Establishment and validation of a standard protocol for the detection of minimal residual disease in B lineage childhood acute lymphoblastic leukemia by flow cytometry in a multi-center setting;

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Design and Methods

MRD by Flow cytometry

The UKALL Flow MRD network was established in 2001 and a common MRD protocol was devised after extensive consultation and includes features previously detailed in reports from St Jude Children's Research Hospital[1] and the Biomed group.[2,3] All the network laboratories followed the same experimental and analysis protocol and attended regular workshops to review data. Results, including dot plot images, were reported to a common website for independent review.

Bone marrow aspirates from patients entered into the UKALL 2003 trial were obtained at diagnosis, at the end of induction (day 28) and at the end of consolidation (week 11). The trial involves randomisation based on levels of MRD, as measured by PCR of patient-specific immunoglobulin rearrangements, at these two time points such that MRD high risk is defined as MRD equal to or greater than 0.01% at day 28, MRD low risk is MRD negative or less than 0.01% at day 28 and negative at week 11. All other patients are classified as indeterminate. The study had appropriate ethical approval. Samples were collected into ACDA and either red cell lysed using a standard ammonium chloride procedure or enriched for mononuclear cells using Lymphoprep (Nycomed, Birmingham, UK), prior to antibody staining according to individual laboratories preference at the start of the study. Paired samples prepared by either method showed good correlation, although Lymphoprep tended to give higher values (*data not shown*). A list of antibodies used in the protocol is shown in *Supplementary Table S1*. At diagnosis, 8 antibody combinations were assessed with a common CD34/CD19/CD10 spine and a minimum of 50,000 events were acquired on a flow cytometer. The flow cytometers used in the study were two FACSCaliburs, a FACSCanto, two Coulter Epics XL, and a Coulter FC500.

For analysis, a sequential gating strategy was used: lymphoid cells were first gated on forward and side scatter, followed by CD19 positive, low side scatter cells, then CD19 positive CD34 negative and CD19/CD34 double positive cells. Finally, the expression of CD10 along with the discriminatory 4th CD antigen was assessed in the $CD34^+CD19^+$ and $CD19^+$ $CD34^-$ populations. The staining pattern of each combination was then compared to that of 'normal' bone marrows which were bone marrows taken from patients in remission in the latter stages of B lineage ALL or during the early and late stages of T-cell ALL therapy or from normal donor bone marrow harvests or from patients with idiopathic thrombocytopenia. Each laboratory was required to acquire 500,000 events for each leukemia associated immunophenotype from a minimum of 6 *normal* samples. Antibody combinations, in which the leukemic blasts fell into empty spaces,[1] distinct from regions housing normal B cell progenitors, were identified as leukemia-associated immunophenotypes (LAIP). At least 2 LAIPs were then tracked in follow up samples and a

Online Supplementary Table S1. Table of antibodies used in the standard protocol.

Antibody	Coulter User Clone (Fluorochrome)	Coulter User Manufacturer	BD User Clone (Fluorochrome)	BD User Manufacturer
CD10	ALB1 (ECD)	IOtest	HI10A (FITC or PE)	BD
CD13	SJ1D1 (PE)	IOtest	L138 (PE)	BD
CD19	J4.119 (PC5)	IOtest	SJ25C1 (APC)	BD
CD20	B9E9 (HRC20) (FITC)	IOtest	L27(FITC)	BD
CD22	SJ10.1H11 (PE)	IOtest	S-HCL-1 (PE)	BD
CD33	D3HL60.251 (PE)	IOtest	P67.6 (PE)	BD
CD34	581 (FITC or PE)	IOtest	8G12 (PERCP)	BD
CD38	T16 (FITC)	IOtest	HB7 (FITC)	BD
CD45	J.33 (FITC)	IOtest	2D1 (FITC)	BD
CD58	A1CD58 (PE)	IOtest	MY31 (PE)	BD
KORSA	KOR-SA3544 (FITC)	Immunotech	KOR-SA3544 (FITC)	Immunotech

Online Supplementary Table S2. Table of percentage agreements on risk category for each lab compared to the consensus risk.

Lab	1	2	3	4	5	6
% Agreement	90	100	80	100	100	100
N=	42	35	35	38	40	18

Online Supplementary Table S3. Table of interassay variability.

	Coulter XL						Coulter FC500					
LAIP	45/10/34/19			38/10/34/19			45/10/34/19			38/10/34/19		
Mock MRD (%)	10%	0.50%	0.05%	10%	0.50%	0.05%	10%	0.50%	0.05%	10%	0.50%	0.05%
Mean (n=10)	7.825	0.561	0.047	8.016	0.572	0.049	7.762	0.555	0.048	7.761	0.560	0.049
Standard deviation	0.174	0.031	0.005	0.329	0.031	0.006	0.283	0.026	0.006	0.253	0.018	0.006
Coefficient of variation (%)	2.217	5.472	10.213	4.101	5.332	11.633	3.646	4.595	13.125	3.259	3.143	11.633

Online Supplementary Table S4. Frequencies of LAIPS detected and used in the study.

Discriminatory CD marker	Expression characteristic	Frequency of detection (%)	Frequency of use in follow-up samples (%)*	Common LAIP pairs	Frequency of detection (%)
CD45	Underexpression	73	78	CD45 and CD38	47
CD38	Underexpression	63	66	CD45 and Korsa	8
CD58	Overexpresssion	55	22	CD45 and CD58	6
Korsa	Aberrant	34	22	CD38 and Korsa	4
CD20	Underexpression	7	3		
CD13	Aberrant	11	4		
CD33	Aberrant	9	0		
CD22	Overrexpression	5	3		

**This is the frequency of the minimum two markers used in this patient cohort.*

minimum of 500,000 events acquired. Samples were considered positive if the number of leukemic cells identified with one or more LAIP combinations was equal to or greater than 0.01% (i.e. the number of leukemic cells/ total number of nucleated cells expressed as a percentage) and at least 50 clustered events were apparent. For those discriminatory antigens which were overexpressed, subtraction of isotype control events was performed.

MRD by molecular analyses

Molecular MRD analysis was performed by the four laboratories of the UKMRD laboratory network (based in Bristol, Glasgow, the Royal London and Sheffield) according to a standard SOP and using centrally purchased reagents and consumables. This SOP was based on the methodology previously reported by the BIOMED-1 and BIOMED-2 Concerted Action Groups.[4,5] Briefly, diagnostic DNA was screened by PCR for rearrangements at the IgH, IgK, TCRD, TCRG, TCRB and Sil-Tal1 loci. Homogeneity of amplified products was determined using heteroduplex analysis and thereafter monoclonal products were sequenced directly. Bi-allelic products were excised from gels, re-amplified and then sequenced. Classification of the junctional region(s) of each rearrangement was performed by comparison relative to germline sequences contained in the NCBI or IMGT reference databases. RQ-PCR-based detection of MRD was undertaken using allele-specific oligonucleotides, designed to span the patient-specific junctional region of the target, in conjunction with consensus dual-labelled probes and reverse primers. Analysis of MRD at day 28 and week 11 was undertaken using two patient-specific real-time assays each capable of detecting disease down to a level of 1/10,000 (quantitative range=10^{-4}). Quantitation of disease level was performed by comparison of the amplification seen relative to that observed in a patient-specific logarithmic dilution series and with reference to that seen in normal template DNA and no template containing controls. To correct for the quantity and quality of DNA, RQ-PCR analysis of the albumin gene was used. All assays were analysed according to ESG-MRD-ALL guidelines.[6]

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