Supplemental Material

S1. Methods

Bacterial strains, culture condition, cell fixation and RNAse digestion

The following strains were used: *Escherichia coli* K-12 (DM1, Invitrogen), *Bacillus subtilis* (AG174, ATCC) and *Streptococcus mutans* (UA159, ATCC). *E. coli* and *B. subtilis* strains were cultured at 37°C in Luria-Bertani medium; the *S. mutans* strain was cultured in Brain Heart Infusion broth. Overnight cultures of the strains were harvested by centrifugation, washed twice with phosphate-buffered saline (PBS; pH 7.4). Cells were centrifuged and fixed by addition of freshly-prepared fixative (methanol/glacial acid, 3:1) and incubated for 15 min at room temperature and washed twice with fresh fixative. After fixation, the cells were resuspended in a small amount of fixative and a drop was placed on a clean poly-lysine microscope slide. The slide was dehydrated through an alcohol series (70%, 90%, 100%) for 2 min each and allowed to air dry. 100 µl RNase A (100 µg/ml) was placed on slides under a 24x50 mm cover slip and incubated at 37°C for 1 hour. The slide was washed twice with PBS and once in 10 mM sodium phosphate buffer (pH 7.0) then dehydrated and dried thoroughly.

Signature sites

Using bacterial genome sequences available from Bacterial Genomes Database (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/) PD-loop sites with different distances and sequences between PNA-binding sites can be selected. We searched for sites $R_kN_nR_l$ (where R is any purine and N is any base) choosing *k*

and *l* between 7 and 8 and *n* between 2 and 8. For three bacteria under study we found that bacterial genomes contain 1913, 4907 and 2077 PD-loop sites for *E.coli* (4.6Mb genome), *B.subtilis* (4.2Mb), and *S.mutans* (2.0Mb), respectively. Thus, assuming that the sequence is random, a PD-loop site is estimated to be found approximately every one thousand base pairs. These PD-loop sites must be tested to select markers that are unique for each chosen bacterial genome according to genomic BLAST (http://www.ncbi.nlm.nih.gov/blast/), which makes it possible to search for all sites in all others genomes with the given pattern. We can expect to find at least 100 signature sites for each of the bacterium under study, which will allow us to distinguish each of them from other sequenced bacteria.

PNA oligomers are commercially available from PANAGENE (Daejeon, South Korea). For the present work we used PNA openers from a large library of PNAs that has been assembled over the years in the Center for Advanced Biotechnology. These PNAs recognize 7-10-bp-long sites on DNA. We used a pair of PNA sequences from the library, with an arbitrary spacer between the PNA binding sites, in order to design unique genomic signature sites for three bacterial species under study. The chosen signature site was confirmed to be present in one copy for only one bacterial genome and absent in genomes of the other two bacteria used in this study. From the list of selected markers, signature sites were chosen that are mostly located within bacterial genes. Thus, each signature site was present in a single copy per the bacterial chromosome. The copy number of chromosomes varied from 1 to 5 copies/cell, depending on the growth stage of the cells.

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2 PNA openers, circularizable probes, RCA primers, and decorator probes

3 Table 1 summarizes the PNAs, circularizable ODNs, primers and decorator
4 probes used in this study. All PNA samples were obtained as a gift from Dr. P.E.
5 Nielsen (Copenhagen University) or Applied Biosystems (Foster City, CA). All
6 primers and labeled probes with a phosphate group (**p**), Cy3 or FITC were
7 supplied by MWG Biotech (High Point, NC). Circularizable probes carried a
8 phosphate group at the 5'-end and sequences complementary to target DNA
9 sites at the 3'- and 5'- ends. They also carried the sequence complementary to
10 the RCA primer and an arbitrary sequence in the middle, which was designed to
11 hybridize specific decorator probes to the RCA product and to discriminate
12 bacterial strains from one another.

13

14 PD-loop assembly, ODN ligation and Rolling Circle Amplification

15 About 100 µl 10mM Na phosphate buffer (pH 7.0) with 0.8 µM of each PNAs was
16 placed on a slide. The slide was covered by 24x50 mm slip and sealed with
17 rubber cement. Binding of a pair of PNA openers to bacterial chromosomal DNA
18 was carried out at 37^0 C overnight (~16 hours). After completion of this step, the
19 rubber cement was gently peeled off and the cover slip was removed. The slide
20 was washed twice in 10mM Na phosphate buffer (pH 7.0) to remove non-bound
21 PNAs. The circularizable probe (2 µM) was then added and the ligation reaction
22 was performed by adding 50 µl of ligation mixture containing 1x T4 ligase buffer
23 with 5 u T4 DNA ligase (USB, Cleveland, Ohio) on the slide. The slide was

incubated under the cover slip at room temperature for 2 hours. Then, the slide was washed twice in 2x SSC buffer (30 mM sodium citrate, 300 mM NaCl, ph 7.0) to remove excess of non-ligated ODN and once in buffer A (100 mM TrisHCl, 150 mM NaCl and 0.05% Tween20).

Next, the RCA reaction was performed by adding 50 µl of the RCA reaction mixture containing 2 µM of primer, 10 u phi29 DNA polymerase (New England Biolabs), 200 µM dNTPs and 1x phi29 DNA polymerase buffer on the slide. In addition, the mixture contained 2 µM of fluorescently-labeled decorator probe. Decorator probes contained fluorescent labels on 3'-ends and could not be extended by DNA polymerase. The slide was covered by a glass slip and sealed with rubber cement. The RCA reaction was performed at 37^0 C, overnight (~16 hours). Then the slide was washed twice in buffer A and once in 2XT (2x SSC and 0.05% Tween20) buffer. After a drop of mounting medium, counterstaining DAPI was added, a coverslip was applied and sealed in place using clear nail polish.

Signal detection

Cells and intracellular amplicons were observed on the slides by using the fluorescence microscope Olympus BX-70 equipped with a Photometrics KAF1400 cooled charge-coupled device camera and multiple narrow band pass filters for cy3, cy3.5, cy5, cy5.5, DAPI, and FITC. Fluorescence signals were captured separately using appropriate filter sets for DAPI, CY3 and FITC. Images were pseudo-colored, processed and merged using the CytoVision image software and then stored as digital files.

S2. Negative Controls

Table 1. Various negative controls performed. In all cases the experiments were performed as described in Methods. All experiments in the table produced no-signal patterns similar to one presented in Fig. 2B.

Bacterium	PNA	Circularizable oligonucleotide (ODN)	Decorator	Nature of Control
E.coli		ODNcspG, ODNrpoN, ODNrnr	decR	No PNA
	PNA1, PNA2, PNA3, PNA4		decR	No Circularizable ODN
	PNA1, PNA2, PNA3, PNA4	ODNcspG, ODNrpoN, ODNrnr		No Decorator
	PNA1, PNA2,	ODNrpoN, ODNrnr	decR	Wrong PNAs
	PNA1, PNA2, PNA3, PNA4	ODNwrong*	decR	Wrong Circularizable ODN
	PNA1, PNA2, PNA3, PNA4	ODNcspG, ODNrpoN, ODNrnr	decW**	Wrong decorator
	PNA1, PNA2,	ODNcspG	decR	No T4 DNA ligase
	PNA1, PNA2,	ODNcspG	decR	No phi29 DNA polymerase
	PNA4, PNA5, PNA6, PNA7	ODNserA, ODNyxjA	decG	Probes specific to one bacterium are applied to the other one
	PNA4, PNA6	ODNhypP	decG	
	PNA7, PNA8, PNA9	ODNdnaK, ODNwapA	decG	
B.subtilis	PNA1, PNA2, PNA3, PNA4	ODNcspG, ODNrpoN, ODNrnr	decR	
S.mutans	PNA1, PNA2	ODNcspG	decR	
	PNA3, PNA4	ODNrpoN, ODNrnr	decR	

*ODNwrong: 5'-**p**-gtactttgag(tcacggaatggttacttgcCAGC)CAGCAGCC(TCACggaatggttacttgccagc)gtctcagtgact-3'
**decW: 5'-CY3-GGTTACTGCGATTAGCACAAGC-BIOTIN-3'

1 **S3. Detection in the clinical/environmental samples**

2 For detection in clinical/environmental samples the *Escherichia coli* (ATCC:
3 10798) strain was used.
4 Despite efforts towards the development of direct methods for rapid diagnostics
5 and detection, a direct microscopic visualization of bacterial cells by FISH is still
6 very difficult, and amplification through culture growth remains the gold standard
7 for bacterial detection. Therefore, we first checked our method for performance
8 on standard culture media (Trek Diagnostic Systems, Cleveland, OH) by the
9 standard smear preparation technique. Toward this goal we performed our
10 approach with the probes corresponding to the 21-nt-target site in *E. coli* cold
11 shock protein gene region (csp) on slides with smears made from the culture
12 media bottles that contains *E. coli* cells (Supplemental Figure 1 A, B). A variety of
13 standard smear preparation methods such as heat fixation (20 min at 55°C),
14 flame fixation, and methanol fixation were all compatible with our technique.
15 Virtually all cells displayed a very bright fluorescent signal indicating that culture
16 media do not effect bacterial detection by our approach.
17 Our approach also carries promise for direct detection in clinical/environmental
18 samples without prior bacterial multiplication. However, to realize this promise
19 our method should be indifferent to various contaminants present in “dirty”
20 samples. Since we are most interested in clinical/biodefense applications of our
21 technique, we tested our method on bacteria immersed in human blood. In so
22 doing we modeled an “infected” blood smear by putting on a polylysine coated
23 microscope slide a pellet of fresh *E.coli* cells resuspended in one milliliter of

1 expired packed human red blood cells (AB^+, courtesy of Dr. Karen Quillen of
2 Transfusion Medicine, Boston Medical Center) (packed red cells). The blood
3 smear was dried and fixed by immersing the slide in freshly-prepared, ice-cold
4 fixative and incubated for 15 minutes. The slide was disinfected and dehydrated
5 through an alcohol series (70%, 90%, 100%) and dried thoroughly. Then the
6 probe corresponding to the 21-nt-target site in *E.coli* cold shock protein gene
7 region (csp) was used (Supplemental Figure 1 C, D, E). Very strong signal was
8 observed for virtually all cells indicating that our approach is capable of detecting
9 bacteria directly in human blood.
10
11
12 **Supplemental Figure 1.**
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14 Images of *E. coli* bacterial cells with the probes corresponding to the 21-nt-target site in
15 *E. coli* cold shock protein gene region (csp) (PNA1, PNA2, ODNcspG and decR)
16 observed by fluorescent microscope in experiments performed directly in culture media
17 **(A, B)** and human blood **(C - E)**.
18 The fluorescent signals were acquired separately using two filter sets: **(A, C)** DAPI only;
19 **(D)** only Cy3 for the labeled RCA product; **(B, E)** superposition of two separate images
20 with DAPI and Cy3. Signals pseudocolored in blue for DAPI and red for Cy3.

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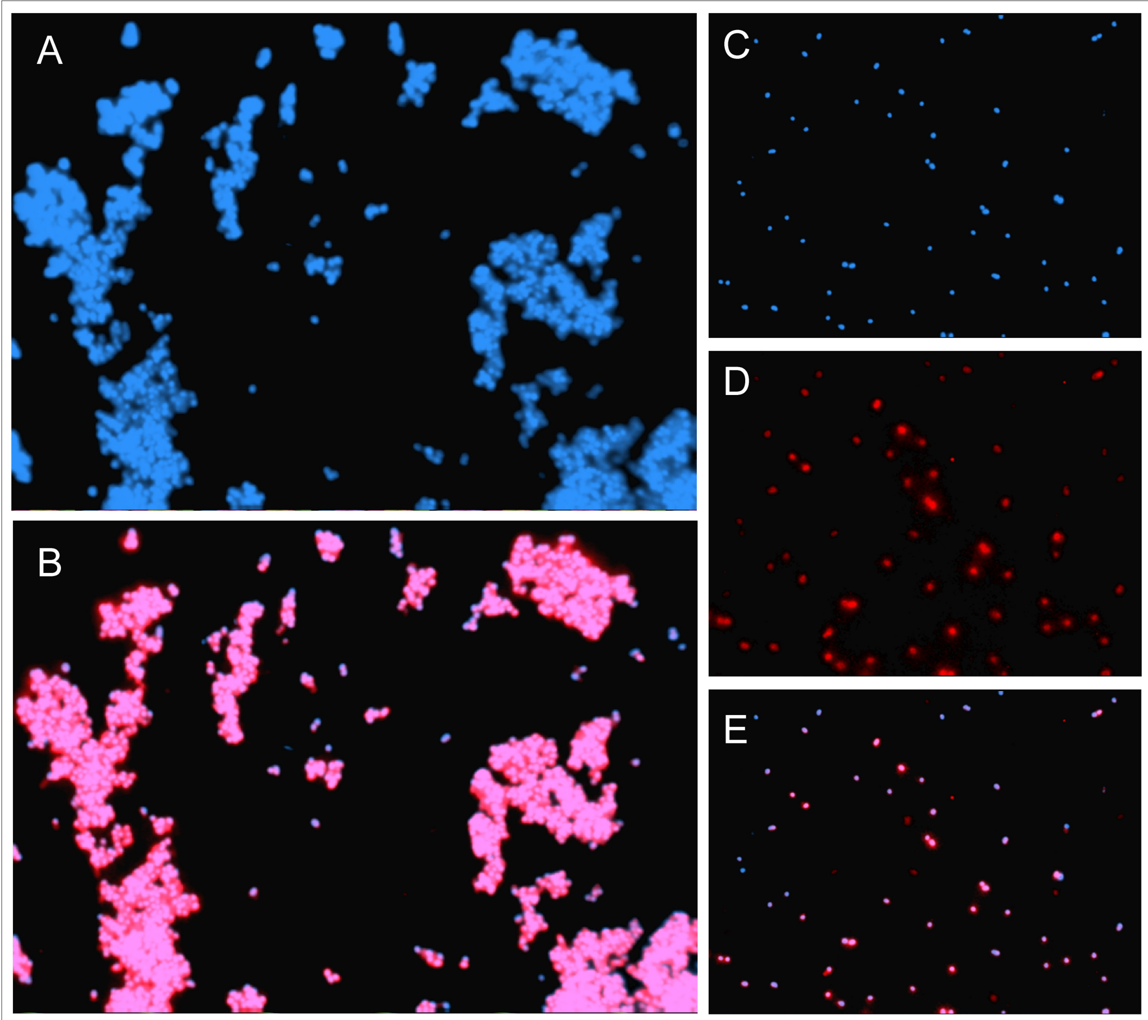

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7 **Supplemental Figure 1 (Smolina et al)**

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