Swiss TPH
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Molecular Detection – Quantification – Genotyping

of P. falciparum

in *in vivo* drug efficacy trials

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Relationship of diagnostic sensitivity and parasite sampling methods

venous ("high volume")	fingerprick	treated filter paper
1 mL blood ⇩ **WBC depletion** 200 µL pellet Spin column preparation ⇩ **Vacuum concentration** ⇩ 10 µL DNA ⇩ 2 µL DNA (1/5) of starting material added to pPCR **≈ 200 µL blood**	**200 µL** blood ⇩ Spin 200 µL pellet Spin column preparation ⇩ 50 µL DNA ⇩ 5 µL DNA (1/10 of starting material added to pPCR **≈ 20 µL blood**	 3 x 3 mm punch ≈ 9 µL blood Dried blood spot on FTA card ⇩ ⇩ DNA extraction (Chelex) 100 µL DNA ⇩ 5 µL DNA (1/20 of 9 µL) in pPCR **≈ 0.45 µL blood** Wash 3 x then added to direct blood kit ⇩ all punches in pPCR **≈ 9 µL blood**

Do sub-microscopic infections matter in a clinical field trial ?

- **Parasite detection** at **enrollment** and **day of recurrence**

  Microscopy: only reliable detection if densities above 50-100 parasites/Ul

  Wongsrichanalai, Wernsdorfer 2007 AJTMH

  ⇩

  RDT
  PCR, LAMP, **qPCR** (see David Saunders presentation)
  Large volume/venous bleeds
  Ultra-sensitive multi-copy markers
  RNA-based

- Antimalarial drug trials in patients with **uncomplicated** falciparum malaria

- **Gametocytes** not affected by treatment may be responsible for positivity

Decision on method depends on
- study population & protocol
- facilities at field site

Is there a consensus among experts on use of molecular detection in field trials?

What is the most sensitive assay for parasite detection in a fingerprick blood sample ?

Template	**RNA** extraction	**DNA** extraction
PCR Target	18S rRNA **transcripts**	18S rRNA **genes**
Copy number	highly abundant	**3**/5 per haploid genome
Limit of Detection	0.002-02 parasites/ µL	1-2 parasites/µL

RNA-based versus DNA-based diagnosis

➡ twice as high prevalence rates in PNG

P. falciparum	Positive samples	Prevalence %
Pf 18S rRNA **DNA**	44/311	14.15
Pf 18S rRNA **RNA**	86*/315	27.30

* cut-off at 10 *Pf* 18SrRNA copies/µl

P. vivax		
Pv 18S rRNA **DNA**	64/311	20.58
Pv 18S rRNA **RNA**	121/315	38.41

(Wampfler et al. 2013 PLoS ONE)

Gametocytes detectable in qPCR-negatives but qRT-PCR-positives?

P. falciparum	Positive samples	Prevalence %
Pf 18S rRNA **DNA**	44/311	14.15
Pf 18S rRNA **RNA**	86*/315	27.30

* cut-off at 10 *Pf* 18SrRNA copies/µl

pfs25 qRT-PCR on **only RNA-positives**: **16% more Pf gametocyte carriers**

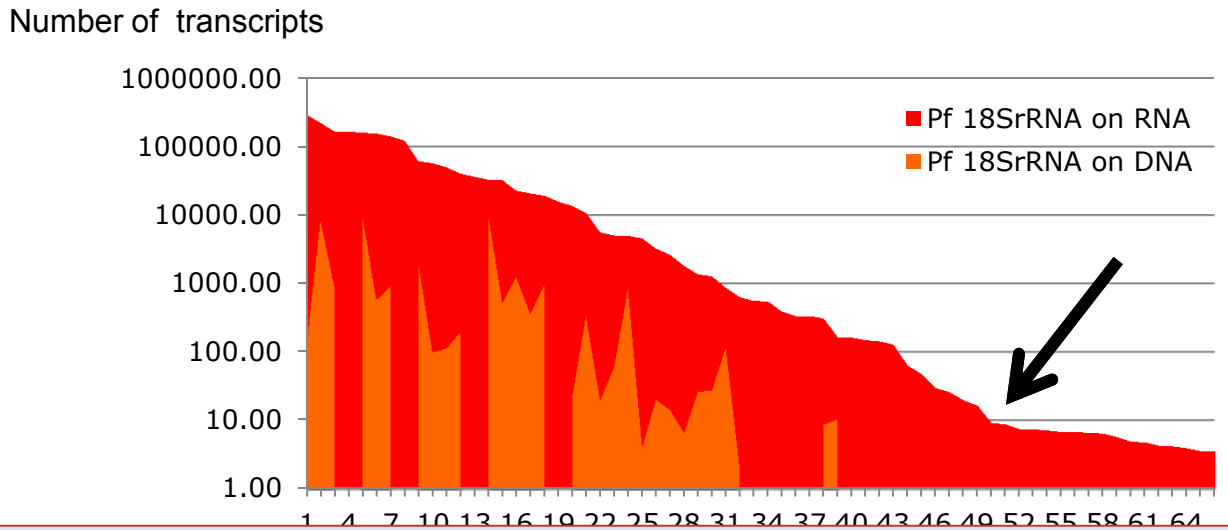
P. vivax		
Pv 18S rRNA **DNA**	64/311	20.58
Pv 18S rRNA **RNA**	121/315	38.41

pvs25 qRT-PCR on **only RNA-positives**: **23% more Pv gametocyte carriers**

(Wampfler et al. 2013 Plos ONE)

What method to chose for field work? DNA-or RNA-based assays?

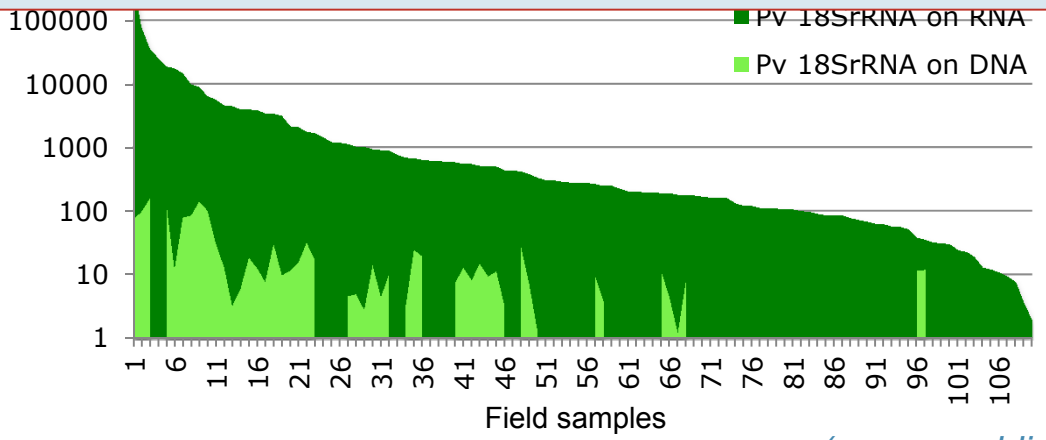
P. falciparum



Decision to use a cut-off as in NASBA

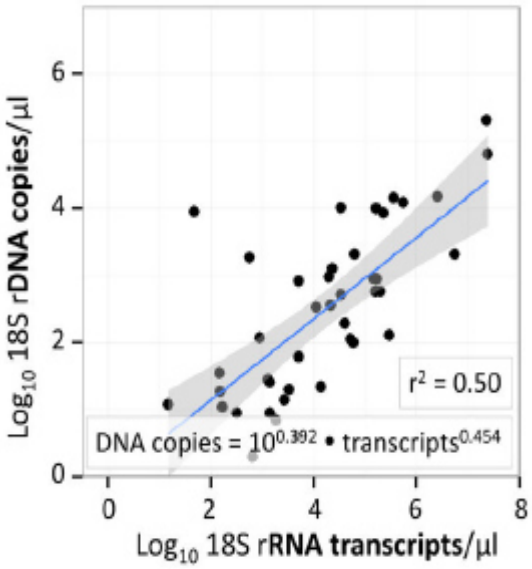
P. vivax

Pv: **8x lower** densities than Pf



(own unpublished results)

RNA-based vs. DNA-based parasite detection

Marker	RNA-based assay	DNA-based assay
18S rRNA	**Abundant** transcripts	**3** copies / genome
	Extremely high sensitivity	„Standard“ sensitivity
	Disadvantages: Quantification imprecise No absolute quantification	**Advantages:** good correlation with LM
	Cross-contamination (aerosols) during RNA extraction; **cut-off necessary**	No contamination issues

Lessons learned from using rRNA transcripts as diagnostic marker

- A large proportion of infections not noticed with standard techniques
- Beware of ribosomal RNA, only use with greatest caution & tight control
- Unlikely field applicable, unless enclosed in fully contained system
- Quantification is not very precise (expression levels/RNA degradation)
- Blood volume matters! Detection limit = 1 parasite in even large volume

- **Ultra-low density infections carry gametocytes**

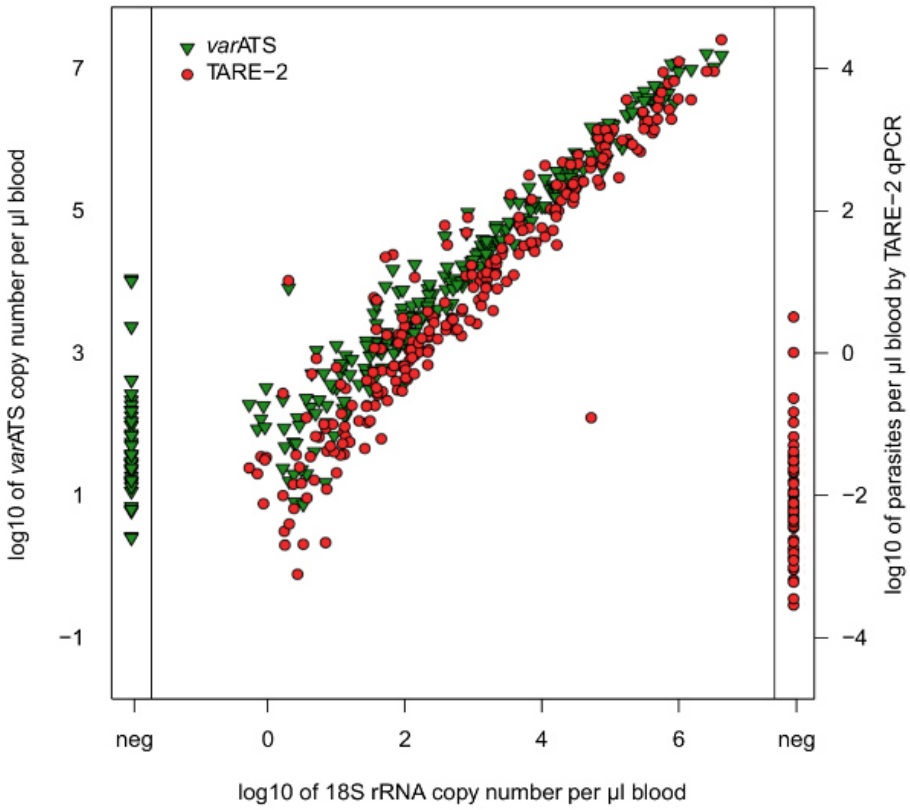
Development of ultra-sensitive DNA-based qPCR

- **TARE-2:** telomere-associated repetitive element 2
- 1.6 kb long blocks of 10-12 **135-bp repeat units** with slightly degenerate sequences
- approx. **250–280 copies /genome**

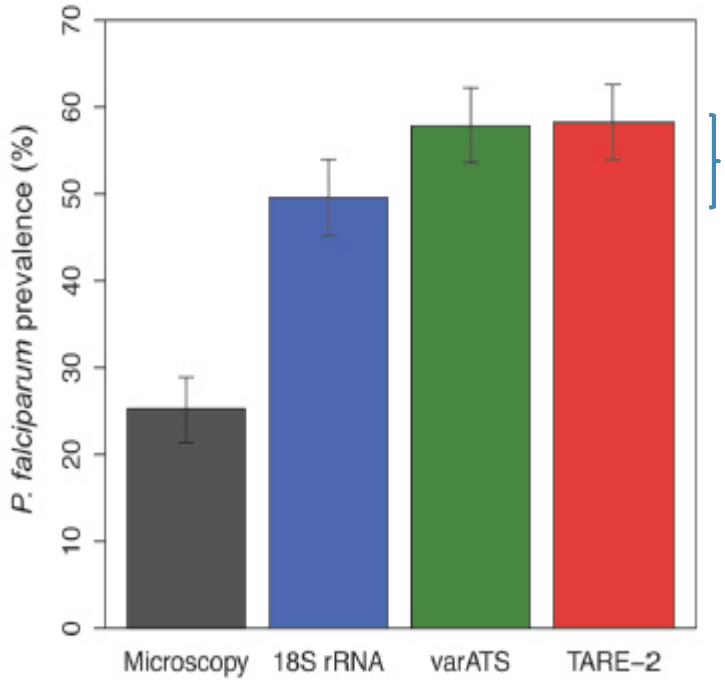
- ***var*-ATS:** acidic terminal segment (semi-conserved)
- 59 var genes in 3D7

Is a multi-copy PCR target suitable for quantification?

YES, good correlation!

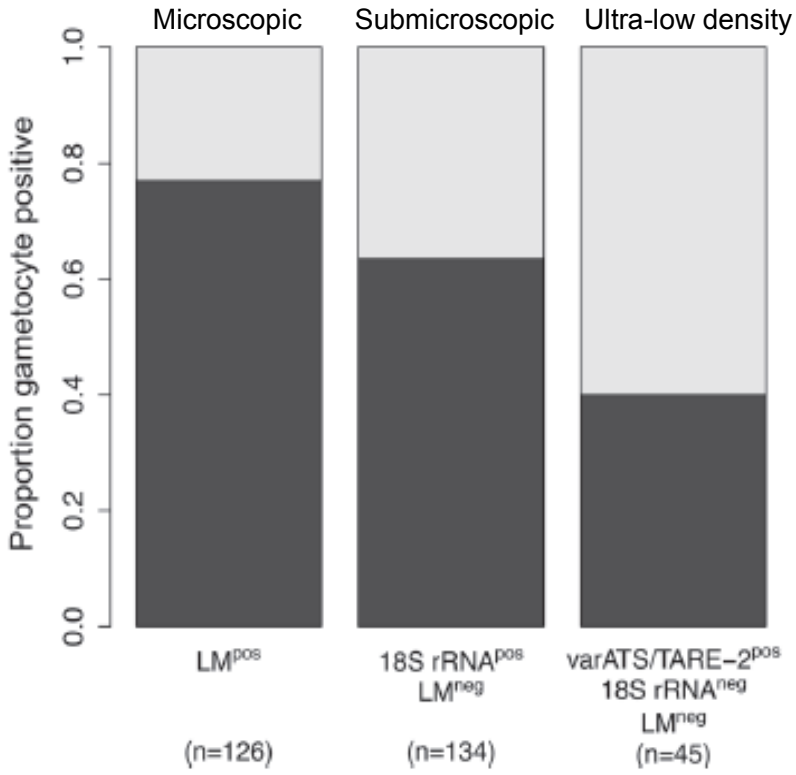


Implication for prevalence rate: **plus 16%**



(*Hofmann et al. 2015 PLOS Medicine*)

***P. falciparum* prevalence** in
498 individuals from Tanzania

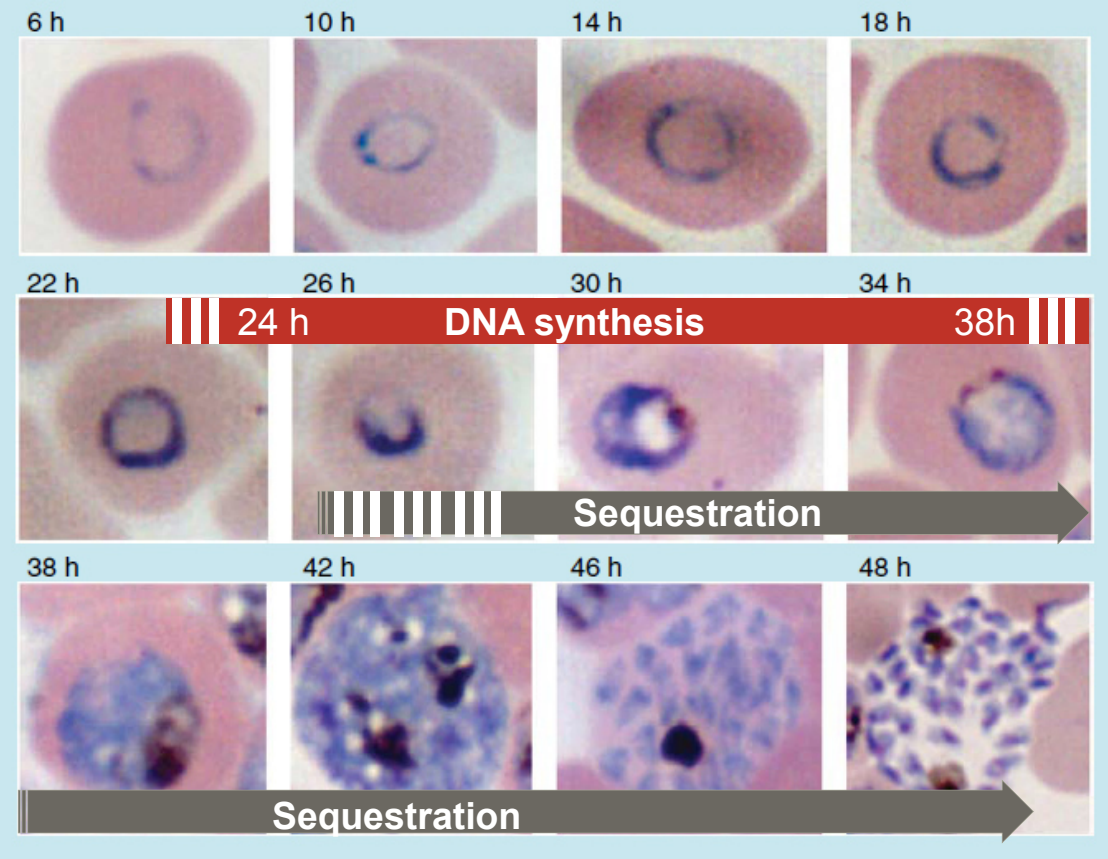


Proportion of gametocyte carriers
by pfs25 qRT-PCR

Are highly sensitivity assays required at all in field trials ?

Task	Use yes/no	Other uses
Day 0: Parasite detection at enrollment	No (symtomatic patents)	Validate LM quanti-fication with qPCR (EQC)
Day X: Detection of recurrence	No (persisting gametocytes) Yes (earlier detection)	Quantification by qPCR (EQC)
Surveillance/Research	Yes (prevalence; low endemicity)	Pooling of samples (multi-copy marker genes!)
In vitro drug assays	Yes (precise quantification for low parasitemia)	

"Absolute" Molecular Quantification?



Sources: Radfar et al. 2009 Nature Protoc; Doerig et al. 2000 Progr Cell Cycle Res

Parasite stages

Ring$_{1\text{-}18\ h}$
Haploid – 1 genome

Trophozoite$_{18\text{-}28\ h}$
Onset of DNA replication varies: 18-28 h p.i.

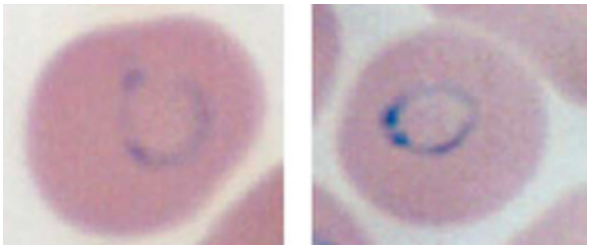
Schizont$_{28\text{-}38\ h}$
Multiple rounds of replication (syncytal cell)

Segmenter$_{38\text{-}48\ h}$
8-32 Merozoites are formed

Pf stages in peripheral blood have 1 (or a few have 2) genomes/parasite

Essentials of molecular quantification by qPCR

- Assay to be validated using a **trend-line** of synchronized **ring stage parasites** from *in vitro* culture (Pf only!)
  1 genome = 1 parasite
  Per genome: 3 or 5 copies of 18S rRNA gene



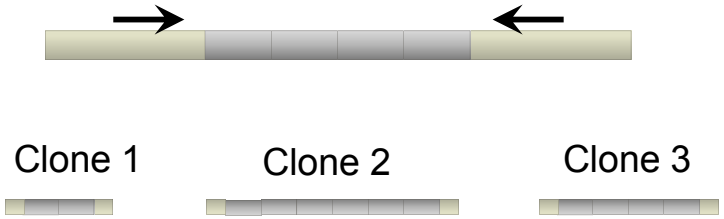
- **Standard curve**:
  - trend-line of ring stages (1 genome)
  - control plasmid with marker gene inserted
    - **supercoil**: copy number **~8-fold overestimated**!!
      Hou et al. 2010 PLOS ONE e9545
    - **restriction digested**: matches well with trendline
  - adust for copy number of the marker gene in genome

- Field samples: Relationship density by **qPCR : microscopy = roughly 1:1**
  If not: DNA stability compromized/nicked
  standard curve: not rings but mixed stages
  standard curve: not digested plasmid

P. falciparum genotyping: length-polymorphic markers

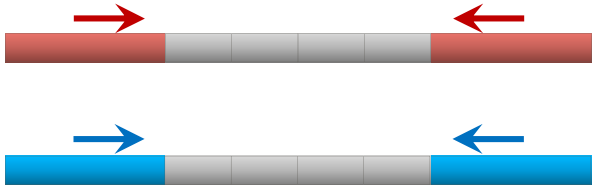
Length-polymorphism

→ Repeat sequences of variable copy number



Allelic families

→ Distinct flanking regions



P. falciparum: *merozoite surface protein 1 (msp1)* → 3 allelic families (2 polymorphic)

merozoite surface protein 2 (msp2) → 2 allelic families

glutamate-rich protein (glurp) → 1 allelic family

PCR method: nested PCR and CE

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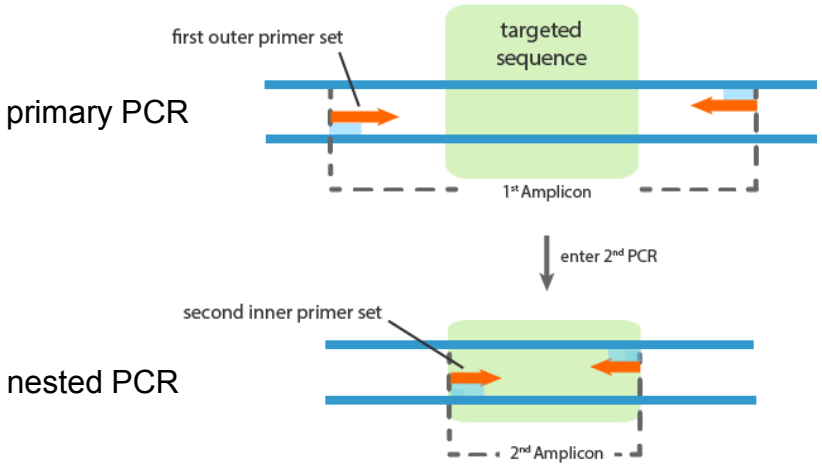
1) **Nested PCR**: increased sensitivity and specificity

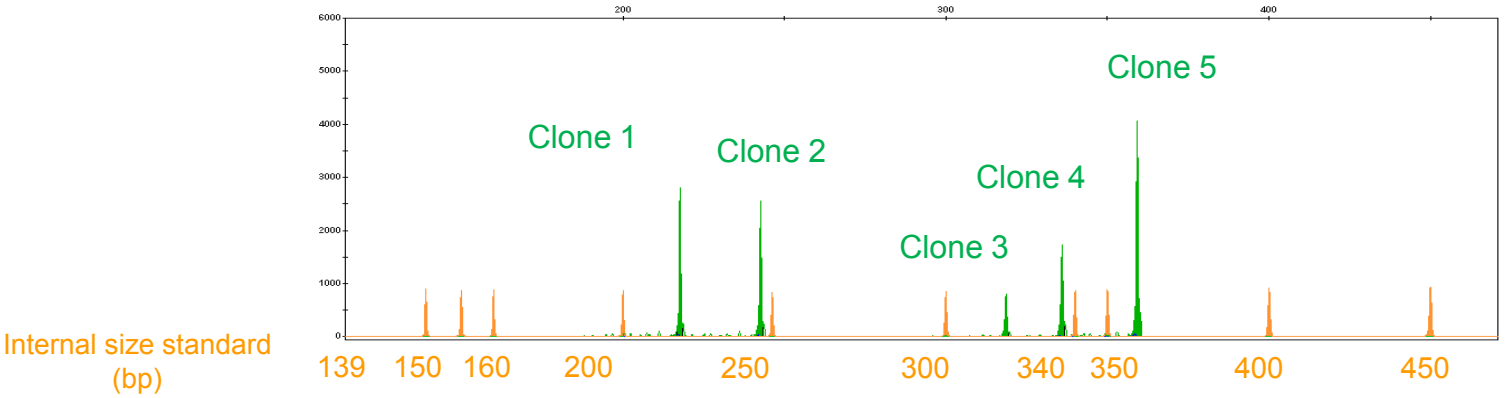


Reduction of time and cost:

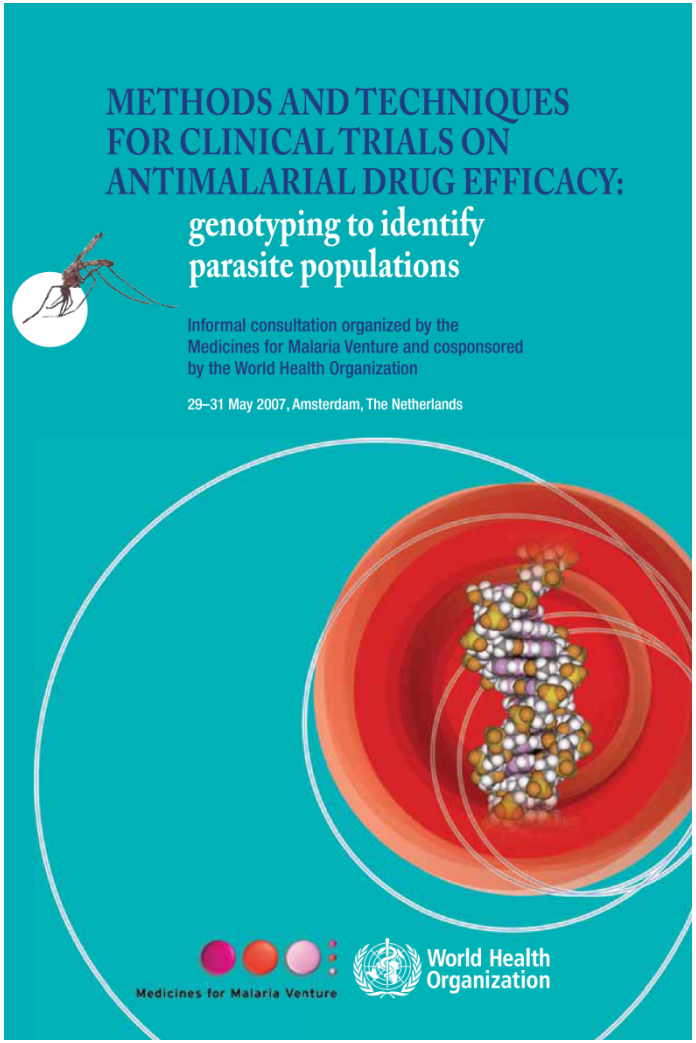
multiplex pPCR of *msp1/2*
multiplex nPCR of allelic families

Revise protocol!

2) **Capillary Electrophoresis (CE):** high-resolution sizing

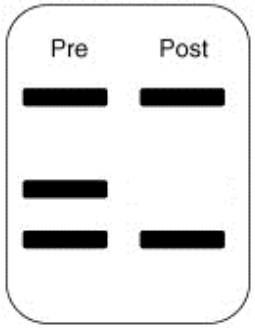


«PCR-correction» of clinical trial outcomes



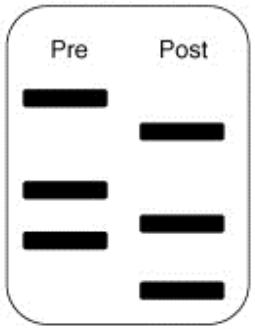
PCR correction:

Comparative genotyping of *Plasmodium* parasites in pre- and post-treatment sample (i.e. day of failure)



Recrudescence:

… at least one allele at each locus is common to both paired samples.



New infection:

… **all** the alleles in […] the post-treatment sample are different from those in the admission sample, **for one or more loci tested**.

Achievements in genotyping and critical issues

CE: improved resolution and reproducibility of fragment sizing

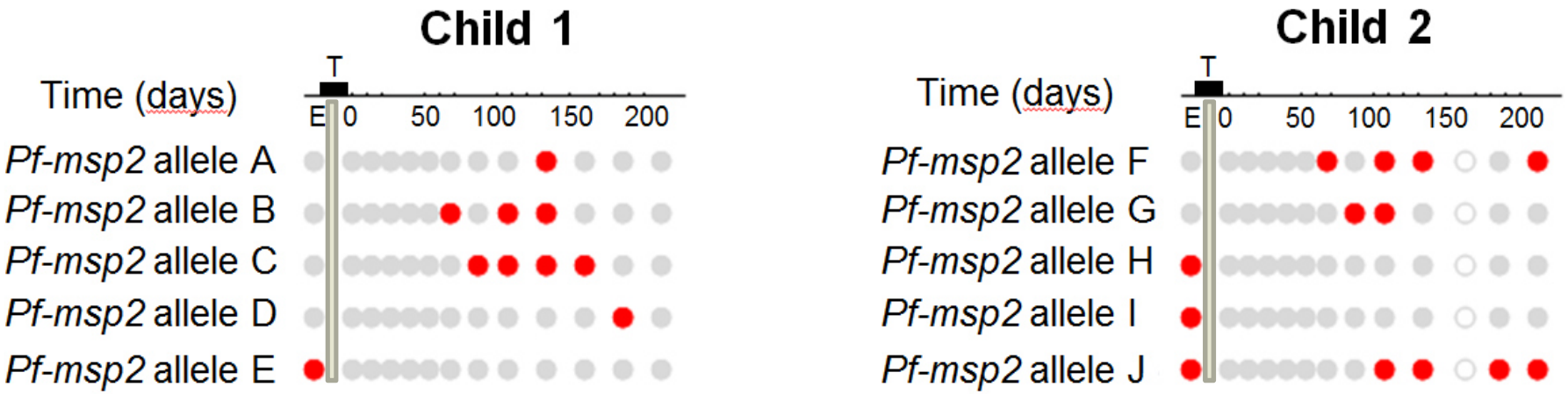
- permits comparison of alleles between separate runs
- allele frequencies in a population can be determined to assess probability of reinfection with same allele

Major critical issues in Genotyping:

? detectability of clones and minority clones (biological & technical causes)

? usefulness in settings with either very low or very high transmission

Detectability of *P. falciparum* clones in natural asymptomatic infections



Dynamics of *msp2* alleles in 2 children from PNG in the course of 8 months follow-up

- Clone detected
- Clone not detected
- Missing sample

Robinson et al. 2015 PLOS Med

msp1/msp2: PCR bias towards short fragments

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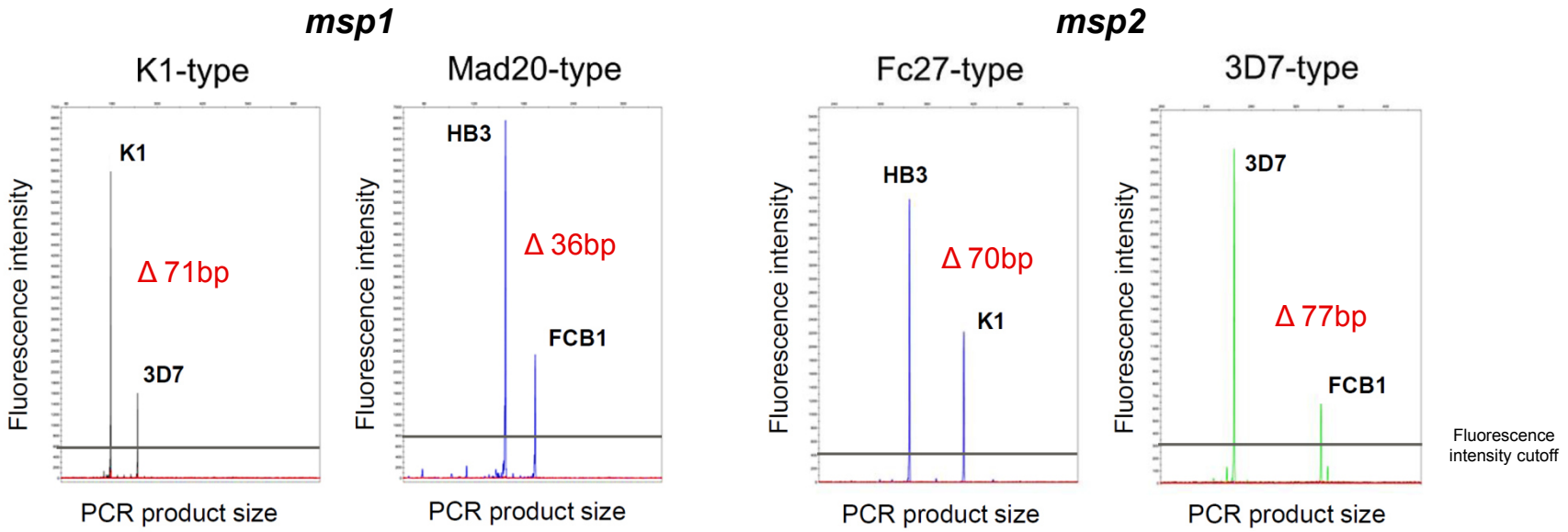
***P. falciparum* culture strains:**

Strain	*msp1*	*msp2*
HB3	Mad20-type 158 bp	Fc27-type 337 bp
3D7	K1-type 248 bp	3D7-type 265 bp
K1	K1-type 177 bp	Fc27-type 407 bp
FCB1	Mad20-type 194 bp	3D7-type 342 bp

Experimental mixtures

- Reciprocal ratios 1:1 to 1:5000
- In human DNA solution
- Minority clone at >10 parasites/µl

***msp1 / msp2* genotyping PCR on 1:1 ratios:**



Messerli, Felger et al. submitted

Template competition in *glurp* PCR

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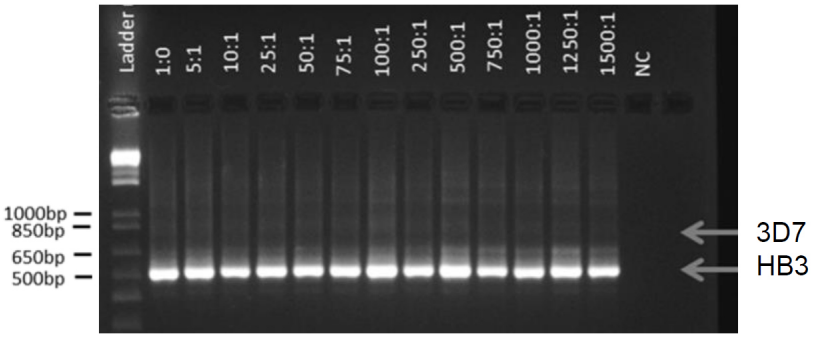
Limitations of marker glurp:

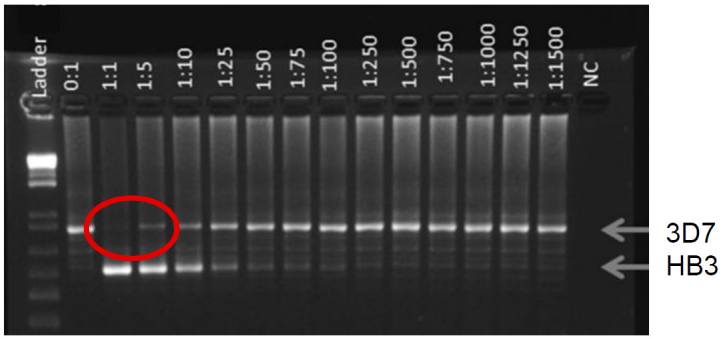
Longest allele sizes	→ increased competition
Only 1 allelic family	→ direct competition between all alleles
Prone to stutter peaks	→ requires increased cutoff

2-strain mixtures

Dominant clone: HB3 (shorter allele)



Dominant clone: 3D7 (longer allele)



norority
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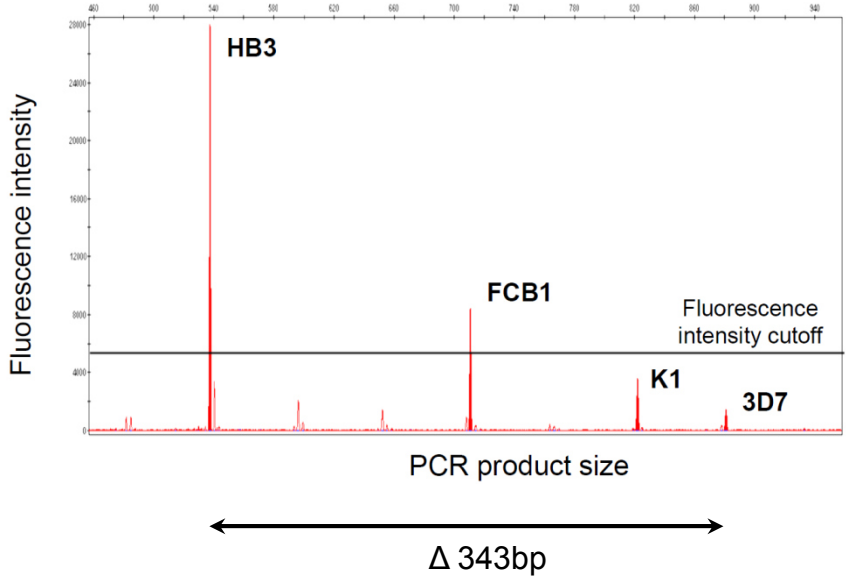
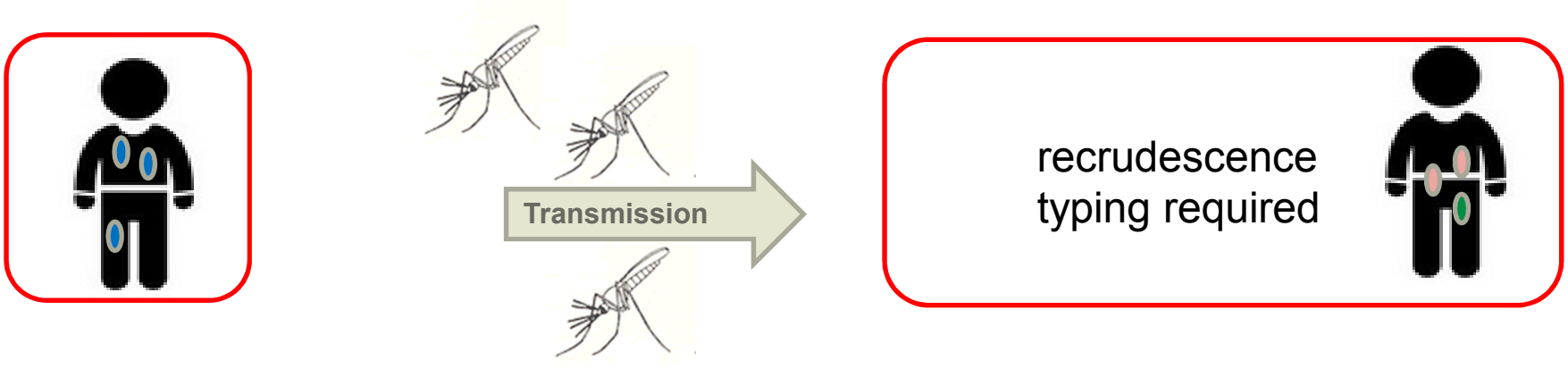
4-strain mixtures



Marker *glurp* is the least useful!

Messerli, Felger et al. submitted

Conclusion 1: genotyping

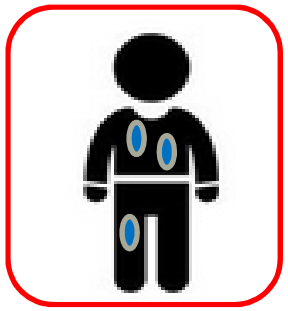


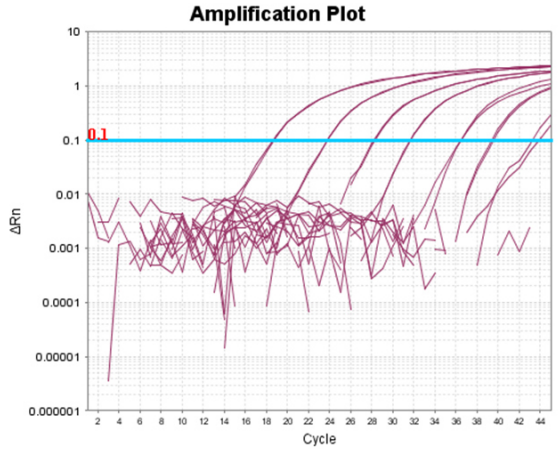
- ✓ Optimized protocols exist
- ✓ EQC established

Needed:
- **revised recommendations**
- **re-assess usefulness for different levels of endemicity**
- **reinforce QA/EQC**

Research Needed
- Validation and EQC of deep sequencing for SNP-based genotyping
- Assess the level of improvement in SNP-based detection of minority clones

Conclusion 2: molecular detection and quantification





- ✓ Good quantification protocols
- ✓ Consensus on epidemiological relevance

Needed:

- **build consensus on potential application in trials**
- **reinforce EQC for absolute quantification**

Research Needed

- Validation and EQC of absolute quantification by digital droplet PCR (ddPCR)
- Contribution of gametocytes (less affected by some drugs) to positivity

Thanks

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