TwistDx™

Unwind DNA's possibilities

TwistAmp® DNA Amplification Kits

Combined Instruction Manual

- TwistAmp® Basic
- TwistAmp® Basic RT
- TwistAmp® exo
- TwistAmp® exo RT
- TwistAmp® fpg
- TwistAmp® nfo

For in vitro use only.
For research and development use only.
Not for diagnostic use.

Contents

Materials provided

- 96 freeze-dried RPA pellets
- 4 x 1 ml TwistAmp® Rehydration buffer
- 1 x 250 µl magnesium acetate (280mM)
- 6 µl of Control template
- 45 µl of Control primer mix

Storage conditions

TwistAmp® reaction pellets: Provided in vacuum-sealed pouches. Store at –20°C upon receipt (full activity is guaranteed for 6 months). Product can tolerate temperatures up to room temperature for days without loss of activity but long term storage at –20°C is recommended. After breaking of vacuum seal, use within 1 day.

TwistAmp® rehydration buffer: Provided as frozen liquid in 4 x 1 ml aliquots. Upon receipt, store at –20°C (full activity is guaranteed for 6 months). Avoid excessive freeze-thaw cycles.

Control primer solution and control DNA template: Provided as frozen liquids. Upon receipt, store at –20°C; refreeze after thawing up to 5 times (full activity is guaranteed for 6 months).

Notice to purchaser

Licence, Use Restrictions and Limitations of Liability

Definitions. As used in this section, “kit” means the items described in this manual (the “Manual”) and supplied by TwistDx to a purchaser (the “Recipient”). “Materials” means all biological and chemical materials supplied as part of the kit. “Information” means all written information supplied as part of the kit, information relating to the kit made available through TwistDx website, and any verbal or written information concerning the kit or its use provided by any employee or agent of TwistDx.

Limitations on Use and Distribution of the Material and Information

Recipient acknowledges and agrees that the Materials and Information are proprietary to TwistDx, may be covered by claims of patents or patent applications of TwistDx, and are supplied subject to the following restrictions: The Materials and Information are non-exclusively licensed to Recipient solely for non-commercial internal research purposes and for applications other than the sequential determination of the identity and relative order of at least 200,000 total nucleotides in a single run on a sequencing apparatus. Any in vitro diagnostic use of the Materials and Information or any use for diagnosing or monitoring any medical condition in a human is expressly prohibited.

No Warranty; Limitation of Liability

Recipient understands and agrees that the materials are experimental in nature and that the materials and information are provided without any warranty as to results, merchantability, fitness for a particular purpose or noninfringement of any patent or other intellectual property right, and without any other representation, warranty or condition, express or implied.

TwistDx shall not be liable in connection with the materials, information or any breach of this agreement under any contract, negligence, strict liability or other theory for (a) loss of revenues, loss of profits, or loss or inaccuracy of data, including test results, regardless of how such damages are characterised (b) for the cost (including procurement costs) of substitute goods, services or technology, or (c) for any special, indirect, incidental or consequential damages. In no event will TwistDx's aggregate liability under this agreement exceed one hundred dollars (US$100). Recipient understands that its use of the materials and/or the information in connection with its activities are entirely at its risk.

Introduction

TwistAmp® DNA amplification kits provide the reagents necessary to amplify nucleic acid (particularly DNA) template material from trace levels to detectable amounts of product (from single template molecules to amplification product in the range of about 10^{12} molecules). The biochemistry of the technology is based on a combination of polymerases and DNA recombination/repair proteins, including recombinases. The resulting enzyme mixture is active at low temperature (optimum around 37°C) and enables the sequence specific recognition of template target sites by oligonucleotide primers, followed by strand-displacing DNA synthesis and thus exponential amplification of the target region within the template. The amplification process is very rapid when optimized and can reach detectable levels of product in even less than 10 minutes using the configurations of the TwistAmp® kits in many cases.

Overview of the TwistAmp® amplification technology

The isothermal TwistAmp® technology is based on the Recombinase Polymerase Amplification (RPA) process developed by TwistDx Inc. [1]. The amplification products generated by RPA can be detected either at endpoint or in real-time by a variety of means, including gel electrophoresis, probe-based fluorescence monitoring or simple non-instrumentation 'sandwich assay' approaches such as lateral flow dipsticks.

The RPA process utilizes enzymes called recombinases, of which E.coli recA is the archetypal member, which can bind to single-stranded nucleic acid backbones - standard oligonucleotides in this case - and stimulate the resulting protein-DNA complex to search for homologous sequences in duplex DNA. Once homology is located, a strand-switching reaction is performed and the oligonucleotide is paired to its complement permitting a polymerase to begin synthesis from the 3' end. The TwistAmp® amplification process uses two opposing oligonucleotide primers to initiate each synthesis event. The design of these primers for a target, in a manner similar to that for PCR, permits the establishment of an exponential amplification process.

TwistAmp® reaction conditions

Like all DNA amplification systems, RPA reaction conditions can be optimized in a number of ways in addition to the selection of good amplification primers and targets. A number of reaction parameters can be influenced by varying reaction component concentrations and these include, amongst others, kinetics, maximum product length and optimal reaction temperature. **However, to simplify end user handling**, TwistAmp® kits are currently formulated specifically to exhibit the following overall performance characteristics:

- very fast amplification (detection capability in 3-10 minutes in most cases)
- amplicon length of under 500bp
- optimal temperature of 37°C - 42°C

Under alternative conditions amplification can proceed with slower kinetics to facilitate quantification, can generate longer amplification products (up to 2 kilobases) and can also operate efficiently at significantly lower temperatures. Interested parties should please refer to the appendix located at www.twistdx.co.uk for a further discussion of special optimisation of TwistAmp® reaction conditions. Alternatively for specialised needs and applications please contact TwistDx™ technical help techsupport@twistdx.co.uk

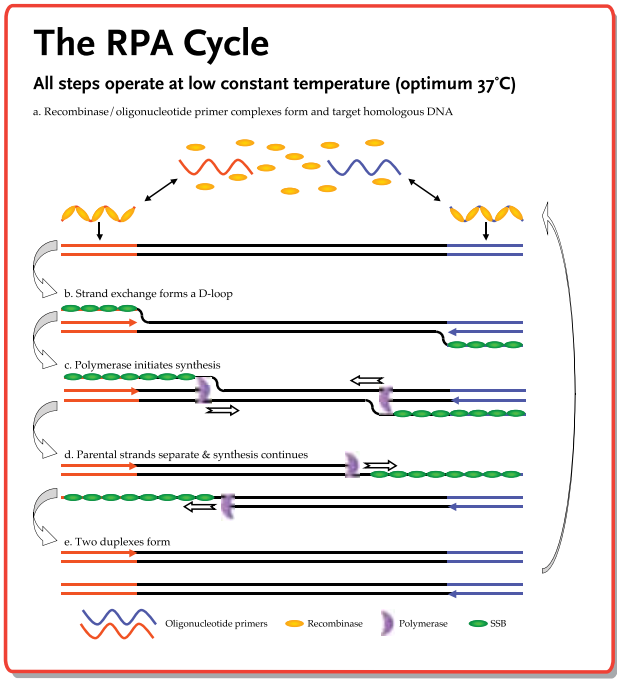


Figure 1 The RPA Cycle

The TwistAmp® application family

The core TwistAmp® amplification 'engine' is utilized in a number of different formulations that are tailored for different applications and detection modes.

TwistAmp® Basic contains all enzymes and reagents necessary for the amplification of DNA, all that has to be supplied by the user are the primers and the template. The amplification success will typically be assessed by an endpoint method, such as gel electrophoresis. Amplified material can also be purified and used for down-stream applications (e.g. subcloning).

TwistAmp® Basic RT is designed for users who want to employ RNA template as the starting material for their amplification in a one-step format. It contains the reagent components of a TwistAmp® Basic kit (see above) as well as a compatible reverse transcriptase, which converts the initially present RNA template into DNA and thus into amplification substrates. The reaction pellets **do not** currently contain RNase inhibitor.

TwistAmp® exo is recommended for users who want to combine RPA amplification technology with the use of TwistDx's proprietary fluorescent TwistAmp® exo probes in a homogeneous format. In addition to the basic components, it contains a powerful nuclease (Exonuclease III) which will process TwistAmp® exo probes during the amplification reaction itself and generate a real-time readout. The presence of Exonuclease III will reduce the final overall yield of amplified material at endpoint and so is not suitable for analysis on gels, however it is the preferred system for generating strong fluorescence signal kinetics in the RPA system.

TwistAmp® exo RT is designed for users who want to employ RNA template as the starting material for their real-time amplification in a one-step format. It contains the reagent components of a TwistAmp® exo kit (see above) as well as a compatible reverse transcriptase, which converts the initially present RNA template into DNA and thus into amplification substrates. The reaction pellets **do not** currently contain RNase inhibitor.

TwistAmp® fpg is tailored for users who want to combine RPA amplification technology with an alternative TwistAmp® reporter probe system, fluorescent TwistAmp® fpg probe, in a homogeneous format. These probes have less design constraints than TwistAmp® fpg probe, but kinetics of fluorescence accumulation may be slower. In addition to the basic components it contains a powerful nuclease (fpg) which will process TwistAmp® fpg probe during the amplification reaction itself and generate a real-time readout. The presence of fpg does not reduce the final overall yield of amplified material, in contrast to the use of Exonuclease III in the TwistAmp® exo kit, allowing endpoint gel analysis.

TwistAmp® nfo is designed for users who want to detect the success of their amplification reaction by means of end-point sandwich assays, such as lateral-flow (LF) strips[1]. Besides the basic amplification reagents it includes a nuclease (nfo) which can generate new polymerase extension substrates with suitable TwistAmp® LF probes – the amplified material can then be used in instrument–free detection formats.

1The TwistAmp® nfo kit can also be used for fluorescence monitoring using fluorescent TwistAmp® exo Probe as an alternative to the TwistAmp® exo kit. The nuclease nfo will process these probes (often with slightly slower kinetics) and will permit product to be analysed by gels at endpoint in addition.

TwistAmp® assay development

The key to TwistAmp® assay optimization is the successful design of amplification primers and, where required, detection probes. Regular oligonucleotide primers can be employed, although they are slightly longer (usually 30 to 35 nucleotides in length) than typical in PCR primers. The primer selection process is straightforward: users of TwistAmp® kits need to identify a suitable target region within their template of interest and design a number of candidate primers (and probes where required). The candidates are then tested for desired parameters, such as sensitivity and kinetic characteristics. A guide to a suitable primer selection strategy is described in more detail in the Appendix to this manual at www.twistdx.co.uk under technical resources. Unfortunately there is currently no automated primer design software available for RPA.

TwistAmp® Basic

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB: Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

Additional materials required

- Amplification primers
- Heating block or other thermal incubator
- DNA fragment purification reagents/equipment
- Agarose gel electrophoresis setup

Protocols

Storage considerations of kit components

The TwistAmp® Basic kit components allow long-term storage (up to 6 months is guaranteed, but much longer stability likely) under the correct conditions. TwistAmp® Basic reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –20°C or lower of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30 minutes.

The TwistAmp® Basic rehydration buffer is provided in four 1 ml aliquots. These should be stored at –20°C to retain full activity.

The TwistAmp® Basic control primer solution and control DNA template are provided. Upon receipt they should be stored at –20°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® Basic reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration. solution. This solution consists of the TwistAmp® Basic rehydration buffer (provided with the kit), amplification primers, and template (and water to a total volume of 47.5 µl per sample).

The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (here according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

Detailed protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10µM)	2.4 µl
Primer B (10µM)	2.4 µl
Rehydration Buffer	29.5 µl
Template and dH2O	13.2 µl
(Total Volume	47.5 µl)

Vortex and spin briefly.

2. For each sample, transfer 47.5 µl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 µl 280 mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8) cap the tubes carefully and spin the magnesium acetate into the rehydrated material to initiate the reactions. Invert vigorously 8-10 times to mix and spin down once again.

4. Insert the tubes into a suitable incubator block (optimum 37-42°C) and incubate for 4 minutes.

5. After 4 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block. (Variation in the exact time of sample agitation can sometimes improve product formation)

6. Continue the incubation/detection for a total incubation time of 20-40 minutes. If a timecourse of TwistAmp® Basic reaction is being taken the incubation time has to be adjusted as required. At the end of the incubation proceed to *Monitoring TwistAmp® Basic amplification reactions*.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step four minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

Warning: TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is advisable to proceed swiftly from the resuspension of the pellet to incubation of the sample at the chosen incubation temperature.

If using a device with a heated lid, this should be **switched off**.

Monitoring TwistAmp® Basic amplification reactions

The outcome of TwistAmp® Basic reactions is typically analysed by an endpoint method after the reaction is completed, such as agarose gel electrophoresis (AGE), which is described in this section. However, alternative methods to AGE can also be used. In this case, the protocol given below has to be modified accordingly. The amplification product should first be purified to remove reaction components that might interfere with downstream applications.

1. Purify the amplification product by following the instructions for commercial PCR-purification kits. Alternatively, the reaction solution (containing the amplification product) can be diluted 1 in 10 in water and then Phenol/Chloroform extracted according to standard molecular biology practices.

2. The required amount of the amplification product can now be resolved by electrophoresis on a suitable agarose-gel following standard protocols and visualized accordingly. These operations are performed much like those for an AGE analysis of PCR products of comparable size.

3. Data analysis: A band of the expected amplification product size should be detectable. Depending on the primers used and if using low target copy number there is the potential for some amount of non-specific products being formed during the reaction and being visible on the gel (see the Appendix at www.

twistdx.co.uk for a discussion of 'primer noise' and other). These artefacts will typically be seen in any no-template controls and at very low target copy number. If necessary, the main product can be isolated from the non-specific products and purified for downstream applications (such as subcloning, sequencing, etc.).

Warning: Be aware that the post-amplification processing of the reaction solutions carries a very great risk of contamination of equipment, work surfaces etc. with amplification product. See the *Preventing template cross-contamination* section below for measures to reduce this risk.

Performing positive control reactions

The TwistAmp® Basic kit contains positive control primers and template, which will allow you to test the activity of the kit components. The positive control material is used with the TwistAmp® Basic reaction pellets and rehydration buffer.

1. Defrost the positive control primer mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (indH2O).

3. Pipette 8 µl primer solution into a fresh 1.5 ml micro centrifugetube.

4. Add 29.5 µl rehydration buffer to the primer solution from step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control DNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

6. Uncap the tubes containing the freeze-dried TwistAmp® Basic reaction pellets, and place the caps upside-down in front of the

tubes.

7. Resuspend each pellet in 47.5 µl Rehydration solution containing primers and template DNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium-acetate solution combines with the rehydrated samples. Invert vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the incubator block (optimum 37-39°C) and incubate for 4 minutes.

10. After 4 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block.

11. Continue the incubation/detection for a total incubation time of 20 minutes. At the end of the incubation proceed to *Monitoring TwistAmp® Basic amplification reactions*.

12. Continue with an AGE analysis of the amplification product of the positive control reactions by proceeding to *Monitoring TwistAmp® Basic amplification reactions*.

The positive control reaction will generate an amplification product of 143 base pairs, that will result in a corresponding band in a gel electrophoresis.

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers. Extra care has to be taken when purifying amplicons and analyzing them on agarose-gels.

TwistAmp® Basic RT

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

TwistAmp® Basic RT **does not** include RNase Inhibitor. If you wish to use this you will need to supply your own and use in accordance with the manufacturer's instructions (using equivalent volumes as if for a 50µl PCR reaction).

Additional materials required

- Amplification primers
- Heating block or other thermal incubator
- DNA fragment purification reagents/equipment
- Agarose gel electrophoresis setup
- RNase Inhibitor

Protocols

Storage considerations of kit components

The TwistAmp® Basic RT kit components allow long-term storage (up to 6 months is guaranteed but much longer stability likely) under the correct conditions. The TwistAmp® Basic RT reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –80°C or lower of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30 minutes.

The TwistAmp® Basic RT Rehydration buffer is provided as frozen liquid in four 1 ml aliquots together with one aliquot of the TwistAmp® Basic RT control primer solution. These should be stored at –20°C to retain full activity.
A single aliquot of the TwistAmp® Basic RT control RNA template is provided as a frozen liquid. Upon receipt it should be stored at –80°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® Basic RT reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp® Basic RT rehydration buffer (provided with the kit), amplification primers, and template (and water to a total volume of 47.5 µl per sample).
The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

Detailed Protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10μM)	2.4 μl
Primer B (10μM)	2.4 μl
Rehydration Buffer	29.5 μl
Template, RNase Inhibitor and dH2O	13.2 μl
(Total Volume	47.5 μl)

   Vortex and spin briefly.

2. For each sample, transfer 47.5 μl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 μl 280 mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8) cap the tubes carefully and spin the magnesium acetate into the rehydrated material to initiate the reactions. Invert vigorously 8-10 times to mix and spin down once again.

4. Insert the tubes into a suitable incubator block (optimum 40-42°C) and incubate for 5 minutes.

5. After 5 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block. (Variation in the exact time of sample agitation can sometimes improve product formulation).

6. Continue the incubation/detection for a total incubation time of 20-40 minutes. If a timecourse of TwistAmp® Basic RT reaction is being taken the incubation time has to be adjusted as required. At the end of the incubation proceed to *Monitoring TwistAmp® Basic RT amplification* reactions.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step five minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

Warning: The TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is advisable to proceed swiftly from the resuspension of the pellet to incubation of the sample at the chosen incubation temperature.

If using a device with a heated lid, this should be **switched off**.

Monitoring TwistAmp® Basic RT amplification reactions

The outcome of TwistAmp® Basic RT reactions is typically analysed by an endpoint method after the reaction is completed, such as agarose gel electrophoresis (AGE), which is described in this section. However, alternative methods to AGE can also be used, and in this case the protocol given below has to be modified accordingly. The amplification product should first be purified to remove reaction components that might interfere with downstream applications.

1. Purify the amplification product by following the instructions for commercial PCR-purification kits. Alternatively, the reaction solution (containing the amplification product) can be diluted 1 in 10 in water and then phenol/chloroform extracted according to standard molecular biology practices.

2. The required amount of the amplification product can now be resolved by electrophoresis on a suitable agarose-gel following standard protocols and visualized accordingly. These operations are performed much like those for an AGE analysis of PCR products of comparable size.

3. Data analysis: A band of the expected amplification product size should be detectable. Depending on the primers used and if using low target copy number there is the potential for some amount of non-specific products being formed during the reaction and being visible on the gel (see the Appendix at www.twistdx.co.uk for a discussion of primer noise and other). These artefacts will typically be seen in any no-template controls and at very low target copy number. If necessary, the main product can be isolated from the non-specific products and purified for downstream applications (such as subcloning, sequencing, etc.)

Warning: Be aware that the post-amplification processing of the reaction solutions carries a very great risk of contamination of equipment, work surfaces etc. with amplification product. See the warning section below for measures to reduce this risk.

Performing positive control reactions

The TwistAmp® Basic RT kit contains positive control primers and template, which will allow you to test the activity of the kit components. The positive control material is used with the TwistAmp® Basic RT reaction pellets and rehydration buffer.

1. Defrost the positive control primer mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control RNA (indH2O and RNase Inhibitor).

3. Pipette 8 µl primer solution into a fresh 1.5 ml micro centrifuge tube.

4. Add 29.5 µl rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control RNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

6. Uncap the tubes containing the freeze-dried TwistAmp® Basic RT reaction pellets, and place the caps upside-down in front of the tubes.

7. Resuspend each pellet in 47.5 µl Rehydration solution containing primers and template RNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium acetate solution combines with the rehydrated samples. Invert vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the incubator block (optimum 40-42°C) and incubate for 5 minutes.

10. After 5 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block.

11. Continue the incubation/detection for a total incubation time of 20 minutes. At the end of the incubation proceed to *Monitoring TwistAmp® Basic RT amplification reactions*.

12. Continue with an AGE analysis of the amplification product of the positive control reactions by proceeding to *Monitoring TwistAmp® Basic RT amplification reactions*. The positive control reaction will generate an amplification product of 141 base pairs, that will result in a corresponding band in a gel electrophoresis.

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre- and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers. Extra care has to be taken when purifying amplicons and analyzing them on agarose-gels.

TwistAmp® exo

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB: Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

Real-time detection of amplification by fluorescence will require special probes compatible with the TwistAmp® exo biochemistry, so called TwistAmp® exo probe. The design of these probes is described in more detail below. Probes intended for the use in PCR and other nucleic acid amplification processes (e.g. Taqman®) will not work in TwistAmp® exo reactions.

Additional materials required

- Amplification primers
- TwistAmp® exo Probe for detection
- Thermal incubator/Fluorometer, e.g. Twista® isothermal fluorometer

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 Appendix at www.twistdx.co.uk for a discussion of primer selection).

The type of oligonucleotide probe that is compatible with the TwistAmp® exo technology is the TwistAmp® exo Probe. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers.

TwistAmp® exo Probe structure and function

TwistAmp® exo Probe typically consist of an oligonucleotide backbone that contains an abasic nucleotide analogue (a tetrahydrofuran residue or THF, sometimes referred to as a 'dSpacer') flanked by a dT-fluorophore and a corresponding dT-quencher group. In addition, probes are blocked from polymerase extension by a suitable 3'–modification group (such as a C3- spacer, a phosphate, a Biotin-TEG or an amine). Any fluorescent signal generated by the fluorophore (typically fluorescein or TAMRA) will normally be quenched by the quencher (typically a suitable black hole quencher (BHQ)) located 2-6 bases 3' to the fluorophore. In a double-stranded context the THF residue presents a substrate for a number of DNA repair enzymes, including 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 step is restricted to cases in which the probe has annealed to its target sequence 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.

The current limited availability of appropriate fluorophore and quencher modifications to T residues restricts optimal probe locations to sequences where two T's can be found with fewer than 6 intervening nucleotides – however, should design of such suitable probes prove problematic, there are some variations to this theme which are detailed further in the Appendix found at www.twistdx.co.uk. At present nucleotide analogues for other bases are not available.

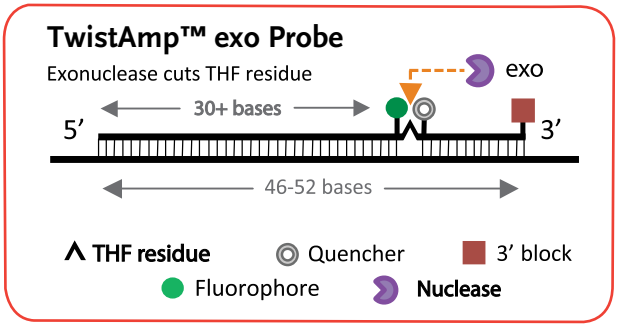


Figure 2 Schematic of the structure of an annealed TwistAmp® exo Probe. The abasic THF residue is cleaved by Exonuclease III only when the probe is bound to its target. This cutting step separates the fluorophore and quencher and generates a fluorescence signal.

TwistAmp® exo Probe can be ordered from various oligonucleotide manufacturers, including Biosearch Technologies (www.biosearchtech.com) and Eurogentec (www.eurogentec.com), using the TwistDx™ TwistAmp® exo Probe order form (available on the TwistDx™ website www.twistdx.co.uk).

TwistAmp® exo Probe length and position

A TwistAmp® exo Probe should typically 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. 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. As a general rule unique sequences present in the amplicon between the amplification primers are employed. However, an amplification primer can overlap the 5’ part of the probe, providing that this overlap does 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. Primers opposing the direction of the probe should not overlap to avoid the occurrence of primer-probe dimers. Secondary structures that could cause probes to fold back on themselves should be avoided.

Example of an TwistAmp® exo Probe

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:

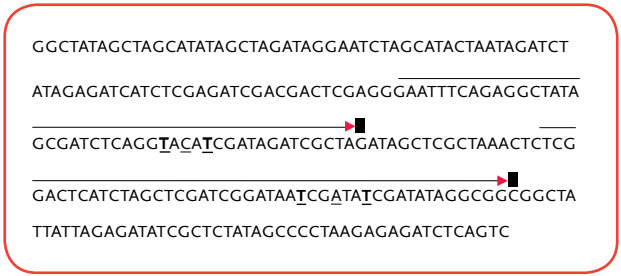


Figure 3 T Residues that are replaced by either dT-fluorophore or dT-quencher are bolded and underlined, while the base replaced with a THF is underlined.

In this case one probe ordered would have the following sequence in which the relevant T residues in the sequence are **replaced** by dTfluorophore 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 [BHQ-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 [BHQ-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.

Note: When using dT-FAM as a fluorescent label we advise the use of dT-BHQ1 as the quencher. When using dT-TAMRA as a fluorescent label we advise the use of dT-BHQ2 as the quencher.

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 = BHQ-dT

Good probe:
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

Bad probes:
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.

TwistAmp® exo Probe candidates

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 Appendix at www.twistdx.co.uk). 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. 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.

Protocols

Storage considerations of kit components

The TwistAmp® exo kit components allow long-term storage (up to 6 months guaranteed, much longer stability likely) under the correct conditions. The TwistAmp® exo reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –20°C of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30 minutes.

The TwistAmp® exo rehydration buffer is provided in four, 1 ml, aliquots. These should be stored at –20°C to retain full activity.

The TwistAmp® exo control primer solution and control DNA template are provided. Upon receipt they should be stored at –20°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® exo reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp® exo rehydration buffer (provided with the kit), amplification primers, the detection probe, and template (and water to a total volume of 47.5 µl per sample).

The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (here according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

Detailed protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
TwistAmp® exo Probe (10µM)	0.6 µl
Rehydration buffer	29.5 µl
Template and dH2O	13.2 µl
(Total volume	47.5 µl)

   Vortex and spin briefly.

2. For each sample, transfer 47.5 µl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 µl 280mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the magnesium acetate into the rehydrated material to initiate the reactions.

Vortex briefly and spin down once again.

4. Place immediately into a suitable incubation/monitoring device such as the Twista® tube scanner (see below for a discussion of the use of alternative fluorometers).

Warning: The TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is essential to proceed swiftly from the resuspension of the pellet to incubation of the sample at 39°C.

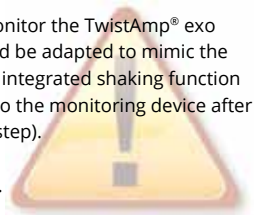
Monitoring TwistAmp® exo amplification reactions

This section describes real-time fluorescence detection with the Twista® tube scanner (for the use of the Twista®, see the manual provided with the instrument). If fluorescence detection equipment other than the Twista® is used, the protocol should be modified accordingly. For instance, the rehydrated sample should be transferred into the appropriate reaction vessel (e.g. a multi-well plate) and incubated/monitored according to the requirements of the alternative device.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step four minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

If alternative devices are used to monitor the TwistAmp® exo reaction, the agitation regime should be adapted to mimic the protocol given below (e.g. use of an integrated shaking function or transfer of the reaction vessel into the monitoring device after a 4 minutes pre-incubation/mixing step).

Heated lids should be **switched off**.



1. Insert the tubes into the Twista® incubator block (39°C).

2. Initiate fluorescence measurements (click on the *start* icon to begin scanning).

3. After 4 minutes, take the samples out of the reader **(do not stop the program)**, invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block (Variation in the exact time of sample agitation can sometimes improve signal strength).

4. Continue the incubation/detection for a total time of 20 minutes.

5. Save data at the end of the program and discard the sample tubes.

Warning: Do not open the tubes after the completion of the amplification reaction, as this carries the risk of contamination of equipment, work surfaces etc. with amplification product.

Performing positive control reactions

The TwistAmp® exo kit contains positive control primers/probe and template, which will allow you to test the activity of the kit components and the detection equipment. The positive control material is used with the TwistAmp® exo reaction pellets and rehydration buffer.

1. Defrost the positive control primer/probe mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (in dH2O).

3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml microcentrifuge tube.

4. Add 29.5 µl Rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control DNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

6. Uncap the tubes containing the freeze-dried TwistAmp® exo reaction pellets, and place the caps upside-down in front of the tubes.

7. Resuspend each pellet in 47.5 µl Rehydration solution containing primers/probes and template DNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium

acetate solution combines with the rehydrated samples. Invert vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the Twista® and start the run.

10. After 4 minutes, take the samples out of the reader **(do not stop the program)**, invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block.

11. Continue the incubation/detection for a total time of 20 minutes.

The internal control uses a probe that is labelled with a fluorescein fluorophore, the excitation optimum is at 488nM and the emission maximum is at 520nM. The expected result of the positive control reaction in a Twista device is an initial base-line readout of about 1000 mV, a detectable onset of signal generation after about 6 to 7 minutes and a final plateau signal of about 3x to 4x the baseline fluorescence. [This baseline is higher than typically found when using Black Hole Quenchers as the quenching dye because these control probes utilise the less efficient dabcyl quencher].

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers.

TwistAmp® exo RT

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

Before you start: Real-time detection of amplification by fluorescence will require special probes compatible with the TwistAmp® exo biochemistry, so called TwistAmp® exo probe. The design of these probes is described in more detail below. Probes intended for the use in PCR and other nucleic acid amplification processes (e.g. Taqman®) will not work in TwistAmp® exo reactions.

Before you start: The TwistAmp® exo RT kit does **not** include RNase Inhibitor. If you wish to use this you will need to supply your own and use in accordance with the manufacturer's instructions (using equivalent volumes as if for a 50µl PCR reaction).

Additional materials required

- Amplification primers
- RNase inhibitor
- TwistAmp® exo Probe for detection
- Thermal incubator/fluorometer, e.g. Twista® isothermal fluorometer

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 different primer pairs and are therefore a very valuable tool in the screening of potential primer candidates (see Appendix at www.twistdx.co.uk for a discussion of primer selection).

The type of oligonucleotide probe that is compatible with the TwistAmp® exo technology is the TwistAmp® exo Probe. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers.

TwistAmp® exo Probe structure and function

TwistAmp® exo Probe typically consist of an oligonucleotide backbone that contains an abasic nucleotide analogue (a tetrahydrofuran residue or THF, sometimes referred to as a *dSpacer*) flanked by a dT-fluorophore and a corresponding dT-quencher group. In addition, probes are blocked from polymerase extension by a suitable 3'–modification group (such as a C3- spacer, a phosphate, a Biotin-TEG or an amine). Any fluorescent signal generated by the fluorophore (typically fluorescein or TAMRA) will normally be quenched by the quencher (typically a suitable Black Hole Quencher (BHQ)) located 2-6 bases 3' to the fluorophore. In a double-stranded context the THF residue presents a substrate for a number of DNA repair enzymes, including 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 step is restricted to cases in which the probe has annealed to its target sequence 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.

The current limited availability of appropriate fluorophore and quencher modifications to T residues restricts optimal probe locations to sequences where two T's can be found with fewer than 6 intervening nucleotides – however, should design of such

suitable probes prove problematic, there are some variations to this theme which are detailed further in the Appendix found at www.twistdx.co.uk. At present nucleotide analogues for other bases are not available.

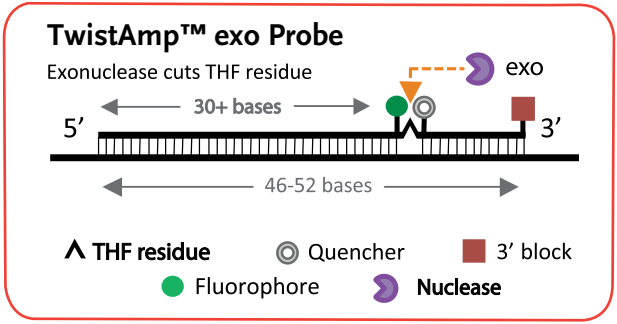


Figure 4 Schematic of the structure of an annealed TwistAmp® exo Probe. The abasic THF residue is cleaved by Exonuclease III only when the probe is bound to its target. This cutting step separates the fluorophore and quencher and generates a fluorescence signal.

TwistAmp® exo Probe can be ordered from various oligonucleotide manufacturers, including Biosearch Technologies (www.biosearchtech.com) and Eurogentec (www.eurogentec.com), using the TwistDx™ TwistAmp® exo Probe order form (available on the TwistDx™ website www.twistdx.co.uk).

TwistAmp® exo Probe length and position

A TwistAmp® exo Probe should typically 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. 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. As a general rule unique sequences present

in the amplicon between the amplification primers are employed. However, an amplification primer can overlap the 5' part of the probe, providing that this overlap does 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. Primers opposing the direction of the probe should not overlap to avoid the occurrence of primer-probe dimers. Secondary structures that could cause probes to fold back on themselves should be avoided.

Example of an TwistAmp® exo Probe

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:

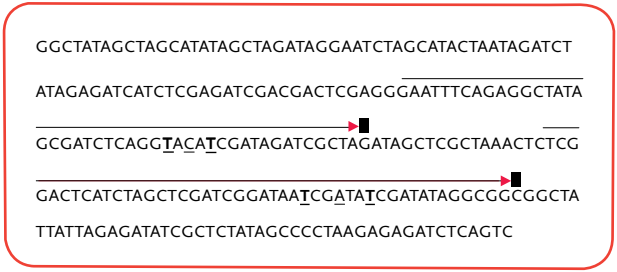


Figure 5 T Residues that are replaced by either dT-fluorophore or dT-quencher are bolded and underlined, while the base replaced with a THF is underlined.

In this case one probe ordered would have the following sequence in which the relevant T residues in the sequence are **replaced** by dTfluorophore 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 [BHQ-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 [BHQ-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.

Note: When using dT-FAM as a fluorescent label we advise the use of dT-BHQ1 as the quencher. When using dT-TAMRA as a fluorescent label we advise the use of dT-BHQ2 as the quencher.
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 = BHQ-dT

Good probe:
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

Bad probes:
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.

TwistAmp® exo Probe candidates

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 Appendix at www.twistdx.co.uk). 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. 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.

Protocols

Storage considerations of kit components

The TwistAmp® exo RT kit components allow long-term storage (up to 6 months guaranteed, much longer stability likely) under the correct conditions. The TwistAmp® exo RT reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –20°C of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30 minutes.

The TwistAmp® exo RT rehydration buffer is provided as frozen liquid in four, 1 ml, aliquots. These should be stored at –20°C to retain full activity.

The TwistAmp® exo RT control primer solution and control RNA template are provided as frozen liquids. Upon receipt they should be stored at –80°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® exo RT reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp® exo RT rehydration buffer (provided with the kit), amplification primers, the detection probe, and template (and water to a total volume of 47.5 µl per sample).

The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (here according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

Detailed Protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
TwistAmp® exo Probe (10µM)	0.6 µl
Rehydration buffer	29.5 µl
Template, RNase inhibitor and dH2O	13.2 µl
(Total volume	47.5 µl)

   Vortex and spin briefly.

2. For each sample, transfer 47.5 µl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 µl 280mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the magnesium acetate into the rehydrated material to initiate the reactions.

Vortex briefly and spin down once again.

4. Place immediately into a suitable incubation/monitoring device such as the Twista® tube scanner (see below for a discussion of the use of alternative fluorometers).

Warning: The TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is essential to proceed swiftly from the resuspension of the pellet to incubation of the sample at 40°C.

Monitoring TwistAmp® exo RT amplification reactions

This section describes real-time fluorescence detection with the Twista® tube scanner (for the use of the Twista®, see the manual provided with the instrument). If fluorescence detection equipment other than the Twista® is used, the protocol should be modified accordingly. For instance, the rehydrated sample should be transferred into the appropriate reaction vessel (e.g. a multi-well plate) and incubated/monitored according to the requirements of the alternative device.

If alternative devices are used to monitor the TwistAmp® exo RT reaction, the agitation regime should be adapted to mimic the protocol given below (e.g. use of an integrated shaking function or transfer of the reaction vessel into the monitoring device after a 5 minute pre-incubation/mixing step).

Heated lids should be **switched off**.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step five minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

1. Insert the tubes into the Twista® incubator block (40°C).

2. Initiate fluorescence measurements (click on the *start* icon to begin scanning).

3. After 5 minutes, take the samples out of the reader (**do not stop the program**), invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block (variation in the exact time of sample agitation can sometimes improve signal strength).

4. Continue the incubation/detection for a total time of 20 minutes.

5. Save data at the end of the program and discard the sample tubes.

Warning: Do not open the tubes after the completion of the amplification reaction, as this carries the risk of contamination of equipment, work surfaces etc. with amplification product.

Performing positive control reactions

The TwistAmp® exo RT kit contains positive control primers/probe and template, which will allow you to test the activity of the kit components and the detection equipment. The positive control material is used with the TwistAmp® exo RT reaction pellets and rehydration buffer.

1. Defrost the positive control primer/probe mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control RNA (in dH2O and RNase inhibitor).

3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml microcentrifuge tube.

4. Add 29.5 µl rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control RNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.
6. Uncap the tubes containing the freeze-dried TwistAmp® exo RT reaction pellets, and place the caps upside-down in front of the tubes.

7. Resuspend each pellet in 47.5 µl rehydration solution containing primers/probes and template DNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium acetate solution combines with the rehydrated samples. Invert

vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the Twista® and start the run.

10. After 5 minutes, take the samples out of the reader (**do not stop the program**), invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block.

11. Continue the incubation/detection for a total time of 20 minutes.

The internal control uses a probe that is labelled with a fluorescein fluorophore, the excitation optimum is at 488nM and the emission maximum is at 520nM. The expected result of the positive control reaction in a Twista device is an initial base-line readout of about 1000 mV, a detectable onset of signal generation after about 6 to 7 minutes and a final plateau signal of about 3x to 4x the baseline fluorescence. [This baseline is higher than typically found when using Black Hole Quenchers as the quenching dye because these control probes utilise the less efficient dabcyl quencher].

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre and post-amplification steps. Use positive displacement pipets or aerosol-resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers.

TwistAmp® fpg kit

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

Before you start: Real-time detection of amplification by fluorescence will require special probes compatible with the TwistAmp® fpg biochemistry, so called TwistAmp® fpg probes. The design of these probes is described in more detail below. Probes intended for the use in PCR and other nucleic acid amplification processes (e.g. Taqman®) will not work in TwistAmp® fpg reactions.

Additional material required

- Amplification primers
- TwistAmp® fpg Probe for detection
- Thermal incubator/fluorometer, e.g. Twista® isothermal fluorometer

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 Appendix at www.twistdx.co.uk for a discussion of primer selection).
The type of oligonucleotide probe that is compatible with the TwistAmp® fpg technology is the TwistAmp® fpg Probe.

Performing positive control reactions

TwistAmp® fpg Probes are used with the TwistAmp® fpg kit, and are intended for fluorescence detection assays. These probes typically are oligonucleotides that are modified at the 5' end with a quencher group and that contain a fluorophore label on 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 ribose of the abasic site via a C-O-C linker (a so-called dR-group). 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 (typically Carboxy-fluorescein) will normally be quenched by the 5' 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 a number of 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.

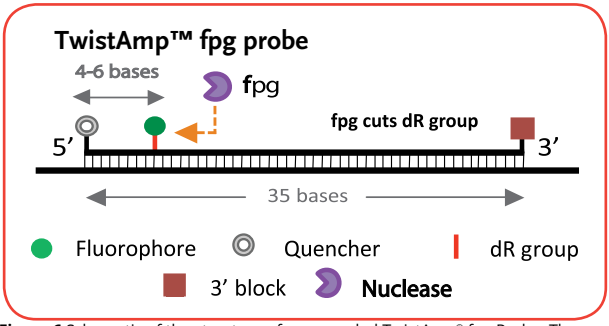


Figure 6 Schematic of the structure of an annealed TwistAmp® fpg Probe. The abasic dR residue is cleaved by fpg only when the probe is bound to its target. This cutting step releases the fluorophore from the probe and generates fluorescence signal.

TwistAmp® fpg Probes can be ordered from various oligonucleotide manufacturers, including Biosearch Technologies (www.biosearchtech.com) and Eurogentec (www.eurogentec.com), using the TwistDx™ TwistAmp® fpg Probe order form (available on the TwistDx™ website www.twistdx.co.uk).

TwistAmp® fpg Probe length and position

A TwistAmp® fpg Probe should typically be 32 - 35 nucleotides long. 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 of an TwistAmp® fpg probe

TwistAmp® fpg Probes should be about 32-35 nucleotides long and have fairly flexible design parameters. As an example a target sequence is shown below along with two suggested probes that could be designed to detect it, in both cases a 5’ quencher is used and then a few bases within the probe a nucleotide is replaced by a

dR-fluorophore. We have used dR-FAM or dR-TexasRed® routinely as fluorophores. The probe is also routinely blocked at the 3' end:

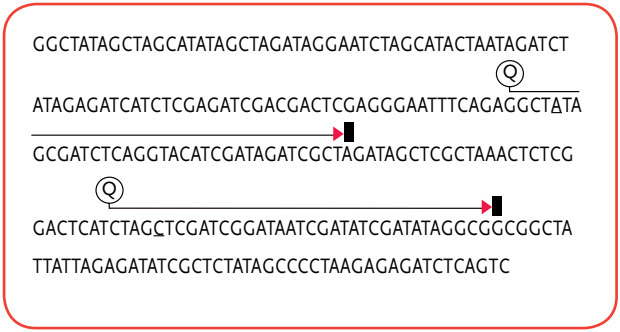


Figure 7 The position of the probe is indicated with an arrow, the 5' end is labelled with a quencher, and the underlined nucleotide is **replaced** with a dR-fluorophore.

In this case one probe ordered would have the following sequence in which the 5' end is labelled with a quencher. Additionally the nucleotide at the position 5 of the sequence is replaced by a dR-fluorophore residue:

[5'BHQ1] GGCT [dR-FAM] TAGCGATCTCAGGTACATCGATAGATCGCT [3'-block]

Based on these principles a second possible probe is shown with sequence:

[5'BHQ1] CTAG [dR-FAM] TCGATCGGATAATCGATATCGATATAGGCG [3'-block]

The exact number of bases between the 5' quencher and the internal [dR-fluorophore] may be varied but we advise positioning the fluorescent group no further than about the 6th or 7th residue from

the 5′ end. Placing the dR-fluorophore further away from the 5′ end will reduce the quenching efficiency. We routinely block the 3′ end of the probe with a group such as C3- spacer, biotin- TEG, or phosphate.

Note: When using dR-FAM as a fluorescent label we advise the use of BHQ1 as the quencher. When using dR-TexasRed as a fluorescent label we advise the use of BHQ2 as the quencher.

TwistAmp® fpg 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 Appendix at www.twistdx.co.uk). The probe can then be used to test the performance of all the compatible primers.

Protocols

Storage considerations of kit components

The TwistAmp® fpg kit components allow long-term storage (up to 6 months guaranteed, much longer stability likely) under the correct conditions. The TwistAmp® fpg reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –20°C of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30 minutes.

The TwistAmp® fpg rehydration buffer is provided in four, 1 ml, aliquots. These should be stored at –20°C to retain full activity.

The TwistAmp® fpg control primer solution and control DNA template are provided. Upon receipt they should be stored at –20°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® fpg reactions are performed by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp® fpg rehydration buffer (provided with the kit), amplification primers, the detection probe, and template (and water to a total volume of 47.5 µl per sample).
The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (here according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

Detailed protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
TwistAmp® fpg Probe (10µM)	0.6 µl
Rehydration buffer	29.5 µl
Template and dH2O	13.2 µl
(Total volume	47.5 µl)

Vortex and spin briefly.

2. For each sample, transfer 47.5 µl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 µl 280mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the magnesium-acetate into the rehydrated material to initiate the reactions. Invert vigorously 8-10 times to mix and spin down once again.

4. Place immediately into a suitable incubation/monitoring device such as the Twista® (see below for a discussion of the use of alternative fluorometers).

Warning: The TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is essential to proceed swiftly from the resuspension of the pellet to incubation of the sample at 39°C.

Monitoring TwistAmp® fpg amplification reactions

This section describes real-time fluorescence detection with the Twista® (for the use of the Twista®, see the manual provided with the instrument). If fluorescence detection equipment other than the Twista® is used, the protocol should be modified accordingly. For instance, the rehydrated sample should be transferred into the appropriate reaction vessel (e.g. a multi-well plate) and incubated/monitored according to the requirements of the alternative device.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step four minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

If alternative devices are used to monitor the TwistAmp® fpg reaction, the agitation regime should be adapted to mimic the protocol given below (e.g. use of an integrated shaking function or transfer of the reaction vessel into the monitoring device after a 4 minutes pre-incubation/mixing step).

Heated lids should be **switched off**.

1. Insert the tubes into the Twista® incubator block (39°C).

2. Initiate fluorescence measurements (click on the 'start' icon to begin scanning).

3. After 4 minutes, take the samples out of the reader (**do not stop the program**), invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block (variation in the exact sample agitation can sometimes improve signal strenth).

4. Continue the incubation/detection for a total time of 20 minutes.

5. Save data at end of the program and discard the sample tubes.

Warning: Do not open the tubes after the completion of the amplification reaction, as this carries the risk of contamination of equipment, work surfaces etc. with the amplification product.

Performing positive control reactions

The TwistAmp® fpg kit contains positive control primers/probe and template, which will allow you to test the activity of the kit components and the detection equipment. The positive control material is used with the TwistAmp® fpg reaction pellets and Rehydration buffer.

1. Defrost the positive control primer/probe mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (in dH2O).

3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml microcentrifuge tube.

4. Add 29.5 µl Rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control DNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

6. Uncap the tubes containing the freeze-dried TwistAmp® fpg reaction pellets, and place the caps upside-down in front of the tubes.

7. Resuspend each pellet in 47.5 µl Rehydration solution containing primers/probes and template DNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium acetate solution combines with the rehydrated samples. Invert vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the Twista® and start the run.

10. After 4 minutes, take the samples out of the reader (**do not stop the program**), invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block.

11. Continue the incubation/detection for a total time of 20 minutes.

The internal control uses a probe that is labelled with a fluorescein fluorophore, the excitation optimum is at 488nM and the emission maximum is at 520nM. The expected result of the positive control reaction in a Twista® is an initial base-line readout of about 1000 mV, a detectable onset of signal generation after about 7 to 8 minutes and a final plateau signal of about 2 - 3x the baseline fluorescence. [This baseline is higher than typically found when using Black Hole Quenchers as the quenching dye because these control probes utilise the less efficient Dabcyl quencher].

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre and post-amplification steps. Use positive displacement pipets or aerosol resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers. Extra care has to be taken when purifying amplicons and analyzing them on agarose-gels.

TwistAmp® nfo

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay will almost certainly not work in TwistAmp® reactions. TwistAmp® primers are longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is not the critical factor for its performance as a primer. Users will have to go through a screening process to define suitable TwistAmp® primers for their application.

NB: Due to current production methods, RPA reactions are not suitable for amplification of standard laboratory strains of E. coli.

Before you start: Endpoint detection of amplification by sandwich assays, such as lateral flow technology based systems, will require special probes compatible with the TwistAmp® nfo biochemistry, so called TwistAmp® LF Probe. The design of these probes is described in more detail below. The probe is an additional oligonucleotide which is typically homologous to sequences between the main amplification primers and can therefore bind to the amplification product. The antigenic label on the 5' end of the probe (typically FAM) becomes conjoined with an antigenic label on the 5' end of the opposing amplification primer (typically biotin, or DIG) and this association can be detected in a sandwich assay.

Additional materials required

- Amplification primers (one of them with a 5' label if using Milenia Genline Hybridetect-1 or Hybridetect-2 lateral flow strips
- TwistAmp® nfo Probe for detection
- Heating block or other thermal incubator

Optional:

- Milenia Genline Hybridetect-1 or Hybridetect-2 lateral flow strips (available from TwistDx™ or Milenia)
- DNA fragment purification reagents/equipmentAgarose gel electrophoresis setup

Probe design considerations

The type of oligonucleotide probe that is compatible with the TwistAmp® nfo technology is the TwistAmp® LF Probe.

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 backbone with a 5'-antigenic label (typically a Carboxyfluorescein/FAM group [FAM]), an internal abasic nucleotide analogue **which replaces a neucleotide** (a tetrahydrofuran residue or THF – sometimes referred to as a 'dSpacer'), and a polymerase extension blocking group (such as a C3-spacer, a phosphate or a dideoxy nucleotide) 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.

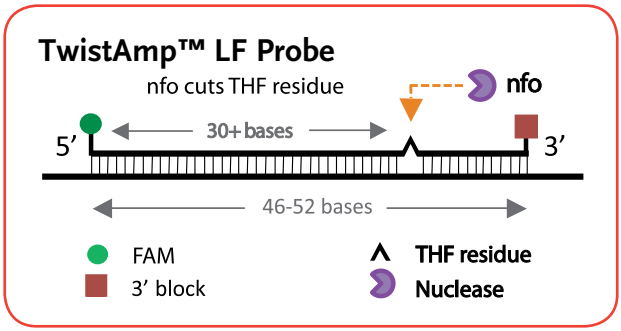


Figure 8 Schematic of the structure of an annealed TwistAmp® LF Probe. The abasic THF residue is cleaved by nfo only when the probe is bound. The 3' OH group generated in the process is a target for extension by the polymerase and enables the 5' label to be intergrated in amplification products.

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 - Rehodl - presents a substrate for a number of DNA repair enzymes, including 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 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. This duplex can then be detected in sandwich assay formats (typically post-amplification, ie. endpoint detection), such as the Genline Hybridetect-1 or Hybridetect-2 lateral flow strips from Milenia GmbH (Germany), that uses anti-FM gold conjugates and anti-Biotin capture antibodies[1], (see figure 9XXXplease checkXX).

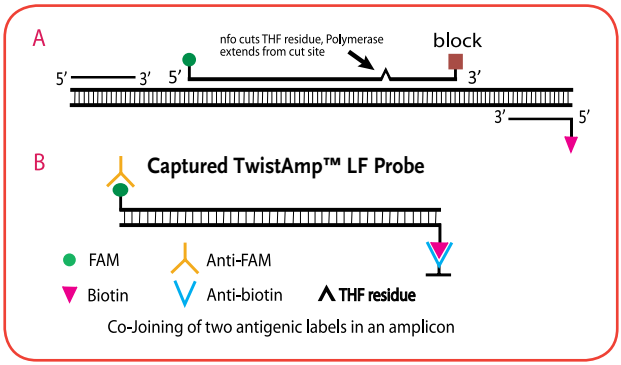


Figure 9 Schematic of the arrangements of amplification primers and TwistAmp® LF Probe. (A)The processed probe and the opposing primer will generate double-stranded amplification products that co-join the two antigenic labels. (B)This product can subsequently be captured using one of the labels (for instance using the anti-biotin test line on Milenia.

Hybridetect-1 or Hybridetect-2 lateral flow strips), and visualised using the other (for example by interaction with gold labelled antibodies). The nfo nuclease reaction and the resulting formation of the labelled amplification product is restricted to cases in which the probe can anneal to its target sequence, chosen to be within the main 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) and Eurogentec (www.eurogentec.com), using the TwistDx™ TwistAmp® LF Probe order form (available on the TwistDx™ website www.twistdx.co.uk).

Note: Lateral flow strips for the detection of nucleic acids (including the Milenia Hybridetect-1 and Hybridetect-2) 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.

TwistAmp® LF Probe length and position

A TwistAmp® LF 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 27 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 of a TwistAmp® LF Probe

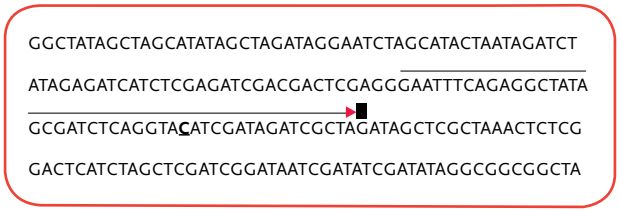


Figure 10 An example of a sequence and a possible TwistAmp® LF Probe that could be generated for this sequence. The position of the possible probe is indicated by an arrow, and the base that will be replaced by a THF residue is underlined.

The probe is blocked (typically with a dideoxy-C, G, A or T, or with a C3-spacer, or a phosphate, **but not 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 10 XXplease checkXX.

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 Appendix at www.twistdx.co.uk). The probe can then be used to test the performance of all the surrounding primers.

Protocols

Storage considerations of kit components

The TwistAmp® nfo kit components allow long-term storage (up to at least 6 months, much longer stability likely) under the correct conditions. The TwistAmp® nfo reaction pellets are provided as strips of 8 reactions in vacuum-sealed pouches. Long term storage at –20°C of the sealed product will ensure full activity of the pellets. After breaking of the vacuum seal the pellets should be used within 30

minutes.
The TwistAmp® nfo Rehydration buffer is provided in four, 1 ml, aliquots. These should be stored at –20°C to retain full activity.
The TwistAmp® nfo control primer solution and control DNA template are provided. Upon receipt they should be stored at –20°C and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium start

TwistAmp® nfo reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp® nfo rehydration buffer (provided with the kit), amplification primers, the detection probe, and template (and water to a total volume of 47.5 µl per sample).
The reaction is initiated by the addition of magnesium acetate solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample.

Detailed protocol

1. For each sample, prepare the rehydration solution as follows:

Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
TwistAmp® LF Probe (10µM)	0.6 µl
Rehydration buffer	29.5 µl
Template and dH2O	13.2 µ
(Total volume	47.5 µl)

Vortex and spin briefly.

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (here according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, rehydration buffer, water) should be prepared as a master-mix, distributed in a corresponding volume into fresh tubes, and be combined with the required volume of the different primer pairs. The different rehydration solutions are then used as normal according to the protocol.

Note: Primers and probes should be added simultaneously to pellets to avoid any bias in recombination filament formation.

2. For each sample, transfer 47.5 µl of the rehydration solution to the reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.

3. For each sample, add 2.5 µl 280mM magnesium acetate and mix well. One way to do this simultaneously for many samples is to place the magnesium acetate into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the magnesium acetate into the rehydrated material to initiate the reactions Invert vigorously 8-10 times to mix and spin down once again.

4. Insert the tubes into a suitable incubator block (optimum 37-39°C) and incubate for 4 minutes.

5. After 4 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block (Variation in the exact time of sample agitation can sometimes improve product formation).

6. Continue the incubation/detection for a total incubation time of 15- 30 minutes. If a timecourse of TwistAmp® nfo reaction is being taken, the incubation time has to be adjusted as required. At the end of the incubation proceed to *Monitoring TwistAmp® nfo amplification reactions.*

Monitoring TwistAmp® nfo amplification reactions

The outcome of TwistAmp® nfo reactions are typically analysed by an endpoint method after the reaction is completed. We recommend determining whether target was present and amplification has occurred by use of simple sandwich assay techniques if a probe was employed (TwistAmp® LF Probe). One approach is the use of Milenia's Genline Hybridetect-1 or Hybridetect-2 strips which have been developed independently for the detection of amplified nucleic

Warning: The TwistAmp® reaction pellets are activated using the rehydration solution and the magnesium acetate solution. The RPA reaction starts as soon as the magnesium acetate is added, even at room temperature. It is advisable to proceed swiftly from the resuspension of the pellet to incubation of the sample at the chosen incubation temperature.
Note: If using device with heated lid, this should be **switched off**.

In order to achieve the best amplification and fluorescent signal generation using the TwistAmp® technology with probes, when ultra-high sensitivity is required, it is advisable to agitate the reaction during the incubation period (as rapid amplification from few copies in a small volume can cause localised substrate depletion). In the protocol described here the agitation is achieved by a manual mixing step four minutes after initiation of the reaction, however slightly longer or slower amplicons may benefit more from later agitation.

acids, including PCR products. TwistAmp® is ideally suited for use of such strips because TwistAmp® nfo kits are designed for use with the TwistAmp® LF Probe system which permits direct interrogation for amplicons on strips without secondary hybridisations or reaction cleanup.
Following a dilution step with PBST (1 x phosphate buffered saline with 0.1% Tween20) amplicons can be detected within a few minutes and signal to noise ratios are superb. Also, when using this kit other methods of detection can be employed such as agarose gel-electrophoresis (AGE), which is also described in this section.
TwistAmp® nfo can also be used with TwistAmp® exo Probe system as an alternative as nfo nuclease can replace exonuclease III to process TwistAmp® exo Probe. Signal generation may be slower and cutting less complete compared with exonuclease III, but the advantage is that amplification products are not destroyed by nfo and so reactions can also be analysed on gels at endpoint.

Assessment of amplification using the TwistAmp® LF Probe system and Milenia Genline Hybridetect-1 or Hybridetect- 2 strips

1. Perform DNA amplification using TwistAmp® nfo kit, amplification primers and TwistAmp® LF Probe as described above. Ensure sufficient time has passed to permit the reactions to approach endpoint typically greater than 10 minutes but less than 20-30 minutes.
2. Employing suitable contamination control measures, remove 2µl of reaction and mix with 98µl PBST running buffer (supplied in the Milenia Genline Hybridetect-1 or Hybidetect-2 kits)(PBS containing 0.1% Tween will work also).
3. Transfer 10µl of the diluted sample to the sample pad of the Hybridetect-1 or 2 strip.
4. Place the sample pad end of strip into 200 µl of running buffer. It is often convenient to dispense the PBST into wells of a 96-well plate and stand the strips in the wells.
5. After 2 - 5 minutes the presence of the amplification product is indicated by the development of a colored test line. A separate control line found further up the strip should always develop

confirming that the strips are functioning correctly.

6. Carefully dispose of tips, strips and excess buffers to avoid amplicon contamination. We advise performing all post amplification work in a seperate area to the RPS reaction setup.

Assessment of amplification by agarose gel-electrophoresis (AGE)

1. Purify the amplification product by following the instructions for commercial PCR purification kits. Alternatively, the reaction solution (containing the amplification product) can be diluted 1/10 in water and Phenol/Chloroform extracted according to standard molecular biology practices.
2. The required amount of the amplification product can now be resolved by electrophoresis on a suitable agarose-gel following standard protocols and visualized accordingly. These operations are performed much like those for an AGE analysis of PCR products of comparable size. Data analysis: A band of the expected amplification product size should be detectable. Depending on the primers used and if using low target copy number there is the potential for some amount of non-specific products being formed during the reaction and being visible on the gel (see the Appendix at www. twistdx.co.uk for a discussion of 'primer noise'). These artefacts will typically be seen in any no-template controls and at very low target copy number. If necessary, the main product can be isolated from the non-specific products and purified for downstream applications (such as sub-cloning, sequencing, etc.).

Warning: Be aware that the post amplification processing of the reaction solutions carries a very great risk of contamination of equipment, work surfaces etc. with the amplification product. See warning below for measures to reduce this risk.

Performing positive control reactions

The TwistAmp® nfo kit contains positive control primers/probe and template, which will allow you to test the activity of the kit ponents and the detection equipment. The positive control material is used with the TwistAmp® nfo reaction pellets and rehydration buffer

1. Defrost the positive control primer/probe mix.

2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (in dH2O).

3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml microcentrifuge tube.

4. Add 29.5 µl Rehydration buffer to the step 3. Briefly vortex and spin down.

5. Add the 10 µl diluted positive control DNA to the solution from step 4. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

6. Uncap the tubes containing the freeze-dried TwistAmp® nfo reaction pellets, and place the caps upside-down in front of the tubes.

7. Resuspend each pellet in 47.5 µl Rehydration solution containing primers/probes and template DNA. Mix by pipetting up and down until the entire pellet has been resuspended.

8. Start the reaction by adding 2.5 µl 280mM of magnesium acetate and mixing well. [This can be done by pipetting 2.5 µl of magnesium acetate solution (provided with the kit) into the appropriate number of tube-caps, carefully re-capping the tubes, ensuring that the magnesium acetate solution remains in the cap, then spinning the tubes to ensure that the magnesium acetate solution combines with the rehydrated samples. Invert

vigorously 8-10 times to mix and spin down again.]

9. Place the tubes in the incubator block (optimum 37 - 39°C) and incubate for a 4 minutes.

10. After 4 minutes, take the samples out of the incubator, invert vigorously 8-10 times to mix, spin down and return the samples to the incubator block.

11. Continue the incubation/detection for a total incubation time of 20 minutes. At the end of the incubation proceed to *Monitoring TwistAmp® nfo amplification reactions*.

If using a lateral flow strip assay as the read-out system, the expected result of the positive control reaction is a clear colored test line on the strip (and the separate control line). The negative control (no template) should in contrast not generate a signal at the position of the test line.

Warning: Take precautions to minimize the potential for carryover of nucleic acids from one experiment to the next. Use separate work areas and pipettors for pre and post-amplification steps. Use positive displacement pipets or aerosol resistant pipet tips. Collect used pipet tips and reaction vessels in airtight containers. Extra care has to be taken when purifying amplicons and analysing them on agarose-gels.

References

Piepenburg et al, PLoS Biol. 2006 Jul;4(7):e204.

End notes

TwistAmp® , Twista® , and TwistAmp® Probe are registered trademarks of TwistDx™. Use of the RPA process and probe technologies are protected by US patents 7,270,981 B2, 7,399,590 B2, 7,435,561 B2, 7,485,428 B2 and foreign equivalents in addition to pending patents.

MSDS information

Material Safety Data Sheet (MSDS) information for TwistDx™ products is provided on the TwistDx™ website at www.twistdx.co.uk. MSDS documents are not included with product shipments.

TwistDx™

Unwind DNA's possibilities

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