samples can be stored for up to 952 days (storage duration of individual 10), however, stability of first 100 days is unknown

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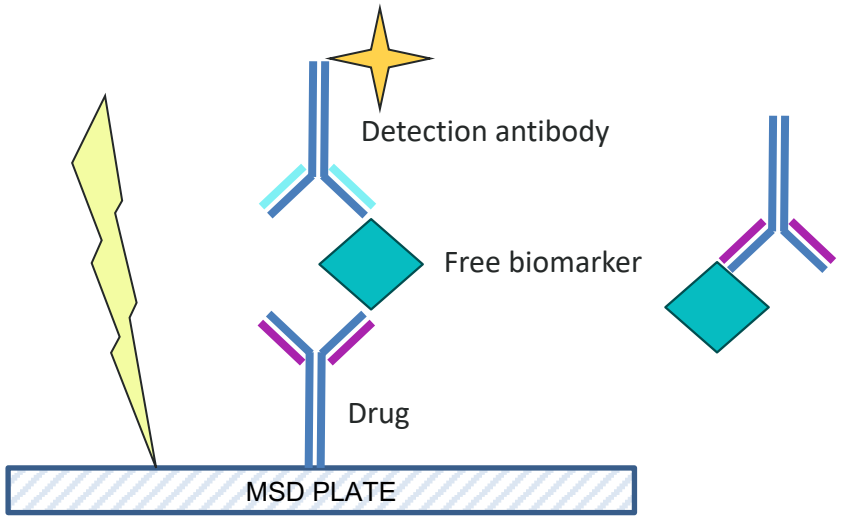
Case 2: Testing stabilities for a free unstable biomarker



Context of use Free Biomarker

Endogenous QCs possible at low level, but not at high level because of low levels in healthy matrix

Levels range from 10.0 – 500 pg/mL in patients and are expected to decrease upon treatment with drug

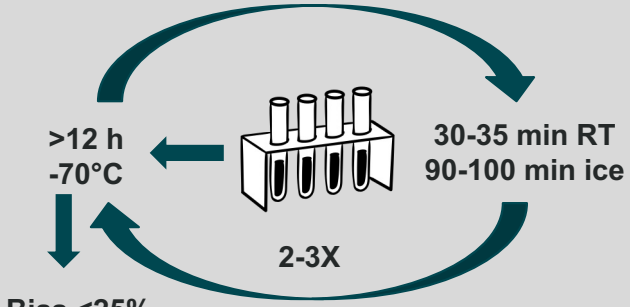
Analyte expected to be unstable on bench, therefore, samples are defrosted for 30 min at RT and then stored on ice

Stability samples to ensure that the ‘free biomarker’ proportion of samples remains the same over time

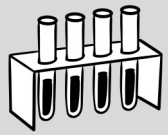
Case 2: Design of stability experiments for free unstable biomarkers

QC	Level (pg/mL)	Established (pg/mL)
Endo Low	~10	8.37
'Endo' Mid + drug Spiked with recombinant biomarker and drug to achieve ~50% inhibition	**Spiked at 100** + 5.00 ng/mL drug	32.6
'Endo' High Spiked with recombinant biomarker	**Spiked at 1500**	1230

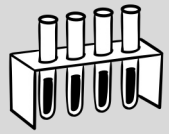
Freeze/thaw stability



Bench top stability

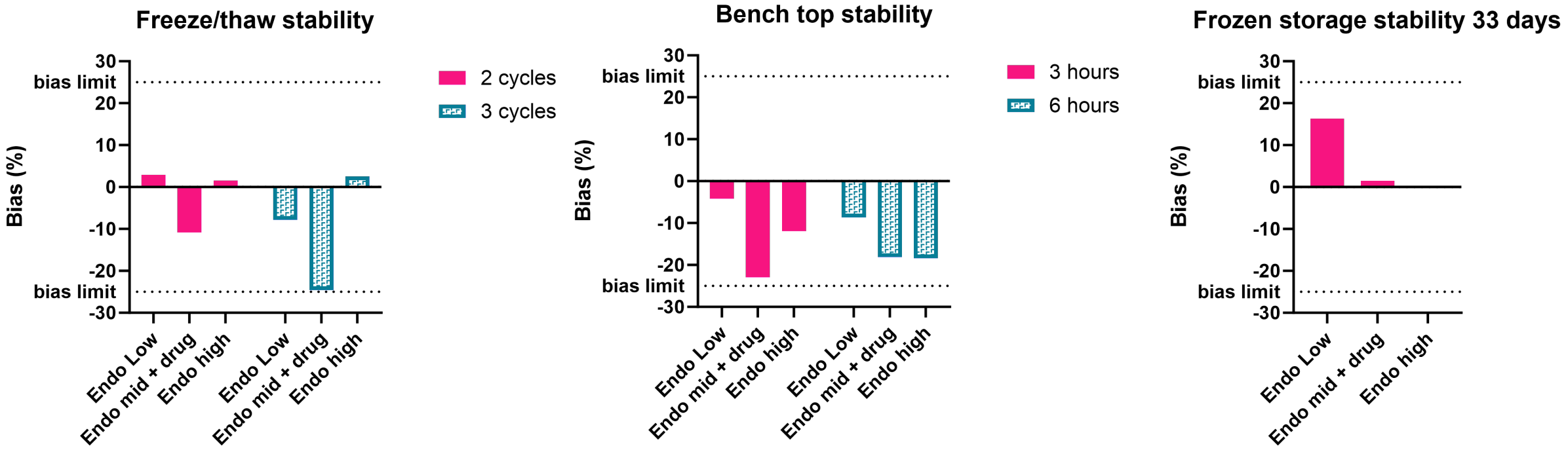


Frozen storage stability



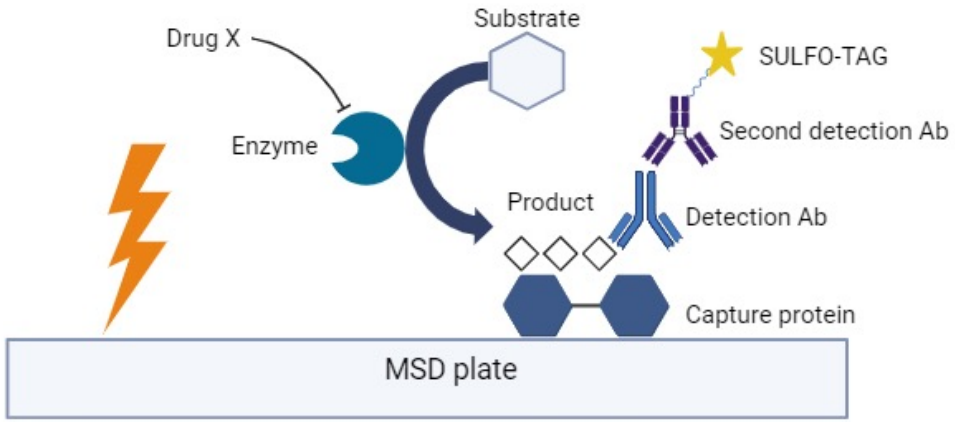
Case 2: Stability results ‘endo’ QCs

Absolute bias <25.0% compared to established nominal concentration



All endo QCs were (just) within criteria using the adjusted stability experiments

Case 3: Enzymatic activity assay; enzyme inhibited by drug



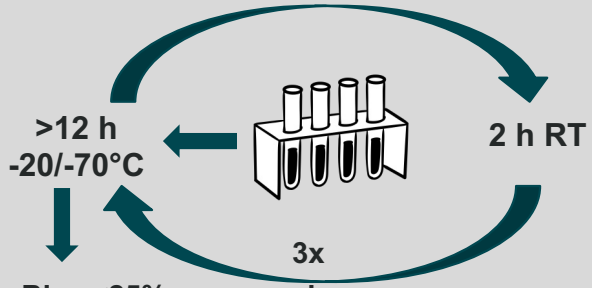
Predose	Predose + drug
Postdose	Postdose +drug
Blank	Blank + drug
Low QC	Low QC + drug
High QC	High QC + drug

- Context of use Enzymatic activity assay
- Read out is product of enzyme activity
- Handling and preparation of matrix was shown to impact activity of the enzyme
- Matrix ordered that was processed immediately after collection and stored at –70°C
- To calculate % inhibition of postdose sample, predose and postdose samples must be analyzed on the same plate, with and without excess drug

Case 3: Activity assay stability design

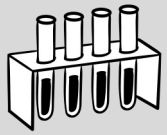
QC	Target % inhibition	Established % inhibition
Unspiked	Calculation % inhibition	NA
QC low	~50% inhibition	51.2%
QC high	~90% inhibition	92.4%

Freeze/thaw stability



Bias <25% compared
to ref sample for 2/3 results

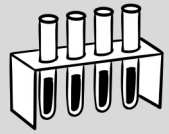
Bench top stability



-70°C → 24 h RT

Bias <25% compared
to ref sample for 2/3 results

Frozen storage stability

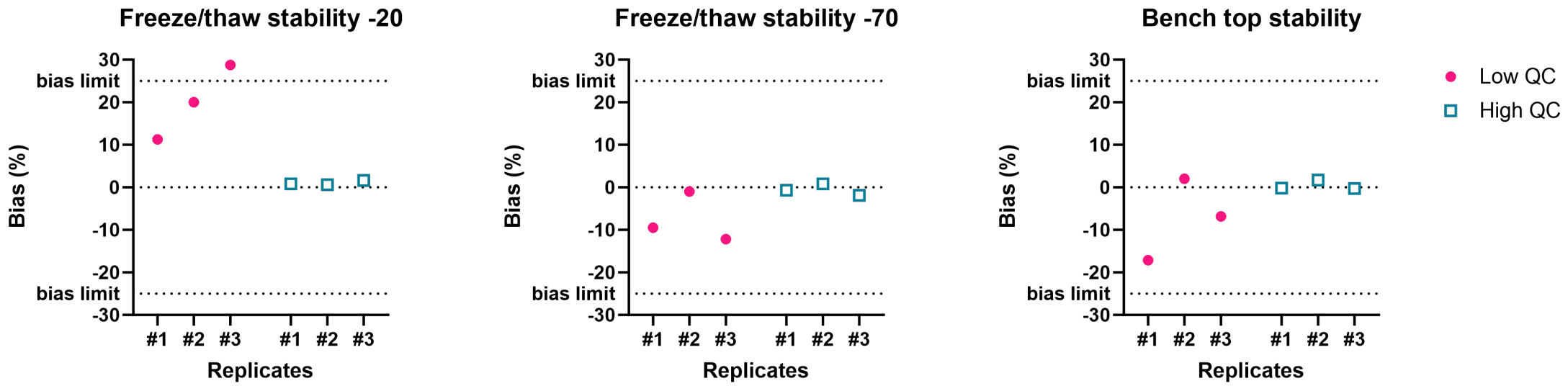


-20°C
-70°C → 30 days

Bias <25% compared
to fresh ref sample for 2/3 results

Case 3: Activity assay stability results

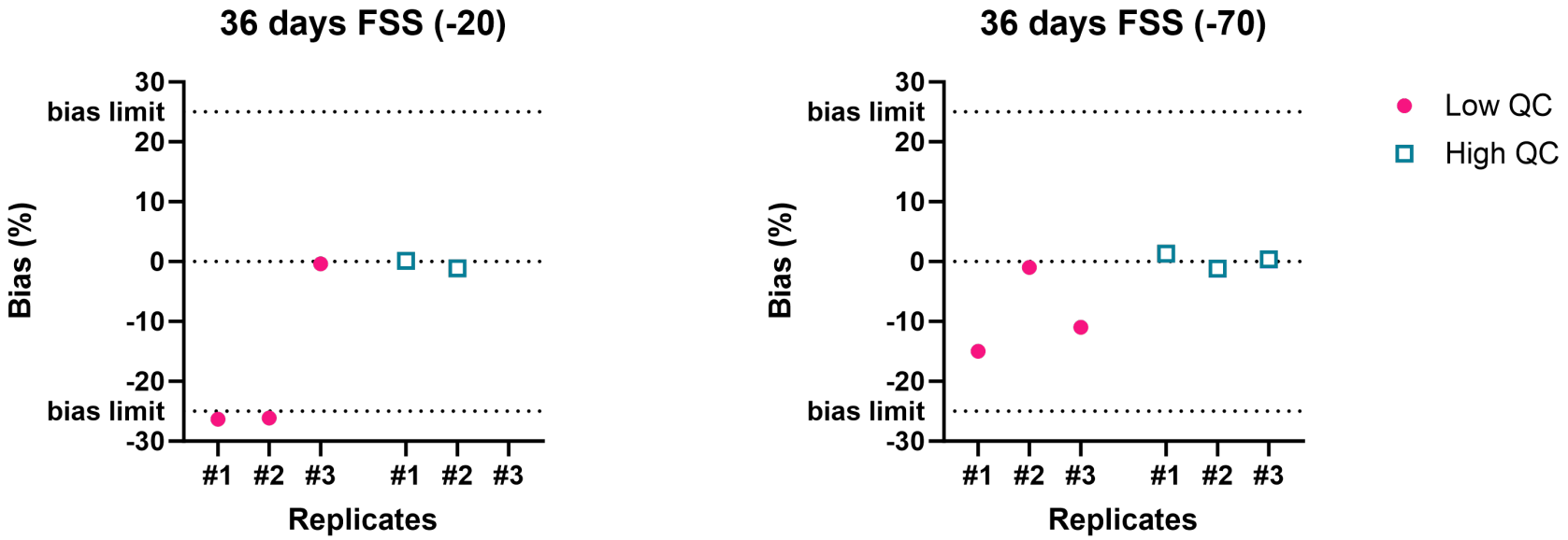
Absolute bias <25.0% compared to reference samples for 2/3 results



Freeze/thaw stability and bench top stability within criteria at low QC and high QC

Case 3: Activity assay frozen storage stability results

Absolute bias <25.0% compared to reference samples for 2/3 results



Frozen storage stability outside criteria at low QC at -20°C, suggesting samples are less stable at this temperature

Summary and conclusion

- For biomarkers, it is often not possible to perform the ideal stability experiment. Therefore, experiments need to be adjusted, depending on the context of use:
  - Case 1: Rare matrix
    - Stabilities with endo QCs not possible: test incurred sample stability
  - Case 2: Free, unstable biomarker
    - Only low levels found in healthy matrix: Use spiked ‘endo’ QCs
    - Unstable biomarker: Reduce bench top time and freeze thaw cycles and keep samples on ice
    - Adjusted QCs to mimic samples: endo QC + drug important for free assays
  - Case 3: Enzymatic activity assay
    - Stabilities tested using reference samples, instead of a nominal established concentration
    - Enzyme unstable and matrix storage conditions adjusted

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