Monitoring Post-Translational Modifications and Protein-Protein Interactions using the Proximity Ligation Assay

Kristin G. Huwiler, Tina Settineri, Rica Bruinsma, Bryan Marks, Mark Shannon, Dave Ruff, & Barry Schweitzer, Life Technologies, 501 Charmany Drive, Madison, WI, USA, 53719

ABSTRACT

Protein post-translational modifications (PTM) and protein-protein interactions (PPI) contribute to the regulation of cellular signaling networks by influencing the function, stability, and localization of proteins (Figure 1). As a result, monitoring changes in select PTMs and PPIs as part of the drug discovery process is important. Although numerous methods exist to measure PTMs or PPIs, most are labor intensive and time-consuming. We have applied quantitative TaqMan® protein assays, which are based on the Proximity Ligation Assay (PLA™), to measure changes in PTMs and PPIs as it offers the following advantages: limited sample requirement, homogeneous assay, robust signal amplification via TaqMan® real-time PCR, and quantitative results within a few hours. We have successfully applied PLA to monitor PTM & PPI changes to the transcription factor p53 within cells subjected to genotoxic stress. Due to the specificity, sensitivity, and small sample requirement, TaqMan® Protein Assays are an appealing alternative that can be applied at multiple stages of the drug discovery process.

INTRODUCTION

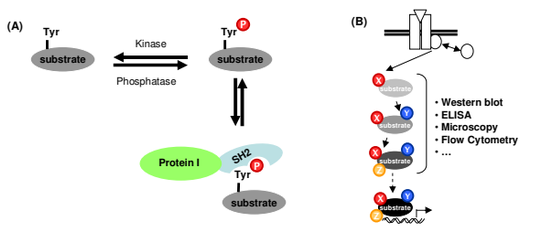
Phospho-protein detection via TaqMan® Protein Assays (TPA) requires two proximity probes, which are antibodies coupled to an oligonucleotide. One antibody is targeted to the phosphorylation site of interest and a second antibody is targeted to another epitope on the protein. Following binding of the proximity probes to the phospho-protein, a bridging oligonucleotide enables oligo hybridization, followed by ligation, and quantitative amplification in a TaqMan® assay (Figure 2). The tumor suppressor protein p53 was chosen for this study for numerous reasons. First, p53 is biologically interesting as it is central to maintaining the integrity of the genome and is found to be mutated in ~50% of all cancers. Second, in response to different cellular stimuli, p53 undergoes changes in post-translational modifications as well as changes in protein-interactions. Third, p53 forms higher order multimers with the tetramer being the active transcription factor (Figure 3).

MATERIALS AND METHODS

Recombinant p53: A recombinant GFP-p53 fusion was expressed using the baculovirus system and purified. Using standard methods, this recombinant p53 was phosphorylated with DNAPK, which is known to phosphorylate Ser15 of p53, and dephosphorylated with lambda phosphatase.
Preparation of Cell Lysates: U2OS cells were grown to confluence and then seeded at 1x10^6 cells/well. Cells, in growth media, were treated with or without compounds known to induce genotoxic stress, resulting in p53 stabilization and phosphorylation of Ser15 (p53 pSer15). After ≥20 hr incubation, the cells were lysed and then processed in TaqMan® Protein Assays or in a commercial ELISA according to the manufacturer instructions.
TaqMan® Protein Assays (TPA): The TaqMan® Protein Assays Open kit was used to generate proximity probes using biotinylated antibodies (Figure 2A). Several polyclonal antibodies (pAb) and/or monoclonal antibodies (mAb) to p53 pSer15, p53, and GFP were tested. The TaqMan® Protein Assay Core Reagents kit with Master Mix was used to run TPA (Figure 2B).

RESULTS

Figure 1. Post-Translational Modifications (PTM), Protein-Protein Interactions (PPI), & Cell Signaling

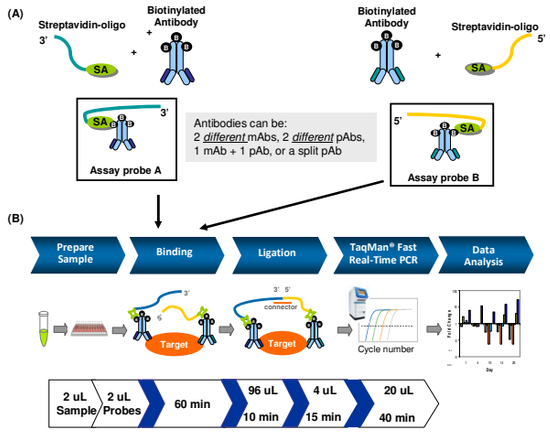


X,Y,Z = post-translational modifications such as phosphorylation, acetylation, ubiquitination, methylation.

(A) Numerous types of protein post-translational modifications (PTM) have been identified, including phosphorylation, acetylation, & ubiquitination. PTMs are important in cellular signaling as they can impact the activity, interaction partners, & stability of a protein. Illustrated is the reversible PTM of phosphorylation on a tyrosine within a protein substrate; the phospho-Tyr moiety allows the substrate to then interact with Protein I that contains an SH2 domain. (B) It is the change in activity & interacting partners that are crucial to cellular signaling networks, which can transmit a change in the extra-cellular environment to changes within the cell such as transcription. Several methods to monitor signaling-induced PTMs are listed.

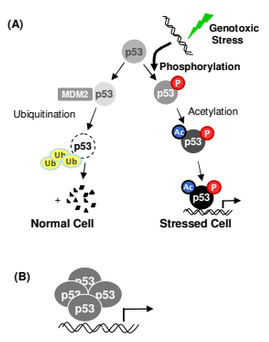
RESULTS

Figure 2. TaqMan® Protein Assay Workflow



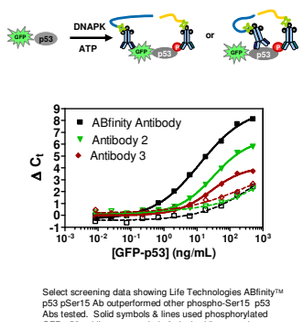
(A) TaqMan® Protein Assays (TPA) are a form of Promixity Ligation that utilize antibody probes with conjugated oligonucleotides. The assay probes (A & B) are formed by the conjugation of streptavidin (SA) oligonucleotides to biotinylated antibodies. (B) Binding of the antibody probes to the target brings the ends of the oligos into proximity. Hybridization of a connector oligo enables ligation. Amplification of the ligated product is accomplished via TaqMan® real-time PCR.

Figure 3. p53 Model System



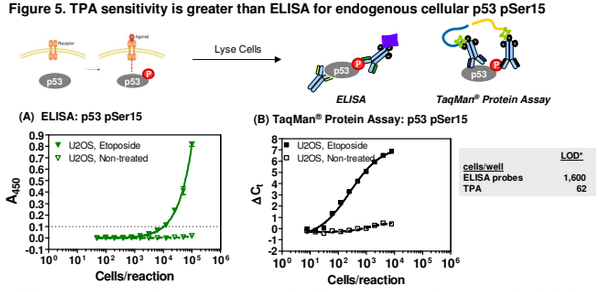
(A) p53 is a transcription factor that is regulated by numerous types of PTM, including phosphorylation (P), acetylation (Ac), & ubiquitination (Ub). In addition, p53 is regulated through protein –protein interactions including it's interaction with MDM2 (A) as well as self interactions (B). The transcriptionally active form of p53 is a homotetramer. For ease of illustration, many figures will depict p53 as a single subunit although that is not meant to imply only monomeric p53 was present.

Figure 4. Antibody Screening for TPA Development using recombinant p53



Select screening data showing Life Technologies ABfinity™ p53 pSer15 Ab outperformed other phospho-Ser15 p53 Abs tested. Solid symbols & lines used phosphorylated GFP-p53, while open symbols & dashed lines used non-treated GFP-p53, which contained some phospho-protein. Abs were screened to develop assays that would measure p53 pSer15 as well as total p53. The total number of Abs screened in TPA: 6 p53 pSer15 & 7 p53.

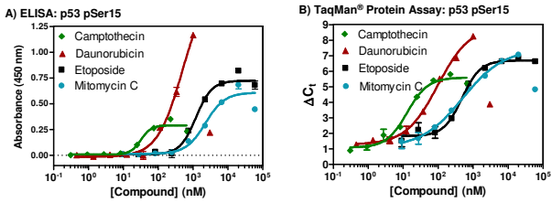
Figure 5. TPA sensitivity is greater than ELISA for endogenous cellular p53 pSer15



cells/well	LOD*
ELISA probes	1,600
TPA	62

U2OS cells were treated with or without etoposide for 20 hrs in 37˚C / 5% CO2 incubator prior to lysis. Sensitivity of p53 pSer15 ELISA Ab pair in ELISA (A) to TaqMan® Protein Assay using p53 pSer15 Ab with p53 Ab (B). Defining the limit of detection (LOD) for each assay as a Z' > 0.5, TPA was >20-fold more sensitive compared to an ELISA.

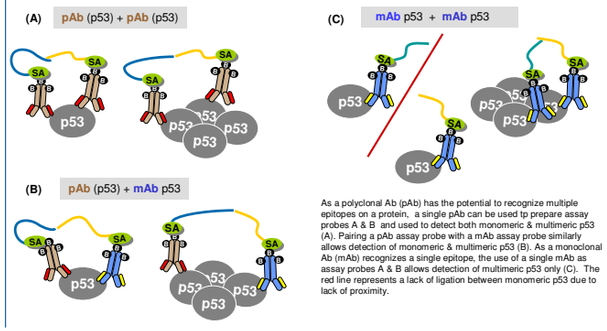
Figure 6. Pharmacology of endogenous p53 phosphorylation obtained with TPA is comparable to ELISA & uses fewer cells



	EC$_{50}$ (nM)			
	Camptothecin	Daunorubicin	Etoposide	Mitomycin C
ELISA	31	ND	1,200	2,200
TPA	13	98	480	500

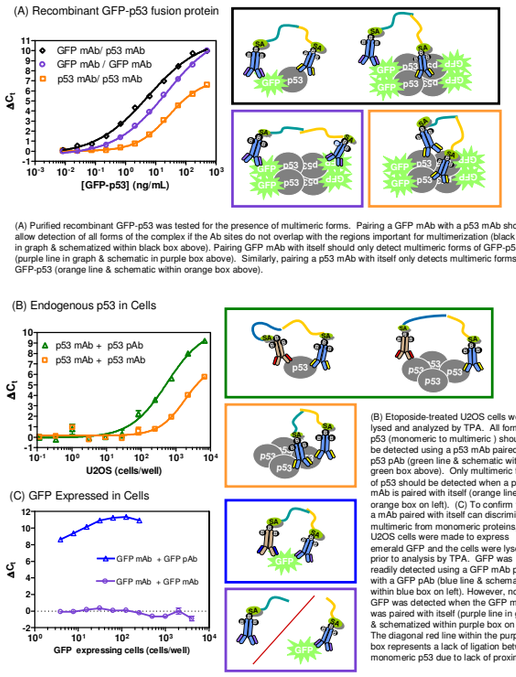
U2OS cells were seeded in 96-well plates (10,000 cells/well) with compounds for 20 hrs. Cells were incubated for 20 hrs in 37˚C/5% CO_2 incubator prior to lysis. The following lysate equivalents used for the two different assays were: ELISA with 30,000 U2OS cells/well; TPA with 3,000 U2OS cells/well. Pharmacology obtained with TPA is comparable to ELISA using 10-fold fewer cells.

Figure 7. Detecting Oligomeric Protein-Protein Interactions with TaqMan® Protein Assays



As a polyclonal Ab (pAb) has the potential to recognize multiple epitopes on a protein, a single pAb can be used tp prepare assay probes A & B and used to detect both monomeric & multimeric p53 (A). Pairing a pAb assay probe with a mAb assay probe similarly allows detection of monomeric & multimeric p53 (B). As a monoclonal Ab (mAb) recognizes a single epitope, the use of a single mAb as assay probes A & B allows detection of multimeric p53 only (C). The red line represents a lack of ligation between monomeric p53 due to lack of proximity.

Figure 8. Detecting Protein-Protein Interactions for p53 via TPA



(A) Purified recombinant GFP-p53 was tested for the presence of multimeric forms. Pairing a GFP mAb with a p53 mAb should allow detection of all forms of the complex if the Ab sites do not overlap with the regions important for multimerization (black line in graph & schematized within black box above). Pairing GFP mAb with itself should only detect multimeric forms of GFP-p53 (purple line in graph & schematic in purple box above). Similarly, pairing a p53 mAb with itself only detects multimeric forms of GFP-p53 (orange line & schematic within orange box above).

(B) Etoposide-treated U2OS cells were lysed and analyzed by TPA. All forms of p53 (monomeric to multimeric) should be detected using a p53 mAb paired with p53 pAb (green line & schematic within green box above). Only multimeric forms of p53 should be detected when a p53 mAb is paired with itself (orange line & orange box on left). (C) To confirm that a mAb paired with itself can discriminate multimeric from monomeric proteins, U2OS cells were made to express emerald GFP and the cells were lysed prior to analysis by TPA. GFP was readily detected using a GFP mAb paired with a GFP pAb (blue line & schematized within blue box on left). However, no GFP was detected when the GFP mAb was paired with itself (purple line in graph & schematized within purple box on left). The diagonal red line within the purple box represents a lack of ligation between monomeric p53 due to lack of proximity.

CONCLUSIONS

- TaqMan® Protein Assays can be used to measure the PTM of phosphorylation with limited cell samples
- The sensitivity of the p53 pSer15 TaqMan® Protein Assay developed using ABfinity™ p53 pSer15 Ab and a pan p53 mAb was better than a competitor p53 pSer15 ELISA by at least 10-20 fold
- TaqMan® Protein Assays were successfully used to measure changes in p53 pSer15 in response to compound treatment, producing compound EC$_{50}$ values comparable to those obtained with an ELISA
- By using a single p53 mAb to make TPA probes, p53 protein-protein interactions were detected in both a recombinant purified p53 protein as well as endogenous p53 from cell lysates

ACKNOWLEDGEMENTS

For helpful discussion, reagents, & encouragement we want to thank:
In Foster City, CA - Shiaw-Min Chen, David Joun, David Ruff, & Elana Swartzman
In Madison, WI- Coby Carlson, Mike Hancock, Spencer Hermanson, Thomas Machleidt, Steve Riddle, Kevin Vedvik, & Kurt Vogel
In Eugene, OR- Ruth Deveny, & Carolyn Petersen

TRADEMARKS/LICENSING