Supplementary Information to:

Mass spectrometry-based *de novo* sequencing of monoclonal antibodies using multiple proteases and a dual fragmentation scheme

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Contents:

anti-FLAG-M2 MS-based sequence (with L51I correction)

```
>anti-FLAG-M2_MS_HeavyChain
QVQLQQSAAELARPGASVKMSCKASGYSFTTYTIHWVKQRPGQGLEWIGYINPSSGYAAYNQNFKDETTLTADPSSS
TAYMELNSLTSEDSAVYYCAREKFYGYDYWGQGATLTVSSAKTTPPSVYPLAPGSAAQTNSMVTLGCLVKGYFPEPV
TVTWNSGSLSSGVHTFPAVLQSDLYTLSSSVTVPSSPRPSETVTCNVAHPASSTKVDKKIVPRDCGCKPCICTVPEV
SSVFIFPPKPKDVLTITLTPKVTCVVVDISKDDPEVQFSWFVDDVEVHTAQTQPREEQFNSTFRSVSELPIMHQDWL
NGKEFKCRVNSAAFPAPIEKTISKTKGRPKAPQVYTIPPPKEQMAKDKVSLTCMITDFFPEDITVEWQWNGQPAENY
KNTQPIMNTNGSYFVYSKLNVQKSNWEAGNTFTCSVLHEGLHNHHTEKSLSHSPGK

>anti-FLAG-M2_MS_LightChain
DVLMTQIPLSLPVSLGDQASISCRSSQSIVHRNGNTYLEWYLLKPGQSPKLLIYKVSNRFSGVPDRFSGSGSGTDFT
LKISRVEAEDLGVYYCFQGSHVPYTFGGGTKLEIRRADAAPTVSIFPPSSEQLTSGGASVVCFLNNFYPKDINVKWK
IDGSERQNGVLNSWTDQDSKDSTYSMSSTLTLTKDEYERHNSYTCEATHKTSTSPIVKSFNRNEC
```

Table S1. Coverage statistics for the Herceptin benchmark and anti-FLAG™-M2 MAb sequences.

		Herceptin	anti-FLAG-M2
# peptide reads (Byonic score >=500)	total	4408	3371
	stepped HCD	2686	1983
	EThcD	1722	1388
depth-of-coverage (median [range])	total	148 [8-394]	84 [0-382]
	CDRH1	163 [158-176]	32 [22-47]
	CDRH2	94 [88-103]	39 [36-43]
	CDRH3	42 [18-67]	66 [50-75]
	CDRL1	210 [208-218]	192 [144-207]
	CDRL2	74 [71-84]	46 [40-60]
	CDRL3	140 [130-143]	127 [109-131]

Table S2. Model statistics for Fab crystal structure.

Refinement statistics		
Resolution (Å)	42.52-1.86	
No. of reflections	39988	
PDB	**2G60 (old)**	**7BG1 (new)**
Total number of atoms	3518	3497
Average atomic displacement parameter (Å^2)	45.0	52.0
$R_{\text{work}}/R_{\text{free}}$	0.235/0.278	0.217/0.255
Bond length RMSZ	0.93	0.28
Bond angle RMSZ	0.96	0.51
Ramachandran favored/outliers (%)	93.0/1.0	97.57/0.24
Molprobity score	3.37	1.60
Clashscore	56	3.61

Table S3. Comparison of CDR sequences from anti-FLAG™-M2 to other known FLAG™-tag binding MAbs (see refs 41-42).

	Heavy Chain		
MAb	CDRH1	CDRH2	CDRH3
anti-FLAG-M2	GYSFTTYT----	LNPSSGYA	AREKFYGYDY
2H8	GFSLNTSGRS--	IYWDDDK	ARRMDY
EEh13.6	GDSLSSFNAGVN	HGAVM-STR	AKSTGRYDF
EEh14.3	GDSLSSYNAGVN	HMAGV-STR	VRNEWSGAF
EEf15.4	GFSIK--GANVN	HVRGDASTR	ADRKMYSFYSGGEA

	Light Chain		
MAb	CDRL1	CDRL2	CDRL3
anti-FLAG-M2	QSIVHRNGNTY	KVS	FQGSHVPYT
2H8	QSLVHSNGNTY	KVS	SQSTHVPYT
EEh13.6	QSIVHSNGNTY	KVS	FQGSLVPPT
EEh14.3	QSIVHSNGNTY	KVS	FQGSLVPPT
EEf15.4	NARSGS	DGN	SAFDQTNKYVG

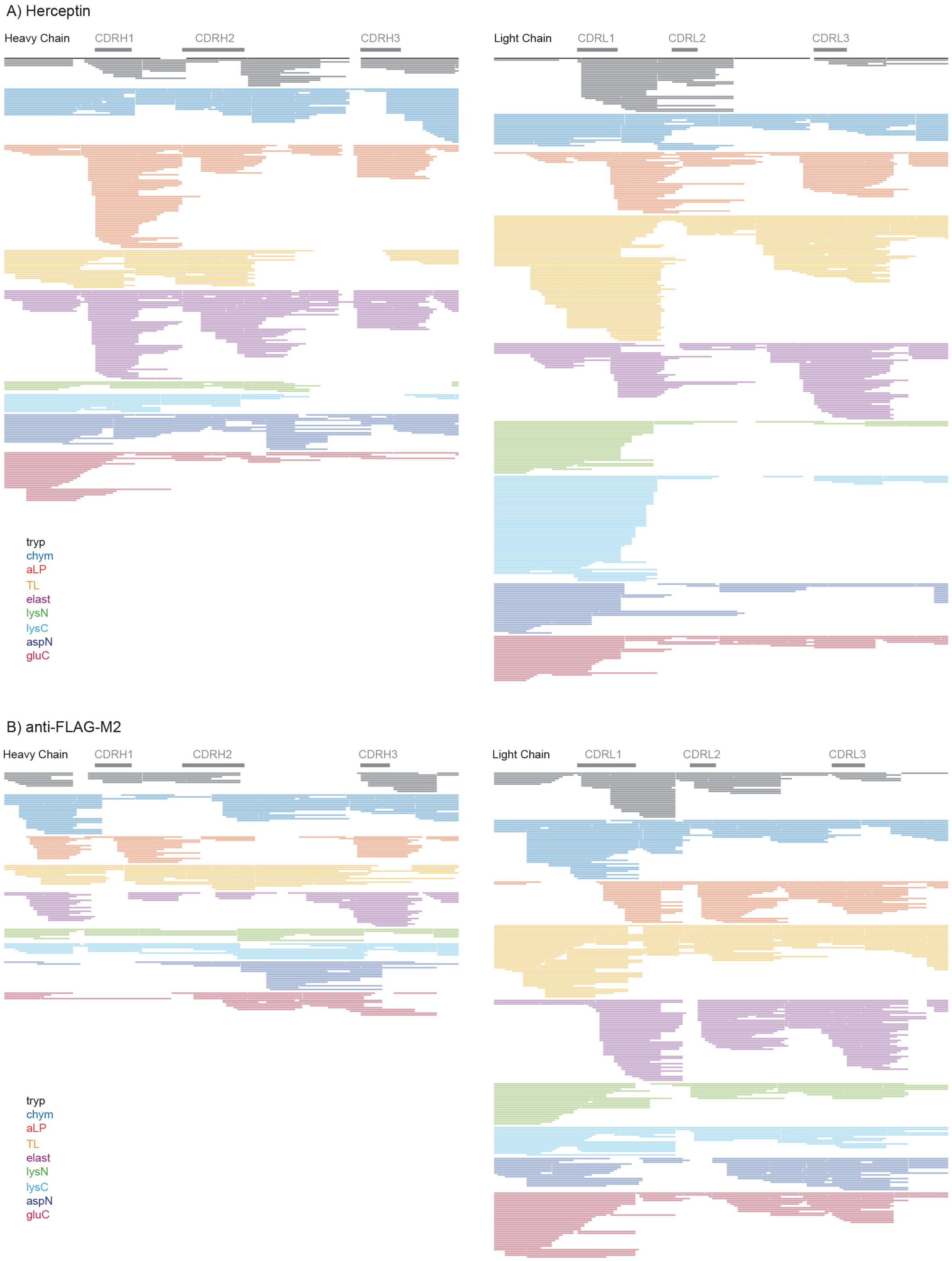


Figure S1. Coverage maps of Herceptin benchmark (A) and anti-FLAG™-M2 MAb (B) sequences. Peptides with Byonic scores of >=500 are shown.

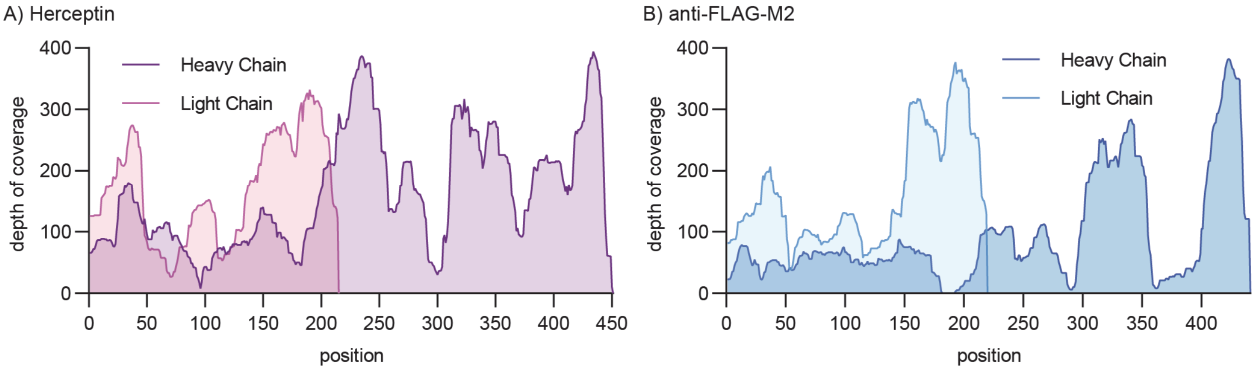
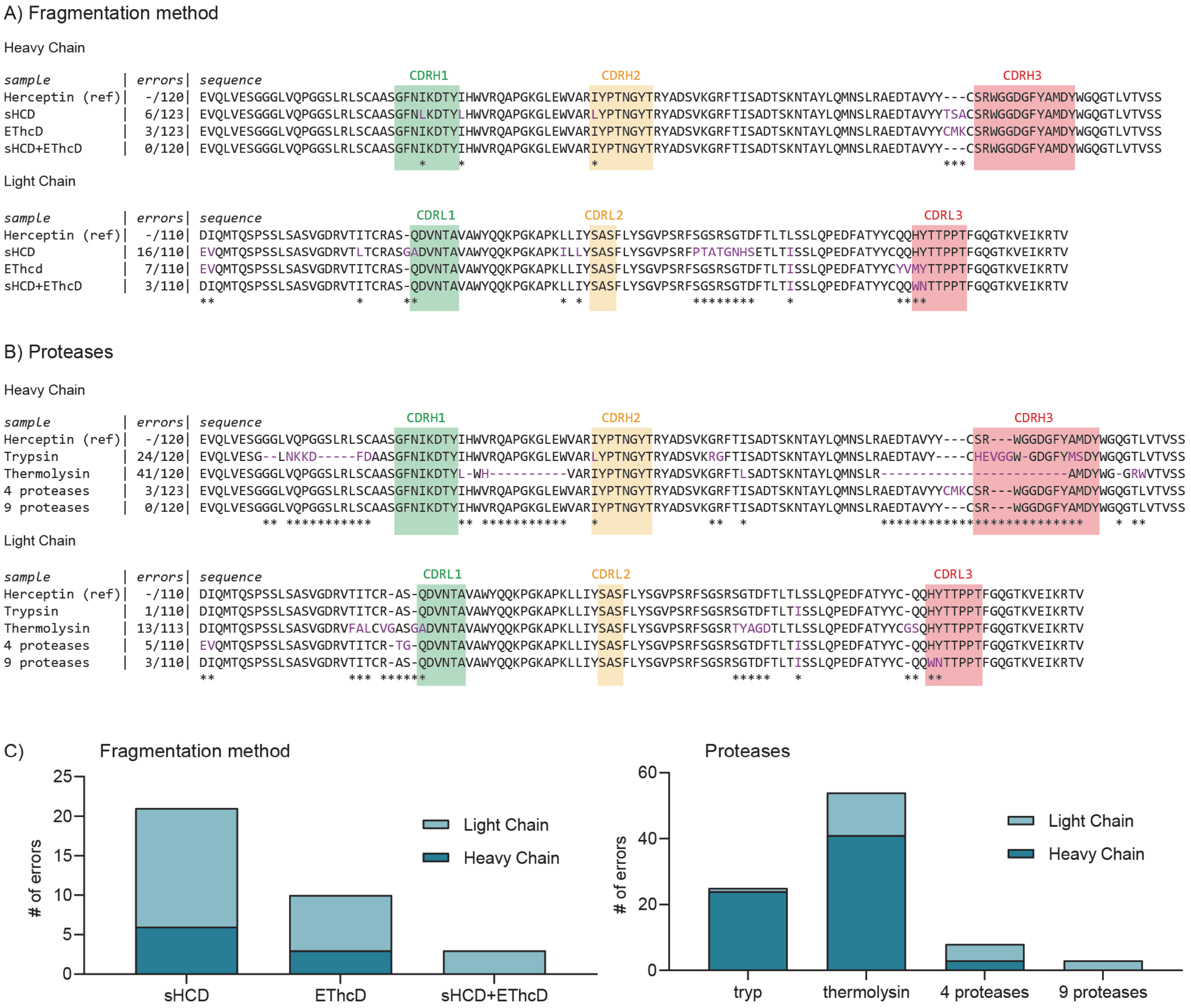


Figure S2. Depth of coverage profiles for Herceptin (A) and anti-FLAG™-M2 (B) sequences, based on peptides with Byonic score >=500, as in Figure S1.



A) Fragmentation method

Heavy Chain

```
                                                     CDRH1                   CDRH2                                               CDRH3
sample          | errors| sequence
Herceptin (ref)|  -/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSRWGGDGFYAMDYWGQGTLVTVSS
sHCD            |  6/123| EVQLVESGGGLVQPGGSLRLSCAASGFNLKDTYLHWVRQAPGKGLEWVARLYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYTSACSRWGGDGFYAMDYWGQGTLVTVSS
EThcD           |  3/123| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCMKCSRWGGDGFYAMDYWGQGTLVTVSS
sHCD+EThcD      |  0/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSRWGGDGFYAMDYWGQGTLVTVSS
                                                      *    *                *                                            ***
```

Light Chain

```
                                                      CDRL1                CDRL2                                       CDRL3
sample          | errors| sequence
Herceptin (ref)|  -/110| DIQMTQSPSSLSASVGDRVTITCRAS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTLSSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTV
sHCD            | 16/110| EVQMTQSPSSLSASVGDRVTLTCRASGADVNTAVAWYQQKPGKAPKILLYSASFLYSGVPSRFPTATGNHSETLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTV
EThcd           |  7/110| EVQMTQSPSSLSASVGDRVTITCRAS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCYVMYTTPPTFGQGTKVEIKRTV
sHCD+EThcD      |  3/110| DIQMTQSPSSLSASVGDRVTITCRAS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQWNTTPPTFGQGTKVEIKRTV
                          **                  *     **                  * *              ********    *             ****
```

B) Proteases

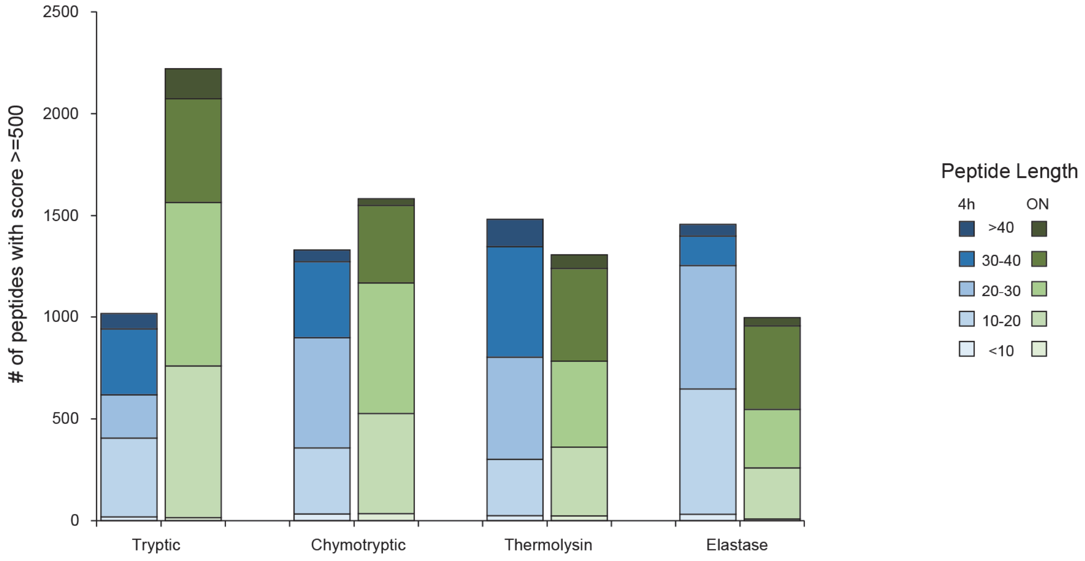
Heavy Chain

```
                                                    CDRH1                    CDRH2                                                CDRH3
sample          | errors| sequence
Herceptin (ref)|  -/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSR---WGGDGFYAMDYWGQGTLVTVSS
Trypsin         | 24/120| EVQLVESG--LNKKD-----FDAASGFNIKDTYIHWVRQAPGKGLEWVARLYPTNGYTRYADSVKRGFTISADTSKNTAYLQMNSLRAEDTAVY----CHEVGGW-GDGFYMSDYWGQGTLVTVSS
Thermolysin     | 41/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYL-WH----------VARIYPTNGYTRYADSVKGRFTLSADTSKNTAYLQMNSLR------------------------AMDYWG-GRWVTVSS
4 proteases     |  3/123| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCMKCSR---WGGDGFYAMDYWGQGTLVTVSS
9 proteases     |  0/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSR---WGGDGFYAMDYWGQGTLVTVSS
                                  ** ***********           ** ***********   *              **  *                 **************************    * **
```

Light Chain

```
                                                       CDRL1                CDRL2                                       CDRL3
sample          | errors| sequence
Herceptin (ref)|  -/110| DIQMTQSPSSLSASVGDRVTITCR-AS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTLSSLQPEDFATYYC-QQHYTTPPTFGQGTKVEIKRTV
Trypsin         |  1/110| DIQMTQSPSSLSASVGDRVTITCR-AS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC-QQHYTTPPTFGQGTKVEIKRTV
Thermolysin     | 13/113| DIQMTQSPSSLSASVGDRVFALCVGASGADVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRTYAGDTLTLSSLQPEDFATYYCGSQHYTTPPTFGQGTKVEIKRTV
4 proteases     |  5/110| EVQMTQSPSSLSASVGDRVTITCR-TG-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC-QQHYTTPPTFGQGTKVEIKRTV
9 proteases     |  3/110| DIQMTQSPSSLSASVGDRVTITCR-AS-QDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYC-QQWNTTPPTFGQGTKVEIKRTV
                          **                 *** ******                                       *****   *             ** **
```

Figure S3. Sequence accuracy of Herceptin by fragmentation type (A) and use of proteases (B). Supernovo analysis was performed using only the specified fragmentation type or proteases as input data. Resulting sequences were aligned to the Herceptin reference sequences to count the number of errors. Every substitution, insertion or deletion was counted as an error as listed before the output sequence; *i.e.* all positions labeled in purple and marked with an asterisks are counted. The '4 proteases' dataset consists of trypsin, chymotrypsin, thermolysin and elastase. The total number of errors is shown for fragmentation strategy and protease datasets in panel C.



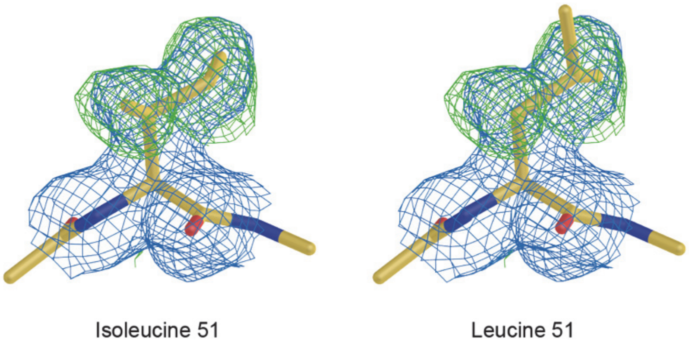
Heavy Chain

```
                                                    CDRH1                   CDRH2                                               CDRH3
sample         | errors| sequence
Herceptin (ref)|  -/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSRWGGDGFYAMDYWGQGTLVTVSS
4 hours        |  3/123| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYYCMKCSRWGGDGFYAMDYWGQGTLVTVSS
overnight      |  0/120| EVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAEDTAVYY---CSRWGGDGFYAMDYWGQGTLVTVSS
                                                                                                                        ***
```

Light Chain

```
                                                     CDRL1                CDRL2                                        CDRL3
sample         | errors| sequence
Herceptin (ref)|  -/110| DI-QMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTLSSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTV
4 hours        |  5/110| EV-QMTQSPSSLSASVGDRVTITCRTGQDVNTAVAWYQQKPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTKVEIKRTV
overnight      |  2/111| DIDQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQKPGKAPKLLLYSASFLYSGVPSRFSGSRSGTDFTLTLSSLQPENFATYYCQQHYTTPPTFGQGTKVEIKRTV
                         ***                      **                      *                          *
```

Figure S4. Peptide length depending on digestion time. Datasets of four proteases were combined for Supernovo analysis. Peptide length distribution is based on peptides with score >=500. Resulting sequences from Supernovo were aligned to the Herceptin reference sequences to count the number of errors. Every substitution, insertion or deletion was counted as an error as listed before the output sequence; *i.e.* all positions labeled in purple and marked with an asterisks are counted.



Isoleucine 51

Leucine 51

Figure S5. Isoleucine/Leucine assignment at Heavy Chain position 51 of anti-FLAG™-M2. (left panel) Electron density around isoleucine 51 at a contour level of 1.0 RMSD in blue and simulated annealing omit map density of the $C_{\gamma1}$, $C_{\gamma2}$ and C_{δ} atoms of this residue at a contour level of 2.5 R.M.S.D. in green. (right panel) A leucine instead of an isoleucine in this location has a poor fit to both maps.

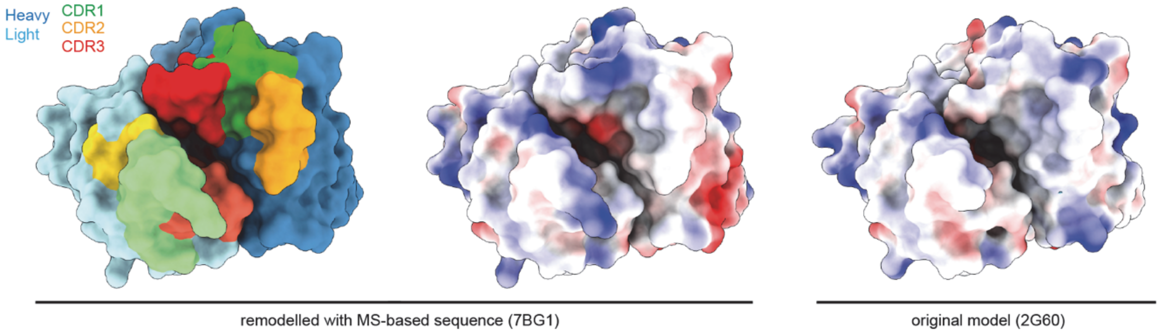


Figure S6. Electrostatic surface potential of the anti-FLAG™-M2 paratope. The revised crystal structure based on the MS-derived sequence (PDB ID: 7BG1) is shown alongside the original model (PDB ID: 2G60). The electrostatic surface was calculated with the default *coulombic* command in ChimeraX.

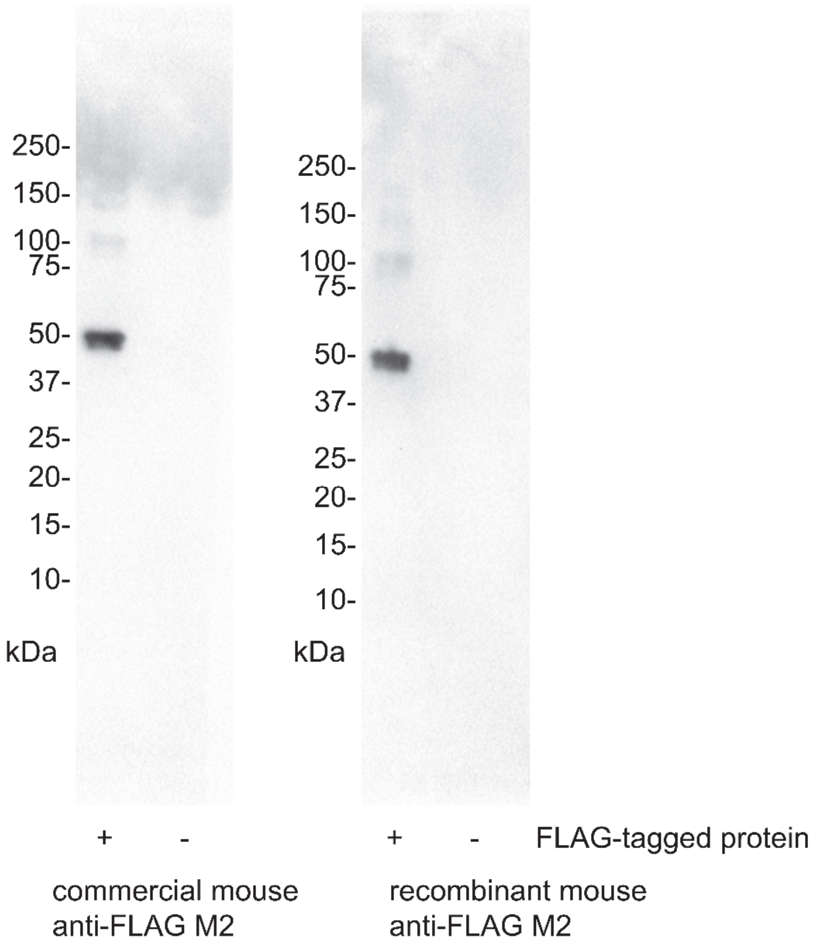


Figure S7. Western blot validation of synthetic recombinant anti-FLAG™-M2 compared to the originally sequenced sample. Same Western blot as shown in Figure 3C, showing complete lanes with marker positions.