Sequence specificity of detection (single-nucleotide mismatch discrimination).

Sequence-specificity of strand-invading homopyrimidine PNAs has been extensively studied *in vitro*, which made it possible to design optimal PD-loop sites (13). Here we report the data on PNA invasion into dsDNA within cells and the data on PNA ability of sequence discrimination while targeting dsDNA *in situ*. This process is essential because the choice of optimal signature sites and conditions for *in situ* applications is crucial to achieve high sequence-specificity necessary in order to avoid false positives and enable accurate detection of bacteria directly in clinical specimens.

To check the tolerance of our *in situ* approach to variously positioned mismatches, we analyzed genome sequences in order to find a clear-cut case of single-mismatch difference between two strains within the PD-loop site. Based on PNA openers available in our collection, we selected a signature site within the *rpo*N gene, which differs for two genomes: *E. coli* and *B. subtilis* in two positions (see Table 1, *E. coli* vs. *B. subtilis*).

Using two bacteria and different combinations of probes shown in Table 1, we performed a series of experiments on mismatch discrimination. The results are shown on Supplementary Figure 4 and summarized in Supplementary Table 2. The observed single nucleotide mismatch discrimination demonstrates high specificity of our approach. Nearly all experiments showed no signal when mismatched probes (with mismatches in PNA or circularizable probe binding site) were used. Only in one experimental design did we observe a discordant signal, from about 20% of cells; this was when a single-nucleotide mismatch was in 4th position from the ligation point at the circularizable probe binding site (case 3) No signal was observed with two single-nucleotide mismatches (case 4). Therefore, in case a single mismatch happens farther from the ligation point, discrimination may be insufficient under our standard conditions and a small modification of the protocol may be necessary. The current protocol can be still applied in case the potential signature site is carefully checked to avoid single-nucleotide replacement located at 4th position or farther from the ligation point. Another possibility to enhance selectivity of detection may be found in simultaneous targeting of two signature sites within the chosen bacterial genome. Different sites can be detected using different fluorophores with resolvable emission bands. Such double-coincidence detection will eliminate the possibility of false positives.

Supplementary Table 2. Results of experiments on mismatch discrimination

Figure	Bacterium	PNAs	Circularizable Oligonucleotide	Type of Mismatch	Signal
1	*E. coli*	PNA8	ODNrpoN1	None (positive control)	+
2	*E. coli*	PNA9	ODNrpoN1	mismatches in both PNAs binding sites	-
3	*E. coli*	PNA8	ODNrpoN2	circular probe binding site (internal) mismatch	+/-
4	*E. coli*	PNA8	ODNrpoN3	circular probe binding site (two) mismatches	-
5	*B. subtilis*	PNA8 & PNA9	ODNrpoN3	None (positive control)	+
6	*B. subtilis*	PNA8	ODNrpoN3	mismatch in PNA binding site	-
7	*B. subtilis*	PNA9	ODNrpoN3	mismatch in PNA binding site	-
8	*B. subtilis*	PNA8 & PNA9	ODNrpoN2	circular probe binding site (end) mismatch	-

Supplementary Figure 4. Sequence specificity of detection. Images of bacterial cells observed by fluorescent microscope in experiments on mismatch discrimination, which results are summarized in Supplementary Table 2. The fluorescent signals were acquired separately using three filter sets (DAPI for DNA and Cy3 or FITC for the RCA product). Each image is a superposition of two separate images, with DAPI and Cy3 or DAPI and FITC. Signals for Cy3, DAPI and FITC were pseudocolored in blue red, blue and green, respectively.