Lyo-ready One-step RT-qPCR System

for one-step RT-qPCR using the Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR and Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase

Catalog Numbers EP215B2SMP1, EP225SMP2

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WARNING! Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves. Safety Data Sheets (SDSs) are available from **thermofisher.com/support**.

Product description

The Invitrogen™ Lyo-ready enzyme system is is specifically developed and designed for one-step RT-qPCR, with both cDNA synthesis and quantitative PCR are performed in a single well. Specially formulated Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR and Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase are both highly processive, next generation enzymes that deliver the same product yield within a shorter reaction time. Multiplexing capability allows testing of more analytes per reaction, while inhibitor-tolerant enzymes reduce the time spent on tedious purification steps.

The enzyme pair and buffer system is intended for the TaqMan™ Multiplex One-Step RT-qPCR application. The RT-qPCR protocol for the one-step RT-qPCR system uses an optimized reaction buffer for quicker and more cost-effective assay development. The specially formulated reaction buffer is also compatible with lyophilization. The system detects and quantifies viruses, as well as human and other RNA or DNA targets using real-time detection instruments together with gene specific primer and probe sets.

For additional information, go to **thermofisher.com/lyo-ready** or contact **MDxenzymes@thermofisher.com**.

Contents and storage

Store all contents at –20°C. Components are designed to withstand at least 20 freeze/thaw cycles.

Table 1 Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase (Cat. No. EP225SMP2)

Component	Concentration	Amount	Storage conditions
Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase	20 U/µL	5000 U/µL	Store at –20°C
Lyo-ready Platinum™ II PCR Buffer [1]	5X	6 × 10 mL	
$MgCl_2$ [2]	50 mM	2 × 4 mL	

[1] The 5X Lyo-ready Platinum™ II PCR Buffer may contain a precipitate. Warm the buffer to room temperature and mix thoroughly to ensure that any precipitate dissolves completely before use.

[2] 50 mM $MgCl_2$ is included for further optimization of the Mg^{2+} concentration.

Table 2 Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR (Cat. No. EP215B2SMP1)

Component	Concentration	Amount	Storage conditions
Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR	200 U/µL	10,000 U/µL	Store at –20°C

System components

Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase

Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase is an engineered *Taq* DNA polymerase that shows increased resistance to reaction inhibitors originating from sample materials or nucleic acid purification steps. The Lyo-ready enzyme formulation offers the option to lyophilize, while retaining all favorable properties of the standard enzyme preparation (with glycerol). The polymerase activity is blocked at ambient temperatures and restored after the initial denaturation step at 95°C. This automatic "hot start" provides increased sensitivity, specificity, and yield, while allowing reaction assembly at room temperature. Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase extends 1 kb in 15 seconds. The extension step can be prolonged without negative effect on specificity. Like the standard *Taq* DNA polymerase, it has both 5′ to 3′ polymerase and 5′ to 3′ exonuclease activities but lacks 3′ to 5′ exonuclease activity.

Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR

Lyo-ready SuperScript™ Reverse Transcriptase, 1-Step RT-qPCR is an engineered version of the Moloney murine leukemia virus (M-MuLV) reverse transcriptase designed especially for the one-step RT-qPCR application, with increased thermal stability and maintained RNase H activity. The lyo-ready enzyme formulation provides the option to lyophilize. The enzyme can be used to synthesize first-strand cDNA at temperatures up to 55°C, providing increased sensitivity, specificity and higher yields of cDNA.

Lyo-ready Platinum™ II PCR Buffer

5X Lyo-ready Platinum™ II PCR Buffer is optimized to achieve the best performance for the lyo-ready reverse transcriptase and Lyo-ready polymerase working as a pair in one-step RT-qPCR. This specially formulated buffer consists of glycerol-free reagents and excipients which provide optimal functionality post-lyophilization and help to achieve high reaction sensitivity, resistance to reaction inhibitors, and compatibility with multiplex application when multiple templates can be detected in the same reaction.

Required materials not provided

Reagents

- dNTP mix (10 mM each) (Cat. No. R0191)
- Primers and probes
- Water, Nuclease-free (Cat. No. R0581)
- (*Optional*) ROX solution, 50 µM (Cat. No. R1371)
- (*Optional*) Lyo-ready RiboLock RNase Inhibitor40 U/µL (Cat. No. EO201SMP)

Plastics and other consumables

- qPCR plates and seals (compatible with your qPCR instrument)
- Disposable gloves
- Pipette tips with filters

Equipment

- Laboratory mixer
- Pipettes
- Microcentrifuge
- Centrifuge for plates
- qPCR instrument

General PCR guidelines

One-step RT-qPCR assays require special laboratory practices to avoid false positive or even false negative reactions. False positive results could be obtained due to amplicon carryover, highly concentrated positive controls or non-specific amplification. No less important is false negative assays when target is degraded or even missed. Therefore, specific precautions are necessary to prevent contamination of equipment, consumable and reagents with RNases.

- Locations for sample preparation, PCR set up and post PCR analysis should be necessarily separated.
- Laboratory equipment, consumables, reagents and lab coats should be dedicated and must not be shared between the areas.
- Wear gloves and clean lab coat. Change gloves whenever you suspect they are contaminated.
- Never bring amplified PCR products into PCR set up area.
- To avoid splashing always centrifuge the reagents and samples before opening. When not in use keep all tubes capped.
- Always use filter pipette tips.
- All used plastics should be RNases free.
- Reaction set up and dilution of highly concentrated solutions should be performed in separate PCR boxes.
- Clean lab surfaces and equipment with RNase*Zap*™ RNase Decontamination Solution (Cat. No. AM9780) and DNA*Zap*™ PCR DNA Degradation Solutions (Cat. No. AM9890M).
- To avoid pipetting errors when template is not added to the reaction mix always use internal positive controls (see troubleshooting guide).

Guidelines for preparing templates

- Use 1 pg to 1 µg of purified total RNA as starting material.
- If using purified mRNA as starting material, the amount of template may be reduced to as litlle as 0.5 pg.
- RNA should be free of RNase contamination and aseptic conditions should be maintained. RNA may be treated with DNase I, RNase-free (Cat. No. EN0521) to remove any contaminating genomic DNA.
- An RNase inhibitor such as Lyo-ready RiboLock (Cat. No. EO201SMP may be added to the reaction mix to safeguard against degradation of target RNA due to ribonuclease contamination.
- Prepared RNA samples should be stored at –70°C. For RNA samples dilution use nuclease-free water.
- The reverse transcription step does not affect thermal cycling performance with DNA, therefore DNA templates could be used along with RNA in multiplex detection.

Guidelines for designing primers

- Primers sequences should not overlap the probe sequence. The optimal primer length is around 20 bases.
- Keep the GC content in the 30–50% range.
- Primers should be free of strong secondary structures and self-complementarity.
- The optimal melting temperature of the primers is around 60°C. The Tm of both primers should not differ by more than 5°C. The annealing temperature should be set no more than 5°C below the lower primer Tm.
- Avoid repeats of identical nucleotides, especially fewer than four consecutive G bases.
- Ensure the last 5 nucleotides at the 3' end contain no more than two G and/or C bases.

About primer concentrations

A final concentration of 200 nM per primer is effective for most reactions. In some cases doubling the amount of reverse primer may improve the performance. Optimal results may require a primer titration between 100 nM and 800 nM, especially setting the multiplex reaction.

Guidelines for designing probes

- The probe should be designed in close proximity to the forward or reverse primer, but should not overlap with a primer-binding site.
- Probes can be designed to either strand of the target.
- Preferably, probes should have a Tm 6–8°C higher than the primers. If the melting temperature of the probe is too low, the primers may amplify a product without the probe resulting in reduced fluorescence signal.
- The annealing temperature should be set no more than 5°C below the lower primer Tm. Optimization may still be necessary.
- As with primer sequences, aim for a GC content of 30-50% and avoid a G at the 5’ end to prevent quenching of the 5’ fluorophore.
- For multiplexing applications, each target must be identified by a separate reporter dye. Dyes emission spectra of them should not overlap.
- Check the compatibility of the dye with the instrument.
- Check self- and cross-complementarity of the primers/probes.
- Check the specificity of designed primers to the target sequences.
- Before the conducting of multiplex test, check the performance of the primers and probes in the singleplex reaction (Cq values should be similar).
- In order to have similar fluorescence level, label rare targets with bright dyes and abundant targets with dim fluorescence dyes.

Recommended ROX dye concentration

Instrument	Final ROX dye concentration
Applied Biosystems™ 7300, 7900HT, StepOne™, StepOnePlus™, ABI PRISM™ 7000, and 7700 systems	300–500 nM
Applied Biosystems™ 7500, ViiA™ 7, and QuantStudio™ real-time PCR systems Stratagene®Mx3000P™, Mx3005P™, and Mx4000™ systems	30–50 nM
Bio-Rad™, iCycler iQ™, iQ™5, MyiQ™, and Opticon™CFX96™ and CFX384™systems Roche™ LightCycler™ 480 and LightCycler™ 2.0 systems QIAGEN™ Corbett™, and Rotor-Gene™ 3000 and 6000 systems Eppendorf™ Mastercycler™ system Cepheid™ SmartCycler™ system	Not required

Perform one-step RT-qPCR

The protocol provides volumes for a single standard 20-µL reaction consisting of 15 µL of master mix, and 5 µL of template RNA.

For larger reaction sizes, component volumes can be scaled as desired (e.g., scaled up to a 25-µL or 50-µL reaction volume).

To prepare a master mix, scale the component volumes according to the number of reactions needed. Include an extra 10% volume to account for volume loss during pipetting.

The master mix consists of all components in the table listed in "Prepare reaction" except the template RNA.

IMPORTANT! Each sample should be tested in triplicate. It is also important to include a no template control (NTC) in every experiment. Replace the template in the NTC with an equivalent amount of water.

1 Prepare reaction

IMPORTANT! Keep the labeled probes and prepared Master Mix protected from light. Excessive exposure to light may affect the assay.

1.1. Thaw all reaction components completely, then vortex briefly, and centrifuge to collect the drops at the bottom of the tubes.

Keep the tubes in cooling blocks at all the time when not in use.

1.2. Combine the components in a sterile, nuclease-free tube on ice in the order listed in the following table.

Component	Volume	Final concentration
Water, nuclease-free	up to 20 µL	—
5X Lyo-ready Platinum™ II PCR Buffer	4 µL	1X
10 mM dNTP mix	1.2 µL	0.6 mM each (0.4–0.6 mM)
50 mM $MgCl_2$	4 µL	10 mM each (6–10 mM)
10 µM forward primer [1]	0.4 µL	0.3 µM each (0.2–1 µM)
10 µM reverse primer [1]	0.4 µL	0.3 µM each (0.2–1 µM)
10 µM probe [1]	0.3 µL	0.2 µM each (0.15–0.5 µM)
(*Optional*) 50 µM ROX reference dye [2]	*x* µL	500 nM (High ROX equipment) 50 nM (Low ROX equipment)
(*Optional*) Lyo-ready RiboLock Inhibitor (40 U/µL)	0.5 µL	1 U/µL (20 U/reaction)
Lyo-ready Platinum™ II *Taq* Hot-Start DNA Polymerase (20 U/µL)	0.12 µL	0.12 µM each (0.10–0.15 U/µL)
Lyo-ready SuperScript™ Reverse Transcriptase (200 U/µL)	0.1 µL	1 U/µL (0.25–1.5 U/µL) [3]
Template RNA [4]	5 µL	1 µg to 0.5 pg/reaction

[1] For guidelines for primer/probe design and primer concentration, see page 3.

[2] See "Recommended ROX dye concentration" on page 3.

[3] Use 0.25–0.5 U/µL for singleplex one-step RT-qPCR. Higher concentrations are recommended for multiplex one-step RT-qPCR.

[4] The volume of template RNA can be increased in conjunction with a corresponding decrease of water in the master mix.

1.3. Dispense 15 µL of prepared master mix to each well of qPCR plate and add the template RNA directly to the well.

1.4. Seal the qPCR plate with optical adhesive film. Invert the plate several time and centrifuge to collect the drops.

2 Perform one-step RT-qPCR

Program your instrument with the following parameters.

Step	Temperature [1]	Time [1]	Cycles
Reverse transcription	50°C	5–15 min	1
RT termination/initial denaturation/polymerase activation	95°C	2 min	1
Denaturation	95°C	5 sec	40-45
Annealing/extension	60°C	15–60 sec [2]	

[1] For specific targets, optimization may be required. For most templates, efficient cDNA synthesis can be accomplished in a 10 minutes incubation at 50°C. For problematic templates, or to increase the specificity of cDNA priming, increase the cDNA synthesis temperature up to 55°C and incubation time to 15 minutes.

[2] Annealing/extension time is dependent on the number of targets and the qPCR instrument used.

Guidelines for data analysis

It should be noted that analysis methods vary between applications. First unselect empty wells and view amplification plot. Set baseline and auto threshold values. If setting threshold manually the values should be above the background and within the exponential growth phase of the amplification curve. Remove outliers from the analysis.

The parameters of standard curve (if present) should fall into the range:

Parameter	Range
Slope	3.1–3.5
Amplification efficiency	90–110%
Linearity over dynamic range (R2 value)	≥0.99

In some cases fluorescence intensity is important and should be assessed.

Sequencing or product size estimation by agarose gel electrophoresis is recommended to confirmation the specificity of targets.

Troubleshooting

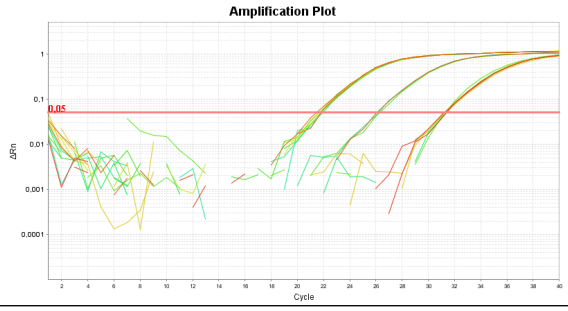
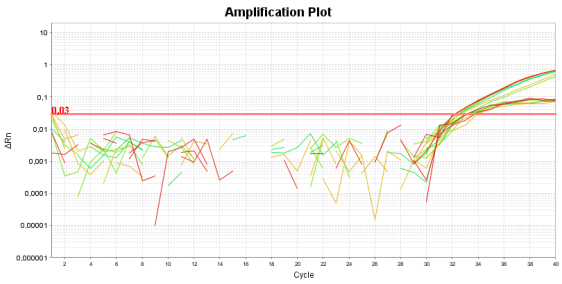
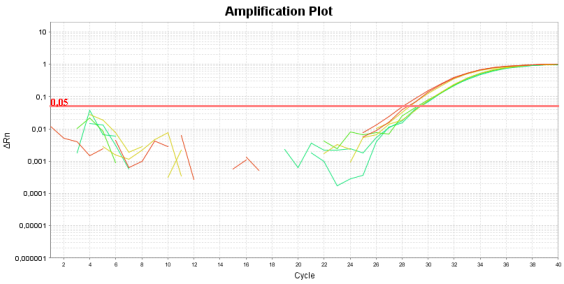
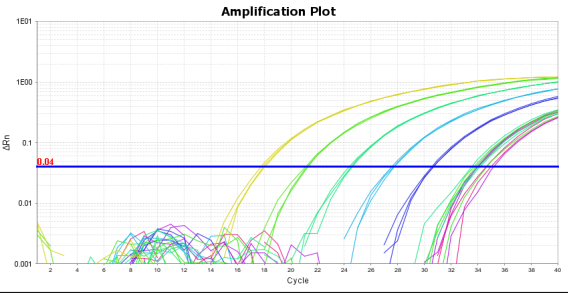
Control reactions

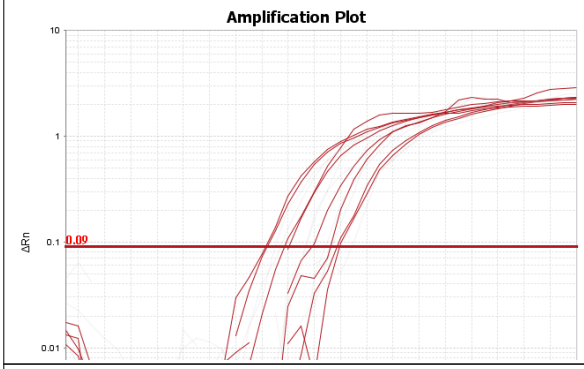
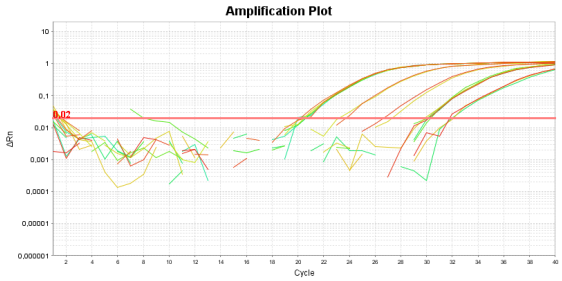
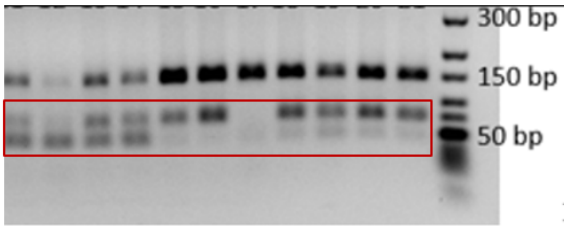
Use of the controls is extremely important for troubleshooting, and it is impossible to analyze problems without examining the result of control reactions.

Control	Description
No template control (NTC)	PCR reaction without DNA or RNA template. Considered as a general control for extraneous nucleic acid contamination.
Positive reaction controls	Commercially available positive controls, or previously diagnosed as positive samples for comparison of Ct values between runs of the same type of experiments, also for verification of reaction conditions and reagents.
Internal positive reaction controls	Exogenous positive controls refer to the use of external DNA or RNA carrying a target of interest. This control assesses whether the tested samples contain any components that inhibit reverse transcription and/or qPCR reaction.
	Endogenous positive controls use different target from the target of interest in the experimental sample. These types of controls are used for correction of quantity and quality differences between samples.
No RT (RT-) control	No reverse transcriptase control involves 1-step RT-qPCR experiment in the absence of reverse transcriptase. This control assesses the amount of DNA contamination present in an RNA template.
Negative control	A sample without target sequence. Positive amplification shows primers dimerization or reaction contamination.

General troubleshooting

One-step RT-qPCR detection is a quite complex system and therefore many things could cause the system improper performance or complete fail. Operator errors and problems with reaction components are the most common cause. General troubleshooting information is provided in the table below.

Observation	Possible cause	Recommended action
Dilution points of standard curve are irregularly spaced (efficiency falls outside the range 90–110 %) 	Pipetting error.	Calibrate pipettes.
	Non-optimal PCR conditions.	Optimize the reaction conditions.
	Incorrect composition of reaction mix.	Check composition of reaction mix. Use fresh stock solutions.
Low Ct values or no amplification of target 	Incorrect RT temperature/cycling protocol, primer/probe sequences, wrong or missing reaction components, presence of inhibitors, degraded or too low concentration of RNA.	Set optimal temperature for reverse transcriptase, re-set cycling protocol, verify the sequences of primers/probes, replace reaction components, repeat RNA purification, verify amplification product on agarose gel.
Variation in technical replicates after target amplification 	Pipetting errors.	Calibrate pipettes.
	Non-optimal qPCR conditions.	Repeat the run using fresh stock solutions. Make sure to set up three technical replicates.
	Limiting reagents. Target concentration is too low.	Increase target concentration in reaction.
No template control (NTC) Cq is near/overlapping the lowest point of the standard curve 	Contamination with the same target sequence.	Use new stocks of reagents, clean surfaces and general lab equipment. See “General PCR guidelines” on page 2).

Observation	Possible cause	Recommended action
Abnormal amplification plots 	qPCR conditions or reagents are not optimal.	Check background fluorescence. Recalculate volumes and final concentrations of reaction components.
	Bad reference dye.	Check reference dye.
Background signal crosses the threshold during early cycles 	Threshold set too low.	Check the raw data. Set threshold value manually.
	Degraded probes.	Keep probes and prepared reaction mix protected from light.
Non-specific products or primer duplex seen in agarose gel 	Low specificity of PCR.	Design the primers/probe that span exon/exon boundaries on the RNA.
	Non-optimal PCR chemistry or conditions.	Review reaction components and concentrations.
	Genomic DNA contamination.	Ensure samples are free of gDNA contamination before PCR.

Multiplex troubleshooting

Observation	Possible cause	Recommended action
Poor or no amplification of some or all loci	Pipetting error/reagent missing.	Repeat experiment. Check volumes and final concentrations of all reagents. Check PCR protocol.
	Polymerase is not activated.	Optimize the reaction conditions.
	Wrong annealing time or temperature.	For most of the primers annealing temperature and time should be carried out as specified in the protocol. But if using short primers avoid high annealing temperature. Too low annealing temperature leads to miss-priming.
	Issue with primers/probes.	Poor primer design. Check the primer sequences. Combine the desired primers in equimolar concentrations. Further increase the concentration of poor amplified locus slightly decreasing concentrations of good working primers. Probes concentrations could be the same or twice decreased compared to primers.
	Issue with dNTPs.	Do not apply freeze-thaw steps on dNTP's stocks, use smaller aliquots instead.
	$MgCl_2$ concentration not optimal.	Optimal final concentration of $MgCl_2$ for multiplex is 6–10 mM. Higher concentration can inhibit polymerase activity. Also use recommended concentration of dNTP as there is close relationship between $MgCl_2$ and dNTP ratio.
Extra bands on agarose gel	Non-specific amplification.	Keep qPCR plate on cooling stand when dispensing master mix and adding template.
	Poor primer design.	Check self and cross dimerization of primers. Use BLAST to analyze the primer sequence to ensure that primers are specific to the target of interest.
	Incorrect annealing temperature.	In case of presence of non-specific products, increase annealing temperature by 2ºC and slightly decrease annealing time.
	Issue with template.	In some case extra amplification bands can occur due to extremely high template concentration. Dilute the template, repeat the assay and check the result.
	Incorrect polymerase concentration.	Too high concentration of polymerase can cause formation of non-specific amplification products. Try not to exceed recommended 0.15 U/µL concentration.

Limited product warranty

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For descriptions of symbols on product labels or product documents, go to thermofisher.com/symbols-definition.

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Revision	Date	Description
A.0	11 April 2022	New user guide for the Lyo-ready One-step RT-qPCR System