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Reverse Transcriptase Core kit Technical Data Sheet

Reference: RT-RTCK-03

Products and procedures describe in this protocol are intended for research purposes only**.**

Storage conditions

For long term storage the Reverse Transcriptase Core kit should be stored at -65 °C to -75 °C in a constant temperature freezer. When stored under these conditions the reagents are stable for 1 year.

For short term storage the Reverse Transcriptase Core kit can be stored at -15 °C to -25 °C for 6 months.

Kit contents

The Reverse Transcriptase Core kit contains enough reagents for up to 300 - 10 µl reactions.

Reagent	Volume	Description
10x reaction buffer (black cap)	1.4 ml	One bottle of RT reaction buffer KCl and Tris-HCl
EuroScript reverse transcriptase (white cap)	75 µl	One tube of Moloney Murine leukemia virus reverse transcriptase, 6250 U at 50 U/µl
RNase Inhibitor (purple cap)	120 µl	One tube of RNase inhibitor 4000U at 20 U/µl
2.5 mM dNTP Mix (green cap)	1.25 ml	One tube of dATP, dCTP, dGTP and dTTP in autoclaved, deionized water titrated with NaOH to pH 7.0
25 mM $MgCl_2$ (orenge cap)	1.5 ml	One tube of 25 mM MgCl2
Oligo $d(T)_{15}VN$ (yellow cap)	150 µl	One tube containing 50 µM oligodeoxynucleotides of sequence $d(T)_{15}VN$ in 10 nM Tris-HCL, pH 8.3
Random nonamers (pink cap)	150 µl	One tube containing 50 µM short oligonucleotides of random sequence $(d(N)_9)$ in 10 mM Tris-HCL pH 8.3
RNase free water (plain cap)	1.75 ml	One tube of DEPC water

Procedure for Two step RT qPCR reaction

1- Thaw all required reagents necessary for the RT step completely and put them on ice, except for the EuroScript, which should be kept in the freezer until required for use. Mix all reagents well by inversion and spin them down prior to pipeting.

2- Prepare the RT Reaction Mix
(sufficient for 200 ng total RNA per 10 µl RT step)

Component	Volume (µl)	Final concentration
10x reaction buffer	1	1x
25 mM $MgCl_2$	2	5 mM (or as required)
2.5 mM dNTP	2	500 µM each dNTP
Random nonamer*	0.5	2.5 µM
RNAse Inhibitor	0.2	0.4 U/µl
EuroScript RT	0.25	1.25 U/µll
RNase free water	3.75	-
Template	1	10 pg - 200 ng Total RNA
Total Mix	**10 µl**	

*Note: random nonamers, oligo $d(T)_{15}VN$ or sequence specific primers can be used for primers. For nonamers and oligo $d(T)_{15}VN$ bthe final concentration should be 200 nM.

2.1- To correct for dispensing losses prepare an excess of reaction mix (for example 100 reactions reaction mix for 96 reactions). Add all components together, except for the template. Mix thoroughly by inversion. Spin down.

2.2- Add the reaction mix to the reaction vial. reaction set up should be done on ice

2.3- Add the template to individual reactions, gently mix by inversion. Spin down. A negative control containing no RNA template should always be included. Optionally a no RT-control should be set up in tubes / wells, which do not contain the EuroScript RT / RNase Inhibitor.

2.4- Program the Real-Time thermocycler using the following recommended parameters:

Initial step*	10 min 25 °C
Reverse Transcriptase step	30 min 48 °C
Inactivation of the RT enzyme	5 min 95 °C

* Only if random nonamers or oligo $d(T)_{15}VN$ are usedare used.

The RT Core kit can be combined with any Eurogentec qPCR kits

3- Thaw all required reagents necessary for the PCR step completely and put them on ice. Mix all reagents well by inversion and spin them down prior to pipeting.

4- Prepare the PCR Reaction Mix

4.1- In case of a probe assay:

Component	Volume (µl)		Final concentration
Reaction buffer	5*	25**	1x
50 mM $MgCl_2$	5	-	5 mM (or as required)
5 mM dNTP	2	-	200 µM each dNTP
Forward primer	5	5	-
Reverse primer	5	5	-
Probe	5	5	-
HotGoldStar	0.25	-	0.025 U / µl
RNase free water	7.75	-	-
Total Mix	**40 µl**		

* If using a qPCR Core kit
** If using a qPCR MasterMix

Add 40 µl of PCR reaction Mix to 10 µl or a dilution of the 1rst strand reaction mix.

4.2- In case of a SYBR® green I assay

Component	Volume (µl)		Final concentration
Reaction buffer	5*	25**	1x
50 mM $MgCl_2$	5	-	5 mM (or as required)
5 mM dNTP	2	-	200 µM each dNTP
Forward primer	5	5	-
Reverse primer	5	5	-
diluted SYBR green I	1.5	-	-
HotGoldStar	0.25	-	0.025 U / µl
RNase free water	7.75	-	-
Total Mix	**40 µl**		

* If using a qPCR Core kit for SYBR® green I

** If using a qPCR MasterMix Plus for SYBR® green I

Add 40 µl of PCR reaction Mix to 10 µl or a dilution of the 1rst strand reaction mix.

4.3- To correct for dispensing losses prepare an excess of reaction mix (for example 100 reactions reaction mix for 96 reactions). Add all components together, except for the template. Mix thoroughly by inversion. Spin down.

4.4- Add 10 µl or a dilution of the first strand reaction mix, 5 µl of the template control (plus 5 µl of water or buffer) for your positive control and 10 µl of water or buffer for your negative control in to your PCR tubes / 96-well plate / 384-well plate.

4.5- Add 40 µl of the reaction mix to the reaction vial, close the vial and mix gently on a stirrer or spin down. Ensure that no bubbles are present in the reaction vial. Reaction set up can be done at room temperature.

4.6- Program the Real-Time thermocycler using the following recommended parameters:

UNG step	2 min. 50 °C
HotGoldStar activation/UNG inactivation	10 min. 95 °C
40 Cycles	15 sec. 95 °C 1 min. 60 °C
Hold	50 °C forever

For any further informations concerning the qPCR step, in terms of primer and probe design, primer and probe concentrations, $MgCl_2$ concentration or concerning optimization of the reaction, please refer to the instruction of the Eurogentec qPCR Core kits or qPCR MasterMixes.

Further information available through Eurogentec web site, **www.eurogentec.com**.

- Troubleshooting Guide for qPCR and RTqPCR (under the "Technical Resources / Troubleshooting Guide" section).

- Primers and probe design (please refer to our Troubleshooting Guide).

- "Your One-stop-shop Real-Time qPCR supplier" handbook (under the "Technical Resources / Documentation" section).

- MSDSs, (under the "Technical Resources / MSDS" section)

- Certificates of Analysis (please contact us).

For any further information required please contact our Customer Help Desk:

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qPCR MasterMix No ROX
Technical Data Sheet

Reference: RT-QP2X-03NR

Products and procedures describe in this protocol are intended for research purposes only**.**

Storage conditions

For long term storage the qPCR MasterMix No ROX should be stored at -15 °C to -25 °C in a constant temperature freezer. When stored under these conditions the reagents are stable for 2 years.

For short term storage the qPCR MasterMix No ROX can be stored at 4 °C to 6 °C for 1 month.

Kit contents

The qPCR MasterMix Plus No ROX contains enough reagents for up to 300 - 50 µl reactions using the hotstart enzyme, HotGoldStar.

Reagent	Volume	Description
2x reaction buffer (green cap)	1.5 ml	Five tubes (1.5 ml) of reaction, buffer, dNTPs (including dUTP), HotGoldStar DNA polymerase, $MgCl_2$ (5mM final concentration), Uracil-N-glycosylase, stabilizers
50 mM $MgCl_2$ (plain cap)	1.5 ml	One tube of 50 mM $MgCl_2$

Procedure

1- Thaw all required reagents completely and put them on ice. Mix all reagents well by inversion and spin them down prior to pipeting.

2- Prepare the reaction mix

Component	Volume (µl)	Final concentration
2x reaction buffer	25	1x
Forward primer	5	(starting with 300 nM)*
Reverse primer	5	(starting with 300 nM)*
Probe	5	(starting with 100 nM)*
Template	5	-
Water	5	(volume is 50 µl minus all other components)
Total Mix	**50 µl**	

*Note that the primer and probe concentrations are recommended as starting concentrations. These concentrations will be correct for many assays, but additional optimization of the primer concentrations and primer-probe ratios may be required to obtain the best results with your primer-probe set.

3- To correct for dispensing losses prepare an excess of reaction mix (for example 100 reactions reaction mix for 96 reactions). Add all components together, except for the template. Mix thoroughly by inversion. Spin down.

4- Pipette 5 µl of the template DNA for your samples, 5 µl of the control DNA for your positive control and 5 µl of water or buffer for your negative control in to your PCR tubes / 96-well plate / 384-well plate.

5- Add 45 µl of the reaction mix to the reaction vial, close the vial and mix gently on a stirrer or spin down. Ensure that no bubbles are present in the reaction vial. Reaction set up can be done at room temperature.

6- Program the Real-Time thermocycler using the following recommended parameters:

UNG step	2 min. 50 °C
HotGoldStar activation / UNG inactivation	10 min. 95 °C
40 Cycles	15 sec. 95 °C 1 min. 60 °C
Hold	50 °C forever

Technical information

Primer and probe design guidelines

Probes:

- avoid runs of identical nucleotides, especially of 4 or more Gs-
- when using Primer Express® software, the Tm should be 65 °C to 67 °C
- avoid 5' end G (quenches the fluorophore)
- for genotyping, position of the polymorphism should be in the center of the probe, and the probe length should be adjusted such that both probes have the same Tm

Primers:

- GC content should be between 30 % and 80 % (ideally 40-60 %)
- avoid runs of identical nucleotides, especially of 3 or more Gs or Cs at the 3' end
- using the Primer Express® sofware the Tm should be 58 °C to 60 °C
- the primer should be placed as close as possible to the probe

Custom assay design

Commonly used concentrations are 300 nM for primers and 100 nM for probes. Optimal results may require titration of primers and probes or adjustment of the primer / probe ratio. The purpose of such a process is to determine the minimum amount of primers and probe required to obtain the most sensitive results with your assay.

Primer titration matrix

Titrate according to the Table 1, perform qPCR and select the concentration, which gives the lowest Ct value.
By doing this type of titration it is also possible to compensate for differences up to 2 °C in melt temperature of the primers.

Table 1: Primer titration matrix

	Forward		
Reverse	**50 nM**	**300 nM**	**900 nM**
50 nM	50 / 50	300 / 50	900 / 50
300 nM	50 / 300	300 / 300	900 / 300
900 nM	50 / 900	300 / 900	900 / 900

Primer-probe ratio matrix

Select optimal primer concentration as described in Table 1 and test with all probe concentrations described in Table 2. Select the concentration, which gives the lowest Ct value

Table 2: Primer-probe ratio matrix

	Probe		
	50 nM	**100 nM**	**250 nM**
Opt. primers	50 / opt	100 / opt	250 / opt

$MgCl_2$ adjustment matrix

Standard $MgCl_2$ concentration is 5 mM but optimal $MgCl_2$ concentration can vary between assay, if necessary use the 50 mM $MgCl_2$ tube. Always prefer optimizing the primer and probe concentrations before the $MgCl_2$ concentration.
Adjust the amount of water if $MgCl_2$ is added to the reaction.

Final $MgCl_2$ concentration (mM)	$MgCl_2$ to add (µl/50 µl)	2x reaction buffer (µl)
5	0	25
5.5	0.5	25
6	1	25
6.5	1.5	25
7	2	25
7.5	2.5	25
8	3	25

3-step protocol instead of 2-step protocol

Increasing extension time or performing a 3-step protocol can increase the ÄRn and / or decrease the Ct of an assay, particularly when the PCR product is longer than 100 bp.
The protocol will be as follows:

UNG step		2 min. 50 °C
HotGoldStar activation / UNG inactivation		10 min. 95 °C
40 Cycles	denaturation	15 sec. 95 °C
	annealing	20 sec. 60 °C
	extension	40 sec. 72 °C
Increase extension time with 10-second steps, if required.		

Further information available through Eurogentec web site, **www.eurogentec.com**.

- Manual for qPCR MasterMix No ROX, reference RT-0000-05 (under "Technical Resources / Manual" section).
- Troubleshooting Guide for qPCR and RTqPCR (under the "Technical Resources / Troubleshooting Guide" section).
- Primers and probe design (please refer to our Troubleshooting Guide).
- "Your One-stop-shop Real-Time qPCR supplier" handbook (under the "Technical Resources / Documentation" section).
- MSDSs, (under the "Technical Resources / MSDS" section)
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