Detection and quantification of rare mutations with massively parallel sequencing

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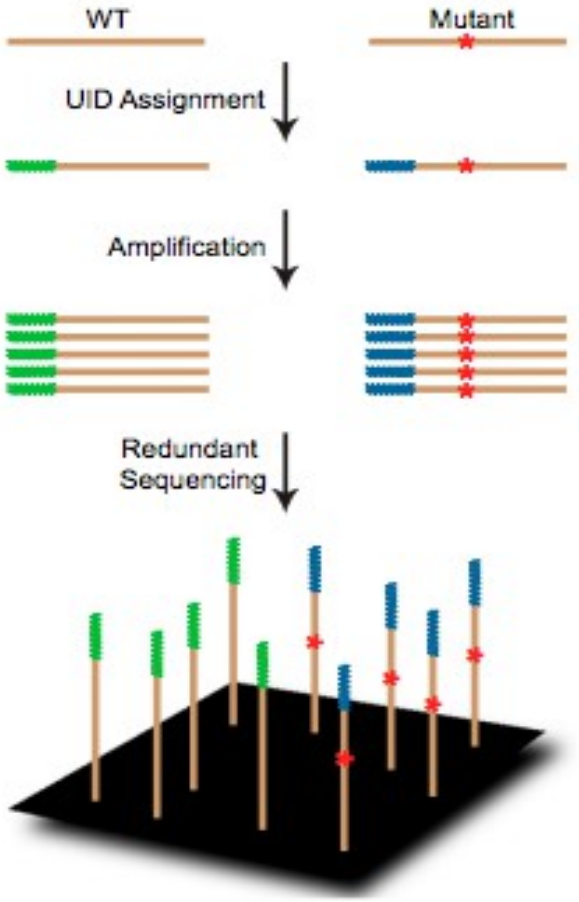
Motivation

- Low-prevalence mutations are difficult to identify with confidence
  - In clinical setting, prevalence can be <0.01%
- Illumina, and sequencing technologies more generally, still have error rates high enough to mask these rare mutations
  - estimates ranged from 1% to 0.05% in 2011
  - ~0.01% in 2015 (1)
- Many aspects of sequencing factor into this error:
  - Library prep
  - Choice of primers
  - Base-calling
  - Read length

Safe-Sequencing System ("Safe-SeqS") Methodology

1. Assign unique id (UID; aka barcodes or indices) to each DNA template
2. Amplify of each uniquely-tagged template:
   - Creates many daughter molecules = "UID family"
3. Sequence
4. If 95% of any UID family has identical mutation → "supermutant"



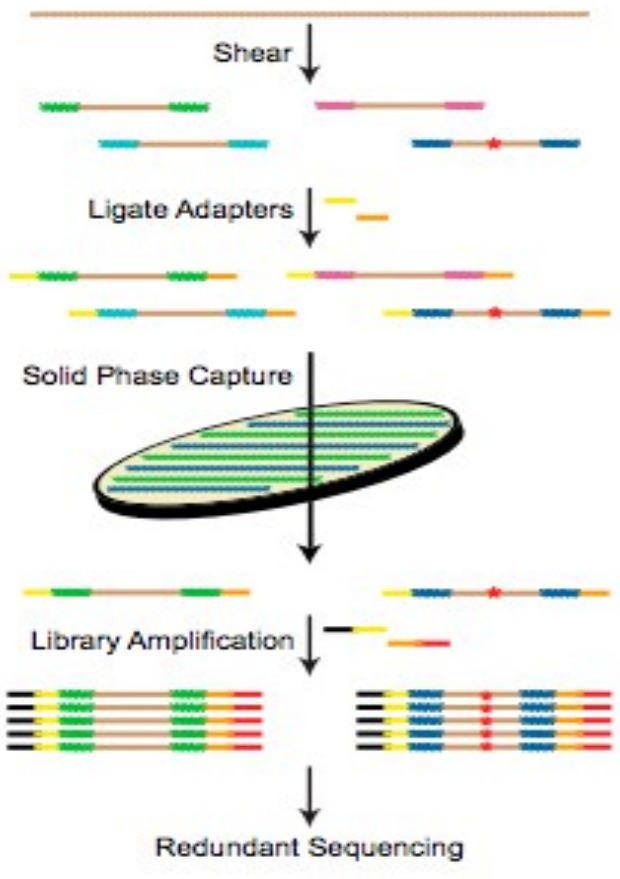
UID Assignment: Endogenous

Randomly sheared DNA inherently contains unique seq at either end → call UID

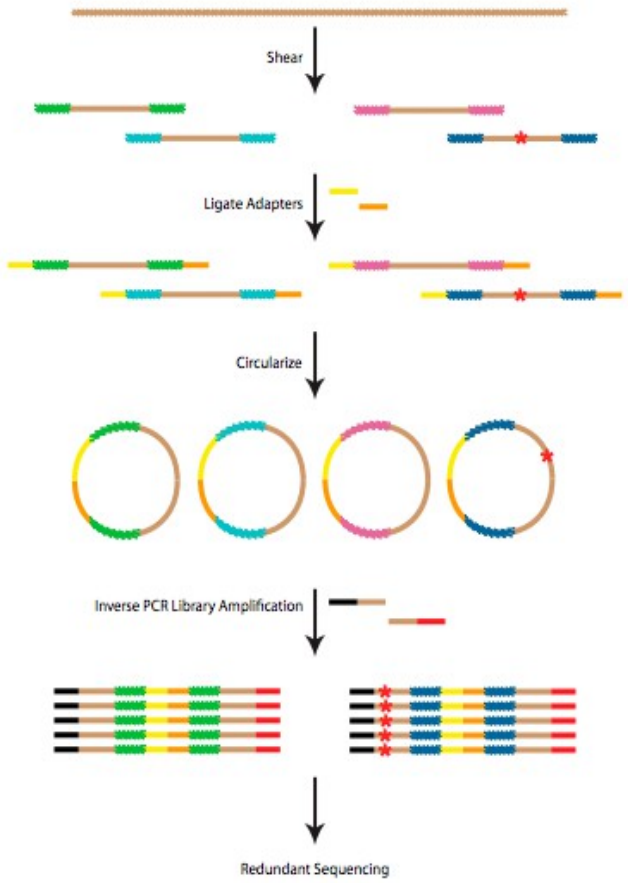
2 approaches:

- Capture: many genes simultaneously
- Inverse PCR: single gene in depth

Capture



Inverse PCR



Endogenous UID Experiments

Capture:

- 15000 normal cells
- 2.6kb from 6 genes
- SafeSeqS: error lowered 70 fold

Inverse PCR:

- 1750 normal cells
- 1 gene of interest
- 1057 unique molecules assessed
- SafeSeqS: conventional results likely all FP

	Capture	Inverse PCR
Conventional analysis		
High-quality base pairs	106,958,863	1,041,346,645
Mean high-quality base pairs read depth	38,620×	2,085,600×
Mutations identified	25,563	234,352
Mutations/bp	2.4*E*-04	2.3*E*-04
Safe-SeqS analysis		
High-quality base pairs	106,958,863	1,041,346,645
Mean high-quality base pairs read depth	38,620×	2,085,600×
UID families	69,505	1,057
Average no. of members/UID family	40	21,688
Median no. of members/UID family	19	4
Supermutants identified	8	0
Supermutants/bp	3.5*E*-06	0.0

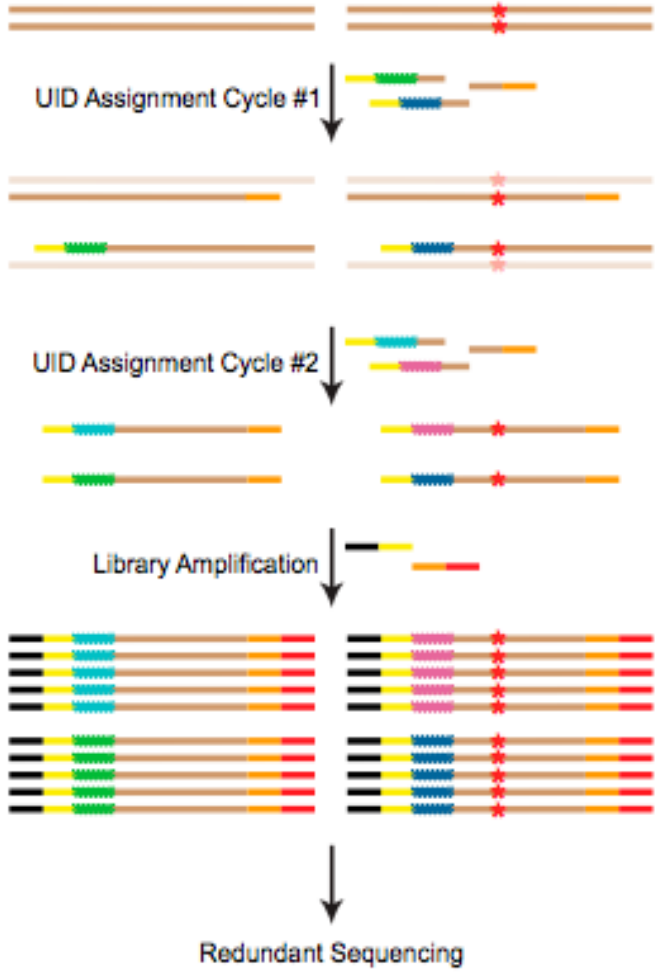
UID Assignment: Exogenous

2 cycles of UID assignment during PCR

12-14 random nucleotides b/t forward primer and seq serves as UID

Amplification: 25 PCR cycles

Gives a uniquely-tagged, dsDNA fragment for each of the original template strands



Mean SD

Exogenous UID Experiments

CTNNB1:

- 3 individuals
- SafeSeqS: reduced apparent error 24 fold

Mitochondrial DNA:

- 7 individuals
- SafeSeqS: 15 fold reduction

Discussion

How do we know conventional analyses represent artifacts rather than true mutations?

- consistency of results
- polymerase fidelity experiment results consistent with previous biological assays

Limitations:

- Exogenous UID strategy cannot be applied to multiple amplicons from sample containing a limited # of templates
- Still does not overcome error introduced in amplification

Future directions

References

(1) Schrimer et al. 2015. Insight into biases and sequencing errors for amplicon sequencing with the Illumina MiSeq platform. *Nuc Acid Res*. doi: 10.1093/nar/gku1341.