Proximity Ligation Assays, A Method For Ultra Sensitive Protein Detection

Stephen Lin, Ph.D.
Staff Scientist
Thermo Fisher Scientific
What Do Proteomic Tools Need To Do?

• Measure protein abundances
  • Protein concentrations can differ by more than 1000-fold

• Measure protein variations
  • Isoforms - the number of mature proteins is estimated to far exceed the number of genes

• Measure protein modifications and protein interactions
  • Can regulate the protein dynamics
    • Activity
    • Localization
    • Stability

• Measure proteins on a large scale
  • Entire pathways
  • Entire cellular systems
PLA® Technology: A Perfect Union

- PLA® combines the best of “two worlds”:
  - The simplicity, sensitivity, and broad assay dynamic range of qPCR
  - The specificity of affinity-based protein detection reagents

- PLA® is flexible, scalable (up and down)

- PLA® enables a multi-dimensional analysis of DNA, RNA, and proteins on a single detection platform
TaqMan® Protein Assays (TPA) utilize PLA® Technology

- Assay based on two antibodies, each conjugated to a different oligonucleotide (one 3’ oligo, one 5’ oligo)
- When the two conjugated antibodies bind and are in close proximity, the oligonucleotides can be ligated, serving as the template for real-time PCR amplification and quantification

PLA® Platform Options: Homogeneous or Solid-phase

• **Option 1: Homogeneous Assays**
  - Rapid (<3hrs TTR), simple (no washes)
  - Dynamic ranges of ~3-4+ logs
  - Ultra-sensitive (e.g. LOD <0.1pg/mL)
  - Low sample input (1-2μL)

• **Option 2: Solid-phase Assays**
  - Ultra-sensitive (e.g. LOD <0.1pg/mL)
  - Broader dynamic ranges, 6+ logs
  - ~5hrs TTR
  - Plate or bead-based assays
  - Higher sample volume input (10-25μL)
Design Elements: The Oligos

• Oligo sequence elements –
  • Oligo A: “left” oligo, ~60mer, 3’ OH
    • Forward primer site
    • Probe site
    • Splint overlap region
  • Oligo B: “right” oligo, ~40mer, 5’ P
    • Reverse primer site
    • Splint overlap region

• Oligo conjugation
  • Indirect – “universal” oligo attachment to streptavidin (SA)
    • SA-oligo to be used in conjunction with biotinylated antibodies
    • Requires that antibodies are biotinylated
Simple, Flexible Homogeneous Workflow: TPA II

**3' oligo**

**5' oligo**

**Target**

**epitope 1**

**epitope 2**

**Room Temp.**

**Ligation-PCR Rxn Mix Addition**

**Addition**

**Single plate**

**4 µl/well**

**20 µl/well**

**30 min.**

**60 min.**

**45 min.**

**Assay Probe Mix**

**Ligase and Fast MMix**

**BINDING in PCR Plate**

**LIGATION & REAL-TIME PCR**

**optional: heat inactivation of ligase before qPCR**

**2 uL sample**

**2 uL Assay Probe Mix**

**Add 16 uL Fast MMix with ligase**

**Ligation (10 min)**

**Cycling protocol (40 min)**

**Alternate protocols available for using 6 uL of sample**
Comparing Workflows: TPA II and ELISA

TaqMan® Protein Assay II

- **Sample Dilution**: 20 min
- **Probe binding**: 1 hr Room Temp
- **Ligation/ qPCR**: 60 min

Homogeneous assay (no washes)

~2.5 hrs

ELISA

- **Sample binding**: 2 hrs
- **4-6 washes**: 15 min
- **Detection Ab binding**: 2 hrs
- **4-6 washes**: 15 min
- **Substrate binding**: 1 hr
- **Amplifier reaction**: 30 min
- **Stop reaction**: <5 min
- **Read OD**: <5 min

~5 hrs
Experimental Design Considerations: Running an Assay

- Sample type – isolated protein, cells, tissues, body fluids (e.g. serum/plasma)
- Assay dynamic range and sample input
- Method for quantification – relative or ‘absolute’

![CSTB Expression in Raji diagram](image1)

Cq vs cell input

NPC (no protein control)

\( \Delta C_q \) vs cell input

Linear dynamic range

"hook"
In vitro interactions between OPG and its ligand (RANKL) demonstrate specificity of TPA probe binding

OPG (Osteoprotegerin) is a circulating ‘decoy’ receptor for RANKL (receptor activator of NF-kappaB ligand) in bone metabolism.

Specific interactions between OPG and RANKL can be detected
Detection of Proteins in Serum Samples Can Be Extremely Sensitive (e.g. IL-6)

- Assay dynamic range
  - 0.128-2000pg/mL
- Sample = diluted serum
  - 4-fold, 10-fold, and 20-fold dilution in sample buffer

### Spike recovery in serum (5, 100, and 1000pg/mL)
- Acceptable range, 70-130%

### Assay performance summary

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Raw Value Average</th>
<th>Raw Value Stdev</th>
<th>Conc. Expected</th>
<th>Conc. Average</th>
<th>% Recovery</th>
<th>Raw Value %CV</th>
<th>% Recovery average</th>
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<tbody>
<tr>
<td>sample buffer</td>
<td>6833.2</td>
<td>2172.3</td>
<td>1000</td>
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<td>100.1</td>
<td>3.2</td>
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<td></td>
<td>9877.7</td>
<td>889.6</td>
<td>100</td>
<td>86.2</td>
<td>86.2</td>
<td>9.0</td>
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<tr>
<td></td>
<td>555.5</td>
<td>21.3</td>
<td>5</td>
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<td>30.9</td>
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<td>25% serum</td>
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<td>8284.1</td>
<td>314.6</td>
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<td>71.0</td>
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<td>836.1</td>
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<td>81.3</td>
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<td>458.4</td>
<td>84.7</td>
<td>0</td>
<td>3.0</td>
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<td>8182.8</td>
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<td>73.8</td>
<td>14.3</td>
<td>89.4</td>
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<td>9130.3</td>
<td>1057.1</td>
<td>100</td>
<td>79.0</td>
<td>90.2</td>
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<td>755.6</td>
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<td>5.2</td>
<td>104.2</td>
<td>3.7</td>
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<td></td>
<td>233.6</td>
<td>36.3</td>
<td>1</td>
<td>1.4</td>
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Detection of Stem Cell Pluripotency Markers

Pluripotent cells:
Self renewal

Loss of Pluripotency:
Differentiation

Embryonic Stem Cells

Key Markers of Pluripotency

<table>
<thead>
<tr>
<th>NANOG</th>
<th>OCT3/4</th>
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<tbody>
<tr>
<td>SOX2</td>
<td>LIN28</td>
</tr>
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</table>

Neural cells (neurons)
Mesenchymal cells (connective tissue)
Hematopoietic cells (blood)
TaqMan® Protein Expression Assays for Stem Cell Markers are Specific and Sensitive

**NTERA2 lysate – positive control**

- **SOX2**
- **NANOG**

**Raji lysate – negative control**

- **OCT3/4**
- **LIN28**

![Graphs showing expression levels of various markers in NTERA2 and Raji lysates](image-url)
Western vs. TaqMan® Protein Expression Assay

<table>
<thead>
<tr>
<th>OCT3/4</th>
<th>Western</th>
<th>TaqMan Protein Expression Assay</th>
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<tbody>
<tr>
<td>100,000 cells</td>
<td>100,000 cells/lane</td>
<td>100-500 cells/well</td>
</tr>
<tr>
<td>33,000 cells</td>
<td>30,000-10,000 cells/lane</td>
<td>10-35 cells/well</td>
</tr>
<tr>
<td>11,000 cells</td>
<td>1-2 days</td>
<td>3 ½ hr</td>
</tr>
<tr>
<td>3,700 cells</td>
<td>Tedious, multiple steps</td>
<td>Simple, no wash steps</td>
</tr>
<tr>
<td>1,200 cells</td>
<td>Gel Image</td>
<td>Numerical</td>
</tr>
<tr>
<td>400 cells</td>
<td>Usually +/-</td>
<td>Relative Quantitation</td>
</tr>
<tr>
<td>137 cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 cells</td>
<td></td>
<td></td>
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</tbody>
</table>

| LIN28           |          |                                |
| NANO2           |          |                                |
| SOX2            |          |                                |

NTERA2 embryonal carcinoma

0CT3/4
LIN28
NANO2
SOX2

TaqMan® Protein Expression Assay

TaqMan® Protein Expression Assay

TaqMan® Protein Expression Assay

TaqMan® Protein Expression Assay
Protein Markers Assayed in Pluripotent and Mesenchymal Stem Cell Lysates

**Pluripotent cells:**
Self Renewal

**Loss of Pluripotency:**
Differentiation

<table>
<thead>
<tr>
<th>Key Markers ↓</th>
<th>Key Markers ↑</th>
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<tr>
<td>LIN28</td>
<td>CSTB</td>
</tr>
<tr>
<td>NANOG</td>
<td>ICAM1</td>
</tr>
<tr>
<td>OCT3/4</td>
<td>CD29</td>
</tr>
<tr>
<td>SOX2</td>
<td>CD105</td>
</tr>
<tr>
<td>DPPA4</td>
<td>CD166</td>
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<tr>
<td>CDH1</td>
<td>TNFR1</td>
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</table>

Mesenchymal cells
TaqMan® Protein Expression - Differentiated/Pluripotent

Summary Relative Quantification Data – After Differentiation

TARGET PROTEINS

NANOG
SOX2
OCT3/4
LIN28
DPPA4
CSTB
CAM1
CD29
CD105
CD166
TNFRI

FOLD CHANGE

-90
-85
-80
-75
-70
-65
-60
-55
-50
-45
-40
-35
-30
-25
-20
-15
-10
-5
0
5
10
15
20

Down-regulated

Up-regulated

CDH1

+15
+10
+5
Analysis of protein and RNA expression: NTERA2 early neuronal differentiation model

NTERA2 cells → RA Induction → NTERA2 - neuronal progenitor

Self renewal

Differentiation to neuronal

Day 10
(-) RA

Day 10
(+ ) RA
NTERA2 cell lysate workflows

Sample Preparation Workflow

<table>
<thead>
<tr>
<th>Protein</th>
<th>mRNA</th>
<th>miRNA</th>
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<tbody>
<tr>
<td>Harvested Cells</td>
<td>Harvested cells</td>
<td>Harvested cells</td>
</tr>
<tr>
<td>Protein Expression Sample Prep kit</td>
<td>PARIS RNA kit</td>
<td>PARIS mirVana kit</td>
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<tr>
<td>TURBO DNA-free DNase treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TaqMan® Protein Expression Assay</td>
<td>One-step RT-qPCR TaqMan® mRNA Assay</td>
<td>Multiplex RT TaqMan® MicroRNA Assay</td>
</tr>
</tbody>
</table>

Harvest and assay at day time points 0, 1, 4, 10 and 14
MicroRNA-145 Regulates OCT4, SOX2, and KLF4 and Represses Pluripotency in Human Embryonic Stem Cells
NTERA2 Loss of Pluripotency: miR-145 and OCT4 expression changes
TaqMan® Protein Assays for Phosphorylation
Endogenous p53 pSer15 in cell lysates

ELISA kit
mAb1, mAb2

Protein Assay
pAb1, mAb2

p53 pSer15 PLA - more sensitive & greater dynamic range vs. ELISA kit
Benchmarking endogenous AKT pSer473 in cells

AKT pSer473 PLA developed was ≥50-fold more sensitive than ELISA.

LOD based on $Z' > 0.5$:
- ELISA-stim: 6,300 cells/well
- PLA-stim: 50 cells/well
**Pharmacology for pS473 AKT: ELISA v. PLA®**

- U2OS starved prior to stimulating with dose responses of 4 agonists for 14 min at 37°C prior to cell lysis.

- **Pharmacology obtained with PLA® comparable to ELISA using 30-fold fewer cells**

<table>
<thead>
<tr>
<th>Compound</th>
<th>PLA</th>
<th>ELISA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>750 cells/well</td>
<td>22,500 cells/well</td>
</tr>
<tr>
<td>PDGF-BB</td>
<td>0.77 nM</td>
<td>1.9 nM</td>
</tr>
<tr>
<td>IGF1</td>
<td>5.7 nM</td>
<td>11 nM</td>
</tr>
<tr>
<td>EGF</td>
<td>0.96 nM</td>
<td>1.9 nM</td>
</tr>
<tr>
<td>HGF</td>
<td>12 nM</td>
<td>22 nM</td>
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</table>
Summary:

- PLA® / TPA technology enables a broad range of protein detection applications, including quantification of:
  - Proteins in cells (including single cells) and tissues
  - Protein modifications
  - Protein-protein interactions
  - Serum/plasma biomarkers

- The simple workflow is ideally suited for high-throughput screening applications
  - All assay steps are completed in a single plate and are compatible with automation
Concluding Remarks:

- PLA® /TPA offers a simple and flexible platform – homogeneous and solid-phase formats

- PLA® is scalable
  - # of assays - Singleplex or multiplex formats
  - # of samples - High throughput screening

- Enabling multi-analyte detection from a single sample on a single platform – DNA, RNA and protein

- PLA® has a low sample input requirement (1-2μL)
Proximity Ligation Assay Application Areas

Genomic/Basic Research

- Single-cell biology/systems biology
- Gene/protein expression correlation
- Pathogen detection
- Prion detection

Drug Development

- Biomarker discovery/validation
- Drug discovery (HTS)
- Pharmacodynamics/pharmacokinetics
- Pharma analytics

- PLA® / TPA

Health and Medical Sciences

- Drug Development
“75% of protein research still focuses on the 10% of proteins that were known before the genome was mapped – even though many more have been genetically linked to disease.”

Acknowledgements

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Kristin Huwiler
Erica Graziosa
Junko Stevens

"For Research Use Only. Not for use in diagnostic procedures."

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## Design Elements: The “Binders”

<table>
<thead>
<tr>
<th>Antibody Type</th>
<th>Details</th>
</tr>
</thead>
</table>
| **Polyclonal Antibody** | • Antigen (affinity) purified  
• Raised against full length or partial antigen (> 100 aa); polyclonals to peptides not extensively tested |
| **Monoclonal Antibody** | • Often used successfully in pairs or in combination with a suitable polyclonal when qualified by ELISA                                              |
| **Aptamers**        | • Single aptamers are suitable for detection of homo-dimers, homo-trimers, etc…  
• Development of aptamer pairs could be challenging  
• Not ideal for detecting modifications such as phosphorylation                                      |
Antibodies typically 2 different mAbs, 1 mAb + 1 pAb, or a split pAb
Design Elements: Assay Buffers and Reagents

- Sample preparation
  - Non-denaturing reagents for cell lysis or tissue homogenization (recommended)
- Binding buffers – containing blocking agents (for non-specific protein and nucleic acid binding and matrix effects)
  - Probe dilution buffer
  - Sample dilution buffer
- Ligation/PCR reagents – fluorescent probe-based chemistry (recommended)
Data Analysis Considerations

• Type of quantification
  • Relative quantification (RQ)
    • Use of sample dilution curves
    • “Single point” RQ using reference assay for normalization
  • “Absolute” quantification
    • Use of standard curves for quantification

• Normalizing for sample input (unit of measure)
  • Cell count – hemocytometer, Countess® cell counter
  • gDNA as a surrogate for cell count (singleplex or duplex reactions)
  • Total protein – determined by microBCA™ protein assay kit (ThermoFisher, PN 23235) or similar method
  • Unit volume
Using Different Antibodies and Combinations

**pSer15 Antibody Screen:**

pSer15-p53 Abs v. fixed GFP pAb

![Diagram of p53 and GFP with antibody binding sites](image)

**Pan p53 Antibody Screen:**

Abfinity® p53 pSer15 v. pan p53s

![Diagram of p53 and GFP with antibody binding sites](image)

**Graphs:**

- **Delta Cq vs. log GFP-p53 (ng/mL):**
  - ABfinity 700439, Ph
  - ABfinity 700439, NP
  - Supplier A, Ph
  - Supplier A, NP
  - Supplier B, Ph
  - Supplier B, NP
  - Supplier C, Ph
  - Supplier C, NP

- **Delta Cq vs. [GFP-p53] (ng/mL):**
  - Supplier A, Ph
  - Supplier A, NP
  - Supplier B, Ph
  - Supplier B, NP
  - Supplier C, Ph
  - Supplier C, NP
  - LIFE AHO-0032 (mAb) Ph
  - LIFE AHO-0032(mAb), NP
Assay Optimization Parameters

• Incubation time and temperature
  • 1-2 hour binding at room temperature is often sufficient
  • Overnight at 4°C can improve performance appreciably for some assays

• Probe concentration
  • 50-200pM probes is recommended

• Trying different antibodies and combinations is highly recommended

• Probe orientation may influence assay performance

• Sample buffer composition may be critical for optimal assay performance
  • Sample or buffer components can inhibit assay or cause false signal
  • Sample or buffer components can affect assay linearity
Effect of Binding Time and Temperature

~10-fold improvement in assay performance with binding at 4°C overnight
Relative Quantification: Normalization to Cell Input

ProteinAssist™ Software

Raji p53 Protein Expression:
5 hours post-irradiation

NTERA2 p53 Protein Expression:
5 hours post-irradiation

\[ \text{p53 Protein Assay Delta Cq} \]

\[ \text{Raji Ctrl (p53)} \]
\[ \text{Raji UV (p53)} \]

\[ \text{Quantitation threshold} \]

p53 no change

p53 RQ cell-intercept: 56/10 cells
5.6 fold induction