Antibody Validation Guide

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1. Key Points

- Life science research is in the midst of a reproducibility crisis with antibodies that are not fit for purpose being a major contributor to this.

- Antibodies are much more complex than first assumed, forming complex protein-protein interactions which can be influenced by an almost limitless number of factors.

- There are numerous ways of validating antibodies ranging in complexity, rigour and practicality for the average researcher.

- We have synthesised current validation guidelines to create a unified guide of how to validate antibodies for research purposes using the guiding principles of application specificity, being as close as possible to experimental conditions and the shared responsibility between supplier and researcher to validate antibodies.

2. Antibodies: A key part of the reproducibility crisis

Life science is in crisis. The scientific method is founded upon on the ability of other researchers being able to precisely repeat previous experiments yet most of the conclusions of previously published studies are false (Button et al., 2013; Ionnidis, 2005). Over 70% of researchers polled by Nature had previously been unable to replicate published work with 50% unable to even replicate their own previous work (Baker et al., 2016). It has been suggested that between 50% and 89% of pre-clinical research is un-reproducible with this not only leading to a monumental wastage of resources (estimated at $28bn a year in the USA alone) but also clinical trials predicated upon false data, the needless sacrifice of animal lives and entire avenues of investigation built on poor foundations (Freedman et al., 2015) Famously Amgen were only able to reproduce 6 out of 53 key cancer biology studies they investigated (Begley and Ellis, 2012). Multiple factors underly this reproducibility crisis including poor reporting (Baker et al., 2016), fraud (Fanelli and Tregenza, 2009), poor statistical practices (Button et al., 2013) and a lack of open data (Miyakawa, 2020). However, one of the most important sources of irreproducibility is from the reagents used in the experiments themselves with it being estimated that up to 36.1% of irreproducibility is due to biological reagents and reference materials (Freedman et al., 2015).

Antibodies are perhaps one of the most ubiquitously used biological reagents. Their highly specific binding to target proteins allows researchers to pick out specific splice variants of a single protein amongst the soup of the proteome while having huge flexibility in the range of techniques they can be used in. Annually researchers spend around $1.5bn on a catalogue of approximately over 5.5 million products from 265 suppliers with the average biology lab spending between $6,000 and $12,000 per year on antibodies (Abcam, 2021; CiteAb, 2021; Goodman, 2018). However this most ubiquitously used of products hides a dark side, a shocking track record of reliability which has destroyed years of research, overturned established knowledge and played a major role in the reproducibility crisis. Scientists producing the human protein atlas (Uhlén et al., 2015) tested over 60,000 antibodies for use in immunohistochemistry (IHC) and immunocytochemistry (ICC) and found that only 50% worked in the conditions that were used to generate the atlas (Älgenäs et al., 2014; Berglund et al., 2008; Taussig et al., 2018). Furthermore a large bio-informatics company tested 6,000 antibodies and found that 75% bound to additional proteins other than the target or did not work at all (Weller, 2016).

While these statistics are deeply worrying they fail to show the devastating impact that bad antibodies can have on a scientific field. Following the release of coding sequences for nicotinic acetylcholine receptor (nAChRs) subunits, researchers spent 10 years investigating the localisation of different nAChR subunits using subunit specific antibodies in a range of tissues. However when Moser et al., 2007 tested these antibodies in nAChR subunit specific knockout mice they found that antibody staining was identical in wild type and knockout mice. Staying with G-protein coupled receptors, Pradidarcheep et al., 2008, 2009 showed the range of muscarinic and adrenergic receptor antibodies they used showed distinct staining patterns in histological preparations but when tested in cell lines expressing their specific receptor none bound to a protein at the expected weight for their target.

However perhaps the case of poorly performing antibodies with the biggest impact was that regarding oestrogen receptor β (ERβ) in breast cancer. ERβ was believed to be a strong biomarker for breast cancer with 8 clinical trials in 2017 using antibodies to ERβ. However, vital work by Andersson et al., 2017 not only reported that 12 out of 13 commonly used antibodies against ERβ were non-selective but using the one good antibody revealed that neither normal nor cancerous human breast tissue even expressed ERβ. As previously alluded to these examples are the tip of an iceberg with antibodies against EpoR, HER2, ERCC1 and CDK1 being used in clinical settings before being found to be unreliable (Voskuil et al., 2020).

These examples show that a renewed focus on antibody validation, the process of proving that an antibody is fit for purpose, is now critical for restoring confidence in research utilising antibodies. Improving the field will be a long and difficult journey involving antibody manufacturers, journals and researchers with a consensus having emerged that validation is not just the responsibility of the supplier but the researcher too (Blow, 2013; Bordeaux et al., 2010; Roncador et al., 2016; Taussig et al., 2018; Voskuil, 2017). It will therefore be critical that researchers have a thorough understanding of both why these problems can occur but also how to perform validation experiments themselves in their own laboratories.

3. Why are antibodies so unreliable?

In order for both researchers and suppliers to begin addressing this challenge it will first be necessary to understand in detail the mixture of technical, cultural and procedural issues that underly antibody irreproducibility.

3.1 Deceptive complexity

Perhaps the biggest misconception regarding antibodies is that they are simple molecules that easily bind their target antigen irrespective of condition. However, this could not be further from the truth; in reality antibody-antigen interactions are dependent on a vast variety of factors such as tissue fixation, subcellular localisation, buffers, application, sample preparation, target posttranslational modifications and interaction partners to name a few (Bordeaux et al., 2010; Lorincz and Nusser, 2008; Taussig et al., 2018; Uhlen et al., 2016; Voskuil, 2017). It is now well understood that just because an antibody works in one application bears no correlation as to whether it will be successful in another (Lorincz and Nusser, 2008; Taussig et al., 2018).

One of the biggest issues is antibody binding to native as opposed to denatured proteins. Often immunogens are created using native proteins, however techniques such as western blotting or immunohistochemistry rely on denaturing proteins through a range of chemicals including SDS and aldehydes. Antibodies can bind to epitopes which are present in the native protein (e.g. conformational epitopes) which are abolished upon denaturing or conversely the 3D shape of a native protein can hide epitopes that are accessible in the denatured peptide (Bordeaux et al., 2010). The degree of protein-unfolding can differ both between applications

and between different buffer conditions meaning that just because an antibody binds denatured proteins in one context does not mean it will bind in a different context (Voskuil et al., 2020). Additionally, fixation during protocols for immunohistochemistry can also present barriers for antibody access to the antigen. Use of aldehyde-based fixatives causes protein cross-links which can prevent progression of the antibody to the target (Holmseth et al., 2006). This is likely to be an even bigger problem in targets with spatially restricted access such as antibodies against synaptic targets.

Subcellular localisation can surprisingly make a big difference to whether an antibody will successfully bind or not. For example there is an epitope on Bcl-2, a key protein involved in apoptosis, which is only accessible in the cytoplasm but once the protein enters the nucleus antibodies can no longer bind due to this epitope being covered by putative binding partners (Bordeaux et al., 2010; Pezella et al., 1990). Proteins can be subject to different posttranslational modifications in different cell-types and subcellular localisations with a common issue being that immunogens are synthesised in recombinant bacterial cells which have different posttranslational modifications to mammalian cells resulting in the antibody being unable to bind (Roncador et al., 2016).

Antigen presentation can also be a big determinant of binding specificity when carrying out IHC. Depending on whether an antigen presentation step is included in the protocol this has been found in the past to change the results so drastically as to make an antibody appearing to be non-specific behave in a specific manner (Lorincz and Nusser, 2008). Watanabe et al., 1998 reported an identical cytoplasmic pattern of NR2A staining between wild type and NR2A KO mice when using standard aldehyde fixed tissue. However, when they included a pepsin pre-treatment step before primary antibody addition this revealed specific staining for receptors in neuropilli which was abolished in KO mice.

Finally, the issue that is perhaps most relevant to antibody use in the laboratory is cross-reactivity. If immunogen sequences are not designed carefully enough or are too short then it is easy for sequences within the immunogen to be present in multiple different proteins within a cell. One example of this is Lukinavičius et al., 2013 who discovered that two popular antibodies against the cyclin dependent kinase Cdk-1 also bound to Cep152, a centromere component. Further investigation revealed that the immunogens used had partially overlapping sequences (Lukinavičius et al., 2013). However another mechanism for cross-reactivity has also been observed with it being found that antibodies are able to exhibit conformational

specificity whereby depending on which conformational structure the antibody adopts (which likely is influenced by subcellular localisation and buffer conditions) different antigens can be bound with high affinity (James et al., 2003). Cross-reactivity is often difficult to predict and can be influenced by the complexity of a sample, target protein concentration and antibody concentration (Baker, 2015a). This means that the only way to rule cross-reactivity out is through carefully designed validation experiments in the target tissue of interest (Lorincz and Nusser, 2008; Taussig et al., 2018; Uhlen et al., 2016).

3.2 Batch variation: A battle of technology

Another large source of problems with antibody irreproducibility is variation between different batches of antibody. The experience of researchers having successfully used an antibody for years then bought a new lot to discover either non-specificity or a lack of binding to target is hardly uncommon (Bordeaux et al., 2010; Couchman, 2009; Goodman, 2018; Perkel, 2014; Skogs et al., 2017). For example, Pozner-Moulis et al., 2007 tested two different lots of the anti-Met antibody (clone 3D4) on nearly 700 breast cancer samples and found completely opposite staining patterns between lots along with a R^2 coefficient of 0.04 when the results of the two lots were correlated. While recent debate in this area has ended up becoming along ideological lines as to which antibody production technology is best there is at its heart a clear cause: a lack of batch specific validation by both suppliers and researchers (Bradbury and Pluckthun, 2015; Pillai-Kastoori et al., 2020). While the different production technologies differ in variation rates between batches, these happen for different reasons and are still critical to be aware of and take account of in testing every new batch of antibodies.

A lot of the blame for poor reproducibility has been laid at the door of polyclonal antibodies with there being pressure in the scientific community to phase out this type of antibody completely (Bradbury and Pluckthun, 2015; Gray et al., 2020; Pillai-Kastoori et al., 2020; Taussig et al., 2018). By their nature polyclonal antibodies are a mixture of antibodies purified from the serum of an immunised animal. As it is impossible to know which epitopes an animal will develop antibodies again it is not possible to know if different batches of the same antibody will bind to similar epitopes. Indeed, it has been found that up to 50% of isolated sera containing antibodies from immunised animals fail to stain the target of interest meaning that without per batch validation there is a good chance that researchers are purchasing non-functional reagents (Saper and Sawchenko, 2003). When the probability distribution of polyclonal antibody production success is assessed, it implies that most polyclonal preparations only have one to

two clones that successfully bind to the target (Saper and Sawchenko, 2003). Polyclonal antibodies do show advantages over monoclonals by them tending to transfer more successfully between applications (Bordeaux et al., 2010; Taussig et al., 2018) and with careful design of small peptide immunogens it is possible to approach the specificity of monoclonals (Voskuil, 2017) when coupled with affinity purification of polyclonal antibodies.

Monoclonal antibodies are now preferred due to their higher reliability between batches with hybridoma cells producing the same antibody clone repeatedly. However they still have well documented issues which harm their reliability. First of which is that hybridoma lines can drift genetically meaning that they have variable antibody expression levels and changes can occur to the antibody coding sequence (Pillai-Kastoori et al., 2020) meaning that unless the antibody was sequenced then the clone is lost forever. Additionally, there are issues with hybridomas expressing supplementary immunoglobulin chains. Bradbury et al., 2018 analysed 185 hybridomas and found that 32% expressed added undesired heavy or light chains in addition to those which bound the target antigen. Due to the way that monoclonals are often generated by implantation into host animals this means that they are often contaminated with host antibodies (Bordeaux et al., 2010). These factors together potentially explain results by Spicer et al., 1994 who found that 7 out of 20 monoclonal antibodies they analysed had non-specific binding with 5 of these not even staining the target antigen.

Recombinant antibodies have been suggested as a solution to these problems by defining the genetic sequence of the antibody and then expressing the protein recombinantly in an expression system of choice (Bradbury and Pluckthun, 2015; Gray et al., 2020). This has the potential to remove any risks of additional immunoglobulin expression alongside guaranteeing batch to batch consistency. While care has to be made to make sure that the expression system chosen reproduces the glycosylation conditions of the host species for the antibody (Frenzel et al., 2013) there are no significant technical drawbacks. However it is not the case that it would be an easy switch from polyclonal to recombinant antibodies as standard practice because this would have significant cost implications. The cost increases steeply with the use of newer technologies with polyclonals costing around $1000 to develop while monoclonals cost around $10,000 and recombinants as high as $50,000 (Weller, 2016). While this might be feasible for popular targets such as loading controls this would not be cost effective for the majority of the proteome.

3.3 Lack of Traceability and Transparency

Another issue that is pervasive across research involving antibodies is a critical lack of information that would enable future researchers to independently replicate published experiments. In an analysis of 238 journal articles Bandrowski et al., 2016 were only able to specifically identify 44% of antibodies from the information given. The issue also extends from researchers to suppliers with the ubiquitous process of companies selling the same products under different product numbers with no linking information perhaps goes some way to explain how a single target such as EGFR can have over 6000 antibodies (Goodman, 2018). It was estimated in 2015 that of the 2 million available antibodies, only 250,000 - 500,000 were unique (Baker, 2015b). These companies may easily close or stop selling a product meaning that any research using their products is irreproducible to future researchers. A good example of this is three anti-EGFR antibodies with over 1000 citations sold by Santa Cruz Biotechnology which are now no longer available (Goodman, 2018). Efforts have been made to improve the traceability through online registries such as Antibodypedia (Björling and Uhlén, 2008) and the Antibody registry (Bandrowski et al., 2016). Corresponding to these issues is the often-poor level of detail provided by suppliers about key antibody parameters. Too often suppliers conceal details of the immunogen used to develop the antibody even though it hamstrings the ability of researchers to independently verify that this is fit for the purpose of their experiment. This has been strongly opposed by prominent voices such as Saper and Sawchenko, 2003 who colourfully warned that “Magic antibodies against magic antigens are not science”. While suppliers often provide some form of validation data they rarely provide enough information to enable researchers to see if the experimental conditions match their planned experiment and as a result are often meaningless. Minimum reporting standards have been proposed (Bourbeillon et al., 2010; Gilda et al., 2015; Saper, 2005) which if followed by both researchers and suppliers would help reproducibility no end.

Finally, too often suppliers do not give details of even how much antibody is contained in a vial. Combined with the fact that often the purification details are not given this means that even if a protein concentration is given that this can be meaningless when trying to relate to the activity of the antibody of interest as the result is derived from a mixture of active and inactive desired antibody amongst multiple others (if polyclonal in nature, (Weller, 2016). It would not be acceptable for suppliers to deliver mystery amounts of NaCl therefore there is no reason why this should be acceptable for antibodies either.

3.4 Researcher Training

With the realisation that it is not possible for suppliers to validate in every single experimental condition, technique and cell type it leads to the conclusion that antibody validation has to be a shared endeavour between supplier and researcher. To this end education of researchers in antibody validation will be critical to improving reproducibility. There is currently a major gap that needs overcoming with only around 40% of junior researchers validating antibodies in their own lab (Freedman et al., 2016). When probed deeper, 27% of junior researchers did not see the value in validating antibodies (Freedman et al., 2016). This lack of self-validation has been suggested to account for large amounts of irreproducibility (Sfanos et al., 2019) with there being no guarantee that because supplier validation data is promising that the antibody will work in the specific application a researcher intends. Standardisation of protocols used for validation will also be critical with one study finding that over 80% of the variance in Western blotting was due to user differences (Koller and Wätzig, 2005).

4. Validation of Antibodies

At its core validation is a simple proposal: does the antibody bind only to its target during the application a researcher wishes to use it in? However, in order to answer this simple question there are a huge range of techniques that can be used as tools for antibody validation. Ranging from simple easily accessible tests to expensive and technically challenging, it will be important for researchers to understand the benefits and pitfalls of each option and how to combine them together to provide well validated antibodies. To this end multiple validation frameworks have been proposed (Holmseth et al., 2006; Lorincz and Nusser, 2008; Pillai-Kastoori et al., 2020; Roncador et al., 2016; Uhlen et al., 2016) with varying degrees of rigour and ease of application. It is challenging for researchers to identify what elements are critical and which are nice to have but not strictly necessary. As argued by Holmseth et al., 2006 it is only ever possible to fail to find cross-reactivity not prove absolute specificity therefore there is no limit to the number of validation experiments that can be performed. It is therefore critical to find an optimal balance between the cost of validation and the risk of publishing with unvalidated antibodies.

4.1 Validation Techniques

The overarching principle for validation is that it should be application specific and in the target tissue prepared in the same way as desired for experimentation (Älgenäs et al., 2014; Holmseth et al., 2006; Lorincz and Nusser, 2008; Lund-Johansen and Browning, 2017). It is also critical to make sure that whatever technique is used that the target protein is expressed as endogenous levels (Pillai-Kastoori et al., 2020). Expression at endogenous levels is critical as when overexpressed this may hide cross-reactivity that is significant when the antibody is used in its intended application (Pillai-Kastoori et al., 2020). Additionally it has been found that some antibodies can only bind over-expressed and not endogenous concentrations of proteins, likely due to conformational changes at high concentrations (Biskup et al., 2007). It should also be clear that before any validation experiments begin it is critical to have a good understanding of the target protein, its different splice variants, subcellular and regional distribution alongside any binding partners and posttranslational modifications.

4.1.1 Traditional basic controls

Traditionally the first step that was carried out when validating antibodies was to perform a Western blot (WB) against either the immunogen or native target. While use of the immunogen reveals almost nothing other than that the antibody binds the immunogen in an isolated mixture the use of native tissue is a useful control if the intended antibody use is for immunoblotting (Bordeaux et al., 2010). In WB only bands corresponding to the target and its splice variants should be seen with any unexplained bands indicating potential cross-reactivity (Wardle and Tan, 2015). However one should be aware of the possibility of cross-reactive proteins with a similar molecular weight to the target. One control often used but no longer recommended is preadsorption of the antibody with an excess of the immunogen (Burry, 2000; Pillai-Kastoori et al., 2020; Roncador et al., 2016). This should lead to an abolition of target staining due to antibody binding site saturation but only in reality shows that the antibody binds the immunogen in conditions of excess and says nothing about it having other lower-affinity cross-reactivities that could be critical in normal tissue (Bordeaux et al., 2010; Burry, 2000). At all points it is critical to remember that success in these experiments only proves that the antibody is specific for those specific conditions in Western blots. It can however be useful as an initial screen to weed out non-binding antibodies if all caveats are kept fully in mind (Lund-Johansen and Browning, 2017).

Technique	Description	Applications	Benefits	Drawbacks
Western blot	WB against target expressed endogenously	WB	Quick and cheap	Not transferable to other techniques and possibility of cross-reactive proteins at a similar size
Pre-adsorption	WB following Ab incubation with immunogen	WB	Not recommended	Only shows that Ab binds antigen no more
Genetic	Comparison of tissue with the target artificially removed and wild type tissue	WB, IHC, ICC, FC, SA, IP, ChIP	High quality and widely accepted	Potential for genes to regulate each other and often poor availability of KO animals
Orthogonal	Comparison of Ab staining with antibody independent methods of protein quantification	WB, IHC, ICC, FC, SA	Accurate and widely accepted	RNA concentrations may not correlate with protein levels.
Tagged protein expression	Correlation of Ab staining to a tagged target and the tag (e.g. HA, GFP)	WB, IHC, ICC, FC	Easy to perform and relatively accurate	Tag may alter target conformation or subcellular localisation
Independent antibody	Correlation of staining between test Ab and independent Ab binding to a different epitope	WB, IHC, ICC, FC, SA, IP, ChIP	Extremely easy to perform and accurate	Requires availability of other antibodies
Regional/cellular distribution	Comparison of test Ab staining with known expression patterns from literature	WB, IHC, ICC	Cost efficient and useful for antibodies used in histological analysis	Assumes accuracy of previous literature and cannot rule out cross-reactivity with co-expressing proteins
Peptide microarrays	Screening of Abs against microarrays containing thousands of peptide sequences	NA	Extremely specific identification of cross-reactivity	Technically challenging and expensive. Peptides don't express conformations seen endogenously.
IP/MS	Ab used to immunoprecipitate target before being analysed by mass spectrometry	IP	Accurate quantification of cross-reactivities	Limited to IP applications only and potentially ignores physiologically relevant cross-reactants.
Targeted analysis	Comparison of related proteins using cell-line expression	WB, IHC, ICC, FC, SA, IP	Allows ruling out reactivity between closely related family members	Isolated subunits/family members may not have endogenous conformations.

Table 1. Overview of different antibody validation techniques. Applications derived from (Uhlen et al., 2016). Abbreviations: WB, western blot; IHC, immunohistochemistry; ICC, immunocytochemistry; FC: flow cytometry; SA, sandwich assays; IP, immunoprecipitation; ChIP, chromatin immunoprecipitation.

4.1.2 Genetic controls

The use of knockout tissue of cell lines has been described as the gold standard for antibody validation (Saper, 2005; Saper and Sawchenko, 2003). When antibodies are tested on tissue without the target of interest this should abolish staining compared to wild type tissue and is one of the best ways to check for a lack of off-target binding in an endogenous mixture of proteins (Lu and Bartfai, 2009; Uhlen et al., 2016). However it is important to be aware of potential issues when utilising this approach; issues that can however be pre-identified through good target knowledge. Firstly many genes regulate the expression of each other, for example Bmal1 knockouts also lead to Bmal2 expression being downregulated to such a degree it creates a functional Bmal1/Bmal2 KO meaning that any potential Bmal2 cross-reactivity cannot be tested (Roncador et al., 2016). Additionally other problems can crop up relating to truncated target proteins being expressed depending on the KO approach (Lorincz and Nusser, 2008). There are also significant practical obstacles with availability of KO tissue being challenging for many research groups, almost exclusively limited to mice and not available where the target protein is critical to cellular survival (Holmseth et al., 2006; Lorincz and Nusser, 2008). Potential ways to overcome some of these issues involve the use of conditional KO animals with the KO spatially restricted to only certain cells to ensure that would reduce issues with compensatory effects (Lorincz and Nusser, 2008). Other genetic technologies such as siRNA have also been used to manipulate protein levels in a more selective manner than knocking out the target (Uhlen et al., 2016). Finally, another widely used genetic approach is to identify cell lines that naturally lack the target protein and use these alongside an expressing cell type (Howat et al., 2014; Schuster et al., 2012). This does however come with risks that there may be differing cross-reactivities between the cell lines.

4.2.3 Orthogonal controls

Orthogonal strategies utilise comparison of antibody staining in multiple tissues or cell lines with variable target expression with an antibody independent method of measuring protein expression such as mass spectrometry or RNA abundance (Uhlen et al., 2016). When antibody staining correlates with the independent measurement then this is a good indicator of specificity within the specific application tested in. This can be carried out for both WB and immunohistochemistry applications with in-situ hybridisation being a powerful technique in histological preparations (Rhodes and Trimmer, 2006). While mass spectrometry provides a more direct measurement of protein levels, the quantification of RNA is also often used.

However it is critical to be aware that mRNA and protein expression may not necessarily correlate due to a variety of physiological phenomenon (Howat et al., 2014; Liu et al., 2016).

4.2.4 Tagged protein expression

Another powerful way of validation is to use recombinant proteins expressing a tag (e.g. Biotin or GFP) and then compare antibody staining with tag expression (Holmseth et al., 2006; Uhlen et al., 2016). If staining is specific and overlaps exclusively then this is good evidence that the antibody is specific. This technique is additionally advantageous as it allows the tissue to be prepared using the same protocols as intended experimentally (Skogs et al., 2017). However one important factor to verify is that the expression pattern of the tagged protein matches that of the endogenous protein (Roncador et al., 2016). It has previously been found that instead of an antibody being specific for the tagged protein it was instead binding to another protein regulated by the promotor for the recombinant protein (Roncador et al., 2016). Additionally it is important to be aware that tagged proteins may exhibit different interactions with other proteins compared to the endogenous target which may interfere with the interpretation of results.

4.2.5 Independent antibody

Independent antibody methods are one of the simplest methods of validation and rely on utilisation of the test antibody alongside another binding to the same target but to a different epitope. Due to the extreme unlikeliness of two independent antibodies showing identical non-specific interactions this means that if the two signals co-localise this is strong evidence for specificity (Rhodes and Trimmer, 2006; Roncador et al., 2016). This technique can be applied to multiple applications with it being easy to assess co-localisation in both immunoblots and immunohistochemistry (Burry, 2000). A pre-requisite of this technique is knowledge of the immunogens used to generate the antibodies to be sure of non-overlapping epitopes alongside the commercial availability of other antibodies. This technique is however widely used and easy to implement, for example being used to find that only 2 out of 6 anti-CB_1 antibodies tested had colocalization with an anti-HA tag antibody (Grimsey et al., 2008; Sivertsson et al., 2020).

4.2.6 Regional and cellular distribution

One simple way to assess antibody specificity is to compare antibody staining with a known pattern of distribution for a target (Goldstein et al., 2007; Pillai-Kastoori et al., 2020; Saper, 2005). The subcellular localisation can be assessed via preparation of different subcellular fractions; if the antibody only binds in fractions known to express the target then this is evidence for specificity. The same principle can also be applied to regional distributions within a tissue where a wide degree of protein targets have well understood distribution (Uhlén et al., 2015). However one thing to be cognizant of is that there is the possibility that previous literature mapping protein distribution may have relied upon antibodies that were not reliable. It should therefore be critical before employing this approach that distributions have been assessed either through other validated antibodies or a multi-pillar approach using non-antibody methods in tandem. Additionally if the target protein is co-expressed with other proteins it would not be possible to rule out cross-reactivity with these proteins either. Finally, this approach cannot be used in targets with either a poorly understood localisation or ubiquitous expression across a range of tissues and subcellular locations.

4.2.7 Peptide microarrays

A recent invention which has shown promise in the field of antibody validation is use of peptide microarrays (Forsström et al., 2014; Sjöberg et al., 2016). By immobilising up to 2.1 million short peptides to a microarray this allows the efficient high throughput screening of antibodies for non-specific interactions (Forsström et al., 2014). While this gives coverage of the entire proteome, enables easy identification of the binding epitope and undoubtably is valuable for detection of sequence based cross-reactivities it cannot replicate the diversity of protein structure and post translational modifications within a cellular environment (Bordeaux et al., 2010). This means that there are a range of cross-reactivities that these assays cannot detect. With the extreme difficulty in manufacturing these peptide arrays (none are yet commercially available) coupled with likely high cost this means that these approaches are beyond the reach of most research groups. The use of these assays also breaks the principle that antibodies should be validated in the techniques that they will be used in (Taussig et al., 2018) which creates questions as to their utility.

4.2.8 Immunoprecipitation / Mass spectrometry

Approaches using immunoprecipitation followed by mass spectrometry provide an accurate way to ensure that the antibody is binding the target of interest (Howat et al., 2014; Marcon et al., 2015; Uhlen et al., 2016). The target protein is precipitated using the antibody to test before being separated using gel electrophoresis or chromatography then fed into a mass-spectrometry analysis (Marcon et al., 2015). There are differing guidelines for when specificity has been achieved to be sufficient with Marcon et al., 2015 suggesting that target antigen should be in the top three most prevalent proteins and Uhlen et al., 2016 requiring that the top three peptides derive from the target antigen. However this process can only prove validation for immunoprecipitation purposes and additionally ignores the significance of non-specific interactions so long as they are lower than target interactions.

4.2.9 Targeted family member analysis

One final technique that can be useful where a target of interest has closely related family members or is only ever found endogenously with other family members (e.g. GluN2B subunits are only ever found with GluN1 subunits (Traynelis et al., 2010)) is expression of family members in cell lines then comparison using the immunochemical technique of choice (Pradidarcheep et al., 2009). This gives the ability to rule out subunit cross-reactivity but must be weighed up against the risk that isolated subunits will not adopt the same conformational structure as in an endogenous situation alongside the fact that the cellular environment will be very different between a typical cell line and the native tissue.

4.2 Validation guidelines

With the large array of validation techniques it is difficult for a researcher to know what to do in each situation and for each application. To this end a variety of validation protocols, guidelines and working groups have been created which are summarised in table 2. However, these different guidelines are to a degree complimentary but also have significant areas of disagreement. Perhaps the most influential proposal has been that by Uhlen et al., 2016 who suggested 5 pillars of antibody validation: genetic, orthogonal, independent antibody, tagged protein expression and IP/MS. While these pillars neglected to include useful techniques such as target distribution they were affirmed by Taussig et al., 2018 who emphasised the importance of application specific validation and user validation. These guidelines have also

been adapted into a workflow for immunohistochemistry by MacNeil et al., 2020 who additionally added the step of target localisation followed by either orthogonal, independent antibody or genetic strategies. However other immunohistochemistry guides have also been created with a mixture of different guidelines and protocols (Bordeaux et al., 2010; Howat et al., 2014; Schuster et al., 2012). While many suggestions are sensible such as using cell lines of multiple expression levels, genetic strategies, regional distribution and independent antibodies all three guides repeatedly rely on non-IHC techniques in their workflows with a heavy emphasis on western blots which are well accepted to be relatively meaningless in relation to antibodies for IHC (Bordeaux et al., 2010; Howat et al., 2014; Schuster et al., 2012; Taussig et al., 2018). Guides have also been created for chromatin immunoprecipitation (Wardle and Tan, 2015) which sensibly suggested using IP/MS and ChIP immunoblotting however also again relied on validation in other applications such as immunofluorescence or western blotting. Finally, Pillai-Kastoori et al., 2020 produced guidelines for validating antibodies for Western blotting. Suggestions included use of positive and negative control cell lines, genetic strategies and independent antibody approaches. However, again the use of alternative applications such as IP/MS was suggested to be appropriate for validation of antibodies for western blot. A mixture of other more generic guides (Roncador et al., 2016; Weller, 2018) have been produced more focussing on guiding researchers through the process of purchasing and validating an antibody in principle have been produced but do not provide specific technical directions on the best way to achieve this.

4.3 Integration of validation proposals

It is clear from the previously discussed validation guidelines that it would be beneficial to integrate update these guides together following current best practice while also emphasising the importance of understanding the target and optimising the experimental conditions for each antibody. With the understanding that validation is a shared process it will be important for researchers to assess the state of validation of an antibody then identify the steps necessary to complete in their own laboratory.

We lay out a proposal for how to validate antibodies in figures 1 to 6 based upon the following key principles:

1. All validation must start with a robust understanding of the target

2. Validation should be done in as close as possible conditions, tissue and cell type to that intended for the experimental use of the antibody.

3. Validation must be application and species specific.

4. Validation should be integrated with the 5 pillars proposed by (Uhlen et al., 2016) but mindful of different practical constraints between laboratories.

5. Validation must be replicable for an antibody to have utility.

6. Validation data needs sharing between users and suppliers using open science approaches.

4.3.1 Pre application specific steps

At the start of the validation process for any antibody the first steps must be to extensively research the target. Specific focusses should include its regional, cellular and subcellular localisation, any splice variants, closely related family proteins, binding partners and the target's known physiological role. The next step should be to perform alignment searches between the immunogen (or epitope if available) and all proteins in the target species. If there is significant homology with proteins known to also express in cells or tissue that the target also expresses in then it is likely futile to carry on further in the validation process.

While we have repeatedly emphasised the importance of application specific performance, we think it is a worthwhile initial step in the validation process to either perform a western blot or ELISA depending on whether the target of interest is denatured or not in the experimental conditions desired. While this does bear the risk of missing antibodies that may be useful in other applications, it acts as a coarse filter to weed out any antibodies that clearly do not bind their target of interest or have obvious cross-reactivities. However due to this then it clearly

depends upon the validator's risk tolerance and a judgement call can be made to proceed even with a failed WB or ELISA experiment.

Before carrying out any more experiments it is critical to identify cell lines and tissue samples that can be used for application specific testing. This has been designed in a pragmatic way appreciating that not all researchers will have access to knockout animals or mass spectrometry facilities. Once the available validation resources are identified then this will inform the validation strategy for each application.

4.3.2 Application specific protocols

The principles behind validation for each application are unified with it being first important to use the same protocol and conditions for validation as aimed for in the intended research experiments. The importance of optimising the experimental conditions is also highlighted here to find the best concentration of antibody, antigen retrieval, buffers and processing steps for signal / noise ratio. For each application a choice of techniques are provided with additional steps if the target protein has closely related expression partners that cannot be controlled for in the other experimental approaches.

4.3.3 Final stages

Following application specific validation the importance of optimising conditions is again highlighted to incorporate both the fact that this late in the process the researcher will be driving the process and need to independently optimise the process using the supplier guidelines as a starting point. Additionally insights learnt from the validation experiments can be included to provide a more accurate optimisation.

The final steps of validation concern making sure that the results are replicable before then disseminating the validation data to the scientific community. Replicability could easily be assessed by re-running one of the validation experiments a few times to check that the results are consistent. There are multiple good platforms to enable researchers to share their validation data (e.g., open science framework (Foster and Deardorff, 2017)) which can then be linked to future publications or pre-prints. Once this process has been complete then we feel confident that an antibody has been proven to be fit for research purposes to a high enough standard. It

is however important for researchers to think carefully about the level of validation they require. The larger, more complex and more expensive study may find it prudent to complete more than one of each validation technique to give additional confidence before embarking on a large experimental program. To finally re-iterate, once an antibody has been through this process for one application if another use is proposed then the validation process needs to go back to before application specific validation.

5. Conclusions

Antibodies are one of the most ubiquitously used yet poorly understood research tools leading to high levels of irreproducibility in antibody research and ultimately contributing to the overall reproducibility crisis. While there have been many validation techniques and guides proposed none have drawn the most recent evidence together into a comprehensive workflow for antibody validation. We have created a validation strategy for antibodies based upon both the principles of application specificity and Uhlen's 5 pillars of validation (Uhlen et al., 2016) while emphasising the joint responsibility of antibody validation between researchers and suppliers. By increasing the level of antibody validation we hope to lead to better research outcomes, reduced animal wastage and an improved trust in the quality of antibody based life science research.

Proposal		Bordeaux et al., 2010	Schuster et al., 2012	Howat et al., 2014	Wardle and Tan, 2015	Roncador et al., 2016	Uhlen et al., 2016	Taussig et al., 2018	Weller, 2018	MacNeil et al., 2020	Pillai-Kastoori et al., 2020
Applications for		IHC//IF	IHC(P)	IHC	ChIP	All	All	All	All	IHC	WB
Techniques suggested as appropriate	Western blot#	Y	Y	Y	Y	Y	N	N	Y	N	Y
	Pre-adsorption	N	Y	N	N	N	N	N	N	N	N
	Genetic	Y	Y	Y	Y	Y	Y	Y	N	Y	Y
	Orthogonal	Y	N	N	N	N	Y	Y	N	Y	Y
	Tagged protein expression	N	N	N	Y	N	Y	Y	N	N	N
	Independent antibody	N	N	Y	Y	Y	Y	Y	N	Y	N
	Regional/cellular distribution	Y	Y	N	Y	Y	N	N	N	Y	N
	Peptide microarrays	N	N	N	N	N	N	N	N	N	N
	IP/MS	N	N	Y	Y	N	Y	Y	Y	N	N
	Targeted analysis	N	N	N	Y	Y	N	N	N	N	N
Replicability required?		Y	Y	N	Y	Y	N	N	N	Y	Y
Reporting standards required?		N	N	Y	Y	Y	Y	Y	Y	N	Y
Target research required?		N	N	Y	N	Y	N	N	Y	N	N
Impact	Citations	396	15	60	9	31	234	19	14	6	10
	Citations/year	36	1.7	8.6	1.5	6.2	46.8	6.3	4.7	6	10

Table 2. Summary of selected proposals for antibody validation. Proposals were marked as to whether they suggested (Y) or not (N) the use of each validation technique in addition to whether they required replicability, reporting standards or specific target research as part of the validation process. Citations were taken from Scopus on 02/06/21. #: WB as a method to prove specificity.

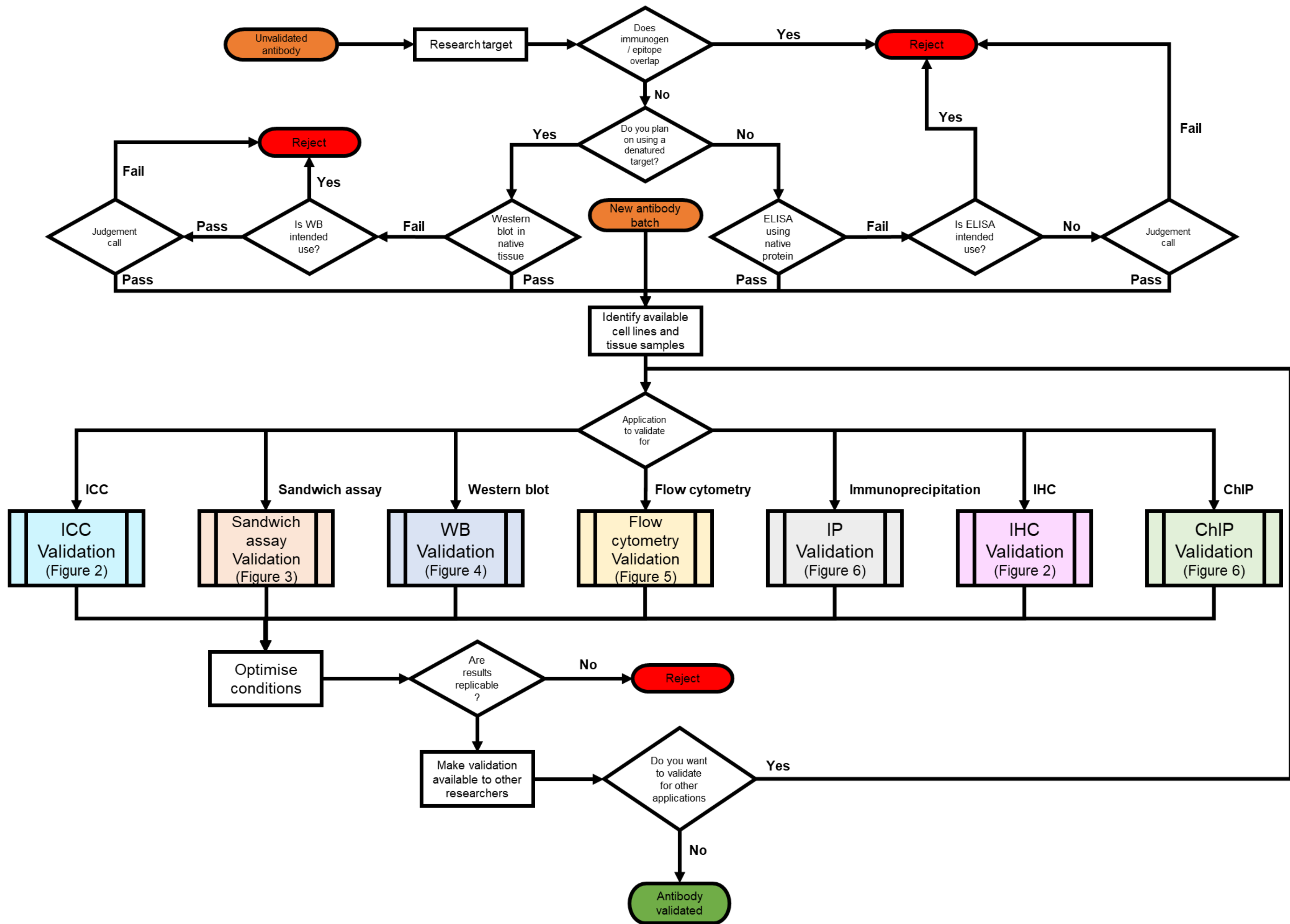

Figure 1. Flowchart of overall suggested antibody validation process. Methodology specific flowcharts are shown in later figures.

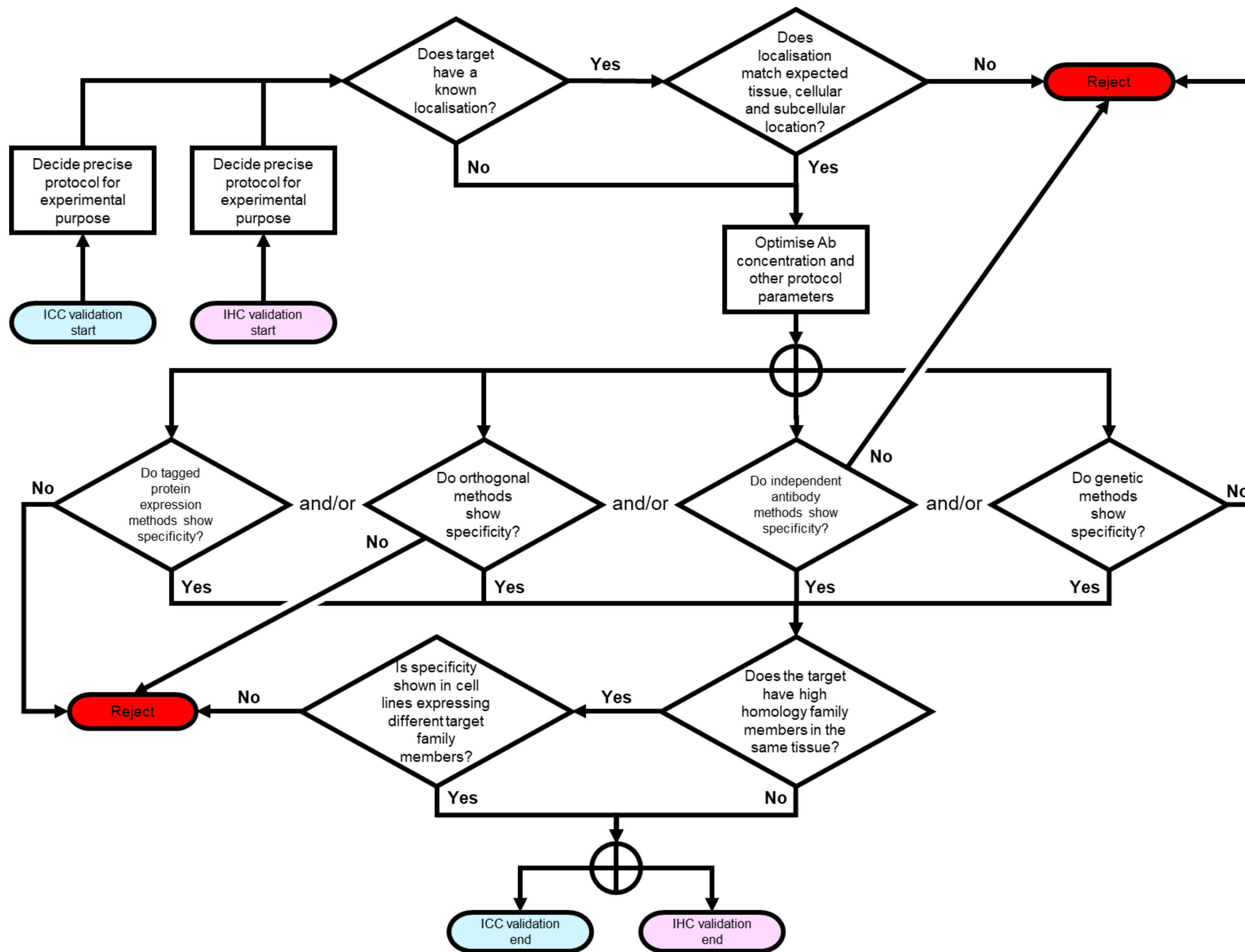

Figure 2. Flowchart for validation of antibodies for immunohistochemistry and immunocytochemistry. It is critical to remember that although the techniques are similar validation for IHC does not imply validation for ICC and vice-versa. Choice of validation strategy should depend upon both practical constraints and what closest aligns with the experimental goals. At all points validation should only use either IHC or ICC techniques as to be validated for. Once completed return to figure 1. Adapted from (MacNeil et al., 2020).

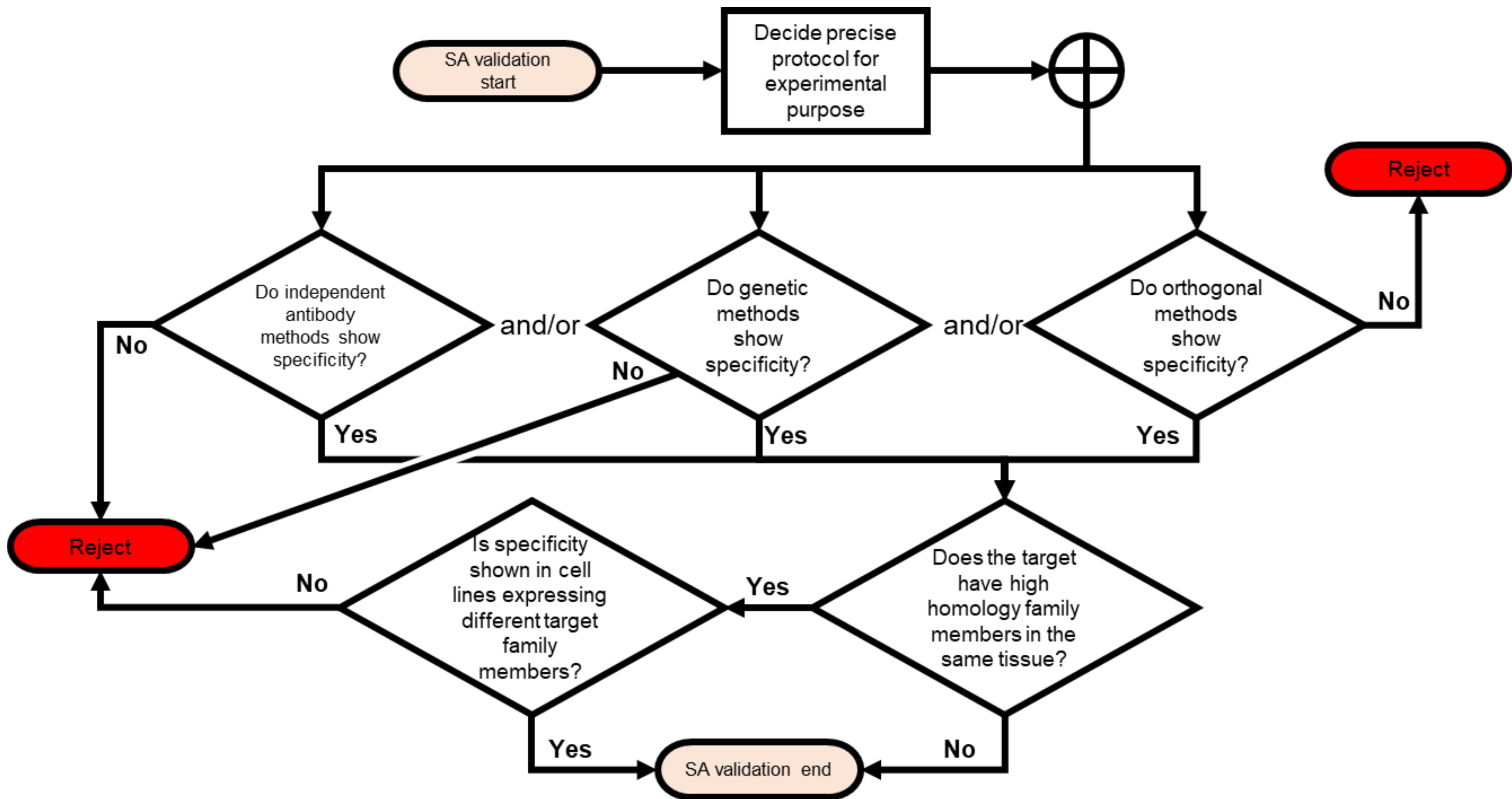

Figure 3. Flowchart for the validation of antibodies for sandwich assays. Choice of validation strategy should depend upon both practical constraints and what closest aligns with the experimental goals. At all points validation should only use the sandwich assay intended for experimental use. Once completed return to figure 1.

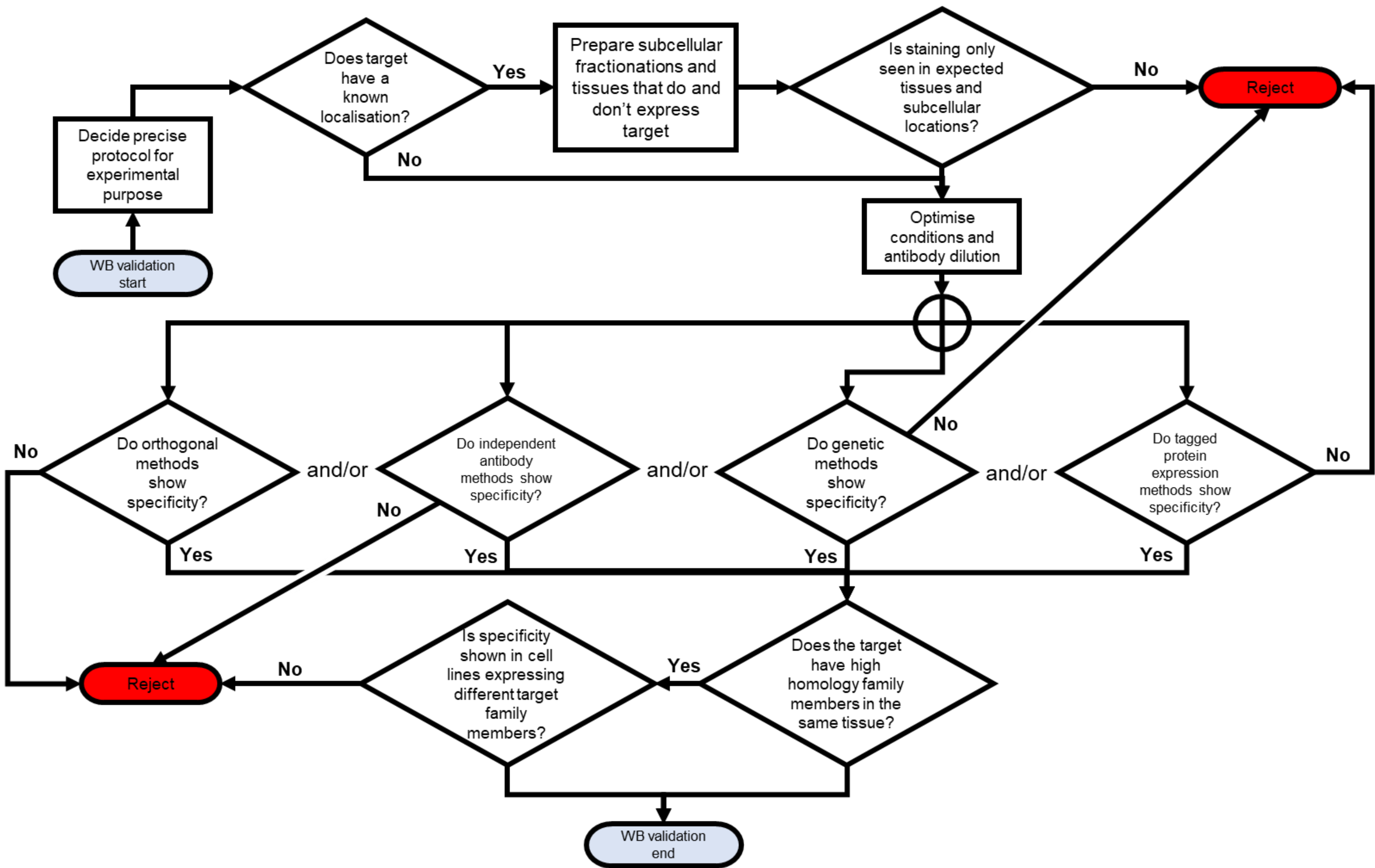

Figure 4. Flowchart for the validation of antibodies for Western blotting. Choice of validation strategy should depend upon both practical constraints and what closest aligns with the experimental goals. At all points validation should only use immunoblotting techniques. Once completed return to figure 1.

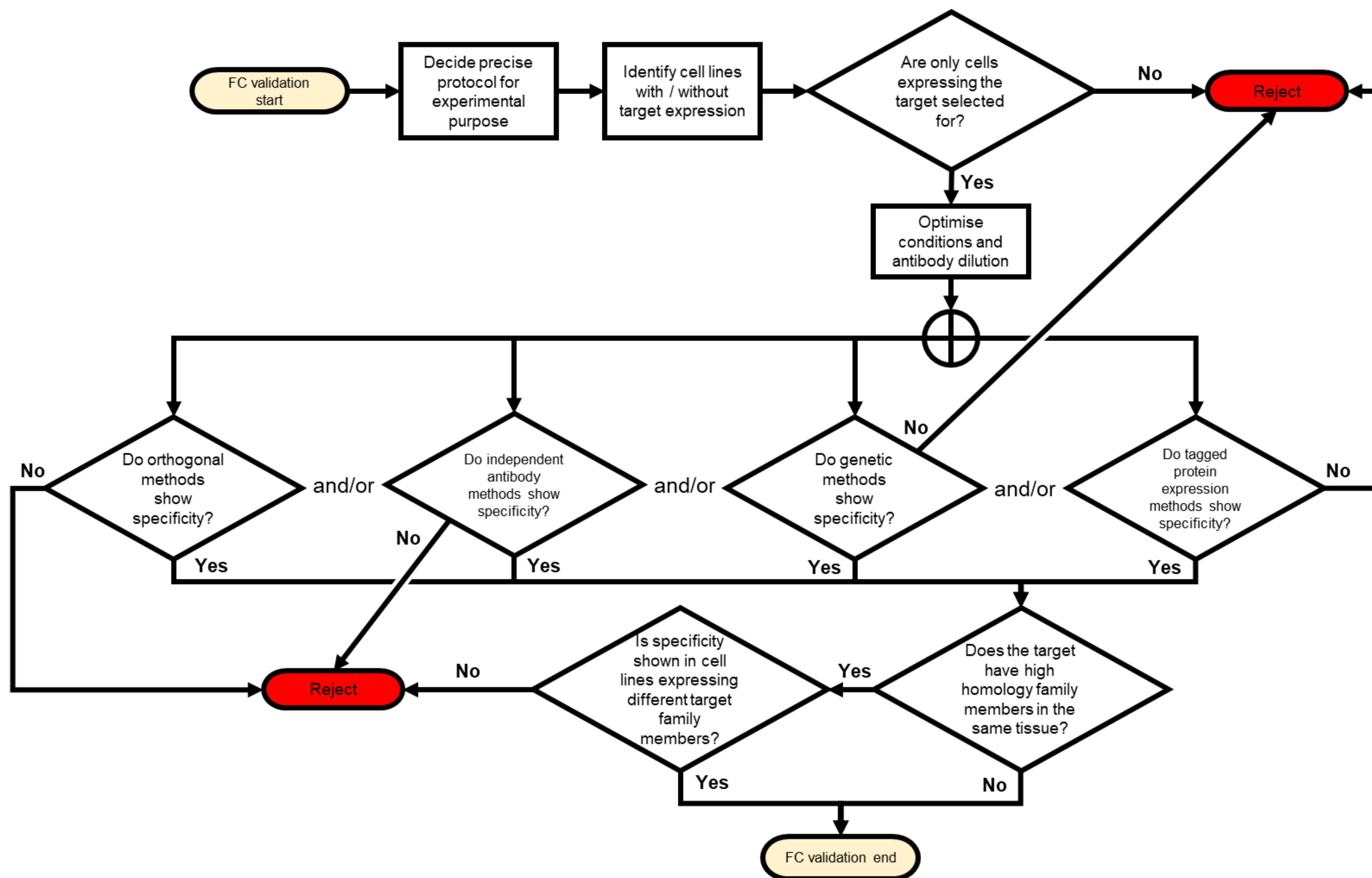

Figure 5. Flowchart for the validation of antibodies for Flow cytometry. Choice of validation strategy should depend upon both practical constraints and what closest aligns with the experimental goals. At all points validation should only use flow cytometry techniques. Once completed return to figure 1.

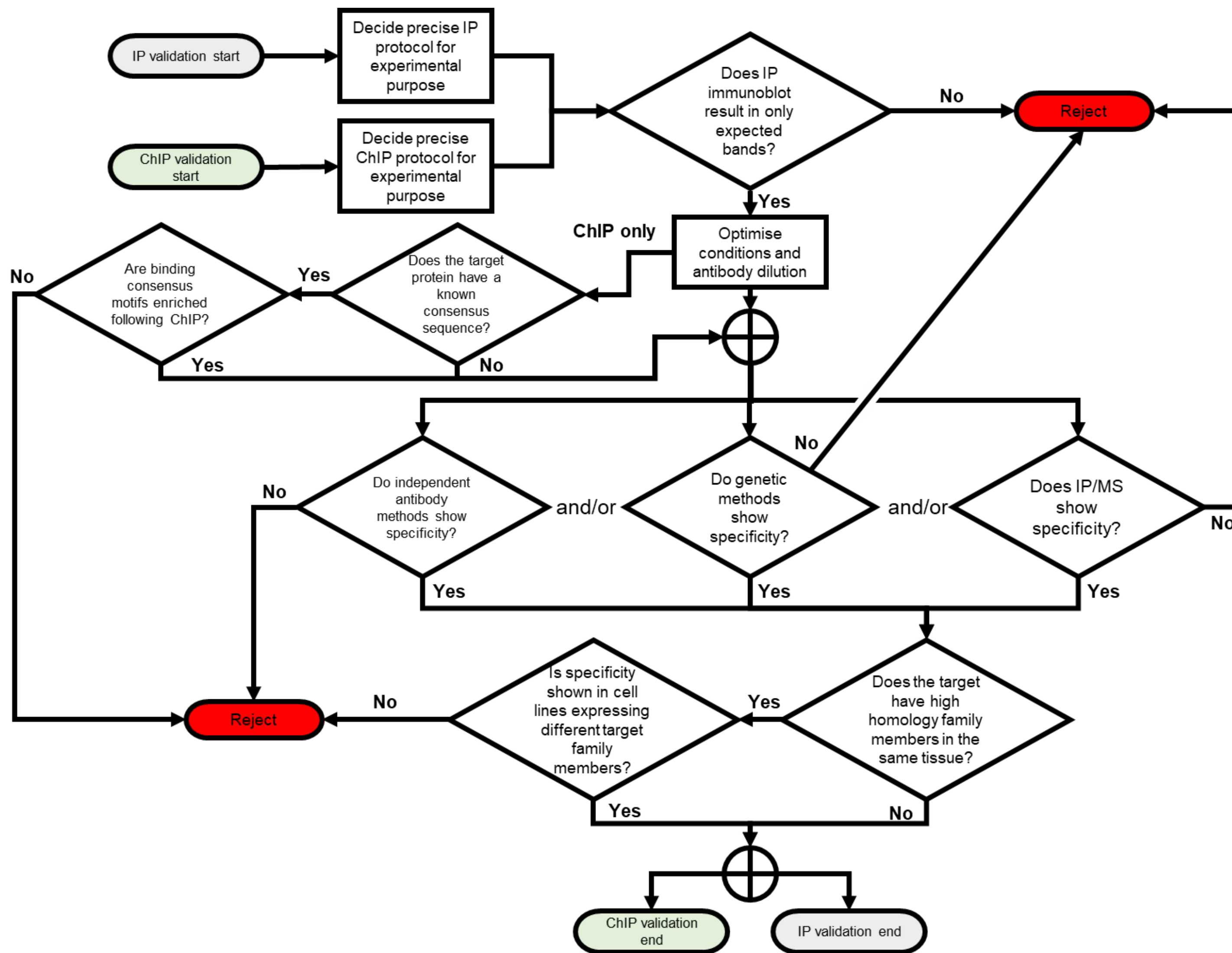

Figure 6. Flowchart for the validation of antibodies for Immunoprecipitation and chromatin immunoprecipitation. It is critical to remember that although the techniques are similar validation for IP does not imply validation for ChIP and vice-versa. Choice of validation strategy should depend upon both practical constraints and what closest aligns with the experimental goals. At all points validation should only use either IP or ChIP techniques as to be validated for. Once completed return to figure 1.

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