## Supplementary Information

# A novel *Plasmodium falciparum* rhoptry associated adhesin mediates erythrocyte invasion through the sialic-acid dependent pathway

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## Supplementary Methods

### Mass Spectrometric analysis of purified recombinant protein

In-gel digestion of the purified recombinant protein band was done to ascertain its identity. The corresponding protein bands from the coomassie stained SDS-PAGE gel were excised, reduced, and alkylated. Proteins were digested overnight with trypsin (Promega) and the corresponding peptides were eluted from the gel using trifluoroacetic acid. Mass spectrometry analysis and protein identification was done as described previously[1]

### ELISA

Antibody responses in mice and rabbit were quantified by ELISA. Briefly, 96-well plates (Costar) were coated overnight with 0.2 μg per well of the recombinant protein in 0.06 M carbonate-bicarbonate buffer, pH 9.6 (Sigma). The plates were washed thrice with 0.05% Tween in PBS and further blocked with 2% skimmed milk in phosphate buffered saline (PBS) at 37°C for 2 h. Washing was sequentially repeated and serial dilutions of the primary sera (1:1,000-fold onwards) were prepared and incubated in the respective wells for 1 hour. The ELISA plate was subjected to stringent washing with PBS containing 0.05% Tween-20 and finally with PBS alone. Thereafter, a 1:10,000 dilution of the horseradish peroxidase conjugated secondary antibody (Sigma, St. Louis, MO) was added to each well and incubated for 60 minutes at 37°C. The enzymatic reaction was developed by the addition of *o*-phenylenediamine dihydrochloride (OPD) and hydrogen peroxide for 25 minutes at 37°C. The reaction was terminated by the addition of 2M sulphuric acid, and $OD_{492}$ was recorded by using an ELISA microplate reader (Molecular Devices). Pre-immune (pre-bleed; PI) sera were used at similar dilutions as a control.

**FACS-based erythrocyte binding assay (EBA)**

Erythrocytes were incubated with 0.5 ml of culture supernatant at 37°C for 3 hours. After incubation, the erythrocytes were washed with PBS and bound PfRA on surface of erythrocytes was detected using anti-PfRA rabbit sera and Alexa 488 conjugated goat anti-rabbit IgG antibodies and were read on a FACS Caliber flow cytometer. The resulting flow cytometry data were analyzed using Cell Quest software.

**Co-Immunoprecipitation and Mass Spectrometric Analysis**

Immunoprecipitation experiments using lysate of the schizont stage parasites were performed as prescribed (Thermo scientific). The trypsin digested samples were analyzed on a nano-LC equipped Orbitrap VELOS PRO (Thermo Fisher Scientific) mass spectrometer as described previously[1]. The proteins were identified by blasting the peptides over a *Plasmodium falciparum* database (Uniprot), using proteome discoverer (Thermofisher) using standardized procedure as described earlier[1].

**Invasion assays**

Invasion assays were done as described previously[2]. Briefly, the parasites were first synchronized by the purification of schizont-stage (40-42 h) parasites on a percoll gradient. 0.4 million schizont stage percoll gradient purified parasites were incubated with 20 million uninfected normal as well as enzymatically treated erythrocytes at 2% haematocrit. After about 40 h post invasion, the parasite infected erythrocytes were stained with ethidium bromide dye and measured by a fluorescence activated cell sorter (FACS)-based assay. Relative invasion in enzyme treated erythrocytes was calculated with respect to the invasion observed in untreated erythrocytes. Two different experiments were performed in triplicate. For each assay Dd2 was

used as control for neuraminidase treated erythrocytes[2]. The error bars represent the standard error of the mean.

**Sequence polymorphism analysis**

To check sequence polymorphisms in the PfRA gene, available nucleotide sequences for the *P. falciparum* laboratory clones and field isolates was retrieved from the Plasmodb database, translated using ExPASy translate tool and aligned using multiple sequence alignment program Clustal Omega.

**References**

1. Reddy, K. S. *et al*. Multiprotein complex between the GPIanchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte invasion. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 1179–1184 (2015).

2. Gaur, D., Storry, J.R., Reid, M.E., Barnwell, J.W., Miller, L.H. *Plasmodium falciparum* Is Able To Invade Erythrocytes through a Trypsin-Resistant Pathway Independent of Glycophorin B. *Infect Immun.* **71**, 6742-6746 (2003).

**Supplementary Figure S1:** Alignment of PfRA protein sequences from 9 *P. falciparum* laboratory strains (Clustal omega).

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IT         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
707A       MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
HB3        MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
CS2        MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
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IT         EEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKEEENHN
707A       EEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKEEENHN
HB3        EEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKEEENHN
CS2        EEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKEEENHN
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T9_94      NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
Dd2-2      NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
7G8        NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
IT         NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
707A       NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
HB3        NDKKTDEQNHVNEKIQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
CS2        NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
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T9_94      DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
Dd2-2      DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
7G8        DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
IT         DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
707A       DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
HB3        DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
CS2        DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
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           241                     267
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Dd2-2      YFNLEKFSMTLIVFNSKINKFIYSQEK
7G8        YFNLEKFSMTLIVFNSKINKFIYSQEK
IT         YFNLEKFSMTLIVFNSKINKFIYSQEK
707A       YFNLEKFSMTLIVFNSKINKFIYSQEK
HB3        YFNLEKFSMTLIVFNSKINKFIYSQEK
CS2        YFNLEKFSMTLIVFNSKINKFIYSQEK
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**Supplementary Figure S2:** Alignment of PfRA protein sequences from 57 *P. falciparum* field isolates (Clustal omega).

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RV_3703         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
RV_3637         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
RV_3729         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
RV_3708         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
UGK_408.2       MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
309.1           MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
UGK_443.2       MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
RV_3736         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
PS122_G11       MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
RV_3687         MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
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SenT101.09      MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
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SenT180.08      MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
SenT142.09      MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKEN
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SenT101.09      EEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKEEENHN
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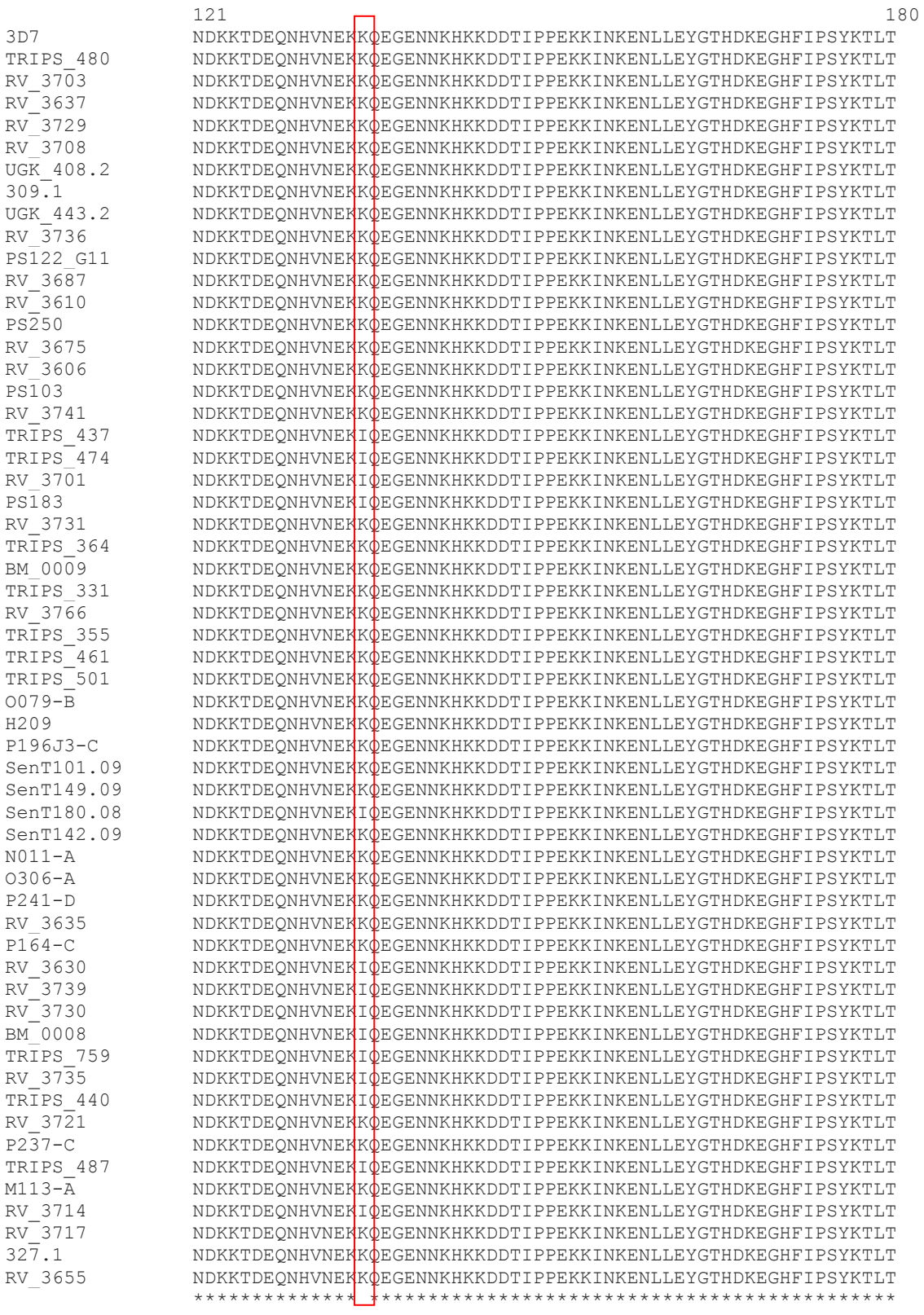
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180
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O079-B NDKKTDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIPSYKTLT
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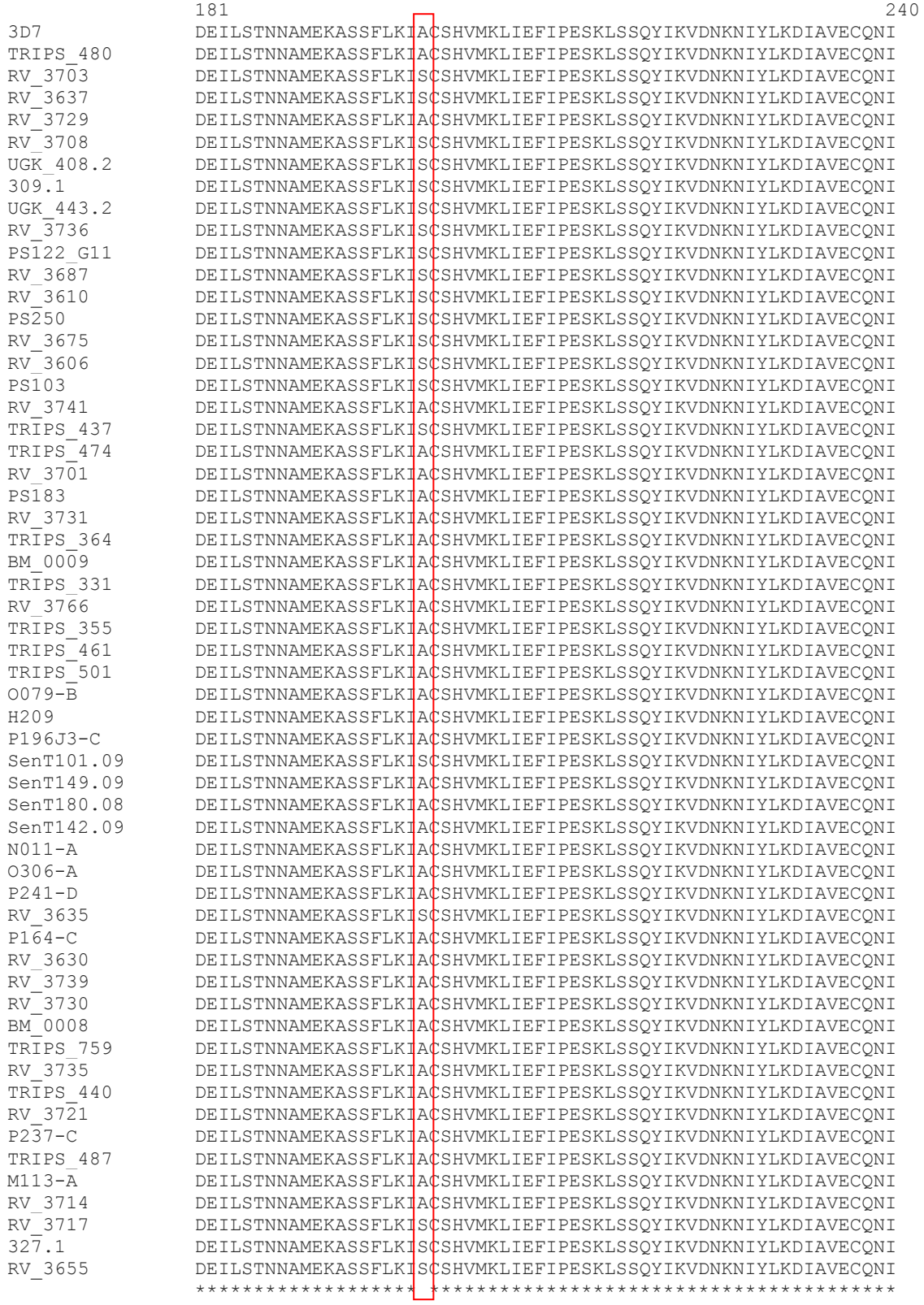
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H209 DEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
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327.1 DEILSTNNAMEKASSFLKISCSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI
RV_3655 DEILSTNNAMEKASSFLKISCSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVECQNI

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RV_3729         YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3708         YFNLEKFSMTLIVFNSKINKFIYSQEK
UGK_408.2       YFNLEKFSMTLIVFNSKINKFIYSQEK
309.1           YFNLEKFSMTLIVFNSKINKFIYSQEK
UGK_443.2       YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3736         YFNLEKFSMTLIVFNSKINKFIYSQEK
PS122_G11       YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3687         YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3610         YFNLEKFSMTLIVFNSKINKFIYSQEK
PS250           YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3675         YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3606         YFNLEKFSMTLIVFNSKINKFIYSQEK
PS103           YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3741         YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_437       YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_474       YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3701         YFNLEKFSMTLIVFNSKINKFIYSQEK
PS183           YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3731         YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_364       YFNLEKFSMTLIVFNSKINKFIYSQEK
BM_0009         YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_331       YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3766         YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_355       YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_461       YFNLEKFSMTLIVFNSKINKFIYSQEK
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H209            YFNLEKFSMTLIVFNSKINKFIYSQEK
P196J3-C        YFNLEKFSMTLIVFNSKINKFIYSQEK
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SenT149.09      YFNLEKFSMTLIVFNSKINKFIYSQEK
SenT180.08      YFNLEKFSMTLIVFNSKINKFIYSQEK
SenT142.09      YFNLEKFSMTLIVFNSKINKFIYSQEK
N011-A          YFNLEKFSMTLIVFNSKINKFIYSQEK
O306-A          YFNLEKFSMTLIVFNSKINKFIYSQEK
P241-D          YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3635         YFNLEKFSMTLIVFNSKINKFIYSQEK
P164-C          YFNLEKFSMTLIVFNSKINKFIYSQEK
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BM_0008         YFNLEKFSMTLIVFNSKINKFIYSQEK
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RV_3721         YFNLEKFSMTLIVFNSKINKFIYSQEK
P237-C          YFNLEKFSMTLIVFNSKINKFIYSQEK
TRIPS_487       YFNLEKFSMTLIVFNSKINKFIYSQEK
M113-A          YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3714         YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3717         YFNLEKFSMTLIVFNSKINKFIYSQEK
327.1           YFNLEKFSMTLIVFNSKINKFIYSQEK
RV_3655         YFNLEKFSMTLIVFNSKINKFIYSQEK
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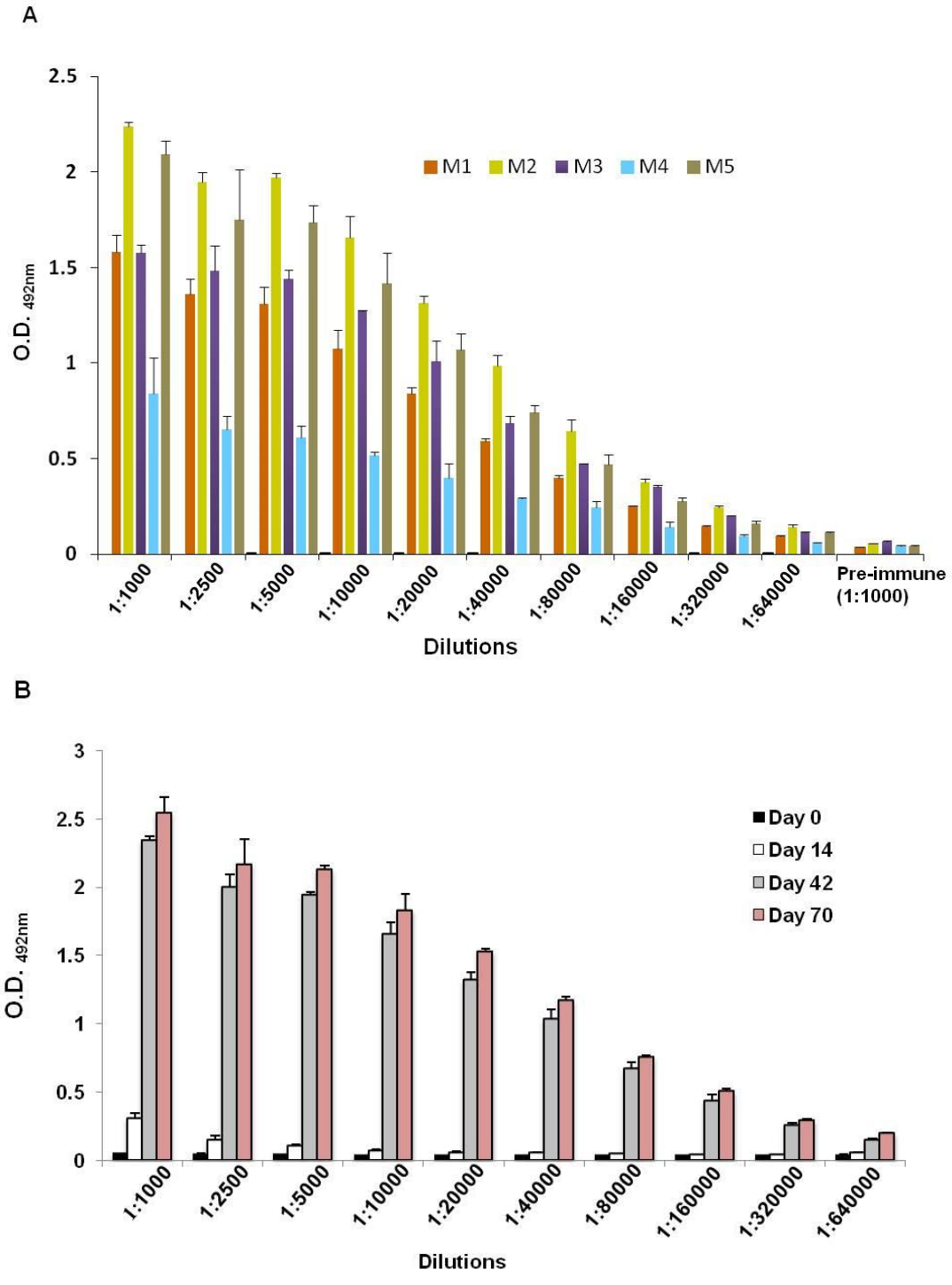
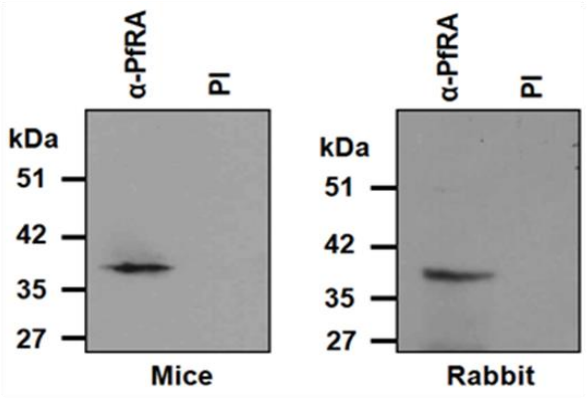
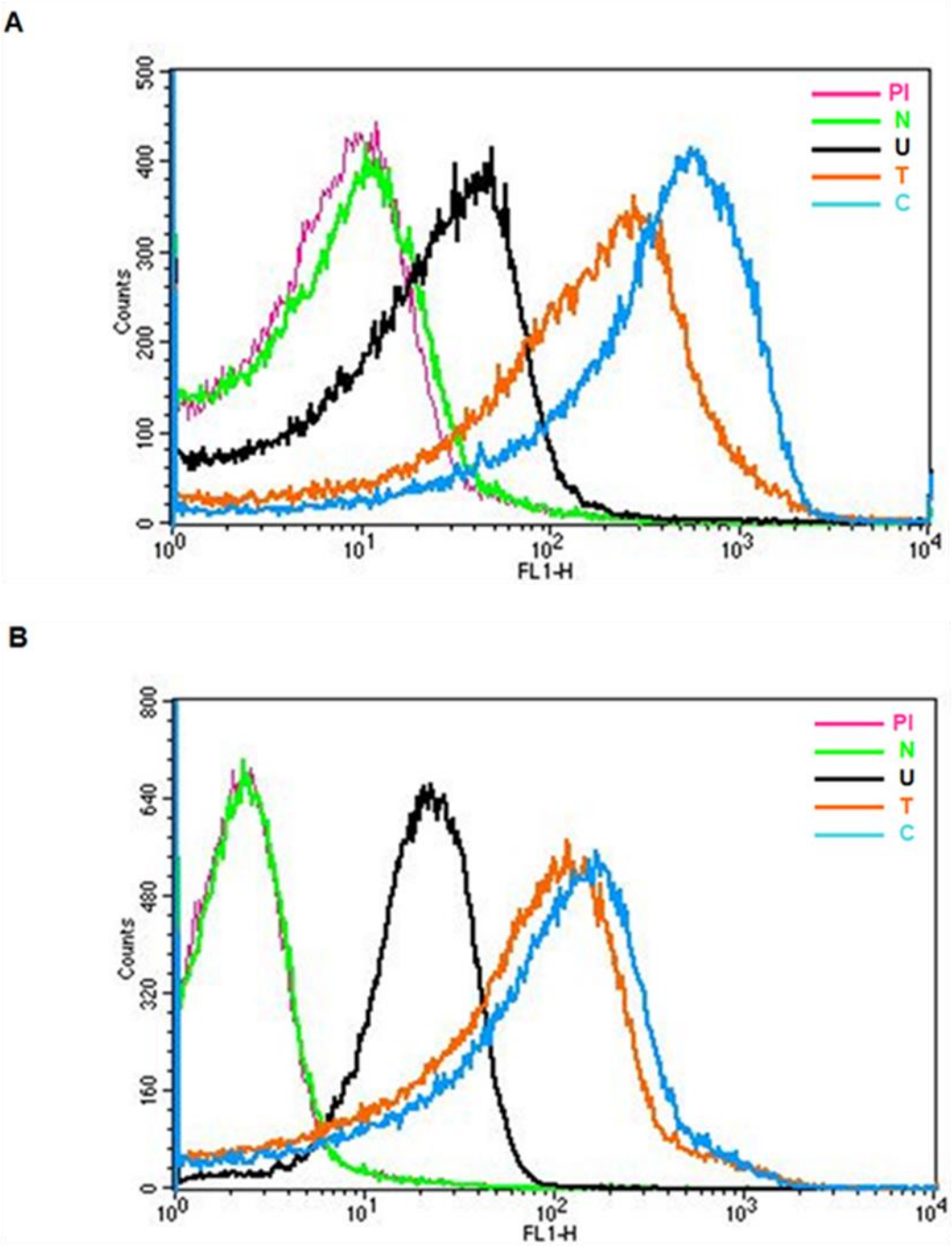
**Supplementary Figure S3:** Measurement of the antibody responses (end point titers) against rPfRA. Immunogenicity of rPfRA in (A) mice and (B) rabbit was analyzed by ELISA. Sera were serially diluted and assessed for end point titers. Pre-immune sera were taken as controls. High titer antibodies (end point observed at dilution of 1:320, 000 in mice and 1:640, 000 in rabbit) against the recombinant PfRA protein were detected. The error bars represent the standard error of the mean.



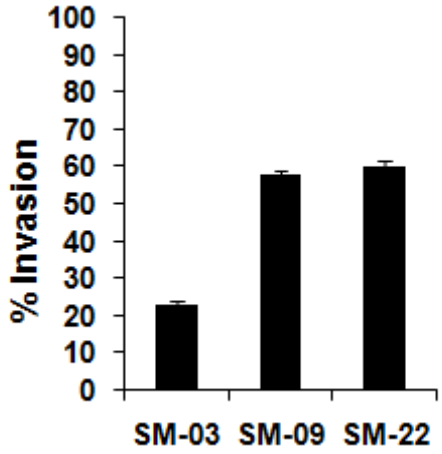
**Supplementary Figure S4:** Immunoblot analysis of rPfRA with immune sera raised in mice and rabbit. I: immune sera; PI: pre-immune sera.



**Supplementary Figure S5:** Binding of (A) native PfRA and (B) rPfRA to untreated (U), neuraminidase-treated (N), trypsin-treated (T) and chymotrypsin-treated (C) human erythrocytes was detected by flow cytometry using anti-PfRA rabbit sera. Pre-immune rabbit sera (PI) was used as a control.



**Supplementary Figure S6:** Invasion phenotype of three freshly cultured *P. falciparum* field isolates from Manhica, Mozambique in neuraminidase-treated erythrocytes.



**Supplementary Table S1:** Geographical origin of *P. falciparum* laboratory clones (www.plasmodb.org).

| No. | *P. falciparum* laboratory clone | Geographical location |
|---|---|---|
| 1. | 3D7 | Africa |
| 2. | Dd2-1 | Laos, Indochina |
| 3. | T9_94 | Thailand |
| 4. | Dd2-2 | Laos, Indochina |
| 5. | 7G8 | Brazil |
| 6. | IT | Brazil |
| 7. | 707A | Unknown |
| 8. | HB3 | Honduras |
| 9. | CS2 | Brazil |

**Supplementary Table S2:** Geographical origin of *P. falciparum* field isolates (www.plasmodb.org).

| No. | *P. falciparum* field isolate | Geographical location |
|---|---|---|
| 1. | TRIPS_480 | Gambia |
| 2. | RV_3703 | Gambia |
| 3. | RV_3637 | Gambia |
| 4. | RV_3729 | Gambia |
| 5. | RV_3708 | Gambia |
| 6. | UGK_408.2 | Uganda, Kampala |
| 7. | 309.1 | Mali |
| 8. | UGK_443.2 | Uganda, Kampala |
| 9. | RV_3736 | Gambia |
| 10. | PS122_G11 | Mali, Bandiagara |
| 11. | RV_3687 | Gambia |
| 12. | RV_3610 | Gambia |
| 13. | PS250 | Mali, Bandiagara |
| 14. | RV_3675 | Gambia |
| 15. | RV_3606 | Gambia |
| 16. | PS103 | Mali, Bandiagara |
| 17. | RV_3741 | Gambia |
| 18. | TRIPS_437 | Gambia |
| 19. | TRIPS_474 | Gambia |
| 20. | RV_3701 | Gambia |
| 21. | PS183 | Mali, Bandiagara |
| 22. | RV_3731 | Gambia |
| 23. | TRIPS_364 | Gambia |
| 24. | BM_0009 | Gambia |
| 25. | TRIPS_331 | Gambia |
| 26. | RV_3766 | Gambia |
| 27. | TRIPS_355 | Gambia |
| 28. | TRIPS_461 | Gambia |
| 29. | TRIPS_501 | Gambia |
| 30. | O079-B | French Guiana, Cayenne |
| 31. | H209 | French Guiana |
| 32. | P196J3-C | Saint-Laurent du Maroni |
| 33. | SenT101.09 | Senegal,Thies |
| 34. | SenT149.09 | Senegal,Thies |
| 35. | SenT180.08 | Senegal,Thies |
| 36. | SenT142.09 | Senegal,Thies |
| 37. | N011-A | French Guiana, Saul |
| 38. | O306-A | French Guiana, Cayenne |
| 40. | RV_3635 | Gambia |
| 41. | P164-C | French Guiana, Cayenne |

| **No.** | ***P. falciparum* field isolate** | **Geographical location** |
|---|---|---|
| 42. | RV_3630 | Gambia |
| 43. | RV_3739 | Gambia |
| 44. | RV_3730 | Gambia |
| 45. | BM_0008 | Gambia |
| 46. | TRIPS_759 | Gambia |
| 47. | RV_3735 | Gambia |
| 49. | TRIPS_440 | Gambia |
| 50. | RV_3721 | Gambia |
| 51. | P237-C | French Guiana, Cayenne |
| 52. | TRIPS_487 | Gambia |
| 53. | M113-A | Saint Georges de l"Oyapock |
| 54. | RV_3714 | Gambia |
| 55. | RV_3717 | Gambia |
| 56. | 327.1 | Mali |
| 57. | RV_3655 | Gambia |

**Supplementary Table S3:** List of Unique Peptides generated from the Mass Spectrometric analysis (LC-MS) of the trypsin digested recombinant PfRA protein.

| **Accession** | **Description** | **Score** | **Coverage** |
|---|---|---|---|
| Q8IJS3 | Conserved Plasmodium protein OS=Plasmodium falciparum (isolate 3D7) GN=PF10_0119 PE=4 SV=1 - [Q8IJS3_PLAF7] | 1226.62 | 79.03 |
| | A2 | Sequence of Unique Peptides | # PSMs |
| | High | INKENLLEYGTHDKEGHFIPSYK | 50 |
| | High | ENEEENKENKENNQNVNK | 4 |
| | High | ENLLEYGTHDKEGHFIPSYK | 31 |
| | High | KQEEVINNNNNNVENKK | 5 |
| | High | EYSFLMLGKENEEENKENK | 8 |
| | High | EYSFLmLGKENEEENKENK | 16 |
| | High | IAcSHVmKLIEFIPESK | 2 |
| | High | LIEFIPESKLSSQYIK | 5 |
| | High | KINKENLLEYGTHDK | 11 |
| | High | KQEEVINNNNNNVENK | 6 |
| | High | EYSFLMLGKENEEENK | 3 |
| | High | QEEVINNNNNNVENK | 2 |
| | High | KDDTIPPEKK | 18 |
| | High | INKENLLEYGTHDK | 12 |
| | High | DIAVEcQNIYFNLEK | 26 |
| | High | TLTDEILSTNNAMEK | 25 |
| | High | EYSFLmLGKENEEENK | 11 |
| | High | KNEEQNHNNEK | 18 |
| | High | KTDEQNHVNEK | 5 |
| | High | TDEQNHVNEK | 12 |
| | High | TLTDEILSTNNAmEK | 60 |
| | High | VDNKNIYLK | 3 |
| | High | KKEYSFLmLGK | 8 |
| | High | ENLLEYGTHDK | 16 |
| | High | KTDEQNHVNEKK | 1 |
| | High | HITNDEEKKK | 14 |

**Supplementary Table S4:** Mass spectrometric identification of native PfRA from immunoprecipitation elutes using specific antibodies.

| Accession | Description | Score | Coverage | Unique Peptides |
|---|---|---|---|---|
| PF3D7_1012200 | Organism = Plasmodium_falciparum_3D7\| product = conserved Plasmodium protein, unknown function \| location = Pf3D7_10_v3:470979 - 471933(+) \| length=267 \| sequence_SO = chromosome \| SO=protein_coding | 44.32 | 22.85 | TLTDEILSTnNAMEK |
| | | | | TLTDEILSTNNAMEK |
| | | | | DIAVECQNIYFNLEK |
| | | | | TLTDEILSTNNAmEK |
| | | | | DIAVEcQNIYFNLEK |
| | | | | FSMTLIVFNSK |
| | | | | LIEFIPESK |
| | | | | HKKDDTIPPEK |
| | | | | FSMTLIVFnSK |

**PfRA protein sequence:** Unique peptides detected by LC-MS highlighted in green

Signal Sequence (1-22)

MKRFFVLFVIFLVHIWSENVDTFKCNYSKKKKNGHHIKRHITNDEEKKKEYSFLMLGKE

NEEENKENKENNQNVNKDNNDNNNNEKKNEEQNHNNEKKQEEVINNNNNNVENKKE

EENHNNDKKDEQNHVNEKKQEGENNKHKKDDTIPPEKKINKENLLEYGTHDKEGHFIP

SYKTLTDEILSTNNAMEKASSFLKIACSHVMKLIEFIPESKLSSQYIKVDNKNIYLKDIAVE

CQNIYFNLEKFSMTLIVFNSKINKFIYSQEK