# THERAPEUTIC MONOCLONAL ANTIBODIES FOR HUMAN USE

Monoclonal antibodies for human use are preparations of an immunoglobulin or a fragment of an immunoglobulin, for example, F(ab')2, with defined specificity, produced by a single clone of cells. They may be conjugated to other substances, including for radiolabelling. They can be obtained from immortalized B lymphocytes that are cloned and expanded as continuous cell lines or from rDNA-engineered cell lines.

Currently available rDNA-engineered antibodies include the following:

*Chimeric monoclonal antibodies:* The variable heavy and light chain domains of a human antibody are replaced by those of a non-human species, which possess the desired antigenic specificity.

*Humanised monoclonal antibodies:* The 3 short hyper variable sequences (the complementarity determining regions) of non-human variable domains for each chain are engineered into the variable domain framework of a human antibody; other sequence changes may be made to improve antigen binding.

*Recombinant human monoclonal antibodies:*The variable heavy and light chain domains of a human antibody are combined with the constant region of a human antibody.

This monograph applies to monoclonal antibodies for therapeutic usages. It does not apply to monoclonal antibodies used as reagents in the manufacture of medicinal products, in vivo diagnostics or in prophylactic usage for which requirements are decided by the competent authority.

This monograph is intended to provide guidance on monoclonal antibody development and manufacturing, covers following aspects:

1. General guidance
2. Product Development
   2.1 Cloning and Cell Line Development
   2.2 Process Development
   2.3 Analytical Method Development
   2.4 Process Characterization
   2.5 Analytical Characterization
3. Non-clinical and Clinical Studies

4. Manufacturing
   4.1 At-scale manufacturing and Process Validation
   4.2 Lot release testing
   4.3 Shelf-Life and storage
   4.4 Reference Standard
   4.5 Stability

**1. General guidance:**

For nomenclature of monoclonal antibodies International Nonproprietary Names (INN) for monoclonal antibodies is followed (Refer, WHO guideline)

2. **Product Development:**

1.1 Cloning and Cell Line Development:

In order to produce a recombinant monoclonal antibody, the protein of interest is sequenced and the same is cloned into desired vector, then a preferred specific clone is developed.

*SOURCE CELLS*

Source cells include fusion partners, lymphocytes, myeloma cells, feeder cells and host cells for the expression of the recombinant monoclonal antibody.

The origin and characteristics of the parental cell are documented, including information on the health of the donors, and on the fusion partner used (for example, myeloma cell line, human lymphoblastoid B-cell line).

Wherever possible, source cells undergo suitable screening for extraneous agents and endogenous agents. The choice of viruses for the tests is dependent on the species and tissue of origin.

*1.2 Cell lines producing the monoclonal antibody.*

The suitability of the cell line producing the monoclonal antibody is demonstrated bydocumentation on the history of the cell line including description of the cell fusion, immortalisation or transfection and cloning procedure; characterisation of the cell line using techniques such as gene copy number, barcoding etc.(for example, phenotype, isoenzyme analysis etc.,); characterisation of relevant features of the antibody; stability of antibody secretion with respect to the characteristics of the antibody and level of expression and glycosylation up to or beyond the population doubling level or generation number used for

routine production; for recombinant DNA products, stability of the host/vector genetic and phenotypic characteristics up to or beyond the population doubling level or generation number used for routine production.

**Host cell line characterization**

The host cell line engineered to express the desired antibody molecule must be subjected to appropriate analyses at genomic level to establish stable integration of the foreign gene. Typically, this analysis should be initiated the moment lead clone is identified. Appropriate analytical tools to be used for this purpose.

*Cell banks.*

The master cell bank is a homogeneous suspension of the cell line producing the monoclonal antibody, distributed in equal volumes in a single operation into individual containers for storage. A working cell bank is a homogeneous suspension of the cell material derived from the master cell bank at a finite passage level, distributed in equal volumes in a single operation into individual containers for storage. Post-production cells are cells cultured up to or beyond the population doubling level or generation number used for routine production.

The following tests are performed on the master cell bank: viability, identity, (bacteria, fungiand mycoplasma), characterisation of the monoclonal antibody produced. Non-endogenous viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination are tested using a suitable range of in vitro tests.

The following tests are performed on the working cell bank: viability, identity, absence of microbes such as bacteria, fungi, and mycoplasma. Adventitious viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. For the first working cell bank, these tests are performed on post-production cells, generated from that working cell bank; for working cell banks subsequent to the first working cell bank, a single *in vitro* and *in vivo* test can be done either directly on the working cell bank or on the post-production cells.

For the master cell bank and working cell bank, tests for specific viruses are carried out when potentially contaminated biological material has been used during preparation of the

cell banks, taking into account the species of origin of this material. This may not be necessary when this material is inactivated using validated procedures.

The following tests are performed on the post-production cells: sterility (bacteria, fungi and mycoplasma). Virus tests are performed on cells or cell culture supernatants. For this, non-endogenous viral contamination is tested with a suitable range of *in vivo* and *in vitro* tests. Retrovirus and other endogenous viral contamination is tested using a suitable range of in vitro tests.

1.3 Process Development:

In order to achieve product quality and titer as per expectations, upstream process in terms media, feed, process parameters and as needed, bioreactor designing to be completed. Further, based on product quality downstream process, where purification is achieved to be developed. A typical monoclonal antibody process is of three chromatography steps where capture, polish and further purification steps are performed.

**CULTURE AND HARVEST**

**Production at finite passage level (single harvest)**. Cells are cultivated up to a defined maximum number of passages or population doublings, or up to a fixed harvest time (in accordance with the stability of the cell line). Product is harvested in a single operation.

**Continuous-culture production (multiple harvest)**. Cells are continuously cultivated for a defined period (in accordance with the stability of the system and production consistency). Monitoring is necessary throughout the life of the culture; the required frequency and type of monitoring will depend on the nature of the production system.

Each harvest is tested for antibody content, bioburden, endotoxin and mycoplasmas. General or specific tests for adventitious viruses are carried out at a suitable stage depending on the nature of the manufacturing process and the materials used. For processes using production at finite passage level (single harvest), at least 3 harvests are tested for adventitious viruses using a suitable range of *in vitro* methods.

The acceptance criteria for harvests for further processing are clearly defined and linked to the schedule of monitoring applied. If any adventitious viruses are detected, the process is carefully investigated to determine the cause of the contamination and the harvest is not further processed. Harvests in which an endogenous virus has been detected are not used for purification unless an appropriate action plan has been defined to prevent transmission of infectious agents.

**PURIFICATION**

Harvests or intermediate pools may be pooled before further processing. The purification process includes steps that remove and/or inactivate non-enveloped and enveloped viruses. A validated purification process, for which removal and/or inactivation of infectious agents and removal of product- and process-related impurities has been demonstrated, is used. Defined steps of the process lead to a purified monoclonal antibody (active substance) of consistent quality and biological activity.

Process Characterization:

Once the process is developed, in order to understand the capability of the process, a detailed process characterization is necessary. This will be important to understand the manufacturing process and to address various issues that may arise during routine commercial processing. Also, these studies will provide insight into design of various attributes used during development.

1.4 Analytical Methods Development:

Analytical methods to be developed that are capable of establishing sufficient information of product quality attributes. Methods required for characterization of product may be extensive than that of methods required for releasing a product from lot to lot. Analytical methods used for product characterization should minimally established suitability, accuracy of the method whereas methods used for in routine lot release should be validated prior to clinical studies.

Wherever possible, compendial procedures should be adapted after establishing suitability. Particular attention to the methods suitability for usage should be shown in safety (microbial) related methods.

**PRODUCT CHARACTERIZATION**

Monoclonal antibody drug molecule should be characterized at various stages of development using combinations of orthogonal techniques to ascertain and ensure purity, physico-chemical characteristics, conformational integrity including quaternary structure and biological activity.

The product is characterized to obtain adequate information including: structural integrity, isotype, amino-acid sequence, secondary structure, carbohydrate moiety, disulphide bridges,

conformation, specificity, affinity, specific biological activity and heterogeneity (characterisation of isoforms). A battery of suitable analytical techniques used includes chemical, physical, immunochemical and biological tests (for example, peptide mapping, N- and C- terminal amino-acid sequencing, amino acid composition, mass spectrometry, chromatographic, electrophoretic and spectroscopic techniques). For those products that are modified by fragmentation or conjugation, the influence of the method used on the antibody needs to be characterized.

**Analytical characterization:**

Various analytical tools are to be used to characterize the products, some of them are listed below:

**(a) Purity and identity of mAb product.** Purity analysis is typically done by SDS-PAGE; this can be considered the first step of physio-chemical characterization, in this technique, purity of heavy and light chain bands under reducing conditions as well as band pattern under non- reducing conditions is analyzed. SDS-PAGE is followed by western blot analysis to establish the identity of the observed bands using primary antibodies specific to human IgG. Further, impurity analysis is done by HPLC based size exclusion chromatography (SEC); this technique is very useful in detecting presence of dimers and aggregates in the purified product. The analysis is done on the basis of differences in time of elution of contaminating products from the main peak. Other methods like Capillary Electrophoresis etc. can be employed where they are relevant.

**(b) Sequence and structural integrity.** Primary sequence of antibody heavy and light chains is recognized by N terminal sequencing using Edman degradation method. In this technique, though sequence of only first ten amino acids can be properly identified, it gives an indication that the protein has been accurately translated in frame as per the gene design. N terminal sequencing coupled with amino acid analysis, in which acid hydrolysis of individual chains followed by capillary electrophoresis (CE)-HPLC is performed, helps in initial identification of the protein. Primary sequence can be further obtained and verified by `de novo' sequencing using mass spectrometry based techniques like ESI/MALDI-TOF/TOF etc. Secondary structure analysis can be done by circular dichroism (Far-UV CD-spectroscopy) for alpha and beta content analysis. Spectrofluorometric analysis based on

fluorescence of hydrophobic amino acid residues is used for detecting improper folding in the recombinant protein.

**(c) Characterization of Charge micro-variants.** Charge related heterogeneity in monoclonal antibodies is examined by IE-HPLC or isoelectric focusing (IEF). Since, most human IgGs have a basic isoelectric point, cation exchange (CEX) - HPLC is typically used. Variants that elute before the main peak are less positively charged and are referred as acidic, whereas, species that elute after the main peak are termed as basic. In IEF, where a mixture of ampholytes is used to establish a pH gradient, antibody molecules migrate to pH equivalent to their pI. As compared to CEX, IEF is generally used for monitoring of variants rather than their characterization, as variants with different surface-charge distribution can merge together during separation due to similar pI.

All human IgGs contain a lysine residue at C-terminus of heavy chain, lysine and arginine residues at carboxyl terminus are removed by cellular enzymes called carboxy peptidases. Usually the removal is incomplete and two variants with lysine on either one or both heavy chains clipping can be expected. Loss of lysine makes the molecule acidic as there is loss of an amino group and can be detected by CEX, also loss of single lysine would result in loss of mass equivalent to 128 Da which can be examined by mass spectrometry. Similarly, C-terminal amidation results in loss of terminal carboxyl group making molecule more basic, amidation leads to a loss of mass by 1 Da.

Another familiar micro-variant involves formation of N-terminal pyroglutamate. In this variant, N- terminal glutamine (Q) or glutamic acid (E) can undergo cyclization forming a pyroglutamic acid, here an amine is converted to neutral amide and Q /E cyclization is detected as acidic variants on CEX. Conversion of N-terminal Q is almost 99%, whereas E conversion is only 3 %. The mechanism is not fully understood for this conversion, although cellular glutaminylcyclase has been implicated in this reaction. Pyroglutamate formation has been observed with elevated temperatures and can be associated with process or storage conditions. Pyroglutamate residue interferes with N-terminal sequencing of heavy chain and N-terminal blocking leads to loss of 17 -18 Da mass.

Oxidation is commonly observed in proteins, and in monoclonal antibodies two methionines located in Fc domain are prone to oxidation. This process is seen in formulated products following incubation at elevated temperatures. Full length antibodies with oxidation on both methionines can be eluted as basic shoulders by CEX.

A very common charge variant is generated by deamidation of asparagine (N) and isomerization of aspartate (D) residues. Usually N or D residues, in complementarity determining regions (CDRs) which are surface exposed regions, are prone to these modifications, and are the main sources of degradation under mild conditions. Both reactions proceed through a common cyclic imide intermediate-succinimide, which hydrolyzes into a mixture of isoaspartate and aspartate in 3:1 ratio. Usually N and D followed by glycine in a sequence are prone to this modification. Deamidated species elutes as acidic species in CEX, whereas, succinimide intermediate and isoaspartate variant elute as basic forms. Deamidation leads to loss of 1 Da mass detected by MS, whereas, succinimide intermediate shows loss of mass by 17 Da. Asparagine deamidation and aspartate isomerization can occur throughout the entire life time of the antibody, including in vivo after administration.

Chemicals present in culture media or in formulation can react with antibodies and can lead to formation of chemical adduct. Non-enzymatic addition of sugars (glycation) is commonly observed in monoclonal antibodies. Glycation occurs by reaction of reducing sugars like glucose and lactose (in culture media) with amine group of lysine or the primary amine in N-terminus. Similar reaction can also occur if a sugar is present in the formulation buffer. Glycation can be observed as an acidic shift in IEF and also detected by mass spectrometry as gain of mass by 162 Da.

Any of the variants that are shown to be present different from that of reference standard either in terms of new species or quantity, it is necessary to understand the impact of these variants on product activity in terms of potency.

**(d) N-linked Glycosylation.** In human IgG, asparagine residue 297 in Fc region is the site for N-linked glycosylation. The most common glycoforms G0F, G1F and G2F are bi-antennary blocks of 8-11 carbohydrate residues comprising of fucose `F' (n=1), N-acetylglucosamine `GlcNac' (n=2), mannose (N=3), galactose`G' (N= 0-2) residues, respectively. Individual moieties of glycan chain are neutral carbohydrates, except sialic acid residues which can be added to terminal galactose residues. In antibodies expressed in human and in the CHO cell lines, the level of sialylation is low. Various glycoforms can be detected efficiently by mass spectrometry after removal of entire glycan chain by peptide N-glycosidase F (PNGase F). Typically, a G0F form has a mass of 1.4 KDa with each additional hexose adding 162 Da. The glycosylation profile needs to be monitored critically

because it can influence the binding of the Fc fragment to receptors that mediate ADCC and CDC. Further, glycosylation profile is prone to changes depending on cell line and fermentation conditions.

Variations in the content/ quantity of glycosylation from that of reference product may be acceptable, provided there is no impact on the product activity.

In addition to the N-glycosylation, there may be specific molecules that have O-glycosylation present on Serine/ Threonine amino acids of monoclonal antibody. Sufficient characterization of the same is required.

**Biological characterization**

ELISA is a very important technique for establishing the binding specificity of antibody to its target antigen. However, strength of binding i.e. affinity can be measured only by surface plasmon resonance method. Availability of purified target antigen in correctly folded form is an absolute requirement for both the techniques and obtaining targets which are cell surface receptors in pure form is a challenging task. Binding to cell surface receptors can be established by FACS analysis, which is another reliable technique complementing ELISA.*In vitro* cell based assays give convincing evidence for efficacy of the monoclonal antibody against their targets, and must be standardized to ensure its reproducibility and robustness.

***3. Non-clinical and Clinical Studies:***

As part of the development studies, once the product of interest is developed various non-clinical and clinical studies to be conducted. These studies are to provide study product toxicity in animals, clearance rate in humans and immunogenicity/ safety/ efficacy in patients. Specific requirements of these studies are prescribed in relevant guidelines.

***4. Manufacturing:***

Production is based on a seed-lot system using a master cell bank and, if applicable, a working cell bank derived from the cloned cells. The production process of the cell bank is executed in a manner to ensure safety during development studies, to prevent transmission of infectious agents by the final product. All biological materials and cells used in the production are characterized and are in compliance withthe limits prescribed by the

competent authorityto minimize the risk of transmitting animal spongiformencephalopathy agents via human and veterinary medicinal products. Where monoclonal antibodies for human use are manufactured using materials of human or animal origin,it should comply with viral safety limits, as prescribed by competent authority. Where an immunogen is used it is characterized and the method of immunization is documented.

While planning for product commercialization, process development/characterization, process validation & process monitoring is of vital importance. A robust system needs to be in place to ensure process consistency over the entire lifecycle of the product.

**SCALE UP**

The scale-up of processes from bench to commercial scale is challenging because of uncertainties about the physiochemical environment experienced by the cells at large scale. However, there are some features of mAb that make the scale-up task significantly easier than for other proteins like, firstly, mAbs are generally very stable molecules that can tolerate relatively harsh treatments, e.g. extremes of pH, shear, etc and secondly, a highly specific affinity ligand, protein A, is available that binds to most classes of therapeutically relevant human antibodies. Process platforms in both upstream cell culture and downstream purification have become widely established in industry for production of large quantities of pharmaceutical-grade mAbs at moderate costs. Large scale production of mAb utilizes mammalian production systems followed by cell removal and purification through sequential chromatographic and membrane filtration steps, to consistently reduce product-(e.g. protein variants) and non-product related variants (e.g. host cell proteins) impurities to acceptable levels.

**PROCESS VALIDATION**

During development studies, the production method is validated for the following aspects:

- consistency of the production process including cell-culture/fermentation, purification and, where applicable, fragmentation method;
- removal or inactivation of infectious agents;
- adequate removal of product- and process-related impurities (for example, host-cell protein and DNA, protein A, antibiotics, cell-culture components);
- specificity and biological activity of the monoclonal antibody;

- absence of non-endotoxin pyrogens, where applicable;
- reusability of purification components (for example, column material), limits or acceptance criteria being set as a function of the validation;
- methods used for conjugation, where applicable.

**Process intermediates**.

Where process intermediates are stored, an expiry date or a storage period justified by stability data is established for each.

**ACTIVE SUBSTANCE**

The test programme for the active substance depends on the validation of the process, on demonstration of consistency and on the expected level of product- and process-related impurities. The active substance is tested for appearance, identity, bioburden and bacterial endotoxins, product-related substances, product- and process-related impurities including tests for host-cell-derived proteins and host-cell- and vector-derived DNA, as well as structural integrity, protein content and biological activity by suitable analytical methods, comparing with the reference preparation where necessary. When the active substance is a conjugated or transformed antibody, appropriate tests must be performed before and after the antibody conjugation/modification.If storage of intermediates is intended, adequate stability of these preparations and its impact on quality or shelf-life of the finished product are evaluated.

**FINAL BULK**

One or more batches of active substance may be combined to produce the final bulk. Suitable stabilizers and other excipients may be added during preparation of the final bulk.The final bulk must be stored under validated conditions with respect to bioburden and stability.

**FINAL LOT**

The final bulk is sterile-filtered and distributed under aseptic conditions into sterile containers, which may subsequently be freeze-dried.

As part of the in-process control each container (vial, syringe or ampoule) is inspected after filling to eliminate containers that contain visible particles. During development of the

product it must be demonstrated that either the process will not generate visible proteinaceous particles in the final lot or such particles are reduced to a low level as justified and authorised.

IDENTIFICATION

The identity is established by suitable validated methods comparing the product with the reference standard, where appropriate. Establishment of identity needs to be shown by more than one method, for ex., peptide mapping as finger print, western blot, biological assay etc.,

TESTS

**Appearance**. Liquid or reconstituted freeze-dried preparations comply with the limits approved for the particular product with regard to degree of opalescence (2.2.1) and degree of coloration (2.2.2). They are without visible particles, unless otherwise justified and authorised.

**Solubility**. Freeze-dried preparations dissolve completely in the prescribed volume of reconstituting liquid, within a defined time, as approved for the particular product.

**pH** (2.2.3). It complies with the limits approved for the particular product.

**Osmolality** (2.2.35): minimum 240 mosmol/kg, unless otherwise justified and authorised.

**Extractable volume** (2.9.17). It complies with the test for extractable volume.

**Total protein** (2.5.33). It complies with the limits approved for the particular product.

**Molecular-size distribution**. Molecular-size distribution is determined by a suitable method, for example size-exclusion chromatography (2.2.30). It complies with the limits approved for the particular product.

**Molecular identity and structural integrity**. Depending on the nature of the monoclonal antibody, its microheterogeneity and isoforms, a number of different tests can be used to demonstrate molecular identity and structural integrity. These tests may include peptide mapping, isoelectric focusing, ion-exchange chromatography, hydrophobic interaction chromatography, oligosaccharide mapping, monosaccharide content and mass spectrometry.

**Purity**. Tests for process- and product-related impurities are carried out by suitable validated methods. Provided that tests for process-related impurities have been carried out on the active substance or on the final bulk with satisfactory results, they may be omitted on the final lot.

**Stabiliser**. Where applicable, it complies with the limits approved for the particular product.

**Water** (2.5.12). Freeze-dried products comply with the limits approved for the particular product.

**Sterility**(2.6.1). It complies with the test for sterility.

**Bacterial endotoxins** (2.6.14).It complies with the limits approved for the particular product.
**Tests applied to modified antibodies**. Suitable tests are carried out depending on the type of modification.

BIOLOGICAL ASSAY

Carry out a suitable biological assay compared to the reference preparation. Design of the assay and calculation of the results are made according to the usual principles (for example, 5.3).

**Reference preparation**

A reference standard obtained from source recognized by national regulatory authority (NRA). If recognized reference standard is not available an "in-house reference preparation" should be established. A properly qualified in-house reference standard for mAbs with known characteristics, specificity, and potency, and that is stored under appropriate conditions and periodically tested to ensure its integrity, should be used for lot-to-lot comparisons. A batch shown to be stable and shown to be suitable in clinical trials, or a batch representative thereof, is used can be as a reference preparation for the identification, tests and assay. The reference preparation is appropriately characterized as defined under Product characterization, except that it is not necessary to examine cross-reactivity for each batch of reference preparation.

**Definition of a batch**. Definition of a batch is required throughout the process.

STORAGE

As stated on the label.

*Expiry date*. The expiry date is calculated from the date of sterile filtration, the date of filling (for liquid preparations) or the date of freeze-drying (where applicable) or as established based on stability data.

LABELLING

The label states:

– amount of monoclonal antibody per millilitre, where applicable;

– as applicable, total quantity of protein per container;

For liquid preparations:

- volume of the preparation in the container

For freeze-dried preparations:

- Name and the volume of the reconstitution liquid to be added, where applicable;
- Period of time within which the monoclonal antibody is to be used after reconstitution;
- Dilution to be made before use of the product, where applicable.

**STABILITY**

The development of a proper long term stability program is critical to the successful development of a commercial product and hence adequate stability studies form an essential part of the product development process and they should be designed to help setting up the shelf life of product. The stability testing protocol should include all stability indicating parameters which provide assurance that change in identity, purity and potency of the product will be detected. Some important tests such as molecular size, potency, residual moisture, protein content, preservative can be considered for evaluation.The product should retain its specifications within established limits for safety, purity and potency throughout the proposed shelf life.

If purified bulk material is to be stored after manufacture but prior to formulation and final manufacturing, atleast 3 batches should be placed on stability at the recommended storage temperature and should be representative of the manufacturing scale of production.The stability of intermediate products (as applicable) before mixing into the final bulk should be demonstrated. The quality of drug substance placed into stability program should be representative of the quality of material used in preclinical and clinical studies. The drug substance entered into stability program should be stored in containers which properly represent the actual holding containers used during manufacture. Containers of reduced size is acceptable for drug substance stability testing provided that they are constructed of the same material and use the same type of containers closure system. Manufacturer is also recommended to consider end of stability of the drug substance should be formulated and the product manufactured thereof should be place on stability till the product claim.

The stability of the product in its final form and at the recommended storage temperatures should be demonstrated using containers from at least three lots of final product made from different independent purified batches of monoclonal antibodies. In addition, real time stability study should be conducted on at least one final container lot produced per year.

Wherever possible, the final product containers should be representative of the quality of the material used in the preclinical and clinical studies.

Accelerated stability studies may provide additional supporting evidence of the stability of the product but cannot replace real time studies.When changes are made in production process or in the primary packaging that may affect the stability of the product, the product manufactured by the new change should demonstrate stability. These will also applicable to different presentations/strengths of the same product, but bracketing can be done if required. The statements concerning storage temperature and expiry date appearing on the label should be based on experimental evidence which should be submitted to NRA for approval.

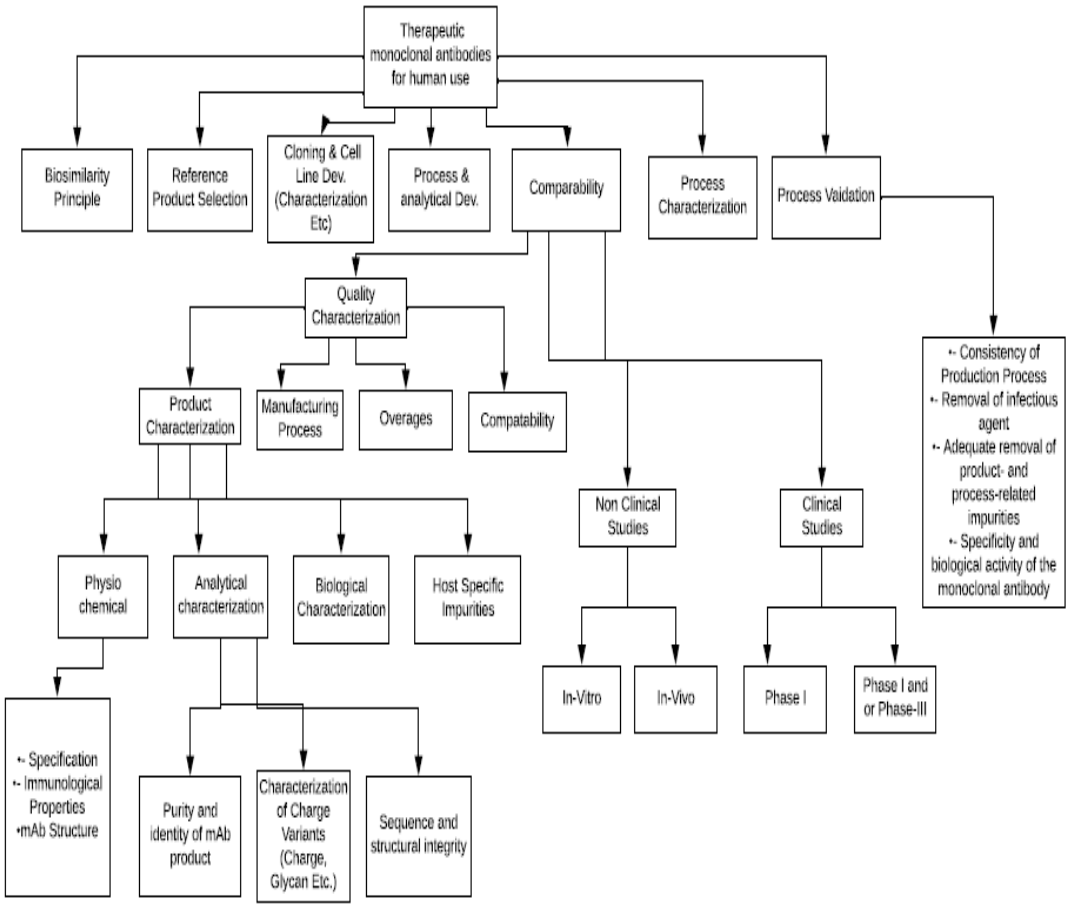


Table- Summary for Monoclonal Antibodies Development
(A brief overview of general steps in monoclonal antibody development is presented in the table above, it may not necessarily consolidate all the steps)