Development of novel histochemical binding assays – unique challenges for unique molecules

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Introduction

The development of antibody-like molecules represents a fast-growing field of bio-therapeutics with potential to transform patient care in a variety of disease indications. Whilst immunohistochemical techniques have long been established for antibodies, the detection and analysis of specific binding of antibody-like molecules represents a new challenge.

Asterand Bio recently developed and validated a histochemical binding assay for an (anonymised) FITC-labelled peptide, with antibody-like characteristics, in human tissue. Using dual-label immunofluorescence, we demonstrated that binding of the labelled peptide overlapped with that of an antibody to the same target protein (Figure 1).

The same approach was then applied to a second antibody-like peptide, designed to bind EphA2 (a member of the Eph receptor tyrosine kinase family that binds Ephrins A1, 2, 3, 4 and 5), However, this resulted in a new set of challenges.

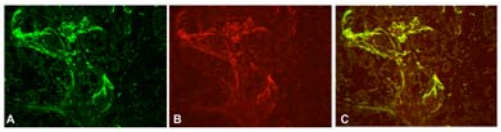

Figure 1. Co-localization of anonymised binding peptide with antibody (x10 mag). Human tumour cryosections were incubated with a binding peptide (A) or an antibody raised against the same target protein (B). Panel C shows the dual label signal where yellow indicates co-localization.

Methods & Materials

Frozen sections from EphA-positive (HT1080 and MDA-MB231) and EphA-negative (HEK293) cell lines were used to develop the assay.

Three detection systems were employed:

- direct detection of the FITC-conjugated peptide
- indirect detection via biotin-conjugated anti-FITC secondary antibodies and AlexaFluor-conjugated streptavidin
- amplified detection via HRP-conjugated anti-FITC antibodies and tyramide amplification.

As before, binding of the peptide was compared to the binding of an anti-EphA2 antibody, using conventional immunofluorescence. In optimising the detection system for both the peptide and the antibody, three fixation techniques and three peptide/antibody concentrations were tested.

The optimal conditions were then to be used to assess EphA2 expression in normal and diseased (tumour) human tissue.

All assays were negatively controlled by the incubation of adjacent sections with a species-specific, non-immune IgG or non-binding peptide at matching concentration to the primary antibody/EphA2-binder.

Table 1. Details of peptides and antibodies

Peptide/Antibody	Description and supplier
EphA2-binder-F	FITC conjugated peptide designed to bind EphA2
Non-binder-F	FITC conjugated peptide with similar design to EphA2-binder but with a mutation in the EphA2 binding sequence
EphA2-binder	Non-labelled peptide designed to bind EphA2
Non-binder	Non-labelled peptide with similar design to EphA2-binder but with a mutation in the EphA2 binding sequence
Anti-EphA2 antibody	R&D Systems #MAB3035, monoclonal mouse IgG2a, clone #371805
Anti-FITC antibody	Acris Antibodies #AP05317BT-N
AlexaFluor ®488/ 546 secondary antibodies	Thermo Fisher Scientific

Result & Discussion

EphA2 antibody optimization is shown in Figure 2. Specific, concentration-dependent immunofluorescence (-if) was observed in the positive control cells with no signal detected in the negative control cells.

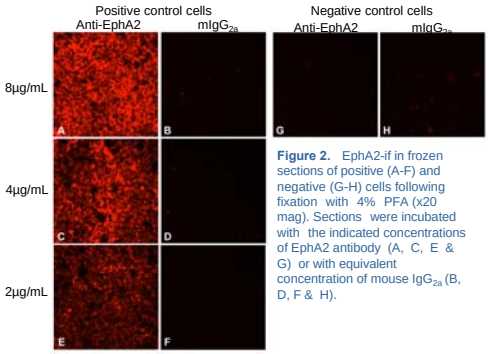

Figure 2. EphA2-if in frozen sections of positive (A-F) and negative (G-H) cells following fixation with 4% PFA (x20 mag). Sections were incubated with the indicated concentrations of EphA2 antibody (A, C, E & G) or with equivalent concentration of mouse IgG_{2a} (B, D, F & H).

Only a very high concentration of EphA2-binder-F resulted in a detectable signal using the direct detection method. However, immunofluorescence was observed in both the positive and negative control cells (Figure 3 upper panels). Lower concentrations of peptide and the indirect detection methodology again resulted in specific binding of the EphA2 peptide when compared to the non-binding peptide, but immunofluorescence was observed in both cell lines. However, binding of the EphA2 peptide in the positive control cells appeared more membrane-associated (Figure 3 middle panels). Further titration of the peptides and use of the amplified detection methodology resulted in similar data to the indirect detection (Figure 3 lower panels).

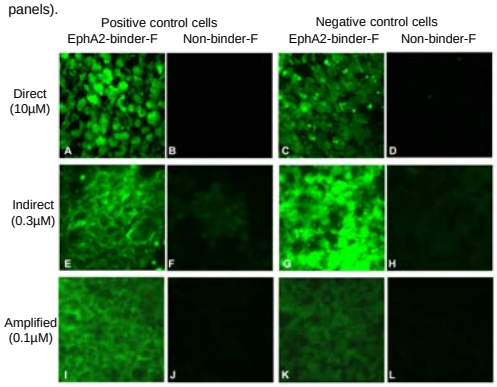

Figure 3. Immunofluorescent detection of the EphA2-binding peptide in frozen sections of positive (left-hand panels) and negative (right-hand panels) control cells (x40 mag). Sections were incubated with the indicated concentration of EphA2-binder-F or the equivalent concentration of non-binder-F and then detected using direct (A-D), indirect (E-H) or amplified (I-L) detection systems. These data show a difference in the characteristics of the binding- and non-binding peptides, but the observed binding did not differentiate positive and negative control cells. *NB. The intensity of the signal varied greatly between the detection systems and so cannot be directly compared.*

A further study evaluated the EphA2 binding peptide in human tissues. Positive and negative control tissues were selected using two methods: literature review and gene expression profiling. Numerous studies have shown that EphA2 is overexpressed in a number of carcinomas, so samples of ovarian and pancreatic tumour were screened. The quantitative rtPCR-derived, XpressWay® profile for EphA2 mRNA, shown in Figure 4, revealed highest EphA2 expression in tonsil. The selected tissue cohort was screened using the EphA2 antibody and a colorimetric (DAB) endpoint in order to allow more detailed analysis. For both tumour types, two out of three samples showed some EphA2 immunoreactivity. No expression was observed in the normal ovary or prostate (Table 2 with selected images in Figure 5). A direct comparison of peptide and antibody binding in tonsil cryosections revealed differing patterns (Figure 6). Peptide binding was observed in the epithelium with membrane-associated staining of lymphoid tissue, antibody binding was only epithelial.

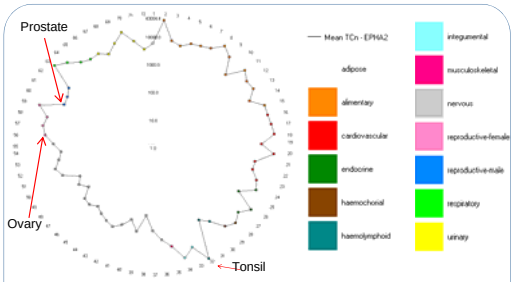

Figure 4. XpressWay® profile for EphA2. Quantitative expression data for EphA2 mRNA across 72 non-diseased human tissue types, each from 3 different donors, covering the major organ systems of the human body.

Table 2. EphA2-immunoreactivity in samples of human normal and tumour tissue

Tissue	EphA2-ir	% tumour	Tissue	EphA2-ir	% tumour
Ovarian Tumour #1	-ve (stroma +ve)	-	Prostate Tumour #1	1+ cytoplasmic	<5%
Ovarian Tumour #2	2+ cytoplasmic 3+ membrane	25-50%	Prostate Tumour #2	3+ cytoplasmic	100% but only in single small discrete region
Ovarian Tumour #3	1+ cytoplasmic (stroma +ve)	<10%	Prostate Tumour #3	-ve	-
Normal Ovary	-ve	-	Normal Prostate	-ve	-

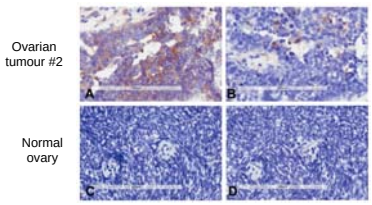

Figure 5. EphA2-ir in frozen sections of ovarian tumour #2 and normal ovary. Sections were incubated with 4µg/mL EphA2 antibody (A & C) or with equivalent concentration of mouse IgG_{2a} (B & D).

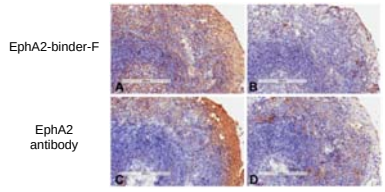

Figure 6. Comparison of peptide and antibody binding in tonsil cryosections. Sections were incubated with 0.3µM EphA2-binder-F (A), 0.3µM Non-binder-F (B), 4µg/mL EphA2 antibody (C) or 4µg/mL mouse IgG_{2a} (D).

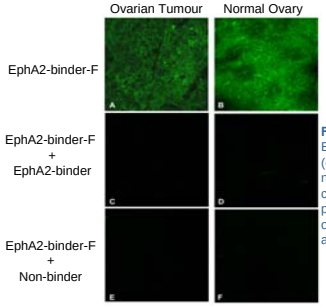

Figure 7. Binding of the EphA2-binder-F to positive (ovarian tumour) and negative (ovary) control tissues in the presence and absence of unlabelled EphA2-binder and non-binder.

The positive (ovarian tumour #2) and negative (normal ovary) control tissues were then used to further assess specificity of the EphA2-binder-F (Figure 7) by co-incubating the peptide with 100-fold molar, unlabelled peptide EphA2-binder and mutated non-binder. EphA2-binder-F bound to both the tumour and normal ovary, although binding in the tumour was one again more membrane-associated. Binding was inhibited by the unlabelled EphA2-binder, but it was also inhibited by the unlabelled non-binding peptide.

Work on developing a reliable and robust histochemical assay for the EphA2 binder is ongoing. The current data highlights some of the potential problems and issues faced when developing assays for novel binding molecules.

Conclusions

- Different approaches are required for individual binding molecules
- Cell lines alone are not always suitable for optimising histochemical assays
- The data generated highlights the importance of negative control cells and/or tissues
- Confirmation of antibody/binding peptide specificity is crucial when developing novel histochemical binding assays