SUPPLEMENTARY DATA

The dynamics of the monomeric restriction endonuclease BcnI during its interaction with DNA

Georgij Kostiuk1,4, Jasmina Dikić2,4, Friedrich W. Schwarz3, Giedrius Sasnauskas1, Ralf Seidel2, and Virginijus Siksnys1*

1Institute of Biotechnology, Vilnius University, Sauletekio av. 7, LT-10257 Vilnius, Lithuania
2Molecular Biophysics group, Institute for Experimental Physics I, Universität Leipzig, Linnéstr. 5, 04103 Leipzig, Germany
3BCUBE, Technische Universität Dresden, Arnoldstrasse 18, 01307 Dresden, Germany
4Equal contribution

* Corresponding authors:
R.S.: Molecular Biophysics group, Institute for Experimental Physics I, Universität Leipzig, Linnéstr. 5, 04103 Leipzig, Germany
Tel. +49 341 97 32550
ralf.seidel@physik.uni-leipzig.de
V.S: Institute of Biotechnology, Vilnius University, Sauletekio av. 7, LT-10257 Vilnius, Lithuania
Tel: +370-5-2234365;
Fax: +370-5-2234367;
siksnys@ibt.lt
Supplementary methods

DNA construct for single-molecule diffusion measurements. The non-specific DNA construct for single molecule diffusion experiments was made by ligating two PCR fragments amplified from T7 phage DNA and digoxigenin-modified handle (Dig-handle) to form a 22.8 kbp nonspecific construct (no recognition sites for BcnI) as depicted in Supplementary Figure S1A. One of the PCR products was modified at the 5’ end with biotin using a biotinylated primer. The ligation of two PCR products was done using overhangs from Nt.BbvCI digestion. The overhangs from NotI digestion were used for ligation of the Dig-handles (Supplementary Table S1).

DNA construct for single-molecule DNA cleavage experiments. A 7.4 kb DNA construct suitable for direct observation of DNA cleavage using magnetic tweezers was made by ligating a 5.9 kb PCR product (template – T7 DNA), a 450 bp PCR product (template – pBluescriptIIISK+) and two PCR products modified with either biotin or digoxigenin (template – pBluescriptIIISK+), serving as Biotin- and Dig-handles, respectively (Supplementary Figure S1B, Supplementary Table S1).

BcnI substrate with two target sites for bulk cleavage experiments. The two-site DNA substrates ‘135’ (100 bp between BcnI sites) and ‘535’ (500 bp between BcnI sites) were made by ligation of Eco31I-digested and gel-purified PCR products (using φX174 phage DNA as template) with [γ-32P]ATP–phosphorylated hairpin oligonucleotides (Supplementary Figure S1C, Supplementary Table S1). The non-ligated fragments were removed by incubating the reaction mixtures with T7 polymerase. T7 polymerase has strong 3’=>5’ exonuclease activity, which degrades all DNA fragments with free 3’-ends. Only the ligated di-hairpin DNA substrates with 2 BcnI recognition sites remain. The final substrates were gel-purified from 1% agarose gel.

Determination of the concentration and labelling efficiency of BcnI conjugates

Concentrations of dyes \(c_{488,s} = c(\text{Alexa488 on single-labelled BcnI proteins})\), \(c_{488,d} = c(\text{Alexa488 on double-labelled protein})\), \(c_{546,d} = c(\text{Alexa546 on double-labelled protein})\) and proteins \(c_{\text{BcnI},s} = \text{single-labelled BcnI}\), \(c_{\text{BcnI},d} = \text{double-labelled BcnI}\) were calculated using equations 4 to 8. Labelling efficiencies were calculated as the ratio of the dye and the corresponding protein concentrations (e.g., \(c_{488,s}/c_{\text{BcnI},s}\) for the single-labelled protein, \(c_{488,d}/c_{\text{BcnI},d}\) and \(c_{546,d}/c_{\text{BcnI},d}\) for the double-labelled protein):

\[
c_{488,s} = \frac{A_{495}}{\varepsilon_{495} * d} \quad (4)
\]

\[
c_{\text{BcnI},s} = \frac{A_{280} - 0.11 * A_{495}}{\varepsilon_{\text{BcnI}} * d} \quad (5)
\]

\[
c_{488,d} = \frac{A_{495} - 0.11 * A_{556}}{\varepsilon_{495} * d} \quad (6)
\]

\[
c_{546,d} = \frac{A_{556}}{\varepsilon_{556} * d} \quad (7)
\]
where $A_x$ and $\varepsilon_x$ are absorption and extinction coefficients (M$^{-1}$×cm$^{-1}$) at $x$ nm wavelength, $d = 1$ cm. All labelled proteins are listed in Supplementary Table S2.

Anisotropy measurements
Anisotropies were determined on a Fluoromax 3 fluorimeter using a constant wavelength analysis mode ($\lambda_{ex}=480$ nm, $\lambda_{em}=515$ nm, integration time 5 s). Measurements were performed in the Reaction Buffer (33 mM Tris-acetate, 66 mM K-acetate, pH 7.9 at 25°C) supplemented with 0.5 µM fluorescently labelled BcnI or DNA. The fluorescence anisotropy ($r$) was calculated according to equations 9-10:

$$
r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (9)
$$

$$
G = \frac{I_{HV}}{I_{HH}} \quad (10)
$$

where $I_{VV}$ is the vertically polarized fluorescence intensity excited with vertically polarized light, $I_{VH}$ is the horizontally polarized fluorescence intensity excited with vertically polarized light, $I_{HV}$ is the vertically polarized fluorescence intensity excited with horizontally polarized light, and $I_{HH}$ is the horizontally polarized fluorescence intensity excited with horizontally polarized light (44). Anisotropy values are listed in Supplementary Table S2.

Quantum Yield measurement and $R_0$ calculation
Quantum yield (QY) of labelled BcnI proteins was measured in the Reaction Buffer using a Fluoromax 3 fluorimeter and a Nicolet Evolution 300 spectrophotometer as described in the instrument manuals. As standard solutions of fluorescein in 0.1M NaOH and Rhodamin6G in water were used for BcnI labelled with Alexa488-C5-maleimide ($\lambda_{ex}=480$nm, $\lambda_{em}=495$-600nm) and for BcnI labelled with Alexa546-C5-maleimide ($\lambda_{ex}=510$nm, $\lambda_{em}=525$-630nm), respectively. The $R_0$ values were calculated using equation (11):

$$
R_0 = \frac{9000 \times \ln 10}{128\pi \times N_A} \times \phi_D \times \kappa^2 \times n^{-4} \times J \quad (11)
$$

where $N_A$ is the Avogadro’s number, $\phi_D$ the donor quantum yield, $\kappa^2$ the dipole orientation factor, $n$ the refractive index of the medium, and $J$ the overlap integral of the fluorescence and absorption spectra of donor and acceptor dyes. The refractive index of the media (1.34) was measured experimentally. The dipole orientation factor was assumed to be 2/3 (equivalent to free rotation of the fluorophores (44)). We note that the obtained anisotropy values for the protein-bound fluorophores are rather high to justify this assumption. The hindered mobility/conformational freedom of the dyes can therefore lead to deviations from the assumed orientation factor causing deviations in the FRET efficiencies. Since the real configurational space of the
dyes is unknown, we used an orientation factor of 2/3 as an estimate. The determined QY and $R_0$ values are listed in Supplementary Table S2.

**Catalytic activity of BcnI conjugates**

The catalytic activity of BcnI-dye conjugates was determined by performing multiple-turnover DNA cleavage reactions in the Reaction Buffer supplemented with 10 mM magnesium acetate. Reactions contained 2 nM φX174 DNA and 0.1 nM wt BcnI. 25 µl aliquots were removed at timed intervals and quenched by adding 10 µl of the non-denaturing loading dye solution (75 mM EDTA, 0.01 % bromphenol blue, 0.1 % SDS, 50 % (v/v) glycerol, pH 8.0). Samples were separated by gel electrophoresis using 0.8% agarose gels to distinguish between supercoiled (uncut), open-coiled (nicked) and full length linear (cut) DNA forms. The initial rate of the full length linear DNA accumulation was determined by linear regression. The determined kinetic constants are listed in the Supplementary Table S3.

**DNA binding studies**

DNA binding by BcnI Cys mutants was analysed by the gel mobility-shift assay, using 33/34 (specific) and 35/36 (non-specific) $^{33}$P-labelled DNA substrates (Supplementary Table S1). Oligonucleotide duplexes (0.1 nM) were incubated with different amounts of proteins in the binding buffer (40 mM Tris-acetate, 5 mM Ca(OAc)$_2$, 0.1 mg/ml BSA, 10% (v/v) glycerol, pH 8.3 at 25°C) in a total volume of 20 µl for 15 min at room temperature. Free DNA and protein-DNA complexes were separated by running non-denaturing 8% polyacrylamide gel electrophoresis for 3 h at 6 V/cm in 40 mM Tris-acetate (pH 8.3 at 25°C) buffer supplemented with 5 mM Ca(OAc)$_2$. In some experiments binding and electrophoresis buffers contained 0.1 mM EDTA instead of 5 mM of Ca(OAc)$_2$.

Radiolabelled DNA were detected and $K_D$ values for cognate oligonucleotide binding by BcnI enzymes were calculated by fitting binding data to equation (12) as described in (45):

$$y = \frac{s_0 - x - K_D + \sqrt{(s_0 + x + K_D)^2 - 4 * s_0 * x}}{2}  \quad (12)$$

where $y$ is the free DNA concentration (in terms of nM) at each protein concentration $x$, $s_0$ is the total DNA concentration in the binding mixture, and $K_D$ is the dissociation constant of protein–DNA complex. Data were analysed using the KYPLOT 2.0 software. The determined $K_D$ constants are listed in the Supplementary Table S3.

**Bulk cleavage experiments with the two-site DNA fragments**

Reactions contained 1 nM BcnI(wt), 2 nM $^{33}$P-labeled 135 or 535 bp di-hairpin substrate (Supplementary Figure S1C, Supplementary Table S1), and 98 nM unlabelled 135 or 535bp 2xCCSGG DNA fragment in the Reaction Buffer supplemented with 10 mM Mg(OAc)$_2$ and 0.1 mg/ml BSA. Samples were collected at timed intervals, quenched by mixing with the loading dye solution (75 mM EDTA, 0.01 % bromphenol blue, 0.1 % SDS, 50 % (v/v) glycerol, pH 8.0), and separated by gel electrophoresis using 6 % (29:1 AA:BAA) polyacrylamide gel for 20 h at 3 V/cm. Radiolabelled DNA was detected and quantified by phosphorimaging (46). The initial rates of uncut DNA cleavage ($v_{SS}$), and formation of single- and double-cut products ($v_{SP}$ and $v_{PP}$ respectively) were determined by linear regression. The processivity of BcnI was determined as the ratio

$$\text{Processivity} = \frac{|v_{PP}|}{v_{SS}}$$
i. e., as the fraction of the double-cut products among all cleaved DNA. Assuming that the maximum processivity of BcnI in our experimental setup is 0.5, the ratio of $k_{\text{cross}}$ and $k_{\text{diss}}$ was determined from eq. (13).

\begin{equation}
\text{Processivity} = 0.5 \frac{k_{\text{cross}}}{k_{\text{cross}} + k_{\text{diss}}} \quad (13)
\end{equation}
Supplementary Figure S1. DNA constructs. (A) 22.8 kbp nonspecific DNA construct used in the study of BcnI sliding and jumping on the DNA. (B) 7.4 kbp DNA construct used in the single molecule DNA cleavage experiments. (C) Radiolabelled 135 and 535 bp 2×CCSGG di-hairpin DNA substrates used in the BcnI processivity measurements, $^{32}$P-phosphate is depicted as a dark red circle. The BcnI specific site is depicted as a red rectangle (A-C).
**Supplementary Figure S2. DNA binding by BcnI.**

(A) BcnI(wt) electrophoretic mobility shift (EMSA) experiments with specific (sp) and non-specific (nsp) DNA performed in the presence of either Ca\(^{2+}\) ions or EDTA. In the presence of Ca\(^{2+}\) ions the \(K_D\) values for BcnI(wt) with sp (specific) and nsp (non-specific) DNA are 0.21 nM and 22 nM respectively. In the presence of EDTA the \(K_D\) values with both sp and nsp DNA exceed 500 nM.

(B) EMSA experiments performed with fluorescently labelled or unlabelled BcnI mutant proteins, specific DNA and 5mM Ca\(^{2+}\) ions. \(K_D\) values are reported in the Supplementary Table S3.
Supplementary Figure S3. Representation of BcnI reactions on the two-site DNA substrates. Steady-state kinetic experiments (Supplementary Methods) were performed with BcnI and 135 and 535 bp two-site DNA (2×CCSGG) substrates (inter-site distances 100 bp or 500 bp, respectively, Supplementary Figure S1C). (A) Uncut (SS), single cut (PS) and double-cut (PP) DNA forms were separated by polyacrylamide gel electrophoresis and (B) amounts of different DNA forms were determined by densitometric analysis of gel images. The initial rates of the SS DNA cleavage and formation of the PP product were calculated by linear regression. (C) Reaction scheme. Processive cleavage of the two-site substrate is possible only if upon cleavage of the first recognition site BcnI remains associated with the DNA fragment containing the second recognition site (probability 1/2) and then locates the second site before dissociation from the DNA [probability \(k_{\text{cross}}/(k_{\text{cross}}+k_{\text{diss}})\)]. The calculated processivity of BcnI (the fraction of processively cleaved DNA) was equal to 0.43 for the ‘135’ and 0.41 for the ‘535’ fragment.
**Supplementary Figure S4. BcnI crystal structures.** (A) apo-BcnI (PDB ID 2ODH). In (B) BcnI-DNA complex (PDB ID 2ODI) the catalytic center contacts a scissile phosphodiester bond on the ‘C’ (5’-CCCGG-3’) strand of the recognition sequence 5’-CCSGG-3’ (where S = C or G). (C) BcnI-DNA complex (PDB ID 3IMB) in the alternative orientation: the catalytic center contacts the ‘G’ (5’-CCGGG-3’) strand.
Supplementary Figure S5. DNA binding orientation of BcnI. Experiments were performed with BcnI(V105C+C202S)-Alexa488 or BcnI(C202S+K209C)-Alexa488 and Alexa546-labelled DNA as in Figure 5. On top of each panel, the schematic representations of BcnI bound to the specific substrate in either the 'C' or 'G' orientations are shown. The measured distances between the Alexa488 (green spheres) and the Alexa546 labels (red spheres), and the predicted FRET efficiencies for each binding orientations are indicated (experimental $R_0$ values determined for BcnI conjugates with Ca$^{2+}$ and DNA are listed in Supplementary Table S2).

(A) Experiment with BcnI(V105C+C202S)-Alexa488 and 'C' strand-labeled DNA.
(B) Experiment with BcnI(V105C+C202S)-Alexa488 and 'G' strand-labeled DNA.
(C) Experiment with BcnI(C202S+K209C)-Alexa488 and 'C' strand-labeled DNA.
(D) Experiment with BcnI(C202S+K209C)-Alexa488 and 'G' strand-labeled DNA.
### Supplementary Table S1. Oligonucleotides used in the study.

| # | Sequence (5’ -> 3’) | Specification | Purpose |
|---|---------------------|---------------|---------|
| 1 | GGTTTTCAAGCGTTATGGCCTGCCGAGGTGCCTCGAGAAAAGG | C-strand for C-DNA-Alexa546, M=dT-Alexa546 | Substrates for BcnI - DNA orientation FRET experiment |
| 2 | CTTTCTTCGAGTACCGGCGGGACTCAACGCTGAAAAACC | G-strand for C-DNA-Alexa546 | |
| 3 | GGTTTTCCAGGTTATGGCCGCGCTACTCGAGAAAAGG | C-strand for G-DNA-Alexa546 | |
| 4 | CCTTTTTCAGTCGAGGGGCAATAACGCTGAAAAACC | G-strand for G-DNA-Alexa546, M=dT-Alexa546 | |
| 5 | GCTGAGCGCCGGATTTTCTCTGCTGCAAGCCAGTTACCTTCGGAACG | C-strand for sp BcnI substrate | |
| 6 | CGTTTCCGAAGGTAACTGGCTCTCAGCAGGGCCTTACCAATACCTCCGTCAGC | G-strand for sp BcnI substrate | |
| 7 | GGTGACGCGGAGTATTTGATGTAGTTCCTCTGCTGCAAGCCAGTTACCTTCGGAACG | non-specific strand #1 for nsp BcnI substrate | |
| 8 | CGTTTCCGAAGGTAACTGGCTCTCAGCAGGGCCTTACCAATACCTCCGTCACC | non-specific strand #2 for nsp BcnI substrate | |
| 9 | GGATTACCTCAGCAAGAAGCAGGGTGGCAGCAT | PCR1 for primer | Oligos for 25.2 kbp non-specific (PCR1+PCR2+ Dig Handle PCR) DNA construct for single molecule translocation experiments |
| 10 | ATGTACCGCAGCCGCTCATTAGAT | PCR1 rev primer (biotin) | |
| 11 | GGTAAATCTCAGCAGAAAGCAGGGTGGGCAGCAT | PCR2 for primer | |
| 12 | CTTCCCGGAGGAGGCACCAGCCTTCTCATGAATT | PCR2 rev primer | |
| 13 | TGATATCGAATTCCTGAGCC | Dig Handle PCR for primer | |
| 14 | CAGGGTCGGAACAGGAGAGC | Dig Handle PCR rev primer | |
| 15 | GGCGTCGCTGAGGTGATTGTAGGCTGAGGCTAGCTGAGGCTGAGGCTGAGGCTGAGTACATGCACTTCAGCG | 76 bp “replace” DNA fragment with 5 BbvCI sites. | Oligos for DNA construct for single BcnI molecule binding dynamics and single DNA molecule |
| 16 | GATCGCTGAGGTGATTGTAGGCTGAGGCTAGCTGAGGCTGAGGCTGAGGATGACATGAGCTGAG | pBluescript II SK+ “replace” for primer with Aval site | |
| 17 | GAACGTCCCGAGAAAGAAGGAGAAGAAGAAAAGC | | |
|   | DNA Sequence          | Biochemical Modification                      | Comments                                           |
|---|----------------------|-----------------------------------------------|---------------------------------------------------|
| 18| AAAAGCTGGAGCTCCACCAGCGGTTGGGCCGC | pBluescript II SK+ replace rev primer with SacI site | hydrolysis experiments |
| 19| TTCATCTCGGTTCTCTATGGTGCTGGT | T7 for primer with Aval site | |
| 20| GGAATCGCCGCGAGCAGCATAGCTCAGG | T7 rev primer with NgoMIV site | |
| 21| CTCTTCCTTTTTCAATATTATTG | Biotin Handle PCR pBluescript II SK+ for primer | |
| 22| GCTCACTGGGCGCTGTTTACAA | Biotin Handle PCR pBluescript II SK+ rev primer | |
| 23| TGATATCGAATTCCTGCAGCC | Digoxigenin Handle PCR pBluescript II SK+ for primer | |
| 24| TTTGTGATGCTGTCAGGG | Digoxigenin Handle PCR pBluescript II SK+ rev primer | |
| 25| TCAGCMCATGTCTATCCACACTTTGACCCTCAGCTGCTAGCCTCAGCTAATACTCACC | Cy5 oligo (with M = T-Cy5) | |
| 26| GAAGTGCCCGGCAATACGTACTGCTACTGAGCAGTACGTATGCCGGCGGCA | dhp 2xCCSGG substrate hp part with BcnI site. | Oligos for 2xCCSGG substrates |
| 27| GCCGCTCGCTCGCTGCTCGG | dhp 2xCCSGG substrate universal dir primer | |
| 28| GTATTGGTCTCGCTTTCAAGCAGCATACCTTGAATG | 135bp dhp 2xCCSGG substrate rev primer | |
| 29| CAAGGTCCTCGCTCTCTGCTCTTCTCTGTCTCTGCTGG | 535bp dhp 2xCCSGG substrate rev primer | |
| 30| TAGCAGTACGTATTGCCGCGGCACTTCTGCTCTGTGCTGG | ds 2xCCSGG substrate universal dir primer | |
| 31| TAGCAGTACGTATTGCCGCGGCACTTCAAGCAGCATACCTTGAATG | 135bp ds 2xCCSGG substrate rev primer | |
| 32| TAGCAGTACGTATTGCCGCGGCACTTCTGCTCTTGTGCATCTCGGC | 535bp ds 2xCCSGG substrate rev primer | |
| 33| CGCAGCAGACTTCCCGGAAGAGCAGCAGC | C-strand for specific DNA duplex | Oligos for EMSA experiments |
| 34| GTTGCATCTCTCCCGGAAAGTCTGTCC | G-strand for specific DNA duplex | |
|   | CGACGACTTGTCACAAGAGCACGC | #1 strand for non-specific BcnI substrate |
|---|--------------------------|-----------------------------------------|
| 35 |                          |                                         |
| 36 | GTTGCCTCCTTGTGACAAGTCGTGCG | #2 strand for non-specific BcnI substrate |
Supplementary Table S2. Labelling efficiencies, quantum yields (QY), Forster radii ($R_0$) and anisotropies of fluorescently labelled Bcni proteins.

| Bcni conjugate                  | Labelling efficiency | Conditions                        | QY   | $R_0$, nm | Anisotropy |
|---------------------------------|----------------------|-----------------------------------|------|-----------|------------|
| Bcni(N18C+C202S)-Alexa488      | 75.9% (Alexa488)     | -                                 | 0.77 | 6.0       | 0.134      |
|                                 |                      | +10mM Ca(OAc)$_2$                | 0.75 | 5.98      | 0.135      |
|                                 |                      | +10mM Ca(OAc)$_2$ + 1μM DNA duplex 2/3 | 0.79 | 6.04      | 0.112      |
| Bcni(V105C+C202S)-Alexa488     | 97% (Alexa488)       | -                                 | 0.80 | 6.03      | 0.190      |
|                                 |                      | +10mM Ca(OAc)$_2$                | 0.75 | 5.97      | 0.182      |
|                                 |                      | +10mM Ca(OAc)$_2$ + 1μM DNA duplex 2/3 | 0.77 | 6.01      | 0.087      |
| Bcni(C202S+K209C)-Alexa488     | 96.5% (Alexa488)     | -                                 | 0.64 | 5.81      | 0.232      |
|                                 |                      | +10mM Ca(OAc)$_2$                | 0.70 | 5.90      | 0.223      |
|                                 |                      | +10mM Ca(OAc)$_2$ + 1μM DNA duplex 2/3 | 0.79 | 6.03      | 0.092      |
| Bcni(N18C+C202S)-Alexa546      | 98.2% (Alexa546)     | -                                 | -    | -         | 0.231      |
|                                 |                      | +10mM Ca(OAc)$_2$                | -    | -         | 0.245      |
|                                 |                      | +10mM Ca(OAc)$_2$ + 1μM DNA duplex 2/3 | -    | -         | 0.251      |
| Bcni(V105C+C202S)-Alexa546     | 100% (Alexa546)      | -                                 | -    | -         | 0.248      |
|                                 |                      | +10mM Ca(OAc)$_2$                | -    | -         | 0.249      |
|                                 |                      | +10mM Ca(OAc)$_2$ + 1μM DNA duplex 2/3 | -    | -         | 0.215      |
| Bcni(N18C+V105C+C202S)-Alexa488-Alexa546 | 94.5% (Alexa488) 57.7% (Alexa546) | - | - | - |
Supplementary Table S3. Binding affinity to specific DNA and catalytic activity of BcnI mutants and BcnI-dye conjugates

| BcnI conjugate                                  | * $K_D$, nM | ** $k_{cat}$, s$^{-1}$ | $k_{cat}$, s$^{-1}$ \(/\) (BcnI protein)/$k_{cat}$, s$^{-1}$ (BcnI (wt)), % |
|------------------------------------------------|
| BcnI(wt)                                        | 0.21        | 0.042                   | 100                                           |
| BcnI(N18C+C202S)                                | 0.29        | 0.022                   | 52                                            |
| BcnI(N18C+C202S)-Alexa488                       | 0.18        | 0.013                   | 31                                            |
| BcnI(V105C+C202S)                               | 0.15        | 0.026                   | 62                                            |
| BcnI(V105C+C202S)-Alexa488                      | 0.16        | 0.026                   | 62                                            |
| BcnI(V105C+C202S)-Biotin                        | -           | 0.02                    | 48                                            |
| BcnI(C202S+K209C)                               | 0.18        | 0.029                   | 69                                            |
| BcnI(C202S+K209C)-Alexa488                      | 0.44        | 0.017                   | 40                                            |
| BcnI(N18C+V105C+C202S)                         | 0.3         | 0.031                   | 74                                            |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546        | -           | 0.015                   | 36                                            |
| BcnI(V105C+C202S)-Biotin labelled with streptavidin-coated quantum dots | -           | 0.004                   | 10                                            |

* $K_D$ values were determined as described in Supplementary methods.

** $k_{cat}$ values were determined as described in Supplementary methods.
| BcnI conjugate | Conditions                          | $E_{\text{FRET}}$ | $\mu \pm \text{s.e.m.}$ | $\sigma \pm \text{s.e.m.}$ |
|---------------|-----------------------------------|-------------------|--------------------------|-----------------------------|
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | -                                | 0.61±0.04         | 0.41±0.05                |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | 10 mM Ca(OAc)$_2$               | 0.72±0.04         | 0.12±0.01                |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | nsp DNA                          | 0.51±0.01         | 0.2±0.02                 |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | nsp DNA + 10 mM Ca(OAc)$_2$      | 0.58±0.02         | 0.21±0.02                |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | sp DNA                           | 0.53±0.01         | 0.14±0.01                |
| BcnI(N18C+V105C+C202S)-Alexa488-Alexa546 | sp DNA + 10 mM Ca(OAc)$_2$       | 0.57±0.01         | 0.09±0.02                |
| BcnI(N18C+C202S)-Alexa488 | Alexa546-C-DNA + 10 mM Ca(OAc)   | 0.35±0.04         | 0.11±0.01                |
| BcnI(N18C+C202S)-Alexa488 | Alexa546-G-DNA + 10 mM Ca(OAc)   | 0.26±0.01         | 0.11±0.01                |
| BcnI(V105C+C202S)-Alexa488 | Alexa546-C-DNA + 10 mM Ca(OAc)   | 0.3±0.02          | 0.1±0.01                 |
| BcnI(V105C+C202S)-Alexa488 | Alexa546-G-DNA + 10 mM Ca(OAc)   | 0.31±0.1          | 0.29±0.1                 |
| BcnI(C202S+K209C)-Alexa488 | Alexa546-C-DNA + 10 mM Ca(OAc)   | 0.24±0.01         | 0.11±0.01                |
| BcnI(C202S+K209C)-Alexa488 | Alexa546-G-DNA + 10 mM Ca(OAc)   | 0.16±0.06         | 0.25±0.06                |
44. Lakowicz, J.R. (2006) Principles of Fluorescence Spectroscopy 3rd ed.
45. Tamulaitis, G., Mucke, M. and Siksnys, V. (2006) Biochemical and mutational analysis of EcoRII functional domains reveals evolutionary links between restriction enzymes. FEBS Lett., 580, 1665–1671.
46. Sasnauskas, G., Halford, S.E. and Siksnys, V. (2003) How the BfiI restriction enzyme uses one active site to cut two DNA strands. Proc. Natl. Acad. Sci. U. S. A., 100, 6410–6415.