DyNAmo™ cDNA Synthesis Kit
for qRT-PCR

Instruction manual

F- 470S Sufficient for 20 cDNA synthesis reactions (20 µl each)

F- 470L Sufficient for 100 cDNA synthesis reactions (20 µl each)

Description 2
Kit Components 2
Shipping and Storage 3
General Considerations 3
- RNA template 3
- M-MuLV RNase H^+ reverse transcriptase 4
- RNase inhibitor 4
- RT primers 4
- Minus RT control 4
- RT efficiency 5

Reaction Setup 5
- Protocol 5

cDNA Synthesis Steps 6
- Predenaturation (optional) 6
- Primer extension 7
- cDNA synthesis 7
- Reaction termination 7

cDNA Template 7
Things to Consider in Planning qPCR 8
- Primers and probe(s) for qPCR step 8
- Absolute quantitation 8
- Relative quantitation 10

Troubleshooting 11
Appendix I: General Molecular Biology Data 13
Appendix II: Related Products 14
Appendix III: Warranty and Trademark Information 14
Ordering Information 15

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. The 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 is a part of DyNAmo 2-step qRT-PCR kits (see Appendix II) and it 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 cDNA synthesis 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 template before the PCR step.

Kit Components

	F-470S	**F-470L**
M-MuLV RNase H^+ reverse transcriptase (includes RNase inhibitor)	1 x 40 µl (sufficient for 20 RT reactions of 20 µl volume)	1 x 200 µl (sufficient for 100 RT reactions of 20 µl volume)
2x RT buffer (includes dNTP mix and 10 mM $MgCl_2$*)	1 x 200 µl	1 x 1 ml
Random hexamers (300 ng/µl)	1 x 20 µl	1 x 100 µl
Oligo$(dT)_{15}$ primer (100 ng/µl)	1 x 20 µl	1 x 100 µl

* Provides 5 mM $MgCl_2$ in 1x reaction concentration

Shipping and Storage

The DyNAmo cDNA Synthesis Kit is shipped in gel ice. 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.

The kit is stable for one year from the date of packaging when stored and handled properly.

General Considerations

Table 1. General recommendations for cDNA synthesis.

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

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 A_{260}/A_{280}. 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.

A suitable template RNA amount for DyNAmo cDNA Synthesis Kit is up to 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.

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.

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.

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).

Minus RT control

A minus RT control should be included in all qRT-PCR experiments to test for DNA contamination (for example genomic DNA or PCR product from a previous run). It 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.

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 made to optimize the amount of the starting material used.

Reaction Setup

- Perform reaction setup in an area separate from nucleic acid preparation or 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 possible bubbles.

Protocol

1. Program the cycler as outlined in table 3.
2. Thaw template RNA, 2x RT buffer and primers. Mix the individual solutions to assure homogeneity.
3. Prepare a cDNA synthesis premix by mixing 2x RT buffer, primers, RNase-free H_2O and reverse transcriptase (see table 2). Mix thoroughly to assure 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 for cDNA synthesis.

Components	Stock	Volume / 20 µl reaction	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 page 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	

Table 3. Cycler protocol for reverse transcription.

Step	Purpose	Temperature	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

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. Raising 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.

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 made to optimize the amount of the starting material used.

Things to Consider in Planning qPCR

Consult your qPCR manual for more detailed instructions.

Primers and probe(s) for qPCR step

Careful primer and probe design is particularly important to minimize non-specific primer annealing and primer-dimer formation. Standard precautions must be used during primer design to avoid primer-dimer or hairpin loop formation. 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. If the intron is long, it prevents 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 primers amplification of genomic DNA will be highly inefficient.

Absolute quantitation

Target nucleic acids can be quantified with qRT-PCR using either absolute quantitation or relative quantitation.

In absolute quantitation, the absolute amount of target nucleic acid (expressed as a copy number or concentration) is determined by comparison of C(t) values of samples to a standard curve created with known amounts of nucleic acid. (C(t) = cycle threshold, the cycle number at which the fluorescence signal reaches the threshold level. The threshold level is set manually or calculated automatically). The external standards should contain the same or nearly the same 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 quantitation to be accurate. 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 C(t) on the x-axis:

PCR efficiency = $((10^{-1 \times slope}) - 1) \times 100\%$
The slope of –0.301 equals 100% efficiency

For a graph where C(t) is on the y-axis and log(RNA copy#) on the x-axis:

PCR efficiency = $((10^{\frac{-1}{slope}}) - 1) \times 100\%$
The 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.

Relative quantitation

Relative quantification is used to determine the ratio between the quantity of a target molecule in a sample and in the calibrator (calibrator being e.g. healthy tissue or untreated cells). The most common application of this method is the analysis of gene expression, e.g. comparisons of gene expression levels in different samples. Target molecule quantity is usually normalized with a reference gene. Examples of commonly used reference genes are β-actin, GAPDH and 18S rRNA. A gene used as a reference should have a constant expression level 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 the efficiency of the target gene, comparative ΔΔC(t) 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 values, ΔC(t)s, are first calculated from following equations:

ΔC(t)sample = C(t)target - C(t)reference
ΔC(t)calibrator = C(t)target - C(t)reference

The ΔΔC(t) is then determined using the following formula:
ΔΔC(t) = ΔC(t)sample –ΔC(t)calibrator

Expression of the target gene normalized to the reference gene and relative to the calibrator = $2^{-\Delta\Delta C(t)}$.

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

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.

No increase in fluorescence signal	
Possible causes	**Comments and suggestions**
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	
Possible causes	**Comments and suggestions**
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 page 6, predenaturation.
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.

Increase in fluorescence signal in negative (no RT) control	
Possible causes	**Comments and suggestions**
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 page 8, primers and probe(s) for qPCR step.

Non-linear correlation between C(t) and log of template amount in the standard curve	
Possible causes	**Comments and suggestions**
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 the 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 1. 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 2. 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}
pU19 DNA	2 686 bp	0.57	3.4×10^{11}
Lambda DNA	48 502 bp	0.03	1.8×10^{10}
Escherichia coli	4.7×10^{6} bp	3.2×10^{-4}	1.9×10^{8}
Human	3.2×10^{9} bp	4.7×10^{-7}	2.8×10^{5}

** for single-copy genes

Appendix II: Related Products

DyNAmo™ cDNA Synthesis Kit is also included in the following DyNAmo 2-step qRT-PCR Kits:
F-430S/L DyNAmo™ SYBR® Green 2-Step qRT-PCR Kit
F-440S/L DyNAmo™ Capillary SYBR® Green 2-Step qRT-PCR Kit
F-460S/L DyNAmo™ Probe 2-Step qRT-PCR Kit

This kit can be used in conjunction with the DyNAmo qPCR Kits:
F- 400S/L DyNAmo™ SYBR® Green qPCR Kit
F- 400RS/L DyNAmo™ SYBR® Green qPCR Kit *with ROX™ passive reference dye*
F-410S/L DyNAmo™ HS SYBR® Green qPCR Kit
F-420S/L DyNAmo™ Capillary SYBR® Green qPCR Kit
F-450S/L DyNAmo™ Probe qPCR Kit

Reverse transcriptase enzymes available separately:
F-570S/L AMV Reverse Transcriptase
F-572S/L M-MuLV RNase H^- (not available in US)

Appendix III: Warranty and Trademark Information

Finnzymes Oy warrants that its products will meet the specifications stated on the technical data section of the data sheets, and Finnzymes Oy agrees to replace the products free of charge if the products do not conform to the specifications. Notice for replacement must be given within 60 days of receipt. In consideration of the above commitments by Finnzymes Oy, the buyer agrees to and accepts the following conditions:

1. That this warranty is in lieu of all other warranties, express or implied;
2. That ALL WARRANTIES OF MERCHANT ABILITY OR OF FITNESS FOR A PARTICULAR PURPOSE ARE HEREBY EXCLUDED AND WAIVED;
3. That the buyer's sole remedy shall be to obtain replacement of the product free of charge from Finnzymes Oy; and
4. That this remedy is in lieu of all other remedies or claims for damages, consequential or otherwise, which the buyer may have against Finnzymes Oy.

Exclusive terms of sale:
Finnzymes Oy does not agree to and is not bound by any other terms or conditions, unless those terms and conditions have been expressly agreed to in writing by a duly authorised officer of Finnzymes Oy. Prices are subject to change without notice.

Recommended Guidelines for Safe Use of the Products:
Finnzymes Oy recommends that the buyer and other persons using the products follow the N.I.H. guidelines published in the Federal Register, Volume 41, No. 131, July 7, 1976, and any amendments thereto. Finnzymes Oy disclaims any and all responsibility for any injury or damage which may be caused by the failure of the buyer or any other person to follow said guidelines.

Research Use Only:
Since these products are intended for research purposes by qualified persons, the Environmental Protection Agency does not require us to supply Premanufacturing Notice.

Notice to User:
The information presented here is accurate and reliable to the best of our knowledge and belief, but is not guaranteed to be so. Nothing herein is to be construed as recommending any practice or any product in violation of any patent or in violation of any law or regulation. It is the user's responsibility to determine for himself or herself the suitability of any material and/or procedure for a specific purpose and to adopt such safety precautions as may be necessary.

The quality system of Finnzymes Oy is certified according to standard SFS-EN ISO9001:2000.

DyNAmo is a trademark of Finnzymes Oy.
SYBR is a registered trademark of Molecular Probes.

Ordering Information

F-470S Sufficient for 20 cDNA synthesis reactions (20 µl each)

F-470L Sufficient for 100 cDNA synthesis reactions (20 µl each)

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Finnzymes Oy
Keilaranta 16 A
02150 ESPOO
FINLAND
Tel: +358-9-584 121
Fax: +358-9-5841 2200
fz@finnzymes.fi
www.finnzymes.com