

# detection of lymphatic endothelial markers using quantitative PCR with 2x Thermo-start® QPCR Master Mix

Our group is interested in the metastatic spread of breast cancer cells. We are particularly interested in the detection of lymphangiogenesis and lymphatic spread of breast cancer cells. One of our approaches is to use quantitative real-time PCR to determine the level of lymphatic markers in breast cancer. LYVE-1 and vascular endothelial growth factor receptor 3 (VEGF-R3) are two molecules that are highly expressed in lymphatic endothelial cells, but are either not expressed or expressed at a very low level in vascular endothelial cells.

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## Methods

### Plasmid preparation

RNA was extracted from human vascular endothelial cells (HUVEC), using the Total RNA Isolation Reagent (TRIR, ABgene®) and quantity determined using a UV spectrometer. cDNA was synthesised using Reverse-iT™ 1st Strand Synthesis Kit (ABgene®), with an anchored oligo dT primer. After a subsequent amplification step, specific products were TA cloned into a promoter-less pCR2.1 vector. Following amplification in *E. coli*, plasmids were extracted, purified and quantified. The number of plasmid copies was determined based on the size of plasmid and insert.

### Quantitative real-time PCR

96-well plates and adhesive films with very low fluorescent background were used. Human LYVE-1 was detected using a Scorpion probe system (Oswel/Eurogentec), in which the forward primer and the molecular beacon probe (FAM™ labelled) were joined with a PCR stopper. Human

VEGF-R3 was analysed with an ABI TaqMan® system, using a FAM™ labelled probe. Quantitation was carried out on an iCycler-iQ™ (Bio-Rad), which incorporates a gradient 96 module, an optical unit, and a four-dye detection system. Two commercially available master mixes were used and compared, i.e. a master mix UDG (2x concentrated) (Supplier L) and a 2x Thermo-Start® QPCR Master Mix (ABgene®, Cat. No. CM-138). Plasmid templates were diluted to obtain a concentration range of 10<sup>9</sup>–10<sup>6</sup> copies/μl. Reactions were carried out in triplicate with the final volume at 16μl using the following reaction conditions: 94°C for 12 minutes, and 50 cycles of 94°C for 15 seconds, 55°C for 15 seconds and 61°C for 50 seconds. Data was analysed using software provided by Bio-Rad.

## Results & Discussion

### The correlation between the starting quantity of the templates and threshold cycles

Reactions using both master mixes have revealed that threshold cycles and the starting quantity of DNA templates are well correlated (correlation coefficient greater than 0.98 for both probe systems) as indicated in figures 1 and 3. Interestingly, both master mixes gave identical results in this correlation (all six replicates are closely mimicking each other).

### Hot-start master mixes and fluorescence signal intensity

Figure 2 shows the fluorescence signal intensity of VEGF-R3, using the ABI TaqMan® probe system. Although the fluorescence signals in both master mixes started at a similar time, the reaction signal with 2x Thermo-Start® QPCR Master Mix became stronger than the Supplier L mix. This did not affect the correlation of starting quantities and threshold cycles (figure 1). The reaction using a molecular beacon-based Scorpion detection system for LYVE-1 resulted in a similar signal intensity between the two master mixes, as shown in figure 4.

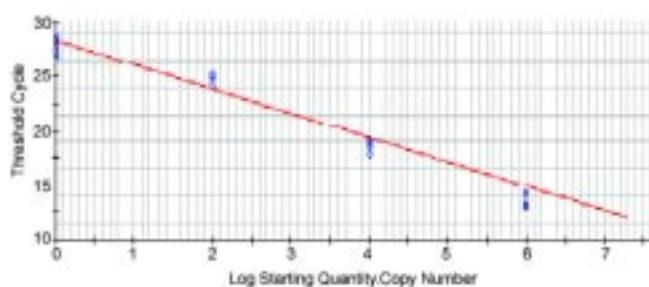


Figure 1: Correlation between threshold cycle numbers and starting quantity of template DNA (VEGF-R3) using ABI TaqMan® detection probes ( $r=0.98$ ).

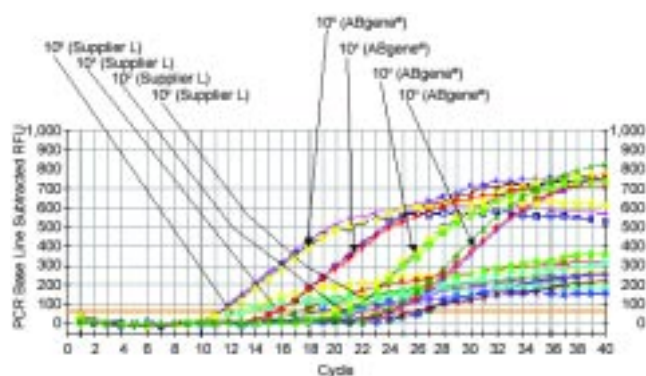


Figure 2: Comparison between ABgene® and Supplier L master mixes using an ABI TaqMan® probe. The template is VEGF-R3 cloned in pCR2.1 cloning vector. Quantities of the standard are given as copies/μl.

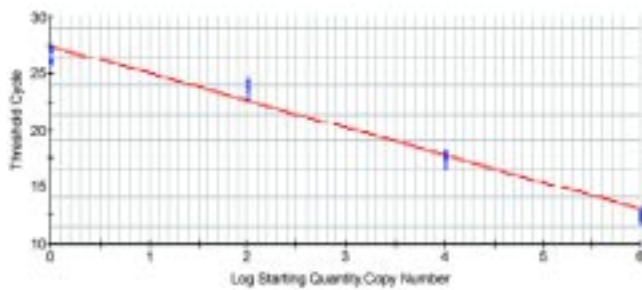


Figure 3: Correlation between threshold cycle numbers and starting quantity of template DNA (LYVE-1) using Scorpion detection probes ( $r=0.981$ ).

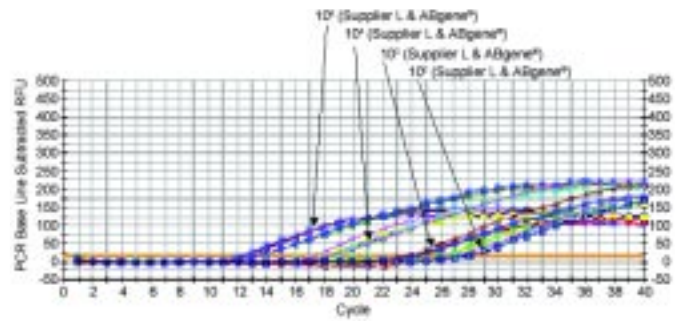


Figure 4: Comparison between ABgene® and Supplier L master mixes using a Scorpion probe. The template is LYVE-1 cloned in pCR2.1 cloning vector. Quantities of the standard are given as copies/μl. The two master mixes yielded identical results.

In summary, we found that the ABgene® 2x Thermo-Start® QPCR Master Mix is a suitable master mix for LYVE-1 and VEGF-R3 quantitation in our laboratory and has been used successfully in a high throughput analysis of these markers in clinical breast cancer samples. In addition to the ABI TaqMan® and Scorpion/Molecular Beacon reactions, we also found that the mix is compatible with the Unifluor probe detection system (Intergen). This master mix has provided us with a reliable, cost effective and reaction-efficient PCR mixture for our quantitative PCR analysis. We are now using

the QPCR mix for examining the impact of lymphangiogenesis in the development and spread of human breast and colon cancer,

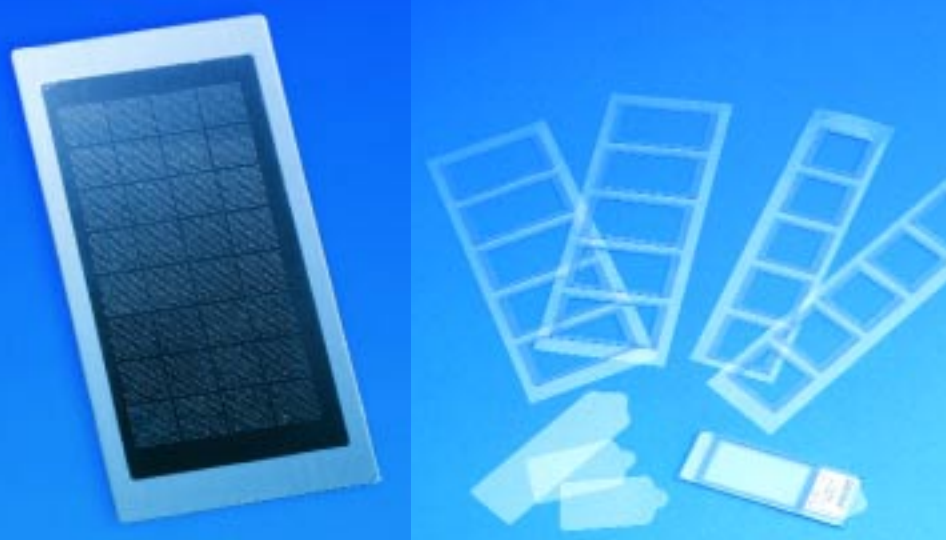
and are also developing methodologies for quantitative analysis of micro-metastasis in these cancers.

Cat. No.	Description	Quantity
CM-138	2x Thermo-Start® QPCR Master Mix*	100 x 50μl rxns
CM-138/b	2x Thermo-Start® QPCR Master Mix*	800 x 50μl rxns
AB-0789	Reverse-iT™ 1st Strand Synthesis Kit	40 reactions
AB-0789/b	Reverse-iT™ 1st Strand Synthesis Kit	100 reactions
AB-0303	Total RNA Isolation Reagent (TRIR)	50ml
AB-0304	Total RNA Isolation Reagent (TRIR)	100ml
AB-0305	Total RNA Isolation Reagent (TRIR)	200ml



\*Products marked "Licensed for PCR" are accompanied by a limited license to use them in the Polymerase Chain Reaction (PCR) process for life science research in conjunction with a thermal cycler whose use in the automated performance of the PCR process is covered by up-front license fee, either by payment to Perkin-Elmer or as purchased, i.e. an authorised thermal cycler. Polymerase Chain Reaction (PCR) is covered by US patents owned by Hoffmann-La Roche. The use of the TaqMan® fluorogenic probes in 5' nuclease assays is covered by U.S. Patent Nos. 5,210,015 and 5,487,972, owned by Roche Molecular Systems, Inc. and by U.S. Patent No. 5,538,848, owned by PE Corporation. Purchase of the product does not provide a license to use this patented technology. A license to practice this technology must be obtained from PE Biosystems, 850 Lincoln Center Drive, Forest City, California 94404, or from Roche Molecular Systems, Inc., 1145 Atlantic Avenue, Alameda, California 94501. TaqMan® is a registered trademark of Roche Molecular Systems, Inc. iCycler-iQ™ is a trademark of Bio-Rad Laboratories. FAM™ is a trademark of Applied Biosystems Corporation.

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