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Supplemental information

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CRISPR-based DNA and RNA detection with liquid-liquid phase separation

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Figure S1

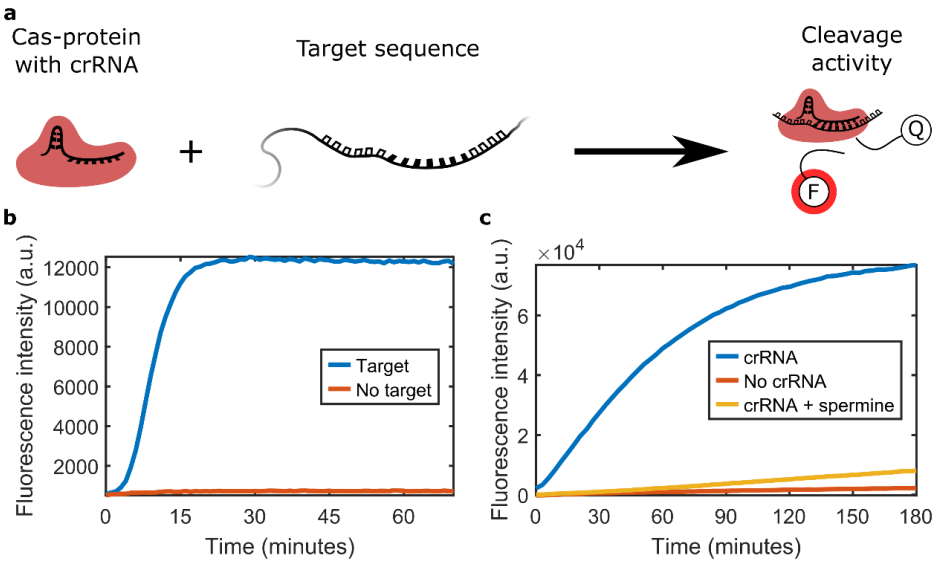

Figure S1: Fluorescence readout of Cas12a and Cas13a cleavage activity. (a) RNA guided Cas-proteins Cas12a and Cas13a target DNA and RNA (respectively) and are activated by a target sequence complementary to a bound crRNA. When active, the enzymes indiscriminately cleave single stranded DNA (Cas12a) and RNA (Cas13a). Their nuclease activity can be measured by fluorescence arising from a DNA or RNA-linked fluorophore-quencher pair. Upon cleavage of the DNA/RNA link between fluorophore (F) and quencher (Q) the fluorophore becomes fluorescent. **(b)** Measurement of Cas12a activity using fluorescence (DNase Alert, IDT). The curves show nuclease activity in the presence (blue) and absence (orange) of DNA target. Fluorescence measurements were done (λ_{ex} = 534 nm, λ_{em} = 585 nm) in a Tecan Infinite M200 PRO plate-reader. Cas12a was loaded with crRNA for a minimum of 15 minutes in 1x C12RB before the measurements and assay conditions were as in the turbidity assay for DNA detection (see Materials and Methods). **(c)** Fluorescence-based assay confirming that in the presence of target, Cas13a activity depends on the presence of crRNA. The curves represent fluorescence measurements with crRNA (blue), without crRNA (orange), and with both crRNA and 1.0 wt% spermine (yellow). The assay consisted of 50 nM Cas13a mixed with reaction buffer (1x C13RB), 0.3 ng/µL target RNA, 0.3 ng/µL crRNA, and 125 nM RNase Alert (IDT). Data was recorded every three minutes using a Synergy H1 (BioTek) fluorimeter (λ_{ex} = 490 nm, λ_{em} = 520 nm). All measurements in the figure were done at 37 °C and in triplicate.

Figure S2

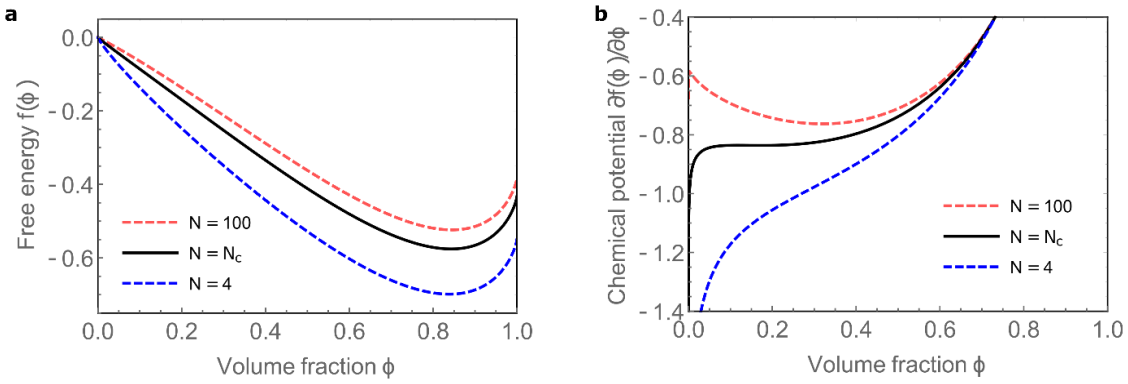

Figure S2: Free energy and chemical potential of a symmetric solution of charged polymers. (a) Free energy as a function of volume fraction ϕ for effective polymer chain lengths N lower than, equal to and larger than the critical length ($N_c \approx 12$). The parameters for this plot are the same as for the phase diagram in **Fig. 1a** ($\chi = 0.5$, $\sigma = 0.22$, $\alpha \approx 3.7$). **(b)** The chemical potential is the derivative of the free energy as displayed in panel a. For effective polymer chain lengths larger than the critical length, a local minimum appears in the chemical potential. For effective polymer chain lengths lower than the critical length, there is no local minimum.

Figure S3

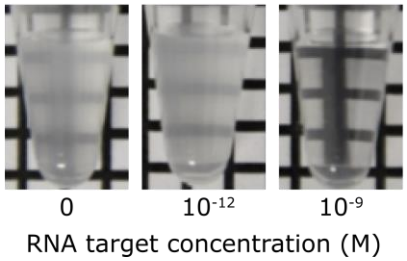

Figure S3: The RNA detection limit with Cas13a lies in the nanomolar range. The tubes contained ~37 nM Cas13a, 0.11 wt% poly(U), 45 nM crRNA, C13RB buffer and varying concentrations of RNA target (molar concentrations indicated below tubes). After 3 hours of incubation at 37 °C, spermine was added to a final concentration of 1.0 wt%. The photographs were converted to grayscale and a colour blindness filter was applied (rod monochromacy).

Length dependent contribution to the free energy in the case of asymmetric polymer lengths.

Here, we briefly investigate the length dependent part of the free energy as given in Eq. 1 in the main text, $f^N(\phi) = \sum_{i=1}^{n} \frac{\phi_i}{N_i} \ln(\phi_i)$. We obtain the following formula by explicitly carrying out the sum and assuming equal volume fractions $\phi_1 = \phi_2 = \frac{\phi}{2}$,

$$\left(\frac{1}{2N_1} + \frac{1}{2N_2}\right) \phi \ln\left(\frac{\phi}{2}\right),$$

and then

$$\left(\frac{N_1 + N_2}{2N_1N_2}\right) \phi \ln\left(\frac{\phi}{2}\right).$$

From here it is possible to identify the interesting limit cases, where $N_1 = N_2 = N$ leads to the expression as in Eq. 2 in the main text. To illustrate what happens when one of the two species has a constant length, we considered the case of $N_2 = 60$ and N_1 to be variable. See Fig. S4 for the resulting phase diagram and a comparison to the case of symmetric length.

Figure S4

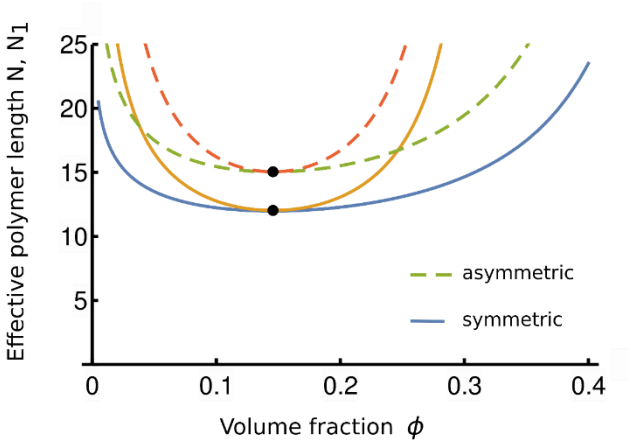

Figure S4: Comparison phase diagrams symmetry in length. Comparison between the phase diagram of the fully symmetric model (solid lines, as Fig. 1a) and the situation where the length of one polymer species is varied (N_1) and the second species' length is kept constant ($N_2 = 60$, dashed lines). There is a quantitative difference between the two cases. This shows that length dependent LLPS does not require a fully symmetric system.

Figure S5

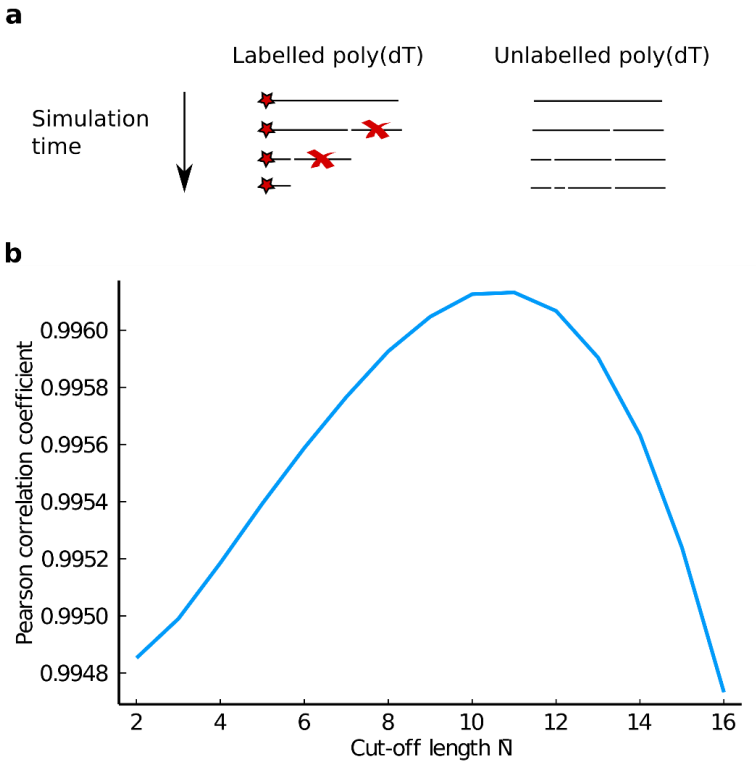

Figure S5: Simulation and fitting routine. (a) Illustration of the two different reaction schemes employed for labelled and unlabelled poly(dT) in the stochastic simulations. **(b)** An optimal cut-off length was found to be $\overline{N} = 10$, at which the simulation data represented the experimental data optimally. To compare the simulation with experimental data we first fitted the experimental data with a single exponential (Ae^{-bt}, which resulted in the parameters $A = 95.1 \pm 3.2$, and $b = 0.101 \pm 0.012$ using Julia LsqFit). Then the Pearson correlation coefficient was calculated between the best fit exponential and the simulation data to which we applied the cut-off length $\overline{N}$. Below the cut-off length the Cy5 labelled poly(dT) did not contribute to the simulated polymer mass shown in **Fig. 3b**.

Protein expression and purification.

Cas12a purification. The plasmid pET21a encoding Cas12a (1) (AsCpf1) was transformed into Rosetta *E. coli* cells, which were grown on agar plate with 100 µg/mL ampicillin and 25 µg/mL chloramphenicol. Cells were grown in LB with addition of 100 µg/mL ampicillin and 25 µg/mL chloramphenicol, to an OD_{600} of 0.6 (37°C, 180 rpm). Cultures were put on ice for 30 minutes, and Cas12a production was induced by the addition of 1 mM IPTG. Cultures were left for 16 hours at 18 °C with rotation (180 rpm). Subsequent steps were done at 4°C. Cultures were spun down at 7000 g for 10 minutes, and the supernatant was discarded. Pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, 500 mM NaCl, 1 mM DTT, 10 mM imidazole, pH 8.0) with addition of 1 protease inhibitor tablet per 50 mL of lysis buffer. Cells were disrupted in a French Pressure Cell at 1000 bar twice and spun down at 16,000 g for 30 min. Supernatant was filtered with a 0.45 µm pore size, applied to His-Select Affinity resin and incubated for 30 minutes. Resin was spun down at 2000 g for 1 minute, and the supernatant was discarded. The resin was loaded onto a gravity column and washed twice with wash buffer (50 mM NaH_2PO_4, 500 mM NaCl, 1 mM DTT, 30 mM imidazole, pH 8.0). Cas12a was eluted with elution buffer (50 mM NaH_2PO_4, 500 mM NaCl, 1 mM DTT, 250 mM imidazole, pH 8.0). The recovered protein was spun down at 14,000 g for 10 min and the supernatant was loaded onto a HiLoad 16/600 Superdex 200 PG column. Fractions of 1 mL were recovered, and the protein content measured at OD280. Protein containing samples were pooled and the elution buffer exchanged with exchange buffer (20 mM Hepes, 150 mM KCl, 10 mM $MgCl_2$, 1% glycerol, 0.5 mM DTT, pH 7.5) using a Millipore Amicon 10,000 NMWL centrifugal filter unit. Sample was spun down at 14,000 g for 10 min, and the supernatant snap frozen in fractions for further use. The final protein concentration was determined using a Bradford assay.

Cas13a purification. The plasmid pC013 (2) encoding Cas13a was transformed into BL21(DE3) *E. coli* cells, which were grown on agar plate with 100 µg/mL ampicillin. Cells were grown in Terrific Broth (TB) medium, containing 12 g/L tryptone, 24 g/L yeast extract, 9.4 g/L K_2HPO_4 and 2.2 g/L KH_2PO_4, with addition of 100 µg/mL ampicillin, to an OD_{600} of 0.6 (37 °C, 180 rpm). Cultures were put on ice for 30 minutes, and subsequently Cas13a production was induced by the addition of 0.5 mM IPTG. Cultures were left for 16 hours at 18 °C with rotation (180 rpm). Subsequent steps were done at 4 °C. Cultures were spun down at 5200 g for 15 minutes, and the supernatant was discarded. Pellets were resuspended in 1xPBS buffer, and centrifuged (3220 g, 10 minutes). Pellets were resuspended in lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM DTT, 25 mM imidazole, pH 8.0) with addition of 100 units/mL benzonase, 0.25 mg/mL lysozyme and 1 protease inhibitor tablet per mL. Cells were French pressed (3 rounds at 100 kbar) and cell lysate was pelleted for 45 minutes at 16,000 g. Supernatant was filtered with a 0.45 µm pore size, applied to His-Select Nickel Affinity gel and incubated for one hour with rotation. Resin was spun down (1 minute, 2000 g), and the supernatant was

discarded. The resin was loaded onto a gravity column and washed with lysis buffer. Cas13a was eluted with elution buffer (20 mM Tris-HCl, 0.5 M NaCl, 1 mM DTT and 250 mM imidazole, pH 8.0), and dialyzed overnight against storage buffer (50 mM Tris-HCl, 0.6 mM NaCl, 5% v/v glycerol, 2 mM DTT, pH 7.5). Final protein concentration was determined using a Bradford assay.

Nucleic acid preparations.

Targets for Cas12a and Cas13a. ssDNA target for Cas12a assays was ordered from Ella Biotech and IDT (5'-Cga gta aca gac atg gac cat cag ATC TAC AAC AGT AGA AAT TCT ATA GTG AGT CGT ATT ACT T-3'). Target RNA for Cas13a assays was produced by Recombinant Polymerase Amplification (RPA, TwistDx) and simultaneous *in vitro* transcription of part of the tetracycline resistance gene from the plasmid pSB1T3. The target amplification was done by mixing 1 ng of plasmid with 1 µM forward primer (5'-AAT TCT AAT ACG ACT CAC TAT AGG gat gcc ctt gag agc ctt caa c-3') containing a T7 promoter overhang, 1 µM reverse primer (5'-cct cgc cga aaa tga ccc a-3'), 4 µL Murine Rnase inhibitor (NEB), 1 µL T7 polymerase (NEB), 5 mM $MgCl_2$, 1 mM ATP, 1 mM CTP, 1 mM GTP and 1 mM UTP and RPA rehydration buffer. This was transferred into an RPA mix and 14 mM MgAc was added to start the reaction, which was left for three hours at 37 °C. Target RNA was purified using Rneasy MinElute (Qiagen), quantified using NanoDrop, and stored at -80 °C.

crRNA preparation. crRNA for Cas12a was bought from IDT (**Table S1**). crRNA for Cas13a (**Table S1**) was prepared by annealing of template DNA oligonucleotides and *in vitro* transcription. Primers 5'- AAT TCT AAT ACG ACT CAC TAT AGG GGA TTT AGA CTA CCC CAA AAA CGA AGG GGA CTA AAA C-3' and 5'- gcc gca ctt atg act gtc ttc ttt atc aGT TTT AGT CCC CTT CGT TTT TGG GGT AGT CTA AAT CCC CTA TAG TGA GTC GTA TTA GAA TT-3' were mixed in 1 µM concentration and annealed by heating to 95 °C for 2 minutes and gradually cooling to 50 °C in steps of 5 °C every ten seconds, and then cooling to 30 °C in ten seconds. The resulting annealed DNA was *in vitro* transcribed in a mix containing 1 mM ATP, 1 mM CTP, 1 mM GTP, 1 mM UTP, 1 µL T7 polymerase, 2 µL murine Rnase inhibitor and RNA polymerase buffer, to a final volume of 100 µL. The reaction was left at 37 °C for 3 hours and cleaned using RNeasy MinElute. The concentration was determined using NanoDrop.

Table S1: Sequences of crRNA used for Cas12a and Cas13a and targets.

crRNA/target	Sequence (5'->3') with target complementarity (lower case)
Cas12a crRNA	UAAUUUCUACUCUUGUAGAUcugaugguccaugucuguuacuc
Cas12a crRNA^{Lba}	UAAUUUCUACUAAGUGUAGAUcugaugguccaugucuguuacuc
Cas13a crRNA	GGGGAUUUAGACUACCCCAAAAACGAAGGGGACUAAAACugauaaag aagacagucauaagugcggc
ssDNA target	cgagtaacagacatggaccatcagATCTACAACAGTAGAAATTCTATAGTGAGTC GTATTACTT
Complement ssDNA target	GGCGAGCTCGGTTTCCTGATGGTCCATGTCTGTTACTCGCCTCCTGT TCAGCTACTGACG
RNA target	*In vitro* transcription from a plasmid, see nucleic acid preparations above

References

1. Zetsche, B., J.S. Gootenberg, ..., F. Zhang. 2015. Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System. *Cell*. 163:759–771.

2. Gootenberg, J.S., O.O. Abudayyeh, …, F. Zhang. 2017. Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science.* 356:438–442.