***Supplemental figures***

**Figure S1.** ***Analysis of proteins produced for TR-FRET assay.*** Coomasie-stained SDS-PAGE analyses of recombinant proteins are shown in top panel. Gel images are shown of purified proteins collected as fractions from size-exclusion chromatography analysis for (A) His-UBC13, ~ 17 kDa (B) His-MMS2, ~ 19 kDa and (C) His-UEV1A, ~ 26 kDa. Peak elution volumes from size-exclusion chromatography experiments are shown in the bottom panel for (D) His-UBC13, ~ 17.25 mL and (E) His-UBC13-His-UEV1A heterodimer, ~ 16.25 mL.

**Figure S2.** ***Characterization of ubiquitin chain topoplogy for UBC13-UEV1A mediated ubiquitination reactions by NanoLC-LTQ mass spectrometry.*** Tryptic digested peptides from 15% SDS-PAGE gels containing ubiquitin chains in enriched reactions were subjected to NanoLC-LTQ mass spectrometry analysis. The peptide tandem spectra (MS2 fragmentations) confirmed GG tag on lysine 63 with good 'b' and 'y' ion coverage. Representative spectrum depicts the characteristic mass increment of +242.14 Da (accounting for the diglycine moiety, +114.1 Da, covalently attached to lysine at position 63, +128.17 Da, of ubiquitin) and fragment ions corresponding to peptide sequence TLSDYNIQK63[242.14]ESTLHLVLR (protein ID: IPI00179330 and IPI00787573), confirming the presence of K63-linked ubiquitin linkages.

**Figure S3.** ***Effects of temperature and time on TR-FRET-based ubiquitination assay using UBC13-UEV1A.*** Time-dependent ubiquitination reactions were performed

at 37℃. Reaction components are indicated. Data are represented as mean ± SEM (n=3). Note that signal:noise ratio remains acceptable for ~8 hr at RT but not at 37 ℃.

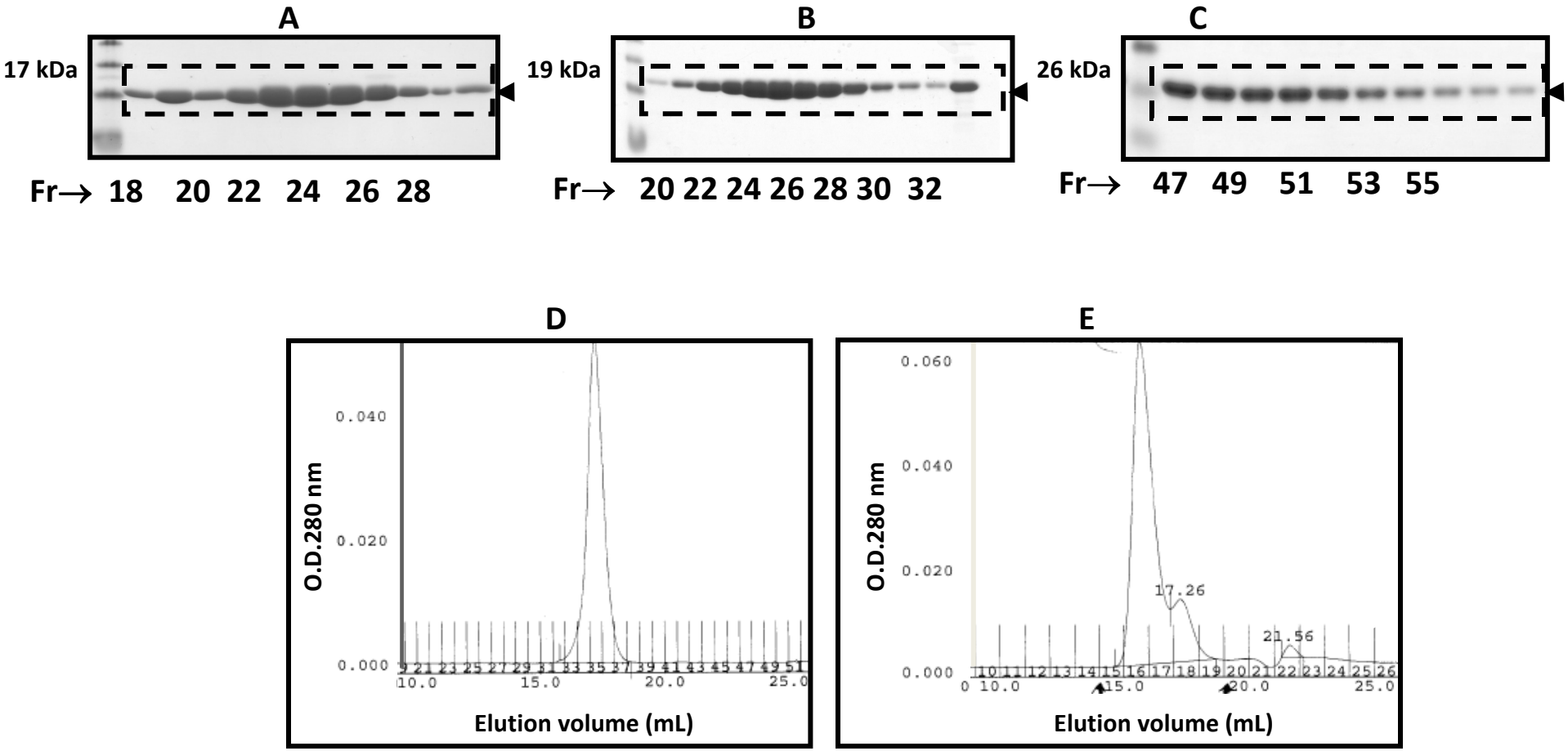
**Figure S4.** ***Z' factor determination of TR-FRET-based UBC13-UEV1A ubiquitination assay.*** Ubiquitination reactions were performed at 37 ºC and TR-FRET measurements taken after 1 hr incubation time point. Assay reproducibility and reliability assessed for complete reaction condition (shown in circles) versus reaction condition lacking UBC13-UEV1A (shown in triangles), yielded a Z' factor of 0.66. Data are represented as mean ± SEM (n=3).

**Figure S5.** ***Concentration-response curves of 'LOPAC Hits' obtained from TR-FRET-based UBC13-UEV1A ubiquitination HTS assay.*** Hits from LOPAC library screen were evaluated for their effectiveness in inhibiting ubiquitin chain formation by TR-FRET at 3 hours. Concentration required to inhibit half of the maximum response shown in the presence of complete reaction system ($IC_{50}$) was determined from log [concentration]-response curves constructed for each inhibitor. Data are expressed as mean ± SEM (n=3). Chemical structures and $IC_{50}$ values are shown for the three LOPAC hits represented as C1 (Tyrphostin AG 537), C2 (Tyrphostin AG 538), and C3 (MRS 2159), respectively.

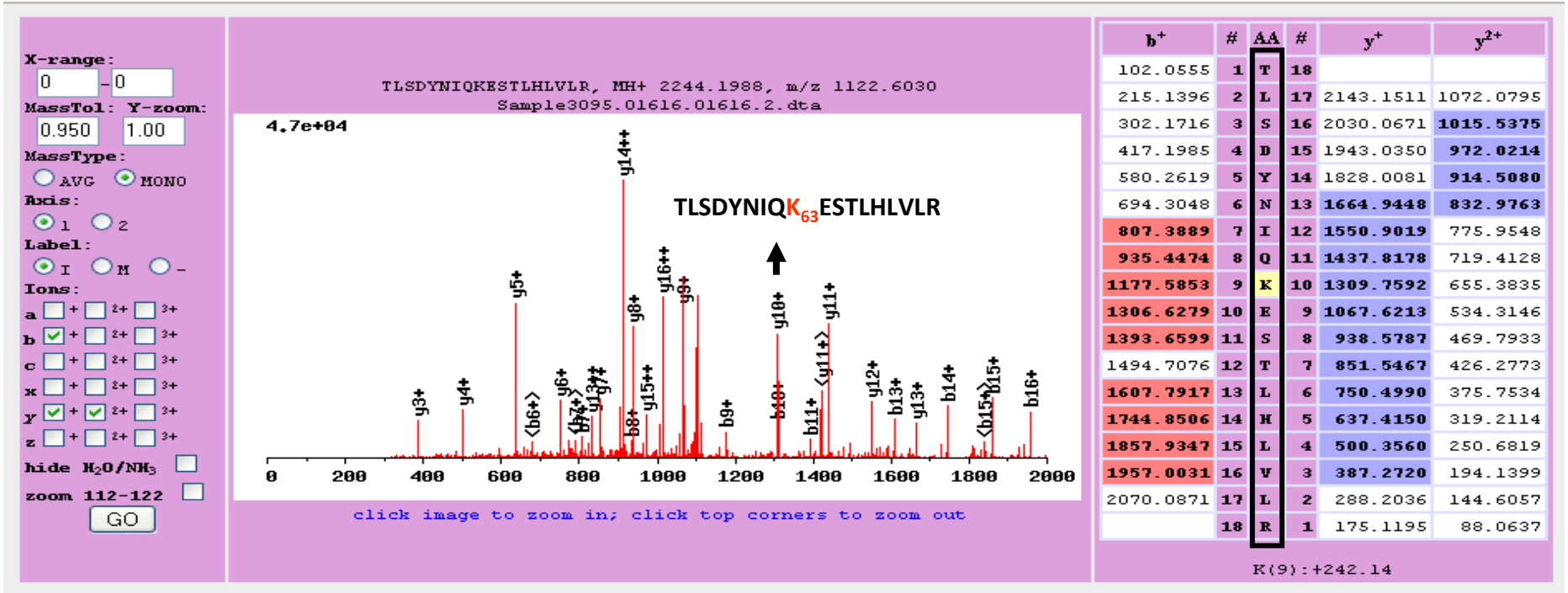
**Figure S6.** ***Miniaturization of TR-FRET assay in 1536-well format.*** ***(A)*** TR-FRET assay reaction volume was miniaturized during optimization procedure in 1536-well format. Low reaction volumes (2-6 uL) yielded optimal TR-FRET signal. ***(B)*** Z' factor

was monitored over a 3 hr time period. Z' factor was observed to be ~ 0.7 within 1 hr of assay incubation performed in 1536-well format and was stable for at least an additional 2 hr.

**Figure S7.** ***Miniaturization of TR-FRET assay in 1536-well plate.*** (A,C) TR-FRET assay performed under original optimized assay conditions in a 384-well plate (A) *versus* 1536-well format (C). (B,D) Evaluation of ubiquitination assay buffer modified with inclusion of 0.1% BSA instead of 0.005% Empigen BB as the detergent.




Supplemental Figure 1




| b+ | # | AA | # | y+ | y2+ |
|---|---|---|---|---|---|
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| 215.1396 | 2 | L | 17 | 2143.1511 | 1072.0795 |
| 302.1716 | 3 | S | 16 | 2030.0671 | 1015.5375 |
| 417.1985 | 4 | D | 15 | 1943.0350 | 972.0214 |
| 580.2619 | 5 | Y | 14 | 1828.0081 | 914.5080 |
| 694.3048 | 6 | N | 13 | 1664.9448 | 832.9763 |
| 807.3889 | 7 | I | 12 | 1550.9019 | 775.9548 |
| 935.4474 | 8 | Q | 11 | 1437.8178 | 719.4128 |
| 1177.5853 | 9 | K | 10 | 1309.7592 | 655.3835 |
| 1306.6279 | 10 | E | 9 | 1067.6213 | 534.3146 |
| 1393.6599 | 11 | S | 8 | 938.5787 | 469.7933 |
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| 1607.7917 | 13 | L | 6 | 750.4990 | 375.7534 |
| 1744.8506 | 14 | H | 5 | 637.4150 | 319.2114 |
| 1857.9347 | 15 | L | 4 | 500.3560 | 250.6819 |
| 1957.0031 | 16 | V | 3 | 387.2720 | 194.1399 |
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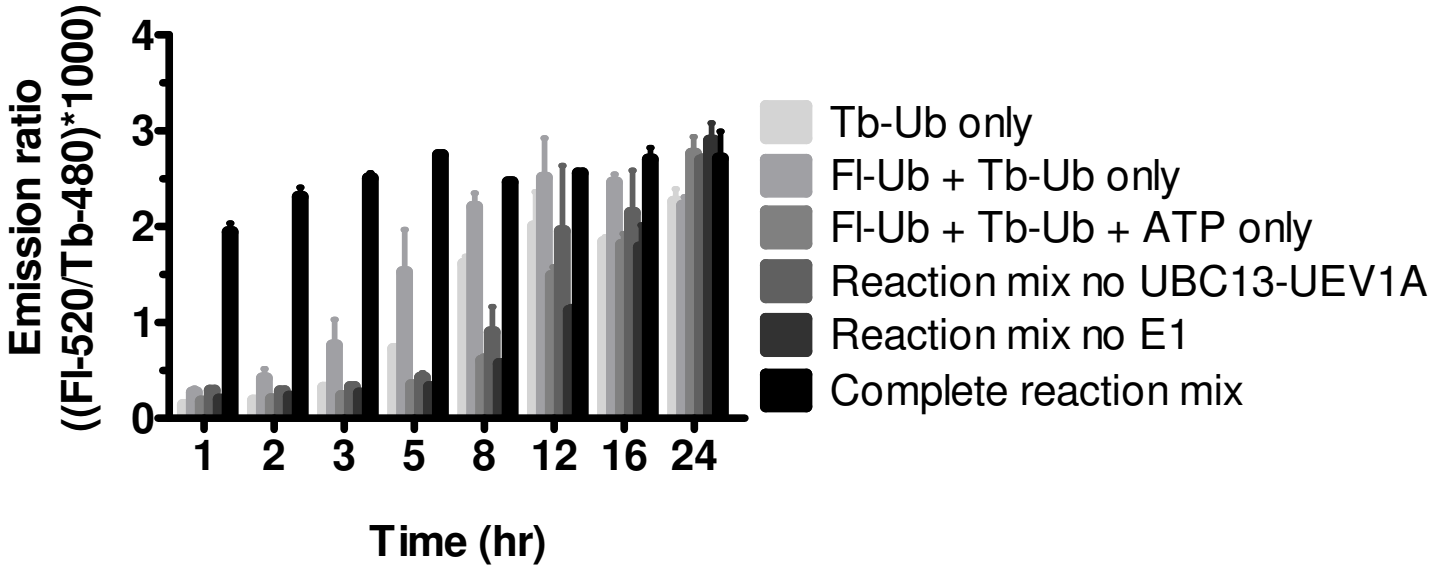
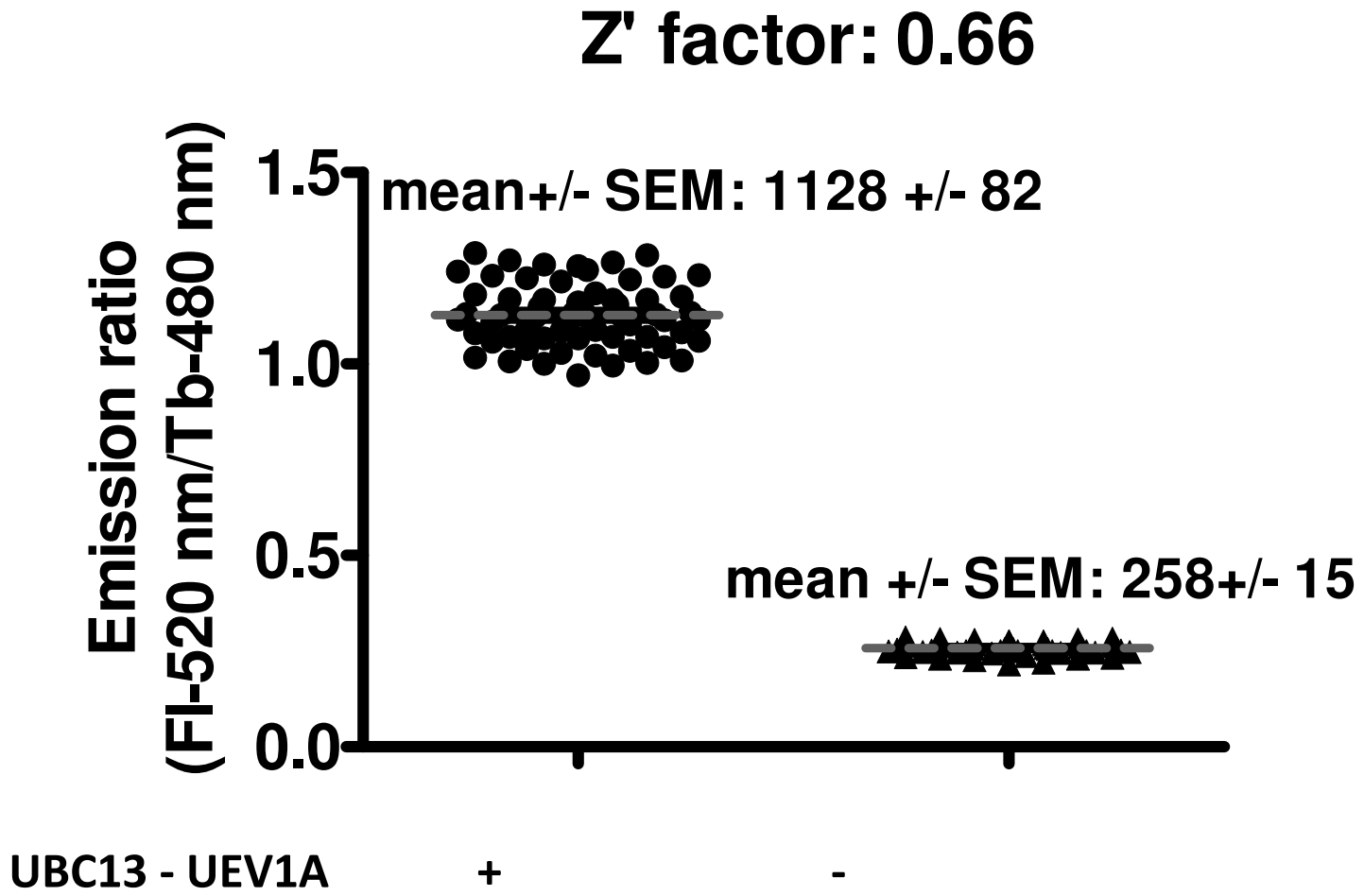
| | 18 | R | 1 | 175.1195 | 88.0637 |

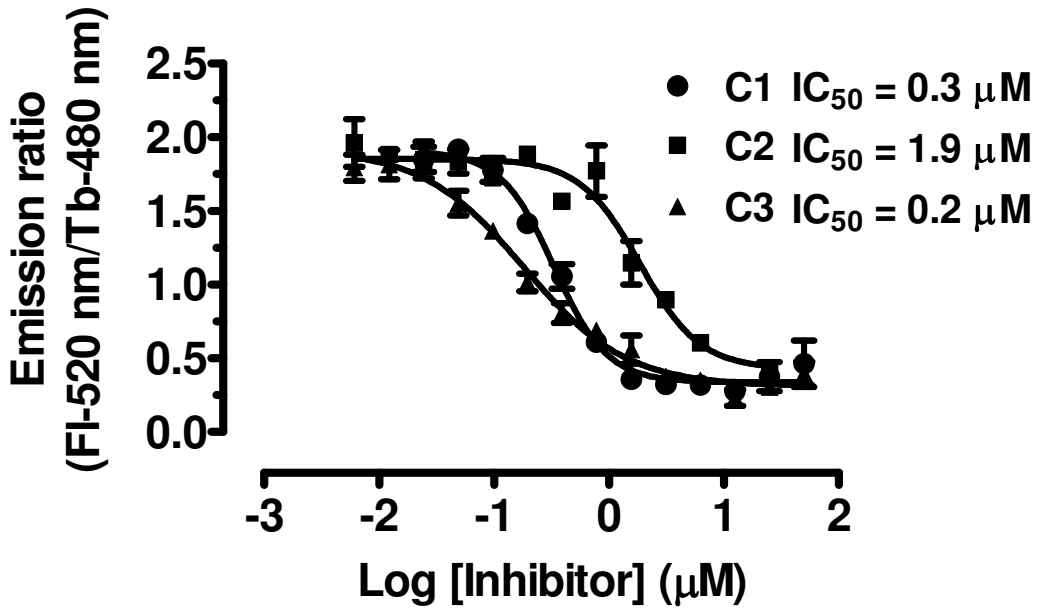
Supplemental Figure 2

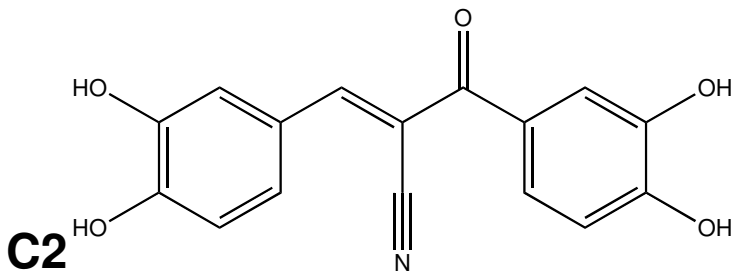


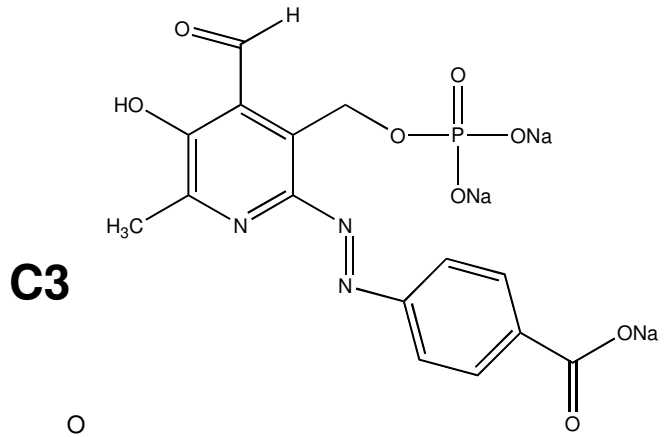

Supplemental Figure 3

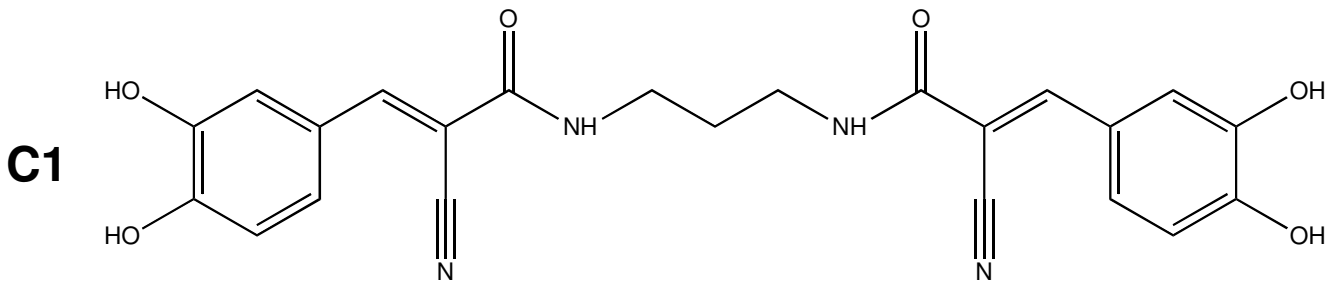



Supplemental Figure 4

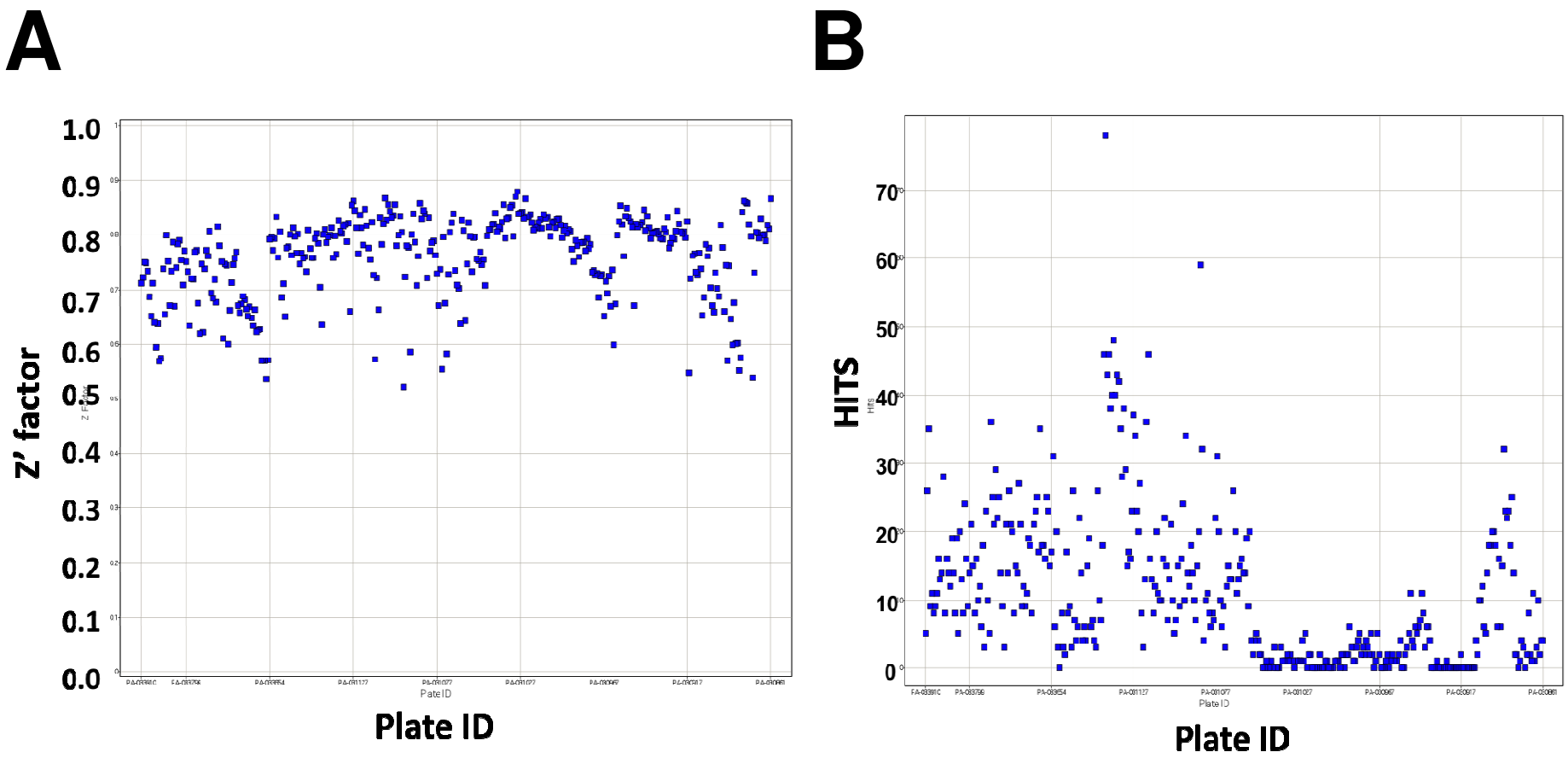


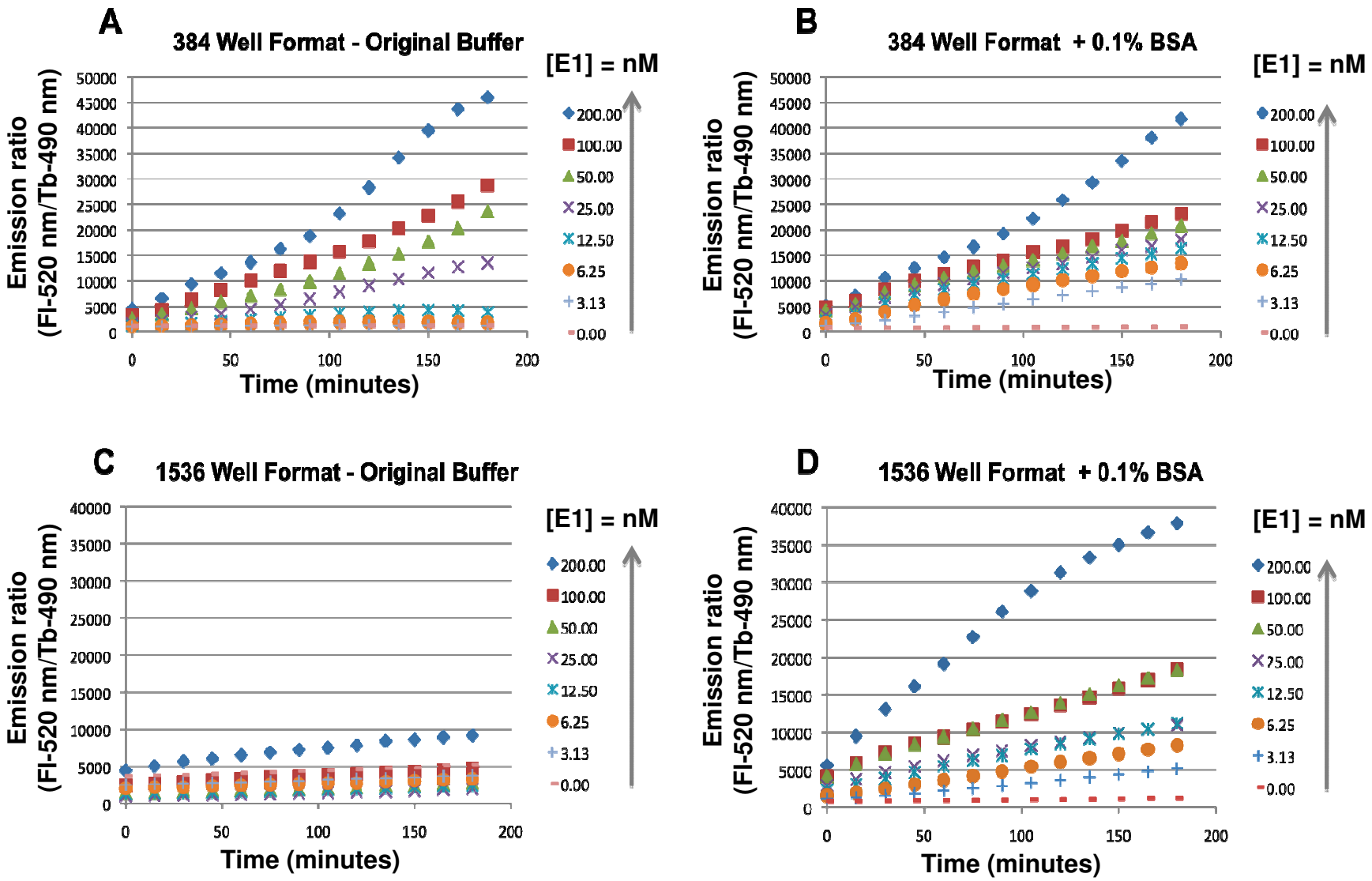




Supplemental Figure 5




Supplemental Figure 6




Supplemental Figure 7