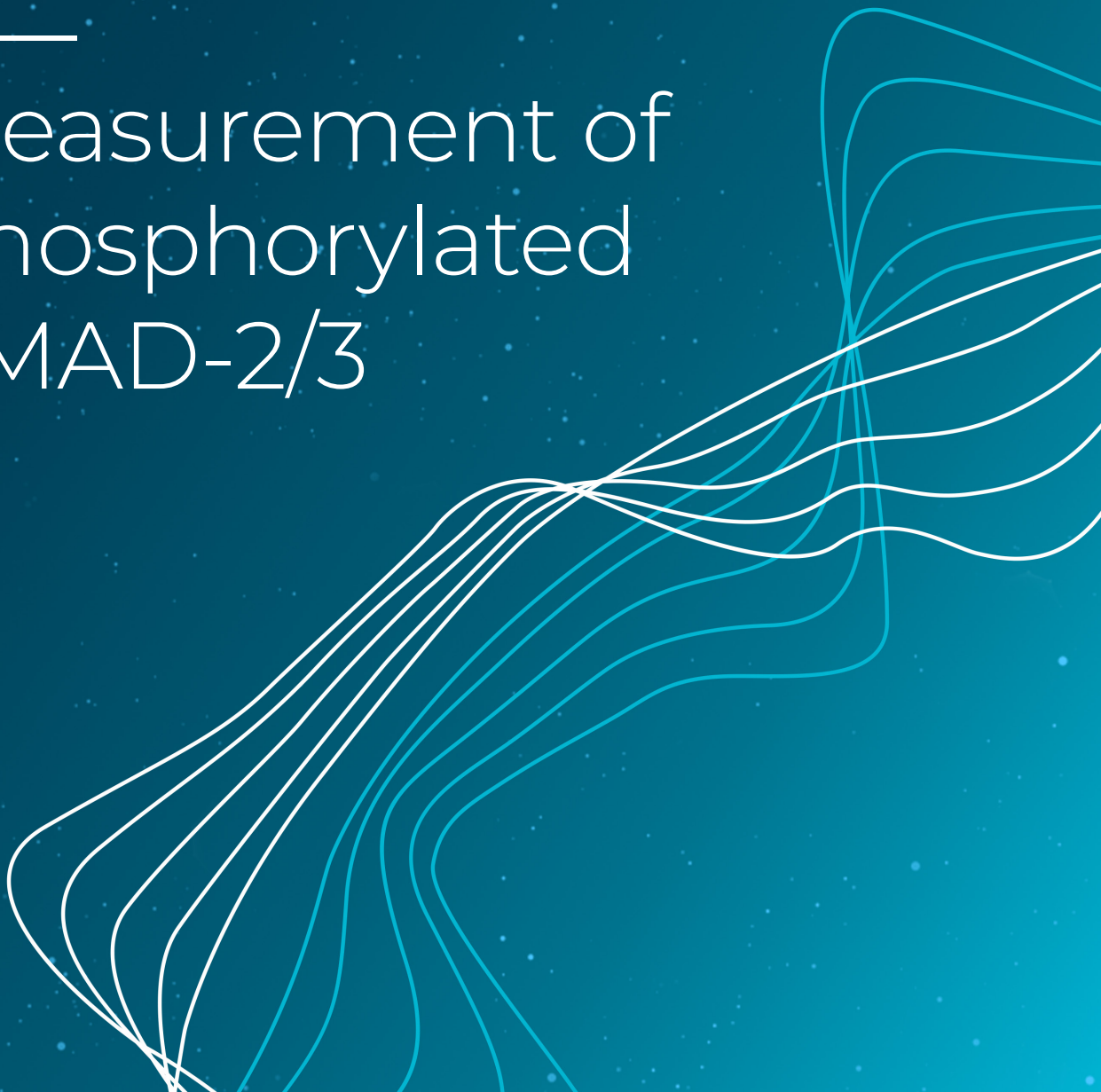
synexa | Life Sciences

Measurement of phosphorylated SMAD-2/3



pSMAD-2/3 is the key signallingprotein in TGF-ß mediated fibrotic disease

Contents

Introduction to Synexa

Synexa Life Sciences is a specialist immunology and translational research firm. Headquartered in the Netherlands, Synexa has a team of over 50 scientists across laboratories in Berlin, Cape Town, and London and is well-positioned to service a global network of clients in the biopharma industry.

For each phase of clinical development, we offer deep expertise in biomarker selection and panel design. Our biomarker programmes are tailor-made to suit each client's needs and involve considerable up-front scientific collaboration.

Our scientists have extensive experience analysing post-translational modifications in biomarkers. This is a key consideration in complex signalling pathways. We have developed methods that guarantee sample integrity and high-resolution data. Client-support is ongoing post-analysis for data interpretation.

Synexa Life Sciences is the pre-eminent laboratory for the analsyis of Phosphorylated and Total SMAD.

TGF-ß mediated SMAD signalling and fibrosis

Phosphorylation and dephos-phorylation of SMADs is a key indicator of target engagement.

In fibrotic disease, deposition of extracellular matrix (ECM) proteins is increased. This compromises tissue architecture and interferes with normal organ function.

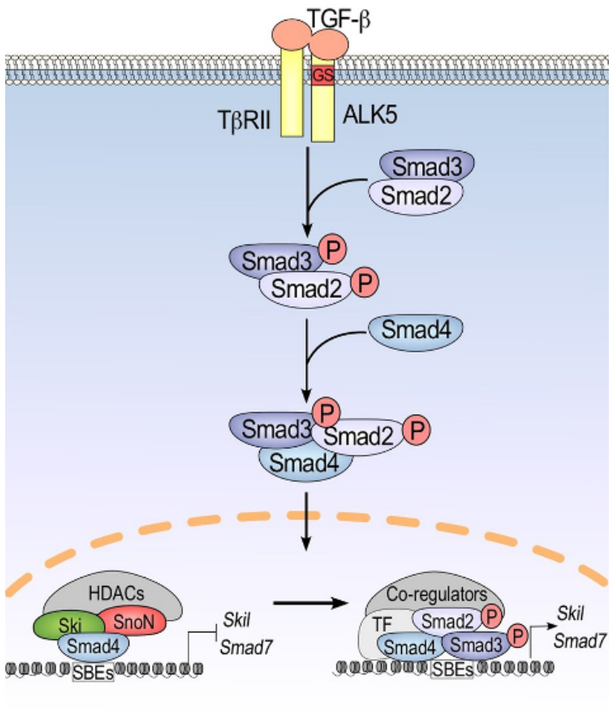
TGF-ß1 drives the expression of profibrotic genes through the SMAD-2/3 signal cascade. TGF-ß1 has a natural affinity for ECM and concentrates in the fibrotic site, thus exacerbating the condition.

Therapies that target the TGF-ß-SMAD signalling pathway are important for the treatment and prevention of fibrosis. Phosphorylation and dephosphorylation of SMADs is a key indicator of target engagement.

The biological pathway

pSMAD regulates fibrosis-related gene expression.

SMAD-2 and SMAD-3 complexes are key signalling molecules in the TGF-ß pathways. TGF-ß activates cell-surface receptors which phosphorylate SMAD-2/3 proteins residing in the cytoplasm. These phosphorylated SMAD complexes translocate to the nucleus where they regulate gene expression.



Tecalco-Cruz *et al.*, 20181

pSMAD-2/3 is a critical target engagement biomarker of anti-fibrotic therapies that modulate the TGF-ß pathway

Assay development & validation

Synexa specialises in custom-designed assays that take into account the priorities of the sponsor, the biology of the drug and known mechanisms of the pathway being targeted. Most of our work involves developing de novo assays or optimising existing commercial assays for a particular matrix or context, allowing the sponsor great flexibility in conducting clinical trials.

Each assay is developed and validated in-house in close partnership with the sponsor, with an emphasis on robustness and sensitivity in new assay development.

Depending on client requirements, assay development and validation can be performed for exploratory research purposes, which is quicker and cheaper, or to meet full FDA/regulatory standards.

Thorough assay development, optimisation and validation is best done well in advance of fieldwork, as the process can deliver significant improvements in assay sensitivity, with big implications for sample volume and sample prep methods.

Well thought-out assay optimisation can ensure the delivery of robust and insightful data and can often save a clinical trial form considerable wasted effort.

The preanalytical phase

Sample matrices

At Synexa, the measurement of pSMAD-2/3 and tSMAD-2/3 has been validated in the following sample types:

- **PRP lysate:** Platelet-rich plasma (PRP) is a concentrate of platelet-rich plasma protein isolated from whole blood. PRP is rich in growth factors, including TGF-ß.
- **HepG2 cell lysate:** HepG2 is a human liver carcinoma cell line with high proliferation rates. HepG2 can be successfully stimulated with growth factors and is an ideal *in vitro* model for cell surface interactions.
- **BAL lysate:** Bronchoalveolar lavage (BAL) is a diagnostic method where fluid is introduced to the lower respiratory airways and then collected for examination. BAL lysate contains diagnostic markers for a range of respiratory conditions, including interstitial lung disease.
- **HT1080 lysate:** HT1080 is a fibrosarcoma cell line with epithelial morphology. It is a prolific cell line that responds well to TGF-ß stimulation.

Sample preparation

The careful treatment of a specimen before analysis ensures a robust representation of *in vivo* biology.

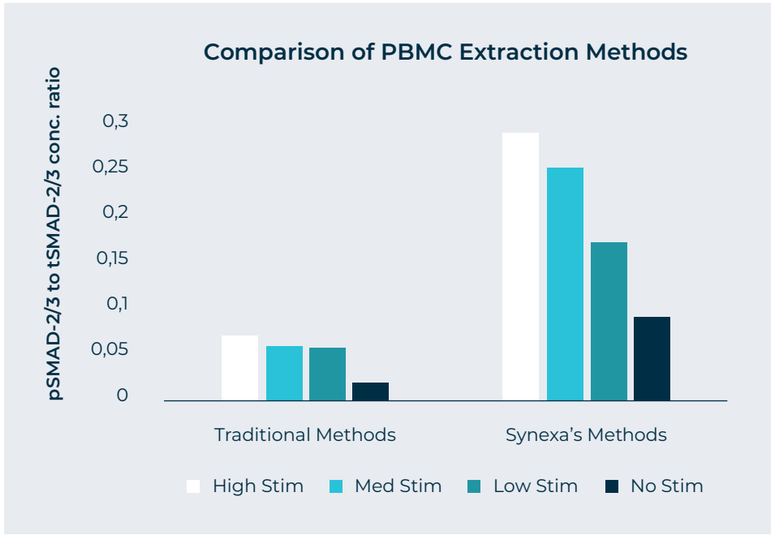
Post-translational modifications, particularly phosphorylation groups, are volatile and will degrade with improper sample handling. The careful treatment of a specimen before analysis ensures a robust representation of *in vivo* biology.

The moment a cell or tissue is removed from its environment, the cells react to *ex vivo* stressors. It is critical to use a protocol that minimises the delay from sample collection to proteome stabilisation.

The recommended maximum elapsed time from removal to stabilisation is 20 minutes before significant phosphoproteome degradation occurs[2]. This is problematic for traditional PBMC isolation methods which require at least 40 minutes in centrifugation alone, with multiple sample handling steps in between. At Synexa we have found a way around this with our faster, more efficient PBMC isolation method. The time from isolation to stabilisation in cell lysis buffer is under 10 minutes.

Synexa has world leading expertise and experience in the stabilization of phosphoprotein biomarkers for clinical research.

The data below demonstrates the importance of reduced sample handling as per Synexa's methods.



Comparison of PBMC Extraction Methods: Whole blood was stimulated with high, medium, low, and zero levels of TGF-ß. PBMCs were extracted from stimulated whole blood by two different methods and analysed for pSMAD-2/3 and tSMAD-2/3.

Sample normalisation

The extraction process introduces technical variation and it is important that this is not mistaken for biologically significant changes between samples. To negate this as a variable, total protein concentration is determined using the reducing compatible / detergent compatible (RCDC) protein assay.

Using the results from the RCDC assay, the same amount of total protein (typically 100 µg) can proceed to analysis for each replicate. This normalises technical variation between samples.

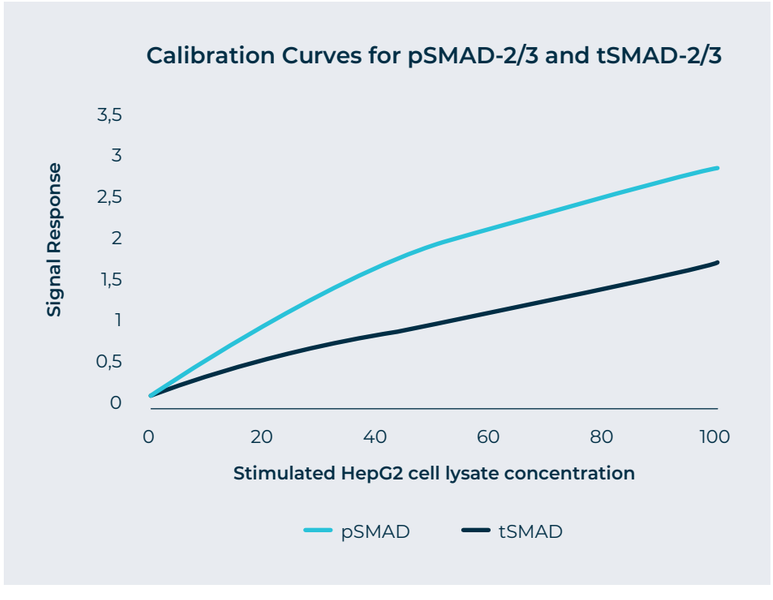
The analytical phase

The SMAD-2/3 assay

Two sandwich ELISA assays are used for the detection of pSMAD-2/3 and tSMAD-2/3. Antibodies for the detection of pSMAD-2/3 are specific to phospho-SMAD2 (Ser465/467) and phospho-SMAD3 (Ser423/425). These are the sites that are phosphorylated specifically by TGF-ß mediated signalling[3,4].

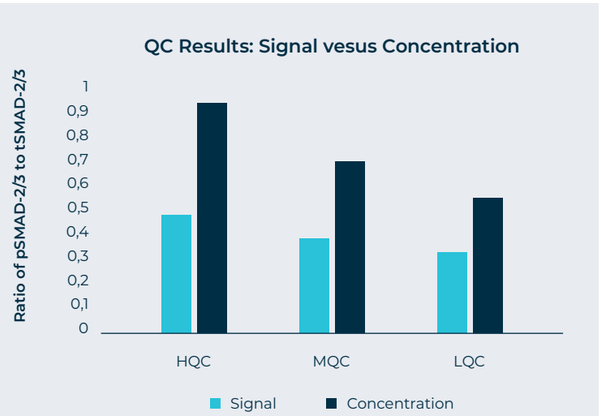
Calibration curve and quality control

Signal response for pSMAD-2/3 and tSMAD-2/3 is not linear which can cause differences in phosphorylation levels to be skewed. To mitigate this, Synexa analyses a calibration curve made from a serial dilution of TGF-ß stimulated HepG2 cell lysates alongside all samples. A relative concentration value for each sample can then be extrapolated from the calibration curve.



Synexa produces SMAD-containing cell lysates as a calibration curve.

To ensure assay precision and resolution, quality control (QC) samples are included in each assay. QC samples are augmented to represent high, medium, and low levels of SMAD-2/3 phosphorylation and are analysed alongside analytical samples. The relative concentration of QC samples is extrapolated from the calibration curve. The graph below shows the importance of concentration data for high-resolution measurement of phosphorylation changes.



Data analytics

Inhibition of TGF-ß results in a decrease of phosphorylated SMAD-2/3, however, the total levels of SMAD-2/3 remain unchanged. The clinically relevant output is the proportion of SMAD-2/3 that is phosphorylated. Thus, two sandwich ELISA assays are performed separately: pSMAD-2/3 and tSMAD-2/3. Data is reported as a signal or concentration ratio of pSMAD-2/3 to tSMAD-2/3.

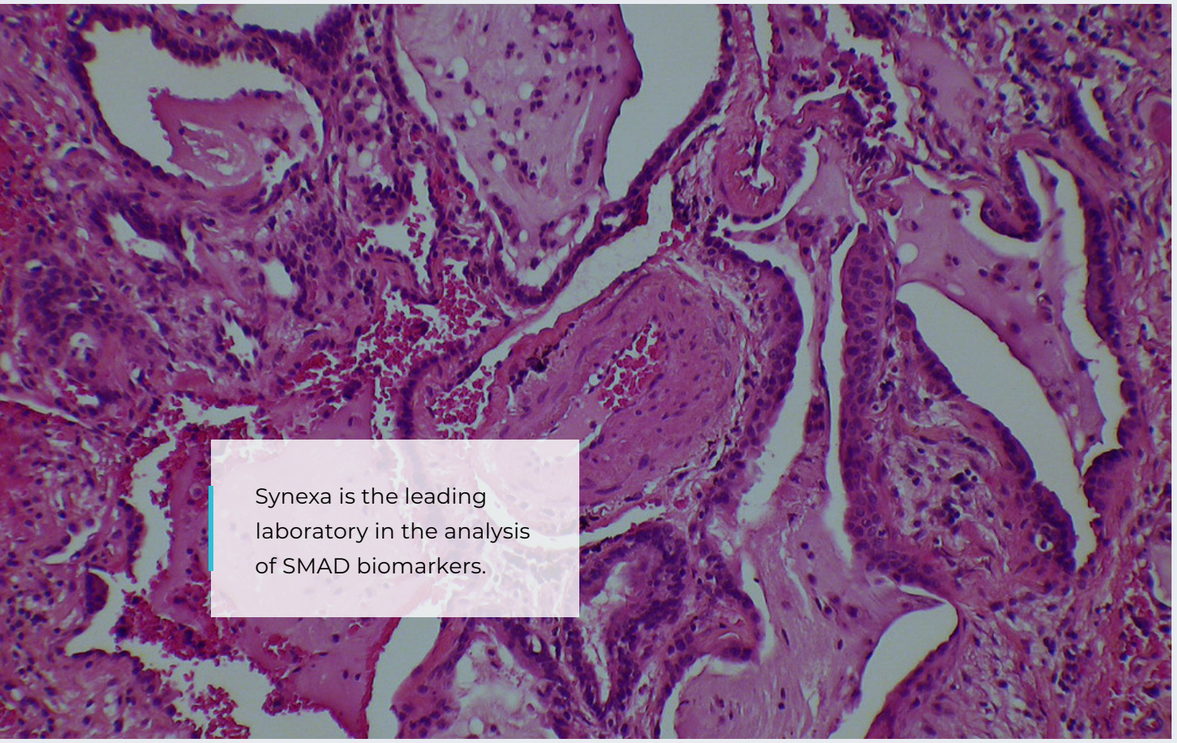
Patient stratification

SMAD phosphorylation data is a vital pharmacodynamic biomarker which gives key insights into therapies that target TGF-ß. Phosphorylation levels of SMADs can indicate appropriate drug dose levels[5], they can serve as a powerful predictor of cell proliferation *in vitro*[6], and they are a reliable marker for disease progression and pathogenesis[7].

Many diseases being tackled with TGF-ß inhibitors are complex in nature and show strong genetic predispositions. It is, therefore, most rational to preselect patient populations before initiating treatment. Circulating PBMCs which are isolated and treated with drug *ex vivo* can be used to predict individual responses to TGF-ß inhibitors by measurements of pSMAD levels[8].

Conclusion

The robustness of Synexa's SMAD-2/3 phosphorylation assay allows clients to monitor a therapeutic across many different clinical stages. The ability to detect pSMAD in a range of tissues allows one to get a broad picture of the effects of a therapeutic.



Synexa is the leading laboratory in the analysis of SMAD biomarkers.

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Dr Justin Devine
jdevine@synexagroup.com

www.synexagroup.com