TwistDx™

Unwind DNA's possibilities

TwistAmp® Liquid DNA Amplification Kits

Combined Instruction Manual

- TwistAmp® Liquid Basic/Basic RT
- TwistAmp® Liquid exo/exo RT

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

Contents

Materials provided as standard

- 250 µl 20x Core Reaction Mix
- 3 ml 2x Reaction Buffer
- 500 µl 280mM Magnesium Acetate (MgOAc)
- 550 µl 10x E-mix ('Basic' or 'Probe' depending on kit)

Kit specific materials

- 100 µl 50x exonuclease III (TwistAmp® Liquid exo/exo RT kits)
- 100 µl 50x reverse transcriptase (RT) (TwistAmp® Liquid Basic RT/exo RT kits)
- 100 µl of Positive control template (DNA with TwistAmp® Liquid exo/ Basic kits, RNA with TwistAmp® Liquid Basic RT/exo RT kits)
- 45 µl of Positive control oligo mix (oligo mix consists of primers for Basic/Basic RT kits, primers plus a fluorescently labelled exo probe in the exo/exo RT kits)

Additional materials required (not provided in the kit)

- molecular grade water
- dNTPs
- assay-specific oligonucleotides
- RNase Inhibitor (for use with RT kits)

Storage conditions

TwistAmp® Liquid kit components: Store at –20°C upon receipt (full activity is guaranteed for 6 months).

2x Reaction buffer: Upon receipt, aliquot and store at –20°C (full activity is guaranteed for 6 months). Avoid excessive freeze-thaw cycles. This can be achieved by dividing into aliquots on receipt, or after first thaw.

Positive control oligo mix and DNA template: Upon receipt, store at –20°C; refreeze after thawing up to 5 times (full activity is guaranteed for 6 months). **Positive control RNA template:** should be stored at –80°C upon receipt.

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 38). The amplification products generated by RPA can be detected - either at endpoint or in real-time - by a variety of means, including gel electrophoresis, probe-based fluorescence monitoring or simple non-instrumentation 'sandwich assay' approaches such as lateral flow dipsticks. The RPA process utilizes enzymes called Recombinases, of which *E. coli* recA is the archetypal member, which can bind to single stranded nucleic acid backbones - standard oligonucleotides in this case - and stimulate the resulting protein-DNA complex to search for homologous sequences in duplex DNA. Once homology is located, a strand-switching reaction is performed and the oligonucleotide is paired to its complement permitting a polymerase to begin synthesis from the 3' end. The TwistAmp® amplification process uses two opposing oligonucleotide primers to initiate each synthesis event. The design of these primers for a target, in a manner similar to that for PCR, permits the establishment of an exponential amplification process.

TwistAmp® reaction conditions

Like all DNA amplification systems, RPA reaction conditions can be 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 the 20x Core Reaction Mix. RPA reactions are not suitable for developing diagnostics for *E. coli* if sequence homology with strains K12 and BL21 (used for our protein production) is present.

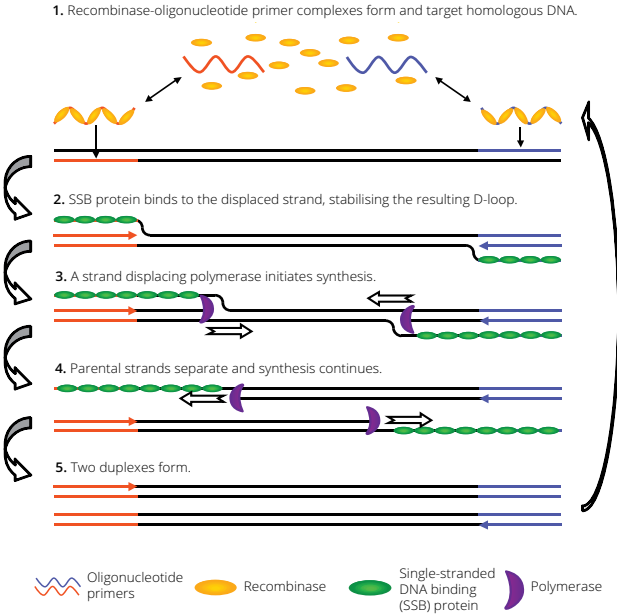

Figure 1 The RPA Cycle

The TwistAmp® Liquid application family

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

TwistAmp® Liquid Basic contains all enzymes and reagents necessary for the amplification of DNA - all that has to be supplied by the user are the primers, the template and dNTPs. 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® Liquid Basic RT is designed for users who want to employ RNA template as the starting material for their amplification in a one-step format. It contains the reagent components of a TwistAmp® Liquid 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.

TwistAmp® Liquid exo is recommended for users who want to combine TwistDx™ amplification technology with the use of TwistDx™ 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® Liquid exo RT is designed for users who want to employ RNA template as the starting material for their real-time amplification in a one-step format. It contains the reagent components of a TwistAmp® Liquid 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.

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 (Tm) 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 Tm 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 Tm 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). TwistAmp® exo oligonucleotide probes are compatible with the TwistAmp® exo kits. These probes are typically designed to have homology to regions within an amplicon between the main amplification primers. The proprietary probe design is 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 using the TwistDx™ TwistAmp® oligonucleotide order forms (available from twistdx.co.uk).

TwistAmp® Liquid Basic/Basic RT

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

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

Protocols

Storage considerations of kit components

TwistAmp® Liquid kit components allow long-term storage (up to 6 months is guaranteed but much longer stability likely) under the correct conditions. After defrosting, all components should be mixed before use. TwistAmp® Liquid 20x Core Reaction Mix and additional enzymes are provided as 50% glycerol stocks and so should be stored at –20°C or lower (if storing at below –20°C, then aliquot into smaller volumes to avoid repeated freeze-thaw cycles). 10x Basic E-mix should be stored at below –20°C. TwistAmp® Liquid 2x Reaction Buffer is provided in a 3 ml aliquot.

This should be stored at –20°C to retain full activity (can be achieved by dividing into aliquots on receipt, or after first thaw).
A TwistAmp® Liquid positive control oligo mix and control DNA (RNA for RT kits) template are provided. Upon receipt they should be stored at –20°C/–80°C (for RNA) and be re-frozen if necessary.

Performing the amplification: Reaction mix preparation and MgOAc start

TwistAmp® Liquid reactions are established by combining the 20x Core Reaction Mix, dNTPs, 10x Basic E-mix, 2x reaction buffer, amplification primers, and template (and water to a total volume of 47.5 µl per sample). The reaction is initiated by the addition of MgOAc solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample. This can be done by pipetting 2.5 µl of MgOAc solution (provided with the kit) into the tube-cap, carefully re-capping the tube, ensuring that the MgOAc solution remains in the cap, then spinning the tube to ensure that the MgOAc solution combines with the sample.
With these liquid reagents, it is very simple to adjust volumes so that users can run smaller or larger volume reactions. If using a different volume, mixing of reagents during reaction preparation should be optimised.

Note: The components of the kit can be combined in a master mix for the number of samples required. **For TwistAmp® Liquid reactions, the order in which these components are combined can be crucial.** The order of addition depends on whether you are trying to amplify multiple templates, or screen multiple primer combinations. This is because primers and probes should be added simultaneously to the 20x Core Reaction Mix to avoid any bias in recombination filament formation.

Incubation mixing

For low copy template amplification (e.g. less than 100 template copies), mixing of reactions during incubation will improve product formation,

and variation in the exact time of sample agitation can sometimes improve product formation. There are a number of mixing methods that can be applied by the user: The simplest being mixing by hand at a single time point by inverting 8-10 times to mix followed by a brief spin down in a micro centrifuge. Some devices enable magnetic mixing during the full incubation time frame by the addition of a micro ball to the reaction (prior to activation with MgOAc). An example protocol for magnetic mixing of TwistAmp® Liquid reactions would be a 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.

Detailed protocols

User protocols are provided for:

- setting up a **primer screen** to determine suitable/optimal oligonucleotides for assay development
- testing different samples **template screen**
- testing kit positive controls

Primer screen set-up (single-plex)[1]

Instructions below are based on use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Add 2.4 µl of each primer at 10µM concentration to 0.2ml PCR tubes (add a single micro ball to each tube if using a magnetic mixing incubation device).

2. Prepare a pre-master mix (per reaction)

2x Reaction Buffer	25 µl
dNTPs[2] + water to	9.2 µl (8.2 µl if using RT[3])[4]
10x Basic E-mix	5 µl

Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

3. To the pre-master mix add 2.5 µl 20x Core Reaction Mix (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. If your template is RNA, add 1 µl 50x RT (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. Master mix is now complete.[5] Pipette mix before use.

4. Add 41.7 µl[3,4] of master mix to primers prepared in tubes (step 1). and pipette mix.

5. Add 2.5 µl of 280mM MgOAc (supplied) and 1 µl template[6] to the lid of your tubes[3,4]. DNA/RNA and MgOAc should be kept separate in the tube lid prior to spin-down, to reduce the formation of tertiary structures. Spin in MgOAc/template and mix well (6x inversions) to start reaction. Spin-down briefly.

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.

6. Incubate at 37-42°C for 20-40 minutes. For low template copy number, remove strip after 4 minutes (5 minutes for RNA), mix by 6x full inversions and spin briefly, and replace in heating device. Alternatively, magnetic mixing using a micro ball may be applied during incubation.

7. After incubation period (step 6), clean amplicons before running on an agarose gel. See 'Monitoring TwistAmp® Liquid Basic/Basic RT amplification reactions.'

Warning: 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.

[1] See TwistAmp® Assay Design Manual at twistdx.co.uk for assay multiplexing information.
[2] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.
[3] If amplifying RNA, users may wish to add RNase Inhibitor together with the template and adjust volumes accordingly.
[4] Volumes should be adjusted if adding more/less template and/or MgOAc.
[5] Please note, the master mix may appear cloudy, this is normal.
[6] When screening oligonucleotides to find the most sensitive combination, we recommend screening with 25-50 copies of template.

Template screen set-up (single-plex) [1]

Instructions below are based on use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Prepare a primer pre-master mix (per reaction)

2x Reaction Buffer	25 µl
dNTPs[2] + water to	9.2 µl[3] (8.2 µl if using RT[3])[4]
10x Basic E-mix	5 µl
Primer A (10µM)	2.4 µl
Primer B (10µM)	2.4 µl

   Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

2. Add 2.5 µl 20x Core Reaction Mix (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. If your template is RNA, add 1 µl 50x RT (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. Master mix is now complete[5]. Pipette mix before use.

3. Add 46.5 µl[3,4] master mix to 0.2ml PCR tubes (add a single micro ball to each tube if using a magnetic mixing incubation device).

4. Add 2.5 µl 280 mM MgOAc (supplied) and 1 µl template to the tube lids[3,4]. DNA/RNA and MgOAc should be kept separate in the tube lid prior to spin-down, to reduce the formation of tertiary structures. Spin in MgOAc/template and mix well (6x inversions) to start reaction, spin-down briefly.

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.

5. Incubate at 37-42°C for 20-40 minutes. For low template copy number, remove strip after 4 minutes (5 minutes for RNA), mix by 6x full inversions and spin briefly, and replace in heating device. Alternatively, magnetic mixing using a micro ball may be applied during incubation. Variation in sample agitation timing can sometimes improve product formation.

6. After incubation period (step 5), clean amplicons before running on an agarose gel. See 'Monitoring TwistAmp® Liquid Basic/Basic RT 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.

[1] See TwistAmp® Assay Design Manual at twistdx.co.uk for assay multiplexing information.
[2] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.
[3] Volumes should be adjusted if adding more/less template and/or MgOAc.
[4] If amplifying RNA, users may wish to add RNase Inhibitor together with the template and adjust volumes accordingly.
[5] Please note, the master mix may appear cloudy, this is normal.

Performing positive control reactions

TwistAmp® Liquid Basic/Basic RT kits may contain positive control primers and template, allowing users to test the activity of the kit components.

Instructions below are based on the use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Defrost the positive control oligo mix.

2. Pipette 7 µl oligo mix into a fresh 1.5 ml micro centrifuge tube.

3. Add 25 µl 2x reaction buffer to the oligo mix from step 2. Briefly vortex and spin-down.

4. Add 5 µl 10x Basic E-mix.

5. Add dNTPs[1] and water up to 5.5 µl (4.5 µl if using RT[2]) . Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

6. Add 2.5 µl 20x Core Reaction Mix to tube lid. Mix by 10x full inversions of tube and spin briefly. Please note, the master mix may appear cloudy, this is normal. If using RNA, add 1 µl 50x RT (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. Transfer to the vessel you will be using for amplification.

7. Add 4 µl 280mM of MgOAc and 1 µl positive control DNA (RNA if using a Basic RT kit) to tube lid. DNA/RNA and MgOAc should be kept separate in tube lids prior to spin-down. Spin in MgOAc/template, mix well (6x inversions) to start reaction. Spin briefly.

9. Incubate at 40°C for 20-40 minutes. For low template copy number, remove strip after 4 minutes (5 minutes for RNA), mix by 6x full inversions and spin briefly, and replace in heating device. Alternatively, magnetic mixing using a micro ball may be applied during incubation. Variation in sample agitation timing can sometimes improve product formation.

10. After incubation period (step 5), clean amplicons before running on an agarose gel. See 'Monitoring TwistAmp® Liquid Basic/Basic RT amplification reactions.'

The positive control reaction will generate an amplification product of 289 base pairs (Basic) or 141 base pairs (Basic RT), which will result in a corresponding band in a gel electrophoresis.

Monitoring TwistAmp® Liquid Basic/Basic RT amplification reactions

The outcome of TwistAmp® Liquid Basic/Basic RT 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, 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, or non-single-unit length duplex forms of an amplicon, being formed during the reaction and being visible on the gel (see the TwistAmp® Assay Design Manual at twistdx.co.uk for a discussion of primer noise). Some such non-specific 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.)

Note: Be aware that the post-amplification processing of the reaction solution carries a very high risk of contamination of equipment, work surfaces etc. with amplification product! See section "Preventing template cross-contamination" for measures to reduce this risk.

Preventing template cross-contamination

Take precautions to minimise the potential for carry-over 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 has to be taken when purifying amplicons and analysing them on agarose gels.

[1] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.
[2] If amplifying RNA, you may wish to add RNase Inhibitor together with your template and adjust volumes accordingly.

TwistAmp® Liquid exo/exo RT

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

- dNTPs
- amplification primers and TwistAmp® exo Probe for detection
- molecular grade water
- RNase Inhibitor (if amplifying RNA template)
- heating block or other thermal incubator with fluorescence detection
- micro centrifuge for reagent spin-down

Protocols

Storage considerations of kit components

TwistAmp® Liquid kit components allow long-term storage (up to 6 months is guaranteed but much longer stability likely) under the correct conditions. After defrosting, all components should be mixed before use.

TwistAmp® Liquid 20x Core Reaction Mix and additional enzymes are provided as 50% glycerol stocks and should be stored at –20°C or lower (if storing at –20°C, then aliquot into smaller volumes to avoid repeated freeze-thaw cycles).

10x Probe E-mix should be stored below –20°C.
TwistAmp® Liquid 2x Reaction Buffer is provided in a 3 ml aliquot. This should be stored at -20˚C to retain full activity (can be achieved by dividing into aliquots on receipt, or after first thaw).
A TwistAmp® Liquid control oligo mix and control DNA/(RNA with RT kits) template are provided. Upon receipt they should be stored at –20°C/–80°C (for RNA) and be re-frozen if necessary.

Performing the amplification: Rehydration of reaction pellets and MgOAc start

TwistAmp® Liquid reactions are established by combining the 20x Core Reaction Mix, 50x Exo, dNTPs, 10x Probe E-mix, 2x reaction buffer, amplification primers, and template (and water to a total volume of 47.5 µl per sample). The reaction is initiated by the addition of MgOAc solution (provided with the kit) in a volume of 2.5 µl, bringing the final reaction volume to 50 µl per sample. This can be done by pipetting 2.5 µl of MgOAc solution (provided with the kit) into the tube-cap, carefully re-capping the tube, ensuring that the MgOAc solution remains in the cap, then spinning the tube to ensure that the MgOAc solution combines with the sample.
With these liquid reagents it is very simple to adjust volumes so that users can run smaller, or larger, volume reactions. If using a different volume, mixing of reagents during reaction preparation should be optimised.

Note: The components of the kit can be combined in a master mix for the number of samples required. **For TwistAmp® Liquid reactions, the order in which these components are combined can be crucial.** The order of addition depends on whether you are trying to amplify multiple templates or screen multiple primer combinations. This is because primers and probes should be added simultaneously to the 20x Core Reaction Mix to avoid any bias in recombination filament formation.

Incubation mixing

For low copy template amplification (e.g. less than 100 template copies), mixing of reactions during incubation will improve product formation. A number of mixing methods can be applied by the user. The simplest being mixing by hand at a single time point by inverting 8-10 times, followed by a brief spin-down in a micro centrifuge. Some devices enable magnetic mixing during the full incubation time frame by the addition of a micro ball to the reaction (prior to activation with MgOAc). An example protocol for magnetic mixing of TwistAmp® Liquid reactions would be a scan duration of 1,200 seconds, and sample rate of 15 seconds. Mixing optimisation is advisable. 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.

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

Detailed protocols

User protocols are provided for:

- setting up a **primer screen** to determine suitable/optimal oligonucleotides for assay development
- testing different samples with a **template screen**
- testing kit positive controls

Primer screen set-up (single-plex) [1]

Instructions below are based on use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Add 2.1 µl of each primer and 0.6 µl of exo probe at 10µM concentration to 0.2ml PCR tubes (add a single micro ball to each tube if using a magnetic mixing incubation device).

2. Prepare a pre-master mix (per reaction)

2x Reaction Buffer	25 µl
dNTPs[2] + water to	8.2 µl (7.2 µl if adding RT[3])[4]
10x Probe E-mix	5 µl

Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

3. To the pre-master mix add 2.5 µl 20x Core Reaction Mix (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly.

4. If your template is RNA, add 1 µl 50x RT (per reaction) to tube lid. Add 1 µl 50x Exo (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. Master mix is now complete[5]. Pipette mix before use.

5. Add 41.7 µl[3,4] master mix to primers and probe prepared in tubes (step 1) and pipette mix.

6. Add 2.5 µl of 280mM MgOAc (supplied) and 1 µl template[6] to the lid of your tubes.[3,4] DNA/RNA and MgOAc should be kept separate in the tube lid prior to spin-down, to reduce the formation of tertiary structures. Spin in MgOAc/template and mix well (6x inversions) to start reaction, spin-down briefly.

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.

7. Place reactions in a fluorometer and start run: 37-42°C, 20 minutes. For low template copy number, after 4 minutes (5 minutes for RNA), take the samples out of the reader **(do not stop the program)**, mix by 6x full inversions, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block. Alternatively, magnetic mixing using a micro ball may be applied during incubation. See 'Monitoring TwistAmp® Liquid exo/exo RT amplification reactions.'

[1] See TwistAmp® Assay Design Manual at twistdx.co.uk for assay multiplexing information.
[2] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.
[3] Volumes should be adjusted if adding more/less template and/or MgOAc.
[4] If amplifying RNA, users may wish to add RNase Inhibitor together with the template and adjust volumes accordingly.
[5] Please note, the master mix may appear cloudy, this is normal.
[6] When screening oligonucleotides to find the most sensitive combination, we recommend screening with 25-50 copies of template.

Template screen set-up (single-plex) [1]

Instructions below are based on use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Prepare an primer and probe pre-master mix (per reaction)

2x Reaction Buffer	25 µl
dNTPs[2] + water to	8.2 µl (7.2 µl if using RT[3])[4]
10x Probe E-mix	5 µl
Primer A (10µM)	2.1 µl
Primer B (10µM)	2.1 µl
Probe (10µM)	0.6 µl

Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

2. Add 2.5 µl 20x Core Reaction Mix (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly.

3. If your template is RNA, add 1 µl 50x RT (per reaction) to tube lid. Add 1 µl 50x Exo (per reaction) to tube lid. Mix by 10x full inversions of tube and spin briefly. Master mix is now complete[5]. Pipette mix before use.

4. Add 46.5 µl[3,4] master mix to 0.2ml PCR tubes (add a single micro ball to each tube if using a magnetic mixing incubation device).

5. Add 2.5 µl of 280mM MgOAc (supplied) and 1 µl template to the tube lids[3,4]. DNA/RNA and MgOAc should be kept separate in the tube lid prior to spin-down to reduce the formation of tertiary structures. Spin in MgOAc/template and mix well (6x inversions) to start reaction. Spin-down briefly.

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.

6. Place reactions in a fluorometer and start run: 37-42°C, 20 minutes. For low template copy number, after 4 minutes (5 minutes for RNA), take the samples out of the reader **(do not stop the program)**, mix by 6x full inversions, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block. Alternatively, magnetic mixing using a micro ball may be applied during incubation. See 'Monitoring TwistAmp® Liquid exo/exo RT amplification reactions.'

[1] See TwistAmp® Assay Design Manual at twistdx.co.uk for assay multiplexing information.
[2] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.
[3] If amplifying RNA, users may wish to add RNase Inhibitor together with the template and adjust volumes accordingly.
[4] Volumes should be adjusted if adding more/less template and/or MgOAc.
[5] Please note, the master mix may appear cloudy, this is normal.

Performing positive control reactions

The TwistAmp® Liquid exo/exo RT kit may contain positive control oligo mix and template which allows users to test the activity of the kit components.
Instructions below are based on use of 50 µl reaction volumes; if using a different volume, quantities should be adjusted appropriately.

1. Defrost the positive control oligo mix.

2. Pipette 8 µl oligo mix into a fresh 1.5 ml micro centrifuge tube.

3. Add 25 µl 2x Reaction Buffer to the oligo mix from step 2. Briefly vortex and spin-down.

4. Add 5 µl 10x Probe E-mix.

5. Add dNTPs[1] and water up to 3.5 µl (2.58 µl if using RT[2]) . Vortex and spin briefly.

Note: The 20x Core Reaction Mix should be warmed to room temperature and pipette mixed slowly to ensure all proteins are in solution and homogenous.

6. To the pre-master mix add 2.5 µl 20x Core Reaction Mix to the tube lid. Mix by 10x full inversions of tube and spin briefly. Please note: The master mix may appear cloudy; this is normal. If using RNA, add 1 µl 50x RT (per reaction) to tube lid. Add 1 µl 50x Exo to tube lid and mix by 10x full inversions of tube and spin briefly. Transfer to the vessel you will be using for amplification.

7. Add 4 µl 280mM of MgOAc and 1 µl positive control DNA (RNA if using a exo RT kit) to tube lid. DNA/RNA and MgOAc should be kept separate in tube lids prior to spin-down. Spin in MgOAc/template, mix well (6x inversions) to start reaction. Spin briefly.

8. Place reactions in a fluorometer and start run: 40°C, 20 minutes. For low template copy number, after 4 minutes (5 minutes for RNA), take the samples out of the reader **(do not stop the program)**, mix by 6x full inversions, spin down and return the samples to the reader ensuring that the tubes are returned to their original positions in the incubator block. Alternatively, magnetic mixing using a micro ball may be applied during incubation. See 'Monitoring TwistAmp® Liquid exo/exo RT amplification reactions.'

Both the exo and exo RT kit positive control uses a probe labelled with a fluorescein (FAM) fluorophore, the excitation optimum is at 488nM and the emission maximum is at 520nM.

[1] Final concentration of 1.8mM (total) dNTPs is suggested. Optimisation of this parameter is recommended.

[2] If amplifying RNA, you may wish to add RNase Inhibitor together with your template and adjust volumes accordingly.

Monitoring TwistAmp® exo/exo RT 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 0.2ml 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. Frequency of fluorescence reading of the reactions can be determined by the user (commonly every 20-30 seconds).

Reaction Agitation

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). Unless you are using very small volume RPA reactions (~5 µl), or a very high copy number of template, mixing is critical. The time of shaking can be varied between 3 minutes and 6 minutes after initiation of the reaction (standard time is 4 minutes – longer or more slowly accumulating amplicons may in particular benefit from slightly later agitation).
For those devices which facilitate mixing by automated magnetic movement of micro balls added to tubes (such as T8, T16), continual mixing intervals during incubation can be deployed. Timing of regularity and intensity of mixing should be optimised for each individual assay developed.

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 devices 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, users can't adjust), which on some models can be switched off. The heat they are normally set to is too high for RPA, and may affect the efficiency of the enzymes. The reason they have heated lids is to prevent PCR reactions evaporating and condensing on the lid, however RPA is run so quickly, at such a low temperature, that this isn't an issue anyway. We recommend switching off the heated lids where possible.

Incubation

For specific use with RPA TwistAmp exo/exo RT reactions, device temperature should be set to 39˚C (which can be later optimised, along with the mixing regime). Reactions should be incubated/ monitored for 20 minutes, data saved, and the reaction tubes should be discarded.

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.

References

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

End notes

SDS information

Safety Data Sheet (SDS) information for TwistDx™ products is provided on TwistDx™ website: twistdx.co.uk/en/support/safety-data-sheets. SDS documents are not included with product shipments.

Ordering information and technical support

TwistDx Limited, Abbott House, Vanwall Business Park, Vanwall Road, Maidenhead SL6 4XE, United Kingdom

orders@twistdx.co.uk
techsupport@twistdx.co.uk

twistdx.co.uk