TwistDx™

Unwind DNA's possibilities

TwistAmp® DNA Amplification Kits

Combined Instruction Manual

Part number: INBAEXNF Revision 1

- TwistAmp® Basic
- TwistAmp® exo
- TwistAmp® nfo

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

Contents

Materials provided as standard

- 1 resealable pouch containing 96 freeze-dried TwistAmp® reaction pellets
- 1 tube x 4 mL Primer Free Rehydration buffer
- 1 tube x 500 µL 280 mM Magnesium Acetate (MgOAc)

Kit specific materials

- 1 tube x 100 µL Positive control template (DNA with exo, Basic, and nfo)
- 1 tube x 75 µL Positive control primer/probe mix (oligo mix consists of primers for Basic kits, primers and a probe in the exo and nfo kits)

Additional materials required (not provided in the kit)

- molecular grade water
- assay-specific oligonucleotides
- Reverse Transcriptase (if amplifying RNA)
- RNase Inhibitor (if amplifying RNA)

Storage conditions

TwistAmp® reaction pellets: Provided in resealable 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. However, for long term storage it is recommended that kit reactions are stored in the resealable pouch at -20°C and that the desiccant sachet remains in the pouch. The expiry date of the reactions still applies on pouch opening.

Primer Free Rehydration buffer and **Magnesium Acetate (MgOAc):** Upon receipt, store at –20°C (full activity is guaranteed for 6 months). Avoid excessive freeze-thaw cycles.

Positive control primer/probe mix and **DNA template:** 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.

Limitations on Use and Distribution of the Material and Information

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

No Warranty; Limitation of Liability

Recipient understands and agrees that the materials are experimental in nature and that the Materials and Information are provided without any warranty as to results, merchantability, fitness for a particular purpose or non-infringement of any patent or other intellectual property right, and without any other representation, warranty or condition, express or implied. TwistDx shall not be liable in connection with the Materials, Information or any breach of this agreement under any contract, negligence, strict liability or other theory for (a) loss of revenues, loss of profits, or loss or inaccuracy of data, including test results, regardless of how such damages are characterised (b) for the cost (including procurement costs) of substitute goods, services or technology, or (c) for any special, indirect, incidental or consequential damages. In no event will TwistDx's aggregate liability under this agreement exceed one hundred dollars (US$100). Recipient understands that its use of the materials and/or the information in connection with its activities are entirely at its risk.

Introduction

TwistAmp® DNA amplification kits provide the reagents necessary to amplify nucleic acid 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 37-42˚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 optimised and can reach detectable levels of product in less than 10 minutes using the configurations of the TwistAmp kits in many cases.

Overview of the TwistAmp® amplification technology

The isothermal TwistAmp® technology is based on the Recombinase Polymerase Amplification (RPA) process developed by TwistDx (see Page 34). 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 utilises enzymes called recombinases, of which *Escherichia coli* recA is the archetypal member, which can bind to single-stranded nucleic acid backbones - standard oligonucleotides in this case - and stimulate the resulting protein- DNA complex to search for homologous sequences in duplex DNA. Once homology is located, a strand-switching reaction is performed and the oligonucleotide is paired to its complement permitting a polymerase to begin synthesis from the 3' end. The TwistAmp® amplification process uses two opposing oligonucleotide primers to initiate each synthesis event. The design of these primers for a target, in a manner similar to that for PCR, permits the establishment of an exponential amplification process.

TwistAmp® reaction conditions

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

- very fast amplification (detection capability in 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 refer to the TwistAmp® Assay Design Manual located at twistdx.co.uk for a further discussion of optimisation of TwistAmp® reaction conditions. For specialised needs and applications not discussed in the TwistAmp® Assay Design Manual please contact TwistDx via technical support at techsupport@twistdx.co.uk

Note: Contaminating *E.coli* DNA is present in all TwistAmp® reaction pellets. RPA reactions are not suitable for developing diagnostics for *E. coli* if sequence homology with strain BL21 (used for our protein production) is present.

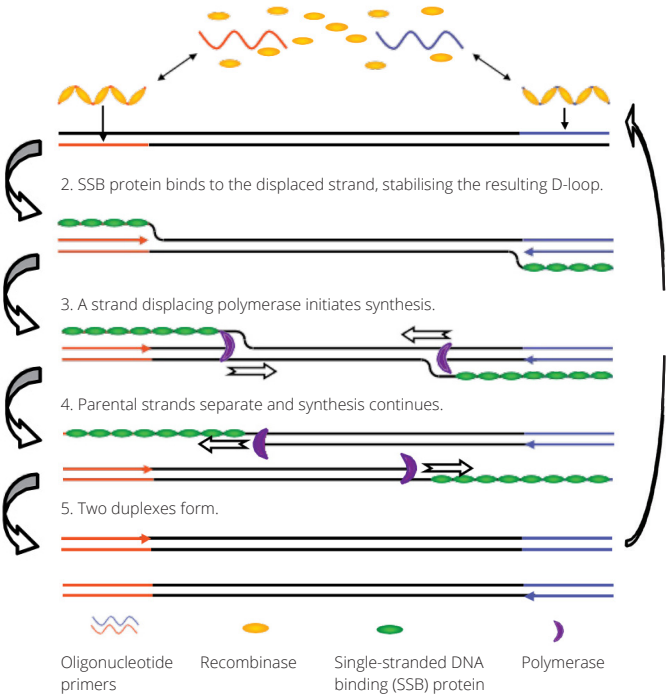

Figure 1 The RPA Cycle

The TwistAmp® application family

A number of different formulations are tailored for different applications and detection modes.

TwistAmp® Basic contains the 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 end-point method, such as gel electrophoresis. Amplified material can also be purified and used for downstream applications (e.g. subcloning).

TwistAmp® exo is recommended for users who want to combine RPA amplification technology with the use of TwistDx's proprietary fluorescent TwistAmp® exo probes. In addition to the basic components, a powerful nuclease (Exonuclease III) is provided 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® nfo is designed for users who want to detect the success of their amplification reaction by means of end-point sandwich assays, such as lateral-flow (LF) strips. Besides the basic amplification reagents it includes a nuclease – endonuclease IV (nfo) which can generate new polymerase extension substrates with suitable TwistAmp® nfo probes – the amplified material can then be used in instrument–free detection formats. 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 nfo will process these probes (often with slightly slower kinetics) and additionally will permit product to be analysed by gels at end-point. Contact Customer Service for product availability.

To amplify RNA: It is possible to use RNA template with a TwistAmp® kit by adding a suitable reverse transcriptase (RT) when setting up a reaction. Such reactions are run as you would a normal TwistAmp® reaction (it's a one-step process) with the exception noted below. If a RT that works at 37–42 °C is added to RPA chemistry, then RNA can be reverse transcribed and the cDNA produced will be amplified all in one step. The complementary DNA sequence will be that of the RNA added. We recommend using similar RT amounts to that of a PCR reaction of the same volume, according to your chosen manufacturer's instructions. Reactions should be run at 40°C with the agitation step at 5 minutes. It's also advisable to add RNase Inhibitor to any reaction where RNA is the target material. Any commercially available RNase Inhibitor will be suitable.

If the M-MLV RT that you are using comes with a separate dilution/reaction buffer, we suggest not using it as it will lower the concentration of the enzyme.

The "agitation step" refers to the first mixing step per the protocols in the manual. Wait 5 minutes instead of 4 minutes before "vortex, spin down briefly, and return the samples to the incubator block."

A titration to determine the optimal amount to add to the reactions is recommended.

TwistAmp® assay development

All TwistAmp assay design is fully described in the separate TwistAmp® Assay Design Manual available from twistdx.co.uk.

There is currently no automated primer design software available for RPA, as we are still learning about optimal design. The reason we do not recommend using PCR primer design software for RPA is because they mainly use melting temperature (T_m) to determine what a good primer pair is. As there is no thermal melting in RPA reactions, (everything is done with enzymes) we do not know how well T_m correlates with RPA primer performance, if at all. Some RPA users have successfully developed primers using design software by changing the default primer length to 32-36 bases and ignoring the T_m parameter. For the most sensitive primers, we recommend following the guidelines in our TwistAmp® Assay Design Manual.

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 valuable tool in the screening of potential primer candidates (see Section 2, TwistAmp® Assay Design Manual). Oligonucleotide probes that are compatible with the TwistAmp® technology come in two different varieties: TwistAmp® exo and TwistAmp® nfo probes. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers. The proprietary probe design of these probes types are described in the TwistAmp® Assay Design Manual, which includes guidelines on structure, function, length, position along with example designs.

TwistAmp® probes, along with assay primers, can be ordered from various oligonucleotide manufacturers.

TwistAmp® Basic

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay may often work, but may not be optimal for TwistAmp® reactions. TwistAmp® primers displaying rapid amplification kinetics are often longer than typical PCR primers, and in contrast to PCR, the melting temperature of an oligonucleotide is perhaps not the most critical factor for its performance as a primer. Users should go through a screening process to define suitable TwistAmp® primers for their application (see TwistAmp® Assay Design Manual at twistdx.co.uk).

Additional materials required

- amplification primers
- molecular grade water
- Reverse Transcriptase (if amplifying RNA template)
- RNase Inhibitor (if amplifying RNA template)
- heating block or other thermal incubator
- DNA fragment purification reagents/equipment
- agarose gel electrophoresis setup
- microcentrifuge for reagent spin-down

Protocols

Storage considerations of kit components

TwistAmp® Basic kit components allow long-term storage (up to 6 months is guaranteed, but much longer stability is likely) under the correct conditions.

TwistAmp® Basic reaction pellets are provided as strips of 8 reactions in a resealable pouch. Long term storage at –20°C or lower of the sealed product will ensure full activity of the pellets. After breaking the pouch seal, reactions should be stored in the closed pouch along with the provided desiccant.

Primer Free Rehydration buffer and Magnesium Acetate (MgOAc) should be stored at -20˚C to retain full activity.

TwistAmp® Basic positive control primer mix and positive control DNA template are provided. Upon receipt they should be stored at -20˚C to retain full activity and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium acetate start

TwistAmp® Basic reactions are established by reconstituting the supplied freeze-dried reaction pellets with a suitable rehydration solution. This solution consists of the Primer Free Rehydration buffer (provided with the kit), amplification primers and template (RT and RNase Inhibitor if using RNA as a template), and water to a total volume of 47.5 µL per sample.

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

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

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

Incubation mixing

For low copy template amplification (e.g. less than 100 template copies), mixing of reactions during incubation will improve product formation (as rapid amplification from few copies in a small volume can cause localised substrate depletion).

There are a number of mixing methods that can be applied by the user: The simplest being mixing by hand (manual mixing) at a single time point by inverting vigorously 8-10 times to mix followed by a brief spin down in a microcentrifuge, or by vortex mixing prior to a brief spin-down as described in the following protocols. The time of manual mixing can be varied between 3 and 7 minutes after initiation of the reaction. A standard time of agitation is 4 minutes (5 for RT-RPA) and longer or more slowly accumulating amplicons may in particular benefit from slightly later agitation.

Some devices enable magnetic mixing during the full incubation timeframe by the addition of a micro ball to the reaction (prior to activation with MgOAc). An example protocol for magnetic mixing of TwistAmp® reactions would be scan duration of 1,200 seconds, and sample rate of 15 seconds. Variation in agitation timing and frequency will also influence product formation. Mixing optimisation is advisable. If using very small volume RPA reactions (less than 5 µL), or a very high copy number of template, mixing may be unnecessary.

Incubation

For specific use with TwistAmp® Basic reactions, incubation device temperature should be set to 39˚C or 40˚C if amplifying RNA. This can be later optimised (37-42˚C is usually optimal), along with the mixing regime. Reactions should be incubated for 20 minutes.

Note: The formulation of TwistAmp® Liquid contains different ratios of core RPA proteins to TwistAmp Basic lyophilised reactions. Assays that have been developed with one kit may perform differently with another.

Detailed protocol

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

Forward Primer (10 µM)	2.4 µL
Reverse Primer (10 µM)	2.4 µL
Primer Free Rehydration buffer	29.5 µL
Template, (RT, RNase Inhibitor if amplifying RNA) and water to	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 (MgOAc) and mix well. One way to do this simultaneously for many samples is to place the MgOAc into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the MgOAc into the rehydrated material to initiate the reactions. Vortex briefly and spin down once again.

Note: The TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as the MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

4. Incubate the tubes in a suitable heated block (39˚C) for 20 minutes. For low template copy number, remove tubes after 4 minutes, vortex and spin briefly and replace in heated block. Alternatively, magnetic mixing using a micro ball may be applied during incubation (see *Incubation mixing*).

5. After incubating, clean RPA product amplicons before running on an agarose gel. See *Monitoring TwistAmp® Basic amplification reactions*.

Note: Take precautions to minimise 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 pipettes or aerosol-resistant pipette tips. Collect used pipette tips and reaction vessels in airtight containers. Extra care should be taken when purifying amplicons and analysing them on agarose-gels.

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 Primer Free Rehydration buffer.

1. Defrost the positive control primer mix and pipette 8 µL into a fresh 1.5 mL microcentrifuge tube.

2. Add 29.5 µL Primer Free Rehydration buffer to the positive control primer mix from step 1. Briefly vortex and spin down.

3. Prepare 10 µL of a 1/10 dilution of the positive control DNA (in molecular grade water).

4. Add the 10 µL diluted positive control DNA template to the solution from step 2. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

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

6. 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.

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

Note: The TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as the MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

8. Insert the tubes into the incubator block (39°C) for 20 minutes. Remove tubes after 4 minutes, vortex, spin down briefly, and return the samples to the incubator block.

9. After incubating, clean RPA product amplicons before running on an agarose gel. See *Monitoring TwistAmp® Basic amplification reactions*.

Note: If tubes are opened after amplification, there is a high risk of contamination of work surfaces with amplicon. Ensure that appropriate avoidance measures are taken.

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

Monitoring TwistAmp® Basic amplification reactions

The outcome of TwistAmp® Basic reactions is typically analysed by an end-point method after the reaction is completed, such as agarose gel electrophoresis (AGE), which is described in this section. However, alternative methods to AGE can also be used. In this case, the protocol given below has to be modified accordingly. The amplification product should first be purified to remove reaction components that might interfere with down-stream 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 amplification product can now be resolved by electrophoresis on a suitable agarose-gel following standard protocols and visualised 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 the formation of non-specific products, or non-single-unit length duplex forms of an amplicon, and being visible on the gel (see TwistAmp® Assay Design Manual at twistdx.co.uk for a discussion of primer noise). Such non-specific artefacts will typically be seen in 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 down-stream applications (such as subcloning, sequencing, etc).

TwistAmp® exo

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay may often work, but may not be optimal for TwistAmp® reactions. TwistAmp® primers are ideally 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 should go through a screening process to define suitable TwistAmp® primers for their application (see TwistAmp® Assay Design Manual at twistdx.co.uk). Real-time detection of amplification by fluorescence will require special probes compatible with the TwistAmp® exo biochemistry, so called TwistAmp® exo probes. The design of these probes is described in the TwistAmp Assay Design Manual. 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
- molecular grade water
- Reverse Transcriptase (if amplifying RNA template)
- RNase Inhibitor (if amplifying RNA template)
- thermal incubator / fluorometer
- microcentrifuge for reagent spin-down

Protocols

Storage considerations of kit components

TwistAmp® exo kit components allow long-term storage (up to 6 months guaranteed but much longer stability is likely) under the correct conditions.

TwistAmp® exo reaction pellets are provided as strips of 8 reactions in resealable pouches. Long term storage at –20°C or lower of the sealed product will ensure full activity of the pellets. After breaking the pouch seal, reactions should be stored in the closed pouch along with the provided desiccant.

Primer Free Rehydration buffer and Magnesium Acetate (MgOAc) should be stored at -20˚C to retain full activity.
TwistAmp® exo positive control primer/probe mix and **positive control DNA template** are provided. Upon receipt they should be stored at –20°C to retain full activity and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium acetate start

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

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

The components of the rehydration solution can be combined in a master-mix for the number of samples required. In some circumstances, for example when performing a primer screen, a number of different rehydration solutions have to be made (according to the number of primer pairs being tested). In that case components common to all reactions (e.g. template, Primer-Free 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 complex formation.

Incubation mixing

For low copy template amplification (e.g. less than 100 template copies), mixing of reactions during incubation will improve product formation (as rapid amplification from few copies in a small volume can cause localised substrate depletion).

There are a number of mixing methods that can be applied by the user:

The simplest being mixing by hand (manual mixing) at a single time point by inverting vigorously 8-10 times to mix followed by a brief spin down in a microcentrifuge, or by vortex mixing prior to a brief spin-down as described in the following protocols. The time of manual mixing can be varied between 3 and 7 minutes after initiation of the reaction. A standard time of agitation is 4 minutes (5 for RT-RPA) and longer or more slowly accumulating amplicons may in particular benefit from slightly later agitation.

Some devices enable magnetic mixing during the full incubation timeframe by the addition of a micro ball to the reaction (prior to activation with MgOAc). An example protocol for magnetic mixing of TwistAmp® reactions would be scan duration of 1,200 seconds, and sample rate of 15 seconds. Variation in agitation timing and frequency will also influence product formation. Mixing optimisation is advisable. If using very small volume RPA reactions (less than 5 µL), or a very high copy number of template, mixing may be unnecessary.

Monitoring TwistAmp® exo amplification reactions

Real-time fluorescence detection can be carried out on various available incubating fluorometers (for example Twista®, T8, T16 devices) or alternatively any plate reader or real-time thermal cycler that can excite and detect the chosen fluorophores and hold a steady temperature of 37-42°C, should be adequate for use for exo probe detection. The rehydrated sample should be transferred into an appropriate reaction vessel if the device does not fit 0.2 mL tubes (e.g. a multi-well plate), and incubated/monitored according to the requirements of the device. Furthermore, the agitation regime should be adapted to mimic the protocol. The frequency of fluorescence reading of the reactions can be determined by the user (commonly every 20-30 seconds).

Thermocycler use

Depending on the choice of thermocycler, you may need to change the supplied reaction tube caps from the domed ones provided. Many fluorometers either read from the base up, or through the side. If however, your thermocycler reads fluorescence from above the tube, flat lids may be required. Thermocyclers usually have heated lids (it's normally a set temperature that users can't adjust), which on some models can be switched off. The temperature they are normally set to is too high for RPA, and may affect the efficiency of the enzymes. Heated lids are used to prevent PCR reactions evaporating and condensing on the lid, however, RPA is run quickly, and at a low temperature, so that this isn't an issue. We recommend switching off the heated lids where possible.

Incubation

For specific use with TwistAmp® exo reactions, the device temperature should be set to 39˚C or 40˚C for RNA. This can be later optimised (37-42˚C is usually optimal), along with the mixing regime. Reactions should be incubated for 20 minutes.

Note: The formulation of TwistAmp® Liquid exo contains different ratios of core RPA proteins to TwistAmp exo lyophilised reactions. Assays that have been developed with one kit may perform differently with another.

Detailed protocol

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

Forward primer (10 µM)	2.1 µL
Reverse primer (10 µM)	2.1 µL
TwistAmp® exo probe (10 µM)	0.6 µL
Primer Free Rehydration buffer	29.5 µL
Template, (RT, RNase Inhibitor if amplifying RNA) and water to	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.

Note: TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

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

4. Insert the tubes into the fluorometer block (39°C) and initiate fluorescence measurements for 20 minutes. For low template copy number, remove tubes after 4 minutes, vortex and spin down briefly and return the samples to the reader ensuring that the tubes are returned to their original positions in the block. Alternatively, magnetic mixing using a micro ball may be applied during incubation (see *Incubation mixing*).

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

Note: 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. Take precautions to minimise 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 pipettes or aerosol-resistant pipette tips. Collect used pipette tips and reaction vessels in airtight containers.

Performing positive control reactions

The TwistAmp® exo kit contains positive control primers/probe mix and positive control DNA template, which will allow you to test the activity of the kit components and the detection equipment (using up to 8 reactions). The positive control material is used with the TwistAmp® exo reaction pellets and Primer Free Rehydration buffer. The positive control probe is labelled with a fluorescein (FAM) fluorophore, the excitation optimum is at 488 nM and the emission maximum is at 520 nM.

1. Defrost the positive control primer/probe mix and pipette 8 µL into a fresh 1.5 mL micro centrifuge tube.

2. Add 29.5 µL Primer Free Rehydration buffer to the positive control primer/probe mix from step 1. Briefly vortex and spin down.

3. Prepare 10 µL of a 1/10 dilution of the positive control DNA template (in molecular grade water).

4. Add the 10 µL diluted positive control DNA template to the solution from step 2. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

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

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

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

Note: TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

8. Insert the tubes into the fluorometer block (40°C) and initiate fluorescence measurements for 20 minutes. Remove tubes after 4 minutes, vortex and spin down briefly, and return the samples to the reader ensuring that the tubes are returned to their original positions in the block.

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

Positive control reactions should result in exponential fluorescence increase or a sigmoidal-like fluorescence curve

TwistAmp® nfo

Before you start: The TwistAmp® amplification process requires suitable oligonucleotide primers to work efficiently. Primers designed for a given PCR assay may often work, but may not be optimal for TwistAmp® reactions. TwistAmp® primers are ideally 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 should go through a screening process to define suitable TwistAmp® primers for their application (see TwistAmp® Assay Design Manual at twistdx.co.uk). 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® nfo probes. The design of these probes is described in the TwistAmp Assay Design Manual. 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
- TwistAmp® nfo probe for detection
- molecular grade water
- Reverse Transcriptase (if amplifying RNA template)
- RNase Inhibitor (if amplifying RNA template)
- heating block or other thermal incubator
- microcentrifuge for reagent spin-down

Optional

- lateral flow strips (see TwistDx™ website for more information)
- DNA fragment purification reagents/equipment
- agarose gel electrophoresis setup

Protocols

Storage considerations of kit components

TwistAmp® nfo kit components allow long-term storage (up to 6 months guaranteed but much longer stability is likely) under the correct conditions.

TwistAmp® nfo reaction pellets are provided as strips of 8 reactions in resealable pouches. Long term storage at –20°C or lower of the sealed product will ensure full activity of the pellets. After breaking the pouch seal, reactions should be stored in the closed pouch along with the provided desiccant.

Primer Free Rehydration buffer and **Magnesium Acetate (MgOAc)** should be stored at -20˚C to retain full activity.

TwistAmp® nfo positive control primer/probe mix and **positive control DNA template** are provided. Upon receipt they should be stored at –20°C to retain full activity and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and magnesium acetate start

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

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

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

Incubation mixing

For low copy template amplification (e.g. less than 100 template copies), mixing of reactions during incubation will improve product formation (as rapid amplification from few copies in a small volume can cause localised substrate depletion).

There are a number of mixing methods that can be applied by the user: The simplest being mixing by hand (manual mixing) at a single time point by inverting vigorously 8-10 times to mix followed by a brief spin down in a microcentrifuge, or by vortex mixing prior to a brief spin-down as described in the following protocols. The time of manual mixing can be varied between 3 and 7 minutes after initiation of the reaction. A standard time of agitation is 4 minutes (5 for RT-RPA) and longer or more slowly accumulating amplicons may in particular benefit from slightly later agitation.

Some devices enable magnetic mixing during the full incubation timeframe by the addition of a micro ball to the reaction (prior to activation with MgOAc). An example protocol for magnetic mixing of TwistAmp® reactions would be scan duration of 1,200 seconds, and sample rate of 15 seconds. Variation in agitation timing and frequency will also influence product formation. Mixing optimisation is advisable. If using very small volume RPA reactions (less than 5 µL), or a very high copy number of template, mixing may be unnecessary.

Incubation

For specific use with TwistAmp® nfo reactions, incubation device temperature should be set to 40˚C. This can be later optimised (37-42˚C is usually optimal), along with the mixing regime. Reactions should be incubated for 20 minutes, although this can also be optimised.

Detailed protocol

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

Forward primer (10 µM)	2.1 µL
Reverse primer (10 µM)	2.1 µL
TwistAmp® nfo probe (10 µM)	0.6 µL
Primer Free Rehydration buffer	29.5 µL
Template (RT, RNase Inhibitor if amplifying RNA template) and water to	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 (MgOAc) and mix well. One way to do this simultaneously for many samples is to place the MgOAc into the lid of the reaction tubes (strip of 8), cap the tubes carefully and spin the MgOAc into the rehydrated material to initiate the reactions. Vortex briefly and spin down once again.

Note: TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as the MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

4. Incubate the tubes in a suitable heated block (40°C) for 20 minutes. For low template copy number, remove tubes after 4 minutes, vortex and spin briefly and replace in heated block. Alternatively, magnetic mixing using a micro ball may be applied during incubation (see *Incubation mixing)*.

5. At the end of the incubation proceed to *Monitoring TwistAmp® nfo amplification reactions.*

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 components and the detection equipment. The positive control material is used with the TwistAmp® nfo reaction pellets and Primer Free Rehydration buffer. 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. The TwistAmp® nfo kit positive control reverse primer is labelled with FAM, and the probe with Biotin.

1. Defrost the positive control primer/probe mix and pipette 8 µL into a fresh 1.5 mL microcentrifuge tube.

2. Add 29.5 µL Primer Free Rehydration buffer to the positive control primer/probe mix from step 1. Briefly vortex and spin down.

3. Prepare 10 µL of a 1/10 dilution of the positive control DNA template (in molecular grade water).

4. Add the 10 µL diluted positive control DNA to the solution from step 2. Briefly vortex and spin down. This mixture constitutes your rehydration solution.

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

6. 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.

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

Note: TwistAmp® reactions are activated using MgOAc. The RPA reaction starts as soon as the MgOAc is added, even at room temperature. It is advisable to proceed swiftly to incubation of the sample at the chosen incubation temperature once MgOAc has been added.

8. Insert the tubes into the incubator block (40°C) for 20 minutes. Remove tubes after 4 minutes, vortex, spin down briefly, and return the samples to the incubator block.

9. At the end of the incubation, proceed to Section: *Monitoring TwistAmp® nfo amplification reactions*.

Note: Take precautions to minimise 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 pipettes or aerosol-resistant pipette tips. Collect used pipette tips and reaction vessels in airtight containers. Extra care should be taken when purifying amplicons and analysing them on agarose-gels.

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 (such as the TwistAmp® nfo probe). One approach is the use of Milenia's Genline Hybridetect-1 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® nfo probe system which permits direct interrogation for amplicons on strips without secondary hybridisations or reaction cleanup. Other lateral flow consumables, such as those described on twistdx.co.uk can also be used.

Following a dilution step with 1 x phosphate buffered saline with 0.1% Tween20 (PBST) amplicons can be detected within a few minutes and signal to noise ratios are superb. Also, when using this kit other methods of detection can be employed such as agarose gel-electrophoresis (AGE), which is also described in this section.TwistAmp® nfo can also be used with TwistAmp® exo probe 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® nfo probe system with Milenia Genline Hybridetect-1

1. Perform DNA amplification using TwistAmp® nfo kit, amplification primers and TwistAmp® nfo 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; PBS containing 0.1% Tween will also work).

3. Transfer 10µL of the diluted sample to the sample pad of the Hybridetect-1 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 coloured test line. A separate control line found further up the strip should always develop confirming that the strips are functioning correctly.

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

Assessment of amplification by agarose gel-electrophoresis (AGE)

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

2. The required amount of amplification product can now be resolved by electrophoresis on a suitable agarose-gel following standard protocols and visualised 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 the formation of non-specific products, or non-single-unit length duplex forms of an amplicon, and being visible on the gel (see TwistAmp® Assay Design Manual at twistdx.co.uk for a discussion of primer noise). Such non-specific artefacts will typically be seen in 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.).

References

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

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.

SDS information

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

Ordering information and technical support

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TwistDX
DNA Amplification Kits
Combined Instruction
Manual

Size:
8.268 in x 5.827 in

CMYK

Part Number: INBAEXNF
Revision 1

Date of Revision:
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