Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific DyNAmo cDNA Synthesis Kit

#F-470L

Lot **Expiry Date**

Store at -20°C

www.thermoscientific.com/onebio

Rev.2

Contents **page**

1. COMPONENTS OF THE KIT

DyNAmo cDNA Synthesis Kit	#F-470L
M-MuLV RNase H$^+$ reverse transcriptase (includes RNase inhibitor)	1 × 200 µL (sufficient for 100 RT reactions of 20 µL)
2X RT buffer (includes dNTP mix and 10 mM $MgCl_2$*)	1 × 1 mL
Random hexamers (300 ng/µL)	1 × 100 µL
Oligo(dT)$_{15}$ primer (100 ng/µL)	1 × 100 µL

* Provides 5 mM $MgCl_2$ in 1X reaction concentration.

2. STORAGE

Upon arrival, store all kit components at -20 °C. All the kit components can be refrozen and stored at -20 °C without affecting the performance of the kit.

3. DESCRIPTION

Quantitative PCR (qPCR) is a useful technique for the investigation of gene expression, viral load, pathogen detection, and numerous other applications. When analyzing gene expression or viral load, the RNA of interest first needs to be reverse transcribed into cDNA. Thermo Scientific™ DyNAmo™ cDNA Synthesis Kit is intended for cDNA synthesis for two-step quantitative reverse transcription-PCR (qRT-PCR) applications, where amplicons are usually around 100 bp in length. This kit can be used in conjunction with DyNAmo qPCR Kits or with any other qPCR kit suitable for the application.

The DyNAmo cDNA Synthesis Kit includes all the necessary reagents for synthesizing cDNA to be used in qPCR. Either total RNA, messenger RNA, viral RNA or *in vitro* transcribed RNA can be used as a template for reverse transcription. The kit includes both random primers and oligo(dT) primers. The user can choose either of these or alternatively use gene-specific primers.

The reverse transcriptase in the DyNAmo cDNA Synthesis Kit is M-MuLV RNase H$^+$, which provides higher sensitivity to qPCR than RNase H$^-$ reverse transcriptases. The Rnase H activity in the RT enzyme facilitates annealing of PCR primers to the cDNA by degrading the RNA in the RNA-cDNA hybrid before the PCR step.

4. NOTES ABOUT REACTION COMPONENTS

Table 1. General recommendations.

Categories	Comments
Reaction volume	20 µL
Amplicon size	50-250 bp
Template amount	Max 1 µg of RNA
Priming options	Random hexamers, oligo(dT) or gene-specific primers

4.1. RNA template

Total RNA, mRNA, viral RNA or *in vitro* transcribed RNA can be used as a template. Successful cDNA synthesis is dependent on the integrity and purity of the template RNA. RNA preparation should be free of any DNA or RNase contamination. The purity of RNA can be determined by measuring the ratio of A260/A280. The optimal ratio is 1.8 - 2.0.
RNA isolation should be performed under RNase-free conditions. Furthermore, any contamination with RNases from other potential sources like glassware, plasticware and reagent solutions has to be avoided. This can be done by wearing gloves and using sterile tubes and pipet tips. Water used for the reactions should also be RNase-free, but not DEPC treated as traces of DEPC can inhibit PCR.
DNA contamination can be removed from the RNA sample by treating the sample with RNase-free DNase I. This should be done especially if primers for the qPCR step cannot be designed in exon-exon boundaries or in separate exons.
The maximum amount of template RNA for DyNAmo cDNA Synthesis Kit is 1 µg. This amount includes all RNA present in the sample, for example carrier RNA and other possible RNAs in addition to the target RNA.

4.2. M-MuLV RNase H$^+$ reverse transcriptase

M-MuLV RNase H$^+$ RT provides good sensitivity in qRT-PCR applications, where amplicons are usually around 100 bp in length. Also, with M-MuLV RNase H$^+$ there is no need to perform separate RNase H treatment after cDNA synthesis, as the RNase H activity in the enzyme degrades RNA in the RNA-cDNA hybrid.

4.3. RNase inhibitor

The RNase inhibitor included in the mix with the reverse transcriptase inhibits contaminating RNases present in the RNA sample. It does not affect the RNase H activity in the M-MuLV reverse transcriptase.

4.4. RT primers

Specific primers, random hexamers or oligo(dT) primers can be used for the RT step. Using specific primers can decrease background, whereas random hexamers and oligo(dT) primers are useful if several different amplicons need to be analyzed from a small amount of starting material.

When choosing primers for cDNA synthesis, a good starting point is to use random hexamers. They transcribe all RNA (mRNA, rRNA, tRNA and *in vitro* transcribed RNA) producing cDNA that covers the whole transcript. The recommended amount of random hexamers per 20 µL RT reaction is 300 ng (can be optimized between 200–400 ng if necessary).

Oligo(dT) primers can be used to transcribe poly(A)$^+$ RNAs. These include eukaryotic mRNAs and retroviruses with poly(A)$^+$ tails. Several different mRNAs are transcribed allowing subsequent qPCR detection of different targets from the same cDNA synthesis reaction. The recommended amount of oligo(dT) primers per 20 µL RT reaction is 100 ng (can be increased up to 1 µg if necessary). If the amplicon is located at the 5' end of the transcript, using random hexamers is recommended.

Gene-specific primers are used to transcribe only the particular RNA of interest, in contrast to oligo(dT)/random primers that transcribe all mRNAs/RNAs in the sample. The recommended amount of specific primer per 20 µL RT reaction is 10 pmol (can be optimized between 5–20 pmol if necessary).

4.5. Minus RT control

A minus RT control should be included in all qRT-PCR experiments to test for DNA contamination (such as genomic DNA or PCR product from a previous run). Such a control reaction contains all the reaction components except for the reverse transcriptase. RT reaction should not occur in this control, so if PCR amplification is seen, it is most likely derived from contaminating DNA.

4.6. RT efficiency

The cDNA synthesis step is very critical in qRT-PCR. The efficiency of reverse transcription varies and can be low in some cases. The expression level of the target RNA molecule and the efficiency of the RT reaction must therefore be considered when determining the appropriate amount of starting template for subsequent PCR steps. The volume of cDNA template should not exceed 10% of the qPCR reaction volume, as elevated volumes of template may reduce the efficiency of the PCR amplification. A dilution series of the template can be done to optimize the amount of the starting material used.

5. REACTION SETUP AND CYCLING PROTOCOLS

- Perform reaction setup in an area separate from nucleic acid preparation and PCR product analysis.
- All plasticware should be RNase-free.
- Use gloves to prevent RNase contamination.
- Pipet with sterile filter tips.
- Minimize pipetting errors by using calibrated pipettes and by preparing premixes to avoid pipetting very small volumes.
- Pipet all components on ice.
- Reaction tubes should be centrifuged before starting the incubations to force the solution to the bottom of the tube and to remove any bubbles.

5.1. Reaction setup

1. Program the cycler as outlined in Table 3.
2. Thaw template RNA, 2X RT buffer and primers. Mix the individual solutions to ensure homogeneity.
3. Prepare a cDNA synthesis premix by mixing 2X RT buffer, primers, RNase-free H_2O and reverse transcriptase (see table 2). Mix the premix thoroughly to ensure homogeneity. Dispense appropriate volumes into reaction tubes.
4. Add template RNA to the reaction tubes.
5. Place the tubes in the thermal cycler and start the program.

Table 2. Reaction setup.

Components	Stock	20 µL rxn	Comments
RT buffer	2X	10 µL	RT buffer includes dNTPs and $MgCl_2$.
Random hexamer primer set	300 ng/µL	1 µL	Alternatively oligo(dT) primer or a specific primer can be used. See Section 4.4.
Template RNA		X µL	Max 1 µg
M-MuLV RNase H^+ reverse transcriptase		2 µL	Includes RNase inhibitor.
RNase-free H_2O		X µL	Add water to fill up to the final reaction volume.
Total volume		20 µL	

5.2 . Incubation protocol

Table 3. Incubation protocol for reverse transcription.

Step	Purpose	Temp	Time	Comments
1	Primer extension	25 °C	10 min	This step is not necessary if gene specific primers are used.
2	cDNA synthesis	37 °C	30 min	Most targets can be synthesized at 37 °C. The temperature can be varied between 37–48 °C if necessary. Incubation time can be extended up to 60 min if needed for long or rare transcripts.
3	Reaction termination	85 °C	5 min	Inactivation of M-MuLV prevents it from inhibiting qPCR reaction.
4	Cooling of the sample	4 °C	Hold	Optional.

5.3. cDNA synthesis steps

Predenaturation (optional)

A separate RNA denaturation step is generally not required, but it can be performed before cDNA synthesis if the template RNA has a high degree of secondary structure. The denaturation step, 5 min at 65 °C, should be performed before adding 2X RT buffer and reverse transcriptase to the reaction mix.

Primer extension

The incubation of 10 min at 25 °C extends random primers or oligo(dT) primers before the actual cDNA synthesis. Without the incubation at 25 °C the primers may dissociate from the template when the temperature is raised to the cDNA synthesis temperature. This preliminary extension step is not necessary for gene-specific primers.

cDNA synthesis

Incubation at 37 °C will work for most templates, but it can be optimized between 37 °C and 48 °C if necessary. Increasing the temperature can be helpful if the template has strong secondary structures. Higher temperature can also improve specificity if gene-specific primers are used. Incubation time of 30 min is sufficient in most cases. If the target is located near the 5’ end of a long transcript and oligo(dT) priming is used, or the target is rare, cDNA synthesis time can be extended up to 60 min.

Reaction termination

The termination step at 85°C inactivates the M-MuLV reverse transcriptase, thus preventing it from inhibiting the qPCR reaction.

6. cDNA TEMPLATE

The cDNA produced with DyNAmo cDNA Synthesis Kit can be quantified with DyNAmo qPCR Kits or with any other qPCR kit suitable for the application.
If the cDNA synthesis reaction will not be used for qPCR immediately, it can be stored at -20 °C. Also, if only part of the reaction volume is needed for qPCR, store the remainder at -20 °C.
The volume of cDNA template should not exceed 10 % of the qPCR reaction volume, as elevated volumes of template may reduce the efficiency of the PCR amplification. Excess salt and random primers in the cDNA synthesis reaction can inhibit the DNA polymerase. A dilution series of the template can be prepared to optimize the volume of the starting material used.

7. THINGS TO CONSIDER IN PLANNING qPCR

Consult your qPCR instruction manual for more detailed instructions.

7.1. Primers and probe(s) for qPCR step

Careful primer and probe design is particularly important to minimize nonspecific primer annealing and primer-dimer formation. Standard precautions must be taken to avoid primer-dimer or hairpin loop formation. Many software tools for designing PCR primers and probes simultaneously are available. Most primer design software tools will yield well-designed primers for use in qPCR.
PCR primers in qRT-PCR experiments should be designed to anneal to sequences in two exons on opposite sides of an intron. This design enables differentiation between amplification of cDNA and contaminating genomic DNA. A long intron inhibits the amplification of the genomic target. Alternatively, primers or probe(s) can be designed to anneal to the exon-exon boundary of the mRNA. With such an assay design the priming of genomic target is highly inefficient.

7.2 Absolute quantification

Target nucleic acids can be quantified with qRT-PCR using either absolute quantification or relative quantification.
In absolute quantitation, the absolute amount of target nucleic acid (expressed as a copy number or concentration) is determined by comparison of Cq values to external standards containing a known amount of nucleic acid. (Cq = quantification cycle, the factional cycle at which the target is quantified in a given sample. The level of Cq is set manually or calculated automatically). The external standards should contain the same or nearly the same nucleic acid sequence as the template of interest. It is especially important that the primer binding sites are identical to ensure equivalent amplification efficiencies of both standard and target molecules.
A linear regression analysis of the standard plot is used to calculate the amount of target nucleic acid in samples. The slope of the equation is related to the efficiency of the PCR reaction. The RT-PCR efficiency should be the same for standards and samples for quantification to be accurate. The PCR efficiency of the samples can be determined by doing a dilution series of these samples.

For a graph where log(RNA copy#) is on the y axis and Cq on the x axis:
PCR efficiency = $((10^{-1 \times \text{slope}}) - 1) \times 100\%$
A slope of –0.301 equals 100% efficiency.

For a graph where Cq is on the y axis and log(RNA copy#) on the x axis:
PCR efficiency = $((10^{-1/slope})-1) \times 100\%$
A slope of –3.322 equals 100% efficiency.

Using RNA molecules as standards for RNA quantification is recommended. The use of RNA standards takes the variable efficiency of the reverse transcription into account. RNA standards can be generated for example by cloning the cDNA of interest to a vector containing RNA polymerase promoter, e.g. T7 or Sp6. From the vector the insert can be *in vitro* transcribed to obtain the final RNA standard with identical sequence to the target amplicon. The vector must then be degraded with RNase-free DNase, and the concentration of the RNA standard determined spectrophotometrically. Alternatively, a defined RNA preparation, e.g. from a cell line or a virus, with known concentration can be used as an RNA standard.

7.3 Relative quantification

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (healthy tissue or untreated cells, for example). The most common application of this method is the analysis of gene expression, such as comparisons of gene expression levels in different samples, for example. The target molecule quantity is usually normalized with a reference gene. Examples of commonly used reference genes are beta-actin, GAPDH and 18S rRNA. A gene used as a reference should have a constant expression level that is independent of the variation in the state of the sample tissue. A problem is that, even with housekeeping genes, the expression usually varies to some extent. That is why several reference genes are usually required, and their expression needs to be checked for each experiment. The amplification efficiency of a reference gene should be the same as the efficiency of the target gene. If this is not the case, the results have to be corrected for the efficiency.
If the amplification efficiency of a reference gene is the same as that of the target gene, the comparative ΔΔCq method can be used for relative quantitation. Both the sample and the calibrator data are first normalized against variation in sample quality and quantity.Normalized (ΔCq) values are calculated by the following equations:

ΔCq(sample) = Cq(target) – Cq(reference)
ΔCq(calibrator) = Cq(target) – Cq(reference)

The ΔΔCq is then determined using the following formula:
ΔΔCq = ΔCq(sample) – ΔCq(calibrator)
The expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta Cq}$

Since RNA quantification involves a number of variables, and each experiment is inherently different, careful experimental design is very important. Useful information and guidelines for experimental design, normalization, RNA standards, etc. can be found in the following review articles:

Bustin S.A. (2000) *Journal of Molecular Endocrinology* 25, 169–193
Bustin S.A. (2002) *Journal of Molecular Endocrinology* 29, 23-39.

8. TROUBLESHOOTING

Possible problems related to the cDNA synthesis reaction are detected after the qPCR reaction is finished. Use this troubleshooting guide to identify and solve problems arising from the cDNA synthesis reaction. Consult your qPCR manual to troubleshoot the qPCR components of the reaction.

	Possibles causes	**Comments and suggestions**
No increase in fluorescence signal	Missing components (e.g. primers or template) or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
	RNA degraded or poor quality	• Check the concentration, integrity, purity and storage conditions of the RNA template. Make new RNA dilutions from the stock if necessary.
	Incorrect temperature in cDNA synthesis reaction	• The recommended temperature in cDNA synthesis step is 37 °C. It can be optimized between 37–48 °C if necessary.
	Missing essential step in the cycler protocols	• Check the cycler protocols for cDNA synthesis and qPCR steps.
	Reverse transcriptase not functional.	• Make sure that the M-MuLV RNase H⁺ enzyme is not heat-inactivated. It should not be used in temperatures higher than 48 °C.
	RT-PCR product too long	• The length of the amplicon should be between 50 and 250 bp. The optimal length is 100–150 bp.
Late increase in fluorescence signal	Missing components or pipetting error	• Check the assembly of the reactions. • Check the concentrations and storage conditions of the reagents.
	RNA template amount too low	• Check the calculation of template stock concentration. • Increase the amount of RNA template (up to 1 µg).
	RNA degraded or poor quality	• Check the concentration, integrity, purity and storage conditions of the RNA template. Make new RNA dilutions from the stock if necessary.
	RNA template contains strong secondary structures	• Perform a predenaturation step on the template before cDNA synthesis. See Section 5.3.
	Incorrect temperature in cDNA synthesis reaction	• The recommended temperature in cDNA synthesis step is 37 °C. It can be optimized between 37–48 °C if necessary.
	Reverse transcriptase not functional	• Make sure that the M-MuLV RNase H⁺ enzyme is not heat-inactivated. It should not be used in temperatures higher than 48 °C.
	RT-PCR product too long	• The length of the amplicon should be between 50 and 250 bp.The optimal length is 100–150 bp.

	Possibles causes	Comments and suggestions
Increase in fluorescence signal in negative (no RT) control	Contaminating genomic DNA in RNA preparation	• Treat the starting RNA template with DNase I before cDNA synthesis. • Redesign qPCR primers to prevent amplification of genomic DNA. See Section 7.1.
Non-linear correlation between Cq and log of template amount in the standard curve	RNA template amount too low	• Increase the amount of RNA template (up to 1 µg).
	RNA template amount too high	• Do not exceed 1 µg of starting RNA template.
	RNA template dilution inaccurate	• Remake dilution series and make sure the samples are well mixed.
	RT-PCR product too long	• The length of the amplicon should be between 50 and 250 bp. • The optimal length is 100–150 bp.
	cDNA template volume too high	• Reduce template amount. The volume of the cDNA template should not exceed 10 % of the qPCR reaction volume. • Increase qPCR reaction volume.
	cDNA template amount too low	• Increase template amount (up to 10% of the qPCR reaction volume).

Appendix I: General molecular biology data

Table 4. Spectrophotometric conversions for nucleic acid templates.

1 A_{260} unit*	Concentration (µg/mL)
Double-stranded DNA	50
Single-stranded DNA	33
Single-stranded RNA	40

* Absorbance at 260 nm = 1 (1 cm detection path).

Table 5. Molar conversions for nucleic acid templates.

Nucleic acid	Size	pmol/µg	Copies/µg*
1 kb DNA	1 000 bp	1.52	9.1×10^{11}
pUC19DNA	2 686 bp	0.57	3.4×10^{11}
Lambda DNA	48 502 bp	0.03	1.8×10^{10}
Escherichia coli DNA	4.7×10^{6} bp	3.2×10^{-4}	1.9×10^{8}
Human DNA	3.2×10^{9} bp	4.7×10^{-7}	2.8×10^{5}

* For single-copy genes.

PRODUCT USE LIMITATION

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

Please refer to www.thermoscientific.com/onebio for Material Safety Data Sheet of the product.