Clinical Significance of Minimal Residual Disease in Leukemia and Lymphoma II

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St. Jude Children’s Research Hospital
# MRD Studies – Applications

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Immunologic Detection of MRD

- T cell
- immature B-cell
- T-ALL
T-lymphoblastic Lymphoma – A Method for Detecting Disease Dissemination

Coustan-Smith et al. J Clin Oncol, 2009
T-lymphoblastic Lymphoma – Disseminated Disease by Flow Cytometry and Staging

Stage II  Stage III  Stage IV

Undetectable 100 10 10.1 0.01 % T-LL cells

J Clin Oncol, 2009
T-lymphoblastic Lymphoma – Disease Dissemination at Diagnosis and Outcome

J Clin Oncol, 2009
T-lymphoblastic Lymphoma – Disease Dissemination at Diagnosis in Marrow and Blood

J Clin Oncol, 2009

Blood, 2002
T-lymphoblastic Lymphoma – Early Response to Treatment
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<td>Risk assignment</td>
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MRD – Technical Issues
Sample Preparation

- Mononuclear cells
  - Clean cell preparation
  - Enrichment of leukemic lymphoblasts
  - Same method that is used for PCR → comparable cell populations for parallel studies
  - Unaltered light scatter and antigen expression

- Erythrocyte-depleted
  - Fits well with leukemia phenotyping lab routine
  - Remaining lysis-resistant red cells
  - Debris, granulocytes, platelets
  - Possible selective elimination of cell subpopulations
Surface Staining (MRD)

- Number of cells per tube: 0.5-1.0 x 10^6 bone marrow mononuclear cells
- Suspended in PBS + 0.2% sodium azide and 0.2% bovine serum albumin (PBSA)
- Add rabbit immunoglobulin to block Fc receptors
- Always add antibodies in the same fluorochrome order
- Incubate for 10 minutes at 18-25°C in the dark
- 2 washes in PBSA
- Fix in 0.5% paraformaldehyde
- Store at 4°C in the dark
Number of cells

- Maximum = $1 \times 10^6$ (1 million) leucocytes per tube.

- Minimum depends on the percentage of target cells in the population being stained and the number of total events to be acquire.

- For MRD a minimum of $0.5 \times 10^6$ leucocytes (0.5 million) per tube are required for sensitivity of 0.01%, acquire at least 100,000 events.
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Add Blocking Serum

- Use rabbit serum or human AB serum to block Fc receptors
  - Cells such as monocytes or activated lymphoid cells have Fc Receptors on the cell surface
  - Monoclonal antibodies can bind via the Fc portion of the immunoglobulin molecule to Fc receptors
  - This can result in false-positives or masking of weakly expressed antigens
Fc Receptor Blocking

- No staining: No serum
- Human AB serum
- Rabbit Ig
- Rabbit serum
- Human AB serum
- Rat Ig
Intracellular Staining

- Antibodies and isotype controls should always be carefully titrated for intracellular staining.

- Re-titration may be necessary for different lots of antibodies or if switching to a different permeabilization reagent.

- Use of internal positive and negative controls are very helpful in the interpretation of analysis.
Properties of permeabilization reagents
Choosing Fluorochromes

• Match brightest fluorochromes to the dimmest antigens

• Antigen density is known to be high, use dim fluorochrome (if low, use bright fluorochrome)

• Intracellular antigens are usually dimmer and/or less discrete populations than surface antigens
CD19 fluorochrome for MRD
Aim of Titrations

- To give maximum separation between positive and negative signals (signal to noise ratio)
- To give maximum fluorescence intensity of the positive population
- To use less antibody than recommended
- Commercial reagents have recommended amounts for staining a fixed number of cells any deviation must be verified by titration experiments
Titration of Antibodies for Surface Staining

- Use a cell type that is strongly expressing the target antigen
- Use $1 \times 10^6$ (1 million) cells per tube
- Begin with manufacturer’s recommended amount followed by decreasing amounts
- Choose the amount that gives the brightest signal without increasing the negative background
- Lots may vary and may need re-titrating
- NEVER reduce amount of antibody without prior titration
Isotype Controls-to use or not to use?

- Primary function is to show presence of non-specific binding of antibodies.

- They should be of the same isotype and conjugated with the same fluorochrome as the antibody being tested.

- Secondary function to serve as a “negative boundary” for analysis:
  - It is not possible to exactly match each antibody (immunoglobulin concentration, fluorochrome to protein ratio etc).
They should be used in exactly the same method as the antibody is used.

Example: stain surface CD22 PE (IgG1 class) followed by intracellular TdT FITC (IgG2a class) staining.

Control: stain surface IgG1 PE followed by intracellular IgG2a FITC.

Isotype controls for intracellular staining often need to be titrated.

In some multi-color experiments it may only be necessary to isotype control one or 2 of the markers.
Instrument Quality Control

- Control Beads
  - Quality control and Levey-Jennings plots
  - Check compensation regularly
- Biological control: Peripheral blood
  - Unstained cells
  - Cells stained with CD3 FITC, CD3 PE, CD3 PerCP, CD3 APC
- Instrument care
  - Regular maintenance service
  - Monthly system cleaning
Quality Control of Reagents and Buffers

- **Monoclonal antibodies**
  - Store at correct temperature
  - Protect from light, especially tandem conjugates
  - Avoid contamination

- **Buffers**
  - Measure pH
  - Check for background noise
  - If using diluting buffer concentrates such as 10xPBS check that the concentrate has been added to the distilled water
Special Precautions for MRD

- **Sample quality**
  - Process as soon as possible
  - Sample with poor viability

- **Sample carry-over**
  - Keep diagnostic and MRD samples separate
  - Avoid situations that may cause tube to tube carry over
  - Test tube to tube carry-over of instrument

- **Background events**
  - Filter buffers and quality control
  - Daily cleaning and monthly system cleaning of instrument
Sample Carry-Over Test
How Many Colors?

2 colors: NO

3 colors: NOT ENOUGH

4 colors: OK

6+ colors: STATE-OF-THE-ART
## Marker Combinations to Study MRD in ALL

<table>
<thead>
<tr>
<th>Leukemia subtype</th>
<th>Marker combinations</th>
<th>Applicability (%)</th>
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<tbody>
<tr>
<td><strong>T-lineage ALL</strong></td>
<td>anti-TdT / CD5 / CD3</td>
<td>90-95</td>
</tr>
<tr>
<td></td>
<td>CD34 / CD5 / CD3</td>
<td>30-50</td>
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<tr>
<td><strong>B-lineage ALL</strong></td>
<td>CD19 / CD34 / CD10 / CD58</td>
<td>40-60</td>
</tr>
<tr>
<td></td>
<td>CD19 / CD34 / CD10 / CD38</td>
<td>30-50</td>
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<tr>
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<td>CD19 / CD34 / CD10 / CD45</td>
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<td>30-50</td>
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<td>CD19 / CD34 / CD10 / anti-NG2</td>
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### Selection of Markers for MRD at Diagnosis
#### B-lineage ALL

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<th>FITC</th>
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<tr>
<td>CD33</td>
<td>CD66c</td>
<td>Mouse IgM</td>
<td>CD15</td>
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Definition of Normal Phenotypes
Gating Strategy Using Templates

38/10/34/19 - Day 46

Ungated

Gated R1

CRU13F.BM.007

CD34 PerCP

CD10 PE

CD19 APC

CRU13F.BM.007

FSC

SSC

CD10 PE

CD34 PerCP

CD19 APC

CD38 FITC

CD38 FITC

CD19 APC

CD10 PE
Methodologic Advances in MRD Detection

Gene profile of ALL cells (~300 samples) and normal CD19+CD10+ cells

Gene profile of AML cells (~200 samples) and normal CD34+CD33+ cells
CD58 as a Marker of MRD

- **De Waele M et al.** Different expression of adhesion molecules on CD34+ cells in AML and B-lineage ALL and their normal bone marrow counterparts. Eur J Haematol 1999; 63:192-201
- **Chen JS et al.** Identification of novel markers for monitoring minimal residual disease in acute lymphoblastic leukemia. Blood 2001; 97: 2115-20
- **Veltroni M et al.** Expression of CD58 in normal, regenerating and leukemic bone marrow B cells: implications for the detection of minimal residual disease in acute lymphocytic leukemia. Haematologica 2003; 88:1245-52
- **Mlcakova A, Babusikova O.** Multiparametric flow cytometry in detection of minimal residual disease in acute lymphoblastic leukemia of early B-cell phenotype. Neoplasma 2003;50:416-21
- **Lee RV et al.** CD58 expression decreases as nonmalignant B cells mature in bone marrow and is frequently overexpressed in adult and pediatric precursor B-cell acute lymphoblastic leukemia. Am J Clin Pathol 2005;123:119-24
Methodologic Advances in MRD Detection

Gene profile of ALL cells (~300 samples) and normal CD19+CD10+ cells

Use of 9+ color flow cytometry

Increase sensitivity of MRD detection

Gene profile of AML cells (~200 samples) and normal CD34+CD33+ cells

Study biologic features of MRD

Stem cells?
Mapping normal and cancer cell signaling networks

Irish, Kotecha, Nolan, Nat Rev Cancer, 2006
Flow Cytometric Assessment of Targeted Signaling Networks

- Enzastaurin (inhibitor of protein kinase C β) in children with CNS tumors
  - Measurement of phospho-PKC substrates in peripheral blood lymphocytes after phorbol ester stimulation

- Temsirolimus (inhibitor of mTOR kinase) in children with high risk neuroblastoma
  - Measurement of mTOR signaling in neuroblastoma cells

- Sorafenib (inhibitor of multiple kinases) in children with high risk AML
  - Measurement of phosphorylation ERK, MAPK, STAT5, etc in AML cells