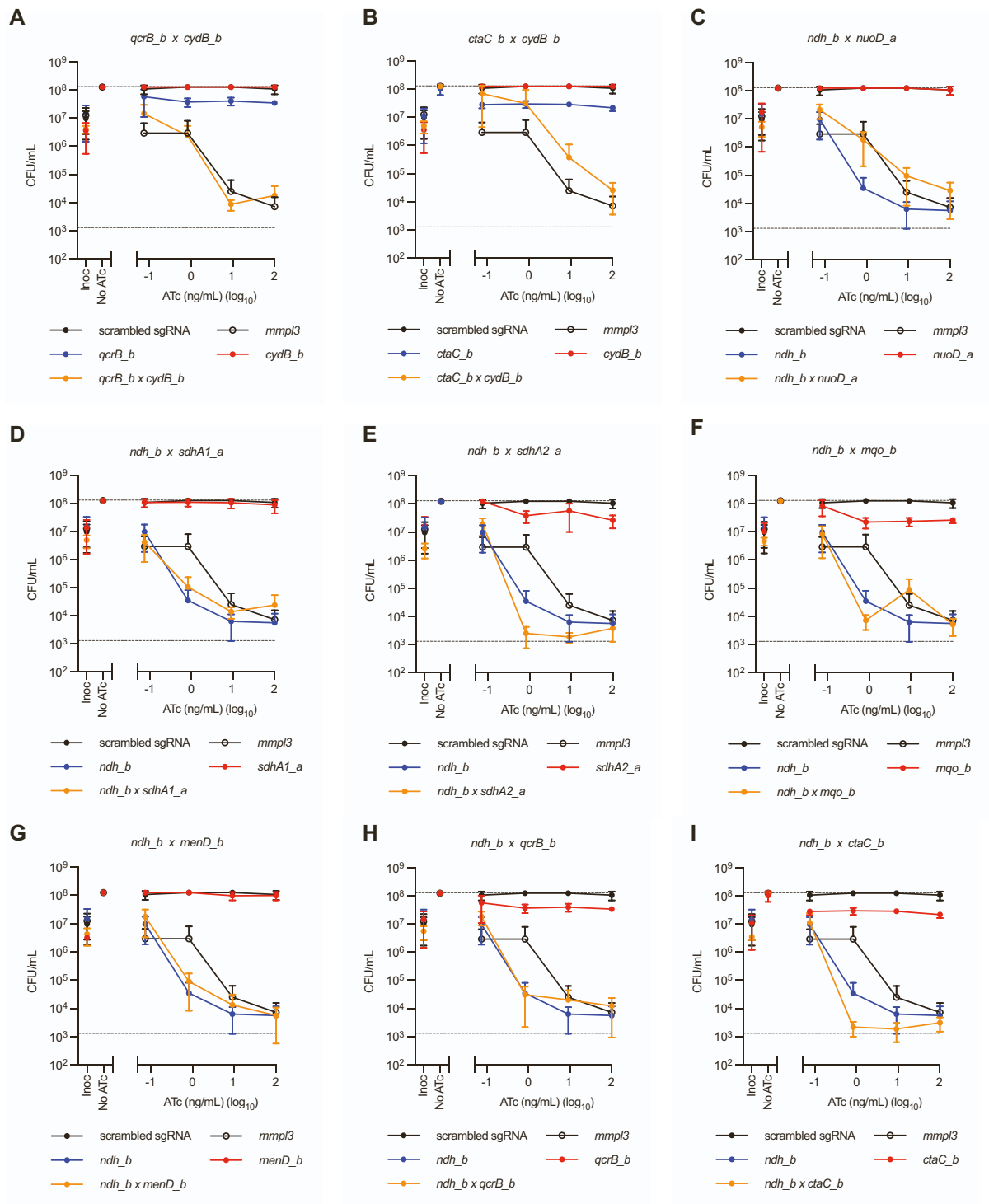


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Supplemental information

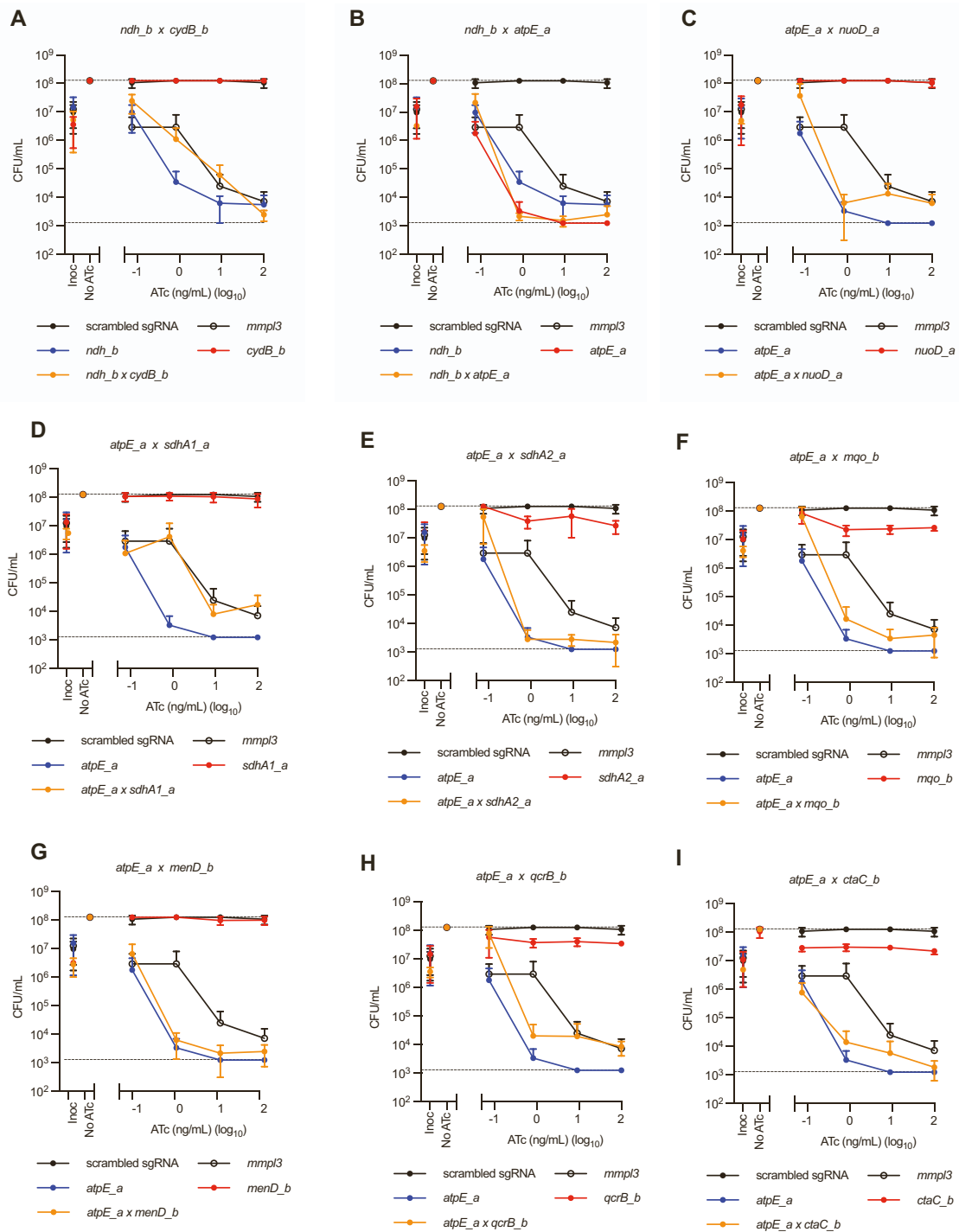
**Multiplexed transcriptional repression identifies
a network of bactericidal interactions
between mycobacterial respiratory complexes**

Matthew B. McNeil, Heath W. Ryburn, Justin Tirados, Chen-Yi Cheung, and Gregory M. Cook



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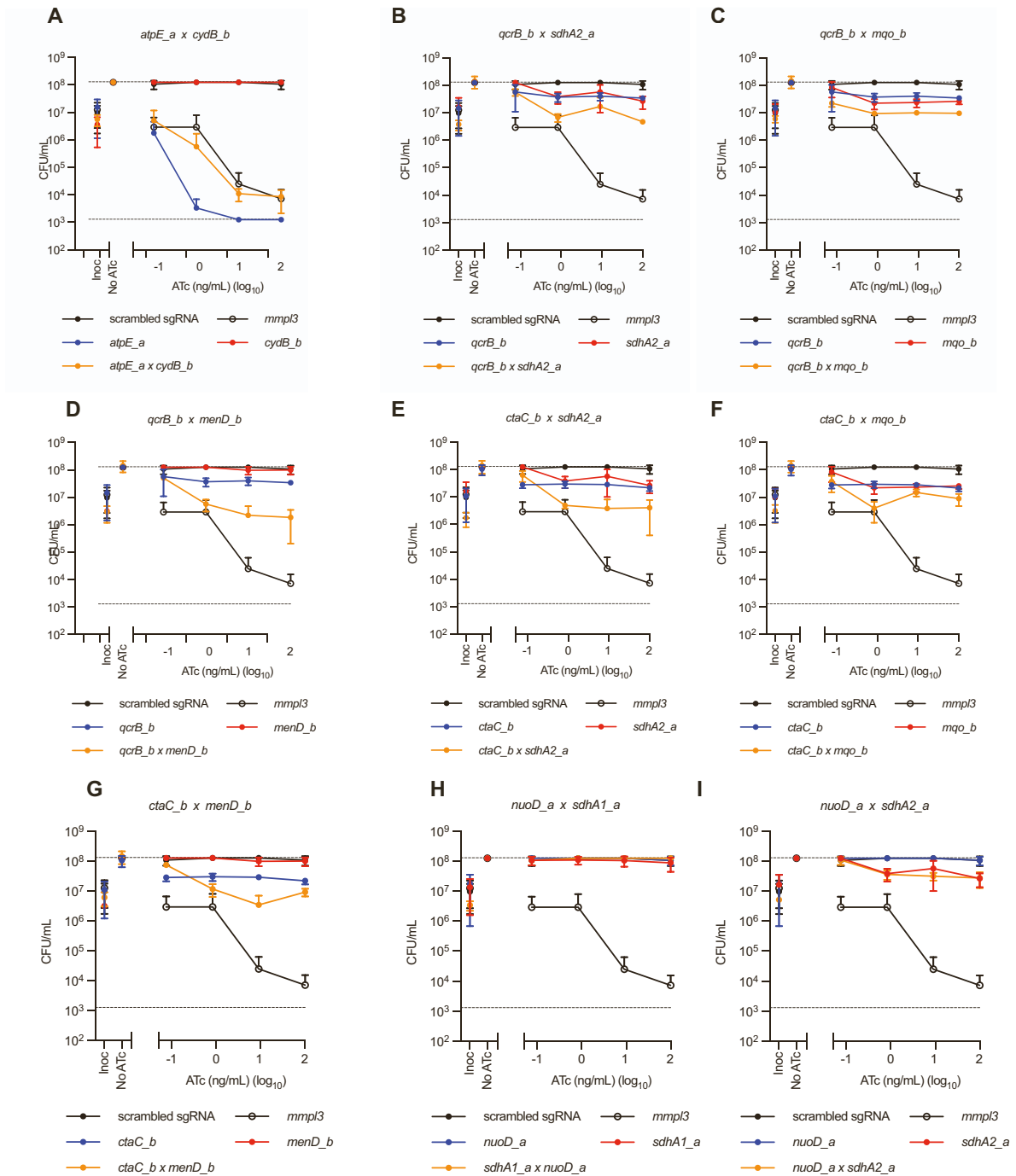
2 **Figure S1| Killing phenotypes of multiplexed, related to figure 1: CFU/ml plots of**
 3 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain
 4 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a
 5 negative and positive control respectively. Results are the mean \pm SD of four biological
 6 replicates



7

8 **Figure S2| Killing phenotypes of multiplexed, related to figure 1: CFU/ml plots of**

9 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain
 10 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a
 11 negative and positive control respectively. Results are the mean \pm SD of four biological
 12 replicates



13

14 **Figure S3| Killing phenotypes of multiplexed, related to figure 1: CFU/ml plots of**

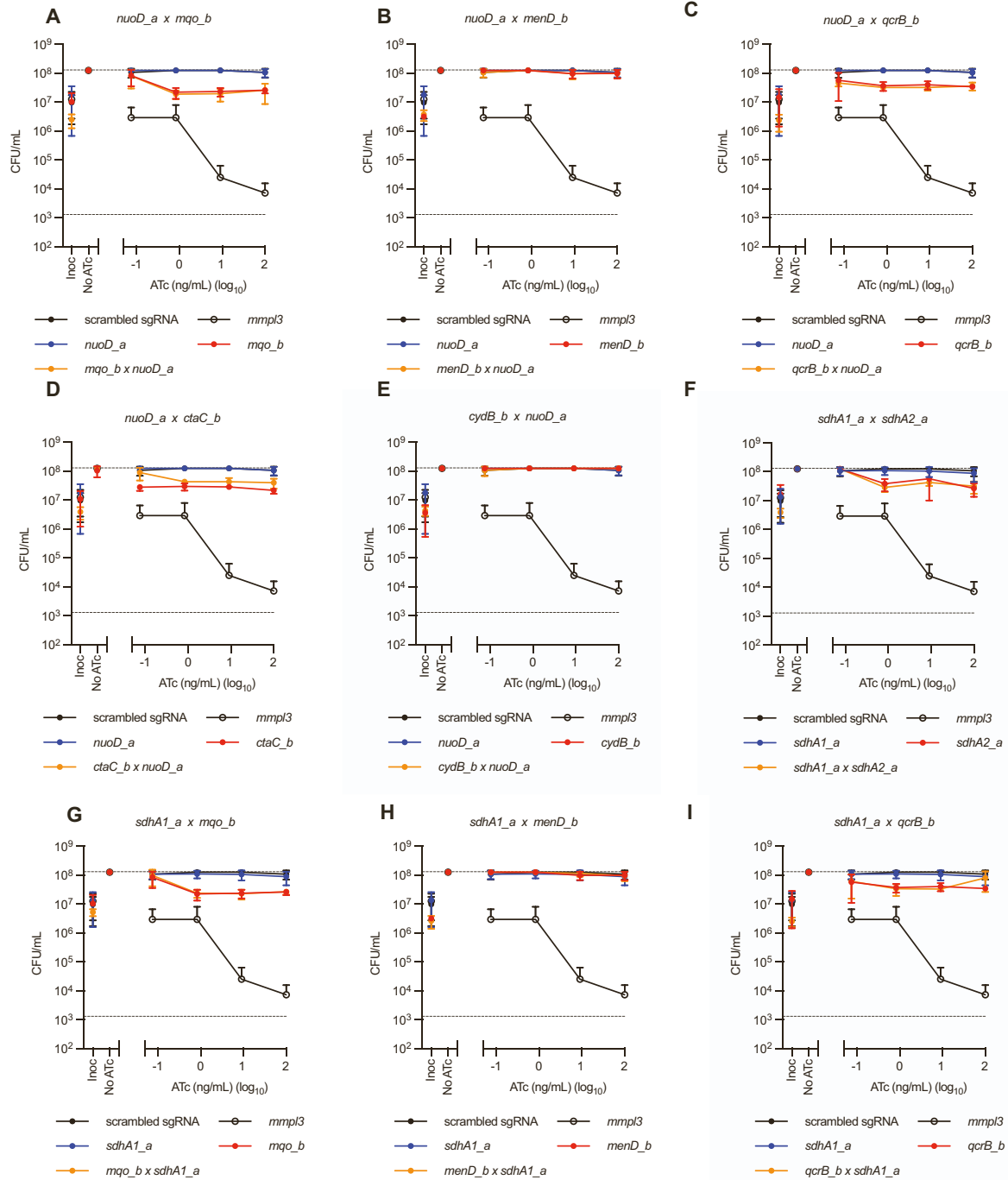
15 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain

16 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a

17 negative and positive control respectively. Results are the mean \pm SD of four biological

18 replicates

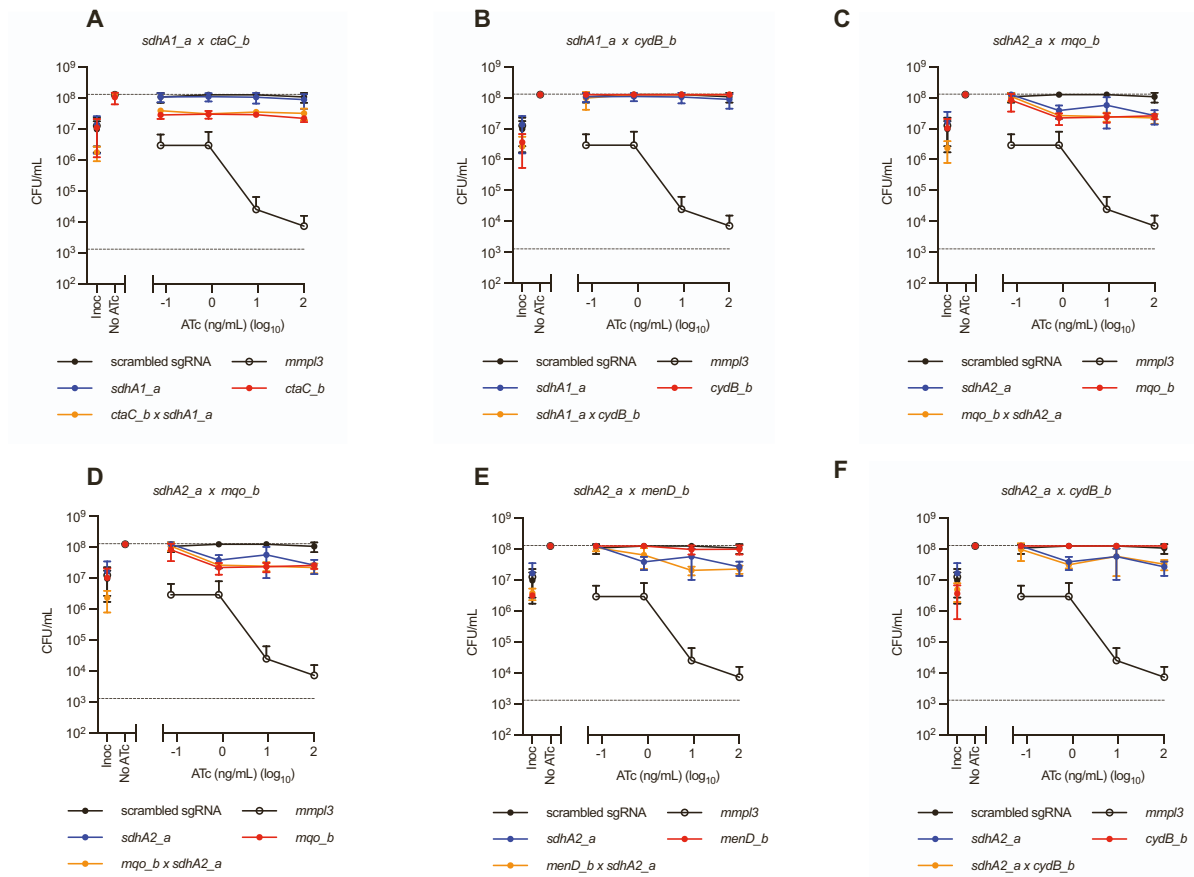
19



20

21 **Figure S4| Killing phenotypes of multiplexed, related to figure 1: CFU/ml plots of**
 22 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain
 23 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a
 24 negative and positive control respectively. Results are the mean \pm SD of four biological
 25 replicates

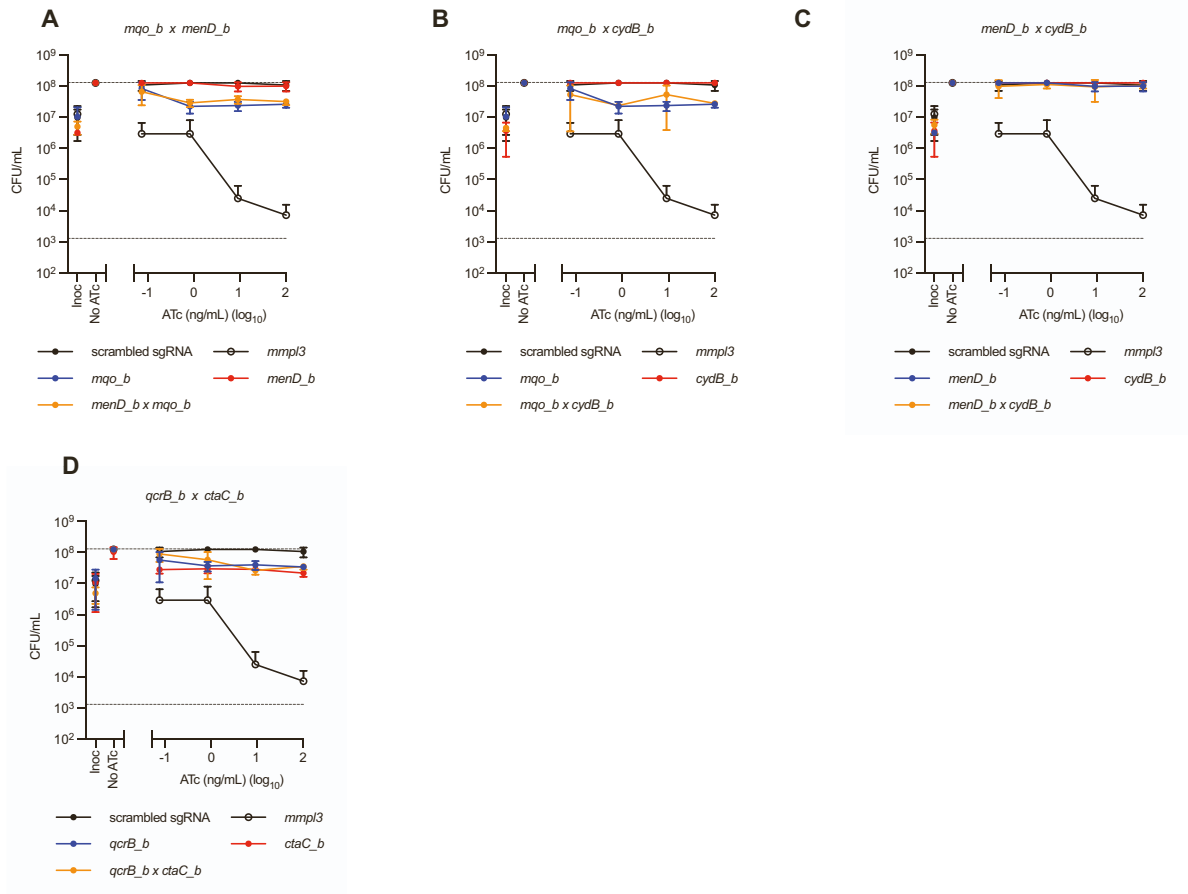
26



27

28 **Figure S5| Killing phenotypes of multiplexed, related to figure 1: CFU/ml plots of**
 29 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain
 30 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a
 31 negative and positive control respectively. Results are the mean \pm SD of four biological
 32 replicates

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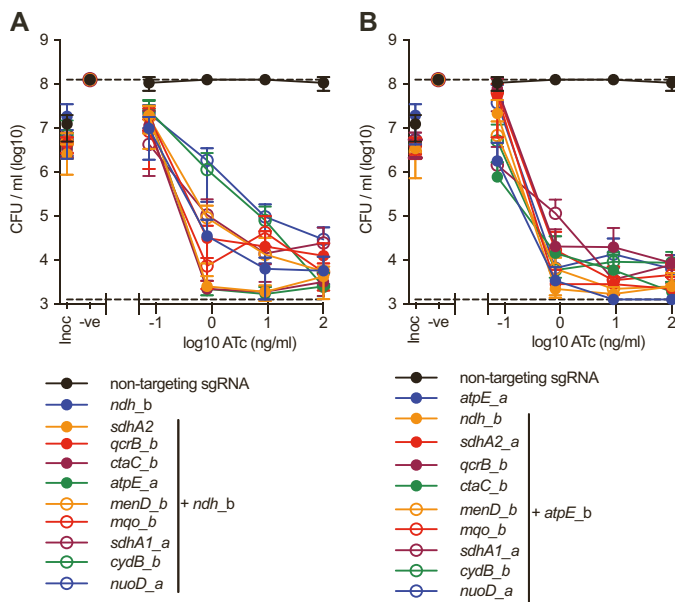
35 **Figure S6| Killing phenotypes of multiplexed, related to figure 1:** CFU/ml plots of
 36 *M. smegmatis* strains expressing stated single and multiplexed sgRNAs. A strain
 37 expressing a non-targeting (NT) sgRNA or sgRNA targeting *mmpL3* is used as a
 38 negative and positive control respectively. Results are the mean \pm SD of four biological
 39 replicates

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46 **Figure S7| Killing phenotypes of sgRNAs targeting *ndh* and *atpE* in multiplexed**

47 **combinations, related to figure 2: (A-B) CFU/ml plots of *M. smegmatis* strains**

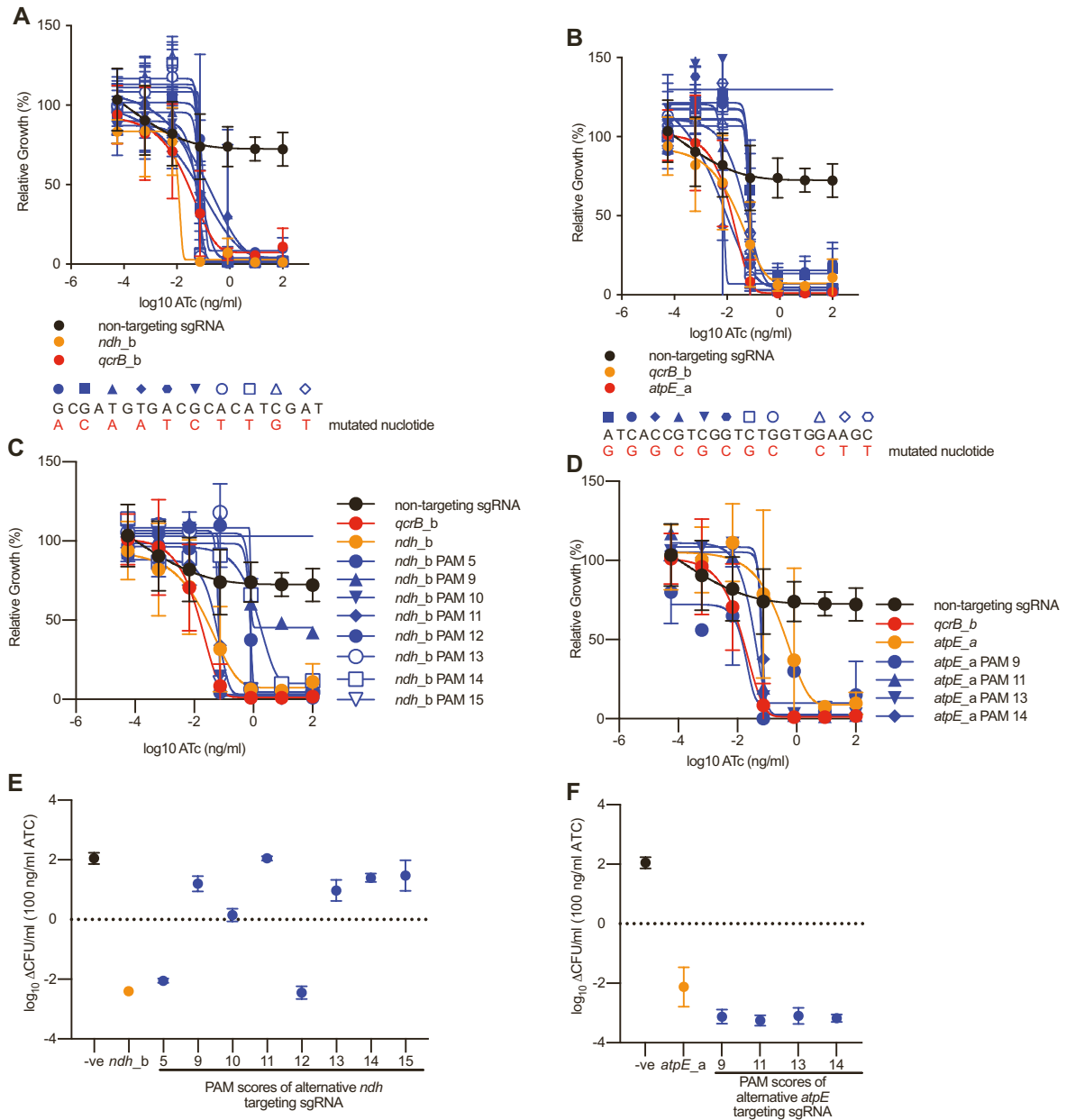
48 **expressing (A) *ndh_b* or (B) *atpE_a* in combination with sgRNAs targeting alternative**

49 **respiratory components across increasing concentrations of ATc. A strain expressing**

50 **a non-targeting (NT) sgRNA is used as a negative control. Results for A-D are the**

51 **mean \pm SD of four biological replicates.**

52



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55 **Figure S8| Phenotypes of alternative PAM variants of sgRNAs targeting *ndh* and**

56 ***atpE*, related to figure 2: (A-D) Growth of *M. smegmatis* strains expressing either (A)**

57 ***ndh_b* mis-matched sgRNAs, (B) *atpE_a* mis-matched sgRNAs, (C) sgRNAs targeting**

58 ***ndh* with weaker PAM variants or (D) sgRNAs targeting *atpE* with weaker PAM**

59 **variants in the presence of increasing concentrations of ATc. Growth is expressed**

60 **relative to a no ATc control. PAM score is based on previous studies that identified 15**

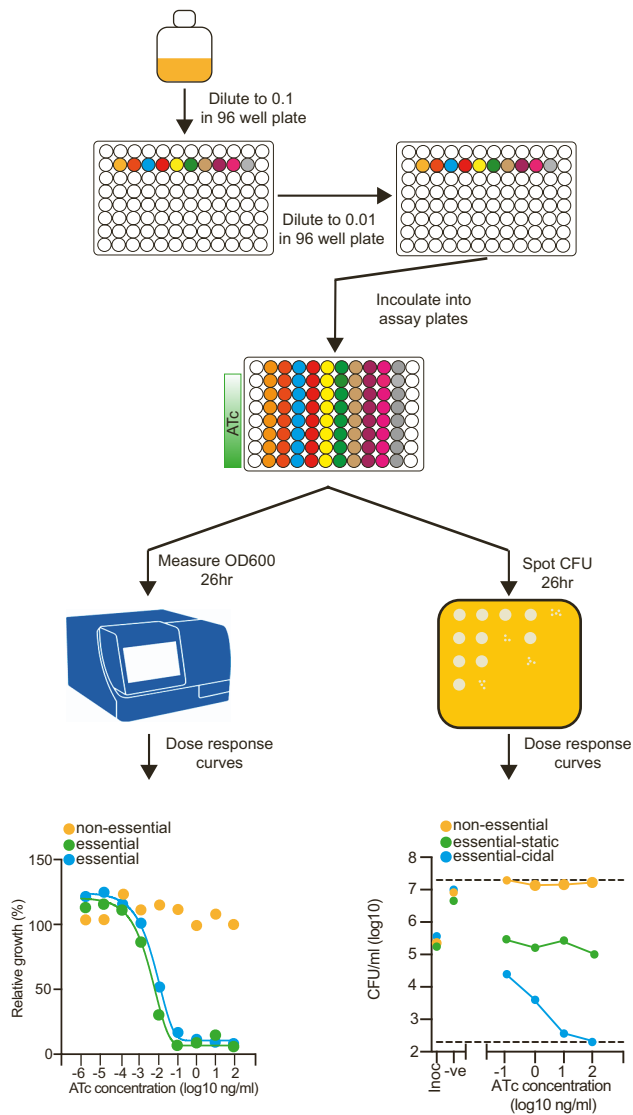
61 **permissible non-canonical PAM variants that retained inhibitory phenotype. (E-F)**

62 Reduction in CFU/ml (CFU/ml at 0 hrs -26hrs) in the presence of 100 ng/ml ATc. For
63 *M. smegmatis* strains expressing sgRNAs targeting *ndh* or *atpE* with weaker PAM
64 variants. Results for A-F are the mean \pm SD of four biological replicates. For all
65 experiments included a negative non-targeting and a parental bactericidal sgRNA (i.e.
66 *ndh_b* or *atpE_a*)

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73 **Figure S9| Workflow for bacterial phenotyping screens of single and multiplex**
74 **sgRNAs, related to figure 1.** Work flow for phenotypic assays, with specific details
75 in materials and methods.

plasmid name	Target-Species	Target-Gene	sgRNA name	Target sequence (Coding (5'-3'))	PAM (non coding 5'-3', NN.....)	PAM Score (Rock et al)	sgRNA target length	Fwd Oligo (5-3') (GGGA_)	Oligo Rev Sequence (AAAC_)
pJLR962	M. smegmatis		non-targeting sgRNA	CGAGACGCATTAATCG TCTCC				GGGAGGAGACGATT AATGCGTCTCG	AAACCGAGACGCAT TAATCGTCTCC
pCi2	M. smegmatis	MSMEG_0250	<i>mmpL3</i>	GACGAGGGCAGCCAG TCTGTCCG	GTAGAAA			GGGAGCGACAGACT GGCTGCCCTCGTC	AAACGACGAGGGCA GCCAGTCTGTCCG
pCi3	M. smegmatis	MSMEG_0418	<i>sdhA1_a</i>	GCGACGAGGTGGCCC GCGCC	GGGGAAG			GGGAGGCGCGGGC CACCTCGTCGC	AAACGCGACGAGGT GGCCCGCGCC
pCi4	M. smegmatis	MSMEG_1670	<i>sdhA2_a</i>	AGGCCAAGCTGCCCC ACATC	CGAGAAC	5	20	GGGAGATGTCGGG CAGCTTGCCCT	AAACAGGCCAAGCT GCCCGACATC
pCi21	M. smegmatis	MSMEG_1670	<i>sdhA2_c</i>	AGACGCTGTACCAAAA CTGC	GCAGGAT	9	20	GGGAGCAGTTTTGG TACAGCGTCT	AAACAGACGCTGTA CCAAACCTGC
pCi46	M. smegmatis	MSMEG_2060	<i>nuoD_a</i>	AGATCGAGGGCGAGA TCATC	CCAGGAT	9	20	GGGAGATGATCTCG CCCTCGATCT	AAACAGATCGAGGG CGAGATCATC
pCi47	M. smegmatis	MSMEG_2060	<i>nuoD_b</i>	GAACCGGAGGATC CTGCGGGT	ACGGAAG	4	23	GGGAACCCGACAG ATCTCCTCGCGTTC	AAACGAACGCGAGG AGATCCTGCGGGT
pCi48	M. smegmatis	MSMEG_3621	<i>ndh_a</i>	CCGCTGCTTACCAG GTGGC	CTGGAAG	4	20	GGGAGCCACCTGGT AGAGCAGCGG	AAACCCGCTGCTCT ACCAGGTGGC
pCi49	M. smegmatis	MSMEG_3621	<i>ndh_b</i>	GCGATGTGACGCACA TCGAT	CGAGAAC	1	20	GGGAATCGATGTGC GTCACATCGC	AAACGCGATGTGAC GCACATCGAT
pCi50	M. smegmatis	MSMEG_4263	<i>qcrB_a</i>	CTGGGTGAGATCGCG CTGTAC	CAGGAAG	4	21	GGGAGTACAGCGC GATCTCACCCAG	AAACCTGGGTGAGA TCGCGCTGTAC
pCi51	M. smegmatis	MSMEG_4263	<i>qcrB_b</i>	GATCCGTGATGGCA CAGT	GAAGAAC	5	20	GGGAACGTGTGCCA TCGACGGATC	AAACGATCCGTGCA TGGCACAGT
pCi52	M. smegmatis	MSMEG_4268	<i>ctaC_a</i>	GATTGCGTCGTTCCG CGTGGGT	ACGGAAC	11	22	GGGAACCCACGGC GAACGACGCAATC	AAACGATTGCGTCCG TTCGCCGTGGGT
pCi53	M. smegmatis	MSMEG_4268	<i>ctaC_b</i>	TTCACCGTCGTCGTGC AGGAAC	GTAGAAC	5	22	GGGAGTTCCTGCAC GACGACGGTGAA	AAACTTCACCGTCCG TCGTGCAGGAAC
pCi54	M. smegmatis	MSMEG_4941	<i>atpE_a</i>	ATCACCGTCGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGACGGTGAT	AAACATCACCGTCCG GTCTGGTGGGAAGC
pCi55	M. smegmatis	MSMEG_4942	<i>atpB_a</i>	CCACTGCCGTACCCG CCGTGAT	CGAGAAT	2	22	GGGAATCACGGCG GTGACGGCAGTGG	AAACCCACTGCCGT CACCGCCGTGAT
pCi56	M. smegmatis	MSMEG_4942	<i>atpB_b</i>	CTGCGGGCCAAGGTC ACCTC	GTAGAAC	1	20	GGGAGAGGTGACCT TGGCCCGCAG	AAACCTGCGGGCCA AGGTACCTC
pCi59	M. smegmatis	MSMEG_2613	<i>mqo_a</i>	GCCCACGCCGTCGAG AACGGT	CCAGAAC	5	21	GGGAACCGTTCTCG ACGGCGTGGGC	AAACGCCACGCCG TCGAGAACGGT
pCi60	M. smegmatis	MSMEG_2613	<i>mqo_b</i>	AACCTGTGCCGCAT GTGAGT	GAGGAAG	4	21	GGGAACCTCACATGC GGCACAGGGTT	AAACAAACCTGTGC CGCATGTGAGT
pCi65	M. smegmatis	MSMEG_3232	<i>cydB_a</i>	CTGCTGGAGGGCTTC GACTT	GAAGAAC	1	20	GGGAAAGTCGAAGC CCTCCAGCAG	AAACCTGTGGAGG GCTTCGACTT

pCi66	M. smegmatis	MSMEG_3232	<i>cydB_b</i>	GGCCGTGCCGCCGAG AAACGC	GAAGAAC	5	21	GGGAGCGTTTCTCG GCGGCACGGCC	AAACGGCCGTGCCG CCGAGAAACGC
pCi67	M. smegmatis	MSMEG_1109	<i>menD_a</i>	TGATCGTGTGAGCG CCAAC	GCGGAAC	11	20	GGGAGTTGGCGCTC AGCACGATCA	AAACTGATCGTGCT GAGCGCCAAC
pCi68	M. smegmatis	MSMEG_1109	<i>menD_b</i>	GCACGGGCGCCAACC AGACC	CGAGCAT	7	20	GGGAGGTCTGGTTG GCGCCCGTGC	AAACGCACGGGCGC CAACCAGACC
pCi266	M. smegmatis	MSMEG_3621	<i>ndh_b_1MM</i>	ACGATGTGACGCACAT CGAT	CGAGAAG	1	20	GGGAATCGATGTGC GTCACATCGT	AAACACGATGTGAC GCACATCGAT
pCi267	M. smegmatis	MSMEG_3621	<i>ndh_b_3MM</i>	GCCATGTGACGCACA TCGAT	CGAGAAG	1	20	GGGAATCGATGTGC GTCACATGGC	AAACGCCATGTGAC GCACATCGAT
pCi268	M. smegmatis	MSMEG_3621	<i>ndh_b_5MM</i>	GCGAAGTGACGCACA TCGAT	CGAGAAG	1	20	GGGAATCGATGTGC GTCACTTCGC	AAACGCGAAGTGAC GCACATCGAT
pCi269	M. smegmatis	MSMEG_3621	<i>ndh_b_7MM</i>	GCGATGAGACGCACA TCGAT	CGAGAAG	1	20	GGGAATCGATGTGC GTCTCATCGC	AAACGCGATGAGAC GCACATCGAT
pCi270	M. smegmatis	MSMEG_3621	<i>ndh_b_9MM</i>	GCGATGTGTGCGACA TCGAT	CGAGAAG	1	20	GGGAATCGATGTGC GACACATCGC	AAACGCGATGTGTC GCACATCGAT
pCi271	M. smegmatis	MSMEG_3621	<i>ndh_b_11MM</i>	GCGATGTGACCCACA TCGAT	CGAGAAG	1	20	GGGAATCGATGTGG GTCACATCGC	AAACGCGATGTGAC CCACATCGAT
pCi272	M. smegmatis	MSMEG_3621	<i>ndh_b_13MM</i>	GCGATGTGACGCTCA TCGAT	CGAGAAG	1	20	GGGAATCGATGAGC GTCACATCGC	AAACGCGATGTGAC GCTCATCGAT
pCi273	M. smegmatis	MSMEG_3621	<i>ndh_b_15MM</i>	GCGATGTGACGCACT TCGAT	CGAGAAG	1	20	GGGAATCGAAGTGC GTCACATCGC	AAACGCGATGTGAC GCACTTCGAT
pCi274	M. smegmatis	MSMEG_3621	<i>ndh_b_17MM</i>	GCGATGTGACGCACA TGGAT	CGAGAAG	1	20	GGGAATCCATGTGC GTCACATCGC	AAACGCGATGTGAC GCACATGGAT
pCi275	M. smegmatis	MSMEG_3621	<i>ndh_b_19MM</i>	GCGATGTGACGCACA TCGTT	CGAGAAG	1	20	GGGAAACGATGTGC GTCACATCGC	AAACGCGATGTGAC GCACATCGTT
pCi276	M. smegmatis	MSMEG_3621	<i>ndh_m</i>	CGGGACCATCCGTGC CATCGAGT	TCGGAAT	12	23	GGGAATCGATGCG ACGGATGGTCCCG	AAACCGGGACCATC CGTCGCATCGAGT
pCi277	M. smegmatis	MSMEG_3621	<i>ndh_n</i>	CGTTCACCGTCGTGC GCGCGGGC	TCAGCAA	10	23	GGGAGCCCGCGCC GACGACGGTGAACG	AAACCGTTCACCGT CGTCGGCGCGGGC
pCi278	M. smegmatis	MSMEG_3621	<i>ndh_o</i>	GCAAGCAGAAGAACG CCCAGGT	GGAGGAT	9	22	GGGAACCTGGGCGT TCTTCTGCTTGC	AAACGCAAGCAGAA GAACGCCAGGT
pCi279	M. smegmatis	MSMEG_3621	<i>ndh_p</i>	TTCGGCAACGACCACT TCGC	GTAGGAC	15	20	GGGAGCGAAGTGGT CGTTGCCGAA	AAACTTCGGCAACG ACCACTTCGC
pCi280	M. smegmatis	MSMEG_3621	<i>ndh_q</i>	GCGGTGGCCAAGGTG GGTCC	GGAGAAC	5	20	GGGAGGACCCACCT TGGCCACCGC	AAACGCGGTGGCCA AGGTGGGTCC
pCi281	M. smegmatis	MSMEG_3621	<i>ndh_r</i>	GTCACACCTACTCGAC GCCC	CGAGCAG	13	20	GGGAGGGCGTCSGA GTAGGTGTGAC	AAACGTCACACCTA CTCGACGCC
pCi282	M. smegmatis	MSMEG_3621	<i>ndh_s</i>	GCACCAAGCGTGGTC AGCTC	TCAGGAA	14	20	GGGAGAGCTGACCA CGCTTGGTGC	AAACGCACCAAGCG TGCTCAGCTC
pCi283	M. smegmatis	MSMEG_3621	<i>ndh_t</i>	AGCACCAAGCGTGGT CAGT	CAGGAAC	11	20	GGGAAGCTGACCAC GCTTGGTGCT	AAACAGCACCAAGC GTGGTCAGCT
pCi284	M. smegmatis	MSMEG_4941	<i>atpE_a_1MM</i>	GTCACCGTCCGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGACGGTGAC	AAACGTCACCGTCCG GTCTGGTGAAGC
pCi285	M. smegmatis	MSMEG_4941	<i>atpE_a_3MM</i>	ATGACCGTCCGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGACGGTCAT	AAACATGACCGTCCG GTCTGGTGAAGC

pCi286	M. smegmatis	MSMEG_4941	<i>atpE_a_5MM</i>	ATCAGCGTCTGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGACGCTGAT	AAACATCAGCGTCTG GTCTGGTGGAAAGC
pCi287	M. smegmatis	MSMEG_4941	<i>atpE_a_7MM</i>	ATCACCTCGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGAGGGTGAT	AAACATCACCCCTCG GTCTGGTGGAAAGC
pCi288	M. smegmatis	MSMEG_4941	<i>atpE_a_9MM</i>	ATCACCGTGGTCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCCACGGTGAT	AAACATCACCGTGG GTCTGGTGGAAAGC
pCi289	M. smegmatis	MSMEG_4941	<i>atpE_a_11MM</i>	ATCACCGTCTGCTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GAGCGACGGTGAT	AAACATGAGCGTCTG GTCTGGTGGAAAGC
pCi290	M. smegmatis	MSMEG_4941	<i>atpE_a_13MM</i>	ATCACCGTCGGTGTG GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA CACCGACGGTGAT	AAACATCACCGTCTG GTGTGGTGGAAAGC
pCi291	M. smegmatis	MSMEG_4941	<i>atpE_a_15MM</i>	ATCACCGTCGGTCTC GTGGAAGC	GAAGAAC	5	23	GGGAGCTTCCACCA GACCGACGGTGAT	AAACATCACCGTCTG GTCTCGTGGAAAGC
pCi292	M. smegmatis	MSMEG_4941	<i>atpE_a_17MM</i>	ATCACCGTCGGTCTG GAGGAAGC	GAAGAAC	5	23	GGGAGCTTCTCCA GACCGACGGTGAT	AAACATCACCGTCTG GTCTGGAGGAAGC
pCi293	M. smegmatis	MSMEG_4941	<i>atpE_a_19MM</i>	ATCACCGTCGGTCTG GTGCAAGC	GAAGAAC	5	23	GGGAGCTTGCACCA GACCGACGGTGAT	AAACATCACCGTCTG GTCTGGTGAAGC
pCi294	M. smegmatis	MSMEG_4941	<i>atpE_a_21MM</i>	ATCACCGTCGGTCTG GTGGATGC	GAAGAAC	5	23	GGGAGCATCCACCA GACCGACGGTGAT	AAACATCACCGTCTG GTCTGGTGGATGC
pCi295	M. smegmatis	MSMEG_4941	<i>atpE_a_23MM</i>	ATCACCGTCGGTCTG GTGGAAGT	GAAGAAC	5	23	GGGAACTTCCACCA GACCGACGGTGAT	AAACATCACCGTCTG GTCTGGTGGAAAGT
pCi296	M. smegmatis	MSMEG_4942	<i>atpB_n</i>	CGCCGATCAACATCGT CGAAGAAGC	CGAGGAA	14	24	GGGAGTTCTTCGAC GATGTTGATCGGCG	AAACCGCGGATCAA CATCGTCGAAGAAGC
pCi297	M. smegmatis	MSMEG_4942	<i>atpB_o</i>	CACGCGGCAGGCATC TGGCGT	GTAGCAG	13	21	GGGAACGCCAGATG CCTGCCGCGTG	AAACCGCGGCGAG GCATCTGGCGT
pCi298	M. smegmatis	MSMEG_4942	<i>atpB_p</i>	TGGTACATCCAGTGGT TCCC	GGGGAAC	11	20	GGGAGGGAACCACT GGATGTACCA	AAACTGGTACATCC AGTGGTTCCC
pCi299	M. smegmatis	MSMEG_4942	<i>atpB_q</i>	TCTCCAAGTGGCTCGC GGTGC	TCAGGAT	9	21	GGGAGCACCGCGA GCCAGTTGGAGA	AAACTCTCCAAGT GCTCGCGGTGC

Table S2: Oligos Used in This Study		
Name- MMO	Sequence	Description
MMO117	TGCGGCGCTTTTTTTTTTGAATTC	Sequencing pJR962
MMO119	CTGCGTTATCCCCTGATTCTG	Sequencing pJR962
MMO120	AATATGCTCTTCAGGATCTGACCAGGGAAAATAGCC	Fwd primer-Cloning into SapI site pJR962/5
MMO121	TTTATGCTCTTCACTGAAAAAAAAAACACCCTGCCATAAAATGAC	Rev primer-Cloning into SapI site pJR962/5
MMO202	GCCTGGCCATCATGGGTATC	Msm_gDNA_check_F1
MMO203	GGAGGATCCGGAGACCAAGC	Msm_gDNA_check_R1
MMO206	CCTCCGTCTTTTCGGCAACA	qPCR-AtpB-MSMEG4942
MMO207	GTCGAAGGTCTTCCACACGG	qPCR-AtpB-MSMEG4942
MMO208	CTGATCTCGGGTATCGCCC	qPCR-AtpE-MSMEG4941
MMO209	GCCAGGTTGATGAAGTACGC	qPCR-AtpE-MSMEG4941
MMO212	GAGTTCGTCCTGAACTCGGC	qPCR-CtaC-MSMEG4268
MMO213	TGTCCGAGTTGTTGGCCTTC	qPCR-CtaC-MSMEG4268
MMO214	GCCTGACGATCTACAACGGA	qPCR-CydB-MSMEG3232
MMO215	GAGATGCGCTTGCTGAACAC	qPCR-CydB-MSMEG3232
MMO216	TCATAGGCGACCTGACGTTT	qPCR-MenD-MSMEG1109
MMO217	TTGTCGTTGGACACCACGAT	qPCR-MenD-MSMEG1109
MMO218	GGTGGGTCTGCTCAAGTACC	qPCR-Mqo-MSMEG2613
MMO219	GCGAATTCACGAAGCGTCTC	qPCR-Mqo-MSMEG2613
MMO220	TACGCCGCGAAGATCATCAA	qPCR-Ndh-MSMEG3621
MMO221	TGTGGAAGTACTCGAACGGC	qPCR-Ndh-MSMEG3621
MMO222	CGAGCACATCGCCAAGATCA	qPCR-nuoD-MSMEG2060
MMO223	CCTTCGGTGACGAGCTTGAA	qPCR-nuoD-MSMEG2060
MMO226	TATCGGCATGGTGGTACTGC	qPCR-qcrB-MSMEG4263
MMO227	GAAGTCGCTTGATGATGCCG	qPCR-qcrB-MSMEG4263
MMO228	CGTCATGGGTGGTATCGAGG	qPCR-sdhA1-MSMEG0418
MMO229	AGATCTGACAGCGAGTTGCC	qPCR-sdhA1-MSMEG0418
MMO230	ACAACACCAACGTCATCCCC	qPCR-sdhA2-MSMEG1670
MMO231	TGATGTCCAGCAGCGAGTTG	qPCR-sdhA2-MSMEG1670
MMO270	TGTGGGACGAGGAAGAGTCC	sigA_Msmeg_Fwd qPCR primer_set 1

MMO271	CACCTCTTCTTCGGCGTTGA	sigA_Msmeg_Rev qPCR primer_set 1
MMO300	GACTCTTCCTCGTCCCACAC	sigA_Msmeg_Fwd qPCR primer_set 2
MMO301	GAAGACACCGACCTGGAAC	sigA_Msmeg_Rev qPCR primer_set 2

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Table S3: Golden gate cloning protocols		
<i>Oligo annealing protocol</i>		
Component	Volume	
Oligo Fwd (100 μ M)	10 μ l	
Oligo Rev (100 μ M)	10 μ l	
H2O	25 μ l	
10x T4 ligase buffer	5 μ l	
Temperature ($^{\circ}$C)	Duration	
95	5	
25 (0.1 $^{\circ}$ C/sec)	Time to ramp	
4	forever	
<i>sgRNA module golden gate cloning protocol</i>		
Component	Volume (μl)	
10 \times T4 Ligase Buffer	1	
Single sgRNA expression plasmid uncut (20 ng/ μ L)	1.25	
Annealed oligo	2.5	
BsmB1 (10,000u/ml)	0.5	
T4 DNA Ligase	0.5	
mQ	4.25	
Temperature ($^{\circ}$C)	Duration	Cycle
42 (digestion)	5 min	30
16 (ligation)	5 min	
4	Forever	1
<i>Multiplex golden gate cloning protocol</i>		
Component	Volume (μl)	
10 \times T4 Ligase Buffer	1	
Single sgRNA expression plasmid uncut (20 ng/ μ L)	1.25	
Cloned and purified target -promoter-sgRNA-scaffold	2.5	
Sap1 (10,000u/ml)	0.5	
T4 DNA Ligase	0.5	
mQ	4.25	
Temperature ($^{\circ}$C)	Duration	Cycle
37 (digestion)	5 min	30
16 (ligation)	5 min	
4	Forever	1

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Table S4: Multiplexed Plasmids Constructed in this Study.						
Plasmid name	Target-Species	pCi plasmid backbone	Second sgRNA	Amplification template for second sgRNA	Fwd Primer for amplification of sgRNA module	Rev primer for amplification of sgRNA module
pCiMX40	<i>M. smegmatis</i>	pCi49(<i>ndh_b</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121
pCiMX41	<i>M. smegmatis</i>	pCi49(<i>ndh_b</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX42	<i>M. smegmatis</i>	pCi49(<i>ndh_b</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX43	<i>M. smegmatis</i>	pCi49(<i>ndh_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX46	<i>M. smegmatis</i>	pCi4(<i>sdhA2_a</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX47	<i>M. smegmatis</i>	pCi4(<i>sdhA2_a</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX48	<i>M. smegmatis</i>	pCi4(<i>sdhA2_a</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX49	<i>M. smegmatis</i>	pCi51(<i>qcrB_b</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX50	<i>M. smegmatis</i>	pCi51(<i>qcrB_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX51	<i>M. smegmatis</i>	pCi53(<i>ctaC_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX52	<i>M. smegmatis</i>	pCi68(<i>menD_b</i>)	<i>ndh_b</i>	pCi49	MMO120	MMO121
pCiMX53	<i>M. smegmatis</i>	pCi68(<i>menD_b</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121
pCiMX54	<i>M. smegmatis</i>	pCi68(<i>menD_b</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX55	<i>M. smegmatis</i>	pCi68(<i>menD_b</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX56	<i>M. smegmatis</i>	pCi68(<i>menD_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX57	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>ndh_b</i>	pCi49	MMO120	MMO121
pCiMX58	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121
pCiMX59	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX60	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX61	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX62	<i>M. smegmatis</i>	pCi60(<i>mgo_b</i>)	<i>menD_b</i>	pCi68	MMO120	MMO121
pCiMX237	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>ndh_b</i>	pCi49	MMO120	MMO121
pCiMX238	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121

pCiMX239	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX240	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX241	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX242	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>menD_b</i>	pCi68	MMO120	MMO121
pCiMX243	<i>M. smegmatis</i>	pCi3 (<i>sdhA1_a</i>)	<i>mqo_b</i>	pCi60	MMO120	MMO121
pCiMX244	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>ndh_b</i>	pCi49	MMO120	MMO121
pCiMX245	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121
pCiMX246	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX247	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX248	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX249	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>menD_b</i>	pCi68	MMO120	MMO121
pCiMX250	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>mqo_b</i>	pCi60	MMO120	MMO121
pCiMX251	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>sdhA1_a</i>	pCi3	MMO120	MMO121
pCiMX252	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>ndh_b</i>	pCi49	MMO120	MMO121
pCiMX253	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>sdhA2_a</i>	pCi4	MMO120	MMO121
pCiMX254	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>qcrB_b</i>	pCi51	MMO120	MMO121
pCiMX255	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>ctaC_b</i>	pCi53	MMO120	MMO121
pCiMX256	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>atpE_a</i>	pCi54	MMO120	MMO121
pCiMX257	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>menD_b</i>	pCi68	MMO120	MMO121
pCiMX258	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>mqo_b</i>	pCi60	MMO120	MMO121
pCiMX259	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>sdhA1_a</i>	pCi3	MMO120	MMO121
pCiMX260	<i>M. smegmatis</i>	pCi46 (<i>nuoD_a</i>)	<i>cydB_b</i>	pCi66	MMO120	MMO121
pCiMX262	<i>M. smegmatis</i>	pCi4 (<i>sdhA2_a</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX263	<i>M. smegmatis</i>	pCi51 (<i>qcrB_b</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX264	<i>M. smegmatis</i>	pCi53 (<i>ctaC_b</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX265	<i>M. smegmatis</i>	pCi54 (<i>atpE_a</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX266	<i>M. smegmatis</i>	pCi68 (<i>menD_b</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX267	<i>M. smegmatis</i>	pCi60 (<i>mqo_b</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX268	<i>M. smegmatis</i>	pCi3 (<i>sdhA1</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX269	<i>M. smegmatis</i>	pCi66 (<i>cydB_b</i>)	<i>ndh_b_3MM</i>	pCi267	MMO120	MMO121
pCiMX271	<i>M. smegmatis</i>	pCi4 (<i>sdhA2_a</i>)	<i>ndh_b_5MM</i>	pCi268	MMO120	MMO121

pCiMX272	<i>M. smegmatis</i>	pCi51 (<i>qcrB</i> _b)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX273	<i>M. smegmatis</i>	pCi53(<i>ctaC</i> _b)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX274	<i>M. smegmatis</i>	pCi54 (<i>atpE</i> _a)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX275	<i>M. smegmatis</i>	pCi68(<i>menD</i> _b)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX276	<i>M. smegmatis</i>	pCi60(<i>mgo</i> _b)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX277	<i>M. smegmatis</i>	pCi3 (<i>sdhA1</i>)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX278	<i>M. smegmatis</i>	pCi66 (<i>cydB</i> _b)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121
pCiMX279	<i>M. smegmatis</i>	pCi49 (<i>ndh</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX280	<i>M. smegmatis</i>	pCi4 (<i>sdhA2</i> _a)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX281	<i>M. smegmatis</i>	pCi51 (<i>qcrB</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX282	<i>M. smegmatis</i>	pCi53 (<i>ctaC</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX284	<i>M. smegmatis</i>	pCi68 (<i>menD</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX285	<i>M. smegmatis</i>	pCi60 (<i>mgo</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX286	<i>M. smegmatis</i>	pCi3 (<i>sdhA1</i>)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX287	<i>M. smegmatis</i>	pCi66 (<i>cydB</i> _b)	<i>atpE</i> _a_3MM	pCi285	MMO120	MMO121
pCiMX288	<i>M. smegmatis</i>	pCi285 (<i>atpE</i> _a_3MM)	<i>ndh</i> _b_3MM	pCi267	MMO120	MMO121
pCiMX289	<i>M. smegmatis</i>	pCi285 (<i>atpE</i> _a_3MM)	<i>ndh</i> _b_5MM	pCi268	MMO120	MMO121

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