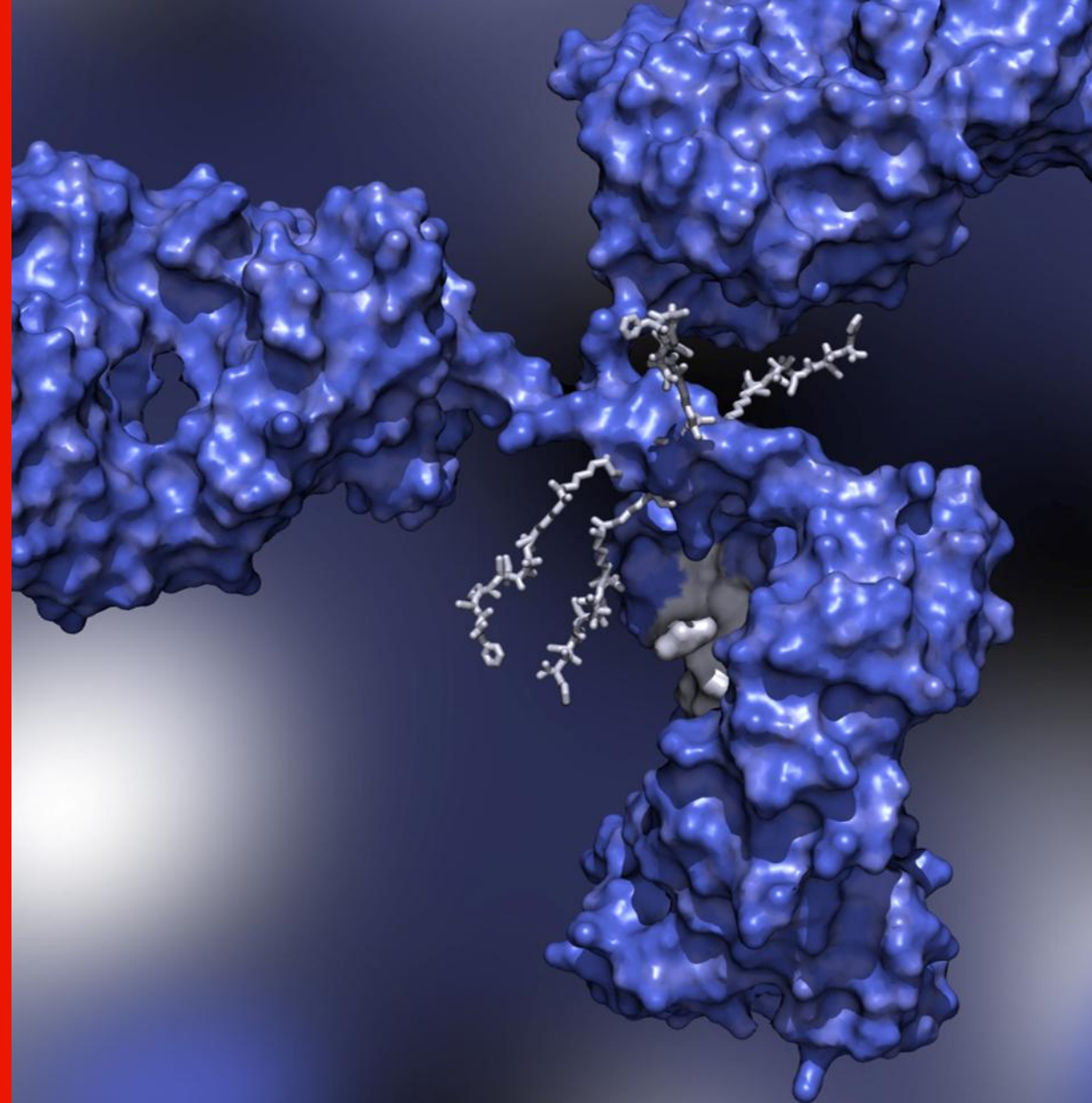


Integrated Native SEC-MS and Subunit Mass Spectrometry Workflow for Rapid Assessment of Chain Pairing and Fc Assembly in Four-Chain Bispecific Antibodies

Mona Goli, Ph.D.
Cell Engineering Sr. Scientist

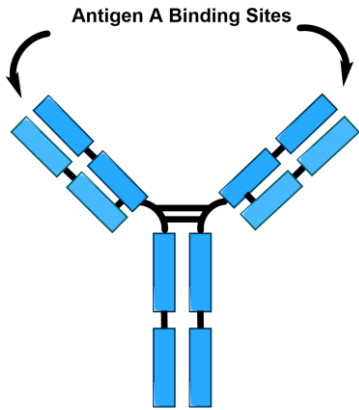
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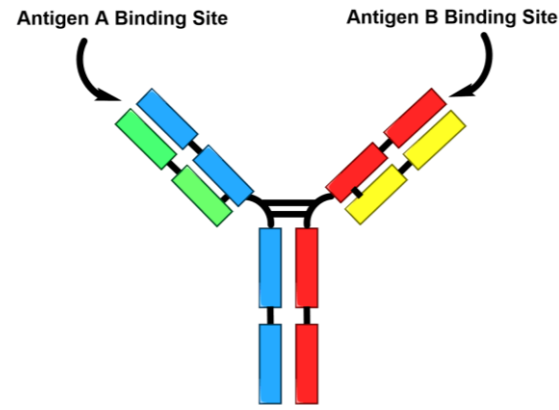
Different Architectures can Create Multispecific Antibodies

Monoclonal Antibody



- ✓ Target a single antigen
- ✓ Incomplete tumor coverage due to heterogeneity
- ✓ Target can mutate
- ✓ More likely for diseases to become resistant

Bispecific Antibody

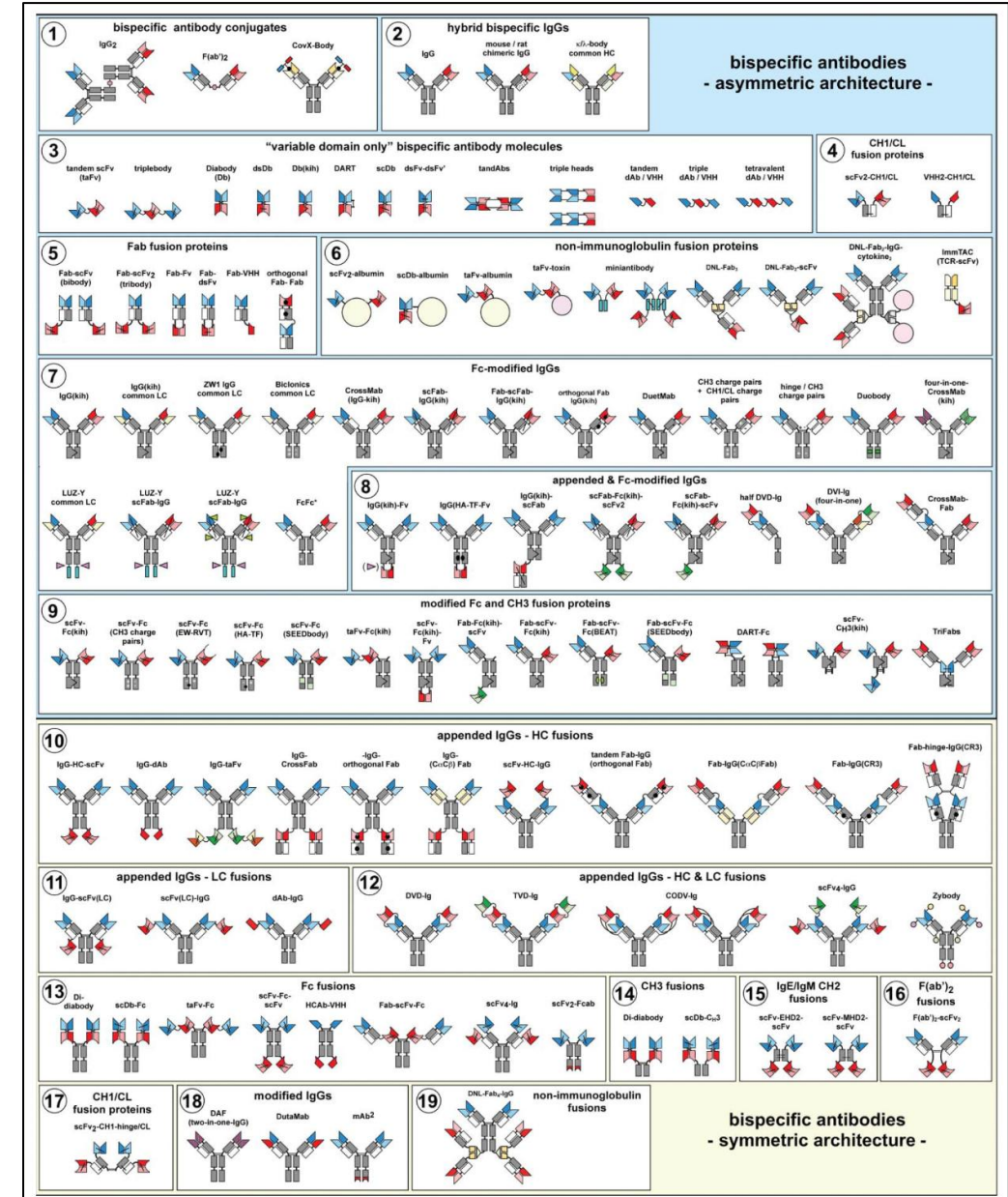


- ✓ Target two different antigens
- ✓ Enhanced precision
- ✓ Greater efficacy
- ✓ Potential to overcome resistance
- ✓ Ability to treat more complex diseases

precisepeg.com

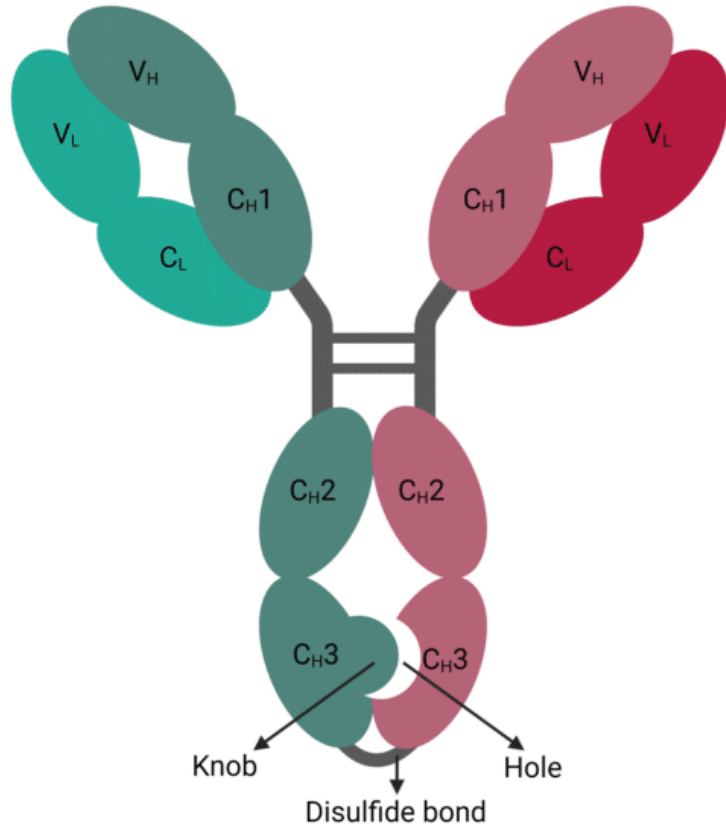
IgG-like Bispecifics

- Two different Fab arms with a heterodimeric Fc
- IgG-like stability, Fc function and development properties
- Requires control of HC heterodimerization and cognate HC-LC pairing



www.dotmatics.com

Four-chain IgG-like bsAbs Need Two Pairing Solutions



1 Direct HC–HC heterodimerization

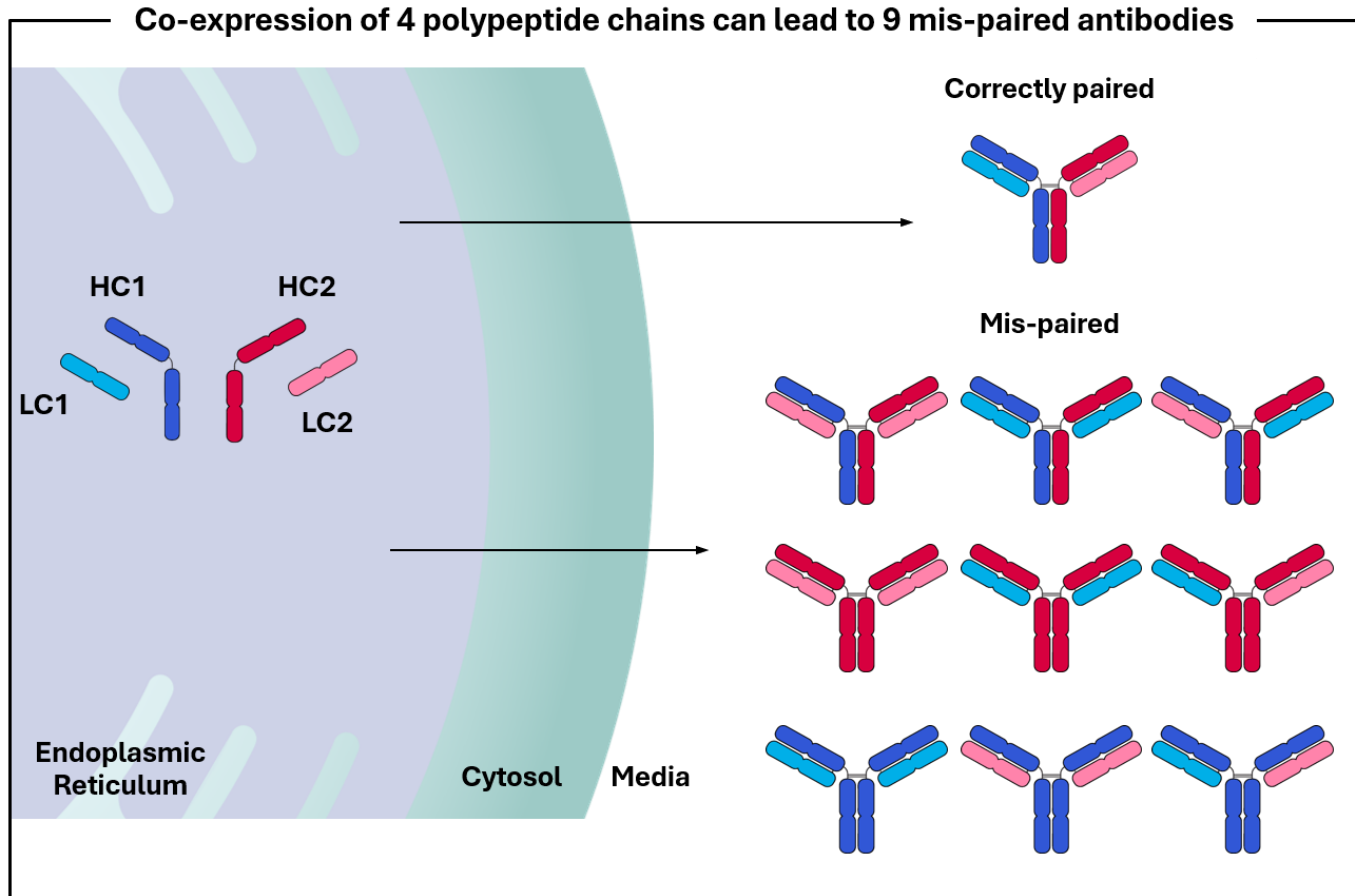
- Knobs-into-holes introduces complementary CH3 steric mutations
- Electrostatic and other heterodimeric Fc interfaces provide alternative routes
- Expression-ratio tuning may still be required

2 Direct cognate HC–LC pairing

- Common light chain removes LC mispairing but restricts variable-region diversity
- CrossMAb or domain exchange redirects pairing
- Engineered orthogonal CH1–CL interfaces favor intended partners
- Variable-region-specific optimization may still be needed

TAKE-HOME Knobs-into-holes solves an Fc-pairing problem; it does not, by itself, solve cognate light-chain pairing.

The Analytical Challenge: Four Chains Creates Multiple Assembly Risks

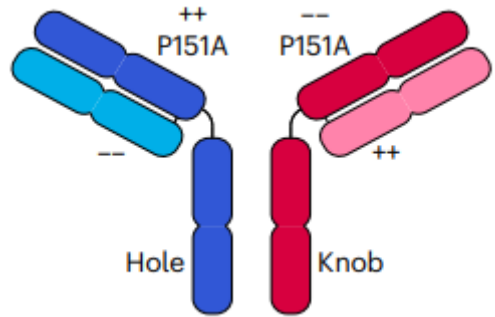


Potential product-quality liabilities

- Fc homodimer formation
- Half-antibody or excess-chain species
- Light-chain swapping and Fab mis-pairing
- High- and low-molecular-weight species
- Chain-ratio imbalance

TAKE-HOME Early, orthogonal mass spectrometry screening helps separate assembly risk from acceptable candidates.

ProAla Couples Correct Assembly to Folding-Mediated Secretion



P151A and Interface Mutations Result in highly efficient HC-LC pairing.

ER Retention

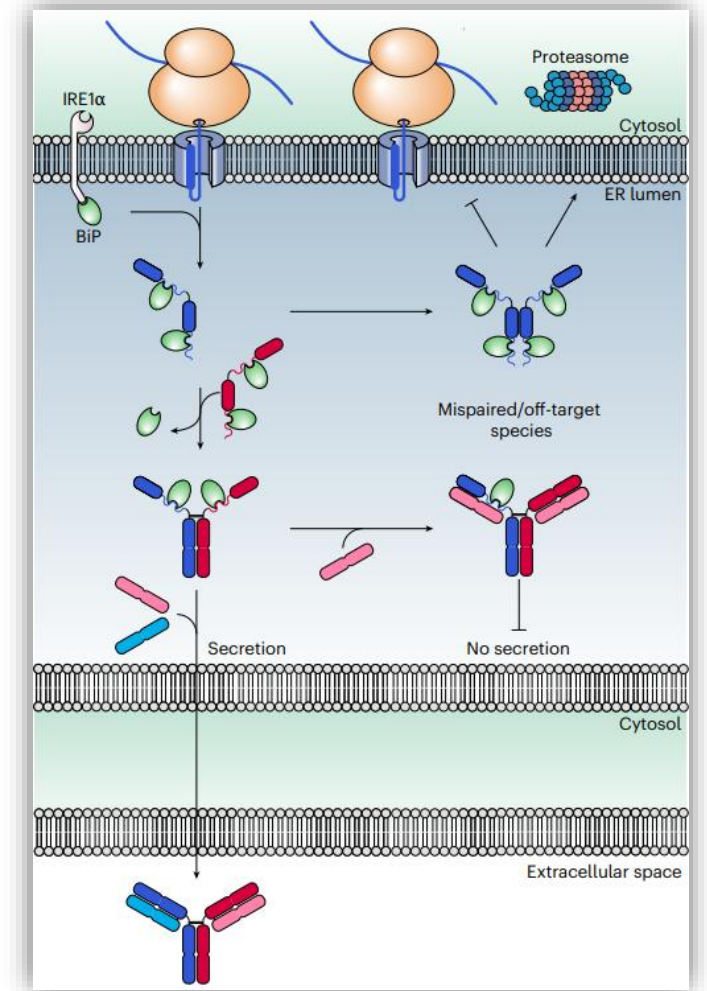
Unpaired or mis-paired chains remain associated with BiP.

Correct Pairing

Cognate HC-LC and HC-HC partners provide the interactions needed for folding.

Selective Secretion

Correctly paired bsIgG displaces BiP and is secreted; off-target species fail to secrete.



TAKE-HOME ProAla is an engineering platform for secretion fidelity; it does not remove the need to verify product quality analytically.

Study Objective

1

Is the Fc correctly assembled?

Differentiate the desired heterodimer from HC1–HC1 and HC2–HC2 homodimers.

2

Are the Fab arms correctly paired?

Screen diagnostic Fab subunits for light-chain swap or other mis-paired species.

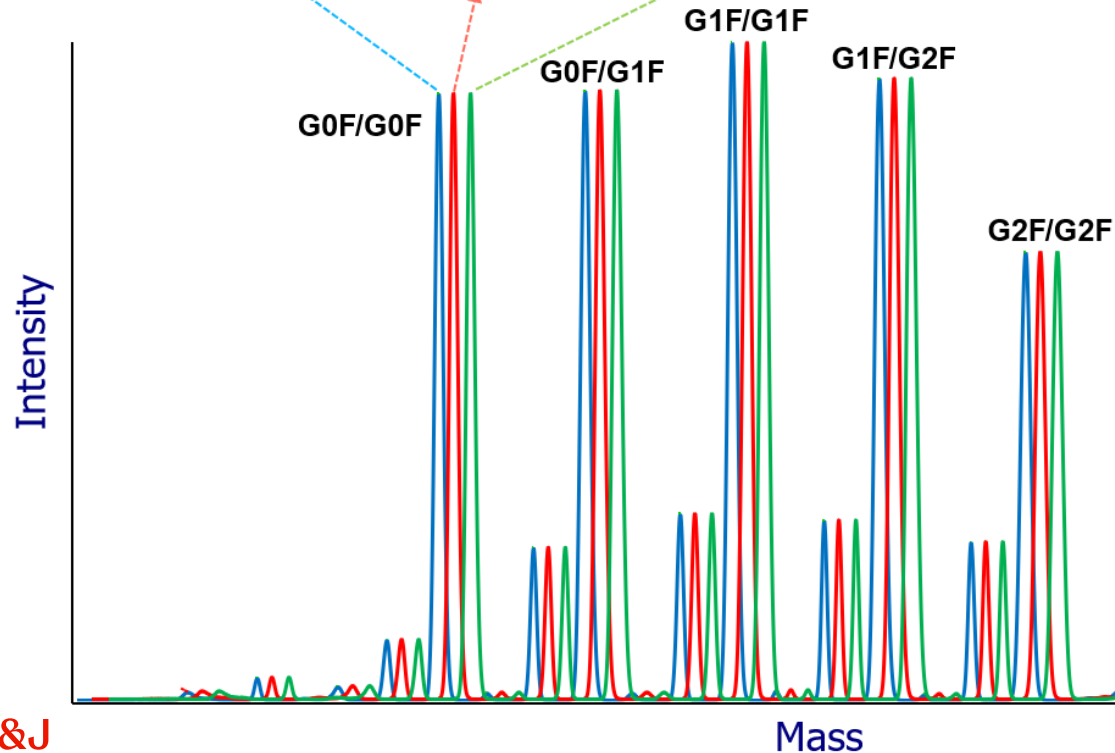
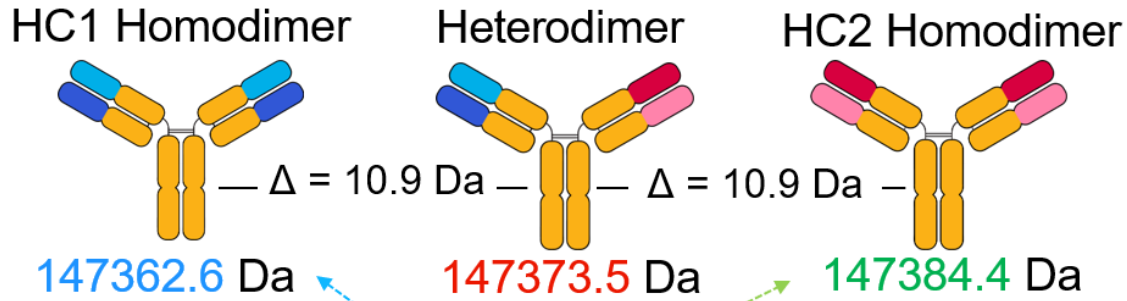
3

What other impurities are present under native conditions?

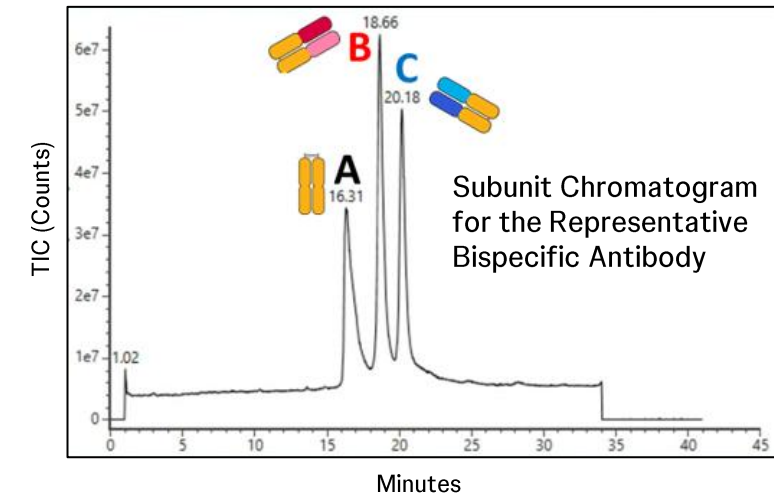
Resolve desired product, high-/low-MW species, aggregates, dimers and excess-chain impurities.

TAKE-HOME Combine chromatographic separation with accurate mass assignment to support fast, decision-oriented screening.

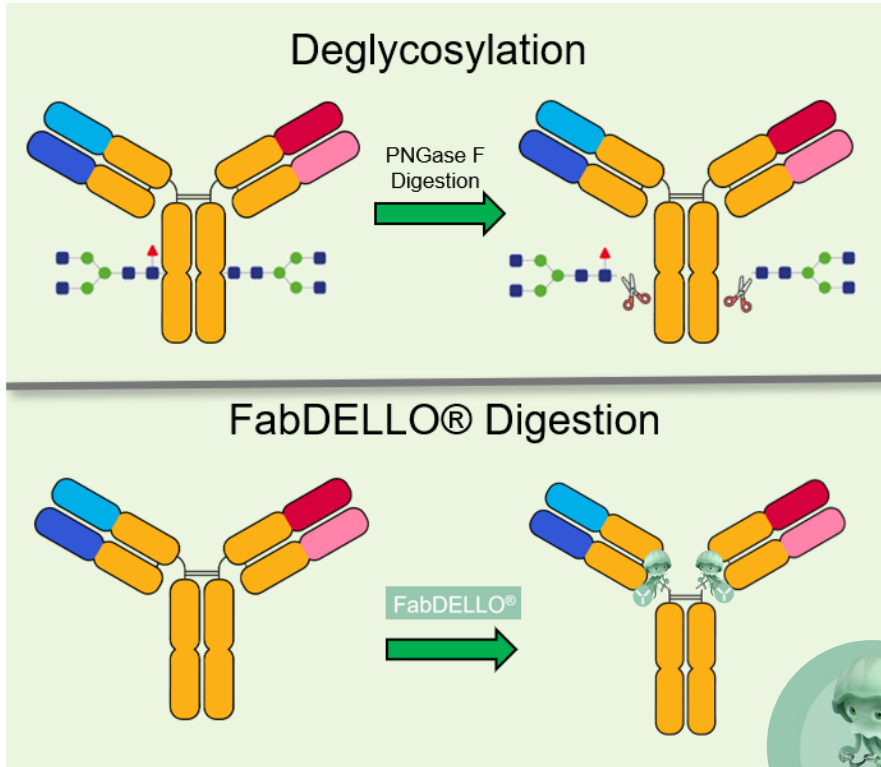
Mass Differences Between Heterodimers and Homodimers



- Mass between heterodimers and homodimers is minimal, leading to possible misidentifications.
- It is necessary to separate the molecule into diagnostic subunits and use orthogonal native analysis rather than relying only on intact mass.



Workflow: Integrated Sample Preparation and LC-MS Strategy



FabDELLO® from Genovis digests bsAbs at a single site above the hinge (...KSCDK/THTCPPCP...), generating intact Fab and Fc fragments.

01 Deglycosylate

Non-reducing conditions simplify the mass profile while preserving the intact protein.

02 Digest

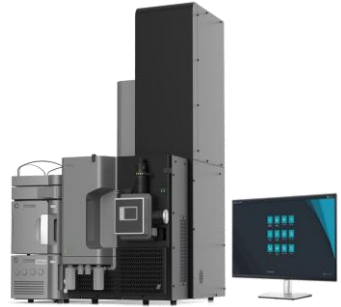
FabDELLO® generates diagnostic fragments while the Fc remains intact.

03 Subunit LC-MS

Xevo G3 QTof screens Fc assembly and light-chain pairing.

04 Native SEC-MS

Cyclic IMS and QE UHMR link separation, fluorescence and accurate mass.



Waters Xevo G3 QTof



Waters Cyclic IMS

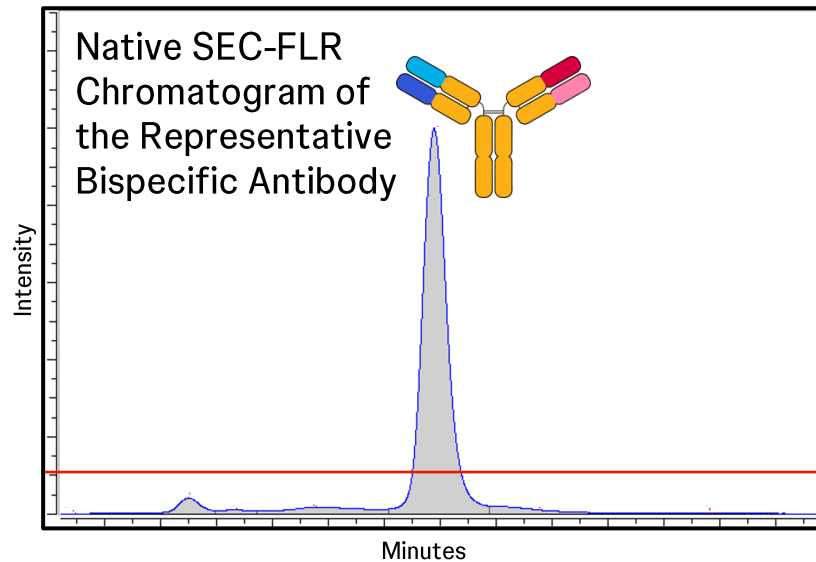


Thermo Fisher Scientific
QE UHMR

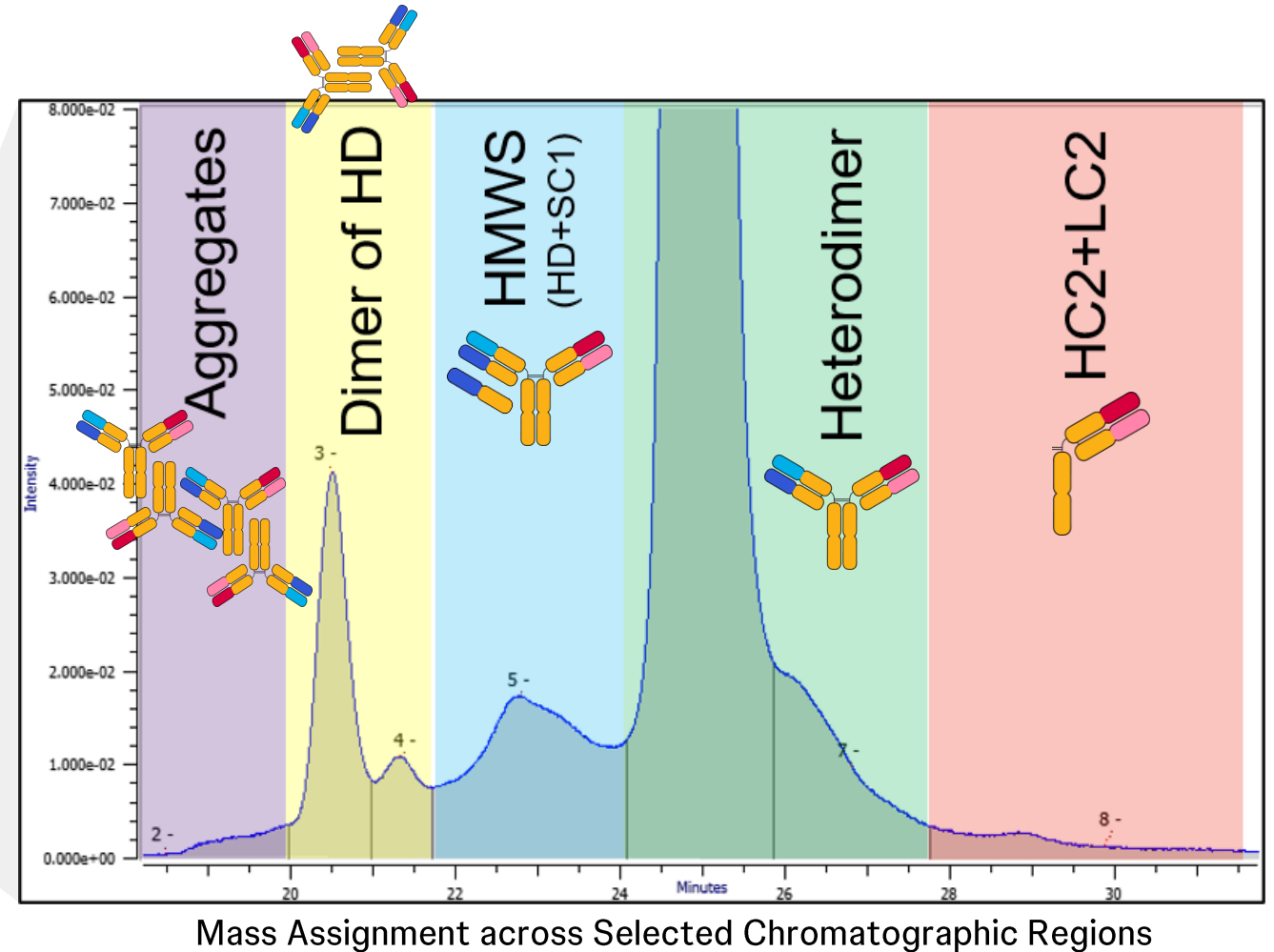
TAKE-HOME Orthogonal readouts are intentionally matched to distinct assembly questions.

Native SEC-MS-FLR Preserves the Assembly Context

- Chromatography separates molecular populations
- Fluorescence supports relative quantitation
- Accurate mass supports species identification

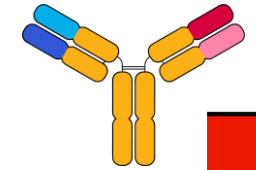


Zoom In



TAKE-HOME The combined readout resolves desired heterodimer alongside high-/low-MW and excess-chain-related species.

Fab Pairing: Diagnostic Fab Subunits Test Light-chain Pairing Fidelity

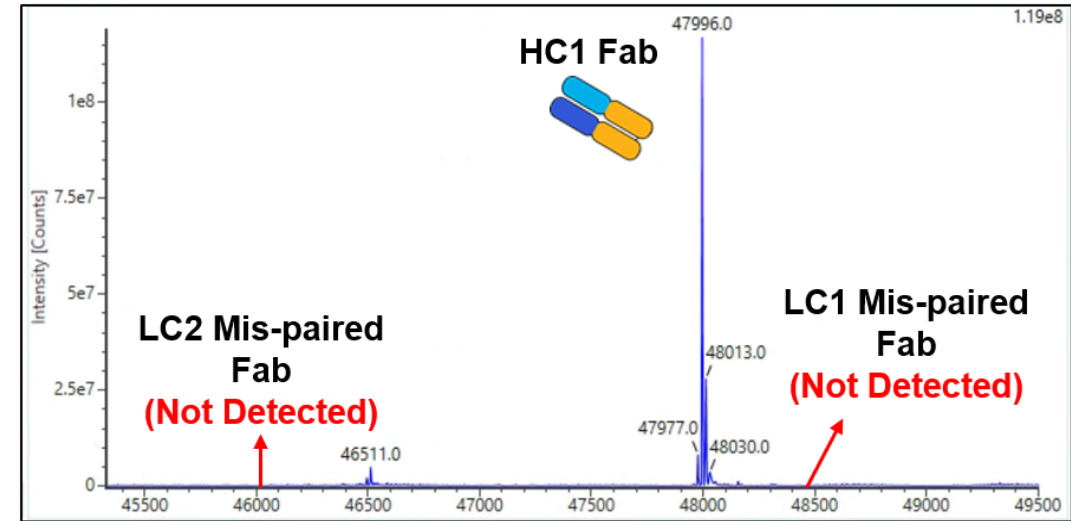


A: Expected Diagnostic Subunit Masses

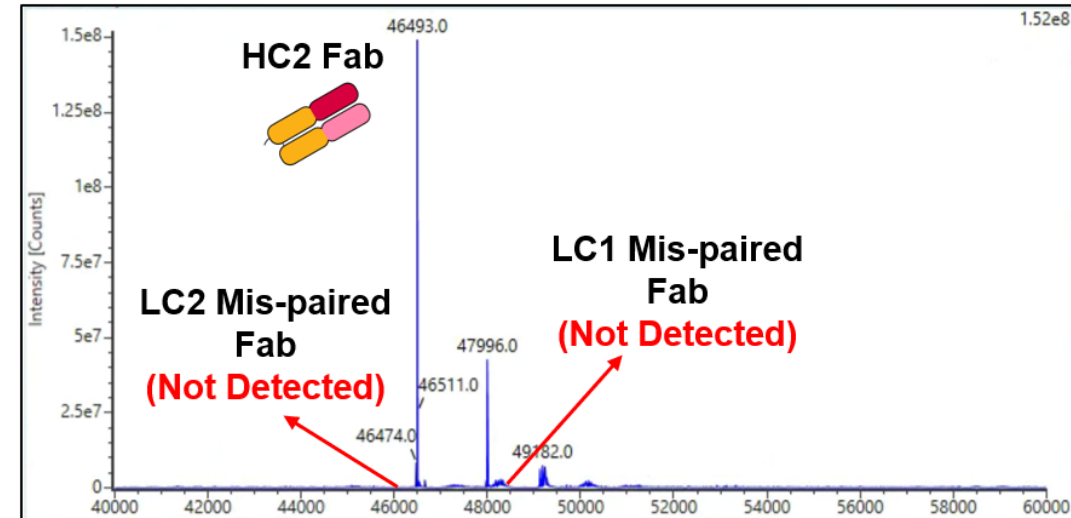
Region	Expected Mass (Da)	
Fab _a (HC1+LC1) (HC1 Fab)	47996.4	
Fab _b (HC2+LC2) (HC2 Fab)	46493.8	
Fab _c (HC1+LC2) (LC2 Mis-paired Fab)	46001.1	
Fab _d (HC2+LC1) (LC1 Mis-paired Fab)	48489.1	

- Expected HC1 and HC2 Fab species were confirmed.
- No detectable LC1- or LC2-associated mis-paired Fab variants were observed.

HC1-associated Fab Region

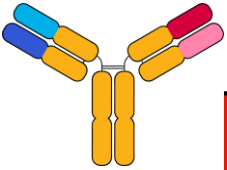


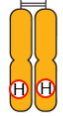
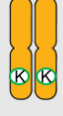
HC2-associated Fab Region

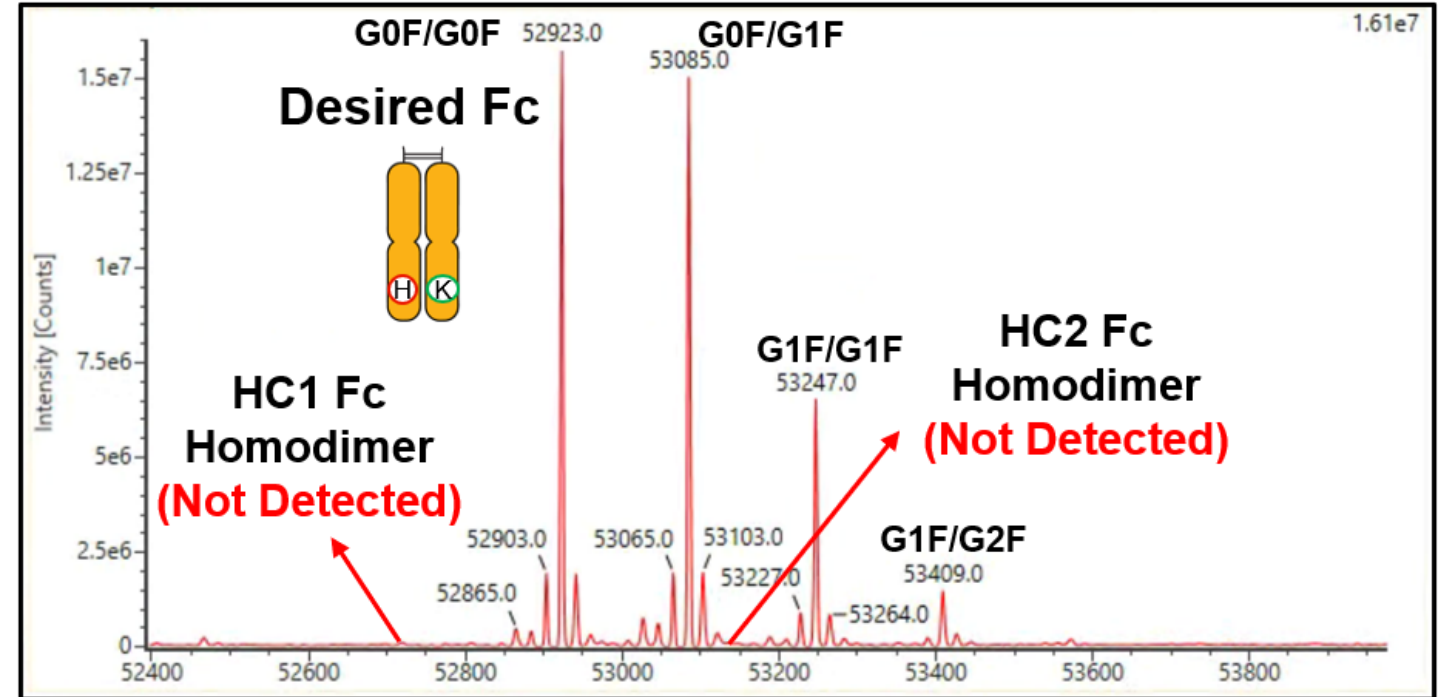


Fc Assembly: Subunit LC-MS Confirms Fc Heterodimerization

A: Representative Bispecific Antibody



Region	Expected Mass (Da)	Fc species
Fc Desired	52923.4	
Hole – Hole (HC1) Fc Homodimer	52721.2	
Knob – Knob (HC2) Fc Homodimer	53125.6	

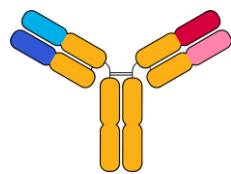


- Mass corresponded to the expected Fc heterodimer and anticipated glycoform pattern.
- HC1-HC1 and HC2-HC2 Fc homodimer masses were not detected in the analyzed molecule.

TAKE-HOME Subunit mass analysis provides a rapid, direct screen for Fc assembly variants.

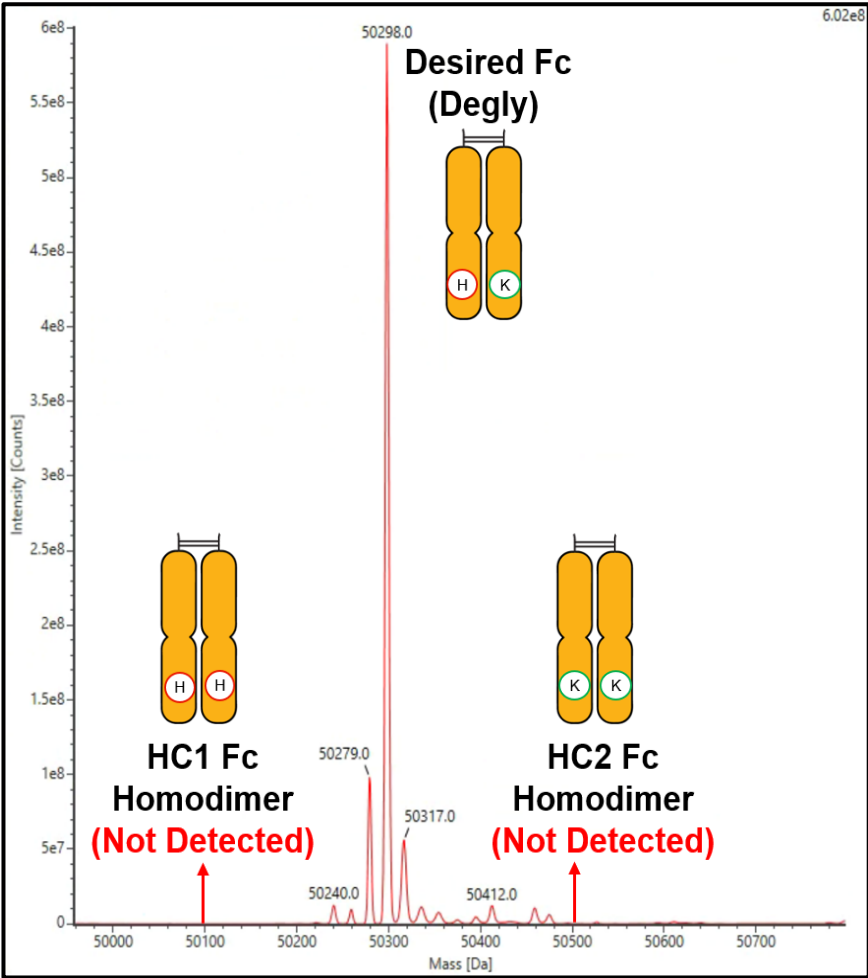
Fc Assembly: Subunit LC-MS Confirms Fc Heterodimerization

B: Representative Deglycosylated Bispecific Antibody



Region	Expected Mass (Da)	Fc species
Fc (Degly) Desired	50297.0	
Hole – Hole (HC1) Fc Homodimer	50096.8	
Knob – Knob (HC2) Fc Homodimer	50501.2	

- Deglycosylated subunit mass analysis was used to distinguish the desired Fc heterodimer from HC1-HC1 and HC2-HC2 Fc homodimers.
- Only the expected Fc heterodimer was detected; no Fc homodimer species were observed.
- Results confirm successful Fc heterodimerization with minimal risk of Fc homodimer impurities.



TAKE-HOME Subunit mass analysis for the deglycosylated bsAb provides a rapid, direct screen for Fc assembly variants.

Key Conclusions

- ✓ In summary, we developed an integrated Native SEC-MS and subunit LC-MS workflow that enables rapid assessment of assembly integrity in four-chain bispecific antibodies.
- ✓ The workflow allows simultaneous evaluation of:
 - Fc heterodimer formation,
 - Fc homodimer impurities,
 - light-chain pairing fidelity,
 - higher-order species such as aggregates.
- ✓ Overall, this approach provides actionable product-quality information that can support clone selection, molecule developability assessment, and advancement of next-generation multi-specific antibody formats.

TAKE-HOME Early analytical clarity helps de-risk complex four-chain formats before downstream development investment.

Acknowledgments

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