Supporting Information

Phase-Separated Multienzyme Biosynthesis

Miao Liu,† Sicong He,‡ Lixin Cheng,§ Jianan Qu,‡ and Jiang Xia†, #, *

†Department of Chemistry, #Center for Cell & Developmental Biology, School of Life Sciences, The Chinese University of Hong Kong, Shatin, Hong Kong SAR, China.
‡Department of Electronic and Computer Engineering, Center of Systems Biology and Human Health, School of Science and Institute for Advanced Study, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong SAR, China.
§Department of Critical Care Medicine, Shenzhen People's Hospital, First Affiliated Hospital of Southern University of Science and Technology, Shenzhen, China.

*Address correspondence to jiangxia@cuhk.edu.hk
Phone: (852) 3943 6165
Fax: (852) 2603 5057
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Detailed experimental procedures

Abbreviations
IPTG: isopropyl β-D-1-thiogalactopyranoside
Tris: Tris(hydroxymethyl)aminomethane
EDTA: ethylenediaminetetraacetic acid
DTT: dithiothreitol
Trx: thioredoxin
NTA: nitrilotriacetic acid
SDS: sodium dodecyl sulfate
ThDP: thiamine diphosphate
TFA: trifluoroacetic acid
Cy5: cyanine5
DMF: N, N-dimethylformamide
DSC: N, N′-disuccinimidyl carbonate
DIPEA: N, N-diisopropylethylamine
DCM: dichloromethane
NHS: N-hydroxysuccinimide
DMSO: dimethyl sulfoxide
TEA: triethylamine

Materials
Unless otherwise noted, all reagents were purchased from commercial sources and used without further purification. Reagents were purchased from commercial suppliers, including Sigma-Aldrich Co. (USA), J&K Scientific Ltd. (Beijing, China), and BBI Life Sciences Co. (Shanghai, China). DNA oligonucleotides were synthesized by BGI (Shenzhen, China). Restriction endonucleases were purchased from Thermo Scientific (USA). Plasmids, PCR products and products of restriction digests were purified by commercial kit from Takara Biotech Co., Ltd (Dalian, China). Sequencing reactions were performed and analyzed at BGI (Shenzhen, China). Sulfo-Cy5 was kindly provided by Professor Jianhao Wang (Changzhou University, China).

Plasmids construction
All plasmids were constructed using traditional cloning methods. Plasmids encoding GKAP, Shank and Homer sequences were kindly provided by Prof. Mingjie Zhang (Hong Kong University of Science and Technology, China). Plasmids encoding CFP and YFP sequences were kindly provided by Prof. Adrian Keatinge-Clay (The University of Texas at Austin, USA). Plasmids encoding MenF, MenD and MenH sequences were kindly provided by Prof. Zhigong Guo (Hong Kong University of Science and Technology, China). Plasmids encoding Idi and IspA sequences were kindly provided by Prof. Tiangang Liu (Wuhan University, China). RI and RIAD sequences were synthesized and inserted into respective plasmids. Sequence information was shown in supporting tables.

Recombinant proteins expression and purification

Protein expression. All constructed plasmids were transformed into E. coli BL21 (DE3) cells and colonies were grown overnight at 37°C in LB media supplemented with 100 μg/mL ampicillin or 50 μg/mL kanamycin. The start culture was grown overnight, and then was used to inoculate LB media supplemented with antibiotics at 1:100 ratio. The cell culture was grown at 37°C to reach OD$_{600}$~0.6 before 0.25 mM IPTG was added to induce protein expression. After grown at 16°C overnight, cells were harvested for protein purification.

Purification of GKAP. Trx-His-GKAP was purified using a nickel-NTA agarose affinity column followed by size-exclusion chromatography (Superdex 200 or Superdex 75, GE Healthcare). Briefly, harvested cells were re-suspended in lysis buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 5 mM imidazole. The cell suspension was lysed by homogenizer and centrifuged. The supernatant was collected, filtered, and loaded on the column. After washs with washing buffer containing 50mM Tris, pH 8.0, 500mM NaCl, 60mM imidazole, the bound protein was eluted by elution buffer containing 50mM Tris, pH8.0, 500mM NaCl, 1M imidazole. Eluted protein was further purified by Superdex 200 column with a column buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA, 2 mM DTT. Then Trx-His tag was removed by HRV 3C protease incubating at 4°C overnight. GKAP without tag was purified by Superdex 75 column with same column buffer.

Purification of Homer and RIAD-Homer. Homer and RIAD-Homer were purified using HisTrap™ HP column followed by Superdex 200 column (GE Healthcare). Briefly, harvested cells were re-suspended in lysis buffer containing 50 mM Tris, pH 8.0, 500 mM NaCl, 2 mM β-mercaptoethanol, 10 mM imidazole. The cell suspension was lysed by sonication and centrifuged. The supernatant was collected, filtered, and loaded on the column. The bound protein was eluted by imidazole gradient from 10 mM to 500 mM. Eluted protein was further
purified by Superdex 200 column with a column buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA.

**Purification of other proteins.** Other proteins were purified using HisTrap™ HP column only. Pure protein fractions eluted with imidazole gradient were collected and exchanged with buffer containing 50 mM Tris, pH 8.0, 100 mM NaCl, 1 mM EDTA. After purification, all proteins were flash frozen under liquid nitrogen and stored at -80°C.

**Fluorescence recovery after photobleaching (FRAP) assay**
FRAP assay was performed on a Leica SP8 confocal microscope at room temperature. GFP signal was bleached using a 488-nm laser beam. The scaffold concentration was set as 5 μM and GFP was 0.2 μM. The fluorescence intensity at pre-bleaching was normalized to 100%.

**Synthesis of Sulfo-Cy5 NHS ester**
Sulfo-Cy5 NHS ester was synthesized using a method modified from Jung et al. and Kvach et al. Briefly, Sulfo-Cy5 acid (0.02 mmol) was dissolved in 1 mL dry DMF. Then DSC (0.03 mmol) and DIPEA (0.1 mmol) were added to the stirred solution, and the mixture was stirred for 2 h at room temperature. TLC was used to monitor the reaction (20% methanol in DCM, v/v). After reaction, product was precipitated by cold diethyl ether, and stored at -20°C for further use.

**Analysis of chorismate and SHCHC by HPLC**
The reactant chorismate and final product SHCHC of menaquinone biosynthesis enzymes were analyzed by Shimadzu HPLC system using RaptorTM C18 column (Restek Corporation, USA). Samples were eluted with the following gradient: Solvent A: water with 0.1% TFA; Solvent B: acetonitrile with 0.1% TFA; 5% B to 30% B in 11 min. Chorismate and SHCHC were monitored by absorbance at 274nm and 293nm respectively. Standard curves were generated by injecting a series of chorismate and SHCHC using same column and HPLC condition and integrating the areas of specific peaks at 274nm or 293nm. The concentration of chorismate and SHCHC was determined by absorbance at 274nm and 293nm respectively.

**Estimation of protein concentration inside condensates**
The concentrations of Idi-RIDD and RIDD-IspA in the terpene biosynthesis enzyme condensates were estimated by confocal microscopy using Cy5 labeled proteins through a
method modified from Zeng. et al. 

In each measurement, only one enzyme was Cy5-labeled and labeled protein was adjusted to 2% by diluting a labeled protein into the unlabeled one. The 2% Cy5-labeled protein was further mixed with other unlabeled proteins at the concentration same in the enzymatic assay (5 μM scaffolds, 0.5 μM Idi-RIDD and 1 μM RIDD-IspA) to form condensates. The mixture was injected into a flow chamber and imaged by Leica SP8 confocal microscope with a 63x objective lens in a z stack mode with the step interval as 0.5 μm. To get the average Cy5 intensity in one droplet, first the fluorescence intensity was analyzed within the selected droplet area using ImageJ software for each z layer. Then the fluorescence intensities of the layers that the intensity <10% deviation from the maximal intensity layer were averaged to get the average Cy5 intensity in that droplet. Average Cy5 intensities from 10 different droplets in each group were analyzed for estimating the protein concentration.

Calibration curve of Cy5 fluorescence intensity was generated by injecting a series of Cy5 into the flow chamber and measuring the fluorescence intensity using the same imaging parameters and confocal settings as for the droplet quantifications. The concentration of Cy5 was determined by absorbance at 646 nm.
Table S1. List of protein sequences

GKAP (aa 328Q-421S + 916Q-992L, UniProt: Q9D415-1)

MSDKIIHTDSFDTDVLKADGAILVDFWAECGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGBTAPKY
GIRGIPTLLLFKNGEVAATKVGALSKGQLKEFBDANLANSQGSHMHMHMMHMHSSGLLEVLFQ/GPSEF
QDEWSGYTPRKGIDDEIPCRRRMSRYIKAMGEDSGDSDTSPKPSKVAARRESYIKATQPSLTLKLTLE
HSPKLQIPRSHYLRAVSEVSQMPLDKERRAPPVPKPAKGPAPLIRERSLESSQREARKRLMAAKRA
ASVRQNSATESAESYEIYPEAQTIR*

HRV3C cut site shown in slash mark. GK binding repeats 328Q-421S were shown in blue;
PDZ binding motif 916Q-992L was shown in green.

Homer (aa 1M-361P, Ser342 is an Arg, UniProt: Q9NSC5-1)

MHHHHHHSGLLEVLFQGPSEGFMSTAREQPFISTREAMFQIDPATKRNWIPAGKHALTSVSYFYDATRN
VYRIISIGGAKAIINSTVTTPMTFTKTSTQKFQWADSRANTVYGLFGASEQHTQFAEKFPVEKKEAALREK
SDQGGELTSPLGSLQHQPSSPLVSANGPEEKLFRSQSSADAPGTERERLKKMLSEGSGVEGEVQWEEAF
FALQDNSNKLAGALREANAAAAQWRQQLQAERQAERLQRQRVAEALAAEVEPTGKEGLGQGQL
EQLEALVQTKDQIQTLSKQTGPPREALEAEREETQKVQDLETRNAELHEQLRAMELLEERERER
ARAEVGRAAQLLDVRLFELSELRGALRAEAP*

Homer3 1M-361P was shown in blue.

RIAD-Homer

MGSSHHHHHHSSHGLPGRSHMRSEQYANQLADQIKEATEGGSGGSEGGSQGGGSEFMSTAREQPFI
STREAMFQIDPATKRNWIPAGKHALTSVSYFYDATRNVRHISIGGAKAIINSTVTTPMTFTKTSTQKFQWADSR
ANTVYGLFGASEQHTQFAEKFPVEKKEAALREKSDQGGELTSPLGSLQHQPSSPLVSANGPEEKLFRSQSS
ADAPGTERERLKKMLSEGSGVEGEVQWEEAFIALQDNSNKLAGALREANAAAAQWRQQLQAERQAERLQRQR
VAEALAAEVEPTGKEGLGQGQLEQLEALVQTKDQIQTLSKQTGPPREALEAEREETQKVQDLETRNAELHEQLRAME
LLEERERERARAEVGRAWAQLLDVRLFELSELRGALRAEAP*

RIAD sequence was shown in red, Homer3 1M-361P was shown in blue.

Shank (aa 533D-665R + 1294D-1323P + 1400A-1426P + 1654G-1730S, UniProt: Q4ACU6-1)

MQYKLALNGKTLKGETTTTEAVDAATAEAKFQKYANDNGVGDEVYDYDDATKFTVTASHMHMHHHSGL
LEVLFQGPSEQFDTRHETREDRTKRLFRHITYGSDLTHSYV1DDKVALQKRHEGFGFLVLRGAKAE
TPIEEFTPTAPALQLYESVDEVGAVARQLRTGDVIENVNVVKGHKGQVVLQGRQGNNLVMKV
SVTREDDEELRALIRGIVPPPPPFAFVALLTTTPADGHTFLEKPPVPKPKLPSLPLKGPGPRFQPQKPLQ
LWSKFDVGDWLESIIHLEHRDFEDHEIEGAHPALTKEFDVFELGVTRGVHRMNIERALRQLDGS*
GB1 tag was shown in red. N-terminal extended PDZ domain 533D-665R was show in green; SAM domain 1654G-1730S was shown in blue.

**RIAD-Shank**

MQYKLALNGKTLGETTTEAVADAATAEKFQKYANDNGVGDGEWTYDDATKFTVTEHMHHHHHSSG
LEVLFQPGPSLEQYANQLADQIKEATEGSGGSEGSSSGGGSEFDTRHETREDRTKRLFRHYTVGYSDSLTS
HSDYVIDDVKVAILQKRDHEGFGFVLRGAKAETPIEFTPTAFPALQYLESVDVEGVAWRASLRTGDFLIEV
NGVNVVKVGHQVVGILQQNGNRLVMKVVSVTREDEETREELARIGLVPPEEFANGILLTTTPADGHTFLEKPPVPPKPLSKPLGKGPGPFRPFQQQKPLQLWSKFDVGDWLESIHGLEHRDFEDHEIEGALPALTKE
DFVELGVTRVGHRMNIELRALRQLDGSG**

EGFP sequences were shown in green. RIDD sequences were shown in red.

**EGFP-RIDD**

MHHHHHHMASMTGGQMMGRGSMVSKGEELFTGVVPILVELDGDVNHKFSVSGEGEDATYGKLTL
KFICTTGKLVPWPWTLVTLTLVGYVQCFSRYPDHMKQHDFDKSAMPEGYVQERTIFFKDDGNYKTRAEVKF
EGDTLVNRIELKGDFDKEDNGILHKEYNHNVYIMADKQKNGIKVNFKIRHNIEDGSVQLADHYQQ
NTPIDGDPVLLPDNHYLSTQSLSKDPNKRDKMDVLLFETFAAGITLGMDELKELRRQASGGGSGGG
GSGGGGCGLRECELYVQKHNNQALLKDSIVQLCTARPMAFLREYFERLEKEAK**

EGFP sequences were shown in green. RIDD sequences were shown in red.

**CFP-RIDD** (CFP is mTurquoise variant 5)

MGSSHHHHHSSSLVPGRHSMVSKGEELFTGVVPILVELDGDVNHKFSVSGEGEDATYGKLTLKFICT
TGKLVPWPWTLVTLTLVGYVQCFSRYPDHMKQHDFDKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEGD
TLMRIELKGDFDKEDNGILHKEYNHNVYFSDVNTADFKQKNGIKANFKIRHNIEDGSVQLADHYQNPIDG
DGKVLLLDDNYLSTQSKLSDPNKRDKMDVLLFFETFAAGITLGMDELKGEFELRRQASGGGSGGG
GSGGGGCGLRECELYVQKHNNQALLKDSIVQLCTARPMAFLREYFERLEKEAK**

CFP sequences were shown in blue. RIDD sequences were shown in red.

**YFP-RIDD** (YFP is mVenus variant 6)

MGSSHHHHHSSSLVPGRHMSKGEELFTGVVPILVELDGDVNHKFSVSGEGEDATYGKLTLKLICTT
GKLVPWPWTLVTLTLVGYVQCFSRYPDHMKQHDFDKSAMPEGYVQERTIFFKDDGNYKTRAEVKFEDGTL
VNRIELKGDFDKEGGNILGHKLEYNYSHNVYITADKQKNGIKANFKIRHNIEDGSVQLADHYQNPIDG
YFP sequences were shown in gold. RIDD sequences were shown in red.

**MenF (aa 1M-431E, UniProt: P38051-1)**

MHHHHHHSSGLVPRGSMQSLTTALENLLLRLSQEIPATPGIRVIDIFPFLKDAFALSWSLASQQTYPQFYWQQRNGDEEAVLGAITRTSFLDAQRQRFLQHPHAVDLRIGWLNAFDSQGNNLPPRLEWRRRCGGKATLRLTFSESSLQHDAAIKEFIATLVSIPKLPLGLHLTTTRQHPDKTGWTQILELAKTIAEGELDKVVLARATDLHFASPVNAAMMAASRRLNLCYHFYMAFDFGENAFLGSSPERLWRRRDRALELAGTVANNPDQDAQQLGLWLMADDKENRENMLVVEDICQRLQADTQLTDVLPQVLRRLRQHLRCIWATSLNADDCICLQHQLQPTAAVGALPRDLQRQFARHEPFREYWAYAGSAGYLSLQSEQFVSLRSAKISGNVRLYAGAGIVRGSDPEQEWQEIDNKAAGLRTLQME*  

MenF 1M-431E was shown in blue.

**MenD (aa 2S-556L, UniProt: P17109-1)**

MHHHHHHHSSGLVPRGSVSVAFRNRRWAIAVILEALTRHGVHRICAPGSRSTPLTTAAAENSAFAHIHTHFDERGLGLHIALGLVSKQPPVAVAIVTSGTAVANYLPAIEAGLTGKILLTADRPEELIDCGANQAIRQPMGMAHPTHSISLPRPTQDIPARWLSTIHALGTLHAGGVTINCPFAEPLYGEMDDTGLSWQQLRGLDWWQDDKPWLRLEAPRLESEKQRDWFFWRQRKVRVGGVEAGRMSEEGKVALWATLGDVPSQTQGQLPDADCWLGNKATSELOQAQQVQLGSSLTKRLLQWQASCEPEEYYWVIDGERLDPAAHHRGRRLIANIDWLELHPAEKRQPWCEVIPRLEAQAMOQAVIAARRDAFGEALAHRICDLYPEQGQLFVGSNLVLRLIDALSLQPAAYPVNSRGASGGIDLSTAAVGQRAkgkPQI0ALVGDLSALNYLNLALRQVQASPLVLIVVNNNGGQIFS5PLPTQSPERFELMPQNPQNHFEHAAMFELKYHRPQNWQELETAFADAWRTPTTTTIEMVNDTDGAQTLQQLAQQVSHL*  

MenD 2S-556L was shown in blue.

**MenH-RIDD (MenH: aa 2I-252F, UniProt: P37355-1)**

MGSSHHHHHSSGLVPRGSHTMAMSTQGQMQRGSLHQAAGKHKPGLPWLVLHGFSGDCEWVEGAEFADYSRLYDLPGHGSAAISVGDFFDVTLRLKTLGVSYNLDFWLYGYSGLGRVAMMAACGQLAGLCGVVEGGMHHGLQNAEQRAERQRSDRWVQRQFTTEPLTSFADWQYPQFVSAKLNDQQRELVALRSNNNGATLAMLETALSQPDRLANSARTFAYLCCGERDESKFRAALAEALACHVIPRAGNAHRENPAGVIALAQLRLFQASGGSGGGSGGSGGGGSGGSGGSGGLRECELYVQKHIQALLKDSIVQLCTRAPERPMAFLREYFERLEKKEAK*  

MenH 2I-252F was shown in blue. RIDD sequences were shown in red.
**Idi-RIDD** (Idi: aa 1M-182K, UniProt: Q46822-1)

MGSSHHHHHHSSGLVPRGSHEQTEHVILLSNAQGVPTGTLEYAAHTADTRLHLAFSSWLFINAKGQLLV
TRRALSKKAWPGVWTNSCGHQLGESNEDAVIRRCRYELGVEITTPESIYPDFRYRATDPSGIVENEVC
PVFAARTTSAQLINDDEVMDYQWCDLADVLHIDATPWAFSPWMVMQATNREARKRLSAFTQLKQA
SGGGGGGGGGGGGGGGCGSLRECELYVKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKEEAK*

Idi 1M-182K was shown in blue. RIDD sequences were shown in red.

**RIDD-IspA** (IspA: aa 1M-299K, UniProt: P22939-1)

MGSSHHHHHHSSGLVPRGSHMASSLRECELYVKHNIQALLKDSIVQLCTARPERPMAFLREYFERLEKE
EAKGGGGGGGGGGGGGSGSDPQQLEACVKQANQLSRFIAPLPQNTPVVETMQYGALLGGKLR
PFLYATGHHMFVSTNLDAPAAAVECHSIYSLIHDDLPAVMDDDDLRGLPTCHVKFGEANAILAGDAL
QTLAFSILSDADMPEVSRDRISMESASGIAGMCQGQALDAEGKHVLDALEIRHRHKTGALIR
AAVRLGALSAGDKGRRALPVLSDKYAEIGLAFLQVQDDLVTVGDTATLGRQGADQQLGKSTYPALLGL
EQARKKARDLDARQSLQAEQSLDTSALEALADYIQRNKQAS*

IspA 1M-299K was shown in blue. RIDD sequences were shown in red.
Figure S1. Assembly of EGFP in the protein condensate. (A) SDS-PAGE analysis showing the distribution of phase scaffolds and EGFP variants between diluted phase/supernatant (S) and condensed phase/pellet (P). More than 50% of EGFP-RIDD added to the mixture was found in P phase when mixed with RIAD-tagged phase scaffolds. Protein scaffolds 5 μM, EGFP or EGFP-RIDD 0.5 μM. (B) Confocal images showing EGFP-RIDD can assembled in the RIAD-tagged condensates at indicated concentration. Scale bar 10 μm, Protein scaffold 5 μM. (C) FRAP analysis showing EGFP-RIDD was dynamically exchanged between condensed protein condensates and solution. Protein scaffolds 5 μM, EGFP-RIDD 0.2 μM. Quantification curve used the results of 10 condensate droplets with diameter of ~5 μm.
**Figure S2.** (A) Exchange of fluorescent proteins between CFP only and YFP only condensates. Two systems, RIAD-tagged condensates with CFP-RIDD only and RIAD-tagged condensates with YFP-RIDD only were formed separately. After 10 minutes two condensates were mixed and injected into a flow chamber. Confocal image of the mixture showing the merge and exchange of fluorescent proteins between condensates with different kind of fluorescent proteins. Scale bar, 5 μm. Protein scaffolds 5 μM, CFP-RIDD or YFP-RIDD 0.2 μM. Yellow arrows point to droplets that in the fusion process. (B) Difference of the fluorescent signal distribution in CFP only and CFP+YFP condensates. Boxplots of the standard deviation (left) and participate coefficient (right) of all the condensates in five CFP only images (numbered 1-5 in cold color) and CFP+YFP images (numbered 6-11 in warm color). The circles indicate outliers.
Figure S3. Centrifugation/SDS-PAGE analysis showing the protein distributions of the menaquinone biosynthetic enzymes in the protein condensate. (A) Assembly of MenD-RIDD disrupts phase formation. When MenD-RIDD was mixed with RIAD-tagged phase scaffolds, most of the MenD-RIDD was found in S phase and about 50% of the scaffold proteins were found in S phase. Protein scaffolds, 5 μM; MenD-RIDD, 0.5 μM. (B) Assembly of MenH-RIDD in RIAD-tagged condensates shown by Coomassie blue stain and Cy5 fluorescent of SDS-PAGE. About 50% of MenH-RIDD was found in P phase. Protein scaffold, 5 μM; MenH-RIDD 1 μM with 5% Cy5 labelled. (C) Spontaneous enrichment of the three enzymes in the protein condensates without RIAD tags in the absence of peptide-peptide interactions. Most of the enzyme were found in S phase when incubated with untagged scaffold proteins. Protein scaffolds, 5 μM; enzymes, 1 μM.
Figure S4. Catalytic property of the menaquinone biosynthetic enzyme condensates. (A) The decrease of substrate chorismate and increase of the final product SHCHC of the whole reaction processes showing the efficiencies of the MenF/MenD/MenH-RIDD cascades. There was significant difference between control group and phase separation group at the indicated time point marked with star. And there was no significant difference between untagged phase group and RIAD-tagged phase group at any time of the whole reaction process. Welch’s t-test, * p < 0.05, n = 3. (B) HPLC data of chorismate showing that MenF turned chorismate into equilibrium faster when protein phase separation formed.
**Figure S5.** Estimating concentration of terpene biosynthesis enzymes inside the protein condensate. (A) Representative confocal images showing the Cy5 fluorescence of labeled enzymes in the condensate. The measured fluorescence intensities of each layer for the droplets marked with yellow arrow were plotted on the right. (B) Standard curve between Cy5 concentration and Cy5 fluorescence intensity measured by confocal microscope. (C) Summary of the average Cy5 intensities analyzed from confocal images and the calculated protein concentrations. Values are represented as mean ± SD of 10 different droplets. (D) Centrifugation/SDS-PAGE analysis showing the protein distributions of the Idi-RIDD and RIDD-IspA in the protein condensate. Protein scaffold, 5 μM; Idi-RIDD 1 μM with 10% Cy5 labelled; RIDD-IspA 1 μM with 5% Cy5 labelled.
References


