Serine-γPNA, Invader probes, and chimeras thereof: Three probe chemistries that enable sequence-unrestricted recognition of double-stranded DNA

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ELECTRONIC SUPPLEMENTARY INFORMATION

Definition of zipper nomenclature. The following nomenclature is used to describe the relative arrangement between two 2′-*O*-(pyren-1-yl)methyl-RNA monomers on opposing strands in an Invader probe. The number *n* describes the distance measured in number of base-pairs and has a positive value if a monomer is shifted toward the 5′-side of its strand relative to a second reference monomer on the other strand. Conversely, *n* has a negative value if a monomer is shifted toward the 3′-side of its strand relative to a second reference monomer on the other strand.

Table S1. MALDI-MS of SγPNAs and Invader strands used in this study.[a]

Strand	Sequence	Observed *m/z* $[M+H]^+$	Calculated *m/z* $[M+H]^+$
SγPNA2u	H-(TMR)-K-ATA CTG GTT TGT GTT C-K-NH_2	5568	5565
SγPNA2d	NH_2-K-TAT GAC CAA ACA CAA G-K-(TMR)-H	5540	5540
SγPNA4u	H-(TMR)-K-AGC CCT GTG CCC TG-K-NH_2	4906	4901
SγPNA4d	NH_2-K-TCG GGA CAC GGG AC-K-(TMR)-H	5038[b]	5037[b]
SγPNA10u	H-(TMR)-K-GTG TAG TGT ATA TG-K-NH_2	5044	5044
SγPNA10d	NH_2-K-CAC ATC ACA TAT AC-K-(TMR)-H	4873	4873
INV2u	5'-Cy3-A**U**AC**U**GGTTTG**U**G**U**TC-3'	6266	6264
INV2d	3'-TA**U**GA**C**CAAACA**C**A**A**G-Cy3-5'	6282	6279
INV10u	5'-Cy3-G**U**G**U**AGTG**U**A**U**ATG-3'	5721	5720
INV10d	3'-CA**C**A**U**CACA**U**A**U**AC-Cy3-5'	5562	5561

[a] MALDI-MS data for **INV4u** and **INV4d** have been previously reported in reference S1.

[b] The K^+-adduct was observed.

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   1 atgcaagccc gggatctcag ccctgtggtc tgggaactgt gaaaccggct tgagtatgtg
  61 tgctgttatc agcactgtgc cctggcgact ctgatactgg tttgtgttca tgtgtgtgtg
 121 tgtgtgtgtg tgtgttgctg ttctcagccc tgtgccctgg cgattgtgca accagtatct
 181 gtatgcctgt gtgtgtgtgt gtgtgtgtgt gtgtgtgtgt gtgtgctgtt ctcaacccat
 241 tgccctggcg attgttcaac cagtttgtgt atatgtgtgt gtagatgtgt gtgccatcct
 301 gagccttgtg ccctggcaac tggggaaacg gtgtgtgtgt gttgtgtgtc tgtgtgtgtg
 361 ctgatttcag ccatgtgccc ttctgactgt gcaactggtt tgtgtgtgtg tgcacgcgat
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 481 gtgctctttt cagccctgtt tcctagagac tgtggaaccg gttggtgtgt gtgtgtgtct
 541 gtgtgtgtgt gtgccattct cagccctgtg ccctggcgac tgtgcaatat tttgtcgtgt
 601 gtgtgtgtgt gtgtatttgt gtgtgcaatt cacagccctg ttccctggcg actgtgcaag
 661 cagattgttg cgtatgtttc tgtgtgtgtg tgtgtgtgtg tgtgtgtgta tgtgctgttc
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 841 tgtgtatgtg tctgtgtgtg catgtgtctg tgtgtgttat atgctgttct cagccctgtg
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1021 tgtgtgtggt ttgaccagtt ttcagccctg tgccttagtg actgtgtaac tggtgtgtgt
1081 gtgtgtgtgt gtgtgtgtgc tcttctcagc cctgtgccct gttgactgtg caagcggttt
1141 gtctgtgtat gtgagtgggt gctgttctca tgcctgtgca ctgg
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Figure S1. Position of target sequences within the *DYZ-1* satellite gene on the bovine (*Bos taurus*) Y chromosome[S2] for the different probes studied herein. Region "2" (grey) is the target for **SγPNA2u**, **SγPNA2d**, **INV2**, **SγPNA2u**:**INV2d** and **SγPNA2d**:**INV2u**. Region "4" (yellow) is the target for **SγPNA4u**, **SγPNA4d**, **INV4**, **SγPNA4u**:**INV4d** and **SγPNA4d**:**INV4u**. Region "10" (red) is the target for **SγPNA10u**, **SγPNA10d**, **INV10**, **SγPNA10u**:**INV10d** and **SγPNA10d**:**INV10u**. Region "4" is present six times within the tandem repeat (~6×10^4 tandem repeats), while regions "2" and "10" are present once.

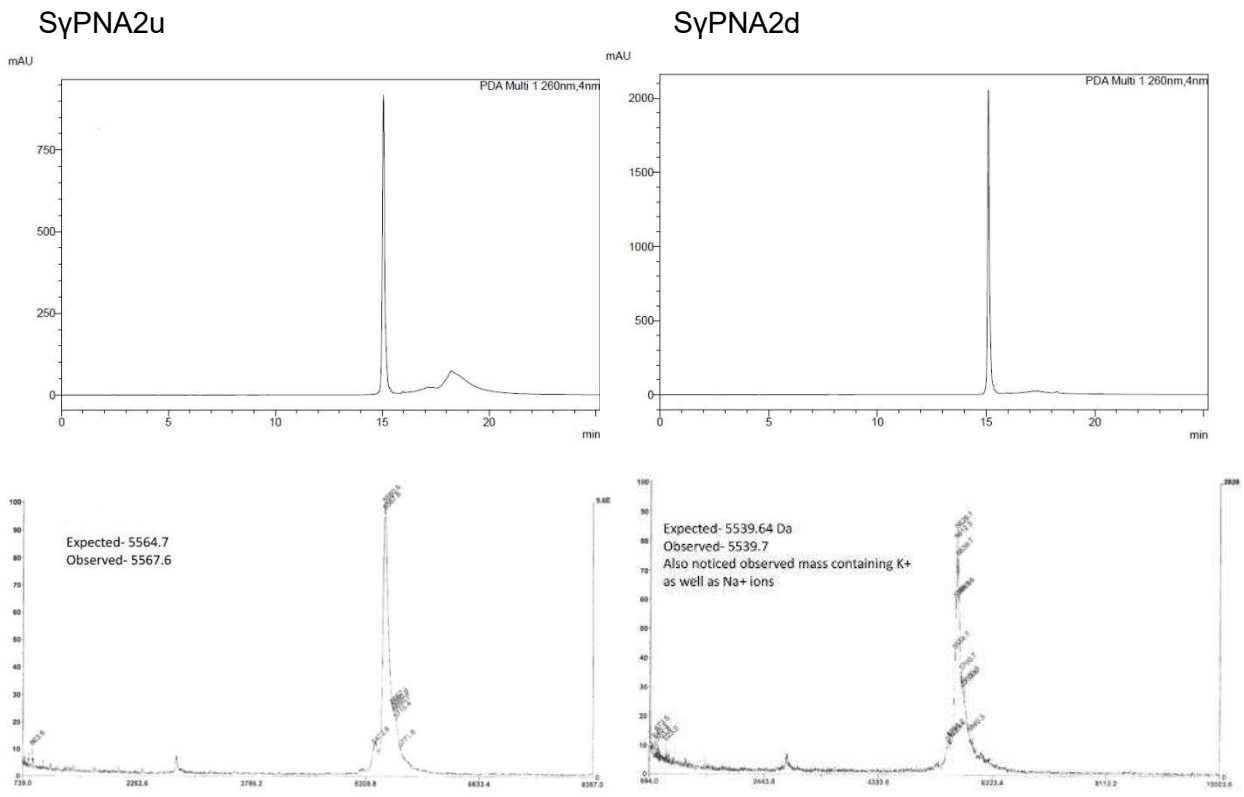


Figure S2. HPLC traces and MALDI-MS spectra for **SγPNA2u** and **SγPNA2d**.

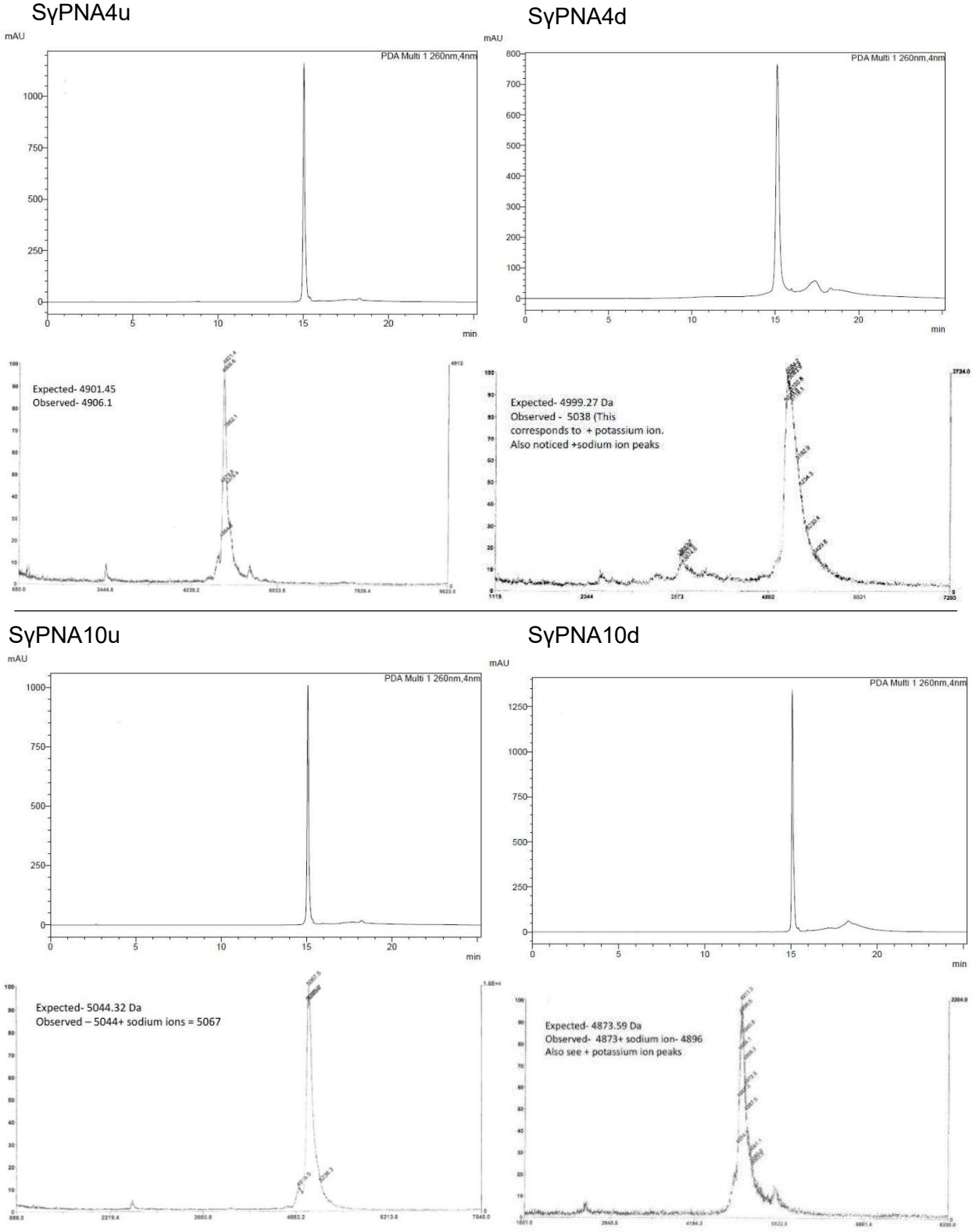


Figure S3. HPLC traces and MALDI-MS spectra for **SγPNA4u/d** and **SγPNA10u/d**.

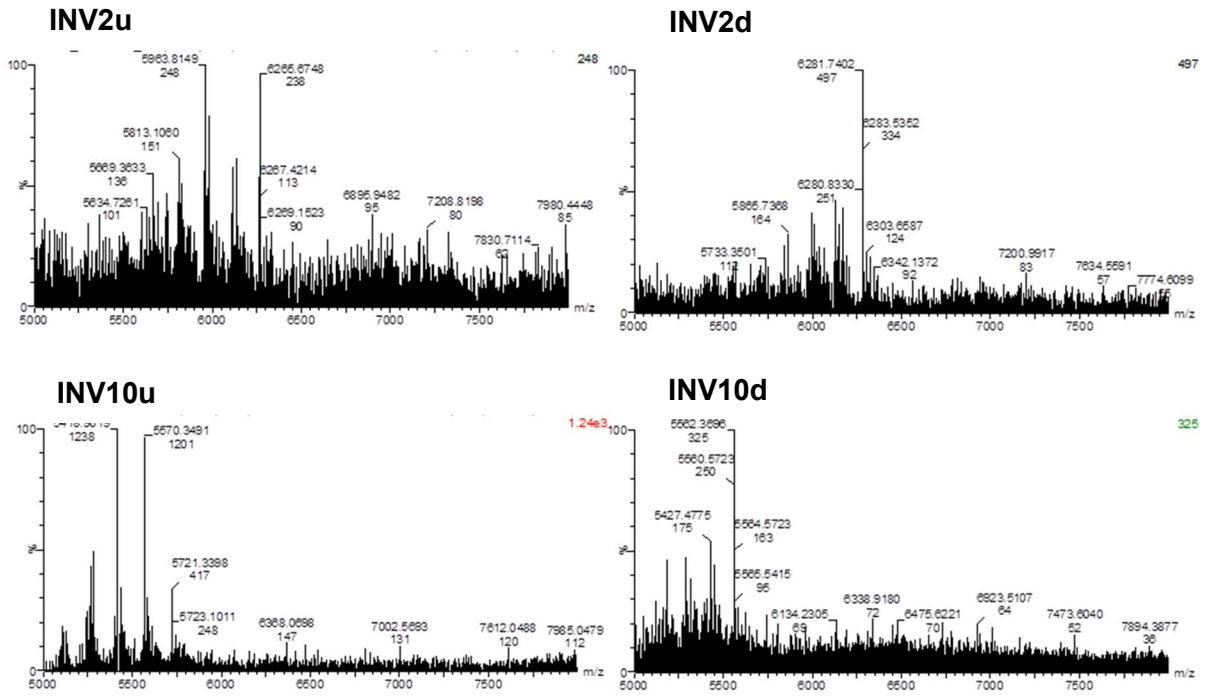


Figure S4. MALDI-MS spectra for **INV2u/d** and **INV10u/d**.

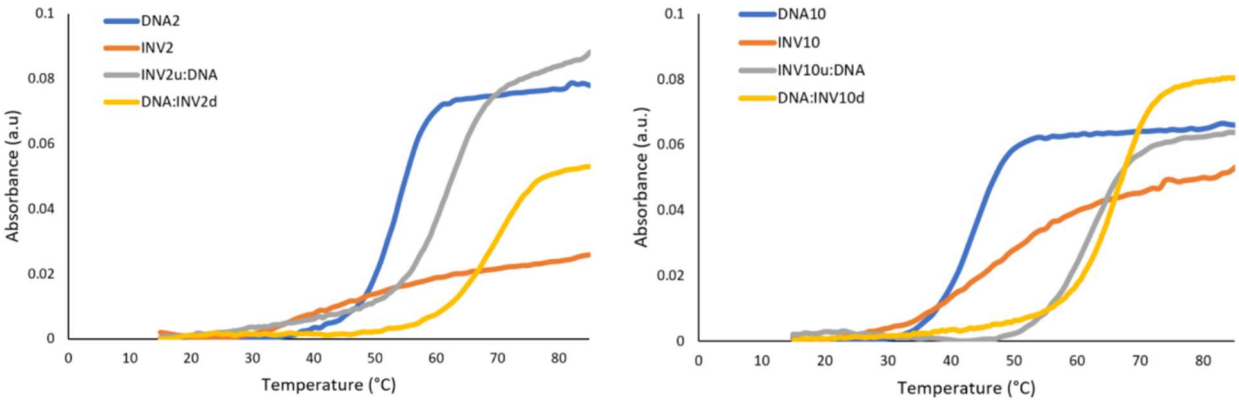


Figure S5. Representative thermal denaturation curves for Invader, Invader:cDNA, and reference DNA duplexes. Experimental conditions are as specified in Table 1.

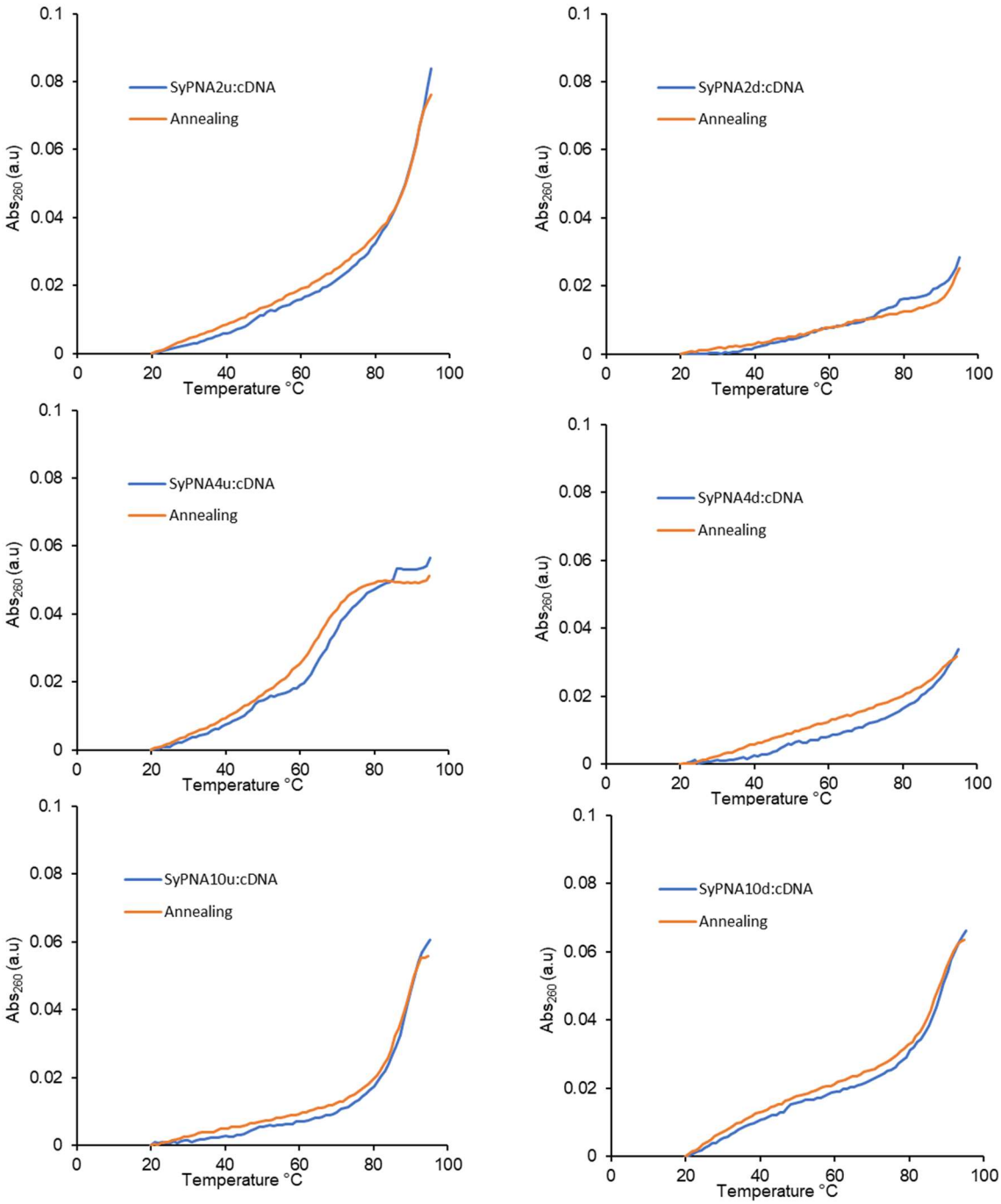


Figure S6. Representative thermal denaturation (heating) and annealing (cooling) curves for SγPNA:cDNA duplexes. Duplexes were heated from 20 °C to 95 °C (blue), followed by cooling to 20 °C (orange). Experimental conditions are as specified in Table 1.

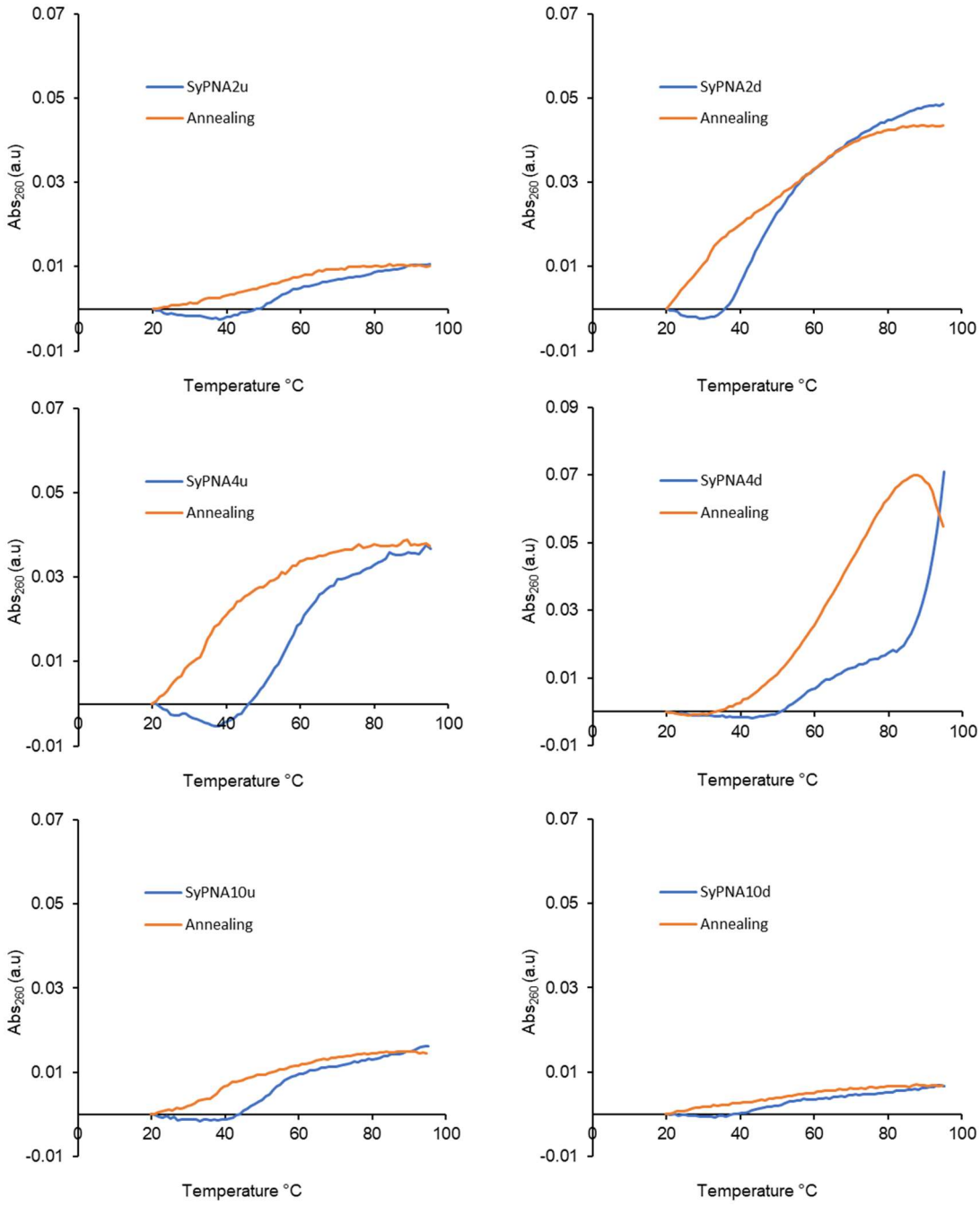


Figure S7. Representative melting (heating) and annealing (cooling) curves for single-stranded SγPNAs. Solutions were heated from 20 °C to 95 °C (blue), followed by cooling to 20 °C (orange). Experimental conditions are specified in Table 1. Pronounced hysteresis is observed rendering T_m determination unreliable.

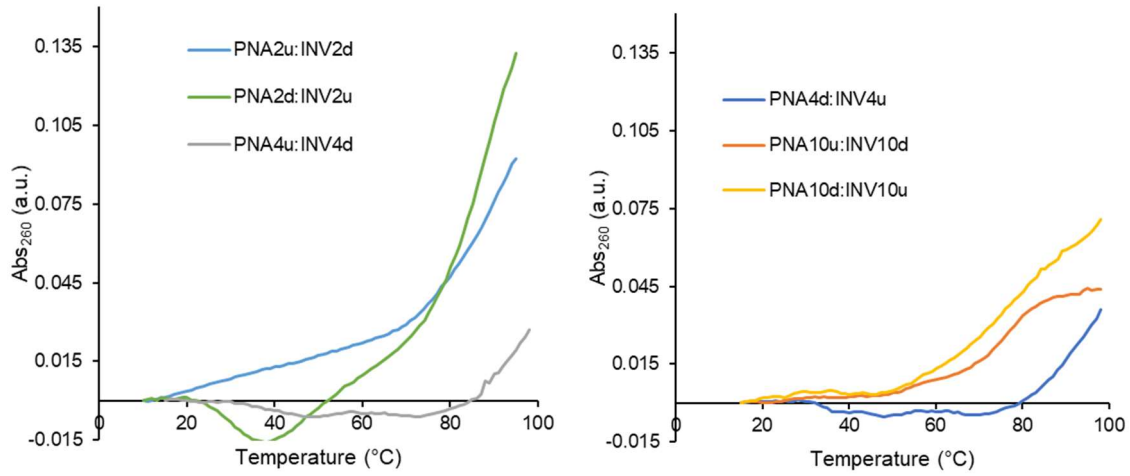


Figure S8. Representative thermal denaturation curves of chimeric duplexes between SγPNA and individual Invader strands. For experimental conditions, see Table 1.

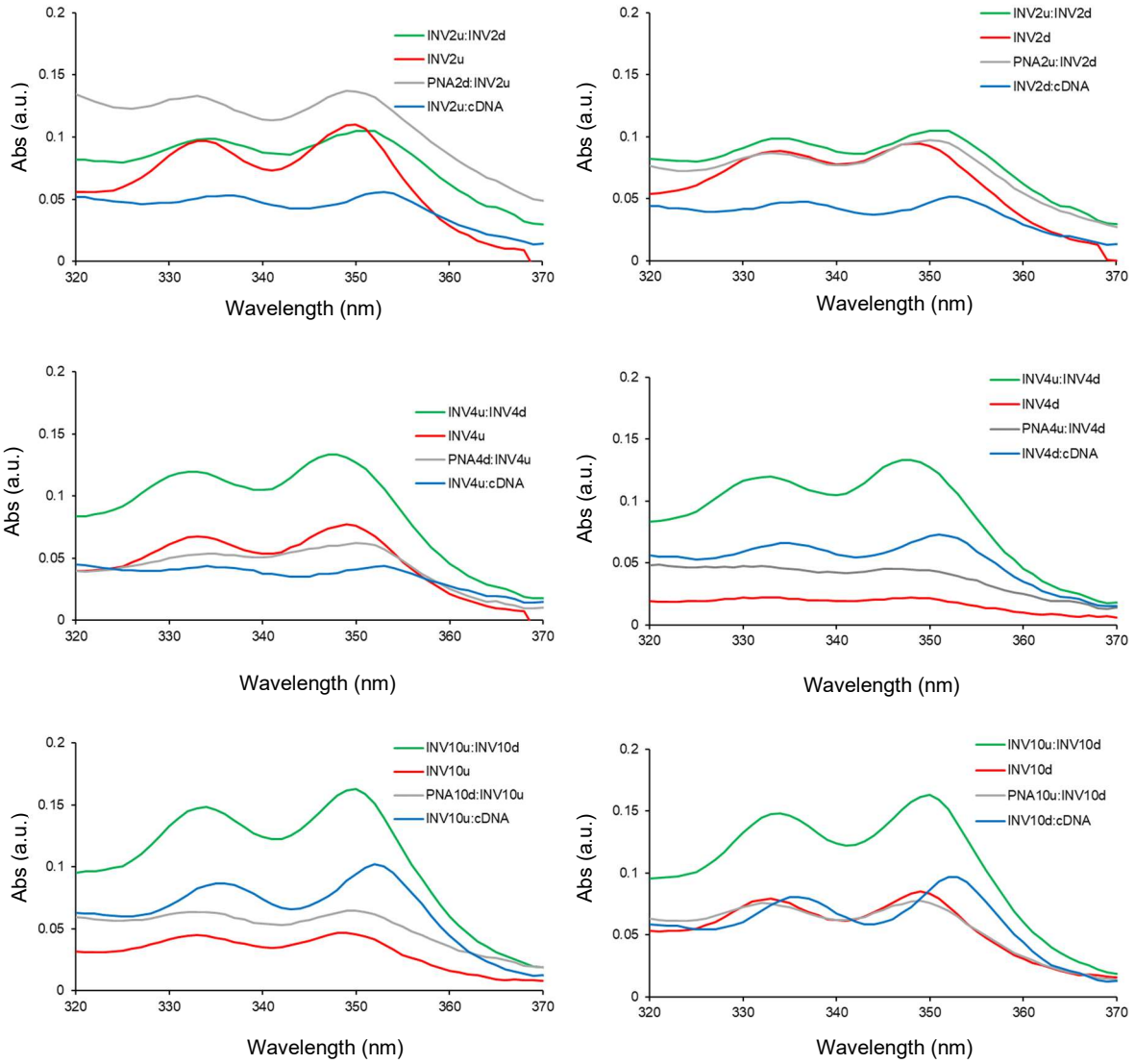


Figure S9. UV-Vis absorption spectra for single-stranded Invader probes and the corresponding duplexes with complementary DNA, SγPNA, and Invader strands. Spectra were recorded at 10 °C with each strand used at 1.0 μM in T_m buffer.

Table S2. Absorption maxima in the 340-365 nm region for single-stranded Invader probes and the corresponding duplexes with complementary DNA, SγPNA, or Invader strands.[a]

	λ_{max} (nm) [$\Delta\lambda_{max}$]			
Probe	SSP	+cDNA	+SγPNA	+ssINV
INV2u	350	353 [+3]	351 [+1]	351 [+1]
INV2d	349	352 [+3]	350 [+1]	351 [+2]
INV4u	349	353 [+4]	350 [+1]	348 [-1]
INV4d	348	351 [+3]	348 [±0]	348 [±0]
INV10u	349	352 [+3]	349 [±0]	350 [+1]
INV10d	349	353 [+4]	349 [±0]	350 [+1]

[a]SSP = single-stranded probe. $\Delta\lambda_{max}$ is calculated relative to the single-stranded Invader strand. Binding partners (listed in parenthesis) are as follows: **INV2u** (**SγPNA2d** and **INV2d**), **INV2d** (**SγPNA2u** and **INV2u**), **INV4u** (**SγPNA4d** and **INV4d**), **INV4d** (**SγPNA4u** and **INV4u**), **INV10u** (**SγPNA10d** and **INV10d**), and **INV10d** (**SγPNA10u** and **INV10u**). Measurements were performed at 10 °C in T_m buffer using quartz optical cells with a 1.0 cm path length. Spectra are shown in Figure S9.

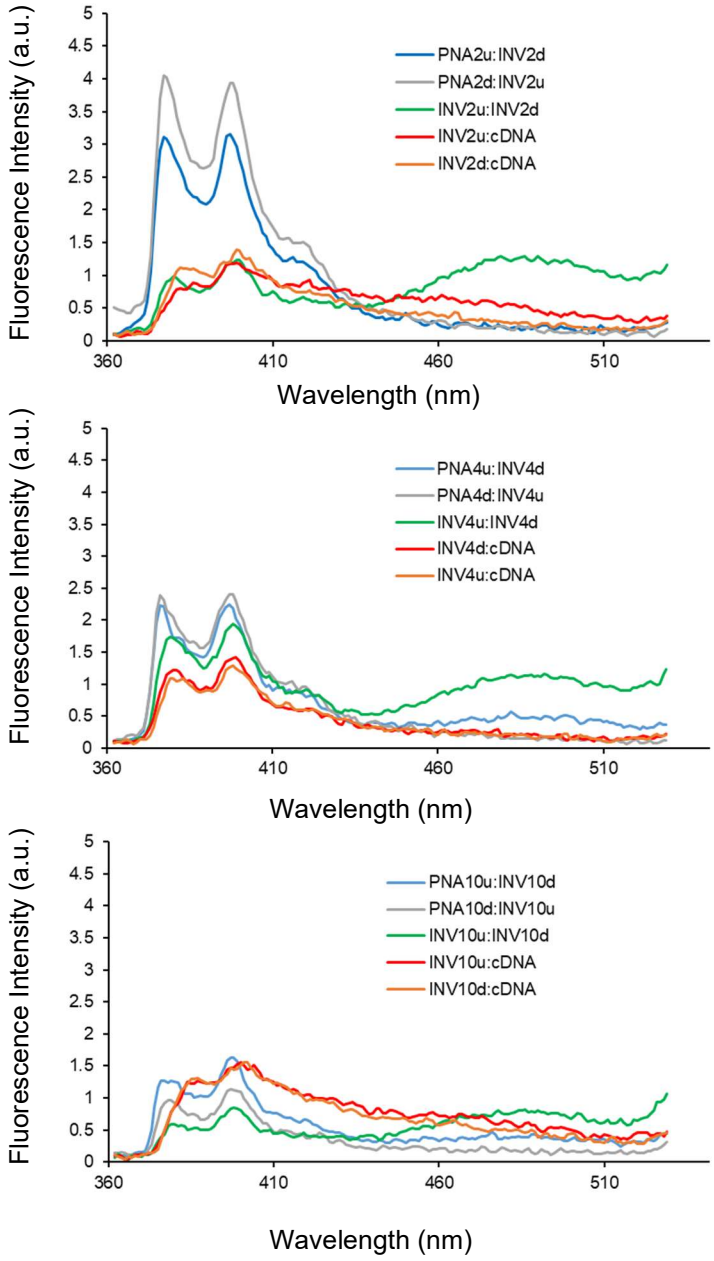


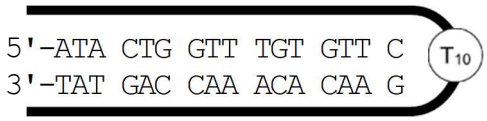
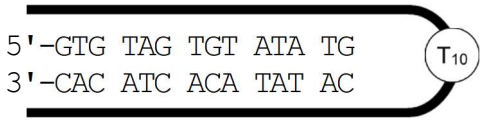
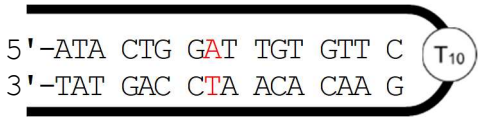
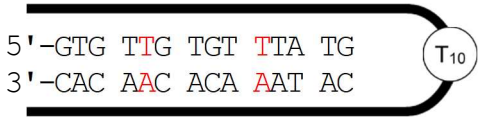
Figure S10. Steady-state fluorescence emission spectra for Invader probes, chimeric SγPNA-Invader probes, and Invader:cDNA duplexes. Spectra were recorded at 5 °C in T_m buffer using λ_{ex} = 350 nm and quartz optical cells with a 1.0 cm path length.

Table S3. I_5/I_1 ratios for Invader probe duplexes and duplexes between individual Invader strands and complementary DNA or SγPNA.[a]

	I_5/I_1 ratios		
Probe	+cINV	+cDNA	+SγPNA
INV2u	1.3	1.3	1.0
INV2d	1.3	1.3	1.0
INV4u	1.1	1.2	1.0
INV4d	1.1	1.3	1.0
INV10u	1.4	1.3	1.2
INV10d	1.4	1.1	1.3

[a]I_5/I_1 ratios were calculated based on observed emission maxima in the 396-400 nm range for I_5 and 376-387 nm range for I_1. Measurements were performed at 5 °C in T_m buffer using quartz optical cells with a 1.0 cm path length. Spectra are shown in Fig. S10.

Table S4. Sequences and select intramolecular T_ms for DNA hairpins used herein.[a]

Hairpin	Sequence	T_m (°C)
DH2		72.0
DH4		82.0
DH10		62.0
DH2m		nd
DH2mm		nd
DH4m		nd
DH4mm		nd
DH10m		nd
DH10mm		nd

[a] T_m were determined as described in Table 1. Hairpins **DH2m/mm, DH4m/mm**, **and DH10m/mm** differ by either one (**m**) or two (**mm**) base pairs relative to the corresponding **DH2**, **DH4**, and **DH10**, as indicated by the red letters. "nd" = not determined.

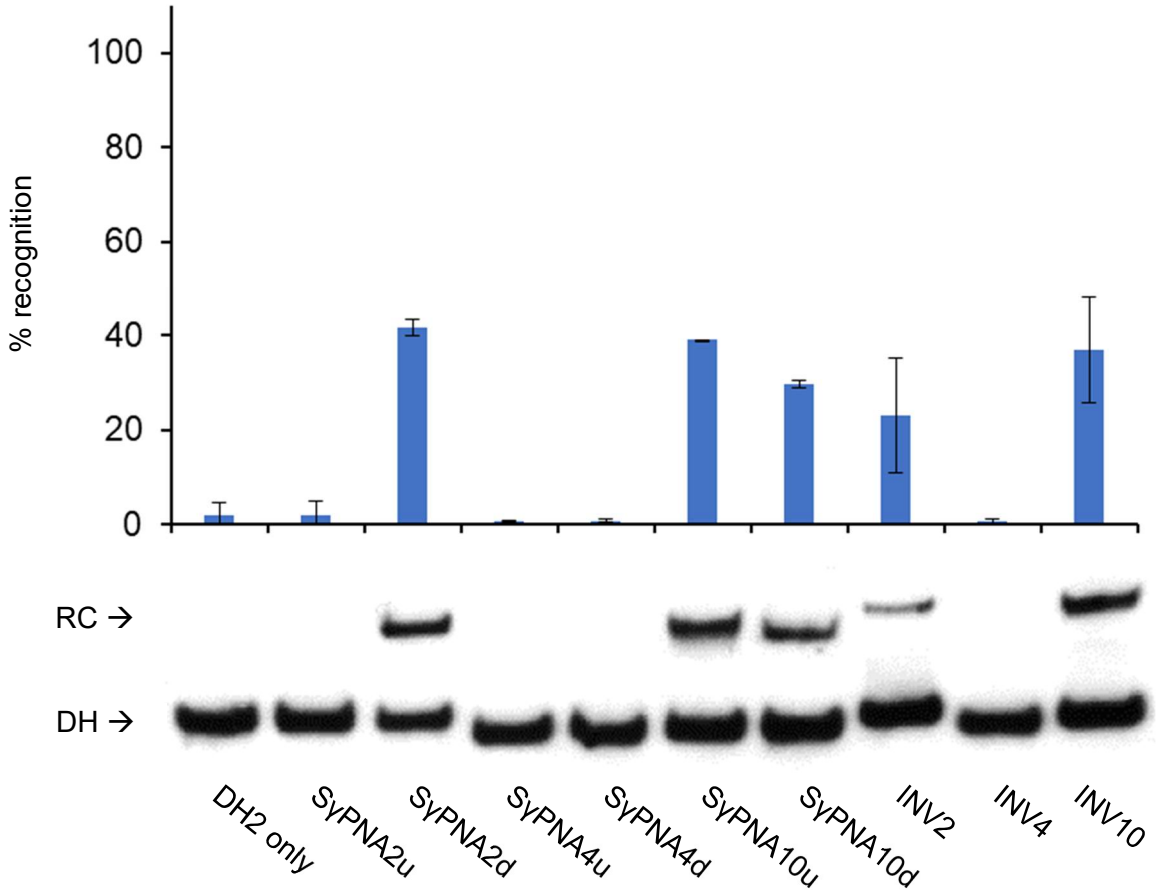


Figure S11. Representative electrophoretograms for recognition experiments entailing a 5-fold molar excess of various probes and their respective DNA hairpin targets **DH2**, **DH4**, or **DH10**. Histograms depict averaged results from at least three experiments with error bars representing standard deviation. RC = band corresponding to recognition complex. DH = band corresponding to DNA hairpin. DIG-labeled **DH2**, **DH4**, and **DH10** (sequences shown in Table S4) were incubated with the specified probe in HEPES buffer (50 mM HEPES, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.2, 10% sucrose, 1.44 mM spermine tetrahydrochloride) at 37 °C for 2.5 h. Binding partners are as follows: **DH2** (target for **SγPNA2u**, **SγPNA2d**, and **INV2**), **DH4** (target for **SγPNA4u**, **SγPNA4d**, and **INV4**), and **DH10** (target for **SγPNA10u**, **SγPNA10d**, and **INV10**). **SγPNA2d**, **SγPNA10u, SγPNA10d, INV2**, and **INV10** resulted in ~42%, ~39%, ~30%, ~23%, and 37% recognition, respectively, whereas no recognition is observed for **SγPNA2u**, **SγPNA4u**, and **SγPNA4d**.

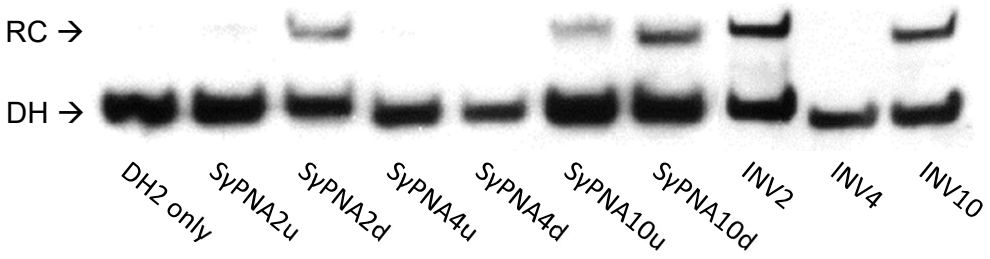


Figure S12. Representative electrophoretograms for recognition experiments entailing DNA hairpin targets **DH2, DH4**, or **DH10** and a 5-fold molar excess of the corresponding probes following 15 h of incubation using the same buffer conditions as described in Figure S11.

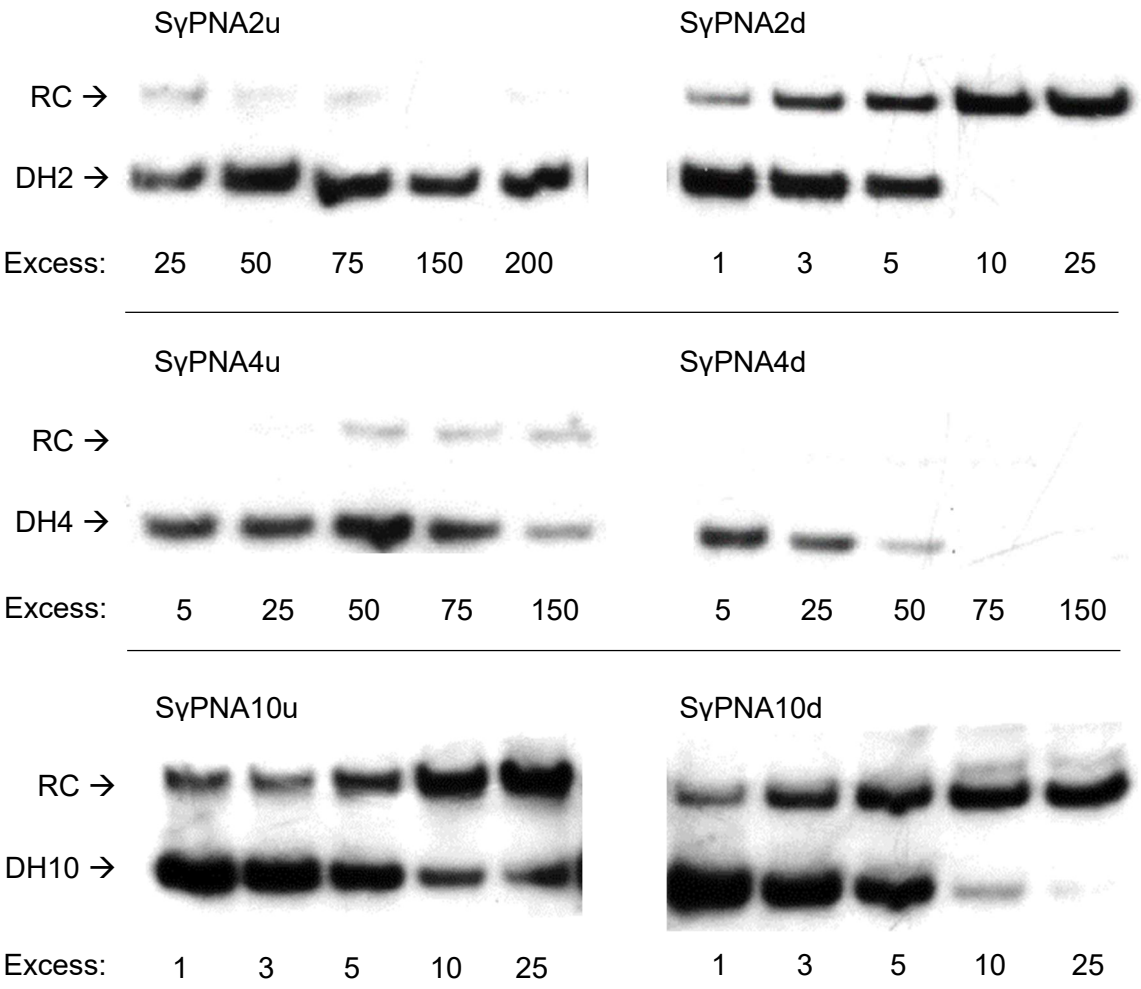


Figure S13. Dose-response experiments. Representative electrophoretograms for recognition of DNA hairpin targets **DH2**, **DH4**, or **DH10** (34.4 nM) using different concentrations of single-stranded SγPNA probes following incubation at 37 °C for 2.5 h. Conditions are otherwise as described in Figure S11.

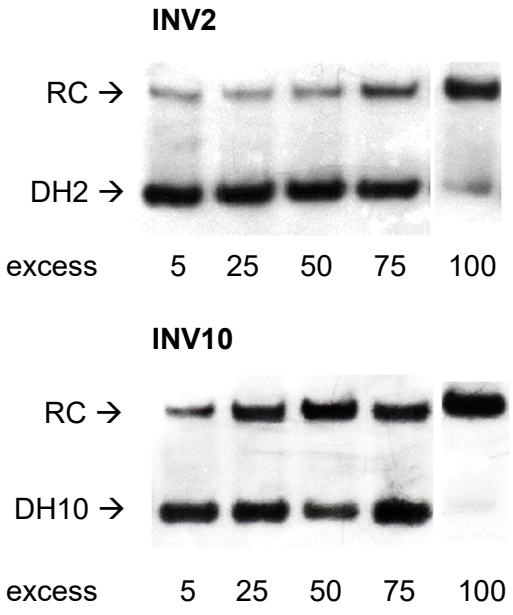


Figure S14. Dose-response experiments. Representative electrophoretograms for recognition of DNA hairpin targets **DH2** or **DH10** (34.4 nM) using different concentrations of Invader probes following incubation at 37 °C for 2.5 h. Conditions are otherwise as described in Figure S11.

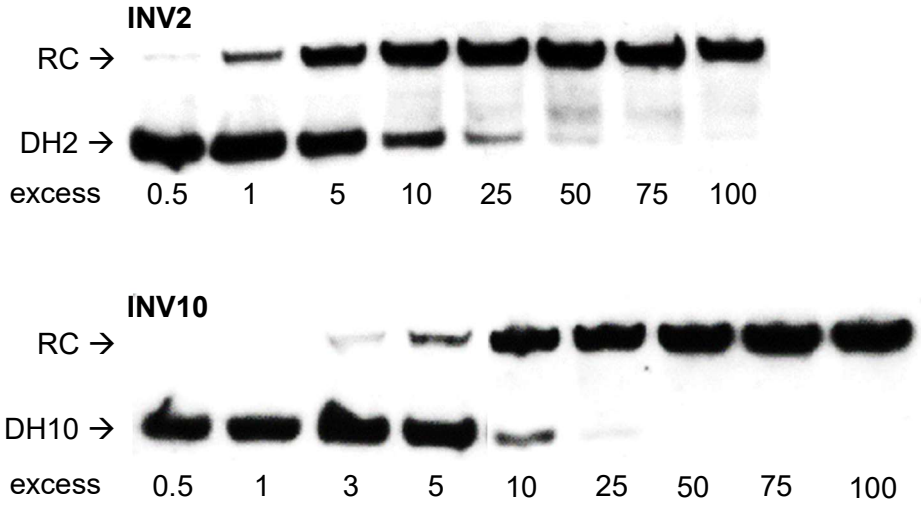


Figure S15. Dose-response experiments. Representative electrophoretograms for recognition of DNA hairpin targets **DH2** or **DH10** (34.4 nM) using different concentrations of Invader probes following incubation at 37 °C for 15 h. Conditions are otherwise as described in Figure S11.

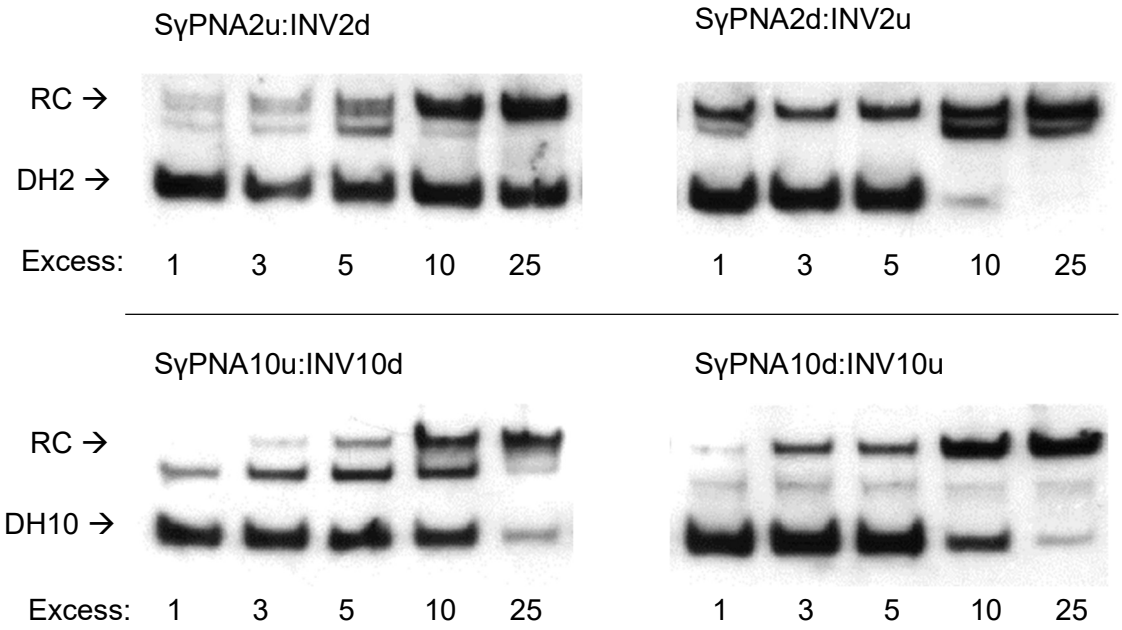


Figure S16. Dose-response experiments. Representative electrophoretograms for recognition of DNA hairpin targets **DH2** or **DH10** (34.4 nM) using different concentrations of chimeric SγPNA:Invader probes at 37 °C for 2.5 hours. Incubation conditions are otherwise as described in Figure S11.

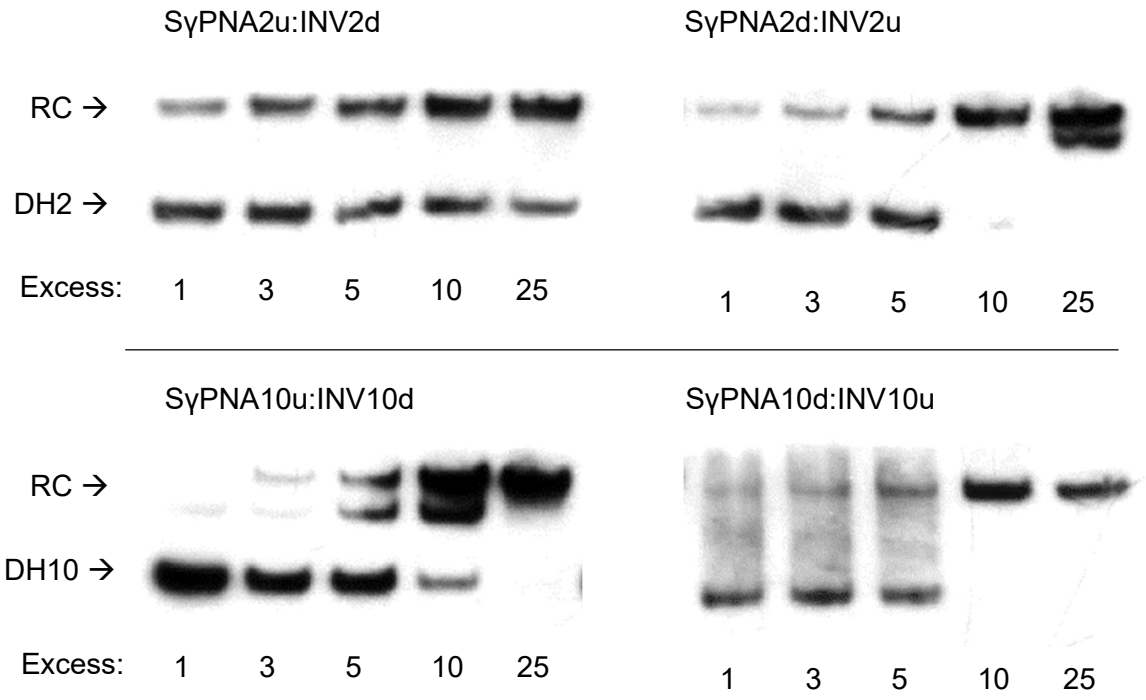


Figure S17. Dose-response experiments. Representative electrophoretograms for recognition of DNA hairpin targets **DH2** or **DH10** (34.4 nM) using different concentrations of chimeric SγPNA:Invader probes following incubation at 37 °C for 15 h. Conditions are otherwise as described in Figure S11.

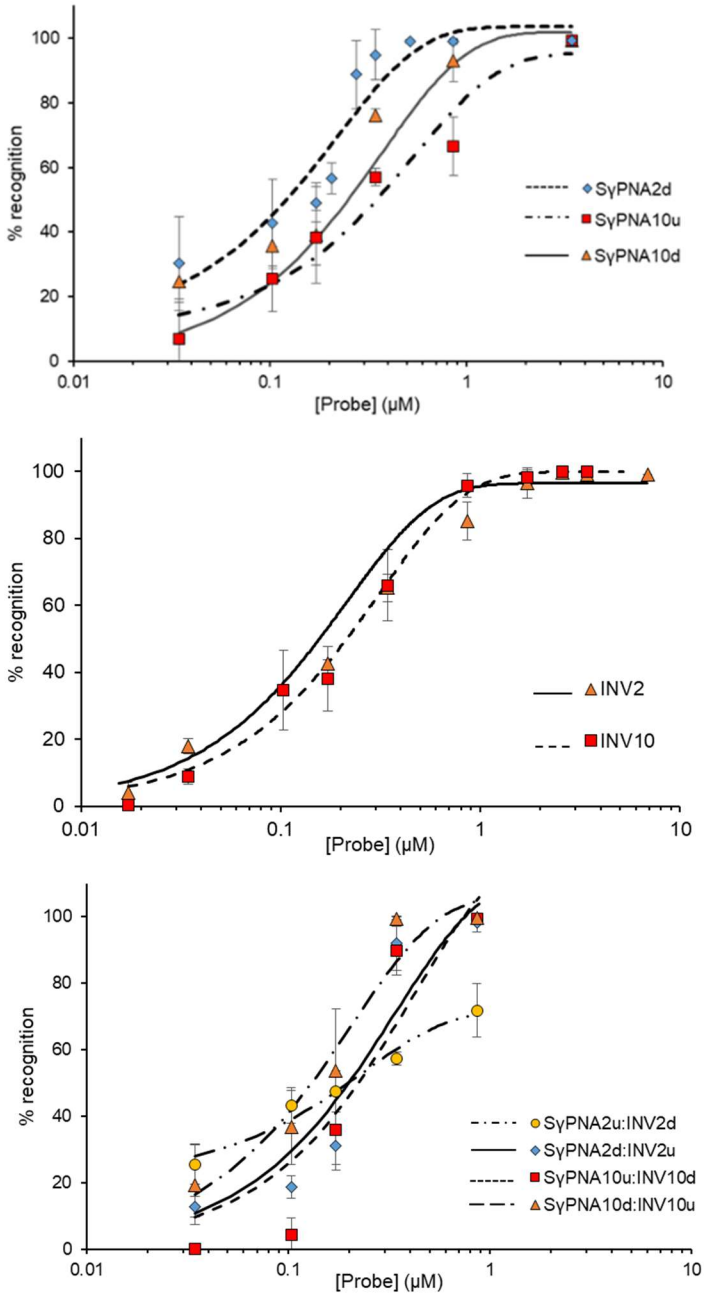


Figure S18. Dose-response curves for recognition of DNA hairpin targets **DH2** or **DH10** using the corresponding SγPNA, Invader or chimeric SγPNA-Invader probes. Incubation conditions are as described in Figure S11 except for variable probe concentrations and different incubation times, i.e., 2.5 h for SγPNAs and 15 h for Invader and chimeric SγPNA-Invader probes. Bars denote standard deviations. For corresponding electrophoretograms, see Figs. S13, S15 and S17.

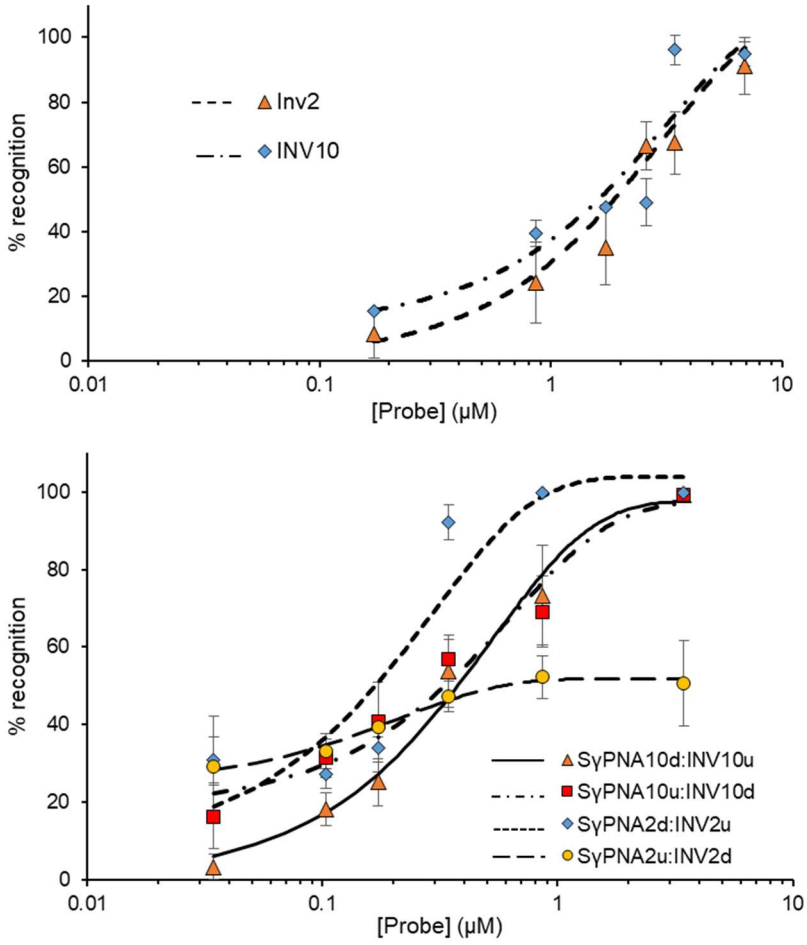


Figure S19. Dose-response curves for recognition of DNA hairpin targets **DH2** or **DH10** using the corresponding Invader and chimeric SγPNA-Invader probes. Bars denote standard deviations. Incubation conditions are as described in Figure S11 except for the use of variable probe concentrations and an incubation time of 2.5 h. For the corresponding electrophoretograms, see Figs. S14 and S16.

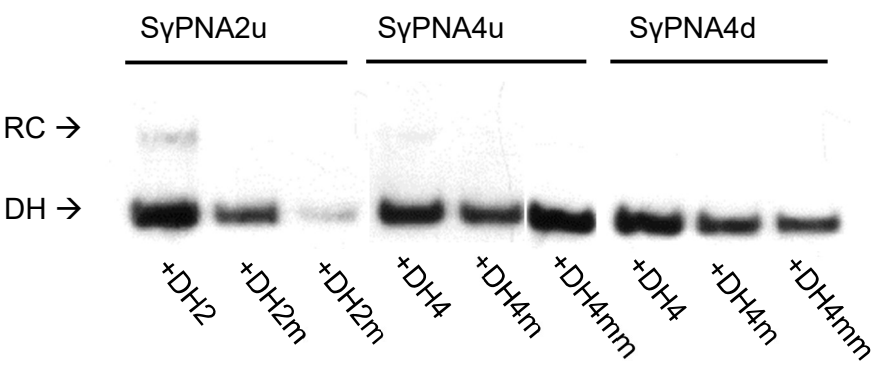


Figure S20. Binding specificity of SγPNAs. A 25-fold molar probe excess was incubated (2.5 h at 37 °C) with the corresponding DNA hairpins featuring stems of identical sequence or differing in sequence at one ("m") or two positions ("mm"), relative to the probes. For sequences of DNA hairpins, see Table S4. Incubation conditions are otherwise as described in Figure S11.

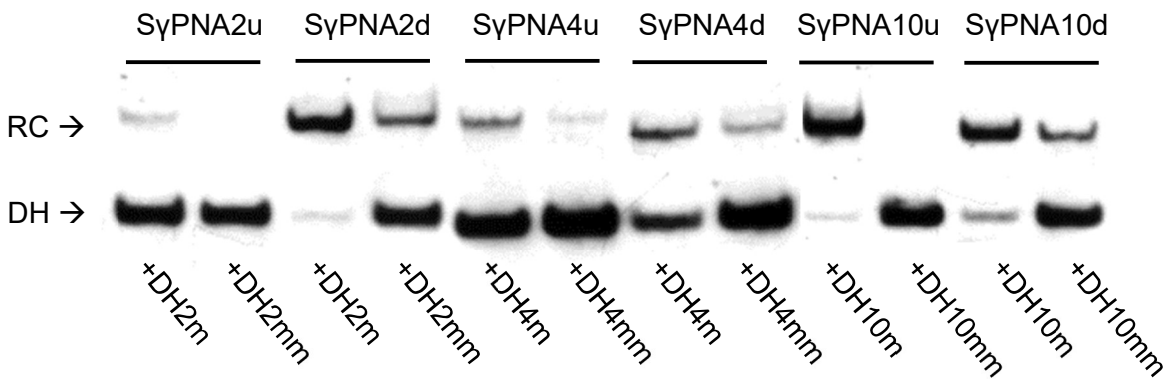


Figure S21. Binding specificity of SγPNAs. A 100-fold molar probe excess was incubated (2.5 h at 37 °C) with the corresponding DNA hairpins featuring stems differing in sequence at one ("m") or two positions ("mm"), relative to the probes. For sequences of DNA hairpins, see Table S4. Incubation conditions are otherwise as described in Figure S11.

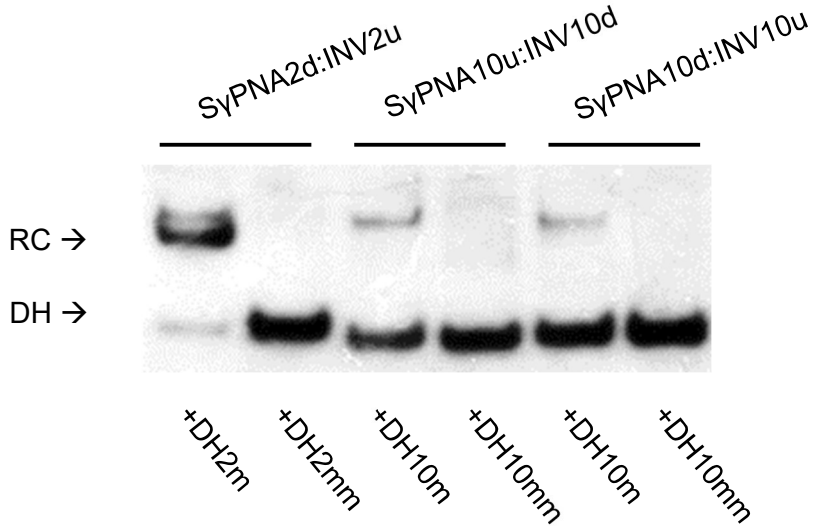


Figure S22. Binding specificity of chimeric SγPNA:Invader probes. A 100-fold molar probe excess was incubated (15 h at 37 °C) with corresponding DNA hairpins featuring stems differing in sequence at one ("m") or two positions ("mm"), relative to the probes. For sequences of DNA hairpins, see Table S4. Incubation conditions are otherwise as described in Figure S11.

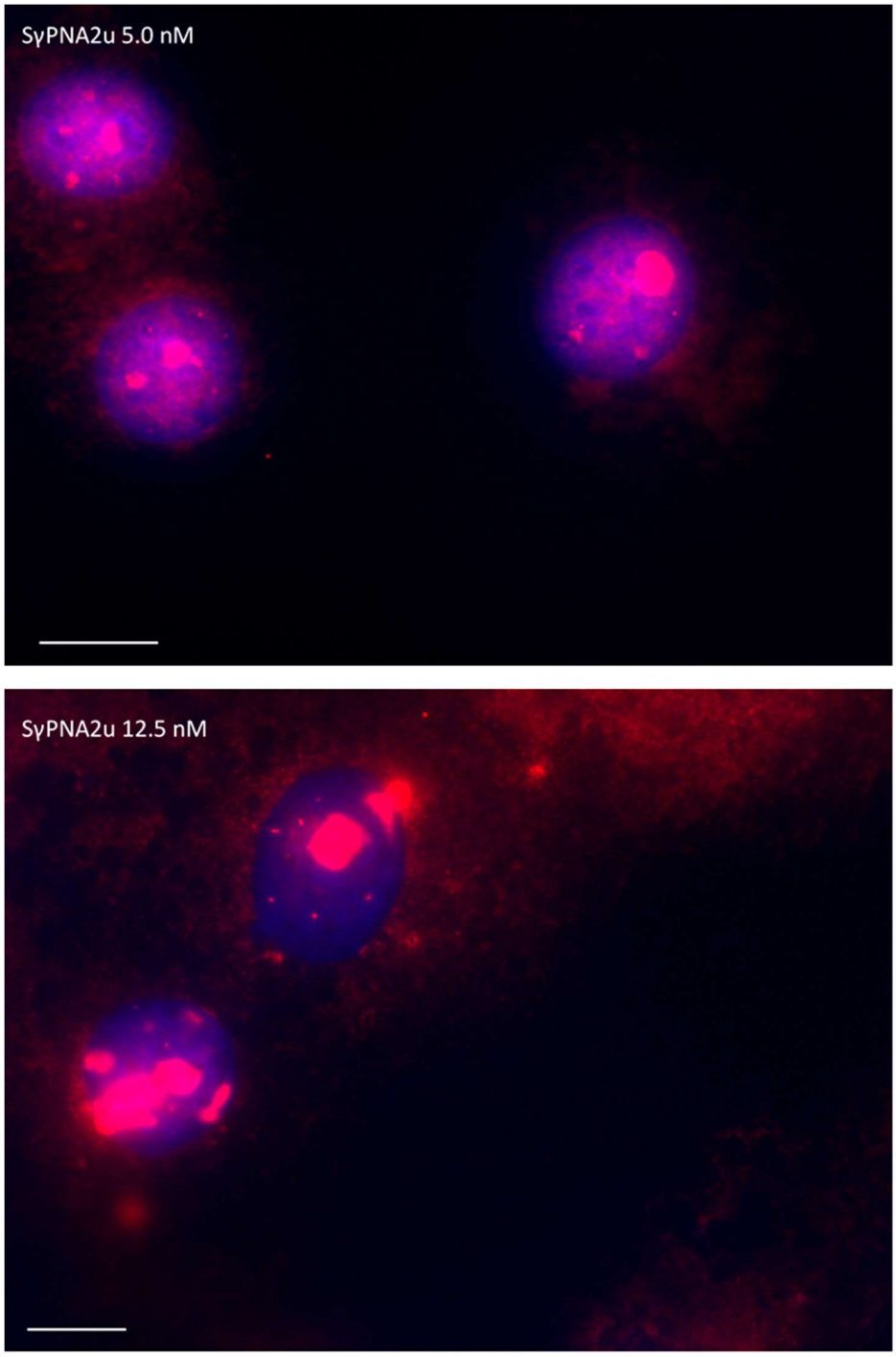


Figure S23. Representative fluorescence microscopy images of **SγPNA2u** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

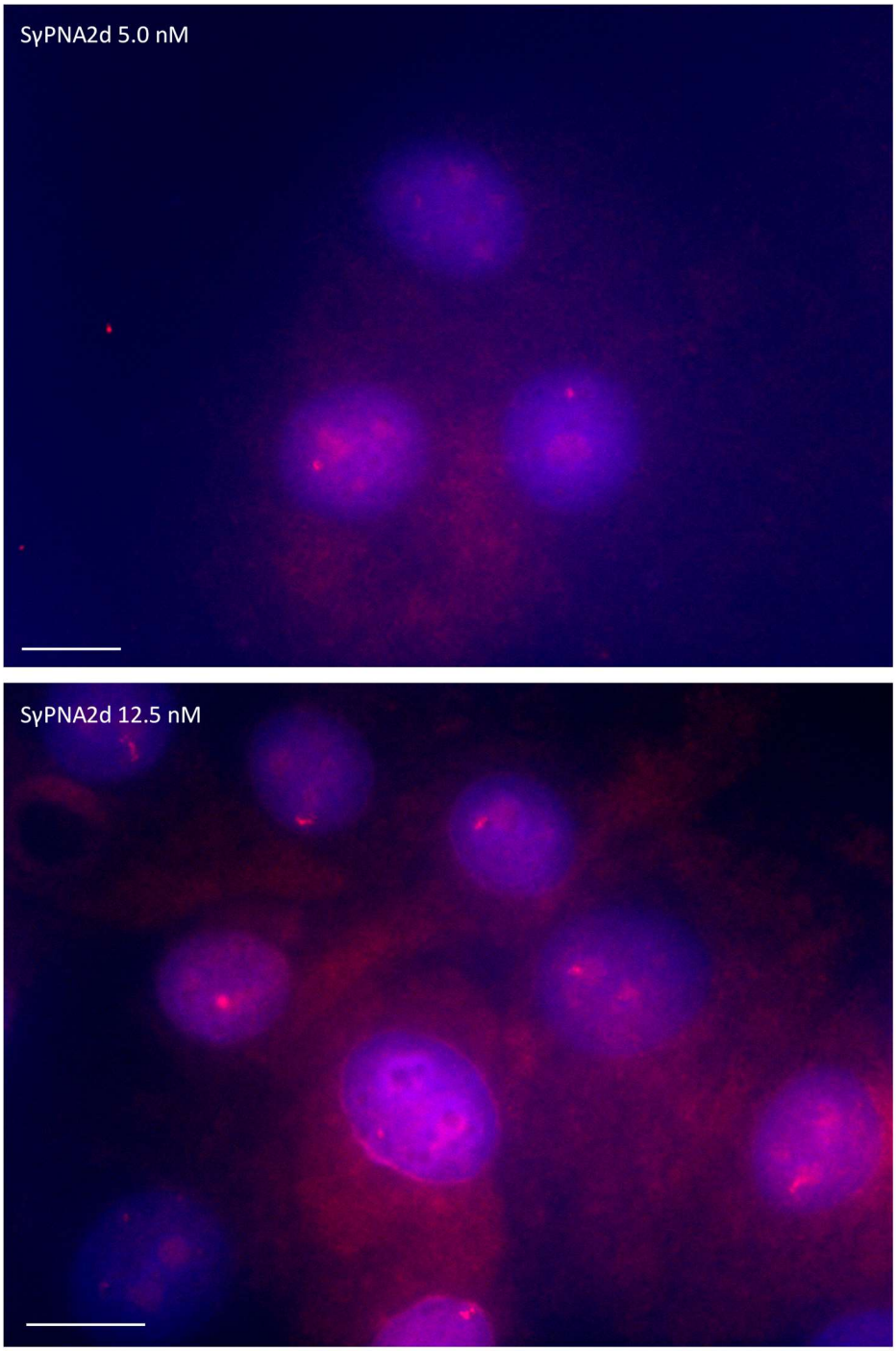


Figure S24. Representative fluorescence microscopy images of **SγPNA2d** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

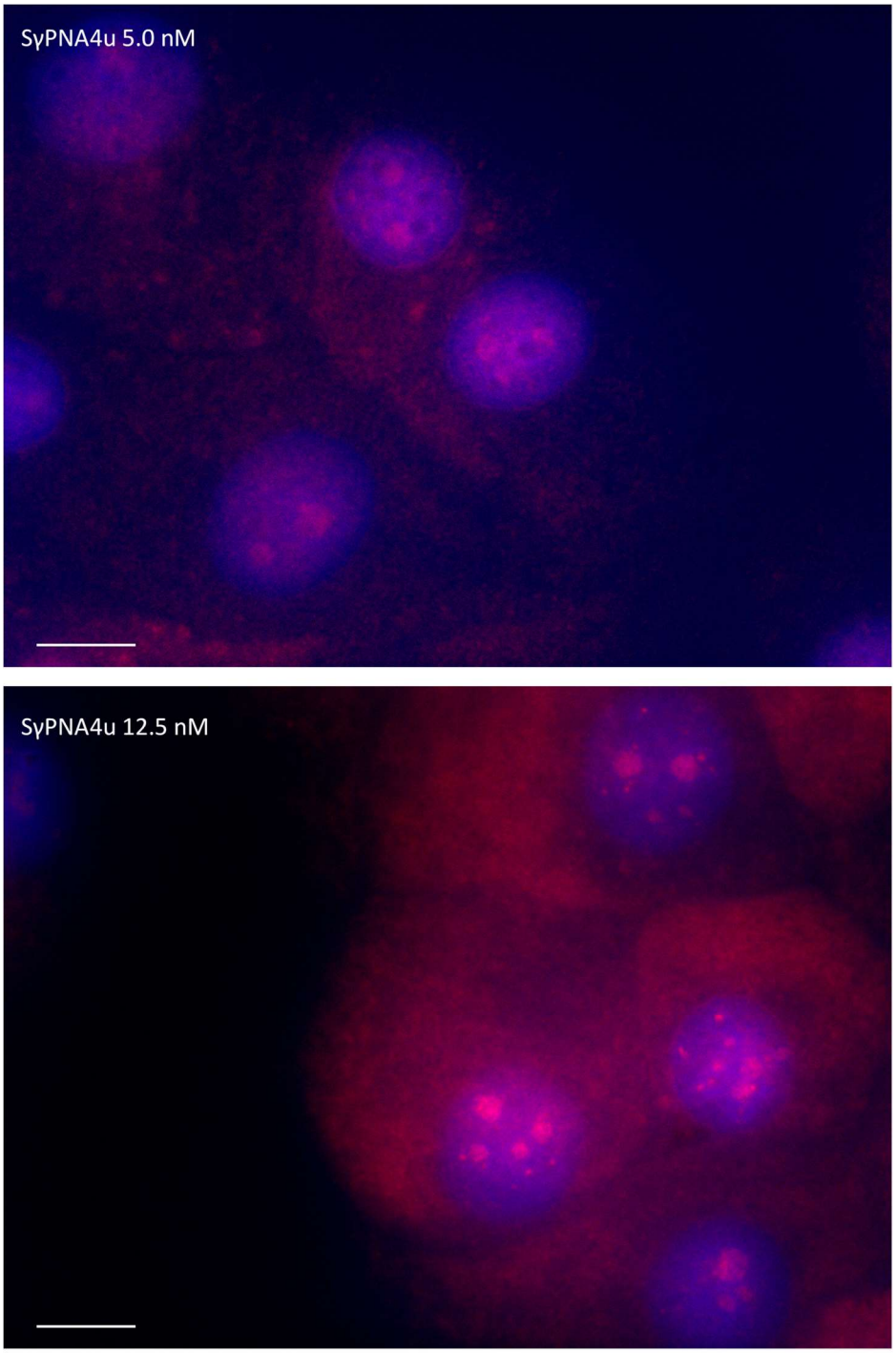


Figure S25. Representative fluorescence microscopy images of **SγPNA4u** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 µm.

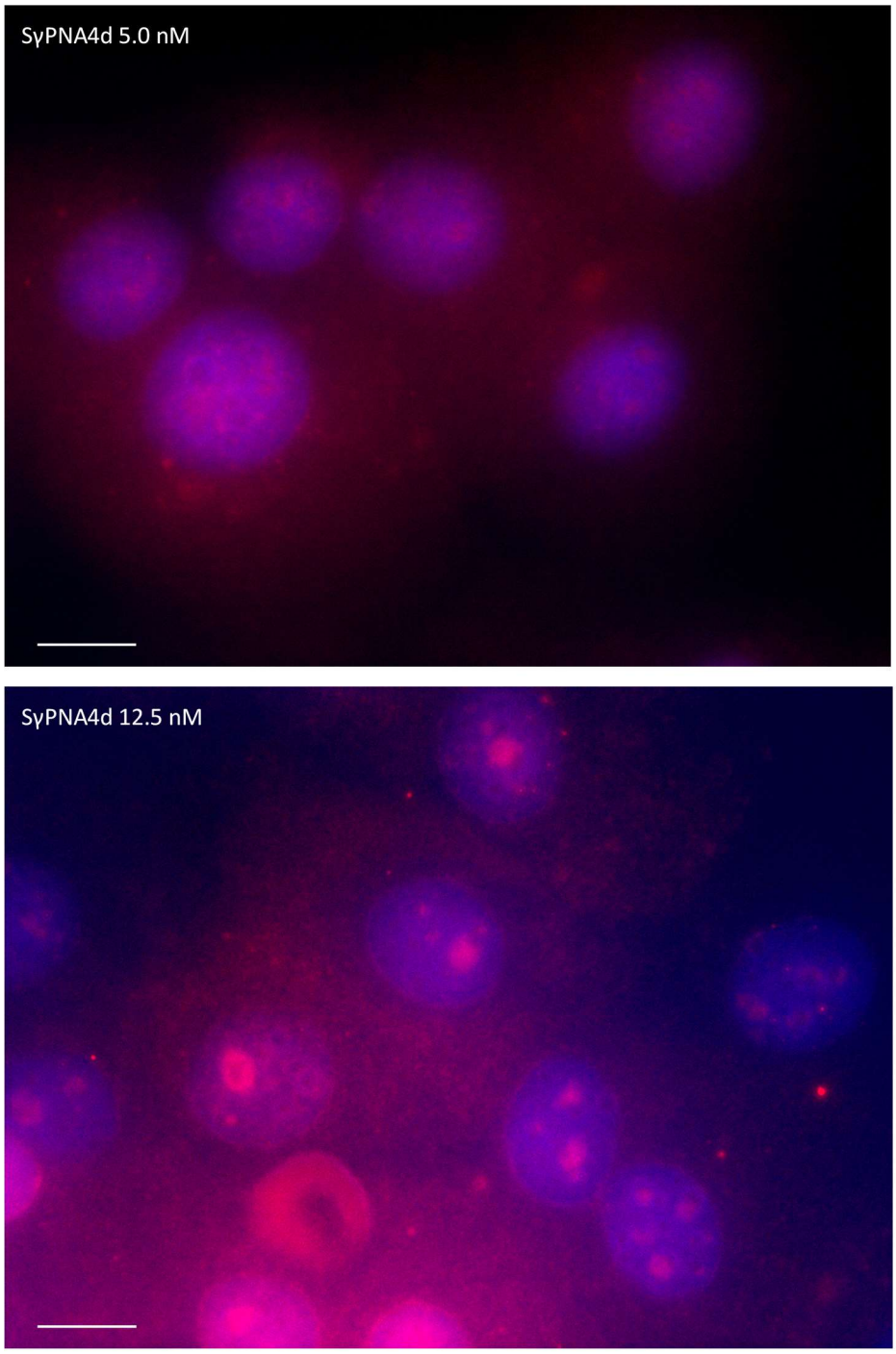


Figure S26. Representative fluorescence microscopy images of **SγPNA4d** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 µm.

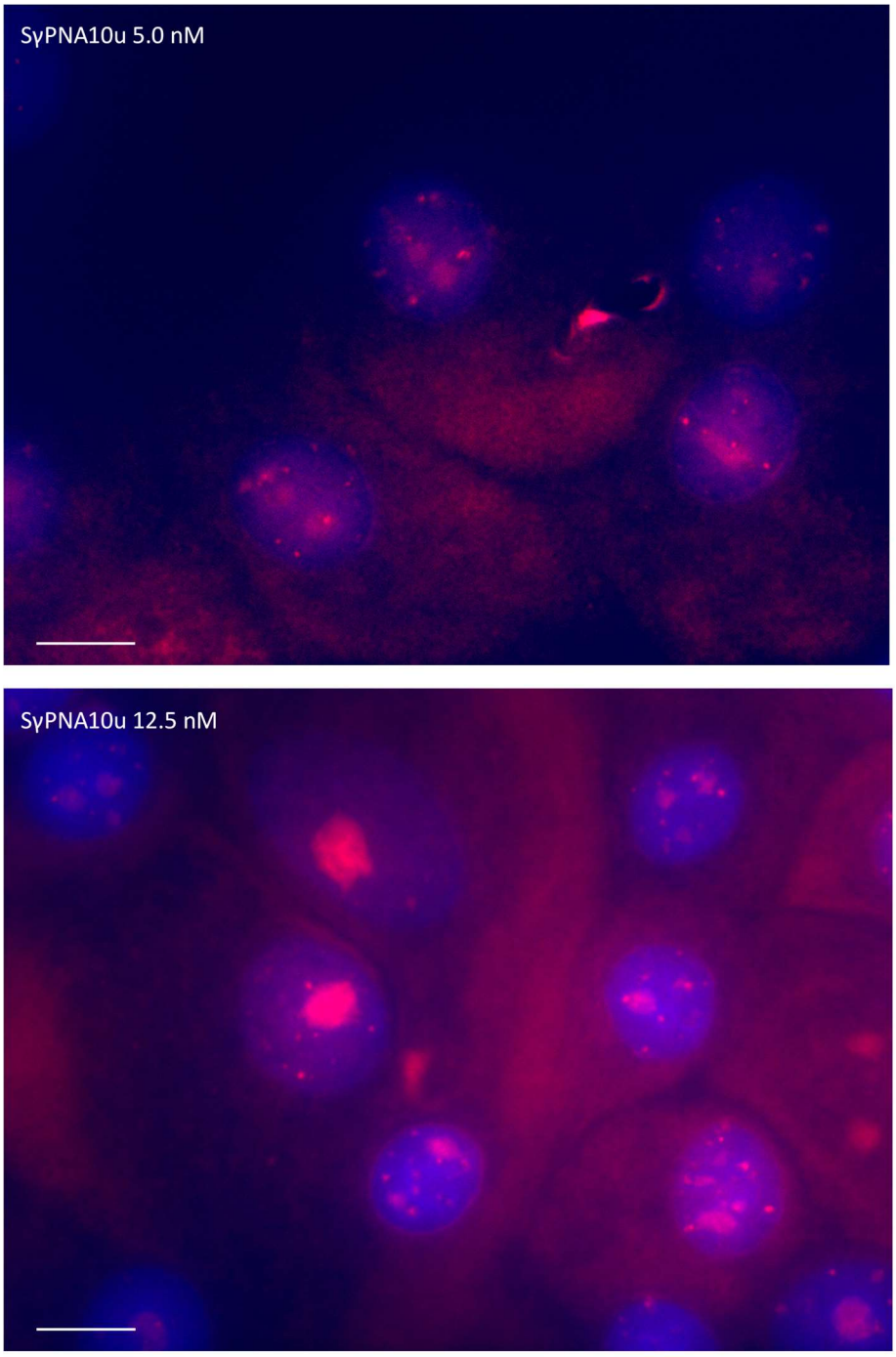


Figure S27. Representative fluorescence microscopy images of **SγPNA10u** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 µm.

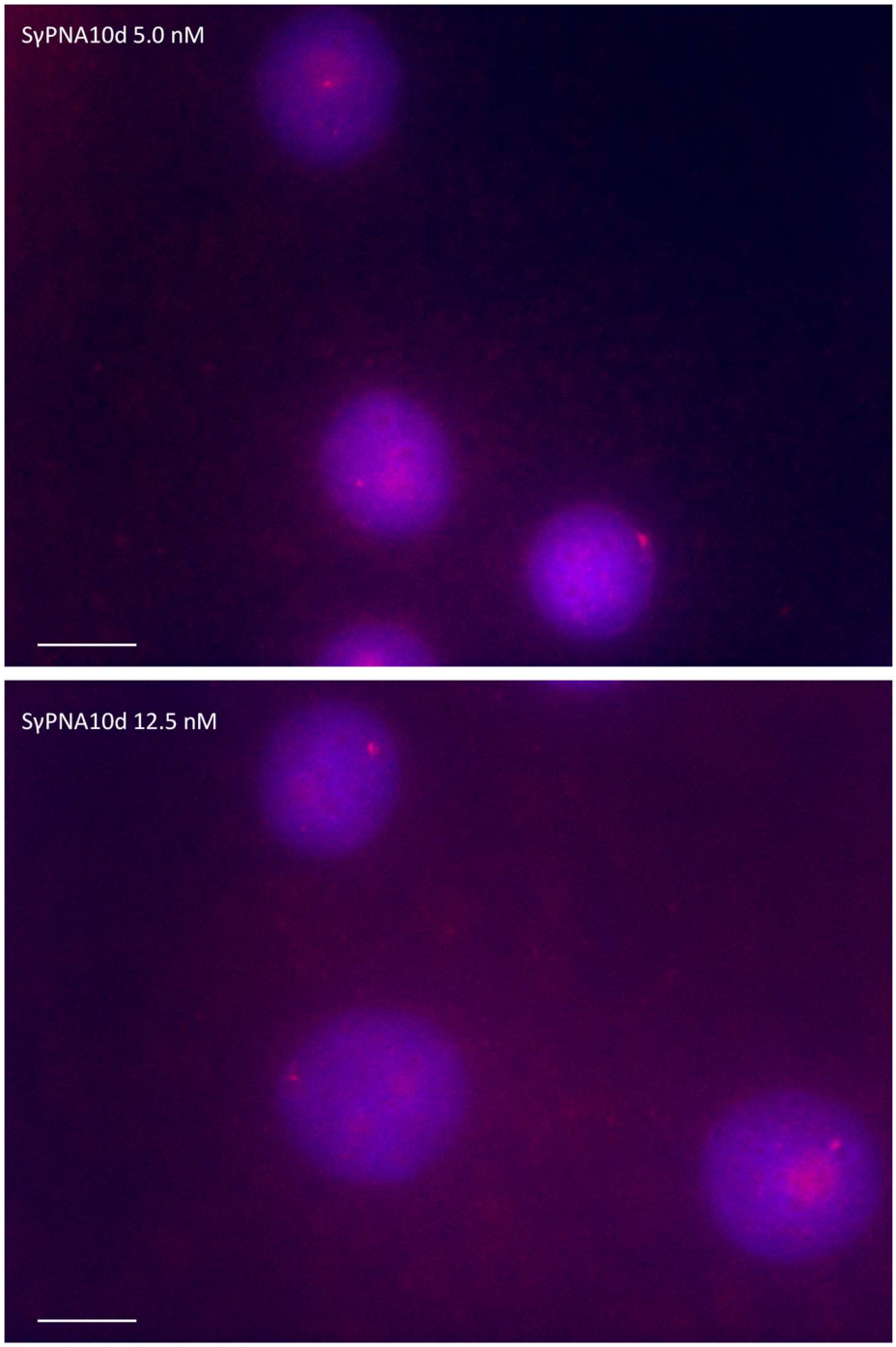


Figure S28. Representative fluorescence microscopy images of **SγPNA10d** incubated (5.0 or 12.5 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

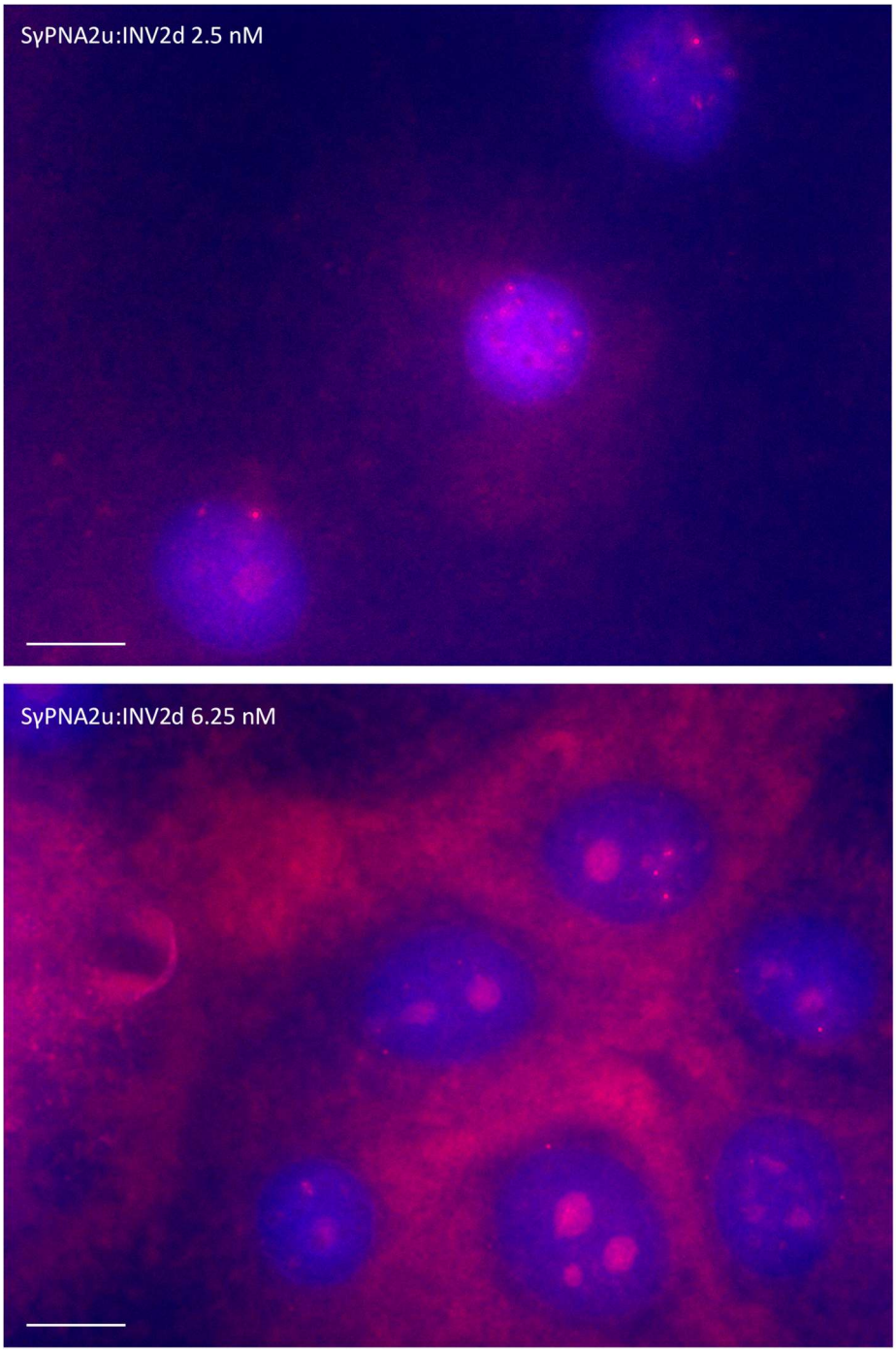


Figure S29. Representative fluorescence microscopy images of **SγPNA2u**:**INV2d** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

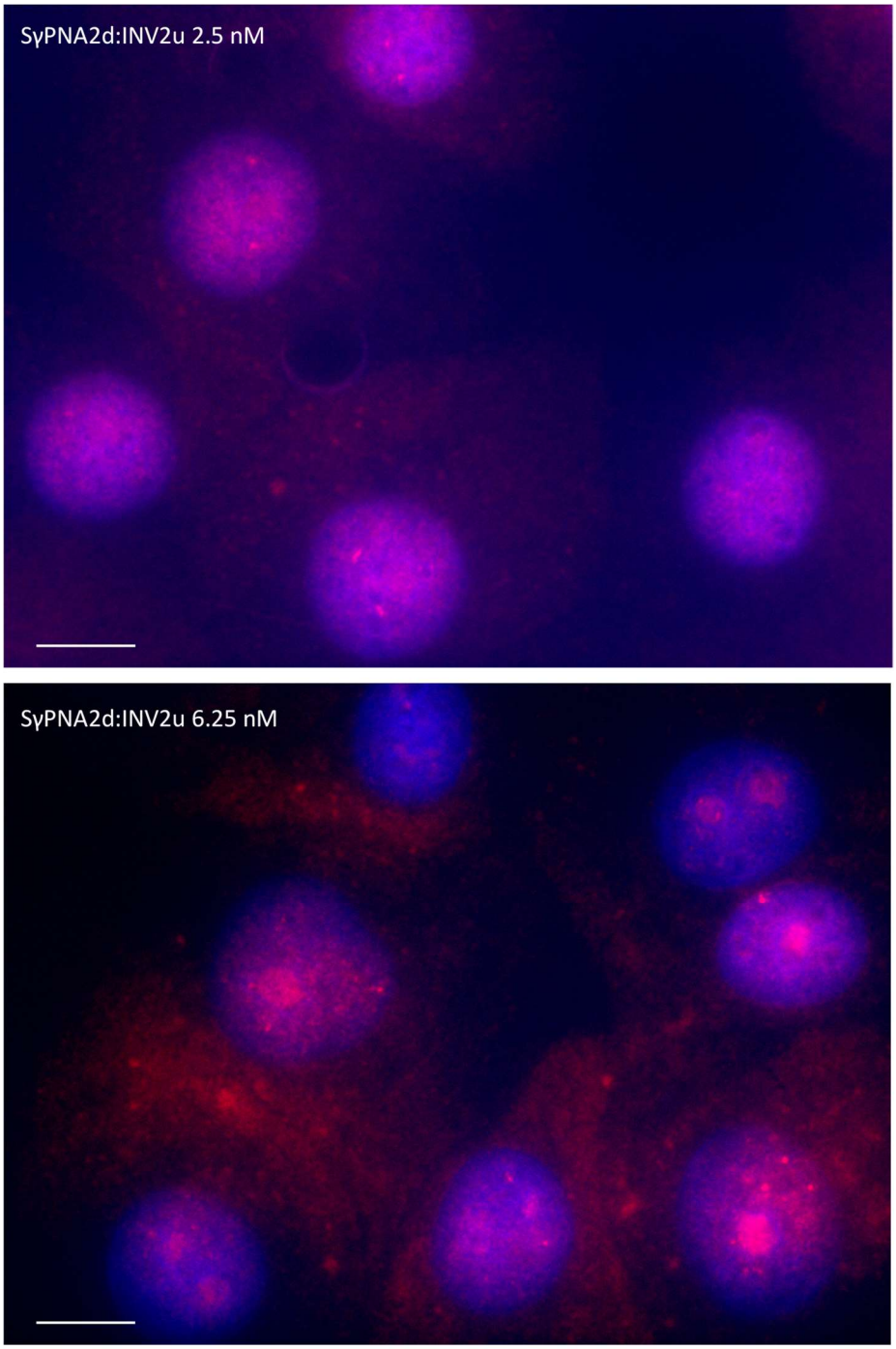


Figure S30. Representative fluorescence microscopy images of **SγPNA2d**:**INV2u** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

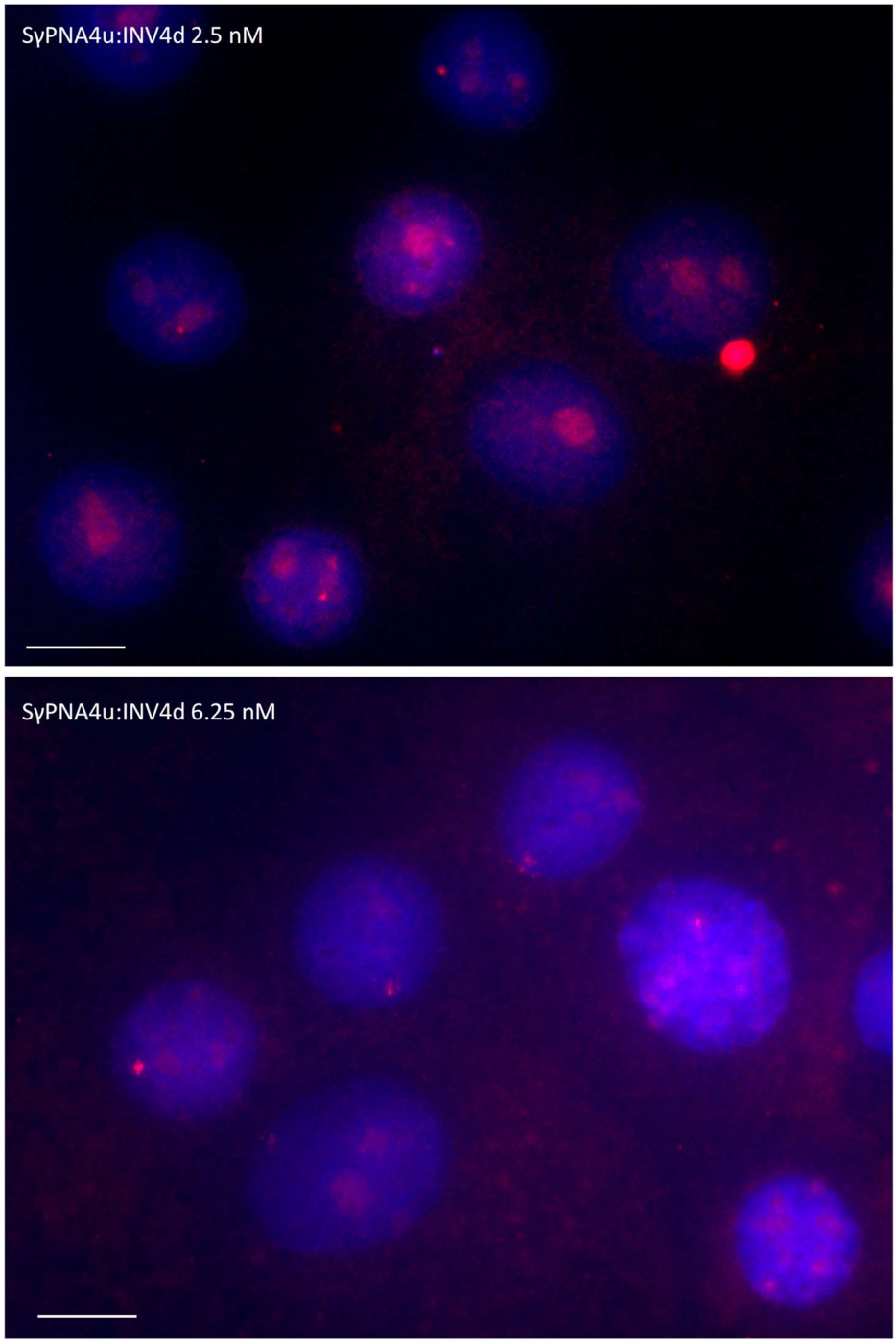


Figure S31. Representative fluorescence microscopy images of **SγPNA4u**:**INV4d** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

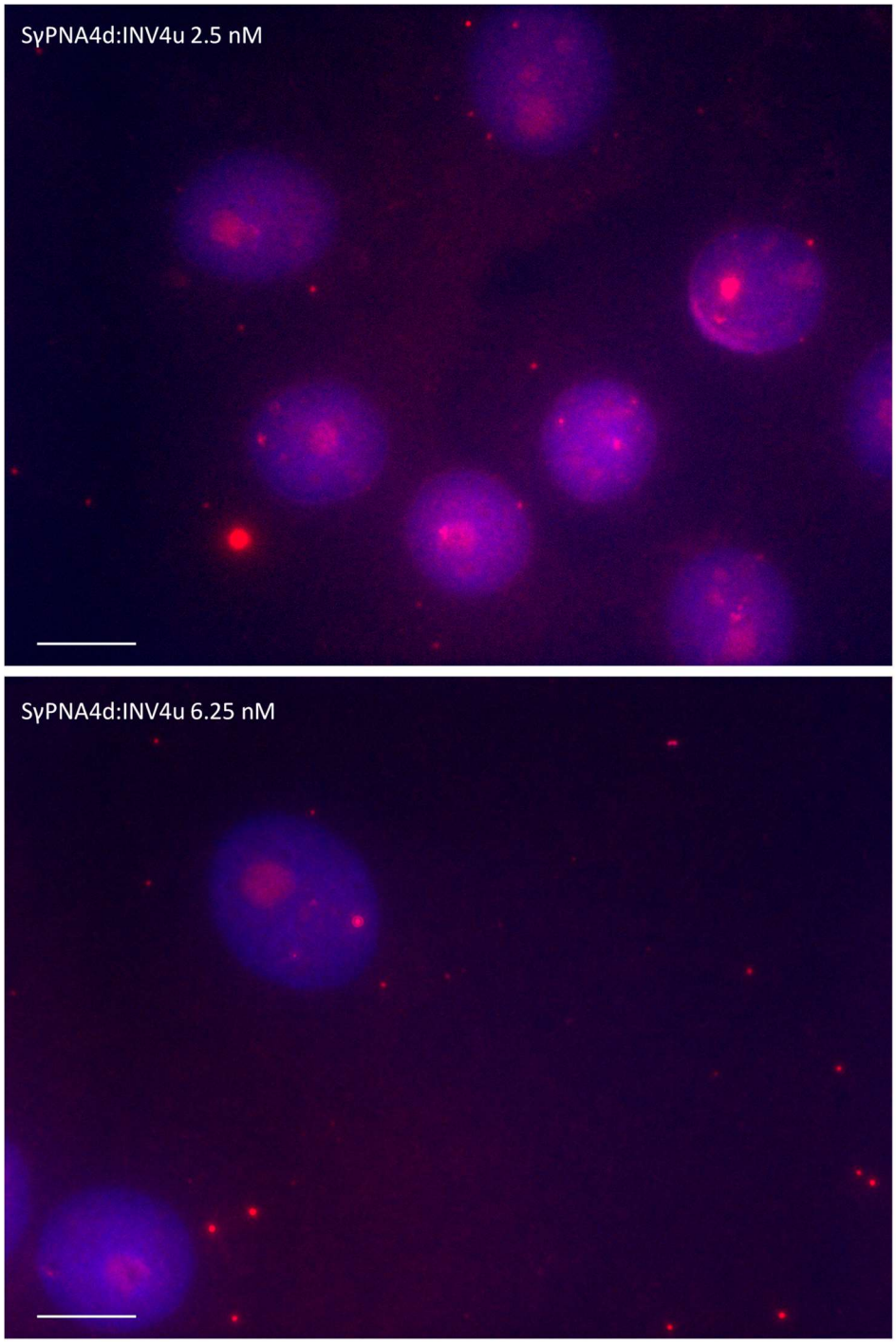


Figure S32. Representative fluorescence microscopy images of **SγPNA4d**:**INV4u** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

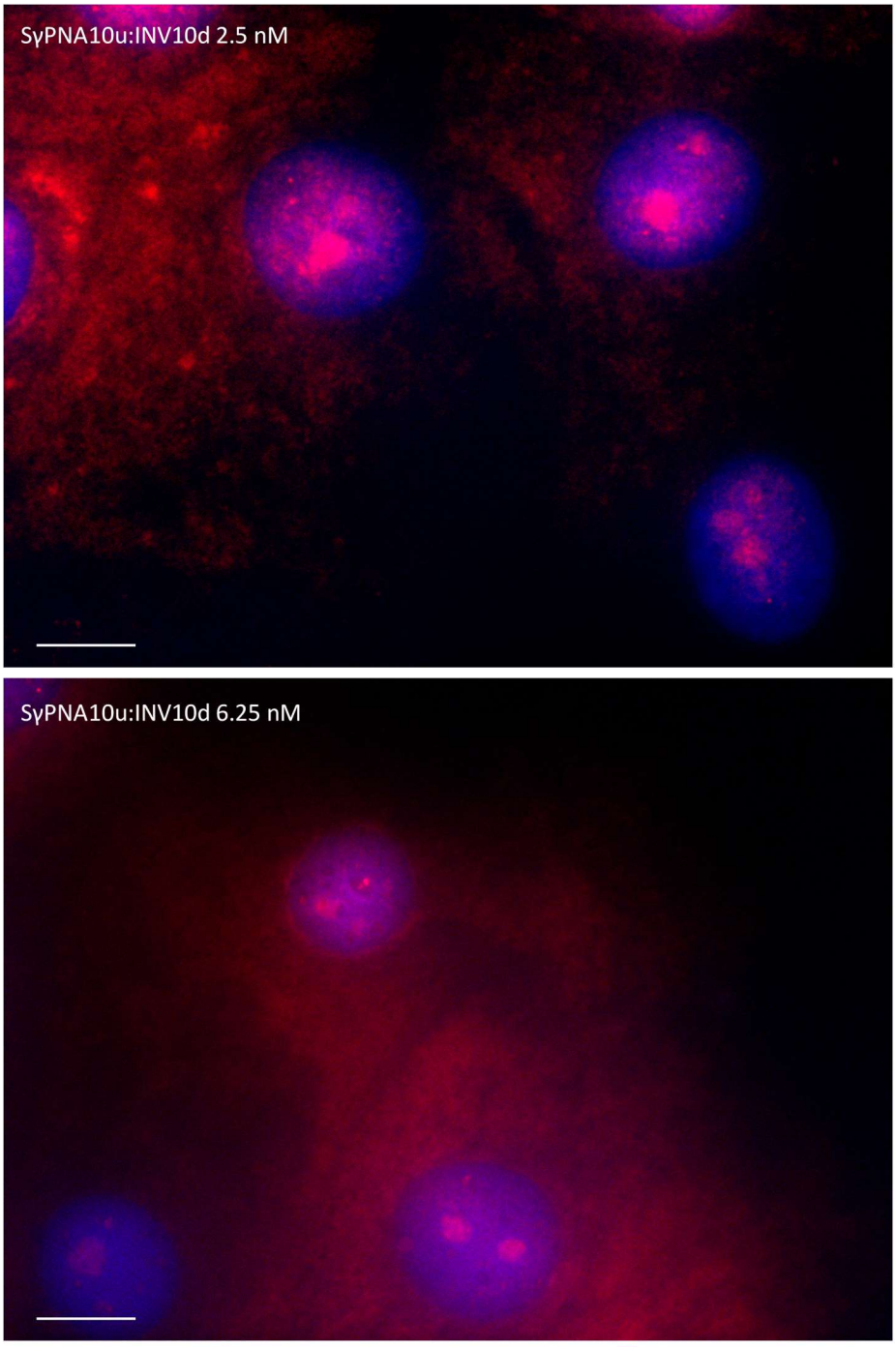


Figure S33. Representative fluorescence microscopy images of **SγPNA10u**:**INV10d** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 µm.

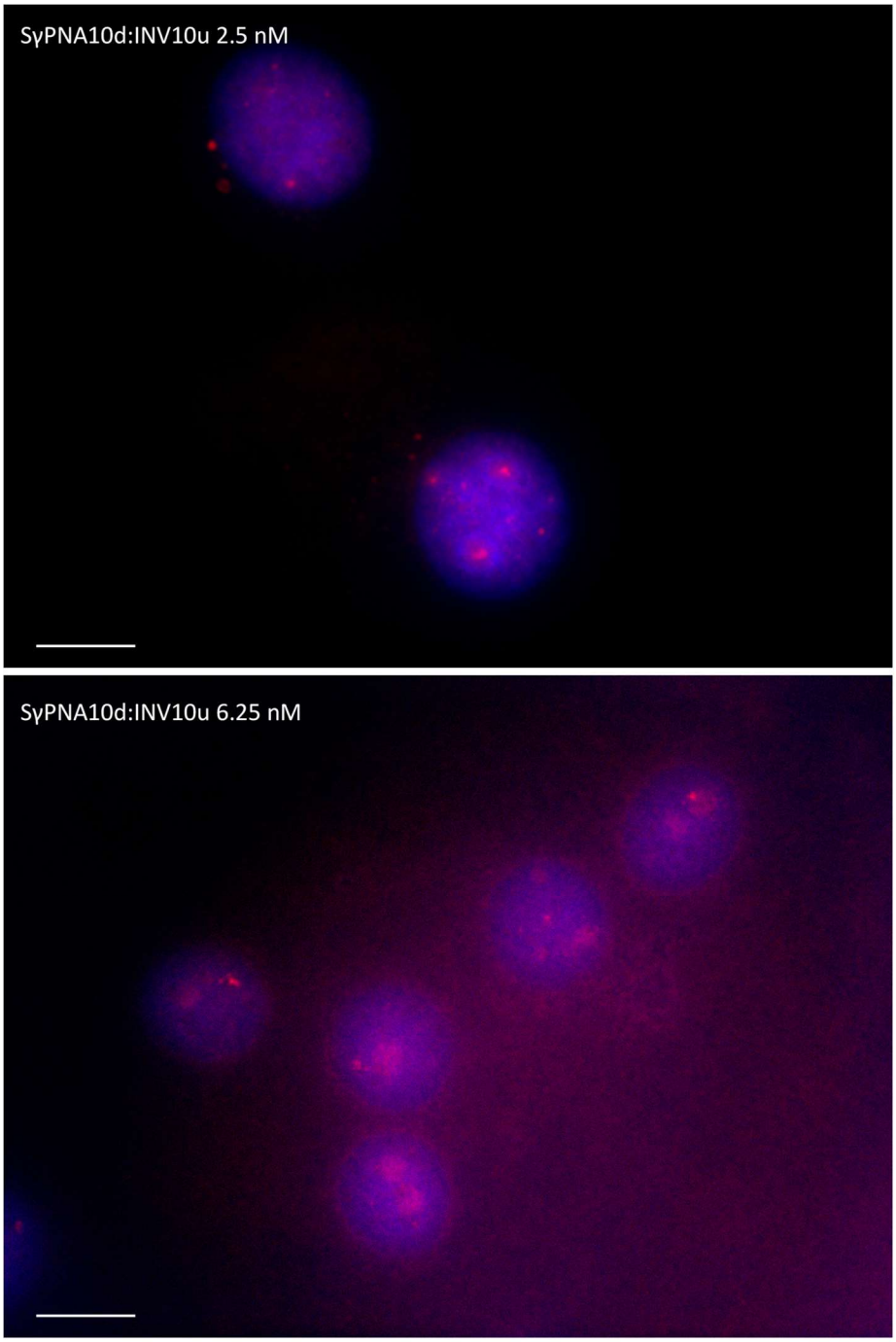


Figure S34. Representative fluorescence microscopy images of **SγPNA10d**:**INV10u** incubated (2.5 or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a male bovine kidney cell line (MDBK). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 10 μm.

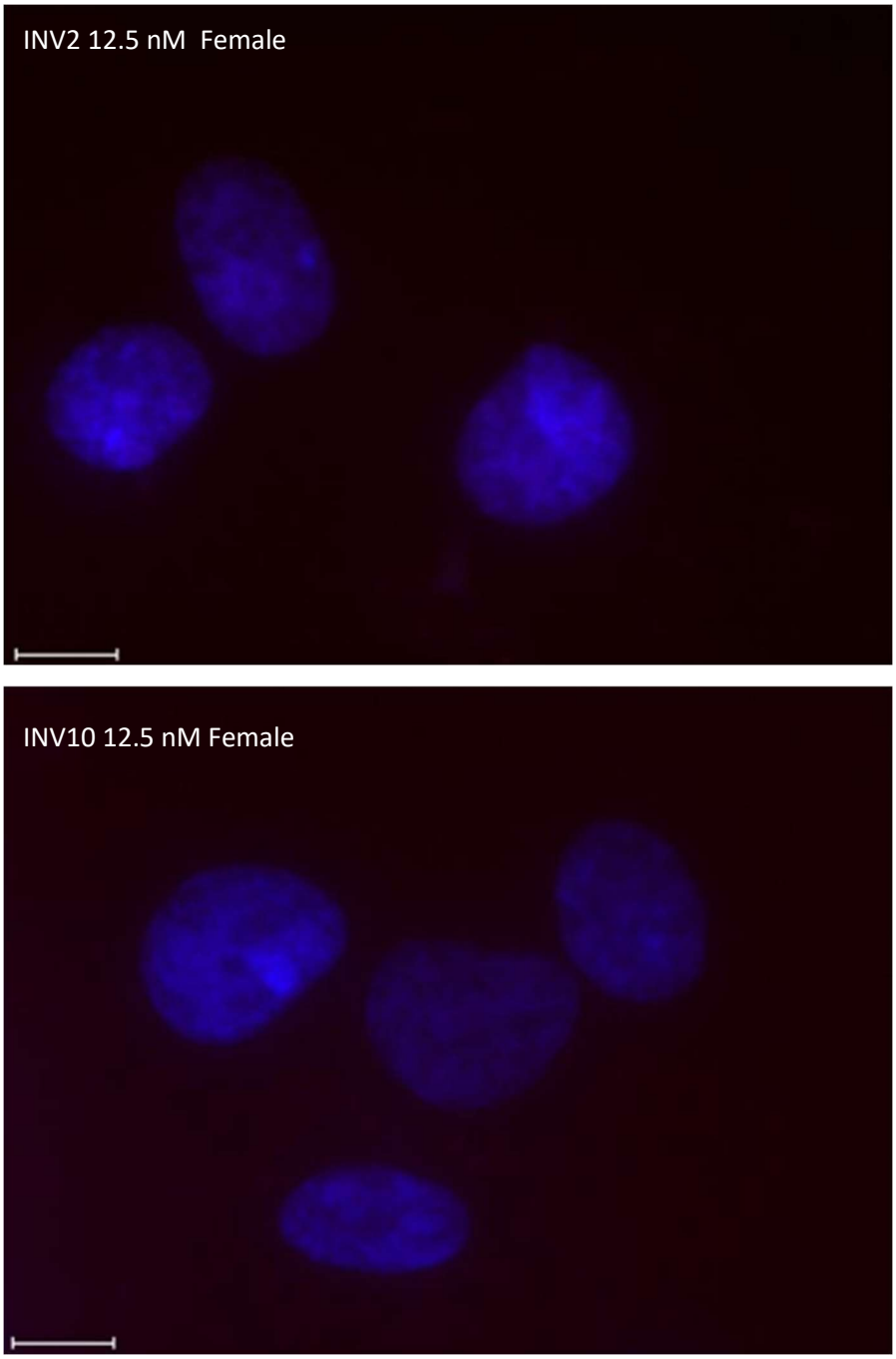


Figure S35. Representative fluorescence microscopy images of **INV2** or **INV10** incubated (12.5 nM, 5 min, 80 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a female bovine endothelial cell line (CPAE). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 16 μm.

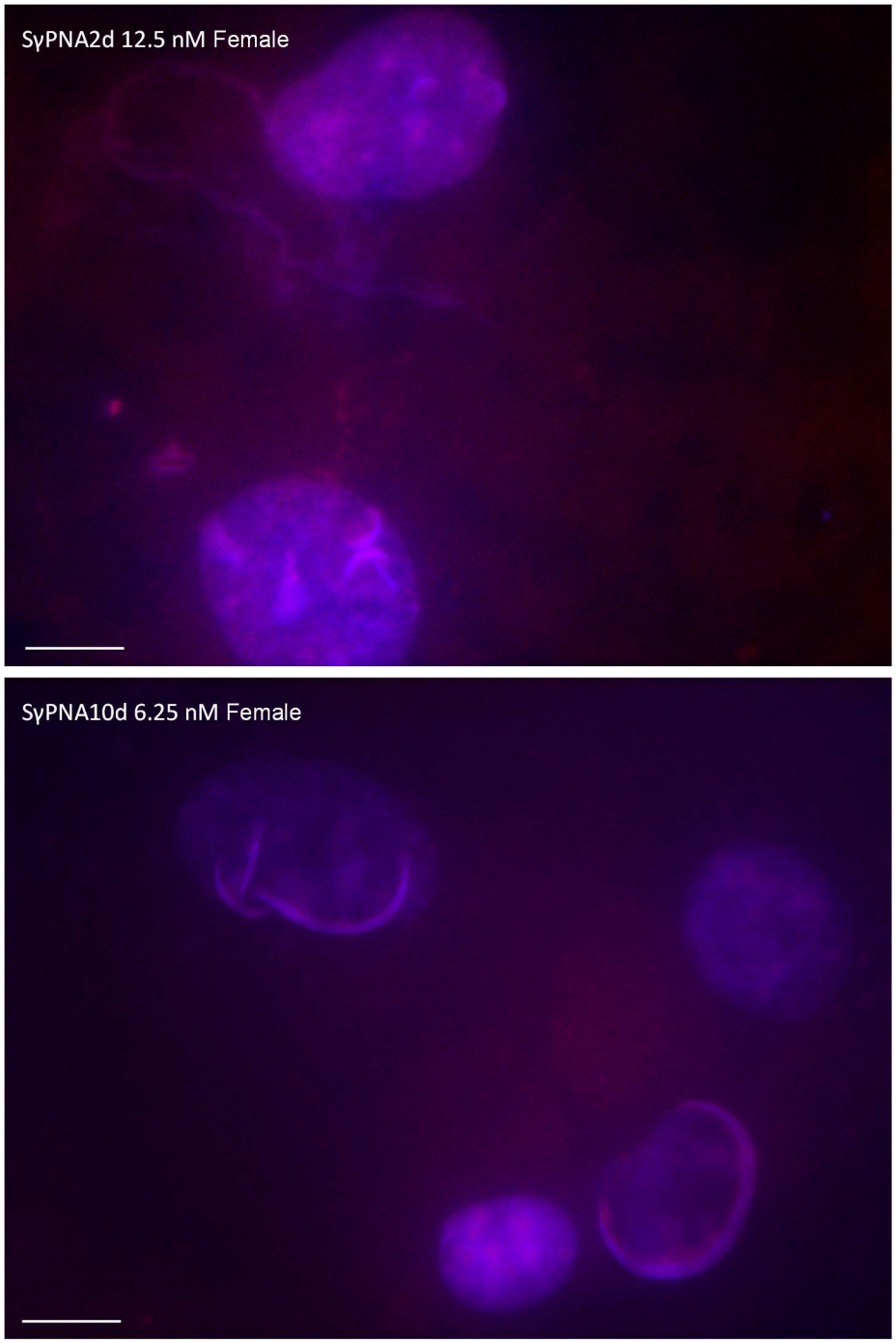


Figure S36. Representative fluorescence microscopy images of **SγPNA2d** or **SγPNA10d** incubated (12.5 nM or 6.25 nM, 3 h, 37 °C, Tris-Cl/EDTA buffer, pH 8.0) with fixed nuclei from a female bovine endothelial cell line (CPAE). Images are overlays of Cy3/TAMRA (red) and DAPI channels (blue). Scale bar denotes 16 μm.

Table S5. Proportion of nuclei presenting either a single or multiple punctate signals per nucleus in nd-FISH experiments when using low or high probe concentration.[a]

	Low Probe Concentration		High Probe Concentration	
	Proportion of nuclei with		Proportion of nuclei with	
Probe	1 Signal	≥ 2 Signals	1 Signal	≥ 2 Signals
SγPNA2u	10%	80%	HB	HB
SγPNA2d	70%	10%	80%	10%
SγPNA4u	10%	0%	10%	40%
SγPNA4d	0%	0%	HB[b]	HB[b]
SγPNA10u	0%	100%	0%	100%
SγPNA10d	70%	10%	30%	70%
SγPNA2u:**INV2d**	40%	50%	10%	80%
SγPNA2d:**INV2u**	50%	30%	20%	70%
SγPNA4u:**INV4d**	60%	10%	80%	0%
SγPNA4d:**INV4u**	HB	HB	HB[b]	HB[b]
SγPNA10u:**INV10d**	30%	40%	40%	30%
SγPNA10d:**INV10u**	40%	10%	60%	10%
INV2	85%	<15%	ND	ND
INV4	90%	<10%	ND	ND
INV10	90%	<10%	ND	ND

[a] HB designates high background rendering it impossible to have a meaningfully signal count. SγPNA, chimeric and Invader probes were used at concentrations of 5.0/12.5 nM, 2.5/6.25 nM, and 12.5 nM (**INV4** ~3.125 nM), respectively. ND = not determined.

[b]The use of **SγPNA4d or SγPNA4d:INV4u** results in the formation of many bright punctate dots scattered across the slide, indicative of poor solubility and aggregation.

SUPPLEMENTARY REFERENCES

S1) D. C. Guenther, G. H. Anderson, S. Karmakar, B. A. Anderson, B. A. Didion, W. Guo, J. P. Verstegen and P. J. Hrdlicka, *Chem. Sci.,* 2015, **6**, 5006-5015.

S2) J. Perret, Y.-C. Shia, R. Fries, G. Vassart and M. Georges, *Genomics*, 1990, **6**, 482-490.