

Analysis of High-Resolution HLA Typing by NGS: Which tools do we need?

Leipzig, 2019

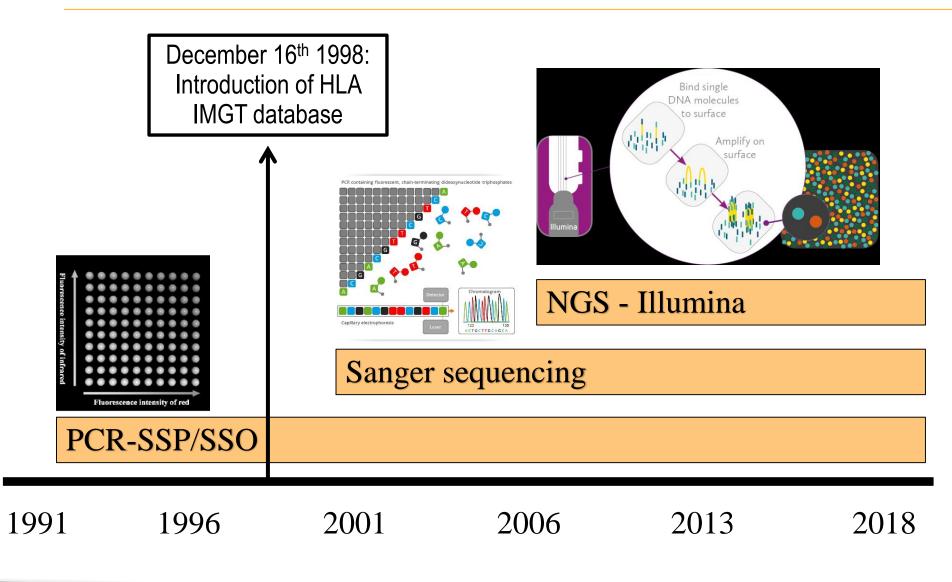
Nina Lauterbach, Senior Field Application Scientist





- Brief History
- SBT vs NGS
- Benefits and challenges of NGS
- Tools in the NGS analysis software HLA Twin
- Summary
- Product update Omnitype
- Questions

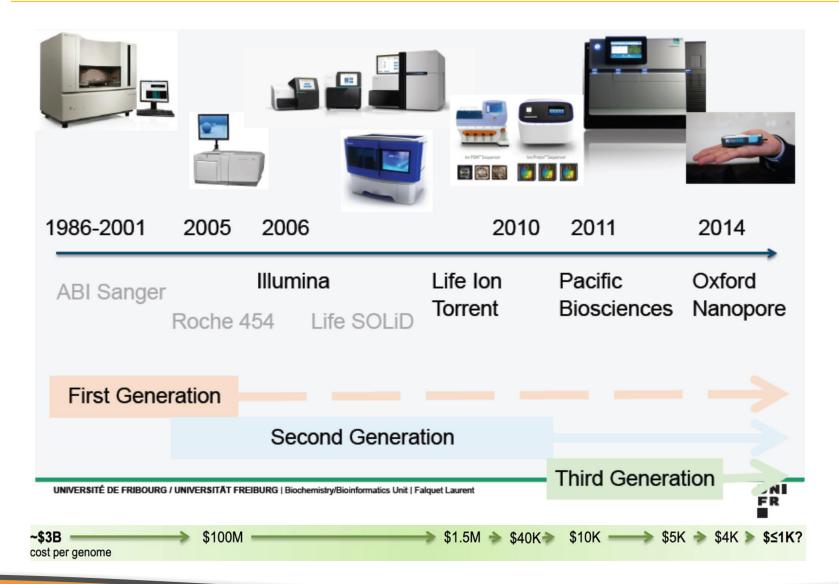
Evolution of molecular HLA typing methods



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Evolution of NGS platforms

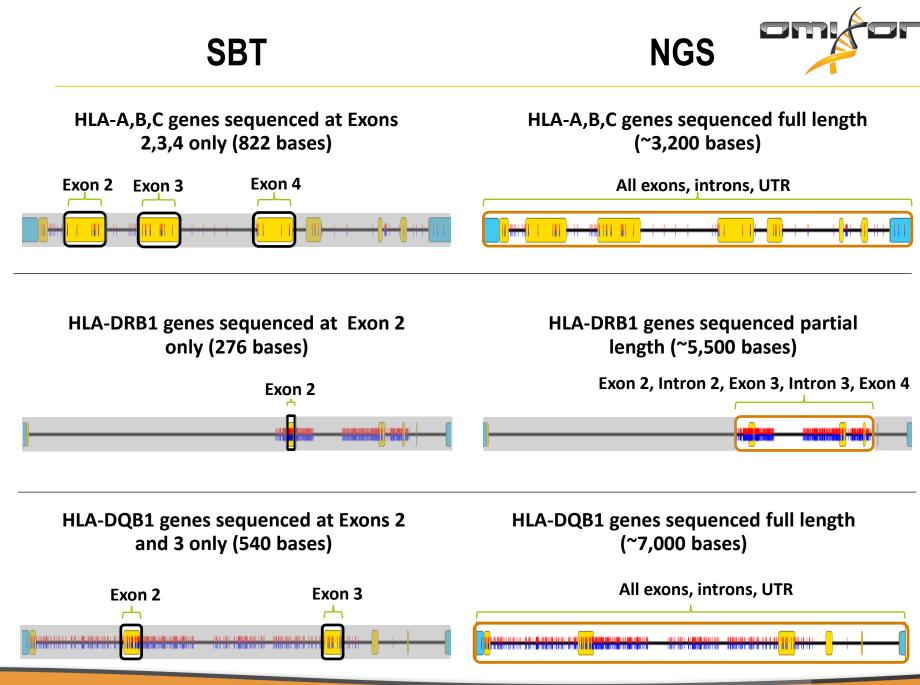




What do we gain from NGS?



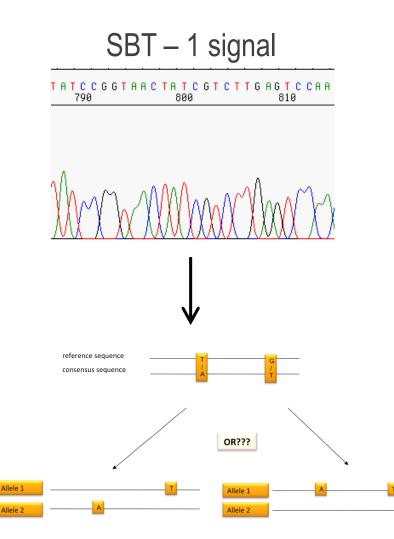
- 1. Full length gene sequencing
- 2. The ability to identify novel/ null alleles
- 3. Typing with (almost) zero ambiguities
- 4. Flexibility in sample number, from low- to high throughput
- 5. Possibility to automate pre- and post-PCR
- 6. Cost efficient possibility to combine different type of libraries



Courtesy of Curt Lind at CHOP

Benefit of phasing with NGS





NGS – 2 signals

	Allele	-	A	-	с 韋	т 韋	G	\$
Н	LA-C*P1:C1				135			1
H	LA-C*P1:C2				1			193

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G G G G G G G		Exon T T T T T T T	G G G G G G	Exe A A A A A A A A A A A A	G G G G G G	E A A A A A A A A A A A A A	G G G G G	3 C C C C C C C C C C	Exo C C C C C C C	n 3 T T T T T T T T T	E A A A A A A A A A	C C C C C C C C	C C C C C C C C C C C C		G G G G G	Ex G G G G G G G G G G G G G G G G G G G	A A A A A A A A A A A A A A A A A A A	E T T T T T	G G G G G G	G G G G G G			EX C C C C C C C C C C C C C C C C C C C	on 3 G G G G G G G G G G

Challenge of HLA typing using NGS

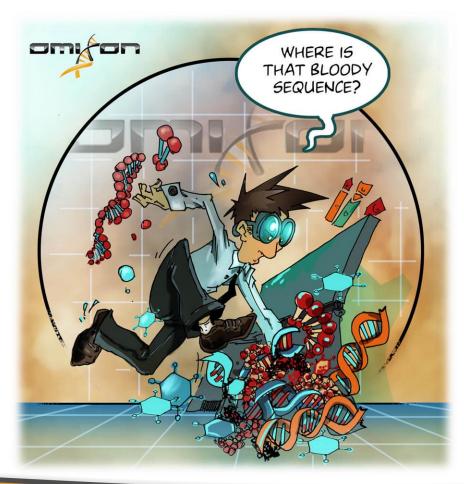


- New definitions (phasing, coverage, Q30 score etc.)
- Complex algorithms
- Incomplete reference allele database
- Increasing allele database (35% increase from 3.33 to 3.38)
- Large and more complex dataset

NGS analysis software



The software is crucial in order to make sense of the huge amount of sequencing data



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Illumina as primary platform





ABI 3730 Capillary sequencer Low volume, high labor



Roche 454 GS Junior Benchtop NGS Expensive per run



Genestudio S5 Benchtop NGS Semi-conductor sequencing Long sample prep Rapid run time



PacBio RS Non Benchtop (large) Fluorophore photocapture S M R T High raw error

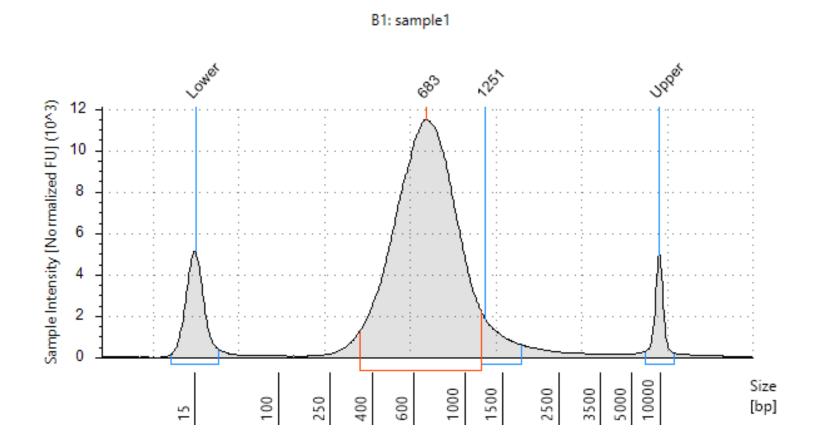


Illumina MiSeq Benchtop NGS Highly automated Quick sample prep Lowest error rate

Library contains various fragment sizes

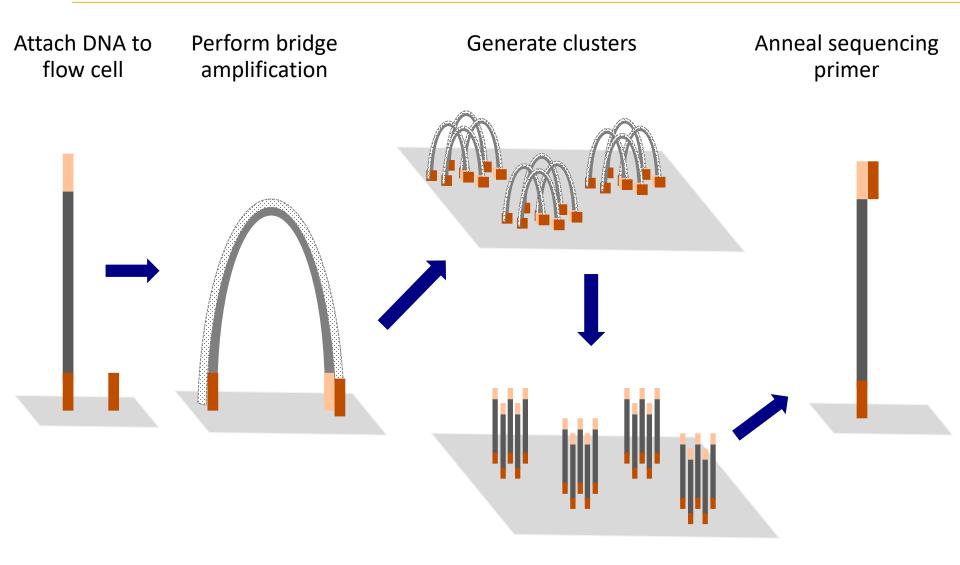


Fragment distribution after the bead-based size selection (500 – 1500 bp)



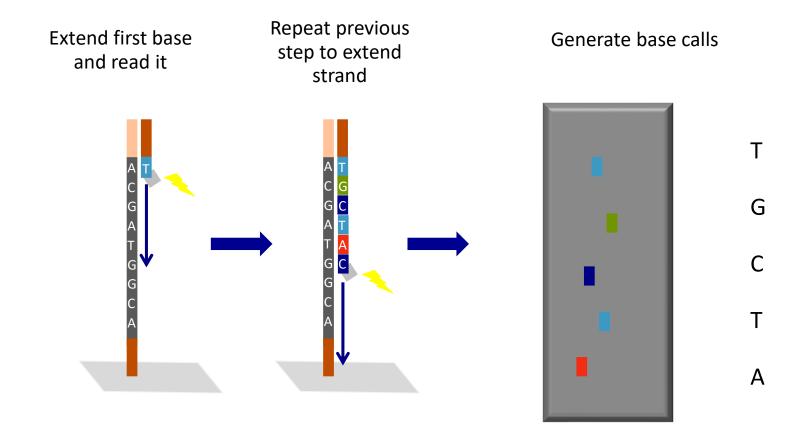
Illumina NGS Chemistry



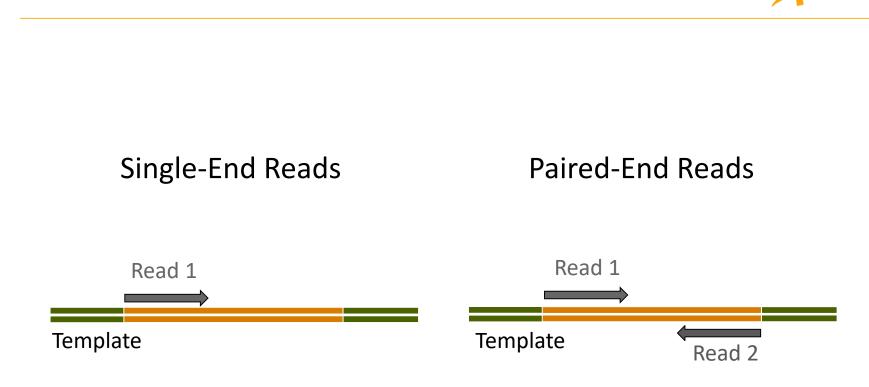


Illumina NGS Chemistry





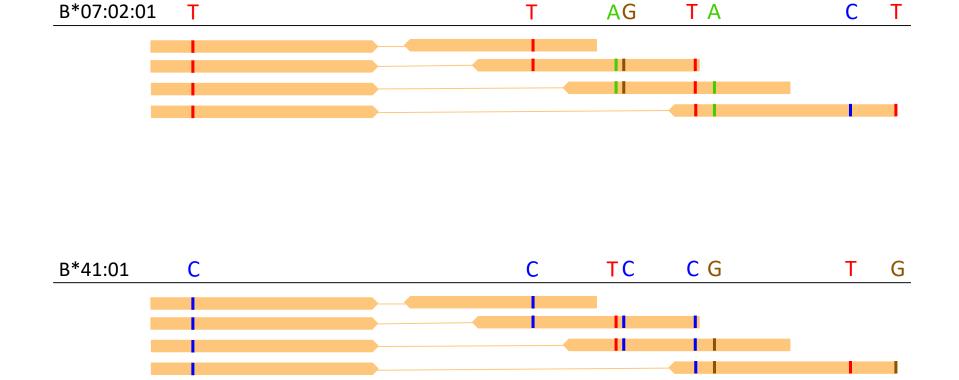




Sequence from the paired reads originate from the same template, and therefore are phased with each other

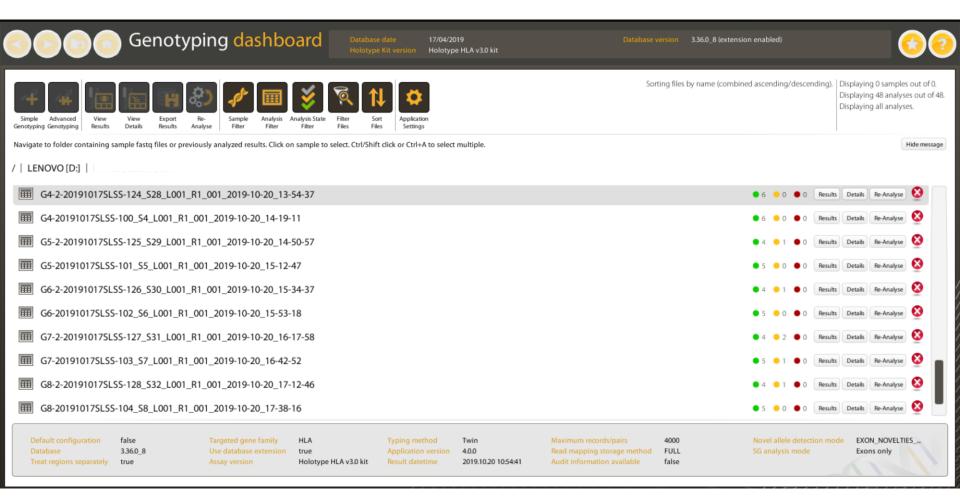
Long Distance Phasing





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Analysis paired-end reads with HLA Twin





Omixon HLA Twin (Software)



- Preconfigured Holotype HLA-specific settings
- Automated genotyping after MiSeq run
- Up to two algorithms for determining HLA genotypes
 - Consensus Genotyping (Assembly)
 - Statistical Genotyping (Alignment to IMGT/HLA)
- Traffic light system for data interpretation and workflow management



Passed Info Inspect Investigate Failed

HLA Twin - The Traffic Light System



HLA-A*01:01:01:01



Fully concordant Concordant to 2nd field Concordant to 1st field

Discordant



All QC Metrics Passed

Passed with extra info

Inspect result

Investigate result

Failed. Rerun or reflexive test



- Two algorithms for determining HLA genotypes:
 - 1. Consensus Genotyping (Assembly)
 - 2. Statistical Genotyping (Alignment to IMGT/HLA)



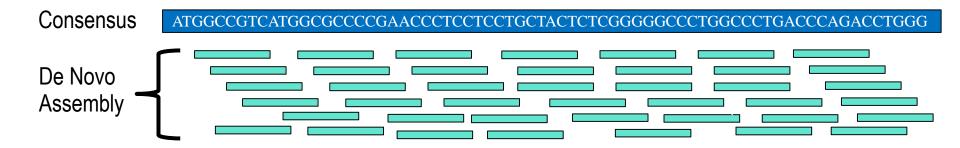
Consensus Genotyping (Assembly) :

- 1. C- collect
- 2. C- construct
- 3. C- compare
- 4. C- check

HLA Twin - Consensus algorithm (1)



- 1. C- collect The HLA Twin will gather all sequencing reads from the Illumina sample file pair and will assign them to a specific locus.
- 1. C- Construct The processed reads per loci will be assembled (De Novo Assembly) to construct a long and continuous sequence called the consensus.



> During the process of De Novo Assembly, a filtering algorithm will detect reads with too low quality, which will be filtered out.

HLA Twin - Consensus algorithm (2)



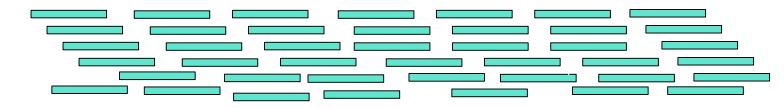
3. C- Compare After completion, the consensus sequence will be compared to all the reference sequences of the alleles (of that specific loci) in the IMGT/HLA database to find a best match allele and to make an allele call.





4. C- Check - Upon the allele call the software will re-check itself (since it used only the consensus sequence to identify the best match): All the processed sequencing reads will be aligned to the reference sequence of the best matched allele (in this example HLA-A*01:01:01:01).

A*01:01:01:01 ATGGCCGTCA TGGCGCCCCG AACCCTCCTC CTGCTACTCT CGGGGGGCCCT GGCCCTGACC CAGACCTGGG



> Potential mismatches (novel sequences) between the reference sequence and sequencing reads will be detected and highlighted.



Statistical Genotyping : Alignment to IMGT/HLA

Works in a " if " scenery:

1. C- confirm



5 – Confirm: If the QC metrics are below a certain threshold (or a novelty is found), the software will try to **confirm the allele call** with the Statistical genotyping.

- The reads from the Illumina sample file pair will be directly aligned to all the alleles of the IMGT/HLA database.
- A complex calculation decides which allele has most reads aligned and therefore will be chosen as best match.

Tools to troubleshoot...





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Quality Control Metrics system



 The 24 quality metrics will guide you towards the cause of a quality issue and help you decide whether or not you need to repeat the run.

Allele 1	Browe Zeles 2 Genotype Show Details Show Show Novelbes Steles Steles Details Steles Deatails Steles Deatails Ste		pprove Rejuct/Revoke Approval			Displ	base version: 3.3 laying 5 loci out ng best matche:
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A-A	Genotype Quality control Data statistics						
	Measure	HLA-A	LI LI	A-B HLA-	C HLA-DC	QB1 HLA-DP	RB1
А-В	Measure	HLA-A					
A-B	Overall ()	INFO		IFO INFO			D
-C	Overall ①						D
-C	Overall © Primary QCs for Interpretation ©	INFO	1	IFO INFO	D PASSI	ED INFO	
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ①	INFO 3844	■ 3424	(FO INFC	D PASSI 0 • 8193	ED INF(0
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ① Noise ratio ①	• 3844 • 0%	 3424 0% 	IFO INFC ■ 3847 ● 0.26%	PASSI 8193 0%	ED INF(0 7635 0 0.05%	0
-C -DQB1	Overall Primary QCs for Interpretation Read count Noise ratio Key exon spot noise ratio	• 3844 • 0% • 0%	 3424 0% 0% 	IFO INFC ○ 3847 ○ 0.26% ○ 9%	 PASSI 8193 0% 0% 	ED INFC 0 7635 0 0.05% 0 0%	0 0
-C	Overall Primary QCs for Interpretation Read count Noise ratio Key exon spot noise ratio Consensus coverage key exon minimum depth	■ 1NFO ● 3844 ● 0% ● 0% ● 66		IFO INFC ○ 3847 ○ 0.26% ○ 0.9% ○ 0.47	PASSI ① 8193 ① 0% ① 0% ① 159	ED 105% 0.05% 0.05% 0.05% 0.41	
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ① Noise ratio ② Key exon spot noise ratio ② Consensus coverage key exon minimum depth ③ Key exon allele imbalance ③	■ 3844 ● 0% ● 0% ● 66 ● 0.54 :0.46		 INFC INFC 3847 0.26% 0.26% 0.9% 47 0.54:0.46 	PASSI 0 8193 0 0% 0 0% 0 159 0 0.5:0.5	ED INFC 0 7635 0 0.05% 0 0.0% 0 41 0 0.54:0.46	
-C -DQB1	Overall Primary QCs for Interpretation Primary QCs for Interpretation Read count Noise ratio Key exon spot noise ratio Consensus coverage key exon minimum depth	■ 1NFO ● 3844 ● 0% ● 0% ● 66		IFO INFC ○ 3847 ○ 0.26% ○ 0.9% ○ 0.47	PASSI ① 8193 ① 0% ① 0% ① 159	ED 105% 0.05% 0.05% 0.05% 0.41	0 0 0
-C -DQB1	Overall Primary QCs for Interpretation Read count Noise ratio Key exon spot noise ratio Consensus coverage key exon minimum depth Key exon allele imbalance Genotype available	■ 3844 ● 0% ● 0% ● 66 ● 0.54 :0.46		 INFC INFC 3847 0.26% 0.26% 0.9% 47 0.54:0.46 	PASSI 0 8193 0 0% 0 0% 0 159 0 0.5:0.5	ED INFC 0 7635 0 0.05% 0 0.0% 0 41 0 0.54:0.46	
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ① Noise ratio ② Consensus coverage key exon minimum depth ① Key exon allele imbalance ① Genotype available ② Secondary QCs for Interpretation ③	INFO		 INFC 3847 0.26% 0.9% 47 0.54:0.46 ○ Yes 	O PASSI ① 8193 ③ 0% ③ 0% ③ 159 ③ 0.5:0.5 ④ Yes	D 7635 ○ 0.05% ○ 0% ○ 0.41 ○ 0.54:0.46 ○ Yes	
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ① Noise ratio ② Key exon spot noise ratio ① Consensus coverage key exon minimum depth ① Key exon allele imbalance ① Genotype available ② Secondary QCs for Interpretation ① Fragment size ③	■ 3844 ● 0% ● 0% ● 66 ● 0.54 :0.46		 INFC INFC 3847 0.26% 0.26% 0.9% 47 0.54:0.46 	PASSI 0 8193 0 0% 0 0% 0 159 0 0.5:0.5	ED INFC 0 7635 0 0.05% 0 0.0% 0 41 0 0.54:0.46	
-C -DQB1	Overall ① Primary OCs for Interpretation ① Read count ① Noise ratio ① Key exon spot noise ratio ① Consensus coverage key exon minimum depth ① Key exon allele imbalance ① Genotype available ① Secondary OCs for Interpretation ① Fragment size ② Read quality ①	■ 3844 ● 3844 ● 0% ● 66 ● 0.54 : 0.46 ● Yes ■ 437	 3424 906 906 906 906 906 9052:048 9 Yes 9435 	 INFC 3847 0.26% 0% 0% 47 0.54:0.46 Yes 426 	 PASSI 8193 0% 0% 0% 159 0.5:05 Yes 	ED 17635 0 0.05% 0 0.05% 0 0% 0 41 0 0.54:0.46 0 Yes	
-C -DQB1	Overall ① Primary QCs for Interpretation ① Read count ① Noise ratio ② Key exon spot noise ratio ① Consensus coverage key exon minimum depth ① Key exon allele imbalance ① Genotype available ② Secondary QCs for Interpretation ① Fragment size ③	 INFO 3844 0% 0% 66 0.54 : 0.46 Yes 	 3424 976 976 976 976 976 976 975 975	IFO INFC ■ 3847 ● 0.26% ● 0% ● 0% ● 0% ● 0% ● 0.54:0.46 ● Yes ① ● 426 ● 3629 	 PASSI 8193 0% 0% 0% 159 0.5:0.5 Yes 	ED INFC 0 7635 0 0.05% 0 0.05% 0 41 0 0.54:0.46 0 Yes 0 418 0 3.741	

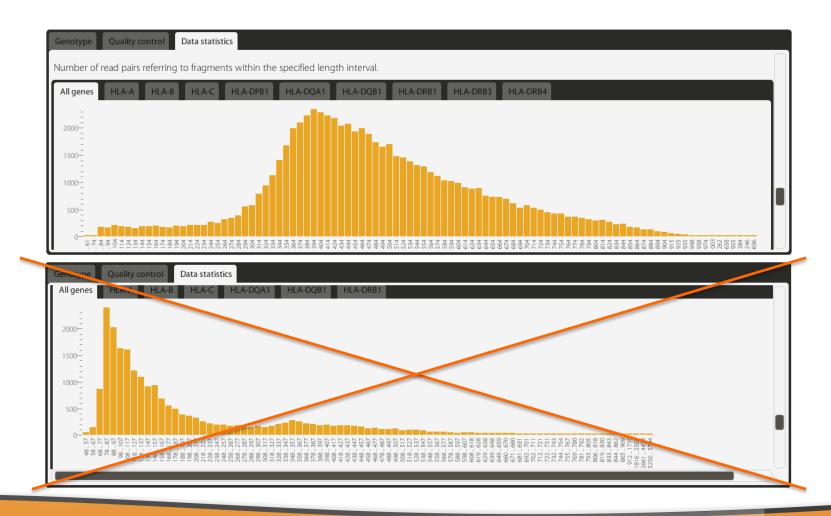
Quality determined according to set thresholds

Measure	HLA-A		HLA-B		HLA-C		HLA-DQB	1	HLA-DRB	1
Overall (1)	INFO	INFO			INFO	PASSE)	INFO		
Primary QCs for Interpretation 🕖										
Read count 🕐	• 3844	0	• 3424	0	3847	0	8193	0	• 7635	0
Noise ratio 🛈	• 0%	0	• 0%	0	0.26%	0	• 0%	0	0.05%	C
Key exon spot noise ratio 🕖	• 0%	0	• 0%	0	• 0%	0	• 0%	0	• 0%	0
Consensus coverage key exon minimum depth 🕐	• 66	0	65	0	• 47	0	• 159	0	• 41	0
Key exon allele imbalance 🛈	• 0.54 : 0.46	0	0.52:0.48	0	PASSED criteria: ≥ 2	25		0	0.54 : 0.46	0
Genotype available	Yes	0	Yes	0	INFO criteria: 25 20			0	Yes	0
					INFO CITIENA: 25 2	.0				
Secondary QCs for Interpretation (1)					INSPECT criteria: 20	15				
Fragment size 🕐	• 437	0	• 435	0	INVESTIGATE criteria	. 15	10	0	• 418	0
Read quality 🛈	• 36.36	0	36.17	0	INVESTIGATE CITIENA	1. 13	10	0	37.41	C
Other exon spot noise ratio 🕖	• 0%	0	• 0%	0	FAILED criteria: ≤ 1	0		0	• 0%	C
PCR crossover artifact ratio 🕐	• 0%	0	• 0.38%	0	0.170	V	070	0	• 1.01%	C
Key exon mismatch count 🕖	• 0	0	• 0	0	• 0	0	• 0	0	• 0	0

DN



Recommended average fragment size of 400 bp



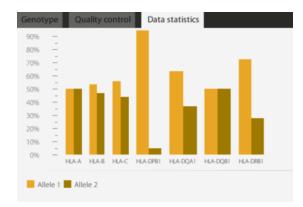
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Allele imbalance?



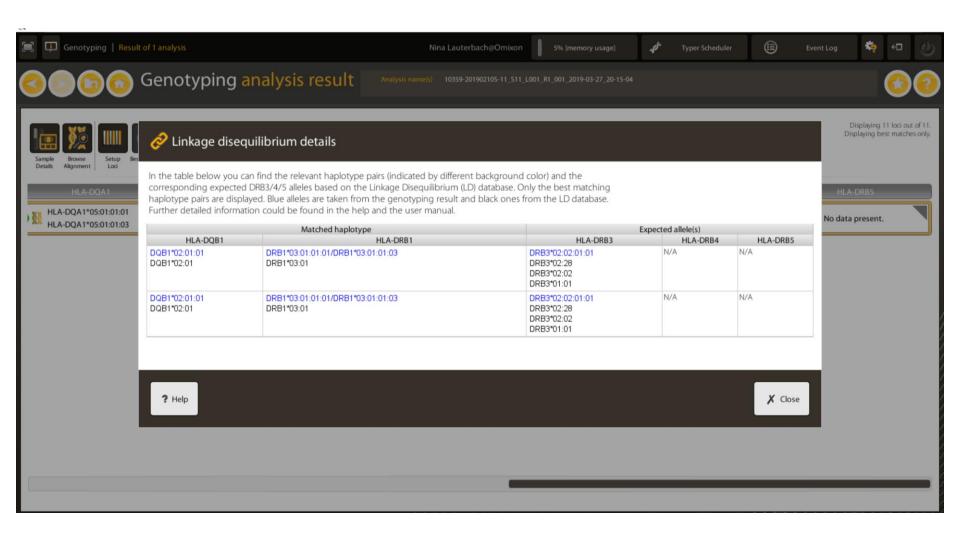
 The software can detect strong allelic imbalance > second allele below 1%

Measure	HLA-A		HLA-B		HLA-C		HLA-DPB1		HLA-DQB	1	HLA-DRB1	
Key exon mismatch count 🕐	• 0	0	• 0	0	• 0	0	• 0	0	• 0	0	• 0	0
Warnings for Troubleshooting 🕐												
Read length 🕐	• 144	0	• 142	0	• 141	0	• 145	0	• 144	0	• 144	0
Crossmapping (intergenic ambiguity) 🕖	• 10.73%	0	• 16.93%	0	• 18.8%	0	0.07%	0	0.02%	0	8.39%	0
Ambiguous layout (intragenic ambiguity) 🛈	• 3.99%	0	10.71%	0	13.17%	0	2.73%	0	• 1.35%	0	• 1.58%	0
Non-exon spot noise ratio 🛈	O%	0	• 7.98%	0	6.27%	0	• 7.45%	0	• 4.72%	0	0 22.31%	0
Continuous consensus 🛈	Yes	0	Yes	0	Yes	0	Yes	0	 Yes 	0	Yes	0
Fully phased consensus 🛛	Yes	0	Yes	0	Yes	0	No	0	• Yes	0	Yes	0
Consensus coverage other exon minimum depth 🕖	• 187	0	• 107	0	• 83	0	• 9	0	• 261	0	• 197	0
Consensus coverage non-exon minimum depth 🕖	• 157	0	64	0	66	0	- 0	0	• 30	0	• 26	0
Other exon allele imbalance 🛈	• 0.5 : 0.5	0	0.55:0.45	0	0.55:0.45	0	0.95 : 0.05	0	0.5:0.5	0	0.5:0.5	0
Non-exon allele imbalance 🕖	• 0.5 : 0.5	0	0.53:0.47	0	0.55:0.45	0	0.95 : 0.05	0	0.5:0.5	0	• 0.72:0.28	0
Other exon mismatch count 🕐	• 0	0	• 0	0	• 0	U		0	• 0	0	• 0	0
Non-exon mismatch count 🕐	• 0	0	• 0	0	• 0	0	• 0	0	• 0	0	• 0	0



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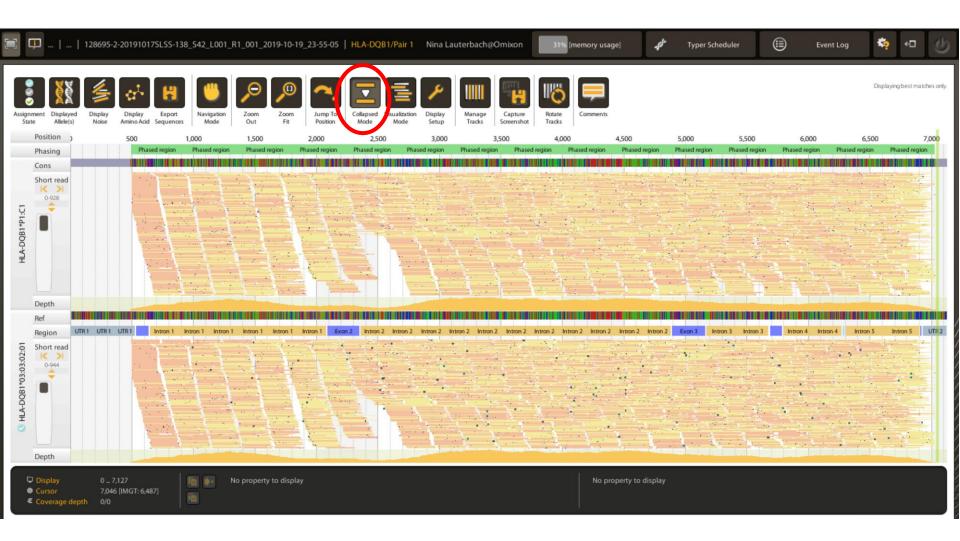
Use of Linkage Disequilibrium database



Check the coverage plot in the Gene Browser

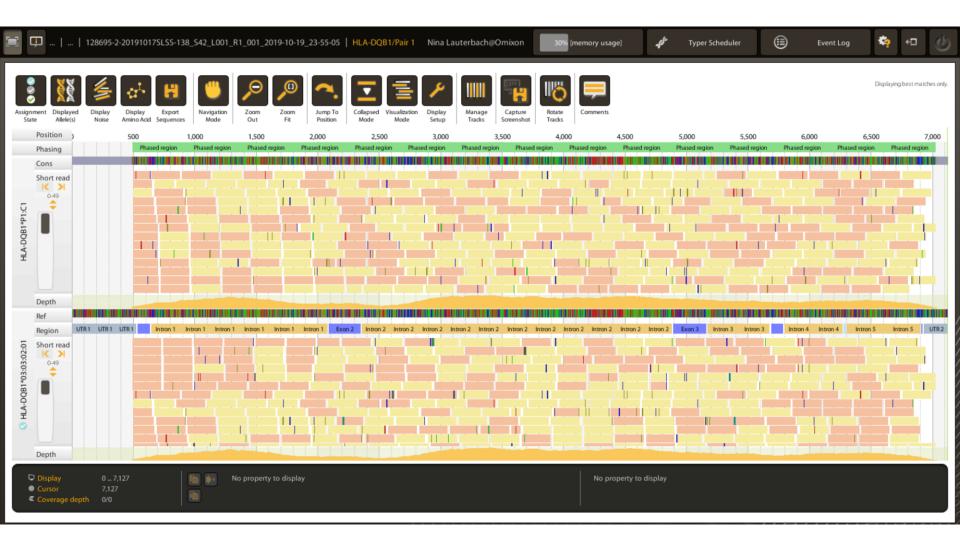


Change the visualization mode





Zoom in on sequencing reads





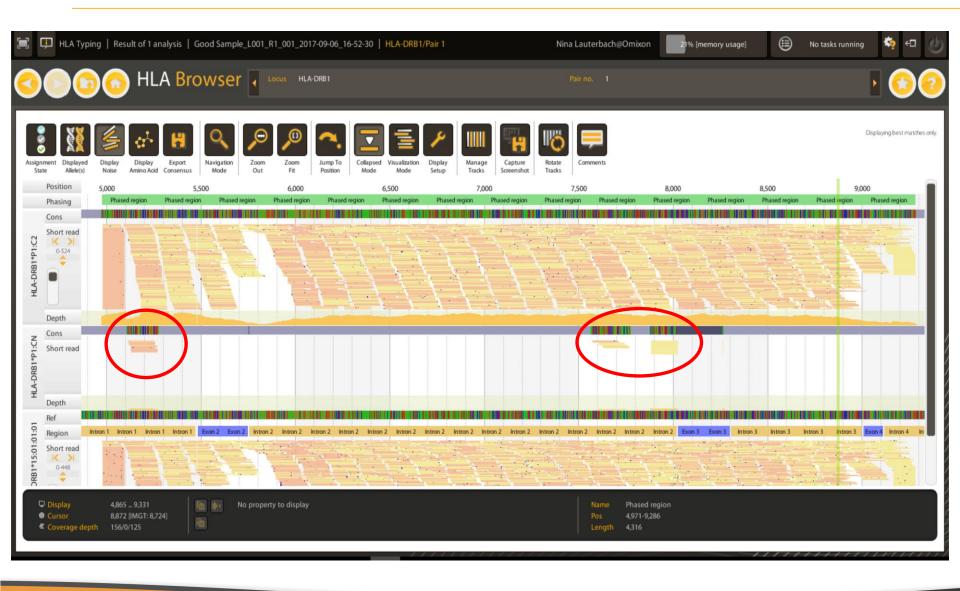
Inspect sequences





Visualize noise reads in the noise track





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How to be alerted on potential contamination?

0		🕞 Ger	notypin	g samı	ple result	Analysis name Application b			e_S1_L001_R1_2017-0 62ba3c26c12e05566fc0		Application version	on 2.1.4	i			@
Browse	Browse Allele 1 Brows		Show Alsmatches		Aatches Assignment State Precis				VRevoke proval Export Result	Turn LD on/off	Show D details				Display	se version: 3.28.0_4 ying 5 loci out of 7. best matches only.
				HLA-A		HLA-B			HLA-C			HLA-DQ	2B 1		HLA-DRB1	
_L001_R1_	_2017-08-15_10	•36-15	● 👯 🗣 HL/	A-A*02:245 A-A*03:01:01:0	1#1	HLA-B*07:33:0 HLA-B*59:04#1	1#1	• * *	HLA-C*01:23 HLA-C*08:04:03#1		● X ♣ H	LA-DQB1 LA-DQB1	*04:02:01:01 *06:146:01#1		HLA-DRB1*08: HLA-DRB1*15:	01:01
HLA-A		Genotype	Quality control	Data statis	tics											
HLA-B				Measur			HLA-A		HLA-B		HLA-C		HLA-DQE	1	HLA-DRB	1
HLA-C		Overall					FAILE	D	FAILED		FAILED		FAILED		FAILED	
HLA-DO	B1	Primary QCs	for Interpretatio	n 🕐												
HLA-DR		Read count (1)					3286	0	3275	0	3352	0	6861	0	6764	0
HLA-DK	ы	Noise ratio 🕐					9 13.06%	0	15.42%	0	7.82%	0	• 29.14%	0	• 29.97%	0
		Key exon spot r					• 26.97%	0	30.84%	0	• 20%	0	• 28.22%	0	38.49%	0
		Consensus cove	erage key exon mi	inimum depth (D		• 0	0	• 0	0	• 3	0	• 5	0	• 26	0
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		Secondary Q	Cs for Interpreta	tion 🛈												
		Fragment size (0				319	0	308	0	• 317	0	• 309	0	307	0
		Read quality 🕐					35.24	0	35.03	0	35.2	0		0	36.48	0
		Other exon spo	ot noise ratio 🕧				19.77%	0	14.46%	0	0%	0	• 32.97%	0	• 22%	0

Check the noise track...





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HLA Twin Novel allele confirmation



• You can confirm a novel sequence in the Gene Browser

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HLA Twin Novel allele confirmation



• You can confirm a novel sequence in the Gene Browser

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- 1. NGS provides us with a large amount of data
 - High resolution typings
 - Complete missing sequences in IMGT database
 - Few to no ambiguities
 - Few to no reflex testing
- 2. NGS analysis software is a crucial part in obtaining trustworthy typings. Therefore HLA Twin provides:
 - Confidence in typing due to strong algorithms
 - Quality Control metric to alert user for potential issues
 - Tools for troubleshooting



Important to give a functional meaning to the high resolution data

- 1. Improve the PIRCHE export function in the software make it bidirectional
- 2. Incorporate HLAMatchmaker algorithm

We are constantly learning and improving









What's new?

New Product: OmniType



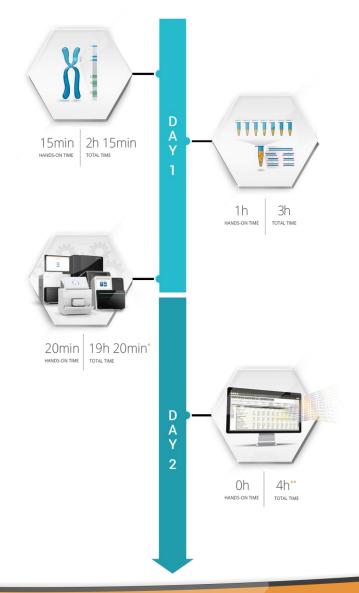
- 11-locus, 1 tube multiplex assay for HLA typing
- Single workday workflow
- Fast PCR
- Efficient and technician-friendly library preparation protocol
- Minimal hands-on time
- Automatable protocol for pre- & post-PCR



OmniType Workflow

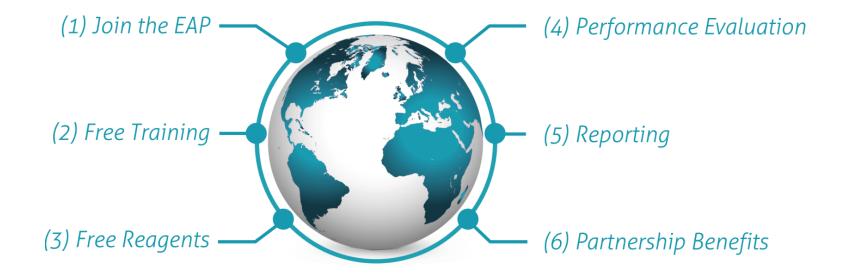


- DNA to library in <7 hours
- ~1 hour hands-on time
- 100 ng total input DNA
- 11 loci, 1 tube
- Fast LR-PCR, ~2 hours
- No amplicon pooling
- Fast library preparation workflow
- Sequence results on Day 2

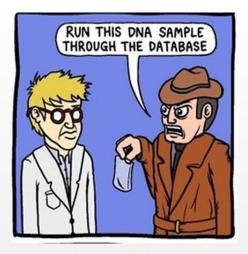


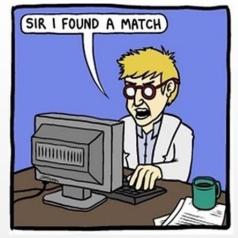
OmniType Early Access Program (EAP)

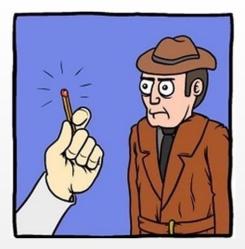




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