**Supplementary information**

## Supplementary methods

### *Plasmid construction*

Streptavidin refers to core streptavidin (Sano *et al*, 1995) with $His_6$ at the C-terminus expressed from pET21a(+) (Howarth *et al*, 2006). The strong mutant was generated by introducing the S52G R53S mutation via QuikChange™ (Stratagene) using the following primer and its reverse complement: 5'-GTTGGTAACGCTGAAGGTAGCTACGTTCTGACCGGTCG. A86D streptavidin was generated in the same way with the primer 5'-GAAAAACAACTACCGTAACGATCACTCCGCTACCAC and its reverse complement. Then the weak mutant, A86D H87G, was generated from A86D streptavidin with the primer 5'- CTACCGTAACGATGGCTCCGCTACCACCTGG and its reverse complement. Such mutants with suitable off-rates were found via combined random and rational mutagenesis, based on previous mutations and screens of streptavidin (Aslan *et al*, 2005; Laitinen *et al*, 2006; Levy and Ellington, 2008). The mutations were confirmed by DNA sequencing.

### *Streptavidin expression and purification*

Streptavidin and its mutants were expressed as described (Howarth and Ting, 2008). Briefly, *E. coli* BL21 (DE3) RIPL (Stratagene) was grown to $OD_{600}$ 0.9 at 37 ºC, induced with IPTG, and incubated for a further 4 hr at 37 ºC. Inclusion bodies were isolated, dissolved in guanidinium hydrochloride, and refolded by rapid dilution into PBS. Soluble protein was then further purified by Ni-NTA and dialyzed into PBS. All versions of streptavidin showed good solubility and were entirely tetrameric (data not shown).

### *Off-rate assay*

The off-rate of biotin-4-fluorescein (B4F) from streptavidin or its strong or weak mutants was measured as described (Howarth *et al*, 2006). The binding of B4F to an excess of binding protein quenches fluorescein emission (Kada *et al*, 1999). As B4F dissociates at 37 ºC in the presence of excess free biotin, the fluorescence of B4F recovers.

## Supplementary references


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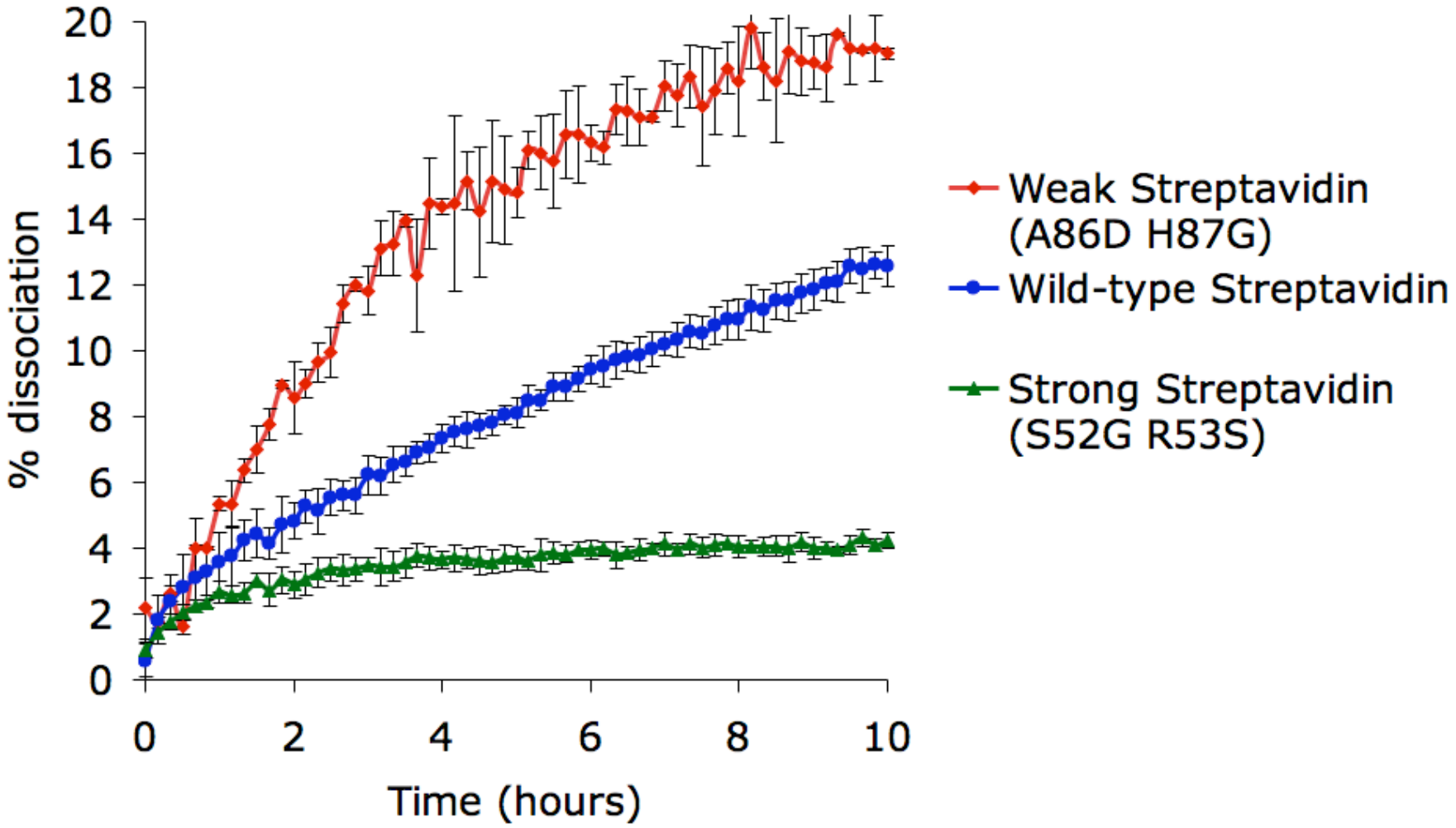
## Supplementary legends

### *Supplementary Figure 1*

Changed biotin-conjugate off-rate for weak and strong mutants of streptavidin. Wild-type streptavidin and weak (A86D H87G) or strong (S52G R53S) mutants were incubated with biotin-4-fluorescein, quenching its fluorescence. On addition of excess free biotin, biotin-4-fluorescein dissociated and the increase in fluorescence was observed at 37 ºC and pH 7.4. Mean of duplicate (weak mutant) or triplicate (WT and strong mutant) measurements ± 1 s.d.


% dissociation
20
18
16
14
12
10
8
6
4
2
0
0
2
4
6
8
10
Time (hours)
Weak Streptavidin
(A86D H87G)
Wild-type Streptavidin
Strong Streptavidin
(S52G R53S)