

Example 1: Humanized antibodies derived from parental IV/18 mAb

This example demonstrates the construction and characterization of humanized antibody variants CA9hu-2 directed to the proteoglycan domain of CA IX.

The humanization process utilized a combination of standard CDR-grafting technologies coupled with the latest research on antibody structure and up-to-date database of mature human IgG sequences. Firstly, the IV/18 murine antibody variable domains were sequenced. The Complementarity Determining Regions (CDRs) were identified using the Immunogenetics Information System® (IMGT®) or the Kabat numbering system (Lefranc et al, *Nucleic Acid Res* 27:209-212, 1999; Lefranc et al, *Dev Comp Immunol* 27:55-77, 2003; Kabat et al, *Sequences of Proteins of Immunological Interest*, 5th edition, 1991). For optimal retention of CDR-loop conformation, both numbering systems were used to identify CDRs within murine variable heavy (V_H) as well as variable light (V_L) domains. Subsequently, a number of human framework sequences were identified and used as “acceptor” frameworks (in the text below) for the CDR sequences. Each of the V_H domains was synthesized in-frame with a human IgG isotype constant domain sequence (allotype G1m17,1). Additionally, each of the V_L domains was synthesized in-frame with a human IgK isotype constant domain sequence (allotype Km3). The entire heavy and light chain sequence was codon optimized and the DNA sequence verified.

The combination of five V_H and five V_L chains resulted in generation of twenty-five humanized variants having humanized variable domains [marked in the following text as heavy (HC) and light (LC) chain] and human Ig constant domains. In order to characterize twenty-five humanized antibody variants, all sequences were screened for MHC Class II binding epitopes, Fv glycosylation motifs and deamidation motifs.

Murine monoclonal antibody IV/18 (isotype IgG2a) directed to the proteoglycan (PG)-like domain of CA IX was generated in the CA IX-deficient mice (WO2003/100029; Zatovicova et al, *J Immunol Methods* 282, 117-134, 2003). Pre-incubation of hypoxic tumor cells with the IV/18 mAb reduced the number of lung metastases in murine lung colonization model (Figure 1A). Metastatic colonies of fluorescently tagged HT1080-red fluorescent protein (RFP) cells in PBS-perfused murine lungs were imaged *ex vivo* after 12 days using IVIS Caliper imaging system (Figure 1B). Total radiant efficiency reflects the amount of cancer cells in murine lungs. Pre-incubation of HT1080-RFP cells with IV/18 mAb, and subsequent administration of three doses of antibody (50 μ g/mouse) during 12 days after the initial tail vein injection (1,500,000 cells/mouse, 10 mice per group) led to a marked decrease in lung colonization by these cells as determined by the fluorescence signal by IVIS. Metastatic colonies were