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Validation of mono-specific antibodies

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Validation of mono-specific antibodies

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Abstract

An important step in the quality control of antibodies is to compare the staining pattern between two antibodies directed towards the same gene product. 98 randomly picked sibling antibodies and 98 randomly picked twin antibodies generated by the HPR-project have been studied as a first step in developing a more objective method for comparison of staining patterns. A standardized immunohistchemical method on consecutive tissue sections has been used and the staining patterns have been manually examined. Results show that there is no greater difference between twin- and sibling antibodies.

Keywords

Validation, monospecific antibodies, immunohistochemistry, PrEST, tissue micro array

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Validation of monospecific antibodies

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Populärvetenskaplig sammanfattning

Proteiner har stor betydelse för alla levande organismer då de förekommer i de flesta av cellen processer och maskinerier. Vissa proteiner fungerar som byggstenar, andra som försvar mot främmande organismer när de invaderar oss osv. Koden till proteinerna finns i organismens DNA som i vårt fall är beläget i våra cellkärnor. Tack vare att koden till våra gener i dag är känd (HUGO-projektet) kan vi med hjälp av bioinformatiska verktyg plocka fram koden för och tillverka ett visst protein. Kartläggningen av alla mänskliga proteiner pågår i dag i The Swedish Human Proteome Resource project (HPR-projektet). I HPR-projektet tillverkas små fragment av antikroppar som sedan används för att framställa antikroppar som kan användas vid kartläggningen av var och när proteinet uttrycks i mänsklig vävnad. Ett viktigt steg i kvalitetskontrollen på de genererade antikropparna är att jämföra olika infärgningsmönster för antikroppar riktade mot samma protein eftersom för ett okänt protein är lika infärgningsmönster en bra validering på att antikropparna känner igen rätt protein.

Syftet med detta examensarbete är att jämföra 100 syskon- och 100 tvillingantikroppar för att kunna studera kvaliteten på de i projektet genererade antikropparna och besvara frågan hur stor del av dessa som är sant specifika. Denna jämförelse görs genom att studera immunohistokemiskt infärgade vävnadsprover.

Resultatet visar att bland syskonantikropparna har 59 % generellt lika infärgningsmönster och motsvarande siffra tvillingantikropparna är 54 %.

Examensarbete 20 p i Molekylär bioteknikprogrammet
Uppsala universitet, december 2007

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1 INTRODUCTION

1.1 Background and aim

The aim of antibody based proteomics is to map proteins on a genome vide scale. The Swedish Human Protein Atlas (HPA) program is such a program. In this project, unique protein fragments corresponding to human genes (Protein Epitope Signature Tags, PrEST), is generated from the human genome (Ensembl database). These protein fragments are then used as immunogen to make antibodies. After affinity purification of polyclonal sera, monospecific antibodies against the original gene product are obtained. By using immunohistochemistry (IHC), these antibodies can be used to map specific proteins pattern of expression in a great number of human cells and tissues. The result is a map of how all human proteins is expressed in different organs and tissues in the human body. This map is the base in the public available Protein Atlas (www.proteinatlas.org).

Today the project generates between 5-10 new antibodies every day, which means that the protein atlas is growing with approximately 2000 genes every year. Important control steps to maintain high quality in a high throughput setup includes validation of production of correct antigen (gene sequence of clones, mass spectrometry of recombinant PrEST protein) and the control of that generated antibodies is against the right protein (PrEST array for control of specificity, Western blot for detection of protein with expected size and immunohistochemistry were results from staining is compared with bioinformatics data, published results and the comparison of two different antibodies directed towards the same protein).

One of the most important evaluation steps of antibodies is to compare different antibodies directed towards the same protein. When working with completely unknown proteins such a comparison is the ultimate control that the antibody recognises right protein because similar staining pattern gives a good validation of both antibodies. In the HPR project antibodies has been named “sibling antibodies” (= two different antibodies made by immunisation of two different PrEST:s from the same protein in two different animals), “twin antibodies” (= two different antibodies made by immunisation of the same antigen in two different animals) and “cousin antibodies” (= two different antibodies where one is commercially produced (mono- or poly clonal) and the other is a PrEST antibody directed towards the same gene product). There are also other more complex cases (combination of sibling, twin and/or cousin antibodies).

The aim is to generate two different PrEST:s from each gene, which in the end means that approximately 20% of all PrEST antibodies have a sibling antibody. Some production of twin antibodies is also made today.

At a first look at the database (June 2006) 141 pairs of sibling antibodies was identified. Statistics from these 141 sibling antibodies shows that 60 pairs (42%) have a similar staining pattern in immunohistochemistry. Remaining 81 pairs (58%) displays differences in the comparison between immunohistochemical staining pattern. From these 81 pairs, one of the two antibodies from 58 pairs (72%) has been accepted on another basis. If the number of genes that have at least one accepted antibody is summed up it gives 118 of the 141 sibling antibodies (84%), and from these 118 genes there are two validated antibodies from 60 genes (one antibody from 58 genes). The hypothesis is that if two sibling antibodies show the same

staining pattern they validate each other, i.e. sibling antibodies with similar staining pattern are true antibodies (specific to the protein they generated from).

The comparison of different antibodies directed towards the same gene product is fundamental important and the aim is to develop a system that minimises subjective evaluation and makes the evaluation reliable and reproducible. A problem is the definition of similar staining pattern. The evaluation made today is done at the microscope and is often subjective and in many cases have different tissues been evaluated. In this work the similarity in 100 pairs of randomly picked sibling- and twin antibodies have been studied.

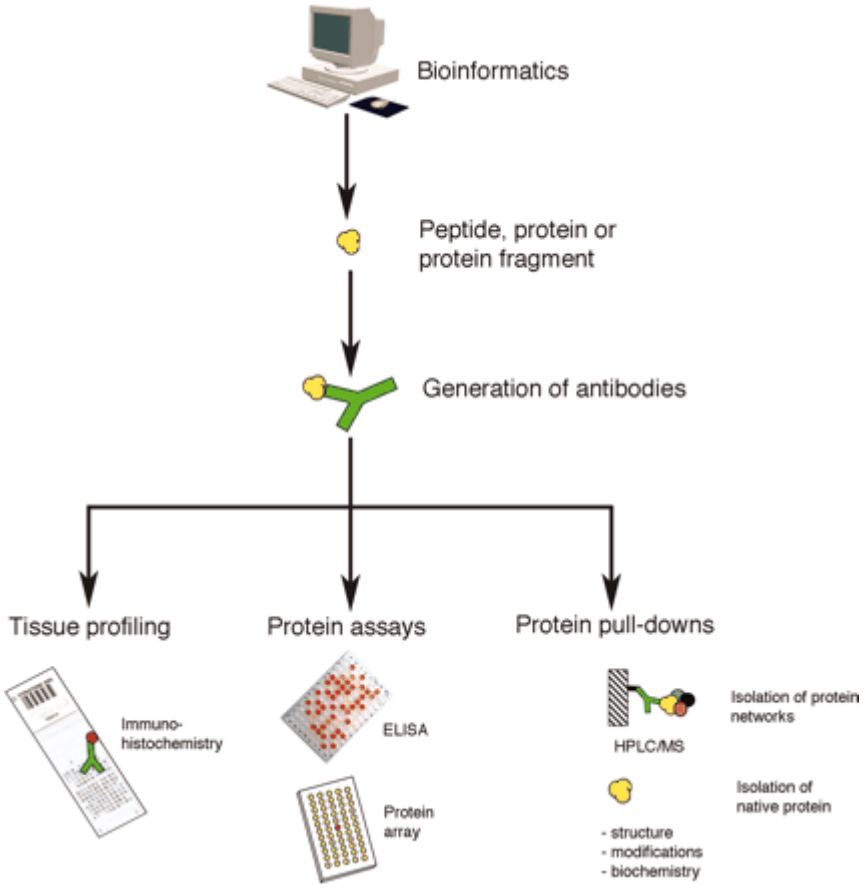

Fig. 1. *The principal of antibody proteomics. Pathway of the generation of an antibody that can be used in several setups.* Illustration used with permission from The Swedish Human Protein Atlas program.

1.2 PrESTs

An antigen is a foreign object or substance that triggers an immune response (production of antibodies) when it enters the body. The part of the antigen where the antibody binds is called epitope. The epitope must be available for recognition and binding of the antibody and not affected by environmental changes (denaturation). Fixation methods, pH changes etc. can

denature the protein and affect the antibodies ability to bind. The HPR-project uses Protein Epitope Signature Tags (PrESTs) as antigen. By using gene sequence information and bioinformatics tools PrESTs are designed. Each PrEST consists of 100-150 amino acid residues and they are selected so that homology regions, transmembrane regions and hairpin loops are avoided. The recombinant protein that is consists of the PrEST sequence, an albumine binding protein and a histidine tag (fig. 2).

Fig. 2. *The generated recombinant PrEST fragment consisting of a histidine-tag, albumine binding protein (ABP) and the PrEST-sequence.*

1.3 Antibodies

An antibody is large glycoprotein belonging to the immunoglobulin super family. It is shaped like a Y and is used by the immune system to identify and neutralize foreign objects. They are produced by B-lymphocytes, either in a membrane bound form or secreted. Secreted antibodies identify antigens that trigger the immune response. The membrane bound form is bound to the B-lymphocytes and has receptor function for antigens.
There are five major classes of antibodies: IgG, IgA, IgM, IgD and IgE. In immunohistochemic use, IgG and IgM are the most commonly used. An antibody has a symmetric core structure composed of two identical light chains and two identical heavy chains. The light-heavy and heavy-heavy chain binding is made up by disulfide bonds. Both heavy chains and light chains consist of amino terminal variable regions that participate in antigen recognition. Antibodies also consist of carboxy terminal constant regions (fig 3).

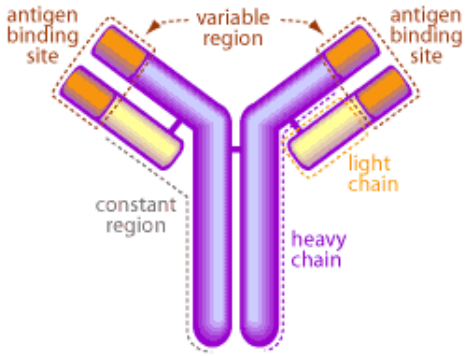

Fig. 3. *Schematic figure of an antibody. Blue parts represent heavy chain and yellow parts light chain. Orange represents the variable, antigen binding region.*
(http://www.scq.ubc.ca/wp-content/uploads/2006/08/mucosalimmunityIgG.gif)

1.3.1 Monospecific antibodies

Monospecific antibodies are generated by using unique PrESTs. The recombinant protein is immunized into a host animal (preferably rabbits) and polyclonal serum is collected and purified (fig 4). Polyclonal antibodies are purified in two steps, first using a depletion column where antibodies towards the histidine tag and albumine binding protein are removed. The

second purification step consists of affinity chromatography using the PrEST protein as ligand. This generates monospecific antibodies recognizing various epitopes on the antigen with less background when used in IHC-staining.

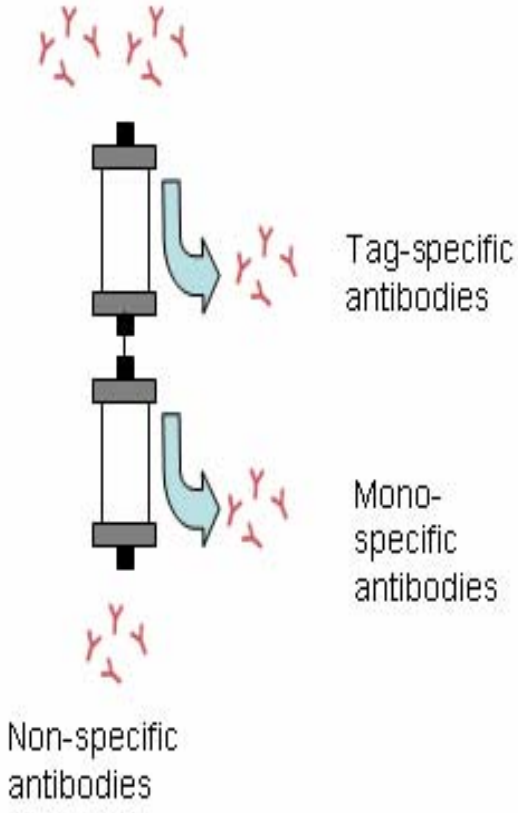

Fig. 4. *The generation of monospecific antibodies. Polyclonal sera are collected and purified in two steps by using affinity chromatography. In the first step is a depletion column used and antibodies towards the his-tag and the ABP are removed. Secondly, the polyclonal antibodies are purified against the recombinant PrEST fragment resulting in msAbs.*

1.4 Immunohistochemistry

Immunohistochemistry (IHC) is a tool used for detection of proteins. It refers to the process of localizing proteins in cells of a tissue section exploiting the principle of antibodies binding specifically to antigens in biological tissues. Immunohistochemical staining is widely used in the clinical diagnosis and treatment of cancer and in research. As an example different cancer types can be charecterized by using specific antibodies.

Tissues used for IHC in this project are fixed and embedded in order to preserve cellular components and stop decomposition of tissue. The most commonly used fixative is neutral-buffered formalin (10%) which contains 4 % formaldehyde. The advantages with formalin are that it´s low cost, it´s easy to use and it preserves the morphology good. The disadvantage with formalin is that it can induce changes, cross-linking, to proteins. The cross-linking involves the formation of hydroxymethylene bridges between proteins or between proteins and nucleic acids. For optimizing the fixation process the surgically removed tissue should emerged in formalin as soon as possible (max 30 min delay). The process takes 24-48 hours depending on size and type of tissue. The bridges can cause changes in the molecular structure of proteins by for example altering epitopes. The changes that occur due to crosslinking can often be reversed by proteolytic digestion or antigen retrieval techniques.

After fixation the tissue must be prepered for embedding. This is done by dehydration (in graded alcohols and xylene). Paraffine is the most frequently used substance for embedding. A probleme with paraffine is that it denatures proteins to some extent which means that some form of antigen retrieval technique must be used prior to IHC staining.

1.4.1 Antigen retrieval

By using antigen retrieval techniques the problem with cross-linking of proteins due to formalin fixation can be fixed. One commonly used method is heating the formalin fixed and paraffin embedded sections in a retrieval solution in a decloaking chamber, microwave or autoclave. This breakes the crosslinking between proteins.

1.4.2 Visualization of proteins

The development of detection systems have made them more sensitive and thanks to automatisation the reproducibility has improved. There are today several detection systems that can be used to yield a strong and specific staining in IHC but the principal is in most cases very much the same. It genarally starts with the binding of a primary antibody (in our case the recombinant PrEST-antibody) towards the antigen of interest. The next step is to add a secondary antibody which is enzyme labeled. This antibody is directed towards the primary antibody. The secondary antibody can be designed in several ways but often is the goal to get a strong signal as possible. To visualize the antibody complex an enzyme-substrate reaction that generates a color is needed. Horserradish (HRP) is a common enzyme that in the presence of an electron donor (peroxidase) forms a complex with the substrate. The electron donors become colored due to oxidation and are therefore called choromogens. Diaminobenzidine (DAB) is a chromogen that generates a brown reaction product that is insoluble in organic solvents.

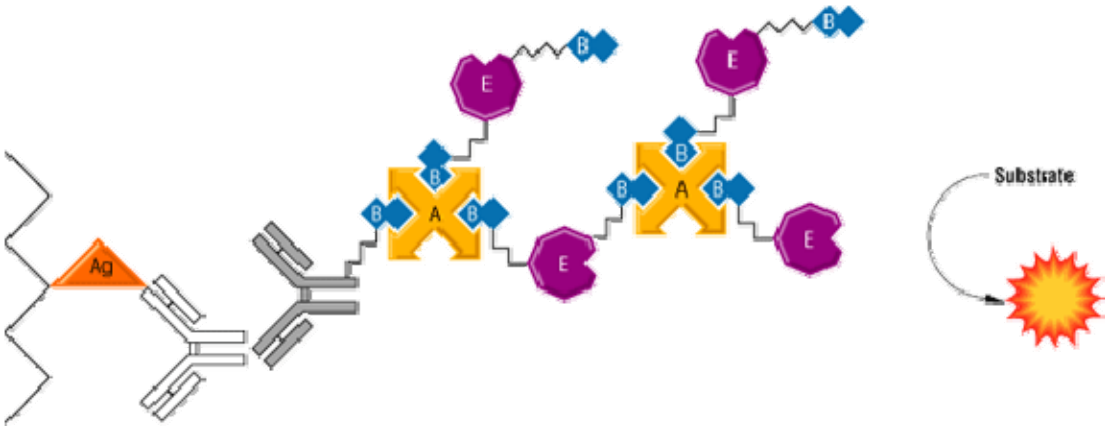

Fig. 5. *The principle of indirect immunohistochemical staining. The primary (white) antibody binds to the antigen. The enzyme labeled secondary (grey) antibody binds to the primary antibody. A change in color occurs when adding a substrate.*

1.5 Validation

Using IHC raises a problem regarding standardization and reproducibility. Variations in preparation and fixation of tissue samples lead to differences in protein detection results. When using IHC as a validation tool the outcome must be reducible and reliable. A tool that can be helpful is the use of databases to search for litterature and get information about splice variants of genes, function of the protein and in what tissue the expression can be expected. To gain reproducebility an automated setup is prefferred because it give the opportunity to stain a large number of tissue sections at the same conditions. The use of tissue micro arrays improves the quality further since a larger number of tissues is being treated the same way. The comparison of two different antibodies directed towards the same protein tells us if the antibody is a good protein marker (exampel in fig 6 and 7).

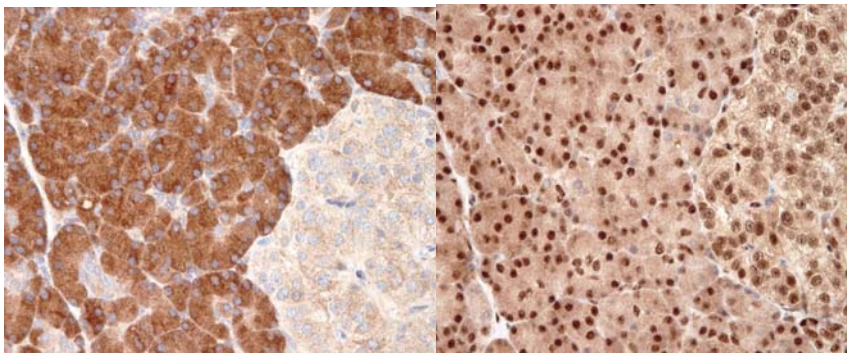

Fig. 6. *Example of an antibody pair with dissimilar staining pattern in pancreas (Lin-52 homolog).*

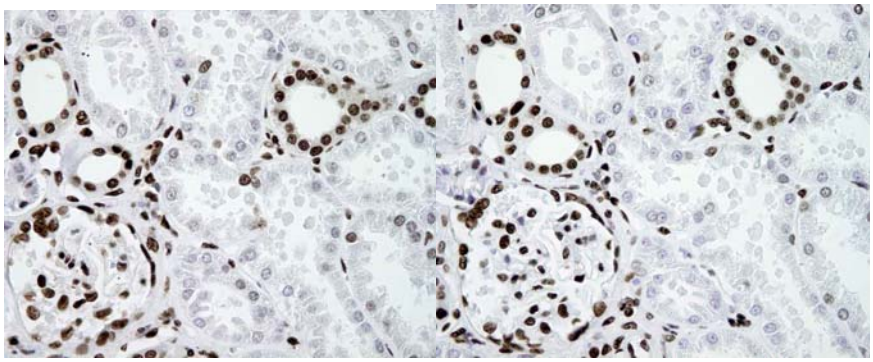

Fig. 7. *Exampel of antibody pair with similar staining pattern in kidney (Methyl-CpG-binding protein 2).*

1.6 Tissue micro array

In diagnostic pathology there is a need for large tissue resources. The introduction of tissue microarray (TMA) technology made it possible to use a smaller amount of material and the method was well suited for a high troughput setup. The idea is to use a recipient block and place tissue cores from a donor block in an organized pattern. Cylindrical cores are punched out from the donor block and transferred to the recipient block. This procedure is done for every core but today it can be done in an automated setup. If the core is deep enough a large number of sections (4µm thick) can taken from each block. The sections can then be used in ordinary IHC. Sections taken from these blocks have aproximately the same structure.

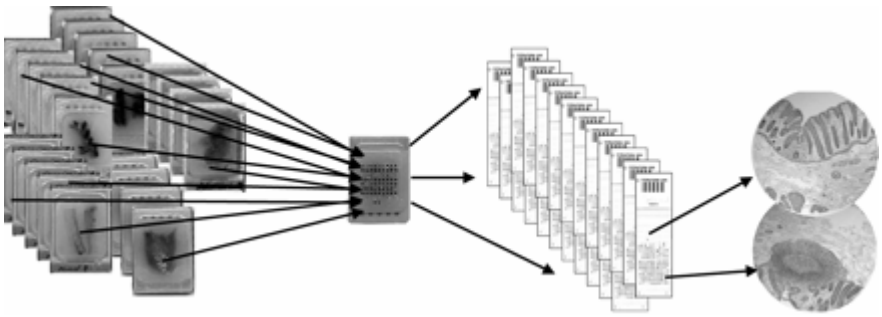

Fig. 8. *A large number of paraffin blocks containing tissues are used as template for the TMA block. From each TMA block can several hundreds of sections be cut.* Illustration used with permission from The Swedish Human Protein Atlas program.

2 OBJECTIVES

The aim of the project is to develop a strategy that can be used in the comparison between different antibodies. The project is divided into three parts: 1. make a test-TMA and analyse the similarity in 100 sibling antibodies and 100 twin antibodies according to immunohistochemical staining pattern, 2. define a reproducible manual scoring model for such analyse and 3. test image analysis as a complement to manual scoring (mainly on cells). In the long run the goal with the project is to make a more objective method for comparison

of staining patterns by using image analysis with the today existing TMAx-system, today used for analysis of cellTMA but in the future perhaps developed to be used for tissues to.

3 MATERIALS AND METHODS

3.1 Tissue micro array design

The TMA was designed as seen in figure 9 with duplicates of each tissue type and with one core of each cell line. The tissues and cell lines were chosen to get a wide range of tissue and cell types. Twelve different normal tissue types were selected. If possible the tissues were selected so that the duplicates were of different sex or age. The cell lines were to represent cell and cancer types not included among the tissues. All tissues were selected from existing archives at the Department of Pathology, University Hospital, Uppsala. The archives consist of formalin fixed and paraffin embedded material. Normal tissue was defined as microscopically normal (non neoplastic) and was often selected from the vicinity of surgically removed tumors. The tissue sections were examined and representative areas in the donor block were selected.

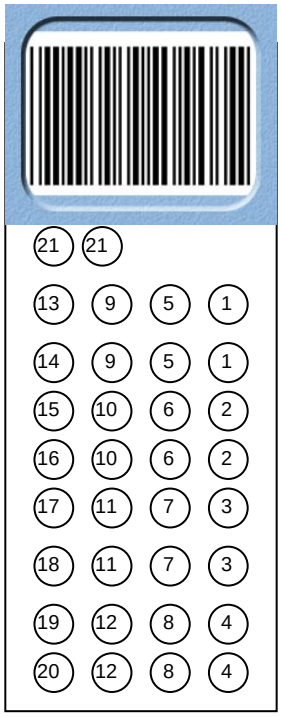

Fig. 9. *Design of tissue microarray; 1- tonsil, 2-endometrium, 3- placenta, 4- small intestine, 5- skeletal muscle, 6- prostate, 7- testis, 8- CNS, 9- skin, 10- liver, 11- kidney, 12- pancreas, 13- cell line A-431, 14- RT-4, 15- U251 MG, 16- K562, 17-U-2 OS, 18- U-937, 19- SK-Mel-30, 20- EFO-21, 21- control.*

3.2 TMA production

Holes were made in the recipient TMA block with an automated tissue arrayer from Beecher instrument (ATA-27, Beecher Instruments, Sun Prairie, CA). A cylindrical core tissue sample (1mm diameter) from the donor block was acquired and deposited in the recipient TMA-block

with a manual tissue arrayer. The TMA block was baked at 50°C for 24 h before sectioning. The TMA blocks were cut into 4µm thick sections and placed on glass slides (SuperFrostplus) for immunohistochemical staining.

3.3 Immunohistochemistry

100 siblingpairs and 100 twinpairs of HPR generated PrEST antibodies were randomly selected from archives. Immunohistochemistry (IHC) was performed according to existing protocols (Lab Vision Corp. Fremont, CA). Glass slides were incubated for 1 h in 60^{o} C, deparaffinized in xylene (2 x 15 min) and hydrated in graded alcohols (EtOH). Endogenous peroxidase was blocked with H_2O_2 in EtOH. As antigen retrieval TRS (Target Retrieval Solution pH6, Dako-Cytomation) were used. The glass slides were immersed in TRS and boiled for 4 min in 125°C in a Decloaking chamber (Biocare Medical). The antibodies were diluted to previously tested and optimized concentrations with antibody diluent (UltrAb Diluent, Lab Vision Corp. Fremont CA). Staining was performed using system for automated immunohistochemistry (Autostainer, Dako Cytomation). Diaminobenzidine (DAB) was used as chromogen and as counterstaining Mayer's hematoxylin (Histolab) was used. The slides were mounted with Pertex (Histolab AB) and manually evaluated using light microscope.

3.4 Validation

The comparison of the twin- and sibling couples was made with a light microscope (Olympus BX51). Results were recorded on a score list (appendix 5). Scores were calculated for comparison of pairs of antibodies (fraction of similar tissues/stained tissues). Depending on the result from comparing the antibody pairs were divided into five groups; 1: 75-100% (number of similar stained tissues divided by number of stained tissues) similarity, 2: 50-74% similarity, 3: 25-49% similarity, 4: 0-24% similarity, 5: special cases. Among the special cases were antibody pairs that in most tissues were similar but showed great differences in a few tissues and therefore considered as dissimilar. Comparison was also made between the given similarity score (IHC-score) and previously done western blots (wb-scoring according to table 1) and status of antibody (pass or fail).

0	Redo (bad quality or technical difficulties)	Uncertain
1	Supportive (single band corresponding to expected size)	Supportive
2	Supportive (protein of expected size detected but additional staining present)	Supportive
3	Supportive (single band not corresponding to expected size but supported by additional data)	Supportive
4	No bands present	Uncertain
5	Uncertain (single band not corresponding to expected size and not supported by other available data)	Not supportive
6	Uncertain (weak interaction with protein of expected size, with additional bands of higher intensity also present)	Not supportive
7	Not supporting (only proteins not corresponding to expected size)	Not supportive

Table 1. *Scoring of western blot results.*

4 RESULTS

All data for sibling antibodies are collected in appendix 1 and 2. All data for twin antibodies are collected in appendix 3 and 4.

4.1 Results for sibling antibodies

4.1.1 Western-blot score when both antibodies are passed

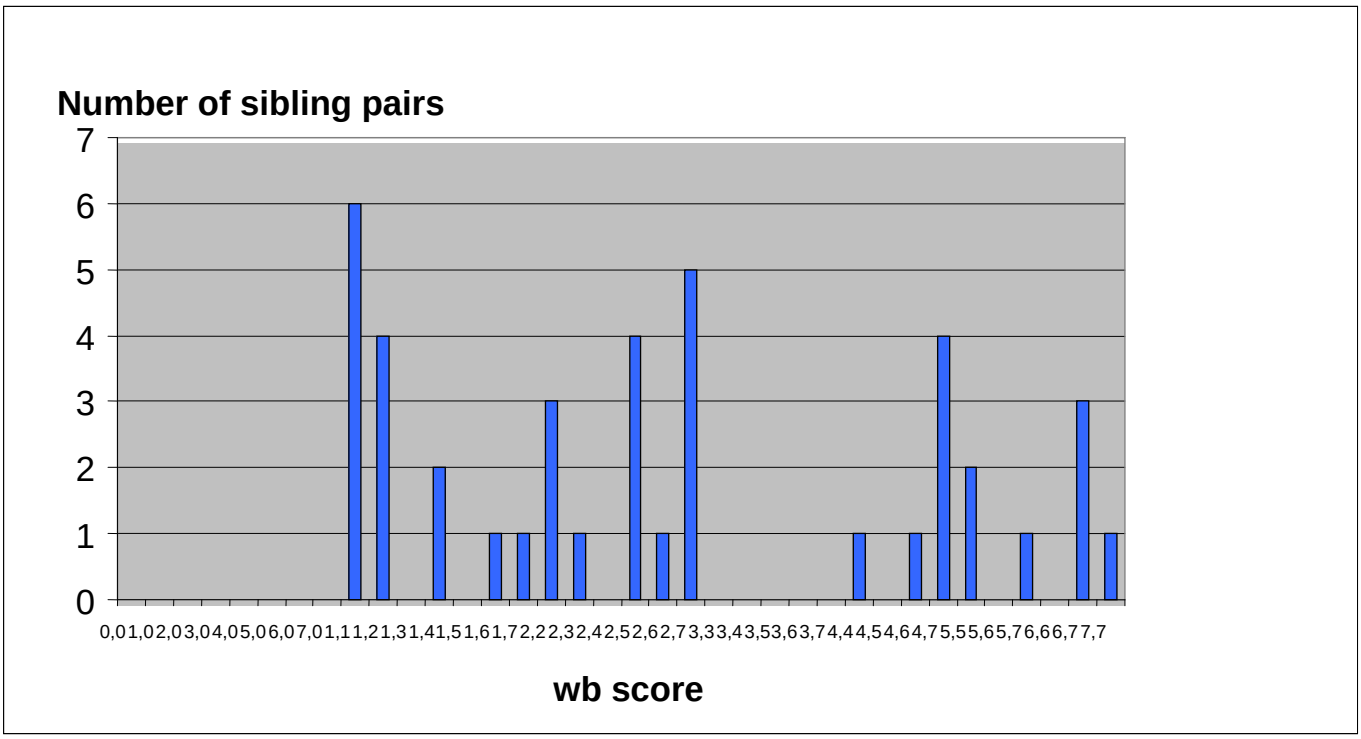

Diagram 1. *Results from comparing western blot score to antibodies were both are passed. Numbers given for western blot are according to table 1.*

Diagram 1 shows that when both antibodies are passed the western blot scores (wb-score) are in most cases supportive but there are also fairly great number of antibodies where one antibody has a supportive wb-score and the other a not-supportive score.

4.1.2 Western-blot score when one antibody is passed and the other failed

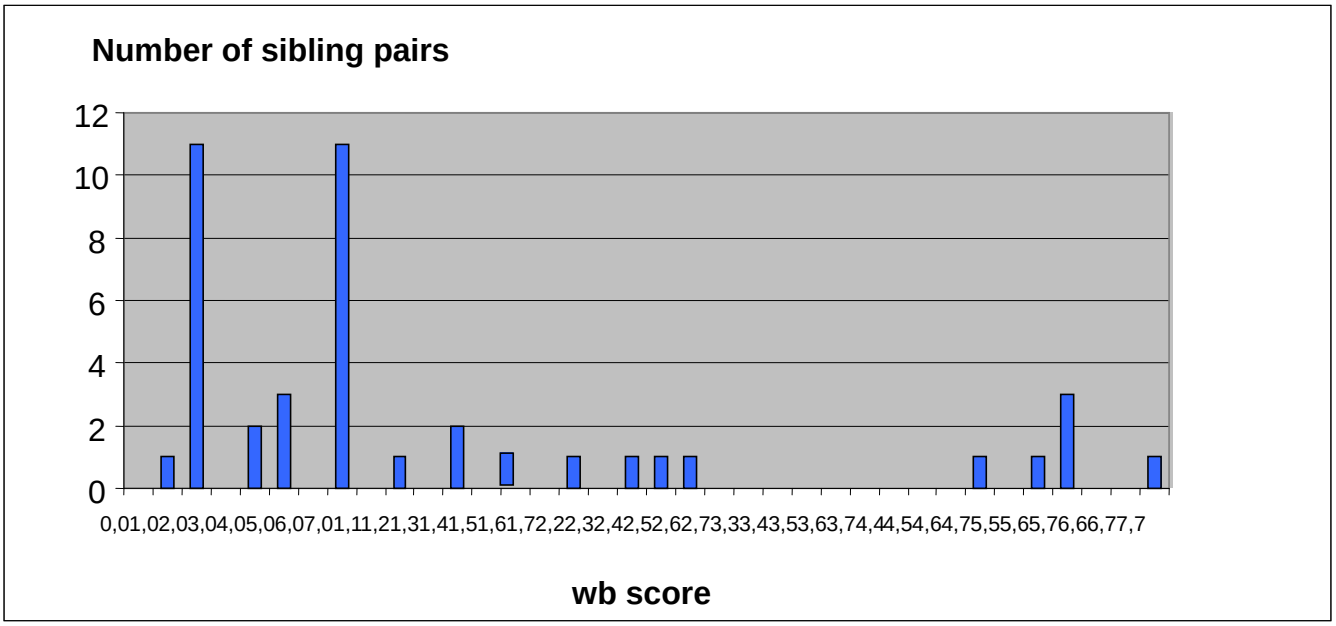

Diagram 2. *Diagram showing results from comparing western blot score to antibodies were one is passed and the other failed.*

In the pass/fail group of antibodies most antibodies have a difference in wb-score, i.e. one is supportive and the other is not-supportive or uncertain.

4.1.3 Western-blot score when both antibodies are passed

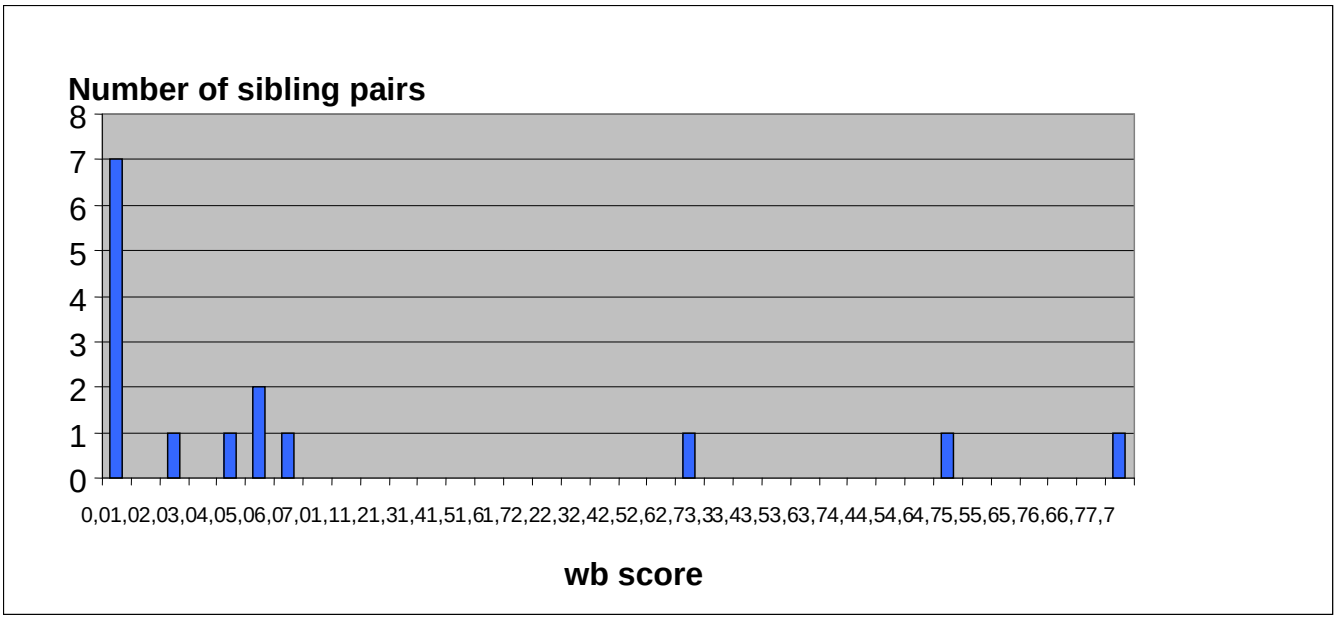

Diagram 3. *Diagram showing results were both antibodies are failed.*

When both antibodies are failed, most of the antibodies have a wb-score that is uncertain. Only a few antibodies have a supportive score.

4.1.4 IHC-score compared to western-blot score

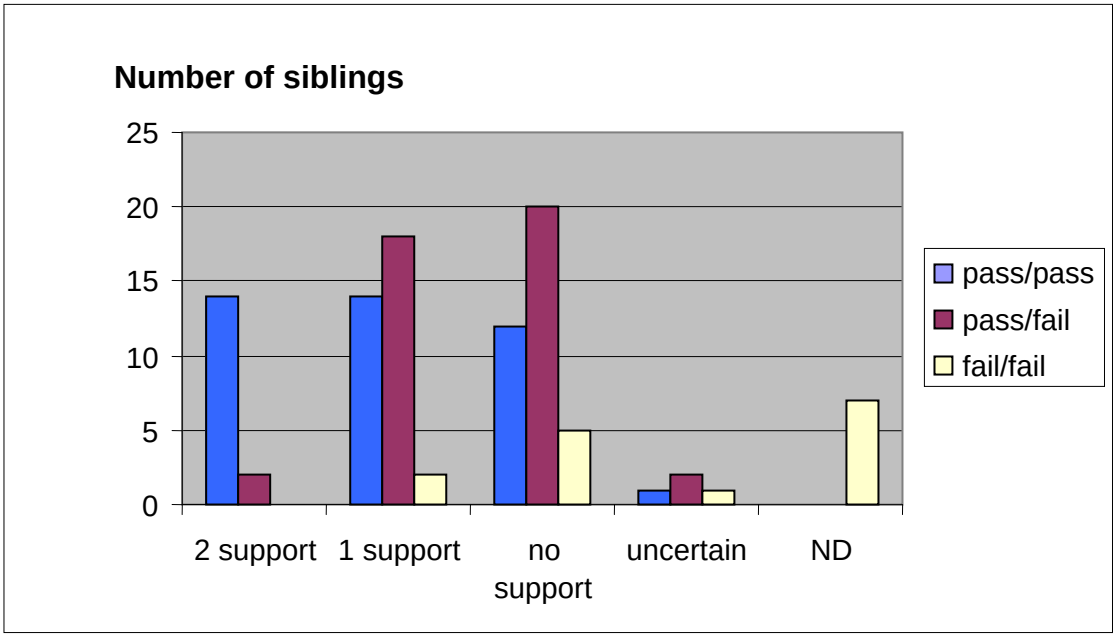

Diagram 4. *Results from comparing western blot and pass/fail data (ND=not done).*

	pass/pass	pass/fail	fail/fail
2 support	14 (34 %)	2 (5 %)	0
1 support	14 (34 %)	18 (43 %)	2
No support	12 (29 %)	20 (48 %)	5
Uncertain	1 (2 %)	2 (5 %)	1
ND	0	0	7

Pass/pass 41 (42%)
Pass/fail 42 (43%)
Fail/fail 15 (15%)

Table 2. *Results from comparing western blot and pass/fail data.*

Most of the antibodies where both are passed have supportive wb-score. When the wb-score is not-supportive the rate of failed antibodies increases (greater number of pass/fail or fail/fail).

4.1.5 IHC-score and pass/fail data

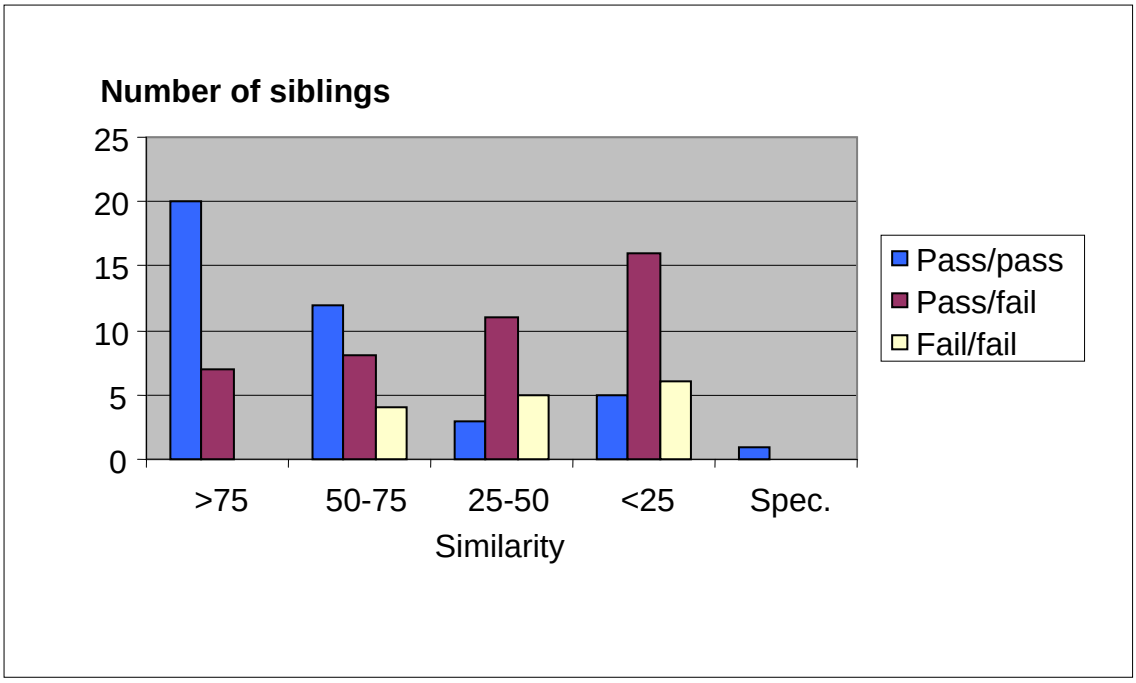

Diagram 5. *Comparison between IHC-score and pass/fail data.*

Comparing IHC-score with pass/fail –data shows that the pass/pass group have the highest number of similarity. When the similarity score decreases the number of pass/fail and fail/fail antibodies increases.

4.2 Results for twin antibodies

4.2.1 Western-blot score when both antibodies are passed

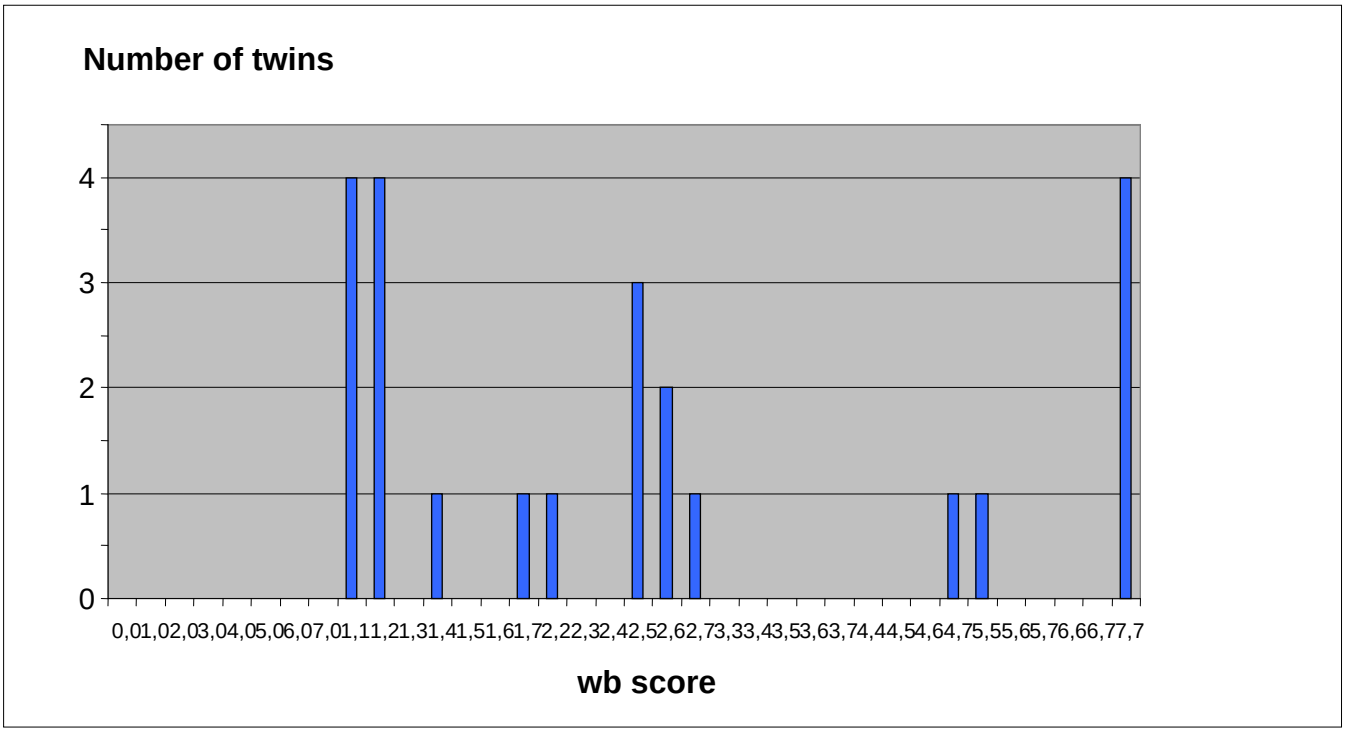

Diagram 6. *Results from comparing western blot score to antibodies were both are passed. Numbers given for western blot are according to table 1.*

Most of the antibodies where both are passed have similar wb-score (supportive or not supportive for both). Only a small part has a wb-score where one is supportive and the other not-supportive.

4.2.2 Western-blot score when one antibody is passed and the other failed

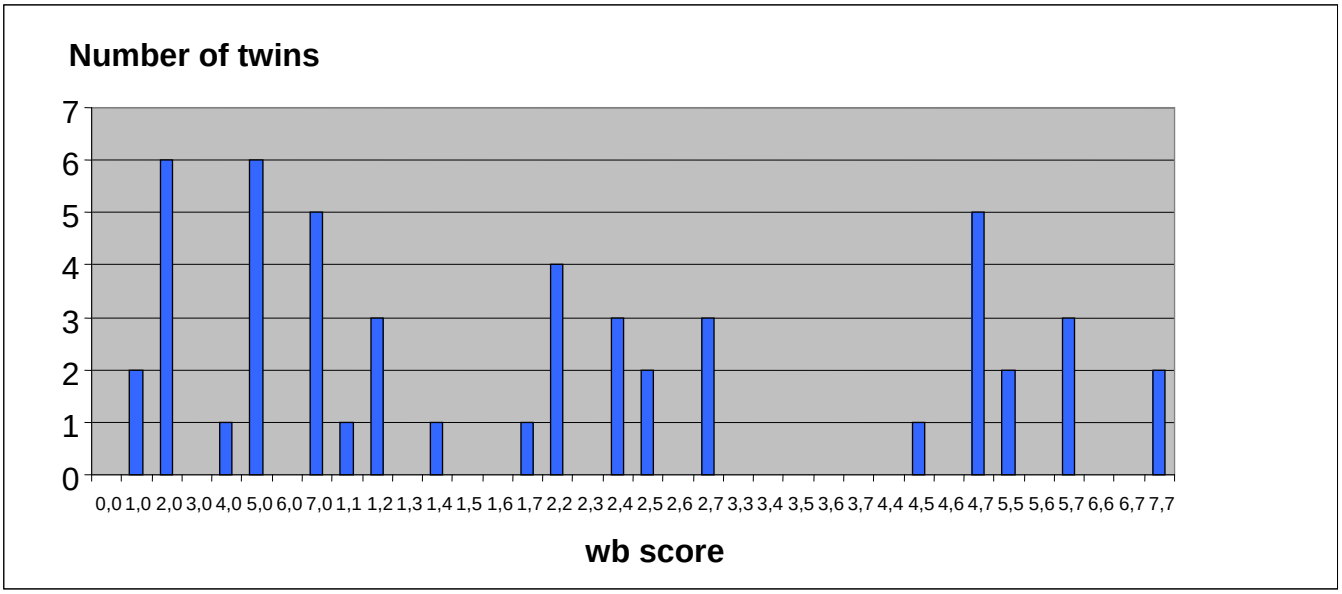

Diagram 7. *Diagram showing results from comparing western blot score to antibodies were one is passed and the other failed.*

The pass/fail group for twin antibodies shows the same pattern as for sibling antibodies in corresponding group. Most of the antibodies have differences in wb-score but there are some that have similar score (supportive or not-supportive)

4.2.3 Western-blot score when both antibodies are failed

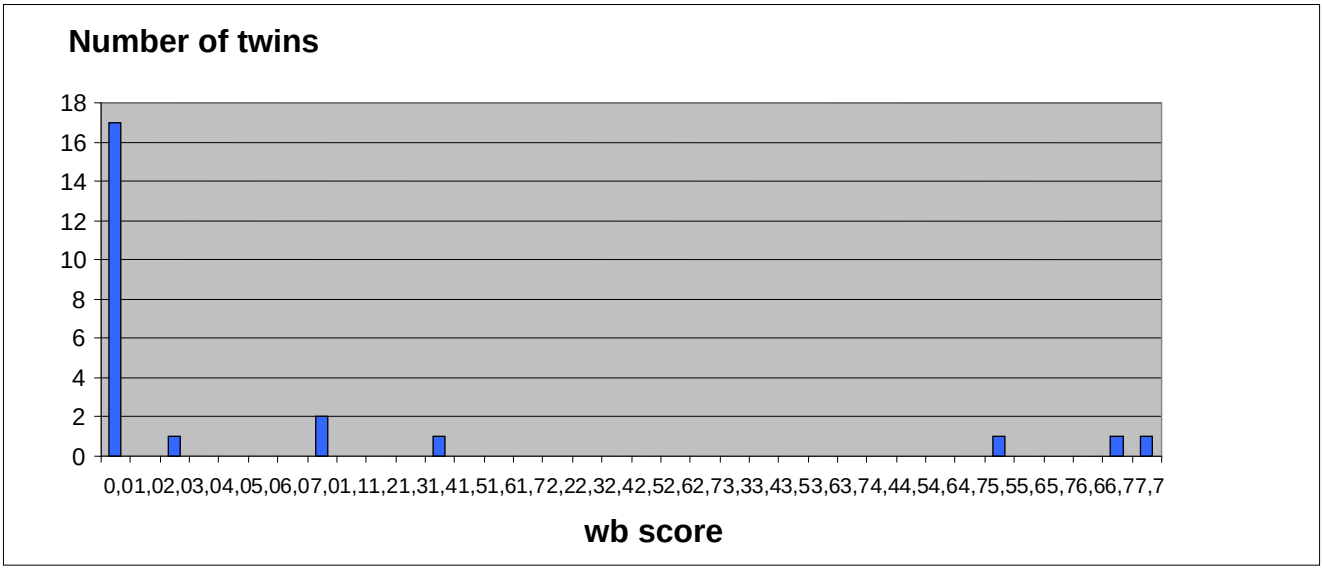

Diagram 8. *Diagram showing results were both antibodies are failed.*

The fail/fail group also shows the same pattern as for sibling antibodies. Most have a wb-score that is uncertain. Some have differences in the wb-score or both have a not-supportive score.

4.2.4 IHC-score compared to western-blot score

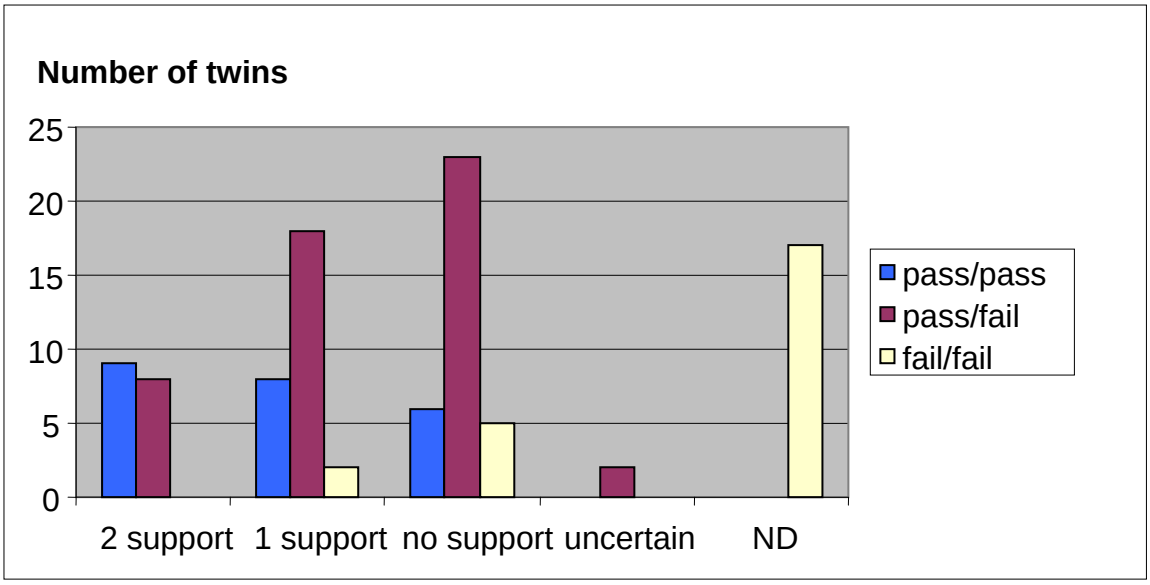

Diagram 9. *Results from comparing western blot and pass/fail data (ND=not done).*

	pass/pass	pass/fail	fail/fail
2 support	9 (39 %)	8 (16 %)	0
1 support	8 (35 %)	18 (35 %)	2
No support	6 (26 %)	23 (45 %)	5

Pass/pass	**23 (23%)**
Pass/fail	**51 (52%)**
Fail/fail	**24 (24%)**

Table 3.*Results from comparing western blot and pass/fail data.*

When the wb-score is two supportive the number of pass/pass antibodies is greatest. The number of pass/pass antibodies decreases when there are no or only one supportive wb-score. For pass/fail or fail/fail antibodies this is inverted.

4.2.5 IHC-score and pass/fail data

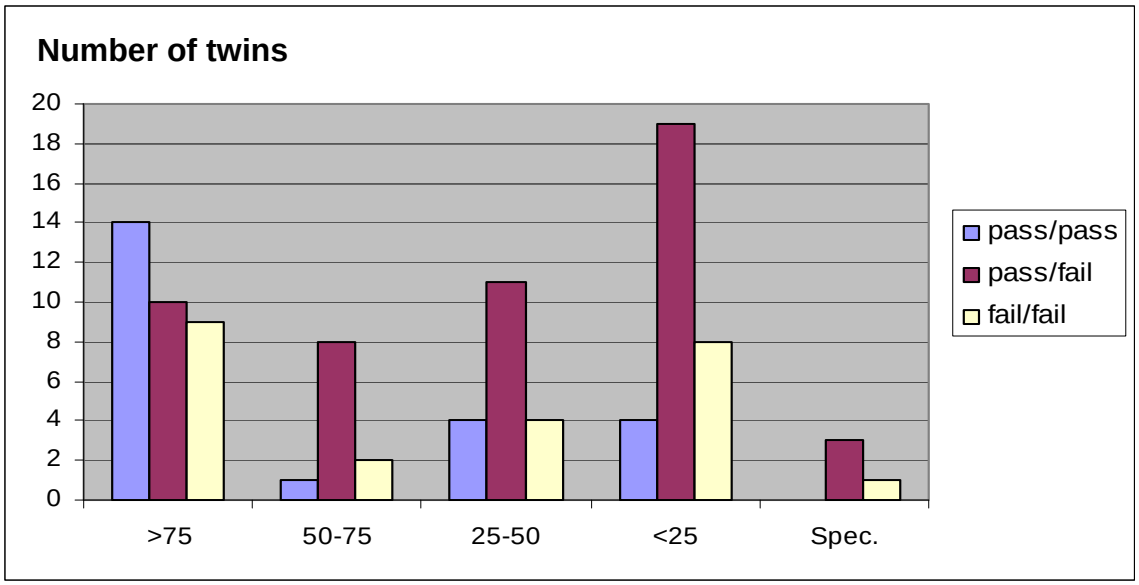

Diagram 10. *Comparison between IHC-score and pass/fail data.*

The results from comparing IHC-score and pass/fail –data is as expected except for the low number of pass/pass antibodies with an IHC-score between 50-75% and the high number of pass/fail and fail/fail antibodies with high IHC-score.

5 DISCUSSION

Manual interpretation of immunohistochemically stained tissue samples is well known method used both in research and clinical practise. It's a fairly simple method and the staining methods prior to evaluation have been developed over several years. The problem is that the

interpretation can shift from time to time and between the evaluators and that the evaluator often needs training to know what to look for.

The making and testing of a testTMA shows that it can be used in many cases but one must remember that very specialized proteins expressed in one or a few tissues can be missed using a TMA that is containing a limited amount of tissues. The small amount of antibodies in this project that showed no immunoreactivity could perhaps have been expressed in tissue not present in this testTMA. In an initial testing step this smaller TMA could work but it is likely that further investigation is necessary.

Why the antibodies show differences when comparing pass/fail-data and wb-score could be due to many things. One can be that the antibodies not are optimized for a certain way of testing them, for example they can't be tested in a western blot set up with good results. As seen in the different diagrams there are antibodies that are passed but they have a not-supporting wb-score and vice versa. The same pattern can be seen for both sibling- and twin antibodies.

The results from the IHC-testing are in some ways surprising. Twin antibodies should have a higher amount of similar antibodies since they are generated towards the same part of the protein but this project shows that there are no greater differences between sibling- and twin antibodies when looking at the quality. The reason why not every antibody pair shows similar staining pattern can be due to several things. Differences because of different handling of tissue samples should be minimized since consecutive sections have been used, i.e. staining has been done on in as similar tissue as possible for the antibody pairs. Perhaps could different antibodies be more or less sensitive to changes in the proteins that occur in the fixation process. The reason for differences is probably due to different transcripts and splicing and perhaps mechanisms not yet known.

6 CONCLUSION

In this project an immunohistochemical method has been used to validate a randomly picked set of sibling- and twin antibodies to answer the question how good the quality of the antibodies generated at the HPR-project is. The TMA-test shows that a smaller TMA can be used but is most likely to be followed by a TMA-test were more tissues are taken in consideration. Results from the IHC-test shows that more than 50 % of the generated antibodies are of such good quality that no further testing is necessary and it's a rather good number since there are many steps on the way making an antibody and lots of things that could go wrong. Why there are differences are to be further tested. The IHC-test also shows that there are no greater differences between sibling- and twin antibodies and therefore can the most benefiting process be used.

7 ACKNOWLEDGEMENT

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Appendix 1

Siblings

Both passed		Passed/failed		Both failed	
wb resultat	**antal**	**wb resultat**	**antal**	**wb resultat**	**antal**
0,0	0	0,0	0	0,0	7
1,0	0	1,0	1	1,0	0
2,0	0	2,0	11	2,0	1
3,0	0	3,0	0	3,0	0
4,0	0	4,0	2	4,0	1
5,0	0	5,0	3	5,0	2
6,0	0	6,0	0	6,0	1
7,0	0	7,0	11	7,0	0
1,1	6	1,1	0	1,1	0
1,2	4	1,2	1	1,2	0
1,3	0	1,3	0	1,3	0
1,4	2	1,4	2	1,4	0
1,5	0	1,5	1	1,5	0
1,6	1	1,6	0	1,6	0
1,7	1	1,7	0	1,7	0
2,2	3	2,2	1	2,2	0
2,3	1	2,3	0	2,3	0
2,4	0	2,4	1	2,4	0
2,5	4	2,5	1	2,5	0
2,6	1	2,6	1	2,6	0
2,7	5	2,7	0	2,7	1
3,3	0	3,3	0	3,3	0
3,4	0	3,4	0	3,4	0
3,5	0	3,5	0	3,5	0
3,6	0	3,6	0	3,6	0
3,7	0	3,7	0	3,7	0
4,4	1	4,4	0	4,4	0
4,5	0	4,5	0	4,5	0
4,6	1	4,6	0	4,6	0
4,7	4	4,7	1	4,7	1
5,5	2	5,5	0	5,5	0
5,6	0	5,6	1	5,6	0
5,7	1	5,7	3	5,7	0
6,6	0	6,6	0	6,6	0
6,7	3	6,7	0	6,7	0
7,7	1	7,7	1	7,7	1

	pass/pass	**pass/fail**	**fail/fail**
2 support	14	2	0
1 support	14	18	2
no support	12	20	5
uncertain	1	2	1
ND	0	0	7

Appendix 2

IHC score (siblings)

Pass/pass		**Pass/fail**		**Fail/fail**
>75	20	**>75**	7	**>75**
50-75	12	**50-75**	8	**50-75**
25-50	3	**25-50**	11	**25-50**
<25	5	**<25**	16	**<25**
Spec.	1	**Spec.**	0	**Spec.**

Appendix 3

Twins

Both passed		Pass/fail		Both failed	
wb resultat	**antal**	**wb resultat**	**antal**	**wb resultat**	**antal**
0,0	0	0,0	0	0,0	17
1,0	0	1,0	2	1,0	0
2,0	0	2,0	6	2,0	1
3,0	0	3,0	0	3,0	0
4,0	0	4,0	1	4,0	0
5,0	0	5,0	6	5,0	0
6,0	0	6,0	0	6,0	0
7,0	0	7,0	5	7,0	2
1,1	4	1,1	1	1,1	0
1,2	4	1,2	3	1,2	0
1,3	0	1,3	0	1,3	0
1,4	1	1,4	1	1,4	1
1,5	0	1,5	0	1,5	0
1,6	0	1,6	0	1,6	0
1,7	1	1,7	1	1,7	0
2,2	1	2,2	4	2,2	0
2,3	0	2,3	0	2,3	0
2,4	0	2,4	3	2,4	0
2,5	3	2,5	2	2,5	0
2,6	2	2,6	0	2,6	0
2,7	1	2,7	3	2,7	0
3,3	0	3,3	0	3,3	0
3,4	0	3,4	0	3,4	0
3,5	0	3,5	0	3,5	0
3,6	0	3,6	0	3,6	0
3,7	0	3,7	0	3,7	0
4,4	0	4,4	0	4,4	0
4,5	0	4,5	1	4,5	0
4,6	0	4,6	0	4,6	0
4,7	1	4,7	5	4,7	0
5,5	1	5,5	2	5,5	1
5,6	0	5,6	0	5,6	0
5,7	0	5,7	3	5,7	0
6,6	0	6,6	0	6,6	0
6,7	0	6,7	0	6,7	1
7,7	4	7,7	2	7,7	1

	pass/pass	**pass/fail**	**fail/fail**
2 support	9	8	0
1 support	8	18	2
no support	6	23	5
Uncertain	0	2	0
ND	0	0	17

Appendix 4

IHC score (twins)

Pass/pass		**Pass/fail**		**Fail/fail**	
>75	14	**>75**	10	**>75**	9
50-75	1	**50-75**	8	**50-75**	2
25-50	4	**25-50**	11	**25-50**	4
<25	4	**<25**	19	**<25**	8
Spec.	0	**Spec.**	3	**Spec.**	1

Appendix 5

Evaluator: **Twin or Sibl** **Antibodypair:**

Tissues	Similar	Dissimilar	Distr	Comments
Tonsil				
Tonsil				
Endomentrium				
Endomentrium				
Placenta				
Placenta				
Small Intestine				
Small Intestine				
Muscle				
Muscle				
Prostate				
Prostate				
Testis				
Testis				
CNS, cortex				
CNS, cortex				
Skin				
Skin				
Liver				
Liver				
Kidney				
Kidney				
Pancreas				
Pancreas				
A-431				
RT-4				
U251 MG				
K5262				
U-2 OS				
U-937				
SK-Mel-30				
EFO-21				

Intensity: **S P D**

Similar tissues: **/12**
Similar cell lines: **/8**

Score: