

Reverse Phase Protein Arrays (RPPA): The Assay Platform of Choice for Pathway Mapping, Drug Mode-of-Action and Multiplex Biomarker Analyses

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Abstract

Reverse Phase Protein Arrays (RPPA) have over the past years evolved as an established protein profiling platform that has proven valuable in translational research, particularly in the oncology area. The uniqueness of RPPA technology lies in its ability to analyze hundreds of cell or tissue samples in parallel, from pre-clinical or clinical samples, at low sample consumption, and for dozens up to hundreds of different protein markers including phosphorylated signaling proteins. The latter distinguishes RPPA from all other multiplex protein profiling methods that focus on total proteins only. In this white paper, we describe fundamentals and technicalities of RPPA technology and illustrate its versatility by highlighting applications from our long-standing RPPA service laboratory experience.

Overview of RPPA Technology

RPPA is an array-based miniaturized immunoassay platform for signaling protein, pathway and biomarker profiling allowing high-throughput analysis of 100s of samples with 10s to 100s of antibodies, at low sample consumption. The platform consists of two essential parts (Fig. 1):

1. Printing of 100s of protein lysate samples as microarrays onto planar chips, and
2. Analysis of printed sample arrays with 10s to 100s of qualified antibodies in a direct immunoassay, using 1 antibody per 1 array.

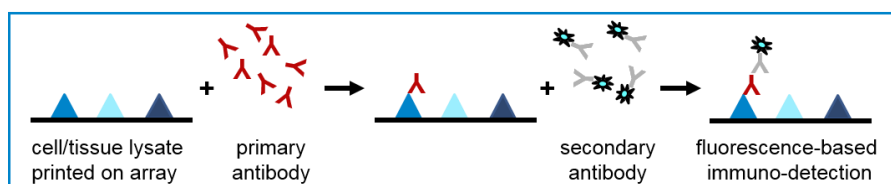


Figure 1. Basic assay principle of Reverse Phase Protein Arrays.

Based on this basic principle, two technological systems have been established:

1. Nitrocellulose on glass chips with contact printing and colorimetric readout, by Prof. Lance Liotta's group (Paweletz *et al.* 2001), and
2. ZeptoCHIP planar waveguide chips with contactless printing and highly sensitive Zeptosens fluorescence readout by Zeptosens AG (Pawlak *et al.* 2002). This approach was adopted by NMI TT Pharmaservices in 2004 (Fig. 2).

- Translation from pre-clinical to clinical results**
- 2004: Implementation of RPPA technology at NMI TT
 - 2006: First protein profiling study performed with Top10 pharmaceutical company
 - 2009: Next generation RPPA sample preparation automation implemented
 - 2013: Protein extraction protocol for FFPE tissue materials established
 - 2014: Panel of 382 antibodies validated for RPPA
 - 2015: Validation of FFPE antibody panel
 - 2015: Collaboration Agreement with a Top 5 clinical CRO
 - 2016: Establishment of new Quality Management system, to support [RPPA analysis of clinical trials/samples](#)
 - 2017: Audit by 2 big pharmas for RPPA and Simoa analyses from clinical samples

Figure 2. History of RPPAs at NMI TT Pharmaservices.

The key features of RPPA technology in terms of sample types, required protein amounts, and applications in research and pharma development are illustrated schematically in Fig. 3.

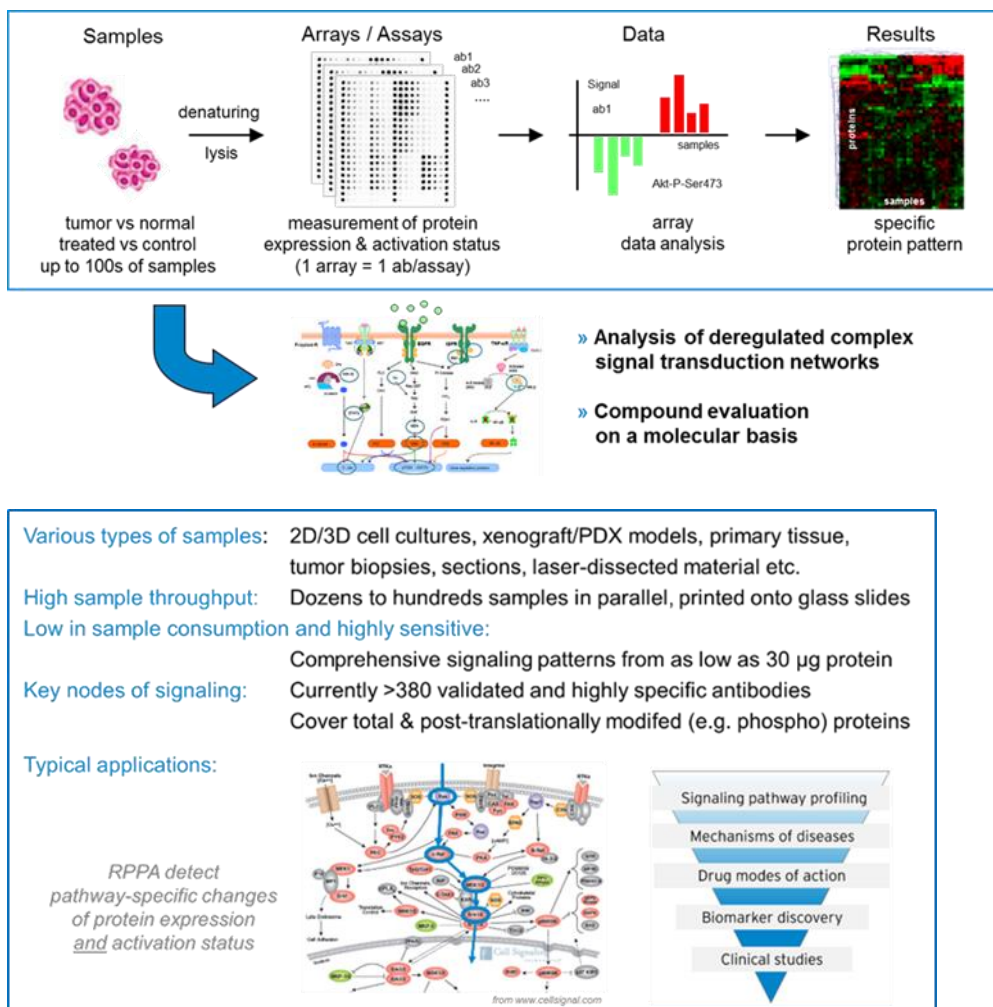


Figure 3. Overview and key characteristics of RPPA technology.

Antibodies

In RPPA assays, a single primary antibody is used to detect the protein expression or activated (phosphorylated) state of the protein of interest. Therefore, antibodies of high quality and specificity are a key requirement for successful RPPA applications.

NMI TT Pharmaservices has established a list of antibodies that have been well characterized and validated for antigen specificity via own Western Blotting (WB) of cell culture and fresh frozen (FF) tissue materials. All antibodies, selected from commercial vendors and specified for WB (since RPPA uses also samples with denatured proteins) have been quality-scored for use with RPPA. Scored antibodies are consolidated in two lists, one human and one mouse list, which are periodically updated. Antibodies of score 1 (single band) and 2 (single band with a minor side band) are classified as “valid”, antibodies of score 4 (specific band not detected due to low expression, but no non-specific bands) are added as “add-to-screen” to the lists. Antibody lists are available in Excel format, and customers are free to fully customize number and type of antibodies for the intended study.

The current RPPA lists for human and mouse materials comprise of 382 and 324 antibodies, respectively (please [contact us](#) to receive the lists), including total proteins but also post-translational modifications like phosphorylations. Additional WB antibodies - either new ones or one that have already been by a customer - can be qualified and included in our RPPA studies upon request.

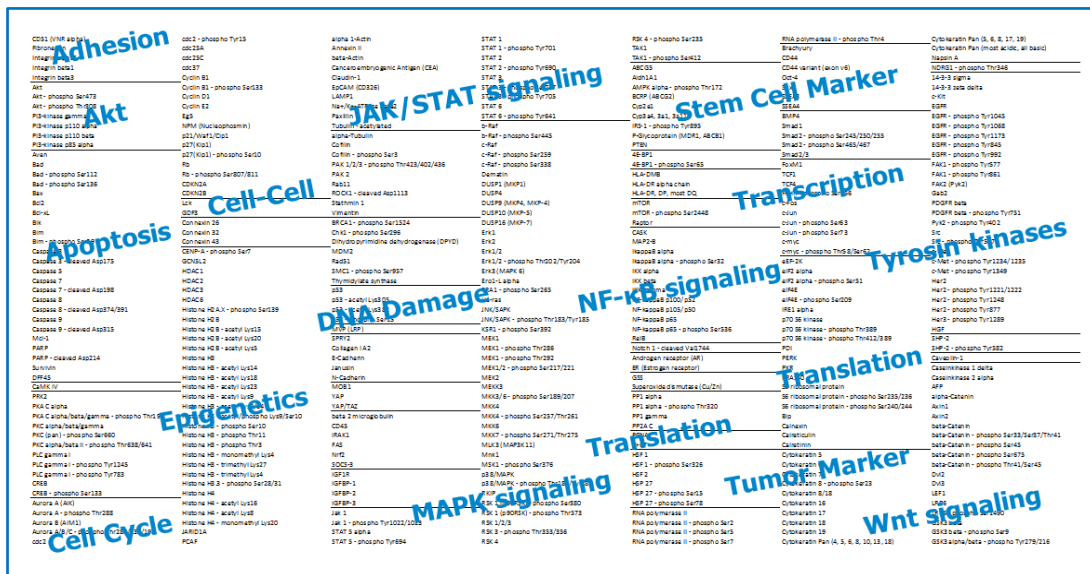


Figure 4. Schematic overview of our RPPA antibody list for use in human sample materials.

Our antibodies have been tested successfully in numerous RPPA studies with pharma customers, some of which have been published (see 'References'). The typical number of antibodies used per study is between 50 and 150, so far up to a maximum of 300 antibodies per study.

Antibodies for use with FFPE Tissues

In a comprehensive analysis in collaboration with Roche, 300 antibodies (pre-validated for use with fresh frozen material), have been validated for use with FFPE tumor tissues. Antibodies with reported good correlation in this study (Bader *et al.* 2015) and in a previous complementary study (Assadi *et al.* 2013) are summarized in the current 'RPPA Antibody List FFPE' of 61 antibodies ([contact us](#)).

RPPA Applications in Drug Development and Clinical Research

Due to its nature, i.e. the multiplexed analysis of total and phospho-proteins from hundreds of samples, RPPA studies are an excellent tool for protein profiling analyses of a wide range of tissue and tumor samples within pre-clinical and clinical drug development. The RPPA format (see 'Array Types') allows for comprehensive study designs, to easily integrate and compare samples treated at different conditions, doses and time points, together with lots of relevant non-treated controls, standards and replicates in the same assay, thus providing biological information with a high level of confidence. RPPAs have shown their validity in generating valuable mechanistic as well as prognostic and predictive information when analyzing cell and tissue materials on the functional protein level, to

- Verify (preclinical) mode-of-action and efficacy in the patient situation,
- Identify and validate predictive biomarkers in human,
- Stratify tumor subtypes and cancer patient populations, to
- Define new and more efficient targeted and combinatorial therapies for the individual patient or well-defined patient sub-populations.

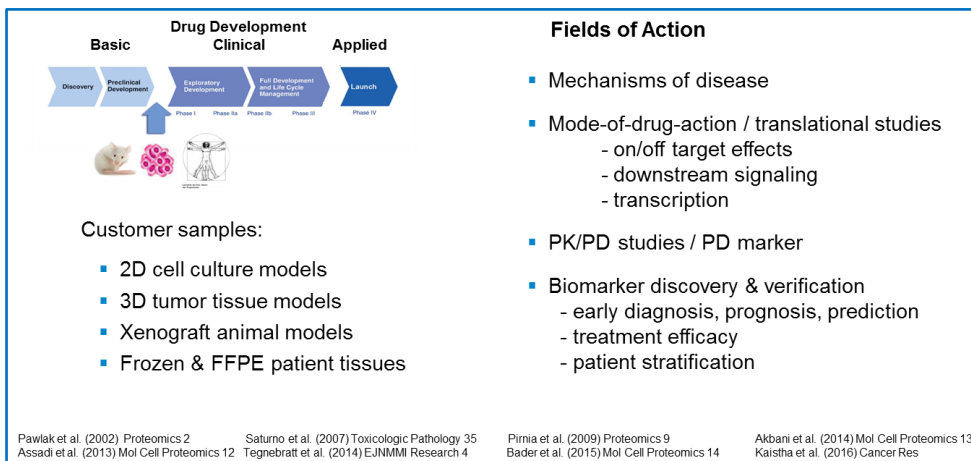


Figure 5. Applicability of RPPA studies along the pharmaceutical value chain.

Study Work Flow

Every RPPA study is a bilateral process that involves close interaction with the customer, to ensure seamless processing according to the customers' needs. The typical study layout is depicted in Fig. 6.

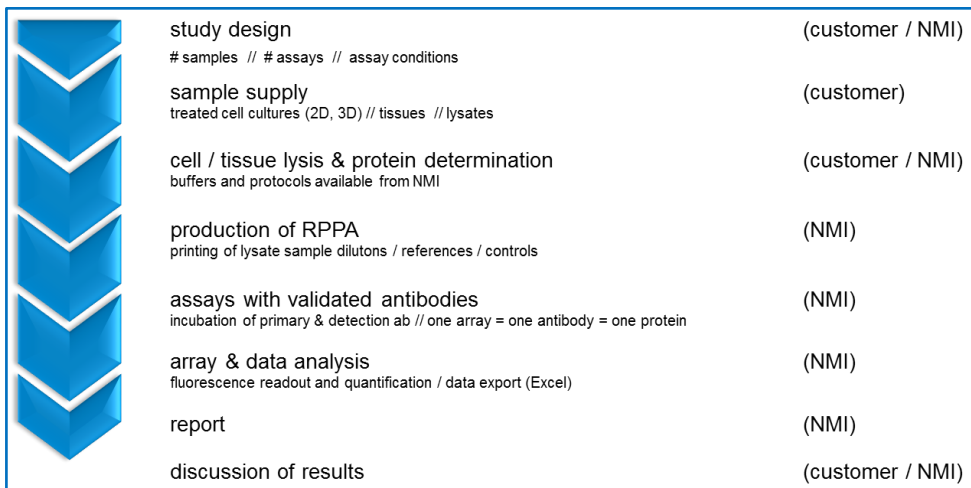


Figure 6. Organizational concept of an RPPA study.

Array Types

Based on the Zeptosens technology, NMI TT Pharmaservices has generated two ZeptoCHIP array options, which are both usable for an unrestricted number of assays:

- Array40** Up to 37 study samples, 4 dilutions, 2 replicates plus validated standards and technical controls
Sample consumption: 8 μ l lysate at 3 μ g/ μ l protein concentration (0.3 μ g/ μ l print conc.)
- Array160** Up to 154 study samples, 2 replicates, plus validated standards and technical controls
Sample consumption: 5 μ l lysate at 3 μ g/ μ l protein concentration (0.3 μ g/ μ l print conc.)

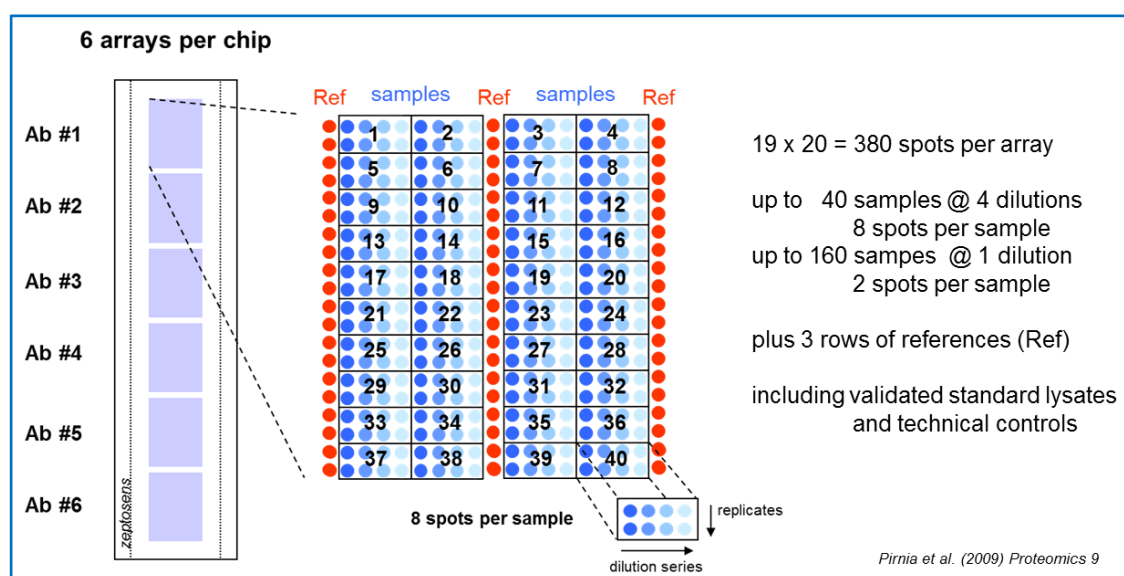


Figure 7. Overview of the ZeptoCHIP RPPA Array40 sample layout.

Sample Requirements for Protein Lysates

Sample preparation is generally considered a key step for successful outcome of a RPPA study. Already before a project starts, the type, size, weight and nature of samples and the transfer of samples from customer to NMI TT are carefully discussed and prepared in detail. For a typical RPPA study, we need the following protein materials:

- Minimum protein conc per lysate: 3 μ g/ μ l in CLB1 lysis buffer (CLB1 will be provided by NMI TT),
- Minimum sample protein: 30 μ g (i.e. 10 μ l lysate),
- A sample amount of typically 150 μ g of protein (i.e. 50 μ l lysate) will allow for additional post-RPPA validation via Western Blot if deemed valuable.

Sample Requirements for Fresh Frozen (FF) Tissues

Based on fresh frozen tissue materials, we require the following sample specs:

- Typically 20 to 50 mg of solid tissue or powder, minimum 8 mg, e.g. a tissue piece of the size of a rice grain (approx. 2 x 2 x 2 mm³),
- If cryo sectioning is available at customer's site: 4 mg, cut as 15 μ m thick tissue sections, 300 mm² total tissue area,
- Core needle biopsy (CNB) samples: principally feasible, e.g. from 14G needle, 1.6 mm diameter x 10 mm length, tissue material OCT free.

Sample Requirements for FFPE Tissues

For clinical FFPE materials, we need:

- 4 mg, cut as 15 µm thick tissue sections, 300 mm² total tissue area.

In close collaboration with NMI and other partners, NMI TT undertakes continuing efforts to further develop and adapt its RPPA processes to meet specific customer needs.

Quality Management (QM) System

To accommodate the additional quality requirements of clinical sample analysis, NMI TT Pharmaservices has set-up a Quality Management (QM) System for RPPA applications, which is compliant with the requests of a big clinical CRO that sub-contracts RPPA studies within their clinical trials to NMI TT Pharmaservices.

The system meets the quality requirements of analyzing clinical patient samples in a GxP-like manner, and includes a dedicated IT system for data storage and analysis system, which was qualified to process and archive protein profiling study data and to fulfill the relevant demands according to CFR, GxP, CAP, ISO17025 guidelines. It covers adequate and functional system design, usability and stability, as well as system and data security, as documented in SOPs and Additional Documents.

Study Prices

NMI TT Pharmaservices offers RPPA studies on a fee-for-service basis. Each study can be fully customized based on our customers' project specs, and study pricing is dependent on the number of samples and antibodies/assays chosen by our customer. As for sample numbers, fully processed arrays (37 or 154 study samples, see 'Array Types' above) provide the most economic pricing per information (i.e. price per sample per analyte), but clients are fully flexible in providing any sample number from minimum 20 to several hundreds. Please contact us to discuss your needs and to receive a quote.

Contact

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References

More than 50 RPPA service studies have been performed by NMI TT Pharmaservices over the past decade. The following lists and comments our publications with some of our collaborators and customers.

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