Reagent Characterisation:

"Reagent Characterisation of BioGenes' generic HEK293|360-HCP ELISA Kit (Type CL)"

Version 01

Preparation, Review and Approval

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1 Introduction

The reagents of BioGenes generic HEK293|360-HCP ELISA Kit Type CL were characterised by 2D fluorescent Western Blotting as well as Immunoaffinity Chromatography in combination with 2D DIGE analysis. Methodology and data evaluation of the study are described in this report.

2 Materials

The following reagents were characterised (**Table 1**):

Table 1: Reagent specification

Reagent	Details
HEK293-HCP (Type CL) kit standard **ID: HEK293|360-HCP-CL**	• HEK293-HCP (Type CL) mock material in stabilised solution; used for preparation of the HCP standard in the HEK293|360-HCP ELISA Kit Type CL
HEK293-HCP (Type CL)-specific antibody **ID: anti-HEK293|360-HCP-CL-IgG**	• polyclonal rabbit HEK293-HCP (Type CL)-specific antibody, used as capture antibody in the HEK293|360-HCP ELISA Kit Type CL

3 Experimental Setup

3.1 2D Fluorescent Western Blot Analysis

For HCP coverage analysis, the HEK293|360-HCP-CL sample was subjected to 2D electrophoresis and Western Blotting. For that, sample clean-up was performed and the proteins were labelled with Sci5 fluorescent dye[1] according to the manufacturer's instructions. The labelled sample was complemented by addition of rehydration buffer before application onto a 24 cm IPG strip via in-gel rehydration overnight. IEF and equilibration were followed by SDS-PAGE. After SDS-PAGE, the sample proteins were transferred onto a nitrocellulose membrane by semi-dry Western Blotting. HCP-specific immunostaining was carried out at room temperature. Total protein fluorescence was detected using a fluorescence scanner at a resolution of 100 µm.

An overview of the 2D Western blot (WB) prepared is given in **Table 2**.

Table 2: Overview of 2D Western blot

Purpose	Sample	Primary antibody / secondary antibody
HCP-specific immunostaining	HEK293|360-HCP-CL	anti-HEK293|360-HCP-CL-IgG / anti-rabbit-IgG-Cy3 conjugate

For the semi-electronic determination of HCP coverage by 2D fluorescent Western Blotting, the software Delta2D (DECODON) was used.

[1] **SERVA Lightning Sci5** (SERVA, Cat. No.: 43406)

3.2 Immunoaffinity Chromatography and Comparative 2D DIGE Analysis

For orthogonal HCP coverage analysis, the HEK293|360-HCP-CL sample was subjected to IAC/2D DIGE analysis.

For preparation of the affinity column, the anti-HEK293|360-HCP-CL-IgG antibody was coupled onto Bromocyan-activated Sepharose. Subsequently, the HEK293|360-HCP-CL sample was applied to the immobilised capture antibody on the column. The chromatographic purification was performed by gravity flow using Tris-based running and washing buffers. Bound HCPs were eluted using an acidic Glycine-HCl buffer. For monitoring purposes, all experimental steps were performed under UV_{280} control. For further analysis, the IAC Eluate was concentrated via ultrafiltration and the protein concentration was determined by Bradford assay.

2D DIGE analysis including the original HEK293|360-HCP-CL sample and the IAC Eluate sample was subsequently performed. The DIGE technique allows for the simultaneous run of differently labelled samples on one single gel. For compensation of natural variation in labelling efficiency and quantum yield of common fluorescence dyes in DIGE experiments (such as Sci3 and Sci5), a 'dye-swap' may be included in the experiment. Alternatively, all samples in the comparison may be labelled with the same fluorescent dye (Sci5) and run on separate gels which will be *warped* by 2D evaluation software based on an internal reference standard, labelled with Sci2[2] fluorescent dye. The latter strategy was applied to the current analysis (**Table 3**).

Table 3: Design of 2D DIGE experiment

DIGE Gel	Samples
1	Sci5: HEK293|360-HCP-CL Sci2: Internal reference standard (HEK293|360-HCP-CL)
2	Sci5: IAC Eluate Sci2: Internal reference standard (HEK293|360-HCP-CL)

2D electrophoresis was performed as previously described (*cf.* section 3.1). Sci dye fluorescence was detected immediately after SDS-PAGE using a fluorescence scanner.

For the semi-electronic determination of HCP coverage by 2D DIGE (qualitative comparison), the software Delta2D (DECODON) was used.

[2] **SERVA Lightning Sci2** (SERVA, Cat. No.: 43404)

4 Results and Evaluation

4.1 2D Fluorescent Western Blot Analysis

Figure 1 shows images of the Sci5- and Cy3-scans of the blotted HEK293|360-HCP-CL sample, immunostained with anti-HEK293|360-HCP-CL-IgG.

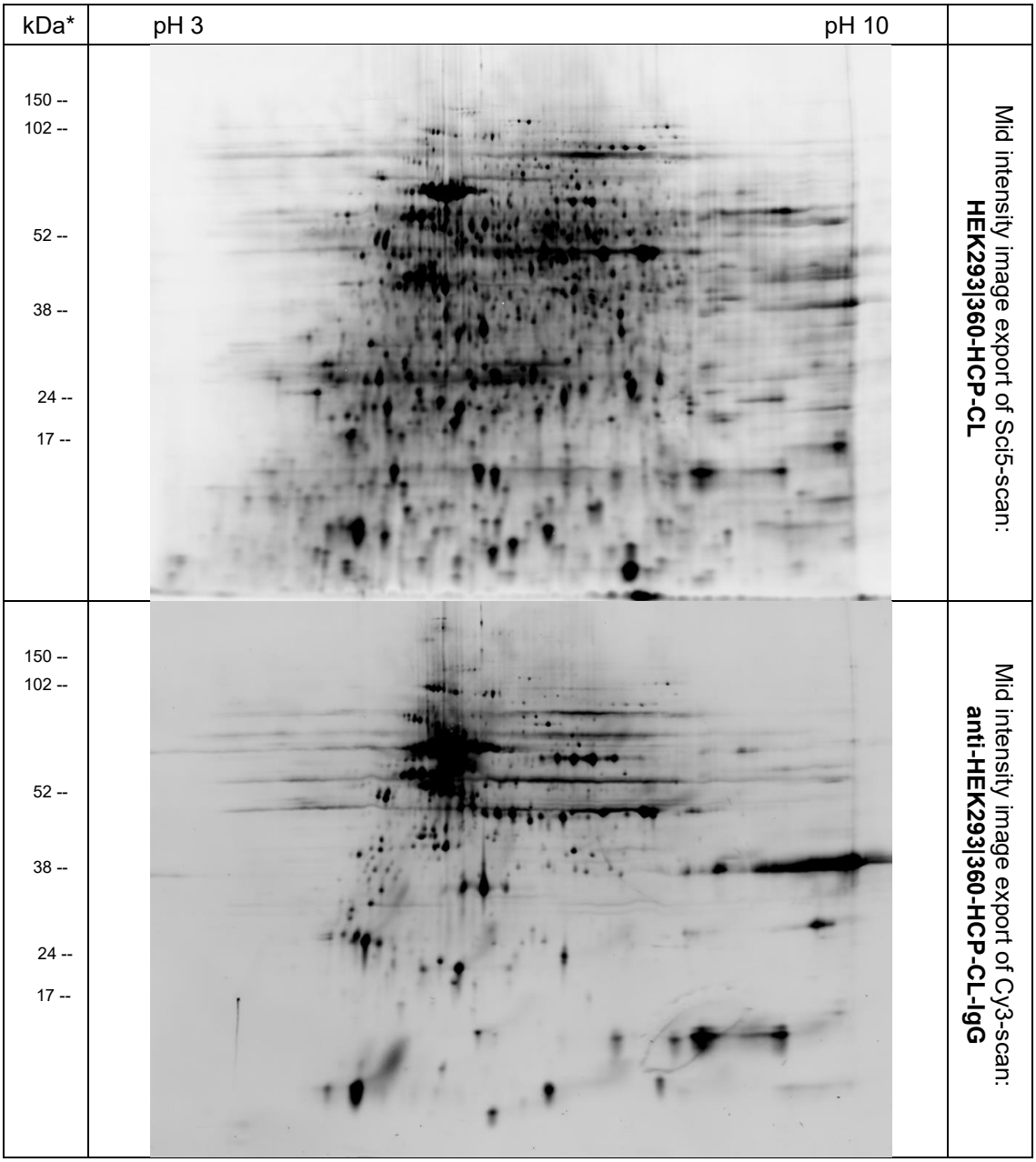

Figure 1: Sci5- and Cy3-scan of 2D fluorescent WB (HEK293|360-HCP-CL and anti-HEK293|360-HCP-CL-IgG)
2D fluorescent Western blot of Sci5-labelled **HEK293|360-HCP-CL** and immunostaining using **anti-HEK293|360-HCP-CL-IgG** detected with anti-rabbit-IgG-Cy3 conjugate;
Scanning settings: photomultiplier: Sci5: 400 V / Cy3: 400 V / Resolution: 100 µm
*Approx. molecular weight ladder

Figure 2 and **Table 4** summarise the HCP coverage analysis results:

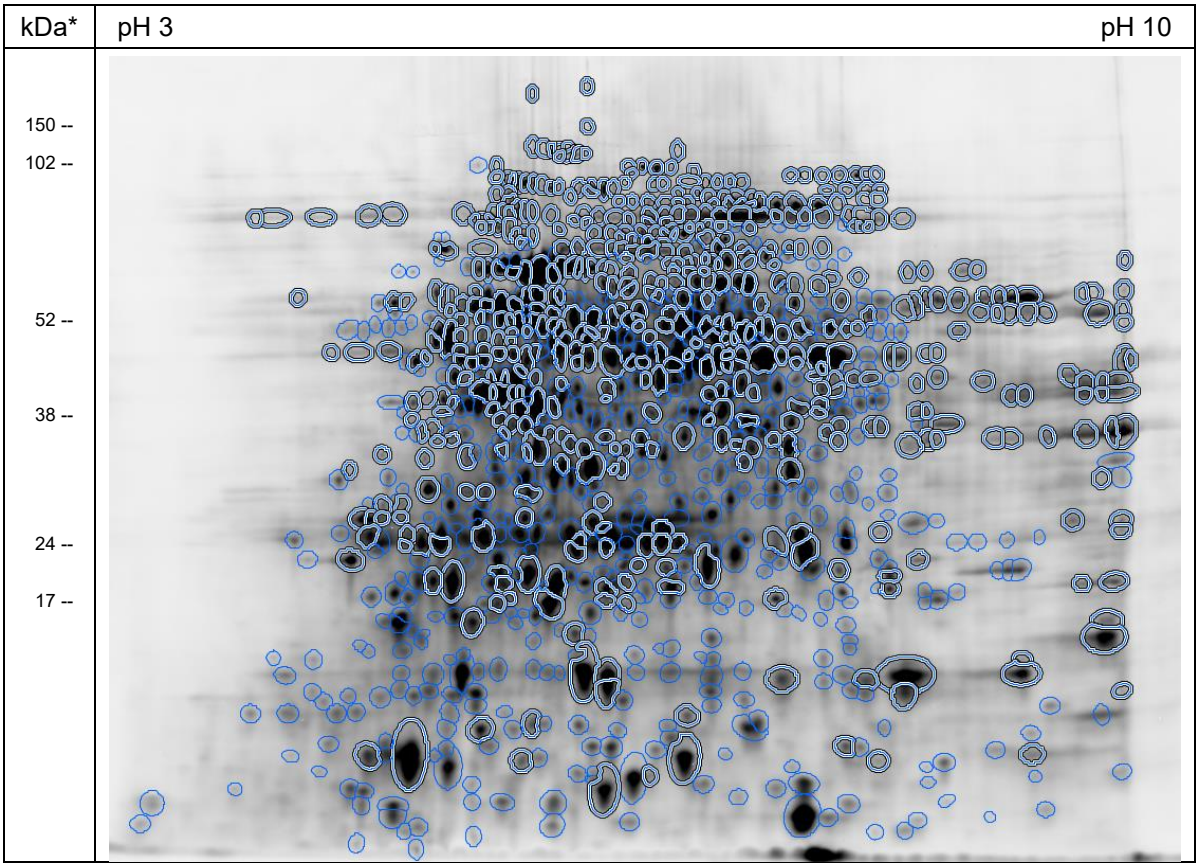

Figure 2: Electronic total protein spot pattern of HEK293|360-HCP-CL with highlighted covered protein spots

560 spots (out of **1025** total protein spots) were successfully detected by **anti-HEK293|360-HCP-CL-IgG** and are circled with thick blue lines in the above image. Non-covered spots are presented with thin blue lines in the above image.
*Approx. molecular weight ladder

Coverage was accordingly calculated to be 55%.

Table 4: Summary of 2D WB coverage results

	HEK293|360-HCP-CL + anti-HEK293|360-HCP-CL-IgG
Total number of spots	**1025**
Number of covered spots by the anti-HCP antibody	**560**
Coverage	**55%**

The results from 2D fluorescent Western blot analysis using the rabbit derived anti-HEK293|360-HCP-CL-IgG capture antibody revealed **55%** coverage of HCPs present in the HEK293|360-HCP-CL sample.

4.2 Comparative 2D DIGE Analysis

Figure 3 shows images of the 2D DIGE gels of the HEK293|360-HCP-CL sample and of the IAC Eluate.

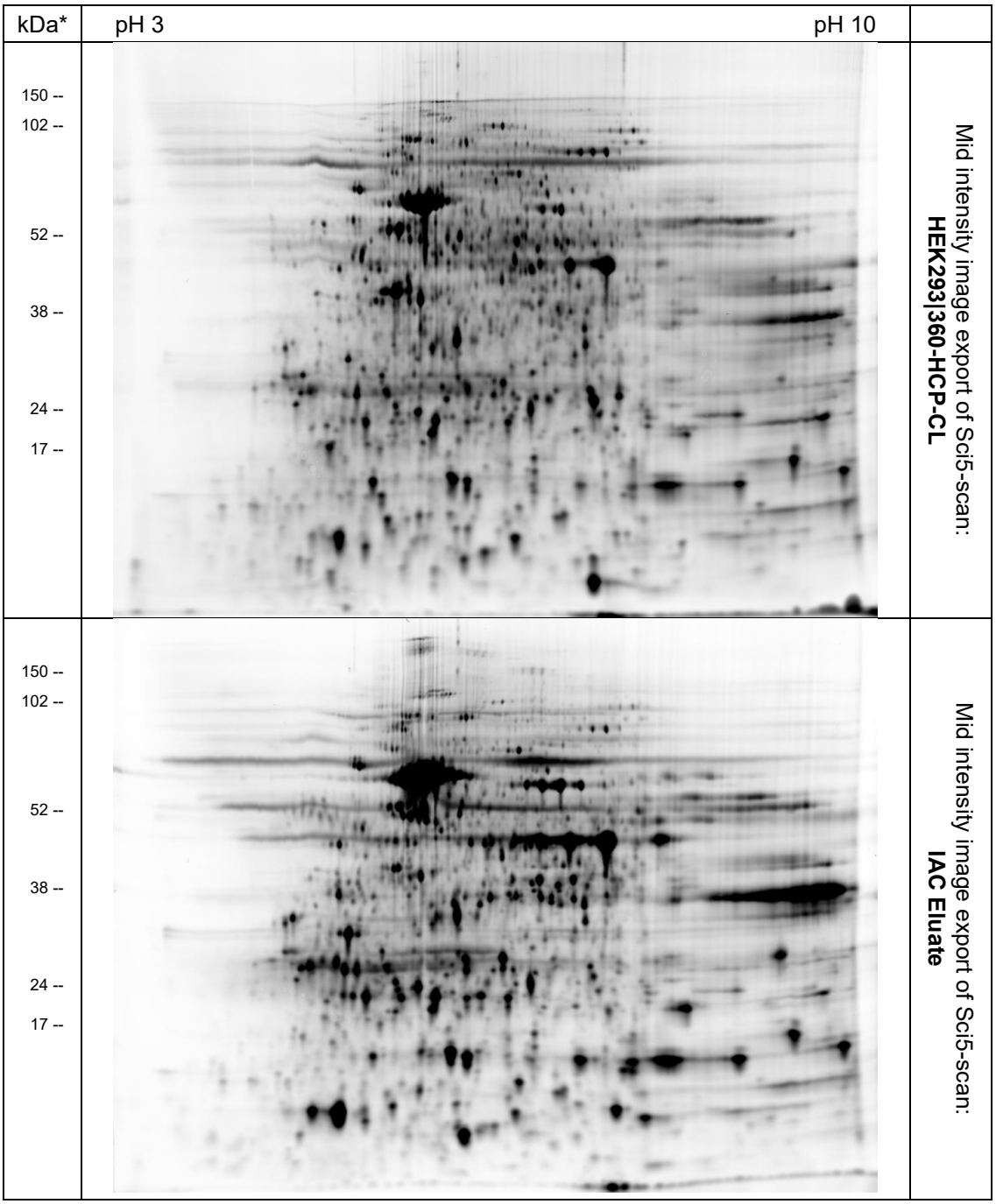

Figure 3: Sci5-scans of HEK293|360-HCP-CL and IAC Eluate

Upper image: Sci5-labelled HEK293|360-HCP-CL
Lower image: Sci5-labelled IAC Eluate
Scanning settings: Photomultiplier: Sci5: 500 V / 450 V, Resolution: 100 µm
*Approx. molecular weight ladder

In a next step, the Sci5-scans of the two samples were warped and fused using Delta2D evaluation software. The resulting overall fusion image (**Figure 4,** upper panel) ideally contains complete spot information from the HEK293|360-HCP-CL sample and the IAC Eluate sample.

In total, **1090** spots were defined on the overall fusion image (**Figure 4**, lower panel)**.**

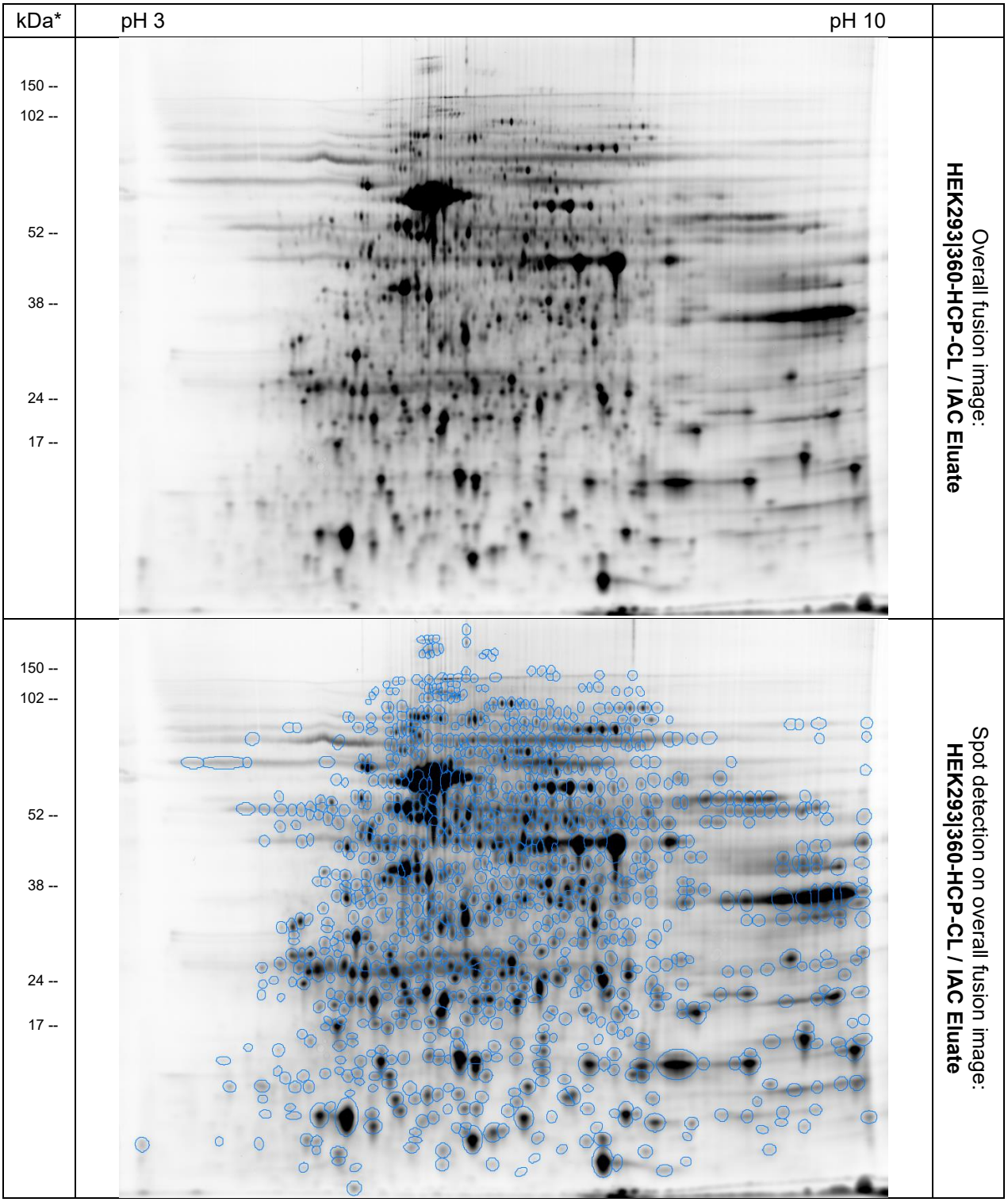

Figure 4: HEK293|360-HCP-CL / IAC Eluate overall fusion image and spot detection

Upper image: Overall fusion image based on HEK293|360-HCP-CL and IAC Eluate Sci5-images
Lower image: Spot detection on overall fusion image including manual spot editing
Total number of spots: 1090
*Approx. molecular weight ladder

Each previously defined spot boundary was manually evaluated for the presence or absence of a visible spot in each of the two samples to identify the **specific spots** of HEK293|360-HCP-CL and of the IAC Eluate.

Finally, **1032** sample-specific spots were identified in **HEK293|360-HCP-CL.**
938 sample-specific spots were identified in the **IAC Eluate.**

880 spots were identified as qualitative matches in both samples (**Figure 5**).

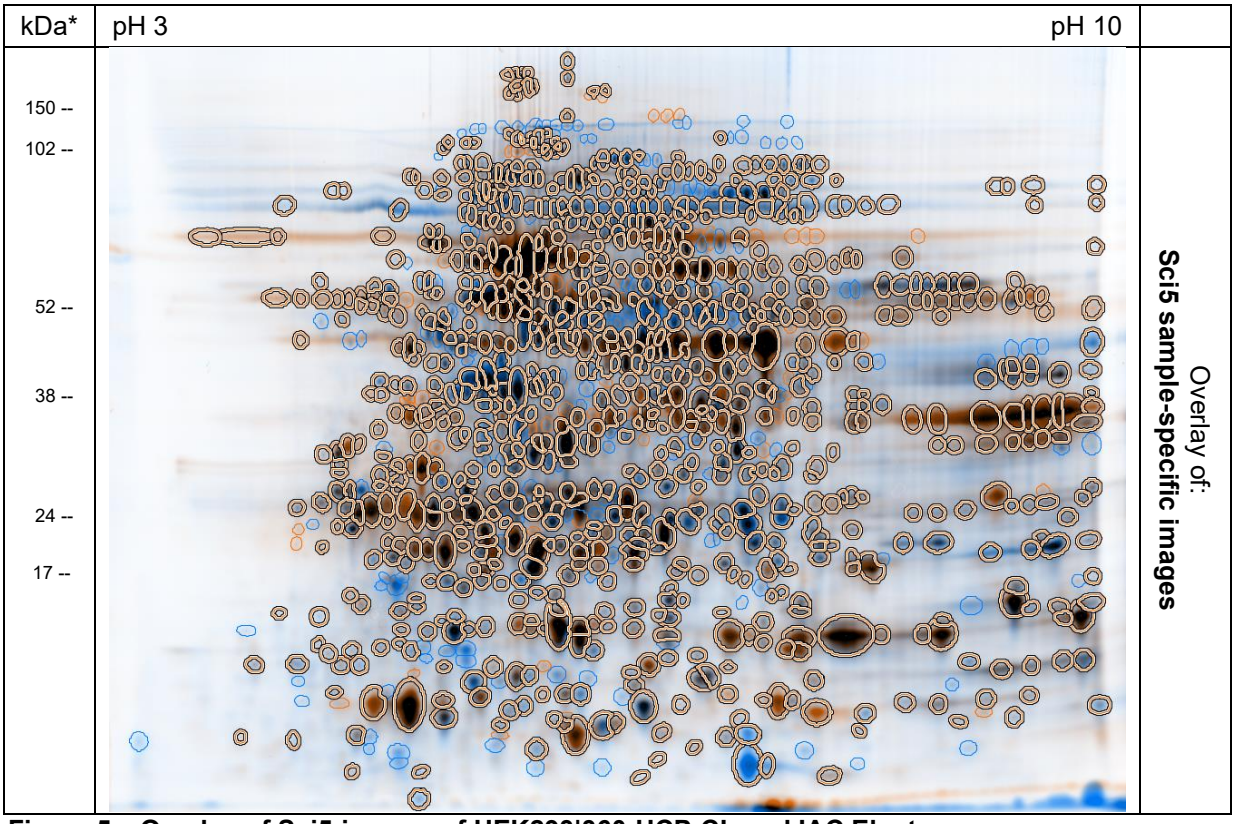

Figure 5: Overlay of Sci5-images of HEK293|360-HCP-CL and IAC Eluate

Blue pseudo-colour: HEK293|360-HCP-CL
Orange pseudo-colour: IAC Eluate

Sci5-image overlay with highlighted **880 matched** spots (circled with thick orange lines);

152 unique spots of HEK293|360-HCP-CL are circled with thin blue lines;
58 unique spots of the IAC Eluate are circled with thin orange lines.

*Approx. molecular weight ladder

In conclusion, **880** specific spots of HEK293|360-HCP-CL had a corresponding match partner in the IAC Eluate, indicating that the respective proteins were successfully bound by the capture antibody.

880 matches / 1032 specific spots of HEK293|360-HCP-CL= **85% Coverage**

Table 5: Summary of IAC / 2D DIGE analysis results

	HEK293|360-HCP-CL	**IAC Eluate**
Number of specific spots:	1032	938
Number of matched spots:	**880**	
Number of unique spots:	152	58
Coverage:	**85%**	

The results from IAC/2D DIGE analysis using the rabbit derived anti-HEK293|360-HCP-CL-IgG capture antibody revealed 85% coverage of HCPs present in the HEK293|360-HCP-CL sample. The observed discrepancy in absolute coverage values between the current IAC/2D DIGE analysis and the corresponding 2D fluorescent Western blot analysis (85% versus 55%) is commonly observed. The IAC method enables antigen-antibody interaction under nearly native assay conditions and allows for enrichment of low abundant proteins. Thus, coverage values tend to be significantly higher as compared to the denaturing 2D Western blot method.

5 Abbreviations

- 2D DIGE Two-Dimensional Difference Gel Electrophoresis
- Aqua bidest. Aqua Bidestillatus
- CL Cell Lysate
- HEK Human Embryonic Kidney
- HCP Host Cell Protein
- IAC Immunoaffinity Chromatography
- SDS-PAGE Sodium Dodecyl Sulfate – PolyAcrylamid Gel Electrophoresis
- WB Western Blot

6 Document History

Release Date	Version	Comments / Changes / Corrections
see page 2	01	first edition