## Combinatorial indexing **Unique Dual Indexing** Nested unique dual indexing (prone to index misassignment) Always keep S index with the same R index Sample 1 Sample 1 Sample 1 R1 N1 Always keep N index with the same F index Sample 2 Sample 2 Sample 2 Sample 3 Sample 3 Sample 3 barcode Sample 4 Sample 4 Sample 4 barcode Sample 5 Sample 5 Sample 5 Sample 6 Sample 6 Sample 6 For 96 samples, need For 96 samples, need For 96 samples, need 12 forward + 8 reverse = 20 primers 96 forward + 96 reverse = 192 primers 12 F + 12 N + 8 R + 8 S = 40 primers B Nested Unique Dual Indexing IRA1-nonsense GPB2 Neutrals 1.0 1.0 Barcode Frequency Proportion of mapped reads to incorrect samples 8.0 8.0 10<sup>-4</sup> Fitness 6.0 0.6 10 Timepoint 0.4 43.2% 10<sup>-1</sup> Combinatorial Indexing 0.4 0.2 10 Barcode Frequency 0.2 0 0.0 0.9% 0.0 **Nested Unique Combinatorial** HiSeq X NextSeq **Dual Indexing** Indexing **Indexing Scheme** Timepoint E Mechanisms for index misassignment "Template Switching" "Index Hopping" **True Samples** Single Swap (inline) Free-floating indices get Homology across amplicons incorporated creates chimeric reads Double Template Switch Single Swap Swap Read out: combinations of 0.2 0.4 0.6 0.8 0.0 1.0 Read out: single-index swaps Proportion of Mapped Reads otherwise correct pairs