

# Appendix to the TwistAmp™ reaction kit manuals

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## 1. 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 full 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 (fairly restricted) recommended range (as amplicon doubling times lengthen more rapidly with temperature drop than energy consumption rates which can lead to premature fuel 'burn-out' before accumulation with some kits). Sensitivity to temperature has been most acutely observed for the TwistAmp™ exo real-time fluorescence kits with the current configuration. 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

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 start' as described in the manual. Alternatively, reactions can also be temporarily 'slowed down' (or halted) by setting them up on ice and then started by simultaneous transfer to the optimal reaction temperature. Methods to slow/alter 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 first generation of TwistAmp™ kits has not been optimized for reduced kinetics and lower temperature and we do not advise running the TwistAmp™ exo system below the recommended temperatures as no amplification to detectable levels may be observed. TwistDx expects to add an additional range of buffers and novel reaction formulations in the near future to address modifications to thermal and kinetic aspects.

### 1.3 Preventing template cross-contamination

It is essential to take precautions to minimize the potential for carryover of nucleic acids 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. 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, such as purification of amplification products or agarose/polyacrylamide gel based analysis.

#### 1.4 Assay optimization

The standard reaction conditions delivered by the current TwistAmp™ kit formulation and protocol (outlined in the manual) create a very good 'compromise' reaction environment for the fast and sensitive amplification and detection of DNA. However, some established assays (i.e. primer/probe sets) can profit in their performance by optimization of the reaction conditions. Parameters that can easily be changed by the user are the reaction temperature, the Magnesium concentration, the agitation regime and the primer/probe (where probes are applicable) concentrations:

- the temperature should be varied within the (limited) recommended range (see 1.1)
- the recommended range of Magnesium-Acetate concentrations in the reaction is between 12mM and 20mM (the standard recommendation of the manual is 14mM – reaction rate can increase with Magnesium concentration)
- the time of shaking can be varied between 3 minutes and 6 minutes after initiation of the reaction (standard time is 4 minutes – longer or more slowly accumulating amplicons may in particular benefit from slightly later agitation)
- the concentration of each primer could be tried between about 150nM and 600nM<sup>1</sup> (Lower concentrations of primer may delay detection onset but benefit longer amplicons and improve real-time resolution, higher concentrations can accelerate kinetics).
- the probe concentration could be varied between 50nM to 150nM

Changes in the concentrations of rehydration buffer components can also improve the performance of a given primer(/probe) combination. TwistDx intends to make available a panel of optimizer buffers in due course. Please check our website from time to time for further updated information.

Modified formulations may be made available in future kits, particularly with an emphasis on longer amplicons, lower temperature operation and resolution in real-time.

<sup>1</sup> Note: TwistDx has observed batch-to-batch variations in the quality of primer preparations from commercial suppliers which can affect reaction performance. For applications where consistency is critical, we would therefore 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 standard first generation TwistAmp™ kit formulations are designed for the fast and sensitive amplification of relatively short target sequences within template DNA. 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 1.3). 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 magnesium concentration can be lowered. However, note that RPA reactions contain a limited 'clock' dictated by the availability of energy regeneration components, and with the first generation products a relatively high

level of ATP-burning recombinase (selected to promote rapid kinetics) means that fuel is consumed typically within about 15-23 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 employed this means that first-generation TwistAmp™ kits are not necessarily perfectly suited for high resolution in real-time as very low amplification rates (for example by lowering temperature) can lead to ATP or dNTP-exhaustion prior to the signal generation caused when sufficient amplicon has accumulated (particularly with the TwistAmp™ exo kits because the exonuclease III introduces some competition with polymerase in regard to product generation).

The TwistAmp™ process may also be employed to generate larger amplification products. However for similar reasons (longer amplicons have longer doubling times) the current first-generation TwistAmp™ kit formulations are not specifically designed for this purpose,

It is possible to some extent to improve the amplification of longer products, or to deliberately slow amplification for real-time analysis, using the optimization steps described earlier (see 1.4) (lower the oligonucleotide concentration, use of optimizer buffers, alter magnesium and temperature within acceptable bounds). We expect to release some specialized kits and buffers for these applications in the near future.

## 2. 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 undergo a simple assay development process including the design and the screening of a series of candidate primers and selecting a preferred primer pair.

### 2.1 Primer length

TwistAmp™ primers typically are 30 to 35 nucleotides long. The ability of recombinase proteins to stimulate and complete recombination/priming with shorter oligonucleotides in the TwistAmp™ reaction environment decreases sharply with size<sup>1,2</sup>.

Oligonucleotides of up to 45 nucleotides have been successfully used as primers in the TwistAmp™ process, and primers could even longer. However, lengthening the oligonucleotide 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.

1 Note: For this reason many PCR primers (those shorter than 30 nucleotides) will not work well in TwistAmp™. 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.

2 Note: Oligonucleotides shorter than 30 nucleotides can still function as hybridization primers even though they have little recombinase-mediated strand-invasion activity. For this reason a single oligonucleotide of greater than 30 nucleotides may be successfully combined with a shorter opposing primer (e.g. 25-mer). However, under these conditions the amplification kinetics are typically slower compared to the use of two opposing amplification primers of 30 residues or greater.

## 2.2 Primer sequence

Oligonucleotides of different sequence perform differently in TwistAmp™ reactions, but there are no fixed rules to predict how good a given amplification primer will work based on the order and composition of its nucleotides. However, some guidelines have evolved based on empirical observations (but should not detract the user from generally trying many sequences). It appears that long tracks of guanines at the 5' end (first 3-5 nucleotides) should be avoided, while cytidines (and perhaps in general pyrimidines) may be beneficial, possibly because this encourages the formation of recombinase filaments. Also, guanines and cytidines at the 3' end of the primer (last 3 nucleotides) tend to improve performance (they might provide a more stable 'clamped' target for the polymerase). 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.), and 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 [Piepenburg et al., 2006]. However, the standard TwistAmp™ kits that are currently available 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 to aim for an amplicon length that does not exceed about 500bp, and ideally is between 100-200bp. This is because shorter products tend to be 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)<sup>1</sup>. 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 a third 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.

<sup>1</sup> Note: The amplicon size will have an effect on amplification sensitivity and speed: while the generation of increasingly longer amplicons takes correspondingly longer periods of time, the 'primer noise' (artefacts)(see section 2.6) produced by a given pair of oligonucleotides is often independent of target size so that genuine amplicons are likely to be superseded by the faster generation of shorter noise fragments the longer the amplicon is. For this reason relatively short amplicons may be desirable for the development of ultra-sensitive assays.

## 2.4 Primer selection

The primer selection process typically consists of the following steps:

### Step1. '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%
- no long homo-polymer tracks
- 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.

### Step2. '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).

### Step3. '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<sup>1</sup>;
- for TwistAmp™ nfo primer pair quality is assessed using LF-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.

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 below).

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. of this appendix). 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.

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

#### Step 4. 'Secondary and tertiary candidate screen'

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.

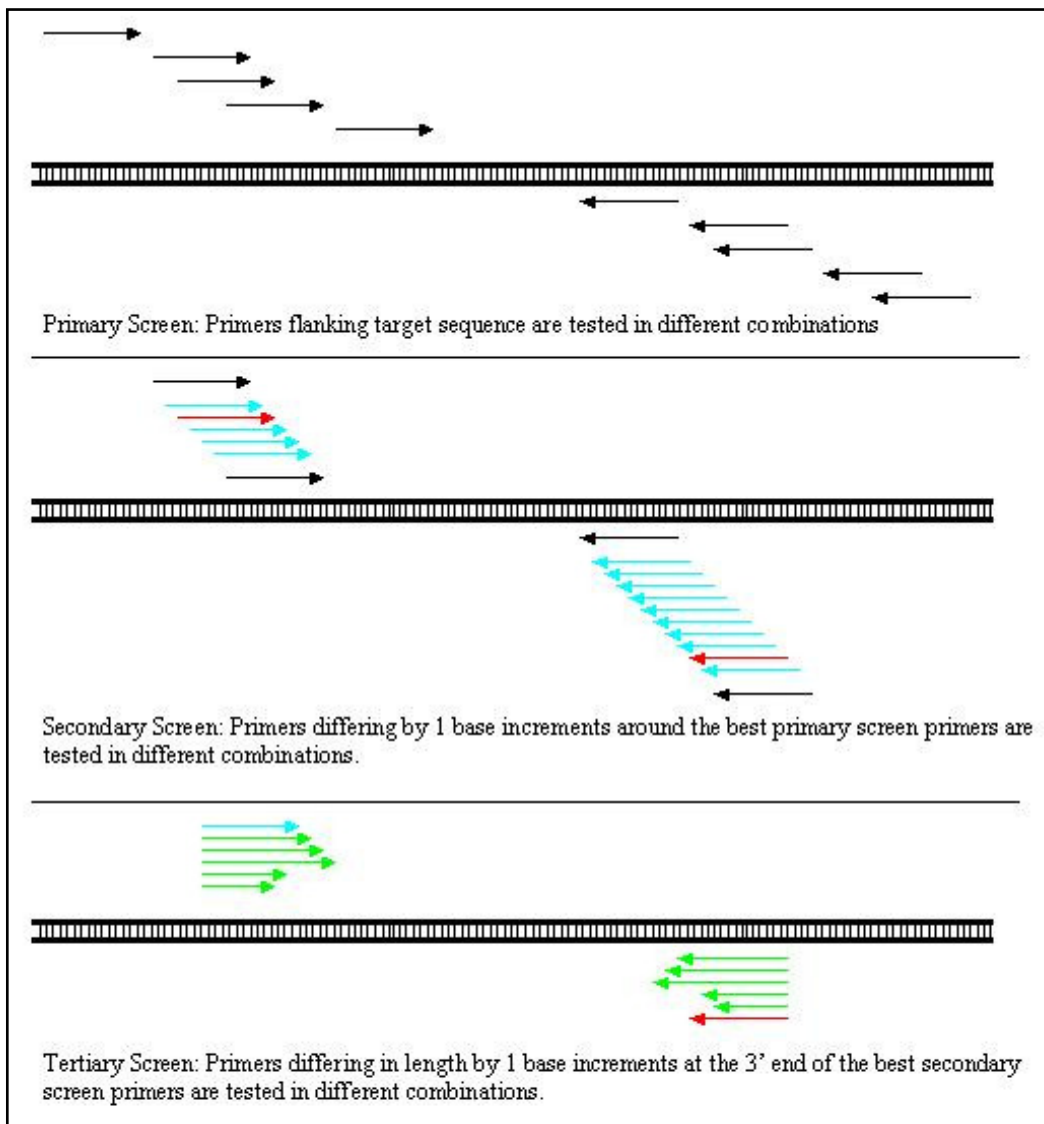


Figure 2. Typical stringent primer screen. Where single molecule sensitivity is not required, the primary screen will usually suffice.

## 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, including 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'). 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.

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.

## 3. PROBE DESIGN CONSIDERATIONS

The use of fluorophore/quencher probes in real-time detection formats is a very convenient way to monitor amplification events in TwistAmp™ reactions. Probes are especially useful to quickly generate comparative data about the speed and sensitivity of different primer pairs and are therefore a very valuable tool in the screening of potential primer candidates (see section 2).

Oligonucleotide probes that are compatible with the TwistAmp™ technology come in three different varieties: TwistAmp™ exo Probes, TwistAmp™ fpg and TwistAmp™ nfo. Each type is used with the TwistAmp™ exo, the TwistAmp™ fpg kit, and the TwistAmp™ nfo kit, respectively. The structures of all three types of probes are discussed separately below.

### 3.1.1 TwistAmp™ exo Probe structure and function

TwistAmp™ exo Probe™ consist of an oligonucleotide with homology to the target amplicon that contains an abasic nucleotide analogue (a tetrahydrofuran residue or THF, sometimes referred to as a 'dSpacer') which replaces a nucleotide in the target sequence flanked by a dT-fluorophore and a corresponding dT-quencher group (these replacing T sequences found within the corresponding target sequence). In addition, probes are blocked from any potential polymerase extension by a suitable 3'-modification group (e.g. a C3-spacer, a phosphate, a biotin-TEG or an amine). Any fluorescent signal generated by the fluorophore (typically Fluorescein or TAMRA as they are available as dT-coupled reagents for oligonucleotide synthesis) will normally be quenched by the quencher (typically a suitable Black Hole Quencher (BHQ)) located 2-4 bases 3' to the fluorophore. In a double stranded context the THF residue – representing the 'gap' in the probe (i.e. no base present where there would normally be one) - 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. Critically, the nuclease activity requires the probe to be 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 6 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.

TwistAmp™ exo can be ordered from various oligonucleotide manufacturers (including Biosearch Technologies ([www.biosearchtech.com](http://www.biosearchtech.com)), Eurogentec ([www.eurogentec.com](http://www.eurogentec.com)), AtdBio and Sigma), using the TwistDx exo TwistAmp™ Probe order form ([www.twistdx.co.uk](http://www.twistdx.co.uk))

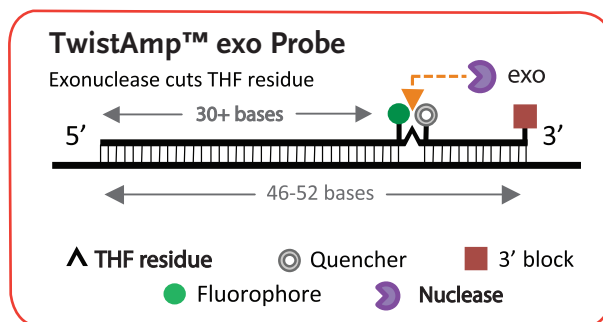


Figure 3 Schematic of the structure of an annealed TwistAmp™ exo Probe. The abasic THF residue is cleaved by exonuclease only when the probe is bound. This separates the fluorophore and quencher and generates fluorescent signal.

### 3.1.2 TwistAmp™ exo Probe length and position

An 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. **REMEMBER THAT THE THF RESIDUE, THE dT-FLUOROPHORE AND dT-QUENCHER MOEITIES ARE USED TO 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. Although primers that have the same direction as the probe can even overlap its 5' part (see 3.1.1), this overlap must not extend up to the fluorophore/abasic-site/quencher 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.

**REPRESENTATIVE EXAMPLES OF TWISTAMP™ exo PROBE DESIGN TO A TARGET SEQUENCE ARE GIVEN IN THE TWISTAMP™ exo KIT MANUAL.**

### 3.1.3 TwistAmp™ exo 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 therefore advisable to test more than one potential probe within a target. However, even without optimisation most probes designed according to the described principle will 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 therefore 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.

In some circumstances it is desirable to test and compare more than one probe in order to increase the overall assay performance. However, the number of suitably spaced thymidines within a given amplicon will limit the number of such alternative probes. It is worth noting that probes can be designed for either strand, increasing the number of possible candidates.

### 3.2.1 TwistAmp™ LF Probe structure and function

TwistAmp™ LF Probe are used with the TwistAmp™ nfo kit, and are intended for the detection by so-called 'sandwich'-assays. The probes consist of an oligonucleotide homologous to the target sequence which incorporates a 5'-antigenic label (typically a Carboxyfluorescein group (FAM)), an internal abasic nucleotide analogue (a tetrahydrofuran residue or THF – sometimes referred to as a 'dSpacer') **WHICH REPLACES A CONVENTIONAL BASE FOUND IN THE TARGET**, and a polymerase extension blocking group (such as a phosphate, C3-spacer or a dideoxy nucleotide, **NOT a biotin-TEG**) at the 3' end. TwistAmp™ LF Probe are used in a configuration in which the opposing amplification primer is labelled at its 5' end with another antigenic label, typically a biotin. The third oligonucleotide present in the reaction (equidirectional with the probe) is a conventional primer. Figure 4 shows a cartoon of a typical TwistAmp™ LF Probe. **NOTE THAT THE THF RESIDUE REPLACES A BASE THAT WOULD BE PRESENT IN THE TARGET SEQUENCE.**

The TwistAmp™ amplification reaction promoted by the two primer oligonucleotides will generate targets for the annealing of the TwistAmp™ LF 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 priming site for polymerase extension, thus transforming the probe into a primer. The amplicon produced by the processed probe and the 5'-labelled amplification primer will effectively co-join the two antigenic residues in one DNA molecule (see figure 5). This duplex can then be detected in 'sandwich' assay formats (typically post-amplification, i.e. as endpoint detection), such as the Hybridetect2 lateral-flow strips from Milenia GmbH (Germany), that uses anti-FAM gold-conjugates and anti-Biotin capture antibodies<sup>1</sup> (available from our website).

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 progress of the TwistAmp™ reaction.

TwistAmp™ LF Probe can be ordered from various oligonucleotide manufacturers (including Biosearch Technologies ([www.biosearchtech.com](http://www.biosearchtech.com)), Eurogentec ([www.eurogentec.com](http://www.eurogentec.com)), AtdBio and Sigma), using the TwistDx TwistAmp™ LF Probe order form.

Note 1: Lateral-flow strips for the detection of nucleic acids (including the Milenia Hybridetect-1 and -2 strips) are typically designed to utilise hybridisation 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.

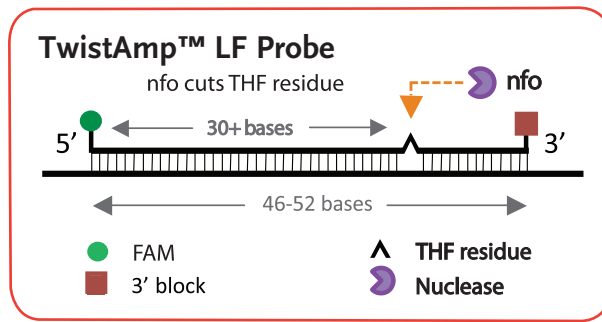


Figure 4 Cartoon of the structure of an annealed TwistAmp™ LF Probe. The abasic THF residue is cleaved by nfo only when the probe is bound.

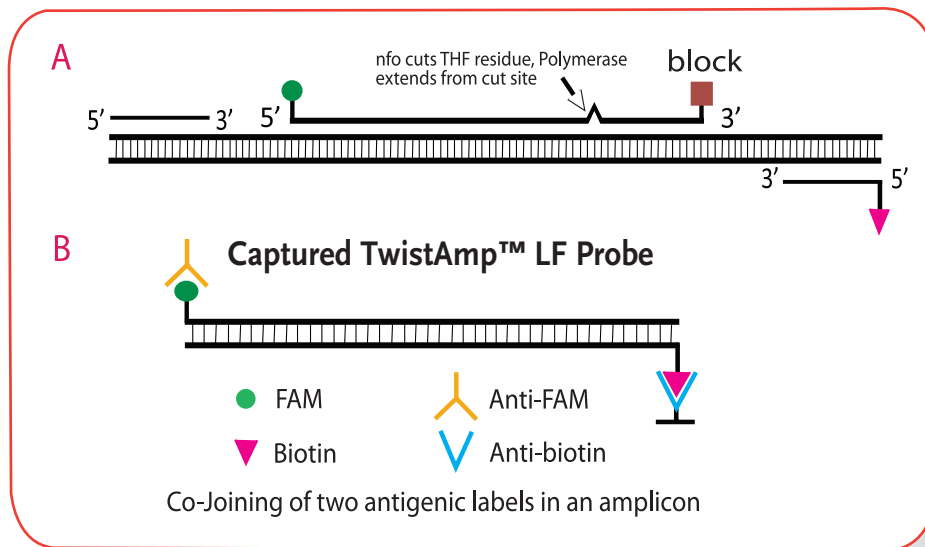


Figure 5: Schematic of the arrangements of amplification primers and TwistAmp™ LF Probe. (A) The processed probe and the opposing primer will generate double-stranded amplification products that co-join the two antigenic labels. (B) This product can subsequently be captured using one of the labels (for instance using the anti-biotin test line on a Milenia Hybridectect-1 or -2 lateral flow strip), and visualised using the other (for example by interaction with gold-labeled antibodies).

### 3.2.2 TwistAmp™ LF Probe length and position

A TwistAmp™ LF Probe are typically 46-52 nucleotides long, at least 30 of which are placed 5' to the THF site (**WHICH REPLACES A BASE FOUND IN THE TARGET SEQUENCE**), and at least a further 15 are located 3' to it.

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.

### 3.2.3 TwistAmp™ LF 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 therefore advisable to test more than one potential probe within a target. However, even without optimisation most probes designed according to the described principle will 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 therefore 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.

### 3.3.1 TwistAmp™ fpg Probe structure

TwistAmp™ fpg Probe™ are used with the TwistAmp™ fpg kit, and are intended for fluorescence detection assays. These probes typically are oligonucleotides homologous to the target amplicon that are modified at the 5'-end with a quencher group and that contain a fluorophore label coupled to an abasic nucleotide analogue 4 to 5 nucleotides downstream of the quencher (i.e. at position 5 or 6). The fluorophore is attached to the deoxyribose of the abasic site via a C-O-C linker (a so-called dR-group) **AND THE dR-FLUOROPHORE REPLACES A BASE FOUND IN THE TARGET SEQUENCE (i.e. IT IS NOT AN INSERTION IN ADDITION TO BASES CONTIGUOUSLY HOMOLOGOUS TO THE TARGET)**. In addition, TwistAmp™ fpg Probe 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 (typically Carboxy-Fluorescein or TexasRed which are available as dR-derivatives) will normally be quenched by the 5'-quencher group (typically a Black Hole Quencher (BHQ) – BHQ1 for Carboxy-fluorescein and BHQ2 for TexasRed). In a double stranded context the dR-fluorophore residue – lacking a base itself and hence constituting the ‘gap’ in the probe - presents a substrate for certain DNA repair enzymes, including 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 6 shows a schematic of a typical TwistAmp™ fpg Probe.

TwistAmp™ fpg Probe can be ordered from various oligonucleotide manufacturers (including Biosearch Technologies ([www.biosearchtech.com](http://www.biosearchtech.com)), Eurogentec ([www.eurogentec.com](http://www.eurogentec.com)), AtdBio and Sigma), using the TwistDx TwistAmp™ fpg Probe order form.

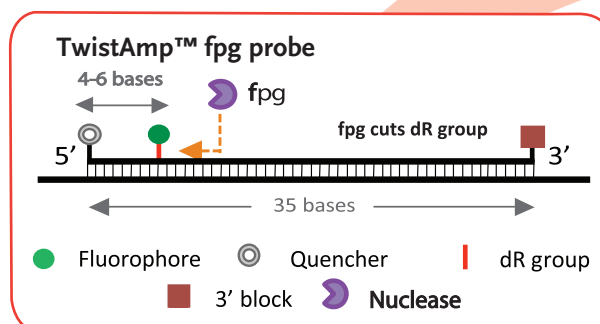


Figure 6: Schematic of the structure of an annealed TwistAmp™ fpg Probe. The abasic dR residue is cleaved by fpg only when the probe is bound to its target. This cutting step releases the fluorophore from the probe and generates fluorescence signal.

### 3.3.2 TwistAmp™ fpg Probe length and position

A TwistAmp™ fpg Probe should be about 35 nucleotides long.

There is no fixed rule describing the best position of a given TwistAmp™ fpg Probe relative to the amplification primers with which it is used. 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.

**REPRESENTATIVE EXAMPLES OF TwistAmp™ fpg Probe DESIGN TO A TARGET SEQUENCE ARE GIVEN IN THE TWISTAMP fpg KIT MANUAL.**

### 3.3.3 TwistAmp™ fpg Probe candidates

Although probes appear to be less sensitive to sequence variations than primers, probes of different sequence can perform differently. If optimal performance is required, it is therefore advisable to test more than one potential probe within a target. However, even without optimisation most probes designed according to the described principle will 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 therefore 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 compatible primers.

## 4. 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 'singleplex' reactions will turn out to perform well in multiplex 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 singleplex 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. In this way one can prevent rapid amplification of the abundant target suppressing the detection of the rare target as it will only reach detectable levels later during the reaction giving the rare target ample time to reach detection before reagents are depleted. 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.