





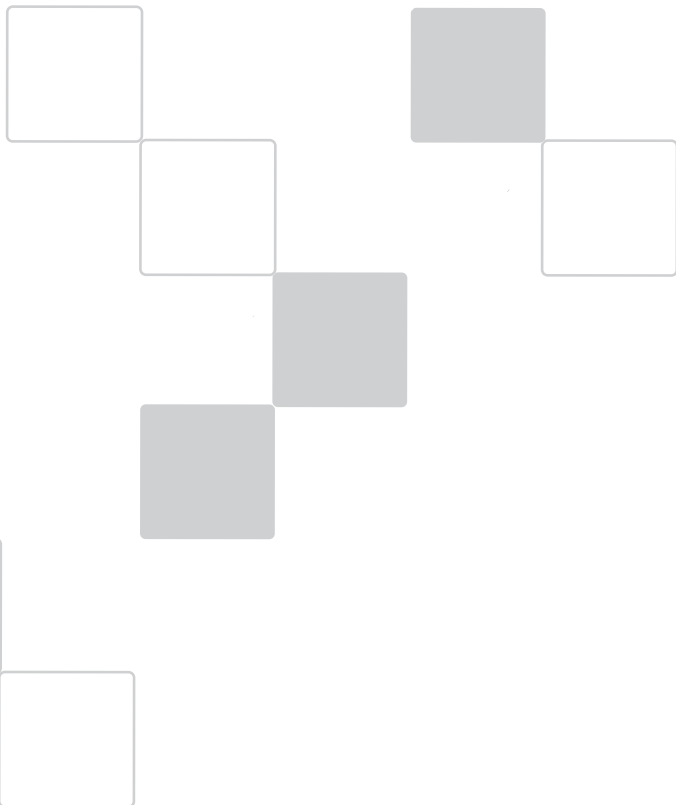


TwistDx

TwistAmp™ DNA amplification kits







COMBINED INSTRUCTION MANUAL

-  TwistAmp™ **Basic** kit
-  TwistAmp™ **RT Basic** kit
-  TwistAmp™ **exo** kit
-  TwistAmp™ **RT exo** kit
-  TwistAmp™ **fpg** kit
-  TwistAmp™ **nfo** kit



For In Vitro Use Only.
For Research and Development use only.
Not for diagnostic use.

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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’s website, and any verbal or written information concerning the kit or its use provided by any employee or agent of TwistDx.

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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™ DNA 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.

The 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, The TwistAmp™ kits are currently formulated specifically to exhibit the following overall performance characteristics:

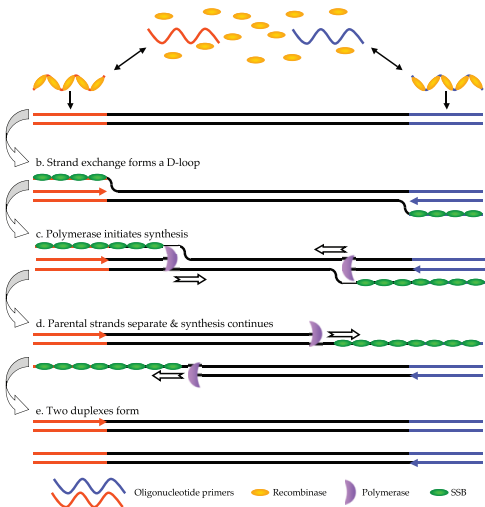
- very fast amplification (detection capability in 10-12 minutes in most cases)
- amplicon length of under 500bp
- optimal temperature of 37°C - 42°C

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 via the technical help website address techsupport@twistdx.co.uk

The RPA Cycle

All steps operate at low constant temperature (optimum 37°C)

a. Recombinase/oligonucleotide primer complexes form and target homologous DNA



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™ RT Basic 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 the Twist amplification technology with the use of Twist'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™ RT exo 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 the Twist 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) strips¹. 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.

¹ The 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.

The background is a solid dark red color. Overlaid on this are several squares of varying sizes and colors. Some squares are white with a thin white outline, while others are a light orange color. They are arranged in a scattered, non-linear pattern across the page. The text 'TwistAmp™ Basic kit' is positioned in the lower right area of the page.

TwistAmp™ Basic 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.**

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. The 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 as frozen liquid 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 as frozen liquids. 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 dH ₂ O	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 37-39°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 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: 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.

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, 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 "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.

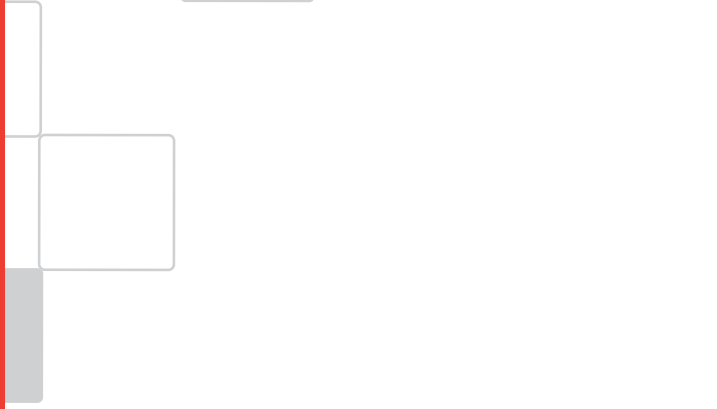
1. Defrost the positive control primer mix.
2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (in dH₂O).
3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml micro centrifuge tube.
4. Add 29.5 µl rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.
5. Add the 10 µl diluted positive control 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 μ l 280mM of Magnesium-Acetate and mixing well. [This can be done by pipetting 2.5 μ l 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.

Preventing template cross-contamination

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™ RT Basic 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.**

BEFORE YOU START... The TwistAmp™ RT Basic 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
- 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™ RT Basic kit components allow long-term storage (up to 6 months is guaranteed but much longer stability likely) under the correct conditions. The TwistAmp™ RT Basic 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™ RT Basic 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™ RT Basic 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™ RT Basic reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp™ RT Basic rehydration buffer (provided with the kit), amplification primers, and template (and water to a total volume of $47.5\ \mu\text{l}$ per sample).

The reaction is initiated by the addition of Magnesium Acetate solution (provided with the kit) in a volume of $2.5\ \mu\text{l}$, bringing the final reaction volume to $50\ \mu\text{l}$ 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 (10 μ M)	2.4 μ l
Primer B (10 μ M)	2.4 μ l
Rehydration Buffer	29.5 μ l
Template, RNase Inhibitor and dH ₂ O	13.2 μ l
(Total Volume)	47.5 μ l)

Vortex and spin briefly.

2. For each sample, transfer 47.5 μ l 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 μ l 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°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 FORMATION).**
6. Continue the incubation/detection for a total incubation time of 20-40 minutes. If a timecourse of TwistAmp™ RT Basic reaction is being taken the incubation time has to be adjusted as required. At the end of the incubation proceed to “Monitoring TwistAmp™ RT 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 **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.

Monitoring TwistAmp™ RT Basic amplification reactions

The outcome of TwistAmp™ RT 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, 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 "Preventing template cross-contamination" section below for measures to reduce this risk.

Performing positive control reactions

The TwistAmp™ RT 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™ RT Basic reaction pellets and rehydration buffer.

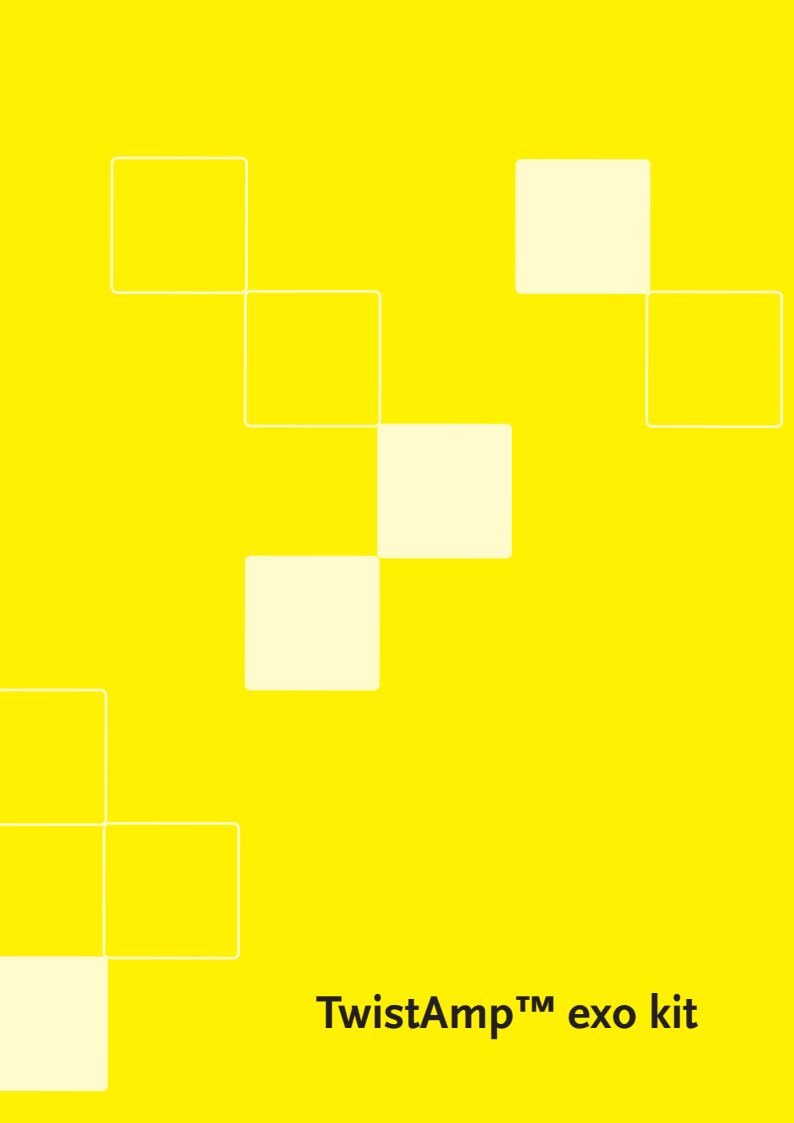
1. Defrost the positive control primer mix.
2. Prepare 10 µl of a 1/10 dilution of the positive control RNA (in dH₂O).
3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml micro centrifuge tube.
4. Add 29.5 µl rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin down.

5. Add the 10 μ l 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 reaction pellets, and place the caps upside-down in front of the tubes.
7. Resuspend each pellet in 47.5 μ l 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 μ l 280mM of Magnesium-Acetate and mixing well. [This can be done by pipetting 2.5 μ l 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°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 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 168 base pairs, that will result in a corresponding band in a gel electrophoresis.

Preventing template cross-contamination

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 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.**

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.

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).

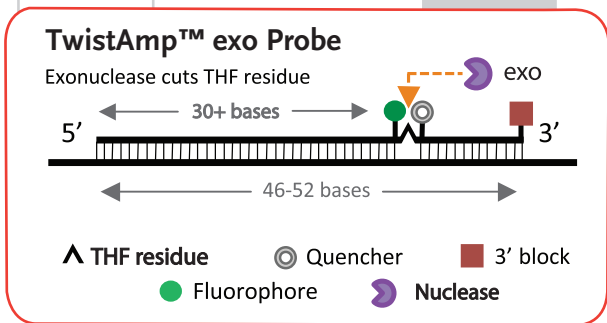
The type of oligonucleotide probe that is compatible with the TwistAmp™ exo technology is the TwistAmp™ exo Probe. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers.

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 1: 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:

```

ATGGCAACTACCCATGAATGGTTCTAAAGAAGGGAGTCAACAAGATGATT
AGAATCGTAAAAGCGAGATCGACGACTCGAGGGAATTCAGAGGCTATA
GCGATCTCAGGTACATCGATAGATCGCTAGATAGCTCGCTAAACTCTCG
GACTCATCTAGCTCGATCGGATAATCGATCGATATCGATATCGGCGGCTA
TTATTAACCTAACGCTGGATATGTCACTGCATCACGTACTTTTGT

```

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:

```

GAATTCAGAGGCTATAGCGATCTCAGG [ 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:

TCCGACTCATCTAGCTCGATCGGATAA [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.

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 as frozen liquid 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 as frozen liquids. 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 $47.5\ \mu\text{l}$ per sample).

The reaction is initiated by the addition of Magnesium-Acetate solution (provided with the kit) in a volume of $2.5\ \mu\text{l}$, bringing the final reaction volume to $50\ \mu\text{l}$ 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 (10 μ M)	2.1 μ l
Primer B (10 μ M)	2.1 μ l
TwistAmp™ exo Probe (10 μ M)	0.6 μ l
Rehydration Buffer	29.5 μ l
Template and dH ₂ O	13.2 μ l
(Total Volume)	47.5 μ l)

Vortex and spin briefly.

2. For each sample, transfer 47.5 μ l 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 μ l 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).

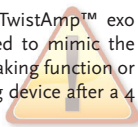
WARNING: The TwistAmp™ reaction pellets are activated using the rehydration solution and the Magnesium Acetate solution. The RPA reaction starts as soon as the Magnesium Acetate is added, even at room temperature. It is essential to proceed swiftly from the resuspension of the pellet to incubation of 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).



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 TwistAmp 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].

Preventing template cross-contamination

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™ RT exo 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.**

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™ RT exo 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
- TwistAmp™ exo Probe for detection
- Thermal incubator/Fluorometer, e.g. Twista™ isothermal Fluorometer
- RNase Inhibitor

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).

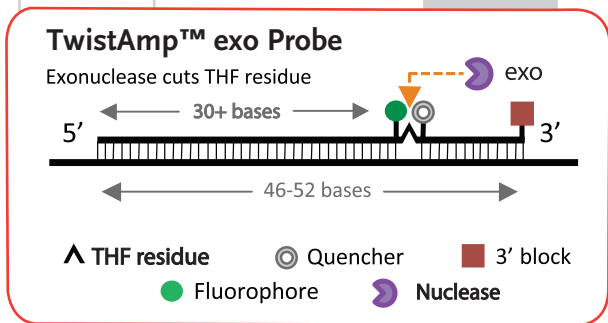
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TwistAmp™ exo Probe typically consist of an oligonucleotide backbone that contains an abasic nucleotide analogue (a tetrahydrofuran residue or THF, sometimes referred to as a 'dSpacer') flanked by a dT-fluorophore and a corresponding dT-quencher group. 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 1: 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:

```

ATGGCAACTACCCATGAATGGTTCTAAAGAAGGGAGTCAACAAGATGATT
AGAATCGTAAAAGCGAGATCGACGACTCGAGGGAATTCAGAGGCTATA
GCGATCTCAGGTTACATCGATAGATCGCTAGATAGCTCGCTAAACTCTCG
GACTCATCTAGCTCGATCGGATAATTCGATAICGATATAGGCGGCGGCTA
TTATTAACCTAACGCTGGATATGTCACGACTCACGACTTTTGT
  
```

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:

```

GAATTCAGAGGCTATAGCGATCTCAGG [ 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:

TCCGACTCATCTAGCTCGATCGGATAA [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.

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™ RT exo kit components allow long-term storage (up to 6 months guaranteed, much longer stability likely) under the correct conditions. The TwistAmp™ RT 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™ RT exo 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™ RT exo 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™ RT exo reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the TwistAmp™ RT exo rehydration buffer (provided with the kit), amplification primers, the detection probe, and template (and water to a total volume of $47.5\ \mu\text{l}$ per sample).

The reaction is initiated by the addition of Magnesium-Acetate solution (provided with the kit) in a volume of $2.5\ \mu\text{l}$, bringing the final reaction volume to $50\ \mu\text{l}$ 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 (10 μ M)	2.1 μ l
Primer B (10 μ M)	2.1 μ l
TwistAmp™ exo Probe (10 μ M)	0.6 μ l
Rehydration Buffer	29.5 μ l
Template, RNase Inhibitor and dH ₂ O	13.2 μ l
(Total Volume)	47.5 μ l)

Vortex and spin briefly.

2. For each sample, transfer 47.5 μ l 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 μ l 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).

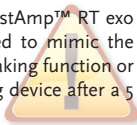
WARNING: The TwistAmp™ reaction pellets are activated using the rehydration solution and the Magnesium Acetate solution. The RPA reaction starts as soon as the Magnesium Acetate is added, even at room temperature. It is essential to proceed swiftly from the resuspension of the pellet to incubation of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

Monitoring TwistAmp™ RT 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 **five** 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™ RT 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 5 minute pre-incubation/mixing step).



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™ RT 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™ RT 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 RNA (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 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™ RT 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 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].

Preventing template cross-contamination

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.**

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 MATERIALS REQUIRED

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

PROBE DESIGN CONSIDERATIONS

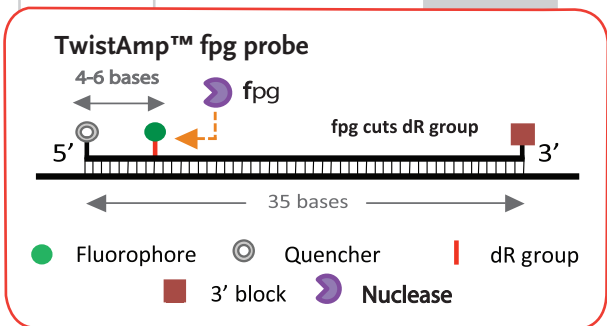
The use of fluorophore/quencher probes in real-time detection formats is a very convenient way to monitor amplification events in TwistAmp™ reactions. Probes are especially useful to quickly generate comparative data about the speed and sensitivity of different primer pairs and are therefore a very valuable 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 1: 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).

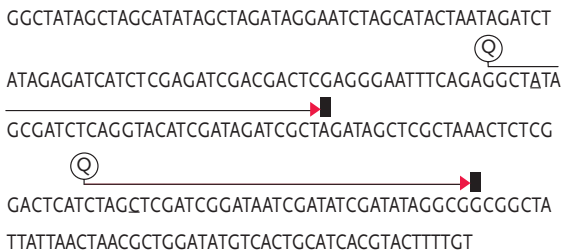
TwistAmp™ fpg Probe length and position

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:



In the above figure 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 as frozen liquid 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 as frozen liquids. 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

- 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 dH ₂ O	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).

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 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.

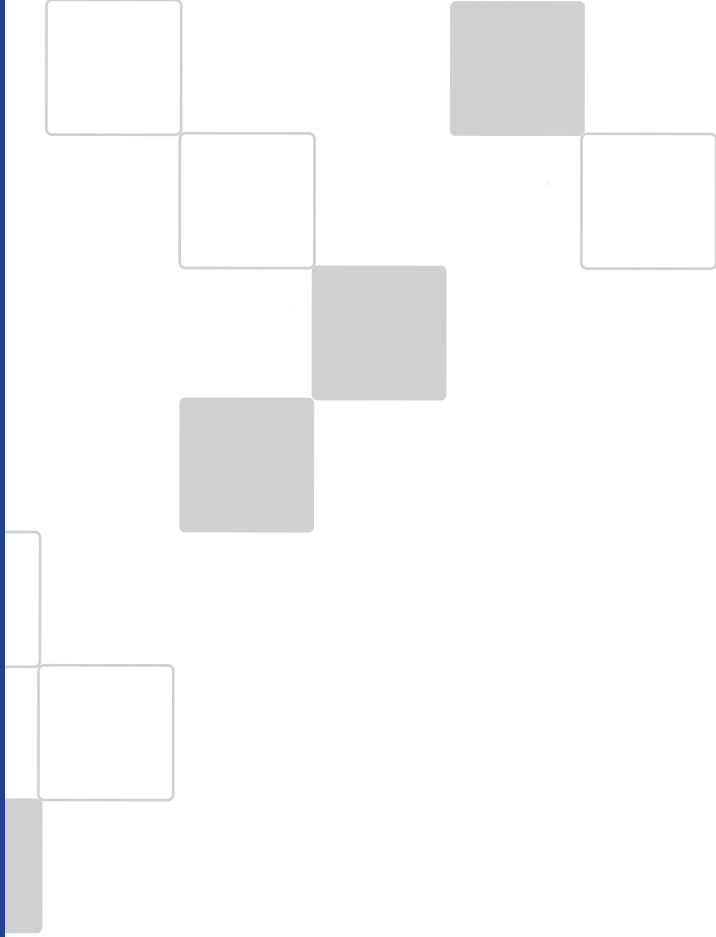
1. Defrost the positive control primer/probe mix.
2. Prepare 10 µl of a 1/10 dilution of the positive control DNA (in dH₂O).
3. Pipette 8 µl primer/probe solution into a fresh 1.5 ml microcentrifuge tube.
4. Add 29.5 µl Rehydration buffer to the primer/probe solution from step 3. Briefly vortex and spin 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.]

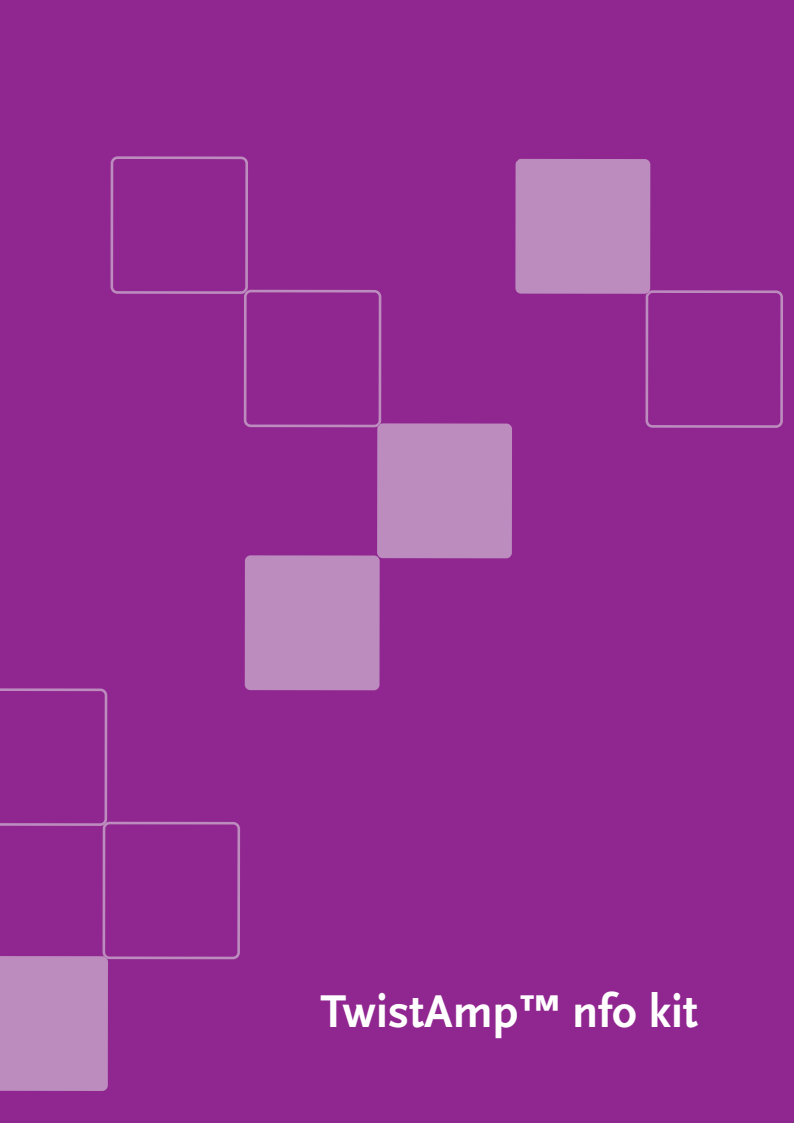
9. Place the tubes in the Twista™ and start the run.
10. After 4 minutes, take the samples out of the reader (DO NOT STOP THE PROGRAM), invert vigorously 8-10 times to mix, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator 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].

Preventing template cross-contamination

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 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.**

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/equipment
- Agarose 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 NUCLEOTIDE (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.

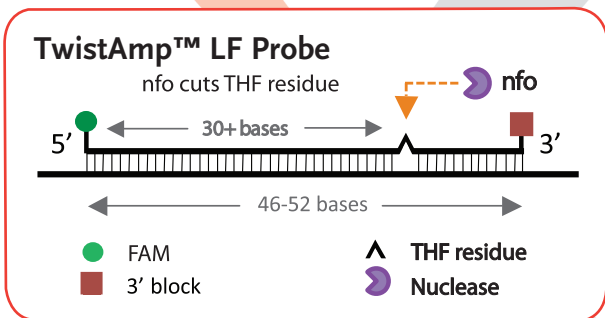


Figure 1: Schematic of the structure of an annealed TwistAmp™ LF Probe. The abasic THF residue is cleaved by nfo only when the probe is bound. The 3' OH group generated in the process is a target for extension by the polymerase and enables the 5' label to be intergrated in amplification products.

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 - Rehdol - 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 Geline Hybridetect-1 or Hybridetect-2 lateral flow strips from Milenia GmbH (Germany), that uses anti-FM gold conjugates and anti-Biotin capture antibodies¹, (see figure 2).

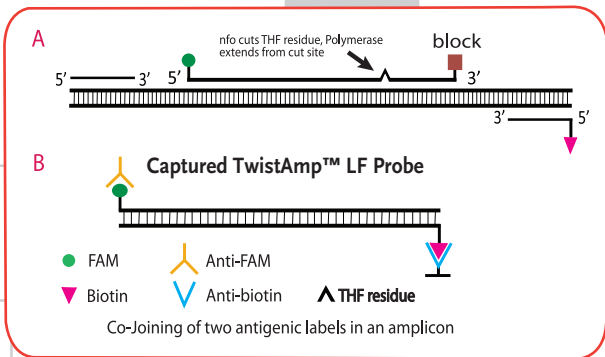


Figure 2: Schematic of the arrangements of amplification primers and TwistAmp™ LF Probe. (A) The processed probe and the opposing primer will generate double-stranded amplification products that co-join the two antigenic labels. (B) This product can subsequently be captured using one of the labels (for 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 1:** 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.

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GGCTATAGCTAGCATATAGCTAGATAGGAATCTAGCATACTAATAGATCT
ATAGAGATCATCTCGAGATCGACGACTCGAGGGAATTCAGAGGCTATA
GCGATCTCAGGTACCATCGATAGATCGCTAGATAGCTCGCTAAACTCTCG
GACTCATCTAAACTAACGCTGGATATGTCACTGCATCACGTACTTTTGT
  
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Example of a TwistAmp™ LF Probe

Below is shown 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:

GAATTT CAGAGGCTATAGCGATCTCAGGTA [THF]
ATCGATAGATCGCTA 3' [BLOCK]

Amplification primers would normally flank the probe on either side as indicated in figure 2.

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 as frozen liquid 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 as frozen liquids. 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 $47.5\ \mu\text{l}$ 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.

The reaction is initiated by the addition of Magnesium-Acetate solution (provided with the kit) in a volume of $2.5\ \mu\text{l}$, bringing the final reaction volume to $50\ \mu\text{l}$ per sample.

DETAILED PROTOCOL

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

Primer A (10 μ M)	2.1 μ l
Primer B (10 μ M)	2.1 μ l
TwistAmp™ LF Probe (10 μ M)	0.6 μ l
Rehydration Buffer	29.5 μ l
Template and dH ₂ O	13.2 μ l
(Total Volume)	47.5 μ l)

Vortex and spin briefly.

2. For each sample, transfer 47.5 μ l of the rehydration solution to he reaction pellet. Mix by pipetting up and down until the entire pellet has been resuspended.
3. For each sample, add 2.5 μ l 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”.

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.

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.

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 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. The TwistAmp™ nfo kit 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 Hybridetect-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. DISPOSE OF TIPS, STRIPS AND EXCESS BUFFERS CAREFULLY TO AVOID AMPLICON CONTAMINATION. WE ADVISE PERFORMING ALL POST AMPLIFICATION WORK IN A SEPARATE AREA TO THE RPA REACTION SET-UP.

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.
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'). 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 the "Preventing template cross-contamination" section 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 dH_2O).
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.

Preventing template cross-contamination

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.

FREQUENTLY ASKED QUESTIONS

Will PCR primers work in RPA?

NO. Most PCR primers will not work in RPA because they are too short. Furthermore there is no established correlation between the qualities of an oligonucleotide as a RPA primer on the one hand and as a PCR primer on the other. See Appendix at www.twistdx.co.uk under Technical Resources for details about primer design.

Can I use my existing PCR probe?

NO. Most popular PCR probe systems are not suited for use with the TwistAmp™ process. In particular those systems employing the 5' to 3' nuclease activity of polymerases cannot be used with the TwistAmp™ system as such enzymatic activity is fundamentally incompatible with the RPA biochemistry.

How do I design RPA primers?

Good RPA primers are identified by a screening process described in detail in the Appendix at www.twistdx.co.uk.

Can I multiplex TwistAmp™ reactions?

YES. It is possible to perform more than one amplification reaction simultaneously in the same tube. However, not all primer pair combinations will work equally well with each other in multiplexing and this format therefore requires careful primer design. Note that the total amount (nmols) of oligonucleotide in the reaction should not significantly exceed that stated in the protocol; if more than two amplification primers are used in a single reaction, then the maximum amount of primer has to be divided between all the oligonucleotides present. Monitoring multiple amplification events at the same time might also require different probes, and limitations on both, the detection equipment and the availability of compatible fluorophores, have to be borne in mind.

How much of my primers do I have to use?

The recommended concentration of primers in the TwistAmp™ Basic reaction is 480nM each. In the TwistAmp™ exo, TwistAmp™ fpg, and Twist Amp™ nfo kits the recommended concentration of

primer is 420nM each. However, the performance of some primer pairs can be improved by slightly varying their amounts in the reaction and a titration strategy (from 200nM to 600nM each) can be employed to identify the optimal concentration conditions for a given primer pair.

How do I reconstitute a lyophilised probe?

Please follow the oligonucleotide manufacturer's instructions for the reconstitution and storage of the probes.

Typically the tube containing the lyophilised oligonucleotide will be spun briefly to collect the DNA at the bottom of the tube and an appropriate volume of To.1E buffer (10mM Tris-HCl pH 8, 0.1mM EDTA) will be added to prepare a stock solution of 100µM. Allow the solution to stand for 10 minutes at room temperature and mix (vortex) for 10 seconds. Reconstituted oligonucleotide probes are typically stored at -20°C for the long term

How much of my probe do I have to use?

The recommended concentration of probe in the reaction is 120nM. However, some TwistAmp™ reactions benefit from being used with slightly different amounts of probe. Testing different concentrations of probe (from 50nM to 150nM) will help to optimise the performance of probe based detection formats of a given assay.

Can I use TwistAmp™ reactions for the quantification of template?

YES. The onset time of detectable amplification for a given assay will depend on the amount of starting template material – the more template copies there are to start with, the earlier the detection time will be. However, exploiting this 'time-based' quantification demands a careful experimental setup ensuring simultaneous initiation of compared reactions (e.g. through 'Magnesium start'). The resolution of quantification is aided by a relatively 'slow' amplification reaction. Strategies to slow the rate of amplification (including the design of suitable primers) are discussed in the Appendix at www.twistdx.co.uk.

Can I add the components of the rehydration solution one at a time directly to the freeze dried reaction pellets?

NO. Adding one primer or probe before another will bias the formation of recombination filaments towards whichever oligonucleotide is added first. This is why primers and probes are added simultaneously.

Can I include the Magnesium-Acetate in my resuspension buffer?

YES. However the reaction components are activated as soon as Magnesium is added. By pipetting Magnesium Acetate into the reaction last, which we recommend, for example by adding it to the lids of strips and spinning it into the reactions, you ensure that reactions start simultaneously and you minimise the risk of cross contamination or RPA products being produced in any reaction residues left in your tips.

Can I make a master-mix?

YES. If you wish to set up multiple reactions, you can make a master mix. If you are screening different DNAs, the resuspension buffer, primers and probe if used can all be mixed together and added to freeze dried reactions to resuspend them. Different DNAs can then be added to reactions before they are started with MgAc as usual. If you are performing a primer screen, it is possible to make a master mix with the resuspension buffer, template DNA, probe (if used) and one of the primers. This should be aliquotted into 1.5 ml tubes and the variable primers added. Only once both primers are present should the freeze dried reactions be resuspended or the formation of recombination filaments will be biased towards the first oligonucleotide added.

Can I perform a fluorescence end-point analysis of the amplification reaction?

YES. The measurement of change of fluorescence can also be used as an endpoint read-out. In this case the fluorescence at the start and at the end of the reaction is compared: an increase of fluorescence signifies a successful amplification event.

Can I analyse the amplification product on an agarose gel?

YES, UNLESS you are using the TwistAmp™ exo kit. The exonuclease present in the reaction mixture will digest most of the amplification product once amplification has ceased. For the analysis of amplicon, use the TwistAmp™ Basic kit. It is also possible to analyse products on gels generated from the TwistAmp™ nfo and TwistAmp™ fpg kits.

How long do RPA primers have to be?

RPA primers should be at least 30 nucleotides long. Typically, primers are between 32 and 35 nucleotides long. In some cases primer can be shorter than 30 residues, but this usually slows overall amplification kinetics. See www.twistdx.co.uk for details about primer design.

How far should RPA primers be apart?

This depends on the application and need. In general we recommend that under the conditions of the standard TwistAmp™ kits the amplicon generated by two RPA primers should typically be no longer than about 500bp. There is probably no lower limit to the size of RPA products, but the minimum size of RPA primers requires that amplicon will typically be longer than about 80bp. For the most rapid real-time kinetics the final amplicons should ideally be 100-200 base pairs. Under specialised conditions amplification products of as much as 2 kilobases have been generated. However, the standard TwistAmp™ kits do not readily permit the generation of such large amplicons. See Appendix at www.twistdx.co.uk for details about primer design and approaches to achieve larger amplicon sizes.

How many primers do I have to screen?

This depends on the sensitivity specifications of the assay. For example, if you only need to detect 1000 molecules per reaction or more, then most primer pairs will be adequate. For very high sensitivity assays, the best way to find good RPA primers is by performing a systematic screen. Typically the number of tested primer pairs will lie between 10 and 20 initially. The more oligonucleotides are tested, the greater the chance of finding good primer pairs capable of detecting single molecules with rapid kinetics.

Where can I get further details on primer design?

For further details on primer design, see the Appendix at www.twistdx.co.uk under Technical Resources / Instruction manuals / Appendix.

Do I need a probe for the primer screen?

NO. Any detection method can be used to evaluate and compare the performance of potential primer pairs. However, real-time monitoring of the amplification with a detection probe has proven to be the fastest and least laborious method for screening high sensitivity primers.

Which conditions should I use for the primer screen?

Ideally the conditions of the screen should mimic as closely as possible the conditions expected in the final assay (approximate template copy number, sample purity, depth of multiplexing, etc.).

Can I improve the performance of a given primer?

Small changes in the sequence of a primer can improve RPA performance. Any given primer can be optimised by slightly varying its length (by single nucleotides) and position (keeping the length but shifting their location in 1bp increments), and re-testing its activity.

What makes a good primer?

The precise rules for this are not yet known, this is why primer screening is so important.

What melting temperature should RPA primers have?

As RPA reactions are performed at a constant temperature and under conditions under which the melting behaviour of DNA is drastically altered by DNA melting proteins, conventionally calculated melting points are not directly applicable to the system.

How do I select a probe?

If using one of the TwistAmp™ kits suitable for probe use, follow the guidelines described in the Appendix at www.twistdx.co.uk for the design of detection probes. Note that there are some sequence restrictions in choosing the position of probes within a target using the preferred TwistAmp™ exo Probe system. If this presents unreasonable limitations TwistDx can help you design alternative detection strategies. The alternative TwistAmp™ fpg Probe system is far more amenable to flexible design, but does not always yield as strong fluorescence signal as TwistAmp™ exo Probe.

Can I use intercalating dyes for real-time monitoring with the TwistAmp™ technology?

YES. It is possible to use intercalating dyes to quantify and monitor the TwistAmp™ reactions in progress. However, as with PCR, the dye typically binds to any double-stranded DNA, so primer noise can generate false positive signals. Moreover, the exonuclease present in the TwistAmp™ exo kit will digest most of the amplification product during the reaction, and the use of intercalating dyes is not recommended with these kits.

Do my primers need any special purification protocols?

When performing a primer screen, one does not normally need to use specially purified oligonucleotides. However, as with use in other techniques, we have noted batch-to-batch variations in the quality of primer preparations. For applications where consistency is critical, we would therefore recommend the use of more purified primers once good primers are identified.

At what temperature should the TwistAmp™ kit be used?

The standard TwistAmp™ kits are configured to operate in the temperature range of 37°C - 42°C. At higher temperatures the system will be compromised as the enzymes progressively lose full activity. The RPA process itself can, under appropriate conditions, be performed at much lower temperatures, but the supplied formulations of the TwistAmp™ kits are optimised for high kinetic rate and not generally compatible with protocols using temperatures below the recommended range.

Do I need to clean up my RPA product before running it on a gel?

Yes. The crowding agent and proteins in an RPA reaction interfere with normal agarose gel electrophoresis, so you will probably get a smear, rather than a clean band if you do not clean up your reaction.

Do I need to dilute my RPA product before running it on a lateral flow strip?

Yes. The crowding agent and proteins in RPA reactions can interfere with the antibodies on a lateral flow strip, so you can get non-specific binding and false positive signals if you do not dilute them sufficiently.

I've tested some primers with a one type of TwistAmp™ kit, will they work with another type of TwistAmp™ kit?

Maybe. If you have developed primers using a TwistAmp™ Basic kit, you will also need to include a probe if you are going to use a TwistAmp™ exo or nfo kit. This and other factors mean that an optimal primer combination for a gel-based approach vs a fluorescence or lateral flow approach may not be the same. In general we have found that switching from TwistAmp™ exo fluorescence monitoring to TwistAmp™ nfo for lateral flow monitoring using a similar sequence probe gives fairly reliable results. The best primer/probe combination for fluorescence monitoring is however not necessarily the primer combination that will give the most attractive gel-based result and vice versa.

Do I have to use a Twista™ if I want to run a TwistAmp™ reaction?

No. For TwistAmp™ Basic and nfo kits, any device that can hold a steady temperature of 37-42°C is fine. For real-time reactions using TwistAmp™ exo kits, any plate reader or real-time thermal cycler that can excite and detect the fluorophores you are using and hold a steady temperature of 37-42°C is fine. The Twista™ is a convenient instrument for running RPA on – it's cheap, can run off a battery, with or without a laptop, you can input parameters for positive/negative outputs and it takes a strip of 8 RPA reactions.

Can I just use labelled primers and a TwistAmp™ Basic kit for lateral flow?

Yes. You can use two modified primers for lateral flow if you wish, but we recommend using a probe and a TwistAmp™ nfo kit. This is because, as with PCR, RPA is subject to primer-dimer formation, so you can get primers cross reacting and giving false positives if you do not have perfectly designed primers. TwistAmp™ nfo probes avoid this problem because they are blocked and cannot be extended to create probe-primer dimers. The nfo enzyme recognises and cuts the abasic site (THF) in the probe only when it has bound to its complementary strand. Cutting the abasic site means that the blocked end of the probe can fall off and the probe can act as a primer, thereby generating a product that can be captured by a lateral flow strip. So, only if you have your desired amplicon can the probe bind, be cut, and extend to form a product with the opposing biotinylated primer..

Does RPA work with biotinylated or fluorescently labelled oligonucleotides?

Yes. Biotinylated primers or ones with fluorophore should work the same as unmodified primers in an RPA reaction. We have yet to see any differences.

I sometimes see a sharp increase in fluorescence towards the end of my TwistAmp™ exo reaction, is this normal?

Yes. The polymerase and exonuclease III in TwistAmp™ exo reactions are in competition. As the reaction runs out of energy, the exonuclease III starts to dominate. This leads to faster probe cleavage and a jump in fluorescence signal.

Can I store the amplicons from my TwistAmp™ reactions?

Yes, if you have used TwistAmp™ Basic or nfo reactions, you can store your amplicons at 4°C for short periods of time and -20°C for longer periods of time.

No, if you have used TwistAmp™ exo reactions. The exonuclease III is likely to digest the amplicon if the reaction is not processed rapidly to inactivate any such activity.

Can I use TwistAmp™ Basic amplicons for TA cloning?

The polymerase used in TwistAmp™ Basic reactions does not have an editing function, so this should be possible. However, we have not tested this ourselves.

Can I add more/less Rehydration Buffer to reactions?

NO. Adding more or less Rehydration Buffer than is recommended can have a detrimental impact on how an RPA reaction works. The buffer contains ingredients that are necessary for RPA reactions, so adding more or less buffer will change their final concentration in a resuspended pellet.



REFERENCES

1. 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

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

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