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(54) Title: TRICHOPLUSIA NI PIGGYBAC TRANSPOSASES WITH REDUCED INTEGRATION ACTIVITY

(57) Abstract: The present invention is directed to nucleic acid and amino acid sequences of a novel piggyBac transposase enzymes created by modifying the transposase of Trichoplusia ni. The piggyBac transposases of the present invention are functionally active or hyperactive for excision and have decreased integration activity compared to wild type Trichoplusia ni piggyBac transposase enzyme. These transposases are ideal for use in methods of transforming cells and organisms. In particular embodiments, the present invention provides methods of transient integration and expression of transgenes.



Trichoplusia ni piggyBac Transposases with Reduced Integration ActivityCross-reference to Prior Applications

[0001] This application claims the benefit of U.S. Provisional Application No. 61/508,386, filed on 15 July 2011, the entire contents of which is incorporated herein by reference to the extent permitted by applicable law and regulation.

U.S. Government Support

[0002] Not Applicable

Background of the Invention

[0003] The ability to generate induced pluripotent stem cells (iPSCs) without a permanent DNA sequence change is extremely important for many subsequent applications. A useful strategy is to integrate the genes necessary for transformation into the target genome using the DNA cut & paste *piggyBac* transposon (1) as the vector. The *piggyBac* transposon has a large cargo size and a high integration efficiency. The *piggyBac* transposase promotes insertion of the *piggyBac* transposon into TTAA target sites by binding to specific sequences at the transposon ends. Upon integration, the element becomes stably associated with the host genome and can serve as a long-term source of transcription factors required for cell transformation. Once iPS cell transformation has occurred, the *piggyBac* vector can then be re-exposed to transposase and excised by its natural "precise excision" pathway, in which the insertion site is restored to its pre-transposon TTAA sequence (2, 3). To avoid further genome modification, it is important that the excised transposon not re-integrate.

[0004] We are interested in developing improved *piggyBac* transposons as more useful tools for generating transgene-free iPSCs. A particularly useful tool for removal of the *piggyBac* vector after iPS cells transformation would be a *piggyBac* transposase that can promote excision at high frequency but is defective for re-integration following excision, *i.e.*, an Exc⁺Int⁻ transposase.

[0005] Using *in vitro* and *in vivo* assays in mammalian cells (4), we have isolated a *piggyBac* Exc⁺Int⁻ transposase. In *Saccharomyces cerevisiae* (4), and confirmed

in mammalian cells, we have also isolated a transposase that is hyperactive for excision but still integration negative, Exc^{+hyper}Int⁻.

Description of the Figures

[0006] FIGURE 1 shows sequences of transposases; Figs. 1A and 1B are sequence alignments of the *T. ni* transposase sequence with other transposases in the same protein family; and Fig. 1C shows the important mutagenized sites of *T. ni* transposase.

[0007] FIGURE 2 shows details of Exc+Int- mutant screening in mammalian cells with Fig. 2A showing the excision assay by PCR; Fig 2B showing results of colony counting assays; and Fig. 2C showing a table of the excision and integration activities;

[0008] FIGURE 3 shows the results of *in vitro* screening of the Ext+Int- candidates; Fig. 3A is a diagram of the steps of the *piggyBac* transposon operation; Fig. 3B shows gels of the purified proteins from the various candidates; Fig. 3C shows the results of the hairpin resolving and target joining assay; Fig. 3D are charts showing quantification of the tests in Fig. 3C; Fig. 3E shows gels depicting the results of the strand transfer assay; and Fig. 3F shows the results of an assay for *in vitro* nick (at 87 nt) and double strand breaks (at 70 nt).

[0009] FIGURE 4 shows the hyperactive R372A/K375A mutant screening in yeast; Fig. 4A is a diagram of the process related to the color from the Cherry gene of the yeast cell; and Fig. 4B shows the retest of the relative excision activities of the hyperactive mutants; and Fig. 4C shows the location of the 35 best candidates.

[0010] FIGURE 5 shows the relative excision activities of the hyper reactive R372A/K375A transposase mutants in mammalian cells; Fig 5A shows a diagram of the test strategy; Fig 5B shows the relative activities of the candidates.

[0011] FIGURE 6 shows how M194V and D450N were combined with 7PB, a hyperactive transposase for screening; Figs. 6A and 6B show the activity levels in bar graphs and Fig. 6C shows the actual cell colonies on plates.

[0012] FIGURE 7 shows integration assays demonstrating that the mutants are still defective in integration; Fig. 7A shows the retest in yeast cells and Fig. 7B shows mutation combinations.

[0013] FIGURE 8 shows retest in mammalian cells of mutant combinations demonstrating hyperactive excision.

[0014] FIGURE 9 shows tables of excision activity of EXc+Int- mutation combinations; Fig. 9A shows mutation combinations with hyperactive excision; Fig. 9B shows mutation combinations with WT-like excision activities; and Fig. 9C shows mutation combination that give low excision activities.

Detailed Description of the Invention

[0015] The following description is provided to enable any person skilled in the art to make and use the invention and sets forth the best modes contemplated by the inventor of carrying out his invention. Various modifications, however, will remain readily apparent to those skilled in the art, since the general principles of the present invention have been defined herein specifically to provide unique Ext+Int- transposases.

[0016] Transient integration and expression of transcription factors are important approaches to generate transgene-free iPSCs as well as directed differentiation tissues for both research and clinical use. Removal of the transgenes is critical for the therapeutic applications of the iPSCs. The *piggyBac* transposon, which was isolated from cabbage loop moth, *Trichoplusia ni*, can efficiently transpose genes of interest into the mammalian genome and more importantly can be precisely removed from the integration sites by *piggyBac* transposase thereby restoring the original sequence at integration sites. However, reintegration of the excised transposon catalyzed by the transposase is a concern (with *piggyBac* about 40-50% of the excised transposons reintegrate (6)). A *piggyBac* transposase mutant, which can only excise without integration would be a useful tool for generating iPSCs without any genetic change.

[0017] To that end, we screened a *piggyBac* transposase target knockout for mutations, which could only catalyze the excision of the transposon but not integration. We further looked for increased excision activity by screening for hyperactive mutants, while still maintaining the integration negative feature.

[0018] **Excision⁺ Integration⁻ mutant screening *in vivo* and *in vitro*.** The alignment of the *piggyBac* family showed that there are some conserved arginines and lysines around the catalytic sites D268, D346 and D447. We mutated these Arg and Lys to Ala. At the same time, we also mutated the other two amino acids, which are located at the conserved second catalytic D of HIV integrase I and asked whether the excision and integration activities of these mutants are abolished. Fig. 1 shows the amino acid residues chosen for making excision⁺/integration⁻ (Exc⁺Int⁻) mutation, R245A, R275A/ R277A, K287A, K287A/ K290A, R315A, R341A, S351E, K356E, K375A, R372A/ K375A, R388A, K409A/ K412A, K432A, R460A/ K461A. To test the excision activity *in vitro*, we co-transfected the transposase plasmids, either wild type (WT) or different mutants made by site-direct mutagenesis, together with the donor plasmid which carries *piggyBac* transposon into HEK293 cells with FuGENE 6 transfection reagent.

[0019] Three days after transfection, the cells were harvested and plasmids were recovered by Hirt methods as described in (5). PCR was then completed with 2 primers against the flanking donor sites (pbexl2, GGAACAGGAGAGCGCACGAG [SEQ ID NO:1] and pbexr2, GAGAGTGCACCATATATGCGGTG [SEQ ID NO:2]) using the following conditions (1x 95°C, 4min; 40x 94°C, 30 sec, 64°C, 30 sec and 72°C, 30 sec; 1x 72°C, 5 min). PCR products were run on 1.8% agarose gel and results were shown by ethidium bromide (EtBr) staining. Fig. 2A shows the excision assay strategy and Fig. 2B shows the results of PCR products from repaired donor DNA after excision. A shorter (277 bp) DNA fragment is shown on the gel if there is excision and the donor side repaired. As shown in Fig. 2B, R245A, R275A/R277A, R315A, R341A, S351E, K356E, K375A, R372A/K375A, R388A showed excision activity at similar level to WT. K409A/K412A and K432A showed decreased excision activity. No excision activity was observed with K287A, K287A/K290A, (F349A) and R460A/K461A either due to abolished excision activity or to flanking donor sites that cannot be repaired. We were interested in the mutants with WT excision activity and wanted to know whether they are defective in the integration step. Colony counting assays were used to test the integration activity of the mutants. HeLa cells were co-transfected with donor

plasmid containing *piggyBac* transposon carrying blasticidin drug resistance marker and transposase (both WT and mutants) expression plasmids.

[0020] Two days later, cells were trypsinized, diluted and seeded on plates with DMEM medium containing 3.5ug/ml blasticidin. Drug selection was continued for 18 to 21 days, surviving cells were stained with 0.2% methylene blue, and the plates were scanned. As shown in Fig. 2C, R275A/R277A, R372A/K375A and R388A have no integration activity (same level as the negative control without transposase) or very low integration activity. Interestingly, S351E showed very low integration activity. S351 is conserved with the Ser119 of HIV integrase I (7) and in a recently solved PFV integrase structure (8), A188 follows the conserved second catalytic D site. Both amino acids are reported to be important for integrase targeting. K356A, R341A and R245A showed decreased integration activity. R315A and K375A showed little change in integration activity as compared with WT.

[0021] Thus, we have several mutant candidates for further study: namely, R275A/R277A, R372A/K375A, R388A and S351E. We also included two mutants with dramatic decreases in integration activity: R341A and R245A. To better understand the mechanism, we performed *in vitro* assays with proteins purified from an *Escherichia coli* strain having an over-expressed transposase.

[0022] ***In vitro* characterization of the Exc⁺/Int⁻ (or Int decreased) mutants.** It has been shown that *piggyBac* transposase catalyzes the transposition in 4-steps (4) as shown in Fig. 3A: 1) *in vitro* nick; 2) hairpin of transposon ends excision; 3) opening of hairpins formed after a double-strand break; and 4) target joining. We know that the excision activity of R275A/R277A, R372A/K375A, R388A, S351E, R341A and R245A mutants is about the same level as WT, this means that nick, hairpin formation and double-strand break steps should be about the same as WT. We started from the hairpin opening/resolution step to see whether they show differences in hairpin opening and/or target joining. To test the kinetics of the hairpin resolution step of different mutants, we used a 5'-³²P - labeled PGB40, a 35 base pair (bp) hairpin with extra TTAA (74 nucleotide (nt) oligo containing 35bp of PB L-end and flanking TTAA in the middle (PGB40:

CATGCGTCAATTTTACGCAGACTATCTTTCTAGGGTTAACCCCTAGAAAGATA
GTCTGCGTAAATTGACGCATG [SEQ ID NO:3]).

[0023] Fig. 3 shows the details of the assays. Fig. 3B shows a gel of the Int⁺ proteins. Fig. 3C shows the results of the hairpin resolved and target joining assay. A 60-bp R-end oligo with TTAA hairpin was labeled with ³²P at the 5'-end used as substrate and reactions were performed as previous described (4), reactions were stopped at different time points by adding EDTA. Samples were run on denaturing PAGE gel. If the hairpin is opened, it will give a shorter (35 nt) DNA fragment on a denaturing sequencing gel. From the reaction, we can also observe target joining products if this step is not defective for the mutants. As shown in Figs. 3E and 3F, R372A/K375A has better hairpin resolving activity as compared with WT, but is defective in target joining. R275A/R277A and S35 1E showed slightly decreased in hairpin resolving, and were defective in target joining. R388A showed dramatic decrease in hairpin opening/resolving and was therefore defective in target joining. The other two mutants--R245A and R341A showed a slight decrease in both hairpin resolving and target joining, which suggest that R245 and R341 are not involved in targeting step. This *in vitro* data agrees with the *in vivo* data shown previously. Based on both *in vivo* and *in vitro* data, we chose R372A/K375A further studies as the best candidate for *piggyBac* transposase mutants with excision plus and integration minus features (Exc⁺/Int⁻).

[0024] To better understand the kinetics of the R372A/K375A mutant, we performed a nick and double-strand break assay with a DNA substrate containing 74 bp flanking sequence with TTAA and 87 bp of *piggyBac* R-TIR and labeled it at 5'-end of both top and bottom strands (Fig 3F). The results showed that the R372A/K375A mutant has slower nick and double-strand break activity in pre-steady state. However, at steady state, R372A/K375A mutant has comparable double strand break products accumulated but cannot further form the target joining product. In the case of WT, partial double strand break products can transform into target joining product.

[0025] ***In vivo* excision assay in yeast, *Saccharomyces cerevisiae*.** To more accurately measure the excision activity *in vivo*, we set up *in vivo* excision systems in both yeast *S. cerevisiae* and mammalian HEK293 cells. In yeast

system, we cloned the *piggyBac* transposon carrying a kanamycin marker into the mCherry gene at TTA site located at the very N-terminus of the mCherry gene (position 80-83) on pGAP/Cherry plasmid (9). This disrupts the expression of red fluorescence protein (RFP) (Fig 4A). The mCherry gene will be fully restored in the presence of *piggyBac* transposase, which can be precisely excised and repaired. RFP signal can be detected under fluorescence microscope (Leica: M165FC). We transformed both WT and R372A/K375A mutants in a pGals expression vector into yeast strain BY4727 with plasmid pCherry::PBTn, 3 days later, colonies were re-suspended in water and spotted onto both SC (synthetic complete) media Trp-Ura +Glucose and Galactose plates. Re-plating plates were scanned under the fluorescence microscope 1, 2 and 3 days later. From Fig. 4B, we can observe that the excision activity of R372A/K375A mutant was weaker than WT, which is in agreement with the *in vitro* results.

[0026] **Excision and integration assay in mammalian cells** A similar strategy was used to measure the excision activity in HEK293 cells. First, we generated a stable HEK293 cell line by Tol2 transposition, in which a Tol2 transposon carrying the Cycle 3-GFP (C3-GFP) under the control of EF-1 α promoter. The C3-GFP gene was first mutated at position 72 from T to C of the C3-GFP gene by site-directed mutagenesis which created a HpaI site for cloning of the *piggyBac* transposon which carries a blasticidin drug resistance gene. The Tol2 transposon will permanently integrate into the chromosome in the presence of Tol2 transposase. With this strategy, we generated a stable cell line—HEK293/T46—to use in mammalian cell excision. Once the *piggyBac* transposon is excised by *piggyBac* transposase, the C3-GFP gene will be restored and the GFP signal can be sorted by FACS (fluorescence activated cell sorter) analysis as shown in Fig. 5A). To compare the R372A/K375A mutant with WT in excision activity, we transiently transfected plasmids of R372A/K375A mutant and WT in pcDNA3.1/myc-HisA mammalian expression vector into HEK293/T46 line which carries C3-GFP::PBTn. Four to five days later, cells were trypsinized and washed with PBS. Then FACS analysis was used to measure the GFP cell population. As showed in Fig. 5B, we also observed the R372A/K375A mutant has about 50-60% of WT excision activity.

[0027] Screening hyperactive piggyBac/Exc⁺Int⁻ mutants in *S. cerevisiae*.

To make a more efficient tool for *piggyBac* transposon excision, we set up to screen excision hyperactive and integration negative mutants. First, we generated PB/ R372A/K375A mutagenesis libraries with 2 primers: pGals-f for priming the 5'-side of the pGals vector cloning site sequence with ATG start codon of the transposase; the second primer, pGals-r for priming the 3'-side of the cloning site with TGA stop codon of the transposase. We used error-prone PCR in the presence of Mn²⁺ to generate three mutagenesis libraries and then co-transformed with linearized pGals vector into *S. cerevisiae* BY4727/Cherry::PBTn. Through homologous recombination, the libraries were constructed in pGals vector in the strain containing Cherry::PBTn. Single colonies were picked and re-suspended in water in 96-well plates and pin replicated to SC-Trp-Ura+Glucose or Galactose omitray plates. Plates were grown at 30°C for 1, 2, 3 and 4 days and scanned by typhoon scanner to see if any colonies showed more fluorescence at the RFP channel on both Glucose and Galactose plates or any of Glucose or Galactose plates. Plasmids were recovered from the yeast strains with Qiagen mini-prep kit and re-transformed into *E. coli* NEB5α. Colonies were sequenced at MCLAB with primers PGB340 and PGB341.

[0028] We screened a total of 6000 colonies from three individual libraries and got 35 candidates to test for further confirmation in the yeast excision assay system. We also tested the candidates in mammalian cells HEK293/T46. As shown in Figure 5B, nine candidates showed higher excision activity than R372A/K375A; they are S103P/R372A/K375A, M194V/R372A/K375A, M194T/R372A/K375A, 1221T/R372A/K375A, T560A/R372A/K375A, S573AJR372A/K375A, M589V/R372A/K375A, S373P/R372AJK375A, D450N/R372A/K375A. As two candidate mutants—M194V/R372A/K375A and D450N/R372A/K375A—showed the highest excision rates, we focused on these two mutants and asked whether we could make them more hyperactive by combining these two mutants into one, or combine them separately or together with 7PB, a combination hyperactive mutant from WT *piggyBac* transposase screening (10). As shown in Figure 6A, when combining M194V and D450N together with R372A/K375A into one ORF, the excision activity went down to about WT PB level. When combining M194V/R372A/K375A or

D450N/R372A/K375A with 7PB, much higher (5-6 fold) excision activity as compared with R372A/K375A was observed. However, when we combined 7PB, M194V, D450N and R372A/K375A all in one, the excision activity decreased to WT level. This result suggests that certain combination of hyperactive mutants might not be better than each one alone of them in excision activity.

[0029] We also tested the integration activity of 7PB/R372A/K375A/M194V and 7PB/R372A/K375A/D450N to make sure none of the combination recovered the integration activity. As shown in Figure 6B, they did not show increases in integration.

[0030] **Shuffle-PCR to screen different mutant combination.** Next we performed a shuffling-PCR screening of the nine different amino acids with R372A/K375A to see which one gave the highest activity. To get all possible combinations of 511 mutants, we used a Shuffle-PCR strategy. Briefly, the first 100 cycle is based on limiting amounts of PCR primers versus two templates: WT with R372A/K375A and all nine amino acid mutations with R372A/K375A at ratio 1:1, 2:1 and 1:2. We used a very short extension time, denatured immediately at 95°C, and then annealed to make them switch templates as described below in materials and methods. Homologous recombination in yeast cell with pGals linearized vector and gel purified Shuffle-PCR generated PCR products were transformed into yeast strain BY4727/Cherry::PBTn. Single colonies were picked and resuspended in water and spotted to SC-TRP-URA+Gal plates. One day after 30°C culture, the plates were scanned for intensity of RFP of the spot. WT PB, PB/R3 72A/K3 7 5A, PB/R372A/K375A/M194V, PB/R3 72A/K3 75A/D45 0N, 7PB/R372A/K375A/M194V+D450N and 7PB, a hyperactive from previous screening in our lab (10) were used as control. Among these controls, PB/R372A/K375A/M194V gives strongest signal. The colonies with increased intensity of RFP signal as compared with PB/R372A/K375A/M194V were chosen and plasmids were recovered, sequenced re-transformed into yeast BY4727/pGAPCherry::PBTn and retested for fluorescence. Because some of the mutants showed fluorescence earlier due to the early excision that occurred in specific colonies, especially with hyperactive mutants, retest can tell the real hyperactive mutants. As shown in Fig. 9A and Fig. 7A, some of the mutant combinations with R372A/K375A show hyperactive excision. Fig. 9B and Fig. 7B

show mutant combinations with R372A/K375A give excision activity at around WT PB level. Fig. 9C shows mutant combinations with R372A/K375A that give low excision activity. We then tested the mutant combinations shown in Fig. 9A in mammalian cell HEK293/T46. Unfortunately, as shown in Figure 8, none of them showed higher activity than 7PB/R372A/K375A/D450N, but HP-8, HP-31, HP-38 and HP-43 showed moderately higher activity than 7PB/R372A/K375A/M194V and 7PB. Since we did not run the FACS at the optimum filter setting (excitation 395nm, emission 507nm) for C3-GFP, the difference between different mutants might be enhanced by changing the filter setting.

[0031] Materials and Methods

[0032] Primers for making Exc⁺Int⁻ mutant candidates: M1 (R245A) 54mer, aga to gca, 5'-gagaaaac gatgtatttactcctgttgcaaaaatatgggatctattatccatcag-3' [SEQ ID NO:4] (5'-ctgatggataaagagatcccatattttgcaacaggagtaaatacatcgtttctc-3' [SEQ ID NO:5])

[0033] M2 (R275A/ R277A) 64mer aga to gca/ cgg to gcc 5'-gaccatagatgaacagttacttggtttgcaggagcctgtccgtttaggatgtatatccc-3' [SEQ ID NO:6] (5'-gggatatacatcctaaacggacaggctcctgcaaaaccaagtaactgttcacatctatggtc-3' [SEQ ID NO:7])

[0034] M3 (K287A) 55mer aag to gcg 5'-ccgtttaggatgtatatcccaaacgcgcc aagtaagtatggaataaaaatcc-3' [SEQ ID NO:8] (5'-ggatttttattccatacttacttggccgc gtttgggatatacatcctaaacgg-3' [SEQ ID NO:9])

[0035] M3+ (K290A) 55mer aag to gcg/ aag to gcg 5'-gtatatccc aaacgcgccaagtgcgtatggaataaaaatcctcatgatgtg-3' [SEQ ID NO: 10] (5'-cac atcatgaggatttttattccataggcacttggcttgttgggatatac-3' [SEQ ID NO:11])

[0036] M4 (R315A) 47mer aga to gca 5'-gataaatggaatgccttatttggg agcaggaacacagaccaacggag-3' [SEQ ID No:12] (5' gttggtctgtgtcctgctccc aaataagg cattccatttatcatat-3' [SEQ ID NO:13])

[0037] M5 (R341A) 40mer cgt to gct 5'-gcctgtgcacggtagttgtgctaatt acgtgtgacaattggtcac-3' [SEQ ID NO:14] (5'-gtgaaccaattgtcacac gtaatattagcacaactaccgtgcacaggc-3' [SEQ ID NO:15])

[0038] M6 (R372A) 35mer cga to gca/ aaa to gca 5'-gtgggaaccgtgcgatc
aaacgcacgcgagataccggaagtac-3' [SEQ ID NO:16] (5'-gtactccggtat
tctcgcgtgcgtttgatcgacggttccac-3' [SEQ ID NO:17])

[0039] M6+ (R372A/ K375A) 36mer 5'-gttaaccattgtgggaaccgtgg
catcaaaacacgcgagatacctaac-3' [SEQ ID NO:18] (5'-ggatatctgcg
gtttgtttgatgccacggttcccacaatgg-3' [SEQ ID NO:19])

[0040] M7 (R388A) 39mer agg to gcc 5'-gtactgaaaaacagtcgctccgccccagt
gggaacatcgatg-3' [SEQ ID NO:20] (5'-catcgatgttcccactgggg
cggagcgcactgttttcagtac-3' [SEQ ID NO:21])

[0041] M8 (K412A) 53mer aag to gcg 5'-
ctcatataaaccgaagccagctgcgatggtatactattatcatcttg-3' [SEQ ID NO:22] (5'-
cacaagatgataataagtataccatggcagctggcttcggttatatgag-3' [SEQ ID NO:23])

[0042] M8+ (K409A/ K412A) 53mer aag to gcg/ aag to gcg 5'-
cttactctcgtctcatataaaccggcgccagctgcgatggtatactta-3' [SEQ ID NO:24] (5'-
ataataagtataccatcttagctggggccggtttatatgagacgagagtaagg-3' [SEQ ID NO:25])

[0043] M9 (K432A) 43mer aaa to gca 5'-cttctatcaacgaaagt
accggtgcaccgcaaagtgtatgtattataatc-3' [SEQ ID NO:26] (5'-taac
catttgcggtgcaccgtactttcggtgatagaagcatc-3' [SEQ ID NO:27])

[0044] M10 (R460A/ K461A) 58mer agg to gcg/ agg to gcg 5'-
ccaaatgtgtctgtgatgacctgcagtcgcgacgaataggtggcctatggcattatg-3' [SEQ ID
NO:28] (5'-caataatgccataggccacctattcgtggcggcactgcaggtcatcacagaacacatttg-3'
[SEQ ID NO:29])

[0045] M11 (R499A) 61mer cgc to gcc (not include in the data) 5'-c
agtagcaagggagaaaaagggtc aaagtgc cgc aaaatttatgagaaac ctttac atg agcctg-3'
[SEQ ID NO:30] (5'-caggctcatgtaaagggttctcataaattttgcggc
actttgaaccttttctcccttgctactg-3' [SEQ ID NO:31])

[0046] M12 (R518A) 42mer cgt to gct (not include in the data) 5'-
gacgtcatcggttatgcgtaaggctttagaagctcctactttgaagag-3' [SEQ ID NO:32] (5'-
ctcttcaaagtaggagcttctaaagccttacgcataaacgatgacgtc-3' [SEQ ID NO:33])

[0047] **Excision assay primers:**

pbexl2, GGAACAGGAGAGCGCACGAG [SEQ ID NO:34]

pbexr2, GAGAGTGCACCATATATGCGGTG [SEQ ID NO:35]

[0048] piggyBac Exc+Int- mutant random mutagenesis library PCR primers:

pGals-f, 57-mer CTTTAACGTCAAGGAGAAAAACCCCGGATTCTAGAA
CTAGTGGAT CCCCCGGGatg [SEQ ID NO:36]

pGals-r, 58mer GATGTGGGGGGAGGGCGTGAATGTAAGCGTGAC
ATAACTAATTACATGACTCGAGtca [SEQ ID NO:37]

[0049] pGals piggyBac mutants sequencing primers:

GTATTACTTCTTATTCAAATG [SEQ ID NO:38]

TCAGGTTGTCTAACTCCTTCC [SEQ ID NO:39]

[0050] **Plasmid constructions** Mammalian expression vectors of the *piggyBac* transposase and its mutants were constructed by PCR. The transposases ORFs (PB-f (KpnI + Kozak) GAggtaccGCCACCATGGGTAGTTCTTTAGACGATGAG [SEQ ID NO:40] and PB-4r (NotI, with stop) CAGgcgccgctcaGAAACAACTTTGGCACATATCAATATTATG [SEQ ID NO:41] with KpnI and NotI sites) were cloned into KpnI and NotI sites of pcDNA3.1/myc-HisA vector (Invitrogen). Excision+ integration- mutant candidates were obtained by site-directed mutagenesis.

[0051] The *piggyBac* transposon plasmids for mammalian cell integration assay contain a green fluorescent protein (Cycle 3-GFP) and blasticidin resistance (BsdR) cassette driven by a CMV promoter, flanked by 662 bp Left and 394 bp Right end sequences.

[0052] Plasmids for expression of *piggyBac* transposase and excision⁺ integration⁻ candidates were constructed in pET22b+(EMD) (PB-112 (NdeI) CAGcatatgGGTAGTTCTTTAGACGATGAGC [SEQ ID NO:42] and PB-2r (XhoI, no stop codon) CAGctcgagGAAACAACTTTGGCACATATCAATATTATGC [SEQ ID NO:43] and cloned in NdeI, XhoI sites without stop codon, which will give (extra peptide: LEHEITIH³H) C-terminal His-tag.

[0053] For yeast hyperactive color screening plasmid Cherry::PBTnKan construct, pYeGAP-Cherry was obtained by homologue recombination with PCR amplified PBTnKan with homologous cherry oligos: TGGCTATTATTAAAGAATTTATGAGATTTAAAGTTCATATGGAAGGTTTCAGTT AACCTAGAAAGATAGTCTGCG [SEQ ID NO:44] and CTCATAT GGTCTACCTTCACCTTCACCTTCAATTTCAAATTCATGACCATTAACCCTAGA AAGATAATCATATT [SEQ ID NO:45]. 6418 colonies were picked and determined by sequencing. The PBTnKan was introduced at position 80-83 TTA site of the Cherry gene.

[0054] **For making mammalian hyperactive color assay plasmid** The pC3GFP::PBTnBsd. Cycle 3-GFP gene from pTracer-CMV/Bsd (Invitrogen) was site-directed mutagenized at position 72 from T to C with primer C3GFHpa CAATTCTTGTTGAATTAGATGGTGATGTTAACGGGCACAAATTTTCTG TCAGTGG [SEQ ID NO:46] and its complimentary strand, which will generate a HpaI site. The *piggyBac* transposon carries a blasticidin resistance gene flanked by 662 by L-end and 394 by R-end sequences with blunt HpaI ends at both 3' and 5' ends that was introduced by ligation. Primers with BseRI and NotI were used to PCR the EF1 α promoter with the Cycle 3-GFP::PBTnBsd out and clone into pCMV/Zeo (Invitrogen). This creates the plasmid of pEF/C3-GFP::PBBsd. To12 transposon L-end 318 by (ApaI, NotI) and R-end 302bp (Psd, Nad) were introduced by PCR To12 L- and R- ends as well as pCMV/Amp + pUC Ori with primers To12L (NotI), GAgcgccgcGTCTGACCAATTTTCATATAATGTG [SEQ ID NO:47] and To12R (SbfI), GAcctgcaggCATTAGATTGTCTGTCTTATAGTTTG [SEQ ID NO:48]. Cut with SbfI and Nod, ligated to SbfI+NotI fragment pEF/C3-GFP::PBBsd containing EF1 α /C3-GFP::PBBsd. This will generate the final plasmid for generating stable cell lines by To12 transposition. pGalsPB/R372A/K375A, pGalsPB, pGals7PB were cloned into pGals vector at XmaI and XhoI sites.

[0055] **Methods**

[0056] **Mammalian cell culture and excision and integration assay** HEK293 and HeLa cells were cultured in DMEM supplemented with 5 % FBS and 2 mM L-glutamine. For excision assay by PCR, about 2×10^6 HEK293 cells were

transfected with 1.6 µg of transposon plasmid — pCMV/PBGB and 0.4 µg of transposase and mutant plasmids with FuGENE 6 transfection reagent (Roche Applied Science). Three days later, cells were harvested and plasmids were recovered by Hirt methods (5). The mixtures (helper, donor, repaired donor plasmids) were used as template to perform PCR with two primers against the flanking donor sides of donor plasmid (pbex12 and pbexr2) with PCR cycles (94°C, 4 mM; 40 cycle of 94°C, 30 sec, 65°C, 30 sec, 72°C, 45 sec; 72°C, 10 min). Reaction mixture: water 30.5 µl, 10 x buffer 5 µl, 10 mM dNTP 1 µl, 20 µM Pbex12, 5 µl, 20 µM Pbexr2, 5 µl, Hirt method recovered DNA 2 µl, High Fidelity Taq DNA polymerase 1 µl) with Expand High Fidelity PCR system (Roche). PCR products were run on 1% agarose gel.

[0057] For integration assay, 3-5 x 10⁵ HeLa cells were transfected with 1.6 µg of transposon plasmid — p CMV/PBGB and 0.4 µg of transposase and mutant plasmids with FuGENE 6 transfection reagent (Roche Applied Science). Two days later, cells were trypsinized and diluted to different dilution and continued culturing in DMEM containing 3.5 µg/ml of blasticidin for 18-21 days. Medium was changed every 2-3 days. Surviving colonies were stained with 0.2% methylene blue. Plates were scanned with scanner.

Protein expression, purification and *in vitro* assay. The *piggyBac* WT and mutant proteins were expressed in BL21 codon plus/DE3 RIL strain (Stratagene) in pET22b vector (Novagen) with C-terminal His-tag. Protein purification and *in vitro* nick, double-strand break, hairpin opening, strand-transfer assays have been described previously (4). For hairpin opening, a 74 nt oligo containing 35bp of PB L-end and flanking TTAA in the middle (PGB40) was synthesized (IDT) and PAGE gel purified and labeled at the 5'-end with γ-³²P-ATP. Reactions were run as previously described and reactions were stopped with EDTA at 1, 3, 10 and 20 min.

[0058] For strand-transfer assay: R-end oligos PGB18 CCCTAGAAAGATAATCATATTGTGACGTACGTTAAAGATAATCATG CGTAAATTGACGCATGTGTTTT [SEQ ID NO:49] and its complimentary strand PGB19 TTAAACACATGCGTCAATTTTACGCATGATTATCTTTAACGTACGT CACAATATGATTATCTTTCTAGGG [SEQ ID NO:50] were annealed and labeled

at 5'-end with γ -³²P-ATP on both strands. Reactions were run as previous described and reactions were stopped with EDTA at 1, 3, 10 and 20 min.

[0059] **Hyperactive PB/Exc⁺Int⁻ mutants screening in yeast** The yeast strain BY4727 has been described previously (4). Donor plasmid pGAP-Cherry::PBTnKan (URA⁺) was used in this study. Random mutagenesis libraries of PB/Exc⁺Int⁻ transposase gene was carried out by error-prone PCR in the presence of manganese and the resulting mutant pools were introduced into a linearized pGALS vector (TRP⁺) by homologous recombination in yeast. Transformant colonies were grown for three days, resuspended in water and spotted onto SC plates lacking tryptophan and uracil and with 2% galactose (SC-Trp-Ura+Gal) for transposase induction. The *piggyBac* excises precisely, allowing us to directly evaluate transposon excision from a gene encoding a *RFP* in a two-plasmid system. The transposon donor plasmid contained a yeast maximized DsRed *RFP* gene (9) in which the *piggyBac* transposon described above was flanked by TTAA at RFP by 80-83. *S. cerevisiae* BY4727 containing this donor plasmid are non-fluorescent. Galactose induction of the transposase from the helper plasmid results in precise excision and colony fluorescence that can be detected using a fluorescence microscope (Leica: M165FC). To screen for hyperactive mutants, we mutagenized the *piggyBac*/R372A/K375A ORF by amplification with error-prone MnCl₂ PCR with primers from the pGalS promoter and *CYC1* terminator region and introduced the PCR product by homologous recombination into pRS414 linearized by *XmaI*-*XhoI* digestion into strain BY4727 containing pGAP-Cherry::PBTnKan. More than six thousand individual transformants were picked in 96 well format on SC (synthetic complete) media containing galactose. We scored transposition by looking for increased fluorescence over 1-3 days, and about 35 hyperactive candidates were found. Yeast plasmid DNAs were isolated and the mutant PB/Exc⁺Int⁻ ORFs were sequenced and retested in BY4727 containing pGAP-Cherry::PBTnKan to exclude the earlier excision events.

[0060] **Mammalian excision assay *in vivo*.** For generating stable cell lines containing pEF1a/cyc-3-GFP::PBTnBsd, a To12 mediated transposition was used to integrate the DNA fragment into genomic DNA of HEK293 cells and colonies were picked and analyzed. To quantitatively analyze the relative excision activity

of different mutants, a color (GFP) excision assay by FACS sorting was used. A HEK293 stable cell line containing pEF1a/cyc-3-GFP::PBTnBsd, T46 was used and transfected with PB transposase and different mutant plasmids with FuGENE 6 transfection reagent (Roche Applied Science). Four days later, cells were trypsinized and FACS analyzed with BD FACSCalibur (BD Biosciences, 488nm excitation, 509nm emission not the best for cycle-3 GFP, should use exc 395nm, 507 nm) to see the percentage of GFP cell population in the whole cell population. Data were analyzed with FlowJo 8.5.3. GFP positive cells were counted against whole cells. GFP cells from WT PB were used to standardize that from mutant PB/Exc+Int-. To detect fluorescent cells, it is important to pick the best filter set to optimize detection. The primary excitation peak of cycle-3 GFP is at 395 nm. There is a secondary excitation peak at 478 nm. Excitation at either of these wavelengths yields a fluorescent emission peak with a maximum at 507 nm. Note that the quantum yield can vary as much as 5- to 10-fold depending on the wavelength of light that is used to excite the GFP fluorophore.

[0061] **Shuffle-PCR:** To generate a library of nine mutations locate at different positions of the PB gene, we used Shuffle-PCR to make different combination. The total number of possible combination is 511. To do the Shuffle-PCR, we used the same forward and reverse primers as we generated Exc+Int- random mutation library and 2:1, 1:1 and 1:2 ration of 2 different templates --- PB/Exc+Int- and 7PB/Exc+Int- + M194V + D450N with low primers/template ratio. The PCR cycle is as follow: 94°C, 4 min; 100 cycles of 94°C, 10 sec, 65°C, 20 sec, 72°C, 6 sec; 10 cycles of 94°C, 30 sec, 65°C, 30 sec, 72°C, 2.5 min; 72°C, 10 min. The PCR reaction mixture was cleaned up by Qiaquick PCR purification Kit (Qiagen). The whole elution was used as templates to amplified by normal PCR reaction. The product was digested by DpnI to remove any template DNA and then gel purified from 1% agarose gel with Qiaquick gel purification Kit (Qiagen). The same procedures were used as previously to generate combination mutation library in the yeast strain BY4727 with donor plasmid pGAP-Cherry::PBTnKan has been described previously. More than 1500 individual transformants were picked in 96 well format on SC media containing galactose. We scored transposition by looking for increasing fluorescence over 1-3 days, and about 50 hyperactive candidates were selected. Yeast plasmid DNAs were isolated and the

mutant combination PB/Exc+Int-ORFs were sequenced and re-tested in BY4727 containing pGAP-Cherry::PBTnKan to exclude the earlier excision events. The ones still showed hyperactive activity in retest were further cloned into pcDNA3.1/myc-HisA mammalian expression vector and examined the relative excision activity in HEK293 stable cell line containing pEF1 α /cyc-3-GFP::PBTnBsd, T46. Four days later, cells were trypsinized and FACS analyzed with BD FACSCalibur (BD Biosciences) to see the percentage of GFP cell population in the whole cell population. Data were analyzed with FlowJo 8.5.3. GFP positive cells were counted against whole cells. GFP cells from WT PB were used to standardize that from mutant PB/Exc+Int-.

[0062] **Acknowledgement** The inventor is an Investigator of the Howard Hughes Medical Institute. This work was supported by Maryland Stem Cell Research Fund.

[0063] **References**

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[0064] The following claims are thus to be understood to include what is specifically illustrated and described above, what is conceptually equivalent, what can be obviously substituted and also what essentially incorporates the essential idea of the invention. Those skilled in the art will appreciate that various adaptations and modifications of the just-described preferred embodiment can be configured without departing from the scope of the invention. The illustrated embodiment has been set forth only for the purposes of example and that should not be taken as limiting the invention. Therefore, it is to be understood that, within the scope of the appended claims, the invention may be practiced other than as specifically described herein.

I claim:

1. An improved transposase comprising a *Trichoplusia ni piggyBac* transposase mutagenized to exhibit enhanced *piggyBac* transposon excision activity and no or significantly diminished *piggyBac* transposon integration as compared to a wild type *Trichoplusia ni piggyBac* transposase.
- 5 2. The improved transposase according to claim 1 comprising replacement of one or more conserved lysines and arginines near catalytic aspartates at amino acid positions 268, 346 and 447 with alanine or glutamate.
3. The improved transposase according to claim 2, wherein one or more of the arginines at amino acid positions 245, 275, 277, 315, 341, 372 and 388
10 are replaced with alanine.
4. The improved transposase according to claim 2, wherein one or more of the lysines at amino acid positions 356 and 375 are replaced with alanine.
5. The improved transposase according to claim 2, wherein the lysine at amino acid positions 356 is replaced with glutamate.
- 15 6. The improved transposase according to claim 1 comprising replacement of the serine at amino acid position 351 with glutamate.
7. The improved transposase according to claim 2, wherein one or more of the arginines at amino acid positions 245, 275, 277, 341, 372 and 388 are replaced with alanine.
- 20 8. The improved transposase according to claim 2, wherein the lysine at amino acid position 375 is replaced with alanine.
9. The improved transposase according to claim 1, wherein a lysine at amino acid position 375 is replaced with alanine and/or an arginine at amino acid position 372 is replaced with alanine.
- 25 10. The improved transposase according to claim 9, wherein a methionine at amino acid position 194 is replaced with valine.

11. The improved transposase according to claim 9, wherein an aspartate at amino acid position 450 is replaced with asparagine.

12. An improved *Trichoplusia ni piggyBac* transposase mutagenized to exhibit enhanced *piggyBac* transposon excision activity and no or significantly diminished *piggBac* transposon integration as compared to a wild type *Trichoplusia ni piggyBac* transposase comprising a *Trichoplusia ni piggyBac* transposase, wherein a lysine at amino acid position 375 is replaced with alanine and/or an arginine at amino acid position 372 is replaced with alanine and a methionine at amino acid position 194 is replaced with valine or an aspartate at amino acid position 450 is replaced with asparagine.

13. A method of reversibly transforming a cell comprising the steps of:

using a *piggyBac* transposon to insert a nucleic acid sequence into the genome of the cell thereby transforming the cell; and

exposing the cell to a *Trichoplusia ni piggyBac* transposase mutagenized to exhibit enhanced *piggyBac* transposon excision activity and no or significantly diminished *piggBac* transposon integration as compared to a wild type *Trichoplusia ni piggyBac* transposase, thereby removing the nucleic acid sequence.

14. The method according to claim 13 wherein the step of exposing employs a transposase comprising replacement of one or more conserved lysines and arginines near catalytic aspartates at amino acid positions 268, 346 and 447 with alanine or glutamate.

15. The method according to claim 14, wherein in the transposon one or more of the arginines at amino acid positions 245, 275, 277, 315, 341, 372 and 388 are replaced with alanine.

16. The method according to claim 14, wherein in the transposon one or more of the lysines at amino acid positions 356 and 375 are replaced with alanine.

17. The method according to claim 14, wherein in the transposon a lysine at amino acid positions 356 is replaced with glutamate.

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18. The method according to claim 13, wherein in the step of exposing employs a transposon having a replacement of the serine at amino acid position 351 with glutamate.

19. The method according to claim 14, wherein in the transposon one or more of the arginines at amino acid positions 245, 275, 277, 341, 372 and 388 are replaced with alanine.

20. The method according to claim 14, wherein in the transposon the lysine at amino acid position 375 is replaced with alanine.

21. The method according to claim 13, wherein in the step of exposing employs a transposon having a lysine at amino acid position 375 replaced with alanine and/or an arginine at amino acid position 372 replaced with alanine.

22. The method according to claim 21, wherein in the transposon a methionine at amino acid position 194 is replaced with valine.

23. The method according to claim 21, wherein in the transposon an aspartate at amino acid position 450 is replaced with asparagine.

AdinetavagaAvPB2/1-559	97	AFICLM-SEKILQKELIYSNMEVERN---IK-LD-----EKEETMIEKASIGLILLAGLIGRLRSDLHSLWRTS---PLESP	167
AdinetavagaLooper/1-436	104	YFCSMF-GKESFNLIKDSNLYSVQT-----NPN-----RPNISDTETROFIQILINSGVSPFQQ--RENMDS---TRIQ	170
AdinetavagaPB1/1-566	105	AFICEL-SEEKLNKIMVYSNIEGHNH---TASNG-----ERKPTMIEELKAFIQLM--GWINEKSRKILIFVARS---PLESP	174
AnophelesAgavPB1/1-575	137	SMRLIT-DNGILNQIRKCTEIEARRV-----LQT-----KNWTLSFAELIEAFALINIRGATESKGMEIDIMWSE---KYGLP	205
Bombyxmori/1-610	142	CNNIEV-TSDMLQELLTHINSSIRHRQTKTAAEN--SS-----AETSFYMOETLCELKALTALLNLAGLIKSNRQSLKDIWRTD---GTGVD	223
CionaCinPB3/1-551	97	YFRUIE-TDELLITLWEEINRYAAQYLOKTSLS--KS-----RAHKWKPVAEELSLFGLITLITGVVHKR-GKLESYMIKN--SMIETP	176
DaphniaPokey/1-487	73	VEKSMLGGEDTLYLIREATINDYIAEFWSSRKTPPSRKVGKPEGRLLIGPRLPAGGFVTDHELVAFFQIQELIGYHRLPEL--SMFWEQQPDAGLGLG	167
Heliothisvirescens/1-581	123	AFFCMF-SMDTVNLVLQQINDYIKSIQEKFORER-----DCKVLEVEEELIAYLGLLNSGVLRSNHLNFKDIWATD---GTGIE	197
Human/1-572	101	AWKLIET-GDDFFEFIVESIRYNNQNRNF-----KL-----SKKSLKWKDITPQEMKKFELGLIVLMGOVRKDRR--DDYMTTE--PWTETP	177
T.nipB/1-594	134	CFKLIFF-TDEIISEINKWNIAEISLKRRESMTGA-----TFRDINEDEIYAFFQILNMTAVRKDNHMSTDDIFDR-----SL-SM	206
TakifuguPigibaku1/1-602	134	SEELIFT-PDSIOEILLDCINLEGRV-----FGE-----RWKELDOTOHAYFGVLLIAGVFRSKGESAESLNDAAE---TGRE	202

FIG. 1A

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AdinetavagaAvPB2/1-559	168	IFKDTISKSPDKLTLACLR	DDKSTREERKK--ADKFAA	IRIWLDFODKLKTCVTPGLNTH	DEQLLGRCKCPFRQ	PIPKPDK	251
AdinetavagaLooper/1-436	171	STASVMSRDELSTKKCFHV	VNTNRPDOND	PNDPAFKVRPL	LNIVKENFRKI-PKEEN	QVDEQIIPKCKSVNKHMPNENR	255
AdinetavagaPB1/1-566	175	NIOSYHVNRFFETLISIQF	DNKTAREKR--TDKFAVR	RIWTFDSRKFKEWNP	SGHGIDERLLGRCKCP	PROYTPSKEDK	258
AnophelesAgapB 1/1-575	206	FKNVMSNRFRFETKFLRF	DEKSTRSORLQ--TDKFA	LISDVFSRFVSNQTNV	PGPHISVDEQLF	PKTRCPTQFMASKPDK	289
Bombyxmori/1-610	224	IFRTMSLQRFOTLONNIRF	DDKSTRDERKQ--TDNMA	HRBIFDQFVOCCONAV	SPSEFTIDEMLLSHR	GRCLFRWYTPNKEAK	307
CionaCinPB3/1-551	177	YFGKCMSRNYQATGFLHF	NDNEKLAENID--NDKLY	KVRPVYDLIVARWKAL	NYLGEHISIDEGMWK	RGRGLFRWYTKDEPIK	260
DaphniaPokey/1-487	168	LIQOAMTRERFKFISKHAC	ASPDWDVDPNSDDPQ--E	PER--POP	IRKIRPLVKRLNERY	HACRPPRGQSIDESWVHKGRSVLRQTMKPKIK	260
Heliothisvirescens/1-581	198	FEQVMSNRFRFETSRVRF	DDKNTKSERLK--TDKLA	VRFFETDLNNNF	INNVCASENVTDEQL	PAFRGRFSGVWVMPNKPDK	281
Human/1-572	178	YFGKMTNRDRFQTMKAWHF	NNVAD--LVNE--SDRL	CKVRPVDYFVPKF	INIYKPHOQSLDEG	IVPWRGRGLFRWYNAKINVK	259
T.niPB/1-594	207	VAVSVMSRDRDFLIRCLRW	DDKSIRPILRE--NDVFT	PRKINDLTHQCIQNT	PGAHITIDEQLLGR	CRCPFRMYTPNKPESK	290
TakifuguPigibaku1/1-602	203	IFPATMSLNFFHITLSRIIRF	DNZDDRPARMQ--RDKLG	VRIRIVWDKWVRRLP	ILLYNPGPNVHIDEQL	MPHRCRCPFLQYLPNKPESKPAK	286

AdinetavagaAvPB2/1-559	252	YGLKFWLCVDAESVNLNAP	FTIGOPGOEK-Q--AHVGE	SVVLELLRPFYGSNR	NVTKDNFFHSVPLAR	NI-Q-TKNLT	LTIGTRNNKPEPIEF	342
AdinetavagaLooper/1-436	256	NGKVFLLIAGGESGLCYDF	VLYTKGSNSTEY-G-F	CTDITLTCETVPPM--M	NKVYDNYFHTIRLQ	VEL-K-KLG	IFSGLVRANAL--PDLT	343
AdinetavagaPB1/1-566	259	YALKFWFCVDVNSYMTF	DAFPMIRPNHR-Q--R	FVGNVVLMLKPMYGS	NRNVTIDNFFHSIHLA	KEL-H-SGK	LTIVGTRNNKPEPIEF	349
AnophelesAgapB 1/1-575	290	YGYVWMAVDVDSKVVNI	IPMLGNDRPA-E--ER	LGDVVKKLVDPYLN	GRNVTCDNFFHSLELA	KEL-K-SKK	TSIVGTRNNKAREVPICV	380
Bombyxmori/1-610	308	YGLKILALVDKMFVNVN	LEVHAGQPSGY-A--V	SNRPFVVERLIQPV	ARSHRNVTDNFFHSI	ELMLHLN-EYR	LTSVGINRNNRQIPESF	400
CionaCinPB3/1-551	261	YGLKSYILLADSHSHKCN	LDNHRNOKTL-----	KETVSQILLTSKCH	FLMSLYDNFTYVSM	SQML-L-AFQ	HSVGTLSNRGE-PRFI	344
DaphniaPokey/1-487	261	SGFKIWSRC-HRGVYKFE	IYQCFRFGKQGR	SRNNEAVERVVDLC	QPLTDQGVVA	DRFFHSIALIDKL-R	ENGNAVGTILHSRVNQTMT	354
Heliothisvirescens/1-581	282	YGLKHYALVDSATFVLL	KFEIYHAGVQPEGV-R	-MPNDTVSLVKRM	TEPIWGTNRNFFHSVPL	ANILLK-DHQL	TWGTTRNNKPEPIPTCF	374
Human/1-572	260	YGLIVRLICESDTHCNWE	IYCCG-----K	RLLE--TIQTV	SPYDTSWYIYMDN	YVNSVANCEAN-M	NNKFRICGTRNNK-IPKDF	342
T.niPB/1-594	291	YGLKILMWDSDGKVMN	QMPILCRGTQF-N-G--	VPLGEYYVKELSK	PHVGSRNITCDNFF	HSIPLAKNLIQEP	YKLTIVGTRNNKAREVPI	382
TakifuguPigibaku1/1-602	287	NGKTIWAACDATTSSV	ANILQVATCKPDGGAP-E	---QWLOHHRHFFHS	HKIGQEL-L-KR	KLTIVGTRNNKSEIPEPQ	L	375

FIG. 1A
CONTINUED

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AdinetavagaAvPB2/1-559	343	ISSKIR--EIG--SS--LFGFE--DNIA--LVSFVPPKNAWVLLISSKHHNH-----V-DN--KTGCPVILLDYNKTKGANDTMDQCHKYT	417
AdinetavagaLooper/1-436	344	MKDEKQLQEGRGATYRLTVV--DGFE--LCATRMFDNNWVWVLSLSCGESKDLVKRWSSEKKHL--QVEFPNVIKWVQYMGVLODMLVSLV--	435
AdinetavagaPB1/1-566	350	QSNKNR--DVG--SS--IFGFS--DNLT--LVSFVPPKNAWVLLISSMHHDSK-----V-DI--GTGCPNVLDYNKSKGANDTIDEMCHKYS	424
AnophelesAgaPB1/1-575	381	KKVKEK--LYF--TK--AFK--S--DDTT--LTVYQCKTKNAWVLLISSMHRDIR-----TGND--KKSQETVAFTNSTKYGVNDVDQCRKYS	455
Bombyxmori/1-610	401	IRTD--R--QPN--SS--VEFGQ--KDJT--LVSFVPPKNAWVWVSTMHHS-----IDESTGEKQPEMITFTNSTKAGVDVDELCAWV	477
CionaCinPB3/1-551	345	RTPPNQ--MKK--GD--ITARQNSMT--VL--AMKDRWKAITSIKHDSVTTITRRQ--RRGEXE--SVEKPVCIAIDYNLHMSGVDQVDQMTSYVP	429
DaphniaPokey/1-487	355	KNESNL--RPD--EFAA--KEGGE--PGTCRKGIFVMDTAFKVASWYHGSNLVKVRRKQ--RDG--SFK--TKSQPKAIDDYANNMGGVDITANQLRSYVE	442
Heliothisvirescens/1-581	375	QPKRTR--TEH--SS--LFGFQ--EDMT--JCSYVPPKSKAVLLISSMHHNN-----IVES--EKKQPEILLVNSTKGGVDINDQCANV	450
Human/1-572	343	QTI--SI--KK--GET--KEIRK--NDIL--LQVWQSKPWWLSSISHSAEWEESQND--R--T--SKK--KIVKPNALIDYNKHKMGVDRADQVLSYVS	423
T.nipB/1-594	383	KNSRR--PVG--TS--MFCFD--GPLT--LVSYPKPKPAWVLLSSCDEEDAS-----I-NE--STGCPQWVWVWVNTKGGVDITLDQCSWMT	457
TakifuguPigibaku1/1-602	376	ITSKNR--PVK--SS--QFAYT--ADTS--LVSFVPPKNAWVWVSTLHRDGR-----MCDQ--EHHQPEIIMDYNTAKGGVDNMDKLVTAYS	451
AdinetavagaAvPB2/1-559	418	VKRGTRWPLCIFYGMIDMAALNAFILWKSKNPVNENKRYQRRFLFEEFLRLSLVTPILL--DFRSKTSNF-----LHKDIQ--NALLIVGHP-----VS-	502
AdinetavagaLooper/1-436	425	VKGRTRWPLCVFYGMIDAAAINAMSLWKKKNPNWANKYKRRLFLEELGTLTTSYLL--DFRIKNSST-----LHKDIQ--NALVRFEGYP---RI-	509
AnophelesAgaPB1/1-575	456	LKSASRRWSVSHFFNILDLAGINAWVLYKELTK-----ENISRRDFLKLGEELAEVY--ENKSANANLP-----	519
Bombyxmori/1-610	478	VSRNSKRWPMTLFYGVLNMAAINACIIYRTNKN-----VTIKRTEFIRSLGLSMIYEHL--HSRNKKKNIPF-----YLRQ--RIEKQ---L-----G	553
CionaCinPB3/1-551	430	CHRKSLWTKKVVFFYFMTISVHNAYILYKSSS-----AKSKTIL--YSFILTIVSOLC--QQDR-----LQPDID--DENLA--GPP---PKS-	502
DaphniaPokey/1-487	443	RDRKAKKWHRLLYSLLETCLVNSWICFNDWVR-----SQMAE-----	480
Heliothisvirescens/1-581	451	VGRRTKRWPWVIFYHLLNVAGINAYVIFKNKID-----HGISRREFLKHIAVDLVKVHQ--QTRSNIPQLPR-----AVQK--RLKRN---AE--VQ	520
Human/1-572	424	ILRRTVKWTKRLAWMINCALNSYAVYKSVRQ-----RKMGMFLKQFAIHWLTDIPEDMDIVPD-----LQ--PVPSTSGMRAXPPTS	503
T.nipB/1-594	458	CSRKTNRWPMALLYMINIACINSFIYSHNVSS--KGEKVQSRKKFMRNLVMSLTSSEF--RKRLFAPTLKR-----YLPD--NISNILLPNEVPGT-	543
TakifuguPigibaku1/1-602	452	CKRRTLWPLVIFFDMLDISAVNAFVWVWALNPENKRVKLQKRRLFLEDLGKALVRQI--ERRKHIPRTPASAMVRRIQENAGALSTOPTPEQSA	547

FIG. 1A
CONTINUED

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		D227	D228	D239	
Adineta	--GWINEKSRKILIFV-NRSPLESPNIQSYHVKNRFETIISSIQF	DNKTAREERKRTDKF			209
Adineta_1	LAGLLGRLRSDLHSLW-RTSPLESPIFKDTISKSRFDKIACLRF	DDKSTREERKKADKF			202
Anopheles	IRGATESKGMEIDLW--SEKYGLPFCKNVMSRNRFRIMKFLRF	DEKSTRSQRLOTDKF			240
Bombyx	LAGLIKSNRQSLKDLW-RTDGTGVDIFRTTMSLQRFQFLQNNIRF	DDKSTRDERKQTDNM			258
Ciona	LTGVVHKRG-KLESYWIKNSMIETPYFGKCMSRNRQAITGFLHF	NDNEKLAENIDNDKL			211
Heliothis	MSGVLRSSHLNFKDLW-ATDGTGIEFFQNTMSFNRLFISRCVRF	DDKNTKSERLKTDKL			232
Takifugu	LAGVFRSKGESAESLW-DAE-TGREIFRATMSLENFHIISRIIRF	DNZDDRPARWORDKL			237
T.ni	MTAVRKDNHMSTDLLF---DRSLSMVYVSVMSRDRDFDLIRCLRM	DDKSIRPTLRENDVF			241
D268					
Adineta	AVSREIWTDFSRKFEMYNPGSHGTID	DERLLGFRGKCPFRQYIPSKPKDYAIKFWFCVDV			269
Adineta_1	AAIREIWLDFQDKLKTCYTPGLNITID	EQLLGFRGKCPFRQFIPTKPKDYGLKFWLCVDA			262
Anopheles	ALISDVFSRFVSNQCOTNYVPGPHISVD	EQLFPSKTRCPFTQFMASKPKDYGQKYWMAVDV			300
Bombyx	AAFRSIFDQFVQCCQAYSPSEFLTID	EMLLSFRGRCLFRVYIPNKPAPYGIKILALVDA			318
Ciona	YKVRPVYDLIVARWKALYNLGEHISID	EGMMKWRGRLGFRVYNKDKPIKYGIKSYILADS			271
Heliothis	AAVREFTDLMNNFINNYCASNVTID	EQLPAFRGRFSGVVYMPNKPTKYGIKHYALVDS			292
Takifugu	GVIRTVDKWVRRLLPLLYNPGPNVTID	EQLMPFRGRCPFLQYLPSKPAKNGIKIWAACDA			297
T.ni	TPVRKIWDLFIHQCIQNYTPGAHLTID	EQLLGFRGRCPFRMYIPNKPISKYGIKILMMCDSD			301
D346					
Adineta	NSYYIFDAFPYIERQP-NEHRQRFVGNVLELMKPMYGSNRNVTD	DNFFTSIHLAKEL-			327
Adineta_1	ESYYVLNAFPYIGRQP-GQEKQAHVGESVLELLRPFYGSNRNVTD	DNFFTSVPLARNL-			320
Anopheles	DSKYVVNIIPYLGKND-ERPAEERLGDFVVKLVDPYLNRRNVTD	DNFFTSLELAKFL-			358
Bombyx	KNFYVVNLEVYAGKQSPGYAVSNRPFVEVVERLIQPVARSHRNVTD	DNWFTGYELMLHLL			378
Ciona	HSYCWNLDMYH-----RVQKTLKETVSOILTSKCHFLWHSLYMD	DNFYNSVMSQML-			323
Heliothis	ATFYLLKFEIYAGVQPEGPYRMPNDTVSLVKRMTEPIWGTGRNVTD	DNWFTSVPLANILL			352
Takifugu	TSSYAWNLOVYTGKPD-GGAPEKNPRNESCPRHVSQTQWTOHH--MRH	DNFFTSKHLGOEL-			353
T.ni	GTKYMINGMPYLGRGTQ--TNGVPLGEYYVKELSKPVHGSCRNITQ	DNWFTSIPLAKNLL			359
D346					
Adineta	-HSGKLTLVGTLRKNKPEIPIEFQSNKNRDVGSSIFGFS-DNLT	LVSYVPKKNKAVILLS			385
Adineta_1	-QTKNLTIGTLRKNKPEIPIEFLLSSKIREIGSSLFGFE-DNL	LALVSFVPKKNKAVLLLS			378
Anopheles	-KSKKTSLVGTINKARREVPICVKKVKEKLYFTKAFK-S-DDT	TTLTVYQGKTCKNVVLLS			415
Bombyx	-NEYRLTSVGTVRKNKRQIPESFIRD-RQPNSSVFGFQ-KDIT	LVSYAPKKNKVVVMS			435
Ciona	-LAFQIHSGTGLRSNRGEP-REIRTPPNQMKKGDIARQNQSVTV	LAW---KDKRVVKAI			378
Heliothis	-KDHQLTMVGTIRKNKPEIPTCFQPKRTRTEHSSLFGFQ-EDV	TLC			410
Takifugu	-LKRKLTI	VGTVRSNKREIPEVLKNSRSPVGTSMFCFD-GPLTLVSYKPKPAKMVILLS			411
T.ni	QEPYKL	TIVGT			418

FIG. 1B

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		D447	D450	W465	
Adineta	SMHHDSKV-DI-----	GTGKPNIVLDYNKSKGAVD	DTIDEMCHKYSVKRGTRRW	P	433
Adineta_1	SKHHDNHV-DN-----	KTGKPVIIIDYNKTKGAVD	DTVDOMCHKYTVKRGTKRW	P	426
Anopheles	SMHRDIRTGND-----	KKSKPETVAFYNSTKYGV	DWVDOMCRKYSLKSSASRW	S	464
Bombyx	TMHHDNSIDEST-----	G--EKQKPEMITFYNSTKAGV	DWVDELANYNVSRNSKRW	P	486
Ciona	STKHDSVTTITRRQRRGGEXESVEKPVC	ADYNLHMSGVD	DQVDMISYYPCRKSLKWT	T	438
Heliothis	SMHNDNNIVES-----	EKKKPEIILYNSTKGGVD	DTNDOMCANYNVGRRTKRW	P	459
Takifugu	TLHRDGRMCDQ-----	EHHKPEIIMDYNATKGGVD	DNDKLVTAYSCKRRTLW	P	460
T.ni	SCDEDASI-NE-----	STGKPQMVMIYNQTKGGVD	DTLDOMCSVMTCSRKTNRW	P	466
Adineta	LCVFGMIDAAAINAMSLWKKKNPNWNANKKYKRRL	FLEELGTLT	SYLLDFRIKNSS--		491
Adineta_1	LCIFYGMIDMAALNAFILWKS	KNPVWNNENKRYQRRL	FLEELRLSLVTPLLDFRSKTSN--		484
Anopheles	VHSFFNILDLAGINAWVLYKELT-----	KENISRDFLFLGEELAE	EYVENKSANAN--		517
Bombyx	MTLFYGVNLMAAINACIIYRTN-----	KNVTIKRTEFIRSLGLSMI	YHLHSRNKKKN--		539
Ciona	KKVFFYFMTISVHNAYILYKSKS-----	SA--KSCKTLYSFILT	LVSQLCQQDRLOPQ--		489
Heliothis	MVIFYHLLNVAGINAYVIFKNK-----	IDHGISRREFLKH	LAVDLVKVHQQTRSNIPQ--		512
Takifugu	LVIFFDMLDISAYNAFVIWMALNPEWKRVKLQKRRL	FLEDLGKALVRPQ	IERRKHIPRTP		520
T.ni	MALLYGMINIACINSFIIYSH-NVSSKGEKVQSRKKFMRNLYMSLTSS	FMKRLEAPT--			523
Adineta	---TLHKDIQ--NALVRFGYPRIETELE----	TFVTDSARSKRKRCSL	CDYSSDRKVSNT		542
Adineta_1	---FLHKDIQ--NALLIVGHPVSKRDSQ----	KSDEDSAQSKRKRCS	ICTSKDRKTSNK		535
Anopheles	---LPM-----	-----TNAGGSRRRYK	VTSCHEGKSANE		544
Bombyx	---IPTYLRO--RIEQLGEPSP-----	RHVNVPGRYVR	QCDYCKDRKTKRS		583
Ciona	---IDD-----ENLAGPPPKSPRIDS----	TKRLKGGFKKHVIALYPT	TKKAAAQRP		535
Heliothis	---LP-RAVQ--KRLKR---NAEVQDPG----	STSRGGPSTSYKRCH	ICPRSKDKKIRFM		559
Takifugu	ASAAMVRRIQKENAGALSTQPTPEQSAEPEVNVZ	XVVNSSNKKKRCEV	CGPKMDRKTQYT		580
T.ni	---LKRYLRD--NISNILPNEV-PGTSD----	DSTEPPVMKKRTYCTY	CPSKIRRKANAS		573
	C574 C577 C582 H585 C590 C593				
Adineta	CYKCSEPICKQHS	SMK-RVFRINC	SK-----		566
Adineta_1	CYNCSAFVCSEHC	CVK-QIFCINC	SK-----		559
Anopheles	CFTCNRPVCKKCTKRISYVC	WSCEPGSDEAM			575
Bombyx	CNACAKPICMEHAK---	FLCENCAE-LDSSL			610
Ciona	CRACMKNCKRKDTIL---	L-----			551
Heliothis	CAKCHHHICHDE	ST---MTCCKCID	-----		581
Takifugu	CIKCKKYICNTH	TVK---LWPS	CVV-----		602
T.ni	CKKCKKVICKREH	NID---MCOS	CIF-----		594

FIG. 1B
CONTINUED

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Blue: catalytic AAs Red: conserved Rs and Ks Green: HIV integrase

FIG. 1C

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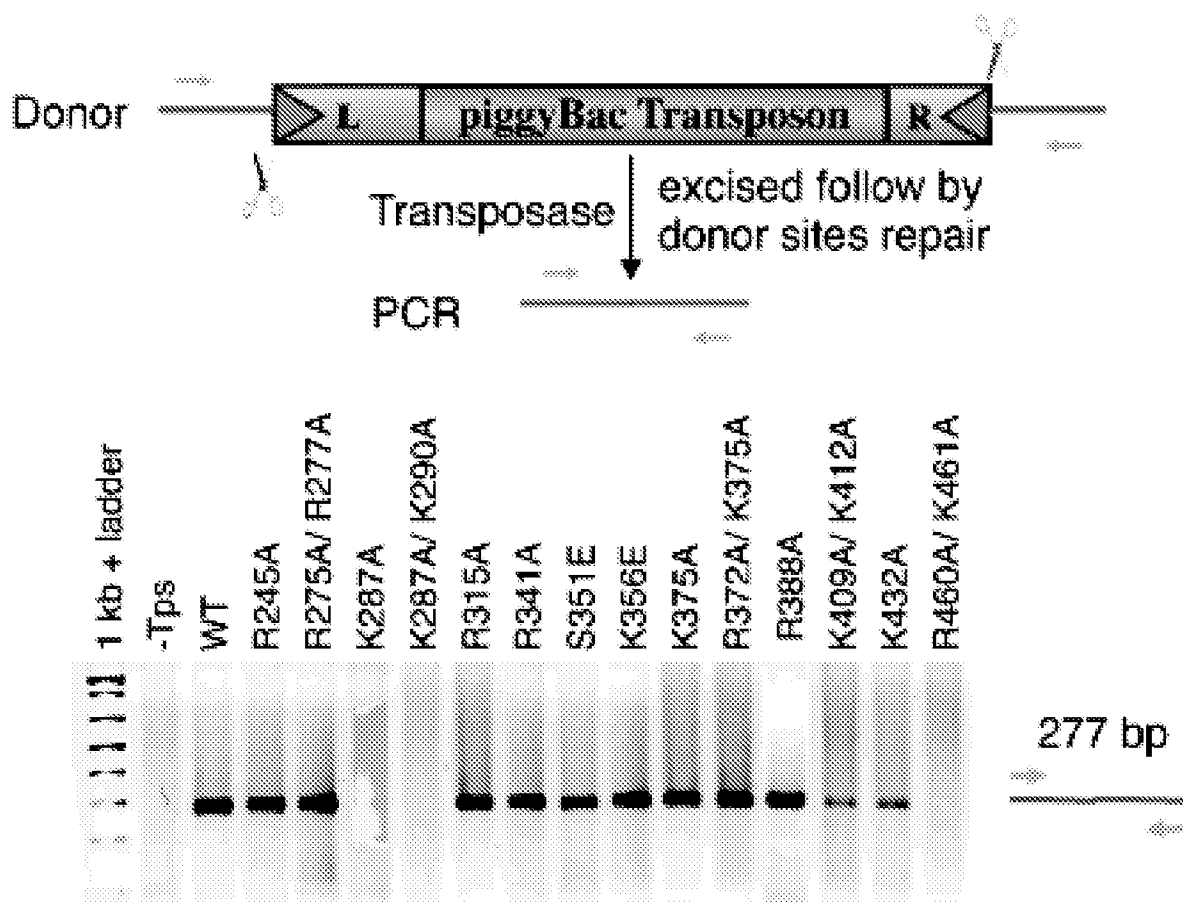
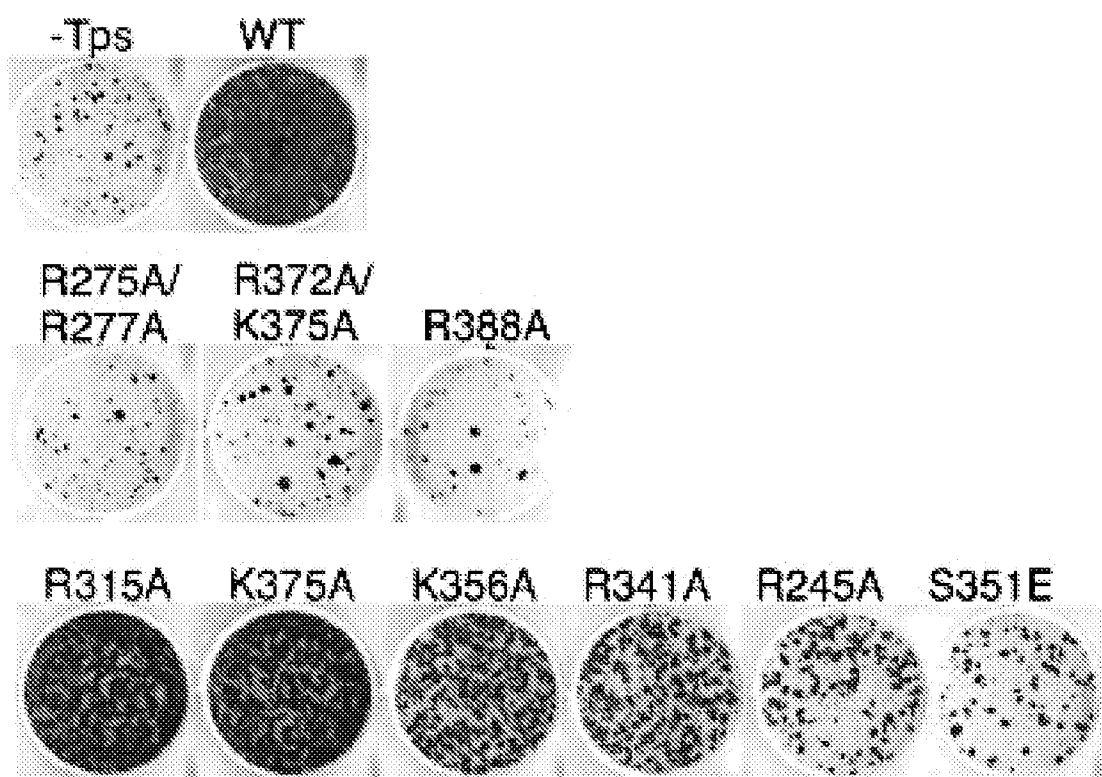


FIG. 2A

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**FIG. 2B**

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	Excision	Integration
<i>piggyBac</i> WT	+++++	+++++
R245A	+++++	++
R275A/ R277A	+++++	-
R315A	+++++	+++++
S351E	++++	++/-
R341A	+++++	+++/-
K375A	+++++	++++
R372A/ K375A	+++++	-
R388A	+++++	-
K409A/ K412A	+++	+++/-
K432A	+++	+++

FIG. 2C

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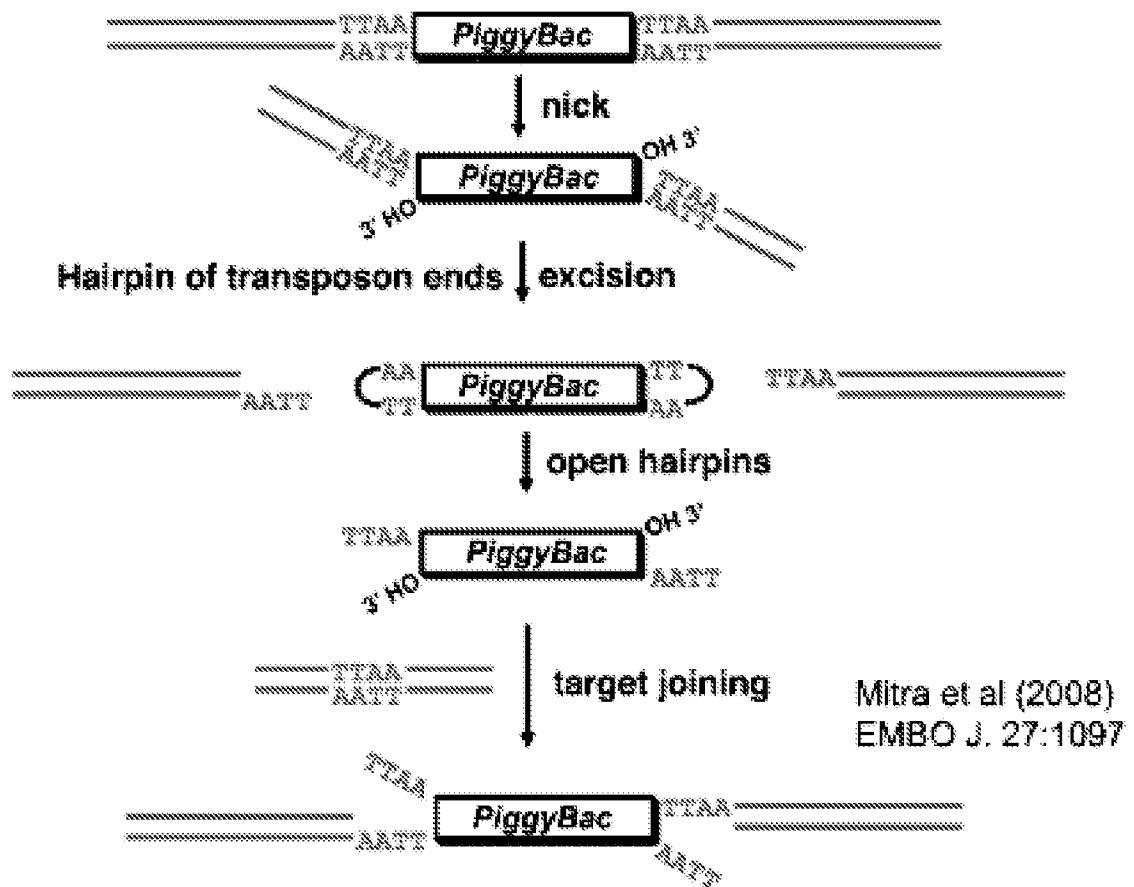


FIG. 3A

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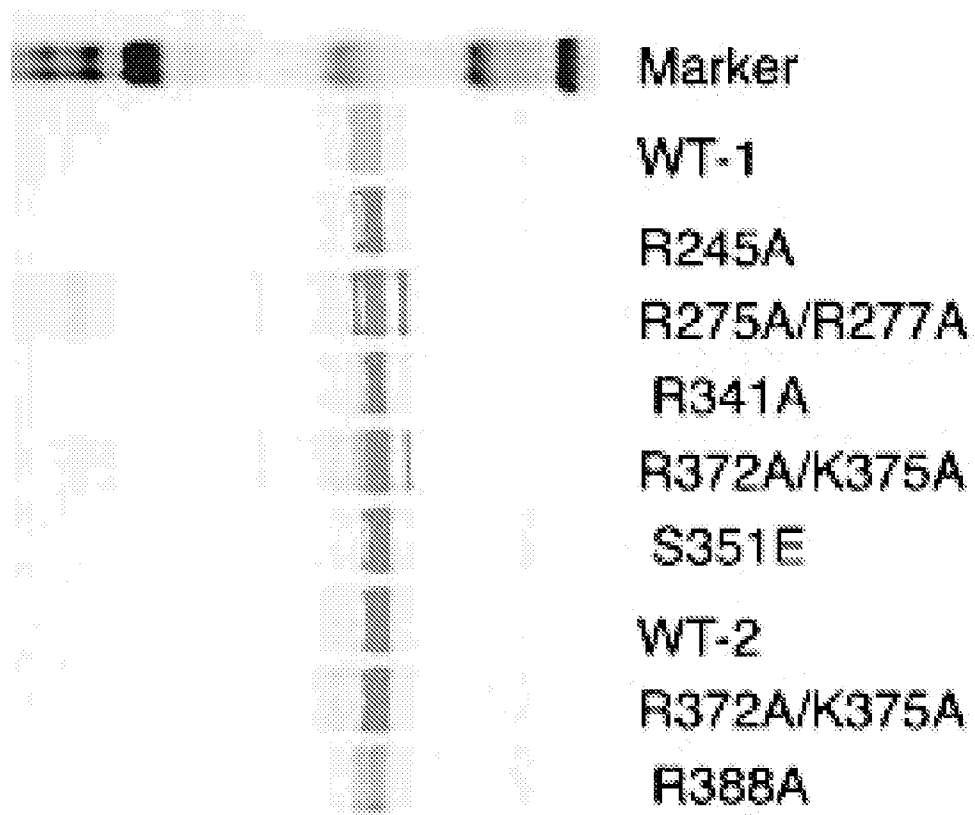


FIG. 3B

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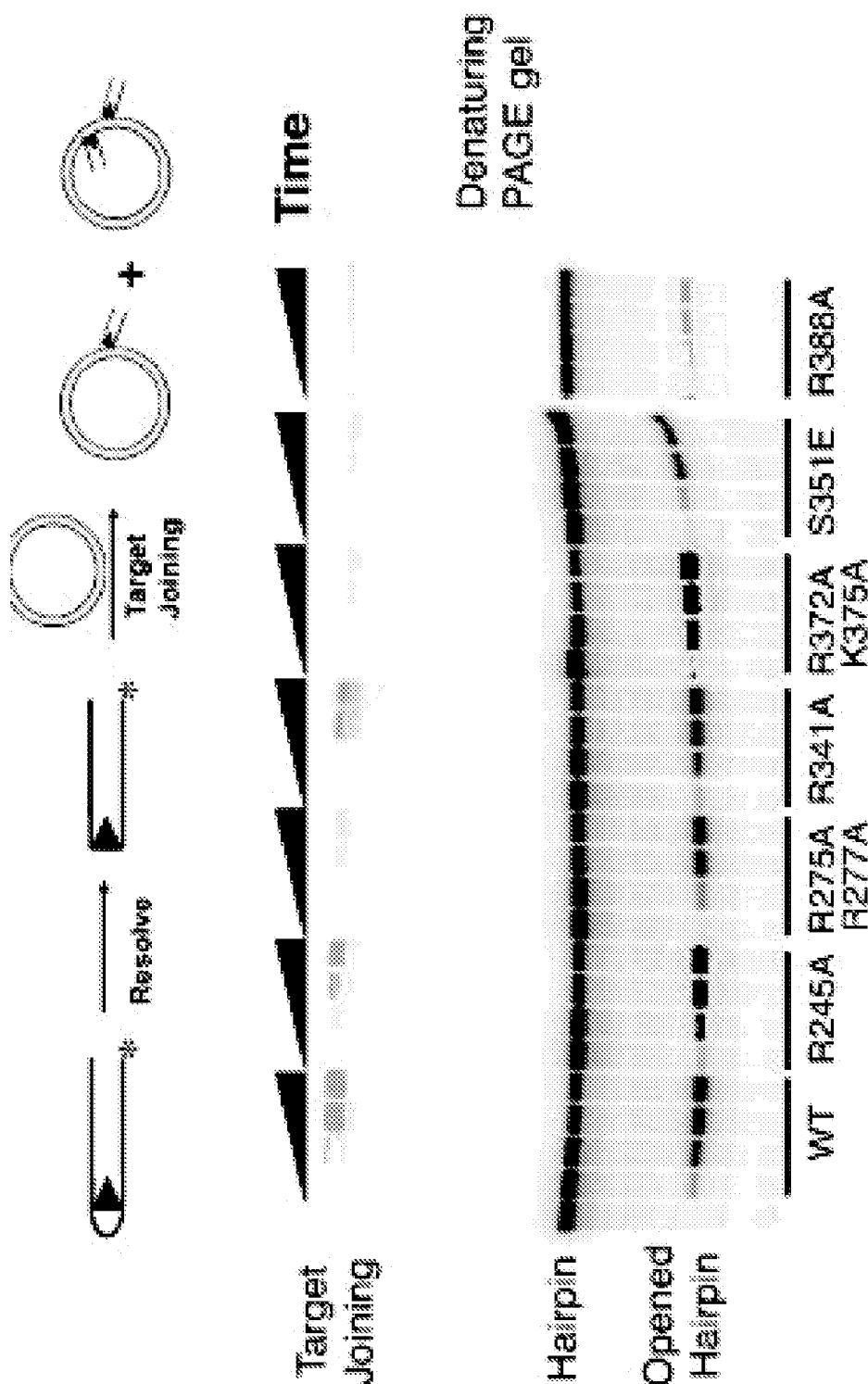


FIG. 3C

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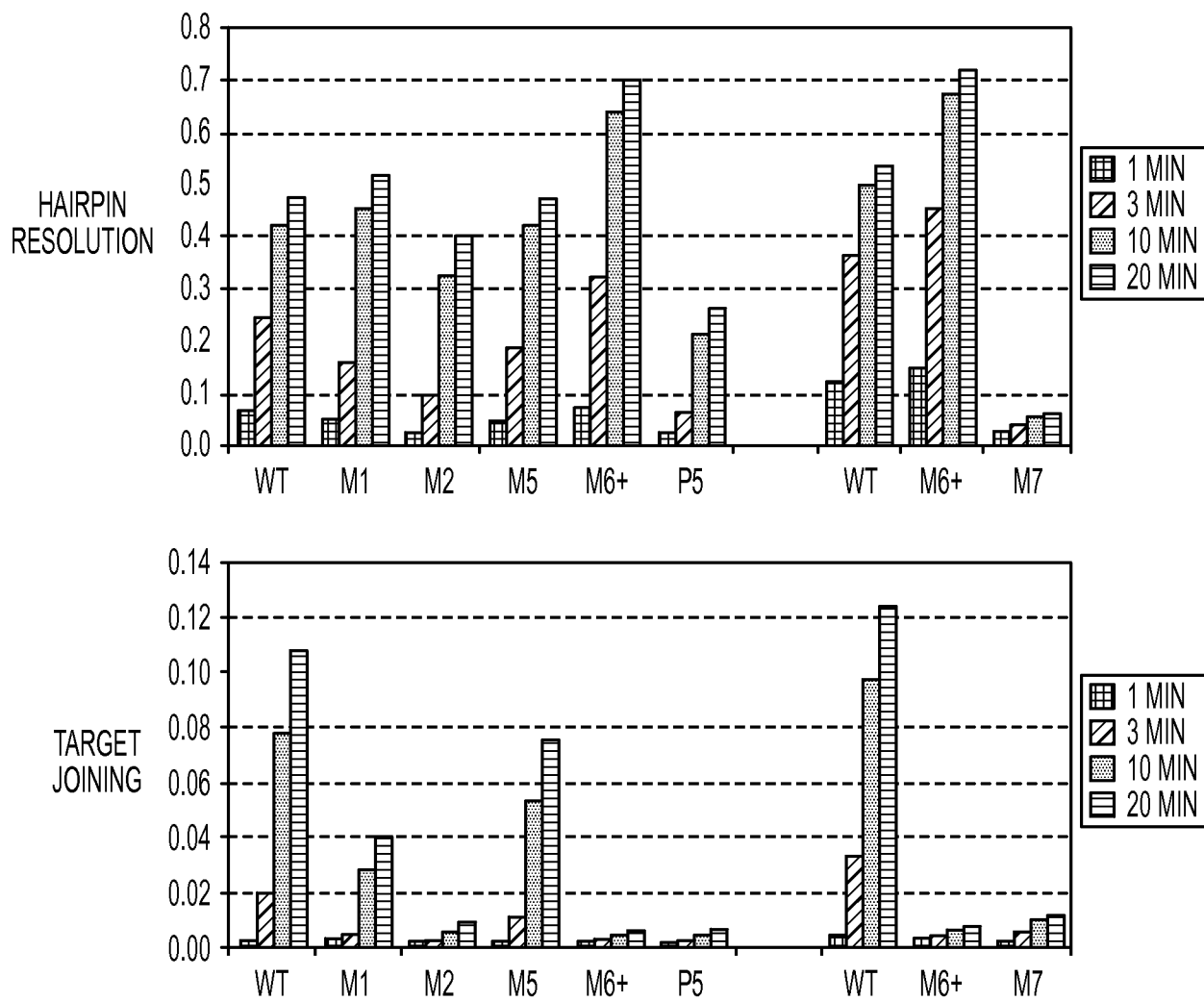


FIG. 3D

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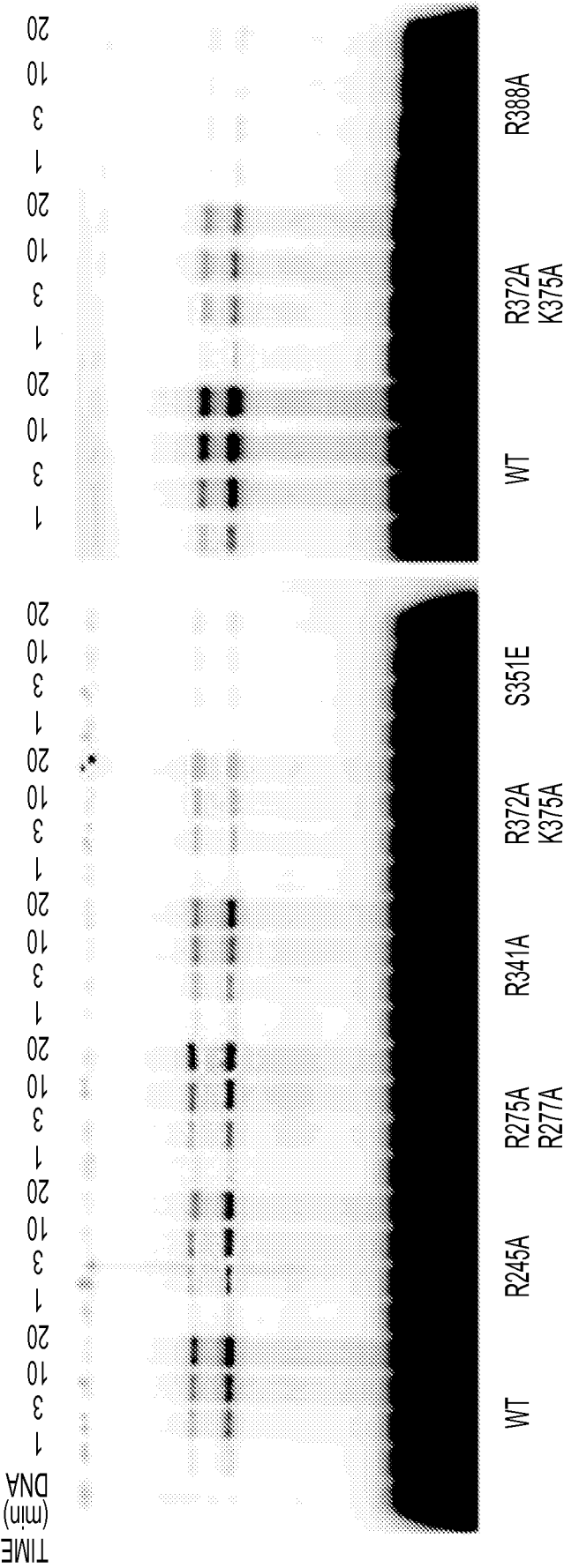


FIG. 3E

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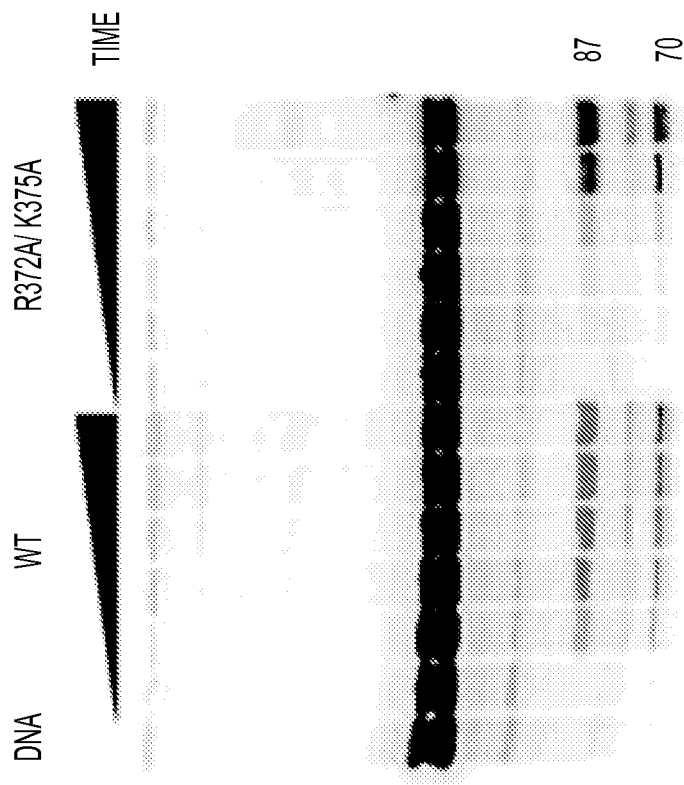
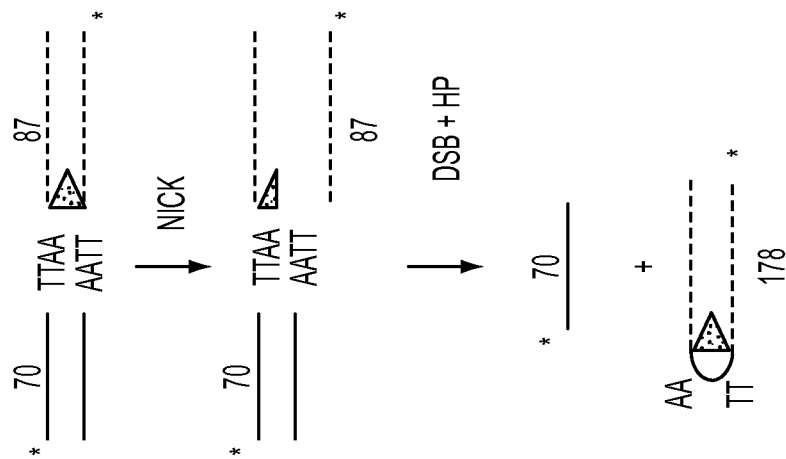


FIG. 3F



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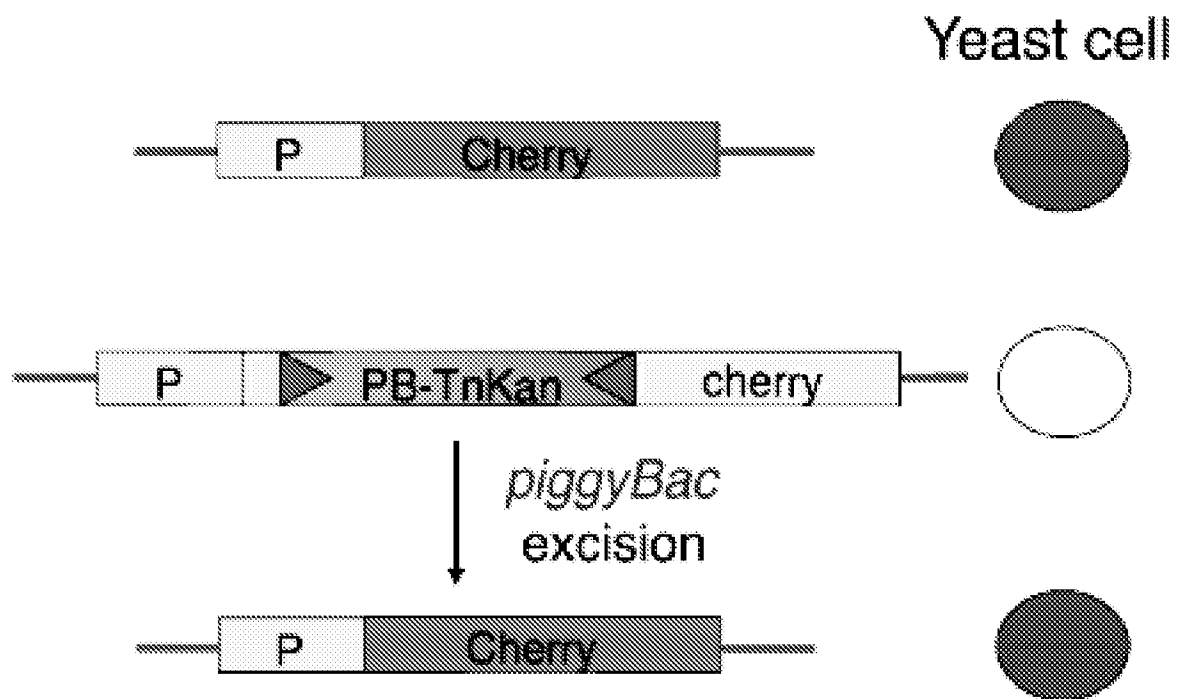


FIG. 4A

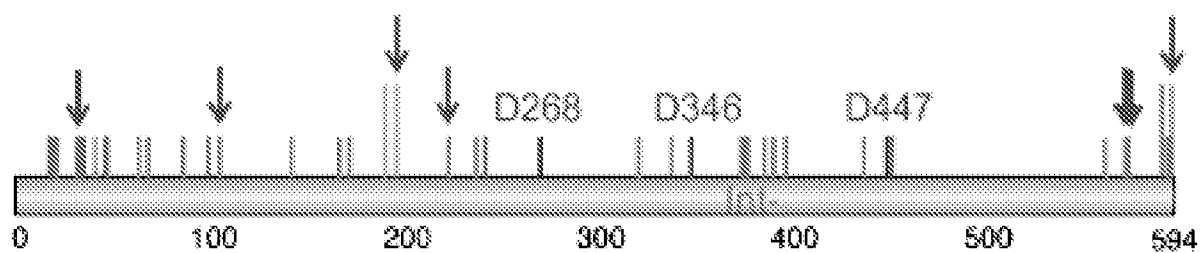
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-Tps	V436I/S213S	E66G	-Tps	M194V
PM6+	S10p/GLESCN	M194V	PM6+	S103P
PB	M589V/D170D	M194T	PB	T319A
F594L	E45G	L15P	4PB	S31P/T164A
F395L	R188K/G120G	L15P	7PB	T56DA
D19N/S461S	S592G	C97R/T242I	m7PB1	M599V
S573A	H33Y/Q360D/N57E/H4K/K334R		m7PB2	F395L
S10p/VWLESCN	N571S	I221T	mPB1	N394T
S17G	S373P		mPB2	.Tps
S584P	R189R/D450N/R526R		PB	PM6+

FIG. 4B

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35 candidates from > 5,000



↓ Also got hit in hyPBase screening

FIG. 4C

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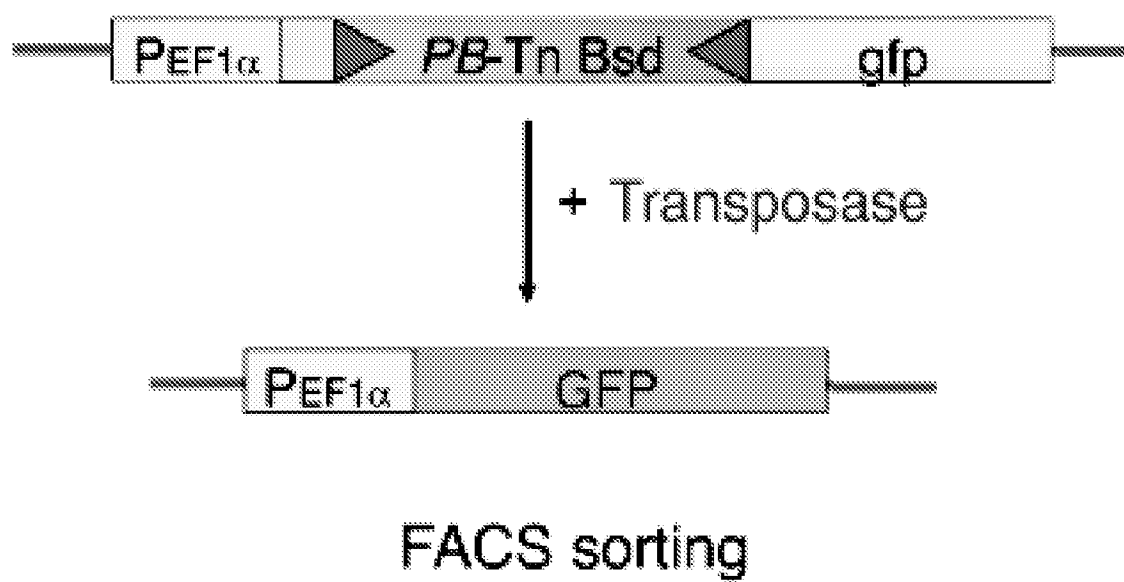


FIG. 5A

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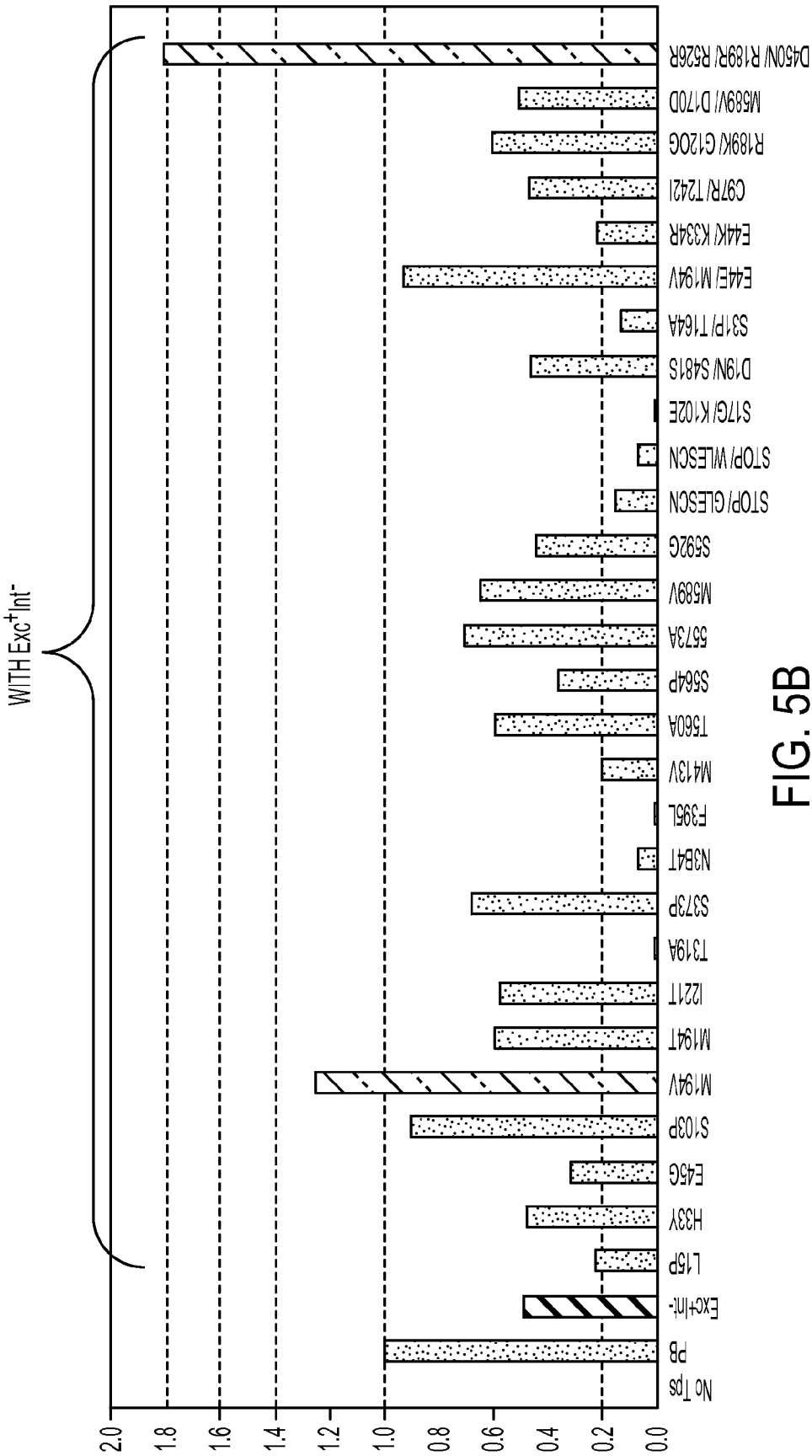
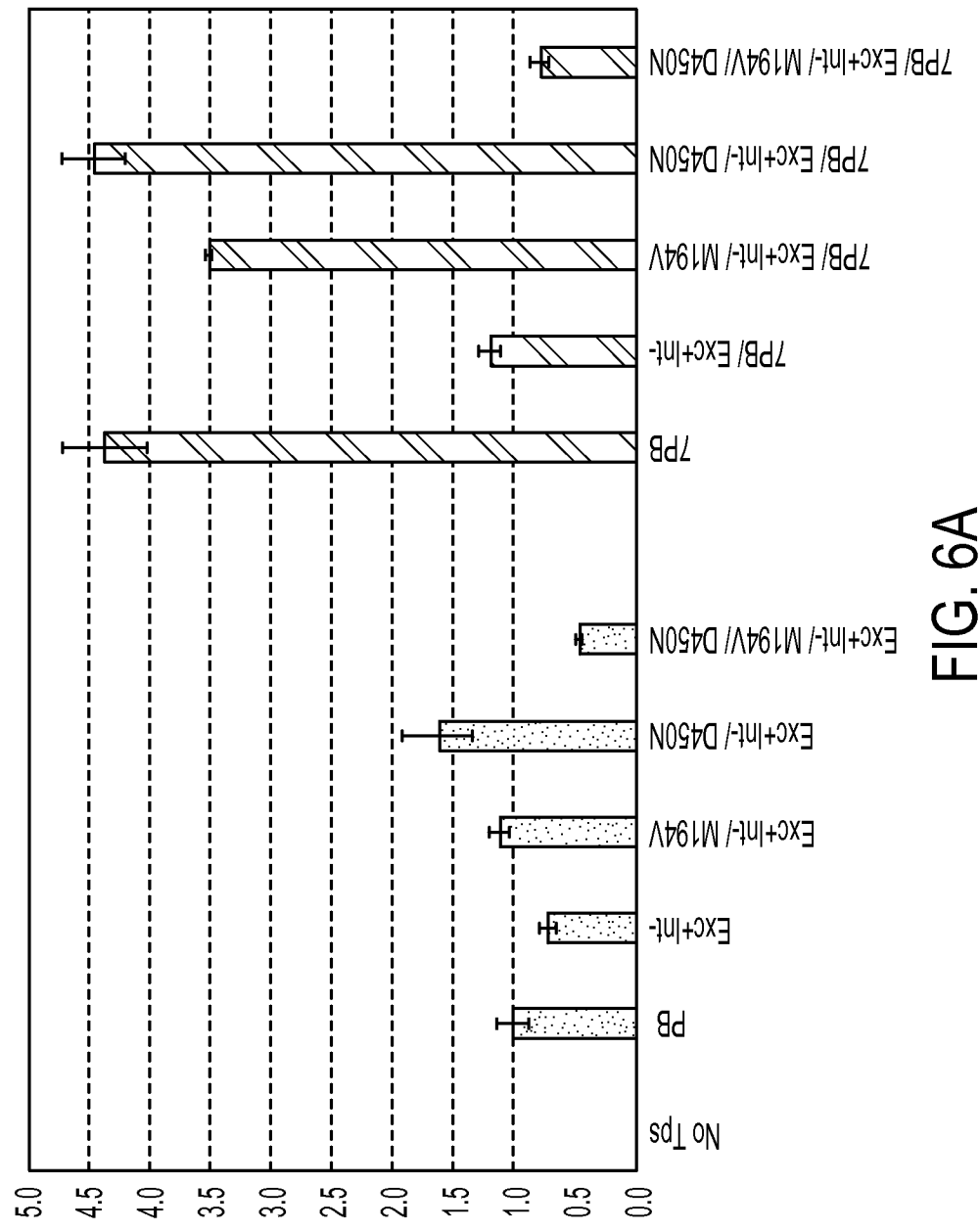


FIG. 5B



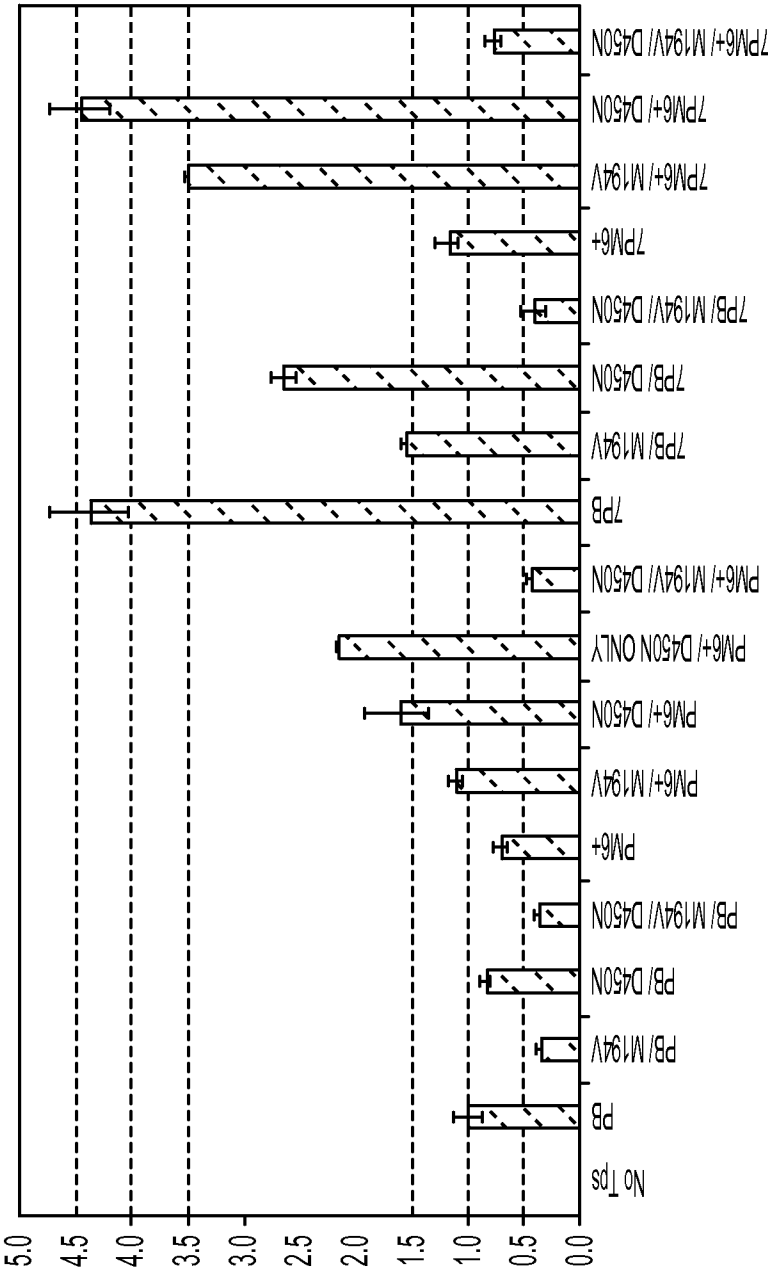


FIG. 6B

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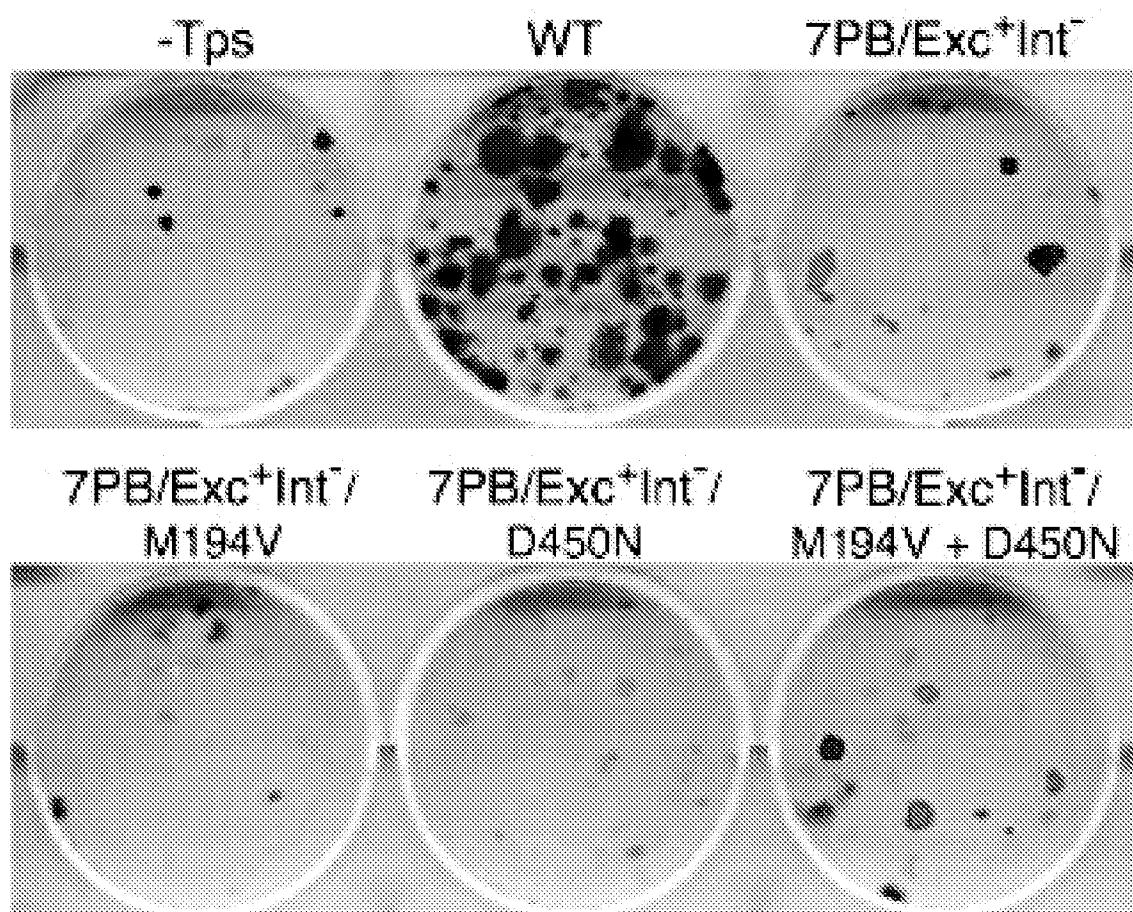


FIG. 6C

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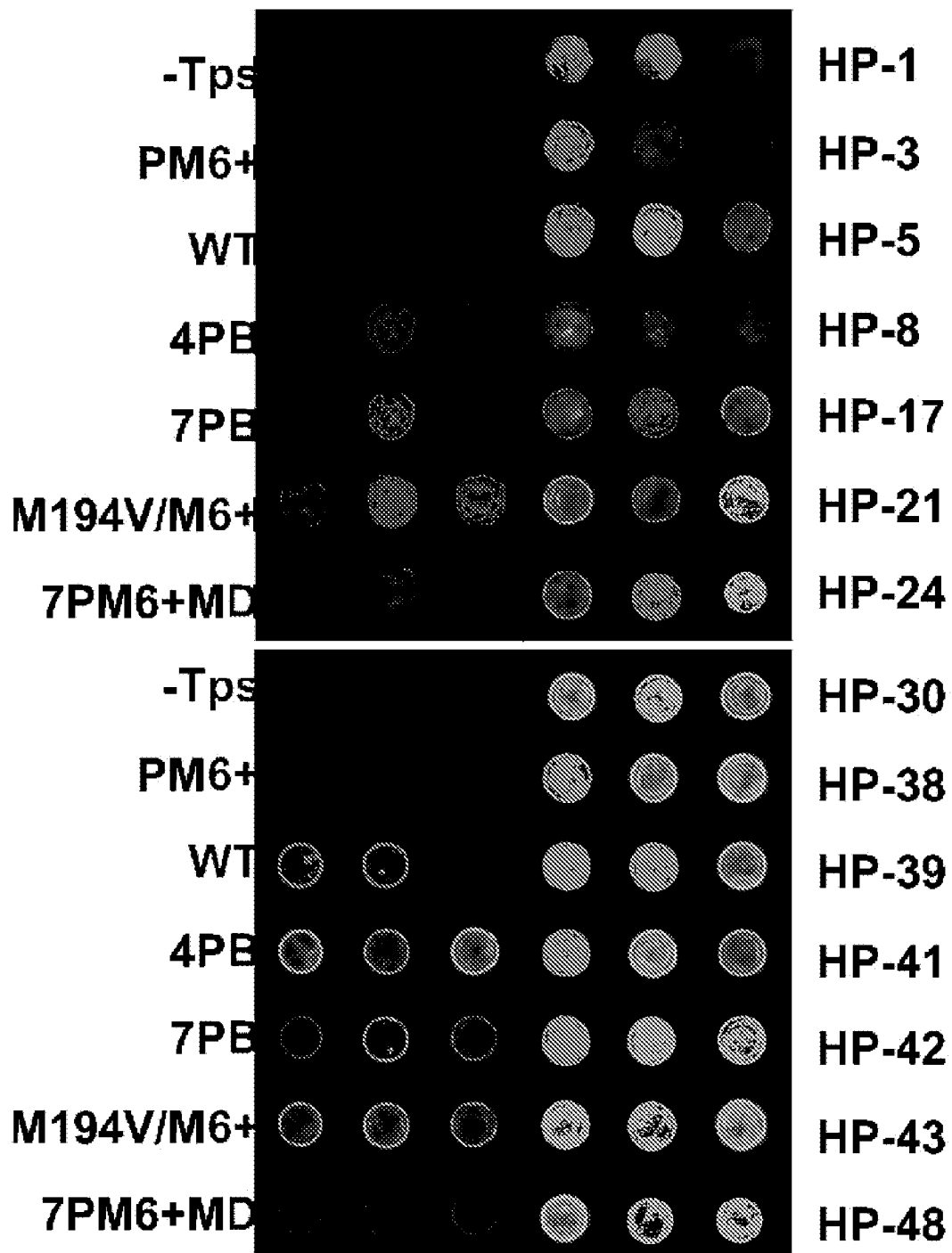


FIG. 7A

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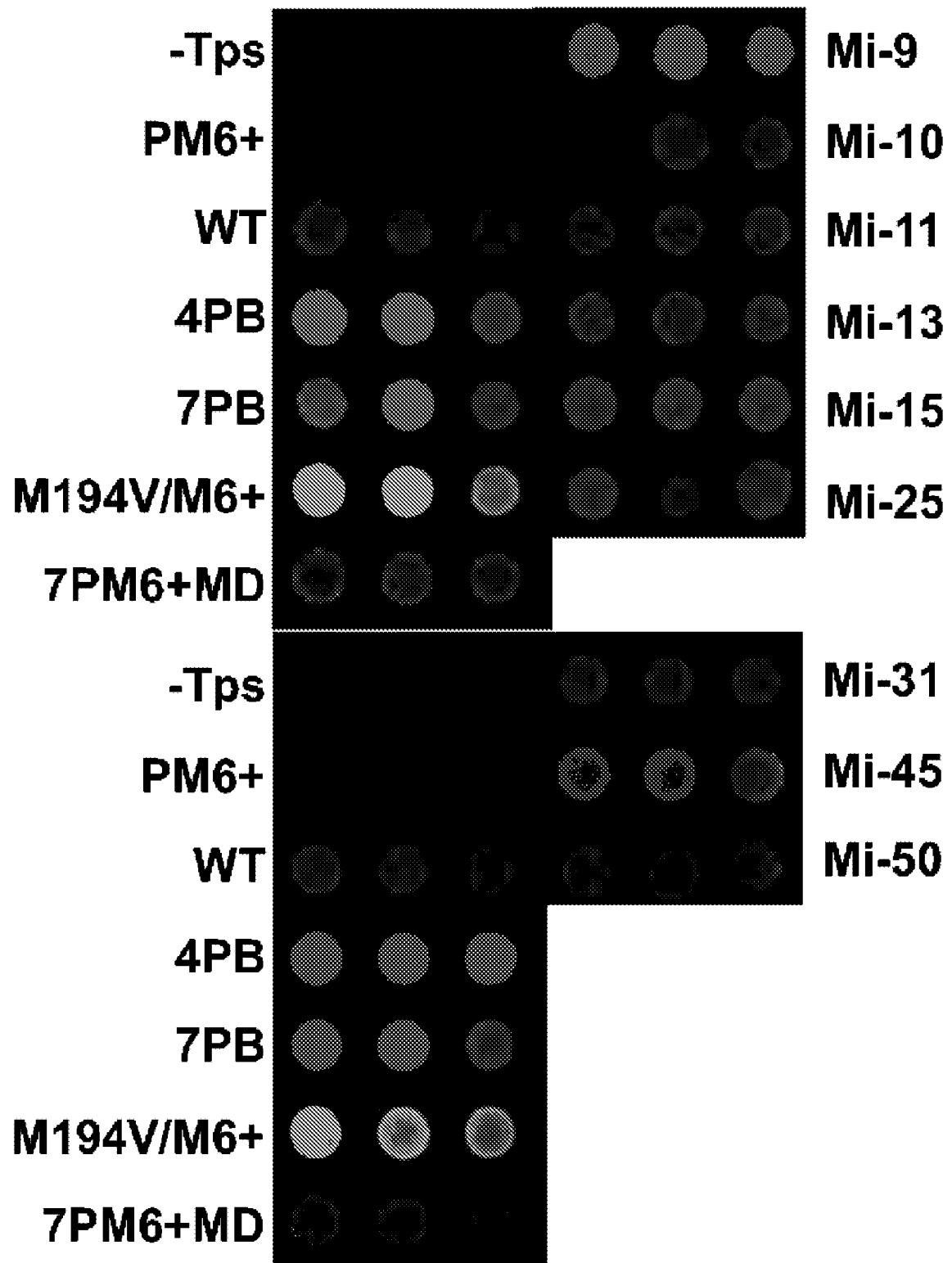
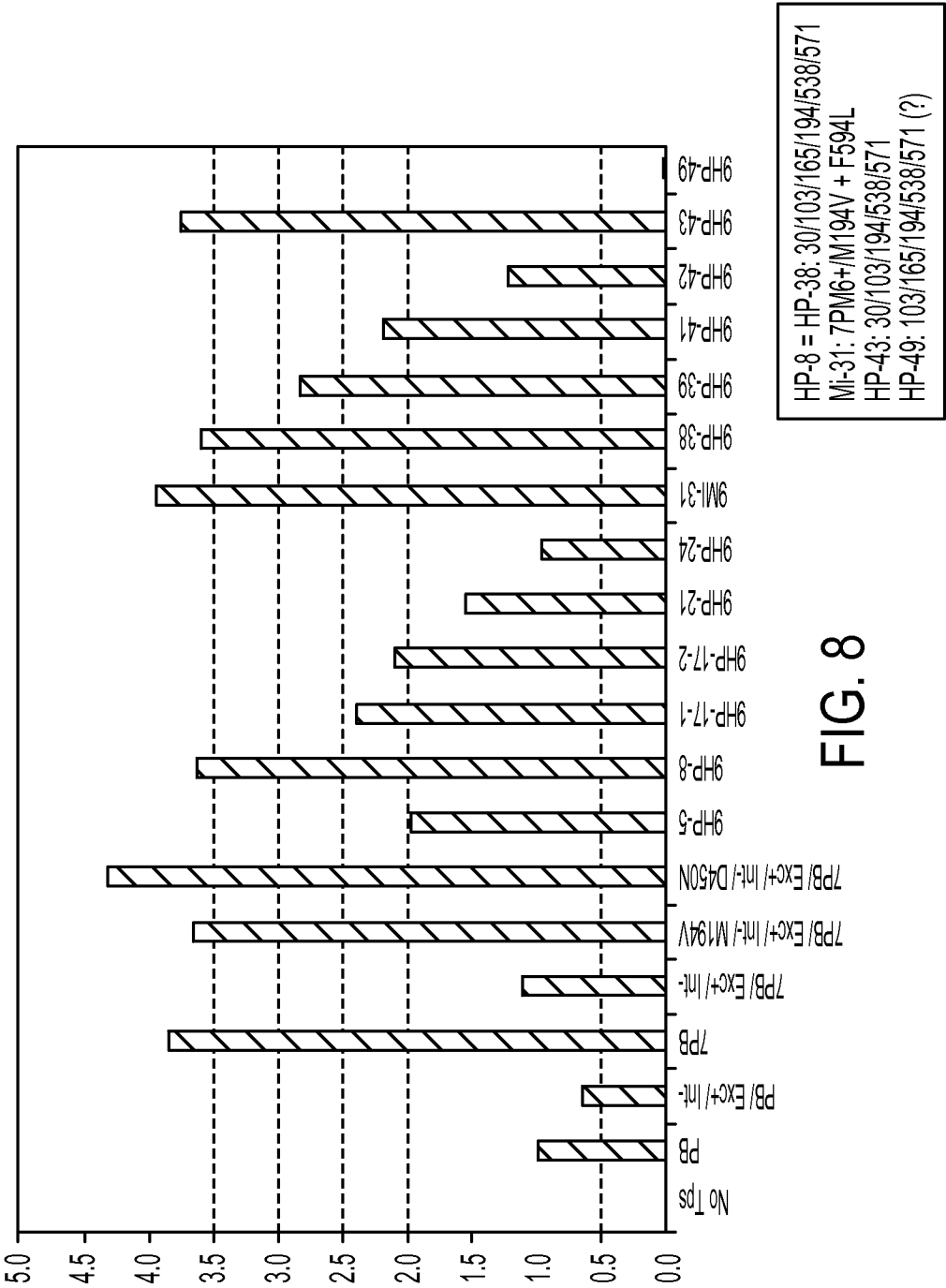


FIG. 7B



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Position	30	103	165	194	282	450	509	538	571	others
HP-1	+	+	-	+	-	-	-	-	-	
HP-3	+	-	+	+	-	-	+	+	+	Stop to W
HP-5	+	+	-	+	-	-	-	+	+	
HP-8	+	+	+	+	-	-	-	+	+	
HP-17	-	-	-	+	-	-	+	+	+	
HP-21	-	-	+	+	-	-	-	-	-	
HP-24	-	-	+	+	-	-	-	-	-	L235H, CTT CAT
HP-30	+	+	+	+	-	-	-	+	+	
HP-38	+	+	+	+	-	-	-	+	+	
HP-39	+	+	+	+	-	-	-	-	+	
HP-41	+	+	+	+	-	-	-	-	+	
HP-42	+	+	+	+	-	-	-	-	+	E45K
HP-43	+	+	-	+	-	-	-	+	+	
HP-48	-	-	+	+	-	-	-	-	-	F594L

HP-8 = HP-30 = HP-38, HP-39 = HP-41,

FIG. 9A

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Position	30	103	165	194	282	450	509	538	571	others
Mi-9	-	+	+	+	+	-	-	-	+	
Mi-10	+	+	+	+	+	-	+	+	+	
Mi-11	+	+	+	+	+	-	+	+	+	
Mi-13	+	+	+	+	+	-	+	+	+	
Mi-15	+	+	+	+	+	-	-	-	+	
Mi-25	+	-	-	-	-	+	-	+	+	F594L
Mi-31	+	+	+	+	+	-	+	+	+	F594L
Mi-45	-	+	+	+	+	-	-	-	+	
Mi-50	-	+	+	+	+	+	+	+	-	F594L

Mi10 = Mi11 = Mi13 = 7PB/ M194V/ Exc+Int-
 Mi15 = M45 = 103/165/194/282/571/ Exc+Int-
 M31 = 7PB/D450N/F594L

FIG. 9B

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Position	30	103	165	194	282	450	509	538	571	others
Low-2	+	-	+	~	-	-	~	-	+	
Low-3	-	+	+	+	+	-	-	-	-	
Low-6	-	-	-	+	+	-	+	+	+	
Low-7	+	+	+	+	+	~	-	~	~	
Low-9	+	+	+	+	+	~	-	~	+	
Low-10	+	-	-	~	-	+	~	-	-	
Low-16	+	-	+	+	-	+	+	-	+	
Low-17	+	+	+	+	+	+	+	+	+	template
Low-22	-	-	-	-	+	+	+	-	-	
Low-26	+	+	-	-	+	+	+	+	+	
Low-27	-	-	-	-	-	-	-	+	+	

FIG. 9C