

Maxim Biotech, Inc.

(Due to many different combinations of a housekeeping gene with a target specific gene, the following is an example of Dual PCR Certificate of Analysis)

Certificate of Analysis

Human Angiopoietin-2 and GAPDH Genes Dual-PCR Kit

Cat. No: DP-10002-G

This Dual PCR Amplification Kit contains the following reagents and materials:

<u>Catalog No.</u>	<u>Kit Component</u>	<u>Amount</u>	<u>Storage</u>
DP-B001	Dual PCR Buffer (containing Buffer, Enhancer, and dNTPs)	1250 µl x 2	-20°C
DP-00003	10X GAPDH PCR Primers (1 µM)	500 µl	-20°C
DP-10002	10X ANG2 PCR Primers (1 µM)	500 µl	-20°C
DP-00003P	10X GAPDH Positive Control (10 ⁶ copies/µl)	100 µl	-20°C
DP-10002P	10X ANG2 Positive Control (10 ⁶ copies/µl)	100 µl	-20°C
MRB-0014	M.W. Marker (100 bp Ladder)	100 µl	-20°C
MRB-0011P	ddH ₂ O (DNase free)	2.0 ml	-20°C

PRIMERS:

DP-10002	PCR Primers for Human Angiopoietin-2	Genebank, XM_034835
	Tm=67°C, 68°C	PCR Product Size: 200 bp
DP-00003	PCR Primers for GAPDH	Genebank, M33197
	Tm=59°C, 59°C	PCR Product Size: 496 bp

**The first time user, please refer to
“Dual Quantitative RT-PCR Kit General Instruction Manual” for details.**

Brief PCR Protocol (for experienced user)

1. Reaction Mixture Preparation:

Set up Dual Quantitative RT-PCR reactions with test samples and Optimized Dual Quantitative RT-PCR buffers provided in the Dual Quantitative RT-PCR kit according to the table below:

- a. Spin all tubes briefly before doing any experiments
- b. Mix 250 µl of premixed target specific primers and 250 µl of premixed internal HKG primers to one tube of optimized dual PCR buffer, total two tubes. Label tubes as **Master Mixture**.
- c. This “**Master Mixture**” should be aliquot and stored at -20°C for one year (long term storage) and “**Ready To Use Master Mixture**” at 4°C for one day.

Due to the minimal volume of Taq enzyme used, “**Ready-to-use Master Mixture**”, including Taq DNA polymerase and all other components except positive control or testing DNA/cDNA, should be prepared to cover daily usage. The “**Ready-to-use Master Mixture**” is stable at 4°C for one day.

<u>Volume (Per assay)</u>	<u>Reagent (Add in order)</u>
35.0 µl	Prepared Master Mixture
0.3 µl	Taq DNA Polymerase(5units/µl)
2.0-5.0 µl	Specimen cDNA or Control cDNA from kit
X µl	Distilled water bring the final volume to 50 µl

2. PCR thermocycle profile:

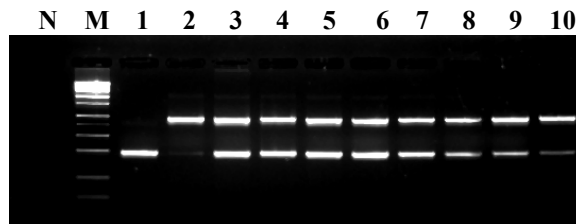
Reaction profiles require optimization according to the machine type and user need. An example of a time-temperature profile for the positive control PCR reaction optimized for Perkin Elmer machine types 2400 and 9600 is provided below:

Temperature	Time	Cycles
96°C	60 sec.	1X
94°C	60 sec.	
58°C	90 sec.	28-32X*
72°C	10 min.	1X
20°C	Soak	

28-32 X*: Use higher number of cycles for low copy genes and lower number of cycles for high copy genes. For some very rare copy number of genes, 40-45 cycles may be used and for some very high copy genes, 20-25 cycles may be used instead.

III. PCR Product Detection:

10 µl of the amplified samples are mixed with 2µ, 6X gel loading buffer and subjected to electrophoresis in a 1% agarose gel containing 0.5 µg/ml ethidium bromide in 0.5-1.0 X TBE buffer. A 100-150 constant voltage is applied for 30-45 minutes or until the dye front reached two-third of the gel before visualized by UV transilluminator.



Line N: Negative Control
 Line 1: 10⁹ copies of ANG2 with Dual Primers
 Line 2: 10⁸ copies of GAPDH with Dual Primers
 Line 3: 10⁸ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 4: 10⁷ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 5: 10⁶ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 6: 10⁵ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 7: 10⁴ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 8: 10³ copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 9: 10² copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers
 Line 10: 10 copies of ANG2 plus 10⁸ copies of GAPDH with Dual Primers

IV. Gene Expression Quantitative:

The amplicons can be quantitative after analysis by various techniques mentioned above. The gel image can be digitized and gene expression quantitative can be calculated accordingly or the chromatogram and gene expression quantitative can be calculated directly. The internal housekeeping gene is served as a normalizer for gene expression quantitative. Under predefined optimization condition, all gene expressions are in a linear range, the relative percentage of target specific gene expression can be calculated as follows:

$$\frac{X}{Y} \times 100\% = \text{Normalized Target Specific Gene Expression \%}$$

Where X = target specific gene expression level, Y = housekeeping gene expression level. Assuming the housekeeping gene selected did not change its expression level before and after treatments and loading the same initial quantity of samples, all Y value from different samples should be the same or very close number.