Calculation of minimal residual disease (MRD)

Normalised Copy Number method (NCN)

Results can be calculated using normalized copy numbers (NCN). This method makes use of standard curves always tested on each PCR plate. In particular, the QUANT-PML-RARA bcr2 kit use three plasmid standard dilutions for the control gene (ABL), and five standard dilutions for the fusion gene (BCR-ABL p190). This methodology has the advantage that degradation of probes can be compensated for, and that data generated on different types of Real time-PCR machines can be compared.
The normalised copy number (NCN) is defined as the copy number (CN) of the fusion gene (FG) per one copy of the control gene (CG) transcript:

mean value of $\log_{10}(FG_{CN})$ - mean value of $\log_{10}(CG_{CN})$.

In the NCN method, the minimal residual disease (MRD) value is a ratio between the CG normalized expression of the FG in follow-up $(FG_{CN}/CG_{CN})_{FUP}$ and diagnostic samples $(FG_{CN}/CG_{CN})_{DX}$.
For calculation of MRD value, Europe Against Cancer (EAC) data (J. Gabert et al. Leukemia 17, 2318-2357 -2003) for the corresponding FG at diagnosis can be used if patient value at diagnosis is not available.

Sensitivity (SENSv) is calculated according to the relative expression of the FG at diagnosis $(FG_{CN}/CG_{CN})_{DX}$ and CG expression $(CG_{CN,FUP})$ in the follow-up sample.

The EAC formula for calculation of MRD value and theoretical sensitivity based on FG and CG Real-Time Quantitative-PCR sets

$$\text{MRD value (MRDv)} = (FG_{CN} / CG_{CN})_{FUP} / (FG_{CN}/CG_{CN})_{DX}$$

$$\text{Sensitivity (SENSv)} = CG_{CN,DX} / (CG_{CN,FUP} \times FG_{CN,DX})$$

References

1) VHJ van der Velden et al. Leukemia **17**, 1013-1034 (2003)
2) E. Beillard et al. Leukemia **17**, 2474-2486 (2003)
3) J. Gabert et al. Leukemia **17**, 2318-2357 (2003)

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QUANT-PML-RARa bcr2

Real-Time Quantitative PCR of bcr2 fusion gene transcripts

Cat 1.004

The t(15;17)(q22;q21) translocation is associated with acute promyelocitic leukemia (APL), a distinct AML subset with M3 cytomorphology, which represents about 10-15% of AML (acute myeloid leukaemia). The two genes involved in t(15; 17) are PML, cod- ing for a novel transcription factor, on chromosome 15 and the retinoic acid receptor-α (RARA) gene on chromosome 17. The chimeric PML-RARA protein created by this translocation is a transcriptional repressor.

The chromosome 17 breakpoints are localized within a 15 kb DNA fragment of the RARA intron 2. By contrast, three regions of the PML locus are involved in the translo- cation breakpoints: intron 6 (bcr1; 55%of cases), exon 6 (bcr2; 5 % of cases) and intron 3 (bcr3; 40% of cases). As a consequence, there are three possible PML-RARA isoforms, type bcr1 or L (for long), type bcr2 or V (for variant) and type bcr3 or S (for small).

In the last decade, all-trans-retinoic acid (ATRA) is used as for the treatment of most APL patients ATRA is a non-toxic agent which activates the retinoic acid receptor (RAR), inhibits proliferation and promotes differentiation of the leukemic promyelocytes. The quantification of PML-RARA transcripts is relevant for monitoring and adapting treatment. There is general agreement that a positive PML-RARA PCR after consolida- tion therapy is a strong predictor of subsequent haematological relapse, whereas repeat- edly negative results are associated with long-term survival. The monitoring of PML- RARA fusion gene transcript levels can be useful to predict relapse while the patient is still in hematological and cytogenetic remission.

In the QUANT-PML-RARA bcr2 kit the real-time PCR is used in the second step of a two-step reverse transcription–polymerase chain reaction (RT-PCR) protocol. The tem- plate, cDNA generated from a reverse transcription reaction, is amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM-TAMRA). During the reaction, cleavage of the probe, by the 5´ nuclease activity of Taq DNA Polymerase, separates the reporter dye (FAM) and the quencher dye (TAMRA), which results in in- creased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. In the QUANT-PML- RARA bcr2 kit, an endogenous control (ABL transcript) is amplified from the sample as well as the fusion transcript of interest. In addition, Standard curves of known amounts of both the endogenous ABL control and the fusion cDNA allow the calculation of the ratio of specific fusion transcript signal to endogenous ABL signal in each sample.

The QUANT-PML-RARA bcr1 allows the quantification of PML-RARA bcr2 tran- scripts in peripheral blood or bone marrow samples of a subgroup of AML patients according to the Europe Against Cancer studies (J. Gabert et al. Leukemia 2003)

Kit contains and storage

NAME	COLOUR CODE	STORAGE
REVERSE TRANSCRIPTION		
5X First -Strand Buffer	**(Green)**	-20°C
DTT 100 mM	**(Violet)**	-20°C
RNase Inhibitor (40U/µl)	**(Purple)**	-20°C
Random hexamer 100µM	**(White)**	-20°C
dNTP 20 mM	**(Red)**	-20°C
Reverse Transcriptase (200U/µl)	**(Yellow)**	-20°C
H_2O RNase/DNase-free	**(Light Blue)**	-20°C
STANDARD DILUTIONS		
St1-ABL-10^3 copies/5µl	**(Green)**	-20°C
St2-ABL-10^4 copies/5µl	**(Green)**	-20°C
St3-ABL-10^5 copies/5µl	**(Green)**	-20°C
St1-PML-RARA bcr2 10^1 copies/5µl (e1-a2 m-bcr)	**(Yellow)**	-20°C
St2-PML-RARA bcr2 10^2 copies/5µl (e1-a2 m-bcr)	**(Yellow)**	-20°C
St3-PML-RARA bcr2 10^3 copies/5µl (e1-a2 m-bcr)	**(Yellow)**	-20°C
St4-PML-RARA bcr2 10^5 copies/5µl (e1-a2 m-bcr)	**(Yellow)**	-20°C
St5-PML-RARA bcr2 10^6 copies/5µl (e1-a2 m-bcr)	**(Yellow)**	-20°C
REAL-TIME PCR		
10X ABL (Primers/FAM-TAMRA probe)	**(Green)**	-20°C
10X PML-RARA bcr2 (Primers/FAM-TAMRA probe)	**(Yellow)**	-20°C
Master Mix 2X	**(Purple)**	**+4°C**
H_2O RNase/DNase-free	**(Light Blue)**	-20°C

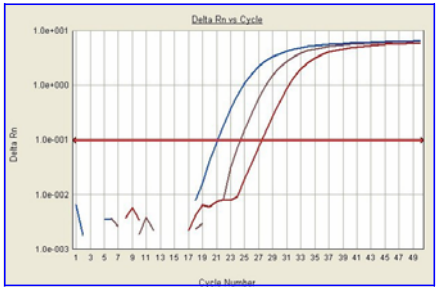
Principle of assay: A) extraction of total RNA B) synthesis of cDNA from total RNA C) real-time PCR
Applicability: On extracted and purified total RNA from peripheral blood or bone marrow cells.
Numbers of tests: 24 (104 PCR reactions)
Stability: over 18 months if correctly stored

Analysis of results

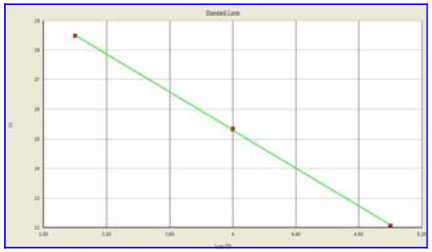
The cycle number at which the reporter dye emission intensities rises above background noise is called the threshold cycle (Ct). The Ct is directly proportional to the copy number of the target template at the beginning of the reaction. (On a TaqMan machine set a threshold at 0.1 and a baseline between cycles 3 and 15). Using Standard curves of known amounts of both the endogenous ABL control and the fusion cDNA allow the calculation of the ratio of specific fusion transcript signal to endogenous ABL signal in each sample.(The theoretical slope of the standard curve is -3,32 for a PCR reaction with a maximum efficiency).
The figures below show an example of Amplification plots and Standard curves of ABL and PML-RARA bcr2 standard dilutions.

STANDARD DILUTIONS - Control gene ABL (St.1 - St.2 - St.3)

Amplification Plots



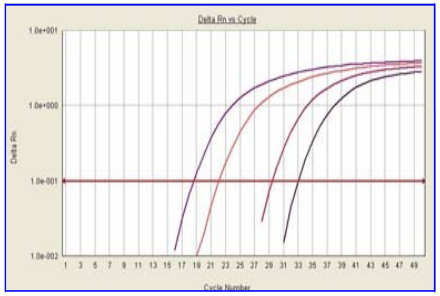
Standard Curve



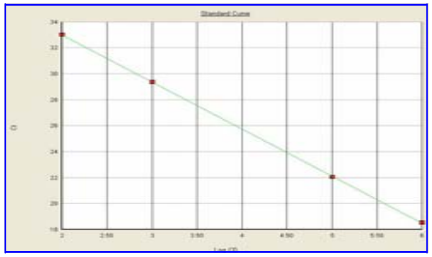
Y = -3.202*LOG(X) + 38.102

STANDARD DILUTIONS - Fusion gene PML-RARA bcr2 (St.2 - St.3 - St.4 - St.5)

Amplification Plots



Standard Curve



Y = -3.624*LOG(X) + 40.236