doi:10.1038/nature14592

Supplementary Information

Supplementary Table 1 – oligonucleotides (attached excel file)
Supplementary Table 2 – sgRNA targets (attached excel file)
Supplementary Table 3 – PAM MiSeq data (attached excel file)

Supplementary Sequences - plasmids used in this study

Supplementary Software - for analyzing PAM depletion MiSeq data

Supplementary Sequences - plasmids used in this study

Name	Addgene ID	Description
BPK764	65767	T7-humanSpCas9-NLS-3xFLAG-T7-BsaIcassette-Sp-sgRNA
MSP712	65768	T7-humanSpdCas9(D10A/H840A)-NLS-3xFLAG-T7-BsaIcassette-Sp-sgRNA
MSP1673	65769	T7-humanSt1Cas9-NLS-T7-BspMIcassette-St1-sgRNA
BPK2101	65770	T7-humanSaCas9-NLS-3xFLAG-T7-BsaIcassette-Sa-sgRNA
p11-lacY-wtx1[15]	-----	BAD-ccDB-Amp^R-AraC-lacY(A177C)
JDS246	43861	CMV-T7-humanSpCas9-NLS-3xFLAG
MSP469	65771	CMV-T7-humanSpCas9(D1135V/R1335Q/T1337R)-NLS-3xFLAG (VQR variant)
MSP680	65772	CMV-T7-humanSpCas9(D1135E/R1335Q/T1337R)-NLS-3xFLAG (EQR variant)
MSP1101	65773	CMV-T7-humanSpCas9(D1135V/G1218R/R1335E/T1337R)-NLS-3xFLAG (VRER variant)
MSP977	65774	CMV-T7-humanSpCas9(D1135E)-NLS-3xFLAG
MSP1594	65775	CAG-humanSt1Cas9-NLS
BPK2139	65776	CAG-humanSaCas9-NLS-3xFLAG
BPK1520	65777	U6-BsmBIcassette-Sp-sgRNA
BPK2301	65778	U6-BsmBIcassette-St1-sgRNA
VVT1	65779	U6-BsmBIcassette-Sa-sgRNA

BPK764: T7-humanSpCas9-NLS-3xFLAG-T7-BsaIcassette-Sp-sgRNA
T7 promoters colored in green, human codon optimized *S. pyogenes* Cas9 colored in blue, NLS underlined, 3xFLAG tag in **bold**, BsaI sites underlined, sgRNA colored in purple, T7 terminator colored in red

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MSP712: T7-humanSpCas9(D10A/H840A)-NLS-3xFLAG-T7-BsaIcassette-Sp-sgRNA
T7 promoters colored in green, human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in pink, NLS underlined, 3xFLAG tag in **bold**, BsaI sites underlined, sgRNA colored in purple, T7 terminator colored in red

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MSP1673: T7-humanSt1Cas9-NLS-T7-BspMIcassette-St1-sgRNA
T7 promoters colored in green, human codon optimized *S. thermophilus1* Cas9 colored in blue, NLS underlined, BspMI sites underlined, sgRNA colored in purple, T7 terminator colored in red

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BPK2101: T7-humanSaCas9-NLS-3xFLAG-T7-BsaIcassette-Sa-sgRNA
T7 promoters colored in green, human codon optimized *S. aureus* Cas9 colored in blue, NLS underlined,
3xFLAG tag in **bold**, BsaI sites underlined, sgRNA colored in purple, T7 terminator colored in red

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JDS246: CMV-T7-humanSpCas9-NLS-3xFLAG
Human codon optimized *S. pyogenes* Cas9 colored in blue, NLS underlined, 3xFLAG tag in **bold**

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MSP469: CMV-T7-humanSpCas9(D1135V/R1335Q/T1337R)-NLS-3xFLAG (VQR variant)
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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MSP680: CMV-T7-humanSpCas9(D1135E/R1335Q/T1337R)-NLS-3xFLAG (EQR variant)
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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MSP1101: CMV-T7-humanSpCas9(D1135V/G1218R/R1335E/T1337R)-NLS-3xFLAG (VRER variant)
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codons in red, NLS underlined, 3xFLAG tag in **bold**

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CAAAGTATAGTCTGTTTGAGTTAGAAAATGGCCGAAAACGGATGTTGGCTAGCGCCAGAGAGCTTCAAAAGGGGAACGAACTCGCACTACCGTCTAAATACG
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GAAATCATAGAGCAAATTTCGGAATTCAGTAAGAGAGTCATCCTAGCTGATGCCAATCTGGACAAAGTATTAAGCGCATACAACAAGCACAGGGATAAACCCA
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ACACTTCTACCAAGGAGGTGCTAGACGCGACACTGATTCACCAATCCATCACGGGATTATATGAAACTCGGATAGATTTGTCACAGCTTGGGGGTGACGGATC
CCCCCAAGAAGAAGAGGAAAGTCTCGAGC**GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG**TGA

MSP977: CMV-T7-humanSpCas9(D1135E)-NLS-3xFLAG (D1135E variant)
Human codon optimized *S. pyogenes* Cas9 colored in blue, modified codon in red, NLS underlined, 3xFLAG tag in **bold**

ATGGATAAAAAGTATTCTATTGGTTTAGACATCGGCACTAATTCCGTTGGATGGGCTGTCATAACCGATGAATACAAAGTACCTTCAAAGAAATTTAAGGTGTT
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ACACTTCTACCAAGGAGGTGCTAGACGCGACACTGATTCACCAATCCATCACGGGATTATATGAAACTCGGATAGATTTGTCACAGCTTGGGGGTGACGGAT
CCCCCAAGAAGAAGAGGAAAGTCTCGAGC**GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG**TGA

MSP1594: CAG-humanSt1Cas9-NLS
Human codon optimized *S. thermophilus1* Cas9 colored in blue, NLS underlined

ATGGGCAGCGACCTGGTGCTGGGCCTGGACATCGGCATCGGCAGCGTGGGCGTGGGCATCCTGAACAAGGTGACCGGCGAGATCATCCACAAGAACAGT
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AGCTGAGCAACGAGGAGCTGTTCATCGCCCTGAAGAACATGGTGAAGCACCGCGGCATCAGCTACCTGGACGACGCCAGCGACGACGGCAACAGCAGCGT
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CAAGATCCGCCTGTGGCACCAGCAGGGCGAGCGCTGCCTGTACACCGGCAAGACCATCAGCATCCACGACCTGATCAACAACAGCAACCAGTTCGAGGTG
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ACCCGCGACACCTACCACCACCACGCCGTGGACGCCCTGATCATTGCGGCTTCTAGCCAGCTGAACCTGTGGAAGAAGCAGAAGAACACCCTGGTGAGCTA
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GGTGCGCACCGACGTGCTGGGCAACCAGCACATCATCAAGAACGAGGGCGACAAGCCCAAGTTGGACTTCAGCAGGGCTGACCCCAAGAAGAAGAGGAAG
GTGTGA

BPK2139: CAG-humanSaCas9-NLS-3xFLAG

Human codon optimized *S. aureus* Cas9 colored in blue, NLS underlined, 3xFLAG tag in **bold**

ATGGGGAAACGGAACTACATCCTGGGGCTTGACATTGGGATAACCAGCGTTGGCTACGGAATTATTGATTATGAGACACGCGATGTGATTGACGCCGGGGT
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GTGAAAAAGCTGCTCTTCGATTACAATCTCCTGACCGATCATAGTGAGCTGAGCGGAATCAACCCCTACGAGGCGCGAGTGAAAGGGCTTTCCCAGAAGCT
GTCCGAAGAGGAGTTCTCCGCCGCGTTGCTGCACCTGGCCAAACGGAGGGGGGTTCACAATGTAAACGAAGTGGAGGAGGACACGGGCAATGAACTTAGT
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GGACGAGAACGAGAAGCTGGAGTACTATGAAAAGTTCCAGATTATCGAGAATGTGTTTAAGCAGAAGAAGAAGCCGACACTTAAGCAGATTGCAAAGGAAAT
CCTCGTGAATGAGGAAGATATCAAGGGATACAGAGTGACAAGTACAGGCAAGCCCGAGTTCACAAATCTGAAGGTGTACCACGATATTAAGGACATAACCGC
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CAAGAAGCTGATCAACAAGTCTCCAGAGAAGCTCCTTATGTATCACCACGACCCACAGACTTATCAGAAATTGAAACTGATCATGGAGCAATACGGGGATGA
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CAAGAAGAAGAGGAAAGTCTCGAGC**GACTACAAAGACCATGACGGTGATTATAAAGATCATGACATCGATTACAAGGATGACGATGACAAG**TAA

BPK1520: U6-BsmBIcassette-Sp-sgRNA

U6 promoter in green, BsmBI sites underlined, *S. pyogenes* sgRNA colored in purple, U6 terminator underlined

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTG
CATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTTGGG
TAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAA
ACACCGGAGACGATTAATGCGTCTCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
TTTTTT

BPK2301: U6-BsmBIcassette-St1-sgRNA
U6 promoter in green, BsmBI sites underlined, *S. thermophilus1* sgRNA colored in purple, U6 terminator underlined

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTG
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TAAGGCTTCATGCCGAAATCAACACCCTGTCATTTTATGGCAGGGTGTTTTT

VVT1: U6-BsmBIcassette-Sa-sgRNA
U6 promoter in green, BsmBI sites underlined, *S. aureus* sgRNA colored in purple, U6 terminator underlined

TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTG
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AGGCAAAATGCCGTGTTTATCTCGTCAACTTGTTGGCGAGATTTTTTTT

Supplementary Software - for analyzing PAM depletion MiSeq data

Run in the command prompt (in the directory containing the file) using the command "python PAM_depletion.py"

```
import numpy as np
import pandas as pd
import glob
import fnmatch
import os
from collections import Counter
from Bio.Seq import Seq
from Bio import SeqIO
import itertools
import re
from pandas import ExcelWriter
import Tkinter, tkFileDialog

__author__ = "Ved V. Topkar"
__version__ = "1.0"


"""
IUPAC_notation_regex describes a mapping between certain base characters and the relavent regex string
(Useful for parsing out ambiguous base strings)
"""
IUPAC_notation_regex = {
    'N': '[ATCG]',
    'Y': '[CT]',
    'R': '[AG]',
    'W': '[AT]',
    'S': '[CG]',
    'A': 'A',
    'T': 'T',
    'C': 'C',
    'G': 'G'
}

def ambiguous_PAMs(length):
    """
    Given an inputted length, return a list of strings describing all possible PAM sequences
    NOTE: Returned strings include ambiguous base characters
    """
    permutations = itertools.product(['N', 'A', 'T', 'C', 'G'], repeat=length)
    PAMs = []

    for item in permutations:
        PAMs.append(''.join(item))

    return PAMs

def unambiguous_PAMs(length):
    permutations = itertools.product(['A', 'T', 'C', 'G'], repeat=length)
    PAMs = []

    for item in permutations:
        PAMs.append(''.join(item))

    return PAMs


def regex_from_seq(seq):
    """
    Given a sequence with ambiguous base characters, returns a regex that matches for
    the explicit (unambiguous) base characters
    """
    regex = ''

    for c in seq:
        regex += IUPAC_notation_regex[c]

    return regex


def regex_match_count(regex, list_of_counts):
    """
    Given a list of strings and a regex, return the number of strings in the list that the regex matches.
```

```
    """
    c = 0

    for item in list_of_counts:
        if re.search(regex, item):
            c += 1

    return c

def tabulate_substring_frequencies(pams, indices):
    """
    Given a list of raw pams and substring indices, tabulates the frequency of tabulate_substring_frequencies
    RETURNS a Pandas Series
    """
    base_PAMs = unambiguous_PAMs(indices[1] - indices[0])
    tmp_PAMs = Counter([pam[indices[0]:indices[1]] for pam in pams])

    c = Counter()
    for base_PAM in base_PAMs:
        c[base_PAM] = tmp_PAMs[base_PAM]

    PAMs = pd.Series(c)
    PAMs.sort(ascending=False)
    excel_PAMs = pd.DataFrame()
    excel_PAMs['PAM'] = PAMs.index
    excel_PAMs['Count'] = PAMs.values
    excel_PAMs['Frequency'] = PAMs.values.astype(float)/sum(PAMs.values)

    return excel_PAMs

def generate_raw_PAM_counts(filepaths, targetsites, PAM_length):
    """
    Here, we get all of our relavent PAM sequences from the inputted files
    by searching for the targetsites and looking at the flanking region
    """

    reverse_target_sequences = {targetsite: str(Seq(targetsites[targetsite]).reverse_complement()) for targetsite in targetsites}
    all_pams = {targetsite: [] for targetsite in targetsites}

    # Iterate through each file and collect the PAMs of each sequence
    # Checks both forward and reverse reads
    for filepath in filepaths:
        print 'Scanning file: ' + os.path.basename(filepath)
        pams = []
        records = SeqIO.parse(filepath, filepath.split('.')[-1])
        for record in records:
            seq = str(record.seq)
            for targetsite in targetsites:
                target_seq = targetsites[targetsite]
                target = seq.find(targetsites[targetsite])
                if target > -1:
                    index = target + len(target_seq)
                    all_pams[targetsite].append(seq[index:index + PAM_length])
                else:
                    target = seq.find(reverse_target_sequences[targetsite])
                    if target > -1:
                        index = target
                        all_pams[targetsite].append(str(Seq(seq[index - PAM_length:index]).reverse_complement()))

    return all_pams


def analyze_PAM_depletion_data(filepaths, targetsites, PAM_length=3):
    """
    Given a directory that contains a given file extension and a target sequence, do the entire PAM depletion analysis
    """

    # Make sure that dirnames and target sequences are inputted
    if filepaths is None:
        raise Exception('Please specify a directory name')

    if targetsites is None:
        raise Exception('Please specify a target sequence')

    if PAM_length is None or PAM_length < 3:
        raise Exception('Please enter a valid PAM length')


    all_pams = generate_raw_PAM_counts(filepaths, targetsites, PAM_length)
```

```
    letters = ['A', 'T', 'C', 'G']

    all_counters = {targetsite: Counter(all_pams[targetsite]) for targetsite in targetsites}

    for targetsite in targetsites:
        pams = all_pams[targetsite]

        base_counters = [Counter() for x in range(PAM_length)]

        for pam in pams:
            for i, c in enumerate(pam):
                base_counters[i][c] += 1

        raw_PAM_counts = pd.Series(all_counters[targetsite])
        raw_PAM_counts.sort(ascending=False)
        raw_counts_df = pd.DataFrame()
        raw_counts_df['PAM'] = raw_PAM_counts.index
        raw_counts_df['Count'] = raw_PAM_counts.values

        single_base_counts = pd.DataFrame(base_counters)
        single_base_frequencies = single_base_counts.divide(single_base_counts.sum(axis=1).ix[0])

        # Prepare substring counts and frequencies
        writer = ExcelWriter('out/' + os.path.basename(filepath).split('.')[0] + '_' + targetsite + '.xlsx')
        single_base_counts.to_excel(writer, 'Single Base Counts')
        single_base_frequencies.to_excel(writer, 'Single Base Frequencies')
        raw_counts_df.to_excel(writer, 'Raw PAM Counts')

        # Designate which windows should be analyzed and name them
        settings = {
                    'XXXNNN': [0,3],
                    'NXXXNN': [1,4],
                    'NNXXXN': [2,5],
                    'NNNXXX': [3,6],
                    'XXXXNN': [0,4],
                    'NXXXXN': [1,5],
                    'NNXXXX': [2,6],
                    'XXNNNN': [0,2],
                    'NXXNNN': [1,3],
                    'NNXXNN': [2,4],
                    'NNNXXN': [3,5],
                    'NNNNXX': [4,6],
                    'XXXXXN': [0,5],
                    'NXXXXX': [1,6],
                    'XXXXXX': [0,6],
                   }

        for item in settings:
            df = tabulate_substring_frequencies(pams, settings[item])
            df.to_excel(writer, item)

        writer.save()

        print 'Saved excel output for ' + targetsite


if __name__ == "__main__":

    # Display the filepicker, accepting only FASTQ files
    root = Tkinter.Tk()
    root.withdraw()
    file_paths = tkFileDialog.askopenfilenames(parent=root, title='Choose FASTQ files', filetypes=[("FastQ files", "*.fastq")])

    # Describe the targetsite(s) to search for
    targetsites = {'EGFP site 1': 'GTCGCCCTCGAACTTCACCT'}

    # Run the analysis on the inputted filepaths and targetsite for a given variable nucleotide region length
    analyze_PAM_depletion_data(file_paths, targetsites, PAM_length=6)
```
