Immunobead technology for detection of fusion proteins in Leukemia

Liesbeth Dekking

Dynamics, spin-off company of Dept. Immunology
Erasmus Medical Center, The Netherlands
Introduction

1) Fusion proteins

2) Bead assay
Chromosomal break and repair

- **Normal:** Adaptive immunesystem:
  - B- and Tcell receptor rearrangements

- **Abnormal:** Chromosomal translocations

Possible results of chromosomal translocations are:

1) Overexpression of proteins
   - c-Myc t(8;14)/ t(2;8)/ t(8;22)/ WT-1/ Tal 1/ Lyl-1

2) Fusion proteins:
Fusion proteins

Gene A

Gene B

Transcription

mRNA

Translation

Fusion proteins
Bead-based flow cytometric assay for fusion proteins

<table>
<thead>
<tr>
<th></th>
<th>Molecular techniques</th>
<th>Flow cytometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Speed</td>
<td>2-3 days (up to weeks)</td>
<td>fast: 4.5 hours !!</td>
</tr>
<tr>
<td>Target</td>
<td>DNA or RNA</td>
<td>protein/cells</td>
</tr>
<tr>
<td></td>
<td>(RNA is an instable target)</td>
<td>(“end-product”)</td>
</tr>
<tr>
<td>Applicability</td>
<td>depends on disease</td>
<td>broad</td>
</tr>
<tr>
<td></td>
<td>(chromosome aberrations)</td>
<td></td>
</tr>
<tr>
<td>Multiplexing</td>
<td>technically demanding</td>
<td>relatively easy</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(even 25 to 100 tests per tube)</td>
</tr>
<tr>
<td>Accuracy</td>
<td>semi-quantitative</td>
<td>quantitative</td>
</tr>
<tr>
<td>Focus</td>
<td>all cells in sample</td>
<td>any subpopulation</td>
</tr>
<tr>
<td></td>
<td>(or: prior purification)</td>
<td></td>
</tr>
<tr>
<td>Facilities</td>
<td>special laboratories needed</td>
<td>only standard lab needed</td>
</tr>
</tbody>
</table>
Advantages of Immuno bead system

- Independent of fusion gene breakpoint
- Multiplex possibilities by use of differential labeling of beads
- Can be run in parallel to standard Immuno-phenotyping
- Easy: no specialized laboratory needed
- Fast: treatment decisions can be made on the same day
Generation of CBA assays by Dynomics and BD-Biosciences
Generation of assays (I)

- Epitopes selected in N- and C-terminal regions of the protein present in all known breakpoint variants
- Immunization mice and fusion (Dynomics and BD Technologies)
- Polydoma’s were selected based on
  A) Cos cell screening (Dynomics)
  B) Elisa (BD)
- Polydoma’s were cloned to monoclonal culture
- Monoclonal cultures were expanded and mAb purified
- Antibodies were tested in matrix bead assay
Cos cell screening

- Full length antigen with tag in vector

- Transfection of vector to Cos cells (eff. 2.5-5 %)

- After 2 days cell culture, fixation and permeabilization of Cos cells with 100% methanol

- Cos cell screening
### Generation of assays (II)

<table>
<thead>
<tr>
<th>Matrix:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bead mAb 1 $\rightarrow$ biotin mAb A</td>
</tr>
<tr>
<td>biotin mAb B</td>
</tr>
<tr>
<td>biotin mAb C $\rightarrow$ streptavidin PE $\rightarrow$ detection</td>
</tr>
<tr>
<td>Bead mAb A $\rightarrow$ biotin mAb 1</td>
</tr>
<tr>
<td>biotin mAb 2</td>
</tr>
<tr>
<td>biotin mAb 3</td>
</tr>
</tbody>
</table>

6 mAb give 18 combinations to test when using standard concentrations!
Antibody combinations were tested against:

1) lysate of transfected Cos cells
   (positive vs negative screening)

2) lysate of leukemic cell lines
   (selection pos. vs neg. and optimalization)

3) lysate of PBMC/ WBC of patient materials vs lysate of controls
   (test for suitability of the assays)
Singleplex milestones
(defined in Third Amendment of the Research Agreement)

- **Milestone 1: Sensitivity**
  “The assay should detect at least a lysate prepared from 10% leukemic cell line in a WBC and PMBC background.”

- **Milestone 2: Specificity**
  “The assay must be specific for the positive leukemic cell line only.”

- **Milestone 3: Suitability of the assay**
  “Only patients with right translocation should be positive.”
  (5 healthy individuals, 5 leukemic patients with no or other translocation, at least 2-3 patient with translocation of interest.)
BCR-ABL Cytometric Bead Array RUO assay
(launched in April 2008)
BCR-ABL RUO assay
(Research Use Only)

The Philadelphia Chromosome

Before translocation

After translocation

ABL #9

BCR #22

der 9

Philadelphia Chromosome

t(9;22)
Eight variants of $BCR$-$ABL$ transcripts from three different $BCR$ breakpoint regions

<table>
<thead>
<tr>
<th>Disease</th>
<th>BCR</th>
<th>ABL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-/pro B-ALL</td>
<td>m-bcr, p190 transcripts</td>
<td>1</td>
</tr>
<tr>
<td>CML</td>
<td>M-bcr, p210 transcripts</td>
<td>11 12</td>
</tr>
<tr>
<td>Neutrophyllic-CML</td>
<td>h-bcr, p230 transcripts</td>
<td>17 18</td>
</tr>
</tbody>
</table>

Eight variants of BCR-ABL transcripts from three different BCR breakpoint regions.
Basic BCR-ABL assay
Procedure BCR-ABL assay

1. Red blood cell lysis
2. Pretreatment cells on ice
3. Cell lysis on ice
4. Incubation with beads and antibodies for 2 hrs
5. Flow cytometry
Sensitivity BCR-ABL assay

K562 diluted in 697 (negative cell line)

s/n ratio 8.0

K562 diluted in PBMC

s/n ratio 3.8

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February 3-4 2009
Aronsborg, Stockholm, Sweden
Detection of all forms of BCR-ABL

697 (t(1;19)) (neg. controle)
TOM-1, BCR-ABL⁺ (p190)
LAMA-84, BCR-ABL⁺ (p210)
AR230, BCR-ABL⁺ (p230)

Bead system
Catching antibody: anti-BCR
Bead: BD-Flex bead (A7)
Detection antibody: biotinylated anti-ABL - SA-PE
Testing of the BCR-ABL RUO kit on clinical samples by EuroFlow*

*European consortium of 9 hematology laboratories that collaborate on the development of improved diagnose and classification of hematologic malignancies by use of flow cytometry.

EuroFlow is coordinated by Prof. Dr. Jacques van Dongen, Erasmus Medical Center, Rotterdam, The Netherlands)
Design BCR-ABL RUO CBA assay testing by EuroFlow*

- BCR-ABL RUO kit provided by BD Biosciences to 9 EuroFlow laboratories
  - Content of the kit:
    - Pretreatment A and B
    - Lysis buffer (including protease inhibitors)
    - beads
    - detector mAb
    - BD wash buffer
  - Controls were provided by Dynomics
    - Pos. control: lysate 10% cell line (K562) in WBC
    - Neg. control: lysate WBC

- Results 1) recorded on patient report forms
  2) analyzed at Erasmus Medical Center, Rotterdam, The Netherlands

- Data 1) report transferred to BD
  2) poster presented on the ASH

*BD Biosciences

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February 3-4 2009
Aronsborg, Stockholm, Sweden
## Results of the BCR-ABL RUO testing by the EuroFlow laboratories

<table>
<thead>
<tr>
<th>Samples</th>
<th>BCR-ABL PCR assay</th>
<th>BCR-ABL immunobead assay*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>negative</td>
<td>p190</td>
</tr>
<tr>
<td>CML (n=19)</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Precursor-B-ALL</td>
<td></td>
<td></td>
</tr>
<tr>
<td>- childhood (n=50)</td>
<td>49</td>
<td>1</td>
</tr>
<tr>
<td>- adult (n=28)</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>T-ALL (n=18)</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>AML (n=27)</td>
<td>27</td>
<td>0</td>
</tr>
<tr>
<td>Reactive (n=1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CMPD (n=1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Mature B-NHL (n=1)</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Healthy controls (n=72)</td>
<td>NT</td>
<td></td>
</tr>
</tbody>
</table>

*negative: MFI value <135; low positive: MFI value 135-1,000; high positive: MFI value ≥1,000
Extra components for BCR-ABL kit

- **Calibrator**: Recombinant BCR-ABL protein
  (shortened at C-terminus) prepared by BD

- **Controls**: 10% K562 in WBC
  100% WBC
  (will be prepared by Dynomics and distributed by BD)

<table>
<thead>
<tr>
<th>Calibr</th>
<th>MFI</th>
<th>s/n</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 ng</td>
<td>26937</td>
<td>316.9</td>
</tr>
<tr>
<td>5 ng</td>
<td>3004</td>
<td>35.3</td>
</tr>
<tr>
<td>500 pg</td>
<td>310</td>
<td>3.6</td>
</tr>
<tr>
<td>100 pg</td>
<td>116</td>
<td>1.4</td>
</tr>
<tr>
<td>blanc</td>
<td>85</td>
<td>1.0</td>
</tr>
</tbody>
</table>
Novel multiplex CBA assay tubes
(precursor B- ALL and AML)
Novel CBA assays suitable for multiplexing

CBA assays for precursor B-ALL tube:
- BCR-ABL t(9;22)
- E2A-PBX1 t(1;19)
- TEL-AML1 t(12;21)
- MLL-AF4 t(4;11)

CBA assays for AML tube:
- AML1- ETO t(8;21)
- PML- RARA t(15;17)
- CBFβ-MYH11 Inv 16
Singleplex milestones
(defined in Third Amendment of the Research Agreement)

- **Milestone 1: Sensitivity:**
  10% leukemic cell line in a WBC and PMBC background.”

- **Milestone 2: Specificity**
  assay specific for the positive leukemic cell line only.”

- **Milestone 3: Suitability of the assay**
  “Only patients with right translocation should be positive.”
Precursor B-ALL multiplex tube: E2A-PBX1 $t(1;19)$

Sensitivity for detection on cell line <10%

Specificity E2A-PBX1 (Dec 12, 2008)

*Exp. Performed on April 27th 2007

Neg. patients
Pos patients
Precursor B-ALL multiplex tube: 
**TEL-AML1 t(12;21)**

Sensitivity for detection REH cell line in:
- **WBC**: 10-50%
- **PBMC**: 1-10%

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**Specificity TEL-AML (July 30th 2008)**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME1</td>
<td></td>
</tr>
<tr>
<td>RS4,11</td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td></td>
</tr>
<tr>
<td>697</td>
<td></td>
</tr>
<tr>
<td>RCH-ACV</td>
<td></td>
</tr>
<tr>
<td>Kasumi</td>
<td></td>
</tr>
<tr>
<td>NB4</td>
<td></td>
</tr>
<tr>
<td>REH</td>
<td>70000</td>
</tr>
</tbody>
</table>

**TEL-AML in patients**

- **donor 1**
- **donor 2**
- **donor 3**
- **donor 4**
- **donor 5**
- **TEL-AML patient 1**
- **TEL-AML patient 2**
- **TEL-AML patient 3**
- **TEL-AML patient 4**
- **B-ALL control 1**
- **B-ALL control 2**
- **B-ALL control 3**
- **B-ALL control 4**
- **B-ALL control 5**
- **healthy control**
- **TEL-AML**
- **precursor B-ALL without translocation**
Precursor B-ALL multiplex tube: MLL-AF4 t(4;11)

Sensitivity for detection MV4;11 line in:
- 697 cell line: 10%
- WBC: 10-50% (close to 10%)
- PBMC: idem 10%

![Graph showing specificity MLL-AF4](image)

![Graph showing patients in MLL-AF4](image)
AML multiplex tube

**AML1-ETO** t(8;21)

Sensitivity for detection Kasumi cell line in:
- WBC: 1%
- PBMC: 1-10%

---

**Specificity AML-ETO**

<table>
<thead>
<tr>
<th>cell line</th>
<th>MFI</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME1</td>
<td></td>
</tr>
<tr>
<td>RS4,11</td>
<td></td>
</tr>
<tr>
<td>K562</td>
<td></td>
</tr>
<tr>
<td>697</td>
<td></td>
</tr>
<tr>
<td>RCH-ACV</td>
<td></td>
</tr>
<tr>
<td>Kasumi</td>
<td>25000</td>
</tr>
<tr>
<td>NB4</td>
<td></td>
</tr>
<tr>
<td>REH</td>
<td></td>
</tr>
</tbody>
</table>

**AML-ETO in patients**

- healthy controls
- AML-ETO
- AML w/ 8;21 translocation

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AML multiplex tube:
PML-RARα $t(15;17)$

Sensitivity for detection NB 4 cell line in:
- WBC: 10%
- PBMC: 10%

![Graph showing specificity for PML-RARA]
AML multiplex tube:
PML-RARα t(15;17)
AML multiplex tube: CBFβ-MYH11 Inv 16

Sensitivity for detection of ME1 cell line in:
- WBC : 1%
- PBMC : 1-10%

Specificity CBFB-MYH11

CBFb-MYH11 in patients
Complexity of multiplexing

- Difficulty; fusion proteins are both cytoplasmatic and nuclear (DNA binding).

- One condition per tube for detection of all fusion proteins
# Multiplexena of AML and precursor B-ALL CBA assays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Cell line</th>
<th>patients</th>
<th>singleplex</th>
<th>multiplexing</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AML tube</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PML-RARA</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>to be tested</td>
</tr>
<tr>
<td><strong>AML-ETO</strong></td>
<td></td>
<td></td>
<td></td>
<td>to be tested</td>
</tr>
<tr>
<td>CBFβ-MYH11</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Pre-B-ALL</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BCR-ABL</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>E2A-PBX</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MLL-AF4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>to be tested</td>
</tr>
<tr>
<td>TEL-AML</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>
Future planning of CBA assays

- April 2009; PML-RARα or one of the multiplex CBA assays will be tested in a small patient trial with material of newly diagnosed patients at various laboratories.

- A report of this study will probably be presented at the ASH December 2009.

- Other assays will follow probably also this year.

- Assays will be optimized and prepared for launch.

- Release of new RUO kits depend on results of these trials and optimization.
Additional CBA assays planned for the future

<table>
<thead>
<tr>
<th>T-ALL tube</th>
<th>MLL tube</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calm-AF10</td>
<td>MLL-AF4</td>
</tr>
<tr>
<td>LMO2</td>
<td>MLL-AF6</td>
</tr>
<tr>
<td>TAL1</td>
<td>MLL-AF9</td>
</tr>
<tr>
<td>Hox11L2</td>
<td>MLL-AF10</td>
</tr>
<tr>
<td></td>
<td>MLL-ENL</td>
</tr>
<tr>
<td></td>
<td>MLL-ELL</td>
</tr>
</tbody>
</table>
People to mention

**Dynomics**
- Anne vd Linde
- Rianne Noordijk
- Linda Gijsbers
- Ilker Makay
- Floor Weerkamp
- Liesbeth Dekking
- Jacques Van Dongen
- Peter Schoevers

**BD Biosciences**
- Hobert Wai and team
- Brian Warner and team
- Alice Ho
- Rudi Varro
- Charlene Bush-Donovan
- Eric Dixon (Tripath)

**BD Europe**
- Frans Nauwelaers
Information BCR-ABL RUO immunobead assay

Additional product information can be provided by
Frans Nauwelaers
(BD Biosciences Europe, Erembodegem Belgium)
Frans_Nauwelaers@bd.com