Anti-Nuclear Antibody (ANA) Testing

Immunofluorescence (IFA) versus Enzyme-Linked Immunosorbent Assay (ELISA)
Systemic Rheumatic Diseases (SRDs):

- Systemic lupus erythematosus (SLE)
- Rheumatoid arthritis (RA)
- Sjogren’s Syndrome (SS)
- Mixed connective tissue disease (MCTD)
- Scleroderma
- Dermatomyositis/polymyositis (DM/PM)
- Drug-induced lupus
Characteristics:

• Variable age at onset
• Greater frequency in females
• Multi-systems involved
• Symptoms develop gradually
• Extensive overlap in manifestations

Disease complexity makes diagnosis difficult
Diagnosis

• Laboratory testing = important tool
• Specific diagnosis = more appropriate treatment
• Earlier detection/identification = better symptom management

Best to detect/identify circulating autoantibodies to the specific cellular antigens
Tests to Detect ANA

• Three main types
  • Immunofluorescence Assay (IFA)
  • Enzyme-linked immunosorbent assay (ELISA)
  • Multiplex assays
IFA Test Antigen

- Whole Hep-2 cells are cultured on slides
- Cytoplasm can mask true nuclear reactivity
- Naturally occurring antigens vary in concentration (SSA low, Scl-70 low, Jo-1 low)
- Antigens in whole cells are soluble, may be leached off during processing
ELISA Test Antigen

- Whole Hep-2 cells are lysed, centrifuged to concentrate the nuclei
- Other purified antigens are added, boost signal for all autoantigens (e.g., SSA, Scl-70, Jo-1)
- Coated onto high-affinity binding wells to retain all signals during processing

Antigen for ELISA over-rides the faults of the IFA antigen
• Individual antigens, cell components are coated onto multiple beads

• All activities are detected and identified in parallel

• High cost of test and automation

• Reimbursement for each analyte may be denied, especially for negative samples (60-80% of total)
IF

- IFA patterns may suggest **but do not identify** specific antibodies
- IFA positive screens must be repeated for titer
- IFA screening/titrating, is labor-intensive
- Microscopic evaluation requires specialized training

Staining patterns/titers will still need to be follow-up tested to identify specific antibody(ies)
ELISA

• ELISA detects the same ANA antibodies as IFA, with improved sensitivity/specificity

• ELISA returns measurable results in one determination (no repeating to titrate)

• ELISA is objective, automatable, requires no specialized training

• ELISA reports out Index Values
ELISA positives can be followup-tested for IFA patterns and titers, and reported the same as IFA.

Staining patterns/titers will still need to be follow-up tested to identify specific antibody(ies).
ELISA Advantages

- Sensitive
- Specific
- Reproducible
- Automatable
- Easy/faster
- Objective
- Index values vary with antibody levels
False Negatives:

- In some SRDs with antibodies to cytoplasmic constituents
- In Sjogren’s Syndrome, scleroderma, polymyositis
- If single antibody, at very low level, is present:
  - SSA, subacute cutaneous lupus
  - dsDNA, ANA negative lupus
IFA Disadvantages

False Positives:

• Known high sensitivity, low specificity
  • 33% of normals can be positive
  • > 20% healthy relatives
  • 75% elderly population

• In other diseases: Chronic Pulmonary Fibrosis- Chronic Infection- Chronic Hepatitis
IFA Difficulties

- Labor-intensive, with microscope screening
- Specialized training required
- Microscope optics can vary
- Tests require standardization
- Inconsistencies in preparation of slides can alter results
- Some laboratories require 2 separate analysts’ readings
IFA Staining Patterns

Homogeneous

Antibodies:
- DNP or Histones (drug-induced lupus)
- dsDNA (SLE)
- ssDNA (not disease-specific)
IFA Staining Patterns

Speckled

Antibodies:
- SSA & SSB (SCLE & neonatal SLE)
- Sm (SLE)
- Sm/RNP (SLE, MCTD)
- Scl-70 (Scleroderma)
IFA Staining Patterns

Nucleolar

Antibodies:

- Scl-70 (Scleroderma)
- Nucleolar (Scleroderma)
IFA Staining Patterns

Centromere

Antibodies:

- Centromere  
  (CREST variant Scleroderma)
A clinical laboratory periodical\textsuperscript{1} published a comparative analysis of Manual and Automated EIA versus IFA testing. The following summary is based on information in that comparison.

\textsuperscript{1} ACL, Vol. 19, Number 9, Nov/Dec 2000
ANA Screen by Automated ELISA versus IFA
Time and Labor Cost Analysis

TIME REQUIREMENTS

MANUAL ELISA
Significant time is required to perform sample dilutions manually. Incubation times, washes, reagent addition, reading, data analysis, reporting of results require hands-on technician time.

Total hands-on time: 2.5 hours

AUTOMATED ELISA
Less than 30 minutes is required for technician to set reagents and samples into instrument. The technician is then free to perform other laboratory functions during the assay.

Total hands-on time: 30 minutes
Cost was estimated for testing 250 samples. Technician time required for each sample by IFA was based on the CAP workload Recording Method and Personnel Management Manual\(^1\). IFA Screen, 11.0 min (2 min. incremental time) #89554.810. Cost of the tests was not included.

# ANA Screen by Automated ELISA versus IFA

## Time and Labor Cost Analysis

<table>
<thead>
<tr>
<th></th>
<th>Immunofluorescence (IFA)</th>
<th>Automated ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIME</strong></td>
<td>8.6 hours, assay</td>
<td>60 minute setup</td>
</tr>
<tr>
<td></td>
<td>plus microscope analysis</td>
<td>for assay for</td>
</tr>
<tr>
<td></td>
<td>for screening and reporting</td>
<td>250 samples.</td>
</tr>
<tr>
<td><strong>COST</strong></td>
<td>$215.00</td>
<td>$18.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>TIME</strong></td>
<td>60 minute shut-down and reporting.</td>
<td></td>
</tr>
<tr>
<td><strong>COST</strong></td>
<td>$18.00</td>
<td></td>
</tr>
</tbody>
</table>

**Total Labor Cost**

- Immunofluorescence (IFA): $215.00
- Automated ELISA: $36.00

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1. Cost of testing 250 samples by IFA. Estimated hourly rate for trained technologist = $25.00.
2. Cost of testing 250 samples by EIA. Estimated hourly rate for laboratory technician = $18.00.
ANA TEST STUDY: COMPARISON OF IFA TO AUTOMATED ELISA

Dr. Gail Woods
University of Texas Medical Branch
Galveston, TX
Test Setup:

- N= 510 samples (no diagnosis)
- ANA ELISA Screen (Diamedix Corp) test
  MAGO® Plus Automated EIA System
- IFA (Kallestad, Sanofi-Pasteur) at 1:160 as the initial screening titer, then...... →
- IFA positives re-tested at titers of 1:160, 1:320, and 1:640. Strong positives were reported as > 1:640.
ANA TEST STUDY:

Test Setup:

• Slides independently read by 2 analysts
• Discrepancies between analysts were reviewed
• Results agreed upon after review were recorded
• IFA results were blinded from ELISA results
Results:

- ELISA Index/interpretation were recorded.
- Equivocals (n=33) were excluded.
- ELISA and IFA results agreed for 437/477 (91.6%) samples.
### ANA TEST STUDY:

#### Initial Correlation:

<table>
<thead>
<tr>
<th>ANA by IFA</th>
<th>ANA ELISA Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
</tr>
<tr>
<td>POS</td>
<td>114</td>
</tr>
<tr>
<td>NEG</td>
<td>26</td>
</tr>
</tbody>
</table>

33 ELISA equivocals were excluded from the calculations

Relative Sensitivity $= \frac{114}{128} = 89.1\%$

Relative Specificity $= \frac{323}{349} = 92.6\%$

Overall Agreement $= \frac{437}{477} = 91.6\%$
ANA TEST STUDY:

Positive/Negative Frequencies:

• 114 / 477 (23.9 %) were positive (+) by IFA and ELISA
• 323 / 477 (67.7 %) were negative (-) by IFA and ELISA
• 40 / 477 (8.4 %) were in disagreement between IFA and ELISA
ANA TEST STUDY:

Discordants were tested:

By ELISA tests for clinically significant antibodies:

- anti-dsDNA
- ENA-6 Screen (6 analytes in one well)
- Profile of separate individual ENA assays:
  - SSA
  - SSB
  - Sm
  - Sm/RNP
  - Scl-70
  - Jo-1

Definitions: Subset of ANAs = anti-ENA group of tests = (Extractable Nuclear Antigen)
Eleven of the 26 EIA(+) but IFA(-) samples were positive or equivocal in one or more of the individual EIA tests.
The remaining twelve of the EIA(+) IFA(-) samples were negative in the individual EIA tests. * 3 samples were unavailable for follow-up testing.

### ANA TEST STUDY:

#### 26 Samples POS by EIA / NEG by IFA

<table>
<thead>
<tr>
<th>Interpretation</th>
<th>EIA Index</th>
<th>ENA-6 Index</th>
<th>SSA EU/ml</th>
<th>SSB EU/ml</th>
<th>Sm EU/ml</th>
<th>Sm/RNP EU/ml</th>
<th>Scl-70 EU/ml</th>
<th>Jo-1 EU/ml</th>
<th>dsDNA IU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pos</strong></td>
<td>&gt; 1.1</td>
<td>&gt; 1.1</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 20</td>
<td>&gt; 35</td>
</tr>
<tr>
<td><strong>Neg</strong></td>
<td>&lt; 0.9</td>
<td>&lt; 0.9</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 25</td>
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<tr>
<td>1</td>
<td>6.57</td>
<td>0.394</td>
<td>4.0</td>
<td>2.4</td>
<td>2.7</td>
<td>2.1</td>
<td>1.8</td>
<td>3.3</td>
<td>9.6</td>
</tr>
<tr>
<td>2</td>
<td>5.88</td>
<td>0.673</td>
<td>5.7</td>
<td>0.9</td>
<td>1.2</td>
<td>3.2</td>
<td>1.3</td>
<td>1.8</td>
<td>9.4</td>
</tr>
<tr>
<td>3</td>
<td>1.84</td>
<td>0.614</td>
<td>11.7</td>
<td>13.8</td>
<td>11.8</td>
<td>8.7</td>
<td>12.5</td>
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<td>14.2</td>
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<tr>
<td>4</td>
<td>1.84</td>
<td>0.214</td>
<td>4.0</td>
<td>0.6</td>
<td>0.8</td>
<td>1.6</td>
<td>1.2</td>
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<td>1.5</td>
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<tr>
<td>5</td>
<td>1.60</td>
<td>0.216</td>
<td>2.0</td>
<td>0.9</td>
<td>1</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>8.0</td>
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<tr>
<td>6</td>
<td>1.59</td>
<td>0.256</td>
<td>2.1</td>
<td>0.4</td>
<td>1</td>
<td>1.5</td>
<td>0.8</td>
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<td>17.1</td>
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<td>0.478</td>
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<td>0.4</td>
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<td>2</td>
<td>1.6</td>
<td>1.9</td>
<td>7.1</td>
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<td>8</td>
<td>1.52</td>
<td>0.302</td>
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<td>0.3</td>
<td>0.5</td>
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<td>1.5</td>
<td>0.6</td>
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<td>9</td>
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<td>0.397</td>
<td>5.6</td>
<td>1.6</td>
<td>2.5</td>
<td>4</td>
<td>2.9</td>
<td>2.6</td>
<td>13.2</td>
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<td>10</td>
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<td>0.211</td>
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<td>1.9</td>
<td>2.1</td>
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<td>2.4</td>
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<td>11</td>
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<td>0.279</td>
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<td>5.3</td>
<td>0.9</td>
<td>1.5</td>
<td>5.2</td>
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<tr>
<td>12</td>
<td>1.18</td>
<td>0.434</td>
<td>3.4</td>
<td>1.2</td>
<td>2</td>
<td>2.8</td>
<td>3</td>
<td>3.1</td>
<td>13.4</td>
</tr>
</tbody>
</table>
8 / 23 ELISA (+) and IFA (-) samples were positive and 3 were equivocal in one or more of the profile of tests for antibodies of clinical significance (e.g., SSA or SSB or dsDNA).

Significant number “false negative” by IFA?

ANA by IFA has been reported not to detect antibodies such as SSA (subacute cutaneous lupus) and dsDNA (ANA negative lupus), especially when they occur alone, in the absence of other antibodies.
# ANA TEST STUDY:

## 14 Samples NEG by EIA / POS by IFA

<table>
<thead>
<tr>
<th>IFA Titer</th>
<th>EIA Index</th>
<th>ENA-6 Index</th>
<th>SSA EU/ml</th>
<th>SSB EU/ml</th>
<th>Sm EU/ml</th>
<th>Sm/RNP EU/ml</th>
<th>Scl-70 EU/ml</th>
<th>Jo-1 EU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pos</td>
<td>≥ 1.1</td>
<td>≥ 1.1</td>
<td>≥ 20</td>
<td>≥ 20</td>
<td>≥ 20</td>
<td>≥ 20</td>
<td>≥ 20</td>
<td>≥ 20</td>
</tr>
<tr>
<td>Neg</td>
<td>&lt; 0.9</td>
<td>&lt; 0.9</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
<td>&lt; 16</td>
</tr>
<tr>
<td>160</td>
<td>0.88</td>
<td>0.274</td>
<td>1.4</td>
<td>1.1</td>
<td>0.6</td>
<td>3.9</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>1280</td>
<td>0.85</td>
<td>0.147</td>
<td>4.8</td>
<td>0.7</td>
<td>1.0</td>
<td>1.7</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>640</td>
<td>0.85</td>
<td>0.202</td>
<td>4.0</td>
<td>0.6</td>
<td>1.0</td>
<td>1.6</td>
<td>1.1</td>
<td>2.0</td>
</tr>
<tr>
<td>320</td>
<td>0.85</td>
<td>0.223</td>
<td>1.9</td>
<td>0.2</td>
<td>0.7</td>
<td>1.8</td>
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<tr>
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<td>0.85</td>
<td>0.316</td>
<td>1.5</td>
<td>0.5</td>
<td>1.1</td>
<td>4.0</td>
<td>1.5</td>
<td>1.8</td>
</tr>
<tr>
<td>640</td>
<td>0.85</td>
<td>0.188</td>
<td>2.4</td>
<td>0.3</td>
<td>0.8</td>
<td>1.2</td>
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<td>0.8</td>
</tr>
<tr>
<td>320</td>
<td>0.85</td>
<td>1.855</td>
<td>18.2</td>
<td>1.3</td>
<td>1.4</td>
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<td>0.493</td>
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<td>5.2</td>
<td>2.8</td>
<td>4.4</td>
</tr>
<tr>
<td>160</td>
<td>0.85</td>
<td>0.283</td>
<td>2.6</td>
<td>1.6</td>
<td>1.0</td>
<td>1.2</td>
<td>3.0</td>
<td>0.9</td>
</tr>
<tr>
<td>160</td>
<td>0.85</td>
<td>0.149</td>
<td>2.9</td>
<td>1.2</td>
<td>0.9</td>
<td>2.0</td>
<td>1.2</td>
<td>0.9</td>
</tr>
<tr>
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<td>0.85</td>
<td>0.131</td>
<td>2.6</td>
<td>0.6</td>
<td>0.9</td>
<td>1.3</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>320</td>
<td>0.85</td>
<td>0.191</td>
<td>4.7</td>
<td>1.5</td>
<td>1.1</td>
<td>2.1</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>320</td>
<td>0.85</td>
<td>0.302</td>
<td>2.4</td>
<td>0.3</td>
<td>1.3</td>
<td>1.9</td>
<td>1.5</td>
<td>1.1</td>
</tr>
<tr>
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<td>0.204</td>
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<td>2.5</td>
<td>1.0</td>
<td>1.3</td>
<td>1.2</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Thirteen of the 14 EIA (-) but IFA (+) samples were negative in the individual EIA tests.
ANA TEST STUDY:

Results:

- 13 of the 14 samples ELISA (-) and IFA (+) were negative by the profile of individual assays.

- The 1 remaining sample was equivocal in the SSA test and positive in the ENA-6 Screen (as a result of the low level of anti-SSA).

- Majority “false positive” by IFA?
ANA TEST STUDY:

Total Discordant Resolution:

- **13 of the 14** samples IFA(+) / ELISA (-) were **negative** by the **profile tests**, in agreement with ELISA.

- **8 of the 26** samples IFA(-) / ELISA (+) were **positive** (and **3 were equivocal**) by the **profile tests**, in agreement with ELISA.

- **3** Samples IFA(-) / ELISA (+) were QNS for discordant testing.
**ANA TEST STUDY:**
Correlation Retabulated:

**Initial Results**

<table>
<thead>
<tr>
<th>ANA by IFA</th>
<th>ANA ELISA Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
</tr>
<tr>
<td>POS</td>
<td>114</td>
</tr>
<tr>
<td>NEG</td>
<td>26</td>
</tr>
</tbody>
</table>

33 Equivocals were excluded

Relative Sensitivity = 114/128 = 89.1 %
Relative Specificity = 323/349 = 92.6 %
Overall Agreement = 437/477 = 91.6 %

<table>
<thead>
<tr>
<th>ANA by IFA</th>
<th>ANA ELISA Screen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POS</td>
</tr>
<tr>
<td>POS</td>
<td>122</td>
</tr>
<tr>
<td>NEG</td>
<td>12</td>
</tr>
</tbody>
</table>

36 Equivocals were excluded from the calculations

Relative Sensitivity = 122/123 = 99.20 %
Relative Specificity = 336/378 = 96.6 %
Overall Agreement = 458/471 = 97.20 %
What to do with Equivocals?

• In this study, 28/33 (84.8%) equivocals were negative by IFA

• 3 were **positive** with a titer of **1:160**, 1 with a titer of **1:640**, 1 was not **titered**.

• Suggestions (per Diamedix package insert):
  
  (1) Report out as ‘equivocal’, or retest; if positive retest, report as ‘positive’; if negative retest, report as ‘negative’.

  (2) Test by another test

  (3) Test a new sample
ANA TEST STUDY:

Benefits of the ANA ELISA Screen

• To ‘rule out’ negatives:
  Approx. 60-80% of the total population tested
• To reduce IFA false positive and negatives
• Positive samples can be tested by IFA for staining pattern and titer
• ELISA Index varies with IFA titer
ANA ELISA Screen Index vs IFA Titer

<table>
<thead>
<tr>
<th>Titer</th>
<th>Mean</th>
<th>Median</th>
<th>High</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>160 Titer</td>
<td>2.14</td>
<td>1.22</td>
<td>12.60</td>
<td>0.39</td>
</tr>
<tr>
<td>320 Titer</td>
<td>2.02</td>
<td>1.83</td>
<td>5.62</td>
<td>0.40</td>
</tr>
<tr>
<td>640 Titer</td>
<td>5.54</td>
<td>4.06</td>
<td>12.60</td>
<td>0.68</td>
</tr>
<tr>
<td>1280 Titer</td>
<td>7.66</td>
<td>8.79</td>
<td>12.60</td>
<td>0.85</td>
</tr>
</tbody>
</table>
ANA TEST STUDY:

Although the IFA test at each titer does not reflect the wide positive response of the ELISA test, there is general correlation between IFA titer and ELISA Index:

- 1:160 versus 1.22 median index
- 1:320 versus 1.83 median index
- 1:640 versus 4.06 median index
- 1:1280 versus 8.79 median index
ANA TEST STUDY:

Conclusions:

**IFA**

- Results subjective (analyst interpretation)
- Multiple dilutions and repeat testing required
- 13/14 discordants (+) by IFA were **negative** in profile tests
- 11/23 (47.8%) discordants (-) by IFA were **positive/equivocal** in profile tests *

**ELISA**

- Results objective (spectrophotometric)
- Index values obtained from a single well
- 13/14 discordants (-) by ELISA were **negative** in profile tests (1 SSA equivocal)
- 11/23 (47.8%) discordants (+) by ELISA were **positive/equivocal** in profile tests *

*11 samples were positive/equivocal for antibody to dsDNA and/or SSA and SSB
Any Questions?
References


- Photo Credits: IFA images courtesy of University of Washington