TwistDx™

Unwind DNA's possibilities

TwistAmp® DNA Amplification Kits

Assay Design Manual

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Assay considerations

1.1 Reaction temperature

The standard TwistAmp® kits are configured to operate optimally in the temperature range of 37°C to 42°C. At higher temperatures the system will be compromised as the enzymes progressively lose activity. At temperatures below the optimal range, Recombinase Polymerase Amplification (RPA) can work excellently albeit at a reduced reaction rate. However, the reagent composition and protocols of the current TwistAmp® kits have been optimised for very fast amplification and are not specifically optimised for protocols using temperatures below the recommended optimal range. With reduced temperatures, amplicon doubling times lengthen more rapidly than energy consumption rates, which can lead to fuel 'burn-out' before accumulation. Sensitivity to temperature has been most acutely observed for the TwistAmp® exo real-time fluorescence kits. The other kits are somewhat more resistant to lower temperatures and amplicons have been successfully generated and detected at typical ambient temperatures with TwistAmp® Basic and TwistAmp® nfo kits.

1.2 Quantification and amplification onset

The onset time of detectable amplification for a given assay will depend on the amount of starting template material: the more template copies there are to start with, the shorter the time to detection. However, exploiting this 'time-based' quantification (as opposed to the cycle-based qPCR approach) demands a careful experimental setup. Firstly, it is critical to ensure the simultaneous initiation of compared reactions, for instance of a test sample versus a standard dilution series. This can be achieved through 'magnesium acetate start' as described in the instruction manuals. Alternatively, reactions can be temporarily slowed down (or halted) by setting them up on ice and then starting by simultaneous transfer to the optimal reaction temperature. Slowing/altering the overall kinetic rate to improve the time resolution of assays may also be achieved by altering reaction composition, and/or temperature. However, as stated above, the current TwistAmp® kits have not been optimised for reduced kinetics and lower temperature and we do not advise running the TwistAmp® exo system below

the recommended temperatures as little, to no detectable levels of amplification may be observed.

1.3 Preventing template cross-contamination

It is essential to take precautions to minimise the potential for carryover of nucleic acid material (in particular amplification product) from one experiment to the next. The use of separate work areas and pipettors for pre- and post-amplification steps and the use of positive displacement pipettors or aerosol-resistant pipette tips are good measures to take, along with the use of PCR hoods where available. Used pipette tips and reaction tubes should be collected in airtight containers or under conditions that destroy potentially contaminating DNA (e.g. acidic conditions, bleach, etc.). Extra care has to be taken with any type of post amplification processing of the reaction whereby tubes containing amplicon are required to be opened, such as when conducting purification, gel electrophoresis, or lateral flow detection.

1.4 Assay optimisation

The standard reaction conditions delivered by the current TwistAmp® kit formulation and protocol (outlined in the instruction manuals) allow both fast and sensitive amplification/ detection for most assays. However, the performance of some assays can be improved by optimisation of the reaction conditions. Parameters that can easily be changed by the user are the reaction temperature, the magnesium acetate (MgOAc) concentration, the mixing regime and the primer/probe (where probes are applicable) concentrations and ratio:

- the temperature should be varied within the recommended range (see Section 1.1).
- the recommended range of MgOAc concentrations in the reaction is between 12mM and 30mM (the standard recommendation of the instruction manuals is 14mM – reaction rate can increase with MgOAc concentration).
- the time of mixing can be varied between 3 minutes and 7 minutes after initiation of the reaction (standard time is 4 minutes – longer or more slowly accumulating amplicons may benefit from slightly later

mixing along with RT assays.

- the concentration of each primer could be varied between approximately 150nM and 600nM and the concentration of probe can be varied between 50nM to 150nM. In addition the ratios of primer 1: primer 2: probe can be varied, however, the total oligonucleotide concentration in the reaction should be maintained in the range 750-2000nM. Using low concentrations of primers may reduce amplification speed, but benefit longer amplicons and improve real-time resolution; whereas high concentrations can accelerate kinetics.

Note: TwistDx has observed batch-to-batch variations in the quality of primer preparations from commercial suppliers which can affect RPA performance. For applications where consistency is critical, we would recommend the use of purified oligonucleotides in established assays. Stock concentrations of primers should also be verified by appropriate means in repeat orders as supplier concentrations can often be misleading.

1.5 Slower rate of amplification and longer amplicons

The current TwistAmp® kit formulations are designed for the fast and sensitive amplification of relatively short target sequences within template DNA (80-500bp). In some cases other parameters may be desired. For example, in some circumstances a slower rate of amplification might be desirable, such as when the TwistAmp® process is going to be used for template quantification (see Section 1.2). There are many ways to engineer decreases in RPA reaction kinetics -for example some primers just amplify more slowly than others and may be selected. Alternatively the reaction temperature or MgOAc concentration can be lowered. However, RPA reactions contain a limited 'clock' dictated by the availability of energy regeneration components, and with the current kits a relatively high level of ATP-burning recombinase (selected to promote rapid kinetics) means that fuel is consumed typically within about 25-30 minutes (depending on primer concentrations used – the complex of DNA and recombinase dictates fuel consumption rate) regardless of modification of reaction conditions within acceptable

parameters. As there is only a certain degree of flexibility in the ratio of primer to recombinase that can be used current TwistAmp® kits may not necessarily be perfectly suited for high resolution in real-time. This is because very low amplification rates (e.g. caused by amplification at low temperature) can lead to ATP or dNTP- exhaustion prior to signal generation (particularly with the TwistAmp® exo kits because exonuclease III introduces some competition with polymerase with regard to product generation).
The TwistAmp® process may also be employed to generate longer amplification products, however, the current TwistAmp® kit formulations are not specifically designed for this purpose. Nevertheless, it is possible to some extent to improve the amplification of longer products, or to deliberately slow amplification for real-time analysis, using the optimisation steps described earlier (see Section 1.4, e.g. lower the oligonucleotide concentration, alter MgOAc and temperature within acceptable bounds). We expect to release some specialised kits for longer amplicons in the near future.

1.6 Reverse Transcriptase use

Current TwistAmp® RT kits contain a reverse transcriptase that allows single-step conversion of RNA into cDNA and subsequent amplification. No additional primers are required for this process to occur.
It's possible to use RNA template with a TwistAmp® kit just by adding a suitable reverse transcriptase (RT) when setting up a reaction. If an RT that works at 37–42 °C is added to RPA chemistry then RNA can be reverse transcribed and the cDNA produced and amplified all in one step. The complementary DNA sequence will be that of the RNA added. We recommend using similar RT amounts to that of a PCR reaction of the same volume, according to your chosen manufacturer's instructions. We also suggest that you run reactions at 40°C and delay agitation of the reaction by a minute to give the RT time to work, otherwise you just run the reaction as you would a normal TwistAmp® reaction (it's a one step process). It's also advisable to add RNase Inhibitor to any reaction where RNA is the target material.

Primer design considerations

Establishing a sensitive and rapid TwistAmp® assay depends on selecting suitable amplification primers. Since it is not yet possible to predict the amplification performance of a given oligonucleotide based purely on its sequence, it is recommended to undertake a simple assay development process including the design and the screening of a series of candidate primers and selecting a preferred primer pair.

Note: The primer design guidance below is advisable for high sensitivity assay development. For assays requiring less strigent detection (greater than 1000 copies per sample tested) only a small number of primers need be screened in most cases, and use of PCR primers may be sufficient.

2.1 Primer length

Primers designed for a given PCR assay may often work in RPA, but may not be optimal for TwistAmp® reactions. TwistAmp® primers are ideally longer than typical PCR primers, 30 to 35 nucleotides long, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. The ability of recombinase proteins to stimulate and complete recombination/priming decreases with oligonucleotide size. Conversely, while many TwistAmp® primers can also be used in PCR amplification, they are not selected or optimised for that purpose and their performance in PCR may not bear a direct relationship to their quality as TwistAmp® primers.

Oligonucleotides shorter than 30 nucleotides can still function, however, under these conditions the amplification kinetics are typically slower compared to the use of two opposing amplification primers of 30 residues or greater.

Oligonucleotides of up to 45 nucleotides have been successfully used as primers in the TwistAmp® process and in principle primers could be even longer. However, lengthening the primer does not necessarily improve the amplification performance and it increases the likelihood of secondary-structures that could lead to primer noise (see Section 2.6). It is therefore advisable not to design excessively long primers.

2.2 Primer sequence

Oligonucleotides of different sequence perform differently in TwistAmp® reactions, but there are no fixed rules to predict how well a given amplification primer will work based on the order and composition of its nucleotides. However, some guidelines have evolved based on empirical observations (although these should not deter the user from generally trying many sequences).

Where possible, it is best to avoid unusual sequence elements within the primer, such as long tracks of one particular nucleotide or a large number of small repeats. Excessively high (>70%) or low (<30%) GC content is likely to be detrimental. Since base-pairing interactions both within and between primers could contribute to artefact generation (primer dimers etc.), oligonucleotides that contain sequence elements that promote secondary structures and primer-primer interactions or hairpins should be discarded.

2.3 Amplification product length

Depending on the reaction conditions RPA allows for the amplification of DNA products of up to at least 1.5kb [http://dx.doi.org/10.1371/journal.pbio.0040204]. However, the current TwistAmp® kits have been configured to favour rapid amplification as opposed to maximising the length of amplification products. For this reason, targets of over about 500bp do not amplify well with the current TwistAmp® formulation.

For ultra-rapid TwistAmp® assays we recommend an amplicon length that does not exceed 500bp, and ideally is between 100 - 200bp.

This is because shorter products are generated in a shorter period of time and therefore tend to have an improved product/noise ratio. As a consequence, the overall amplification performance is improved (although a main factor in amplification speed is a characteristic of the chosen primer pair).

Note: Amplicon size in RPA directly affects the amplification sensitivity and speed: due to the effects of primer noise (artefacts; see Section 2.6). Whilst longer amplicons require increased time to amplify, primer noise tends to be independent of target size. Therefore, the longer an amplicon is, the more likely primer noise will outcompete target amplification. For this reason, short amplicons are preferred for ultra-sensitive assays.

If probes are to be used for detection, then care must be taken when defining amplification primers to leave enough sequence space for the design of an oligonucleotide probe (see Section 3). The lower limit to the size of RPA products is mainly determined by the size of RPA primers. Typically this requires that amplicons will be longer than about 70-80bp.

2.4 Primer selection

The primer selection process typically consists of the following steps:

Step 1. Choice of target region

It is advisable to select a region within the template that is characterised by relatively 'average' nucleotide sequence composition:

- GC content of between 40% and 60%
- repetitive sequences
- few direct/inverted repeats, palindromes, etc.

Repetitive elements within a given genome should be avoided in order to preserve the uniqueness of the target. In this respect the preferred sequences are identified in much the same way as those for PCR.

Step 2. Primer candidates

After choosing a suitable target region, two groups of staggered oligonucleotides facing each other (i.e. having forward and reverse direction) are selected that serve as primer candidates (see Figure 2). Primers in the same orientation can, but need not, be overlapping. Each primer from the forward group can later be paired with each primer from

the reverse group. A medium scale screen designed to detect template DNA with single molecule sensitivity with faster than 20 second 'doubling times' (the length of reaction time during the exponential phase of RPA in which the amount of product doubles) typically has 8 to 10 primers per direction (i.e. creating 64 to 100 possible primer combinations).
When under strict time constraints and where optimal primer design is not essential (such as for an assay target found in relatively high concentration in samples), the following value inputs are suggested as PCR software settings (e.g. Primer 3, Primer-BLAST):

- primer size minimum of 30 and maximum 36
- primer GC% minimum of 20 and maximum 70
- primer Tm minimum 50 and maximum 100
- maximum allowable length of a mononucleotide repeat, e.g. 'CCCCC' set to 5.

Step 3. Screening of candidates

Once candidate primer pairs are defined, their relative performances have to be assessed and compared.
The quality of each primer pair depends on the context of the intended assay, and for this reason the method of read-out for the screening procedure differs accordingly:

- for TwistAmp® Basic each primer pair should be used as described in the protocol, the various amplification products should be purified (e.g. standard PCR-product purification methods) and then resolved by agarose-gel- electrophoresis. The various primer pairs can then be classified according to sensitivity, product yield, product/noise ratio and amplification time (if a time course is taken).
- for TwistAmp® exo and TwistAmp® fpg the primer pairs are tested using the relevant fluorescence detection probes according to the standard protocol for a real-time read-out and the fluorescence data is compared. The key performance parameters are sensitivity, time of amplification onset and total fluorescence signal strength.
- for TwistAmp® nfo primer pair quality is assessed using TwistAmp®

nfo probes and the corresponding lateral-flow strip analysis as read-out system. Suitable performance indicators are total signal strength and absence of background signal on the lateral-flow strip, as well as sensitivity and amplification time (if a time course is taken).

It should be noted that in practice the most practical assay method for primer screening is probably by use of a real-time fluorescence read-out which rapidly provides both sensitivity and kinetic data. Primer pairs and probes can then be adapted for appropriate readout i.e. nfo probe, Basic. Requalification may be performed to confirm choice. It is not always necessary to test every possible primer combination. For example, by screening all reverse primers against a single forward primer, picking the best reverse primer and then using it to screen all the forward primers, a good primer pair can be found in 16 to 20 reactions (see Figure 1a and 1b).

	R1	R2	R3	R4	R5
F1					
F2					
F3	--	+	+	+++	++
F4					
F5					

Figure 1a. *Example of a 5x5 primer candidate matrix and the results of a first screening experiment. All reverse primers (designated R1 to R5) are screened with forward primer 3 (F3) and scored for amplification performance ('-' for failed amplification, '+++' for best amplification, etc.). Here, reverse primer 4 (R4) gives the best results.*

	R1	R2	R3	R4	R5
F1				--	
F2				++	
F3	--	+	+	+++	++
F4				++++	
F5				++	

Figure 1b. *Example of a second screening experiment based on the result shown in figure 1a. R4 is screened with all forward primers. F4 gives the best results. The combination F4/R4 is the best performing primer pair. Further screening experiments can be performed to confirm this finding, e.g. screening all the reverse primers against F4.*

The test conditions for the candidate screen (copy number of template, template purity) should be sufficiently stringent to make it possible to distinguish primer quality at the top end of the performance list. On the other hand, initial conditions should not be so challenging that none of the primer pairs tested is successful at this stage of the assay development process: even if the goal is to detect one molecule, finding the limited set of primers that detect 25 molecules will narrow the field to those that are generally well-behaved and can potentially be improved (see Step 4).

In many cases Step 3 of the screening strategy will already yield sufficiently good primer pairs for the intended assay. If so, the best primer pairs (typically the best 3) can then be further characterised for amplification speed and sensitivity by repeated testing at different starting template concentrations. It is advisable to decide on a short-list of suitable primer pairs at this stage (rather than just one pair) if multiplexing is required (see Section 4). If none of the primer pairs tested at this stage of the assay development process performs satisfactorily, it is necessary to return to Step 2 and design new primers.

Note: The success of a primer screen setup can also depend on the quality of the probe; see Section 3 for a discussion of detection probe design.

Step 4. Secondary and tertiary candidate screen (for high sensitivity assays)
Even small variations in the sequence of oligonucleotides can sometimes result in significant differences in primer activity (possibly due to a different propensity to form primer dimers or other artefacts). A strategy to improve the performance of a given assay is therefore to generate a second generation of primers by creating variants of the best primers identified in Step 3 and re-screen these for improved amplification performance. A good first step is to fill in the gaps around the primers selected in the initial screen. Screen primers of the same length as those selected, but moved in 1 base increments around them up to rejected primers. Further refinements can be made by adding and subtracting bases from the 3′ end of the chosen primers (sometimes a 32-mer will perform better than a 35-mer, sometimes a 38-mer will perform best). Step 4 of the primer selection procedure should yield good primer pairs for the intended assay. If this is not the case and none of the primer pairs tested during the development process performs satisfactorily, it is necessary to return to Step 2 and design new primers.

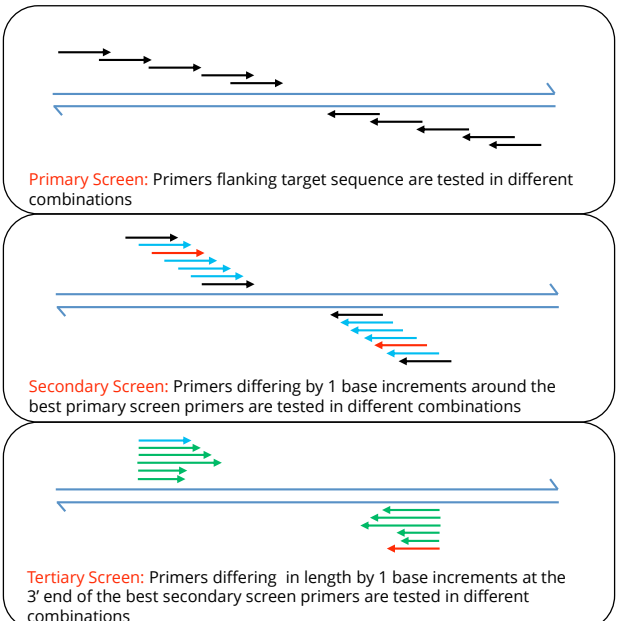

Figure 2. *Typical stringent primer screen. Where single molecule sensitivity is not required, the primary screen will usually suffice.*

2.5 Difficult amplicons

On rare occasions sequence elements within an amplicon (i.e. the sequences between primers) are detrimental to rapid amplification and all primer pairs producing amplicons sharing such a sequence motif will perform poorly. Should attempts to design suitable primer pairs for a given target region consistently yield results outside of the normal range of expected or desired behaviour, it may be worth seeking another, non-overlapping target region elsewhere in the template. The

normal expectation for a first-round screen would be to detect as low as 25-50 copies and achieve detection threshold in real-time within 15 minutes of attaining optimal reaction temperature using most standard fluorescence monitoring systems.

2.6 Primer noise

In addition to promoting genuine amplification events, the TwistAmp® reaction environment also allows undesired primer interactions to occur (similar phenomena are common to other nucleic acid amplification techniques, for example PCR). Such interactions can be intramolecular (hairpins, etc.) or result from primer dimer formation, both between identical or different oligonucleotides. These structures can provide substrates for extension by DNA polymerase and some of the artefacts so generated will serve as template for further recombination/extension events and thus enter a phase of exponential amplification. Processes of this type will generate detectable levels of relatively low molecular weight DNA consisting of primer derived sequence ('primer noise'). Noise reactions are in competition (for primers, nucleotides, polymerase binding and energy) with the genuine amplification process, and will eventually inhibit the latter. The propensity to generate noise will limit the sensitivity of a given primer pair. For this reason, it is important to select primers, which will minimise the competitive production of primer artefacts. In practice this requires screening of candidate primers for those with the highest sensitivity. Primer noise will be unrelated in sequence to that of the detection probes used with the TwistAmp® exo, TwistAmp® fpg and TwistAmp® nfo kits. Such artefacts will therefore not generate an erroneous signal with these kits.

2.7 Degenerate primers and mismatches

There are no specific suggested limits on use of degenerate bases with RPA other than minimal use is probably advisable. RPA is generally quite robust to mismatches. It has been shown that the performance of RPA assays is not affected when primers and probe carry 5–9 base pair mismatches (http://dx.doi.org/10.1371/journal.pone.0071642 ; http://dx.doi.org/10.1128/mBio.00135-13).

Probe design considerations

RPA utilises proprietary probe systems designed for providing specific and sensitive detection of amplicons. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers. Oligonucleotide probes that are compatible with the TwistAmp® technology come in three different varieties: TwistAmp® exo, TwistAmp® fpg and TwistAmp® nfo probes. Each type is used with the TwistAmp® exo, the TwistAmp® fpg kit, and the TwistAmp® nfo kit, respectively.

Probe ordering

TwistAmp® probes can be ordered from various oligonucleotide manufacturers, using the TwistDx™ TwistAmp® probe order forms (available at twistdx.co.uk).

Fluorophore choice

A range of fluorophores of different wavelength are applicable for use. Fluorophore labels previously used include FAM, SIMA/HEX, ROX, TAMRA, Texas Red, and CalFluor610. When using FAM fluorophore it is recommended to use 6FAM, as the emission band from the 6-carboxyfluorescein oligonucleotide has been shown to be substantially sharper than that of the 5-carboxyfluorescein analogue (https://doi.org/10.1021/bc7001874). When using biological samples, it is recommended to avoid FAM use as these samples may exhibit a high level of background fluorescence.
Suggested suitable fluorophore quenchers are listed below:

Fluorophore	**Quencher**
FAM	BHQ1
SIMA/HEX	BHQ1
ROX	BHQ2
TAMRA	BHQ2
TexasRed/CalFluor610	BHQ2

The structures of all three types of TwistAmp® probe are discussed separately below.

Probe candidates

Although probes appear to be less sensitive to sequence variations than primers, probes of different sequence will perform differently. If optimal performance is required it is advisable to test more than one potential probe within a target. However, even without optimisation most probes designed according to the described principle will work adequately and be suitable for the purpose of distinguishing the performance of different primer pairs. If a probe is to be used for a primer screen, it is a good strategy to design it so that it is located within the smallest candidate amplicon (defined by the innermost primers in the forward and reverse groups of candidate primers, see Section 2). The probe can then be used to test the performance of all the surrounding primers. It is worth noting that probes can be designed for either strand which increases the number of possible candidates that can be designed for a given target.

3.1.1 TwistAmp® exo probe structure and function

The type of oligonucleotide probe that is compatible with the TwistAmp® exo kit is the TwistAmp® exo probe. TwistAmp® exo probe consist of an oligonucleotide backbone that contains:

- an abasic nucleotide analogue (a tetrahydrofuran (THF) residue, sometimes referred to as a 'dSpacer').
- a flanking dT-fluorophore (typically fluorescein, but any fluorophore available as dT-coupled reagents for oligonucleotide synthesis can be used).
- a corresponding dT-quencher group (typically a suitable Black Hole Quencher (BHQ)) on the other side of the THF group.
- a suitable 3'–modification group (such as a C3-spacer, a phosphate, a biotin-TEG or an amine) to block the probe from polymerase extension.

Any fluorescent signal generated by the fluorophore will normally be quenched by the quencher located 2-5 bases 3' to the fluorophore. In a double stranded context the THF residue presents a substrate for the DNA repair enzyme Exonuclease III present in the TwistAmp® exo

kit, which will cleave the probe at the THF position, thereby separating the fluorophore and the quencher and generating a fluorescent signal. This nuclease activity is restricted to when the probe has annealed to the target sequence within the amplification product. The cutting of the probe is therefore indicative of the specific target amplification event and can be used to monitor specific amplicon accumulation.

As the internal labels used in TwistAmp® exo probe are currently only readily available on thymines, there is generally a restriction of ideal probe locations to sequences in which two thymines can be found with fewer than about 5 intervening nucleotides (greater separations decrease the quenching efficiency and at present nucleotide analogues for other bases are not available). However in most cases, and given that either strand of the target can be used, there are usually appropriate design sites available. Furthermore, there are two additional approaches that may be used to expand the available sites for TwistAmp® exo probe design. (1) The TwistAmp® exo probe can be identical to one of the amplification primers but possess an additional 3' sequence extension containing the THF residue and the further 15 residues of homology 3' to the THF residue. The THF residue must be positioned further 3' to the sequence of the related main amplification primer to avoid the probe detecting 'primer noise' events that may be generated from it. (2) It is also possible to tolerate a mismatch in the probe such that if two conveniently separated thymines cannot be located one can simply ignore the mismatch of one of the thymines in the probe with the target sequence. There may be a reduction in the efficiency of the probe, the extent to which cannot be predicted; however such configurations have been effective in the past. Figure 3 shows a schematic of a typical TwistAmp® exo probe.

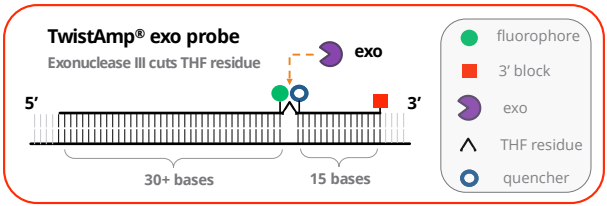

Figure 3 *Schematic of the structure of an annealed TwistAmp® exo probe. The abasic THF residue is cleaved by Exonuclease III only when the probe is bound to its target. This cutting step separates the fluorophore and quencher and generates a fluorescent signal.*

3.1.2 TwistAmp® exo probe length, position and examples

A TwistAmp® exo probe should be 46-52 nucleotides long, at least 30 of which are placed 5′ to the THF site, and at least a further 15 are located 3′ to it.

Note: The THF residue, the dT-fluorophore and dT-quencher **replace bases** found within the target amplicon sequence and are **not additional insertions.**

There is no fixed rule describing the best position of a given probe relative to its corresponding amplification primers. Care must be taken to avoid the possibility that primer artefacts can be detected by the probe, as could occur if the probe overlaps the amplification primers. Primers opposing the direction of the probe should not overlap to avoid the occurrence of primer-probe dimers. However, primers in the same direction as the probe can overlap its 5′ part (see Section 3.1.1) this overlap must not include the abasic-site and more 3′ parts of the probe (i.e. the overlap of the primer should be restricted to the 5′-most 27-30 nucleotides of the probe). This will prevent the inadvertent generation of artefactual hybridisation targets for the sensitive cleavage sequence element of the probe. Secondary structures that could cause probes to

fold back on themselves should also be avoided.

Example TwistAmp® exo probe designs

Given an appropriate target sequence the most important factor is to identify a pair of T residues in close proximity to one another (with only 1-5 intervening nucleotides). As an example a target sequence is shown below along with two suggested probes that could be designed to detect it:

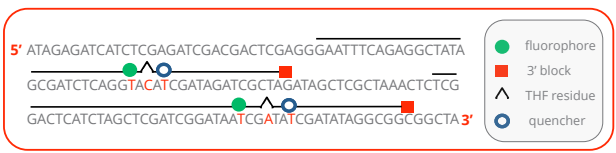

Figure 4 *An example of a sequence and possible TwistAmp® exo probes that could be generated for this sequence. The positions of the possible probes are indicated with a bold line, probe attachments and structures are labelled accordingly. Corresponding bases to be replaced are indicated in red.*

In this case one probe ordered would have the following sequence in which the relevant T residues in the sequence are replaced by dT-fluorophore residues or dT-quencher residues, and one base (a C in this case) is replaced by the THF residue:

GAATTTCAGAGGCTATAGCGATCTCAGG [FAM-dT] A [THF] A [BHQ1-dT] CGATAGATCGCTA [3'-block]

The number of nucleotides between the dT-fluorophore, or the dT-quencher, and the THF can be 0, 1 or 2 and there is no known sequence requirement for these intervening nucleotides, nor for the base which is replaced with a THF. Based on these principles a second possible probe is shown with sequence:

TCGGACTCATCTAGCTCGATCGGATAA [FAM-dT] CG [THF] TA [BHQ1-dT] CGATATAGGCGG [3'-block]

We have routinely blocked the 3'-end of the probe with a group such as C3- spacer, biotin- TEG, or phosphate.
Amplification primers are designed, in most cases, to flank the probe sequences, however there can be some overlap between the 5' portion of the probe and an amplification primer as detailed above.

Common TwistAmp® exo probe design errors:

Target sequence for probe:

GAATTTCAGAGGCTATAGCGATCTCAGGTCAATCGATAGATCGCTA

F = FAM-dT H = Tetrahydrofuran Q = BHQ1-dT

Good probe design:

GAATTTCAGAGGCTATAGCGATCTCAGGFAHAQCGATAGATCGCTA
[3'-block]
≥30 bases 5' of THF
≥15 bases 3' of THF
≤5 bases between fluorophore and quencher Single THF, between fluorophore and quencher

Poor probe designs:

GAATTTCAGAGGCTATAGCGATCTCAGGFHHHQCGATAGATCGCTA
[3'-block]
Only one THF needed per probe.

GAATTTCAGAGGCTATAGCGATCTCAGGTACATCGAFAHAQCGCTA
[3'-block]
There should be ≥15 bases 3' of the THF if the exonuclease is going to cut it efficiently.

GAATTTCAGAGGCTATAGCGATCFCAHGQACATCGATAGATCGCTA
[3'-block]
There should be 30-38 bases 5' of the THF so that the probe can act as a primer when it is cut.

GAATTTCAGAGGCTATAGCGATCTCAGGFAHATCGAQAGATCGCTA
[3'-block]
Distance between fluorophore and quencher is too large – quenching may be poor.

GAATTTCAGAGGCTATAGCGATCTCAGGFACHAQCGATAGATCGCTA
[3'-block]
THF has been added as an additional base, rather than replacing an existing base.

3.2.1 TwistAmp® nfo probe structure and function

TwistAmp® nfo probes are used with the TwistAmp® nfo kit, and are intended for detection by so-called sandwich assays, on lateral flow consumables. The probes consist of an oligonucleotide backbone with:

- a 5'-antigenic label.
- an internal abasic nucleotide analogue (a tetrahydrofuran residue or THF – sometimes referred to as a 'dSpacer') which replaces a nucleotide.
- a polymerase extension blocking group (such as a phosphate, C3-spacer or a dideoxy nucleotide, but **not a biotin-TEG**) at the 3' end.

TwistAmp® nfo probes are used in a configuration in which the opposing amplification primer is labelled at its 5' end with another antigenic label (different to that of the probe). The third oligonucleotide present in the reaction (equidirectional with the probe) is a conventional primer. Figure 5 shows a schematic of a typical TwistAmp® nfo probe. In practise, it makes no difference which way round the labels are used i.e. which label is on the probe, and which one is on the primer. However, for ease of further test development, it may prove useful to put the label for the conjugate antibody on the probe (Biotin for PCRD). This way, users who proceed to use a two line strip for multiplexing, can change which line on the strip they detect at by ordering a new labelled primer rather than ordering a new probe.

Note: The THF residue **replace a base** that would be present in the target sequence and is **not an additional insertion.**

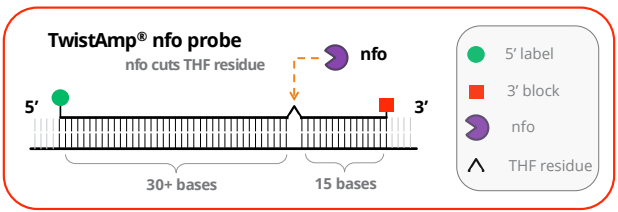

Figure 5. *Schematic of the structure of an annealed TwistAmp® nfo probe. The abasic THF residue is cleaved by nfo only when the probe is bound. The 3' OH group generated in the process is a target for extension by the polymerase and enables the 5' label to be integrated in amplification products.*

The TwistAmp® amplification reaction promoted by the two primer oligonucleotides will generate targets for the annealing of the TwistAmp® nfo probe. In the resulting double strand context the THF residue presents a substrate for the enzyme nfo (also known as Endonuclease IV) present in the TwistAmp® nfo kit. nfo will cleave the probe at the THF position and thereby generate a new 3'-hydroxyl group (effectively de-blocking the probe) that can act as a priming site for polymerase extension, thus transforming the probe into a primer.
The amplicon produced by the processed probe and the 5'-labelled opposing amplification primer will effectively co-join the two antigenic residues in one DNA molecule (see Figure 6). This duplex can then be detected in sandwich assay formats (typically post-amplification, i.e. endpoint detection), using consumables such as those listed below:

Consumable	**Designed to detect amplicons labelled with**
Milenia Hybridetect-1	biotin + FITC/FAM
U-Star units	biotin + FITC/FAM
PCRD	DIG + biotin and/or FITC/FAM + biotin

Further information about the above consumables can be found at twistdx.co.uk.

Lateral-flow strips for the detection of nucleic acids (including the Milenia Hybridetect-1) are typically designed to utilise products (for instance of PCR products and antigen-labelled probes) as their substrate and therefore require extensive sample processing procedures. In contrast the TwistAmp® nfo reaction mechanism generates the double-labelled reporter molecule simultaneously with the amplification reaction and only requires minimal post-amplification processing.

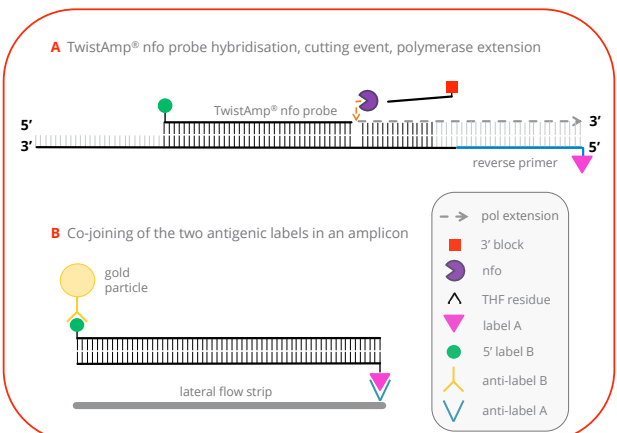

Figure 6. *Schematic of the arrangements of amplification primers and TwistAmp® nfo probe. (A) The processed probe and the opposing primer will generate double- stranded amplification products that co-join the two antigenic labels. (B) This product can subsequently be captured using one of the labels (for instance using the anti- biotin test line on a lateral flow strip), and visualised using the other (for example by interaction with gold-labeled antibodies).*

The nfo nuclease reaction and the resulting generation of the double-labelled amplicon are restricted to cases in which the probe can anneal to its target sequence, chosen to be within the original amplification product. Cutting of the probe is therefore indicative of the amplification event itself and can be used to monitor the success of the TwistAmp® reaction.

3.2.2 TwistAmp® nfo probe length, position and examples

A TwistAmp® nfo probe should typically be 46-52 nucleotides long, at least 30 of which are placed 5′ to the THF (tetrahydrofuran) site and at least a further 15 are located 3′ to it. The THF residue replaces a nucleotide that would normally base pair to the complementary sequence.

There is no fixed rule describing the best position of a given probe relative to its corresponding amplification primers. Care must be taken to avoid the possibility that primer artefacts can be detected by the probe. Although primers that have the same direction as the probe can even overlap its 5′ part, this overlap must not extend up to the abasic-site portion of the probe (i.e. the overlap of the primer should be restricted to the 5′-most 30 nucleotides of the probe or so). This will prevent the inadvertent generation of hybridisation targets for the sensitive sequence element of the probe by primer artefacts. Primers opposing the direction of the probe should not overlap to avoid the occurrence of primer-probe dimers. The opposing amplification primer has to be labelled with an antigenic group, usually a biotin.

Example TwistAmp® nfo probe design

As an example a target sequence is shown below along with a suggested probe that could be designed to it:

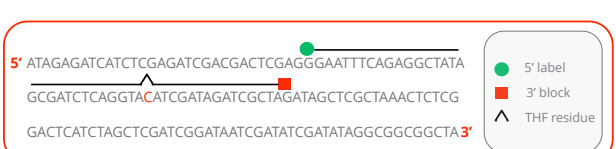

Figure 7. *An example of a sequence and possible TwistAmp® nfo probe that could be generated for this sequence. The position of the possible probe is indicated with a bold line, probe attachments and structures are labelled accordingly. Corresponding base to be replaced is indicated in red.*

The probe is blocked (typically with a dideoxy-C, G, A or T, or with a C3-spacer, or a phosphate, **but <u>not</u> a biotin**). The sequence of this probe would thus be:

GAATTTCAGAGGCTATAGCGATCTCAGGTA[THF]ATCGATAGATCGCTA [3' block]

Amplification primers would normally flank the probe on either side as indicated in Figure 6.

3.3.1 TwistAmp® fpg probe structure and function

TwistAmp® fpg probe is used with the TwistAmp® fpg kit, and is intended for fluorescence detection assays. These probes typically are oligonucleotides homologous to the target amplicon that contain:

- a fluorophore label replacing an A or G nucleotide with a dR-fluorophore (typically FAM or ROX) positioned about 15 nucleotides upstream from the 3' end of the probe. The fluorophore is attached to the deoxyribose of the abasic site via a C-O-C linker (a so- called dR-group).
- a dT-quencher (specific to the chosen fluorophore type) is then positioned 1 to 3 nucleotides either upstream, or downstream of the fluorophore.
- In addition, TwistAmp® fpg probes are blocked from polymerase extension by a suitable 3' modification (such as a C3-spacer, a phosphate, a Biotin-TEG or an amine).

The fluorescent signal generated by the fluorophore (any fluorophores that are available as dR-derivatives such as Carboxy-fluorescein) will normally be quenched by the quencher group (typically a Black Hole Quencher (BHQ)). In a double-stranded context the dR-fluorophore

residue, the 'gap' in the probe, presents a substrate for the enzyme fpg present in the TwistAmp® fpg kit. fpg will cleave the probe at the dR position, thereby separating the fluorophore and the quencher and generating a fluorescent signal. This nuclease step is restricted to cases in which the probe can anneal to its target sequence, chosen to be within the amplification product. Cutting of the probe is therefore indicative of the amplification event itself and can be used to monitor the progress of the TwistAmp® reaction. Figure 8 shows a schematic of a typical TwistAmp® fpg probe.

Note: the dR-fluorophore and dT-quencher **replace bases** found within the target amplicon sequence and are **not additional insertions.**

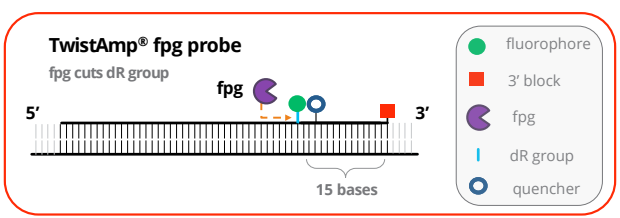

Figure 8. *Schematic of the structure of an annealed TwistAmp® fpg probe. The abasic dR residue is cleaved by fpg only when the probe is bound to its target. This cutting step releases the fluorophore from the probe and the quencher proximity and generates fluorescence signal.*

3.3.2 TwistAmp® fpg probe length, position and examples

A TwistAmp® fpg probe should typically be 46-52 nucleotides long, but shorter probes may also be successful. There is no fixed rule describing the best position of a given TwistAmp® fpg probe quencher relative to the amplification primers with which it is used. However, care must be taken to avoid the possibility that primer artefacts can be detected by the probe, so any overlap between primers and the probe should be avoided.

Example TwistAmp® fpg probe design

As an example a target sequence is shown below along with two suggested probes that could be designed to it:

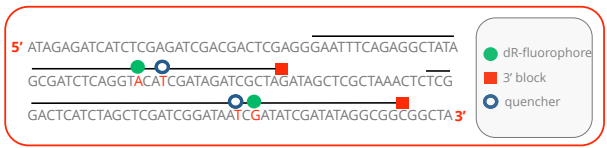

Figure 9. *An example of a sequence and possible TwistAmp® fpg probes that could be generated for this sequence. The positions of the probes are indicated with bold lines, probe attachments are labelled accordingly. Corresponding bases to be replaced are indicated in red.*

In this case one probe ordered would have the following sequence in which a fluorophore label attached via a dR-group is positioned 15 bases from the 3' probe end, with a dT-quencher positioned 2 nucleotides downstream of the fluorophore, and blocked with a 3' C3-spacer:

GAATTTCAGAGGCTATAGCGATCTCAGGT [dR-FAM] CA [BHQ1-dT] CGATAGATCGCT [3'-block]

Based on these principles a second possible probe is shown with dR-fluorophore positioned 16 bases from the 3' probe end, with a dT-quencher positioned 1 nucleotide upstream of the fluorophore, and blocked with a 3' C3-spacer:

TCGGACTCATCTAGCTCGATCGGATAA [BHQ1-dT] C [dR-FAM] ATATCGATATAGGCGG [3'-block]

The exact number of bases between the internal dT-quencher and the dR-fluorophore is advised to be kept to a maximum of 3 bases to maintain the quenching efficiency.

Multiplexing

A multiplex TwistAmp® reaction is the simultaneous amplification of more than one target in a single reaction. The primers used for the amplification of each of the multiplexed targets have to be tested and potentially optimised for compatibility. Not all primer pairs that prove to be sufficiently active in single assay reactions will turn out to perform well in multiplex assay reaction setups.

4.1 Primer compatibility and concentration ratios

A good strategy for identifying good primers for multiplex reactions is to first define a number of good candidate primer pairs for each target individually. Subsequently, the candidate pairs for each target are successively combined with the candidate pairs of the other targets in the final multiplex format. After having defined the best combination, differences in the relative performance of the primer pairs for the various targets can be adjusted by changing the ratios of the amount of primers used in the reaction (for instance, in a hypothetical duplex reaction the proportion of primers used to amplify target A might account for 65% of the total primer amount in the reaction, while the proportion of those used for target B will only be 35%).

4.2 Inhibitory primers

In some cases of multiplexing it can appear difficult to pair certain primer pairs effectively together even when the ratios of primers are adjusted. Oftentimes this is because one primer in the system has a dominating and/or inhibiting activity on other primers and amplicons in the system. In this case it can be worthwhile to add even only one primer of a pair into an otherwise effective system to determine which single primer under analysis has this undesirable activity. Once the primer has been identified approaches may be taken to exclude it from future multiplexing experiments.

4.3 Total primer concentration

It should be noted that the total amount of oligonucleotides in the reaction (i.e. the sum of all primers added to the reaction mixture)

should not exceed the recommended amount. Multiplexing will therefore lead to a reduction of the yield of individual amplicons for the various targets compared to single assay reactions.

4.4 Different amplification rates

Different amplicons in RPA can amplify at different rates. Under circumstances in which target amplicons must be co-amplified and their expected target numbers in a sample are similar, then equal amplification rates of the two targets may be optimal. However, in other circumstances it may be the case that one target will always be in significant excess over the other, and sensitivity to the less abundant target must be maintained. RPA, in contrast to PCR, offers advantages in this case as it is possible to tune one's primer selections and develop a multiplex system in which the amplification rate of the less abundant target is faster than the more abundant species. This flexibility is very useful when detection of a rare event is desired in a background of an abundant target which is to be used as an internal control.

Sample input compatibility

RPA compatible template has been prepared from a wide range of sources, including blood, nasal swabs, plant material and culture media, by basic lysis methods (such as weak alkali or heat treatment). Often further isolation and purification of the nucleic acid material post lysis is unnecessary. RPA is relatively robust to sample inhibitors in comparison to PCR. The best approach has to be specifically defined for each type of application (and assay) and is dependent on factors such as pathogen titre, presence of inhibitors and lysis requirements.

5.1 Example lysis methods

Simple methods such as using non-ionic detergents (1% Triton-X 100 for example), or heating can be employed. Sample dilution with 0.2M NaOH or KOH can also work well for lysis.

5.2 Buffer compatibility

Buffers commonly used with PCR without dilution, are also likely to be

compatible with RPA. All the obvious suspects that disrupt PCR are also likely to affect RPA – i.e. EDTA/EGTA, strong detergents, high salt, acid/base etc.

5.3 Termination of amplification

RPA reactions will run out of fuel and reagents eventually during the amplification process; however termination of amplification may be a desired function for some applications. In those scenarios a number of strategies can be applied as follows:

- heat the reactions (above or equal to 85oC) to denature the proteins.
- add EDTA or similar to chelate the MgOAc present.
- dilute the reactions with water so that the crowding agent is ineffective (at least 1/4 dilution). This method is not recommended for TwistAmp® exo or fpg products due to the nucleases present which degrade amplicon over time.

Other RPA methods

Although not directly supported through the instruction manuals, many RPA users have applied the technique in other formats for their specific applications; those include digital, nesting, microfluidics, solid phase, template generation, electrochemistry, colorimetric or SNP detection. More information as to how these methods have been applied with, or in combination with RPA can be found in the publications listed at twistdx.co.uk.

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