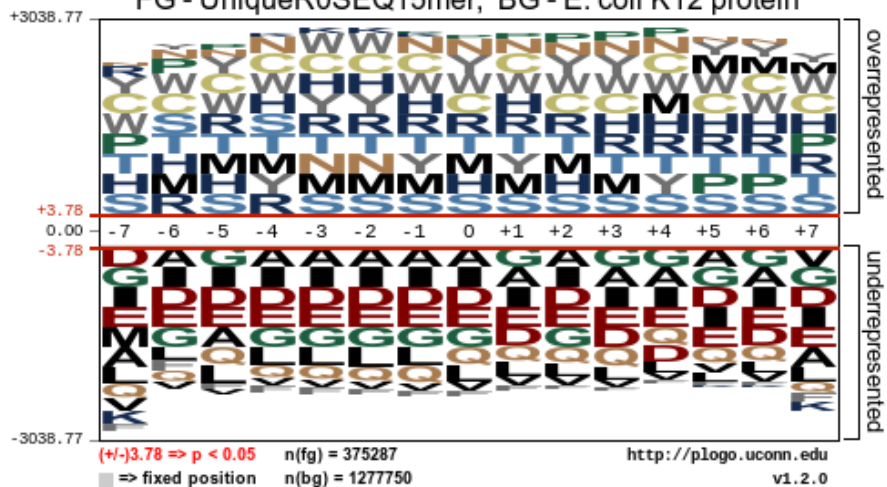


Supplementary Figure S1. Amino acid representation within all unique sequences within the library excluding (A) and including (B) the effects of frequency of sequence occurrence, for Round 0 (blue), Round 1 (orange) and Round 2 (grey).

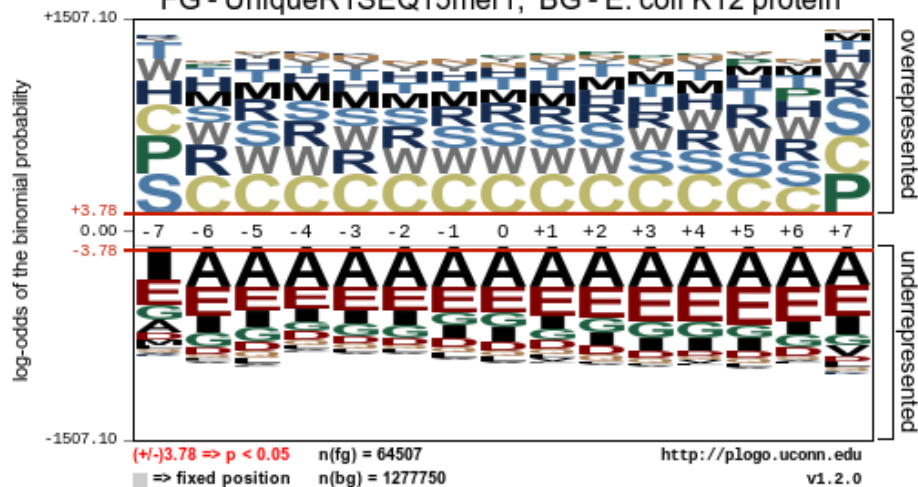
A)

FG - UniqueR0SEQ15mer; BG - E. coli K12 protein



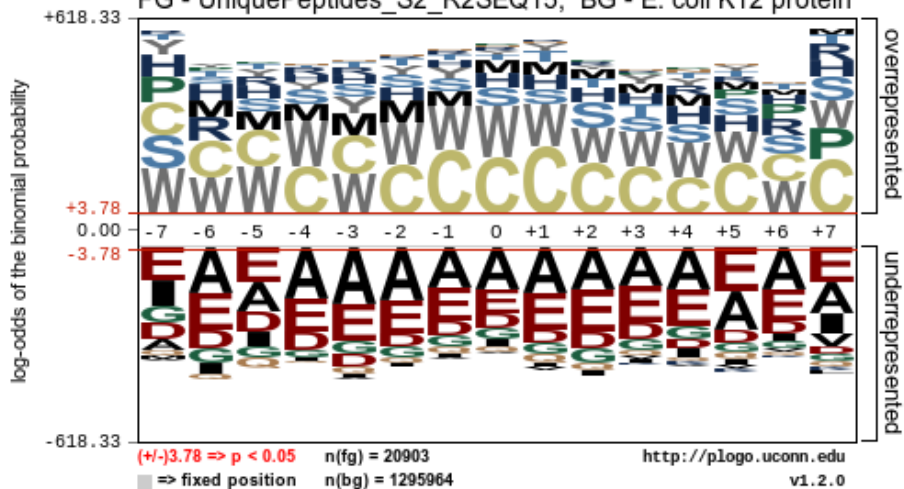
B)

FG - UniqueR1SEQ15mer1; BG - E. coli K12 protein

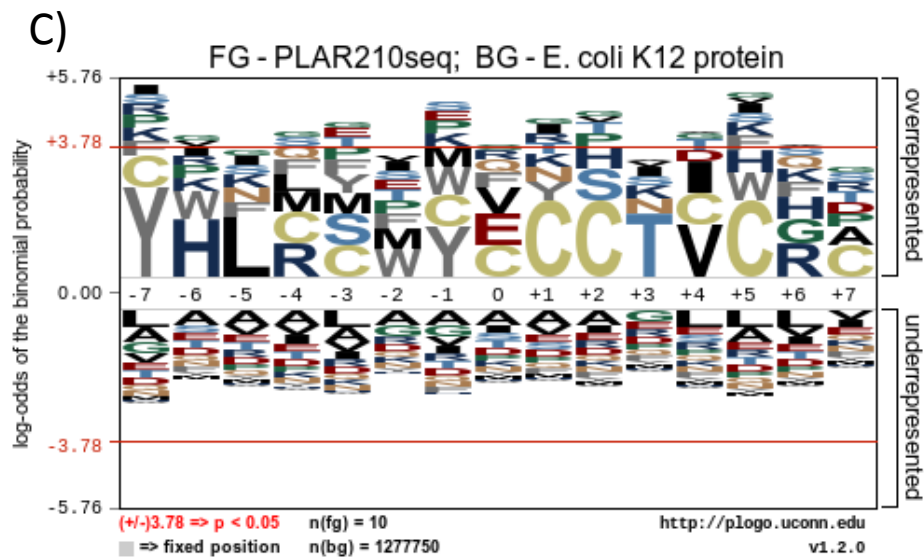
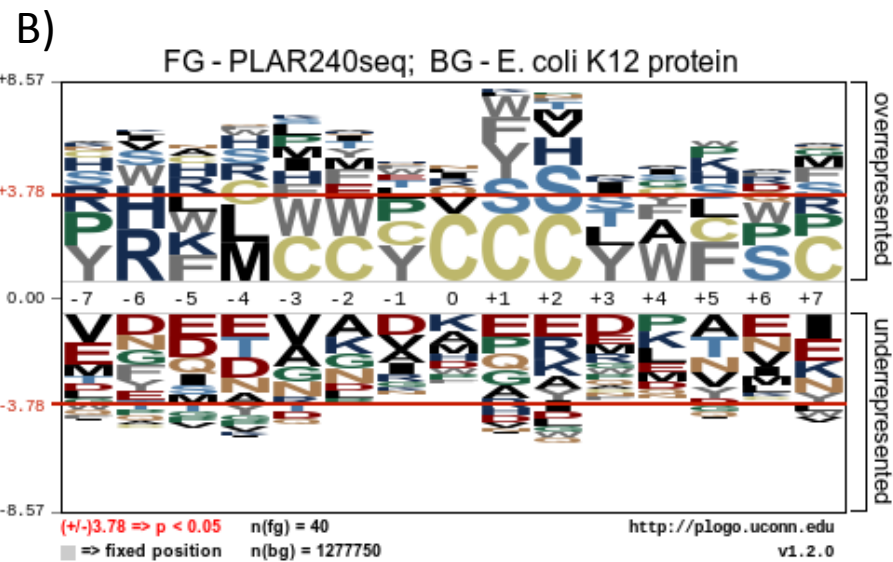
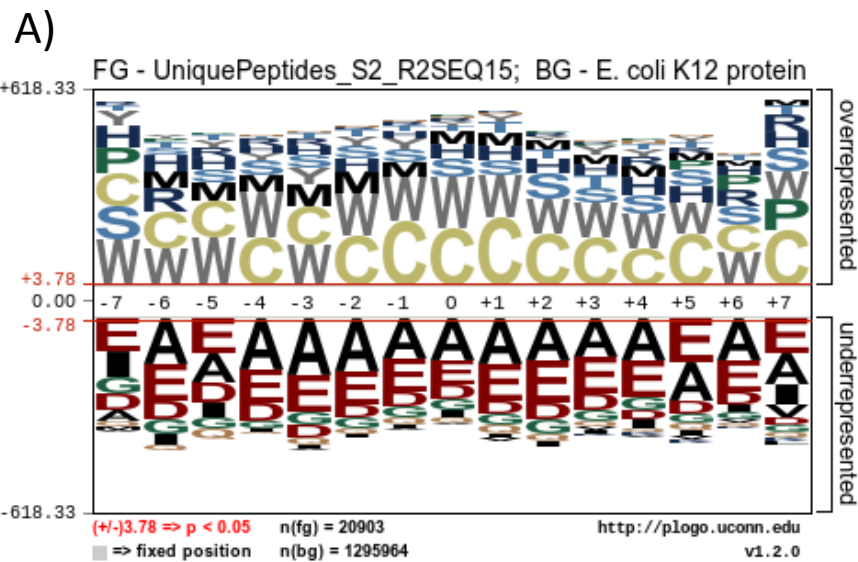


C)

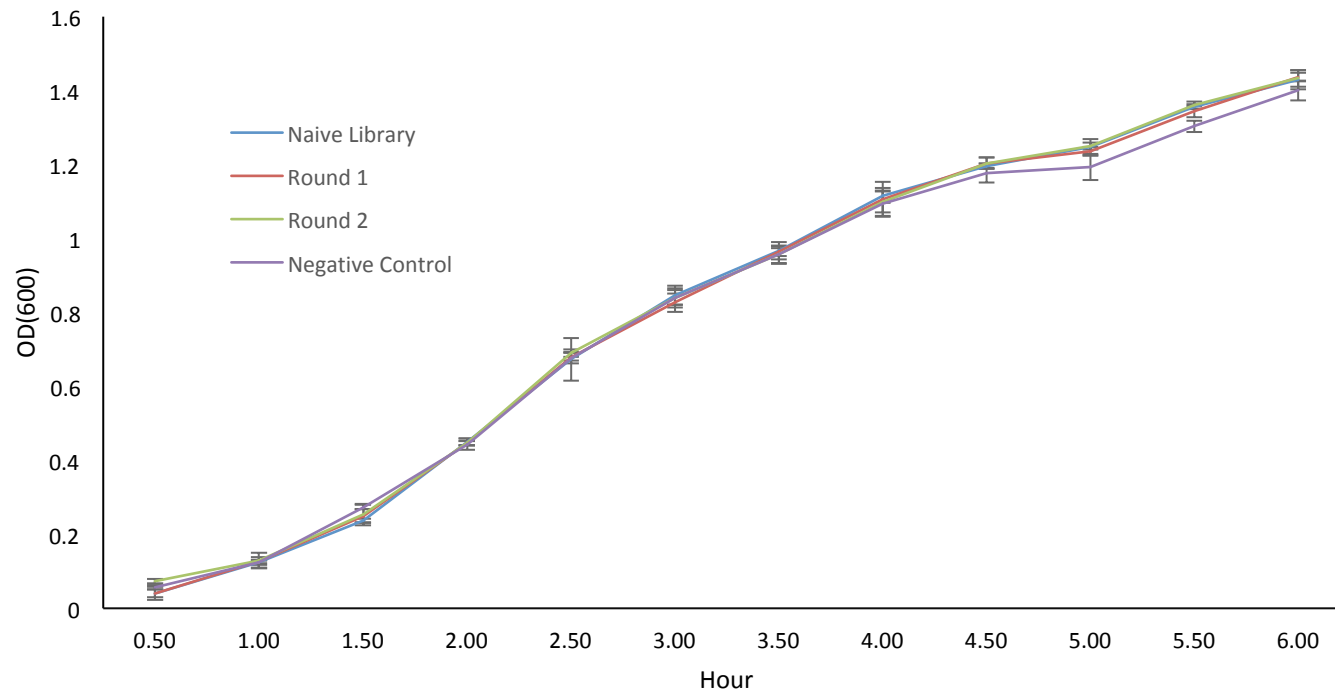
FG - UniquePeptides_S2_R2SEQ15; BG - E. coli K12 protein



Supplementary Figure S2. Visualization of motifs of unique 15-mer sequences calculated against background probabilities for *E. coli* K12 in (A) Round 0 (B) Round 1 and (C) Round 2 libraries, as generated by the pLogo software (<http://plogo.uconn.edu>) of O'Shea *et al.* [21]



Supplementary Figure S3. Visualization of motifs of unique 15-mer sequences calculated against background probabilities for *E. coli* K12 in Round 2 library (A) all 15-mers (B) 40 most frequently occurring 15-mers and (C) 10 most frequently occurring 15-mers, as generated by the pLogo software (<http://plogo.uconn.edu>) of O'Shea *et al.* [21]



Supplementary Figure S4. Growth curves over 6 hours for the naive library, both sorting rounds, and the negative control after a 1:100 dilution from overnight cultures in fresh media.

| | | Valid Inserts | Frame Shift | Stop Codon | Empty Scaffold | Total # of Sequences |
|----------------|---------------|---------------|-------------|------------|----------------|----------------------|
| Round 0 | Total | 466,476 | 331,377 | 255,658 | 158,791 | 1,212,302 |
| | Unique | 396,641 | 313,601 | 224,685 | --- | --- |
| Round 1 | Total | 591,400 | 350,783 | 299,598 | 176,237 | 1,418,018 |
| | Unique | 67,885 | 208,322 | 42,017 | --- | --- |
| Round 2 | Total | 770,853 | 279,786 | 201,774 | 152,954 | 1,405,367 |
| | Unique | 21,931 | 133,599 | 7,966 | --- | --- |

Supplementary Table S1. Raw count of total and unique sequences for all sequence categories of each library.

| | | 0 cysteines | 1 cysteine | 2 cysteines | 3 cysteines | 4 cysteines |
|----------------|------------------|--------------------|-------------------|--------------------|--------------------|--------------------|
| Round 0 | raw count | 252165 | 116024 | 24846 | 3275 | 309 |
| | decimal | 0.635751 | 0.292516 | 0.062641 | 0.008257 | 0.000779 |
| Round 1 | raw count | 42299 | 20649 | 4305 | 564 | 65 |
| | decimal | 0.623098 | 0.304176 | 0.063416 | 0.008308 | 0.000958 |
| Round 2 | raw count | 12344 | 7809 | 1497 | 263 | 18 |
| | decimal | 0.562856 | 0.356071 | 0.06826 | 0.011992 | 0.000821 |

Supplementary Table S2. Raw count and decimal of total sequences in library containing 0-4 cysteines, round by round.