

1    **Development and qualification of a high-yield recombinant**  
2    **human Erythropoietin biosimilar**

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15

16 **ABSTRACT**

17 Recombinant human erythropoietin (rhEPO) has been saving millions of lives worldwide as a  
18 potent and safe treatment for the lack of erythrocyte, which is caused by chronic kidney  
19 disease (CKD) and other issues. Several biosimilars of rhEPO have been approved since the  
20 expiry of the relevant patents to provide cost-effective options but the price of rhEPO is still  
21 high for the affordability of global community. Therefore, development of biosimilar of  
22 rhEPO at a lower price is highly necessary. Here we report the development and  
23 characterization of a biosimilar of rhEPO with high-yield satisfying regulatory requirements.  
24 The hEPO-expressing cDNA was stably expressed in CHO cells with successive transfection.  
25 The master cell bank (MCB) and working cell bank (WCB) were established from the best  
26 selected clone and characterized for 50 passages. The rhEPO was expressed from the WCB in  
27 single-use suspension culture system with a high-titer ( $1.24\pm 0.16$  g/L). To the best of our  
28 knowledge this is the highest reported rhEPO titer to date. The rhEPO was purified using a  
29 series of validated chromatography unit processes including virus inactivation and filtration.  
30 The purified EPO was formulated in serum-free buffer, sterile filtered, and analyzed as the  
31 biosimilar of reference product Eprex<sup>®</sup>. Physicochemical analysis strongly suggested  
32 similarities between the developed rhEPO (GBPD002) and the reference. The *in vitro* and *in*  
33 *vivo* functional assays confirmed the similar biofunctionality of the GBPD002 and Eprex<sup>®</sup>.  
34 GBPD002 could provide a less-expensive solution to the needful communities as an effective  
35 and safe biosimilar where rhEPO treatment is necessary.

36

37

## 38 1. INTRODUCTION

39

40 Erythropoietin (EPO) is an essential hormone for erythrocyte or red blood cell (RBC)  
41 production in mammals [1]. It is also known as haematopoietin or hemopoietin. EPO is a  
42 glycoprotein and composed of 165 amino acids with an estimated molecular weight of 34  
43 kDa [2]. It has multiple isomeric forms; 5 – 8 isoforms were identified over the isoelectric  
44 point (pI) range of 4.4 – 5.2 [3]. The 3 potential N-linked glycosylation sites were predicted  
45 located at Asn24, Asn38, Asn83, whereas the single O-glycosylation site was predicted on  
46 Ser126 [4-7]. Approximately, 40% molecular weight of the protein has been attributed to the  
47 glycosylation. EPO has two disulfide bonds as well, which are formed between the cysteines  
48 of 7-161 and 29-33 amino acids; these cysteine-cysteine bridges are essential for maintaining  
49 biological activity [2, 8].

50 EPO is secreted from the kidney, which is enhanced in response to cellular hypoxia  
51 [9]. It promotes the division and differentiation of bone marrow resident committed erythroid  
52 progenitors to maintain erythrocyte population in blood for oxygen supply [10]. EPO is the  
53 primary erythropoietic factor that cooperates with various other growth factors like,  
54 interleukin (IL)-3, IL-6, glucocorticoids, and stem cell factors (SCF), which are involved in  
55 the development of erythroid lineage from multipotent progenitors. EPO works on RBC-  
56 progenitor by binding to the EPO receptor located on the surface of these cells, which triggers  
57 JAK2 signaling cascade [11, 12]. Activated JAK2 then initiates the STAT5, PIK3 and Ras  
58 MAPK signaling cascades resulting in differentiation, survival and proliferation of the  
59 erythroid cell [13].

60 Compromised EPO production is the main reason of anemia in human with chronic  
61 renal failure [14]. It has been demonstrated that the human recombinant epoetin (rhEPO)  
62 stimulate erythropoiesis in anemic patients with chronic kidney disease (CKD), including  
63 patients who need and do not need dialysis [15-18]. The administration of rhEPO has been  
64 found beneficial for the treatment of chemotherapy-induced anemia in cancer patients and to  
65 reduce the requirement for allogenic blood transfusions in patients with mild anemia who are  
66 undergoing surgery [19-21]. Furthermore, rhEPO is recommended for patients who are at  
67 high risk for perioperative transfusions due to substantial blood loss. Though, the use of  
68 rhEPO was initially restricted to dialysis patients with most severe forms of anemia, however,  
69 it has been currently administered to most dialysis patients with renal anemia and to  
70 predialysis patients [22].

71 The entry of rhEPO into clinical practice was a milestone for the treatment of renal  
72 anemia [23], followed by alfa, beta, zeta and other formulations. Johnson & Johnson's  
73 Eprex®/Procrit was the first rhEPO preparation that was approved by the US FDA and the  
74 European Medicine Agency (EMA) in June 1989; Amgen's Epogen was followed later with  
75 the approval of US FDA in February 1999 [24, 25]. The patents on Epogen/ Eprex® already  
76 expired in both the US and in Europe in 2013 [26], and opened up the opportunity for  
77 developing biosimilars for rhEPO to supply cost-effective medication to the market. Three  
78 biosimilars of rhEPO, *viz.*, Binocrit® from Sandoz, Abseamed® from Medice and Epoetin  
79 Alfa Hexal® from Hexal AG were approved by the EMA, and Retacrit (epoetin alfa-epbx)  
80 from Hospira (Pfizer) was approved in the US in May 2018 [24].

81 More than 850 million people worldwide were estimated who have been suffering  
82 from CKD [24]. The prevalence of anemia in patients with CKD (15.4%) in the USA is  
83 double compared with the overall population (7.6%); the prevalence rate increased to 53.4%  
84 with stage 5 of the disease [27]. CKD patients with anemia are at a higher risk of health-  
85 related quality of life impairment, cardiovascular disease, end-stage renal disease,  
86 hospitalization and mortality, compared with CKD patients without anemia [1, 28, 29]. The  
87 high prevalence of CKD-related anemia has been creating growing demand for the rhEPO but  
88 limited supply of qualified rhEPO has made the treatment expensive. The cost to treat anemia  
89 by rhEPO per quality-adjusted life-year was estimated at US \$24,128.03 and US \$28,022.33  
90 for the Hb level 9-10g/dl and 11-12g/dl, respectively [30]. The average GDP per capita of the  
91 global population, which was US \$11570 in 2019 [31], is below the average medication cost  
92 of EPO treatment.

93 Biosimilar is the fastest-growing segment in the biological drug market, owing to the value  
94 points of comparatively low-cost therapeutics than the originator with desired efficacy [25,  
95 32]. Despite introduction of few biosimilars in the market [26], the price of rhEPO is still out  
96 of reach for global community, and inviting the introduction of more cost-effective and  
97 affordable biosimilars to the world. Therefore, we have developed a biosimilar for rhEPO  
98 with the objective to reduce the cost by exploiting the science and technology. Here, we  
99 describe the development and characterization of GBPD002, a candidate biosimilar of  
100 rhEPO, with a superior productivity that will help to cater the product for better  
101 socioeconomic benefits to the global community.

102

## 103 **2. MATERIALS AND METHODS**

### 104 *Amplification of the gene of interest:*

105 Human spleen total RNA (Thermo Fisher Scientific, USA) was used as template for cDNA  
106 synthesis using Superscript IV 1<sup>st</sup> strand (Thermo Fisher Scientific, USA) cDNA synthesis  
107 kit. Primers were designed against EPO amino acid sequence obtained from DrugBank  
108 (accession number DB00016) and nucleic acid sequence obtained from GenBank (accession  
109 number X02158.1), for the cDNA synthesis and subsequent amplification of the gene of  
110 interest. Platinum Pfx DNA polymerase (Thermo Fisher Scientific, USA) was used for the  
111 amplification using standard PCR protocol (Denaturation: 94 °C for 30 secs, annealing: 66 °C  
112 for 30 secs, extension: 68 °C for 42 secs and total number of cycles: 35) in ProFlex 3x32-well  
113 PCR system (Applied Biosystems, USA). Desired amplified product was excised from 1.2%  
114 agarose gel and purified using GeneJET gel extraction and DNA cleanup kit following  
115 supplier's protocol (Thermo Fisher Scientific, USA).

116

### 117 *Cloning:*

118 pcDNA5/FRT (Thermo Fisher Scientific, USA) mammalian expression vector was used for  
119 construction of the rDNA. Amplified gene of interest and expression vector was digested  
120 using FastDigest XhoI and HindIII (Thermo Fisher Scientific, USA) restriction endonuclease  
121 following standard protocol. The digested sticky end insert and vector was purified from  
122 agarose gel excision using GeneJET gel extraction and DNA cleanup kit. The insert was then  
123 ligated with the pcDNA5/FRT vector using T4 DNA ligase (Thermo Fisher Scientific, USA)  
124 following standard sticky end ligation protocol. After ligation, the constructed rDNA was  
125 transformed into Top10 chemically competent *E. coli* cells (Thermo Fisher Scientific, USA)  
126 for rDNA amplification. The rDNA purification was done using PureLink quick mini and  
127 PureLink plasmid midi kit (Thermo Fisher Scientific, USA) following supplier's protocol for  
128 mini and midi scale of rDNA, respectively.

129

### 130 *Digestion and Sequencing:*

131 XhoI and HindIII was used to digest the gene of interest from the purified rDNA and insert  
132 size was confirmed by agarose gel electrophoresis. BigDye Terminator v1.1 Cycle  
133 sequencing kit (Thermo Fisher Scientific, USA) was used for sequence amplification, 3500  
134 genetic analyzer (Applied Biosystems, USA) was used for sequencing and analysis was done  
135 using ABI Sequencing Analysis v5.4 software.

136

137 *Cell transfection:*

138 Flp-In system (Thermo Fisher Scientific, USA) was used for stable transfection. Flp-In CHO  
139 cells were chemically co-transfected with the linearized rDNA and circular pOG44 Flp-  
140 recombinase vector using Lipofectamine 3000 (Thermo Fisher Scientific, USA) following  
141 their protocol. Constructed rDNA was linearized using FastDigest LguI (SapI) (Thermo  
142 Fisher Scientific, USA). Approximately,  $0.75 \times 10^5$ ,  $1 \times 10^5$  and  $1.5 \times 10^5$  cells in FBS containing  
143 Ham's F-12 nutrient media were seeded in one 24-well TC-treated sterile cell culture plate  
144 (SPL, Korea) 24 hours before transfection. Wells seeded with  $1 \times 10^5$  and  $1.5 \times 10^5$  cells  
145 reached at desired 60 to 70% confluency with a viability of around 90% and transfected with  
146 2.5  $\mu\text{g}$  linear rDNA and 22.5  $\mu\text{g}$  pOG44 in Opti-MEM media. After 6 hours of transfection,  
147 the cells were transferred in Ham's F-12 media for protein expression. Another expression  
148 construct was made using the pLVX-EF1a/puromycin (+) plasmid (Genemedi, China)  
149 containing EF1a promoter. This plasmid also includes puromycin resistance gene and the  
150 ampicillin resistance gene as known selectable markers of stable mammalian transfectants.  
151 This plasmid was used for second transfection to obtain high-copy number of insertions in the  
152 target cells.

153

154 *Antibiotic selection:*

155 20,000 cells per well were seeded in a 24-well TC treated plate for applying the selection  
156 pressure. Hygromycin B (Thermo Fisher Scientific, USA) of different concentrations (200  
157  $\mu\text{g}/\text{mL}$  to 1000  $\mu\text{g}/\text{mL}$ , with 100  $\mu\text{g}/\text{mL}$  gradual increment) was used in 3 replicate wells for  
158 each concentration with negative control 24 hours post seeding. Antibiotic containing media  
159 of respective concentration was changed in every 48 hours for 14 days. The selection  
160 pressure was withdrawn from the wells on day 14, where 600  $\mu\text{g}/\text{mL}$  of Hygromycin B was  
161 maintained and the cells were split into a fresh 24-well plate. Western blot analyses of all  
162 representative wells were performed to check the expression of desired protein. After second  
163 transfection, different concentration of puromycin antibiotic (25  $\mu\text{g}/\text{mL}$  to 200  $\mu\text{g}/\text{mL}$ , with  
164 25  $\mu\text{g}/\text{mL}$  gradual increment) challenge was applied. After that 100  $\mu\text{g}/\text{mL}$  of puromycin was  
165 maintained and the cells were split into a fresh 24-well plate.

166

167 *Clonal isolation and stable expression analysis:*

168 After antibiotic selection and expression analysis, clonal isolation following serial dilution  
169 method was performed in a 96-well plate, with a starting cell of 10,000 at A1 well. Serial  
170 dilution was done in 10% FBS containing Ham's F12 media in treated plate. After that,

171 western blot was performed for expression analysis. Four top performing clones were  
172 selected and were subjected for re-transfection followed by clonal isolation. For expansion,  
173 cells were cultured in 75 cm<sup>2</sup> TC-treated flask. The stable expression of the desired proteins  
174 was periodically checked by Western blotting using the media in culture [32, 33].  
175 After second antibiotic selection, clonal isolation was performed by automated cell sorter  
176 BD FACSAria™ Fusion Flow Cytometers (BD Bioscience, USA). After that, western blot  
177 was performed and top eight clone were selected.

178

179 *Best clone selection and MCB development:*

180 Four clones (Clone No. GB002EC001, GB002EC002, and GB002EC003) were processed  
181 forward. These clones were systematically adapted to suspension culture in serum free  
182 medium. The serum-free suspended cells were cultured in microbioreactor (Ambr® 15 cell  
183 culture bioreactor; Sartorius, Germany) to determine the specific productivity ( $Q_p$ ) and  
184 cellular growth rate of clones. Based on the productivity and process suitability the best  
185 clone was selected. Cell viability, growth rate, specific expression kinetics, cell  
186 morphology, stability of the desired protein in culture etc. were considered for selection.  
187 The dot blot and Western blot analyses were performed routinely for evaluation. The  
188 stability of the selected clone was confirmed up to 50<sup>th</sup> passages (generally, 4 days/passage)  
189 over a period of 200 days.

190

191 *Cell bank identity:*

192 The GBPD002 (rhEPO) expressing suspended CHO (expressing clone GB002EC003) cells  
193 were seeded into 24-well TC-nontreated plate containing 600 µL of 100% ActiPro medium  
194 and four replicates was prepared for seeding density of approximately 4.80E+04 cells/600µl  
195 or 3.0E+05 cells/mL. Cells were counted every 24 hours for up to day 18, with the daily  
196 replenishment of media. The media was collected and analyzed for the expression of desired  
197 protein by Western blot. Growth rate ( $\mu$ ) and population doubling time (PDT) is calculated  
198 from the projected graph. Cell morphology and cell viability analysis were done by  
199 microscopic evaluation of MCB. The growth kinetics of the cells were measured over the 50  
200 passages.

201

202 *Dot blot analysis:*

203 Dot blot analysis was performed to identify the best clone. PVDF membrane (Thermo Fisher  
204 Scientific, USA) was taken and grid was drawn by pencil to indicate the region of blot. To



205 activate the membrane, it was submerged into 100% methyl alcohol for 3 minutes at room  
206 temperature. Then the membrane was incubated in transfer buffer (pH 8.3) for 12 minutes.  
207 After proper incubation the membrane was taken on a wet Whatman filter paper. 10  $\mu$ L  
208 sample from each clone was added on each grid of the membrane. Then the membrane was  
209 dried at 40 °C for 5 minutes and activated the membrane again by incubating it into 100%  
210 methyl alcohol for three minutes at room temperature without any shaking. Then membrane  
211 was transferred into transfer buffer (pH 8.3) for 12 minutes. After that, the membrane was  
212 blocked with blocking buffer for 1 hour at room temperature. After successful blocking, 1  $\mu$ L  
213 EPO specific primary antibody (Thermo Fisher Scientific, USA) was added with 10 mL  
214 blocking buffer for the preparation of primary antibody solution and membrane was  
215 submerged into it for 1 hour at room temperature. The membrane was washed with TBST  
216 buffer for 10 minutes. This process was performed three times. After that, 1  $\mu$ L goat anti  
217 rabbit secondary antibody (Thermo Fisher Scientific, USA) was added with 10 mL blocking  
218 buffer for the preparation of secondary antibody solution and membrane was submerged into  
219 it for 1 hour at room temperature. Again, membrane was washed with TBST buffer for 10  
220 minute and performed this step three times. Then, membrane was rinsed with type-1 waster  
221 for two minutes and placed into a clean plastic sheet. Novex® ECL chemiluminescent  
222 substrate (Thermo Fisher Scientific, USA) was added over the entire blotting paper and  
223 incubated for two minutes and finally image was captured by Amersham Imager 600 RGB  
224 [34].

225

#### 226 *SDS-PAGE analysis:*

227 SDS-PAGE was performed under reducing conditions. In short, the samples and the reference  
228 product Eprex® [ $\text{@}6.5 \mu\text{L}$  (1  $\mu\text{g}$ ) in 2.5  $\mu\text{L}$  NuPAGE® LDS Sample Buffer (4 $\times$ ) and 1  $\mu\text{L}$   
229 NuPAGE® Sample Reducing Agent (10 $\times$ )] were loaded in Bolt 4-12% Bis-Tris Plus Gels  
230 (Thermo Fisher Scientific, USA). Samples were denatured at 95°C for 3 mins, and cold on  
231 ice, and spin them down (for 1min, at 12,000 rpm) before loading them in gel. Proteins were  
232 separated using 90 mA for 45 min. Proteins were visualized by Coomassie brilliant blue  
233 staining. Novex sharp pre-stained protein marker (Thermo Fisher Scientific, USA) was used  
234 as reference for molecular weight.

235

#### 236 *Western blot:*

237 Comparative analysis was done between reference product Eprex® and GBPD002 protein  
238 sample using Western blot at reducing conditions. Bolt 4-12% Bis-Tris Plus Gels (Thermo



239 Fisher Scientific, USA) was used for separation of the proteins by molecular weight and then  
240 separated proteins were transferred in iBot 2 PVDF membrane (Thermo Fisher Scientific,  
241 USA). Anti-Epo polyclonal antibody (Thermo Fisher Scientific, USA) was used as a primary  
242 antibody and HRP conjugated Goat anti-rabbit (H+L) IgG (Thermo Fisher Scientific, USA)  
243 was used as a secondary antibody for detecting target proteins. Molecular size was  
244 determined by using novex sharp pre-stained protein marker (Thermo Fisher Scientific,  
245 USA). Novex<sup>®</sup> ECL Chemiluminescent Substrate (Thermo Fisher Scientific, USA) for HRP  
246 was used to detect the signal and the imaging was done using Amersham Imager 600 RGB  
247 (GE Healthcare, USA).

248

249 *Two-dimensional gel electrophoresis:*

250 Two-dimensional (2D) gel electrophoresis was performed using 20 µg of each sample for  
251 charge variant analysis. In short, isoelectric focusing (IEF) was performed in ZOOM<sup>®</sup> Strip  
252 pH 3–10L (Thermo Fisher Scientific, USA) followed by SDS PAGE in Novex<sup>™</sup> 4–20%  
253 Tris–Glycine ZOOM<sup>™</sup> Protein Gels (Thermo Fisher Scientific, USA) with XCell  
254 SureLock<sup>™</sup> Mini-Cell Electrophoresis System (Thermo Fisher Scientific, USA). Gels were  
255 stained with Coomassie blue and evaluated against Novex sharp pre-stained protein marker  
256 (Thermo Fisher Scientific, USA). The imaging was done using Amersham Imager 600 RGB  
257 (GE Healthcare, USA).

258

259 *Insert sequence and the copy number of insert:*

260 The DNA sequencing of inserted EPO gene in MCB and WCB were performed as described  
261 elsewhere in the article using the Chromosomal DNA and cDNA. The copy number of inserts  
262 in MCB and WCB were analyzed using QuantStudio 12K Flex system qRT-PCR system  
263 (Thermo Fisher Scientific, USA) and genomic DNA from sample cells. Comparative C<sub>t</sub> and  
264 melt-curve analysis method was used to identify the relative copy number of inserts in  
265 relevant genome. A Flp-In-CHO stably transfected with rhEPO is considered as the positive  
266 reference for relative quantitation of the copy number of the inserted rhEPO DNA in WBC. A  
267 serial dilution of the reference cells was considered as scale for calculating copy numbers of  
268 the experimental sample.

269

270 *Mycoplasma detection:*

271 Comparative  $C_t$  and melt-curve analysis were done for mycoplasma contamination check for  
272 MCB and WCB. Analysis was done by using MycoSEQ™ Mycoplasma Real-Time PCR  
273 Detection Kit (Thermo Fisher Scientific, USA) and QuantStudio 12K Flex system qRT-PCR  
274 system (Thermo Fisher Scientific, USA). Positive control reaction, negative control reaction,  
275 inhibition-control reaction and extraction spike control were used for method suitability test.  
276

277 *Upstream processing:*

278 Batches were started from WCB at an N-4 step. Three consecutive batches were performed  
279 in 5 L bioreactor using ReadyToProcess WAVE 25 system (GE Healthcare, USA). A single-  
280 use 10 L Cell bag™ bioreactor (working volume: 0.5-5 L; GE Healthcare, USA) was placed  
281 on the rocker. Cell cultures were controlled using the UNICORN™ system control software  
282 (GE Healthcare, USA). Cells were seeded at  $\sim 7.93 \times 10^8$  total viable cells (around  $7.93 \times 10^5$   
283 viable cells/mL) in a chemically defined media (ActiPro media; GE Healthcare, USA). The  
284 cultures were maintained with setpoints for pH at  $7.2 \pm 0.05$ , dissolved oxygen (DO) at 40%  
285 air saturation and stage-specific variable rocking speeds from 18 – 28 with rocking angle 5 –  
286 8 degrees. Culture pH was controlled by either addition of 7.5%  $\text{NaHCO}_3$  and/or by sparging  
287 of  $\text{CO}_2$  gas. Bioreactor cultures were provided with gas flow from 0.10 – 0.5 lpm through air  
288 sparger, and DO was controlled by sparging with air and oxygen. Culture temperature was  
289 kept at 37 °C from day 0 to day 7, and shifted to 32 °C on the day 7. Required amounts of  
290 media and nutrients were added to the cultures on regular basis. The cultures were harvested  
291 at day 17. Samples were taken at every 24-hour interval for measurement of viable cell  
292 density (VCD), viability, and productivity. The media were harvested in 2D sterile bags after  
293 filtration through 0.6  $\mu\text{m}$  followed by 0.2  $\mu\text{m}$  PES filter (GE Healthcare, USA) and  
294 proceeded for downstream processing.

295

296 *Downstream processing & formulation:*

297 The binding and elution conditions in each of chromatography processes were screened out in  
298 96 deep-well plate format and adapted in small scale column format. The optimum conditions  
299 (volumetric flow rate, linear flow rate, residence time, dynamic binding capacity of each  
300 resin, operating temperature etc.) for each chromatography step were determined in small  
301 scale. The unit processes were scaled up, and the conductivity and pH of samples were  
302 considered as in-process check (IPC). The rhEPO was enriched onto 0.40 L Capto blue (GE  
303 Healthcare, USA) column with AKTA Avant 150 system (GE Healthcare, USA) followed by  
304 washing the unbound potential contaminants, and then eluted using gradient of 20 mM Tris-

305 HCl, pH 7.4 and 1.5 M NaCl. FPLC systems and columns were sanitized with 1.0 N NaOH  
306 prior and after each purification step. The in-line UV<sub>280nm</sub>, conductivity and pH of the sample  
307 were considered as IPC. The eluate from Capto blue was diafiltered against Tris-HCl, pH 7.0  
308 to remove the salt and undesired low molecular weight (LMW) molecules (<10 kDa) from  
309 sample using 0.10 m<sup>2</sup> Sartoclon slice PES cassette (Sartorius Stedim, Germany) and AKTA  
310 flux 6 system (GE Healthcare, USA). The system and cassette were sanitized with 1.0 N  
311 NaOH before and after diafiltration. The conductivity and pH of retentate were considered as  
312 IPC. The retentate was loaded onto 0.25 L Q Sepharose HP (GE Healthcare, USA) column to  
313 bind desired isoforms. The rhEPO was separated from some host proteins (HCPs), nucleic  
314 acid (DNA) and adventitious viruses using gradient of Tris-HCl, pH 7.0 and 1.5 M NaCl. The  
315 in-line UV<sub>280nm</sub>, conductivity and pH of the sample were considered as IPC. The elute from Q  
316 Sepharose was loaded on to 0.20 L source 15RPC (GE Healthcare, USA) column to separate  
317 non-glycosylated and glycosylated rhEPO. The eluate was collected using gradient of 0.1%  
318 TFA in WFI and 95% acetonitrile. The in-line UV<sub>280nm</sub>, pH and conductance of the eluate  
319 were considered as IPC.

320 The eluate of source 15RPC was incubated for virus inactivation at low pH. The  
321 virus-inactivated RPC eluate was loaded on to 0.10 L Macrocap SP Sepharose (GE  
322 Healthcare, USA) column to remove RPC buffers and rhEPO aggregates followed by  
323 reconstitution into pre-formulation buffer. After washing with 20 mM glycine buffer, the  
324 sample was eluted using gradient of 25 mM sodium phosphate, 0.00023 mM polysorbate 80,  
325 pH 7.0 and 1.0 M NaCl. The in-line UV<sub>280nm</sub>, conductance and pH of the eluate were  
326 considered as IPC. The eluate of Macrocap SP was filtered through 20 nm Virosart HF  
327 (Sartorius Stedim, Germany). The rhEPO concentration was checked and diluted to desired  
328 concentration in formulation buffer. After sterile filtration through 0.45|0.2-micron PES  
329 Sartopore 2 filter (Sartorius Stedim, Germany) the formulated bulk sample was collected in 3  
330 L Flexboy 2D bag (Sartorius Stedim, France) and filled into pre-sterile syringe (Schott,  
331 Switzerland) for dose preparation. The pre-filled syringes were stored at 5 ± 3 °C and were  
332 subjected for analysis as per specification.

333

#### 334 *Enzyme-linked immunoassay (ELISA)*

335 Microtiter plates (Nalge Nunc International, USA) were coated with polyclonal anti-human  
336 EPO (4 mg/mL; Thermo Fisher Scientific, USA) in 0.1 M sodium bicarbonate buffer (pH  
337 8.3) at 4°C overnight. The plates were blocked by 3% BSA/PBS for 2 h and then incubated  
338 with serial dilutions of an EPO standard (Thermo Fisher Scientific, USA) or culture

339 supernatant samples for 4 hours at room temperature. After washing, the bound EPO was  
340 incubated with monoclonal mouse anti-EPO antibody (1 mg/ mL; Thermo Fisher Scientific,  
341 USA) at 4°C overnight and then alkaline phosphatase (AP) conjugated anti-mouse IgG  
342 adsorbed with rat serum protein) (Thermo Fisher Scientific, USA) for 2 h diluted 1: 15,000 in  
343 1% BSA/PBS/0.05% Tween20. For the detection of the antigen–antibody reaction, p-  
344 nitrophenyl phosphate was added as a substrate, and incubated at 4°C overnight. The optical  
345 absorbance at 405 nm was measured by an ELISA reader. Each incubation step was followed  
346 by washing four times with PBS/0.05% Tween20.

347

#### 348 *Chromatography for determination of assay and impurities:*

349 The quantitative analysis (assay) for rhEPO of the samples were performed using a Vanquish  
350 UHPLC system (Thermo Fisher Scientific, USA). The reference product Eprex<sup>®</sup> and samples  
351 (20 µL of each) were applied in RPC column (Hypersil GOLD C8, 175 Å, 2.1× 100 mm, 1.9  
352 µm column; Thermo Fisher Scientific, USA) using an auto injector system. A gradient of  
353 media A (water with 0.1% FA) and media B (90% ACN in water with 0.1% FA) was used as  
354 mobile phase at a flowrate of 0.3 mL/min. The formulation buffer was considered as  
355 reference for base line signal. The impurity profile of the samples was analyzed using size  
356 exclusion chromatography (SEC). A 50 µL of each sample and reference product Eprex<sup>®</sup>  
357 were loaded in a Biobasic SEC-300 column (300 mm\*7.8mm, 5µm; Thermo Fisher  
358 Scientific, USA), and the analyses were performed using an Ultimate 3000 RSLC system  
359 (Thermo Fisher Scientific, USA). Phosphate buffer with pH 7.4 was used as carrier phase at a  
360 flow rate of 1.0 ml/min. All analyses were performed with a 25 min run time and at a fixed  
361 column temperature of 70 °C. The chromatograms were obtained from the absorbance at 280  
362 nm, and relevant values were used for calculations.

363

#### 364 *Peptide mapping:*

365 40 µg of rhEPO preparation was diluted in 50 mM ammonium bicarbonate (Wako Pure  
366 Chemicals Industries Ltd., Japan), at pH 8 containing 8 M urea (Thermo Fisher Scientific,  
367 USA). To reduce the sample, 500 mM DTT (Thermo Fisher Scientific, USA) was added to  
368 the solution to a final concentration of 20 mM (1:25 dilution), mixed briefly, and then  
369 incubated at 60 °C for 1 hour. For alkylation, 1 M IAA (Sigma-Aldrich, USA) was added to  
370 the reduced protein sample to a final concentration of 40 mM (1:25 dilution), and incubated  
371 the reaction mixture for 30 minutes protected from light. The reaction was stopped by adding  
372 500 mM DTT solution to a final concentration of 10 mM (1:50 dilution). Trypsin (Thermo

373 Fisher Scientific, USA) was added to the sample solution to a final trypsin to protein ratio of  
374 1:23 (w/w). Samples were incubated at 37 °C for 16 – 24 hours. Reaction was stopped by  
375 adding formic acid up to bringing the pH 2.0. C18 spin column (Thermo Fisher Scientific,  
376 USA) was prepared as per manufacturer’s instruction to purify the peptide-pool. Columns  
377 were activated by adding 200 µL 50% acetonitrile (Wako Pure Chemicals Industries Ltd.,  
378 Japan), and equilibrated using 200 µL of 0.5% formic acid (Wako Pure Chemicals Industries  
379 Ltd., Japan) in 5% acetonitrile (Wako Pure Chemicals Industries Ltd., Japan). Samples were  
380 applied to the column and eluted using 20 µL of 70% acetonitrile. Samples were dried under  
381 low temperature and vacuum and processed for experiments.

382 rhEPO preparation was digested with Serine Protease (MS grade, Pierce, Thermo  
383 Fisher, USA) and purified according to the supplier’s instructions. 2 µg of digested peptides  
384 were loaded into mass spectrometry system (Q Exactive Hybrid Quadrupole-Orbitrap MS,  
385 Thermo Fisher Scientific, USA). Hypersil gold C18 (100x2.1 mm; particle size: 1.9 µm,  
386 Thermo Fisher Scientific, USA) column was used for separation of peptides. Column oven  
387 temperature was set at 40 °C and samples were eluted in 95– 60 % mobile phase A (0.1%  
388 formic acid in water) and 5 – 40 % mobile phase B (0.1 % formic acid in acetonitrile)  
389 gradient with 0.300 mL/min flow rate for 100 minutes. Peptide elution were checked by  
390 absorbance at 214 nm. For peptide identification, data-dependent mass spectrometry was  
391 performed where full-MS scan range was 350 m/z to 2200 m/z, resolution was 70000, AGC  
392 target was 3E6, maximum IT was 100 milliseconds (ms). Data-dependent mass spectrometry  
393 resolution was 17500, AGC target was 1E5, and maximum IT was 100 ms. Data analysis was  
394 performed in BioPharma Finder (Thermo Fisher Scientific, USA) using variable parameters  
395 to get confident data, and then data were combined in one map to visualize complete  
396 fragmentation.

397

#### 398 *Glycosylation pattern analysis:*

399 20µg of rhEPO and reference product Eprex<sup>®</sup> were treated with 20 units of recombinant  
400 PNGase F (Thermo Fisher Scientific, USA) at 37 °C for hours. Samples were subjected for  
401 SDS PAGE and Western blot analyses as described elsewhere in the article. The imaging was  
402 done using Amersham Imager 600 RGB (GE Healthcare, USA).

403

#### 404 *Host Cell DNA:*

405 Host cell DNA were analyzed using resSEQ<sup>™</sup> CHO DNA Quantification Kit (Thermo Fisher  
406 Scientific, USA) following supplier’s protocol. Briefly, six 10-fold serial dilutions of control

407 CHO DNA were prepared using DNA dilution buffer such that the final amount of CHO  
408 DNA in each reaction ranged from 3000 – 0.03 pg. Forward and reverse primers and a FAM-  
409 labeled probe that were designed to amplify a hamster-specific region of a multi-copy gene  
410 were provided. Each 30- $\mu$ l PCR reaction mix contained 2  $\mu$ l of negative control, 3  $\mu$ l of 10x  
411 primer/probe mix, 15  $\mu$ l of 2x Environmental Mastermix, and 10  $\mu$ l of diluted CHO DNA.  
412 All reactions were performed on the QuantStudio 12K Flex System (Thermo Fisher  
413 Scientific, USA) using the following cycling conditions: 10 minutes at 95 °C to activate the  
414 enzyme, followed by 40 cycles of 15 seconds at 95 °C and then 1 minute at 60 °C.

415

#### 416 *Host Cell Protein:*

417 Host cell Protein were analyzed using ProteinSEQ™ CHO HCP Quantitation Kit (Thermo  
418 Fisher Scientific, USA) as per supplier's protocol. Briefly, seven 5-fold serial dilutions of  
419 control CHO HCP standard were prepared using DNA dilution buffer such that the final  
420 amount of HCP standard in each reaction ranged from 3125 – 0.2 ng/mL. Then, MagMAX  
421 express-96 magnetic particle processor (Thermo Fisher Scientific, USA) was used for capture  
422 the HCP automatically followed by several steps; i) preparing wash plate ii) preparing qPCR  
423 plate, iii) preparing probes plate, iv) preparing capture plate. Finally, QuantStudio 12K Flex  
424 System (Thermo Fisher Scientific, USA) using the following cycling conditions: 10 minutes  
425 at 37 °C to activate the enzyme, 20 seconds at 95 °C followed by 40 cycles of 15 seconds at  
426 95 °C and then 30 seconds at 60 °C.

427

#### 428 *Particle size distribution:*

429 To stabilize the system, the equipment was turned on minimum 30 – 60 minutes before taking  
430 the reading. Samples were prepared in 1 $\times$  PBS (pH 7.2) and passed through 0.22-micron  
431 filter. Respective buffers were used as dispersant. The samples were allowed for stabilization  
432 at 20 °C for 20 min, and then analyzed in disposable plastic cuvette using a Zetasizer Nano  
433 ZSP (Malvern Panalytical Ltd., UK). The refractive index (RI), viscosity and dielectric  
434 constant of dispersion buffer (1 $\times$  PBS, pH 7.2 at 20 °C) was considered 1.33, 0.88 cPs and  
435 79, respectively.

436

#### 437 *Receptor binding:*

438 The Biacore T200 equipment (GE Healthcare, USA) was used for relevant experiments. EPO  
439 receptor protein (Fc chimera active; Abcam, USA) was immobilized on Series S Sensor  
440 Chips CM5 (GE Healthcare, USA) using amine coupling kit (GE Healthcare, USA). First, the



441 flow-cell surface of Series S Sensor Chips CM5 was activated by injecting a mixture of  
442 EDC/NHS (1:1) for 7 minutes. Then 70  $\mu$ L of 50  $\mu$ g/mL EPO receptor protein was prepared  
443 in sodium acetate at pH 5.0 and injected over the activated surface at 10  $\mu$ L/min flow rate.  
444 Residual NHS-esters were deactivated by a 70  $\mu$ L injection of 1 M ethanolamine, pH 8.5.  
445 The immobilization procedure was performed in running buffer HBS-EP, pH 7.4 (GE  
446 Healthcare, USA). rhEPO samples were passed over the active flow cell surface of CM5 chip  
447 for kinetics analysis. Glycine-HCl of pH 2.5 was used for regeneration. All samples were  
448 diluted in 1 x HBS-EP running buffer (pH 7.4).

449

450 *In vitro functional assay using cell culture:*

451 TF-1 cell line, which was originally derived from a patient diagnosed with erythroleukemia,  
452 were used for the *in vitro* functional assay of rhEPO. Cells were maintained in RPMI 1640  
453 medium (Gibco, USA) supplemented with 10 % FBS (Thermo Fisher Scientific, USA), 1%  
454 PS (Thermo Fisher Scientific, USA), and 5 ng/mL human recombinant GM-CSF (Thermo  
455 Fisher Scientific, USA). The incubator condition was set at 37 °C with 5 % carbon dioxide  
456 environment. During the experiment, GM-CSF were eliminated from the cells by washing  
457 twice in PBS. Cells were then seeded at a density of  $10^5$  cells/well in 24-well TC-nontreated  
458 cell-culture plate. Cells were allowed to grow for 72 h in the presence or absence either  
459 rhEPO samples at indicated concentrations. Eprex<sup>®</sup> at indicated concentrations were used as  
460 positive control. Cells were collected after 72 hours through centrifugation for 10 min at 800  
461  $\times$ g. After washing with PBS, cells were counted using an automated cell counter (Countess 2,  
462 Thermo Fisher Scientific, USA)

463

464 *In-vivo assay by mice model:*

465 64 albino normocytic mice (8-12 weeks old) were randomly assigned to one of eight the  
466 (groups) cages at day (-)2. The groups were as follows: control, placebo (formulation buffer),  
467 treatment I (20IU/mL, GBPD002), treatment II (40IU/mL, GBPD002), treatment III  
468 (80IU/mL, GBPD002), treatment I (20 IU/mL, reference), treatment II (40 IU/mL, reference),  
469 and treatment III (80 IU/mL, reference). There were eight mice in each cage. Animals from  
470 each group received a pre-defined 0.05 mL subcutaneous injection at day 0. After the  
471 injections, on day 4, the blood samples were collected from the animals. RBC and  
472 Hemoglobin were analyzed using auto hematology analyzer (BK-6190 VET, Biobase,  
473 China). For reticulocyte analysis, blood samples were diluted 500 times in the buffer and  
474 thiazole orange (Sigma-Aldrich, USA) was added. After staining for 3–10 min, cells are



475 washed and the reticulocyte count was analyzed using a flow cytometer (BD FACSLyric™  
476 Flow Cytometer, BD Bioscience, USA).

477

478 *Sterility and endotoxin testing:*

479 Bacterial and fungal sterility were tested using direct inoculation technique. Samples (1 mL)  
480 were inoculated in 10 mL of Tryptic Soya Broth (TSB) media for 14 days and absorbance  
481 were measured at 600 nm. Endotoxin in samples were tested using Pierce LAL Chromogenic  
482 Endotoxin Quantitation Kit (Thermo Fisher, USA) as per supplier's instructions.

483

### 484 **3. RESULTS**

485 Development and characterization of a biosimilar is a meticulous process that has to be  
486 aligned with the regulatory framework. A snap shot of the overall workflow for the study is  
487 shown in figure-1. The activities mentioned in the article have gone through according to the  
488 work breakdown structure (WBS) of (Fig.: 1A), which is designed to comply regulatory  
489 requirements for a biosimilar.

490

491 *rhEPO expression construct and high-level rhEPO-expressing cell line development:*

492 The amplified cDNA of rhEPO from human spleen was cloned into pcDNA5/FRT plasmid.  
493 Through a tBLASTn search against human genome, the DNA sequence of the insert data was  
494 confirmed to have an amplicon of 611 nucleotide (nt) with the desired 501 nt-long ORF of  
495 hEPO with start and stop codon (accession no.: X02158). The coding sequence was found  
496 100% match to translate the hEPO protein (DrugBank reference no.: DB00016) with the pre-  
497 sequence. After transfection of the expression plasmid in Flp-In-CHO cells followed by  
498 challenge against Hygromycin B at 600 µg/mL concentration for 14 days, we have observed  
499 the existence of high-level of rhEPO-expressing cells. Lower concentration of Hygromycin B  
500 challenges was associated with low-level of rhEPO expression. We have screened the cells  
501 against 800 µg/mL of Hygromycin but no cells were survived. Several single clones were  
502 identified (Fig.: 1B) as rhEPO-expressing cells from the surviving pool of 600 µg/mL  
503 Hygromycin B challenge. They were amplified in separate well and the expression of the  
504 rhEPO was confirmed by Western blot. Most of them were found equipotent in terms of the  
505 rhEPO expression level (Fig.: 1C). After second transfection on these cells, we were able to  
506 identify surviving cells even in 100 µg/mL puromycin challenge, which represents higher  
507 level of antibiotic resistivity in these cells. Several single colonies were identified and

508 allowed to grow. After dot blot analysis we found several of these clones were having  
509 significant higher level of rhEPO expression (Fig.: 1D).  
510 We have selected eight of the similar high-level expressing cells with desired cellular  
511 morphology of a round shape isolated single cell in suspension from this set of clones. On a  
512 culture system with daily media replenishment, we found that four of the clones were  
513 growing above the other cells suggesting that they have higher expression level and higher  
514 cell growth kinetics as well. These cells were tested again for expression level of rhEPO with  
515 daily media replenishment and cell population correction to have the similar cells altogether  
516 throughout the experiment period of 7 days. We found that the relative specific expression of  
517 rhEPO for these cells were more or less similar (Clone no. 1: 5.4 pg/cell/day; Clone no. 2: 5.8  
518 pg/cell/day; Clone no. 3: 5.5 pg/cell/day; and Clone no. 4: 5.7 pg/cell/ day. Since these clones  
519 have similar growth kinetics and expression profile therefore, they were further subjected for  
520 stress-screening mimicking mid- to end-stage fed-batch environment. These cells were  
521 allowed to grow in fixed media (no media change over 7 days) to check the tolerance  
522 capacity of the individual clones against media deprivation and media toxicity due to the  
523 continuous accumulation of cellular metabolic load. We found that though they have similar  
524 morphology, growth kinetics in fresh media and expression level of rhEPO but their growth  
525 rate and survivability were astoundingly different in stressed condition (Fig.: 2A-D). Clone  
526 no. 2 grew at the lowest rate and Clone no. 3 grew at the highest rate, where the two other  
527 clones stayed in between. This data clearly showed the supremacy of the Clone no. 3 as the  
528 candidate for master cell bank (MCB) over the others.

529

#### 530 *Master cell bank and working cell bank:*

531 The selected clone (clone no.: 3) were further adapted for 4 more weeks under constant  
532 shaking and serum-free media. The cells were split and media were replenished on every 4<sup>th</sup>  
533 day. The cellular morphology was remained similar over the period but the growth kinetics  
534 and protein expression level of rhEPO were increased for the first 5 splits and then remain  
535 constant (Fig.: 2E-F). The final growth rate for the cells were found 0.67/day and the specific  
536 productivity of cells were found 5.89 pg/cell/hr. At this point, the copy number of the rhEPO  
537 gene in cells were determined as 4 copies/cell, which remained unchanged throughout the  
538 adaptation process (Fig.: 2G). These cells were declared as MCB, and WCB were prepared  
539 from this MCB. The MCB and WCB were stored in liquid nitrogen as well as in -150 °C  
540 freezer and were subjected for characterization.

541 Cells from MCB were further maintained in culture for 50 passages with a split and media  
542 replenishment on every 4<sup>th</sup> day. These media showed consistent expression of the rhEPO with  
543 same molecular migration pattern detected by Western blot (Fig.: 3A). Cells from all  
544 passages were stored as mentioned before. The cellular growth kinetics and the copy number  
545 of the rhEPO gene also were found consistent (Fig.: 3B-C). Separate batches of cell culture  
546 were run (1L batch size in wave bioreactor) using the thawed cells from the frozen stocks  
547 (MCB) of passage no. 1, 25, and 50. Cell growth kinetics, copy number of rhEPO, and the  
548 molecular identity of rhEPO protein by Western blot were found similar among these three  
549 batches (Fig.: 3D). The cells were found free from mycoplasma and bacterial contaminations  
550 (Fig.: 3E-F).

551

### 552 *Manufacturing of rhEPO:*

553 Through a systematic approach, three consecutive batches were successfully performed in  
554 wave bioreactor using the WCB stock. During culture, maximum viable cell density (VCD)  
555 was above 18 million cells/ mL (total viable cell:  $6.73 \times 10^{10} \pm 5.85 \times 10^8$ ) at day 9 with  
556 viability 92% to 94%. Specific growth rate of cells was 0.0148 h<sup>-1</sup> (day 2 to day 6), and  
557 decay rate were 0.0001 h<sup>-1</sup> (day 6 to day 12) (Fig.: 4A-G). After harvesting the batches, the  
558 mass balance (amount of the total harvested media) for all these batches were found  
559 comparable (Batch 01: 4965 mL, Batch 02: 4980 mL, and Batch 03: 5010 mL). Relevant titer  
560 for the batches were found 1.23 g/L, 1.31 g/L, and 1.21 g/L, respectively for Batch 01, Batch  
561 02, and Batch 03. The metabolite in the media were analyzed to check whether these batches  
562 might have any differences in relevant properties that may reflects differences in metabolic  
563 loads among the batches. We have tested ammonia, lactate, glucose, glutamate, and  
564 glutamine; all the trendlines were found in close proximity with no eccentric data point (Fig.:  
565 5A-E). After the filtration, the appearance of the clarified samples was found brown with the  
566 absorbances of 1.79, 1.77 and 1.72, respectively at 600 nm for Batch 01, Batch 02, and Batch  
567 03. The clarified sample did not produce any turbidity on standing at room temperature for 72  
568 hours though the titer was reduced after 12 hours (Fig.: 5F), and suggested that the samples  
569 must be processed for the downstream within the next 12 hours.

570 Accordingly, all batches were processed for the AFC to enrich the rhEPO and remove  
571 the majority of the unwanted materials as soon as possible after obtaining the media from the  
572 upstream processing. The rhEPO in final formulation were obtained by following the  
573 workflow shown in (Fig.: 6A.) At AFC unit process step, approximately 5.0 L of rhEPO-  
574 containing media sample was captured and eluted from resin; where sample pH, conductance,

575 volume and UV280 were 7.34, 124.61 mS/cm, 5.0 L and 483,873 respectively. The eluate  
576 was buffer exchanged; where sample pH, conductance and volume were 7.12, 1.82 mS/cm,  
577 and 5.0 L, respectively. At AEX unit process step, approximately 1.5 L sample was eluted  
578 from resin; where sample pH, conductance, volume and UV280 were 7.05, 16.3 mS/cm, 1.5  
579 L, and 305,150, respectively. At RPC unit process step, approximately 1.5 L sample was  
580 loaded and eluted from resin; where sample pH, conductance, volume, acetonitrile percentage  
581 and UV280 were 2.22, 2.79 mS/cm, 0.5 L, 41.6%, and 105,403, respectively. At VI-unit  
582 process step, 0.5 L RPC eluted sample was incubated for inactivation of virus or virus like  
583 particles; where incubation time and incubation temperature were 90 minutes and  $22 \pm 3$  °C,  
584 respectively. At CEX unit process step, approximately 1.0 L sample was bound and eluted  
585 from resin; where sample pH, conductance, volume and UV280 were 6.98, 13.35 mS/cm,  
586 0.84 L and 68,067, respectively. A set of relevant chromatograms are shown in (Fig.: 6B-E)  
587 The final yield for the three consecutive batches were found as follows: Batch 01: 0.724 gm,  
588 Batch 02: 0.728 gm, and Batch 03: 0.715 gm; the corresponding yield percentages were  
589 13.30, 14.62, and 13.31, respectively.

590

#### 591 *Qualification of the rhEPO formulation:*

592 The finished rhEPO was analyzed against the given specification (Table: 1). Since rhEPO is a  
593 parenteral preparation, therefore, the microbial sterility and endotoxin test were attempted  
594 first to conform with the specification and proceed forward with other critical quality  
595 attributes (CQA). No growth was found for microbial sterility testing, and the endotoxin  
596 values were found below 2.5 EU/mL for reference and GBPD002. To claim Eprex<sup>®</sup>  
597 biosimilar, GBPD002 (Erythropoietin alfa) were analyzed by several identification tests. SDS  
598 PAGE and Western blot were performed for molecular mass and immunochemical detection  
599 analysis. High intensity similar banding pattern were found at near around 34 kDa for both  
600 reference and GBPD002 (Fig. 7A-B). two-dimensional gel electrophoresis showed well  
601 separated four isoform spots focused in the acidic region (pH 4.78-6.54) of the strips for both  
602 reference and GBPD002; though there were few other similar minor bands observed for both  
603 samples (Fig. 7C-D).

604 Peptide mapping was performed by LC/MS-MS to obtain GBPD002 protein sequence  
605 coverage analysis. The results demonstrate 296 MS peaks, spanning 100% protein sequence  
606 coverage and 100% abundance (mol) (Fig. 7E). The pattern of MS peaks was very similar  
607 like those obtained from the reference product Eprex<sup>®</sup> (Fig. 7F-G). Chromatographic pattern  
608 was also analyzed by reverse phase chromatography and results demonstrated similar

609 retention (RT) time for both reference (9.550 min) and GBPD002 (9.558 min) (Fig. 7H), and  
610 suggested similar molecular identity for both proteins. Deglycosylation of N-linked sugars  
611 reduced the protein to 18 kD from the original protein of ~37kD; the reference protein also  
612 showed similar protein bands on deglycosylation as well as for full form (Fig. 8A-B).  
613 Macromolecular aggregation and particle size distribution were analyzed. For Particle size  
614 analysis, Z-average were found  $11.50 \pm 0.81$  nm and  $11.49 \pm 0.28$  nm with the PDI values  
615 0.294 and 0.218 for reference and GBPD002, respectively (Fig.8C-D). Host cell DNA and  
616 host cell protein (HCP) were analyzed by real-time quantitative PCR (RT-qPCR). For Host  
617 cell DNA, three consecutive batches were analyzed and results demonstrated <10 ng/sample  
618 for all 3 batches (Fig. 8E). Three consecutive batches were analyzed and HCP concentrations  
619 were found below the detection limit (Fig. 8F), which has indicated that the rhEPO  
620 preparations were having non-detectable HCP. SEC revealed a single peak in chromatogram  
621 for the reference and GBPD002 product (Fig.8 Gi). There was a noticeable secondary peak  
622 observed for both samples, which can be attributed to the buffer components (Fig. 8 Gii). No  
623 other aggregated and/or degraded peak was found for any samples (Fig. 8 Giii). RPC analyses  
624 revealed the assay for the GBPD002 preparations for 3 consecutive batches were  $103.0 \pm$   
625  $2.0\%$  in comparison with the reference (Fig. 8H).

626

#### 627 *Biofunctional activity:*

628 Comparative functional study was performed by receptor binding assay, *in vitro* cell culture  
629 assay, and *in vivo* bioassay between reference and GBPD002 to prove the similar bio-  
630 functionality between the reference and GBPD002. Receptor binding assay against  
631 immobilized EPO receptor revealed similar types of interaction kinetics for both reference  
632 and GBPD002. The association rate ( $k_a$ ) was found  $2.916E5$  and  $3.124E5$  for reference and  
633 GBPD002, respectively; whereas the dissociation rate was found ( $k_d$ ) were  $2.538E-8$  and  
634  $2.838E-8$  for reference and GBPD002, respectively (Fig. 9A-B). The experiment U-value was  
635 9.

636 The rhEPO-induced proliferation data of TF-1 cell clearly revealed that the GBPD002 and  
637 reference products responded similarly. At a 2 ng/ml and higher concentration, both of the  
638 rhEPO formulations assisted the growth of experimental cells by 3 times of the originally  
639 seeded cell numbers (Figure 9C). The mock-controlled cells, on the other hand, were reduced  
640 to half by number from its original seeding population ( $10^5$ /well). This data has clearly  
641 established that the rhEPO preparations GBPD002 and Eprex<sup>®</sup> are similarly active.

642 For *in vivo* bioassay, mice reticulocytes (Ret) were counted as the primary marker. The mean  
643 values of Ret were found  $3.5 \pm 0.54$  % and  $3.9 \pm 0.85$  % for placebo and control group,  
644 respectively. As expected, for treatment-I (20 IU/ mL), the mean values of Ret were found  
645 increased to  $10.0 \pm 1.17$  % and  $10.3 \pm 0.45$  % for reference and GBPD002, respectively. For  
646 treatment-II (40 IU/ mL), the mean values of Ret were found  $12.5 \pm 0.60$ % for reference and  
647  $12.9 \pm 0.53$  % for GBPD002, respectively. For treatment-III (80 IU/ mL), the mean value of  
648 Ret was found ( $13.5 \pm 0.54$ ) and ( $13.9 \pm 0.45$ ) % for reference and GBPD002, respectively  
649 (Fig. 9D).

650

#### 651 4. DISCUSSION

652 Two rhEPO products were compared to determine biosimilarity. These were the reference  
653 product Eprex<sup>®</sup> (International Nonproprietary Name: epoetin alfa) and a candidate biosimilar  
654 of rhEPO designated as GBPD002. Both were prepared using rDNA technology, expressed in  
655 suspension culture of CHO cells, and purified using a series of validated unit processes. We  
656 have chosen CHO cells because the reference product is also CHO cell derived. Furthermore,  
657 it has been shown that CHO cell has similar level of glycosylation-related transcripts to  
658 human cells [35], which may help to obtain the expressed protein with desired post-  
659 translational modification (PTM). CHO cells can also evade infection of human virus through  
660 minimizing the expression of many important viral entry gene that improves final product  
661 safety [35].

662 Several techniques have been in use for development and isolation of stable mammalian cell  
663 lines, e.g., FACS, automated colony picker, serial infinite dilution etc. FACS needs addition  
664 of foreign biological reagent like antibody as well as application of photo-beam, which may  
665 harm cell viability and cellular integrity [36]. FACS and automated clone picker (such as  
666 Clonepix and CellCelector) also need highly expensive instrumentation. On the contrary,  
667 classical limiting dilution technique, though time consuming but does not require expensive  
668 instrumentation, is the best method to harvest single clones satisfying the regulatory  
669 requirements [37]. Therefore, this classical method for clonal isolation of desired cells was  
670 followed in the study. A common practice is to use antibiotic challenge at a suitable  
671 concentration to select the stably-transfected cells. Some low-expressing or unstably-  
672 transfected or non-transfected cells also can survive in this process, albeit with a lower  
673 frequency. In this study we have used highest possible concentration of antibiotic challenge  
674 to identify the highest-expressing cells with only a few cells surviving on challenge. Such



675 high level of selection challenge was also applied with Methotrexate (MTX) selection for  
676 dihydrofolate reductase (DHFR) compromised cells resulting selection of high-level protein  
677 expressing clones [38-40].

678 The cell growth rate was compromised during the high-concentration antibiotic challenge,  
679 which is a common phenomenon for such protocol [41] however, during the subsequent  
680 adaptation process, the growth rates for the clones were revived. The isolated clones usually  
681 go for evaluation of growth rate and the specific productivity per cell to identify the best  
682 desired clone for further development to MCB and WCB. We have applied a new screening  
683 technique where the clones whose growth kinetics and specific expressions were comparable  
684 were subjected for a capacity test in a fixed media depriving fresh supplements and with  
685 accumulating load of metabolites. This stress-test revealed a superior clone over the others,  
686 which performed exceptionally in batch format to produce high level of expression for rhEPO  
687 ( $1.24 \pm 0.16$  g/L). To the best of our knowledge, this is the highest level of titer for rhEPO  
688 expression using a standard fed-batch wave bioreactor system. Searching of available  
689 information in web space revealed the highest level of rhEPO titer was  $\sim 696$  mg/L for a  
690 transient expression system [42].

691 In 2013, the World Health Organization (WHO) published a Technical Report Series (TRS  
692 978: Recommendations for the Evaluation of Animal Cell Cultures as Substrates for the  
693 Manufacture of Biological Medicinal Products and for the Characterization of Cell Banks)  
694 that states, “For proteins derived from transfection with recombinant plasmid DNA  
695 technology, a single, fully documented round of cloning is sufficient provided product  
696 homogeneity and consistent characteristics are demonstrated throughout the production  
697 process and within a defined cell age beyond the production process”. Accordingly, we have  
698 successfully completed the isolation of single clones and expanded them, and finally  
699 characterized them to qualify as the MCB. Cell cultures are maintained for not more than 60  
700 days for a N-6 systems (generally, each step may consume 7 days each for up to N-5 steps  
701 ( $5 \times 7 = 35$  days), and the 6<sup>th</sup> step may consume 18 days, total  $35 + 18 = 53$  days) in  
702 manufacturing train of a commercial batch (2000 L) in fed-batch process. Continuous batch  
703 run time in perfusion mode also generally does not run for more than 6 months (180 days).  
704 Here, we have characterized the MCB for around 200 days, which can be used for  
705 manufacturing of rhEPO either in fed-batch or continuous mode without compromising yield  
706 and product quality.



707 Genomic plasticity is inherent to any immortalized mammalian cell lines [43-45]. Recently,  
708 CHO genome drafts revealed significant chromosomal heterogeneity among different CHO  
709 cell lineages and genomic landscapes [35, 46]. Therefore, clonally isolated production cells  
710 will undergo some genetic heterogeneity over the age, though different clones will withstand  
711 original property for different ages[44, 45, 47-49]. We presumed that the exceptional stability  
712 of our MCB for product substrate is due to the acquired fitness of the clone associated with  
713 the sequence of multiple stringent screening methods during clonal isolation. This notion is  
714 supported by a recent study, which has reported that a secondary screening of an established  
715 MCB produced high-titer stable clones without affecting the quality attributes of the desired  
716 product [50].

717 A working guideline has been suggested for setting the specification for the qualification of  
718 biological drugs [51]. Further, through a case study, the quality assessment framework has  
719 been suggested for rhEPO biosimilar characterization by regulatory experts [52]. We have  
720 followed these suggestions and performed the characterization with the scientifically  
721 reasonable experimental methods. One of the first critical quality attributes was the cell  
722 substrate. As mentioned in other reference, [53] ICH guideline Q5D Section 2.1.3 states that  
723 “For recombinant products, the cell substrate is the transfected cell containing the desired  
724 sequences, which has been cloned from a single cell progenitor”, we have deduced the gDNA  
725 and cDNA sequences for the rhEPO insertion in the MCB, which was completely matched  
726 with the reference sequence. Amino acid sequencing from the formulated rhEPO sample also  
727 showed 100% match with the protein sequence in data bank. These data confirmed the cell  
728 substrate conformity for the MCB and WCB for rhEPO production.

729 The identification of the rhEPO was done with orthogonal methods SDS-PAGE, Western  
730 blot, dot blot, Elisa, as well as UHPLC. All methods explicitly confirmed the similar  
731 molecular pattern and immunochemical properties for GBPD002 and Eprex®. The biosimilar  
732 comparison included peptide mapping and mass spectrometry procedures to assess amino acid  
733 sequence and mass spectra, which were found highly similar with a few minor variations.  
734 Sialic acid content is very important components among the glycosylation matrix, and is  
735 directly connected to the half-life of the rhEPO for in vivo functionality. Similar charge  
736 variant profiles of GBPD002 and Eprex® suggested likely similar sialic acid contents for the  
737 both rhEPO preparations. Since both products likely have similar sialic acid content,  
738 therefore, such minor variations can be attributed to differences in sugar residues related to  
739 glycosylation. PNGase F digestion of protein followed by molecular mobility detection has

740 been used for analysis of glycosylation pattern of proteins [54]. Accordingly, we have also  
741 applied the same method for determining comparability for glycosylation profile of  
742 GBPD002 and Eprex®. The molecular mobility and immunodetection patterns of full-form  
743 and deglycosylated form were found highly similar for GBPD002 and Eprex® thereby  
744 suggesting that the differences in glycosylation between the two products are minimum, if  
745 there is any.

746 Glycosylation profile has been found important for protein folding that affects protein  
747 stability and receptor interaction. However, for rhEPO exact glycosylation between two  
748 products seems not rational as discussed below: (1) Non-glycosylated Escherichia coli-  
749 expressed rEPO have demonstrated that it is well folded and possess the same *in vitro*  
750 biological activity as the CHO cell-derived glycosylated rEPO [55, 56]. (2) Multiple different  
751 EPO-glycoforms were identified in circulating systems of human [57]. (3) The glycosylation  
752 profile of human serum EPO is remarkably different from the rhEPO [58]. (4) There were no  
753 significant differences found for *in vivo* biofunctionality for several approved rhEPO  
754 preparations (12 different sources) despite having differences in glycosylation profile [59].  
755 (5) Several clinical studies did not find any significant differences between pharmacokinetic  
756 and pharmacodynamic properties for different rhEPO [60-66]. (6) Confident glycoform  
757 analysis is a very critical process, and multiple parallel methods are preferred for obtaining  
758 confident PTM profiles using mass spectrometry analysis [67, 68], which might not be  
759 supportive for cost-benefit perspective. (7) Further, it has been suggested that the separation  
760 of rEPO into pure glycoforms is not feasible even with the most advanced methods due to the  
761 heterogeneity of the three N-glycans [69]. (8) Minor differences in glycosylation may not be  
762 affecting the final product quality significantly. In reality, such type of minor variations are  
763 not infrequent for EMA and US FDA approved biosimilars [70]. A recent study reported the  
764 batch-to-batch variability in quality parameters of marketed rhEPO reference medicines,  
765 Eprex®/Erypo® (Janssen-Cilag, High Wycombe, UK) and NeoRecormon® (epoetin beta;  
766 Roche Registration Limited, Welwyn Garden City, UK), and two biosimilars, Binocrit®  
767 (Sandoz GmbH, Kundl, Austria) and Retacrit® (Hospira UK Limited, Maidenhead, UK), and  
768 found batch-batch minor variability for experimental products [71]. Therefore, it has been  
769 suggested that, it is not essential to have identical glycosylation pattern for two molecules to  
770 be considered biosimilar though the biofunctionality must conform to offset the residual  
771 uncertainty [32, 52].

772 To offset the residual uncertainty, we have performed the receptor binding assay, and bio-  
 773 functional assay in mammalian cell as well as in mice model. The receptor binding assay, and  
 774 the *in vitro* and *in vivo* biofunctionality assays clearly demonstrated very similar receptor  
 775 binding affinity and biological activities for GBPD002 and Eprex<sup>®</sup>. EPO-specific antidrug  
 776 antibodies (ADAs) are generated *via* immunogenicity of an administered rhEPO preparation,  
 777 and they likely cross-react with endogenous EPO in human. Aggregation present in rhEPO  
 778 preparation were held responsible for the ADA effect. These ADAs, in severe cases, lead to  
 779 clinically relevant autoimmunity known as pure red cell aplasia (PRCA) [72-74]. Three  
 780 orthologous analysis procedures, *viz.*, Western blot, dynamic light scattering and SEC-HPLC  
 781 were applied for detecting any high molecular aggregates in our final products, and the  
 782 results confirmed that GBPD002 did not contain any such macromolecular entity. Together,  
 783 all the results clearly demonstrated the biosimilarity of GBPD002 and Eprex<sup>®</sup>.

## 784 5. CONCLUSION

785 The study showed systematic development of a rhEPO biosimilar from the DNA preparation  
 786 to the formulation following regulatory guidelines. Higher yield of the relevant process can  
 787 help reducing the product cost and may socioeconomically support the global communities  
 788 who are in need of rhEPO at a comparatively cheaper price. The physicochemical and  
 789 biofunctionality results suggested the comparable biosimilarity between GBPD002 and  
 790 Eprex<sup>®</sup>. Nevertheless, the complete similarity for biofunctionality should be investigated in a  
 791 clinical study with ‘head-to-head’ comparability between GBPD002 and the reference and  
 792 should include appropriate endpoints, *e.g.*, hematocrit and hemoglobin. Such a study would  
 793 reveal clinically noteworthy variance in the *in vivo* quality attributes for the rhEPO  
 794 preparations.

796 **Table 1:** Head-to-Head analysis of Eprex<sup>®</sup> and GBPD002.

Test Parameters	Eprex <sup>®</sup>	GBPD002
<b>Structural Analysis</b>		
Molecular mass and immunochemical properties analysis by SDS PAGE and Western Blot	High intensity similar banding pattern were found at near around 37 kDa	High intensity similar banding pattern were found at near around 37 kDa
Peptide Mapping by LC-MS/MS	100% protein sequence coverage and 100% abundance (mol)	100% protein sequence coverage and 100% abundance (mol)

Isoform pattern analysis by 2D gel electrophoresis (2D GE)	Well separated, four isoform spots focused in the acidic region (pH 4.78-6.54) of the strip	Well separated, four isoform spots focused in the acidic region (pH 4.7-6.5) of the strip
Chromatographic pattern analysis by HPLC	Chromatogram pattern and retention time are similar Retention time (RT) time: 9.550	Chromatogram pattern and retention time are similar  Retention time (RT) time: 9.558
<b>Impurity Analysis</b>		
Aggregation pattern analysis by size-exclusion chromatography (SEC)	Single similar type of peak was found	Single similar type of peak was found
Particle size analysis by Malvern zeta sizer	No aggregated and degraded peak was found Z-average was found 11.50 ±0.81 nm with the PDI values 0.294	No aggregated and degraded peak was found Z-average was found 11.49 ± 0.28 nm with the PDI values 0.218
<b>Functional Analysis</b>		
Kinetics analysis by surface plasmon resonance (SPR)	The association rate (ka) was 2.916E <sup>5</sup> and the dissociation rate was (kd) 2.538E <sup>-8</sup>	The association rate (ka) was 3.124E <sup>5</sup> and the dissociation rate was (kd) 2.838E <sup>-8</sup>
Cell culture-based assay	High cell proliferation was found at dose concentration 2 ng/mL	High cell proliferation was found at dose concentration 2 ng/mL
In-vivo study by mice model	Reticulocytes (Ret) were counted as the primary marker. Ret: Placebo: 3.5 ± 0.54 %. Control: 3.9 ± 0.85 %. Treatment-I (20 IU/ mL: 10.0 ± 1.17 %. Treatment-II (40 IU/ mL: 12.5 ± 0.60 %. Treatment-III (40 IU/ mL: 13.5 ± 0.54 %.	Reticulocytes (Ret) were counted as the primary marker. Ret: Placebo: 3.5 ± 0.54 %. Control: 3.9 ± 0.85 %. Treatment-I (20 IU/ mL: 10.3 ± 0.45 %. Treatment-II (40 IU/ mL: 12.9 ± 0.53 %. Treatment-III (40 IU/ mL: 13.9 ± 0.45 %.
Assay by reverse phase chromatography	Chromatographic pattern complies the testing acceptance criteria	Chromatographic pattern complies the testing acceptance criteria (103.0 ± 2.0%) for 3 consecutive batches were 103.0 ± 2.0%.

799 **Author contribution:** Kakon Nag and Naznin Sultana conceptualized the project. Md. Jikrul  
800 Islam, Md. Maksusdur Rahman Khan, Md. Mashfiqur Rahman Chowdhury, Md. Enamul Haq  
801 Sarker and Samir Kumar designed experiments and analyzed data. Md. Maksusdur Rahman  
802 Khan, Rony Roy and Md. Shamsul Kaunain Oli contributed to cell line development, and  
803 Md. Mashfiqur Rahman Chowdhury contributed to cell bank development and scale-up of the  
804 cell culture. Md. Enamul Haq Sarker, Samir Kumar, Sourav Chakraborty and Habiba Khan  
805 performed downstream process development and manufacturing steps. Md. Jikrul Islam and  
806 Ratan Roy performed quality control experiments. Kakon Nag, Naznin Sultana, Md. Jikrul  
807 Islam, Md. Maksusdur Rahman Khan, Md. Mashfiqur Rahman Chowdhury, Md. Enamul Haq  
808 Sarker and Samir Kumar wrote the manuscript. Bipul Kumar Biswas, Md. Emrul Hasan  
809 Bappi and Mohammad Mohiuddin assured the quality management system of relevant  
810 activities.

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825

## 826 **7.0 DISCLOSURE**

827 The authors have nothing to disclose.

828

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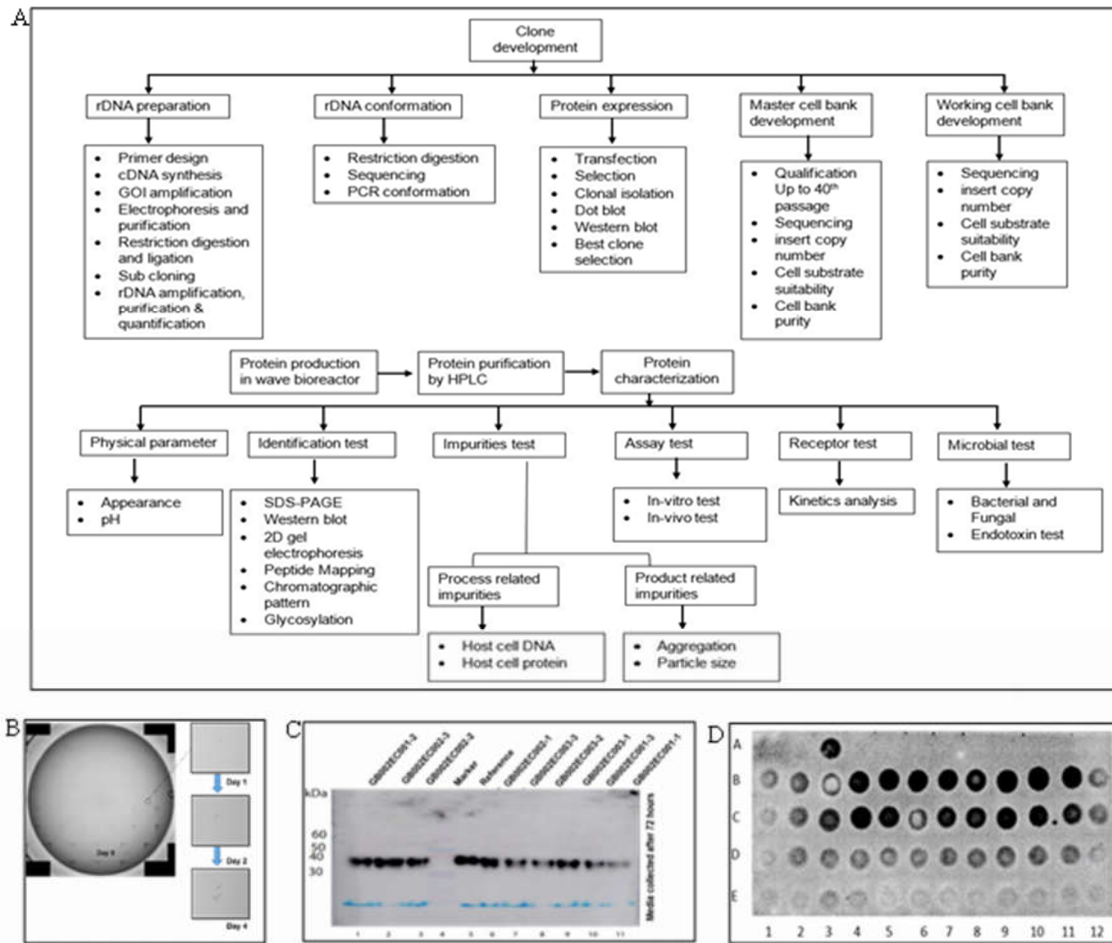
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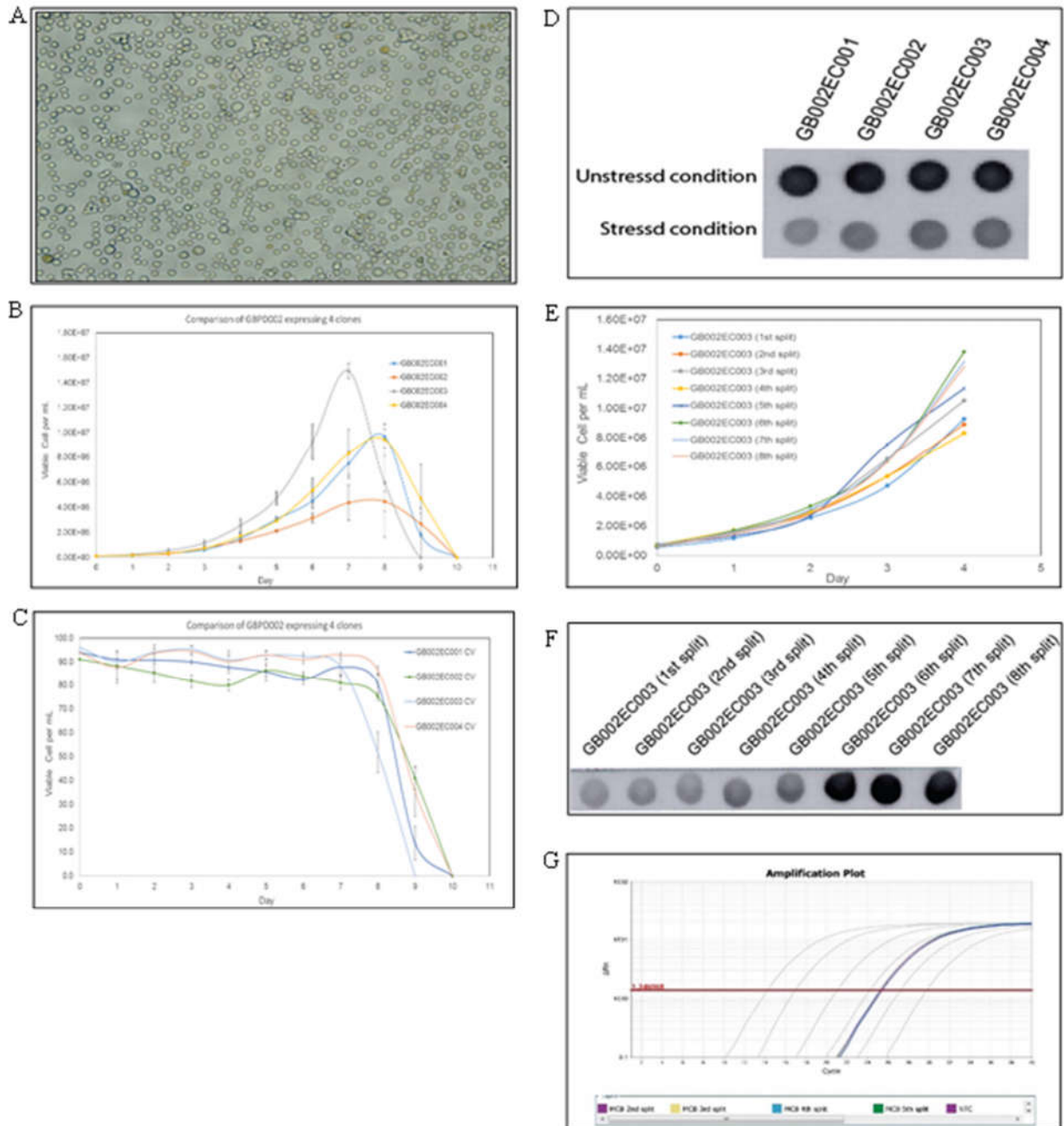
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1025 **Figure 1:** (A) Work break down structure of development and qualification of GBPD002 (B)  
 1026 Clonal isolation, (B) Western blot analysis of isolated expression clones. (C) Dot blot analysis  
 1027 for protein expression to select the best clone.

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1030 **Figure 2:** (A) Microscopic morphology of the clone, (B) Growth curve analysis of selected  
 1031 clones, (C) Viable percentage analysis of selected clones, (D) Expression level of rhEPO in  
 1032 stressed and unstressed condition (E) Growth curve analysis of MCB of first 8 splits (F)  
 1033 Protein expression analysis of MCB of first 8 splits (G) Copy number analysis amplification  
 1034 plot of the rhEPO gene in cells from first 5 splits.

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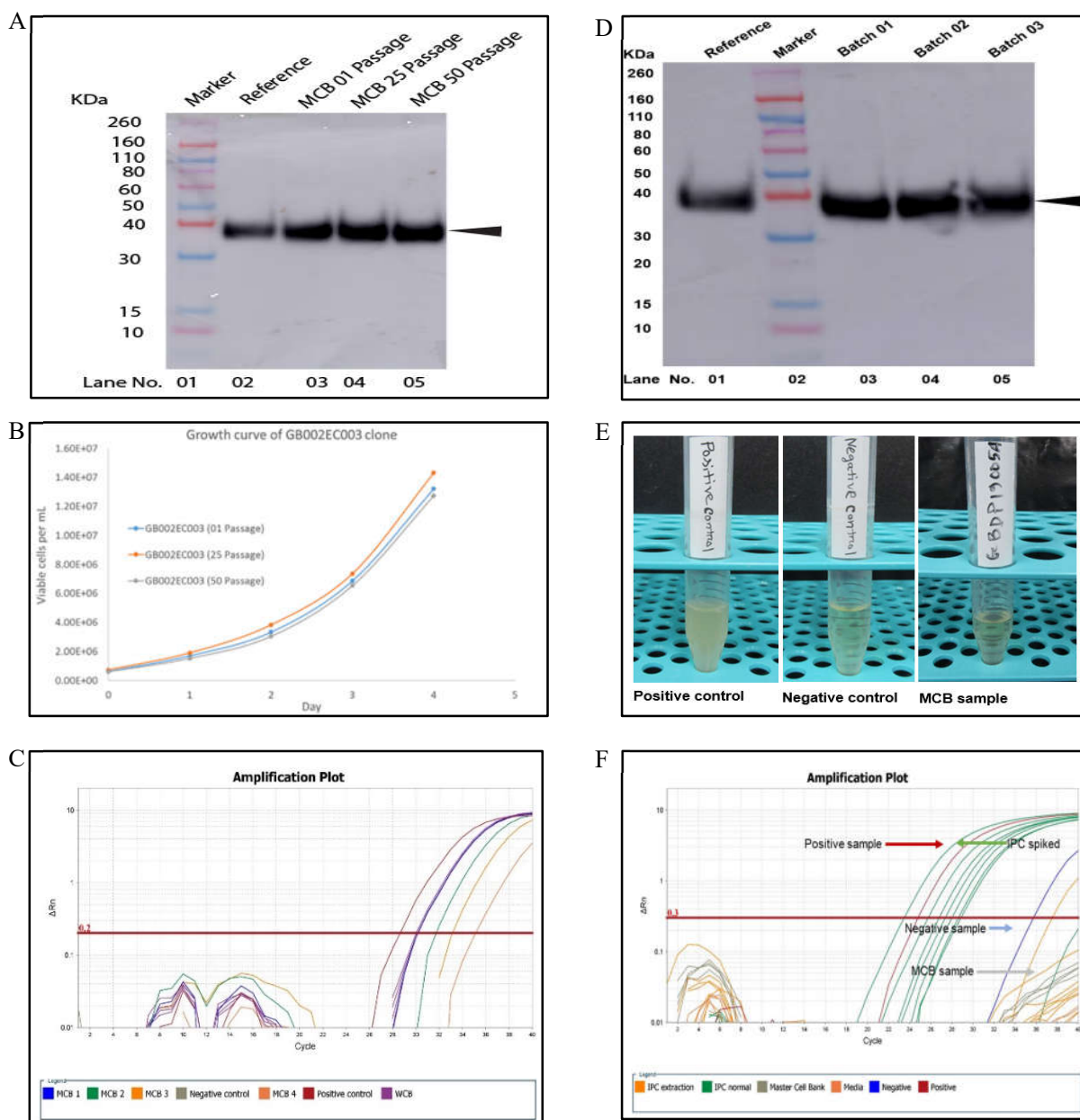
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1058 **Figure 3:** Cell line qualification of GBPD002. (A) Stability analysis for protein expression of  
 1059 MCB up to 50<sup>th</sup> passages, (B) Growth kinetics of MCB for different passage, (C)  
 1060 Amplification plot of copy number analysis for rhEPO-insert of MCB, (D) Western blot  
 1061 analysis of 3 consecutive batches of MCB, (E) Microbial purity analysis of MCB by sterility  
 1062 testing, (G) Mycoplasma analysis of MCB.

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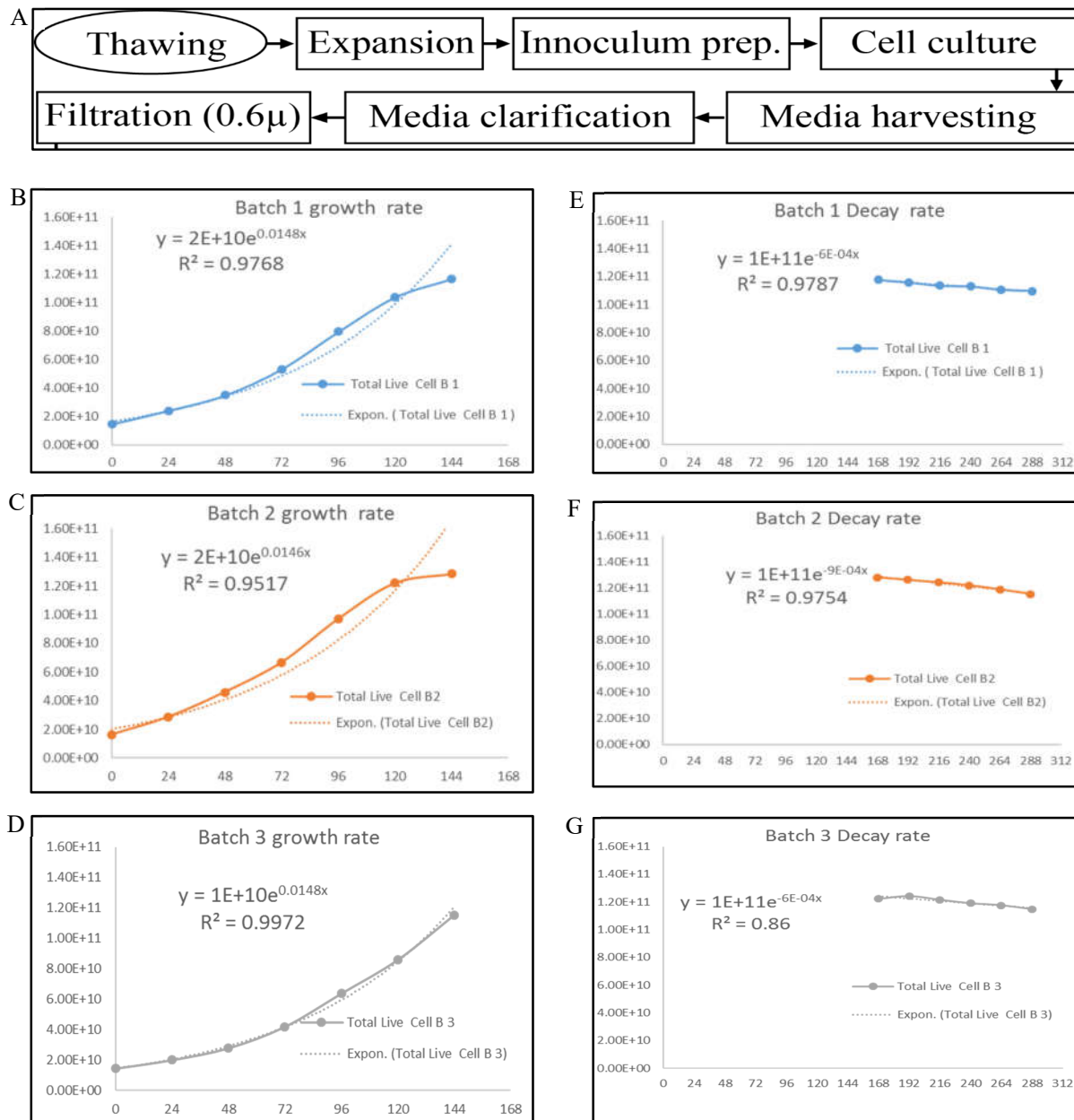
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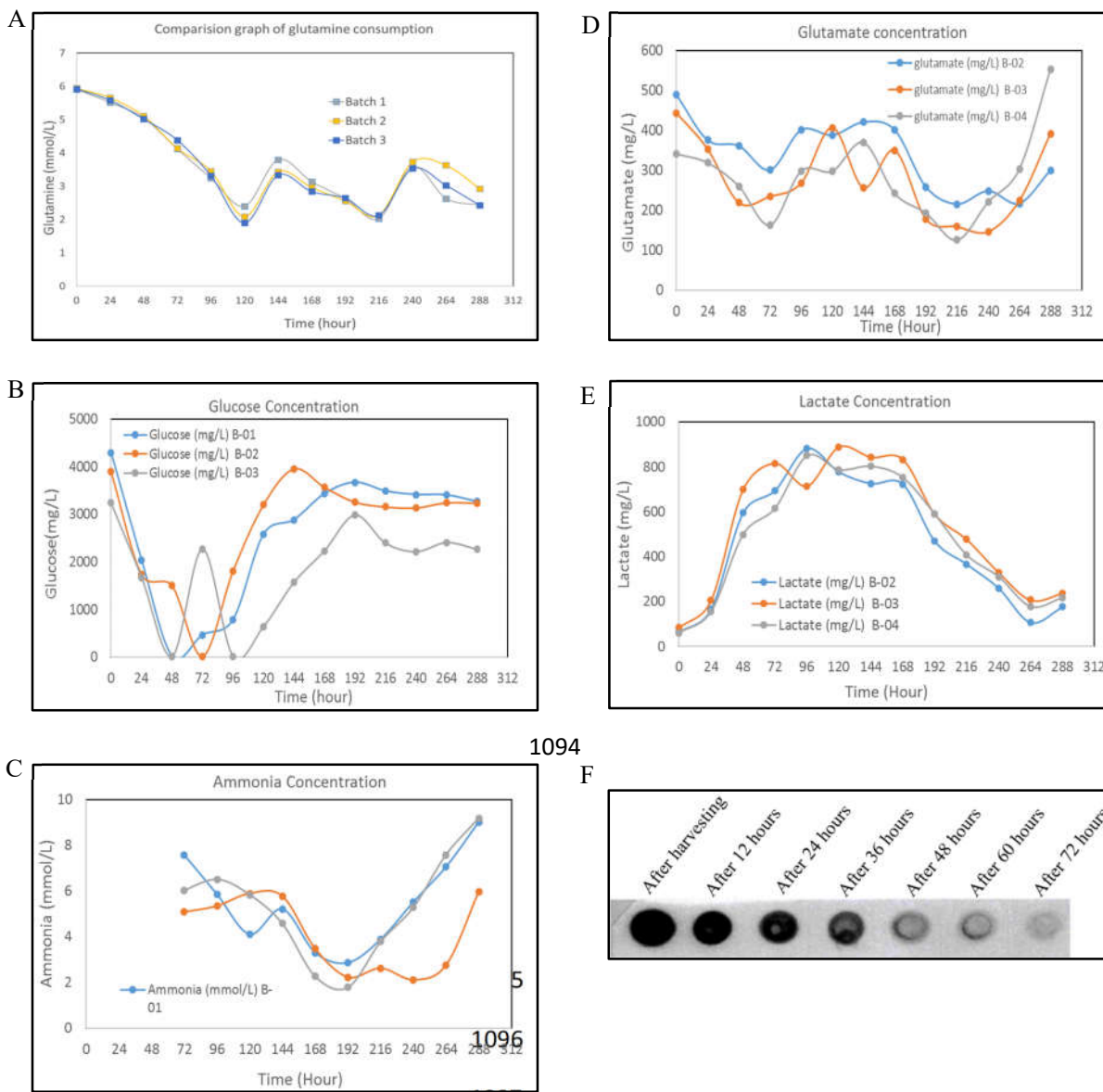
**Figure 4:** (A) Flow chart of Upstream process from thawing of WCB to harvest of cell culture media, (B) Growth rate of Batch 1 (C) Growth rate of Batch 2, (D) Growth rate of Batch 3, (E) Decay rate of Batch 1, (F) Decay rate of Batch 2, (G) Decay rate of Batch 3.

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1098 **Figure 5:** Metabolites analysis of batch 1 to 3. (A) Glutamine (B) Glucose (C) Ammonia, (D)  
 1099 Glutamate (E) Lactate (F) Sample holding stability after 12-hour interval at room temperature.

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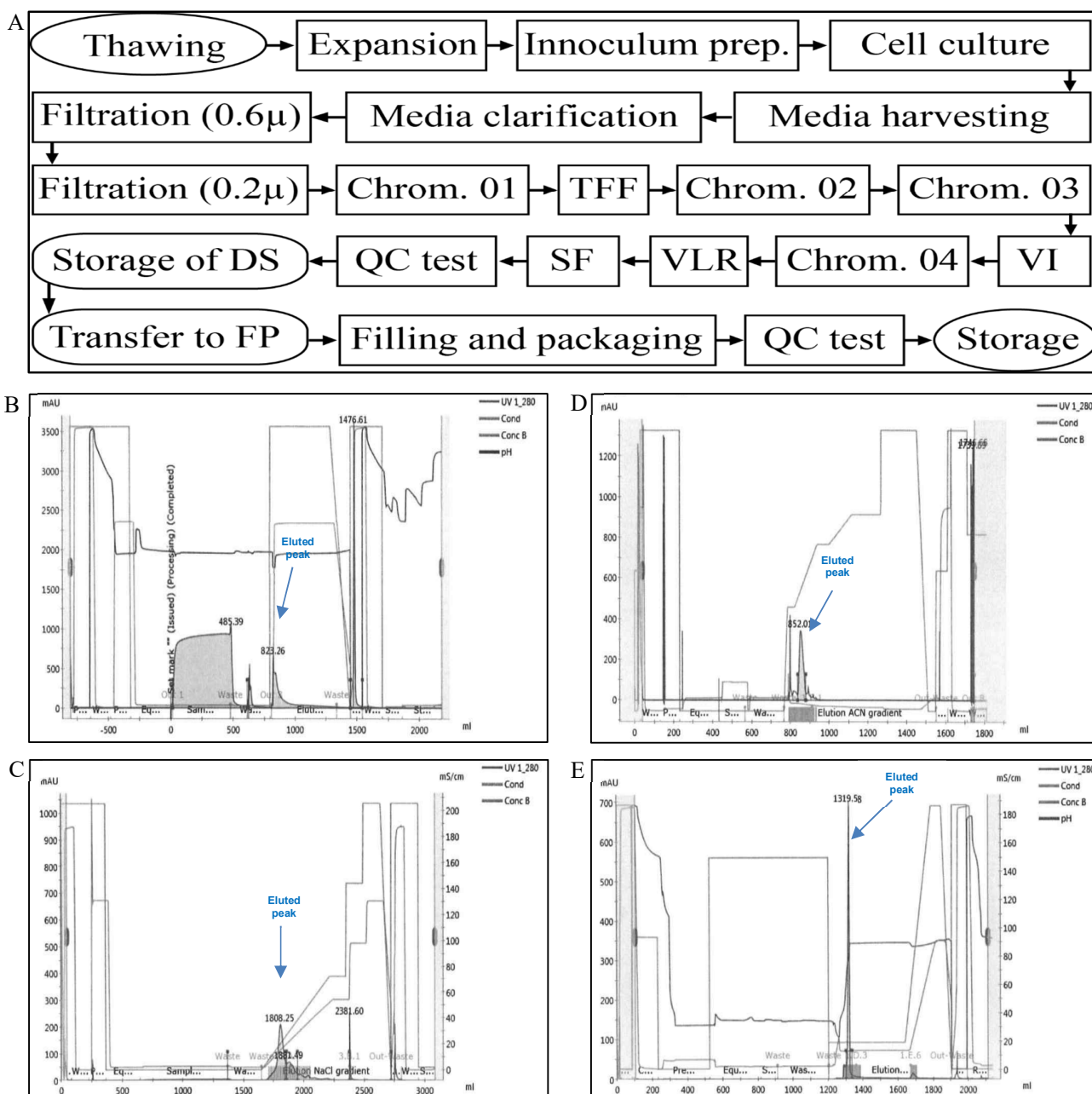
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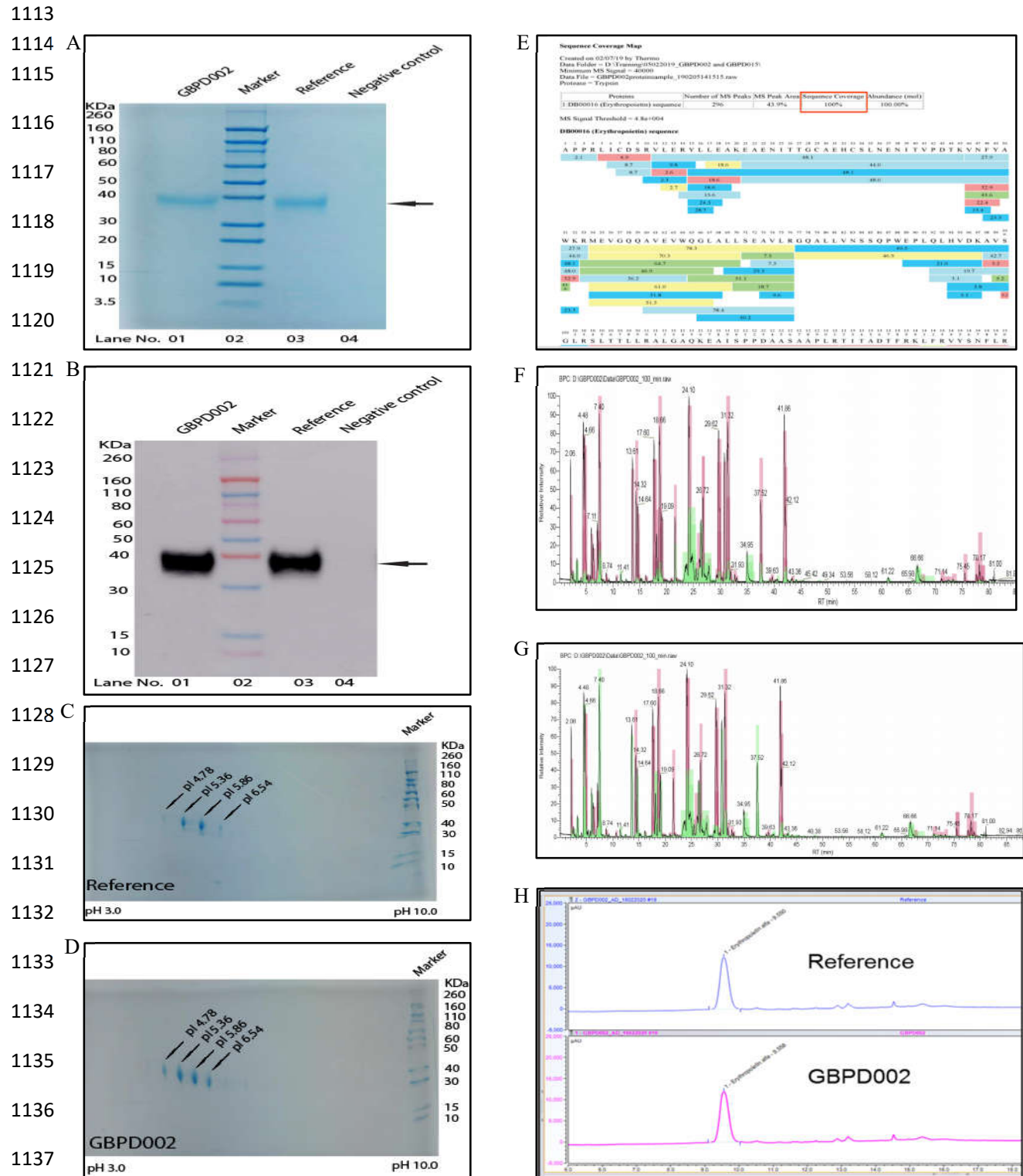
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1106 **Figure 6:** (A) Process flow of downstream process for GBP D002, (B) Representative  
 1107 chromatogram of affinity chromatography (AFC), (C) Representative chromatogram of anion  
 1108 exchange chromatography (AEX), (D) Representative chromatogram of reversed-phase  
 1109 chromatography (RPC), (E) Representative chromatogram of cation exchange  
 1110 chromatography (CEX) in the FPLC purification process.

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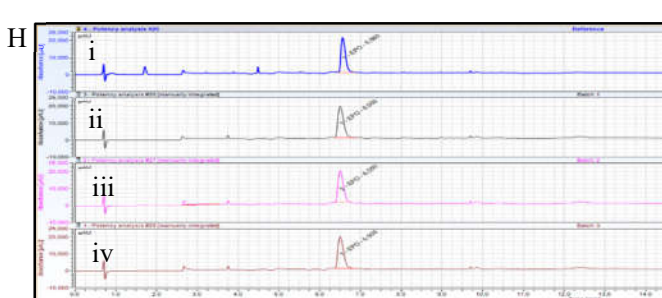
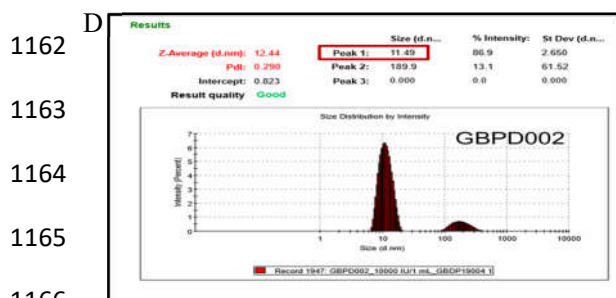
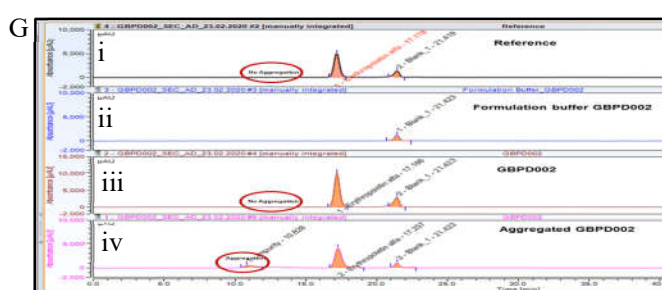
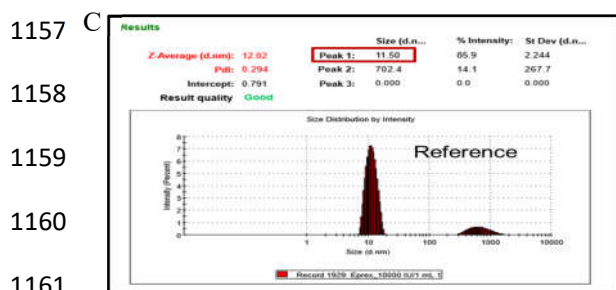
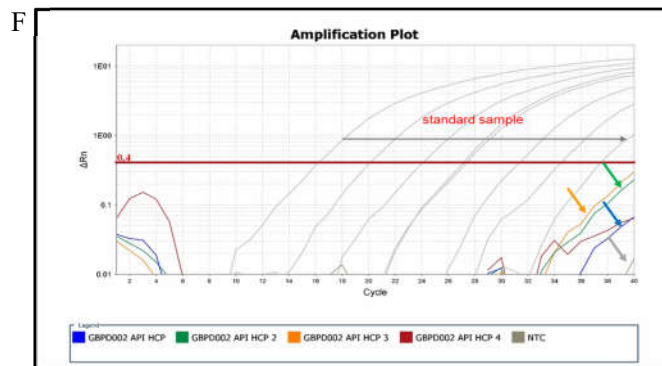
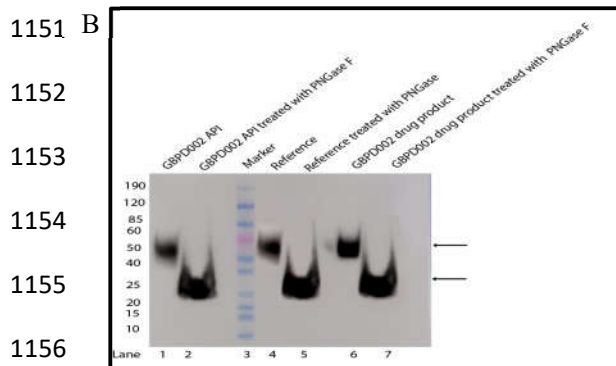
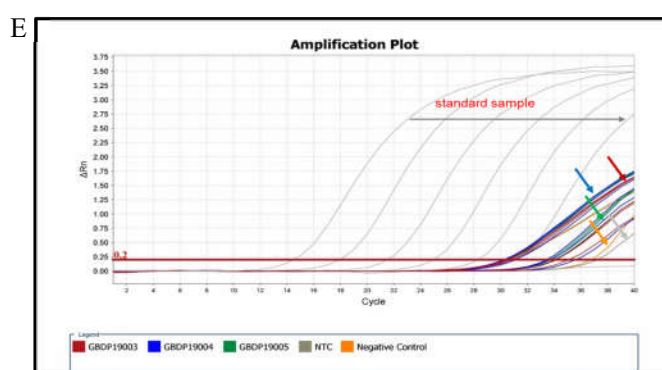
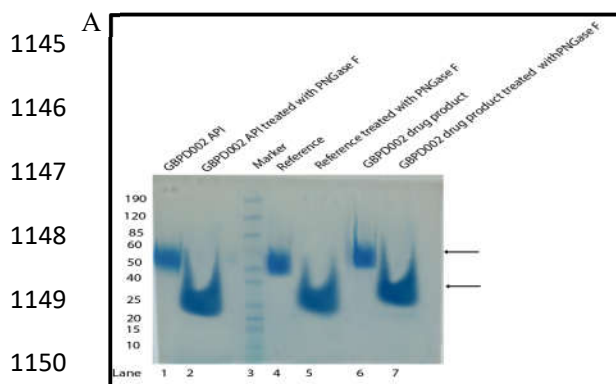
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1138 **Figure 7:** Comparative analysis for identification test between reference product Eprex<sup>®</sup> and  
1139 GBP D002. (A) SDS PAGE analysis, (B) Western Blot analysis, (C) Isoform pattern analysis  
1140 of Eprex<sup>®</sup>, (D) Isoform pattern analysis of GBP D002, (E) Peptide mapping (amino acid  
1141 sequence) of GBP D002, (F) Peptide mapping chromatogram (relative intensity) of Eprex<sup>®</sup>,

1142 (G) Peptide mapping chromatogram (relative intensity) of GBPD002, (H) Comparative study  
 1143 for chromatographic pattern analysis by Reverse-phase chromatography (RPC).

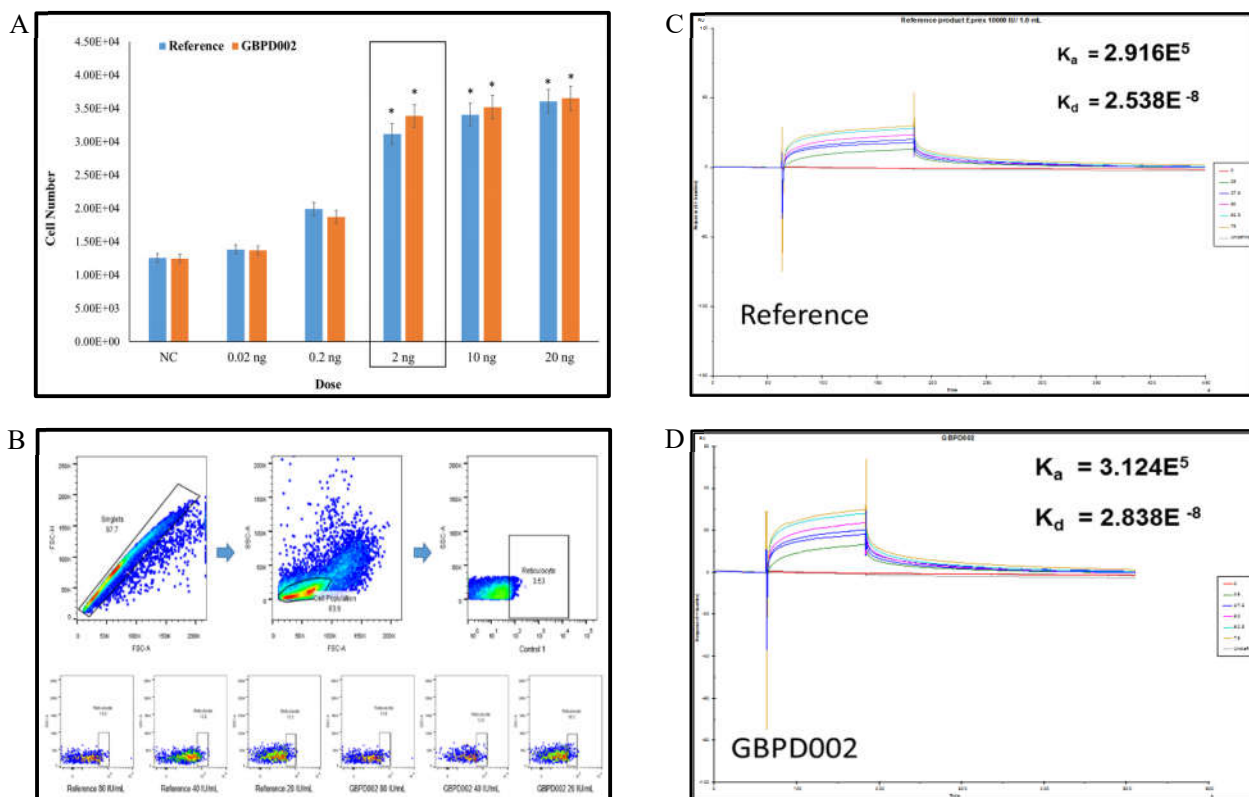
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1168 **Figure 8:** Comparative analysis for glycosylation pattern, process- and product-related  
 1169 impurities between Eprex<sup>®</sup> and GBPD002. (A) Glycosylation pattern analysis by SDS PAGE,  
 1170 (B) Glycosylation pattern analysis by western blot, (D) Particle size analysis by Malvern zeta  
 1171 sizer of Eprex<sup>®</sup>, (E) Particle size analysis by Malvern zeta sizer of GBPD002, (E) Host cell

1172 DNA analysis (process-related impurities), (F) Host cell protein analysis (process-related  
1173 impurities), (G) Aggregation pattern analysis (product-related impurities) by size-exclusion  
1174 chromatography (SEC), (H) Potency and quantity analysis by Reverse-phase chromatography  
1175 (RPC).

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1177 **Figure 9:** Comparative analysis of bio-functionality between Eprex® and GBP002. (A)  
1178 Graphical presentation of cell culture-based assay, (B) *In-vivo* study in mice model, (C)  
1179 Analysis of receptor binding kinetics by SPR of Eprex®, (D) Analysis of receptor binding  
1180 kinetics by SPR of GBP002.