## 2.3.S.3 Characterization

The characterization of KN035consists of: a) the primary, secondary and higher-order structure; posttranslational modification forms; b) biological activity; c) purity;d)immunochemical properties. The lots used in these studies are 3 lots of GMP DS that are used for nonclinical, reference standard, stability and clinical studies. 3 lots of DP derived from these 3 lots of DS were also used in some characterization studies. The characterization study results are summarized in Table 2.6.

**Table 2.6 Summary of characterization results of KN035**

| Characterization | Test method | Results |
|---|---|---|
| Amino acid sequence | Reducing peptide mapping with LC-MS/MS after trypsin or chymotrypsin digestion | 100% matches to the theoretical sequences for all three lots of DS. |
| N/C-terminal amino acid sequencing | Reducing peptide mapping with LC-MS/MS | 99.7% of N-terminal sequences are blocked by pyroglutamate. Approximately89.0-93.3% of C terminal has no lysine. The sequence matches the theoretical terminal sequence. |
| Amino acid composition | LC | Closely matches the theoretical composition with low RSD |
| Disulfide bond position | Non-reducing LC-MS/MS | Correct disulfide bond formation, and match the theoretical disulfide bonds. |
| Free thiol content | Colorimetric method | In the range of 0.16- 0.28mol/mol of KN035 |
| N-glycosylation site | Reducing peptide mapping with LC-MS/MS | One N linked glycosylation site is detected at N210 for all 3 lots of DS. |
| N-glycan | 2AB-labeled NP-UPLC-MS/FLD | Mainly G2F and G1F with approximately 30% of sialylatedglycans. |
| Monosaccharide content | IEX-PAD | Fucose, glucosamine, galactosamine, galactose and mannose are detected. |
| Sialic acid | RP-HPLC | NeuGc< 0.05 mol/mol<br>NeuAc≤ 0.90 mol/mol |
| O-linked glycosylation | LC-MS/MS | May present ~ 2.0% of O-linked glycosylation based on MW analysis |
| Protein secondary and tertiary structure | Circular dichroism (CD) | Has secondary and tertiary structures |
| Higher order structure | Differential scanning calorimetry (DSC) | KN035 has two melting temperatures at approximately 65°C and 85°C. |

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| Apparent molecular weight | SDS-PAGE | 100 kD (non-reducing)<br>50 kD (reducing) |
| | Western blot | 50 kD |
| Accurate molecular weight | LC- MS | Different MW was obtained for different glycoforms in the range of 82.3 KDa to 84.0KDa. |
| Charge profile | WCX-HPLC | Acidic peaks 1 and 2, K0，K1 and K2, and other basic peaks (basic peak 1, 2 and 3) are noted. |
| Size purity | SE-HPLC | > 99.0% monomer with less than 1% of aggregate. |
| | CE-SDS (reducing) | Monomer > 98.0% and Fragments < 2.0% |
| Binding affinity | ELISA | EC50 ~ 57 -69 ng/mL |
| | Octet K2:<br>Binding affinities to PDL1 | KD ~ 3 nM |
| Blocking activity | Competitive ELISA | IC50 ~ 458 -474ng/mL |
| Binding affinity to FcRn | Fortebio BLI | KD ~ 0.5 μM |
| ADCC/CDC | Cell based assays | No ADCC or CDC activity |
| Binding to FcγR | ELISA and cell based assay | No binding to FcγRI, FcγRIIa and FcγRIIIa |