VIVA-NUS CenTRAL
Centre for Translational Research in Acute Leukaemia (Molecular)
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Standard Operating Procedures (SOP)

Title: Quantitation of Oncogene Fusion Transcripts by Droplet Digital PCR using Taqman Hydrolysis Probes

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1. BACKGROUND

Gene fusions are chimeras resulting from juxtaposition of two previously separate genes usually on different chromosomes. This is by translocations or deletions of neighbouring genes. Some recurrent gene fusions act as driver mutations in certain leukaemias and have prognostic outcome. These are known as oncogene fusion transcripts. The new WHO classification 2016 for acute leukaemias have incorporated many of the recurrent gene fusions[1].

Oncogene Fusion Transcripts (OFTs) are important markers for leukaemia diagnosis, sub-classification and risk stratification of haematological malignancies[2]. Leukaemia sub-classification and molecular characterization allow us to administer the right therapy based on patient's genetic risk profile, which includes the detection of oncogene fusions. The *ETV6-RUNX1*, commonly present in 20% of childhood Acute Lymphoblastic Leukaemia (ALL), has good outcome on Standard-risk treatment arm[2-3]. Conversely, *KMT2A-AFF1* (f.k.a *MLL-AF4*) and *BCR-ABL1* fusions are associated with poor prognosis[2-3,5].

In Acute Myeloid Leukaemia (AML), *RUNX1-RUNX1T1* and *CBF*β*-MYH11* are associated with favourable outcome on high-dose cytarabine therapy[2]. Patients with Acute Promyelocytic Leukaemia (APML) are associated with *PML-RAR*α and have increased risk of developing Disseminated Intravascular Coagulation (DIC)[6]. Accurate and timely detection of *PML-RAR*α is pertinent, as treatment with ATRA or arsenic needs to start as soon as APML is suspected.

Apart from detecting oncogene fusion for leukaemia diagnosis and molecular characterization, it can also be used to monitor patients' response to therapy; guide clinicians to intervene therapy in a timely manner on a subset of patients for better outcome e.g. the use of imatinib (TKI) on *BCR-ABL1* positive patients[7-8] the use of arsenic trioxide in *PML-RAR*α positive APML patients[6].

Droplet digital polymerase chain reaction (ddPCR™) was developed to provide high-precision, absolute quantification of nucleic acid target sequences with wide-ranging applications for both research and clinical diagnostic applications. ddPCR measures absolute quantities by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions. Its technical advantages enable

detection and analysis of nucleic acids at a level of sensitivity and precision beyond the capabilities of previous methods such as real-time quantitative PCR (qPCR)[4]. A typical ddPCR application that focuses on the lower limits of nucleic acid detection is to measure OFT level particularly in post-treatment samples, which generally uses either a single assay to measure fusion concentration (i.e. copies/µL) or two independent, non-competitive assays to quantify a fusion transcript as a proportion of a reference sequence (**Figure 1**).

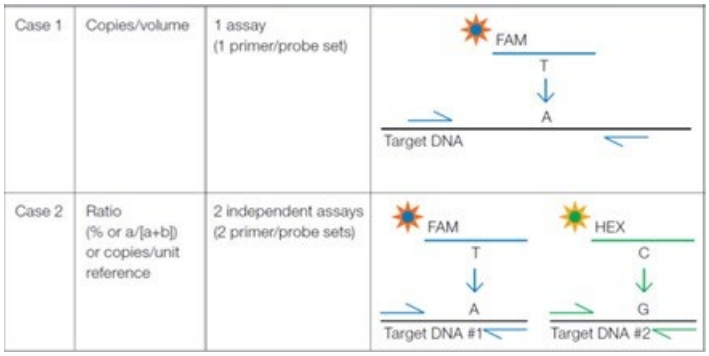


Figure 1. OFT Quantitation using ddPCR.

2. PURPOSE

This Standard Operating Procedure describes the steps to perform Quantitation of Oncogene Fusion Transcripts (OFTs) by droplet digital PCR (ddPCR) using TaqMan hydrolysis probes.

3. SCOPE

The procedure applies to CenTRAL (Molecular) staff performing Quantitation of Oncogene Fusion Transcripts by ddPCR™ using TaqMan® hydrolysis probes.

4. DEFINITIONS

4.1 Leukaemia: Cancer of white blood cells, tissue mainly affecting bone marrow.

4.2 **Acute Lymphoblastic Leukemia (ALL):** Cancer of the white blood cells characterized by overproduction of immature, malignant lymphoblasts.

4.3 **Acute myeloid leukemia** (**AML**): Leukaemia of the myeloid lineage white blood cells, characterized by the rapid growth of abnormal white blood cells that accumulate in the bone marrow and interfere with the production of normal blood cells.

4.4 **Oncogene Fusion Transcripts (OFTs):** Gene transcripts of fusion of different genes resulting from translocation, inversion or interstitial deletion. These transcripts give rise to a different product that causes cancer.

4.5 **Breakpoint:** refers to the positions or places where the hybrid chromosomes break. E.g. t(9;22) – a section of chromosome 9 breaks and translocates to chromosome 22.

4.6 **Housekeeping Gene:** refers to a Control gene, a constitutive gene that is transcribed or expressed at a relatively constant level under normal and patho-physiological conditions. GUSB (β-glucuronidase) is a common housekeeping gene.

4.7 **cDNA:** Complimentary double-stranded DNA synthesized using messenger RNA as a template

4.8 **Droplet Digital Polymerase Chain Reaction (ddPCR):** ddPCR is a method for performing digital PCR that is based on water-oil emulsion droplet technology. A sample is fractionated into maximally 20,000 droplets, and PCR amplification of the template molecules occurs in each individual droplet.

4.9 **Normalization:** A prerequisite for correct measurement (quantitation) of the Target Gene, it involves standardization to a single constitutively expressed control gene (housekeeping gene)

4.10 **Absolute Quantitation:** Without using a standard curve method, the quantitation of the unknown concentration (copies/µL) is based on two elements: positive and negative fluorescence data from the sample droplets and data fitting to a Poisson distribution.

4.11 **Absolute OFT Level:** The actual normalised copy number of the studied OFT against the housekeeping gene (*GUSB*) in the test sample.

4.12 **Relative OFT Level**: The normalised copy number of the OFT in the test sample is compared against the normalised copy number of the OFT at diagnosis/relapse. It is expressed as a ratio.

4.13 **Event:** A eligible droplet detected and recorded by the reader.

4.14 **Threshold:** The cut-off amplitude value (i.e. the signal intensity) to call a positive event.

4.15 **Cluster:** Group of droplets (e.g. positive droplets) formed closely enough to be distinguished clearly from other groups (e.g. negative droplets).

4.16 **Positive (POS):** The quantity of OFT measured in the tested sample is significantly higher than that measured in the negative control ($p<0.0001$).

4.17 **Negative (NEG):** The quantity of OFT measured in the tested sample is NOT significantly different from that measure in the negative control ($0.05>p>0.0001$)).

4.18 **Limit of Quantitative Range (LOQ):** The quantity range in which the OFT measured in the tested sample is literally higher than that measured in the negative control but is not high enough to qualify as a Positive call ($p>0.05$).

4.19 **Unqualified:** Invalid result resulting from failure to meet the minimum QC criteria of RNA input or RNA quality.

4.20 **Quantifying Limit:** The positive and negative limits are the cut off levels for the samples to qualify as positive and negative respectively.

4.21 **Normalisation:** Normalise the fusion gene copy per housekeeping gene (*GUSB*) copy. A prerequisite for correct measurement (quantitation) of the target gene by standardizing to a single constitutively expressed control gene (housekeeping gene).

5. RESPONSIBILITIES

5.1 Laboratory Director (or Designee)

5.1.1 Ensure Scientific Advisors and/or Technical Team Leaders and/or Competent Trainer (Staff) conduct adequate training to all laboratory staff involved in delivering the laboratory test including quality control checks, laboratory safety and the use of equipment as listed in the respective SOPs.

5.1.2 Ensure adequate resources for Laboratory Staff to perform the work tasks using this SOP.

5.1.3 May delegate selected duties and/or responsibilities to Scientific Advisors and/or Technical Team Leaders to assist in verifying the quality control checks of the test performance, laboratory results and patient demographics.

5.1.4 Ensure the laboratory results are accurate.
5.1.5 Provides clinical advice with respect to:
5.1.5.1 Interpretation of laboratory results where appropriate.
5.1.5.2 Choice of examination and use of the laboratory test services where appropriate.
5.1.5.3 Generating clinically relevant information on laboratory report where appropriate.
5.1.6 Approve the laboratory report generated.
5.1.7 Shall maintain the ultimate responsibility for the laboratory report generated.
5.1.8 Review and approve Quality Improvement Plan when required.

5.2 Scientific Advisors/Technical Team Leaders

5.2.1 To conduct adequate training and ensure the competency of all Laboratory Staff involved in using this SOP.
5.2.2 Inform all Laboratory Staff involved of this SOP and/or any related SOPs and the risk assessments conducted.
5.2.3 Ensure all Laboratory Staff are compliant with good laboratory practices and any applicable regulatory requirements.
5.2.4 Address any laboratory procedure or any arising issue raised by Staff.
5.2.5 Review and monitor the progress of the corrective and preventive action taken where applicable.
5.2.6 Assist to verify the quality control checks of the sample and laboratory test performance, laboratory results, reports and patient demographics.
5.2.7 Ensure Laboratory Staff meet the expected quality control standards written in this SOP.
5.2.8 Investigate and document incidents such as failed quality control checks or discrepant laboratory test results. Review workflow processes and implement Quality Improvement Plan if required.
5.2.9 Ensure laboratory reports are released within the stipulated turnaround time (+ 1-2 days deviation is acceptable).
5.2.10 May delegate selected duties and/or responsibilities to Laboratory Staff to assist in recording and documenting all relevant information, laboratory test results and reports; including routine equipment maintenance and housekeeping.

5.2.11 Ensure critical equipment used for laboratory examinations (tests) have yearly preventive maintenance plans conducted.

5.3 Laboratory Staff

5.3.1 Ensure adequate training is given; and have passed work competency assessment to perform the assigned task.
5.3.2 Inform respective Team Leader (Technical Manager) or Laboratory Management on the resources required in order to perform the task where applicable.
5.3.3 Understand and follow the instructions in this SOP including fulfilling the Quality Control and any applicable regulatory requirements.
5.3.4 Ensure patient demographics and laboratory results are reliable and accurate.
5.3.5 Submit timely laboratory results and reports (drafts) for verification by the Scientific Advisors and/or Technical Team Leaders.
5.3.6 Ensure laboratory reports generated meet the expected turnaround time.
5.3.7 Proper recording and documenting of all relevant laboratory information, results and reports.
5.3.8 Raise any laboratory procedure, quality control measure, or any arising issue with respective Team Leader (Technical Manager) or Laboratory Management; resolve together where applicable.
5.3.9 Ensure that critical equipment used for laboratory tests have yearly preventive maintenance conducted.
5.3.10 Conduct routine equipment maintenance where applicable.
5.3.11 Maintain good housekeeping.
5.3.12 Participate in external quality control assessments at least once in two years.

6. MATERIALS AND EQUIPMENT

6.1 Sample Source

6.1.1 cDNA (complementary DNA)
6.1.1.1 from Bone Marrow (BM)
6.1.1.2 from Peripheral Blood (PB)

Note: Recommended to use 1.5µg RNA to reverse transcribe to cDNA per reaction tube (30µl).

Additional Information can be found in the following SOP and RA	
MOL-07	Reverse Transcribe Total RNA to cDNA using Random Primers Preparation of cDNA

6.2 Consumables and Reagents

Items	Brand	Catalogue No	Comment
AutoDG Oil For Probes	Bio-Rad	1864110	
ddPCR™ Droplet Reader Oil	Bio-Rad	1863004	
ddPCR™ Supermix For Probes (No dUTP)	Bio-Rad	1863024	
TaqMan Probes	IDT/Sigma Aldrich	--	Custom Order
Nuclease-Free Water	Any	--	Molecular Grade Water
ddPCR™ Plates 96-Well, Semi-Skirted	Bio-Rad	12001925	
DG32 Cartridge	Bio-Rad	1864108	
Pipet Tips for AutoDG	Bio-Rad	1864120	
Pierceable Foil Heat Seal	Bio-Rad	1814040	
1.5 mL Micro-Centrifuge Tubes	Any	--	
0.6 mL Micro-Centrifuge Tubes	Any	--	
Synthesized Ultramer® DNA oligonucleotides	Integrated DNA Technologies	--	

6.3 Equipment

Items	Brand	Catalogue No.	Comment
Automated Droplet Generator	Bio-Rad	1864101	
QX200™ Droplet Reader	Bio-Rad	1864003	
C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module	Bio-Rad	1851197	
PX1™ PCR Plate Sealer	Bio-Rad	1814000	
Micro-centrifuge	Any	--	
Vortex	Any	--	

7. SAFETY PRECAUTIONS

7.1 Chemical and Biological Hazards

7.1.1 RNA may pose as a potential biohazardous material. Make sure appropriate Personal Protective Equipment (PPE) are worn/used at all times e.g. gloves and lab coats.

7.1.2 Chemicals used may be hazardous, always refer to SDS prior to conducting experiments.

7.1.3 Safety datasheets (SDS) on the chemicals should be readily available for references.

7.2 Equipment Associated Hazards

7.2.1 General electrical safety precautions for normal equipment are applicable for any electrical equipment involved – avoid handling equipment with wet hands, do not overload electrical sockets and label each plug with equipment type.

7.2.2 Extra safety precautions are to be taken for operation of micro-centrifuge and centrifuge. Make sure centrifuge is balanced. Always ensure it comes to a complete halt before handling.

7.2.3 When handling fridge or freezer with more than 1 door, please ensure that the overhead door is completely closed before commencing on other activities (i.e. picking up items from floor).

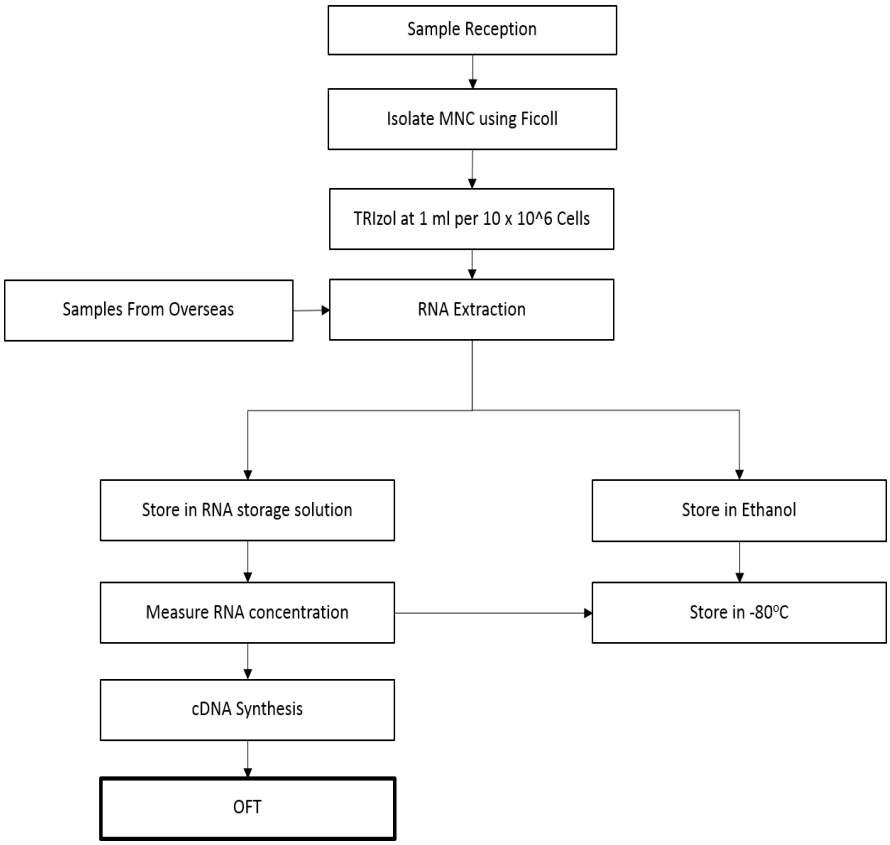
7.2.4 The plate sealer may heat up the metal plate holder and plate cover when left inside the sealer for extended period. Remove the plate holder and plate cover from the plate sealer to avoid handling heated items.

7.2.5 Additional equipment-associated hazards are found in the following documents.

Additional Information can be found in SOP and/or RA	
EQP-08	Operation and Maintenance of NanoDrop™ 2000
EQP-13b	Operation and Maintenance of Bio-Rad C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module
EQP-32	Operation and Maintenance of Bio-Rad Automated Droplet Generator (AutoDG™)
EQP-32a	Operation and Maintenance of Bio-Rad PX1™ PCR Plate Sealer
EQP-32b	Operation and Maintenance of Bio-Rad QX200™ Droplet Reader (with QuantaSoft Software)

8. PROCEDURES

8.1 Flowchart



Additional Information can be found in SOP and/or RA	
MOL-12	Isolation of Mononuclear cells by Ficoll Paque
MOL-06	RNA Extraction from BM or PB using TRIzol
MOL-17	Measuring RNA Concentration and Quality Assessment
MOL-07	Reverse Transcribe Total RNA to cDNA using Random Primers

8.2 Determining & Designing OFT breakpoint primer pairs using Oligo 6 software

8.2.1 Obtain the OFT breakpoint information from available lab tests:
- 8.2.1.1 Real-Time PCR (ALL panel, AML panel)
- 8.2.1.2 RNA sequencing
- 8.2.1.3 Sanger Sequencing (For sequences obtained from external sources).

8.2.2 If existing primer/probes for the breakpoints are not available, proceed to design primers and probes for the novel fusions.
- 8.2.2.1 TaqMan probe should have a melting temperature (Tm) of 65–70°C
- 8.2.2.2 TaqMan probe should have a melting temperature (Tm) of 60°C
- 8.2.2.3 short amplicon length (about 200bp)
- 8.2.2.4 GC% in the range of 20-80%
- 8.2.2.5 No dimers
- 8.2.2.6 No hairpin stems

Additional Information can be found in SOP and/or RA	
TEST-02	Detection of Oncogene Fusion Transcripts in ALL and AML patients

8.3 Reagent Preparation for Primer and Probe Mixes

8.3.1 Order FAM probe and primers for the appropriate OFT breakpoint and HEX probe and primers for housekeeping gene, *GUSB*.

8.3.2 Prepare working stock of both OFT and *GUSB* primer/probe mixes at 4.0µM for the primers and 2.0µM for probe (Refer to Appendix 13.1 for the available probes and sequences).

8.4 Experimental set up for ddPCR OFT Quantitation

8.4.1 ddPCR OFT Assay Worksheet Preparation

8.4.1.1 Indicate the name of fusion gene that is to be examined and fill in the required details appropriately.

8.4.1.2 Each OFT assay should include:

8.4.1.2.1 Positive Control - at least 1 replicate of Diagnostic (Dx) cDNA (100x diluted)

Note: If the Dx sample is not available, 1x10-9uM ($1x10^{-10}$ dilution from 10uM) of oligos with the same breakpoint may be used as the technical positive control.

8.4.1.2.2 Negative Control - 1 replicate of HL60

8.4.1.2.3 No template Control (NTC) - 1 replicate

8.4.1.2.4 Follow-up samples – at least 2 replicates

8.4.1.3 Each *GUSB* assay should include:

8.4.1.3.1 Inter-assay control - at least 1 replicate of HL60 (100x diluted)

8.4.1.3.2 Positive Control - 1 replicate of Dx cDNA (100x diluted)

8.4.1.3.3 No template Control (NTC) - 1 replicate

8.4.1.3.4 Follow-up sample – at least 2 replicates (100x diluted)

8.4.1.4 Fill in the required number of OFT and *GUSB* controls and samples in the 96-well table (12 columns by 8 rows).

8.4.1.5 Calculate the number of reactions required for each OFT and *GUSB* assay respectively. To include one extra reaction for each assay in the calculation to ensure sufficiency.

8.4.2 Thaw all reagents to room temperature. Vortex and centrifuge briefly to ensure the reagents are well mixed.

8.4.3 Prepare the OFT and *GUSB* master-mixes according to the volume of reagents required.

Components	Final Concentration	Volume per reaction well (µl)
2× ddPCR Supermix for Probes	1×	11

Primer Probe mix	0.4 µM Primer 0.2 µM Probe	4.4
cDNA or controls	-	5.5
Nuclease-free water	-	Top up to final 22 µl after adding controls/samples.

8.4.4 Aliquot the appropriate volume of the master-mixes into the 96-well plate according to experimental set-up indicated on the ddPCR OFT assay worksheet.

8.4.5 Add in 5.5µl of the controls to the respective wells, followed by the samples (cDNA).

8.4.6 Seal the 96-well sample plate with an adhesive film seal. Vortex the 96-well plate and centrifuge briefly. Allow the plate to equilibrate to room temperature.

8.4.7 The sample plate is ready for droplet generation.

8.5 Droplet Generation

8.5.1 Switch on the Bio-Rad Automated Droplet Generator and load in the sufficient amount pipette tips and cartridges (Each well requires 2 pipette tips and one cartridge well).

8.5.2 Load in a new PCR reaction plate on the cooling block for droplet collection, followed by the sample plate, refer to 8.4.6.

8.5.3 Remove the adhesive film seal from the sample plate.

8.5.4 Ensure the Automated Droplet Generator's lid is closed properly before proceeding to start droplet generation.

8.5.5 Select the AutoDG oil type – "AutoDG oil for probes".

8.5.6 Select the appropriate rows or columns containing the controls and samples.

8.5.7 Start the droplet generation. Check the droplet collection plate for the layer of droplets formed before proceeding to PCR. As the droplets may not be stable at room temperature, proceed to run PCR immediately or keep at 4^{o}C.

Additional Information can be found in the following SOP and RA

EQP-32	Operation and Maintenance of Bio-Rad Automated Droplet Generator (AutoDG™)

8.6 Plate Sealing

8.6.1 Pre-heat the Bio-Rad PX1 PCR Plate Sealer by switching it on.
8.6.2 Lay the pierce-able foil seal on the plate and load the reaction plate on the accompanying metal block. The red line of the seal should be facing up and on the upper side of the plate. Place the metal cover over the foil.
8.6.3 Press the seal button.
8.6.4 Upon sealing the plate, remove the reaction plate and metal accessories from the sealer.
8.6.5 Switch off the sealer to prevent overheating the sealer.

Additional Information can be found in the following SOP and RA	
EQP-32a	Operation and Maintenance of Bio-Rad PX1™ PCR Plate Sealer

8.7 PCR

8.7.1 Switch on the C1000 Touch™ Thermal Cycler and load the sealed 96-well reaction plate containing the droplets and start the program.
8.7.2 Start the ddPCR OFT program on the C1000 Touch™ Thermal Cycler.

Temperature	Time	Cycles	Remarks
95ºC	10 min		
94ºC	30 s	40	
60ºC	1 min		3'-5'exonuclease activity that hydrolyses the TaqMan probe is most active at 60°C and the short amplicon (~200bp+/-) ensure a high reaction efficiency making it possible to have a 2-step PCR cycle
98ºC	10 min	1	
12ºC	Hold	infinity	

8.7.3 Upon completion, transfer the plate to the droplet reader. As the droplets may not be stable at room temperature, proceed to read the droplets immediately or keep at 4ºC until ready to run PCR.

Additional Information can be found in the following SOP and RA	
EQP-13b	Operation and Maintenance of Bio-Rad C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module

8.8 Droplet Reading

8.8.1 Switch on the Bio-Rad QX200™ Droplet Reader.
8.8.2 Click open the QuantaSoft software.
8.8.3 Create a new experiment template in QuantaSoft software.
8.8.4 Select "Absolute Quantification (ABS)" for the type of experiment set-up.
8.8.5 Double click each well on the setup page to label each well. Input/Enter:
- 8.8.5.1 Names of control/samples. For HL60 samples used as controls, indicate the batch number.
- 8.8.5.2 Experiment type – ABS
- 8.8.5.3 Supermix type – ddPCR Supermix for probes (no dUTP).
- 8.8.5.4 Label name of target gene for each well. Target 1 should be the OFT of interest (FAM) and Target 2 should be the *GUSB* (HEX).
- 8.8.5.5 Select the corresponding control type for each well (positive, negative and NTC).
- 8.8.5.6 Follow-up sample for quantitation should be set as unknown.

8.8.6 Load the Post-PCR plate into the droplet reader, secure it with the plate cover and click run to start reading.

Additional Information can be found in the following SOP and RA	
EQP-32b	Operation and Maintenance of Bio-Rad QX200™ Droplet Reader (with QuantaSoft Software)

8.9 Data Analysis and Calculation

8.9.1 Visual Inspection

Check the 1D and 2D amplitude views for each well after the droplet reading.

8.9.1.1 Positive control for the specific target gene shows a distinct cluster of positive droplets and clear separation from the baseline formed by the negative droplets.

8.9.1.2 The negative droplets should be well aligned across the different wells.

8.9.1.3 To exclude any outlier droplets which are out of the cluster.

8.9.2 **Determining Thresholds**

8.9.2.1 Export out the amplitude and cluster data for the selected wells from the QuantaSoft software.

8.9.2.1.1 For OFT, export the data for the positive, negative and NTC controls.

8.9.2.1.2 For *GUSB*, export the data for all the *GUSB* wells in that run, including samples.

8.9.2.2 To determine the threshold for OFT

8.9.2.2.1 Run the "threshold for positive events R" script for the FAM channel on R Studio software

8.9.2.2.2 Select the downloaded data files.

8.9.2.2.3 Round off the threshold value generated to whole number.

8.9.2.3 Apply the threshold to all the OFT wells in the Quantasoft software. Save the 1D amplitude view of the assay in the patient's run folder.

8.9.2.4 To determine the threshold for *GUSB*

8.9.2.4.1 Run the "threshold for positive events.R" script for the HEX channel on R studio software.

8.9.2.4.2 Select the downloaded data files.

8.9.2.4.3 Round off the threshold value generated to whole number.

8.9.2.5 Apply the threshold to all the *GUSB* wells in the Quantasoft software.

8.9.2.6 Visually check that set thresholds separates the positive and negative clusters appropriately. Otherwise, manual adjustments of the thresholds may be required.

8.9.2.7 Save the 1D amplitude view of the OFT and *GUSB* assays in the patients' run folder.

8.9.3 **Calculating OFT Level**

8.9.3.1 The OFT level calculation would be performed on the "Quantitation Result" spreadsheet.

8.9.3.2 Enter the concentration, merge concentration, positive events and total events of the samples and controls onto the appropriate columns on the "Quantitation Result" spreadsheet. Outlier droplets identified in 8.9.1.3 should be excluded manually from the positive events and subsequent calculations.

8.9.3.3 In cases where the merge concentrations have to be recalculated due to exclusion of outlier droplets or require manual input, the formula is:

Merged Concentration = -ln [1-(Total positive events/Total events)]/0.00085

8.9.3.4 The Absolute OFT level (normalised to *GUSB*) will be computed.

8.9.3.5 The Relative OFT level will also be computed by normalising the absolute OFT level of the sample to that of the Diagnostic sample (if available).

8.9.4 **Calculating Quantifying Limit and Limit of Quantitation**

8.9.4.1 Update the number of events obtained for OFT assay on the "OFT baseline odd quantitation" spreadsheet for the particular OFT run.

8.9.4.2 Update the newly computed OFT baseline odd into the "Quantitation Result".

8.9.4.3 Baseline odd is computed based on the formula: Concentration of OFT/Concentration of *GUSB* in negative control (HL60). If the total event is zero, a value of "1" is used.

8.9.4.4 Run the "LOQ range.R" script on R studio. Enter **N1** as the total *GUSB* copies of the follow-up sample and **P** as the newly computed baseline odd.

8.9.4.5 The value generated for 95.00% cumulative probability is the copies for the quantifying limit for negativity.

8.9.4.6 The value generated for 99.99% cumulative probability is the copies for the quantifying limit for positivity.

8.9.4.7 If the sample's OFT level above or equal to the positive quantifying limit, it is positive.

8.9.4.8 If the sample's OFT level below the negative quantifying limit, it is negative.

8.9.4.9 If the sample's OFT level is between the negative and positive quantifying limits, the sample is within the limit of quantitation range (LOQ).

8.10 Recommendations

8.10.1 **Limited RNA**

If the total RNA is less than 2µg, add the entire volume (max volume 5ul) of RNA during the cDNA synthesis.

Note: Staff to inform Team Leader of inadequate RNA. May have to request for additional samples from the requesting clinician.

8.10.2 **OFT DNA breakpoints**

In exceptional cases, the ddPCR OFT DNA levels may be monitored instead of RNA expression levels. In this case, DNA primers should be optimised beforehand and 1µg DNA should be loaded per sample well in triplicates. Albumin is recommended as the housekeeping gene for DNA.

Additional Information can be found in the following SOP and RA	
Mol-22	Quantitation of Target Gene by Droplet Digital PCR using TaqMan® Hydrolysis Probes

8.10.3 **Alternative relapse sample for OFT level quantitation**
In cases where Diagnostic sample is not available, Relapse sample of known blast count (more than 30%) can be used to compute relative OFT level instead.

8.11 Troubleshooting

8.11.1 If the controls does not amplify as per QC criteria (refer to section 10), the run should be repeated by replacing with another control aliquot.
8.11.2 Resynthesizing new batch of cDNA controls or changing the reagents.
8.11.3 If the test sample has poor RNA quality based on *GUSB* concentration (Section 9.1.2), the assay should be repeated with a newly synthesized cDNA aliquot and/or using new aliquots of ddPCR reagents.

9. REPORTING

Reports should be generated following the guidelines from WP-08 SOP.

Additional Information can be found in SOP and RA	
WP-08	Preparation and Release of Lab Results Reports

9.1 Verifying Results

9.1.1 **Assay Controls**
9.1.1.1 Ensure positive control has positive droplet cluster.
9.1.1.2 Ensure the negative and NTC controls have ≤ 3 positive droplets.

9.1.2 **RNA Quality of sample**
Expected *GUSB* concentration for different RNA qualities:
9.1.2.1 Good Quality: ≥ 20 copies/µl
9.1.2.2 Acceptable Quality: 10-20 copies/µl
9.1.2.3 Poor Quality: ≤10 copies/µl

9.2 Result Interpretation

Result	Result Interpretation
Positive (POS) (Report actual value, e.g. 1.03E-05)	The sample is **POSITIVE** for the OFT (e.g. TCF3-PBX1 (E2A-PBX1)/t(1;19)).
Negative (NEG)	The sample is **NEGATIVE** for the OFT (e.g. TCF3-PBX1 (E2A-PBX1)/t(1;19)).
Limit of Quantitation (LOQ)	The sample is within the **Limit of Quantitation** range for OFT (e.g. TCF3-PBX1 (E2A-PBX1)/t(1;19)). The sample may be positive but does not satisfy the criteria for positivity.
Unqualified (Report as N.A.)	The sample is **unqualified** for quantitation. The sample does not meet the minimum RNA input requirement for quantitation. OR The sample does not meet the minimum RNA quality requirement for quantitation. (*Report as unqualified only if no positivity is detected.)

9.3 Graph Plotting

9.3.1 Include graph of results in the report if more than one time-point is measured. Plot the graph of Absolute or Relative OFT levels against Time (weeks). Indicate the OFT as the title of the graph.

9.3.2 Baseline level at diagnosis (if sample available) is defined as 100%

9.3.3 Negative result(s) is/are plotted as 1E-08.

9.4 Turnaround Time

The stipulated TAT of this Lab Test is 1 week.

10. QUALITY CONTROL

10.1 RNA Yield and OD ratio

10.1.1 There should be at least 1.5µg of RNA available for cDNA synthesis.
10.1.2 The A260/280 ratio between 1.8-2.1.

Additional Information can be found in SOP and RA	
MOL-08	Preparation and Release of Lab Results Reports

10.2 Assay Controls and Housekeeping Gene

10.2.1.1 As per 9.1.2, *GUSB* should be of Good Quality.

10.3 Reagent Preparation

10.3.1 All working reagents and master-mixes should be aliquot out to prevent contamination of the primary stock and to maintain quality performance.
10.3.2 Date of preparation (DD/MM/YYYY) should be indicated on all reagent working stock and log-sheets.
10.3.3 Ensure all reagents used are within stipulated shelf life. Order primers and probes from authorised manufacturer. The COAs of the reagents should be satisfactory.
10.3.4 When reagents are running low or the previous batches are not working properly, in-house prepared reagents are to be prepared according to the preparation sheet. To monitor the run number which the reagents are tested. Record the information on the reagent preparation logsheets.

10.4 TAT

Release Lab test reports within TAT

10.5 Proficiency Testing

10.5.1 Training records of staff
10.5.2 Internal and/or External Quality Assessment

10.6 Equipment Preventive Maintenance

10.6.1 Automated Droplet Generator
10.6.2 QX200 Droplet Reader
10.6.3 C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module.

11. RECORD MANAGEMENT

11.1 Records

11.1.1 Primers/Probes Order Date
11.1.2 HL60 run records
11.1.3 OFT baseline odd quantitation
11.1.4 Reagent preparation log-sheet
11.1.5 COA of reagents
11.1.6 Training Records
11.1.7 Other QC records

11.2 Worksheets

11.2.1 ddPCR OFT Assay worksheet
11.2.2 ddPCR cDNA synthesis worksheet

11.3 Results

11.3.1 Printouts of ddPCR results
11.3.2 ddPCR OFT quantitation results record

11.3.3 ddPCR OFT lab report

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13. APPENDICES

13.1 Primer Probe Sequences

13.2 R Scripts for ddPCR (“Threshold for positive events” and “LOQ Range”)

14. AMENDMENT/REVIEW HISTORY

Version	Date	Amendment/Review	Recorded by
V1.1	18/10/21	Changed run condition in 8.7.2. 6.1.1 – Change to 1.5ug of RNA. 8.4.1.2.4 and 8.4.1.34 – at least 2 replicates 10.1 – Changed to RNA yield 1.5ug 9.4 – Changed TAT to 2 weeks. Added in 10.3.4	Evelyn Lim