Handhold-Mediated Strand Displacement: A Nucleic Acid-Based Mechanism for Generating Far-from-Equilibrium Assemblies Through Templated Reactions

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ABSTRACT: The use of templates is a well-established method for the production of sequence-controlled assemblies, particularly long polymers. Templating is canonically envisioned as akin to a self-assembly process, wherein sequence-specific recognition interactions between a template and a pool of monomers favour the assembly of a particular polymer sequence at equilibrium. However, during the biogenesis of sequence-controlled polymers, template recognition interactions are transient; RNA and proteins detach spontaneously from their templates to perform their biological functions and allow template reuse. Breaking template recognition interactions puts the product sequence distribution far from equilibrium, since specific product formation can no longer rely on an equilibrium dominated by selective copy-template bonds. The rewards of engineering artificial polymer systems capable of spontaneously exhibiting non-equilibrium templating are large, but fields like DNA nanotechnology lack the requisite tools; the specificity and drive of conventional DNA reactions rely on product stability at equilibrium, sequestering any recognition interaction in products. Here we introduce handhold-mediated strand displacement (HMSD), a DNA-based reaction mechanism suited to producing out-of-equilibrium products. HMSD decouples the drive and specificity of the reaction by introducing a transient recognition interaction, the handhold. We measure the kinetics of 98 different HMSD systems to prove that handholds can accelerate displacement by four orders of magnitude without being sequestered in the final product. We then use HMSD to template the selective assembly of any one product DNA duplex from an ensemble of equally stable alternatives, generating a far-from-equilibrium output. HMSD thus brings DNA nanotechnology closer to the complexity of out-of-equilibrium biological systems.

KEYWORDS: reaction mechanism, self-assembly, fluorescence, nucleic acids, kinetics

Over the last 25 years, DNA has proven a versatile engineering material for developing synthetic molecular systems. Achievements have included self-assembled structures, information-processing architectures, and molecular motors.¹⁻⁵ However, a class of processes essential to biology – the chemically-driven far-from-equilibrium templating – has been largely neglected.

Far-from-equilibrium templating underlies the processes that constitute the central dogma of molecular biology.⁶⁻⁸ In a templated process, a pool of monomers assembles into a product of a specific sequence by interacting with the sequence elements of a template.⁹ For example, during RNA transcription, ribonucleotides use sequence-specific recognition interactions with a DNA template to polymerise into a sequence-complementary RNA strand.⁶ The essence of templating is illustrated in the context of dimerization in Fig.1, where it is contrasted with conventional self-assembly.

Therefore, as in any templating process, the sequence of the product (the RNA) is determined solely by the information transmitted by the recognition interactions with the template, rather than by interactions within the product itself (the key determinant in conventional self-assembly, Fig.1-a). This information transferred by the template is essential to biological complexity. For example, there is simply not enough information stored in the interactions of amino acids alone to direct their assembly into tens of thousands of distinct proteins, and only those proteins.¹⁰

A crucial feature of natural templating processes is that the product must eventually detach from the template, breaking all recogni-

tion interactions (Fig.1-c). Firstly, this decoupling allows the product to operate independently of the template – proteins would be extremely restricted if they were anchored to their template polynucleotides. Secondly, the decoupling of the product allows the template to be used repeatedly. Without template recovery, templates would not solve the mechanistic problem of directing the assembly of proteins, since a new template would need to be assembled *de novo* from its monomers for each protein produced.

It is product detachment the step that necessarily pushes the products of natural templating processes far from equilibrium. Before separation, templated processes can rely on highly specific recognition interactions between template and monomers to assemble the correct product at equilibrium (Fig.1-b).¹¹ However, if interactions between template and product are transient, there can be no systematic equilibrium bias for a template-matching sequence in the final product, since no specific interactions remain to provide this bias (Fig.1-c).^{11, 12} Sequence-matched products and templates are therefore necessarily far from equilibrium in this setting.

We emphasize that “far from equilibrium” in this sense does not mean the creation of steady states with fuel-consuming cycles. Rather, the reactants are directed by the templates into assemblies that are effectively long-lived kinetic traps. Template-favoured product sequences are formed faster, and the slow conversion into alternative sequences means that the system does not relax to equilibrium on accessible timescales. Unlike the unintentional kinetic traps that often impede reliable self-assembly,¹³ these kinetic traps are the required product. Moreover, each template sequence must reliably direct the reactants towards a distinct trapped state.

Figure 1: Dimer formation mechanisms. **a) Equilibrium self-assembly.** The introduction of complementary recognition interactions between monomers biases the formation of specific products at equilibrium. **b) Equilibrium templating.** Templates use complementary recognition interactions to determine the spatial organisation of the two interacting monomers. Once bound to the template, the monomers bind to each other through a generic bond. The template-complementary dimer is favoured at equilibrium due to its specific interactions with the template. **c) Catalytic far-from-equilibrium templating.** After the production of the generic bond between monomers, the dimer and template can detach spontaneously. The template can then be reused to catalyse again the formation of that dimer and push its concentration out-of-equilibrium. Since only the generic bond persists, all possible dimers are equally probable in the unwanted equilibrium ensemble. **d) Non-catalytic far-from-equilibrium templating as implemented in this work.** A process simpler than (c) in which templates are pre-loaded with one monomer, with the focus on the selective production and detachment of a dimer once the first monomer is bound. Although simpler, the products are still in a far-from equilibrium ensemble driven by transient recognition interactions with the template.

Specifically, the distribution $p(s)$ of product sequences, s , must be sharply peaked around the complement of the template sequence s_0 . Even though the equilibrium distribution $p_{eq}(s)$ is necessarily independent of s_0 , and essentially uniform. For a perfectly matching product of length N , and an alphabet of M distinct monomers,

the excess free energy stored in this non-equilibrium product distribution is approximately $NRT \ln M$ per mole of product.¹¹

Given the fundamental significance of far-from-equilibrium templating, achieving similar functionality in synthetic systems would provide a platform for the development of more complex chemical and biomolecular systems. The success of DNA nanotechnology suggests that short, synthetic oligonucleotides may be the ideal engineering material for this challenge.¹⁴⁻¹⁶ Yet, it is far more challenging to engineer systems to reach these far-from-equilibrium target states than to design a system that relaxes to a specific structure at equilibrium.¹⁷ Moreover, the central reaction underlying dynamic DNA nanotechnology – toehold-mediated strand displacement (TMSD) – is poorly suited to non-equilibrium templating.

TMSD is a spontaneous reaction in which an invader nucleic acid strand (I) replaces an incumbent strand (N) in a duplex with a target strand (T) (Fig.2-a).¹⁸ TMSD is driven by toeholds, overhanging single-stranded domains in T recognised by the I strand. Toeholds increase TMSD reaction rates by inhibiting the detachment of the I strand from T during the competition with N ,¹⁹ a process known as branch migration. Thus, TMSD reaction rates increase exponentially with toehold length until saturating at approximately six orders of magnitude faster than toehold-free displacement, at a toehold length of 6-7 nucleotides (nt).²⁰ Toehold versatility and ease of design have made TMSD a great building block for constructing an enormous range of chemical reaction networks.^{21, 22}

The toehold and the contiguous displacement domain act cooperatively to ensure the stability of the IT complex. Toehold and displacement domain cooperativity remains even for more complex TMSD topologies – like associative²³ (Fig.2-b) and remote²⁴ toeholds – that locate the toeholds in positions that are non-contiguous with the displacement domain. The result is that toeholds – the recognition domains of strand displacement – are typically tightly sequestered in products after the reaction takes place. TMSD is therefore poorly suited to far-from-equilibrium templating, in which recognition interactions are transient interactions with a template, rather than permanent interactions incorporated into the product. As a result, attempts at far-from-equilibrium templating using TMSD are inherently limited.

Other nucleic acid-based approaches to templating that rely directly on hybridisation between DNA strands have faced related challenges. The tendency of assembled products to bind cooperatively to templates and “inhibit” their function^{25, 26} restricts its output to the production of dimers.²⁷ unless non-chemical mechanisms such as mechanical agitation,²⁸ or cyclically varying conditions, are used to separate the product from the template.^{14-16 29} However, these externally-driven approaches to reuse the template are much harder to implement as an autonomous reaction networks in general contexts. Other, non-nucleic-acid-based synthetic templated systems have faced similar challenges.^{30, 31}

Here, we introduce HMSD (Fig.2-c), a strand displacement topology capable of introducing far-from-equilibrium templating to DNA reaction networks. HMSD increases the rate of displacement of N from a complex NT through recognition of an overhanging “handhold” domain located in N , rather than a toehold in T . The molecular logic of recognising one molecule N and binding to another T is more naturally suited to templating than TMSD. Moreover, since the handhold is not cooperatively sequestered in the product IT , it can instead function as a transient bond with a template N .

Figure 2: Strand displacement topologies. **a) Toehold-mediated strand displacement.** The presence of a proximal toehold (t) in the target strand (T) mediates the displacement of the incumbent strand (N) by the invader strand (I). After displacement, t remains cooperatively sequestered in the product IT . **b) Associative toehold.** An auxiliary strand (M), complementary to the strand I and T , accelerates the displacement by effectively increasing the length of t . The reaction results in the formation of a stable three-stranded product ITM that cooperatively sequesters the associative toehold. **c) Handhold-mediated strand displacement (HMSD).** The incorporation of a handhold (h) – an independent overhang in N – increases the effective concentration of the I strand in the vicinity of T , enhancing the efficiency of displacement with short proximal toeholds. Reversible handhold binding for handholds of moderate length results in a displacement rate increase for handhold-complementary I strands, while still allowing the detachment of IT from N when the reaction takes place.

In this work, we first thoroughly characterise the effect of handhold complementarity in the displacement rate. We then demonstrate that the displacement rate can be increased by several orders of magnitude when using I -strands with handhold complementarity (I_{hc}) over identical strands but without handhold complementarity (I_{wc}). Next, we show that it is possible to both increase the displacement rate and allow the IT product to detach from N when using handholds of moderate length. Finally, we demonstrate the templating of specific, far-from-equilibrium IT complexes *via* transient handhold interactions. The process we demonstrate – illustrated schematically in Fig.1-d – is a simplified non-catalytic version of far-from-equilibrium templating which, nonetheless, retains the central challenge of producing a non-equilibrium distribution of products through transient recognition interactions with a template.

Results and discussion

Handhold-mediated strand displacement. The proposed handhold mechanism is as follows (Fig.2-c): First, I_{hc} recognises and reversibly binds to the handhold, producing a transient 3-stranded complex that colocalises I_{hc} and the duplex TN ; then I_{hc} displaces N from T . A short proximal toehold between I_{hc} and T can further ac-

celerate the displacement step. The distinguishing feature of handholds relative to toeholds is that I recognises N but uses this recognition to strongly bind to T while, in the process, weakening the interaction between N and T . It is this reversal of the reaction topology relative to TMSD that inspires the name HMSD. The crucial functional difference between TMSD and HMSD is that the formation of the IT product does not produce binding cooperativity between the handhold recognition interaction (between N and I) and the displacement domain binding (between I and T). Hence, the product IT can spontaneously detach from N 's handhold, breaking the recognition interaction.

We therefore hypothesize that handholds can provide reaction specificity by increasing the displacement rate of a particular I . But, in contrast to toeholds, for short enough handholds, the handhold detachment reaction can dominate the reverse re-binding reaction, resulting in the production of IT complexes free in solution, instead of bound to N . Since the detached IT product resultant from just HMSD have similar stability as TN , the displacement process can be driven by introducing a small proximal toehold, mismatches in N ,³² or coupling it with a downstream displacement reaction that acts as a product sink.

Figure 3: Handhold complementarity substantially enhances displacement rates for short toeholds. a) Handhold-mediated strand displacement (HMSD) characterisation and fluorescent reporter (RQ) reaction: During HMSD characterisation, several target-incumbent duplexes (TN) with different combinations of proximal toehold (t) and handhold (h) lengths are tested. To test the handhold contribution to the kinetics, each TN is triggered with different concentrations of invader strands, with (I_{hc}) and without (I_{wc}) handhold complementarity. Completion of the displacement reaction is followed *via* the external reporter RQ. Once T is displaced from N, the first 6 nucleotides (nt) of the 5' edge of the displacement domain (rt) can trigger the displacement of Q from the reporter, independently of the handhold occupation, producing a fluorescence signal. **b) Measured and fitted fluorescence traces:** Representative results for a reaction mediated by a 2 nt t-T strand and 7 nt h-N strand. A single set of reaction parameters is fitted simultaneously to all the I_{hc} trajectories (red), as outlined in Supplementary Note XVI. Conditions: $[RQ]_0=15$ nM, $[TN]_0=10$ nM, $[I_{hc}]_0=[7-4]$ nM, 1M NaCl in 1x TAE at 25°C. Final fluorescence signal is used to confirm experimentally the precise reagent concentrations as described in Supplementary Note I. Inset: Kinetics for 6nM of I_{wc} (grey), alongside the data from the main figure.

We first test the influence of different handhold and proximal toehold lengths on strand displacement kinetics. We monitor strand displacement with an external fluorescence reporter to minimise fluorophore interference³³ in the reaction system. The external reporter is a DNA duplex (RQ) labelled with a quenched fluorophore. As shown in Fig.3, N triggers RQ by conventional TMSD, producing fluorescence only after IT formation. The toehold in RQ is complementary to 6 nucleotides (nt) in the 5' edge of N's displacement domain, and so, reporter triggering does not happen unless N has been displaced. Clamp domains are also added to both sides of RQ to minimise any leak reactions, which are indeed negligible as shown in Supplementary Note VIII.³⁴

Typical HMSD reactions consist of adding I_{hc} to a solution containing TN and RQ. We also compare HMSD kinetics to those of a TMSD reaction triggered by I_{wc} in the same conditions. We analyse the recorded kinetics by modelling HMSD as a two-step process, where I_{hc} reversibly attaches to the handhold and once bound, triggers the displacement (Fig.2-c):

Here, X/Y indicates that species Y is bound to the handhold of X. k_b and k_d are rate constants for 2nd order binding and 1st order detachment of I_{hc} from N's handhold, respectively. k_m is the 1st order rate constant for the strand displacement once I_{hc} is bound to N by its handhold.

Once displacement is completed, the incumbent strand N can trigger the reporter. At this stage, we are agnostic as to whether IT has detached from N, assuming that both N and N/IT activate the reporter with the same 2nd order rate constant k_r .

We treat all displacement reactions as effectively irreversible, even in the absence of a proximal toehold to drive the reaction, because RQ acts as a sink for displacement products.

Figure 4: Handhold-complementarity systematically enhances strand displacement rates for short toeholds, as inferred from the fits shown in Supplementary Table 15. a) Half-lives ($t_{1/2}$) for HMSD. The analysis shows three different reaction regimes: 2nd order reactions with reversible handhold binding (from 4 to 8 nt handhold) and with effectively irreversible handhold binding (outlined in purple); Also a 1st order reaction regime, where the limiting factor is the displacement once bound to the handhold (outlined in green). Reactions in the absence of a handhold are outlined in grey. Displacement is systematically favourable even in the absence of toehold due to the downstream RQ reaction acting as a sink. $t_{1/2}$ calculated for $[I]_0=6$ nM and $[TN]_0=10$ nM with parameters inferred at 25°C. **b) Half-lives for I strands without handhold-complementarity ($t_{1/2}^{wc}$).** Handhold presence in N produces negligible displacement rate changes when I -strands lack handhold-complementarity. $t_{1/2}^{wc}$ is calculated in the same conditions as $t_{1/2}$. **c) Comparison between $t_{1/2}$ and $t_{1/2}^{wc}$.** HMSD can reduce displacement $t_{1/2}$ up to four orders of magnitude when compared to displacement triggered with I -strands without handhold-complementarity. $t_{1/2}$ difference translates as an increment of the displacement rate of more than 4 orders of magnitude. The displacement rate increment produced by handhold-complementarity is substantially higher in the regime of toeholds below 3 nt.

To complete the model, we also consider displacement of N from I by direct interactions with the toehold, bypassing the handhold. These reactions, with a 2nd order rate k_t , could occur for both I_{hc} and I_{wc} . We estimate the value of k_t for every HMSD system from experiments triggered with I_{wc} . Toehold-only triggering is essentially a leak reaction, and our results will identify a regime where these leaks are extremely slow compared to the intended handhold-triggered reaction. See Supplementary Note XI for the full implementation of the reaction model as an ODE system.

We numerically solve the ODE system to fit a single set of values for k_d , k_m and k_b to the fluorescent traces produced for each reaction system at a range of I_{hc} concentrations. We obtain the values for k_r , the constraints for k_b , and the competing TMSD rate k_t , from the results of complementary experiments (See *Experimental methods*). The fitted results (Supplementary Table 15) reveal that HMSD can operate in three different reaction regimes determined by the behaviour of the intermediate species TN/I . The three defined reaction regimes are 1st order and 2nd order with either reversible or effectively irreversible handhold-binding. For the tested HMSD systems, the most frequent reaction regime is the 2nd order rate with reversible handhold-binding (Condition: $k_m, k_b[I]_0 < k_d$, with $[I]_0$ as the initial invader concentration). In this reaction regime, the complex TN/I is short-lived, reaching a quasi-steady state concentration of $[TN/I] = [I][TN]k_b / (k_m + k_d)$ that balances detachment and migration events with binding. Branch migration events occur from this state at a rate k_b , and so combining the overall HMSD rate with the direct TMSD rate yields an apparent 2nd order displacement rate:

$$k = k_b \frac{k_m}{k_m + k_d} + k_t \quad (4)$$

When handholds become longer than 8 nt ($\Delta G^{\circ}_{binding} < -12.39$ Kcal/mol)³⁵ we observe effectively irreversible handhold binding. For systems with a non-zero proximal toehold the kinetics are of 2nd order. In this regime, TN/I is quickly formed and consumed by the branch migration reaction. The above analysis holds, but with $k_m \rightarrow \infty$: all handhold-binding events are stable on the timescale of branch migration, which is fast relative to binding. (Condition: $k_d, k_b[I]_0 < k_m$). The resultant k is insensitive to changes in k_m and k_d ($k \approx k_b + k_t \approx 10^6$ M⁻¹s⁻¹). The 1st order reaction regime occurs in systems with handhold binding irreversibility and no proximal toehold (Condition: $k_b[I]_0 > k_d, k_m; k_t \approx 0$). In this regime, TN/I is quickly and irreversibly formed, accumulating due to the handhold-binding stability and the reduced branch migration rate. The intermediate thus reaches a concentration of $[TN/I] = \min([TN]_0, [I]_0)$, and then undergoes a first order transition to the displaced state with rate constant k_m (Mean fitted k_m at 25°C = $1.21 \pm 0.29 \cdot 10^{-3}$ s⁻¹, see Supplementary Tables 15 and 16).

Given the existence of reaction regimes with 1st and 2nd order rates, we cannot use each HMSD system's apparent rate constant for direct comparison of the different systems tested. Instead, we use the fitted rate constants to infer each HMSD system half-life ($t_{1/2}$) at a default reference concentration, as described in the *Experimental methods*, to use it as a comparable measure of each system reaction speed (Fig.4-a). From these results, we prove that handhold complementarity decreases $t_{1/2}$ by several orders of magnitude, analogously to toeholds in conventional TMSD. For HMSD systems with a 1 nt-long toehold and saturating handhold lengths (>8 nt), the obtained $t_{1/2}$ is consistent with conventional TMSD at saturating toehold lengths. In Fig.4-b, we report the half-life for systems triggered with I_{wc} ($t_{1/2}^{wc}$). As expected, these systems only react by conventional TMSD, with rate k_t and $t_{1/2}^{wc}$ is insensitive to handhold presence. Hence, we can conclude that $t_{1/2}^{wc}$ is mainly determined by proximal toehold length.

To estimate the contribution of handholds to the kinetics, we plot the ratio $t_{1/2}/t_{1/2}^{wc}$ for every system in Fig.4-c. The handhold contribution to the kinetics is especially notable for systems with proximal toeholds smaller than 3 nt and handholds longer than 6 nt, where handhold-complementarity can reduce $t_{1/2}$ by four orders of magnitude. This reduction of $t_{1/2}$ in our experimental conditions translates to k increments of more than 4 orders of magnitude (Supplementary Figure 20).

Figure 5: Handholds of moderate length accelerate displacement while allowing for detachment. **a) Kinetic analysis.** HMSD triggered under the same experimental conditions as described in Fig.3 with $[I]_0=4$ nM and a 3 nt proximal toehold. Once HMSD reaches steady state, reporter R_2Q_2 is added to react with any detached IT by an associative toehold strand displacement. The rate of triggering is negatively correlated with handhold length, saturating for handholds shorter than 9 nt. Inset: The unbound fraction of IT after HMSD is complete is inferred from fitting K_{eq} to R_2Q_2 kinetics as outlined in Supplementary Note XVII. **b) Native polyacrylamide gel electrophoresis.** 12.5 mM $MgCl_2$ in 0.5x TBE at 25°C. A qualitative confirmation that, for handholds up to 8 nt, IT is mostly detached from the handhold at equilibrium. V: TN duplex. W: N -strand. X: TN duplex + random strand with handhold-complementarity (results in a 3-strand complex since it cannot displace N). Y: $TN + I$ without handhold-complementarity (results in $IT + N$). Z: IT duplex. All controls have a handhold length of 20 nt.

Product Separation. For HMSD to underlie far-from-equilibrium templating, handhold binding must be transient and release the majority of IT after the displacement. To verify release, we design a second reporter DNA duplex (R_2Q_2) to detect detachment of IT from N . Like RQ , we label R_2Q_2 strands with a quenched fluorophore. R_2Q_2 has an associative toehold²³ formed by two single-stranded domains brought into proximity by a hairpin (Fig.5-a).

As shown in Supplementary Figure 14, R_2Q_2 only reacts at high-speed with IT duplexes free in solution. The reaction that describes the kinetics of R_2Q_2 is:

where k_{r2} is a 2nd order rate constant. We infer k_{r2} value from systems with no handhold, where IT is completely detached ($[IT]=[I]_0$, $[N/IT]=0$). For systems with a handhold, it is possible to extrapolate the amount of detached IT from the kinetics resultant from adding R_2Q_2 after HMSD reaches completion. We estimate the equilibrium constant:

$$K_{eq} = \frac{[N][IT]}{[N/IT]}, \quad (6)$$

assuming that IT remains in instantaneous equilibrium with the N/IT complex, which is reasonable given that $k_b > k_{r2}$. Hence, we fit the value of K_{eq} with the experimental R_2Q_2 traces as described in the *Experimental methods* section. The fitted values for K_{eq} reveal that, when HMSD reactions have reached completion, >99% of the produced IT is free in solution for handholds shorter than 9 nt (See *Experimental methods*, Fig.5-a). For handhold lengths of 9 and 10 nt, the three-stranded complex N/IT becomes more prevalent, as evidenced by the slowdown in the reporter kinetics. For handholds 20 nt long, all IT is sequestered in the N/IT form and reporter triggering is minimal.

We further perform a qualitative confirmation of HMSD products detachment with PAGE.³⁶ To prevent sample heating, the gel is not resolved using identical buffer conditions: buffer NaCl content is exchanged for a lower concentration of $MgCl$. Although the stability of the complexes could slightly vary between buffer conditions, the gel results confirm the substantial presence of three-stranded N/IT complexes only for handholds longer than 8 nt (Fig.4-b). Furthermore, we show in Supplementary Figure 5 that the PAGE buffer does not qualitatively alter the detachment observed with R_2Q_2 .

Figure 6: Selective production of a single complex from approximately equally stable alternatives $I_A T$, $I_B T$, and $I_C T$ through handhold-mediated templating. a) The specific production of $I_x T$ complexes is determined by the handhold sequence of three different strands N_x . Complementarity between I_x and T is identical for all the possible $I_x T$ products. Therefore, product selectivity cannot rely on a bias in equilibrium. The transient interaction with a complementary handhold (h) determines the rate of the reaction, without altering the relative product stability, and thereby pushing product distributions out of equilibrium. b) Three reporters are used to detect the formation of free $I_x T$ complexes via HMSD (8 nt handhold/2 nt proximal toehold). Sequence differences in h result in slightly different trajectories, but the unambiguous ability to select any product is confirmation of the success of non-equilibrium templating. The shaded area represents the standard error of the mean fluorescence for each experiment ($n=4$). Conditions: $[TN_x]=10$ nM, $[I_x]=25$ nM for all three I_x strands, and 30 nM of each specific reporter, 1M NaCl in 1x TAE at 25°C.

Templating non-equilibrium assembly. We next harness HMSD to produce a far-from-equilibrium ensemble of IT products through templating. We perform an iteration of the experiment depicted in Fig. 5-a, but with three competing invading strands I_A , I_B and I_C (Fig. 6-a). Each invader I_A , I_B and I_C is equally complementary to a T strand and its 2 nt toehold, and complementary to one of the different 8 nt handholds located in templates N_A , N_B and N_C .

From the law of mass action, the equilibrium yields of free IT products would be related by expressions of the form:

$$\frac{[I_A T]/[I_A]}{[I_B T]/[I_B]} = \exp((G_B - G_A)/RT), \quad (7)$$

in which G_i is the standard free energy of formation of $I_i T$. These ratios are independent of which template N is present, and predict relatively similar yields of all products, since the free energies of formation of all IT are similar. Indeed for an equimolar mixture of all the strands in our experimental conditions, NUPACK³⁷ predicts a ratio of product formation of 0.21/0.27/0.52 for the products $I_A T$, $I_B T$ and $I_C T$, respectively, equivalent to a free-energy difference of only around 1 RT between the products.

However, we use handhold complementarity with either N_A , N_B or N_C to produce product ensembles that are heavily biased towards either $I_A T$, $I_B T$ or $I_C T$, respectively – as illustrated schematically in Fig. 1-d. To do so, we mix all three invaders with initial solutions containing one species of the duplexes $N_A T$, $N_B T$ or $N_C T$. Since

handholds of this length produce only transient binding, the products $I_A T$, $I_B T$ and $I_C T$ will overwhelmingly dissociate from N_A , N_B and N_C respectively.

We, add three different reporters, analogous to $R_2 Q_2$, that detect the possible products $I_A T$, $I_B T$ and $I_C T$, when free in solution. Although the presence of N_x cannot provide an equilibrium bias towards a detectable complementary $I_x T$ product free in solution, we nonetheless observe in Fig. 6-b, the selective formation of any single $I_x T$ product, free in solution, can be templated by the appropriate transient handhold interaction from a solution containing all three invaders. HMSD is therefore capable of templating the system intentionally into specific kinetic traps for the formation of far-from-equilibrium product ensembles.

Conclusion

We have introduced HMSD not only as a simple and rational method to control strand displacement kinetics, but to add an important feature to DNA nanotechnology: templating of far-from-equilibrium assemblies. Other superficially similar processes^{23-25, 38} do not involve a transient recognition interaction; when using toeholds as the source of specificity, they are permanently sequestered in the product complex due to cooperativity with the remainder of the base pairs in the product. In HMSD, this cooperativity does not arise; the base pairs between N/T and NT are essentially independent in the product state.

In the current article, we have used transient recognition interactions with a template to selectively assemble a single DNA duplex from an ensemble of equally stable alternatives. The product state is far from the equilibrium distribution of approximately equal concentrations of all duplexes; HMSD thus successfully channels the system towards this non-equilibrium state of high free energy. We have also characterised the kinetics of HMSD and introduced a simple model to predict it. We hope our work will establish HMSD as an orthogonal reaction in DNA nanotechnology that can be used to create non-equilibrium assemblies for a great variety of uses.

The production of non-equilibrium assemblies is essential to biological processes such as transcription and translation. The second crucial requirement of these processes in nature is that the templates act catalytically – a single template molecule can be used to produce multiple products, whose assembly is kinetically suppressed in the absence of the template. The next challenge is to incorporate HMSD into a genuinely catalytic mechanism for dimerization (Fig. 1-c), rather than initiating the templates with the *T* monomer pre-loaded as in this work (Fig. 1-d). A pool of functionalised strands in solution could then be continuously templated, working as a synthetic analogue of aminoacyl-tRNA synthetases, the molecules responsible for selectively combining tRNAs with their cognate amino acids.³⁹

Eventually, HMSD has the potential to underlie the catalytic production of sequence-specific polymers from longer templates, to create synthetic analogues of transcription and translation. Although many challenges remain, HMSD solves an important problem in this context. A central challenge of engineering such systems in a purely chemically-driven context has always been “product inhibition”: the cooperativity of interactions between long products and templates tends to prevent detachment unless the complexes are disrupted by another source of energy.^{14–16, 25, 29, 31} HMSD is explicitly designed to overcome product inhibition; much of the free energy released by the binding between *I* and *T* is diverted into weakening the binding between *T* and the template *N*, so that the *IT* complex does not bind more strongly to the template than *I* alone. This mechanism – using the free energy of a reaction to enhance dissociation from a template – was recently postulated to be ideal in a theoretical analysis of minimal catalysts.⁴⁰

Other systems have been proposed in which the dimerization process weakens the interaction of the product with the template,^{14–16} but a particular advantage of HMSD is that the binding of *I* to *N* is not weakened by the branch migration process, only the binding of *T* to *N*. Thus, it is possible to imagine a long template with multiple handhold sites; an invader *I* can bind to a target *T* via HMSD, then, remain bound by a long handhold and in turn becoming the target for the next displacement. This way, creating a templated polymer of DNA strands step by step. A shorter handhold for the final step would allow full detachment of the complete copy at the appropriate time.

Experimental methods

DNA sequence design. DNA sequences that minimise undesired interactions during HMSD were designed with bespoke scripts using the NUPACK server (<http://www.nupack.org>).³⁷ All strands were purchased from Integrated DNA Technologies (IDT) with HPLC purification and normalized at 100 μ M in LabReady® buffer. All used sequences are listed by function (Incumbent (*N*), Target (*T*), Invader (*I*) and reporters) are collected in Supplementary Tables 20 to 23.

DNA duplex preparation. *TN* duplexes were formed by combining 200 nM of *T* with a 10% excess of *N* to ensure the anneal of every *T*. For *Reporter characterisations*, the *TN* anneal was prepared with a 10% excess of *T* since *T* is inert during those assays. Annealing was performed in the experimental buffer (TAE 1X and 1M NaCl, pH 8.3), except for PAGE samples which were annealed in a solution of TAE 1X and 12.5 mM MgCl₂. Strands forming

reporter complexes, except *R₂Q₂*, were mixed at a concentration of 300 nM. *RQ* was annealed in experimental buffer with a 10% excess of the quencher-labelled strand. *Rep_A*, *Rep_B* and *Rep_C* were annealed with a 60% excess of the quencher-labelled strand to minimise the crosstalk between reporter complexes. Each 100 μ L of *TN* or reporter solution, except *R₂Q₂*, was annealed by heating to 95°C for 4 min and cooling to 20°C at a rate of 1°C/min. *R₂Q₂* reporter was annealed at a concentration of 60 nM with a 10% excess of the quencher-labelled strand. Each 500 μ L of *R₂Q₂* was heated up to 95°C for 5 minutes and then cooled to room temperature over 1 hour.

Bulk fluorescence spectroscopy. Bulk fluorescence assays were carried out in a Clariostar Microplate reader (BMG LABTECH) using flat μ Clear bottom 96-well plates (Greiner) and reading from the bottom. Kinetics were recorded, unless stated otherwise, after injecting 50 μ L of the reaction trigger in 150 μ L of experimental buffer containing the reactant species and the reporter complex (pump speed: 430 μ L/s). The final mixture was shaken for 3 seconds (double-orbital, at 400 rpm). Injected and reacting volumes were previously preheated to the experiment temperature. The samples were contained in Eppendorf® Lobind tubes, and the plate reader’s injector system passivated by incubating with BSA 5% during 30 minutes to maximise concentration reproducibility during the assays⁴¹.

RQ reporter was labelled with Cy3 and FQ IowaBlack™ quencher (Excitation: 530/20 nm; emission: 580/30 nm). *R₂Q₂* reporter was labelled with Cy5 and RQ IowaBlack™ quencher (Excitation: 610/30 nm; emission: 675/50 nm). Signal was averaged for 20 flashes per data point. Experiments with durations over 12 hours were averaged for 100 flashes in a spiral area scan per data point. *Reporter characterisation* assays were measured individually with 1 flash/datapoint due to the fast rate of the reaction. Every system was assayed with at least three different reaction trigger concentrations.

Fluorescence calibrations. Fluorescence calibrations were made for the reporter DNA duplexes used during characterisation assays (*RQ*/Cy3 and *R₂Q₂*/Cy5). Calibrations aimed to estimate the units of fluorescence produced per nM of the fluorophore-labelled strand to quantify the amount of reacted reporter during the assays. Calibration curves ranged from 1 to 15 nM in 200 μ L and were made by triplicate from stock solutions at 100 μ M. One replica included both fluorophores (Cy3 and Cy5) to rule out bleed-through fluorescence. The calibration protocol and results are collected in Supplementary Notes IV and V and Supplementary Figures 7 to 10.

General protocol for characterisation experiments. HMSD systems with a combination of 9 different handhold lengths (0, 4, 5, 6, 7, 8, 9, 10 and 20 nt) and 6 different proximal toeholds (0, 1, 2, 3, 5 and 7 nt) were tested at 25°C. A smaller set of systems were also characterised at 37°C (Supplementary Table 17). The effect of a spacer sequence located after the handhold of *N* was also tested. Spacer lengths of 0, 1, 2, 3, 5 and 8 nt were included for systems with handhold lengths of 0, 5 and 20 nt and proximal toehold lengths of 0, 2 and 7 nt. The addition of a spacer sequence did not have a clear effect on HMSD except for systems with 5 nt handholds and proximal toeholds of 0 or 2 nt, where a spacer length of 2 nt was found optimal (Supplementary Table 16). Based on this finding, all systems reported in the main text contain a 2 nt spacer in *N*.

Characterisation protocols were adapted from the method employed by Srinivas *et al.*⁴² Each experiment consisted of the system kinetics and a set of complementary measurements. These complementary measurements quantified the fluorescence baseline and estimated the concentration of each species in the system from the fluorescence signal measured after sequentially triggering the reaction of all the species (Supplementary Figures 1 and 2). Intended species concentrations during the experiments, unless specified otherwise, were [*RQ*]₀=15 nM, [*TN*]₀=10 nM and [*I*]₀=[4–8] nM, at

a reference reaction volume of 200 μL . The excess of $[RQ]_0$ and $[TN]_0$ over $[I]_0$ was used to ensure that all invader strands reacted; this allowed us to estimate reagents' concentrations from fluorescence signals.

The purpose of considering a range of concentrations for the I -strand was to test the robustness of the reaction kinetics for their 2nd order and pseudo-1st order approximations. Simultaneously, the experimental replicas provided an estimate of the statistical error in the inferred reaction kinetics. Supplementary Notes I to III contain a detailed description of the quantification method and the measurements during each type of characterisation assay.

General fitting procedure. All fittings were performed with MATLAB® R2019a Optimization Toolbox. Besides the relevant rate constants, each fitting also allowed a variation of the fitted curve's reaction initiation time (t_0). This correction amended the initial leak of fluorescence signal produced by defective duplexes that can trigger instantaneously the reporter RQ . t_0 fitting became especially relevant for slow reactions where the initial increment of fluorescence could be equal to that after several hours of reaction. Also, for reactions that consumed at least 90% of its reaction trigger, the estimated trigger concentration was fitted to correct any discrepancy with the fluorescence signal of the kinetics equilibrium plateau. Supplementary Notes XI to XVII contain a detailed description of the fitting procedures.

Reporter characterisation. The value of k_r was obtained experimentally for every species of N . Experiments consisted of a solution of RQ triggered by N/IT ($[N/IT]_0 = [4-8] \text{ nM}$). The injected N/IT solution was obtained by mixing TN (toehold 7 nt long) with a 20% excess of I_{hc} . Each individual fluorescence trace was used to fit the analytical solution of the ODE describing equation 2:

$$[RN] = [N/IT]_0 \frac{(1 - e^{(k_r(t-t_0)([N/IT]_0 - [RQ]_0)})})}{1 - \frac{[N/IT]_0}{[RQ]_0} e^{(k_r(t-t_0)([N/IT]_0 - [RQ]_0)})}} \quad (8)$$

with RN being the fluorescent species. Modifications in the size and sequence of the N strands produced moderate variation of the fitted k_r value. k_r variation was particularly relevant during the fitting of HMSD systems with high reaction rates. To minimise the fitting error, subsequent fits used the k_r value obtained for the N present in each fitted system. All fitted k_r are contained in Supplementary Tables 9 and 10.

Toehold-mediated reaction characterisation. The experimental value of k_t was obtained for most of the combinations of handhold and toehold lengths assayed. Otherwise, k_t was inferred from the mean of the k_t obtained for systems with the same toehold length. Experiments consisted of a solution of RQ and TN triggered by I_{wc} . Each individual fluorescence trace was used to fit a reduced ODE model describing equation 4 and a TMSD 2nd order reaction:

Subsequent fits used the k_t obtained for each system to account for any unpredicted effect of the handhold length on k_t . All fitted k_t values are collected in Supplementary Tables 15 to 17.

HMSD rate characterisations. A solution of RQ and TN was triggered by I_{hc} . For each system, their traces were used simultaneously to fit the ODE model implied by equations 1, 2, 9 and the additional handhold binding/unbinding reactions:

The species TN/IT resultant from equation 10 is unable to undergo displacement *via* the handhold due to the bound IT complex. At the same time, invasion of the free proximal toehold in the TN duplex still occurs with the rate k_t :

The complete mass-action-based ODE model (equations 1, 2, 9, 10, 11, 12 and 13) is described in Supplementary Note XI. During the fitting, parameters k_r and k_t were fixed at the values obtained as described in *Reporter characterisation* and *Toehold-mediated reaction characterisation*, respectively. k_b was constrained to a certain range, determined by fittings done in systems confidently located in the 2nd order regime with irreversible handhold binding (handhold=20 nt; toehold>1 nt).

For the proposed reaction mechanism, most systems operated in the 2nd order rate reaction regime with reversible handhold-binding, where the reaction rate was defined by relative values of k_b , k_d and k_m (equation 4). This fact precluded the fitting algorithm from obtaining comparable absolute values for the rate constants across all tested systems. Constraining k_b to a fixed range eased this problem while still producing low error fittings for all tested systems; however, compensatory errors in the ratio $\frac{k_m}{k_m + k_d}$ could mitigate for an inaccurate k_b . Thus, the effective bimolecular rate constant (equation 4) is considered the most reliable descriptor of system kinetics in the 2nd order rate reaction regime. For the 1st order rate reaction regime, the rate is described with k_m . For all tested systems, the mean values of its fitted parameters and their effective bimolecular rates are collected in Supplementary Tables 15 to 17.

HMSD half-life ($t_{1/2}$) calculation. Using the rate constants obtained from *HMSD rate characterisations*, new trajectories were generated for each system with the complete ODE reaction model (Supplementary Note XI). Trajectories were generated for initial conditions $[I]_0 = 6 \text{ nM}$, $[TN]_0 = 10 \text{ nM}$ and $[RQ]_0 = 1 \mu\text{M}$. The $t_{1/2}$ of a system was defined as the time at which 3 nM of $[I]$ had reacted. The high concentration of simulated $[RQ]_0$ made the reporter's reaction effectively instantaneous, removing variability produced by k_r . Obviating the reporter reaction also allowed calculation of the analytical expression for $t_{1/2}^{wc}$ as:

$$t_{1/2}^{wc} = \frac{\ln(2 - \frac{[I]_0}{[TN]_0})}{k_t([TN]_0 - [I]_0)} \quad (14)$$

All calculated $t_{1/2}$ and $t_{1/2}^{wc}$ are collected in Supplementary Tables 15 to 17.

Detachment assays. I_{hc} was added to a solution containing RQ and TN to produce a final mixture of IT and N/IT ($[IT] + [N/IT] = [I]_0 = [4-8] \text{ nM}$). After the system reached steady-state, 50 μL of R_2Q_2 was injected into the experimental solution ($[R_2Q_2]_0 = 15 \text{ nM}$). During these assays, the T strand contained an additional domain in 3' to trigger reporter R_2Q_2 . The kinetics of IT triggering R_2Q_2 were measured for 10 minutes. The initial concentrations for all the strands present in the solution (I , N and T) were inferred from the signal of reporter RQ . The results of the controls without reporter RQ , shown in Supplementary Table 19, confirmed that the presence of RQ did not significantly alter the equilibrium between IT and N/IT . For controls in the absence of RQ , concentrations were inferred from the signal of reporter R_2Q_2 .

To value of k_{r2} was fitted, analogously to k_r , by using the traces of a system without handhold with the analytical solution of the ODE describing equation 5 (See *Reporter characterisation* and

Supplementary Figure 21). For systems with a handhold, each individual trace was used to fit the value of K_{eq} with the ODE describing equation 5, and concentrations constrained by equation 6. The percentage of detached IT was calculated from the fitted K_{eq} for concentrations $[I]_0/[N]_0=10\text{nM}$. All fitted K_{eq} are collected in Supplementary Table 18.

Polyacrylamide gel electrophoresis (PAGE). The gel for de-attachment analysis (Figure 5-b) was casted with 12% 37.5:1 acrylamide:bisacrylamide (Millipore®) and 12.5 mM MgCl_2 (effective concentration 12 mM MgCl_2) in TBE 0.5x and used in the same day. Samples were annealed in the presence of MgCl_2 and saturated afterwards with I_{hc} while incubating at 25°C (See *Duplexes annealing*). Controls and O'range ruler 5bp ladder (Thermo Scientific™) were incubated simultaneously. The gel was pre-ran for 10 min at 120 V. Then, the samples were loaded and ran for 2 hours at the same voltage. 15 μL of each sample were mixed with 3 μL of orange DNA loading dye (6x) (Thermo Scientific™) and 17 μL of the mixture were loaded in each well. The gel ran in a Mini-Protean® electrophoresis chamber (Bio-Rad) keeping the running buffer (0.5xTBE and 12.5 mM MgCl_2) at 25 °C using a water bath. Gels were stained for 30 min with a GelRed® 3x solution (Biotium, Inc) and visualised in a FluorChem® FC2 imager (Alpha Innotech). Excitation: 300 nm. Bandpass filter: 620/40 nm.

Selective production of complexes far-from-equilibrium. The experimental system contained three reporter complexes: Rep_A (Identical to R_2Q_2) and two analogous reporters: Rep_B and Rep_C . Each reporter differed in its distal toehold sequence, which enabled them to specifically recognise the handhold of one of the product complexes $I_A T$, $I_B T$ or $I_C T$ when free in solution (Supplementary Note X). Rep_B was labelled with Black Hole Quencher® 2 and Rhodamine Red™-X (Excitation: 550/20 nm; emission: 605/40nm). Rep_C was labelled with FQ IowaBlack™ quencher and Alexa Fluor® 488 (Excitation: 488/14 nm; emission: 535/40 nm).

Each experiment consisted of 190 μL of experimental buffer at 25°C, containing the three reporters (Rep_A , Rep_B and Rep_C) and the three invader species (I_A , I_B and I_C). The reaction kinetics were triggered by adding 10 μL of TN_x complex solution with a specific handhold (TN_A , TN_B , or TN_C). Initial concentrations were 25 nM of each species of I_x , 30 nM of each reporter and 10 nM of TN_x complex. An excess of $[I_x]_0$ over $[TN_x]_0$ was used to minimise the effect produced by any discrepancies between $[I_A]_0$, $[I_B]_0$ and $[I_C]_0$.

The traces for each different TN_x were assayed 4 times, and the fluorescence of each reporter normalised with its mean maximum fluorescence during the assays.

ASSOCIATED CONTENT

Supporting information: Extended description of experimental and computational protocols, experimental controls and calibration curves, complete kinetic modelling of the handhold-mediated strand displacement mechanism, DNA sequences, and tables containing the fitting results of k_r , k_{r2} , K_{eq} , k_t , k_b , k_m and k_d for all tested conditions at 25° and 37° C. This material is available free of charge via the Internet at <http://pubs.acs.org>.

All experimental files and MATLAB® scripts used for its analysis are available for download through the file repository Zenodo at www.zenodo.org/record/4389212.

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‡These authors conceived the project and planned the experiments. † This author performed the experiments and analysed experimental data. All authors interpreted the results and co-wrote the paper.

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Notes

The authors declare no competing interests.

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Handhold-mediated DNA strand displacement

Selective DNA duplex production out of equilibrium

“For Table of Contents Only”

Handhold-Mediated Strand Displacement: A Nucleic Acid-Based Mechanism for Generating Far-from-Equilibrium Assemblies Through Templated Reactions

Supplementary Information

Javier Cabello-Garcia Wooli Bae, Guy-Bart V. Stan and Thomas E. Ouldridge

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Supplementary Note I: General procedure for measuring reaction kinetics

All experiments contained at least one negative control (buffer only) and one positive control per reporter used, which allowed correction of changes in fluorescence by temperature and reaction volume. Positive controls contained a variable concentration of the reporter's fluorophore-labelled strand in the experimental buffer (TAE 1x, 1M NaCl). The volume contained in the controls changed consistently with the volume of the main assays. The function of the controls during data analysis is further described in **Supplementary Note VI**.

All measurements consisted of at least 10 data points. The number of measurements per experiment depended on the class of characterisation experiment; However, they all followed a similar structure that helped to quantify all the species in the experimental solution from the steady-state fluorescence, as outlined below. Specific procedures for each type of experiment are collected in **Supplementary Tables 1 and 2**. Examples of their fluorescence traces are shown in **Supplementary Figures 1 and 2**. For convenience, in all Supplementary Tables and Figures, we report the quantity of each species in the reaction volume at any time as the concentration that would be present if the same amount was solvated in a reaction volume of 200 μL .

Measurement 1. Fluorescence baseline produced by residual fluorescence of RQ:

The experiments started by mixing 10 μL of a standard solution of RQ (300 nM) with experimental buffer. A measurement was made after 30 mins of incubation at the experimental temperature, when the fluorescence value of the positive controls became stable.

Measurement 2. Estimation of $[N]_{\text{excess}}$:

10 μL of a standard solution of TN (200 nM) were added to the solution, except during *Reporter characterisations*. After adding TN, the excess of N resultant from the annealing of TN ($[N]_{\text{excess}}$) immediately reacted with RQ, producing an increment of the fluorescence baseline (See **Supplementary Note XI**). The new baseline was measured and used to infer $[N]_{\text{excess}}$.

Measurement 3. Experimental kinetics:

50 μL of a solution with the reaction trigger (I or N/IT) was added for a final reaction volume of 200 μL . Intended reagent concentrations at 200 μL were $[RQ]_0=15$ nM, $[N]_0=10$ nM and $[I]_0$ ranging from 4 to 8 nM unless specified otherwise. The system kinetics were measured after the injection, when possible until the reaction reached equilibrium. For kinetics that took more than 2 hours to reach equilibrium, several systems, with their respective controls, were run in parallel in a single 96-well plate. The kinetics of the experiments in a 96-well plate were always measured for at least 100 hours. After measuring, the plate was sealed and incubated for 1 month at the experimental temperature, and preserved from light, to wait for the reaction to reach equilibrium.

Measurement 4. Estimation of reaction trigger concentration from the steady-state fluorescence:

After the reaction reached equilibrium, a measurement recorded the fluorescence plateau. The total amount of reaction trigger added was inferred from this measurement. Some systems had such slow displacement rate that they did not reach equilibrium after 1 month (proximal toehold=0 nt; short handholds). For the slow-rate systems, trigger concentration was inferred from the mean concentration of all the experiments that reached equilibrium in the same plate. All experiments in a plate were triggered with the same aliquot of reaction trigger, so significant variability was not expected between experiments. Experiments that reacted till at least 80% of the highest concentration of trigger measured in that same plate were considered to have reached equilibrium.

Measurement 5. Saturation and quantification of the rest of the strands:

Finally, the experimental solution was sequentially saturated. High concentrations of single strands were added to trigger unreacted substrates and infer their actual initial concentrations from the resultant change in fluorescence. For example, to quantify $[TN]_0$, all the TN in the solution was triggered with 10 μL of a saturating solution of I_{hc} (Measurement 5 i)). $[RQ]_0$ was estimated similarly with a saturating solution of N (Measurement 5 ii)).

Supplementary Table 1 | Detailed experimental procedure for reporter RQ characterisation.

Due to the high rate of the reaction, the four iterations of each experiment were triggered and measured individually. The triggering species was a solution of N/IT . Since no TN was added, no $[N]_{\text{excess}}$ existed, and no measurement 2 was needed.

Steps	Addition	Purpose	Expected concentrations in 200 μL (nM)	Volume (μL)
Measurement 1	10 μL RQ (300 nM) 140 μL Buffer	Estimate residual fluorescence baseline	RQ :15	150
Measurement 3	[45-20] μL N/IT (40 nM) + [5-30] μL Buffer	Experimental kinetics	Evolve with time	200
Measurement 4	-	Estimate $[N]_0$	RQ : [6-11]; RN : [9-4]; IT : [9-4];	200
Measurement 5 ii)	10 μL N (400 nM)	Estimate $[RQ]_0$	RN :15; IT : [9-4]; N : [14-9]	210

Supplementary Table 2 | Detailed experimental procedure for toehold/handhold-mediated strand displacement characterisation.

The four trajectories of each reaction system and their controls were triggered by mechanical injection and measured simultaneously. To characterise the handhold-mediated strand displacement, the reaction was triggered with I_{hc} . To characterise the toehold rate k_t , I_{wc} was used. The species I_{wc} have, instead of the handhold-complementary domain, a random sequence of the same length.

Steps	Addition	Purpose	Expected concentrations in 200 μL (nM)	Volume (μL)
Measurement 1	10 μL RQ (300 nM) 130 μL Buffer	Estimate residual fluorescence baseline	RQ :15	140
Measurement 2	10 μL TN (200 nM) + N ~20 nM	Estimate $[N]_{\text{excess}}$	RQ :14; TN :10; RN :~1;	150
Measurement 3	[45-20] μL I (40 nM) + [5-30] μL Buffer	System kinetics	Evolve with time	200
Measurement 4	-	Estimate $[I]_0 + [N]_{\text{excess}}$	RQ : [5-10]; TN : [1-6]; RN : [10-5]; IT : [9-4]	200
Measurement 5 i)	10 μL I_{hc} (400 nM)	Estimate $[N]_0$	RQ :4; RN :11; IT :10; I : [19-14]	210
Measurement 5 ii)	10 μL N (400 nM)	Estimate $[RQ]_0$	RN :15; IT :10; I : [19-14]; N :16	220

Supplementary Figure 1 | Example of reporter complex *RQ* reporter characterisation.

Raw data from the triggering reaction of *RQ* with an *N/IT* complex with a 5 nt handhold, 8 nt spacer and 7 nt toehold at 25°C (5/8/7_25). Measurement 1 contains the baseline of the experiment produced by 15 nM of quenched *RQ*. In measurement 3, a variable amount of trigger *N/IT* was added, between 9 and 4 nM (8 nM in this case). After the reaction finished, the total amount of trigger *N/IT* was estimated from measurement 4. After that, 20 nM of *N* was added to trigger the rest of *RQ* and quantify it in measurement 5 ii). All concentrations are defined for a reaction volume of 200 μ L. The positive controls contained more than 10 nM of the Cy3-labelled reporter strand. The negative controls consisted of only the experimental buffer.

Supplementary Figure 2| Example of a handheld-mediated strand displacement characterisation experiment.

Raw data from the reaction system with a 7 nt handheld, 2 nt spacer and 5 nt toehold at 25°C (7/2/5_25) triggered with I_{hc} . Measurement 1 contains the baseline of the experiment produced by 15 nM of quenched RQ . Then, 10 nM of TN was added. $[N]_{excess}$ was inferred from the annealing increase the baseline, which was recorded in measurement 2. In measurement 3, a variable amount of trigger I_{hc} was added, between 9 and 4 nM (8 nM in this case). After the reaction finished, measurement 4 was used to estimate the total amount of trigger I_{hc} . Measurement 5 i) saturated the solution with 20 nM of I_{hc} to estimate the total amount of N in the solution. After that, 20 nM of N was added to trigger the rest of RQ and quantify it in measurement 5 ii). All concentrations are defined for a reaction volume of 200 μ L. The positive controls contained more than 10 nM of the Cy3-labelled reporter strand. The negative controls consisted of only the experimental buffer.

Supplementary Note II: Experimental procedure for the kinetic analysis of handhold detachment

The design of detachment experiments had to be modified to minimise R_2Q_2 leak reactions (**Supplementary Figure 3**). The triggering mechanism for the reporter R_2Q_2 is composed of an associative toehold with an 8-nt distal toehold and a 2-nt proximal toehold. The distal toehold is complementary to the handhold-complementary domain of I_{hc} and is unable to bind R_2Q_2 until IT detaches. However, the proximal toehold is complementary with a 3' additional domain in T . This 2-nt toehold-mediated displacement reaction can always take place when R_2Q_2 and T are together in the solution.

To reduce the time that R_2Q_2 and the T strand were together, R_2Q_2 was added as the trigger of the reaction. Steps of the experiments and an example of the fluorescence traces are shown in **Supplementary Table 3** and **Supplementary Figure 4**. The modification required the addition of a new control to the experiment. Besides the negative control and the positive controls for Cy5 and Cy3 fluorescence, a 4th control was added to quantify the residual fluorescence resultant produced by quenched R_2Q_2 . This “quenching control” was composed of 15 nM of the reporter complex R_2Q_2 from the same aliquot used during the experiments. This control was eventually saturated with IT complex to measure the total fluorescence and calculate the efficiency of quenching for R_2Q_2 . Then, the quenching efficiency was used to infer the fluorescence baseline for the experimental trajectories.

Control without RQ reporter complex:

The signal of reporter RQ was used to infer the concentrations of all the species in the solution. The Cy3 signal had a higher signal-to-noise ratio and informed about $[N]_{\text{excess}}$, which is relevant for the calculation of K_{eq} (eq.15). To prove that RQ did not modify K_{eq} , a subset of the reactions (7,8,9 and 10 nt handhold) was repeated without including RQ in the solution. In this control without RQ , the concentration of each species was inferred only from the signal produced by R_2Q_2 . The use of R_2Q_2 resulted in a more inexact determination of the species concentration, like N_{excess} , which could not be quantified (it was assumed to be 0). Results from this control, collected in **Supplementary Table 19**, showed that rate saturation still occurred for handholds shorter than 9 nt. The obtained K_{eq} slightly differed, but that effect could be attributed to the limitations during the quantification of the assay species.

Control at PAGE conditions:

To avoid excessive heating, during PAGE electrophoresis, NaCl was exchanged with a smaller amount of MgCl₂. The expected decrease of Gibbs free energy for the magnesium buffer condition is approximately 0.1 kcal/basepair.mol^{1, 2}. A new control was assayed to demonstrate that handhold detachment is comparable between both buffer conditions. The kinetic analysis experiment described in **Supplementary Table 3** was repeated for a buffer condition of 12.5 mM [MgCl₂] in 0.5 X TAE for a subset of the reactions (7,8,9,10 and 20 nt handhold). Due to the presence of divalent cations, the behaviour of the reporter system could change, hindering reaction species quantification and comparison between conditions. The fluorescence signal of R_2Q_2 reaction traces was normalised to the value obtained in measurement 5, except for the condition with a 20 nt handhold, which was normalised with measurement 6. The fluorescence signal was not processed with the positive controls either. The obtained trajectories are shown in **Supplementary Figure 5**. The result of the control show qualitatively the same behaviour observed in the NaCl buffer condition. The traces of the systems with 7 and 8 nt handhold have comparable rates, but the initial reaction rate decreases for 9 and 10 nt. Also, the system with a 20 nt handhold shows no detachment at all.

Supplementary Table 3| Detailed experimental procedure for the kinetic analysis of handheld detachment.

The four trajectories of each reaction system and their controls were triggered by mechanical injection and measured simultaneously. All the tested *TN* contained 3 nt proximal toeholds and a 2 nt spacer.

Conditions	Addition	Purpose	Expected concentrations in 200 μL (nM)	Volume (μL)
Measurement 1	10 μL <i>RQ</i> (300 nM) 80 μL Buffer	Estimate residual fluorescence baseline	<i>RQ</i> :15	90
Measurement 2	10 μL <i>TN</i> (200 nM) + <i>N</i> ~20 Nm	Estimate $[N]_{\text{excess}}$	<i>RQ</i> :14; <i>TN</i> :10; <i>RN</i> :~1;	100
Measurement 3	[45-20] μL <i>I_{hc}</i> (40 nM) + [5-30] μL Buffer	Trigger <i>TN</i> and estimate $[I]_0 + [N]_{\text{excess}}$	<i>RQ</i> : [5-10]; <i>TN</i> : [1-6]; <i>RN</i> : [10-5]; <i>IT</i> : [9-4]	150
Measurement 4	50 μL <i>R₂Q₂</i> (60 nM). Reporter is also added to the quenched control.	<i>R₂Q₂</i> kinetics	Evolve with time	200
Measurement 5	-	Estimate $[IT]_{\text{Total}}$	<i>RQ</i> : [5-10]; <i>TN</i> : [1-6]; <i>RN</i> : [10-5]; <i>R₂IT</i> : [9-4]; <i>R₂Q₂</i> : [6-11]	200
Measurement 6	10 μL <i>I_{hc}</i> (400 nM)	Estimate $[N]_0$	<i>RQ</i> : 4; <i>RN</i> : 11; <i>R₂IT</i> : 10; <i>R₂Q₂</i> : 5; <i>I</i> : [19-14]	210
Measurement 7	10 μL <i>T</i> (400 nM)	Estimate $[R_2Q_2]_0$	<i>RQ</i> : 4; <i>RN</i> : 11; <i>IT</i> : [10]; <i>R₂IT</i> : 15	220

Supplementary Figure 3| Reaction mechanisms of *R₂Q₂*

Raw data from *R₂Q₂* characterisation experiments. **Main reaction)** Intended reaction mechanism for *R₂Q₂*. A solution of 15 nM of *R₂Q₂* is triggered by 10 nM of *N/IT*. The detached *IT* triggered an associative toehold reaction with a rate of $7.14 \pm 0.23 \cdot 10^5 \text{ M}^{-1} \text{ s}^{-1}$ (k_{r2} ; **Supplementary Figure 21**). **Leak reaction)** Leak produced after adding 15 nM of *R₂Q₂* to a solution with 10 nM *TN*. The mere presence of *R₂Q₂* and *T* in the same solution triggered a 2 nt toehold-mediated strand displacement with an estimated rate of $4.66 \pm 0.89 \cdot 10^2 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Rate inferred from the mean of the assayed 2 nt toehold-mediated strand displacement reactions at 25° C). All concentrations are defined for a reaction volume of 200 μL . The positive controls contained more than 10 nM of the Cy5-labelled reporter strand. The negative controls consisted of only the experimental buffer.

Supplementary Figure 4| Example of the kinetic analysis of handheld detachment

Raw data from the reaction system with a 7 nt handheld, 2 nt spacer and 3 nt toehold at 25°C (7/2/3_25) triggered with I_{hc} . T strands in these experiments always contain an extra domain at 3' that triggers reporter R_2Q_2 . Measurement 1 contains the baseline of the experiment produced by 15 nM of quenched RQ . Then, 10 nM of TN was added. $[N]_{excess}$ was inferred from the increase in Cy3 baseline, which was recorded in measurement 2. After that, a variable amount of trigger I_{hc} was added, between 9 and 4 nM (8 nM in this case). After the reaction finished, measurement 3 estimated $[I]_0$, which is equal to the total amount of produce IT . During measurement 4, 15 nM of R_2Q_2 was added, and only red fluorescence was recorded. From this measurement, the signal of the quenched R_2Q_2 control was also measured. Measurement 5 recorded again the baseline of Cy3 and Cy5 fluorescence after the reaction finished. Measurement 6 saturated the solution with 20 nM of I_{hc} to estimate the total amount of N in the solution. After that, 20 nM of T was added to trigger the rest of R_2Q_2 and quantify it in measurement 7. Quenched control was triggered with 20 nM of IT . All concentrations are defined for a reaction volume of 200 μ L. Cy3 fluorescence was pre-eminently used for species quantification due to its higher signal-to-noise ratio and Cy5 fluorescence to follow the actual reaction kinetics. The positive controls contained more than 10 nM of the labelled reporter strands. The negative controls consisted of only the experimental buffer.

Supplementary Figure 5 | Kinetic analysis in PAGE buffer condition.

This control followed the experimental procedure contained in **Supplementary Table 3**, using a reaction buffer 12.5 mM MgCl₂ in 0.5X TAE. Mean of the normalised fluorescence data of the addition of *R2Q2* (Measurement 4) after the reaction of systems with variable handhold lengths, a 2 nt spacer, and a 3 nt toehold that has reacted with *Ihc*. Each trajectory included its standard error of the mean (shaded) for n=4. Fluorescence traces were normalised with the median value obtained in measurement 5 (except the 20 nt handhold condition, which was normalised with measurement 6). As observed in main text **Figure 5**, the reaction rate is still inversely correlated with handhold length, saturating at 8 nt long.

Supplementary Note III: Selective production of complexes far-from-equilibrium

The aim of the experiment was to determine the rate difference between the three competing displacement reactions. To minimise the effect produced by any concentration uncertainty, all *I*-strands and reporter complexes were present at much higher concentrations than the reaction trigger (10 nM TN_x). Each set of three different experiments was assayed simultaneously using the same aliquots of *I*_x strands and reporter complexes to ensure consistency between the different conditions. At the end of each experiment, all wells were triggered by adding *T* strand to check that all *I*_x and reporter complexes were present. Steps of the experiments and an example of the fluorescence traces are shown in **Supplementary Table 4** and **Supplementary Figure 6**. Fluorescence signal in this class of experiments was not calibrated as in previous ones because they did not need to be fitted. The gain was set to arbitrary values that do not saturate for 30 nM of each reporter strand. No bleed-through fluorescence was detected between channels during the experiments. The handheld sequences used during the experiments were: the first 8 nt of sequence used during the HMSD characterisations for RepA, a similar sequence with the same GC content for RepB, and the sequence complementary to *I*_{wc} for RepC (**Supplementary Table 23**).

Supplementary Table 4 | Detailed experimental procedure to produce far-from-equilibrium complexes

The three TN_x conditions and their controls were triggered by mechanical injection and measured simultaneously. All the tested TN were triggered with a 2 nt proximal toehold and contained an 8 nt handheld and a 2 nt spacer.

Conditions	Addition	Purpose	Expected concentrations in 200 μ L (nM)	Volume (μ L)
Measurement 1	20 μ L of each Rep (300 nM) + 130 μ L Mix of <i>I</i> (38.46 nM each)	Estimate residual fluorescence baseline	Rep _A :30; Rep _B :30; Rep _C :30; <i>I</i> _A :25; <i>I</i> _B :25; <i>I</i> _C :25;	190
Measurement 2	10 μ L TN_x (200 nM)	System kinetics	Evolve with time	200
Measurement 3	-	Give max. fluorescence value for each Rep	The <i>I</i> and Rep with the same handheld complementarity as N_x will have reacted.	200
Measurement 4	20 μ L <i>T</i> (1000 nM)	Trigger Rep to test that all species were present	Rep _x / <i>T</i> :~25; Rest of Rep _x could react with <i>T</i> free in solution.	220

Supplementary Figure 6| Example of raw experimental data from the production of complex $I_A T$

The experiment consisted of three competing reaction systems with different 8 nt handholds, 2 nt spacer and 2 nt toehold at 25°C (8/2/2_25). Measurement 1 contains the baseline of the experiment produced by 30 nM of each quenched reporter and 25 nM of each I_x . Then, 10 nM of TN_A were added, and the kinetics recorded in measurement 2. After the reaction finished, measurement 3 estimated the total amount of produced IT . The fluorescence signal observed for Rep_A during this measurement was then used to normalise the Cy5 fluorescence of all the experiments. After the experiment, in measurement 4, the wells were saturated with 20 nM of T to trigger all the reporter complexes. All concentrations are defined for a reaction volume of 200 μ L. The positive controls contained more than 30 nM of the labelled reporter strands. The negative control consisted of only the experimental buffer.

Supplementary Note IV: Experimental protocol for fluorescence calibration curves

Two fresh aliquots of 1 μM of the fluorophore-labelled reporter strand were created from a stock solution of 100 μM . The aliquots contained 10 μL of the stock solution in 990 μL of TAE 1x buffer (pH=8.3). From each 1 μM aliquot, two more were created with a final concentration of 200 μM , producing a total of four 200 μM aliquots. Four replicas of the calibration curve were pipetted from the 200 μM aliquots, for a range of 1 to 15 μM and a final volume of 160 μL . The 200 μM aliquot used for each point was chosen randomly. The volume was increased to 200 μL with 40 μL of a solution of 5M NaCl in TAE 1x, for a final experimental buffer of 1M NaCl in TAE 1x. The plate was incubated at the assay temperature for 30 minutes until the fluorescence signal stabilized. Each calibration point was obtained from the median value of 10 data points. For each replica of the calibration curve, calibration points were treated as outliers when deviating more than 2 nM from its expected value in a preliminary linear regression.

The photomultiplier gain was set so that its dynamic range was 1.25x the highest fluorescence value in the calibration experiments. Two replicas for the calibration curve of Cy5 contained concentration values up to 16 nM. No bleed-through fluorescence between Cy5 and Cy3 was observed during these calibration experiments.

Supplementary Note V: Fitting procedure for fluorescence calibration curves

The value of a negative control consisting of only the experimental buffer was subtracted from all the points in the calibration curves. All calibration curves were fitted with a weighted linear regression to a model of the form $f(x)=ax$. The residuals were weighted with the inverse of the fluorescence reading to account for the Poisson distribution of the photomultiplier counts³. Regression coefficient value is reported with 95% confidence bounds. Fitting results and their residuals are shown in **Supplementary Figures 7 to 10**.

Supplementary Figure 7 | RQ reporter complex (Cy3) calibration curve at 25°C. Gain 2251

Used to extrapolate concentrations for all the characterisation experiments at 25°C. Left: Regression line. Right: Fit residuals. $a = 1.498e+04$ ($1.469e+04$, $1.527e+04$). Goodness of fit: SSE: $6.915e+04$. R-square: 0.9915. Adjusted R-square: 0.9915. RMSE: 27.87.

Supplementary Figure 8 | RQ reporter complex (Cy3) calibration curve at 37°C. Gain 2336

Used to extrapolate concentrations for all the characterisation experiments at 37°C. Left: Regression line. Right: Fit residuals. $a=1.461e+04$ ($1.44e+04$, $1.481e+04$). Goodness of fit: SSE: $3.386e+04$. R-square: 0.9955. Adjusted R-square: 0.9955. RMSE: 19.5.

Supplementary Figure 9| RQ reporter complex (Cy3) calibration curve at 25 degrees. Gain 2230

Used to extrapolate concentrations for detachment assays. Gain was reduced during this calibration to avoid the frequency of detector saturation events. Left: Regression line. Right: Fit residuals. $a = 1.319 \times 10^4$ (1.298×10^4 , 1.339×10^4). Goodness of fit: SSE: 2.189×10^4 . R-square: 0.9923. Adjusted R-square: 0.9923. RMSE: 19.26.

Supplementary Figure 10| R_2Q_2 reporter complex (Cy5) calibration curve at 25 degrees. Gain 2816

Used to extrapolate concentrations for detachment assays. Left: Regression line. Right: Fit residuals. $a = 1.4 \times 10^4$ (1.371×10^4 , 1.43×10^4). Goodness of fit: SSE: 4.667×10^4 . R-square: 0.993. Adjusted R-square: 0.993. RMSE: 27.22.

Supplementary Note VI: Fluorescence data processing

To compare calibration lines with the fluorescence signal produced during the experiments, the experimental fluorescence signal was processed as follows:

Step 1. Removing background fluorescence produced by experimental buffer:

The value of the negative control at each time point was subtracted from the fluorescence value of all wells.

Step 2. Merging all measurements in a single file:

All measurements, except the actual kinetics, were converted in a single data point per well, with its fluorescence value given by the median of all the data points in that set. Then, all the single data points corresponding to the same experiment were merged into a file that also contained the complete kinetics of the experiment.

Step 3. Calculation of the fluorescence variation in the positive control signal:

During experiments, the plate could be taken out of the plate reader or liquids at slightly different temperatures could be injected. These temperature fluctuations produced distortions in the fluorescence signal, especially for assays at 37°C. The fluorescence value of the positive control for each data point was used to track such distortions of the fluorescence signal due to temperature changes. The positive control value during the 1st measurement was the reference value for the experiment since all wells were at the expected experimental temperature, and any photobleaching was assumed to be negligible.

Step 4- Correction of all trajectories with the positive control ratio:

All data points were multiplied by the ratio between the positive control reference value and its value at each time. When more than one positive control of the same species was present in the plate, the positive control ratio at each time was obtained by calculating the mean of the ratio of all positive controls in the plate. This approach could be applied because the variation of fluorescence produced by temperature changes is proportional to the fluorescent signal⁴. **Supplementary Figure 11** contains an example of the signal processing result.

Step 4.1- For quick measurements (*Reporter Characterisation kinetics*):

The correction described in step 4 was detrimental to the precision of the measurements when positive control fluorescence traces had a lower signal-to-noise ratio, and signal fluorescence artefacts were more common. This happened particularly during quick measurements, like *RQ* reporter characterisations. Besides, since all the kinetics were measured individually, positive controls could not be measured in parallel. For these cases, all the data points in the kinetics section were just multiplied by the ratio between the reference control value and control value obtained in measurement 4, after the kinetics finished (See **Supplementary Note I**).

Step 5- Correction for residual fluorescence:

A difference between the signal of the calibration curves and the experiments is that in the former, no quenched *RQ* is present. To compare both fluorescence signals to infer *RQ* concentrations, the background due to the residual fluorescence of quenched *RQ* complexes needed to be subtracted from the experimental measurements. The residual fluorescence was subtracted from the signal F_i at data point i using the formula:

$$F_i^* = F_{max} \frac{F_i - F_1}{(F_{max} - F_1)} \quad (1).$$

Here, F_i^* is the data point i with the corrected background fluorescence, F_1 the residual fluorescence background value during measurement 1 and F_{max} the final fluorescence value in measurement 6 (**Supplementary Note I**). The method assumes that any difference of fluorescence with the negative control during measurement 1 is due to unquenched RQ , being that increment proportional to $[RQ]_0$ (**Supplementary Note I**). The fluorescence was corrected in such a way that F_1 became 0 and F_{max} did not change.

Step 6- Transformation of fluorescence arbitrary units to nM:

Once all the corrections are applied, all the values of the data points are transformed to nM by dividing them by the slope of their corresponding calibration curve (**Supplementary Note V**).

Supplementary Figure 11| Example of fluorescence data processing

Four traces from a reaction system with a 20 nt handheld, 5 nt spacer and 2 nt proximal toehold (20/5/2_25), triggered with [4-7] nM of I_{wc} . **Left)** Raw data. Irregularities during the experiment can produce bumps in the fluorescence that tampers the fitting process. These fluctuations appeared in all trajectories and controls contained in the plate. This example contained three different positive controls for Cy3. Even when controls did not have the same Cy3 concentrations, their variations in fluorescence were proportional. **Right)** The same data after applying the fluorescence processing pipeline. Fluorescence units were transformed to nM, and the bumps disappeared. All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Note VII: Additional fluorescence processing

Out of range fluorescence:

The photomultiplier gain was set so that its dynamic range was 1.25x the fluorescence signal produced by 15 nM of Cy3. However, during some assays, the total amount of RQ was slightly over that concentration. This resulted in a fluorescence signal over the photomultiplier detection limit when measuring the fluorescence produced by $[RQ]_0$. To correct these out-of-range data points, the gain was slightly decreased until no saturation events appeared. After that, the fluorescence values from the saturated and the low-gain measurement were compared. The fluorescence change ratio between the measurements was calculated with the positive controls and the samples that did not saturate. The corrected fluorescence values of the saturated data points were estimated by multiplying the data point at a low gain by the mean of the obtained ratios. This correction assumed that linearity is maintained at the two different gains. Any error introduced in the inferred $[RQ]_0$ using this approximation is considered negligible since variations of $[RQ]_0$ had a small impact in the fitting procedure for most of the systems. All files modified this way are listed in the file `Original_files/Raw_files_modifications.txt` found in www.zenodo.org/record/4389212.

Out of range fluorescence during kinetics:

When out-of-range outliers appeared during the kinetics, that sample was discarded. For experiments with kinetics longer than 2 hours, the datapoint with the outlier was removed from all samples instead.

Estimation of residual fluorescence during kinetic analysis of handheld detachment:

During detachment assays R_2Q_2 was used to trigger the reaction. This fact precluded the measurement of the Cy5 fluorescence baseline of each fluorescence trace. Thus, Cy5 fluorescence baselines were calculated by using the quenched control of each experiment. The quenching efficiency for R_2Q_2 was estimated from the quenched control by calculating the ratio between its initial fluorescence value and its fluorescence value once fully triggered. The quenching efficiency was multiplied by the maximum fluorescence of each trace to calculate its Cy5 fluorescence baseline. The obtained Cy5 baselines were used to correct the fluorescence traces with **Supplementary equation 1**, in the same way as with RQ .

Supplementary Note VIII: Reaction controls for RQ

The control aim was to prove that no leak reaction occurred between *TN* and *RQ*. A plate with 18 reactions was set. The 18 wells contained a solution of the *RQ* reporter and *TN* with a 7 nt toehold. 6 different systems were tested in triplicate: 0, 5 and 20 nt long handholds with spacers of 0 and 8 nt. The solution of *RQ* and *TN* was incubated for more than 24 hours without producing any fluorescent signal. After that, three different concentrations of I_{hc} (4,6 and 8 nM) were added to each system to prove the proportionality between the fluorescence signal and $[I_{hc}]_0$. Steps of the reaction control for *RQ* and the obtained results are shown in **Supplementary Table 5** and **Supplementary Figure 12**. An additional control was assayed to test if the reporter could not be triggered by handhold complementarity alone. The modification included to the additional control was that the added *I*-strand only had complementarity with the handhold domain in *N* and not with *T* (**Invader-random 3'**, **See Supplementary Table 22**). Fluorescence was measured for more than 30 hours without any increase of signal observed. Steps of the control using *I* without *T* complementarity and the obtained results are shown in **Supplementary Table 6** and **Supplementary Figure 13**.

Supplementary Table 5 | Steps of the reaction control for RQ

All the trajectories in the plate and their controls were triggered by mechanical injection and measured simultaneously. All the tested *TN* contained a 7 nt proximal toehold.

Steps	Addition	Description	Purpose	Volume (μL)
Measurement 1	10 μL <i>RQ</i> (300 nM) + 10 μL <i>TN</i> (200 nM) + 130 μL Buffer	Kinetics during 16.5 hours of the <i>RQ</i> basal fluorescence	Prove that the presence of <i>TN</i> does not trigger <i>RQ</i>	150
Measurement 2	-	Measurement after ~24 hours	Prove signal has not changed	150
Measurement 3	[40/30/20] μL I_{hc} (40 nM) + [10/20/30] μL Buffer	Trigger <i>TN</i>	Prove that <i>I</i> can trigger the reaction, and check proportionality of fluorescence signal.	200
Measurement 4	10 μL <i>I</i> (400 nM)	Trigger all <i>TN</i>	Prove triggering of total <i>TN</i>	210

Supplementary Table 6 | Steps of the control for I without T-complementarity

All the trajectories in the plate and their controls were triggered by mechanical injection and measured simultaneously. All the tested *TN* contained a 7 nt proximal toehold.

Steps	Addition	Description	Purpose	Volume (μL)
Measurement 1	10 μL <i>RQ</i> (300 nM) + 130 μL Buffer	Measure baseline	Remove the residual fluorescence	140
Measurement 2	10 μL <i>TN</i> (200 nM)	Measure new baseline	Check the signal produced by $[N]_{excess}$	150
Measurement 3	50 μL <i>I</i> (40 nM)	Add <i>I</i> without <i>T</i> complementarity.	Prove that handhold interaction does not trigger the reporter.	200
Measurement 4	-	Measure after one day	Prove signal has not changed	200

Supplementary Figure 12| Reporter *RQ* control, proving the absence of leak when mixed with unreacted TN

Processed fluorescence data of 6 different systems tested in triplicate: 0, 5 and 20 nt long handholds with spacers of 0 and 8 nt, and a proximal toehold of 7 nt. Mean of the signal produced for each handhold length with its standard error (shaded). The baseline of fluorescence produced by $[N]_{\text{excess}}$ was removed to clearly show the scale of the fluorescence signal noise. **Left)** After the kinetics of measurement 1, measurement 2 is taken after approximately one day. No significant increase in the fluorescence signal could be observed. **Right)** For each replica of each system, a different concentration of I_{hc} was added: 8, 6 and 4 nM. The produced signal is proportional to the concentration of I_{hc} , which allows the quantification of the strand. All concentrations are defined for a reaction volume of 200 μL .

Supplementary Figure 13| Reporter *RQ* control using *I* without *T*-complementarity, proving the absence of reaction when using an *I* strand that is only handhold complementary

Processed fluorescence data of 6 different systems tested in triplicate: 0, 5 and 20 nt long handholds with spacers of 0 and 8 nt, and a proximal toehold of 7 nt. Mean of the signal produced for each handhold length with its standard error (shaded) for $n=9$. Measurement 1 set the baseline produced by 15 nM of quenched *RQ*. Measurement 2 set a new baseline produced by the $[N]_{\text{excess}}$ after adding 10 nM *TN*. In measurement 3, 10 nM of *I* without *T* complementarity is added and measured for at least 30 hours. No increase in the fluorescence signal was observed. Measurement 4 is taken after approximately one day, and no increase is observed either. All concentrations are defined for a reaction volume of 200 μL .

Supplementary Note IX: Reaction controls for R_2Q_2

Five different conditions were assayed in duplicate, testing different qualities of R_2Q_2 . The used T contained a 3 nt proximal toehold and, to trigger the reporter R_2Q_2 , an extra 5' domain. Condition 1 proved that the reporter could be quickly triggered by the addition of the complex IT . During the experiments of condition 2, R_2Q_2 was quickly triggered when a TN with a short handhold (6 nt) reacted with I_{hc} . Conditions 3 and 4 showed that the leak reaction of R_2Q_2 with TN (reported in **Supplementary Note II**) is independent of handhold length. Besides, condition 4 proved that systems with 20 nt handholds could not trigger R_2Q_2 quickly after displacement since the produced IT cannot detach from the handhold. Finally, condition 5 showed that R_2Q_2 does not change its fluorescence signal for 3 hours in the presence of only I_{hc} . Concentrations of R_2Q_2 differed between conditions due to an unoptimized annealing process of R_2Q_2 . However, the qualitative effect of each condition can still be observed. Steps for each condition and the obtained results are shown in **Supplementary Table 7** and **Supplementary Figure 14**.

Supplementary Table 7 | Steps of the R_2Q_2 control

All the trajectories in the plate and their controls were triggered by mechanical injection and measured simultaneously. All the tested TN contained a 3 nt proximal toehold. Each column of the table contains the species added to the conditions before each measurement.

Conditions	Measurement 1	Measurement2	Measurement 3	Measurement 4
Condition 1	75 μ L R_2Q_2 (40 nM) + 75 μ L Buffer	10 μ L IT (200 nM) + 40 μ L Buffer	10 μ L I_{hc} (400 nM)	10 μ L IT (400 nM)
Condition 2	75 μ L R_2Q_2 (40 nM) + + 75 μ L Buffer	10 μ L $TN(6/2/3)$ (200 nM) + 40 μ L I_{hc} (40 nM)	10 μ L I_{hc} (400 nM)	10 μ L IT (400 nM)
Condition 3	75 μ L R_2Q_2 (40 nM) + + 75 μ L Buffer	10 μ L $TN(0/2/3)$ (200 nM) + 40 μ L I_{hc} (40 nM)	10 μ L I_{hc} (400 nM)	10 μ L IT (400 nM)
Condition 4	75 μ L R_2Q_2 (40 nM) + + 75 μ L Buffer	10 μ L $TN(20/2/3)$ (200 nM) + 40 μ L I_{hc} (40 nM)	10 μ L I_{hc} (400 nM)	10 μ L IT (400 nM)
Condition 5	75 μ L R_2Q_2 (40 nM) + + 75 μ L Buffer	50 μ L I_{hc} (40 nM)	10 μ L I_{hc} (400 nM)	10 μ L Buffer

Supplementary Figure 14| Reporter R_2Q_2 controls

Processed fluorescence data of five different conditions tested in duplicate (except condition 1). Mean of the signal produced for each condition with its standard error (shaded). The purpose of each condition is described in **Supplementary Note VIII**. Measure 1 set the baseline produced by 15 nM of quenched R_2Q_2 and measure 2 contains the kinetics of every condition (Described in **Supplementary Table 7**). Measurement 3 was taken after adding 20 nM I_{hc} to all conditions. During measurement 3, condition 3 did not reach saturation due to the slow reaction rate of the 3 nt proximal toehold reaction. In the case of condition 4, it did not reach saturation because the produced IT could not detach from the 20 nt handhold and trigger the reporter. Measurement 4 was taken after adding 20 nM IT to all conditions, except condition 5. All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Note X: Specificity controls for Rep_A, Rep_B and Rep_C

For the selective production of complexes far-from-equilibrium, not only must the handhold do its part, but the reporters that monitor the reactions (Rep_A, Rep_B, and Rep_C) must be able to discern between the IT_x complexes. The purpose of these controls was to confirm that the designed reporters react only with the IT_x with which they had handhold complementarity.

Since the design of the reporters was the same as R_2Q_2 (Rep_A being the same reporter) a slow leak reaction was expected in the presence of strand T . This leak was slower for Rep_B and Rep_C, since these reporters contained a non- T -complementary 2 nt clamp at the end of the displaced domain that helped to maintain quencher and fluorescence strands hybridised. For Rep_A this clamp was not needed since the additional unspecific interaction between Cy5 and the quencher was strong enough to stabilize the hybridisation of Rep_A strands⁵.

The controls tested the rate at which the specific reporters reacted with each TN complex and if any leak could be produced by the interaction of the I_x strands alone with the reporter. The results prove that during the time that took the reporters to consume 10 nM of complementary TN_x , the amount of non-complementary TN_x consumed is less than 10%. The additions during each step of the control experiments are described in **Supplementary Table 8**. Results for Rep_A, Rep_B and Rep_C are shown in **Supplementary Figures 15, 16 and 17**, respectively.

Supplementary Table 8 | Steps of the selectivity controls for Rep complexes

All the trajectories in the plate and their controls were triggered by mechanical injection and measured simultaneously. The used T contained a 3 nt proximal toehold and an extra 5' domain to trigger the reporter R_2Q_2 . Each column of the table contains the species added to the conditions before each measurement.

Conditions	Measurement 1	Measurement 2	Measurement 3	Measurement 4
$I_x + TN_x$	20 μ L Rep (300 nM) + 5 μ L I_x (1 μ M) + 165 μ L Buffer	10 μ L TN_x (200 nM)	20 μ L T (1 μ M)	5 μ L I -complementary to the reporter (1 μ M)
$I_A + I_B + I_C$	20 μ L Rep (300 nM) + 5 μ L I_A (1 μ M) + 5 μ L I_B (1 μ M) + 5 μ L I_C (1 μ M) + 155 μ L Buffer	10 μ L Buffer	20 μ L T (1 μ M)	5 μ L I -complementary to the reporter (1 μ M)

Supplementary Figure 15| Control to prove Rep_A specificity for $I_A T$

Processed fluorescence data normalised to the max value of $I_A + TN_A$ condition during measurement 2. Measurement 1 set the baseline produced by 30 nM of quenched Rep_A and 25 nM of I_x (75 nM in total in condition $I_A + I_B + I_C$). Measurement 2 contains the kinetics of every condition after adding 10 nM of the specified TN_x or just buffer for condition $I_A + I_B + I_C$. Condition $I_A + TN_A$ is the only one capable of reacting at high speed. Measurement 3 was taken after saturating each condition with 100 nM of T , so only the wells containing I_A managed to produce fluorescence at an increased rate. In measurement 4, all wells were saturated with 40 nM of I_A to trigger all Rep_A. Rep_A, in comparison with the other two reporters, had more leakiness, especially with TN_B since handhold A and B have a similar sequence. However, in competition with the other two reporters, the leakiness was greatly reduced. All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Figure 16| Control to prove Rep_B specificity for $I_B T$

Processed fluorescence data normalised to the max value of $I_B + TN_B$ condition during measurement 2. Measurement 1 set the baseline produced by 30 nM of quenched Rep_B and 25 nM of I_x (75 nM in total in condition $I_A + I_B + I_C$). Measurement 2 contains the kinetics of every condition after adding 10 nM of the specified TN_x or just buffer for condition $I_A + I_B + I_C$. Condition $I_B + TN_B$ is the only one capable of reacting at high speed. Measurement 3 was taken after saturating each condition with 100 nM of T , so only the wells containing I_B managed to produce fluorescence at an increased rate. In measurement 4, all wells were saturated with 40 nM of I_B to trigger all Rep_B. Rep_B showed less leakiness than Rep_A, but the similarity between handholds A and B still resulted in a quicker leak reaction for I_A than for I_C . All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Figure 17| Control to prove Rep_C specificity for $I_C T$

Processed fluorescence data normalised to the max value of $I_C + TN_C$ condition during measurement 2. Measurement 1 set the baseline produced by 30 nM of quenched Rep_C and 25 nM of I_x (75 nM in total in condition $I_A + I_B + I_C$). Measurement 2 contains the kinetics of every condition after adding 10 nM of the specified TN_x or just buffer for condition $I_A + I_B + I_C$. Condition $I_C + TN_C$ is the only one capable of reacting at high speed. Measurement 3 was taken after saturating each condition with 100 nM of T , so only the wells containing I_C managed to produce fluorescence at an increased rate. In measurement 4, all wells were saturated with 40 nM of I_C to trigger all Rep_C. Rep_C showed no detectable leakiness. All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Note XI: Implementation of handhold-mediated strand displacement reaction network as an ODE system

The handhold-mediated strand displacement chemical reaction network is composed of the following reactions:

Binding of I to TN :

N displacement by I :

Competing TMSD:

Blocking and unblocking of TN 's handhold by free in solution IT :

TN with blocked handhold reacting via TMSD:

RQ triggering by TMSD.

Produced quencher strand (Q) is inert.

Free in solution IT attaching and detaching from the handhold in RN

Free-in-solution I attaching and detaching from the handhold in RN

RN/I triggering TN and TN/IT by toehold interaction.

The ODE model did not include inert species (Q), or those which concentration were negligible for all reaction systems (N). Considering the N molecule produced by direct TMSD (**Supplementary equation 4**) as a distinct species greatly increased the complexity of the ODE model that results from this reaction network. Since k_r and k_b are always large, we assume for simplicity that direct TMSD instead produces the complex N/IT , which swiftly binds to the reporter (**Supplementary equations 7 and 10**).

The subsequent detachment of IT from the triggered reporter complex RN is then accounted for by the backwards reaction in **Supplementary equation 8**.

The introduced approximation could fail when both the toehold and handhold are long. In this limit, toehold-based displacements will produce N quicker, and this N can sequester I -strands with its handhold, either before or after triggering the reporter. In our approximation, the N species will instead remain bound to the reacted invader-target complex and cannot perform sequestration of the unreacted invader. However, regions of long proximal toeholds are not the primary focus of this work, and any discrepancies introduced are likely small on the scale of over four orders of magnitude considered here.

No base-pair-level intermediates were considered explicitly for any of the branch migration reactions. The resultant ODE model was composed of the mass-action law based differential equations:

$$\frac{d[TN/I]}{dt} = k_b[TN][I] - k_d[TN/I] - k_m[TN/I], \quad (12)$$

$$\frac{d[TN/IT]}{dt} = k_b[TN][IT] - k_d[TN/IT] - k_t[TN/IT][I], \quad (13)$$

$$\frac{d[N/IT]}{dt} = k_m[TN/I] + k_t[TN][I] + k_t[TN/IT][I] - k_r[N/IT][RQ] + k_t[RN/I][TN] + k_t[RN/I][TN/IT], \quad (14)$$

$$\frac{d[IT]}{dt} = k_t[TN/IT][I] + k_d[TN/IT] + k_d[RN/IT] - k_b[TN][IT] - k_b[RN][IT], \quad (15)$$

$$\frac{d[RN]}{dt} = k_d[RN/I] + k_d[RN/IT] - k_b[RN][I] - k_b[RN][IT] + k_t[RN/I][TN], \quad (16)$$

$$\frac{d[RN/I]}{dt} = k_b[RN][I] + k_b[RN][IT] - k_d[RN/I] - k_d[RN/IT] - k_t[RN/I][TN] - k_t[RN/I][TN/IT], \quad (17)$$

$$\frac{d[RN/IT]}{dt} = k_r[N/IT][RQ] + k_b[RN][IT] - k_d[RN/IT] + k_t[RN/I][TN/IT]. \quad (18)$$

With conservation laws for the initial species:

$$[I] = [I]_0 - [TN/I] - [TN/IT] - [N/IT] - [IT] - [RN/I] - [RN/IT], \quad (19)$$

$$[TN] = [TN]_0 - [TN/I] - 2[TN/IT] - [N/IT] - [IT] - [RN/IT], \quad (20)$$

$$[RQ] = [RQ]_0 - [RN] - [RN/I] - [RN/IT], \quad (21)$$

And initial conditions: $[TN/I]_0, [TN/IT]_0, [N/IT]_0, [IT]_0, [RN/I]_0, [RN/IT]_0 = 0$ and $[RN] = [N]_{\text{excess}}$, being $[N]_{\text{excess}}$ the excess of N resultant from the inexact stoichiometry when annealing TN with a 10% excess of N . $[N]_{\text{excess}}, [I]_0, [N]_0 = [TN]_0 + [N]_0$ and $[RQ]_0$ were inferred from the fluorescence signal produced during characterisation experiments as described in **Supplementary Note I**.

Supplementary Note XII: General fitting procedure

All fluorescence data were first corrected as described in **Supplementary Note VI** and the baseline produced by $[N]_{\text{excess}}$ subtracted. All fitting procedures included, in addition to their rate constants, the reaction trigger concentration and t_0 for each trajectory.

The initial guesses for the reaction trigger concentrations were inferred from the fluorescence value of measurement 4. The last value of the recorded kinetics in measurement 3 was compared with measurement 4 to determine the percentage of completion of the reaction during the kinetics recording. For kinetics that did not reach at least 90% completion, the concentration of reaction trigger was only allowed to fluctuate $\pm 10\%$ from its initial guess.

The initial guess for t_0 (t_{0_init}) was obtained from the cutting point of a regression line with the x-axis, except during *reporter RQ fitting* (**Supplementary Note XIII**). The regression line was calculated by fitting the values of the kinetics that were under 50% completion, where the trajectories were almost linear.

Fittings were performed with the MATLAB® function *fminsearch*, based on the simplex search method by Lagarias et al.⁶ The common logarithm of the error function was minimised for all the fluorescence traces of a given system:

$$\log_{10}(\text{mean weighted error}) = \log_{10} \frac{\sum_i \sum_t \frac{(f_{\text{exp}}(i,t) - f_{\text{model}}(i,t))^2}{f_{\text{model}}(i,t)}}{n}, \quad (22)$$

where $f_{\text{exp}}(i,t)$ is the inferred concentration at time t of the trace i , and $f_{\text{model}}(i,t)$ is the sum of concentrations of all fluorescent species predicted by the model at time t at the reagent conditions of the trace i . The error at each point was weighted by $f_{\text{model}}(i,t)$ to account for the Poisson distribution of the photomultiplier detection error as done by Zhang et al.³. When more than one trace was fitted simultaneously, the mean total weighted error was calculated by dividing the sum of the error by the number of fitted traces (n).

All MATLAB fitting scripts are available for download at www.zenodo.org/record/4389212.

Supplementary Note XIII: Reporter RQ fitting

The experimental value of k_r was obtained for every species of N used during the characterisation experiments. **Equation 8** in the main text was fitted to each trajectory individually. Since all reactions were completed in a minute, the effect of the initial leak on t_0 was not significant. Thus, the time of N/IT injection was considered t_{0_init} .

The model was fitted in a three-step iteration. During the 1st round of fittings, the initial guess k_{r_init} was inferred from the time at which the reaction reached 90% completion:

$$k_{r_init} = \frac{\ln(10 - 9 \frac{[N]_0}{[RQ]_0})}{t_{90\%}([RQ]_0 - [N]_0)}, \quad (23)$$

with $t_{90\%}$ being the time at which 90% of $[I]_0$ reacted, and $[N]_0$ the total amount of strand N injected in the form of N/IT . During the 2nd round of fittings, k_{r_init} was the mean of all the k_r obtained during the first round at the same temperature (k_{r_init} at 25°C: $5.10^6 \text{ s}^{-1}\text{M}^{-1}$; k_{r_init} at 37°C: $4.8 \cdot 10^6 \text{ s}^{-1}\text{M}^{-1}$). For the 3rd round, all systems were fitted using as initial guesses their results of the second round for k_r , t_0 and $[N]_0$. All fitted k_r at 25 °C and 37 °C are collected in **Supplementary Tables 9 and 10**, respectively

Supplementary Table 9 | Fitted k_r at 25°C

Data obtained using procedures described in **Supplementary Notes I and XIII**. The reactant strands used were all N strands “Incumbent x hh/x sp” (**Supplementary Table 20**) with “Target (7 th)” (**Supplementary Table 21**) and “Invader (I_{hc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (R)” and “Reporter 1 quencher (Q)” (**Supplementary Table 23**). Mean of all fitted $k_r = 5.068 \cdot 10^6 \text{ s}^{-1}\text{M}^{-1}$; Standard deviation = $0.505 \cdot 10^6 \text{ s}^{-1}\text{M}^{-1}$; Standard mean error (SEM) = $0.052 \cdot 10^6 \text{ s}^{-1}\text{M}^{-1}$.

Condition (handhold/spacer)	Individual experiments $k_r \cdot 10^6 \text{ (s}^{-1}\text{M}^{-1})$				Mean $k_r \cdot 10^6 \pm \text{SEM}$ ($\text{s}^{-1}\text{M}^{-1}$)
0/0	5.46	5.42	5.55	5.60	5.506±0.041
0/1	5.41	6.11	5.54	6.02	5.77±0.17
0/2	5.18	5.18	5.24	5.09	5.17±0.03
0/3	5.45	4.99	5.08	5.04	5.14±0.1
0/5	5.42	5.63	5.63	5.79	5.617±0.076
0/8	5.51	5.29	5.43	5.21	5.359±0.069
4/2	5.47	5.25	5.44	4.08	5.06±0.33
5/0	5.32	5.06	5.63	6.11	5.55±0.22
5/1	3.95	4.21	4.27	4.58	4.25±0.13
5/2	4.66	4.35	4.33	5.68	4.76±0.32
5/3	5.42	5.69	4.49	5.38	5.24±0.26
5/5	5.63	5.61	5.98	5.69	5.728±0.086
5/8	5.08	4.63	5.03	4.65	4.85±0.12
6/2	4.46	4.91	4.53	4.52	4.61±0.1
7/2	5.00	4.85	4.94	5.10	4.97±0.51
8/2	4.82	4.55	4.72	5.05	4.78±0.1
9/2	4.60	4.54	4.71	4.83	4.67±0.064
10/2	4.80	4.83	6.01	4.80	5.11±0.30
20/0	3.97	4.13	3.77	4.14	4.004±0.087
20/1	5.24	5.06	5.45	5.31	5.265±0.081
20/2	4.87	4.96	5.11	4.75	4.922±0.078
20/3	4.91	5.01	5.00	4.80	4.931±0.049
20/5	5.13	5.06	5.47	5.58	5.31±0.13
20/8	5.12	5.12	5.12	4.9	5.08±0.041

Supplementary Table 10| Fitted k_r at 37°C

Data obtained using procedures described in **Supplementary Notes I and XIII**. The reactant strands used were all *N* strands “Incumbent x hh/x sp” except the ones with spacer 1,3,5 nt (**Supplementary Table 20**) with “Target (7 th)” (**Supplementary Table 21**) and “Invader (I_{hc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (**Supplementary Table 23**). Legend: **Red highlight**: k_r values that were not obtained from direct experimental evidence. These values were estimated from the mean of all the obtained k_r at 37°C. Mean of all $k_r = 4.52 \cdot 10^6 \text{s}^{-1} \text{M}^{-1}$; Standard deviation = $0.53 \cdot 10^6 \text{s}^{-1} \text{M}^{-1}$; Standard mean error (SEM) = $0.11 \cdot 10^6 \text{s}^{-1} \text{M}^{-1}$

Condition (handhold/spacer)	Individual experiments $k_r \cdot 10^6 (\text{s}^{-1} \text{M}^{-1})$				Mean $k_r \cdot 10^6 \pm \text{SEM}$ ($\text{s}^{-1} \text{M}^{-1}$)
0/0	4.38	4.61	4.92	5.15	4.76±0.17
0/8	4.66	4.87	4.93	5.27	4.94±0.13
4/2	-	-	-	-	4.52±0.11
5/0	4.65	4.57	4.84	4.64	4.677±0.057
5/2	-	-	-	-	4.52±0.11
5/8	4.07	4.26	4.54	4.88	4.44±0.18
6/2	-	-	-	-	4.52±0.11
7/2	-	-	-	-	4.52±0.11
8/2	-	-	-	-	4.52±0.11
9/2	-	-	-	-	4.52±0.11
10/2	-	-	-	-	4.52±0.11
20/0	3.29	3.52	3.76	3.54	3.525±0.096
20/2	-	-	-	-	4.52±0.11
20/8	4.63	4.84	4.76	4.95	4.794±0.069

Supplementary Note XIV: TMSD fitting

The value of k_t was fitted using the individual trajectories of each system with a simplified ODE model consisting of **Supplementary equations 14 and 18**, assuming $[TN/IT]$, k_b , k_d , and $k_m = 0$.

$$\frac{d[N]}{dt} = k_t[TN][I] - k_r[N][RQ], \quad (24)$$

$$\frac{d[RN]}{dt} = k_r[N][RQ]. \quad (25)$$

The model was fitted iteratively in three steps. During the 1st round of fittings, k_{t_init} was obtained using a modification of **Supplementary equation 23**, exchanging $[N]_0$ for $[I]_0$, and $[RQ]_0$ for $[TN]_0$. For kinetics that did not reach 90% of completion, k_{t_init} was obtained from the generalized form of **Supplementary equation 23**:

$$k_{t_init} = \frac{\ln\left(\frac{[I]_0}{[I]_{end}} + \frac{[I]_0}{[TN]_0} \left(1 - \frac{[I]_0}{[I]_{end}}\right)\right)}{t_{end}[TN]_0 - [I]_0}, \quad (26)$$

with $[I]_{end}$ being the maximum $[I]$ value recorded during the kinetics and t_{end} the final time of the measurement (corrected with t_{0_init}). During the 2nd round of fittings, k_{t_init} was the median of all the k_t obtained for that system during the 1st round. For the 3rd round, all systems were fitted using as initial guesses its results of the second round for k_t , t_0 and $[I]_0$.

Reactions triggered with I_{hQ} but in the absence of handholds in N also followed this fitting procedure to confirm the value of k_t obtained with I_{wC} . All fitted k_t are collected in **Supplementary Tables 15 to 17**.

Supplementary Note XV: k_b estimation

For reaction systems located in the 2nd order regime with irreversible handhold-binding (handhold=20 nt; toehold>1 nt), k_d is considered negligible and k_m very large. Thus, the reaction rate is solely determined by k_b (equation 4). To obtain an estimation of k_b , the complete ODE model, described in **Supplementary Note XI**, was fitted to individual fluorescence traces of systems confidently located in the 2nd order regime (20 nt handhold; >1 nt toehold).

During the fitting, k_m was fixed to an arbitrarily large value (10^8 s^{-1}) and k_d to $1.10^{-22} \text{ s}^{-1}$. The data was fitted in a two-step iteration. During the 1st round of fittings, k_{b_init} was arbitrarily set to $10^7 \text{ s}^{-1}\text{M}^{-1}$. For the 2nd round, all systems were fitted using as initial guesses its results of the 1st round for k_b , t_0 and $[I]_0$.

For the subsequent fittings, the value of k_b at each temperature was constrained to a range of twice the standard deviation of the fitted k_b , centred on its mean value. All k_b and their ranges at 25°C and 37°C are collected in **Supplementary Tables 11 and 12**, respectively.

Supplementary Table 11 | k_b fitting at 25 °C

Data obtained using procedures described in **Supplementary Notes II and XV**. The reactant strands used were all *N* strands “Incumbent 20 hh/x sp” (**Supplementary Table 20**) with *T* strands “Target (x th)” from 2 to 7 nt (**Supplementary Table 21**) and “Invader (I_{hc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (**Supplementary Table 23**). Mean of all $k_b = 5.14.10^6 \text{ s}^{-1}\text{M}^{-1}$; Standard deviation = $1.27.10^6 \text{ s}^{-1}\text{M}^{-1}$; Standard mean error (SEM) = $0.17.10^6 \text{ s}^{-1}\text{M}^{-1}$.

Condition (handhold/spacer/toehold)	Individual experiments $k_b \cdot 10^6 (\text{s}^{-1}\text{M}^{-1})$				Mean $k_b \cdot 10^6 \pm \text{SEM}$ ($\text{s}^{-1}\text{M}^{-1}$)
20/0/7	6.41	6.31	5.87	7.87	6.62±0.44
20/0/2	3.47	3.49	3.21	3.51	3.421±0.071
20/1/7	7.68	6.87	6.92	6.83	7.07±0.2
20/1/2	5.44	5.22	5.77	5.24	5.42±0.13
20/2/7	5.74	5.74	5.44	5.95	5.72±0.1
20/2/5	7.60	7.66	7.88	6.92	7.52±0.21
20/2/3	4.91	4.86	4.87	4.47	4.78±0.1
20/2/2	5.55	5.28	5.52	5.29	5.411±0.071
20/3/7	6.22	4.89	5.00	4.52	5.16±0.37
20/3/2	4.90	4.69	4.59	4.46	4.659±0.092
20/5/7	3.87	3.55	3.61	3.40	3.609±0.098
20/5/2	5.17	4.23	4.39	3.98	4.44±0.26
20/8/7	4.12	3.99	4.19	4.12	4.105±0.044
20/8/2	4.47	3.82	3.84	4.08	4.05±0.15

Supplementary Table 12 | k_b fitting at 37 °C

Data obtained using procedures described in **Supplementary Notes II and XV**. The reactant strands used were all *N* strands “Incumbent 20 hh/x sp” (**Supplementary Table 20**) with *T* strands “Target (x th)” from 2 to 7 nt (**Supplementary Table 21**) and “Invader (I_{hc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (**Supplementary Table 23**). Legend: **Red**: Rates with a standard error bigger than the 10% of the mean. Corresponds with long experimental conditions (toehold = 0 nt) or systems at the boundary between different kinetic regimes. Mean of all $k_b = 1.10^7 \text{ s}^{-1}\text{M}^{-1}$; Standard deviation = $0.45.10^7 \text{ s}^{-1}\text{M}^{-1}$; Standard mean error (SEM) = $0.11.10^7 \text{ s}^{-1}\text{M}^{-1}$.

Condition (handhold/spacer)	Individual experiments $k_b \cdot 10^6 (\text{s}^{-1}\text{M}^{-1})$				Mean $k_b \cdot 10^6 \pm \text{SEM}$ ($\text{s}^{-1}\text{M}^{-1}$)
20/0/7	13.73	15.82	17.81	17.84	16.3±0.98
20/0/2	10.47	10.66	10.18	11.25	10.64±0.23
20/8/7	6.09	6.68	2.90	3.07	4.69±0.99
20/8/2	7.94	8.98	9.23	8.59	8.68±0.28

Supplementary Note XVI: HMSD fitting

The parameters k_d , k_m , k_b , $[I]_0$ and t_0 were fitted simultaneously for each set of HMSD trajectories. A single set of values for k_d , k_m and k_b was fitted for each system condition. Constraints for the value of k_b and the value for the rest of the rate constants in the model were obtained experimentally (See **Supplementary Notes XIII, XIV and XV**). The model was fitted iteratively in three steps. During the 1st round of fittings, k_{d_init} was calculated with an estimation of handhold-binding free energy for each handhold and spacer combination¹, using the equilibrium equation:

$$k_{d_init} = \frac{k_b}{e^{-\frac{\Delta G_{hb}}{RT}}}, \quad (27)$$

where R is the gas constant ($1.987 \text{ cal mol}^{-1} \text{ K}^{-1}$), T is the experimental temperature, and ΔG_{hb} is the estimation of the handhold-binding Gibbs free energy in calories per mol¹. All the estimated ΔG_{hb} and k_{d_init} at 25°C and 37°C are collected in **Supplementary Tables 13 and 14**, respectively. The value of k_d was constrained during the fitting to a range $\pm\sqrt{10} k_{d_init}$. k_{m_init} was changed iteratively in the range of $[10^{-6} - 10^4]$ one order of magnitude at a time, and the best fitting was stored. For the 2nd round, each system was fitted without constraining k_d , using the best result of the 1st fitting round as initial guesses for k_d , k_b , k_m , t_0 and $[I]_0$. The 3rd fitting round used the results of the 2nd fitting round as initial guesses. Results from this fitting procedure and their calculated reaction rates and half-lives at 25°C and 37°C are collected in **Supplementary Tables 15, 16 and 17**.

To estimate and error for the fitted constants, the model was fitted to each individual trajectory and the standard error of the mean of each rate is reported. The results of the previous fitting process were used as initial guesses for k_d , k_b , k_m , t_0 and $[I]_0$. For reactions with 2nd order rate kinetics with reversible handhold binding, the standard error for the obtained 2nd order rate k (**equation 4**) is reported, due to compensatory errors between the ratio $\frac{k_m}{k_m + k_d}$ and k_b . For reactions with 2nd order kinetics and irreversible handhold binding, the standard error for the obtained k_b is reported. For reactions with 1st order kinetics, the standard error for the obtained k_m is reported.

Supplementary Table 13 | Estimated ΔG_{hb} and k_d for each handhold at 25 °C

Data obtained as described in **Supplementary Notes II and XVI**. The reactant strands used were all *N* strands “Incumbent x hh/x sp” (**Supplementary Table 20**) with all *T* strands “Target (x th)” (**Supplementary Table 21**) and “Invader (*I_{hc}*)” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (**Supplementary Table 23**).

Condition (handhold/spacer)	ΔG_{hb} (Kcal M^{-1})	k_{d_init} (s^{-1})	Mean fitted k_d for each condition (s^{-1})							Mean k_d $\pm$ SEM (s^{-1})
4/2	-7.24	2.48E+01	7.82E+00	1.29E+01	1.13E+01	3.07E+01	4.03E+01	8.43E+00		1.86 $\pm$ 0.55E+1
5/0	-7.26	2.40E+01	1.34E+01	-	-	2.11E+01	-	7.67E+00		1.41 $\pm$ 0.39E+1
5/1	-7.96	7.35E+00	2.11E+00	-	-	7.80E+02	-	2.57E+00		2.6 $\pm$ 2.6E+2
5/2	-8.03	6.53E+00	8.27E+00	1.70E+01	3.19E+00	1.96E+01	9.81E+00	2.46E+00		1.01 $\pm$ 0.29E+1
5/3	-7.96	7.35E+00	2.63E+00	-	-	9.16E+00	-	9.56E+00		7.1 $\pm$ 2.2E+0
5/5	-7.74	1.07E+01	6.00E+00	-	-	7.62E+00	-	1.37E-01		4.6 $\pm$ 2.3E+0
5/8	-7.96	7.35E+00	2.59E+00	-	-	4.70E+00	-	2.79E+00		3.36 $\pm$ 0.67E+0
6/2	-10.22	1.62E-01	5.14E-01	1.56E-01	2.87E-02	2.07E-01	8.92E-01	7.95E-02		3.1 $\pm$ 1.4E-1
7/2	-11.18	3.21E-02	1.63E-02	5.90E-02	9.77E-02	4.72E-02	2.07E-03	8.26E-02		5.1 $\pm$ 1.5E-2
8/2	-12.39	4.16E-03	1.60E-02	9.74E-03	7.65E-02	1.23E-01	1.76E-02	2.63E-04		4.1 $\pm$ 2E-2
9/2	-15.04	4.75E-05	8.88E-14	2.37E-11	9.01E-09	6.09E-09	3.73E-09	4.08E-06		6.8 $\pm$ 6.8E-7
10/2	-15.83	1.25E-05	2.89E-12	5.80E-03	6.13E-03	3.79E-03	4.90E-04	1.52E-03		3 $\pm$ 1.1E-3
20/0	-29.72	8.22E-16	1.04E-20	-	-	6.59E-16	-	3.81E-16		3.5 $\pm$ 1.9E-16
20/1	-30.42	2.52E-16	3.01E-16	-	-	3.72E-16	-	1.17E-16		2.63 $\pm$ 0.76E-16
20/2	-30.5	2.20E-16	2.39E-16	6.68E-16	1.46E-16	5.41E-16	8.03E-17	8.14E-17		2.9 $\pm$ 1E-16
20/3	-30.42	2.52E-16	9.14E-17	-	-	4.10E-16	-	1.27E-16		2.1 $\pm$ 1E-16
20/5	-30.21	3.59E-16	1.69E-16	-	-	2.58E-16	-	1.67E-16		1.98 $\pm$ 0.3E-16
20/8	-30.42	2.52E-16	2.65E-16	-	-	5.73E-16	-	2.17E-16		3.5 $\pm$ 1.1E-16
Toehold lengths			7 nt	5 nt	3 nt	2 nt	1 nt	0 nt		

Supplementary Table 14 Estimated ΔG_{hb} and k_d for each handhold at 37 °C

Data obtained as described in **Supplementary Notes II and XVI**. The reactant strands used were all *N* strands “Incumbent x hh/x sp” except the ones with spacer 1,3 and 5 nt (**Supplementary Table 20**) with “Target (x th)” with 0, 2 and 8 nt (**Supplementary Table 21**) and “Invader (*I_{hc}*)” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (**Supplementary Table 23**).

Condition (handhold/spacer)	ΔG_{hb} (Kcal M^{-1})	k_{d_init} (s^{-1})	Fitted k_d (s^{-1})			Mean k_d $\pm$ SEM (s^{-1})
4/2	-6.02	5.72E+02	-	-	1.95E+02	1.95E+02
5/0	-6.26	3.88E+02	3.37E+02	1.82E+02	1.28E+02	2.16 $\pm$ 0.51E+02
5/2	-6.87	1.44E+02	-	-	5.01E+01	5.01E+01
5/8	-6.84	1.51E+02	1.19E+02	3.59E+01	4.79E+01	6.8 $\pm$ 2.1E+01
6/2	-8.53	9.75E+00	-	-	9.13E+00	9.13E+00
7/2	-9.45	2.19E+00	-	-	3.8E+00	3.8E+00
8/2	-10.22	6.28E-01	-	-	1.05E+00	1.05E+00
9/2	-12.49	1.59E-02	-	-	1.37E-02	1.37E-02
10/2	-13.34	3.98E-03	-	-	1.53E-03	1.53E-03
20/0	-24.65	4.26E-11	4.36E-10	1.04E-10	1.2E-11	1.8 $\pm$ 1.1E-10
20/2	-25.26	1.58E-11	-	-	1.6E-11	1.6E-11
20/8	-25.23	1.66E-11	6.98E-11	1.52E-06	8.7E-12	5.1 $\pm$ 4.14E-07
Toehold lengths			7 nt	2 nt	0 nt	

Supplementary Table 15| Handhold Characterisation at 25 °C

Data used for **Figure 3** in the main text. All the relevant characterisation constants are accompanied by the standard error of the mean of individual fittings (**Supplementary Note XVI**). Data obtained as described in **Supplementary Notes II, XIV (to obtain k_t) and XVI**. The reactant strands used were all N strands “Incumbent x hh/2 sp” (**Supplementary Table 20**) with all “Target (x th)” (**Supplementary Table 21**) and I strands “Invader (I_{hc})”, and to obtain the value of k_t “Invader random-5’ (I_{wc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (R)” and “Reporter 1 quencher (Q)” (**Supplementary Table 23**). Legend: **Bold**: Relevant reaction rate for the system. **Yellow highlight**: 1st order reaction rates. **Green**: k_b with a negligible standard error of the mean. This artefact is produced by the boundaries imposed to k_b during its fitting. **Red**: Rates with a standard error bigger than the 10% of the mean. Corresponds to long experimental conditions (toehold=0 nt) or systems at the boundary between different kinetic regimes. **Red highlight**: k_t values that were not obtained from direct experimental evidence but the mean of all the systems with the same toehold length. Experiments omitted to reduce the number of extremely slow experiments. The standard error of the mean for each parameter was calculated from the values obtained in individual fittings.

System hh/sp/toe	k_m (s^{-1})	k_d (s^{-1})	k_b ($s^{-1} M^{-1}$)	k_t ($s^{-1} M^{-1}$)	2 nd order rate (k) (eq.3) ($s^{-1} M^{-1}$)	$t_{1/2}^{wc}$ (min)	$t_{1/2}$ (min)
0/2/7	0	0	0	2.942±0.085E+6	2.942±0.085E+6	5.45E-01	5.45E-01
0/2/5	0	0	0	2.101±0.037E+5	2.101±0.037E+5	6.56E+00	6.56E+00
0/2/3	0	0	0	1.267±0.025E+4	1.267±0.025E+4	1.08E+02	1.08E+02
0/2/2	0	0	0	1.65±0.15E+2	1.65±0.15E+2	9.63E+03	9.63E+03
0/2/1	0	0	0	8.59±0.26E+1	8.59±0.26E+1	2.84E+04	2.84E+04
4/2/7	1.12E+0	8.19E+00	6.41E+06	1.053±0.007E+6	1.823±0.052E+6	1.77E+00	7.80E-01
4/2/5	9.52E-1	1.26E+01	5.24E+06	1.136±0.009E+5	4.82±0.11E+5	1.23E+01	2.93E+00
4/2/3	3.37E-1	1.32E+01	4.51E+06	1.599±0.011E+4	1.28±0.017E+5	8.77E+01	1.10E+01
4/2/2	1.34E-2	2.42E+01	5.72E+06	1.884±0.015E+2	3.357±0.066E+3	7.44E+03	4.19E+02
4/2/1	1.13E-2	2.79E+01	4.62E+06	1.097±0.045E+2	1.984±0.076E+3	1.28E+04	7.08E+02
4/2/0	1.78E-5	7.55E+00	4.76E+06	8.3±1.3E+0	1.95±0.41E+1	1.70E+05	7.31E+04
5/2/7	1.79E+0	8.57E+00	3.87E+06	6.123±0.093E+5	1.282±0.025E+6	2.29E+00	1.10E+00
5/2/5	4.18E-2	3.23E-01	3.87E+06	8.69±0.16E+4	4.958±0.068E+5	1.61E+01	2.85E+00
5/2/3	4.27E-1	8.46E+00	5.24E+06	1.674±0.008E+4	2.723±0.043E+5	1.82E+06	5.29E+00
5/2/2	9.66E-3	7.28E+00	3.87E+06	2.354±0.048E+2	5.348±0.027E+3	4.82E+05	2.63E+02
5/2/1	4.08E-3	8.54E+00	6.41E+06	1.116±0.006E+2	3.18±0.1E+3	1.26E+04	4.45E+02
5/2/0	1.49E-5	2.11E+00	5.33E+06	9.36±0.62E+0	4.607±0.064E+1	1.52E+05	3.13E+04
6/2/7	6.62E-1	5.14E-01	3.93E+6	1.072±0.021E+6	3.285±0.06E+6	1.31E+00	4.55E-01
6/2/5	1.55E-1	1.56E-01	3.76E+6	1.066±0.011E+5	1.98±0.21E+6	1.32E+01	8.45E-01
6/2/3	3.75E-2	2.87E-02	3.76E+6	2.135±0.015E+4	2.152±0.078E+6	6.57E+01	1.12E+00
6/2/2	2.70E-3	2.07E-01	3.77E+6	2.641±0.029E+2	4.87±0.12E+4	5.31E+03	3.66E+01
6/2/1	4.92E-3	8.92E-01	4.04E+6	1.335±0.01E+2	2.227±0.037E+4	1.05E+04	6.74E+01
6/2/0	7.18E-6	7.95E-02	4.53E+6	9.36±0.62E+0	4.19±0.18E+2	1.52E+05	6.03E+03
7/2/7	8.16E-2	1.63E-02	3.76E+6	9.99±0.17E+5	4.13±0.13E+6	1.40E+00	5.17E-01
7/2/5	1.92E-1	5.90E-02	3.76E+6	1.146±0.009E+5	2.991±0.031E+6	1.22E+01	5.71E-01
7/2/3	1.23E-1	9.77E-02	6.29E+6	2.433±0.032E+4	3.53±0.11E+6	5.76E+01	5.55E-01
7/2/2	5.63E-3	4.72E-02	3.76E+6	2.987±0.045E+2	4±1.3E+5	4.69E+03	6.87E+00
7/2/1	7.75E-4	2.07E-03	6.28E+6	1.247±0.006E+2	1.71±0.11E+6	1.12E+04	1.74E+01
7/2/0	4.09E-5	8.26E-02	3.76E+6	9.36±0.62E+0	1.87±0.12E+3	1.52E+05	1.24E+03
8/2/7	7.27E-2	1.60E-02	3.76E+6	8.548±0.09E+5	3.94±0.14E+6	1.64E+00	5.59E-01
8/2/5	7.79E-2	9.74E-03	3.76E+6	1.11±0.007E+5	3.45±0.1E+6	1.26E+01	6.41E-01
8/2/3	4.65E+0	7.65E-02	3.81E+6	2.281±0.018E+4	3.768±0.047E+6	6.15E+01	3.88E-01
8/2/2	3.78E-2	1.23E-01	6.29E+6	2.52±0.032E+2	1.478±0.044E+6	5.56E+03	1.47E+00
8/2/1	5.58E-3	1.76E-02	6.29E+6	1.28±0.017E+2	1.51±0.39E+6	1.10E+04	3.68E+00
8/2/0	4.84E-5	2.63E-04	6.29E+6	9.36±0.62E+0	9.78±0.83E+5	1.52E+05	2.70E+02
9/2/7	2.01E+0	8.88E-14	4.04±0.16E+6	2.384±0.069E+6	6.42±0.3E+6	5.88E-01	2.27E-01
9/2/5	3.90E+0	2.37E-11	3.828±0.048E+6	2.529±0.038E+5	4.08±0.056E+6	5.54E+00	3.51E-01
9/2/3	1.99E+0	9.01E-09	4.92±0.1E+6	6.137±0.024E+4	4.99±0.1E+6	2.28E+01	2.93E-01
9/2/2	4.01E+0	6.09E-09	5.29±0.34E+6	4.66±0.89E+2	5.29±0.34E+6	3.03E+03	2.73E-01
9/2/1	6.57E+1	3.73E-09	4.405±0.084E+6	1.69±0.36E+2	4.406±0.084E+6	8.28E+03	3.22E-01
9/2/0	6.007±0.021E-4	4.08E-06	6.29E+6	9.36±0.62E+0	6.29E+6	1.52E+05	1.96E+01
10/2/7	1.26E+0	2.89E-12	3.76E+6	1.586±0.049E+6	5.347±0.098E+6	8.84E-01	2.75E-01
10/2/5	6.83E+1	5.80E-03	3.95±0.18E+6	2.417±0.034E+5	4.2±0.19E+6	5.80E+00	3.39E-01
10/2/3	1.83E+0	6.13E-03	4.41±0.14E+6	5.054±0.009E+4	4.44±0.14E+6	2.77E+01	3.29E-01
10/2/2	3.85E-2	3.79E-03	4.5±0.1E+6	6.97±0.058E+2	4.1±0.1E+6	2.01E+03	8.00E-01
10/2/1	8.53E+1	4.90E-04	3.76E+6	3.584±0.02E+2	3.76E+6	3.91E+03	3.76E-01

10/2/0	8.85±0.25E-4	1.52E-03	3.76E+6	9.36±0.62E+0	3.76E+6	1.52E+05	1.54E+01
20/2/7	1.09E+1	2.39E-16	5.7±0.11E+6	1.277±0.024E+6	6.98±0.16E+6	1.10E+00	2.06E-01
20/2/5	5.16E+1	6.68E-16	6.29E+6	2.85±0.41E+5	6.579±0.071E+6	4.92E+00	2.17E-01
20/2/3	2.08E+0	1.46E-16	4.75±0.11E+6	5.201±0.045E+4	4.8±0.11E+6	2.70E+01	3.04E-01
20/2/2	4.68E+0	5.41E-16	5.412±0.072E+6	8.64±0.1E+2	5.413±0.072E+6	1.63E+03	2.66E-01
20/2/1	2.34E+0	8.03E-17	3.761±0.012E+6	3.03±0.16E+2	3.762±0.012E+6	4.63E+03	3.83E-01
20/2/0	2.038±0.015E-3	8.14E-17	6.29E+6	9.36±0.62E+0	6.29E+6	1.52E+05	6.09E+00

Supplementary Table 16| Spacer Characterisation at 25 °C

Data used to infer the effect of a spacer sequence between the handhold and the displaced domain. All the relevant characterisation constants are accompanied by the standard error of the mean of individual fittings (Supplementary Note XVI). Data obtained as described in Supplementary Notes II, XIV (to obtain k_t) and XVI. The reactant strands used were all *N* strands “Incumbent x hh/x sp” excluding 2 nt spacers (Supplementary Table 20) with *T* strands “Target (x th)” with toeholds of 0, 2 and 8 nt (Supplementary Table 21) and *I* strands “Invader (I_{hc})”, and to obtain the value of k_t “Invader random-5’ (I_{wc})” (Supplementary Table 22). Reporter strands were “Reporter 1 (*R*)” and “Reporter 1 quencher (*Q*)” (Supplementary Table 23). Legend: **Bold**: Relevant reaction rate for the system. **Yellow highlight**: 1st order reaction rates. **Green**: k_b with a negligible standard error. This artefact is produced by the boundaries imposed to k_b during its fitting. **Red**: Rates with a standard error bigger than the 10% of the mean. Corresponds to long experimental conditions (toehold=0 nt) or systems at the boundary between different kinetic regimes. **Red highlight**: k_t values that were not obtained from direct experimental evidence but the mean of all the systems with the same toehold length. Experiments omitted to reduce the number of extremely slow experiments. The standard error of the mean for each parameter was calculated from the values obtained in individual fittings.

System hh/sp/toe	k_m (s^{-1})	k_d (s^{-1})	k_b ($s^{-1} M^{-1}$)	k_t ($s^{-1} M^{-1}$)	2 nd order rate (<i>k</i>) (eq.3) ($s^{-1} M^{-1}$)	$t_{1/2}^{wc}$ (min)	$t_{1/2}$ (min)
0/0/7	0	0	0	4.39±0.12E+6	4.39±0.12E+6	2.79E-01	2.82E-01
0/0/2	0	0	0	1.99±0.1E+2	1.99±0.1E+2	7.52E+03	7.51E+03
0/1/7	0	0	0	3.43±0.12E+6	3.43±0.12E+6	4.20E-01	4.23E-01
0/1/2	0	0	0	1.79±0.029E+2	1.79±0.029E+2	7.48E+03	7.47E+03
0/1/0	0	0	0	1.041±0.067E+1	1.041±0.067E+1	3.78E+05	-
0/3/7	0	0	0	2.274±0.057E+6	2.274±0.057E+6	5.74E-01	5.77E-01
0/3/2	0	0	0	1.37±0.12E+2	1.37±0.12E+2	9.08E+03	9.07E+03
0/5/7	0	0	0	5.503±0.095E+5	5.503±0.095E+5	2.34E+00	2.35E+00
0/5/2	0	0	0	1.407±0.094E+2	1.407±0.094E+2	9.16E+03	9.15E+03
0/8/7	0	0	0	3.25±0.11E+5	3.25±0.11E+5	3.83E+00	3.83E+00
0/8/2	0	0	0	1.093±0.068E+2	1.093±0.068E+2	1.32E+04	1.32E+04
5/0/7	1.46E-1	1.34E+01	6.25E+6	3.09±0.068E+5	3.76±0.17E+5	4.54E+00	3.76E+00
5/0/2	1.98E-2	2.11E+01	6.29E+6	3.112±0.043E+2	6.21±0.096E+3	4.55E+03	2.27E+02
5/0/0	1.31E-4	7.67E+00	5.73E+6	9.36±0.62E+0	1.069±0.048E+2	1.52E+05	1.33E+04
5/1/7	8.91E-2	2.11E+00	3.76E+6	4.764±0.07E+5	6.29±0.17E+5	2.94E+00	2.29E+00
5/1/2	2.28E-1	7.80E+02	5.12E+6	2.758±0.028E+2	1.774±0.023E+3	5.04E+03	7.90E+02
5/1/0	1.82E-6	2.57E+00	4.51E+6	9.36±0.62E+0	1.24±0.13E+1	1.52E+05	1.16E+05
5/3/7	3.27E-1	2.63E+00	4.16E+6	9.13±0.16E+5	1.373±0.054E+6	1.54E+00	1.05E+00
5/3/2	1.19E-3	9.16E+00	4.03E+6	3.855±0.029E+2	9.12±0.11E+2	3.62E+03	1.55E+03
5/3/0	5.96E-7	9.56E+00	3.76E+6	9.36±0.62E+0	9.5±1.4E+0	1.52E+05	1.49E+05
5/5/7	2.12E-1	6E+00	3.76E+6	3.64±0.14E+5	4.92±0.19E+5	3.85E+00	2.88E+00
5/5/2	1.80E-4	7.62E+00	6.22E+6	2.66±0.025E+2	4.14±0.11E+2	5.27E+03	3.43E+03
5/5/0	1.76E-9	0.14E+00	6.16E+6	9.36±0.62E+0	9.33±0.58E+0	1.52E+05	1.86E+05
5/8/7	2.07E-1	2.59E+00	5.40E+6	4.23±0.11E+5	8.22±0.49E+5	3.32E+00	1.76E+00
5/8/2	1.78E-4	4.7E+00	6.29E+6	1.46±0.12E+2	4.05±0.23E+2	8.43E+03	3.53E+03
5/8/0	6.19E-7	2.79E+00	6.18E+6	9.36±0.62E+0	1.062±0.058E+1	1.52E+05	1.36E+05
20/0/7	1.46E+1	1.04E-20	6.29±0.11E+6	6.284±0.085E+5	6.92±0.12E+6	2.23E+00	2.08E-01
20/0/2	1.03E+1	6.59E-16	3.76E+6	1.43±0.044E+3	3.76E+6	1.02E+03	3.78E-01
20/0/0	4.015±0.033E-4	3.81E-16	6.29E+6	9.36±0.62E+0	6.29E+6	1.52E+05	2.92E+01
20/1/7	2.67E+0	3.01E-16	6.29E+6	1.34±0.02E+6	7.634±0.02E+6	1.05E+00	1.92E-01
20/1/2	2.02E+0	3.72E-16	5.41±0.13E+6	1.436±0.012E+3	5.41±0.13E+6	9.81E+02	2.71E-01
20/1/0	2.62±0.11E-3	1.17E-16	6.29E+6	9.36±0.62E+0	6.29E+6	1.52E+05	4.84E+00
20/3/7	1.39E+0	9.14E-17	4.8±0.43E+6	1.417±0.063E+6	6.21±0.49E+6	9.89E-01	2.38E-01
20/3/2	1.66E+1	4.1E-16	4.672±0.089E+6	1.36±0.014E+3	4.673±0.089E+6	1.03E+03	3.04E-01
20/3/0	1.28±0.02E-3	1.27E-16	5.46E+6	9.36±0.62E+0	5.46E+6	1.52E+05	9.50E+00
20/5/7	5.48E-1	1.69E-16	3.761±0.032E+6	5.291±0.033E+5	4.29±0.035E+6	2.65E+00	3.58E-01
20/5/2	1.27E-1	2.58E-16	4.52±0.26E+6	7.387±0.033E+2	4.53±0.26E+6	1.90E+03	4.62E-01
20/5/0	6.191±0.028E-4	1.67E-16	3.96E+6	9.41±0.73E+0	3.96E+6	1.52E+05	1.93E+01
20/8/7	1.74E+1	2.65E-16	4.047±0.045E+6	7.752±0.044E+5	4.822±0.049E+6	1.81E+00	2.95E-01
20/8/2	1.68E-1	5.73E-16	4.2±0.13E+6	6.429±0.045E+2	4.21±0.13E+6	2.21E+03	4.49E-01
20/8/0	5.686±0.03E-4	2.17E-16	3.76E+6	9.36±0.62E+0	3.76E+6	1.52E+05	2.10E+01

Supplementary Table 17| Handhold Characterisation at 37 °C

All the relevant characterisation constants are accompanied by the standard error of the mean between all the obtained fluorescence traces for that reaction system. Data obtained as described in **Supplementary Notes II, XIV (to obtain k_t) and XVI**. The reactant strands used were N strands “Incumbent x hh/x sp” with spacers 0, 2 and 8 (**Supplementary Table 20**) with T strands “Target (x th)” with toeholds of 0, 2 and 7 nt (**Supplementary Table 21**) and I strands “Invader (I_{hc})”, and to obtain the value of k_t “Invader random-5’ (I_{wc})” (**Supplementary Table 22**). Reporter strands were “Reporter 1 (R)” and “Reporter 1 quencher (Q)” (**Supplementary Table 23**). Legend: **Bold**: Relevant reaction rate for the system. **Yellow highlight**: 1st order reaction rates. **Red**: Rates with a standard error bigger than the 10% of the mean. Corresponds with long experimental conditions (toehold=0 nt) or systems at the boundary between different kinetic regimes. **Red highlight**: k_t values that were not obtained from direct experimental evidence. Their value was estimated from the k_t for a toehold=0 nt at 25°C. The standard error of the mean for each parameter was calculated from the values obtained in individual fittings.

System hh/sp/toe	k_m (s^{-1})	k_d (s^{-1})	k_b ($s^{-1} M^{-1}$)	k_t ($s^{-1} M^{-1}$)	2 nd order rate (k) (eq.3) ($s^{-1} M^{-1}$)	$t_{1/2}^{wc}$ (min)	$t_{1/2}$ (min)
0/0/7	0	0	0	5.14±0.11E+6	5.14±0.11E+6	2.38E-01	2.41E-1
0/0/2	0	0	0	4.33±0.2E+2	4.24±0.19E+2	3.63E+03	3.63E+3
0/8/7	0	0	0	4.27±0.053E+5	4.27±0.053E+5	2.53E+00	2.53E+0
0/8/2	0	0	0	2.71±0.16E+2	2.65±0.16E+2	5.43E+03	5.42E+3
5/0/7	2.3E+0	3.37E+02	8.34E+06	3.746±0.048E+5	4.312±0.127E+5	3.74E+00	3.26E+0
5/0/2	3.61E-2	1.82E+02	7.66E+06	4.32±0.17E+2	1.955±0.187E+3	3.31E+03	7.18E+2
5/0/0	1.22E-3	1.28E+02	5.73E+06	9.36±0.62E+0	6.419±0.073E+1	1.52E+05	2.18E+4
5/8/7	1.82E+0	1.19E+02	1.04E+07	4.915±0.08E+5	6.472±0.133E+5	2.85E+00	2.17E+0
5/8/2	2.85E-4	3.59E+01	5.57E+06	3.4±0.19E+2	3.841±0.284E+2	4.21E+03	3.66E+3
5/8/0	4.36E-5	4.80E+01	7.97E+06	9.36±0.62E+0	1.661±0.123E+1	1.52E+05	8.45E+4
20/0/7	1.24E+1	4.36E-10	1.459±0.003E+7	6.424±0.083E+5	1.5229±0.003E+7	2.18E+00	9.82E-2
20/0/2	7.93E+0	1.04E-10	1.054±0.025E+7	5.33±1E+2	1.054±0.025E+7	2.76E+03	1.40E-1
20/0/0	6.456±0.081E-4	1.18E-11	1.19E+07	9.36±0.62E+0	1.19E+7	1.52E+05	1.81E+1
20/8/7	6.37E+0	6.98E-11	5.57±0.27E+6	6.187±0.078E+5	6.19±0.28E+6	2.27E+00	2.32E-1
20/8/2	1.31E+1	1.52E-06	8.59±0.75E+6	3.77±0.4E+2	8.59±0.75E+6	3.79E+03	1.68E-1
20/8/0	1.323±0.008E-3	8.75E-12	1.44E+07	9.36±0.62E+0	1.44E+7	1.52E+05	8.9E+0
4/2/0	3.58E-4	1.95E+02	8.67E+06	9.36±0.62E+0	2.527±0.104E+1	5.58E+04	5.55E+4
5/2/0	1.39E-4	5.01E+01	7.66E+06	9.36±0.62E+0	3.798±0.11E+1	3.71E+04	3.70E+4
6/2/0	1.41E-4	9.13E+00	6.41E+06	9.36±0.62E+0	1.08±0.11E+2	1.31E+04	1.31E+4
7/2/0	1.78E-4	3.80E+00	7.07E+06	9.36±0.62E+0	3.395±0.099E+2	4.35E+03	4.25E+3
8/2/0	2.80E-4	1.06E+00	5.57E+06	9.36±0.62E+0	1.486±0.031E+3	1.02E+03	1.02E+3
9/2/0	1.85±0.17E-4	1.37E-02	9.02E+06	9.36±0.62E+0	1.2E+5	8.20E+01	9.73E+1
10/2/0	5.65±0.36E-4	1.53E-03	9.60E+06	9.36±0.62E+0	2.59E+6	2.27E+01	2.27E+1
20/2/0	1.78±0.28E-3	1.60E-11	5.57E+06	9.36±0.62E+0	5.57E+6	7.02E+00	6.96E+0

Supplementary Figure 18| Inferred 2nd order rate constants for handhold-mediated strand displacement.

k values collected in **Supplementary Table 15**. Reaction rates for toeholds = 0 nt do not correspond with their real 1st order reaction rate.

Supplementary Figure 19| Inferred 2nd order rate constants for *I*-strands without handhold complementarity.

The reaction rates are the ones of a toehold mediated strand displacement (k_t , **Supplementary Table 15**). Handhold presence in *N* produces negligible rate changes for strand displacement reactions when *I*-strands lack handhold-complementarity.

Supplementary Figure 20| Comparison between k and k_t

Our inferred 2nd order rate constants suggest that HMSD can increase displacement rate by up to 5 orders of magnitude compared to displacement triggered with I -strands without handhold-complementarity. The reaction rate increment obtained by the addition of handhold-complementarity is sensibly higher in the regime of toeholds below 3 nt.

Supplementary Note XVII: Fitting of the kinetic analysis of handheld detachment

R_2Q_2 controls shown in **Supplementary Note IX** proved that the presence of strand T in the solution triggered R_2Q_2 associative toehold at a slow rate, except when free in solution as a product IT . Due to the high reaction rate of R_2Q_2 with IT , the slow-rate leak was ignored when fitting the kinetics of R_2Q_2 . The obtained trajectories were fitted to obtain the value of K_{eq} (**equation 6**). Each trajectory was fit individually to the ODE describing **equation 5**:

$$\frac{d[R_2IT]}{dt} = k_{r2}[R_2Q][IT], \quad (28)$$

With constraints:

$$[R_2Q_2] = [R_2Q_2]_0 - [R_2IT], \quad (29)$$

$$[IT] = \frac{-(K_{eq} + [N]_0 + [R_2IT] - [I]_0) + \sqrt{([N]_0 + K_{eq} + [R_2IT] - [I]_0)^2 - 4K_{eq}([R_2IT] - [I]_0)}}{2}. \quad (30)$$

Supplementary equation 30 describes free IT assuming an instantaneous equilibrium between N/IT and $IT+N$. The detailed derivation of **Supplementary equation 30** from **equation 6** of the main text is included in the **Supplementary Note XIX**. The values of k_{r2} were obtained from individually fitting Supplementary equations 24 and 25 to the kinetics of a system in the absence of handheld (**Supplementary Figure 21**)

For each system, the model was fitted to individual fluorescence traces to obtain the value of K_{eq} , $[I]_0$ and t_0 . K_{eq_init} was set arbitrarily to 5.10^{-9} nM. For the obtained K_{eq} , the percentage of expected detached $[IT]$ in the equilibrium was calculated with **Supplementary equation 30** for $[N]_0; [I]_0 = 10$ nM and $[R_2IT] = 0$ nM. Fitted K_{eq} and the expected percentage of detached $[IT]$ are collected in **Supplementary Table 18**. Results for the control without RQ reporter are collected in **Supplementary Table 19**.

Supplementary Table 18 | Fitting results from the kinetic analysis of handheld detachment

Data obtained as described in **Supplementary Notes III and XVII**. The reactant strands used were all N strands "Incumbent x hh/2 sp" (**Supplementary Table 20**) with "Target(3 th)- R_2Q_2 trigger" (**Supplementary Table 21**) and "Invader (I_{hc})" (**Supplementary Table 22**). Reporter strands were "Reporter 1 (R)", "Reporter 1 quencher (Q)", "Rep_A (R_2)" and "Rep_A quencher (Q_2)" (**Supplementary Table 23**).

Condition (handhold/spacer)	K_{eq} (M)	% of detached displacement products
4/2	1.27E-1	99.99
5/2	1.19E-1	99.99
6/2	9E-1	99.99
7/2	1.01E-1	99.99
8/2	2.1E-1	99.99
9/2	5.6E-8	83.3±2.8
10/2	3.945E-9	45.6±1.7
20/2	~0	0

Supplementary Table 19| Fitting results from the kinetic analysis of handheld detachment in the absence of reporter RQ

Data obtained as described in **Supplementary Notes III and XVII**. The reactant strands used were N strands “Incumbent x hh/2 sp” with handholds 7, 8, 9 and 10 nt (**Supplementary Table 20**) with “Target(3 th)- R_2Q_2 trigger” (**Supplementary Table 21**) and “Invader (I_{hc})” (**Supplementary Table 22**). Reporter strands were “Rep_A (R_2)” and “Rep_A quencher (Q_2)” (**Supplementary Table 23**). The standard error of the mean for each parameter was calculated from the values obtained in individual fittings.

Condition (handhold/spacer)	$K_{eq} \pm SEM$ (M)	% of detached displacement products
7/2	1.01E-1	99.99
8/2	1.77E-1	99.99
9/2	2.381±0.02E-8	75.84±0.14
10/2	5.22±0.23E-9	50.68±0.82

Supplementary Figure 21| Triggering of reporter R_2Q_2 in the absence of handheld.

Processed fluorescence data of the addition of R_2Q_2 (Measurement 4) after the reaction of a system without handheld, a 2 nt spacer, and a 3 nt toehold that has reacted with I_{hc} . Each trajectory was fitted individually with **Supplementary equations 28 and 29** to obtain the value of k_{r2} . In the absence of a handheld, all IT was assumed to be detached. The obtained values for k_{r2} were 6.51, 7.14, 7.58 and 7.33.10⁵ M⁻¹s⁻¹, which produces a mean of 7.14±0.23.10⁵ M⁻¹s⁻¹. All concentrations are defined for a reaction volume of 200 μ L.

Supplementary Note XVIII: Derivation of HMSD bimolecular rate (equation 4)

To calculate the effective rate of IT production, we omit the reporter reaction and assume the species TN/I to be in quasi-steady state, since k_d and k_m are assumed to be large:

$$\frac{d[TN/I]}{dt} = k_b[TN][I] - k_d[TN/I] - k_m[TN/I] = 0$$
$$[TN/I] = \frac{k_b}{k_d + k_m} [TN][I] \quad (31)$$

So, the rate of production of IT for handhold mediated strand displacement is defined:

$$\frac{d[IT]}{dt} = k_m [TN/I] = k_m \frac{k_b}{k_d + k_m} [TN][I] \quad (32)$$

Toehold-mediated strand displacement also contributes to the reaction with a rate equal to $k_t[TN][I]$. By adding the contribution of handhold and toehold mediated displacements we obtain the final rate:

$$\frac{d[IT]}{dt} = (k_m \frac{k_b}{k_d + k_m} + k_t) [TN][I] = k [TN][I] \quad (33)$$

Supplementary Note XIX: Derivation of Supplementary equation 30

From the equilibrium equation (**equation 6**), it is possible to obtain a boundary for the value of $[IT]$ that depends on the concentrations of the species in the solution. The first step is to substitute $[N]=[N]_0-[N/IT]$ and $[N/IT]=[I]_0-[IT]-[R_2IT]$:

$$K_{eq} = \frac{[N][IT]}{[N/IT]} = \frac{([N]_0+[IT]+[R_2IT]-[I]_0)[IT]}{[I]_0-[IT]-[R_2IT]}, \quad (34)$$

$$([I]_0-[IT]-[R_2IT])K_{eq} = ([N]_0+[IT]+[R_2IT]-[I]_0)[IT],$$

$$K_{eq}([I]_0-[R_2IT]) - K_{eq}[IT] = [IT]^2 + ([N]_0+[R_2IT]-[I]_0)[IT],$$

$$0 = [IT]^2 + (K_{eq}+[N]_0+[R_2IT]-[I]_0)[IT] + K_{eq}([R_2IT]-[I]_0) \quad (35),$$

$[IT]$ (**Supplementary equation 30**) is the positive solution of the quadratic equation:

$$[IT] = \frac{-(K_{eq}+[N]_0+[R_2IT]-[I]_0) + \sqrt{(K_{eq}+[N]_0+[R_2IT]-[I]_0)^2 - 4K_{eq}([R_2IT]-[I]_0)}}{2}$$

Supplementary Note XX: Sequences

All sequences used during the experiments are collected in **Supplementary Table 20 to 23**. Strands are named by their handhold (hh), spacer (sp) and toehold (th) sequences length. The combination of these strands produces the complexes referred during the supplementary as “X/X/X_Temperature”. For example, the combination of Incumbent(5hh/2sp) with Target(7nt) reacting at 25°C, is referred to as the system 5/2/7_25. The domains of each strand are coded in the same colour as in **Figures 1 and 3**, specifically:

Grey: *R* binding domain in *N* strands and binding domain between *R* and *Q* strands.

Orange: *N* strands domain. Displaced domain during the strand displacement. Same sequence as **green**.

Green: *I* strands domain. Same sequence as **orange**.

Blue: *T* strands domain. Complementary to **orange/green**

Gold: Handhold domain of variable length in *N* strands. Variable 8 nt sequence in reporters. The handhold-complementary sequence in *I* strands.

Light blue: Toehold domain. Always the same sequence, but with variable length in *T* strands. 7 nt for *I* strands and 2 nt for *I_{A/B/C}* strands

Black: Spacer domains between orange and gold domains in *N*. Hairpins, poly-T spacers and clamps in Rep_{A/B/C} strands.

Purple: Rep_{A/B/C} binding domain in *N* strands and binding domain between Rep_{A/B/C} and their quenchers.

Supplementary Table 20| Incumbent strands (N)

	Sequence	Length (nt)
Incumbent (0 hh/0 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CG - 3'	38
Incumbent (0 hh/1 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA - 3'	39
Incumbent (0 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA T - 3'	40
Incumbent (0 hh/3 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TA - 3'	41
Incumbent (0 hh/5 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC C - 3'	43
Incumbent (0 hh/8 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC CTA A - 3'	46
Incumbent (4 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TG - 3'	44
Incumbent (5 hh/0 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGG ATG T - 3'	43
Incumbent (5 hh/1 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA GAT GT - 3'	44
Incumbent (5 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT - 3'	45
Incumbent (5 hh/3 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAG ATG T - 3'	46
Incumbent (5 hh/5 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC CGA TGT - 3'	48
Incumbent (5 hh/8 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC CTA AGA TGT - 3'	51
Incumbent (6 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT G - 3'	46
Incumbent (7 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT GA - 3'	47
Incumbent (8 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT GAA - 3'	48
Incumbent (9 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT GAA G - 3'	49
Incumbent (10 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT GAA GT - 3'	50
Incumbent (20 hh/0 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGG ATG TGA AGT GAA TGA G - 3'	58
Incumbent (20 hh/1 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA GAT GTG AAG TGA ATG ATG AG - 3'	59
Incumbent (20 hh/2 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TGA TGT GAA GTG AAT GAT GAG - 3'	60
Incumbent (20 hh/3 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAG ATG TGA AGT GAA TGA TGA G - 3'	61
Incumbent (20 hh/5 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC CGA TGT GAA GTG AAT GAT GAG - 3'	63
Incumbent (20 hh/8 sp)	5' - CCC TAT TAC CCT TGC CGA CCC TAC CTA CCT ACC CTG CGA TAC CTA AGA TGT GAA GTG AAT GAT GAG - 3'	66
Template A (8 hh/2 sp)	5' - CCG ACC CTA CCT ACC TAC CCT GCG ATG ATG TGA A - 3'	34
Template B (8 hh/2 sp)	5' - CCG ACC CTA CCT ACC TAC CCT GCG ATG ATA GCA T - 3'	34
Template C (8 hh/2 sp)	5' - CCG ACC CTA CCT ACC TAC CCT GCG ATT TAG GTT T - 3'	34

Supplementary Table 21 | Target strands (7)

	Sequence	Length (nt)
Target (0 th)	5' - CGC AGG GTA GGT AGG TAG GGT CGG TAA CTT ATC G - 3'	34
Target (1 th)	5' - GCG CAG GGT AGG TAG GTA GGG TCG GTA ACT TAT CG - 3'	35
Target (2 th)	5' - TGC GCA GGG TAG GTA GGT AGG GTC GGT AAC TTA TCG - 3'	36
Target (3 th)	5' - GTG CGC AGG GTA GGT AGG TAG GGT CGG TAA CTT ATC G - 3'	37
Target (5 th)	5' - AAG TGC GCA GGG TAG GTA GGT AGG GTC GGT AAC TTA TCG - 3'	39
Target (7 th)	5' - GGA AGT GCG CAG GGT AGG TAG GTA GGG TCG GTA ACT TAT CG - 3'	41
Target (3 th) - R_2Q_2 trigger	5' - GTG CGC AGG GTA GGT AGG TAG GGT CGG CTC AAC ATA CAT CG - 3'	41

Supplementary Table 22 | Invader strands (I)

	Supplementary Note XXI: Sequence	Length (nt)
Invader (I_{hc})	5' - CTC ATC ATT CAC TTC ACA TCC CGA CCC TAC CTA CCT ACC CTG CGC ACT TCC - 3'	51
Invader-random 5' (I_{wc})	5' - ACC TAT AAC CTG AAA CCT AAC CGA CCC TAC CTA CCT ACC CTG CGC ACT TCC - 3'	51
Invader-random 3'	5' - CTC ATC ATT CAC TTC ACA TCA ACT CCC ACT CAA CTC ACC TCA ACT CAA CCC - 3'	51
Invader A (2 th/ 8 hh)	5' - TTA TGC TCC CGA CCC TAC CTA CCT ACC CTG CGC A - 3'	34
Invader B (2 th/ 8 hh)	5' - ATG CTA TCC CGA CCC TAC CTA CCT ACC CTG CGC A - 3'	34
Invader C (2 th/ 8 hh)	5' - AAA CCT AAC CGA CCC TAC CTA CCT ACC CTG CGC A - 3'	34

Supplementary Table 23 | Reporter strands

	Sequence	Length (nt)
Reporter 1 (R)	5' - AGG GTC GGC AAG GGT AAT AGG GAC ttt-Cy3 - 3'	27
Reporter 1 quencher (Q)	5' - IowaBlackFQ-ttt GTC CCT ATT ACC CTT GCC - 3'	21
Rep _A (R_2)	5' - Cy5-ttt CGA TGT ATG TTG AGG CGT GCC GTT TTC GGC ACG CGA TGT GAA - 3'	45
Rep _A quencher (Q_2)	5' - CAA CAT ACA TCG ttt-IowaBlackRQ - 3'	15
Rep _B fluorescence	5' - RhodamineRed TM X-ttt GTC GAT GTA TGT TGA GGC GTG CCG TTT TCG GCA CGC GAT AGC AT - 3'	47
Rep _B quencher	5' - CAA CAT ACA TCG AC ttt-BlackHoleQuencher2	17
Rep _C fluorescence	5' - AlexaFluor [®] 488-ttt GTC GAT GTA TGT TGA GGC GTG CCG TTT TCG GCA CGC TTA GGT TT - 3'	47
Rep _C quencher	5' - CAA CAT ACA TCG AC ttt-IowaBlackFQ - 3'	17

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