

Time is short
The list is not

del(1)(p32) (STIL-TAL1)
t(1;11) (p32;q23) (MLL-EPS15)
t(1;11) (q21;q23) (MLL-MLLT11)
t(1;19) (q23;p13) (TCF3-PBX1)
t(3;5) (q25;q34) (NPM1-MLF1)
t(3;21) (q26;q22) (RUNX1-MECOM)
t(4;11) (q21;q23) (MLL-AFF1)
t(5;12) (q33;p13) (ETV6-PDGFRB)
t(5;17) (q35;q21) (NPM1-RARA)
t(6;9) (p23;q34) (DEK-NUP214)
t(6;11) (q27;q23) (MLL-MLLT4)
t(8;21) (q22;q22) (RUNX1-RUNX1T1)
t(9;9) (q34;q34) (SET-NUP214)
t(9;11) (p22;q23) (MLL-MLLT3)
t(9;12) (q34;p13) (ETV6-ABL1)
t(9;22) (q34;q11) (BCR-ABL1)
t(10;11) (p12;q23) (MLL-MLLT10)
t(11;17) (q23;q21) (MLL-MLLT6)
t(11;17) (q23;q21) (ZBTB16-RARA)
t(11;19) (q23;p13.1) (MLL-ELL)
t(11;19) (q23;p13.3) (MLL-MLLT1)
t(12;21) (p13;q22) (ETV6-RUNX1)
t(12;22) p13;q11) (ETV6-MN1)
t(15;17) (q24;q21) (PML-RARA)
inv(16) (p13;q22) (CBFB-MYH11)
t(16;21) (p11;q22) (FUS-ERG)
t(17;19) (q22;p13) (TCF3-HLF)
t(X;11) (q13;q23) (MLL-FOXO4)

Run a
HemaVision®-7Q

7 Translocations
40+ Breakpoints
In just 4 hours

CE IVD

Cat. No. HV01-7Q

USER MANUAL

Revision date 2015.09.30

USER MANUAL

HEMAVISION[®]-7Q

Cat. No. HV01-7Q

A MULTIPLEX RT-QPCR TEST FOR SCREENING OF 7 LEUKEMIA-ASSOCIATED TRANSLOCATIONS

12 TESTS PER KIT

CE IVD

FOR PROFESSIONAL USE ONLY



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1. PURPOSE OF THE TEST

HemaVision®-7Q is a 4 hour CE IVD marked in vitro diagnostic test for qualitative screening of 7 frequent chromosome translocations involved in chronic and acute leukemia. The amplification plot allows professionals to react rapid and with precision in terms of treatment planning, while profiting from the cost- and labour-effective screening process.

HemaVision®-7Q provides a rapid screen for 7 translocations with more than 40 clinically relevant chromosomal breakpoints. The test requires only limited hands on time and the procedure can be completed in 4 hours after RNA extraction.

HemaVision®-7Q detects RNA transcripts from fusion genes using a RT-qPCR procedure. Alternative splice variants are also detected.

HemaVision®-7Q detects the following 7 translocations:

t(1;19) (q23;p13) (TCF3-PBX1)
t(4;11) (q21;q23) (MLL-AFF1)
t(8;21) (q22;q22) (RUNX1-RUNX1T1)
t(9;22) (q34;q11) (BCR-ABL1)
t(12;21) (p13;q22) (ETV6-RUNX1)
t(15;17) (q24;q21) (PML-RARA)
inv(16) (p13;q22) (CBFB-MYH11)

2. PRINCIPLES OF THE TEST

HemaVision®-7Q is a RT-qPCR based assay for detection of leukemia associated fusion gene transcripts in total RNA from whole blood or bone marrow samples. Included in the kit are ready to use cDNA and qPCR Master Mixes. cDNA is synthesized by adding purified RNA to the HemaVision®-7Q ready to use cDNA reaction mix. The resulting cDNA is added to 7 ready to use qPCR reaction tubes, which contain specific PCR primers and probes for detection of fusion genes, one reference gene and an internal amplification control (IAC). The qPCR is performed in a real-time qPCR instrument with optical filters for detection of FAM, ROX, and CY5 fluorescence. Amplification plots and Ct (cycle at threshold) values are used for identification of the translocation and fusion gene transcript using an easy to use interpretation table.

HemaVision®-7Q detects fusion gene transcripts using specific PCR primers and probes. The translocation specific primers bind to exons in the fusion gene enabling amplification of the region containing the breakpoint. The primers are designed to detect multiple clinical relevant breakpoints and splice variants (Figure 1).

The qPCR master mix probes are dual labeled with a fluorophore and a quencher molecule at each end of the oligoes. During the annealing step of the PCR the probe bind to the PCR products of the previous rounds. Signals are generated in the subsequent elongation step as the 5'->3' exonuclease activity of the Taq polymerase enzyme degrades the hybridized probe. This liberates the fluorophore from the quencher, which thereby increases the fluorescence. The fluorescence is measured at the end of the elongation step of every PCR cycle.

When the fluorescence for a translocation exceeds the threshold level before cycle 35 (Ct <35) the test is positive. The qPCR's are multiplexed by the use of FAM, ROX and CY5 labeled probes. This permits two

translocation tests and the IAC to run in the same tube. The identity of a positive translocation test is easily deduced from the Interpretation Table.

As a control for the functionality of the qPCR reaction and for correct transfer of cDNA aliquots to the 7 qPCR reactions, an Internal Amplification Control (IAC) is included in the cDNA reaction mix.

HemaVision®-7Q also includes primers and probes for detection of the reference gene transcripts ABL-1. Detection of the reference gene is a control for the integrity of the RNA sample and functionality of both cDNA and qPCR reactions.

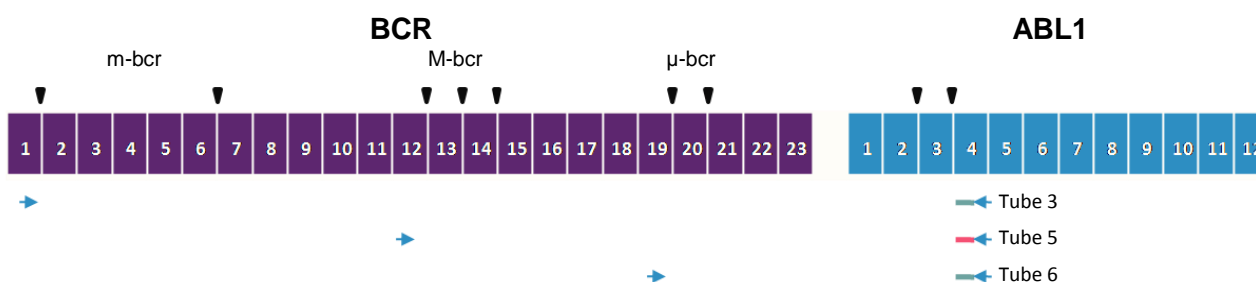


Figure 1. Primers and probes in HemaVision®-7Q are designed to detect multiple clinical relevant breakpoints. In this example primers and probe for detection of t(9;22) fusion gene transcript BCR-ABL1 are shown. Primers are depicted as blue arrows, FAM and ROX labeled probe as green and red lines and breakpoints as black triangles. Exons are numbered for the fusion genes BCR and ABL1. The three breakpoint regions (m-bcr, M-bcr, μ-bcr) of BCR are detected with the primer and probe combination of qPCR tube 3, 5, 6, respectively.

3. KIT COMPONENTS AND STORAGE

HemaVision®-7Q contains reagents for 12 tests.

Included in HemaVision®-7Q kit is the following components:

- 12 cDNA tubes with 28 uL reaction mix (0.65 mL tubes with yellow screw cap)
- 12 strips of 8 qPCR tubes containing 23 uL qPCR reaction mix.
- 12 extra strips of optical caps for the qPCR tubes.
- Two tubes with RNase free H₂O (0.65 mL tubes with blue screw cap)
- One user manual

HemaVision®-7Q is produced in 4 formats of plastic to suit various qPCR apparatus. The qPCR mix can be supplied in 0.1 mL white low profile (WLP), 0.2 mL white regular profile (WRP), 0.2 mL frosted regular profile (FRP) or 0.2 mL clear regular profile (CRP) PCR tubes. Adaptor plates can be supplied with the kit if the qPCR instrument requires a 96-tube plate format (e.g. Roche LightCycler 480 and some ABI models). For the Qiagen Rotorgene instrument, the CRP 8-tube strip can be divided in 2x4 tubes before insertion in the instrument rotor.

The cDNA reaction tubes contain reverse transcriptase, nucleotides, buffer, cDNA primers and IAC oligo template.

The qPCR tubes contain hot start Taq DNA polymerase, nucleotides, buffer and primers/probes.

Tube no. 1-6 contain primers and probes for both fusion genes and the IAC template.

Tube no. 8 contain primers and probes for a reference gene ABL1 and the IAC template.

Tube no. 7 is empty.

To confirm the orientation of the PCR strips after qPCR analysis check for FAM amplification signals in tube no. 8 and the absence of CY5 amplification signal in tube no. 7.

The kit must be stored at -20°C.

Avoid thawing and freezing of the kit.

The qPCR tubes must be protected from strong light to avoid bleaching of the probes.

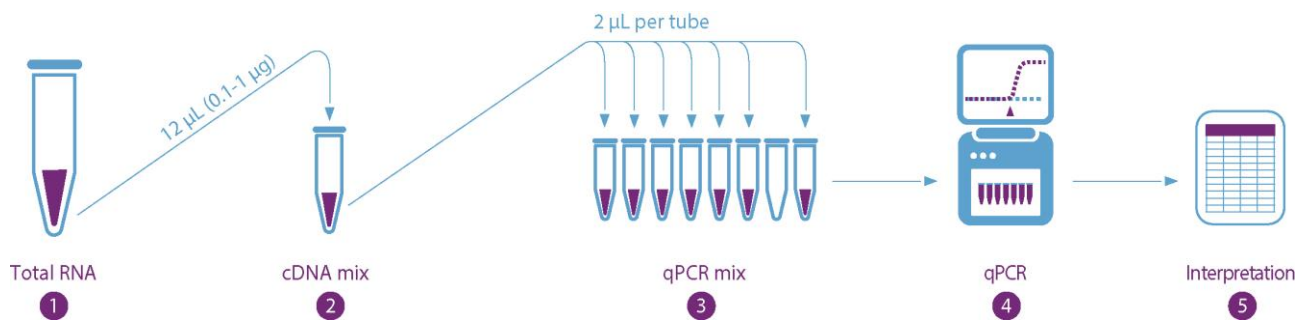
4. REQUIRED EQUIPMENT

- Centrifuge for 96 well plates or PCR 8-tube strips.
- Thermal Heating Block.
- A qPCR instrument with filters for FAM (Abs 495 nm, Em 520 nm), ROX (Abs 585 nm, Em 605 nm) and CY5 (Abs 635 nm, Em 665 nm).
- Pipettes and sterile RNase free filtered tips.
- Gloves

5. PRECAUTIONS

- The quality and concentration of the RNA sample greatly affects the results of this test. To minimize the risk of degradation by ribonucleases, we strongly recommend purification of total RNA immediately after blood (bone marrow) extraction. Do not freeze Ficoll purified cells without adding a denaturing solution [e.g. containing guanidinium isothiocyanate (GTC)] immediately after isolation and before freezing. Always store cell samples and aqueous RNA solutions at -80°C. Even an overnight storage at -20°C may result in RNA degradation.
- When working with RNA always use gloves, as hands are a major source of ribonuclease contamination.
- The high sensitivity of DNA amplification techniques makes the reactions susceptible to DNA contamination from previous amplification reactions, potentially resulting in false positive results. To minimize the risk of contamination avoid opening qPCR tubes after amplification.
- Laboratory workbenches and pipettes must be cleaned with bleach on a regularly scheduled basis.
- Use aerosol barrier pipette tips.
- Always use gloves.

6. PROTOCOL AT A GLANCE



1: RNA denaturation	2: cDNA synthesis	3: Add cDNA to qPCR tubes	4: Program qPCR Instrument	5: qPCR analysis
12 µL total RNA (0.1-1 µg) ▼ 65°C / 5 min ▼ 0°C / 1 min ▼ Spin / 1 min	Transfer to one cDNA tube ▼ Mix and spin / 1 min ▼ 42°C / 60 min ▼ 95°C / 5 min ▼ 0°C / 1 min ▼ Spin / 1 min	Spin 8-tube strip 10 seconds ▼ Remove and discard lids ▼ Add 2 µL cDNA to each of the 7 qPCR mixes ▼ Close tubes with new optical lids ▼ Spin / 1 min	1 cycle: 95°C / 15 min ▼ 40 cycles: 95°C / 30 sec 60°C / 50 sec 72°C / 80 sec* *read FAM, ROX, CY5	Generate amplification plots and use Interpretation Table

Figur 2. Overview of HemaVision®-7Q protocol.

7. DETAILED PROTOCOL

7.1 RNA Preparation

Total RNA is extracted from blood or bone marrow cells. Minimum 0.1 µg total RNA with a minimum concentration of 8 ng/µL RNA is needed for the subsequent steps.

Due to the inherent instability of RNA use patient samples as fresh as possible.

Blood samples can be stabilized in Paxgene Blood RNA tubes, Cat. No. BD762165. Do NOT use Heparin for stabilization of blood samples.

Total RNA should be stored at -80°C.

7.2 cDNA Synthesis

- Incubate total RNA at 65°C for 5 minutes and cool immediately on ice. Spin tube for 1 minute to collect condensate.
- Take one yellow capped tube with cDNA master mix from the HemaVision®-7Q kit stored at -20°C. Add 12 µL (0.1-1 µg) of denatured total RNA to the cDNA master mix tube (tube with yellow cap). If necessary the RNA sample can be diluted with the provided RNase free H₂O (tube with blue cap). Mix and spin for 1 minute.

- Incubate the cDNA reaction at 42°C for 60 minutes.
- Inactivate Reverse Transcriptase enzyme by heating to 95°C for 5 minutes.
- Cool the cDNA reaction tube on ice for 1 minute and spin it again for 1 minute to collect condensate.

7.3 qPCR

- For each cDNA sample use one 8-tube strip with qPCR master mix. Spin the 8-tube strip for 10 second to collect qPCR master mix at the bottom of tubes.
- Remove and discard the caps from 8-tube strip. Be careful to avoid cross contamination and spill of reaction mixes while removing the caps.
- Add 2 µL cDNA reaction to each of the 7 tubes containing 23 µL qPCR master mix (tube 1-6 and 8). Tube number 7 is empty and is used for orientation of the 8-tube strip, see figure 3.

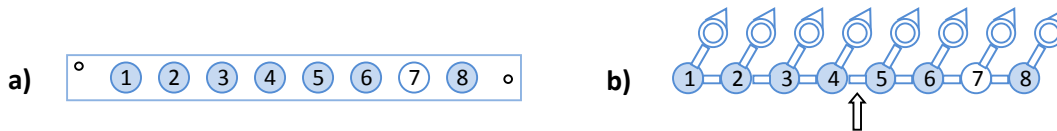


Figure 3. Orientation of qPCR tubes in 8-tube strips.

a) Positions of the 7 qPCR master mixes in the 8-tube strip formats **WLP and WRP**. Tube number 7 is empty to help orientation of the 8-tube strip. The hole in the corner and the middle of each 8-tube strip (depicted as °) marks the beginning and end respectively of the strip. The tube numbering refers to the tube numbering in the Interpretation table.

b) Positions of the 7 qPCR master mixes in the 8-tube strip format **CRP** for use in RotorGene instruments. Tube number 7 is empty to help orientation of the 8-tube strip. The tube numbering refers to the tube numbering in the Interpretation table. The arrow marks the hinge to be broken before insertion into the rotor since only 4-tube strips and not 8-tube strips can be inserted in the rotor.

- Close the 8 tubes with a fresh set of optical caps supplied with the kits (WLP, WRP) or the hinged caps (CRP). Use gloves to avoid grease and dust on the optical caps or optical tubes (CRP).
- Insert the 8-tube strip into the qPCR instrument. The empty tube (#7) is intended for orientation of the tubes in the qPCR instrument.
- Run the qPCR reaction with the following program:

Step	Time/Temperature	Cycles	Comment
1	15 minutes / 95°C	1	Activation of Taq Polymerase
2	30 seconds / 95°C 50 seconds / 60°C 80 seconds / 72°C *	40	DNA amplification * Read fluorescence for FAM, ROX and CY5 at the end of each extension step

Note: As the HemaVision®-7Q kit contains ROX labeled probes make sure that the instrument software is not set for using ROX as a passive reference dye.

7.3.1 Important notes for ABI 7500 and ViiA7 users

- Ramp speed: Use standard ramp speed (not fast mode).
- Targets: While defining the 3 targets FAM, ROX and CY5 choose for all targets “None” as Quencher.
- Passive reference dye: Select “None” instead of “ROX” as passive reference dye.

7.3.2 Important notes for Roche LightCycler 480 users

A Color Compensation (CC) file should be generated before the first run on the Light Cycler 480 and if the lamp has been replaced. A CC-kit with FAM, ROX and CY5 dye is available from DNA Diagnostic.

qPCR software setup:

- Set the ramp speed to 2.2 C/sec for annealing step and 4.4 C/sec for the remaining steps.
- For the amplification step of the PCR program, under Analysis Mode choose Quantification and Acquisition Mode: Single.
- Filter choice: Either choose 4 color hydrolysis probe or manually choose FAM, Red 610 and CY5.

7.4 Analysis and Interpretation

Generate amplification plots showing:

- Thermal cycles on the X-axis.
- Background subtracted fluorescence on the Y-axis. The exact name on the Y-axis differs among qPCR instruments. See table 1.

Instrument	Y-axis on amplification plot with background subtracted fluorescence
ABI: 7500, ViiA7	ΔRn
BioRad: CFX96	RFU
Roche: LightCycler 480	Fluorescence (using Analysis option: Abs Quant / 2nd Derivative Max for All Samples)
Qiagen: Rotor-Gene	Norm. Fluoro.
Agilent: Mx3000P / Mx3005P	dR

Table 1. Examples of instrument nomenclature for the Y-axis when the amplification plot shows background subtracted fluorescence.

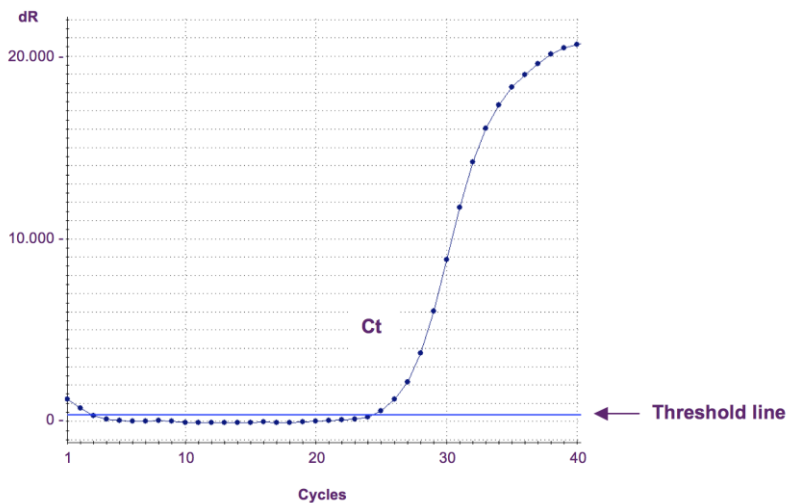


Figure 4. Amplification plot from Agilent Mx3005P with X-axis showing cycle number and Y-axis showing background subtracted fluorescence.

The threshold line is used to find the Ct value. Ct is the intersection between the amplification curve and the threshold line. The threshold line is set just above the base line in this example resulting in a Ct value of 24.4.

Ct values are calculated automatically by the instrument. The algorithms used to calculate Ct values differ between qPCR instrument software packages. The threshold lines in the amplification plot are set manually to allow accurate Ct determination. The threshold lines are set just above the background fluorescence. The threshold lines are set sufficiently high to allow for fluctuations in background fluorescence and background fluorescence drift (a linear increase in fluorescence). The threshold level can be set manually using the following the steps:

ABI; 7500, 7500 Fast, ViiA 7: In the Analysis window, choose “Plot Type: deltaRn vs Cycle”, and “Graph Type: Linear”. Unmark “Auto Threshold” and “Auto Baseline”. Then click “Show Threshold” and with the mouse move the threshold line above the baseline.

Bio-Rad CFX96: Click the “Settings” tab, choose “Baseline Setting” and “Baseline Subtracted Curve Fit”. Using the mouse adjust the threshold level above baseline.

Qiagen Rotor-Gene Q: Click “Analysis”, choose the fluorescence to analyze, click “Linear Scale”, “Slope Correct” and “Auto-Scale”. In the right side panel, under “CT Calculation”, click the button next to “Threshold” and mark the threshold level on the amplification plot.

Roche LightCycler 480: Choose “Absolut Quantification” and “Fit Points”. Then click the tab “Noise Band” and click the “Noiseband” drop down button and choose “Noiseband (Fluoresc)”. Use the mouse to set the Noise Band.

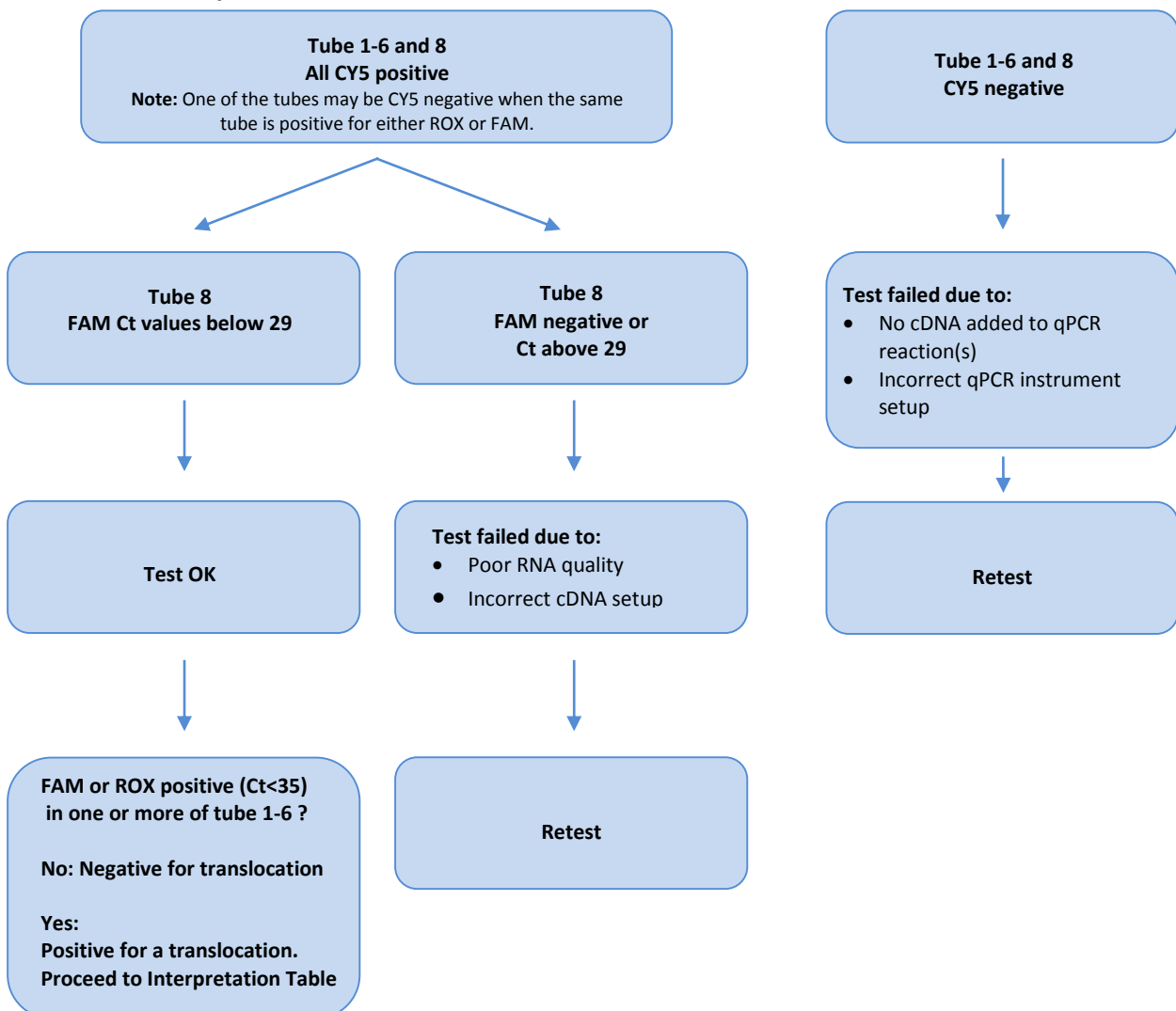
Agilent MxPro: The automatic threshold level can be used. Choose “Adaptive Baseline Correction” and “Background-based Threshold” using cycle 6-11 and a sigma multiplier of 10.

Note! The Ct values cannot be used for exact quantification of the fusion transcripts level since the fusion gene amplicons differ in length, resulting in different PCR efficiencies.

- Check that a CY5 signal is present from the Internal Amplification Control (IAC) in tubes no. 1-6 and 8. The IAC CY5 fluorescence should yield Ct values between 29 and 33. If no CY5 signals are detected the test has failed either due to no addition of cDNA to the qPCR tube(s), incorrect instrument settings or evaporation from the qPCR tube(s). Repeat the test. Due to competition for PCR constituents CY5 Ct value above 33 may occur when the tube is also positive for FAM or ROX fluorescence.

- Check the Ct values for ABL1. The Ct values should be below: 29 in tube 8 (ABL1). Detection of the reference gene ABL1 shows RNA extraction, cDNA synthesis and qPCR have been functional.
ABL1 Ct values higher than 29 indicates the quality of the RNA may be too low to generate a valid test result. Repeat test with fresh RNA.
- Check for a “translocation” FAM or ROX signal with a Ct value below 35 in the tubes 1-6.
- Translocation tests with Ct values below 35 for FAM and ROX signals and amplification curves with exponential growth can be considered as true positive. Use the Interpretation Table to identify the specific translocation.
- Ct values above cycle 35 for FAM and ROX signals may be the result of unspecific amplification (false positive). Repeat the test to confirm or reject the result. When the second test is negative, the sample is negative for the translocation observed in the first test. When the second test is also positive, the sample may be positive for the corresponding translocation. However, we highly recommend using other diagnostic techniques to confirm the result from positive tests having Ct values above 35.

Flowchart for interpretation of data:



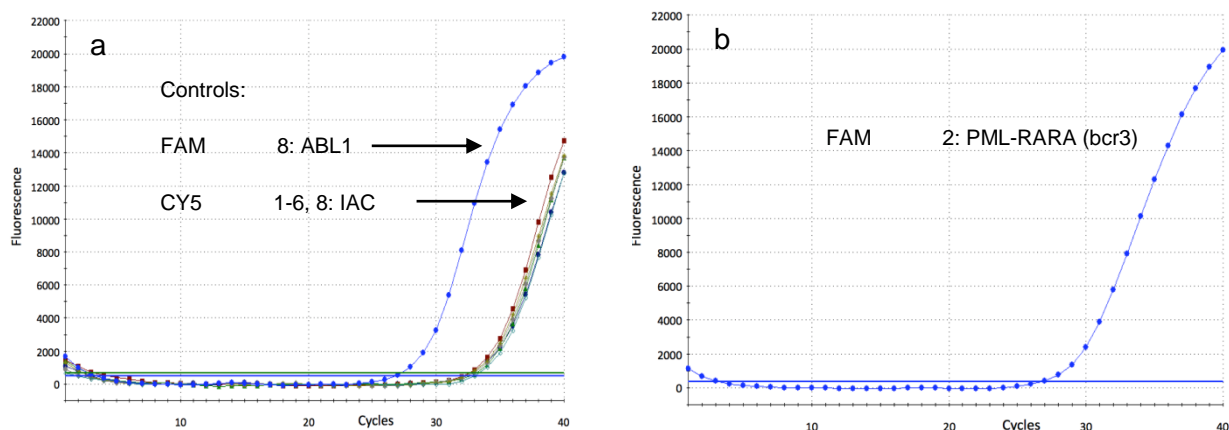


Figure 5. Example of amplification plots from a HemaVision®-7Q test.

a) The CY5 amplification plot shows all 7 IAC control curves are present and has Ct values around 32. This serves as a positive control for correct transfer of 2 μ L cDNA to all 7 qPCR tubes and functionality of the qPCR reactions.

The FAM amplification plot for the reference gene ABL1 in tubes 8 with Ct at 26. This is a positive control for both RNA quality and functionality of RT-qPCR reactions.

b) The FAM amplification plot for tube 2 shows amplification curve with Ct of 27. Therefore, the test is positive for t(15;17)(q24;q21)(PML-RARA, bcr3, S-form). See Interpretation Table.

8. INTERPRETATION TABLE

Tube	Translocation	Fusion Gene	Fw primer - Rev primer	Flouochrome	
1	t(1;19)(q23;p13)	TCF3-PBX1	TCF3 ex16-PBX1 ex3	FAM	CY5
	t(12;21)(p13;q22)	ETV6-RUNX1	ETV6 ex5-RUNX1 ex5	ROX	CY5
2	t(15;17)(q24;q21)	PML-RARA (bcr3, S)	PML ex3-RARA ex3	FAM	CY5
	t(4;11)(q21;q23)	MLL-AFF1	MLL ex7-AFF1 ex9	ROX	CY5
3	t(9;22)(q34;q11)	BCR-ABL1 (m-bcr, P190)	BCR ex1-ABL1 ex3	FAM	CY5
	t(15;17)(q24;q21)	PML-RARA (bcr2, V)	PML ex5-RARA ex3	ROX	CY5
4	inv(16)(p13q22)	CBFB-MYH11	CBFB ex4-MYH11 ex34	FAM	CY5
	t(8;21)(q22;q22)	RUNX1-RUNX1T1	RUNX1 ex6-RUNX1T1 ex3	ROX	CY5
5	t(9;22)(q34;q11)	BCR-ABL1 (M-bcr, P210)	BCR ex12-ABL1 ex3	FAM	CY5
	inv(16)(p13q22)	CBFB-MYH11	CBFB ex4-MYH11 ex31	ROX	CY5
6	t(9;22)(q34;q11)	BCR-ABL1 (μ -bcr, P230)	BCR ex19-ABL1 ex3	FAM	CY5
	t(15;17)(q24;q21)	PML-RARA (bcr1, L)	PML ex6a-RARA ex3	ROX	CY5
7					
8	ABL1	Reference gene	ABL1 ex2-ABL1 ex3	FAM	CY5

When more than one of the tubes 1-6 are positive for FAM or ROX interpretate as follows:

Positive tubes	Interpretate as tube
2 (FAM) 3 (ROX) 6 (ROX)	6 (ROX): t(15;17)(PML-RARA), bcr1, L-form
3 (ROX) 6 (ROX)	6 (ROX): t(15;17)(PML-RARA), bcr1, L-form
2 (FAM) 3 (ROX)	3 (ROX): t(15;17)(PML-RARA), bcr2, V-form
3 (FAM) 5 (FAM)	5 (FAM): t(9;22)(BCR-ABL1), M-bcr, P210
4 (FAM) 5 (ROX)	4 (FAM): Inv(16)(CBFB-MYH11)

Fusion genes targeted with more than one pair of amplification primers may result in positive signals in more than one tube. Use table above in combination with Interpretation Table to identify breakpoint.

9. HGNC GENE NAMES AND NCBI ACCESSION NUMBERS

Old Abbreviation	HGNC Abbreviation	Chromosome	HGNC ID	NCBI Accession
ABL	ABL1	9q34.1	HGNC:76	NG_012034.1
AF4	AFF1	4q21.3	HGNC:7135	NM_001166693.1
AML1	RUNX1	21q22.3	HGNC:10471	NG_011402.2
BCR	BCR	22q11	HGNC:1014	NG_009244.1
CBFB β	CBFB	16q22.1	HGNC:1539	NG_009281.1
E2A	TCF3	19p13.3	HGNC:11633	NG_029953.1
ETO	RUNX1T1	8q22	HGNC:1535	NG_023272.2
MLL	MLL	11q23	HGNC:7132	NG_027813.1
MYH11	MYH11	16p13.11	HGNC:7569	NG_009299.1
PBX1	PBX1	1q23.3	HGNC:8632	NG_028246.1
PML	PML	15q24	HGNC:9113	NG_029036.1
RAR α	RARA	17q21	HGNC:9864	NG_027701.1
TEL	ETV6	12p13	HGNC:3495	NG_011443.1

The HUGO Gene Nomenclature Committee (HGNC) approves a unique and meaningful name for every known human gene. The table contains a list of all relevant genes for the HemaVision[®]-7Q kit, with the old gene name abbreviation and the corresponding HGNC abbreviation. Furthermore the table contains the NCBI (www.ncbi.nlm.nih.gov/nucleotide) accession numbers for the gene or transcript sequences targeted by HemaVision[®]-7Q primers and probes.



Run a
HemaVision®-7Q

Availability & questions

Our team and distributors are always at hand to answer all your questions.
Contact us to find your nearest HemaVision® partner.

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DNA Diagnostic A/S (previously named DNA Technology A/S) was established in 1992. DNA Diagnostic A/S is an ISO 13485:2012 certified developer, manufacturer, and worldwide supplier of PCR based CE IVD marked in vitro diagnostic kits.