VIVA-NUS CenTRAL
Centre for Translational Research in Acute Leukaemia (Molecular)
Department of Paediatrics, Yong Loo Lin School of Medicine

Standard Operating Procedures (SOP)

Title: Droplet Digital™ PCR MRD using TaqMan® Hydrolysis Probes

Document Ref No.:	TEST-13
Version No.:	1.1
Issue Date:	Jul 2021
Review Date:	03 Sep 2021

Reviewed by:
Name: Evelyn Lim

Approved by:
Name: A/Prof Allen Yeoh Eng Juh

1. BACKGROUND

V(D)J recombination of IgH/TCR genes is a mechanism of genetic recombination in the early stages of immunoglobulin (Ig) and T cell receptors (TCR) production of the immune system. V(D)J recombination takes place in the primary lymphoid tissue (the bone marrow for B cells, and Thymus for T cells). V(D)J recombination randomly combines **V**ariable, **D**iverse, and **J**oining gene segments in lymphocytes. Due to the random nature of combination between different genes, this results in diversely encoded proteins to match antigens from leukaemia cells. V(D)J gene rearrangements are specific to every leukaemia cell and can be used as a surrogate biomarker for measuring minimal residual disease to monitor treatment response.

Minimal Residual Disease (MRD) refers to trace amounts of sub-microscopic leukaemia cells remaining in the patient after treatment or after bone marrow transplantation to confirm molecular remission[1-3]. It is well established that MRD is an independent prognostic factor in acute lymphoblastic leukaemia (ALL) for treatment stratification for most contemporary ALL protocols in developed countries[4-9]. Molecular remission is pre-requisite for cure.

Real-time Quantitative PCR (RQ-PCR) is the gold standard for MRD monitoring. However, it is limited by the availability of the diagnostic materials. A significant fraction of patients with low MRD levels below the level of sensitivity, which may be difficult to interpret in clinical practice.

Droplet Digital Polymerase Chain Reaction (ddPCR™) was developed to provide high precision, absolute quantitation of nucleic acid target sequences. ddPCR measures absolute quantities by counting nucleic acid molecules encapsulated in discrete, volumetrically defined water-in-oil droplet partitions (Figure 1). Its technical advantages enable detection and analysis of nucleic acids at a level of sensitivity and precision beyond the capabilities of RQ-PCR. A typical ddPCR application that focuses on the lower limits of nucleic acid detection is to measure MRD 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 2).

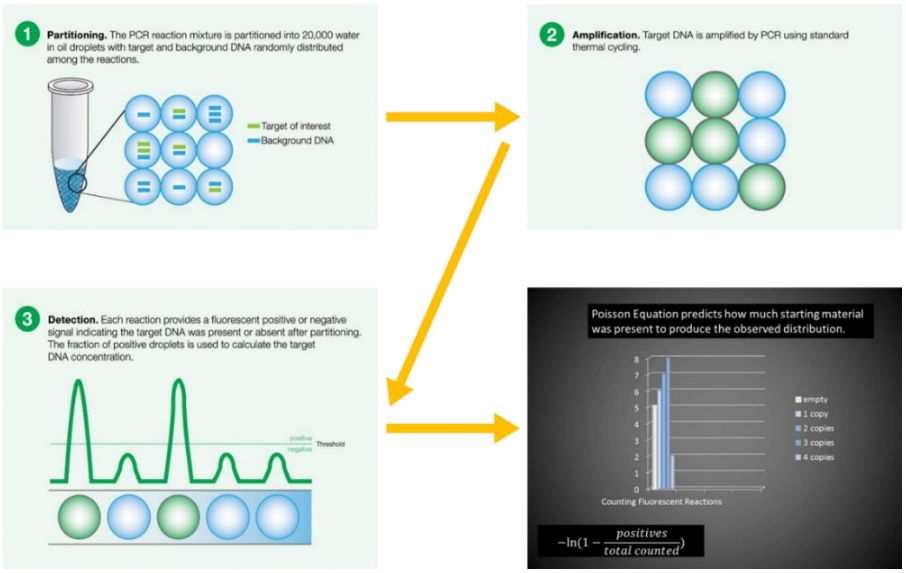


Figure 1 Illustration of ddPCR workflow (Adapted from BioRad).

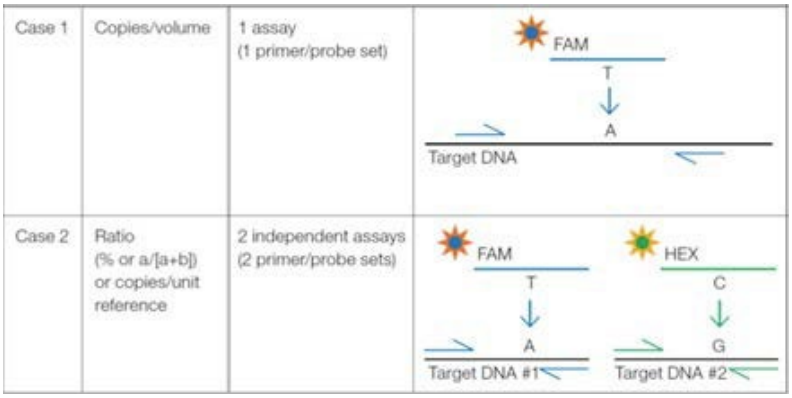


Figure 2. OFT Quantitation using ddPCR (Adapted from BioRad).

2. PURPOSE

This Standard Operating Procedure describes the steps in performing quantitation of Minimal Residual Disease (MRD) by droplet digital PCR (ddPCR) using TaqMan® hydrolysis probes.

3. SCOPE

The procedure applies to CenTRAL (Molecular) staff and personnel performing MRD quantitation by ddPCR using TaqMan® hydrolysis probes.

4. DEFINITIONS

4.1. Acute Lymphoblastic Leukaemia (ALL): Cancer of white blood cells characterised by overproduction of immature, malignant lymphoblasts that accumulate in the bone marrow. They interfere with the product of normal white blood cells.

4.2. Minimal Residual Disease (MRD): Monitor sub-microscopic minimal or traces of residual disease or in ALL.

4.3. Ig/TCR rearrangements: Specific rearrangements in the B-cells and T-cells resulting from V(D)J recombination.

4.4. Mononuclear Cells (MNCs): Pooled mononuclear cells obtained from at least 5 healthy subjects.

4.5. Droplet Digital Polymerase Chain Reaction (ddPCR): a methodology 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.

4.6. Albumin gene: A housekeeping gene to monitor DNA integrity and quality.

4.7. Event: An eligible droplet detected and recorded by the reader.

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

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

4.10. Normalisation: Normalise the fusion gene copies per housekeeping gene (ALB) copy.

4.11. 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.

4.12. Absolute MRD level: The actual normalised copy number of Ig/TCR rearrangement of interest against the housekeeping gene (Albumin) in the test sample.

4.13. Relative MRD level: The normalised copy number of the Ig/TCR rearrangement in the test sample is compared against the normalised copy number of the Ig/TCR rearrangement at diagnosis/relapse. It expressed as a ratio.

4.14. Positive (POS): The MRD level in the test sample is significantly different from that measure in the negative control ($p>0.0001$).

4.15. Negative (NEG): The MRD level in the test sample is not significantly different from that measure in the negative control ($0.05>p>0.0001$).

4.16. Limit of Quantitative Range (LOQ): The quantity range in which the MRD level 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 ($p>0.05$).

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

5. RESPONSIBILITIES

5.1. Laboratory Director

5.1.1. Ensure Laboratory Management informed Staff involved in this area of work on this SOP and they understand the Risk Assessments conducted.

5.1.2. Ensure Laboratory Management provides a safe laboratory environment that complies with good laboratory practice and applicable NUS OSHE and/or regulatory requirements.

5.1.3. Review and approve SOP and Risk Assessments.

5.2. Laboratory Management (Quality & Technical Managers)

5.2.1. Ensure Staff involved in this area of work understand this SOP and the Risk Assessments conducted.

5.2.2. Maintain and/or update SOP and Risk Assessments when there is a change in process.

5.2.3. Provide adequate training and ensure availability of resources to ensure Staff is competent to perform the task.

5.2.4. Ensure a safe laboratory environment that complies with good laboratory practice and applicable NUS OSHE and/or regulatory requirements.

5.2.5. Address any laboratory procedure, quality control measure, or any arising issue raised by Staff.
5.2.6. Review and monitor the progress of corrective and preventive action taken where applicable.

5.3. Laboratory Staff

5.3.1. Understand this SOP and the Risk Assessments conducted.
5.3.2. Ensure adequate training is provided to perform task competently.
5.3.3. Raise any laboratory procedure, quality control measure, or any arising issue to the Laboratory Management. Address and resolve issues appropriately.

5.4. Research Staff/Graduate or Attachment Students and Visitors

5.4.1. Understand this SOP and the Risk Assessments conducted.
5.4.2. Ensure adequate training is provided to perform task competently.
5.4.3. Raise any arising issue with immediate Supervisor.

6. MATERIALS AND EQUIPMENT

6.1. Sample Source

6.1.1. DNA Extracted from:
6.1.1.1. Bone Marrow aspirate
6.1.1.2. Peripheral Blood
Note: Samples collected in EDTA and NaHep collection tubes are acceptable, EDTA is the preferred choice.

Additional Information can be found in the following SOP and RA	
MOL-02	DNA Extraction of Mononuclear Cells – TRIzol
MOL-12	Isolation of Mononuclear Cells by Ficoll-Paque Density Gradient

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		

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

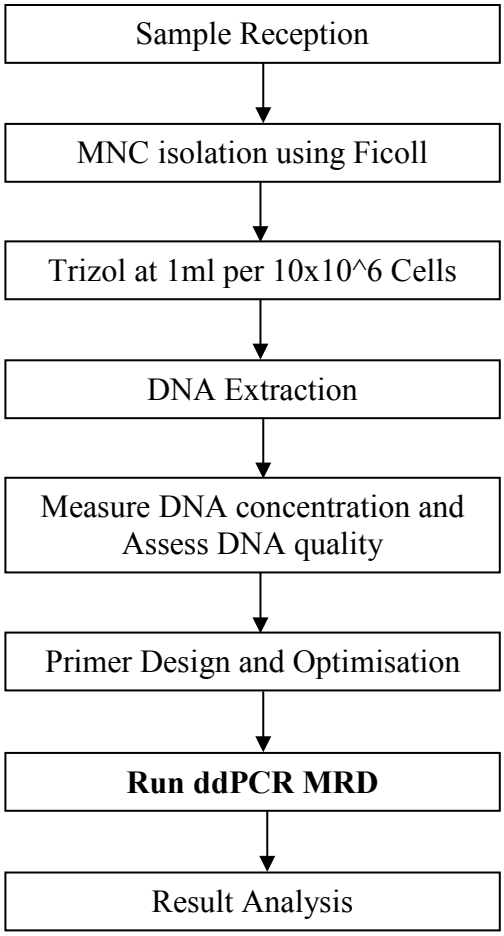
7.1.1. DNA may pose as a potential biohazardous material. Make sure appropriate Personal Protective Equipment (PPE) are worn/used at all times e.g. lab coat, gloves, covered shoes and safety glasses.
7.1.2. Chemicals used may be hazardous, always refer to SDS prior to conducting experiments.
7.1.3. Safety 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. When using the centrifuge, balance the rotor when loading tubes/plates and before centrifugation. Always close the lid securely when centrifuge is in use. Make sure centrifuge is balanced. Always ensure it comes to a complete halt before handling. Imbalanced centrifuge rotor may cause damage to equipment and injury to user.
7.2.3. When removing PCR plate from C1000 Touch™ Thermal Cycler, take caution that the plate may be hot (60-95 °C). Allow the plate to cool for 10 minutes before removal. It may cause minor burns.
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. 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).
7.2.6. Additional equipment-associated hazards are found in the following SOP and RA listed in the table below.

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



Additional Information can be found in SOP and/or RA	
MOL-12	Isolation of Mononuclear cells by Ficoll Paque
MOL-02	DNA Extraction of Mononuclear Cells – TRIzol
MOL-16	Measuring DNA Concentration and DNA Quality Assessment
TEST-01	Minimal Residual Disease

BP-05	DdPCR MRD Analysis Pipeline

8.1. Primer design and optimisation

8.1.1. Design and optimise primers on RQ-PCR as according to TEST-01 SOP.

8.1.2. Next, optimise the ddPCR annealing temperature by running a temperature gradient (58ºC to 63ºC) of the Diagnostic/Relapse DNA (10ng).

8.1.3. Prepare and run the primer according to the set up from 8.2 to 8.6. Only 1 replicate of 10ng Diagnostic DNA and 1µg of healthy donors' MNC is required for each temperature (58ºC to 63ºC).

Run condition:

Temperature	Time	Cycles
95ºC	10 min	1
94ºC	30 s	40
58-63ºC	1 min	
98ºC	10 min	1
12ºC	Hold	Infinity

8.1.4. Indicate the run as an Optimisation Run in the file name by including the label "OPT".

8.1.5. Select a suitable annealing temperature with minimal raining droplets and a distinct positive cluster.

8.1.6. Save the 1D-amplitude view onto the patient's ddPCR MRD run folder in CenTRAL folder.

8.2. Reagent Preparation for master-mixes

8.2.1. Order FAM probe and primers for the appropriate Ig/TCR targets and HEX probe and primers for housekeeping gene, Albumin.

8.2.2. Prepare working stock of both Ig/TCR and Alb primer/probe mixes at 10.0µM (Refer to Appendix 13.1 for the available probes and sequences).

8.3. Experimental set up for ddPCR MRD quantitation

8.3.1. **Assay Preparation (in PCR cabinet)**

8.3.1.1. Two assays should be run – the MRD marker assay and the Albumin assay. Prepare the ddPCR MRD assay worksheet. Indicate the samples/controls type and the Ig/TCR marker name on the 96-well template. Calculate the number of reactions required for both the MRD marker assay and Albumin assays, including at least one extra reaction for pipetting allowance.

Each MRD marker assay should include at least one replicate of Dx DNA (10ng) as positive control, at least four replicates of pooled healthy donor MNC DNA as negative control, one replicate of non-template control and at least four replicates of the follow-up sample (1µg) for quantitation.

For the Albumin assay, include at least one replicate of healthy donor MNC DNA, one replicate of Dx DNA (10ng), one replicate of non-template control and at least two replicates of follow-up sample (200x diluted).

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

8.3.1.3. Prepare the MRD marker and Albumin master-mixes according to the volume of reagents required.

Master-mix preparation for MRD marker run:

Components	Final Concentration	Volume per reaction well (µL)
2× ddPCR Supermix for Probes	1×	12.5
Primer and Probe Mix	0.50 µM Primer/ 0.25 µM Probe	1.10
DNA sample	-	Variable (Up to 9.0µL)

Autoclaved Milli-Q water	-	variable (top up to total 22µL per reaction)

Master-mix preparation for Albumin run:

Components	Final Concentration	Volume per reaction well (µL)
2× ddPCR Supermix for Probes	1×	12.5
Primer and Probe Mix	0.50 µM Primer/ 0.5.0 µM Probe	1.10
DNA sample	-	Variable (Up to 9.0µL)
Autoclaved Milli-Q water	-	variable (top up to total 22µL per reaction)

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

8.3.1.5. Add in 6.25µl of the controls to the respective wells, followed by the follow-up samples DNA (200x diluted).

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

8.3.1.7. The sample plate is ready for droplet generation.

8.3.2. **Droplet Generation**

8.3.2.1. 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).

8.3.2.2. Load in a new PCR plate on the cooling plate for droplet collection, followed by the sample plate from 8.3.1.7.

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

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

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

8.3.2.6. Start the droplet generation. Check the 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°C until ready to run PCR.

Additional Information can be found in the following SOP and RA	
EQP-32	Operation and Maintenance of Bio-Rad Automated Droplet Generator (AutoDG™)

8.3.3. **Plate Sealing**

8.3.3.1. Pre-heat the Bio-Rad PX1 PCR Plate Sealer by switching it on.

8.3.3.2. Remove the used adhesive film seal from the sample plate.

8.3.3.3. Lay the pierce-able foil seal on the plate and load the sample 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.

8.3.3.4. Press the seal button.

8.3.3.5. Upon sealing the plate, remove the sample plate and metal accessories from the sealer. 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

8.3.4. **PCR**

8.3.4.1. Switch on the C1000 TouchTM Thermal Cycler and load the sealer 96-well plate containing the droplets and start the programme.

8.3.4.2. Start the ddPCR MRD programme on the C1000 Touch™ Thermal Cycler.

Run condition:

Temperature	Time	Ramp Rate (C/s)	Cycles
95°C	10 min	2	1
94°C	30 s		40
58-63°C*	1 min		
98°C	10 min		1
12°C	Hold		infinity

*This is recommended range. To use optimised annealing temp.

8.3.4.3. 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 4°C 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

8.3.5. **Droplet Reading**

8.3.5.1. Switch on the Bio-Rad QX200™ Droplet Reader.

8.3.5.2. Create a new experiment sheet in QuataSoft software. Select "Absolute Quantificaiton (ABS)" for the type of experiment set-up.

8.3.5.3. Double click each well on the setup page to label each well. Enter the sample/control names, experiment type – ABS, Supermix type – ddPCR Supermix for probes (no dUTP). The target name and type for each well should also be labelled if included. Target 1 should be the IgTCR marker and Target 2 should be the Albumin housekeeping gene. Select the corresponding controls type for each well. Follow-up sample for quantitation should be set as unknown.

8.3.5.4. 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)

8.3.6. **Data Analysis**

8.3.6.1. Prepare the sample sheet in 96-well format and save it as text file (tab delimited) using the following labelling format (Note: Input amount to be entered as XXXXng all empty wells must also be labelled) :

Reaction well	Run Type	Labeling
Diagnostic Sample	MRD Marker	Dx_Mk_<Patient ID>_<Marker>_<Input Amount>
Follow-up Sample		FU_Mk_<Patient ID>_<Marker>_<Input Amount>
MNC		MNC_Mk_<Patient ID>_<Marker>_<Input Amount>
NTC		H2O_Mk_<Patient ID>_<Marker Name>_0
Diagnostic Sample	Albumin	Dx_ALB_<Patient ID>_<Input Amount>
Follow-up Sample		FU_ALB_<Patient ID>_200x
MNC		MNC_ALB _200x_MNC<Batch Number>
NTC		H2O_ALB _0
Empty	Both	EMPTY

8.3.6.2. Run the algorithm for ddPCR MRD monitoring in silent or interactive mode (Refer to BP-05 ddPCR MRD Analysis Pipeline).

8.3.6.3. Enter the dilution factor (default: 200x) for MNC (for Albumin assay)

8.3.6.4. Enter the MNC batch's albumin value (at the same temperature)

8.3.6.5. Enter the Diagnostic (or Relapse) sample's Baseline Threshold value

8.3.6.6. When running the interactive mode, the user may check the excluded droplets (crossed) to include them back in the analysis. They may also cross out droplets to exclude them from analysis.

8.3.6.7. Save the generated report and 2D figures files in the patient folder.

8.3.7. **Recommendations**

8.3.7.1. Limited DNA

If the total follow-up DNA for MRD assessment is insufficient to run 1µg DNA per replicate, add the maximum possible volume. Note: Staff to inform Team Leader of inadequate DNA. May have to request for additional samples from the requesting clinician.

8.3.7.2. Use of relapse sample for MRD assessment.

In cases where Diagnostic samples are not available, relapse sample of high known blast count (more than 30%) can be used to compute relative MRD level instead.

8.3.8. **Troubleshooting**

8.3.8.1. If the run does not amplify as per QC criteria, the run should be repeated by changing another aliquot of controls and/or changing the reagents.

8.3.8.2. If the sample has poor DNA quality, the DNA A260/280 ratio should be rechecked and the Albumin housekeeping gene assay rerun again. Otherwise, the DNA sample may be re-precipitated using ethanol and the assay should be repeated using new reagents.

Additional Information can be found in SOP and RA	
MOL-02	DNA Extraction of Mononuclear Cells -TRIzol®
MOL-16	Measuring DNA Concentration and DNA Quality Assessment
MOL-16a	Re-purification of DNA by Ethanol Precipitation

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 Visual Inspection

- Check the 1D and 2D amplitude views for each well after the droplet reading.
- There should be proper clustering of the positive droplets and negative controls.
- The negative droplets (baseline) should be well aligned across the different wells.
- Check for any outlier droplets which are out of the clusters that should be excluded.

9.1.2 Minimum Events

- ≥ 8,000 events for all wells

9.1.3 Minimum Follow-up Albumin Concentration

- Good: >10,000copies/µL
- Acceptable: 2,000-10,000copies/µL
- Poor:<2,000 copies/µL

9.1.4 Assay Controls

- Ensure positive control has positive droplet cluster (≥10 copies/ µL).
- Ensure the negative and NTC controls have only negative cluster and no positive droplets (≤0.1copies/µL).

9.1.5 Assay Controls for Albumin Assay

- Ensure positive control has positive droplet cluster (≥50 copies/ µL).
- Ensure the negative and NTC controls have only negative cluster and no positive droplets (≤0.1copies/µL).

9.1.6 **Overall QC**

- Pass

Additional Information can be found in SOP and/or RA	
MOL-22	Droplet Digital PCR using TaqMan Hydrolysis Probes for Detecting and Quantifying the Gene of Interests

9.2 Result Interpretation

9.2.1 The following table shows the ddPCR result interpretation to report.

Result	Result Interpretation
Positive (POS) (Report actual value, e.g. 1.03E-05)	MRD **positive**. Detectable disease at 1.03E-05.
Negative (NEG)	MRD **negative**. No detectable disease above the negative threshold.
Limit of Quantitation (LOQ)	MRD is within the **Limit of Quantitation (LOQ)** range. The sample may be positive but does not satisfy the criteria for positivity.
Unqualified *report as unqualified only if no positivity detected. (Report as N.A. Indicate reason in Remarks column)	The sample is **unqualified** for quantitation. (1) The sample does not meet the minimum DNA input requirement for quantitation. (2) The sample does not meet the minimum DNA quality requirement for quantitation.

10. QUALITY CONTROL

10.1 DNA yield and A260/280 ratio

10.1.1 There should be at least total 5µg of DNA available for ddPCR MRD. The A260/280 ratio should be 1.6 to 1.94 (Refer to MOL-16 for more information).

10.2 QC statuses (from the ddPCR algorithm report)

10.2.1 The QC statuses should be pass/good for all components.

10.3 Reagents Preparation

10.3.1 All working reagents and master-mixes should be aliquot out to prevent contamination of main (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 form 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 Result verification and reporting

10.4.1 Results are to be verified and reporting by trained, authorised personnel.

10.4.2 Ensure details and results are accurate.

10.5 TAT

10.5.1 Release lab test reports within TAT.

10.6 Proficiency Testing of staff

10.6.1 Training records of staff.

10.7 Quality Assessment

10.7.1 Intra-laboratory comparison

10.8 Equipment Preventive Maintenance

10.8.1 Automated Droplet Generator
10.8.2 QX200 Droplet Reader
10.8.3 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 Log-sheets
11.1.3 Dx Tumorload Database
11.1.4 Intra-laboratory comparison records
(Instructions, Results Submission and Performance Report)
11.1.5 Validation Reports
11.1.6 Templates
11.1.7 Measurement of uncertainty
11.1.8 Certificates of Analysis (COAs)
11.1.9 MNC batch testing record (Albumin)
11.1.10 Non conformity record
11.1.11 Report Amendment record
11.1.12 Staff Training Records
11.1.13 DNA databases
11.1.14 TAT record

11.2 Worksheets

11.2.1 ddPCR MRD Assay worksheet

11.3 Results

11.3.1 ddPCR results (algorithm generated report)

11.3.2 ddPCR MRD lab report

12. REFERENCES

12.1 van Dongen JJM, Langerak AW, Bruggemann M, et al. Design and standardization of PCR primers and protocols for detection of clonal immunoglobulin and T-cell receptor gene recombinations in suspect lymphoproliferations: Report of the BIOMED-2 Concerted Action BMH4-CT98-3936. Leukemia, 2004:17;2257-23

12.2 Pongers-Willemse MJ, Verhagen OJHM, Tibbe GJM, et al. Real-time quantitative PCR for the detection of minimal residual disease in acute lymphoblastic leukemia using junctional region specific TaqMan probes. Leukemia 1998:12;2006-2014.

12.3 Pongers-Willemse MG, Seriu T, Stolz F, et al. Primers and protocols for standardized detection of minimal residual disease in acute lymphoblastic leukemia using immunoglobulin and T cell receptor gene rearrangements and TAL1 deletions as PCR targets: report of the BIOMED-1 CONCERTED ACTION: investigation of minimal residual disease in acute leukemia. Leukemia 1999:13;110-118.

12.4 Sutton R, Shaw PJ, Venn NC, Law T, Dissanayake A, Kilo T, Haber M, Norris MD, Fraser C, Alvaro F, Revesz T, Trahair TN, Dalla-Pozza L, Marshall GM, O'Brien TA. Persistent MRD before and after allogeneic BMT predicts relapse in children with acute lymphoblastic leukaemia. Br J Haematol. 2015 Feb;168(3):395-404.

12.5 **Yeoh AEJ**, Hany Ariffin, Elaine Li Leng Chai, Cecilia Sze Nga Kwok, Yiong Huak Chan, Kuperan Ponnudurai, Dario Campana, Poh Lin Tan, Mei Yoke Chan, Shirley Kow Yin Kham, Lee Ai Chong, Ah Moy Tan, Hai Peng Lin, Thuan Chong Quah. Minimal Residual Disease-Guided Treatment De-Intensification for Children with Acute Lymphoblastic Leukemia: Results from the Malaysia-Singapore (Ma-Spore) ALL 2003 study. J Clin Oncol 2012 Jul 1:30(19):2384-92.

12.6 Kwok CS, Kham SK, Ariffin H, Lin HP, Quah TC, **Yeoh AEJ**. Minimal residual disease (MRD) measurement as a tool to compare the efficacy of chemotherapeutic drug regimens using Escherichia Coli-asparaginase or

Erwinia-asparaginase in childhood acute lymphoblastic leukemia (ALL). Pediatr Blood Cancer, 2006 Sep:47(3):299-304.

12.7 Pieters R, de Groot-Kruseman H, Van der Velden V, et al. Successful therapy reduction and intensification for childhood acute lymphoblastic leukemia based on minimal residual disease monitoring: Study ALL10 From the Dutch Childhood Oncology Group. J Clin Oncol. 2016; 34(22):2591-2601.

12.8 Vora A, Goulden N, Wade R, et al. Treatment Clinical utility of sequential minimal residual disease measurements in the context of risk-based therapy in childhood acute lymphoblastic leukaemia: a prospective study. Lancet Oncol. 2015;16(4):465-474.

12.9 Borowitz MJ, Devidas M, Hunger SP, et al; Children's Oncology Group. Clinical significance of minimal residual disease in childhood acute lymphoblastic leukemia and its relationship to other prognostic factors: a Children's Oncology Group study. Blood. 2008; 111(12):5477-5485.

12.10 Sun L, Quah TC, **Yeoh AEJ**, Lee BW. T-cell receptor gene rearrangements in childhood acute lymphoblastic leukaemia and its implications in detection of minimal residual disease. Cancer Research, Therapy and Control, 1996;5:59-65.

12.11 **Yeoh AEJ**, Quah TC. Minimal residual disease in childhood acute lymphoblast leukemia – An Asian perspective. J Am Med Assoc (South East Asia Ed), 1998:14:9.

12.12 Szczepański T, Flohr T, van der Velden VH, Bartram CR, van Dongen JJ. Molecular monitoring of residual disease using antigen receptor genes in childhood acute lymphoblastic leukaemia. Best Pract Res Clin Haematol. 2002 Mar;15(1):37-57. Review

12.13 van der Velden VHJ, Wijkhuijs JM, Jacobs DCH, et al. T cell receptor gamma gene rearrangements as targets for detection of minimal residual disease in acute lymphoblastic leukemia by real-time quantitative PCR analysis. Leukemia 2002:16;1372-1380.

12.14 van der Velden VHJ, Willemse MH, van der Schoot CE, et al. Immunoglobulin kappa deleting element rearrangements in precursor-B-acute lymphoblastic leukemia are stable targets for detection of minimal residual disease by real-time quantitative PCR. Leukemia 2002:16;928-936

12.15 Bruggerman M, van der Valden VHJ, Raff T, et al. Rearranged T-cell receptor beta genes represent powerful targets for quantification of minimal residual

disease in childhood and adult T-cell acute lymphoblastic leukemia. Leukemia 2004:18;709-719.

12.16 Szczepanski T, van der Velden VH, Hoogeveen PG, et al. Vdelta2-Jalpha rearrangements are frequent in precursor-B-acute lymphoblastic leukemia but rare in normal lymphoid cells. Blood, 2004:103;3798-804.

12.17 Vincent H.J. van der Velden et al. EuroMRD Book Chapter 15: Analysis of minimal residual disease by Ig/TCR gene rearrangements: Guidelines for interpretation of real-time quantitative PCR data. 2012; 42-49.

12.18 Della Starza, I., Nunes, V., Cavalli, M., De Novi, L.A., Ilari, C., Apicella, V., Vitale, A., Testi, A.M., Del Giudice, I., Chiaretti, S., Foà, R. and Guarini, A. (2016), Comparative analysis between RQ-PCR and digital-droplet-PCR of immunoglobulin/T-cell receptor gene rearrangements to monitor minimal residual disease in acute lymphoblastic leukaemia. Br J Haematol, 174: 541-549. https://doi.org/10.1111/bjh.14082

12.19 Irene Della Starza, Vittorio Nunes, Federica Lovisa, Daniela Silvestri, Marzia Cavalli, Andrea Garofalo, Mimma Campeggio, Lucia Anna De Novi, Carlotta Oggioni, Andrea Biondi, Anna Guarini, Maria Grazia Valsecchi, Valentino Conter, Giuseppe Basso, Robin Foà, Giovanni Cazzaniga; Digital-Droplet PCR, an Accurate Method for IG/TR PCR-MRD Stratification in Childhood Acute Lymphoblastic Leukemia. *Blood* 2018; 132 (Supplement 1): 1544. doi: https://doi.org/10.1182/blood-2018-99-117954

13. APPENDICES

13.1 Primer and Taqman hydrolysis probes for ddPCR MRD analysis

14. AMENDMENT/REVIEW HISTORY

Version	Date	Amendment/Review	Recorded by
V1.1	3 Sep 2021	Amended referencing 8.1.3 and 8.3.4.2 – Change PCR conditions	Evelyn Lim

		8.3.1.3 – Change PCR mix	