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Directed evolution using dCas9-targeted somatic hypermutation in mammalian cells

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Abstract

Engineering and study of protein function by directed evolution has been limited by the requirement to introduce DNA libraries of defined size or to use global mutagenesis. Here, we develop a strategy to repurpose the somatic hypermutation machinery used in antibody affinity maturation to efficiently perform protein engineering *in situ.* Using catalytically inactive Cas9 (dCas9) to recruit variants of the deaminase AID (CRISPR-X), we can specifically mutagenize endogenous targets with limited off-target damage. This generates diverse libraries of localized point mutations, in contrast to insertions and deletions created by active Cas9, and can be used to mutagenize multiple genomic locations simultaneously. With this technology, we mutagenize GFP and select for spectrum-shifted variants, including EGFP. In addition, we mutate the target of the cancer therapeutic bortezomib, PSMB5, and identify known and novel mutations that confer resistance to treatment. Finally, we utilize a hyperactive AID variant with dramatically increased activity to mutagenize endogenous loci both upstream and downstream of transcriptional start sites. These experiments illustrate a powerful new approach to create highly complex libraries of genetic variants in native context, which can be broadly applied to investigate and improve protein function.

Directed evolution employs successive rounds of mutation and selection to engineer biomolecules with enhanced, novel or non-natural functions, such as improved antibodies[1], more efficient enzymes[2], or mutant proteins with altered activity[3]. A major limitation to these experiments is the generation and maintenance of a diverse mutant population.

Author Contributions

The research was conceived by G.T.H. and M.C.B. G.T.H. conducted experiments with aid of A.L. L.F. performed sequencing data analyses with the aid of G.T.H. K.H. aided in the fluorescence microscopy. C.H.L. aided in the design of PSMB5 mutation validation experiments. K.A.C. aided in design of mutagenic approach. S.B.M. aided in developing analysis methods. G.T.H., L.F., and M.C.B. wrote the paper with help from all authors.

Radiation and chemically-induced DNA damage have been used to mutate the entire genome, but this requires maintaining a large number of cells since the majority of mutations are located outside the target of interest. Alternatively, diverse plasmid libraries can be introduced into cells; however, these proteins are often expressed at inappropriate levels and without normal regulation. Importantly, these libraries are of limited size, both in terms of total diversity and length of diversified region, which greatly restricts the potential for evolution experiments. Due to these limitations, the majority of these engineering experiments have been performed in bacteria, bacteriophage, and yeast due to the relative ease of generating diverse libraries in these organisms[4–6]. However, mammalian proteins engineered in these systems often change behavior in their native host environment. Hence, generating a diverse library of mutants in native context would have enormous advantages.

Nature has a built-in mechanism for generating diversity at a specific genetic locus, which is used with exquisite precision during the process of antibody maturation. After V-D-J recombination, B cells create point mutations in their immunoglobulin (Ig) regions through the process of somatic hypermutation (SHM) to perform affinity maturation on the antibody[7,8]. SHM is mediated by an enzyme called activation induced cytidine deaminase (AID), which deaminates cytosine (C) to a uracil (U) initiating a DNA repair response which causes errors in the Ig locus at a rate of 1/1000 bp^{-1} [9], compared to the normal rate of mutation during cellular DNA replication of $1/10^9$ bp^{-1} [10]. The process generates point mutations rather than insertions/deletions, and favors transition mutations (i.e. pyrimidine to pyrimidine or purine to purine) over transversions[8]. After deamination, mutations can be generated in three ways: the uracil-guanine (U-G) mismatch can be misread resulting in a (C>T) or (G>A) transition, the U can be removed by base excision repair and replaced by any base, or an error-prone translesion polymerase can be recruited through the mismatch repair pathway, generating transitions and transversions near the lesion[7].

Although sequence elements flanking the immunoglobulin locus have been linked to SHM targeting[11], the mechanisms by which SHM is regulated and targeted are not completely understood. It has been proposed that AID migrates with RNA polymerase II complex during transcription of the Ig locus and mutates specific hotspot sequence motifs[12,13]. Cell lines that misregulate or overexpress AID have the mutagenic capacity to evolve both fluorescent proteins[14,15] and antibodies[16], but these strategies create mutations throughout the genome.

With the recent advent of the CRISPR/Cas9 system, it has become possible to target functional proteins to specific genomic loci using catalytically dead Cas9 (dCas9). This approach has been used for both repression and activation of transcription[17–20] as well as targeting fluorescent proteins[21,22] and modifying enzymes[23–26], and most recently for the efficient conversion of C>T as a means of therapeutic targeted editing[23,26]. Here, we use dCas9 to target hyperactive AID to induce localized, diverse point mutations (CRISPR-X). This process differs markedly from mutagenesis using active Cas9[27], which predominantly generates insertions and deletions[28–30], or the introduction of mutations via externally generated oligonucleotide donor libraries by homologous recombination following Cas9 cleavage[31,32]. We show that AID-induced mutations can be generated in cells that express AID constitutively or transiently via electroporation, and can be targeted to multiple loci in

the same cell. Furthermore, we show two proof of principle examples of protein engineering using CRISPR-X: the alteration of the absorption/emission spectrum of genomically integrated wild-type GFP, and the evolution of variants of PSMB5 that are resistant to bortezomib, a widely used chemotherapeutic drug. In the latter example, we not only generate mutations that have previously been observed in resistant cell lines, but also identify novel drug-resistant mutants that may reveal new properties of PSMB5 and its interaction with bortezomib. Finally, we demonstrate that a hyperactive AID enzyme introduces mutations at a higher rate and can generate variants in both protein coding regions as well as regulatory regions upstream of the transcription start site. Together, this work illustrates the potential of a novel targeted mutagenesis strategy for the engineering and evolution of new protein function in normal cellular context.

Results

Targeted mutagenesis through dCas9 recruitment of AID

In order to recruit the AID protein to a genetic locus, we used dCas9[30] combined with a single guide RNA (sgRNA) bearing an MS2 hairpin binding site (Fig. 1a) that has been previously used to recruit MS2 fused effector proteins to activate transcription[20]. In this system, the sgRNA contains two MS2 hairpins that each recruit two MS2 proteins (four in total) fused to AID. For our initial test, we generated MS2 fused to three AID variants (Supplementary Data Fig. 1a): wild-type AID, a truncated version without the last three amino acids (AIDΔ) which ablates its nuclear export signal (NES) while increasing SHM activity[33], and a catalytically inactive truncated version (AIDΔDead)[34]. The deletion of the NES resulted in primarily nuclear localization of the MS2 fusion protein as observed by immunofluorescence staining in K562 cells (Supplementary Data Fig. 1b), with minimal change in protein expression (Supplementary Data Fig. 1c).

We generated K562 cells stably expressing dCas9 along with GFP and mCherry, which, when used together with sgRNAs targeting GFP, serve as phenotypic readout for on-target (GFP) and off-target mutations (mCherry). These cells were transiently electroporated with plasmids coding for either a GFP-targeting sgRNA (sgGFP.1) or a scrambled non-targeting sgRNA (sgNegCtrl) paired with plasmids coding for MS2-AID, MS2-AIDΔ, or MS2-AIDΔDead. After 10 days, cells were analyzed by flow cytometry to measure GFP and mCherry fluorescence (Supplementary Data Fig. 1d). As expected for on-target mutation resulting in non-fluorescent protein, we observed an increase in the GFP negative population for MS2-AIDΔ treatment when comparing sgGFP.1 to sgNegCtrl (1.64% vs. 0.55%). However, we did not see this effect with MS2-AID (0.71% vs. 0.78%). At the same time, the mCherry negative population showed little change (1.02% vs. 0.91%), indicating that targeting AIDΔ to GFP resulted in specific mutagenesis. Additional fluorescence measurements made beyond 10 days did not change, suggesting mutation had stabilized following electroporation (data not shown).

Based on the observed change in fluorescence, we performed a more detailed analysis of the population by sequencing the locus. To quantify mutations in the GFP negative population, we collected the GFP low population from the AIDΔ;sgGFP.1, AΓDΔ;sgNegCtrl, and AIDΔ-Dead:sgGFP.1 samples via FACS and sequenced the GFP locus. Enrichment of

mutations was calculated by comparing collected samples to parental cells that had not been exposed to a mutagenic agent. We observed enrichment of mutations only in the AIDΔ:sgGFP.1 (Supplementary Data Fig. 1e). The most enriched position for mutations was base pair 280 which had over 500 fold enrichment in mutations and 41.2% of sequences at that base showed a G>A transition. This transition resulted in the introduction of a tyrosine in place of cysteine in GFP at amino acid 48. Reduced fluorescence of GFP due to this alteration is consistent with previous work showing that cysteine thiol binding by dTNB quenches GFP fluorescence[35].

Given the superior performance of AIDΔ, we continued with this variant. To more accurately estimate the mutation rate without considering transient electroporation efficiency, we integrated the CRISPR-X system into our reporter cells. MS2-AIDΔ or MS2-AIDΔDead were stably integrated in cells together with sgGFP.1 or sgNegCtrl, and GFP and mCherry negative populations were monitored 14 days after infection (Supplementary Data Fig. 2a). As before, in the presence of MS2-AIDΔ, we observed an increase in the GFP negative population (1.88%) when compared to either the sgNegCtrl (0.75%) or MS2-AIDΔDead (0.47%). By contrast, the mCherry low population was minimally changed (0.67% MS2-AIDΔ:sgGFP.1, 0.34% MS2-AIDΔ:sgNegCtrl, 0.43% MS2-AIDΔDead:sgGFP.1) (Supplementary Data Fig. 2a). We sequenced both GFP and mCherry loci from these cells (Fig. 1b and Supplementary Data Fig. 2b), and observed an enrichment of mutations in the 270–290bp region of GFP only in cells expressing MS2-AIDΔ:sgGFP.1. We did not detect any enrichment of mutations in the mCherry locus.

Defining the region of mutagenesis for CRISPR-X

To determine the region of mutagenesis with respect to the sgRNA, we selected an additional 11 sgRNAs (sgGFP.2–12) tiling the GFP locus on both strands (Fig. 1c and Supplementary Data Fig. 3a). Since AID mutagenesis has been shown to require transcription[12], we hypothesized the strand of the guide relative to the direction of transcription may change the targeting of mutations. We sequenced the GFP locus in each of these samples and mapped the mutations relative to the end of the PAM sequence of each sgRNA (Fig. 1c). While different sgRNAs exhibited a range of mutation efficiencies (Supplementary Data Fig. 3b), we observed a mutational hotspot region from +12 to +32 bp downstream of the PAM relative to the direction of transcription that was independent of the strand targeting (Fig. 1c). The mutational hotspot was defined to include any base with at least 10-fold increased mutation over all three biological replicates for a given sgRNA. Mutations in this region were measured for the 12 sgGFP guides, and a median mutation frequency of 0.0104 was observed (Supplementary Data Fig. 3c). This translates to a mutation rate of ~1/2000 bp^{-1}, which is similar to that observed for somatic hypermutation[8], and is an order of magnitude higher than the observed frequency of 0.0014 for a negative control sgRNA (MS2-AIDΔ:sgNegCtrl) and 0.0015 for catalytically inactive AID (MS2-AIDΔDead:sgGFP.1). Given the ability of this system to generate targeted point mutations, we sought to apply it for directed evolution experiments.

Evolution of wtGFP to EGFP using CRISPR-X

As an initial proof of principle experiment, we tested whether we could alter an integrated copy of wild-type GFP (wtGFP) from *Aequorea victoria* (excitation 395nm/emission 509nm) to EGFP (490/509nm)[36]. EGFP has two mutated residues from wtGFP: S65T, which shifts the ex/em spectrum, and F64L, which improves the folding kinetics of GFP[36–38]. We designed four guides (sgwtGFP.1–4) that target this region and introduced them via electroporation along with MS2-AIDΔ into K562 cells expressing dCas9 and wtGFP. As a negative control, we also electroporated four 'safe harbor' sgRNAs that target regions of the genome that are annotated as non-functional. Cells were grown for 10 days to allow for mutations to be introduced, and then sorted by FACS to collect cells expressing spectrum-shifted GFP (Fig. 2a). In biological replicate experiments, we observed a population with decreased signal in the Pacific blue channel and increased GFP signal (0.076% replicate 1, 0.025% replicate 2) (Fig. 2b and Supplementary Data Fig. 4a), which was not observed in the safe harbor samples (0.002%, 0.002%) (Fig. 2b and Supplementary Data Fig. 4a). After another round of sorting, the safe harbor samples did not have any cells pass the sorting gates, while the spectrum-shifted population had increased to 2.29% and 1.16% in the GFP-targeted replicates.

The GFP locus was sequenced to identify mutations enriched by the sorting process (Fig. 2b and Supplementary Data Fig. 4a), revealing enrichment of mutations at positions 331 (G>C) and 377 (G>C). The former mutation introduces the known S65T mutation from EGFP (Fig. 2b). The latter mutation generated a Q80H substitution, which we suspected was a passenger mutation since the majority of sequences containing the mutation also showed the S65T transition. In order to determine the contribution of each individual mutation to changes in GFP fluorescence, we introduced each mutation into GFP separately, and confirmed that the S65T mutation alters the fluorescence spectrum of GFP while Q80H does not, either alone or in conjunction with S65T (Fig. 2c). We did not observe the F64L mutation in our selection, which was shown in an inducible expression system to affect protein stability[36], but did not change fluorescence intensity under constitutive expression when coupled with the S65T mutation (Fig. 2c). We performed a similar selection experiment with the integrated CRISPR-X system and a single integrated guide (sgwtGFP.1 or sgSafe.2) and recovered the same S65T transition but did not observe the Q80H mutation (Supplementary Data Fig. 4b).

Identification of bortezomib-resistant PSMB5 variants

Another potential application of CRISPR-X is the investigation of mechanisms of drug resistance. Mutations are a common escape pathway for cancer cells to develop resistance to drug treatment[39], and understanding which mutations can arise is important for the design of new drugs or drug combinations. To test this, we mutagenized PSMB5, a core subunit of the 20S proteasome, which is the target of the proteasome inhibitor bortezomib[40]. We generated a library of 143 guides tiling all coding exons of PSMB5, as well as a control library of 705 safe harbor guides (Extended Dataset 2). Both libraries were lentivirally integrated into K562 cells expressing dCas9 and MS2-AIDΔ, given 14 days to develop mutations, and pulsed with bortezomib three times (Fig. 3a). After selection, genomic DNA was extracted, the PSMB5 exonic loci of both libraries were sequenced, and variant frequencies were

quantitated at each base (Fig. 3b–c and Supplementary Data Fig. 5a–b). The screen was performed in biological replicate, and mutants were selected for further analysis that showed enrichment of at least 20 fold in both replicates (Fig. 3c and Supplementary Data Fig. 5b). We identified 11 such mutations (Fig. 3d), including two (A108T/V) altering a residue known to be involved in binding bortezomib[41]. Novel mutations were identified near a threonine (residue 80) that also binds bortezomib (A74V, R78M/N, A79T/G, and G82D). We suspect these mutations disrupt the position of the threonine, destroying the binding pocket for bortezomib. Beyond mutations expected to affect the binding pocket, we identified two mutations in Exon 1 (L11L, G45G), an intronic mutation before Exon 2, and a mutation in Exon 4 (G242D) that is located on the side of the protein distal to the bortezomib binding pocket. No resistant mutations were identified in Exon 3, an alternate exon that is not expressed in K562 cells (data not shown). In the safe harbor control library, we identified one mutation (A79T) that was also found with the PSMB5 targeted library, and was likely present at undetectable levels in the parent K562 population (Fig. 3c and Supplementary Data Fig. 5b).

We chose to functionally validate 8 of these mutations by knocking each one into the genome separately at the native PSMB5 locus using active Cas9 cutting followed by HDR mediated by a DNA donor oligo[28,29]. To control for the effect of Cas9 cutting and HDR, we knocked in a synonymous mutation not identified in our screen in each exon. We electroporated Cas9 expressing K562 cells with donor oligo and sgRNA and waited for six days followed by subsequent selection with bortezomib. After 14 days, the viability of the cells was measured (Fig. 3e). Five of the mutations (R78N, A79G, A79T, A108V, and G242D) were strongly protective against bortezomib-induced cell death, while the other three (L11L, Intronic, and G82D) showed more modest protection when compared to controls. For the most resistant mutations, the PSMB5 locus was sequenced following bortezomib selection and the presence of the expected mutation was verified in the majority of non-frameshifted sequences (Supplementary Data Fig. 6). Together, these experiments show that CRISPR-X can be used to selectively mutagenize an endogenously expressed protein target, identifying known and novel mutants that confer drug resistance.

Enhanced mutagenesis using a hyperactive AID mutant

We observed variable mutation efficiency with AIDΔ, and therefore investigated whether this could be improved even further with AID variants previously shown to have increased SHM activity[42]. We selected one of the strongest mutants (AID*) and removed its NES as we had with wild-type AID (Supplementary Data Fig. 1a). AID*Δ was integrated along with one of three sgRNAs (sgGFP.3, sgGFP.10, and sgSafe.2), and the enrichment of mutation in GFP and mCherry loci was measured (Fig. 4a and Supplementary Data Fig. 7a). Despite lower expression of the protein (Supplementary Data Fig. 1c), we observed an approximate 10-fold increase in mutation at the most enriched base position for GFP-targeting sgRNAs when compared with AIDΔ, with no noticeable increase in mCherry off-target mutation (Fig. 4b). sgSafe.2 samples did not show mutation at either locus. We aligned these mutations relative to the PAM and observed an increase in the size of the hotspot to span from −50 to +50 bp (Fig. 4b). Within the hotspot window, we still observe the most highly mutated bases are located within the +12 to +32 region. This suggests that the targeting

pattern of AID*Δ has not changed compared to AIDΔ, but that the increased activity of AID*Δ allows for detection of mutation over a larger window. Within this region, we observed a substantial increase in mutation rate (2.25 fold for sgGFP.3 and 6.52 fold for sgGFP.10) reaching over 20% of reads for sgGFP.10 (Fig. 4b), as well as a modest increase in sequences that contained multiple mutations per read (1.32 mutations/read for AID*Δ vs. 1.07 for AIDΔ, Supplementary Data Fig. S7b). Given that we can measure mutations in up to 10– 20% of sequences (which is ~ 1 mutation per 500–1000 bp within a hotspot), we estimate that CRISPR-X is capable of mutagenesis on par with somatic hypermutation ($1/1000\ bp^{-1}$)[9].

To further explore the capacity of AID*Δ-induced mutagenesis, we targeted three classes of endogenous loci: protein coding genes, promoter regions, and safe-harbor regions. For the protein coding genes, we targeted five sgRNAs to 3 highly expressed genes, FTL, HBG2, and GSTP1, sequenced the respective loci, and quantitated mutation enrichment (Fig. 4c). Additionally, we quantified the frequency of mutations at each base position relative to the PAM site (Supplementary Data Fig. 8a and Fig. 4d). We observed mutated bases in each of the three genes with similar targeting in the −50 to +50 hotspot relative to the sgRNA PAM. To determine whether we could mutagenize genes with more moderate expression levels, as well as associated promoter regions, we targeted PTPRC, CD274, and CD14. For each gene, we targeted both the transcribed region as well as sequences upstream of the transcription start site (TSS). For each locus, we observed mutated bases for sgRNAs located both upstream and downstream of the TSS (Fig. 4c and Supplementary Data Fig. 8a). For CD274, we observe mutations targeted up to 3.2kb upstream of the TSS, suggesting some types of non-transcribed regions can be investigated using CRISPR-X. Lastly, we tested sgRNAs targeting four safe harbor regions (non-functional genomic regions) but we did not observe mutations in these samples (data not shown), although we cannot rule out that this was because of ineffective sgRNA choice or other factors.

We compared the mutation types observed for both AIDΔ and AID*Δ within their respective hotspots (Fig. 4b,d). The mutation rates were normalized by alternative allele frequencies observed in the parental samples within targeted hotspot regions. In addition, we calculated the standard deviation of the alternative allele frequency in the parent samples when compared to reference sequence ($5.68 \cdot 10^{-4}$ for AIDΔ and $3.74 \cdot 10^{-4}$ for AID*Δ), and used this as a noise threshold for the transition/transversion frequencies. For both AID variants, we observe a preference for G>A and C>T transitions with the most highly mutated bases being G or C (Fig. 4d), consistent with the preference of AID deaminase activity. Importantly, we find a significant increase in mutation frequencies for all possible base changes except A>T for the AID*Δ treated samples. For both variants, low levels of insertions (maximum frequency of $1.98 \cdot 10^{-3}$ for AID*Δ and $7.44 \cdot 10^{-4}$ for AIDΔ) and deletions (maximum frequency of $5.15 \cdot 10^{-4}$, $3.01 \cdot 10^{-4}$) are observed, suggesting that mutation induced frame shifts are rare. Thus, the increased activity of AID*Δ can greatly expand the sequence space that can be mutagenized by a single sgRNA, including both coding and promoter regions of genes.

To quantitate the rate of mutation for AID*Δ over a range of sgRNAs, we tabulated mutation frequencies for each sgRNA over their respective 100bp hotspots. Consistent with previous

observations for Cas9, certain sgRNAs did not show activity and we thus removed these (4/34 sgRNAs). To consider an sgRNA, we required that each replicate contained a base position in the hotspot that was enriched at least 10-fold over the parent population. For the remaining 30 sgRNAs, we calculated the percent of reads containing a mutation, and found that the median frequency was 0.0163, with ~25% of sgRNAs giving a frequency of >0.05 and up to 0.22 (Supplementary Data Fig. 8b).

To estimate the range of mutations that can be sampled using CRISPR-X in a population of cells, we quantified the diversity generated by AID*Δ. Using data from the sgRNAs targeting GFP and the endogenous loci (Fig. 4b–c), we scanned across the region next to the PAM with a 21bp window, which was the size of the AIDΔ hotspot. In each window, we calculated the percentage of all 63 possible single base variants (21 bases and 3 possible changes at each position) measured above the noise threshold in the population (Fig. 4e). While the efficiency of mutation varied with different sgRNAs, a window spanning from +20 to +40 from the PAM displayed the highest median percentage of possible variants (20.6%), and we observe up to 77.8% of all possible transitions in some cases. The +20 to +40 window is similar to the observed targeting hotspot for AIDΔ (Fig. 4b), suggesting that this region is the most highly mutagenized for both AIDΔ and AID*Δ.

Simultaneous mutation of multiple loci using CRISPR-X

Independent mutagenesis at multiple locations is typically not possible with traditional directed evolution experiments. However, the CRISPR/Cas9 system can target multiple loci using different sgRNAs[28,29]. We incorporated two guides, one targeting GFP (sgGFP.10) and the other targeting mCherry (sgmCherry.1), both individually and in combination. We measured GFP and mCherry fluorescence and observed ~15% GFP or mCherry low populations for each sgRNA individually (Supplementary Data Fig. 9), thereby demonstrating that these sgRNAs were effective in generating mutations that ablated fluorescence. Upon the addition of both sgRNAs, we observed a slight decrease in mutation of GFP or mCherry separately (~12%) perhaps due to sharing of CRISPR-X machinery, but an increase in cells with mutations at both loci to 1.92% compared to 0.26% or 0.30% for cells with either sgGFP.10 or sgmCherry.1 incorporated individually. These results demonstrate that CRISPR-X can be used to simultaneously mutagenize two sites within the same cell, suggesting that co-evolution of two genomic loci should be possible.

Discussion

Here we demonstrate that hyperactive AID targeted with dCas9 can be used to generate localized sequence diversity within the mammalian genome at a rate comparable to somatic hypermutation, and that these mutagenized populations can be subjected to selection to evolve new protein function. This system, CRISPR-X, can simultaneously mutagenize multiple genomic loci, and preserves reading frame by avoiding insertions/deletions observed with active Cas9. While the activity of AID in antibody maturation has been shown to require transcription[12], we observed mutations above background for sgRNAs targeting both upstream and downstream of the TSS. Although regions upstream of the TSS may be transcribed at lower levels, these findings suggest that CRISPR-X is not bound to regions

downstream of annotated transcription start sites and could allow for the engineering and investigation of promoters, enhancers, and other regulatory elements.

Using CRISPR-X, we highlight several examples of directed evolution. First, we show that GFP can be readily evolved to EGFP with the simple electroporation of an appropriately designed sgRNA and targeted AID. In addition, we demonstrate that mutagenesis of the target of the chemotherapeutic bortezomib (PSMB5) could reveal both known and novel mechanisms of resistance. In this experiment, we find the canonical A108V/T mutation, which was identified in bortezomib resistant cell lines[41,43] and observed in colorectal cancer patient samples[44], along with many others that are consistent with the disruption of the binding pocket of bortezomib. Interestingly, we uncover a mutation located in Exon 4 (G242D), which had not been previously connected to bortezomib resistance, and is located on the side of the protein opposite the bortezomib pocket (Fig. 3d). This could suggest additional mechanisms of resistance, and may inform study of PSMB5 function as well as future drug design. Additionally, we identified synonymous and intronic mutations which require further study.

CRISPR-X represents an efficient strategy to create a diverse library of point mutations *in situ*, which expands the repertoire of methods for genome engineering using Cas9. Mutagenesis using active Cas9 has been effective for inducing insertions and deletions, which can disrupt functional elements[27,45,46], and inactivate protein function[28–30,47]. During the preparation of our manuscript, two elegant studies by Komor et al. and Nishida et al. demonstrated that dCas9 can be used to recruit deaminases for the remarkably precise conversion of C > T within a 5 bp window, as a way to correct single base changes observed in disease[23,26]. Here, we show that a hyperactive AID variant can create dense, highly variable point mutations within a region of 100bp surrounding an sgRNA target site at a rate of up to ~1/500–1/1000 bp^{-1} (Fig. 4b), compared to the normal mutation rate during cellular DNA replication of ~$1/10^9$ bp^{-1} [10]. As in antibody somatic hypermutation, we observe a large variety of transitions and transversions from C and G bases to all possible bases (rather than just C>T and G>A described in previous studies), and a low level of all base changes (Fig. 4d). Using this diverse population of mutants, we demonstrate that we are able to select for the evolution of new function.

CRISPR-X presents a number of significant advantages over existing methods used to engineer proteins or introduce diversity, which make it a highly complementary strategy for genome engineering. Previous work has demonstrated a powerful strategy by which active Cas9 can be used to introduce mutant oligonucleotide donor libraries by homologous recombination; the resulting cell populations can be used to study RNA and protein function in mammalian cells[32] or select for improved fermentation in yeast[31]. However, this strategy requires the separate synthesis of a mutant donor library for each engineered site. In contrast, CRISPR-X repurposes the somatic hypermutation machinery, making it possible to generate a library of point mutations *in situ* using a single sgRNA, and even greater diversity through multiplexing. In addition, the targeting of AID should allow continuous mutagenesis and evolution of protein function as is observed in antibody affinity maturation, as opposed to introducing a synthetic library of defined size.

Previous efforts to use AID for mutagenesis used overexpression of both AID and the target protein. In those studies, the target is present at non-physiological levels, and cells have significant genome instability and potentially confounding off-target mutations due to promiscuous AID activity[48,49]. While elegant work has been done to understand the targeting of somatic hypermutation to the Ig locus[11,50], the known control elements would be difficult to install systematically throughout the genome. CRISPR-X overcomes both of these limitations by using dCas9 to target somatic hypermutation, which should facilitate both engineering of new biomolecules as well as an understanding of the SHM process itself. Importantly, the ability to introduce the CRISPR-X system by electroporation, use multiple sgRNAs, and potentially induce repeated rounds of mutagenesis should allow exploration of a virtually limitless sequence space, since combinations of mutations observed with single sgRNAs can be multiplied by simultaneously targeting multiple genomic locations. We envision that this system should make it possible to study the co-evolution of two interacting proteins expressed at endogenous levels, and could provide a streamlined strategy for selection of enhanced antibody and enzyme function via mutagenesis in native context.

Methods

Design and construction of CRISPR-X and fluorescent protein plasniids

A list of the plasmids and primers used are listed in Extended Dataset 1. Lenti dCAS-VP64_Blast, lenti MS2-P65-HSF1_Hygro, and lenti sgRNA(MS2)_zeo backbone were a gift from Feng Zhang (Addgene plasmids #61425–61427). The VP64 effector was removed from the dCas9 construct by digesting with BamHI and EcoRI followed by Gibson assembly to re-insert PCR amplified blasticidin resistance marker (pGH125). For the MS2 fusions, the P65-HSF1 was removed using restriction digest with BamHI and BsrGI. AID (pGH156) and AIDΔ (pGH153) were PCR amplified from a FLAG-AID expressing plasmid, courtesy of the Cimprich Lab, and Gibson assembled into the digested vector. Catalytically inactive (pGH183) and hyperactive mutants (pGH335) were generated using PCR primers containing the desired mutations. Subunits of AID were amplified using those primers and then joined using overlapping PCR. The mutant AID PCR product was Gibson assembled into the digested MS2 expression vector. GFP, mCherry, and wtGFP expressing plasmids driven by an Ef1α promoter were generated using pMCB246 which was digested with NheI and XbaI, removing a puromycin resistance-T2A-mCherry cassette. GFP (pGH045) and mCherry (pGH044) were PCR amplified and inserted into the digested vector using Gibson assembly. Variants of GFP (wtGFP (pGH220) and identified mutants (pGH311-S65T, pGH312-Q80H, pGH314-S65T + Q80H) were constructed using the previously described overlapping PCR method followed by Gibson assembly. Plasmids maps of these constructs are available upon request. For dual guide experiments, a second sgRNA expressing plasmid was constructed by removing the zeocin resistance (digestion of lenti sgRNA(MS2)_zeo with BsrGI and EcoRI) and replaced with puromycin resistance with a removed BsmBI cut site by Gibson assembly (pGH224). sgRNA vectors were generated by digesting the either lenti sgRNA(MS2)_zeo or pGH224 with BsmBI. Oligonucleotides with overhangs compatible with subsequent ligation were designed and annealed followed by ligation into the digested vector. The sequence for the sgRNAs are listed in the Extended Dataset 1. All plasmid

sequences were verified using Sanger sequencing. All oligonucleotides were ordered from Integrated DNA Technologies (IDT).

Cell Culture and generating parent cell lines

Lentiviral production as well as infection and culturing of K562 cells (ATCC) were performed as described[51]. Parental K562 cell lines were generated by infecting dCas9-Blast (pGH125) followed by blasticidin selection (10μg/mL, Gibco) for 7 days. Cells were subsequently infected with both GFP (pGH045) and mCherry (pGH044) expression vectors or with a wtGFP (pGH220) expression vector and sorted via FACS for fluorescence. These cell lines were used as the parental samples in the sequencing assays. For integrated CRISPR-X experiments, these cells were infected with MS2-AID (pGH153, 156, 183, and 335) expressing vectors followed by selection with hygromycin B (200μg/mL, Life Technologies) for 7 days. All cell lines were maintained in a humidified incubator (37°C, 5% CO2), and checked regularly for mycoplasma contamination.

Fluorescence Microscopy of MS2-AID localization

K562 cells were lentivirally infected by constructs expressing an MS2-AIDΔ (pGH153) and MS2-AID (pGH156) and selected with hygromycin B for 7 days. 1 million cells were harvested and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were washed 3 times with PBS and then permeabilized with 0.1% Triton-X in PBS for 10 min at 4°C. Cells were incubated in blocking solution (3% BSA in PBS) for 1h at room temperature. They were centrifuged at 500 g for 5 minutes and resuspended in 1:500 dilution of rabbit anti-MS2 antibody (Millipore, cat no. ABE76) in blocking solution for 2h at room temperature. The cells were washed 3 times with PBS and resuspended in 1:1000 dilution of Alexa Fluor 488 conjugated goat anti-rabbit antibody (Life Technologies) in blocking solution and incubated for 2h at room temperature. Cells were washed in PBS 3 times and resuspended in Vectashield (Vector Laboratories) containing DAPI. The samples were deposited on a glass coverslip and imaged using an inverted Nikon Eclipse Ti confocal microscope with 488nm (AlexaFluor488) and 405nm (DAPI) lasers, an oil immersion objective (Plan Apo λ, N.A. = 1.5, 100X, Nikon), and an Andor Ixon3 EMCCD camera. Images were processed using ImageJ (National Institutes of Health).

Comparison of MS2-AID variant expression

K562 cells were infected with constructs expressing MS2-AID (pGH156), MS2-AIDΔ (pGH153), and MS2-AID*Δ (pGH335) and selected with hygromycin B for 7 days. 1.2 million cells were harvested and rinsed once with ice cold PBS before being lysed in lysis buffer (1% Triton X-100, 150mM NaCl, 50mM Tris pH 7.5, and 1mM EDTA) for 20 minutes on ice. Debris was removed by centrifugation for 10min at 21,000g at 4°C. The supernatant was collected and protein was quantified for each sample using DC Protein Assay (Bio-Rad). For each sample, 100μg of protein was denatured under reducing conditions (NuPAGE® LDS Sample Buffer (4X), Life Technologies, cat no. NP0007, and 100mM DTT), loaded on a 4–12% Novex BisTris SDS-PAGE gel (Life Technologies), and analyzed by immunoblot using a rabbit anti-MS2 antibody (1:1000 dilution, Millipore, cat no. ABE76) and mouse anti-GAPDH antibody (1:4000 dilution, Life Technologies, cat no. AM4300). Donkey anti-mouse IRDye 680 LT and goat anti-rabbit IRDye 800CW (1:20000

dilution, LI-COR Biosciences, product nos: 925–68022 and 925–32211) were used as secondary antibodies. Immunoblots were imaged using an Odyssey infrared imaging system (LI-COR Biosciences).

Transient electroporation of K562 cells and testing MS2-AID variants

Nucleofection of K562 cells were performed as described[52]. 1 million K562 cells were harvested for each electroporation. Cells were centrifuged at 300 g for 5 min and resuspended in 100μL of nucleofection solution and mixed with plasmid DNA (5μg MS2-AID expressing plasmid and 5μg sgRNA expression vector) and loaded into a 2mM cuvette (VWR). Electroporations were performed using the T-016 program on the Lonza Nucleofector 2b. After electroporation, cells were rescued in warm supplemented RPMI media. Cells were grown for 10 days and the GFP and mCherry fluorescence was measured using the BD Accuri C6 flow cytometer. The scatter plots shown were generated in FlowJo. The cells were sorted for low GFP fluorescence and the cells were grown before preparation of sequencing.

Generating mutations from individual and dual sgRNA experiments

For integrated CRISPR-X experiments, three days after infection, selection was applied and continued for 11 days using blasticidin for dCas9, hygromycin B for MS2-AID variants, and zeocin (200μg/mL, Life Technologies) for sgRNA. For dual sgRNA experiments, the sgGFP. 10 plasmid was further selected using puromycin (1μg/mL, Sigma-Aldrich). For GFP and mCherry targeting sgRNAs, the GFP and mCherry fluorescence were measured after selection using a BD Accuri C6 flow cytometer. Scatter plots shown were generated in FlowJo. Experiments targeting GFP or mCherry were performed with 3 biological replicates while endogenous loci were performed with 2 biological replicates.

Preparation of sequencing samples

To sequence the targeted loci, genomic DNA was extracted from 0.5–1.5 million cells using the QiaAmp DNA mini kit (Qiagen). The targeted loci were PCR amplified from 0.5–1μg of genomic DNA using primers shown in Extended Dataset 1. The product was purified on a 0.8–1% TAE agarose gel. The concentration was measured by Qubit (Life Technologies) and then prepared for sequencing following the Nextera XT kit protocol (Illumina). For PSMB5 experiments, DNA was extracted from 20 million cells and PCR amplification was performed on 5μg of genomic DNA. After individual gel purification of PCR product from each exon, PCR products were mixed in equimolar amounts before beginning the Nextera XT preparation.

Sequences were measured on a NextSeq 500 (Illumina) with paired end reads of length 76 or 151bp. Every sequencing run included a parental sample for each locus that was being sequenced.

Analysis of Sequencing data

Sample sequencing and Alignment—Over all sequenced samples, 4.5 million reads were produced on average. Sequencing adapters (5′ adapter: *CTGTCTCTTATACACATCTCCGAGCCCACGAGAC;* 3′ adapter:

CTGTCTCTTATACACATCTGACGCTGCCGACGA) were trimmed using cutadapt (version 1.8.1[53]), also discarding reads under 30 bp and nucleotides flanking the adapters with Illumina quality score lower than 30 (leaving only flanking sequences for which the base call accuracy is over 99.9%). Alignment on respective reference loci was performed using bwa aln (v0.7.7) and bwa samse[54]). A maximum number of 3 or 5 mismatches was allowed for samples with read length of 76 bp and 151 bp respectively. Aligned files were then sorted using samtools (v0.1.19[55])

Only reads aligned to their respective references with mapping quality over 30 were kept for further analysis. On average, 90% of sequenced reads (Standard Deviation 16%) were successfully mapped to the provided reference genome. From these aligned reads, 96% (Standard Deviation 5.7%) were remaining after filtering on mapping quality.

Tabulation of mutations per base—We computed allelic counts at each position with a custom script, after filtering for nucleotides with Illumina base quality score over 30 using samtools mpileup (version 1.2). The parental sample was used to estimate the mutations introduced through sample preparation and sequencing. Using the parental as a reference, we calculated the mutation enrichment at each base by taking the percentage of reads with alternative alleles in comparison to the same proportion calculated in the parental sample. For frequency of mutation calculated at each base, we subtracted the frequency of alternative alleles in the parental sample from the frequency calculated for the mutated sample. The first and last 50 bases of each locus were excluded from these enrichments given the ends had lower read coverage that was a byproduct of the Nextera XT preparation. We calculated the transitions/transversions/indels observed in the hotspots by looking at the distribution of frequencies of every possible alternative nucleotide at each position. We then subtracted the parental cell line respective frequencies in the hotspots to take into account the background noise. Negative values were set to 0. To estimate the remaining noise resulting from sequencing and variability between samples, we calculated the standard deviation of the frequency of alternative alleles in all parental samples from the studied batch (Fig. 4d). Reported medians, maximums, and distributions result from this calculation.

Calculation of mutation frequency in hotspot regions—The number of mutations per read was limited during the alignment step (see above). We performed mutation counts from the filtered aligned data to compute the enrichment of reads carrying mutations within the hotspot. After selecting all reads overlapping the hotspot using samtools view (version 1.2[55]), each read was screened for mutations with their respective positions. These results were then summarized for each sample by calculating the ratio between the number of reads with mutations spanning the hotspot and the total number of reads spanning the hotspot. The frequency of mutations enrichment was calculated by subtracting the results from the parental cell line as background.

Calculation of the observed percentage of possible transitions—To estimate mutant diversity in a population of cells, we analyzed the hyperactive AID*Δ mutant samples (Fig. 4b,c) with a custom R script. For each sgRNA-targeted sample, we selected the mutation hotspot (+/− 50bp with respect to the PAM) and computed the frequency of each observed alternate allele. At each position we subtracted the respective allelic

frequency observed in the corresponding parent sample. Using a sliding window of 21 bp over the hotspot and the 20bp flanking each end, we calculated the percentage of all 63 possible transitions in the window that were observed above noise. Noise was defined as the standard deviation of the alternative allele frequency among all parent samples. Results were then output by window as a boxplot representing the combination of all considered sgRNA-targeted samples.

Evolution of wtGFP to EGFP using CRISPR-X

For transient electroporation wtGFP experiments, K562 cells expressing dCas9 and wtGFP were electroporated as described earlier with 5μg of MS2-AIDΔ and either 1.25μg for each of wtGFP.1–4 or Safe.2,4–6 sgRNA expressing vectors. Cells were grown for 10 days after electroporation before sorting. For integrated experiments, K562 cells expressing dCas9, MS2-AIDΔ, and wtGFP were infected with either wtGFP.1 or Safe.2 sgRNA expressing vectors. After 3 days, cells were selected with blasticidin, hygromycin B, and zeocin for 11 days. Cells were sorted via FACS to obtain spectrum-shifted GFP variants. For the electroporation experiments, cells were grown for 7 days between sorting rounds. Samples were prepared for sequencing as described previously.

Flow cytometry of wtGFP variants

HEK293T (ATCC) cells were cultured in DMEM with 10% FBS, penicillin/streptomycin, and L-glutamine. For each transfection, 1 million HEK293T cells were plated in 2 mL of supplemented DMEM media. 1.5μg of wtGFP expressing plasmid (pGH045, 220, 311, 312, and 314) was mixed with 200μL serum-free DMEM and 10μL of polyethylenimine (PEI, 1mg/mL, pH 7.0, PolySciences Inc.) and incubated at room temperature for 30 minutes. The mixture was added to the cells and grown for 72 h with an additional 3 mL of DMEM supplemented media added after 24 h. The samples were trypsinized and analyzed using a FACScan flow cytometer (BD Biosciences). Additional analysis of the data was performed using FlowJo.

Design and construction of PSMB5 Tiling libraries

The PSMB5 tiling library was generated using CHOPCHOP online tool[56] for the three PSMB5 isoforms (NM_0011449632, NM_00130725, and NM_002797). sgRNAs for each isoform were combined. sgRNAs having any genomic off-target matches, more than 1 off-target when allowing one mismatch in the sgRNA sequence, or 5 or more off-targets when allowing one or two mismatches within the sgRNA sequence were removed. The sgRNAs were further filtered by removing any containing a BsmBI cut site, which interferes with the library cloning strategy. The final library contained 143 sgRNAs. Safe harbor sgRNAs were designed to target genomic loci that have not been annotated to include gene exons or UTRs, have signal in biochemical assays (DNaseI, CHIP-Seq, etc.) or have signal in sequence-based analyses (conserved elements, transcription factor motif searches, etc). The design and selection of these sgRNAs will be discussed in more detail in future work. 705 sgRNAs targeting safe harbor regions were selected to serve as a control library. The sgRNA sequences for both libraries are included in Extended Dataset 2.

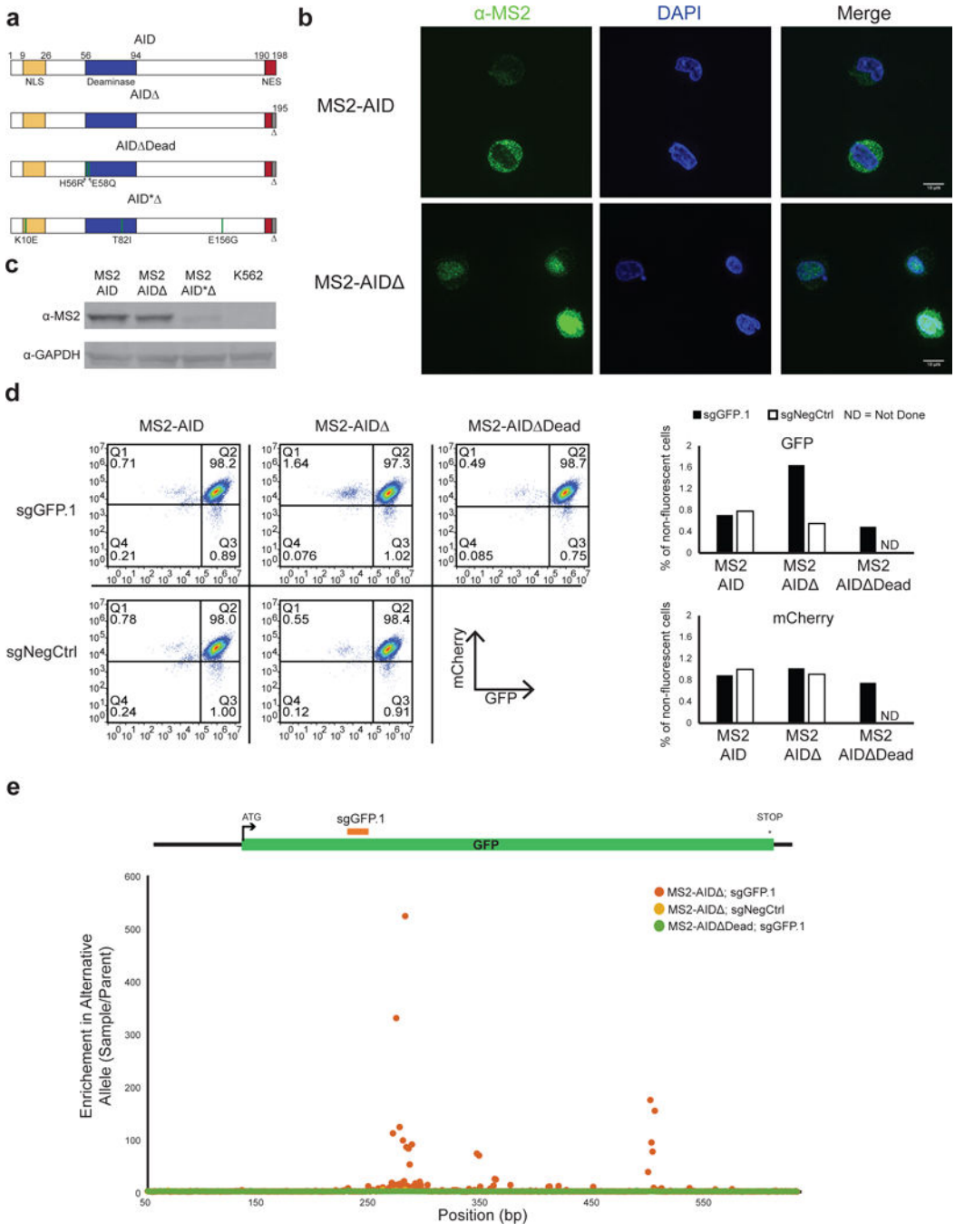
Oligonucleotide libraries were synthesized by Agilent and cloned into the sgRNA expression vector as previously described[57–59]. Vector and sgRNA inserts were digested with BsmBI. Large scale lentivirus production and infection of K562 cells were performed as described[57,59]. Three days after infection, selection began with blasticidin, hygromycin B, and zeocin for 11 days. Cells were expanded to 20 million cells for each treatment (safe harbor and PSMB5 libraries in duplicate) and were pulsed with 20nM bortezomib (Fisher Scientific) for three days followed by recovery until log growth was restored (5–10 days) before the next pulse. The cells were pulsed a total of three times. After the final pulse, cells were harvested and prepared for sequencing as described earlier.

Installation and validation of bortezomib resistant PSMB5 mutations

sgRNAs were designed to target near the location of the installed SNP and 101nt donor oligos were designed to be centered around the installed mutation. Oligonucleotides with proper overhangs were ordered from IDT and annealed before ligation into BbsI digested pGH020, a hu6 driven sgRNA expression vector. All plasmids were verified by Sanger sequencing. The sgRNA and ssDNA donor oligo sequences are listed in Extended Dataset 1, respectively.

K562 cells expressing Cas9 were electroporated with 5μg of sgRNA expressing vector and 100 picomoles of donor oligo. Cells were grown for 6 days before 300,000 cells were placed under selection with 20nM bortezomib for 14 days. The viability of the cells was measured by flow cytometry using a live cell gate (FSC/SSC). After selection, 750,000 cells were harvested and genomic DNA was extracted using the QiaAmp DNA Mini Kit (Qiagen). The PSMB5 exonic locus containing the mutation was PCR amplified, gel purified, and ligated into the pCR-Blunt vector using the Zero-Blunt cloning kit (Life Technologies). 8–15 colonies were Sanger sequenced for each sample.

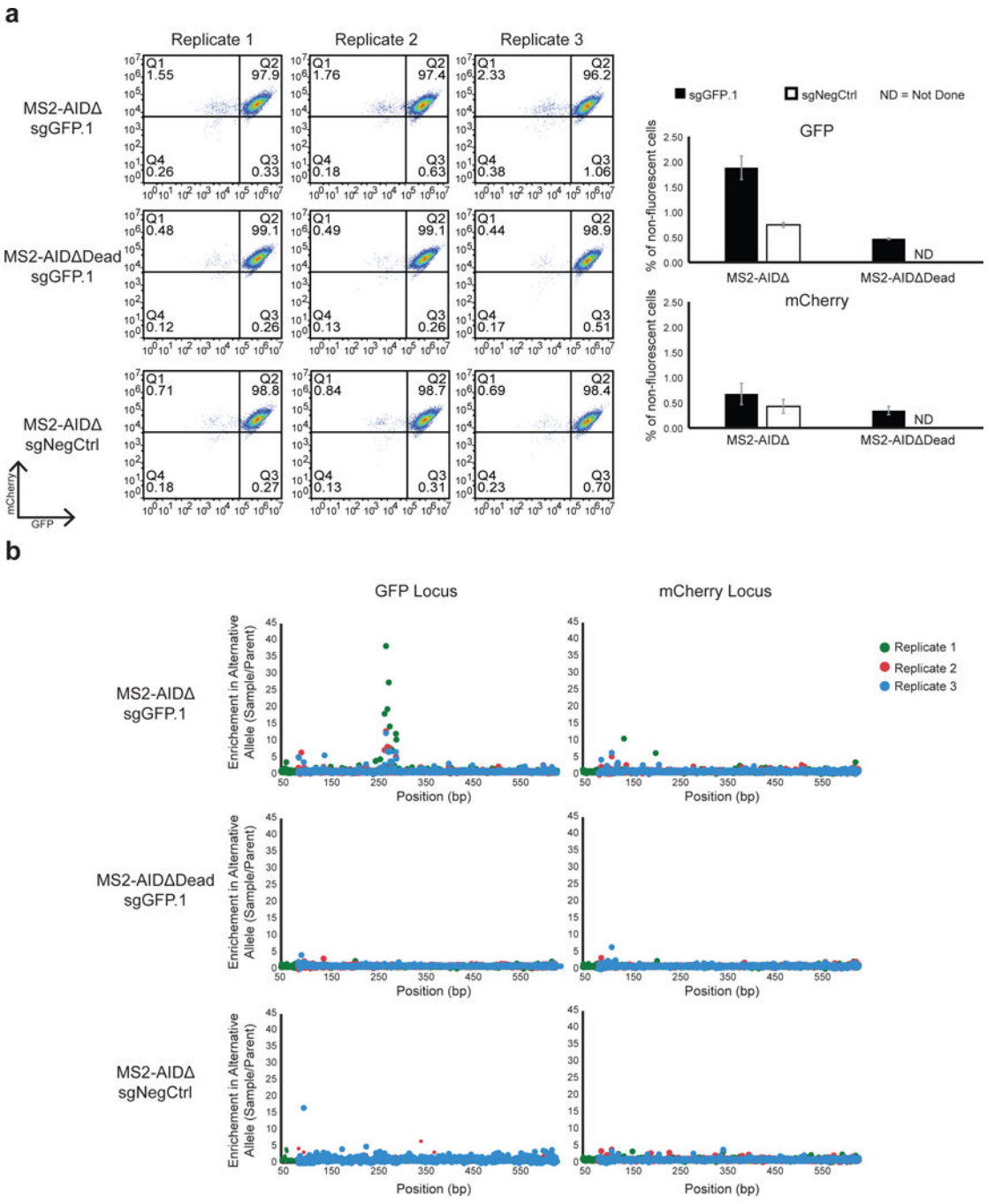
Extended Data



Extended Data Figure 1. Characterization of AID variants

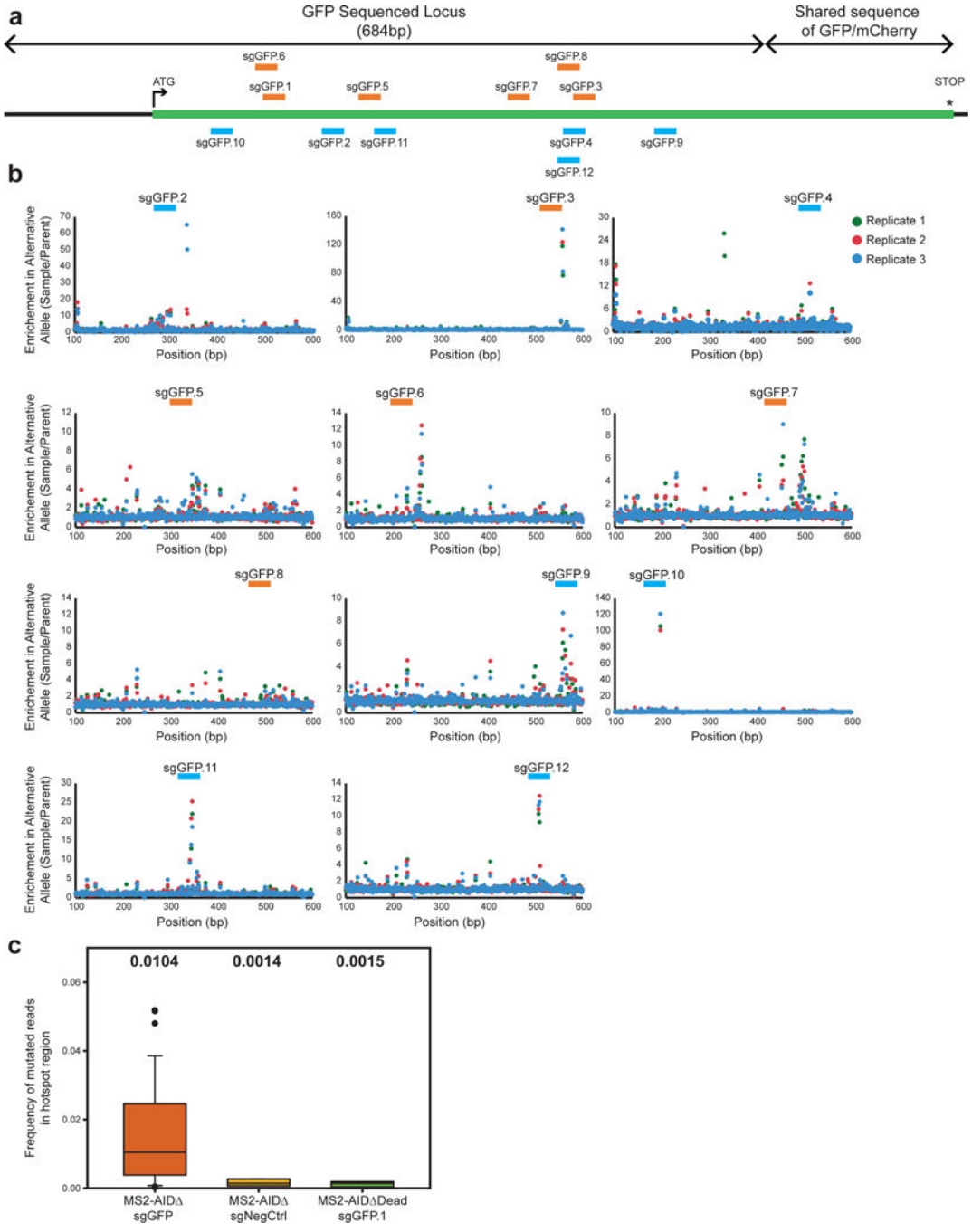
a) Diagram of AID variants. NLS, NES, deaminase domain, truncations, and activity-altering mutations are indicated. b) Fluorescence microscopy of MS2-AID and MS2-AIDΔ constructs in K562 cells is shown. Cells were fixed and stained with an MS2 antibody (green) and the nuclear stain DAPI (blue). c) A comparison of the expression of different MS2-AID variants is shown. K562 cells expressing the variants were lysed and analyzed on an SDS-PAGE gel followed by immunoblotting with an MS2 antibody (top) or GAPDH

antibody (bottom). d) K562 cells containing dCas9, GFP, and mCherry were transiently electroporated with indicated combinations of MS2-AID, MS2-AIDΔ, or MS2-AIDΔDead and either sgGFP.1 or sgNegCtrl. GFP and mCherry fluorescence of the cells were measured by flow cytometry as a proxy for mutation rate. Shown are the scatter plots from the flow cytometry and a graph summarizing the non-fluorescent populations. e) Cells were sorted for low GFP expression and the GFP locus was sequenced. A graph of the enrichment of mutation at each base is shown here.



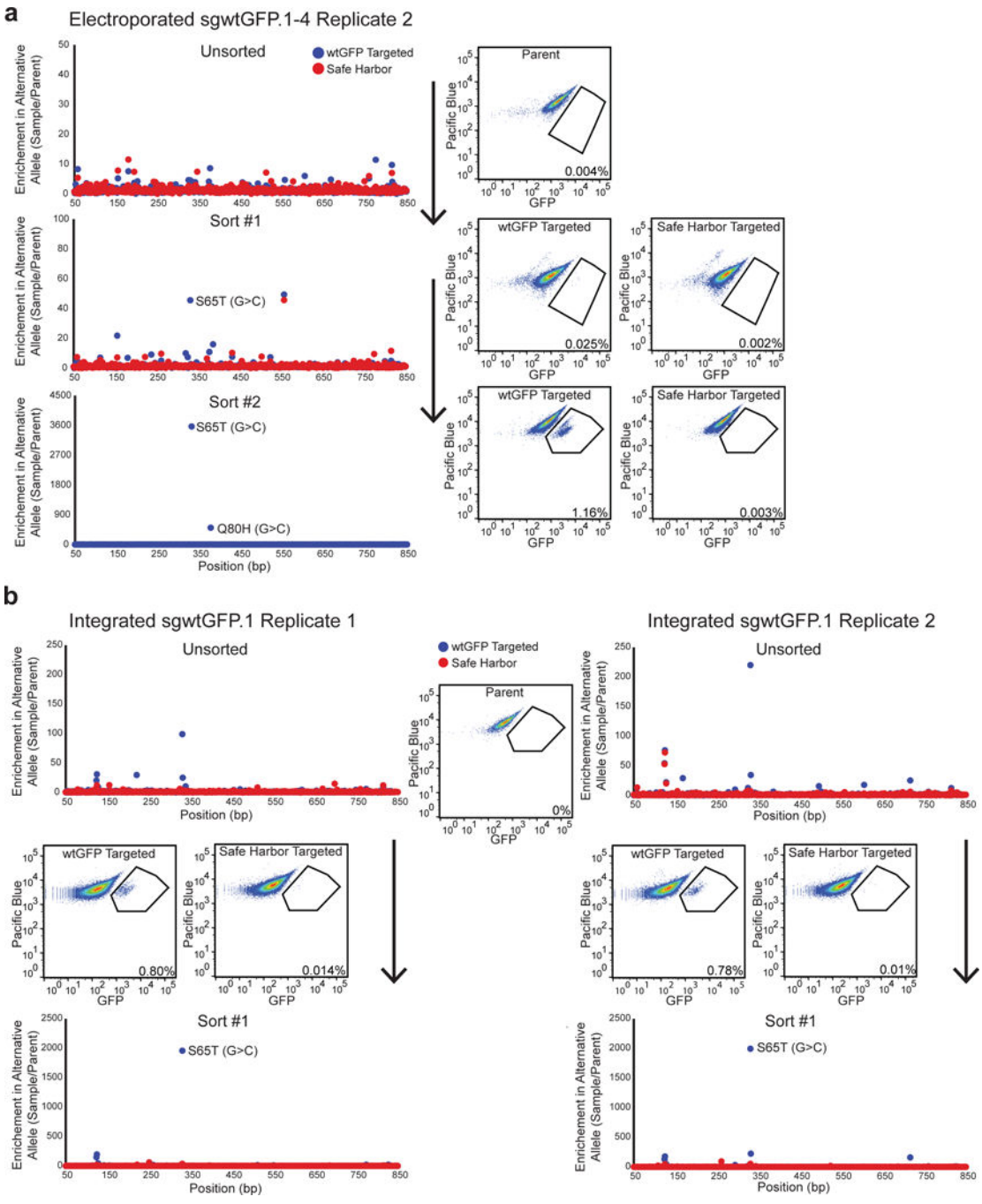
Extended Data Figure 2. On-target mutagenesis using CRISPR-X with limited off-target effect

a) Cells were infected with indicated combinations of MS2-AIDΔ or MS2-AIDΔDead and sgGFP.1 or sgNegCtrl and the GFP and mCherry fluorescence of the cells was measured by flow cytometry as a proxy for mutation rate. Shown are the scatter plots from flow cytometry and graphs summarizing the non-fluorescent populations. Error bars represent standard error. b) GFP and mCherry loci of the infected cells were sequenced and enrichment of mutation was calculated at each base position for three replicate experiments.



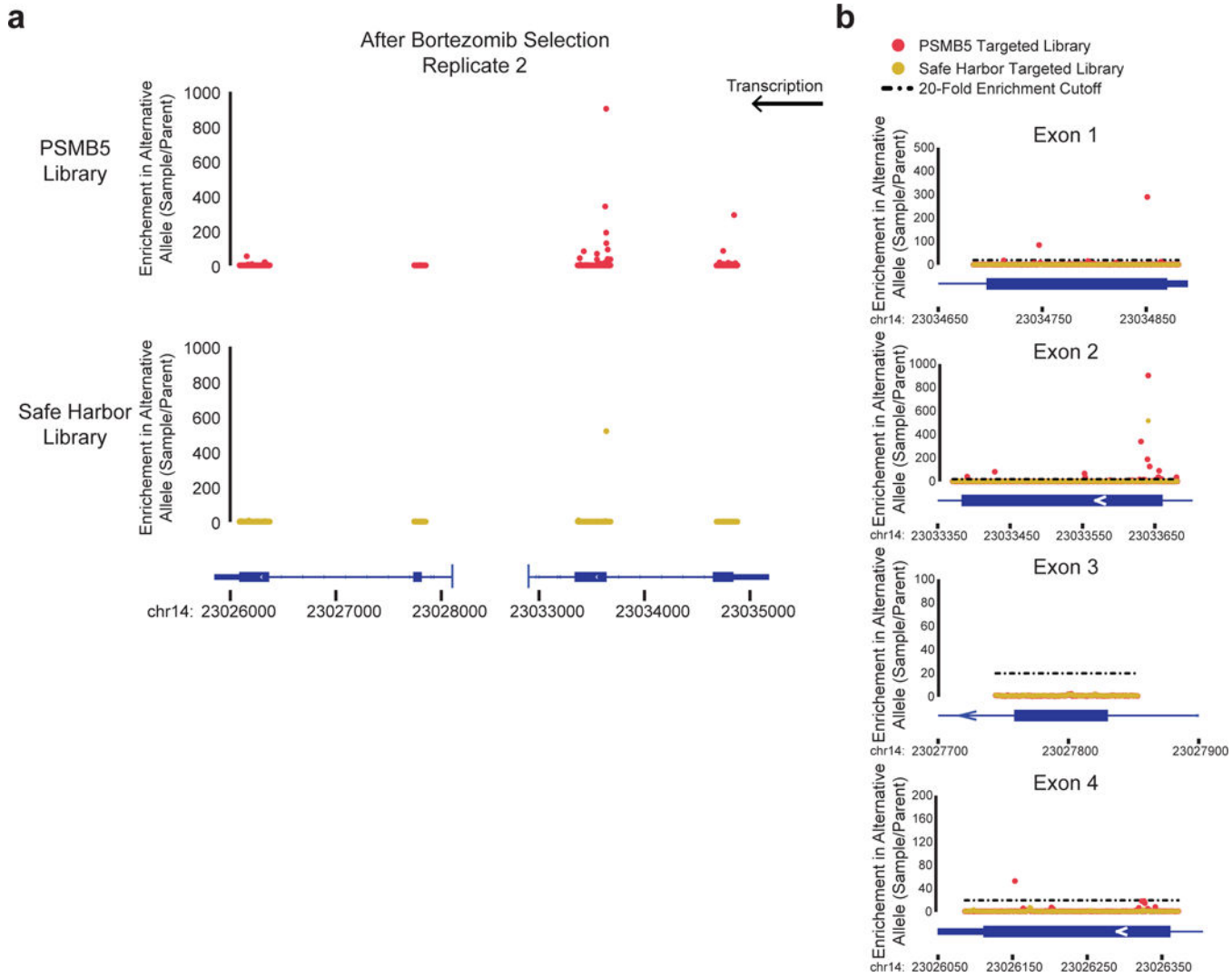
Extended Data Figure 3. CRISPR-X tiling of GFP locus

a) Map of sgRNAs tiling the GFP locus. b) sgRNAs targeting GFP were integrated into cells expressing dCas9, MS2-AIDΔ, GFP, and mCherry, and the GFP locus was sequenced. Enrichment of mutations at each base position is shown for three replicates of each sgRNA. c) A box plot indicating the frequency of mutated reads observed in the respective hotspot of each sgRNA is shown. The median value for the conditions is listed above each sample. The box plot lines represent the 1.5 of the interquartile range.



Extended Data Figure 4. Directed evolution of wtGFP to EGFP using CRISPR-X

a) A replicate of the wtGFP evolution experiment (Fig. 2a) was performed using electroporated sgRNAs and MS2-AIDΔ. Flow cytometry scatter plots are shown for the wtGFP parent and samples before each round of sorting. The wtGFP locus was sequenced for the unsorted condition and after both sorting rounds. Enrichment of mutation was calculated at each base position. The graphs of enrichment are shown for both wtGFP targeted and safe harbor targeted libraries except after Sort #2 where no safe harbor cells were recovered after sorting. Identified mutations are labeled in the graphs. b) wtGFP cells expressing dCas9, MS2-AIDΔ, and wtGFP were lentivirally infected with sgwtGFP.1 or sgSafe.2 in replicate and sorted once, enriching for spectrum-shifted GFP cells. Scatter plots for the parent and unsorted populations are shown for both replicates. The wtGFP locus was sequenced pre- and post-sorting, and enrichment of mutations at each base position is shown. The S65T mutation is labeled in the graph for the sorted condition.

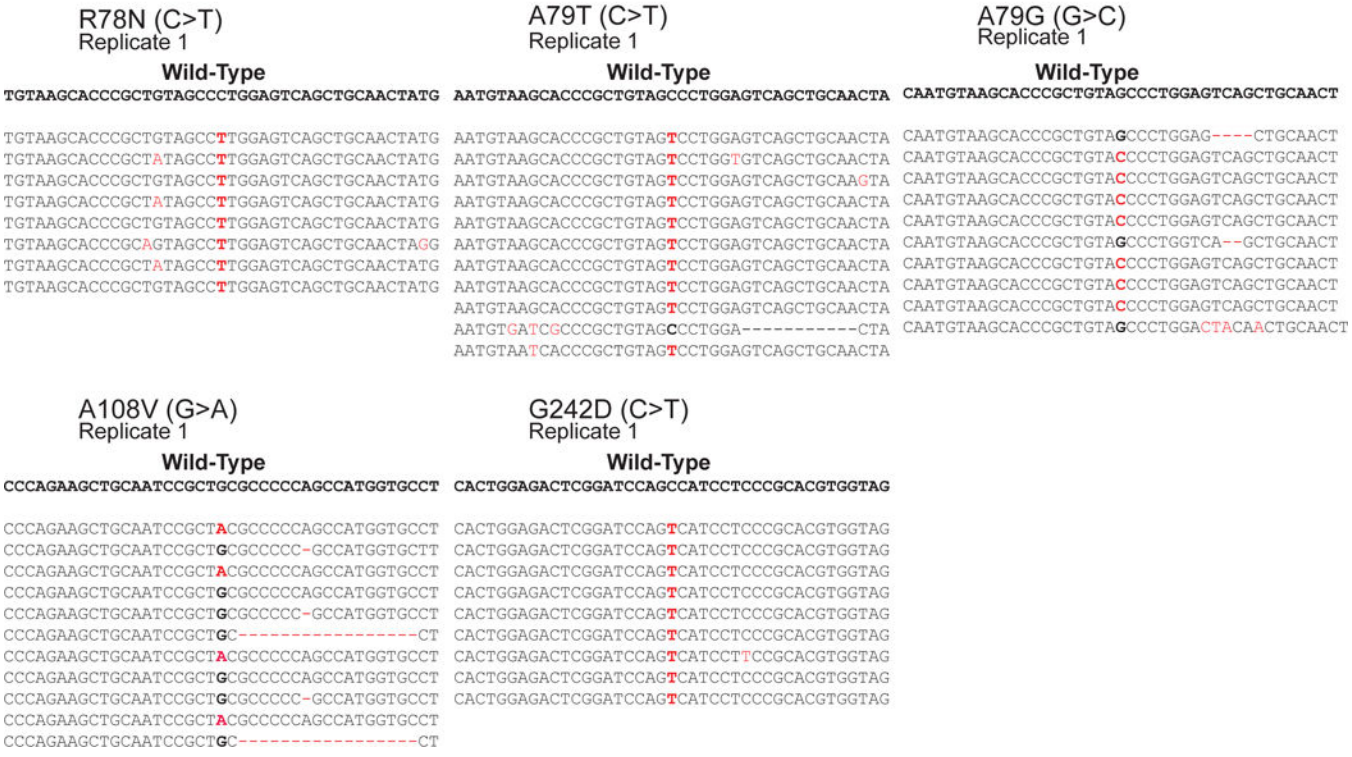


Extended Data Figure 5. Identifying bortezomib resistant mutations in PSMB5

a) A replicate experiment was performed for directed evolution of bortezomib-resistant PSMB5 mutations (see Fig. 3). The PSMB5 exonic loci were sequenced after selection with

bortezomib for both the PSMB5 and Safe Harbor libraries and enrichment of mutations at each base position is shown. b) Graphs of mutation enrichment are shown for individual exonic loci of PSMB5. Mutations that were enriched beyond the 20-fold cutoff (dashed black line) are observed in Exons 1, 2, and 4.

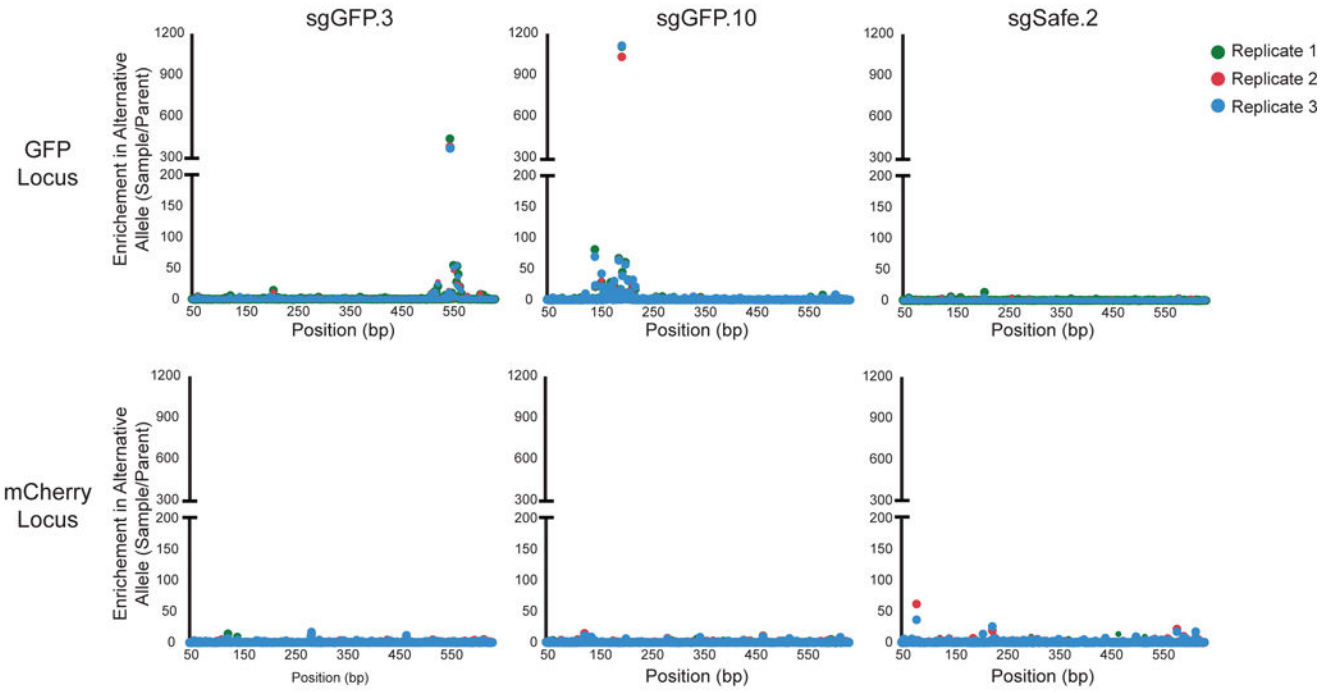
Mutation	Sequences with Mutation			Sequences with Wild-type			Sequences with Indels		
	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3	Rep1	Rep2	Rep3
R78N (C>T)	8/8	6/8	2/8	0/8	1/8	0/8	0/8	1/8	6/8
A79T (C>T)	10/11	6/7	4/7	0/11	1/7	1/7	1/11	0/7	2/7
A79G (G>C)	7/10	4/8	2/8	0/10	0/8	0/8	3/10	4/8	6/8
A108V (G>A)	4/11	5/7	4/8	2/11	0/7	2/8	5/11	2/7	2/8
G242D (C>T)	9/9	4/7	7/8	0/9	0/7	1/8	0/9	3/7	0/8



Extended Data Figure 6. Knock-in and validation of novel bortezomib-resistant PSMB5 variants

Bortezomib resistant mutations observed in PSMB5 (Fig. 3d) were knocked-in to K562 cells and populations were selected with bortezomib. The corresponding PSMB5 exons for the five most viable mutations were amplified, cloned into pCR-Blunt, and sequenced individually. Shown is a table summarizing the sequences of individual colonies with mutations or insertions/deletions shown in red; the targeted base is in bold.

a



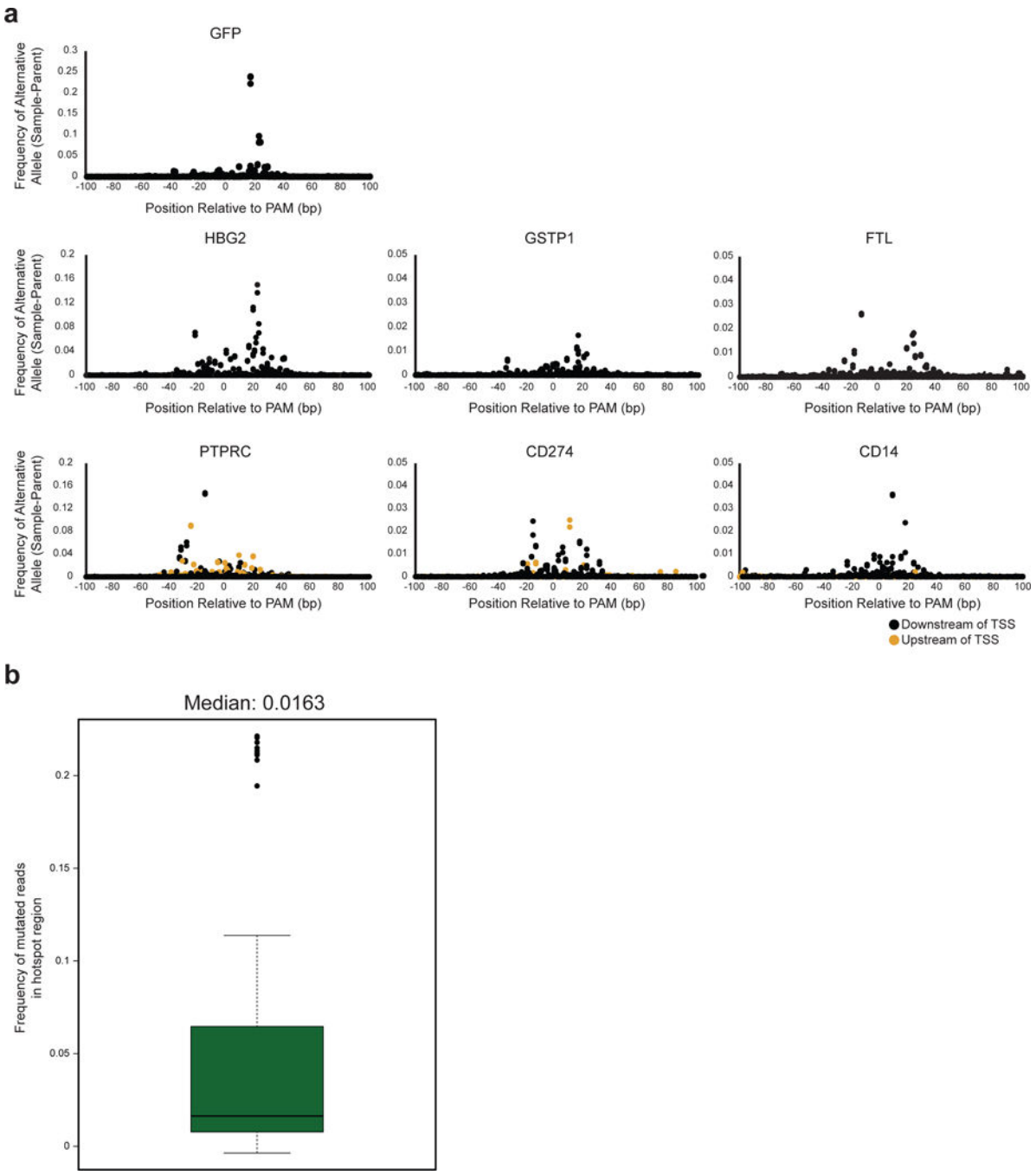
b

Number of mutations per mutated sequence

	AIDΔ	AID*Δ
sgGFP.3	1.07±0.26	1.31±0.60
sgGFP.10	1.07±0.28	1.32±0.61

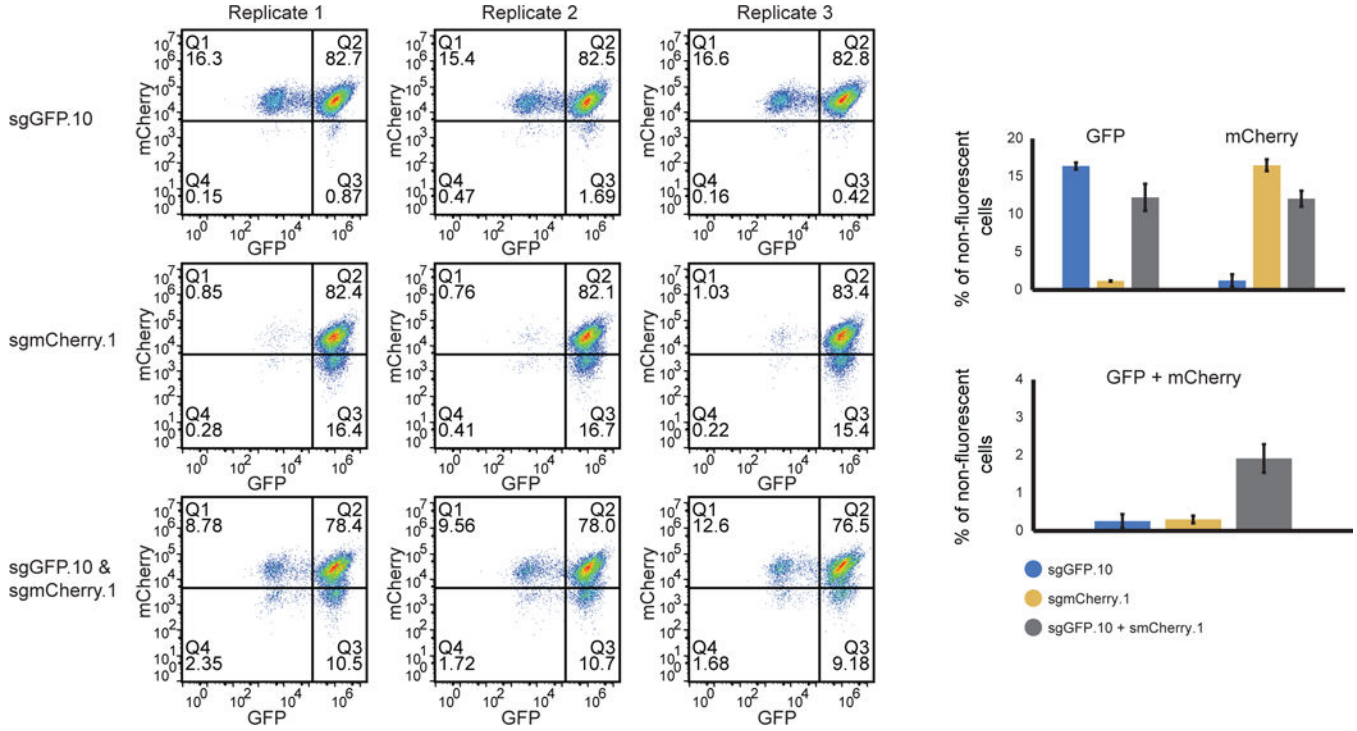
Extended Data Figure 7. Improved mutagenesis using AID*Δ

a) sgRNAs targeting either GFP (sgGFP.3 and sgGFP.10) or a safe harbor locus (sgSafe.2) were integrated into cells expressing dCas9, MS2-AID*Δ, GFP, and mCherry. The GFP and mCherry loci were sequenced. Enrichment of mutation at each base position is shown. b) For sgGFP.3 and sgGFP.10 paired with either AIDΔ or AID*Δ, sequences were filtered for those containing a mutation, and the average number of mutations per sequence was calculated. The average and standard error are shown.



Extended Data Figure 8.

a) sgRNAs targeting either GFP or endogenous loci were integrated into cells expressing dCas9, MS2-AID*Δ, GFP, and mCherry. The respective targeted loci were sequenced. Graphs showing the frequency of alternative alleles at each base position relative to the PAM of the sgRNA are shown. b) Box plot indicating the range of frequency of mutated reads over the 100bp region for 30 sgRNAs is shown. The lines represent 1.5 times the interquartile range. Median value is indicated above graph.



Extended Data Figure 9.

sgGFP.10 and sgmCherry.1 were integrated separately or in combination into cells expressing dCas9, MS2-AID*Δ, GFP, and mCherry. The GFP and mCherry fluorescence of the cells were measured. The scatter plots of the flow cytometry for each of the samples are shown (left). A graph summarizing the percentage of GFP negative or mCherry negative cells is shown (top left). In the bottom left panel, a graph displaying the percentage of cells that have neither GFP nor mCherry is shown. Error bars indicate standard error.

Extended Dataset 1

Complete list of plasmids, oligonucleotides, and sgRNA sequences used.

PLASMIDS

Name	Description
pGH125	dCas9-Blast
pGH153	MS2-AIDΔ-Hygro
pGH156	MS2-AID-Hygro
pGH183	MS2-AIDΔDead-Hygro
pGH224	sgRNA_2xMS2_Puro
pGH044	mCherry
pGH045	GFP
pGH220	wtGFP
pGH311	wtGFP S65T
pGH312	wtGFP Q80H
pGH314	wtGFP S65T, Q80H

PLASMIDS

Name	Description
pGH335	MS2-AID*Δ-Hygro
pGH020	sgRNA_G418-GFP

OLIGONUCLEOTIDES

Vector	Name	Sequence (5′–3′)
dCas9	dCas9-Blast For (oGH255)	AAAAAGAGGAAGGTGGCGGCCGCTGGATCCGAGGGCAGAGGAAGTCTGCTAACAT
	dCas9-Blast Rev (oGH256)	AGGTTGATTACCGATAAGCTTGATATCGAATTC
MS2-AID	MS2-AID For (oGH272)	AAGAGGAAGGTGGCGGCCGCTGGATCCATGGACAGCCTCTTGATGAACCG
	MS2-AID Rev (oGH273)	TTCCTCTGCCCTCTCCACTGCCTGTACAAAGTCCCAAAGTACGAAATGCGTC
	MS2-AIDΔ Rev (oGH274)	TTCCTCTGCCCTCTCCACTGCCTGTACAAGTACGAAATGCGTCTCGTAAGTC
	AIDΔDead Mut For (oGH315)	GAACGGCTGCCGCGTGCAATTGCTCTTCCTCCGCTACATCTCG
	AIDΔDead Mut Rev (oGH316)	AAGAGCAATTGCACGCGGCAGCCGTTCTTATTGCGAAGATAAC
	AID*Δ K10E For (oGH456)	AAGAGGAAGGTGGCGGCCGCTGGATCCATGGACAGCCTCTTGATGAACCGGAGGG
	AID*Δ E156G For (oGH457)	TACTGCTGGAATACTTTTGTAGAAAACCACGGAAGAACTTTCAAAGCCTGGGAAGC
	AID*Δ E156G Rev (oGH458)	CCTTCCCAGGCTTTGAAAGTTCTTCCGTGGTTTTCTACAAAAGTATTCCAGCAGTA
	AID*Δ T82I For (oGH459)	GCTGCTACCGCGTCACCTGGTTCATCTCCTGGAGCCCCTGCTACGAC
	AID*Δ T82I Rev (oGH460)	GTCGTAGCAGGGGCTCCAGGAGATGAACCAGGTGACGCGGTAGCAGC
Fluorescent Proteins	GFP/mCherry For (oGH144)	CATTTCAGGTGTCGTGAGCTAGCCCACCATGGTGAGCAAGGGCGAGGAG
	GFP/mCherry Rev (oGH146)	CTGGCTTACTAGTCGGTTCAACTCTAGATTACTTGTACAGCTCGTCCATGCCG
	wtGFP Mut For (oGH363)	GTGACCACCTTCAGCTACGGCGTGCAGTGC
	wtGFP Mut Rev (oGH364)	GCACTGCACGCCGTAGCTGAAGGTGGTCAC
	wtGFP Q80H For (oGH447)	ACCCCGACCACATGAAGCACCACGACTTCTTCAAGTCC
	wtGFP Q80H Rev (oGH448)	GGACTTGAAGAAGTCGTGGTGCTTCATGTGGTCGGGGT
	wtGFP S65T For (oGH449)	CCTCGTGACCACCTTCACCTACGGCGTGCAGTGCT
	wtGFP S65T Rev (oGH450)	AGCACTGCACGCCGTAGGTGAAGGTGGTCACGAGG
Puromycin Resistance	Puro For (oGH375)	TTTCTTCCATTTCAGGTGTCGTGATGTACAATGACCGAGTACAAGCCCACGG
	Puro Rev (oGH376)	ATTACCGATAAGCTTGATATCGAATTCTCAGGCACCGGGCTTGCGGGTCATG
	Puro BsmBI For (oGH377)	TCCTGGCCACCGTCGGCGTATCGCCCGACC
	Puro BsmBI Rev (oGH378)	GGTCGGGCGATACGCCGACGGTGGCCAGGA

sgRNA Sequences

Name	sgRNA Sequence (5′–3′)	Genomic Position
sgGFP.1	GGCGAGGGCGATGCCACCTA	
sgNegCtrl	GCTCAAGAACGCCTTCCCCAGTC	
sgGFP.2	GGCACGGGCAGCTTGCCGG	
sgGFP.3	AAGGGCATCGACTTCAAGG	
sgGFP.4	CGATGCCCTTCAGCTCGATG	
sgGFP.5	CTCGTGACCACCCTGACCTA	
sgGFP.6	CAAGTTCAGCGTGTCTGGCG	
sgGFP.7	CAACTACAAGACCCGCGCCG	

sgRNA Sequences

Name	sgRNA Sequence (5′–3′)	Genomic Position
sgGFP.8	GGTGAACCGCATCGAGCTGA	
sgGFP.9	CGGCCATGATATAGACGTTG	
sgGFP.10	CGTCGCCGTCCAGCTCGACC	
sgGFP.11	AGCACTGCACGCCGTAGGTC	
sgGFP.12	TCAGCTCGATGCGGTTCACC	
sgwtGFP.1	CCGGCAAGCTGCCCGTGCCC	
sgwtGFP.2	GCTTCATGTGGTCGGGGTAG	
sgwtGFP.3	CGTGCTGCTTCATGTGGTCG	
sgwtGFP.4	GTCGTGCTGCTTCATGTGGT	
sgSafe.2	TCCCCCTCAGCCGTATT	chr12: 114129110-114129129
sgSafe.4	GATTGATATTGCCTTCT	chr12: 17350231-17350250
sgSafe.5	TCTGACTCCTAATGGAG	chr12: 114127368-114127387
sgSafe.6	ATTACTTTAGAGTAAGA	chr13: 105390313-105390332
sgHBG2.1	GGTCCATGGGTAGACAACC	chr11: 5249566-5249584
sgHBG2.2	GTGAGATTGACAAGAACAGT	chr11: 5249593-5249612
sgHBG2.3	AGGTCGCTTCTCAGGATTTG	chr11: 5249633-5249652
sgHBG2.4	GAGATCATCCAGGTGCTTTG	chr11: 5249437-5249456
sgHBG2.5	GCTACTATCACAAGCCTGTG	chr11: 5249758-5249777
sgGSTP1.1	GGAGATGTATTTGCAGCGG	chr11: 67585205-67585223
sgGSTP1.2	GGACATGGTGAATGACGGCG	chr11: 67585175-67585194
sgGSTP1.3	AGCCACCTGAGGGGTAAGGG	chr11: 67585310-67585329
sgGSTP1.4	CTGCACCCTGACCCAAGAAG	chr11: 67585341-67585360
sgGSTP1.5	TGATCAGGCGCCCAGTCACG	chr11: 67585090-67585109
sgFTL.1	GCCGAGGAGAAGCGCGA	chr19: 48965833-48965849
sgFTL.2	GCGCGAGGAGCCTTGATTTG	chr19: 48965963-48965982
sgFTL.3	CTCTATTTCCAGCGGTTAAG	chr19: 48966038-48966057
sgFTL.4	TAGCGGGAGGCGAGGCCAAG	chr19: 48965721-48965740
sgFTL.5	ACGCGCCAGCCTTCTTTGTG	chr19: 48965673-48965692
sgPTPRC.1	GTTTGTTCTTAGGGTAACAG	chr1: 198639077-198639096
sgPTPRC.2	TATCCTTGTGAAGCTAGGAG	chr1: 198638504-198638523
sgPTPRC.3	TGTTCTTGGCGCTACTGATG	chr1: 198638409-198638428
sgPTPRC.4	GGCGAGTGTGTATAGATCAG	chr1: 198697174-198697193
sgPTPRC.5	TAATGCATGTTGTTAGGGAG	chr1: 198697085-198697104
sgPTPRC.6	TGGGGAGTTAGTATACTGGG	chr1: 198696623-198696642
sgPTPRC.7	ATACACACTATAGTGGACTG	chr1: 198696605-198696624
sgCD274.1	AACTCCCACAGCATTTATCC	chr9: 5447248-5447267
sgCD274.2	ATGGGAAAATGAATGGCTGA	chr9: 5448598-5448617
sgCD274.3	CACCACCAATTCCAAGAGAG	chr9: 5462979-5462998
sgCD274.4	CAATGCAGGCTGGTTCTCAG	chr9: 5462727-5462746
sgCD274.5	TTTCATAGCCGGGAAACCTG	chr9: 5463466-5463485
sgCD14.1	TCAGGGAGGGGGACCGTAAC	chr5: 140633319-140633338
sgCD14.2	GGAGGGGGACCGTAACAGGA	chr5: 140633323-140633342

sgRNA Sequences

Name	sgRNA Sequence (5′–3′)	Genomic Position
sgCD14.3	ATTCAGGGACTTGGATTTGG	chr5: 140633606-140633625
sgCD14.4	CCTCATCTGTTGGCACCAAG	chr5: 140633670-140633689
sgCD14.5	AGGAGAGAGCAACGTGCAAG	chr5: 140634212-140634231
sgmCherry.1	GCGGTCTGGGTGCCCTCGTA	

Genomic Amplification Primers

Locus	Direction	Sequence (5′–3′)
GFP	For (oGH072)	AGGCCAGCTTGGCACTTGATGT
	Rev (oGH046)	TGTTGTGGCGGATCTTGAAGTTC
mCherry	For (oGH072)	AGGCCAGCTTGGCACTTGATGT
	Rev (oGH343)	GCTTCAGCCTCTGCTTGATCTC
Safe.2	For (oGH371)	CACTATGACCACAGCCACTCAC
	Rev (oGH372)	CTTTCTGAAAAGTAACCCAGCCTCA
Safe.4	For (oGH397)	GAACTGTGAATAATAAGCAATCATCCAG
	Rev (oGH398)	GCTTGCCAAAAATTGTGTACCCTTTCC
Safe.5	For (oGH399)	TAGGTAACCCATCTGAGGTTTTCAAATAT
	Rev (oGH400)	GAGAAAAGAACATGACTTCCAGCAGC
Safe.6	For (oGH401)	CCAAATTGCAGCCACACTTGAAAACC
	Rev (oGH402)	TAGGAAGCAGTGTAGGAGGATTGG
wtGFP	For (oGH072)	AGGCCAGCTTGGCACTTGATGT
	Rev (oGH029)	AAGCAGCGTATCCACATAGCGT
PSMB5 Exon 1	For (oGH468)	GCAAGGGGGCTGGCTCCACAC
	Rev (oGH469)	TTAGTTCTTTCTGCCCACACTAGAC
PSBM5 Exon 2	For (oGH470)	CATGTGGTTGCAGCTTAACTCAC
	Rev (oGH471)	GTGTTTTTGTGGTCTTATGTGGCC
PSMB5 Exon 3	For (oGH472)	ACAACATACCACCCCATCTCACC
	Rev (oGH473)	CAAAGTGCTGGGATTACGGGTTTG
PSMB5 Exon 4	For (oGH474)	CAAGCAGCTGCATCCACCCTCTT
	Rev (oGH475)	CTGCTAACCTCATCTCCCTTTCCAG
HBG2	For (oGH440)	GTATCTTCAAACAGCTCACACCC
	Rev (oGH441)	GTCTTAGAGTATCCAGTGAGGCC
GSTP1	For (oGH442)	CACTGAGGTTACGTAGTTTGCCC
	Rev (oGH443)	CGACAAATCCTCCTCCACCTCT
FTL	For (oGH454)	TTCCTCTCCGCTTGCAACCTCC
	Rev (oGH455)	CGGCACATAGAACTAAACCTACATTTC
PTPRC Locus 1	For (oGH500)	GCCAGTAAGCATTTTCCTAATAGATGGAC
	Rev (oGH501)	GCCAAATGCCAAGAGTTTAAGCC
PTPRC Locus 2	For (oGH502)	TCATCCTTCTGAACTCAATTGCTTTG
	Rev (oGH503)	CAATGATGCAAATGCTCTTAAAAGAAACTC
CD274 Locus 1	For (oGH504)	GGTGACTATTTCATTTGTGTGACACTC
	Rev (oGH505)	GAAAGCAGTGTTCAGGGTCTACC
CD274 Locus 2	For (oGH508)	GAAAACCTGAACAAATGGAGAGGG

Genomic Amplification Primers		
Locus	**Direction**	**Sequence (5′–3′)**
CD14	Rev (oGH509)	GCTTGCTCAGTAGATTATAATCCTACAGG
	For (oGH510)	GGTCGATAAGTCTTCCGAACCTC
	Rev (oGH511)	GCGAAACTGGTGAGTTACTAATTAATCC

Reagents for PSMB5 Variant Installation by HDR	
sgRNAs	
Mutation	**sgRNA Sequence (5′–3′)**
L11L, Exon 1 Control	CCGCGCTGGTTCACCGGTAG
Intronic	CTGCAACTATGACTCCATGG
R78N, A79TG, Exon 2 Control	TCATAGTTGCAGCTGACTCC
G82D	AGCTGACTCCAGGGCTACAG
A108V	CTGCTAGGCACCATGGCTGG
G242D	CAACCTCTACCACGTGCGGG
Exon 4 Control	TGAAGGGAACCGGATTTCAG

ssDNA donor oligonucleotides	
Mutation	**Sequence (5′–3′)**
L11L (oGH512)	CAGATCTGCACGACCCCCAAGTCCGAAAAACCCGCGCTGGTTCACCGGTAACGGTCTCTCCAACACGCTGGCAAGCG
Exon 1 Control (oGH513)	CTCCCTGGACCTAGATCCAGCAGATCTGCACGACCCCCAAGTCCGAAAAATCCGCGCTGGTTCACCGGTAGCGGTCTC
Intronic (oGH520)	ACCCGCTGTAGCCCTGGAGTCAGCTGCAACTATGACTCCATGGCGGAACTATTAAGATCAGAGGAAAACACAAAACAC
R78N (oGH518)	CTATCACCTTCTTCACCGTCTGGGAGGCAATGTAAGCACCCGCTGTAGCCTTGGAGTCAGCTGCAACTATGACTCCATG
A79T (oGH517)	CTCTATCACCTTCTTCACCGTCTGGGAGGCAATGTAAGCACCCGCTGTAGTCCTGGAGTCAGCTGCAACTATGACTCCA
A79G (oGH516)	TCTCTATCACCTTCTTCACCGTCTGGGAGGCAATGTAAGCACCCGCTGTACCCCTGGAGTCAGCTGCAACTATGACTCC
G82D (oGH515)	ATGGGTTGATCTCTATCACCTTCTTCACCGTCTGGGAGGCAATGTAAGCATCCGCTGTAGCCCTGGAGTCAGCTGCAAC
A108V (oGH514)	AGATTCGACATTGCCGAGCCAACAGCCGTTCCCAGAAGCTGCAATCCGCTACGCCCCCAGCCATGGTGCCTAGCAGGT
Exon 2 Control (oGH519)	ATCTCTATCACCTTCTTCACCGTCTGGGAGGCAATGTAAGCACCCGCTGTCGCCCTGGAGTCAGCTGCAACTATGACTC
G242D (oGH521)	TATACTTCTCATGTAGATCAGCCACATTGTCACTGGAGACTCGGATCCAGTCATCCTCCCGCACGTGGTAGAGGTTGAC
Exon 4 Control (oGH523)	TCCATGACCCCATATGCATACACAGAGCCAGAACCTACAGAGAAGGTGGCACCTGAAATCCGGTTCCCTTCACTGTCCA

Extended Dataset 2

Complete list of sgRNA sequences of PSMB5 Tiling and Safe Harbor libraries (Microsoft Excel file available online).

PSMB5 Tiling Library	
sgRNA Name	sgRNA sequence
PSMB5_001144932.23	AAAAACCCGCGCTGGTTCAC
PSMB5_001144932.36	AACAACCACCCTGGCCTTCA
PSMB5_00130725.83	AACATGGTGTATCAGTACAA
PSMB5_001144932.101	AAGGTAGTTATTATAATATA
PSMB5_001144932.107	AAGTACATTCCAAATGACTT

PSMB5 Tiling Library	
PSMB5_00130725.84	AATCTATGAGCTTCGAAATA
PSMB5_00130725.60	ACCACGTGCGGGAGGATGGC
PSMB5_00130725.47	ACCTGCTAGGCACCATGGCT
PSMB5_00130725.29	ACGTAGTAGAGGCCTGGAAA
PSMB5_00130725.52	ACGTGGACAGTGAAGGGAAC
PSMB5_00130725.36	AGAAGGTGGCCCCTGAAATC
PSMB5_001144932.29	AGACCATCACTGAGACTCCC
PSMB5_00130725.78	AGAGCCAGAACCTACAGAGA
PSMB5_001144932.59	AGAGGATCGGCAACATGGCA
PSMB5_001144932.97	AGCCTGGCCGCGCCAGGCTG
PSMB5_001144932.27	AGCGCGGGTTTTTCGGACTT
PSMB5_001144932.9	AGCTGACTCCAGGGCTACAG
PSMB5_00130725.61	AGCTGCATCCACCCTCTTTC
PSMB5_00130725.67	AGGCATCTCTGTAGGTGGCT
PSMB5_00130725.44	AGTCAACCTCTACCACGTGC
PSMB5_00130725.34	AGTGAAGGGAACCGGATTTC
PSMB5_00130725.80	AGTGGAGCAGGCCTATGATC
PSMB5_00130725.19	ATCCGCTGCGCCCCCAGCCA
PSMB5_001144932.90	ATCTGCTGGATCTAGGTCCA
PSMB5_00130725.70	ATCTGTGGCTGGGATAAGAG
PSMB5_00130725.39	ATGCATATGGGGTCATGGAT
PSMB5_001144932.33	ATTTCGATTCCTGGCTCTTC
PSMB5_00130725.24	CAAAGGCATGGGGCTGTCCA
PSMB5_00130725.9	CAACCTCTACCACGTGCGGG
PSMB5_001144932.25	CAAGTCCGAAAAACCCGCGC
PSMB5_00130725.2	CACCATGGCTGGGGGCGCAG
PSMB5_00130725.50	CACCATGTTGGCAAGCAGTT
PSMB5_001144932.99	CACCCCAGCCTGGCGCGGCC
PSMB5_001144932.10	CACCTTCTTCACCGTCTGGG
PSMB5_00130725.30	CACGTAGTAGAGGCCTGGAA
PSMB5_001144932.26	CAGCGCGGGTTTTTCGGACT
PSMB5_001144932.39	CAGCTGCAACTATGACTCCA
PSMB5_00130725.23	CAGCTTCTGGGAACGGCTGT
PSMB5_00130725.8	CAGTCAACCTCTACCACGTG
PSMB5_00130725.79	CATAGGCCTGCTCCACTTCC
PSMB5_001144932.70	CATAGTTGCAGCTGACTCCA
PSMB5_00130725.16	CATCCTCCCGCACGTGGTAG
PSMB5_001144932.19	CATGGCGCTTGCCAGCGTGT
PSMB5_00130725.3	CATGTTGGCAAGCAGTTTGG
PSMB5_001144932.6	CCACACCTTGAAGGCCAGGG
PSMB5_00130725.76	CCACATTGTCACTGGAGACT
PSMB5_001144932.34	CCATGAAGCATTTCGATTCC

PSMB5 Tiling Library	
PSMB5_00130725.18	CCATGGTGCCTAGCAGGTAT
PSMB5_00130725.48	CCCCAGCCATGGTGCCTAGC
PSMB5_001144932.2	CCGCGCTGGTTCACCGGTAG
PSMB5_00130725.21	CGCAGCGGATTGCAGCTTCT
PSMB5_001144932.4	CGCGGGTTTTTCGGACTTGG
PSMB5_001144932.22	CGCTACCGGTGAACCAGCGC
PSMB5_00130725.22	CGGATTGCAGCTTCTGGGAA
PSMB5_001144932.28	CGTGCAGATCTGCTGGATCT
PSMB5_001144932.21	CGTGTTGGAGAGACCGCTAC
PSMB5_00130725.64	CTAACCTCATCTCCCTTTCC
PSMB5_001144932.45	CTATCACCTTCTTCACCGTC
PSMB5_00130725.56	CTATGACCTGGAAGTGGAGC
PSMB5_00130725.14	CTATTCCTATGACCTGGAAG
PSMB5_00130725.59	CTCTACCACGTGCGGGAGGA
PSMB5_00130725.11	CTCTACCCCCTGAAAGAGGG
PSMB5_00130725.32	CTCTACTACGTGGACAGTGA
PSMB5_001144932.8	CTGCAACTATGACTCCATGG
PSMB5_00130725.13	CTGCATCCACCCTCTTTCAG
PSMB5_00130725.1	CTGCTAGGCACCATGGCTGG
PSMB5_00130725.55	CTGCTCCACTTCCAGGTCAT
PSMB5_00130725.65	CTGGCTCTGTGTATGCATAT
PSMB5_00130725.31	CTGTCCACGTAGTAGAGGCC
PSMB5_00130725.26	CTTATCCCAGCCACAGATCA
PSMB5_00130725.5	CTTCACTGTCCACGTAGTAG
PSMB5_00130725.4	CTTTCCAGGCCTCTACTACG
PSMB5_001144932.17	CTTTCTGCCCACACTAGACA
PSMB5_001144932.72	GAGATCAACCCATACCTGCT
PSMB5_001144932.102	GAGCCTGGCCGCGCCAGGCT
PSMB5_00130725.85	GATCTACATGAGAAGTATAG
PSMB5_001144932.94	GATCTGCTGGATCTAGGTCC
PSMB5_001144932.18	GCAAGCGCCATGTCTAGTGT
PSMB5_00130725.7	GCATATGGGGTCATGGATCG
PSMB5_00130725.63	GCCACAGATCATGGTGCCCA
PSMB5_00130725.37	GCCACCTTCTCTGTAGGTTC
PSMB5_00130725.71	GCCAGAACCTACAGAGAAGG
PSMB5_00130725.62	GCCATGGTGCCTAGCAGGTA
PSMB5_00130725.20	GCGCAGCGGATTGCAGCTTC
PSMB5_001144932.3	GCGCGGGTTTTTCGGACTTG
PSMB5_001144932.69	GCTCCACACCTTGAAGGCCA
PSMB5_001144932.71	GCTGACTCCAGGGCTACAGC
PSMB5_00130725.46	GCTGCATCCACCCTCTTTCA
PSMB5_001144932.35	GCTTCATGGAACAACCACCC

PSMB5 Tiling Library	
PSMB5_001144932.1	GGCAAGCGCCATGTCTAGTG
PSMB5_001144932.7	GGCGGAACTGTTAAGATCAG
PSMB5_001144932.95	GGCTCCACACCTTGAAGGCC
PSMB5_00130725.41	GGCTCGACGGGCCAGATCAT
PSMB5_00130725.75	GGCTGGGATAAGAGAGGCCC
PSMB5_00130725.42	GGCTTGGTAGATGGCTCGAC
PSMB5_001144932.37	GGGCTGGCTCCACACCTTGA
PSMB5_001144932.67	GGTCCAGGGAGTCTCAGTGA
PSMB5_001144932.30	GGTCTGAGCCTGGCCGCGCC
PSMB5_00130725.51	GGTGTATCAGTACAAAGGCA
PSMB5_00130725.27	GGTTGCAGCTTAACTCACCA
PSMB5_001144932.41	GTAAGCACCCGCTGTAGCCC
PSMB5_001144932.24	GTGAACCAGCGCGGGTTTTT
PSMB5_00130725.35	GTGAAGGGAACCGGATTTCA
PSMB5_00130725.10	GTGGCTCTACCCCCTGAAAG
PSMB5_00130725.73	GTGTATCAGTACAAAGGCAT
PSMB5_00130725.58	GTTGACTGCACCTCCTGAGT
PSMB5_00130725.77	TAGATCAGCCACATTGTCAC
PSMB5_001144932.20	TAGCGGTCTCTCCAACACGC
PSMB5_001144932.44	TATCACCTTCTTCACCGTCT
PSMB5_001144932.40	TCATAGTTGCAGCTGACTCC
PSMB5_00130725.17	TCCAGCCATCCTCCCGCACG
PSMB5_00130725.25	TCCATGGGCACCATGATCTG
PSMB5_00130725.54	TCGGGGCTATTCCTATGACC
PSMB5_00130725.33	TCTACTACGTGGACAGTGAA
PSMB5_001144932.81	TCTCAGTGATGGTCTGAGCC
PSMB5_00130725.53	TCTGGCTCTGTGTATGCATA
PSMB5_00130725.49	TCTGGGAACGGCTGTTGGCT
PSMB5_00130725.57	TCTGTAGGTGGCTTGGTAGA
PSMB5_001144932.31	TCTTCTGGGACACCCCAGCC
PSMB5_00130725.6	TGAAGGGAACCGGATTTCAG
PSMB5_001144932.68	TGAGCCTGGCCGCGCCAGGC
PSMB5_00130725.15	TGAGTAGGCATCTCTGTAGG
PSMB5_001144932.38	TGATCTTAACAGTTCCGCCA
PSMB5_00130725.40	TGCATATGGGGTCATGGATC
PSMB5_00130725.12	TGCATCCACCCTCTTTCAGG
PSMB5_001144932.43	TGCCTCCCAGACGGTGAAGA
PSMB5_001144932.58	TGCTGAGAGGATCGGCAACA
PSMB5_001144932.42	TGCTTACATTGCCTCCCAGA
PSMB5_001144932.104	TGCTTGAAACCTAAGTCATT
PSMB5_00130725.45	TGGCTCTACCCCCTGAAAGA
PSMB5_00130725.38	TGGCTCTGTGTATGCATATG

PSMB5 Tiling Library	
PSMB5_00130725.43	TGGCTTGGTAGATGGCTCGA
PSMB5_001144932.5	TGGGACACCCCAGCCTGGCG
PSMB5_001144932.80	TGGGGGTCGTGCAGATCTGC
PSMB5_001144932.82	TGGGGTGTCCCAGAAGAGCC
PSMB5_00130725.28	TGGTTGCAGCTTAACTCACC
PSMB5_001144932.57	TGTGGGTGTGCTGAGAGGAT
PSMB5_00130725.66	TGTGTATGCATATGGGGTCA
PSMB5_001144932.78	TGTTTTGTGGGTGTGCTGAG
PSMB5_001144932.105	TTGGAATGTACTTGTTTTGT
PSMB5_001144932.32	TTTCGATTCCTGGCTCTTCT
PSMB5_001144932.98	TTTGGAATGTACTTGTTTTG
PSMB5_00130725.82	TTTGTACTGATACACCATGT

Safe Harbor Library	
sgRNA Name	sgRNA sequence
SafeHarbor.1	GGCTAAATTCCTCTTATTCA
SafeHarbor.2	GTAACCAAGAGTCAGGACTG
SafeHarbor.3	GGGATAATATAAGGCATTCT
SafeHarbor.4	GGATCTTATAATCTAGTTAT
SafeHarbor.5	GTTAATGCCTTGGTCAAATG
SafeHarbor.6	GTGTAAACTAAGACCTAAGT
SafeHarbor.7	GCTAAAGTTGTCATTGATTT
SafeHarbor.8	GTGCTTCCGACAAACTACAA
SafeHarbor.9	GGAACGTAGGTAATAAGGTC
SafeHarbor.10	GATTCTTCATATCTTTCTCA
SafeHarbor.11	GCTCATGAGACACTTCACAG
SafeHarbor.12	GTCAGCATTAAACATGCTTA
SafeHarbor.13	GTGAAAGTTCTCATCTTCTT
SafeHarbor.14	GCATGAGAAGAGGAGATTGA
SafeHarbor.15	GACTGTTCATAGGACCCTAA
SafeHarbor.16	GCCCTGTCTGTATCCAGTCC
SafeHarbor.17	GGGATCTTTCAGTGTAGGTA
SafeHarbor.18	GATTCTGTATAATGGAAATC
SafeHarbor.19	GACATGTCCTAATTGTATGG
SafeHarbor.20	GTGTGCTTTGAAGAATAATG
SafeHarbor.21	GCAATATGATCTCATTTGTG
SafeHarbor.22	GAGTTTAGAGGTTTGAGATT
SafeHarbor.23	GTGGTCCTGGACTGGTCTCA
SafeHarbor.24	GTTATGCCAACACATTTGTA
SafeHarbor.25	GTTACATACAAAAATTGGAT
SafeHarbor.26	GCATATTATCACTCCAGTGA
SafeHarbor.27	GACATTGGGATTAAATTTGG
SafeHarbor.28	GGTGGCCGCCATCATGGCTG

Safe Harbor Library	
SafeHarbor.29	GGCAGATCAGAATGTGAGCT
SafeHarbor.30	GAGGAAGGAGTTATATTGAC
SafeHarbor.31	GAGCCAAAGATAAGCATGAG
SafeHarbor.32	GGCTACTCAGATATAGTCAT
SafeHarbor.33	GTTATTTGATGAGCAGCTAT
SafeHarbor.34	GACGTAGTAAGGTAGAGACA
SafeHarbor.35	GTGATGAAGAGTGCTACAGC
SafeHarbor.36	GCTAGGGACTTCAAAGTTAT
SafeHarbor.37	GATATCTTCCCAATGATGAC
SafeHarbor.38	GAGTAGTTTCTGACGTCCGA
SafeHarbor.39	GAGCATAATGAAGGTTCTTG
SafeHarbor.40	GCGTTTCCAATCCCAGAGAG
SafeHarbor.41	GGCCTAATAGCTTTGGTAGA
SafeHarbor.42	GACAGGAGGAACTTGTAACC
SafeHarbor.43	GAGAGCACTCAGCAAAATCA
SafeHarbor.44	GCGTTGGTGAAATTACAATT
SafeHarbor.45	GTTAATGATCAAAAGTTACA
SafeHarbor.46	GAGAGAATTGCTATTCTGAG
SafeHarbor.47	GATTGTATGAAAACATAGAT
SafeHarbor.48	GGCTACCTGTCTATTGGCAC
SafeHarbor.49	GGCATGTGTGTCTGAATACA
SafeHarbor.50	GCTGAAGCTCTGGCAAGAGC
SafeHarbor.51	GTACCTTAATCACACCTTTG
SafeHarbor.52	GTTCACATAGCAGTACTTGT
SafeHarbor.53	GACTGACCTTTCTTTGAGAG
SafeHarbor.54	GACTTGAATGATCAATTACT
SafeHarbor.55	GTTCTGAGTTACTGGAACCC
SafeHarbor.56	GCAAGATCAGGTAAGTATCT
SafeHarbor.57	GTCGTGAAGCTGTGTTTGAC
SafeHarbor.58	GGTCTTGAAATAAAATTTAG
SafeHarbor.59	GACTGCTTCTTAGTTAGGTA
SafeHarbor.60	GGAAATCCTTGAGTTTCAGG
SafeHarbor.61	GCCCAAGCAGGCTACATTGC
SafeHarbor.62	GAGGTGGCAAAGAATGTGCC
SafeHarbor.63	GTTCAAATAATAGGGTGCAT
SafeHarbor.64	GAGGGGATACTCAAGCTAGG
SafeHarbor.65	GGGTATCAGCTCACCTCCTC
SafeHarbor.66	GAAGTACTGGCAATGCAACT
SafeHarbor.67	GACATAGCCTGCAATTGTTT
SafeHarbor.68	GGGCAGATTGGAAGAGCCCT
SafeHarbor.69	GTGTACAACATCACAGCATA
SafeHarbor.70	GGGTGGTTCTGAATGGGAGC
SafeHarbor.71	GCTATCCTTAAATTGGCCTG

Safe Harbor Library	
SafeHarbor.72	GCCTGAATATAGTGAAAGTC
SafeHarbor.73	GGGAAGTCCTGGGGTTTGAT
SafeHarbor.74	GTCAGTTATTCTTTCCTCTA
SafeHarbor.75	GCATGGTCACAATAATCTTG
SafeHarbor.76	GGGAGGATAAGAGACACTTT
SafeHarbor.77	GCTTATTTAGTTTGGTTCAA
SafeHarbor.78	GTCTCTACTAGAACTCAATC
SafeHarbor.79	GGAGCTTGGTATCTAAAATT
SafeHarbor.80	GATGTTCACTGTTAATTGAT
SafeHarbor.81	GCTACTTAAATCATTGCCAT
SafeHarbor.82	GCACTTCACCTGAGAAAAAC
SafeHarbor.83	GCTTGCTTGTCTCTGTTTCG
SafeHarbor.84	GTCAACAGCAAGGCTACTGA
SafeHarbor.85	GACAGAAGAAGCTAGAAGTC
SafeHarbor.86	GTACAACCCAAAGTATATGG
SafeHarbor.87	GAATCCCGGGCTTTCTCTGT
SafeHarbor.88	GATAATTTCAGGAGTGAGAT
SafeHarbor.89	GTATTGTGATCAAGTAATTT
SafeHarbor.90	GAACCTAAAAATATAGTTGT
SafeHarbor.91	GCATTGGTGCCCAGTAGGAG
SafeHarbor.92	GAATACTGTGAGAAATTTCA
SafeHarbor.93	GTCAAGATATACCTAGCAAA
SafeHarbor.94	GACCTCACTTACTGTTGCCA
SafeHarbor.95	GCATACCATAGGGTAAAGGC
SafeHarbor.96	GGTGACAATCAAACTGGCAA
SafeHarbor.97	GGTATTGTCAATGTAAAAAG
SafeHarbor.98	GCACAGTAAATATACGTGTG
SafeHarbor.99	GTGTGCCCCTCCAAAAGAGA
SafeHarbor.100	GACATATGCTATGCAGAGTT
SafeHarbor.101	GTAAGAATCAAATCATCATG
SafeHarbor.102	GGAAATTGCTTCTGGTTTAT
SafeHarbor.103	GTAGATGAGCTCTTATCAGT
SafeHarbor.104	GGCTTTGTTCATGACTTTGA
SafeHarbor.105	GCACCAGTCTATGCCACCAC
SafeHarbor.106	GTAATGACTTGGGGGAGATA
SafeHarbor.107	GAGTCTGTCTCTAATGAGAC
SafeHarbor.108	GTGGTCCACAGACAATGCAT
SafeHarbor.109	GGTTAAGAAAAGACACTCAG
SafeHarbor.110	GGTAATCATAAGTTGTATAA
SafeHarbor.111	GGCCCTCCTTAGAAGTTGCA
SafeHarbor.112	GAAATTGGTCCCCACCTTCA
SafeHarbor.113	GTCCAAGAACAAAGCAAAGA
SafeHarbor.114	GATGAGCCAATCTTTAGCAA

Safe Harbor Library	
SafeHarbor.115	GTGAATCAAGAAGCAATGTC
SafeHarbor.116	GAAAGGCAGACATGGCTAAA
SafeHarbor.117	GACAAAAGCAGAATACCAGA
SafeHarbor.118	GCACACAAAATATCGTTATT
SafeHarbor.119	GAGAAAGGCCCAGCTCTGAT
SafeHarbor.120	GCCAGTCTACCCACTGTCCC
SafeHarbor.121	GCAGGGTGAAGGTCCTCCTC
SafeHarbor.122	GAAGAGACTACAATTATTCT
SafeHarbor.123	GATATCCTTTGTGTTAACTT
SafeHarbor.124	GAATGACTCGCATGACTTTA
SafeHarbor.125	GGATGTTCAAACCTTCAAAA
SafeHarbor.126	GAGAATATATGTTTCCATTA
SafeHarbor.127	GGAAAAGTAATGAATCATAC
SafeHarbor.128	GTTACACGAAGCACAGGGTG
SafeHarbor.129	GAACTAGGTGCTCAAGGAAT
SafeHarbor.130	GGCAAAGACCAGTCTGATAC
SafeHarbor.131	GTCTAGTTTCACAATAATTT
SafeHarbor.132	GCTTTATATAAGATATGAGA
SafeHarbor.133	GCATAGGATATTATATTTCG
SafeHarbor.134	GACCTTGACTGCTCCTGAAC
SafeHarbor.135	GCAGCTCCCTAGTTCACAGA
SafeHarbor.136	GTCTGACCAGAGGTGGAGAG
SafeHarbor.137	GAATCACATTGTACCACAAA
SafeHarbor.138	GACAAAATTGATACAACAGC
SafeHarbor.139	GAATTCCAAGACTTCACATT
SafeHarbor.140	GACAGGGACCGCCATCCACT
SafeHarbor.141	GTTGTATGGTTCCTAAGGAT
SafeHarbor.142	GAATATCCACTACTAGCTTT
SafeHarbor.143	GCCATTAATCATGATCTGGA
SafeHarbor.144	GGTGAATAGGTAGGTATTGA
SafeHarbor.145	GCTCATCAAAGGTAGTAAAC
SafeHarbor.146	GGGACCCAGCCCTTGGGCTG
SafeHarbor.147	GTGCACCTTTCTATAAATGT
SafeHarbor.148	GACTTCATTAAAAGCAGTCT
SafeHarbor.149	GTTGAACTTGTGAACACAAA
SafeHarbor.150	GGGTCCTCACCAGGAAATTT
SafeHarbor.151	GTAGCCTATTGGCAATTGGC
SafeHarbor.152	GCATAAATAAAATCGATTCC
SafeHarbor.153	GAAGGGCAATAATTGGTACA
SafeHarbor.154	GAGTTCTTAATAACATTCTA
SafeHarbor.155	GCTTTCTACTTGCCTTAGAT
SafeHarbor.156	GCTTCTTATTTCTCTCCAGT
SafeHarbor.157	GCATTCTGTCCTAATAAGAA

Safe Harbor Library	
SafeHarbor.158	GCTTAAGCTAGTTTAAAGAA
SafeHarbor.159	GGTTTCCAGTGTTTATCTGT
SafeHarbor.160	GAGAGTCTAGGTACGTTCTC
SafeHarbor.161	GCTTTCAAGTTAACATAGCT
SafeHarbor.162	GTAAAATGAACCGAGCTTTA
SafeHarbor.163	GTAAGATTATTAACCCCTTC
SafeHarbor.164	GGGTCCTCACGATAGAAGAA
SafeHarbor.165	GATTACACTCAAGAAAGCGA
SafeHarbor.166	GATGTAGACGTAGAAGTGAT
SafeHarbor.167	GTGAGTTACAGAAATTAGCA
SafeHarbor.168	GCAGGGGGACACGGGCACAT
SafeHarbor.169	GACAATTGTGTTGCAGACAA
SafeHarbor.170	GTCAATGGGAAATTATAAAC
SafeHarbor.171	GAGTTATAGCACACTTAGAA
SafeHarbor.172	GATTGAAACCAGAAAATAAG
SafeHarbor.173	GGAGTCTAGTGATAGGGGTA
SafeHarbor.174	GGGATAGTCTTAGAAGGCTT
SafeHarbor.175	GTCAATTGATTCACTGGAAT
SafeHarbor.176	GTATTCCTGCAAGATAATTC
SafeHarbor.177	GGTCAAGCAACAGGCATAAT
SafeHarbor.178	GACATCCATAACTTCCTAAC
SafeHarbor.179	GTCAAACAAAAGCGTCTATA
SafeHarbor.180	GCTAGATTAATATGAATGAG
SafeHarbor.181	GAACCCCATAGGAGGTTTAG
SafeHarbor.182	GCCTCTTTCCCCTGCCGGCA
SafeHarbor.183	GGTAAGGGCTGCTTATCTTT
SafeHarbor.184	GTATTCAGTATAATCAAGGA
SafeHarbor.185	GTTGTCTTATGGGACTGCAT
SafeHarbor.186	GTATACGATATGATTGACTC
SafeHarbor.187	GGTAGAGACAAAATATATTT
SafeHarbor.188	GTACCTATGTCCTTGAGGCT
SafeHarbor.189	GGCAAAAGAACGTCTGTAAT
SafeHarbor.190	GGACTAGTTTACCTAGGGAG
SafeHarbor.191	GGAGGGTGGAGCAAAGAAAG
SafeHarbor.192	GAGCCATATTATGTCCTTTA
SafeHarbor.193	GTGCACTCTATGCACCAAAG
SafeHarbor.194	GGTCTCCCGAGTCATTGTTG
SafeHarbor.195	GCAATCATTCTGGTTCAGGC
SafeHarbor.196	GCACAGGTTCCCCTCCTAAC
SafeHarbor.197	GATCAGGGAATCTTTGAGAA
SafeHarbor.198	GAACCCAGCTGTCCTCGCTG
SafeHarbor.199	GCTAACTGTGTTACAAGCAG
SafeHarbor.200	GTGATCAAAGAGAGAGGTGT

Safe Harbor Library	
SafeHarbor.201	GGAAAGCCCGTTGTATTTAT
SafeHarbor.202	GGTCCCCCACTTTCTCCTTG
SafeHarbor.203	GCCAGATGACCATAGAAACT
SafeHarbor.204	GGTGCAATCCAAAGGTGGGC
SafeHarbor.205	GTGTAAAATCACTTTAAACT
SafeHarbor.206	GTCACATGTTCAAGTTTAAC
SafeHarbor.207	GAAGCTTAGTCCTGAATTGT
SafeHarbor.208	GGGTCTGTTTCCTTGTGTTA
SafeHarbor.209	GATAGAGACTGGATGAAGTT
SafeHarbor.210	GCAACAAGGCAAATGTGGTA
SafeHarbor.211	GCTATTTAGCTCAACCTTGT
SafeHarbor.212	GTGCCATTATCATTTCCTCA
SafeHarbor.213	GCAAATAGAAGAGACAATCT
SafeHarbor.214	GAAAATATATGGACTGGGAT
SafeHarbor.215	GAATAGAACTCCTGCCATCA
SafeHarbor.216	GCTTTCTACCTGGATGTTTA
SafeHarbor.217	GCTAACTTGAGGGCAAAAGA
SafeHarbor.218	GTGGTAAAAATGTGCTTTGT
SafeHarbor.219	GAGCCTCAGCTGGTGCATGG
SafeHarbor.220	GCCTATGCCGCAATACCCTC
SafeHarbor.221	GACCTGTGTAAACCAGCTAA
SafeHarbor.222	GACCTCATTCCTGAGTGTGT
SafeHarbor.223	GTGTTTGCCTCATAATAACC
SafeHarbor.224	GACTGGGCATACAGCCATTT
SafeHarbor.225	GGCATACTACATTGGCTTTA
SafeHarbor.226	GCAAACATATTGGAGTACTG
SafeHarbor.227	GGGGAGTAGGGAAGAGCTTA
SafeHarbor.228	GGGCTCGTATGTCGTTCTTC
SafeHarbor.229	GTGCCTTATCTATTTCCACA
SafeHarbor.230	GGTAATTACCTGCTCTCTGC
SafeHarbor.231	GTCTGATAACTTGTGTTACT
SafeHarbor.232	GACTGAGTTAATAATAGCGG
SafeHarbor.233	GAATATTGTGCACTGTATTT
SafeHarbor.234	GTTTCTAAATGTGATCTGTG
SafeHarbor.235	GCACACTGGCTAGTTAAGGA
SafeHarbor.236	GGAGGAGTGTGCAATGAAGC
SafeHarbor.237	GAGGACGGGTGGGAAGTTAG
SafeHarbor.238	GATACTGTAGCAGTTACTGA
SafeHarbor.239	GATTCTAAGCAAAGGACAGA
SafeHarbor.240	GGAGCTTAGACCATATTTGG
SafeHarbor.241	GTGTCCGTGGGTCTGTTCCC
SafeHarbor.242	GCAATAGCTGTGAGCTCATA
SafeHarbor.243	GGGATGGGCCATCCAGCTGT

Safe Harbor Library	
SafeHarbor.244	GACAGATTACTTAATAAAAG
SafeHarbor.245	GTGGCAAGGTTAAGTACAAT
SafeHarbor.246	GGAGGAAACAGAATAATGGC
SafeHarbor.247	GTGAATTAATGTCATTTCAC
SafeHarbor.248	GTGAACTAGAACACTGAGAG
SafeHarbor.249	GATGCTGTGGCCAATGTGCA
SafeHarbor.250	GACTGTAAGCATTCCTGACA
SafeHarbor.251	GTCCTAATTCCATGCCTAAA
SafeHarbor.252	GTGGGTTCGTTGTCTACTAC
SafeHarbor.253	GAGACTATTAGATCGTATGT
SafeHarbor.254	GGTGTAGTATCAAAAATTGA
SafeHarbor.255	GATAGCTCTTAAGGATAAAT
SafeHarbor.256	GATTCAGTCACATCACAATA
SafeHarbor.257	GTCTAAGAAAGACTTCTAGG
SafeHarbor.258	GATTTGGGTCTTTGCGCATC
SafeHarbor.259	GACCTTAAAGTTATAGTTAA
SafeHarbor.260	GCTCTGCATCTTTCCCCAGG
SafeHarbor.261	GACCTAAGTTTGAGAATGAG
SafeHarbor.262	GAAAGTACATTCATTAGCAT
SafeHarbor.263	GGAGAACGTGGTGATAAAGC
SafeHarbor.264	GGCAACATGGCAAAATAGTT
SafeHarbor.265	GATAATAGCAGAGAGAGGTG
SafeHarbor.266	GGACTTTAAGGAATTCAGCT
SafeHarbor.267	GAATATTGGGGGGTGGATGG
SafeHarbor.268	GGAGTAAGTATGTGTGTTGA
SafeHarbor.269	GTATTGGATAAGGGAGCTCA
SafeHarbor.270	GTGAGTTGGGAGATGTACTG
SafeHarbor.271	GTTTACAATTTCATTTGTAC
SafeHarbor.272	GTCCATTCAATTTGGACATG
SafeHarbor.273	GAGTGCTTACTGGGAATGAG
SafeHarbor.274	GCTAATTGTTCAAAAAGCCC
SafeHarbor.275	GCTTTCAAGAGTTTATTTGA
SafeHarbor.276	GATATTCTGTGCAATCTGTT
SafeHarbor.277	GTGTAGGACTACGCTGGCAC
SafeHarbor.278	GTCTTAAAGAGTAAAGTACA
SafeHarbor.279	GTTAGACTGCAAACACCCAC
SafeHarbor.280	GCCTAGGAGAAGCCCTGGCA
SafeHarbor.281	GTCGAGTATTTCTAATCTTT
SafeHarbor.282	GAATCTGAGACATCATTCAT
SafeHarbor.283	GACAAAAGATTATGCTTCCC
SafeHarbor.284	GAGAATTACATTCATGATCT
SafeHarbor.285	GAACTGAGCTTCTACCATGC
SafeHarbor.286	GGTAAGATTGTAATAGCTTG

Safe Harbor Library	
SafeHarbor.287	GTCAGAAATGATCTCGTCCT
SafeHarbor.288	GACATATCTAAGAACTGAGC
SafeHarbor.289	GCTTCAATATGACAGAACTC
SafeHarbor.290	GGAGAGCAAATCAGCATATC
SafeHarbor.291	GCAAAATAGCCGCACAGAAA
SafeHarbor.292	GCATATTTCTATACAATACA
SafeHarbor.293	GATGCAAATTCATGGTGGTA
SafeHarbor.294	GAACTGTAATAGTCTTGAGC
SafeHarbor.295	GAACTCACTACATTAAGGCT
SafeHarbor.296	GAGGTAAATCAGTACAAACA
SafeHarbor.297	GTTGTTTCTAAGATTAAAAG
SafeHarbor.298	GTGGTAGTCAGTTTCACAAA
SafeHarbor.299	GGTTTCAAATAGTTGGATCA
SafeHarbor.300	GAATATGAAAGACATCATAA
SafeHarbor.301	GAAGTAGGAAGGAGATTGCC
SafeHarbor.302	GGAAAAGTGCTGTTTGCATT
SafeHarbor.303	GAGCATTAGGCTGGGGCCTT
SafeHarbor.304	GTCTAGGTATGATTAGAAGA
SafeHarbor.305	GAGTTATAATCTTCAGAAAA
SafeHarbor.306	GCTGTAATGAGACTTCAGCT
SafeHarbor.307	GTGTGCAATCTGAAGGAAAT
SafeHarbor.308	GTGATGAGGTCGCTGAAGTT
SafeHarbor.309	GTGGAGCCCTTATAACCCTG
SafeHarbor.310	GTTGGATTATTTCTTCTATA
SafeHarbor.311	GGATTTCTACATTATATACT
SafeHarbor.312	GCTAATGTAGATCAAGTTAT
SafeHarbor.313	GATTGCAAGAGACTGAACTC
SafeHarbor.314	GGGTGAACTTGAGTGAACTT
SafeHarbor.315	GGGCTCAAATCCCTATAATT
SafeHarbor.316	GATAGAAGGTATTAACTCCC
SafeHarbor.317	GGCTATAAGCACAAATGTAA
SafeHarbor.318	GATTCCCATTGCATGCCAGT
SafeHarbor.319	GCAAATTACAATTATGTTTC
SafeHarbor.320	GAATTAAATTCACTTTGAAC
SafeHarbor.321	GAGCAGACAGGAAATAAAGC
SafeHarbor.322	GCCCACCAGTCCTTCTCACT
SafeHarbor.323	GTTAAGAAGTGAAAGAAATT
SafeHarbor.324	GTTGAATTGAATGGGTCATT
SafeHarbor.325	GTAGACACAAACTTGTGTAA
SafeHarbor.326	GAGCGTACTATATTCTTAAA
SafeHarbor.327	GGTGGTACATCGTTGAAGGA
SafeHarbor.328	GATGAACTCCCAATCACAGG
SafeHarbor.329	GTATAAATAAGGATAAGGTA

Safe Harbor Library	
SafeHarbor.330	GGAAATAATCTTGGAACATA
SafeHarbor.331	GGTAGTTAATCTTCTACTTT
SafeHarbor.332	GAGAAGAGAACATTCTAGTT
SafeHarbor.333	GTCGGAGCTCAGTGTTGCAT
SafeHarbor.334	GAAGAGACATGTTTCAGTGA
SafeHarbor.335	GTCATATCTGACTTAAATTG
SafeHarbor.336	GGAGAATATGCTAAAAGCGT
SafeHarbor.337	GATTGTTGTAGTAGAATAAA
SafeHarbor.338	GTAAGCAGCACCACCACTTA
SafeHarbor.339	GTCTTGTGCTGACATGCTCA
SafeHarbor.340	GCAGACTTTATTAGCTAGTG
SafeHarbor.341	GAGGTATTTGATATGACTCA
SafeHarbor.342	GCAGGTTGCCCATTCTCCCA
SafeHarbor.343	GAGGGGACGTTGACCTGTGG
SafeHarbor.344	GAACCCAAGGATTTATAAAG
SafeHarbor.345	GTGTTCAGGACATGTACTCA
SafeHarbor.346	GGTGATGATAGTCAAATACC
SafeHarbor.347	GCTTTACAGCTAATTTCTAA
SafeHarbor.348	GGTATCTACATTAACACTCA
SafeHarbor.349	GACAGTTTGCTTACTATGGA
SafeHarbor.350	GAAAAACTCTTAGCTTAATG
SafeHarbor.351	GTCATCTTAACTTCAGTAGA
SafeHarbor.352	GATCACTGGTAGGCCACAGT
SafeHarbor.353	GAGAAAGGCAAGTGCATCAA
SafeHarbor.354	GAACTGATAAAGATTCAGTA
SafeHarbor.355	GCCATTCAAAAGCAGCTATA
SafeHarbor.356	GACAGAACTTCTTTGAGCTA
SafeHarbor.357	GGGTGACATTGAAATTTAAC
SafeHarbor.358	GACTATAAACTGCACACTAT
SafeHarbor.359	GCTATGGTGGGAAAGCTCAT
SafeHarbor.360	GACTAACTTGCTAATGGCTA
SafeHarbor.361	GAGAGTCACTTCAAAGTGTG
SafeHarbor.362	GAGTGTATTTGTGGACAATA
SafeHarbor.363	GAAGAATTAGGGTTCCATTT
SafeHarbor.364	GAGGAGTGGCACTTTATACT
SafeHarbor.365	GAAGGATGCAGTAGCCATTG
SafeHarbor.366	GTGCATTGTTGGTGGTTGTG
SafeHarbor.367	GAGAAGTTATGCAAATTTAT
SafeHarbor.368	GAAATAGATTGGCAGAGTGT
SafeHarbor.369	GTGGGGTGGGCTCCCTGCCT
SafeHarbor.370	GTCTCTAACAAGACTGAAAT
SafeHarbor.371	GCAGAGTAGATCTACATCTT
SafeHarbor.372	GTGCCAGCTAAGATGAAATT

Safe Harbor Library	
SafeHarbor.373	GATGGTGATGCACCAACTTT
SafeHarbor.374	GAAGTGTTGCCATTCAATTC
SafeHarbor.375	GAGAGAGTTGGAATAAGCTA
SafeHarbor.376	GAGGGTACTTATTTCAACTT
SafeHarbor.377	GCTACATGTTCTAGAATACA
SafeHarbor.378	GAGAAATCTCTTTGAGCTGG
SafeHarbor.379	GGCTTTGTGTCTGACTTTCC
SafeHarbor.380	GGATTAGATCAATTATTCTA
SafeHarbor.381	GATTCTGGAAATAAGTACCT
SafeHarbor.382	GAGATAAAATTGCGAGACCA
SafeHarbor.383	GACAAAATTTAGCAACTCAG
SafeHarbor.384	GCAGATACTCACCATTACCC
SafeHarbor.385	GGTGATTGTTGCAGCTGTCA
SafeHarbor.386	GATAGACTTGTGAAGGAAAC
SafeHarbor.387	GAGTCACTGGATTGTTGTCC
SafeHarbor.388	GGATTATATGGGAGGTACAC
SafeHarbor.389	GCTTAAAAATACTATCTGCT
SafeHarbor.390	GACAAGGAGGACCAAAGTTG
SafeHarbor.391	GGCAGTGATTTACTCCTATC
SafeHarbor.392	GATCTTCCAGGACTGTTAGA
SafeHarbor.393	GAAACAAGCTAATATTATCA
SafeHarbor.394	GTCAGTCTTTACAAATCACT
SafeHarbor.395	GGCAGTTGAGTAAACGTAAG
SafeHarbor.396	GCCTCTACTGCTAACTCTAT
SafeHarbor.397	GTTGTAATTTAAAGCACTCA
SafeHarbor.398	GCATAAAGAGAACAAGCAAT
SafeHarbor.399	GGTAGTTGGTCTAATCAGTA
SafeHarbor.400	GGCTAACACCTGCCAACTTT
SafeHarbor.401	GTCTAATCTAGCATCAAACT
SafeHarbor.402	GAGAGAGACTATTTCAGGAT
SafeHarbor.403	GACCTAGACCAAGCTACGAA
SafeHarbor.404	GTTACTGATACCAGTCCCTG
SafeHarbor.405	GCCCTACTGTGGTAACTTTG
SafeHarbor.406	GTGTAAAGGAATCTTAGCTT
SafeHarbor.407	GGTGAGACTATTATATTTAT
SafeHarbor.408	GCTTCAGAGAACTATTTGGT
SafeHarbor.409	GATGTGTTCGTTGAGGCATA
SafeHarbor.410	GTTGACTCTAACTATAGAGT
SafeHarbor.411	GGACAGCCATTGAAGATATG
SafeHarbor.412	GATGGAGAGCCTGGAGCATA
SafeHarbor.413	GCATGATTAAAGGTGAGCAT
SafeHarbor.414	GGAACCCACAGATATAGCTA
SafeHarbor.415	GCATAGCTTCAGAGTTCAGA

Safe Harbor Library	
SafeHarbor.416	GAGAAAAGACGTGTATTTCC
SafeHarbor.417	GCTAGAGCTTCCTTATGTTT
SafeHarbor.418	GATGGGCAGTCAGGACTACG
SafeHarbor.419	GTTCTGCATGAGAAGCACTA
SafeHarbor.420	GACTCCACCTATCTCAAAAT
SafeHarbor.421	GATATTTGACAGTGGATAAA
SafeHarbor.422	GAAAGATTATGGATCATAGT
SafeHarbor.423	GCATCAATGTACACTGTGGC
SafeHarbor.424	GCAGCAAGCTATGGTCCATG
SafeHarbor.425	GGTTGTTTGAATTAAAGACT
SafeHarbor.426	GAACCCCTGGCTAGTTTCCC
SafeHarbor.427	GGATAAAGAGTGAACCTGTA
SafeHarbor.428	GTAGATTTCACTAAATTGTT
SafeHarbor.429	GTGTAGTTAGAATAAGAAGG
SafeHarbor.430	GTGGCAATGTCCTGGAGAAA
SafeHarbor.431	GTGAAGTGCTTTATCTGTAC
SafeHarbor.432	GAGTTTATATAGGTATGAAA
SafeHarbor.433	GACCTCATAAACAAATCACT
SafeHarbor.434	GAAACGTCTGTATGCAAAGC
SafeHarbor.435	GGTGTGGTGCAAGGGTGAGT
SafeHarbor.436	GAGAATCTGCTATTGCCAAT
SafeHarbor.437	GTACTAAGTATCTTGAAATG
SafeHarbor.438	GTCATGACATGAGTTGCATG
SafeHarbor.439	GCAGTGATCAGAGACAGTTG
SafeHarbor.440	GGCAAAATAACTTCATCTAT
SafeHarbor.441	GCCTGGCCTTCTGTGGAATT
SafeHarbor.442	GGTGGCCTTTGTTTGCAGGC
SafeHarbor.443	GAGATGGTATATTTGTCAGA
SafeHarbor.444	GGGACACCCAGCATCTCAAC
SafeHarbor.445	GTATATGACAGTAGGGTTGG
SafeHarbor.446	GGACCCCAGAACTGAAATCA
SafeHarbor.447	GGGCACCACTGAGAATGTAT
SafeHarbor.448	GGGACTACAAATATGAAAAA
SafeHarbor.449	GTAAAATTATGAGCTCCAGT
SafeHarbor.450	GATTGTGAGTGATGAGAATC
SafeHarbor.451	GAGACTGAGGGTTGCTCTTA
SafeHarbor.452	GCATAGAGTGAACACTTTGG
SafeHarbor.453	GAAGTTCTCCTTTAACCAAT
SafeHarbor.454	GACCTTGACCAAAGATATTA
SafeHarbor.455	GTGTGGGCAAGAGACAGTCC
SafeHarbor.456	GTTGGGGGCTCTCTTGCCAC
SafeHarbor.457	GGATAAAACTCTAACAGAAC
SafeHarbor.458	GGAAACATATTACCCCTCCA

Safe Harbor Library	
SafeHarbor.459	GCACTATTACTCCACTGAGA
SafeHarbor.460	GTGAGCAGAGATCACCTTAG
SafeHarbor.461	GGGTTCATATAGGTCGGAAT
SafeHarbor.462	GTGCCCCCGATTCTTCCATG
SafeHarbor.463	GGAACAAAATTTGCACATAA
SafeHarbor.464	GAGAAAGTCCAAGGGTAAAA
SafeHarbor.465	GCAATTAACTCTACAAGGAA
SafeHarbor.466	GTTTCAACCATTAGGGGGCT
SafeHarbor.467	GGCAGGGGTAGTAAGCTTAG
SafeHarbor.468	GTACACATCTTCCCAATCAG
SafeHarbor.469	GTTACTTGGAAAAATGACCA
SafeHarbor.470	GTACCCGGTAAATCATAGAG
SafeHarbor.471	GTGTATTATCCTGCATTCCA
SafeHarbor.472	GGGTAAAACAAATGCATCAT
SafeHarbor.473	GTGTGTTGGCCTAGGGATGA
SafeHarbor.474	GGTGTGATAAAACCTCAGAG
SafeHarbor.475	GAGCTAATTGGTCAGATTCT
SafeHarbor.476	GTACCAGAGTACAGTGTCCG
SafeHarbor.477	GGTCAGTGCTCTATCATTTA
SafeHarbor.478	GTTGCCTATCTTCAGAGTAC
SafeHarbor.479	GAAGATGCATGGACCTACCA
SafeHarbor.480	GAATAGACACTGGTTCTCTG
SafeHarbor.481	GTCAGCTCTTAACATCTGGT
SafeHarbor.482	GATAACAAGGCTCAGAAGGC
SafeHarbor.483	GTCAAAACACAGTGAGCTGT
SafeHarbor.484	GAGAATATAGCTGAAGGTGG
SafeHarbor.485	GGGATTGACCATCAATACAG
SafeHarbor.486	GAAACCCCCATCTCAGTCTT
SafeHarbor.487	GTACAGATACCACTATTTGG
SafeHarbor.488	GAGTAGCTAGAGGCACTCTT
SafeHarbor.489	GAGATTTGCAGTGCATGAAT
SafeHarbor.490	GTTCAACTAAAGGTCTTATG
SafeHarbor.491	GTGTTTCACTGTTCTCTTCA
SafeHarbor.492	GTGAAGTAGAGATTATGTAA
SafeHarbor.493	GTCAAACCAAGTTGAATTCA
SafeHarbor.494	GATGCTAAAAATCTAAACCT
SafeHarbor.495	GGCCCTTATTACCAGATTTG
SafeHarbor.496	GTGGAGATTTGCTTACGAGC
SafeHarbor.497	GAACCTTGGAGAATTGAATA
SafeHarbor.498	GATAGAAAAGAGCAGCTACA
SafeHarbor.499	GCAAGAAGAAACTGCTATTA
SafeHarbor.500	GTAATGTTGCCGAAGCAATT
SafeHarbor.501	GAATTTCATTACAGGAAGTA

Safe Harbor Library	
SafeHarbor.502	GAAAACACACCTTATCACAG
SafeHarbor.503	GTTATCTTTGAGAGAACATT
SafeHarbor.504	GAACTCTTAAGGTTAATAAG
SafeHarbor.505	GAACCATCCATCCTCACCTG
SafeHarbor.506	GGAGATGCACTGGTAAAAAG
SafeHarbor.507	GCTCATCTCCACAGCCATCC
SafeHarbor.508	GAGTGGCCGGTGCCATTTCT
SafeHarbor.509	GCTACTAGCGAAGAAGAAGG
SafeHarbor.510	GTAAGCTTAAAACATTAGTA
SafeHarbor.511	GTTTACAGGAAGGAGAAGGA
SafeHarbor.512	GTAATATTTGAGGTATGAAT
SafeHarbor.513	GATGGCTCACACTTGCTGTA
SafeHarbor.514	GAAACTGGGAACAAGCTTTA
SafeHarbor.515	GCTAATGCTTTGCCTACCCC
SafeHarbor.516	GCCTTACCCTCAGTAGTGAA
SafeHarbor.517	GAACTGAAGTTTAGAAGTAA
SafeHarbor.518	GAAATATCATGATGGTGAAG
SafeHarbor.519	GTGTTGATTCTGAACAAGTT
SafeHarbor.520	GGCCCTGTCCTGGACATAAA
SafeHarbor.521	GCACATTCTAATTTGTGGAT
SafeHarbor.522	GAAGTTAACATGGAATTAAA
SafeHarbor.523	GTCCTTAGGCTTGCAATGCT
SafeHarbor.524	GAGAGACAATTTGGGTCTAG
SafeHarbor.525	GTTAAATCCAATGGATTCCT
SafeHarbor.526	GTTCTCAATTTACTGGGATT
SafeHarbor.527	GCAGCTGTGCTCAAAAGACC
SafeHarbor.528	GAGGCTTAGTTGTAATAATG
SafeHarbor.529	GCCCCTCAATTCCAGTGTAA
SafeHarbor.530	GACTGGCAAATACAATTTGC
SafeHarbor.531	GAATGCAATATAGTGATCTT
SafeHarbor.532	GGAGAGGGTGGTTTAAAAGC
SafeHarbor.533	GGGTATACCTTAGGAAAGCT
SafeHarbor.534	GATGCATTCAATAGCTCTGT
SafeHarbor.535	GGGCTAAATAAAGCAATGTT
SafeHarbor.536	GTTATTCATAAATTGTAAGC
SafeHarbor.537	GTGACATAGTGGGATAGCCC
SafeHarbor.538	GGGAACATTTCTTCATAGGG
SafeHarbor.539	GGTATGTGTCCATATGTGTC
SafeHarbor.540	GAAGAATTAACACATTGTCT
SafeHarbor.541	GATGCCTGGTTAACAATTCA
SafeHarbor.542	GCCTTAAAGCTCCTATAGAA
SafeHarbor.543	GGGCCCACATTTATCTCTAT
SafeHarbor.544	GCAGGTGTCTAAATTCACTC

Safe Harbor Library	
SafeHarbor.545	GAACAATAAGTCAAGCAAGT
SafeHarbor.546	GGGACAATCTAAATGTCCTA
SafeHarbor.547	GGATATAAAAGCATACAAAA
SafeHarbor.548	GAGTCACCCCAGGGACAAAC
SafeHarbor.549	GGACCCTAAGGGAAGCTTGA
SafeHarbor.550	GTACTCACTGATACACAGCT
SafeHarbor.551	GTTTATAAATATTCCGACTA
SafeHarbor.552	GGTGACTAGGAAGTTTCTGC
SafeHarbor.553	GACTTAGAAACAGTTAATAA
SafeHarbor.554	GTTATTATTGAGTTGGTATA
SafeHarbor.555	GAACACTTTCACTGGGAATA
SafeHarbor.556	GGGATTCTCCTAGAATAAAT
SafeHarbor.557	GCCCACTTATGCAGTATAAG
SafeHarbor.558	GTGCATACCAAATTAGTGTC
SafeHarbor.559	GTATTCACAGCCAAAAAGTA
SafeHarbor.560	GTTCTGCTTCTAACATAGTA
SafeHarbor.561	GGAAAAGCTATGTTAAACCT
SafeHarbor.562	GTATCTGCATATTAAACACA
SafeHarbor.563	GGCCCTTAAAACATGGAACC
SafeHarbor.564	GTAGCCTATGTCAGAATGAG
SafeHarbor.565	GAGTTGCTAGACAGCTACCA
SafeHarbor.566	GAAGCAACACAGATTCTCAC
SafeHarbor.567	GGTTAGCAAAATTGCAAGAG
SafeHarbor.568	GGAACCTGGAGAATGTTAAG
SafeHarbor.569	GTGTTCTCATTCTTCACTCA
SafeHarbor.570	GAGTCACGGTCAAACAGTCG
SafeHarbor.571	GAGAACATACACATAATGAC
SafeHarbor.572	GCTTCAAATGTGTGTGCTTC
SafeHarbor.573	GAGAAATTAACTCACTTTAT
SafeHarbor.574	GTATTTAGGCTATGCTTGAA
SafeHarbor.575	GTCTTTGGAAACAACCATGT
SafeHarbor.576	GCCCATCATGACAGGACAGG
SafeHarbor.577	GGTAGAGCAGGGGTATTACT
SafeHarbor.578	GGAAGTGCATGCATGACCTT
SafeHarbor.579	GTTGAAATCAACATAAGGAA
SafeHarbor.580	GGGGTGGCACTGGGTTAATT
SafeHarbor.581	GGGCAGATCGACAACTGCCG
SafeHarbor.582	GTTGAATTATGTTACCTCCA
SafeHarbor.583	GAAAAATGACCCATGATTAA
SafeHarbor.584	GGTAGAGGGATAATGCACTG
SafeHarbor.585	GAAAGTCAAGCAGAGGGGCA
SafeHarbor.586	GGAGAGAATTAATCTTATTT
SafeHarbor.587	GGAGACACCAGTCACGGAGT

Safe Harbor Library	
SafeHarbor.588	GAGCCAAAGTGGCAAAGTGG
SafeHarbor.589	GTGGGAGGACAGGCAGCAGA
SafeHarbor.590	GATTAAAGACTTGCTTAGTT
SafeHarbor.591	GAGCTTATTTGACATGTTAG
SafeHarbor.592	GGATTAATGTAGCTGTAAAT
SafeHarbor.593	GTAAGAGACCAAGCCCAAGT
SafeHarbor.594	GGTTCACTGAGTATGTGCCC
SafeHarbor.595	GGATGCAGCCACTCTCAGAG
SafeHarbor.596	GAGGTACCTCACAATTTGAA
SafeHarbor.597	GTATCAACAGAGTGTCAGAT
SafeHarbor.598	GTACCTCAAAGTGTTCCCTG
SafeHarbor.599	GGCCTCTGTAAGAGGGGAGT
SafeHarbor.600	GATATATAAAGTAAGTGGAG
SafeHarbor.601	GATCCTTATTGCTCCATTCT
SafeHarbor.602	GAACTTATAAAGTGCCCACA
SafeHarbor.603	GGTAGGGTTGGAAGGGTAAC
SafeHarbor.604	GTGATGCATAGCATAGTTTC
SafeHarbor.605	GGGAGGCAACCTGTCCCTGC
SafeHarbor.606	GGTACAATAGATGCCTGAAA
SafeHarbor.607	GGGAGTGACTCAGCTACATG
SafeHarbor.608	GGTCATGATGCCACTGGGAG
SafeHarbor.609	GACCAGTAAGATTAAAAATG
SafeHarbor.610	GGCACTGGTTTGTGCACTTC
SafeHarbor.611	GAAATATTCAAGTTTATGAG
SafeHarbor.612	GTTTGCAGCACACAGGTAGA
SafeHarbor.613	GTTTGGTACAGTATAACCAA
SafeHarbor.614	GATCATAACAGAAGCTCCAA
SafeHarbor.615	GCAAGAGCAATTCTCAGGCT
SafeHarbor.616	GGGCCATGGAAAACAGCCCA
SafeHarbor.617	GTGTTATGACTTTAAAGTTA
SafeHarbor.618	GCAGGTCAAAAGCTCTAGAC
SafeHarbor.619	GAAACCTAAACAATAGCTCC
SafeHarbor.620	GCCAAGTGGACTAGAAGCCG
SafeHarbor.621	GTGTCATCATGCTAAGTAAT
SafeHarbor.622	GCTCTAGATTAGTTGGCTTA
SafeHarbor.623	GACCTCTAATTCACAGAGAG
SafeHarbor.624	GACTGAGGGTGGATAATCCA
SafeHarbor.625	GAGTCGAATGTAAGAAATTC
SafeHarbor.626	GATATGAGAGATAATTAAAG
SafeHarbor.627	GAATACCTACCCATTAGTGA
SafeHarbor.628	GTGTTAAGTAGGGAATATAC
SafeHarbor.629	GAGAAATGAGGCGCTTGTTA
SafeHarbor.630	GATTCACTTAGTTGCTCCCC

Safe Harbor Library	
SafeHarbor.631	GAATATGAGCTCCTAACATA
SafeHarbor.632	GTACTCAGCAGAAACAAAGG
SafeHarbor.633	GTGTACATAAACAAAAAGTT
SafeHarbor.634	GCAGGTGCAATATTTAGTAG
SafeHarbor.635	GTAAGGCCATGACACCAATT
SafeHarbor.636	GTCTTAGGTGCACAATTCCC
SafeHarbor.637	GTGTTATCTTTCACTCATAT
SafeHarbor.638	GATTTAAGTCCTCCATGCTT
SafeHarbor.639	GATTTGACATGCTTTAATAA
SafeHarbor.640	GTTTCCAGGTGACTCAGTTA
SafeHarbor.641	GGTCTGTGTGTGGATTTCCA
SafeHarbor.642	GTCAAGCCTTATGCAATTTC
SafeHarbor.643	GTCACTGGAGAAGCAACTTC
SafeHarbor.644	GAGACTAAATGCGGGAAAGA
SafeHarbor.645	GAACTAATCAATGTGCATCA
SafeHarbor.646	GGCAGCCCTAAGGCAGTCAC
SafeHarbor.647	GGGATTGTTAATGTCCAAGC
SafeHarbor.648	GCATAAACATTCATGAGTTT
SafeHarbor.649	GCACTCACGGAGTGCTAGGG
SafeHarbor.650	GTGCTTAATATGAATGCTGG
SafeHarbor.651	GGAACATGAAAATAACGTTG
SafeHarbor.652	GTGACTTCATTTGATTTCAC
SafeHarbor.653	GCCATCCACCATGCTATCAA
SafeHarbor.654	GAGAATGGAGCTGAAAATAC
SafeHarbor.655	GCTTGCTCTGTATGACTGTC
SafeHarbor.656	GTCATCAGGATAAATCAGCG
SafeHarbor.657	GTCTTAGTCAGGGAAGGAGT
SafeHarbor.658	GGATCTCAAGAGCTACCTAA
SafeHarbor.659	GAAATTACATCCCTAGATAG
SafeHarbor.660	GAAGCAAAACTACCTTTGTT
SafeHarbor.661	GCTTCATCTGGGGTGAAACC
SafeHarbor.662	GCATTACTAACCATGGAAAG
SafeHarbor.663	GTGGGTCATTCAAGTGGAGC
SafeHarbor.664	GTTCCATAAGTGGAAGCGTT
SafeHarbor.665	GAAATAGGAAGGGAATATAA
SafeHarbor.666	GTAACACTCAGCAGCTGAGA
SafeHarbor.667	GCTATTCCAGGAGAACACAT
SafeHarbor.668	GTGTTGATAACAGAAGATCC
SafeHarbor.669	GGATCACATATACATGCCTG
SafeHarbor.670	GTCAAACTCTTCAATATTCT
SafeHarbor.671	GCAACTTGAACTCCAACTTA
SafeHarbor.672	GAGACTGAATATAAGATGTA
SafeHarbor.673	GTGTCAAAAAACCTCAGAAA

Safe Harbor Library	
SafeHarbor.674	GTTAGGAAGTATTCGGAGTT
SafeHarbor.675	GTATCAAGTAAATAGGTGGA
SafeHarbor.676	GTAAAGCAACAGGTAATTAA
SafeHarbor.677	GATGTTTATTGTAGGGCATG
SafeHarbor.678	GACCACTCAATTTATATATT
SafeHarbor.679	GGCCATTATTTGTTGATCAT
SafeHarbor.680	GGAGAAACTGGATTTAAAGA
SafeHarbor.681	GTCTACAGACCACAGAAGAA
SafeHarbor.682	GGTATCCCTTAAGAATTTAA
SafeHarbor.683	GGTAGATTAATATTCTGGAA
SafeHarbor.684	GTAGTTATCCAAGGTAACAG
SafeHarbor.685	GGATTTGCGCAGGTCCCTCT
SafeHarbor.686	GCATGTTAGCCAGCAGAACA
SafeHarbor.687	GTCACCTAAAACGATGTATG
SafeHarbor.688	GATACTAATCAATAAGTGGG
SafeHarbor.689	GAAGGTTATGGGAGGGGTAC
SafeHarbor.690	GCAGAAAGTGATCTTTACAT
SafeHarbor.691	GAAGAGGTTTAGGTTGTCAG
SafeHarbor.692	GAGCCACAGTTAGAGTAACT
SafeHarbor.693	GTATTGGCTAGTTAAGTGCA
SafeHarbor.694	GGTCACCTTAAAAACATCTA
SafeHarbor.695	GTGCATTTGGGTATTAGATT
SafeHarbor.696	GAATAATAGCTATGGCTGCT
SafeHarbor.697	GGGCATTGCCTGTTTAATCT
SafeHarbor.698	GACTTTGTCACTAACACGCA
SafeHarbor.699	GTAAGCATGTACGAAGTAAC
SafeHarbor.700	GTTTGCCTTCCAGATAGGAG
SafeHarbor.701	GGGAGTGTATGTTCATTGGA
SafeHarbor.702	GGGTGACTACTGGTTGCTTT
SafeHarbor.703	GTTAAACCTGTTTATGCTCT
SafeHarbor.704	GGATTCTGAATTAATTGTAG
SafeHarbor.705	GATTCTATAGTCTATAGTTA

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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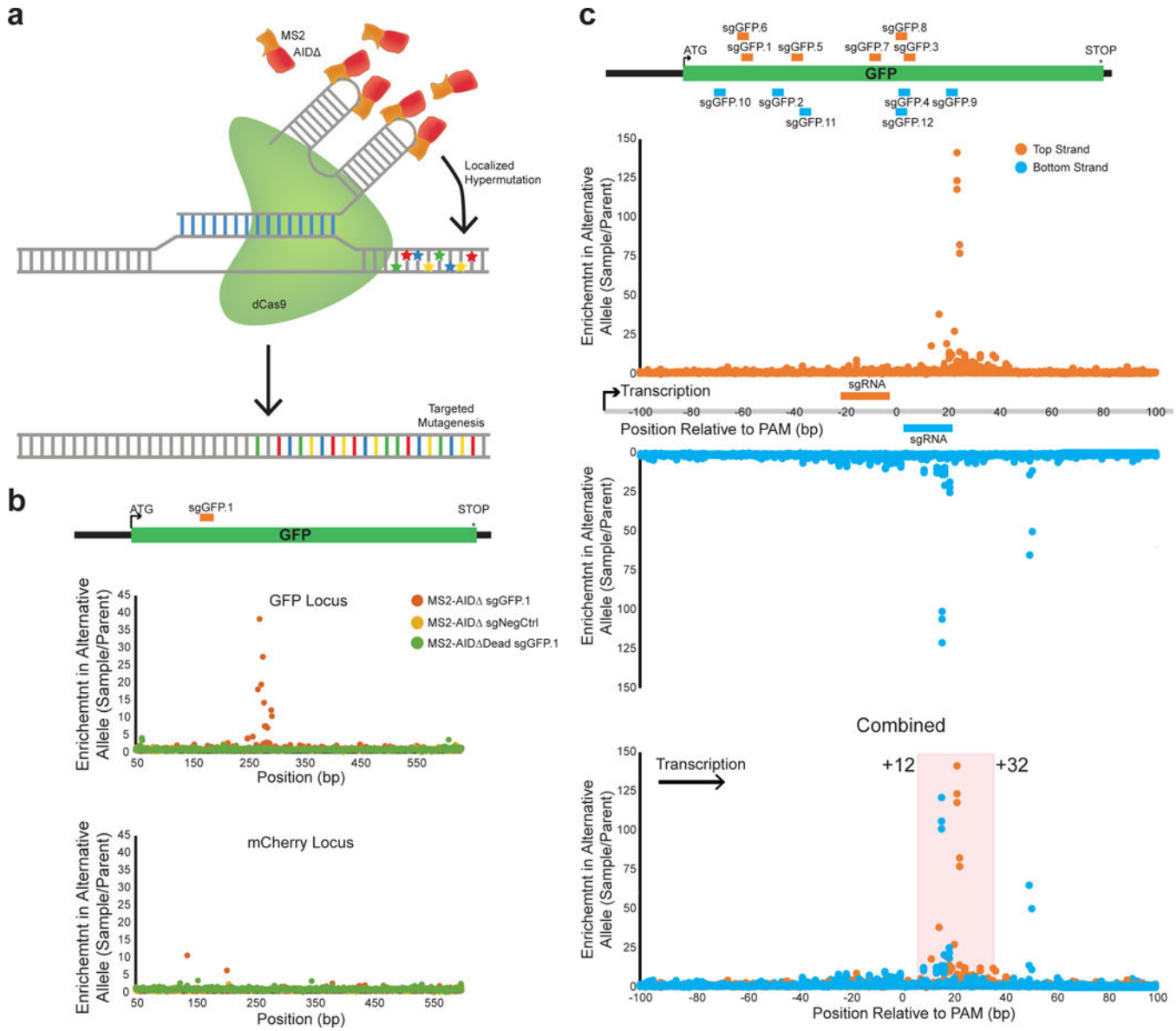

Figure 1. CRISPR-X generates targeted mutations

a) Schematic of CRISPR-X. dCas9 (160 kDa) complexes with an sgRNA containing MS2 hairpins in its stem loop, which recruit AIDΔ fused to MS2 binding protein (40 kDa). The deaminase induces local DNA damage which in turn introduces mutations. b) Cells expressing dCas9, GFP and mCherry were infected with indicated combinations of MS2-AIDΔ or MS2-AIDΔDead and sgGFP.1 or sgNegCtrl, and the GFP and mCherry loci were sequenced. Enrichment of mutations at each base position are shown for one replicate each. Additional replicates are shown in Supplementary Data Fig. 2b. c) 12 guides targeting GFP were infected into cells expressing dCas9, MS2-AIDΔ, GFP and mCherry. The targeting locations of the guides in the GFP locus are shown on the top panel. The GFP locus was sequenced for each sample. Enrichment of mutation relative to the position of the PAM of the sgRNAs is shown on the lower panel. The direction of transcription was defined as the positive direction as indicated by the arrow.

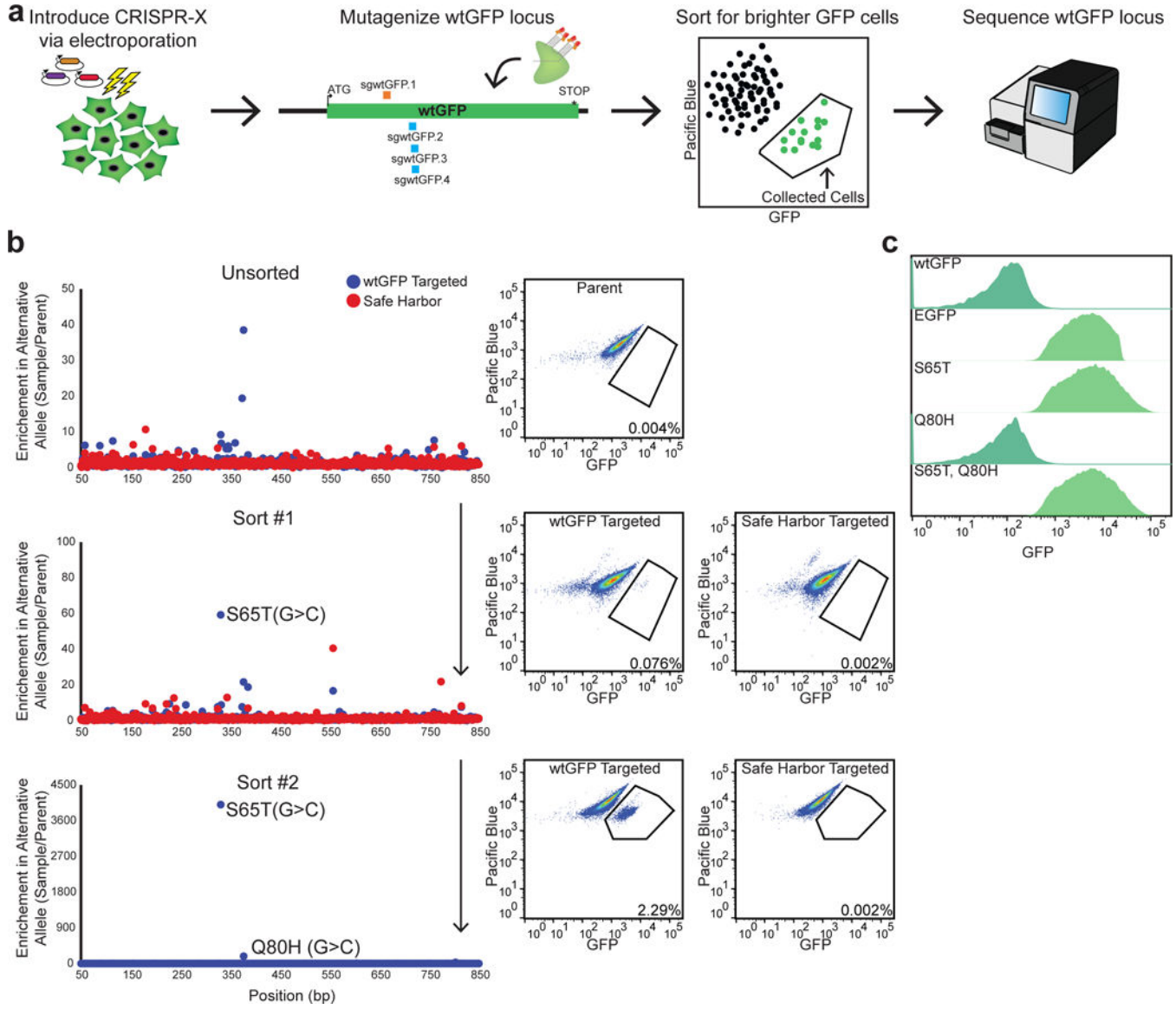

Figure 2. Evolution of wtGFP to EGFP using CRISPR-X

a) Schematic of wtGFP evolution experiments. wtGFP expressing cells were transiently electroporated with MS2-AIDΔ and 4 sgRNAs either targeting GFP or safe harbor regions. Cells were sorted for spectrum shifted GFP bright cells followed by sequencing of the wtGFP locus. b) Cells were collected from unsorted populations and after each round of sorting, and the wtGFP locus was sequenced. (left) Enrichment of mutations at each base position for both wtGFP targeted and safe harbor targeted libraries are shown except after the Sort #2 condition where no safe harbor cells were recovered. Identified mutations are labeled. (right) Scatter plots of the flow cytometry and gating are shown for the wtGFP parent and pre-sorting populations. c) Lentiviral expression constructs were generated containing each of the S65T and Q80H mutations separately and together. Plasmids encoding these variants along with wtGFP and EGFP controls were lipid transfected into 293T cells and the GFP fluorescence was measured by flow cytometry.

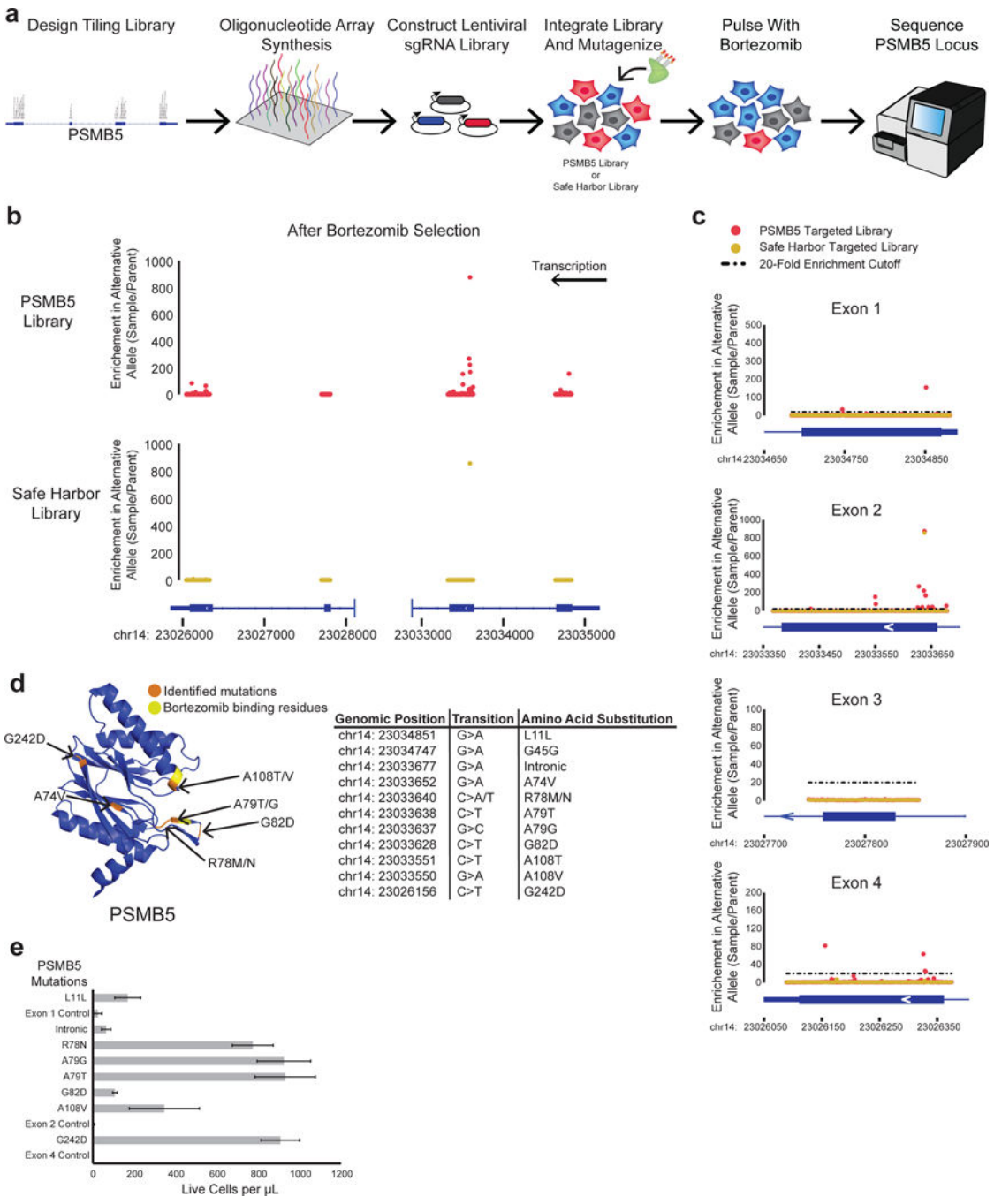

Figure 3. Directed evolution of bortezomib resistant mutations in PSMB5

a) Schematic for PSMB5 mutagenesis and bortezomib selection. Libraries targeting the exons of PSMB5 or control safe harbor regions were designed and synthesized on an oligonucleotide array and cloned into an sgRNA expressing vector. This vector was integrated into cells expressing dCas9 and MS2-AIDΔ to generate mutations. Cells were pulsed with bortezomib, after which the PSMB5 exonic loci were sequenced. b) Graphs of the enrichment of mutation at each base position are shown for the PSMB5 locus in both PSMB5 and safe harbor targeted libraries for one biological replicate. c) Graphs of the

enrichment of mutations are shown for individual PSMB5 exons. Positions that were above 20-fold enriched (black dashed line) in both replicates were identified as possible candidates. d) PSMB5 structure is shown. Identified mutations (orange) and residues involved in binding bortezomib (yellow) are indicated. A table summarizing the mutations is included. e) Mutations were installed into K562 cells and selected with bortezomib. A graph summarizing the density of live cells after selection is shown. Error bars indicate standard error.

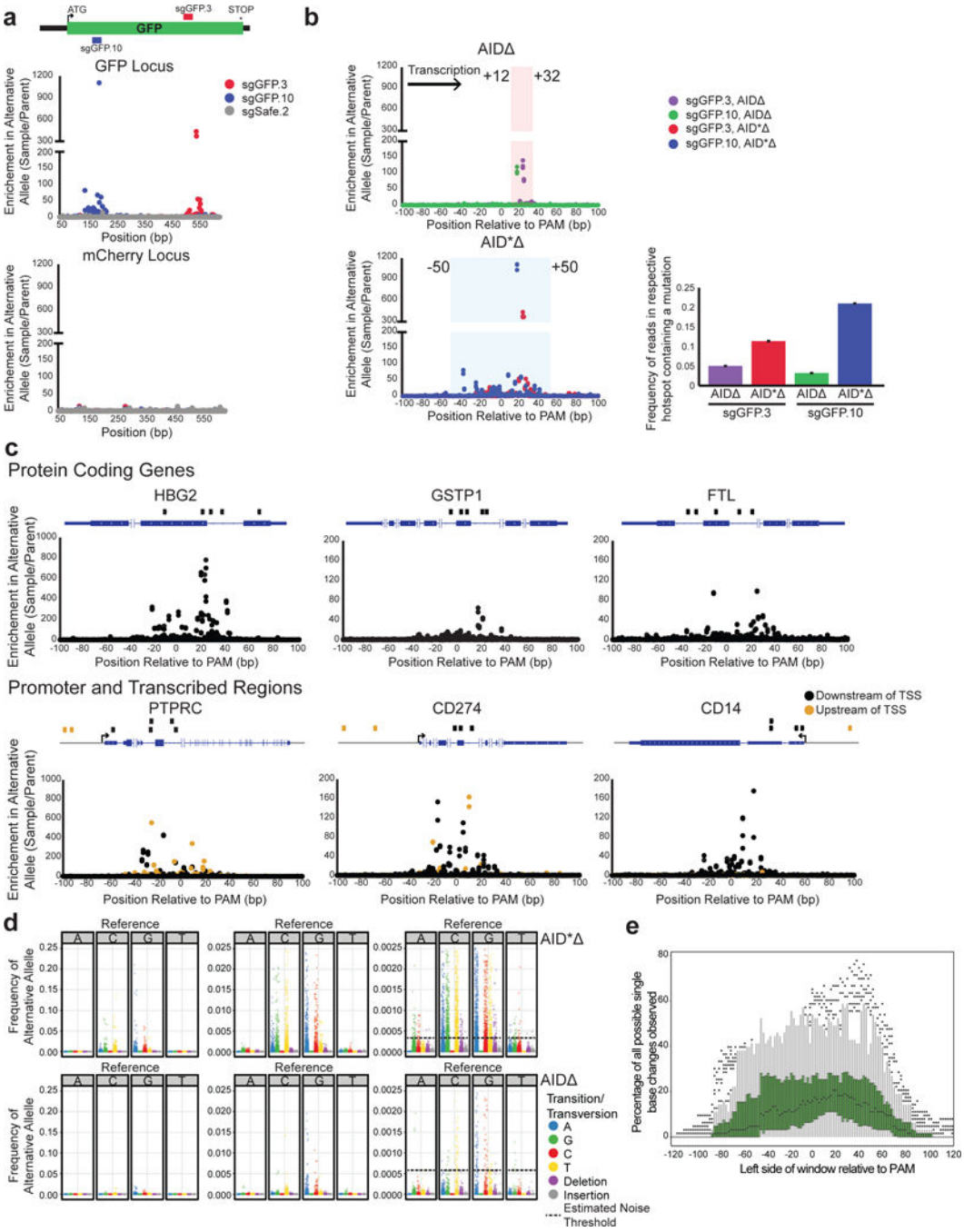

Figure 4. Enhanced mutagenesis of genes, promoters, and multiple loci with hyperactive AID*Δ

a) sgGFP.3, sgGFP.10, and sgSafe.2 were infected into cells expressing dCas9, MS2-AID*Δ, GFP, and mCherry. The GFP and mCherry loci were sequenced. Enrichment of mutations at each base position in both loci is shown. b) Enrichment of mutations at positions relative to the sgRNA PAM is shown for 2 GFP-targeting sgRNAs, sgGFP.3 and sgGFP.10, using either AIDΔ (top graph) or hyperactive AID*Δ (bottom graph). The shaded rectangles highlight the respective hotspot regions. (right) The frequencies of mutated sequences in the respective hotspots are shown. Error bars indicate standard error. c) sgRNAs were designed to target six

endogenous loci. Gene diagrams for each locus are shown indicating the position of the respective guides. Cells expressing dCas9 and MS2-AID*Δ were infected with the sgRNAs, and the loci were sequenced. Shown are graphs of the enrichment of mutations at positions relative to the PAM at each of the loci. Samples with sgRNAs targeting upstream of the transcription start site are shown in orange. d) Transition and transversion mutations observed using AID*Δ and AIDΔ are shown at three different scales. At each base in the hotspot region, the frequency of each transition was calculated and normalized to the parent population. The AID*Δ transitions were tabulated from mutations generated with sgGFP.3, sgGFP.10, and sgRNAs targeting endogenous loci. The mutations induced by AIDΔ were tabulated from sgGFP.1–12. The standard deviation of alternative allele frequencies in the parental samples were calculated and indicated by the dashed black line. e) Graph of the percentage of all possible single base changes observed for AID*Δ targeted with sgRNAs (described in Fig. 4a,c) in a 21bp sliding window. Single base changes with a frequency above the estimated noise were counted over a 21bp window beginning at the indicated position relative to the PAM, and the measured fraction of all possible changes is reported for each window. Box plots at each position are shown summarizing the distribution observed over all sgRNAs. The box plot lines represent 1.5X the interquartile range.