Determination of the affinity constants for phage display albumin-binding peptides

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Background. Phage display technology has been established as a powerful screening approach to select ligands or peptides for binding to proteins. Despite rapid growth in the field, there has been a relative dearth of quantitative criteria to measure the effectiveness of the process of phage display screening. Since Human serum albumin (HSA) has been extensively studied as a drug carrier to extend the plasma half-life of protein therapeutics, use of phage display technology is required for identifying albumin-binding peptides as the very promising strategy of albumin-binding against albumin fusion. The construction of albumin-binding drug requires assessment of enough number of HSA-binding peptide (HSA binder) candidates for conjugation with therapeutic proteins. The use of the linear epitope mapping method has allowed researchers to discover many HSA-binding peptides. However, it may be inefficient to select these peptides based on sequence identity via randomly sequencing individual phage clones from enrichment pools.

Method. Here a simple assessment method to facilitate phage display selection of HSA-binding peptides was recommended. With experimentally determined phage titer, one can calculate the specificity ratios, the recovery yields and the relative dissociation constants, which are defined as quantitative criteria for panning and characterization of the binding phage fused peptides.

Results. Consequently, this approach may not only enable more rapid and low-cost phage display screening, but also efficiently reduce pseudo-positive phages selected as HSA binders for conjugation with therapeutic proteins.

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21   **Abstract**

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23   to select ligands or peptides for binding to proteins. Despite rapid growth in the field, there has

24   been a relative dearth of quantitative criteria to measure the effectiveness of the process of phage

25   display screening. Since Human serum albumin (HSA) has been extensively studied as a drug

26   carrier to extend the plasma half-life of protein therapeutics, use of phage display technology is

27   required for identifying albumin-binding peptides as the very promising strategy of albumin-

28   binding against albumin fusion. The construction of albumin-binding drug requires assessment of

29   enough number of HSA-binding peptide (HSA binder) candidates for conjugation with

30   therapeutic proteins. The use of the linear epitope mapping method has allowed researchers to

31   discover many HSA-binding peptides. However, it may be inefficient to select these peptides

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33   pools.

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35   peptides was recommended. With experimentally determined phage titer, one can calculate the

36   specificity ratios, the recovery yields and the relative dissociation constants, which are defined as

37   quantitative criteria for panning and characterization of the binding phage fused peptides.

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39   display-screening, but also efficiently reduce pseudo-positive phages selected as HSA binders for

40   conjugation with therapeutic proteins.

Introduction

42

43   Human serum albumin (HSA) is a 67 KDa globular protein with a concentration is of 3.4 to

44   5.4 g/dL in blood, accounting for about 60% of the total protein in blood serum. Its long half-life,

45   19~20 days, makes HSA an ideal carrier to extend the half-lives of therapeutic proteins

46   (Varshney et al, 2010; Dennis et al, 2002; Larsen et al, 2016; Cho et al, 2022). Both HSA fusion

47   and HSA binding technologies have been developed to reduce the clearance rate of peptide and

48   protein drugs. HSA fusion technologies, e.g. granulocyte colony-stimulating factor-HSA

49   (Albugranin) (Halpern et al, 2002), growth hormone-HSA (Albutropin) (Osborn et al, 2002),

50   interleukin 2-HSA (Albuleukin) (Melder et al, 2005), interferon-HSA (Albuferon-α) (Osborn et

51   al, 2002), glucagon-like peptide-1 receptor agonists-HSA (Yu et al, 2018) and clotting factor IX-

52   HSA (Pasca et al, 2022) have been achieved through expression of HSA-fused proteins via gene

53   recombination. In contrast, HSA-binding technologies attach an HSA binding peptide or protein

54   domain rather than HSA itself to therapeutic proteins, enabling them to bind to HSA through

55   non-covalent interactions (Hoogenboezem et al, 2018). For example, AB-Fab is trastuzumab-Fab

56   fused to HSA binding peptide (AB) (Nguyen et al, 2006), scDb-ABD is the albumin-binding

57   domain of streptococcal protein G (ABDs) fused to a bispecific single chain diabody (Stork et al,

58   2007) and AlbudAb/IL-1ra is interleukin-1 receptor antagonist fused to serum albumin-binding

59   domain antibodies (AlbudAbs) (Holt et al, 2008).

60   Which HSA technology is right for extending in vivo life of a protein drug? Walker *et al*.

61   compared such two forms of recombinant IFN-α2b: serum albumin-binding domain antibodies

62   fused to IFN-α2b (AlbudAbs- IFN-α2b) and HSA directly fused to IFN-α2b (HAS-IFN-α2b).

63 They found that AlbudAbs-IFN-α2b showed 18-fold higher potency in cell reporter assays, 1.5-

64 fold increased half-life in rat PK studies and 5.8-fold greater antiviral efficacy in the EMCV

65 assays (Encephalomyocarditis virus assay for antiviral activity) (Walker et al, 2010). Shi *et al.*

66 compared a series of protein drugs created by fusion (covalent) and binding (non-covalent) to

67 HSA and found that the HSA-fusion proteins showed a more significant loss in binding capacity

68 to their receptors, which related directly to their pharmacological action. The decreased

69 pharmaceutical effects of HSA-fusion drugs were different than what was observed for HSA

70 binding proteins because the HSA molecule is much larger than HSA binders resulting in steric

71 hindrance and structural heterogeneity. In addition, the HSA-binding peptides are smaller in size

72 and do not greatly increase the size of recombinant proteins, thus often present an advantage in

73 their ability to diffuse rapidly into tissues (Shi et al, 2008).

74   Due to the binding properties with various ligands, HSA generally transports endogenous and

75 exogenous compounds, which might be toxic in the unbound state, but non-toxic as albumin-

76 bound. Thus HSA may serves as a circulating depot for improving the pharmacokinetic behavior

77 of a variety of drugs, including: drug half-life in the bloodstream, regulating drug efficacy and

78 decreasing drug toxicity, which based on the unbound substances are the pharmacologically

79 active moieties. Since HSA-binding technologies have been shown to efficiently improve the

80 half-life of protein drugs, it is essential to obtain high affinity and specificity of HSA binding

81 peptides or HSA binders. Phage display technology has been successfully applied to antibody

82 library and random peptide library technologies since its emergence in 1985 (Zhang et al, 2010;

83 Smith, 1985; Kehoe et al, 2005), and provides a convenient platform for screening HSA- binding

84   peptides with the required properties. The use of the linear epitope mapping method has allowed

85   researchers to discover many HSA-binding peptides (Dennis et al, 2002). However, it may be

86   inefficient to select these peptides based on sequence identity via randomly sequencing

87   individual phage clones from enrichment pools. In this study, the protocols to isolate HSA-

88   binding peptides based on phage titer and identify the relative affinity of albumin-binding

89   peptide were developed.

90   Phage display technology has been established as a powerful screening approach to select

91   ligands or peptides for binding to proteins. Despite rapid growth in the field, there has been a

92   relative dearth of quantitative criteria to measure the effectiveness of the process of phage

93   display panning. It is also necessary to measure directly the target-binding-affinity constants of

94   the phage-fused-peptide rather than the artificial binding-peptide synthesized after phage display

95   screening. This study focus on developing general criteria for the performance of rapid and cost-

96   effective phage display screening.

97

98   **Materials and Methods**

99   **Phage library and bacteria strains**

100   The Ph.D.-7 Phage Display Library Kit including the Heptapeptide Phage Display Library

101   and *E. coli* ER2738 host strain were purchased from New England Biolabs. ER2738 with F

102   factor, which confers tetracycline resistance, can grow in LB-Tet medium (1% bacto-tryptone,

103   0.5% yeast extract, 0.5% NaCl, 20 g/mL tetracycline) and is used for M13 phage propagation.

104   Since the library phage carries the *lacZ* gene, phage plaques appear blue when the phage-

105   infected ER2738 cells are plated on LB-Tet-IPTG-X-gal media (1% bacto-tryptone, 0.5% yeast

106   extract, 0.5% NaCl, 20 μg/mL Tetracycline, 50 μg/mL IPTG, 40 μg/ml X-gal, 1% agarose).

107   **Phage titer analysis**

108   A single *E. coli* ER2738 colony was inoculated into 10mL LB-Tet medium and incubated at

109   37 °C by shaking until mid-log phase ($OD_{600} \sim 0.5$) for phage titering. Phage solutions were

110   diluted 10-fold serially in TBS (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) and aliquots of these

111   solutions (50 μL) were added to a suspension of the prepared ER2738 cells (450 μL), 1 for each

112   phage dilution. The phage infected *ER2738* cells (100 μL) were then poured from each dilution

113   tube to a LB-Tet-IPTG-X-gal plate and the plates were incubated overnight at 37 °C. We

114   determined the number of phage particles contained in the original stock phage culture by

115   counting the number of plaques formed on the seeded agar plate and multiplying this by the

116   dilution factor.

117   **Panning procedure**

118   96-well microplates (Polypropylene, Xinhua Glass Instruments Factory, Sanhe, Haimen,

119   China) were coated overnight at 4 °C with 100 μL of the HSA target (Sigma, 100 μg/mL in 0.1

120   M $NaHCO_3$, pH 8.6) and blank control (0.1 M $NaHCO_3$, pH 8.6) respectively. After rapid

121   washing 3 times with TBST (TBS + 0.1% [v/v] Tween-20), 100 μL of the diluted phage library

122   ($\sim 2 \times 10^{11}$ phage) was added to the well of the coated plate and rocked gently to let them absorb

123   for 60 min. Following 10 washes with TBST, the bound phages were eluted with 0.2 M Gly-HCl,

124   pH 2.2. The 100 μL that was eluted was neutralized with 15 μL Tris-HCl (1 M, pH 9.1)

125   immediately. A small amount of eluted phage was titered as described above and the rest of

126 elutes were added to 20 ml ER2738 culture ($OD_{600} \sim 0.5$) to be amplified 4.5 h at 37°C for the

127 next round.

Recovery yield and Specificity ratio

129 To evaluate the efficiency of the phage display panning procedure, we created two selection

130 criteria: the recovery yield and the specificity ratio. The recovery yield is the ratio of the titer of

131 the eluted phage to the input phage. The specificity ratio is the ratio of the titer of the eluted

132 phage from the target coated well to the blank control well.

$$\text{Recovery Yield (\%)} = \frac{\text{Elute phage titer from target}}{\text{Input phage titer}} \times 100$$

133

$$\text{Specificity Ratio} = \frac{\text{Elute phage titer from target}}{\text{Elute phage titer from blank}}$$

134

Isolation and purification of phage

136 The amplified culture of phage clones was transferred to a 50 mL centrifuge tube and

137 centrifuged for 10 min at 10,000 rpm at 4 °C with a high-speed refrigerated centrifuge (Himac

138 CR21G). The supernatant was decanted to fresh tube and precipitated by 1/5 volume of PEG/ -

139 NaCl (20% [w/v] Polyethylene Glycol–8000, 2.5 M NaCl) at 4 °C overnight and centrifuged for

140 20 min at 10,000 rpm. The supernatant was discarded and the sediment containing phage was

141 suspended with TBS. To precipitate the phage again, a 1/5 volume of PEG/ NaCl was added to

142 the culture and it was stored at 4 °C for at least 60 min. The PEG suspension was centrifuged for

143 10 min at 10,000 rpm, the phage precipitate was suspended in 500 μL of TBS.

Measurement of the relative dissociation constant of selected peptide fused phage binding

145 **to target**

146   The dissociation constant (K_d) can be determined from a double-reciprocal plot of eluted phage

147   concentrations $[\phi]_e$ against a variety of input phage concentrations $[\phi]_t$ at a fixed target

148   concentration of HSA [T] according to following equation (see Results & Discussion in detail):

149
$$\frac{1}{[\phi]_e} = \frac{K_d}{[T][\phi]_t} + \frac{1}{[T]}$$

150   The K_d value is equal to the slope of the line $K_d/[T]$ divided by the intercept on the vertical axis

151   1/[T], in which the target concentration of HSA immobilized in the microplate well is not

152   necessary for a K_d determination.

153   **DNA sequencing for characterization of specific binding peptides**

154   After 3 rounds of bio-panning, individual phage clone (blue plaque) was isolated, amplified

155   and purified to check the specificity ratio of binding to HSA. The sequences of HSA specific

156   binding peptides were determined by DNA sequencing of the selected phage using -96 gIII

157   sequencing primer 5'-HOCCC TCA TAG TTA GCG TAA CG-3 ' in the Ph.D-7 phage library.

158

159   **Results**

160   **HSA-immobilizing microplates and blocking agents**

161   Unlike the instructions on many phage display peptide library kits, we do not need to coat the

162   well by a blocking agent protein such as BSA, skim milk, et al. which process is common with

163   ELISA assays though. Blocking proteins are a mixture that may cause more complex binding

164   results in phage display screening. If the number of phages binding to blocking proteins is larger

165   than to target proteins, the recovery yield still rises in each round of panning and may cause

166   pseudo-positive results in phage display screening. Thus we used a blank well as the negative

167   control. The specificity ratio is used to measure how much the number of target binding phages

168   is larger than the number of blank binding phage in each round of the panning process. Therefore

169   no matter what kind of microplate, with low or high protein-binding capacity, is used to

170   immobilize the target protein molecule, the specificity ratio is generally suited for assessment of

171   the presence of target-binding phages.

172   **Panning against HSA**

173   We examined the enhancement effects of using the titer-based criteria in monitoring panning

174   HSA-binding peptides from the Ph.D.-7 phage display peptide library. The phage titer results in

175   the panning were shown in Table 1 and the corresponding recovery yields and specificity ratios

176   were shown in Table 2. Our data in 200 μg/mL HSA coated microplate demonstrated both a

177   recovery yield and specificity ratio increase by each round panning. Specifically, the recovery

178   yield increased 1.45 fold in the second round and 3.6-fold in the third round, as in the first round,

179   and the specificity ratios improved significantly from 2.35 in the second round up to 7.78 in the

180   third round. Importantly, the specificity ratio in panning rounds 2 and 3 that is larger than 1

181   indicated that the eluted phages preferred binding to HSA in the target coated well over the blank

182   in the target-uncoated well. The increase of specificity ratio after each round of panning also

183   implies that phages with high affinity for HSA have been amplified. Because blank micro-plates

184   only function to distinguish target-binding phages from microplate--binding phages in panning

185   results, the first round of panning using the original phage display peptide library against the

186   blank microplate is not necessary and should be excluded from the panning process. The increase

187   in the recovery yields after each round indicates that the pools of high affinity phages binding to

188   HSA have been enriched.

189   **Identification of positive HSA-binding phages**

190   The specificity ratio was used to determine if positive phage clones with high HSA-binding

191   affinity were isolated. From the final round of panning plates, we isolated individual clones to

192   amplify, purify and measure the specificity ratio for binding to HSA. We tested the selected

193   clones with significant specificity ratios as potential HSA-binders, after screening many phage

194   clones that consisted of microplate-surface-binding phages, the specific and non-specific target-

195   binding phages, from the target-binding plate as shown in Table 3. The two selected clones,

196   HSA-3 and HSA-4, show specificity ratios of 7.4 and 9.8, respectively, confirming that our

197   method in the panning process is effective.

198   **Phage binding assay by the relative dissociation equilibrium constants**

199   For confirming the binding peptides to HSA, the dissociation equilibrium constant (K_d), which

200   is commonly used to describe the interaction between a ligand (such as a drug) and a protein, is

201   also employed to explore how tightly the selected peptide which fused with phage binds to the

202   target molecule. The formation of the binding complex (ϕT) of phage fused peptide and HSA can

203   be described in a simple one-to-one association of a phage fused peptide (ϕ) and a target

204   molecule HSA (T):

$$\phi + T \rightleftharpoons \phi T \qquad (1)$$

205   The corresponding dissociation constant (K_d) is defined:

$$K_d = \frac{[T][\phi]}{[\phi T]} \qquad (2)$$

206   where [T], [ϕ] and [ϕT] represent molar concentrations of the target molecule HSA, phage fused

207   peptide and their binding complex, respectively.

208   The total target concentration $[T]_t$ is equal to the concentration of bound phage target $[\phi T]$ and

209   free target $[T]$:

$$[T]_t = [T] + [\phi T] \qquad (3)$$

210   Substituting this expression for $[T]$ in in equation of dissociation constant (K_d):

$$K_d = \frac{[\phi]\,([T]_t - [\phi T])}{[\phi T]} \qquad (4)$$

211   Thus, the bound and total target and the free phage concentration are related by rearranging

212   above equation of dissociation constant (K_d):

$$\frac{[\phi T]}{[T]_t} = \frac{[\phi]}{K_d + [\phi]} \qquad (5)$$

213   The concentration of bound phage $[\phi T]$ and free phage $[\phi]$ can be calculated from the mass

214   conservation equation, where $[\phi]_T$ is the total concentration of input phage and the concentration

215   of target bound phage $[\phi T]$ can be estimated by eluted phage concentration $[\phi]_e$, which is very

216   much less than $[\phi]_T$ in our experiment.

$$[\phi] = [\phi]_t - [\phi]_e \approx [\phi]_t \qquad (6)$$

$$[\phi T] \approx [\phi]_e \qquad (7)$$

217   A double reciprocal plot of phage-target affinity measurement is generated by plotting $1/[\phi]_e$ as a

218   function of $1/[\phi]_t$. The slope is $K_d/[T]$, the intercept on the vertical axis is $1/[T]$, and the intercept

219   on the horizontal axis is $-1/K_d$. Thus, the dissociation constant (K_d) can be readily derived from

220   eluted phage concentrations at variety of total input concentrations at a fixed target concentration

221   of HSA.

$$\frac{1}{[\phi]_e} = \frac{K_d}{[T]\,[\phi]_t} + \frac{1}{[T]} \qquad (8)$$

222   This method is used to evaluate the relative dissociation constants of selected phage fused

223   peptide HSA-3 and HSA-4 against target molecule HSA respectively. Figure 1 & 2 shows a

224   double reciprocal plot for determination of relative dissociation constant of selected peptide

225   fused phage HSA-3 and HSA-4 binding to HSA respectively. In the experiment, a constant

226   concentration of target molecule HSA coated in the wells of microtiter plates was incubated with

227   varying concentration of input HSA-3 or HSA-4 peptide fused phage until equilibrium was

228   reached. Then TBST buffer wash the unbound phages, followed by the elution of the bound

229   phages to the well with glycine-HCl, pH2.2. The concentration of input and eluted phage was

230   quantitated by titration. The K_d value HSA-3 and HSA-4 were conveniently calculated from

231   linear regression of the plot. The relative order of affinity of the selected peptides HSA-3 and

232   HSA-4 displayed on phage for the target HSA are shown in Table 3. These phage-fused peptides

233   were subjected to DNA sequencing and their corresponding heptapeptide sequences are also

234   shown in Table 3.

235

236   **Discussion**

237   **The recovery yield and the specificity ratio**

238   To analyze the effectiveness of our phage display experimental technique, two operating

239   parameters, the recovery yield and the specificity ratio, were developed. These parameters

240   assessed the initial efficiency of phage display screening. The recovery yield was calculated by

241   the titer of eluted phages divided by the titer of input phages in the same target-coated well. The

242   value of recovery yield quantifies the enrichment of target-binding phages. Ideally, the recovery

243   yield will rise as the phage display proceeds and the enrichment of phages after each round of

244   panning progresses so that a greater and greater quantity of target-binding phages is achieved.

245 However, the specificity ratio is more necessary than the recovery yield in monitoring the phage

246 display screening experiment. The specific ratio is employed to identify positive clones of

247 target-binding phages, as compared with the blank control. It is calculated by the titer of eluted

248 phages from the target-coated well divided by the titer of eluted phages from the blank control

249 well. The specificity ratio should be larger than 1, for a positive result indicating that target-

250 binding phages are present. High specificity ratios indicate large amount of target-binding

251 phages and imply that there may be high affinity of phage clones present.

252   It is very necessary to use specificity ratio to avoid background interference generated

253 by non-specific binding (Vodnik et al, 2011). This is because eluted phages often include both

254 target-binding and microplate surface-binding phages, resulting in false-positive panning. Low

255 specificity ratios and recovery yields may result from the presence of a large number of plate-

256 surface-binding phages.

257   According to the instruction manual for the phage display peptide library kit by the

258 New England Biolabs (New England Biolabs.), large-scale randomized DNA sequencing in the

259 enrichment pool of eluted phages is required to uncover the binding motif among target-binding

260 peptides. But the surface of HSA for binding drugs may not be limited to the motif-specific area.

261 This method focusing on phage-fused peptide clones with greater binding capacity rather than

262 the enriched HSA-binding motif may be a more rapid and cost-efficient means of determining

263 which HSA-binding peptides are candidates for conjugation with a bioactive protein.

264 **Surrogate measure of intended HSA-binding affinity**

265   The indirect ELISA methods were also reported to determine the affinity constants of phage-

266   displayed peptides (Thong et al, 2018; Parakasikron et al, 2021), but our method is direct and

267   short protocol, which saves the phage antibody employed. Although biosensors such as Surface

268   Plasmon Resonance (SPR) or Quartz Crystal Microbalance (QCM) are popular methods for the

269   determination of affinity of protein-protein interaction, the relative dissociation constants (k_D)

270   between phage displayed peptides and their respective targets via phage titer developed by us

271   and others (Dyson et al, 1995) still can be employed as surrogate measure of affinity of protein-

272   protein interaction, which method may be cost-effective and user-friendly for rapid validation of

273   candidates of a target binder. In fact, when the HSA-binding peptides are fused with bioactive

274   proteins, their affinity for HSA would most likely decrease owing to shielding effects and

275   folding problems in their corresponding fusion proteins. The accurate affinity of free-HSA

276   binding peptide binding to HSA, as measured by their SPR or QCM-measured K_D, does not

277   necessarily have a guaranteed affinity in HSA binding peptide-drug fusions for HSA. Thus, the

278   relative dissociation constants (k_D) or even the specificity ratio can be employed as an initial

279   criterion to select HSA-binding peptides as candidates for fusion with the intended protein drugs,

280   then subjected HSA binding peptide-drug fusions to SPR or QCM to exam or validate the K_D

281   between the fusion protein and HSA.

282

283   **Conclusions**

284   Phage display is a general practical tool for selecting or isolating binding molecules against

285   the desired targets in phage libraries. In the case of targeting the protein with epitope mapping

286   method, conventional bio-panning has limitations on the efficient screening of the functionally

287 relevant peptides. With experimentally determined phage titer instead of sequencing individual

288 phage clones from enrichment pools, some quantitative criteria are characterized for bio-panning

289 and binding affinity of phage display of screening binding peptides in this study.

290   In summary, both the recovery yield and the specificity ratio are designed to monitor the

291 phage display panning process. The specificity ratio is a critical recognition feature used to

292 discriminate target binding from background interference. The relative dissociation constant of a

293 peptide fused phage also serves as surrogate endpoint to demonstrate the extent of binding

294 affinity and relative specificity of a HSA binder. Use of such criteria facilitates phage panning

295 and avoids pseudo-positive phage clones selected for further test procedures. Therefore, these

296 quantitative parameters based on phage titer are simply amenable to a wide variety of phage

297 display screening applications.

298   The selected HSA-binding peptides will be attached to bioactive proteins to prolong their

299 half-lives through non-covalent association with HSA in vivo. The small size makes HSA-

300 binding peptides ideal fusion partners for recombinant proteins because they avoid decreasing

301 biological activity and maintain the native capacity to penetrate tissues. In addition, the

302 extremely high plasma concentration of HSA confers the fusion proteins more opportunities to

303 bind HSA, even with a relatively low affinity HSA-tag. Thus, small HSA-binding peptides are

304 attractive candidates for the engineering of pharmacokinetic properties of protein therapeutics.

305

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379   Table Captions

380   **Table 1** Phage titers during panning against HSA

381   **Table 2** Recovery yields and specificity ratios during panning against HSA

382   **Table 3** Specificity ratios, relative dissociation constants and sequences of phage clones selected

383   for binding to HSA

384

385   Figure Captions

386   **Figure 1**. A double reciprocal plot for relative affinity constant measurement of Human Serum

387   Albumin binding to peptide HSA-3 fused phage.

388   **Figure 2**. A double reciprocal plot for relative affinity constant measurement of Human Serum

389   Albumin binding to peptide HSA-4 fused phage.

390

391   Caption of graphical abstract

392   Using specificity ratio, recovery yield and relative dissociation constant based on phage-titer,

393   phage-display-screening HSA binding peptides can perform more efficiently and cost-effectively

Phage titers during panning against HSA

Table 1 Phage titers during panning against HSA (In 200 μg/mL HSA coated microplate)

Panning	Round 1	Round 2	Round 3
Input			
HSA (pfu/ml)	4.40×10^{11}	4.24×10^{11}	2.40×10^{11}
Blank (pfu/ml)	/	4.24×10^{11}	2.40×10^{11}
Elute			
HSA (pfu/ml)	5.20×10^{4}	7.24×10^{4}	1.12×10^{5}
Blank (pfu/ml)	/	3.08×10^{4}	1.44×10^{4}

The recovery yields and the specificity ratios during panning against HSA

1   **Table 2** The recovery yields and the specificity ratios during panning against HSA (In 200 µg/mL HSA coated microplate)

Panning	Round 1	Round 2	Round 3
Recovery yield (%)	1.18×10^{-7}	1.71×10^{-7}	4.25×10^{-7}
Specificity ratio	/	2.35	7.78

2   The recovery yields were calculated by the titers of eluted phage divided by the titers of input phage; the specificity ratios were calculated
3   by the titers of eluted phage from target molecule coated plate divided by the titers of eluted phage from blank control plate.

Specificity ratios, relative dissociation constants and sequences of phage clones selected for binding to HSA

1 **Table 3** Specificity ratios, relative dissociation constants and sequences of phage clones selected for binding to HSA

Phage displyed peptide	Input phage titre (pfu/ml)	Elute phage titre (pfu/ml)	Blank control phage titre (pfu/ml)	Specificity ratio	Relative dissociation constant (μmol/L)	DNA sequence	Peptide sequence
HSA-3	1.16×10^{12}	9.16×10^5	1.24×10^5	7.4	0.529	GCG GAG AGT TAG AAG CTT AAT	AESQKLN
HSA-4	1.34×10^{13}	5.12×10^6	5.22×10^5	9.8	0.388	CTT ACG CCG CTT CCG TTG CGT	ITPLPLR

Figure 1

A double reciprocal plot for relative affinity constant measurement of Human Serum Albumin binding peptide HSA-3 fused phage.
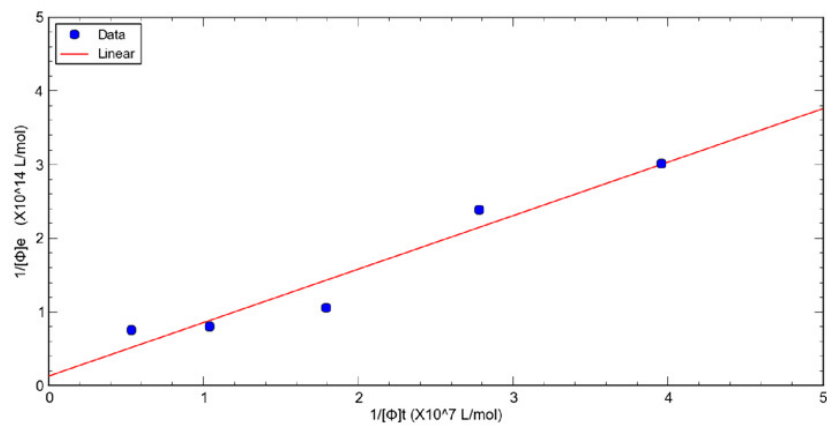
Figure 1. A double reciprocal plot for relative affinity constant measurement of

Human Serum Albumin binding peptide HSA-3 fused phage. $[\Phi]_e$ is the eluted phage,

$[\Phi]_t$ is the total input phage.

Figure 2

A double reciprocal plot for relative affinity constant measurement of Human Serum Albumin binding peptide HSA-4 fused phage
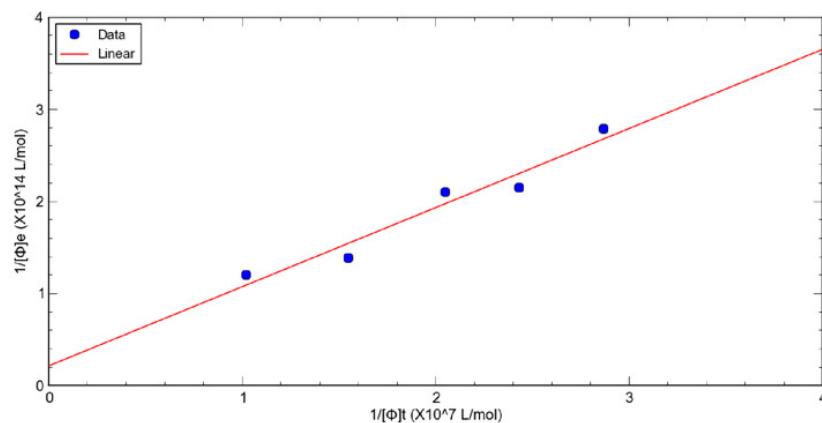
Figure 2. A double reciprocal plot for relative affinity constant measurement of

Human Serum Albumin binding to peptide HSA-4 fused phage. $[\Phi]_e$ is the

concentration of eluted phage, $[\Phi]_t$ is the concentration of total input phage.