ANTIBODY VALIDATION ARTICLE

Referencing cross-reactivity of detection antibodies for protein array experiments [version 1; peer review: 1 approved, 2 approved with reservations]

Darragh Lemass^{1,2}, Richard O'Kennedy^{1,2}, Gregor S. Kijanka¹

¹Biomedical Diagnostics Institute, National Centre for Sensor Research, Dublin City University, Dublin, Ireland

²School of Biotechnology, Dublin City University, Dublin, Ireland

V1 First published: 18 Jan 2016, 5:73
<https://doi.org/10.12688/f1000research.7668.1>

Latest published: 23 May 2017, 5:73
<https://doi.org/10.12688/f1000research.7668.2>

Abstract

Protein arrays are frequently used to profile antibody repertoires in humans and animals. High-throughput protein array characterisation of complex antibody repertoires requires a platform-dependent, lot-to-lot validation of secondary detection antibodies. This article details the validation of an affinity-isolated anti-chicken IgY antibody produced in rabbit and a goat anti-rabbit IgG antibody conjugated with alkaline phosphatase using protein arrays consisting of 7,390 distinct human proteins. Probing protein arrays with secondary antibodies in absence of chicken serum revealed non-specific binding to 61 distinct human proteins. The cross-reactivity of the tested secondary detection antibodies points towards the necessity of platform-specific antibody characterisation studies for all secondary immunoreagents. Secondary antibody characterisation using protein arrays enables generation of reference lists of cross-reactive proteins, which can be then excluded from analysis in follow-up experiments. Furthermore, making such cross-reactivity lists accessible to the wider research community may help to interpret data generated by the same antibodies in applications not related to protein arrays such as immunoprecipitation, Western blots or other immunoassays.

Keywords

Protein arrays , Whole-cell immunisation , Antibody profiling , Cross-reactivity , Chicken IgY , Reference list , Secondary antibody , Detection antibody

This article is included in the **Antibody**
Validations gateway.

Open Peer Review

Approval Status ✓ ? ✓

	1	2	3
version 2 (revision) 23 May 2017			✓ view
version 1 18 Jan 2016	✓ view	? view	? view

1. **Brigitte Hantusch**, Medical University of Vienna, Vienna, Austria
2. **Carsten Grötzinger**, Charité – Universitätsmedizin Berlin, Berlin, Germany
3. **Konrad Büssow** , Helmholtz Center for Infection Research, Braunschweig, Germany

Any reports and responses or comments on the article can be found at the end of the article.

Corresponding author: Gregor S. Kijanka (gregor.kijanka@gmail.com)

Competing interests: The authors do not declare any competing interests.

Grant information: This material is based upon works supported by the Irish Cancer Society Research Fellowship Award CRF10KIJ (GSK), the Science Foundation Ireland under CSET Grant no. 10/CE/B1821 and the Enterprise Ireland Dairy Processing Technology Centre award.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Copyright: © 2016 Lemass D *et al.* This is an open access article distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

How to cite this article: Lemass D, O'Kennedy R and Kijanka GS. **Referencing cross-reactivity of detection antibodies for protein array experiments [version 1; peer review: 1 approved, 2 approved with reservations]** F1000Research 2016, 5:73 <https://doi.org/10.12688/f1000research.7668.1>

First published: 18 Jan 2016, 5:73 <https://doi.org/10.12688/f1000research.7668.1>

Introduction

Secondary label-conjugated and non-conjugated detection antibodies are frequently used in a wide range of research applications. However, they are often affinity-isolated, polyclonal reagents that may lack the highest standard of antibody validation. The antibodies characterised in this study are a polyclonal anti-chicken IgY antibody produced in rabbit (31104, Thermo Fisher) and a polyclonal goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (AP) (A3687, Sigma-Aldrich). Although the use of the rabbit anti-IgY antibody in the literature is limited, the goat anti-rabbit IgG AP was extensively utilised in research for over 15 years^{1,2}.

The research conducted in this laboratory examines complex antibody repertoires in humans and animals by means of protein arrays. Protein arrays are frequently used to profile antibody binding to human proteins in autoimmune disease³, cancer⁴ and in healthy individuals⁵. Other protein array applications include recombinant⁶ and hybridoma-derived⁷ antibody characterisation studies. This article investigates the cross-reactivity of a rabbit anti-chicken IgY and an alkaline phosphatase-conjugated goat anti-rabbit IgG, which were used for the profiling of IgY antibody responses to human antigens in chickens immunised with human cancer cells. The protein array technology applied here, developed by Büssow and colleagues⁸, is comprised of a fully annotated set of 7,390 distinct human proteins, in its current version, that may serve as potential antigens. The aim of this study is to define a cross-reactivity reference list for the two described secondary antibodies, which can then be used to eliminate non-specific binders from ongoing chicken IgY profiling studies. Furthermore, publication of the cross-reactivity reference list may support other researchers using these antibodies in the evaluation of their experiments.

Materials and methods

Antibody details

Rabbit anti-chicken IgY (H+L) secondary antibody (Thermo Fisher Scientific, Product code 31104, Lot code PK19380211) is a polyclonal antibody that targets the variable heavy and light chains of chicken IgY immunoglobulins (Table 1). The antibody was isolated from the serum of the antigen-immunised rabbit through immunoaffinity chromatography using antigen coupled to agarose beads.

The antibody was added to the protein array at a 1/1,000 dilution in 2% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, A2153) in tris-buffered saline (TBS, Trizma® Base, Sigma-Aldrich, T6066 and sodium chloride, Fisher Scientific, S/3160/68) with 0.1%, v/v, Tween 20 (Sigma-Aldrich, P1379).

Alkaline phosphatase-conjugated goat anti-rabbit IgG (whole molecule) (Sigma-Aldrich, Product code A3687, Lot code SLBJ6146V) is a polyclonal antibody that targets all rabbit IgGs (Table 1). The antibody was isolated through immunospecific purification of antisera from a rabbit IgG-immunised goat. Following isolation, the anti-rabbit IgG was conjugated to alkaline phosphatase using glutaraldehyde-based cross-linkage. The antibody was added to the protein array at a 1/1,000 dilution in 2% (w/v) BSA in tris-buffered saline (TBS) with 0.1%, v/v, Tween 20.

Protein arrays

Unipex protein arrays were obtained from Source Bioscience Life Sciences (Nottingham, UK). The Unipex arrays comprise of 15,300 fully annotated *E. coli* clones expressing a total of 7,390 distinct in-frame ORF human recombinant proteins. The Unipex proteins are immobilized under denaturing conditions directly on the PVDF membrane surfaces exposing linear sequence epitopes ideally suited for epitope mapping, antibody profiling and antibody cross-reactivity analyses. The details of protein arrays utilised in this study are provided in Table 2. For general information on Unipex protein arrays please refer to: (http://www.lifesciences.sourcebioscience.com/media/290406/sbs_ig_manual_proteinarray_v1.pdf).

Cross-reactivity assessment

Antibody cross-reactivity was assessed using Unipex protein arrays. The detailed experimental protocol is provided in Table 3. Briefly, secondary rabbit anti-chicken IgY and goat anti-rabbit IgG AP were validated in preparation for a chicken IgY antibody profiling experiment of a chicken immunised with human cancer cells. Protein arrays were probed with secondary antibodies in the absence of IgY-containing chicken serum, as described in Table 3. Signal generation for array-bound secondary antibodies was obtained using AttoPhos AP fluorescent substrate system (Promega, S1001).

Table 1. Details of characterised antibodies.

Antibody	Manufacturer	Catalogue number	RRID
Rabbit anti-chicken IgY (H+L)	Thermo Fisher Scientific	31104	AB_228382
Goat anti-rabbit IgG alkaline phosphatase-conjugated	Sigma-Aldrich	A3687	AB_258103

Table 2. Details of protein arrays.

Protein array	Library number	Array number	Manufacturer
Unipex 1 pt.1	9027	633.4.730	Source Bioscience
Unipex 2 pt.1	9028	634.5.737	Source Bioscience

Table 3. Secondary antibody protein array analysis protocol.

Protocol steps	Objective	Reagent	Time
Protein array preparation	Rinse array	70% (v/v) ethanol (Sigma-Aldrich, E7023)	5 min
	Remove ethanol and rinse	dH ₂ O for 2	2 min
	Wipe off all <i>E. coli</i> colonies	laminar tissue	As appropriate
Wash 1	Wash off any <i>E. coli</i> debris	TBST-T	10 min (×3)
		TBS	2 min (×2)
		TBS	10 min
Array blocking	Block arrays by shaking	5% (w/v) Milk Marvel (Dried skim milk, Premier Foods Group (UK)) in TBS-T	2h
Wash 2	Wash off any blocking solution	TBS-T	15 min (×3)
Incubate first antibody	Rabbit anti-chicken IgY	1 in 1,000 in 2% (w/v) BSA TBS-T	2h
Wash 2	Wash off any unbound antibody	TBS-T	15 min (×3)
Incubate second antibody	Goat anti rabbit IgG AP conjugated	1 in 1,000 in 2% (w/v) BSA TBS-T	2h
Wash 3	Wash off any unbound antibody	TBS-T	10 min (×2)
		TBS	10 min (×2)
Protein array signal detection	Signal generation for array bound goat anti-rabbit IgG AP-conjugated	AttoPhos AP Fluorescent Substrate diluted 1 in 8 in AP buffer (1mM MgCl ₂ , 100mM Tris base, pH 9.5)	10 min
	Protein array image acquisition	FujiScanner Fla5100 (Settings Laser: 473, Filter: LPB, Resolution 50µm)	18 min

diluted 1 in 8 in AP buffer (1mM MgCl₂, Sigma-Aldrich, M4880 and 100mM Tris base, pH 9.5). Protein array image acquisition was conducted using a Fuji scanner Fla5100. Positive signals were localized according to the manufacturer's protocol. Protein annotations were retrieved from the Unipex database provided by the manufacturer and updated using the National Cancer Institute's UniGene CGAP Gene Finder tool (<http://cgap.nci.nih.gov/Genes/GeneFinder>).

Results

Probing protein arrays with antibodies enables the assessment of specificity and cross-reactivity on large numbers of potential antigens in parallel. Here we investigated the cross-reactivity of secondary anti-chicken IgY from rabbit and anti-rabbit IgG AP from goat using human protein arrays in the absence of chicken serum. The analysis revealed antibody binding to human proteins in the absence of chicken serum and hence chicken IgY immunoglobulins. The identified positive signals varied in strength, as shown in **Figure 1**, with intensity 3 being the strongest and 1 the weakest. The difference in signal intensities may relate to varying protein quantities on the array and differences in antibody affinities

to corresponding proteins. A total of 63 binding events were visible on the protein arrays, of which 61 corresponded to unique proteins (**Table 4**). Five of the identified signals were scored as intensity 3, twelve signals were scored as intensity 2 and remainder were scored as intensity 1. The original protein array images are shown in **Figure S1** and **Figure S2** (**Supplementary material**) and protein array images with highlighted positive signals, which correspond the cross-reactive proteins listed in **Table 4**, are shown in **Figure S3** and **Figure S4** (**Supplementary material**).

The investigated antibodies were found to bind to a wide range of human proteins (**Table 4**). However, it is worth noting that a total of six identified binding events correlated to human immunoglobulin proteins, with four scored at the highest intensity (Intensity 3). Such cross-reactivity is not surprising considering the antibodies are polyclonal and the immunogens of both hosts were immunoglobulins. In addition, the data sheet provided with the anti-chicken IgY antibody produced in rabbit (31104, Thermo Fisher) has specified that this antibody may cross-react with immunoglobulins from other species. The data sheet for the goat anti-rabbit IgG AP antibody (A3687, Sigma-Aldrich) has specified binding to all rabbit immunoglobulins.

Figure 1. Cross-reactivity of rabbit anti-chicken IgY and goat anti-rabbit IgG identified by protein array screening. (A) Image of a whole protein array and a representative section illustrating antibody-antigen binding at three different signal intensities; 3 = strong, 2 = intermediate and 1 = weak. (B) The proteins are arranged in a 3x3 pattern on the array and all proteins are arrayed twice and appear as duplicate spots in a particular pattern within a block after a successful hybridization. (C) Description of proteins chosen as examples provided on the representative array image above; signal intensities, patterns, Unigene IDs and protein names are listed.

Conclusion

This work illustrates the cross-reactivity of an antibody-based detection system for IgY binding. The polyclonal anti-IgY rabbit antibody in combination with an anti-rabbit IgG alkaline phosphatase-conjugated antibody was shown to bind to 61 human proteins present on Unipex protein arrays comprising of 7,390 human proteins. Characterisation of this cross-reactivity provides a 'false-positive' database for future chicken antisera characterisation on protein array systems not limited to the Unipex protein array used here. These results, in combination with 'false-positives' from earlier research investigating antibody cross-reactivity by this

group⁹ and others¹⁰ may provide valuable information for future protein array-based experiments. Reference lists provided by such experiments would be further strengthened by arrays that include additional portions of the human proteome and/or post-translational modifications. Using antibodies that have been extensively characterised on protein arrays will reduce the risk of identifying irrelevant cross-reactive secondary antibody binding to the array as a host-antigen response.

Overall, the antibodies tested here showed cross-reactivity to unrelated human proteins as well as to human immunoglobulin proteins,

Table 4. Reference list of antibody cross-reactivity identified by protein array analysis.

Protein array clone ID	Signal intensity	GenBankID	UnigenelID	Name
IMGSp9028F0610D	3	BM914329	Hs.533963	Clone SFV019_2F05H immunoglobulin heavy chain variable region
IMGSp9028H079D	3	BQ711793	Hs.547404	Clone IgA-MZ-aa42c-2 immunoglobulin alpha heavy chain variable region (IgA)
IMGSp9028F0316D	3	BQ709082	Hs.620437	IGH mRNA for immunoglobulin heavy chain VHDJ region, partial cds, clone:TRH1-16
IMGSp9028G0921D	3	BX417981	Hs.698070	Immunoglobulin heavy constant gamma 1 (G1m marker)
IMGSp9027F0514D	3	118471	Hs.15951	Proline-rich acidic protein 1
IMGSp9027H0434D	2	BC044933	Hs.135094	Kinesin family member 18B
IMGSp9027G0658D	2	BC010132	Hs.445893	KH domain containing, RNA binding, signal transduction- associated 1
IMGSp9027F0369D	2	AK092483	Hs.470417	Penta-EF-hand domain containing 1
IMGSp9027D1015D	2	NM_006814	Hs.471917	Proteasome (prosome, macropain) inhibitor subunit 1 (PI31)
IMGSp9028C0313D	2	DA970556	Hs.510650	Clone IP80 immunoglobulin heavy chain variable region
IMGSp9027G0525D	2	NM_002228	Hs.525704	Jun proto-oncogene
IMGSp9027E0966D	2	BC041022	Hs.584909	SCAN domain containing 1
IMGSp9027F0171D	2	BC018708	Hs.632706	Zinc finger CCCH-type containing 10
IMGSp9027F0625D	2	BC018708	Hs.632706	Zinc finger CCCH-type containing 10
IMGSp9028G0311D	2	BM920476	Hs.633485	Enhancer of polycomb homolog 1 (Drosophila)e
IMGSp9028G027D	2	BX417981	Hs.698070	Immunoglobulin heavy constant gamma 1 (G1m marker)
IMGSp9028F099D	2	BG754662	Hs.698202	Transcribed locus, strongly similar to XP_001167979.1 PREDICTED: uncharacterized protein LOC749354 [Pan troglodytes]
IMGSp9027H0728D	1	NM_001978	Hs.106124	Erythrocyte membrane protein band 4.9 (dematin)
IMGSp9027C0116D	1	NM_080881	Hs.130316	Drebrin 1
IMGSp9027D129D	1	NM_001012426	Hs.131436	Homo sapiens forkhead box P4 (FOXP4), transcript variant 1, mRNA
IMGSp9027G0310D	1	BX647115	Hs.173381	Forkhead box P4
IMGSp9027G0172D	1	AF479827	Hs.182081	BR serine/threonine kinase 1
IMGSp9028E0623D	1	NM_022489	Hs.24956	Inverted formin, FH2 and WH2 domain containing
IMGSp9027C0164D	1	BC000786	Hs.25584	ADP-ribosylation factor GTPase activating protein 1
IMGSp9027A0339D	1	BC008343	Hs.292493	X-ray repair complementing defective repair in Chinese hamster cells 6
IMGSp9027C1211D	1	BC000459	Hs.306791	Polymerase (DNA directed), delta 2, accessory subunit
IMGSp9027E0916D	1	BC040880	Hs.315568	Chromosome 10 open reading frame 114

Protein array clone ID	Signal intensity	GenBankID	UnigeneID	Name
IMGSp9027H0366D	1	NM_003260	Hs.332173	Transducin-like enhancer of split 2 (E(sp1) homolog, <i>Drosophila</i>)
IMGSp9028A0867D	1	AL833379	Hs.333388	Eukaryotic translation elongation factor 1 delta (guanine nucleotide exchange protein)
IMGSp9027F1049D	1	NM_006548	Hs.35354	Insulin-like growth factor 2 mRNA binding protein 2
IMGSp9027G1059D	1	AK097073	Hs.361323	ATP-binding cassette, sub-family F (GCN20), member 3
IMGSp9027H0825D	1	AK127401	Hs.407368	LSM14A, SCD6 homolog A (<i>S. cerevisiae</i>)
IMGSp9028A0819D	1	BC036307	Hs.465929	Calponin 1, basic, smooth muscle
IMGSp9027D1063D	1	BC146654	Hs.493796	RUN and SH3 domain containing 2
IMGSp9028D0712D	1	BC022890	Hs.511149	Synaptosomal-associated protein, 23kDa
IMGSp9027F0118D	1	NM_002087	Hs.514220	Granulin
IMGSp9027B0725D	1	NM_032627	Hs.515259	Single stranded DNA binding protein 4
IMGSp9027G1020D	1	AK127255	Hs.515364	Rho GTPase activating protein 33
IMGSp9027F0926D	1	BC090883	Hs.516160	Splicing factor 3b, subunit 4, 49kDa
IMGSp9027H0318D	1	NM_003768	Hs.517216	Phosphoprotein enriched in astrocytes 15
IMGSp9027B0425D	1	AB002328	Hs.517478	Calcineurin binding protein 1
IMGSp9027D0732D	1	AK096320	Hs.517543	Pescadillo ribosomal biogenesis factor 1
IMGSp9027E0219D	1	NM_015695	Hs.520096	Bromodomain and PHD finger containing, 3
IMGSp9027B0369D	1	NM_007371	Hs.522472	Bromodomain containing 3
IMGSp9027H1010D	1	NM_014866	Hs.522500	SEC16 homolog A (<i>S. cerevisiae</i>)
IMGSp9027B0757D	1	NM_031372	Hs.527105	Heterogeneous nuclear ribonucleoprotein D-like
IMGSp9027C0965D	1	NM_014811	Hs.533260	Protein phosphatase 1, regulatory subunit 26
IMGSp9027E0964D	1	NM_001098800	Hs.571729	Melanoma antigen family D, 4
IMGSp9027G0156D	1	BC037307	Hs.590990	Anoctamin 8
IMGSp9027C1216D	1	AB208876	Hs.592082	Axin 1
IMGSp9027F0322D	1	BF110897	Hs.612694	Transcribed locus
IMGSp9027E0122D	1	BC004352	Hs.613351	Kinesin family member 22
IMGSp9027G0312D	1	AK225632	Hs.631593	Protein phosphatase 1, regulatory subunit 15A
IMGSp9027E104D	1	AL133055	Hs.636446	Zinc finger protein 853
IMGSp9027A1171D	1	NM_032329	Hs.645460	Inhibitor of growth family, member 5
IMGSp9027B0415D	1	XR_015693	Hs.654404	Major histocompatibility complex, class I, B
IMGSp9027A0861D	1	BC006105	Hs.654798	Alpha tubulin acetyltransferase 1
IMGSp9027F0471D	1	NM_002140	Hs.695973	Heterogeneous nuclear ribonucleoprotein K
IMGSp9027D0731D	1	NM_001270	Hs.696018	Chromodomain helicase DNA binding protein 1
IMGSp9027B0439D	1	AK124880	Hs.696054	Protein phosphatase 1, regulatory subunit 18
IMGSp9027C0140D	1	AB209272	Hs.76662	Zinc finger, DHHC-type containing 16
IMGSp9027C1264D	1	CR606311	Hs.77100	General transcription factor IIE, polypeptide 2, beta 34kDa
IMGSp9027E1075D	1	AK128584	Hs.79110	Nucleolin

which are homologous to the original immunogens. Despite the identified non-specific binding, the tested antibodies are suitable for use in protein array experiments as the cross-reactive binding partners can be readily excluded from further analysis. As both antibodies were used as a pair in this study, the possibility to deduce the exact cross-reactivity profile for each individual antibody may be limited. However, the cross-reactivity reference list provided in this paper can be further utilised to validate research using those antibodies in applications other than protein arrays.

Author contributions

ROK and GSK designed the study, DL performed the protein array experiments and GSK conducted data analysis. GSK wrote and DL

and ROK critically reviewed and edited the article. All authors have agreed to the final content of the manuscript.

Competing interests

The authors do not declare any competing interests.

Grant information

This material is based upon works supported by the Irish Cancer Society Research Fellowship Award CRF10KIJ (GSK), the Science Foundation Ireland under CSET Grant no. 10/CE/B1821 and the Enterprise Ireland Dairy Processing Technology Centre award.

I confirm that the funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Supplementary material

Figure S1. Unipex 1 pt.1 protein array image. Original image of protein array (Number 633.4.730) probed with rabbit anti-chicken IgY and alkaline phosphatase-conjugated goat anti-rabbit IgG, visualised using AttoPhos AP Fluorescent Substrate.

Figure S2. Unipex 2 pt.1 protein array image. Original image of protein array (Number 634.5.737) probed with rabbit anti-chicken IgY and alkaline phosphatase-conjugated goat anti-rabbit IgG, visualised using AttoPhos AP Fluorescent Substrate.

Figure S3. Unipex 1 pt.1 protein array image with highlighted positive signals. Cross-reactive proteins listed in Table 4 are highlighted corresponding to their intensity as red (intensity 3 = strong), green (intensity 2 = intermediate) and yellow (intensity 1 = weak) circles.

Figure S4. Unipex 2 pt.1 protein array image with highlighted positive signals. Cross-reactive proteins listed in Table 4 are highlighted corresponding to their intensity as red (intensity 3 = strong), green (intensity 2 = intermediate) and yellow (intensity 1 = weak) circles.

References

- Cibelli G, Corsi P, Diana G, *et al.*: Corticotropin-releasing factor triggers neurite outgrowth of a catecholaminergic immortalized neuron via cAMP and MAP kinase signalling pathways. *Eur J Neurosci.* 2001; 13(7): 1339–1348.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Bartos A, Majak I, Diowks A, *et al.*: Omega-3 Fatty Acids Used as Cross-Linkers to Reduce Antigenicity of Wheat Flour. *J Food Process Preserv.* 2015.
[Publisher Full Text](#)
- Fathman CG, Soares L, Chan SM, *et al.*: An array of possibilities for the study of autoimmunity. *Nature.* 2005; 435(7042): 605–611.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Kijanka G, Hector S, Kay EW, *et al.*: Human IgG antibody profiles differentiate between symptomatic patients with and without colorectal cancer. *Gut.* 2010; 59(1): 69–78.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Nagele EP, Han M, Acharya NK, *et al.*: Natural IgG autoantibodies are abundant and ubiquitous in human sera, and their number is influenced by age, gender, and disease. *PLoS One.* 2013; 8(4): e60726.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Holt LJ, Büssow K, Walter G, *et al.*: By-passing selection: direct screening for antibody-antigen interactions using protein arrays. *Nucleic Acids Res.* 2000; 28(15): E72.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kijanka G, Barry R, Chen H, *et al.*: Defining the molecular target of an antibody derived from nuclear extract of Jurkat cells using protein arrays. *Anal Biochem.* 2009; 395(2): 119–124.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Büssow K, Cahill D, Nietfeld W, *et al.*: A method for global protein expression and antibody screening on high-density filters of an arrayed cDNA library. *Nucleic Acids Res.* 1998; 26(21): 5007–5008.
[PubMed Abstract](#) | [Publisher Full Text](#) | [Free Full Text](#)
- Kijanka G, Ipcho S, Baars S, *et al.*: Rapid characterization of binding specificity and cross-reactivity of antibodies using recombinant human protein arrays. *J Immunol Methods.* 2009; 340(2): 132–137.
[PubMed Abstract](#) | [Publisher Full Text](#)
- Michaud GA, Salcius M, Zhou F, *et al.*: Analyzing antibody specificity with whole proteome microarrays. *Nat Biotechnol.* 2003; 21(12): 1509–1512.
[PubMed Abstract](#) | [Publisher Full Text](#)

Open Peer Review

Current Peer Review Status:

Version 1

Reviewer Report 04 April 2016

<https://doi.org/10.5256/f1000research.8257.r12773>

© 2016 Büssow K. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Konrad Büssow

Structural Biology, Helmholtz Center for Infection Research, Braunschweig, Germany

In the present work, the authors have tested direct binding of secondary antibodies to arrays of human proteins.

Readers who use array technology may benefit from the present work, since they will become aware of the problem of signals caused by secondary antibodies and not by the primary antibody. It appears that human immunoglobulins are frequently detected by secondary antibodies, which is a useful finding that would likely also be relevant for other secondary antibodies.

The authors have included the original images in the supplement, which is useful for users of the technique.

Issues

1. In the Results section, it should be made clear that the arrays were probed with both antibodies in the same experiment, not one antibody at a time.
2. It would be interesting how strong the signals caused by the secondary antibodies are in comparison to signals obtained in the presence of a primary antibody.
3. In comparison, the part 1 image has a much higher background than part 2. It appears that very clear signals were obtained from part 2, but not from part 1. In the part 1 image, there is considerable background and almost all positions have been slightly stained. I would recommend repeating the experiment to verify whether the weak signals obtained on part 1 can be reproduced.
4. Two secondary antibodies were used in the same experiment. Therefore, it cannot be determined which of the two antibodies gave rise to the signals on the array. This problem should be discussed.

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 16 May 2017

Gregor Kijanka, Dublin City University, Dublin, Ireland

The authors would like to thank Dr. Konrad Büssow for his thorough review of this article and his helpful comments. Dr. Büssow points out that the authors should stress that both secondary antibodies were used in the same experiment using one single set of protein arrays. This experimental design issue entails that it cannot be determined which signals are caused by which antibody. We have highlighted and discussed both issues throughout the text and we performed an additional sequence analysis in an *in silico* approach to clarify the origin of the signals on the protein array. The results of these analyses are presented in the new Table 5 and Supplementary table 1 and are further discussed in the text.

Dr. Büssow has furthermore highlighted the differences in background signal between the two arrays of the protein array set. The authors have encountered similar background differences when using other sets of antibodies and serum samples and find similar discrepancies in background noise being likely due to different tissues and vectors used for the generation of the distinct expression clone libraries utilized for array 1 and 2. This issue is now specifically highlighted in the article.

Competing Interests: The authors do not declare any competing interests.

Reviewer Report 30 March 2016

<https://doi.org/10.5256/f1000research.8257.r12776>

© 2016 Grötzinger C. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Carsten Grötzinger

Department of Gastroenterology and Molecular Cancer Research Center, Charité –
Universitätsmedizin Berlin, Berlin, Germany

This article describes an experiment performed to characterize the background signals in a particular combination of three commercially available research tools: a protein macroarray on PVDF membranes used in conjunction with two antibodies used for detection. As no first antibody or serum was used in this experiment, all the signals could be attributed to unwanted, unspecific reactivity of the detection antibody combination used. A list of genes was generated from these signals that is proposed as a reference database of for other researchers.

In general, the approach is scientifically sound and feasible. Background reactivities may limit

antibody-based assays and need to be accounted for. So performing a control experiment without serum or first antibody on a protein array and with just the detection antibodies makes perfectly sense to control for unspecific binding. The title of the paper is appropriate, the abstract gives enough information on the setting. The background information about the antibodies is described in enough detail. However, the narrow focus of the paper and a number of technical issues limit the quality of the paper and its utility for the readership.

Major issues

- The experiment was performed only once. Consequently, the reliability of the results will be limited.
- Only one specific combination of a protein macroarray with two consecutive detection antibodies was analyzed. It remains unclear, whether the results obtained would apply to other lots of the antibodies or whether they are specific for a certain preparation, limiting the benefit of this protein list as a reference database and also limiting the replication of results by other groups.
- The authors suggest that their results may also apply to other protein array systems. This claim needs substantiation, especially in the case of *E.coli* proteins derived from high-throughput cloning that do not show authentic posttranslational modification patterns and often contain extra amino acid sequences that may cause unspecific binding.
- The paper discusses cross-reactivity with human Ig genes. A sequence analysis of the other cross-reactive proteins with IgY and rabbit Ig sequences may provide evidence for the mechanisms behind this phenomenon, expanding scope and depth of this so far rather descriptive study.

Minor issues

- Antibody concentrations should be given explicitly, e.g. as µg/ml rather than as dilutions.
- The procedure of signal quantification and scoring needs to be described in more detail. The description states "Positive signals were localized according to the manufacturer's protocol" - what exactly was done to identify positive signals? The pictures provided show varying background intensities as well as a number of very dark spots that do not appear in the analysis. Which algorithm was used to include or exclude signals? How were the different signal intensities attributed to the score values 1, 2 and 3?
- It would be interesting to know why this specific combination of two detection antibodies was used here: a polyclonal anti-chicken IgY antibody produced in rabbit and then a polyclonal goat anti-rabbit IgG antibody conjugated with alkaline phosphatase. Was there no conjugated anti-chicken antibody available? Every additional antibody will add to the number of unspecific reactions, so using just one instead of two may help reduce background.
- The abstract does not provide a conclusion on whether the antibodies should be used in a particular setting (see Article Guidelines For Antibody Validation Articles).

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 16 May 2017

Gregor Kijanka, Dublin City University, Dublin, Ireland

The authors would like to thank Dr. Carsten Grötzinger for his very helpful observations which prompted us to perform additional *in silico* analysis resulting in an improvement of this paper. Dr Grötzinger points out that the paper has its limitations due to the fact that only one experiment has been performed leading to questions regarding reliability of data, lot-to-lot reproducibility and combinations of antibody pairs. As those issues are certainly important, however, not feasible to address in this specialized antibody validation paper, we have discussed those within the text; For instance, the lot-to-lot reproducibility of polyclonal antibodies is an important issue that needs to be taken into consideration during the experimental design of a study, it goes however, beyond the scope of this particular article. The important issue of determining the origin of the identified signals to either of the secondary antibodies tested in a single protein array experiment is now, however, addressed in more detail. We have performed an additional *in silico* analysis comparing sequence similarities between the antibody immunogens used to produce the secondary antibodies and the human proteins identified on the arrays. The analysis shed some light into the possibility that all immunoglobulin (Ig) related signals were caused by both tested secondary antibodies and others were caused by either of the two antibodies. These findings are particularly interesting, as the binding patterns of the non-labelled secondary antibody are difficult to show unless additional labelling is performed directly on the antibody. Such additional labelling might, however, impact on the antibody binding specificity. The results of those analyses, as discussed in a similar manner in the Reviewer 1 response, are presented in a new Table 5 and Supplementary Table 1 and further discussed in the text.

The authors have also addressed minor issues related to post-translational modification, antibody concentrations, signal quantification and others throughout the text.

In addition, we concluded that the antibodies should be used in a particular setting and highlighted this in the abstract as required in the Article Guidelines For Antibody Validation Articles.

Competing Interests: The authors do not declare any competing interests.

Reviewer Report 03 February 2016

<https://doi.org/10.5256/f1000research.8257.r12247>

© 2016 Hantusch B. This is an open access peer review report distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Brigitte Hantusch

Clinical Institute of Pathology, Medical University of Vienna, Vienna, Austria

This study presents data concerning the issue of secondary antibody cross-reactivity towards antigens other than desired immunoglobulins. By screening a high-throughput protein array, the authors establish the amount and identity of proteins detected by commercially available secondary antibodies, a rabbit anti-chicken antibody combined with an AP-conjugated goat anti-rabbit antibody.

Title and Abstract: The title might contain the information that two detection antibodies were used. The abstract represents a sound summary of the work performed.

Article: The methods used are described clearly, especially by showing a concise work flow as seen in table 3.

Data: Results are described appropriate and sufficiently. Supplementary Figures S1 and S2 have very huge size and are dispensable. The sentence about signal intensity differences due to varying protein amounts should be part of the conclusion section and also discussed more extensively.

Conclusion: The conclusions drawn are appropriate and concise. Briefly can some information be drawn from the kind / category of proteins falsely detected?

Competing Interests: No competing interests were disclosed.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 16 May 2017

Gregor Kijanka, Dublin City University, Dublin, Ireland

The authors would like to thank Dr. Brigitte Hantusch for kindly reviewing this manuscript and the helpful and detailed comments. We have discussed the signal intensity differences in more detail in the text as highlighted by Dr. Hantusch. We decided to retain the current title of the article as it points to a more general applicability of our validation approach to other antibodies.

Furthermore, we have extensively addressed Dr. Hantusch comments regarding categories of proteins detected on the protein array as part of the new *in silico* analysis as presented in Table 5 and in the supplementary Table 1

Competing Interests: The authors do not declare any competing interests.

The benefits of publishing with F1000Research:

- Your article is published within days, with no editorial bias
- You can publish traditional articles, null/negative results, case reports, data notes and more
- The peer review process is transparent and collaborative
- Your article is indexed in PubMed after passing peer review
- Dedicated customer support at every stage

For pre-submission enquiries, contact research@f1000.com

F1000Research