NIPT: Non-Invasive Prenatal Testing

Presentation by Ben Caljon, adapted by Pieter Pannus

Universitair Ziekenhuis Brussel

BRIGHT core
Brussels Interuniversity Genomics High Throughput core

Overview

- Detection of cell-free DNA
- NIPT technique
- Claimed accuracy
- Indications and limitations

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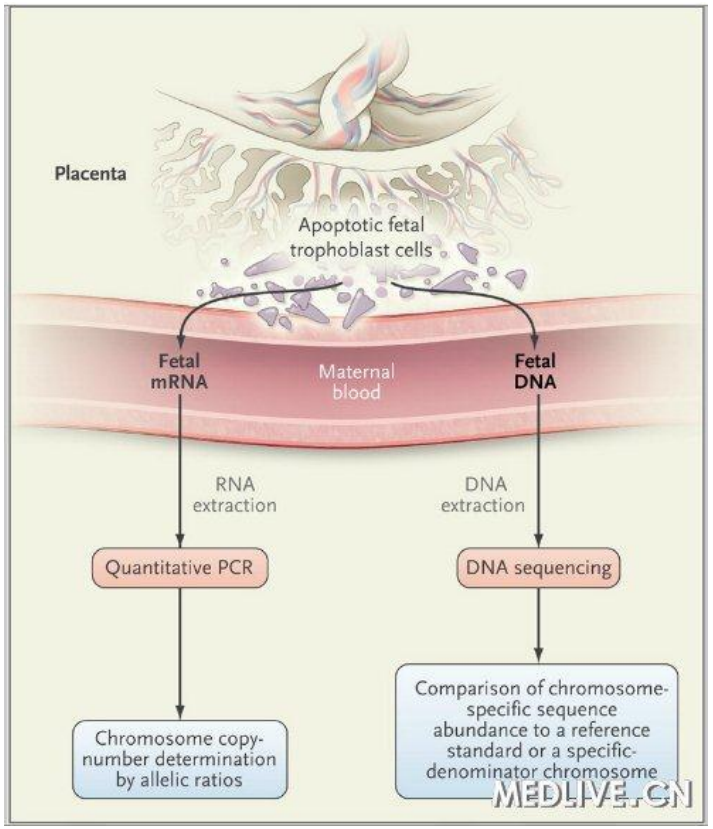
Detection of cell-free DNA (cfDNA)

Detection of cell-free fetal DNA in maternal plasma in 1997[1]

- → Shedding of trophoblast cells
- → Micro-particles of fragmented DNA into maternal bloodstream
- → Short half life (2 hour clearance)

- Median prevalence of 3% (RT-PCR) to 10%[2] (dPCR) in 1st and 2nd trimester
- Reliable detection from 11 to 12 weeks onwards
- Increasing % during 2nd and 3rd trimester



1. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, et al. (1997) Presence of fetal DNA in maternal plasma and serum. Lancet 350: 485–487.
2. Lun FMF, Chiu RWK, Chan KCA, Leung TY, Lau TK, Lo YMD. 2008 Microfluidics digital PCR reveals a higher than expected fraction of fetal DNA in maternal plasma. Clin. Chem. 54, 1664–1672.

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Centrum voor Medische Genetica

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Overview of NIPT technique

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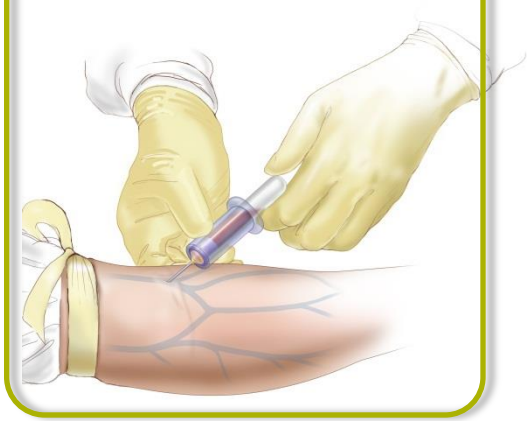

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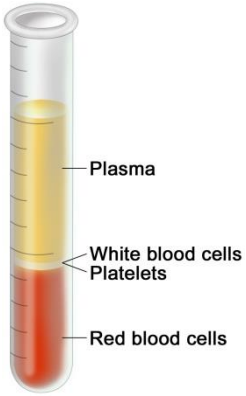

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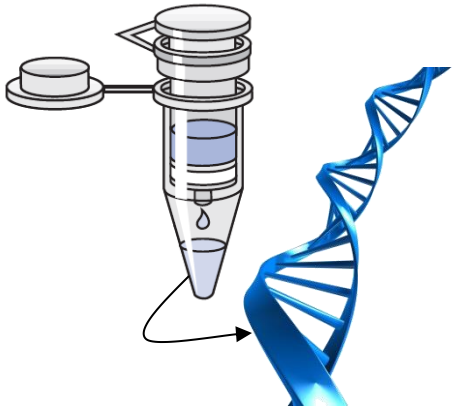

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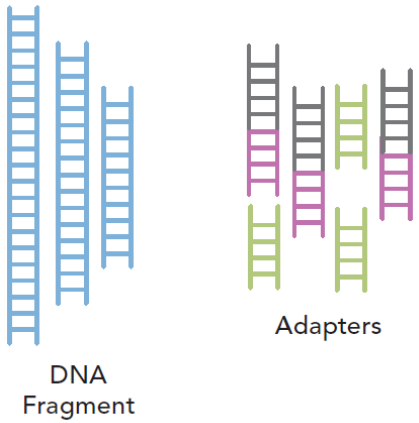

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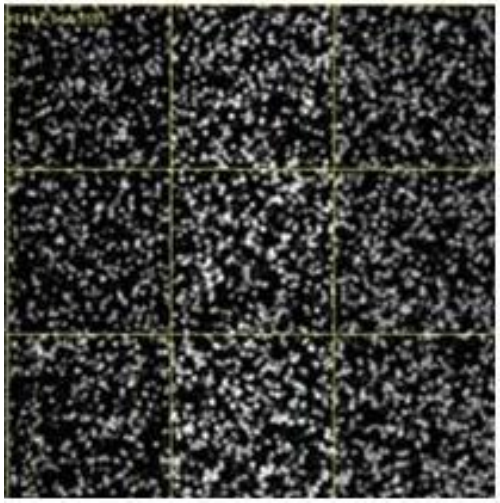

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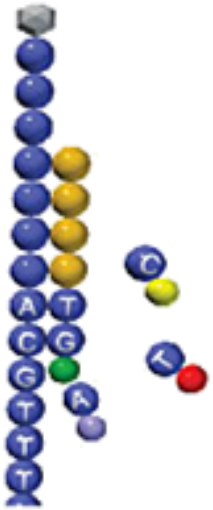

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NIPT technique – Phlebotomy (1)

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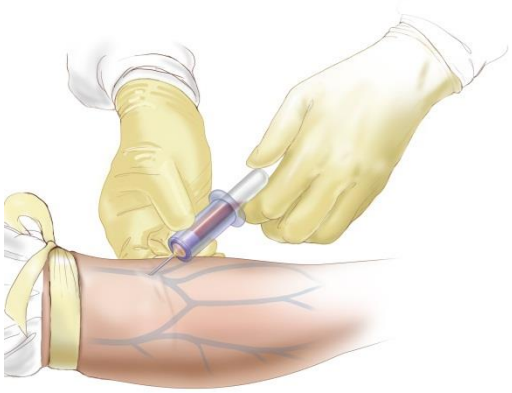
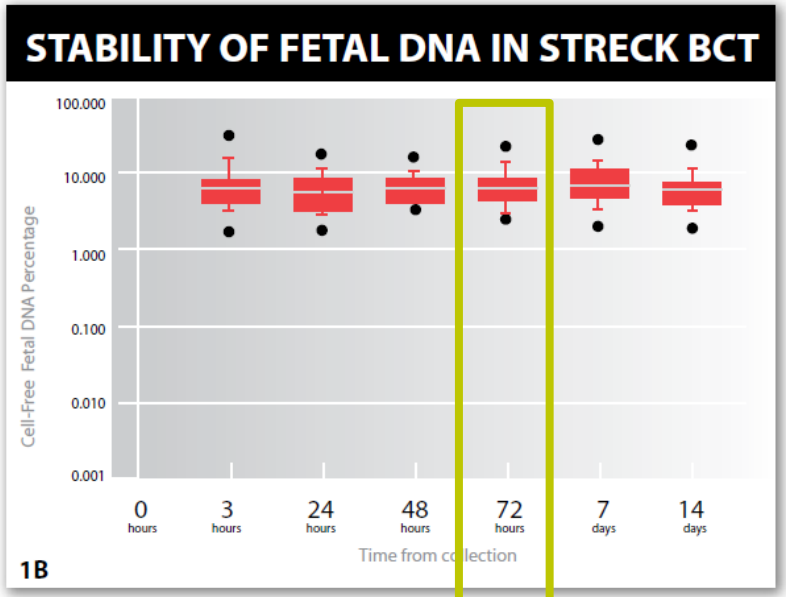
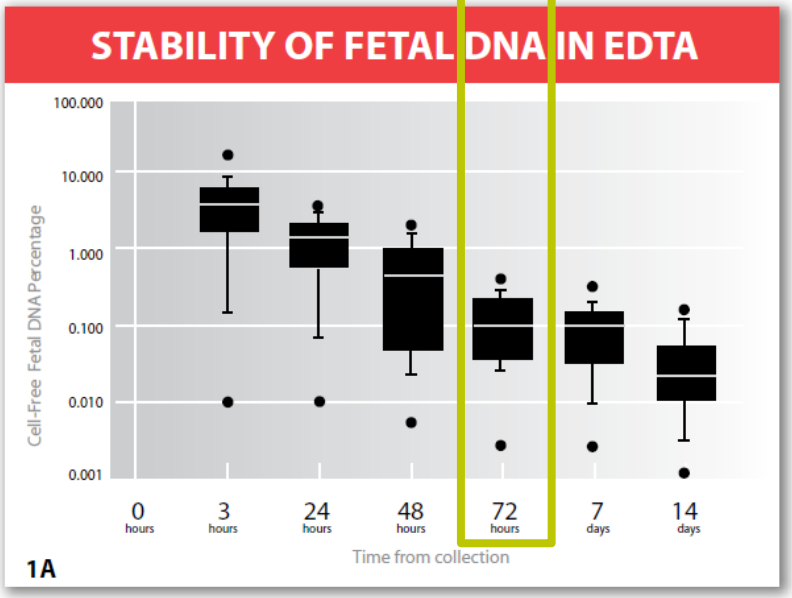


Maternal plasma + DNA isolation

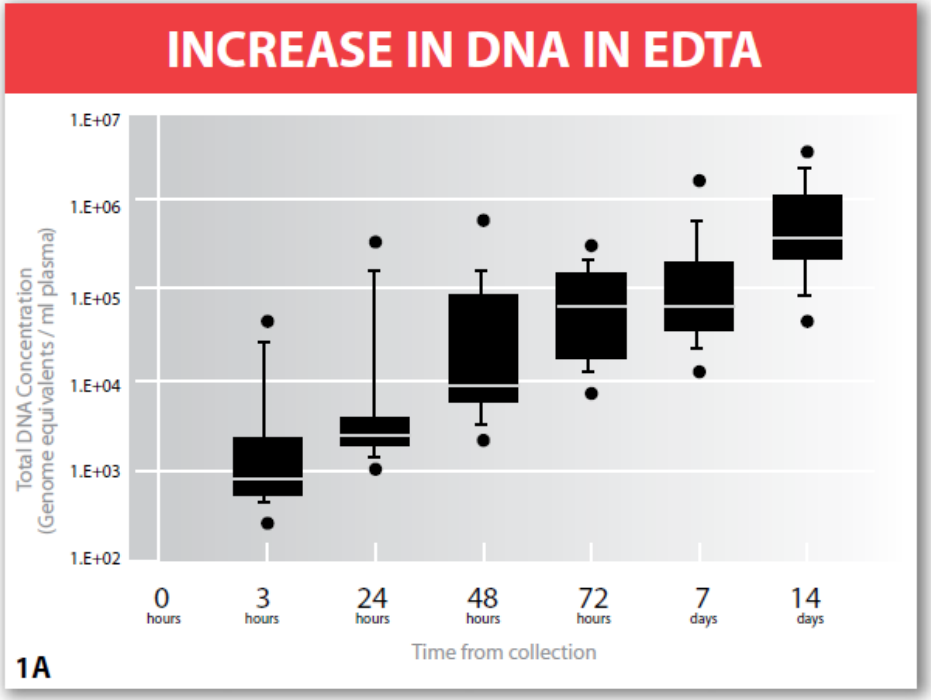
- Collection of maternal peripheral blood (from 12 weeks gestation) in 10ml EDTA tubes (Streck tubes) with a proprietary stabilizing agent:
  - Inhibits gDNA release from nucleated cells
  - Inhibits nuclease activity

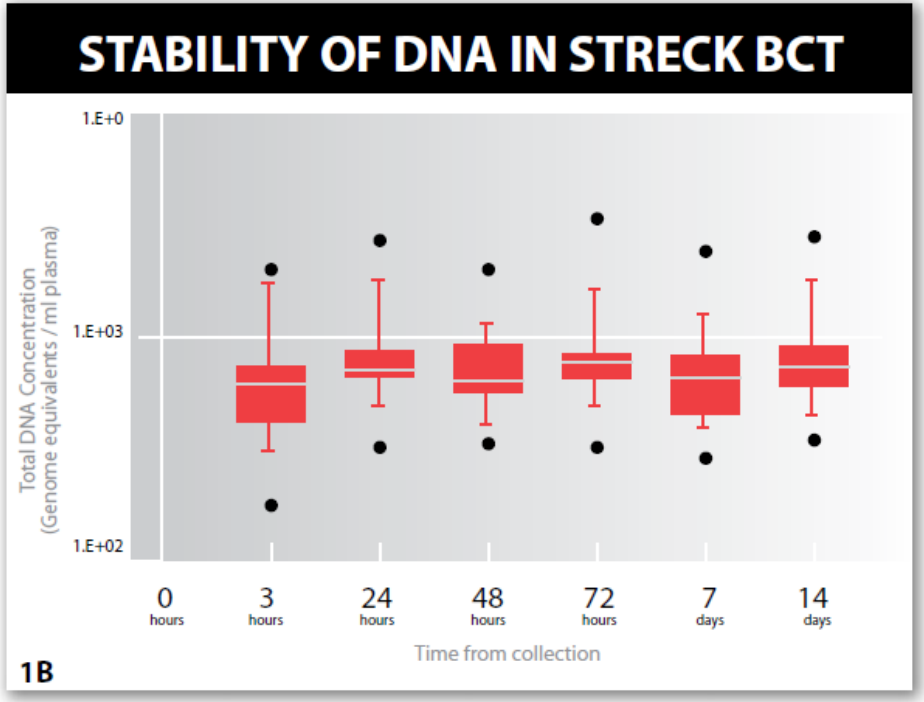




NIPT technique – Phlebotomy (2)

- gDNA release from nucleated (maternal) cells over time (0-14 days)





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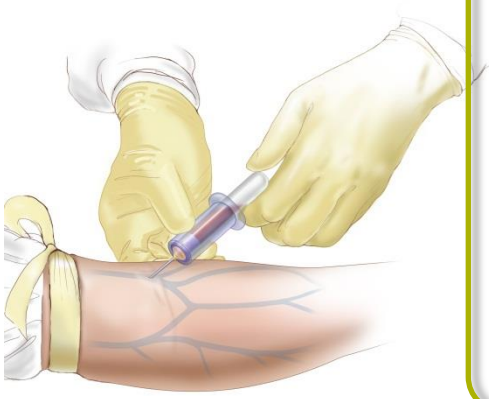

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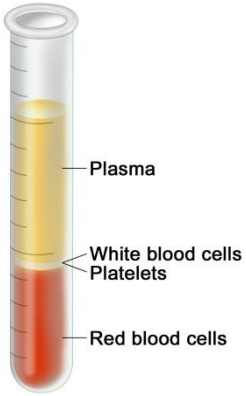

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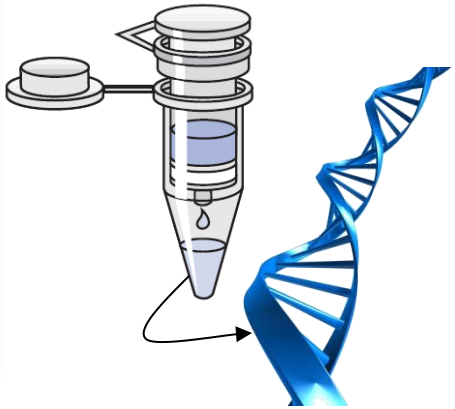

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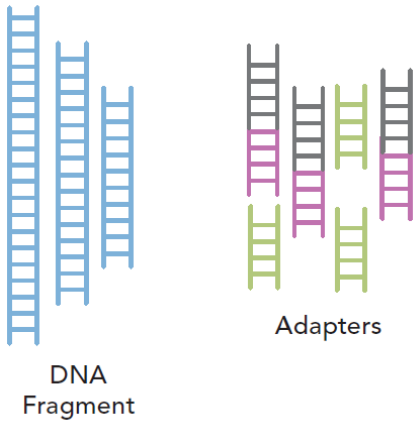

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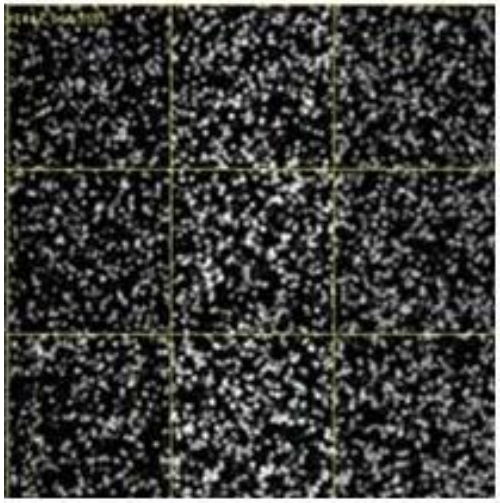

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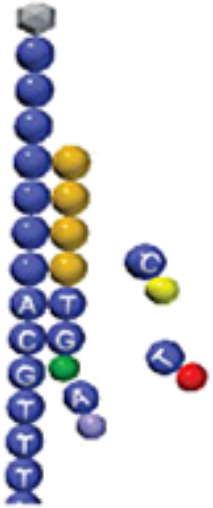

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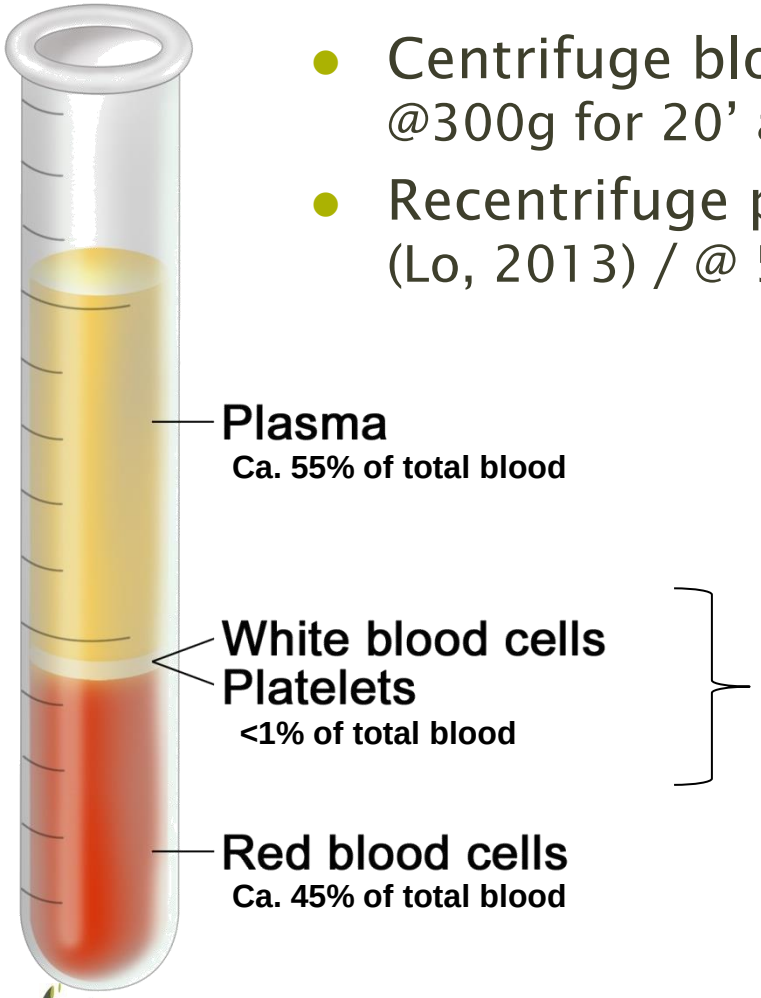

- Centrifuge blood @ 1600g for 10’ at 4°C (Lo, 2013) / @300g for 20’ at 22°C (Streck)
- Recentrifuge plasma fraction @ 16.000g for 10’ at 4°C (Lo, 2013) / @ 5000g for 10’ at 22°C (Streck)

Removal of nucleated cells, as a source of maternal DNA

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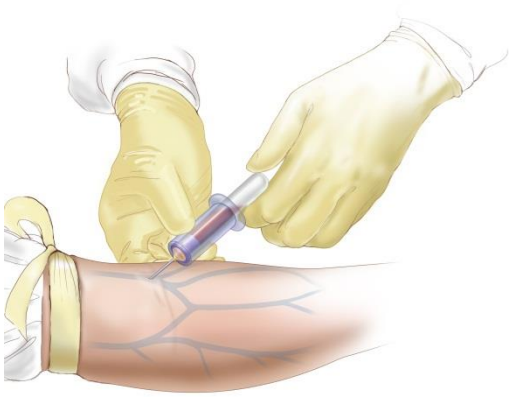

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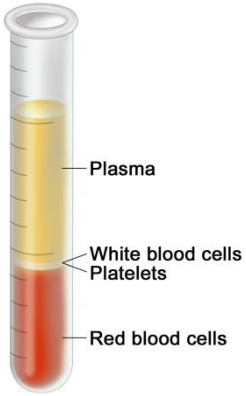

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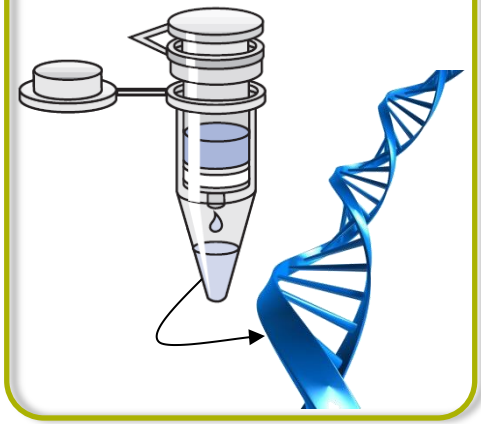

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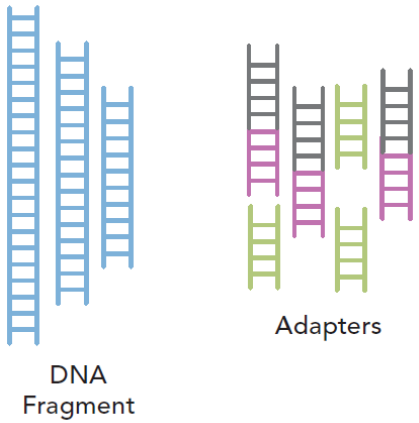

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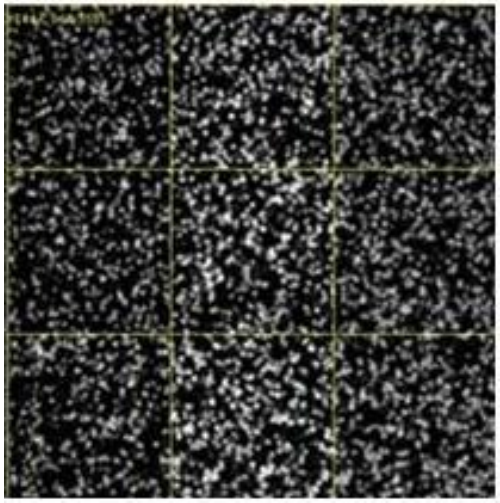

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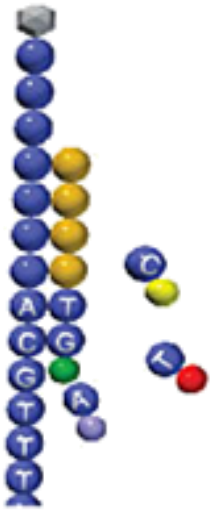

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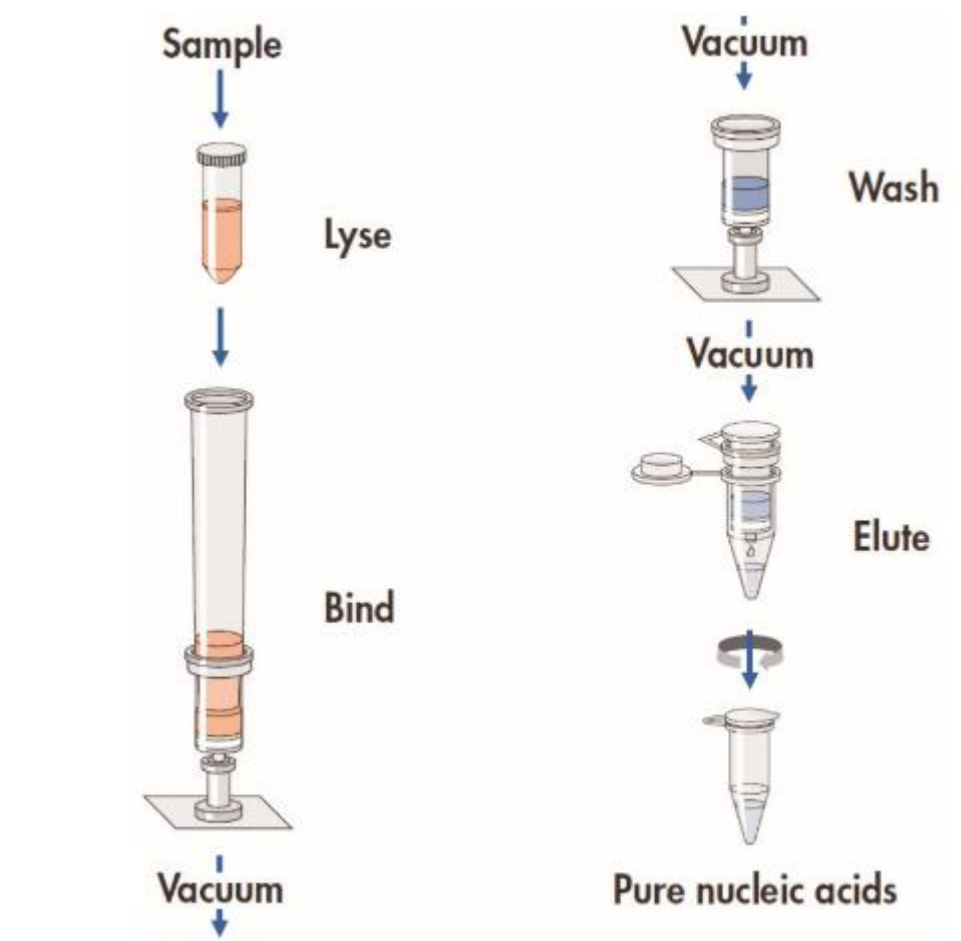

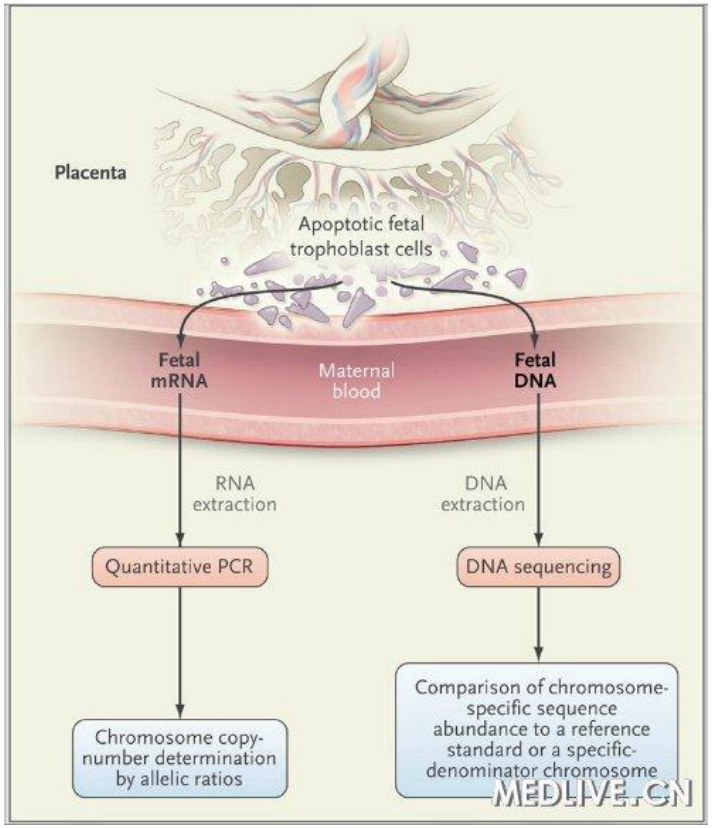

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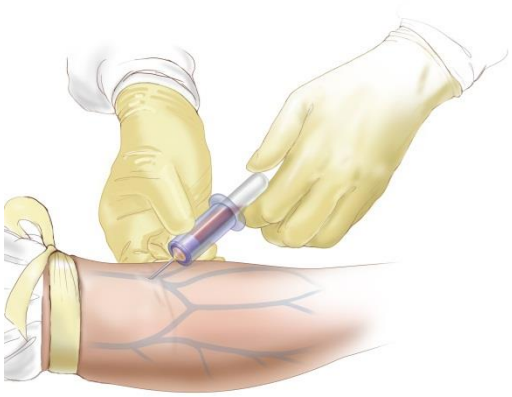

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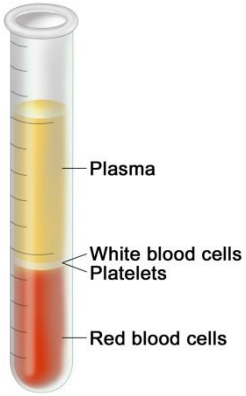

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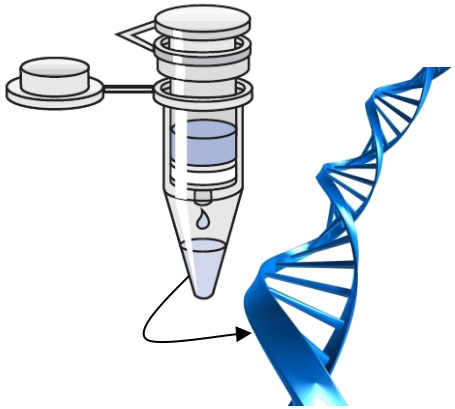

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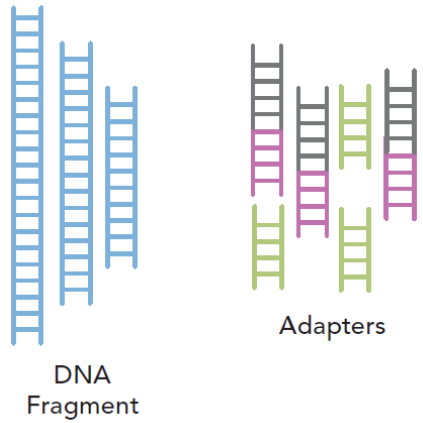

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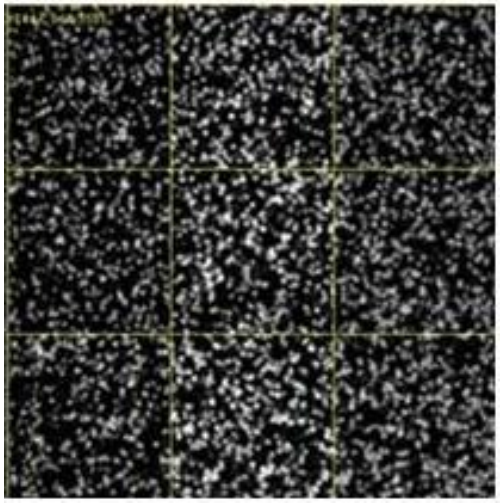

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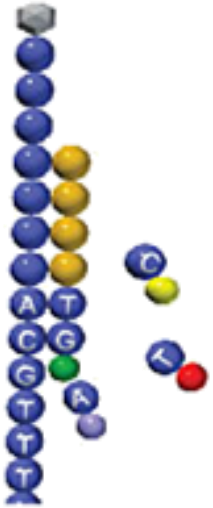

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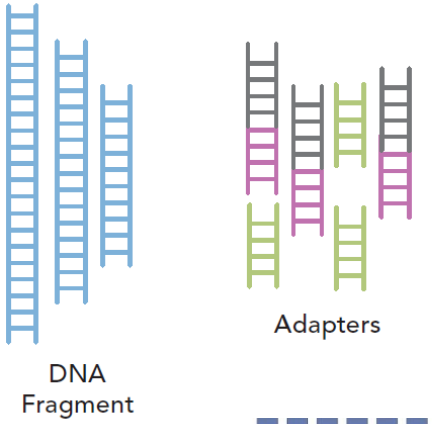
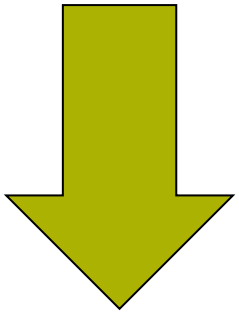


The cfDNA has to be modified for the sequencing instrument (eg. HiSeq) to be able to read the DNA sequences of each individual fragment. The following actions are required :



- **End repair**
- **Adenylation (3')**
- **Adapter ligation**
- **Library purification (x2)**
- **PCR amplification**
- **Library purification**
- **Library validation**

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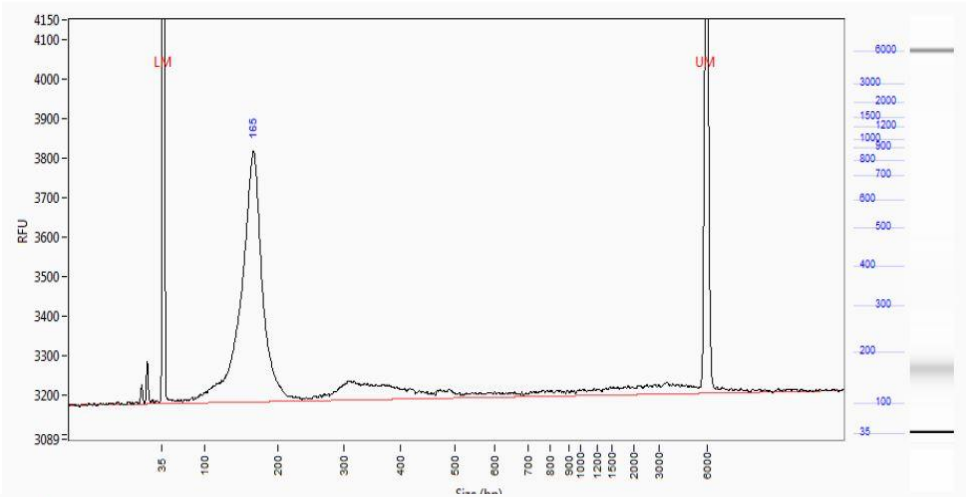
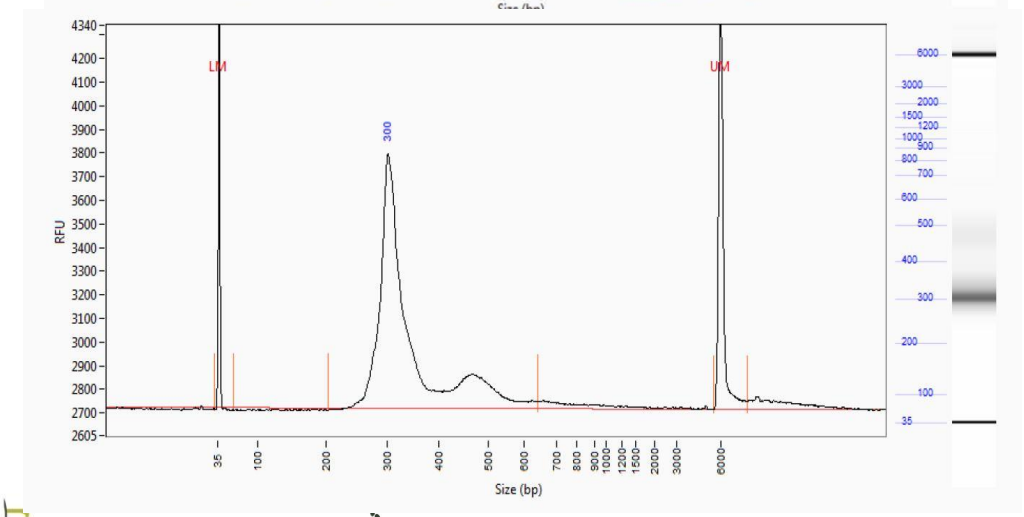
NIPT technique – Library prep. (2)

Library validation





Average size cfDNA fragments = 150-170 bp

Average size cfDNA fragments + adapters = ca. 300 bp

- ***Validated libraries are pooled in equal ratios (equimolar).***
- ***Because of the unique adapter (1 adapter per cfDNA sample), the instrument can discriminate***

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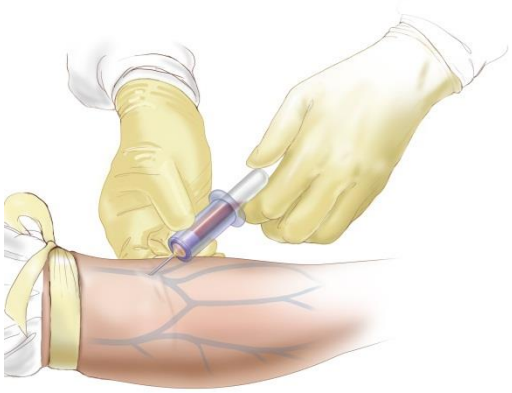

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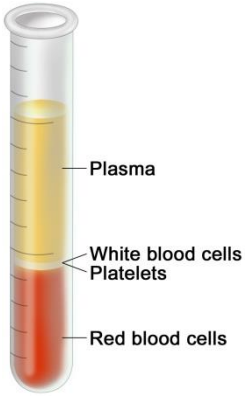

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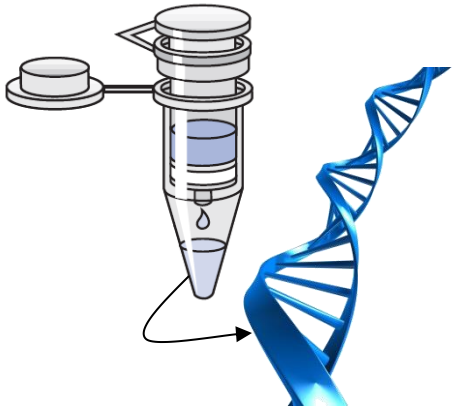

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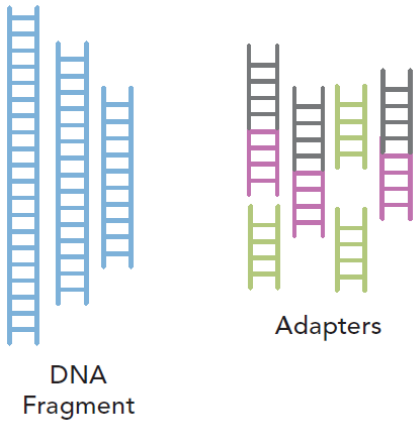

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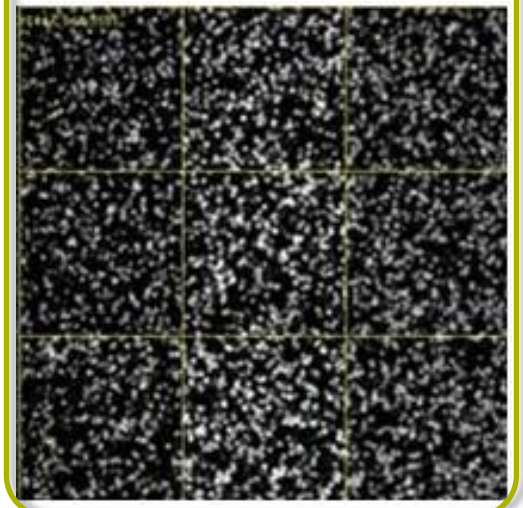

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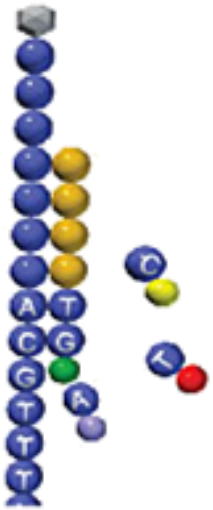

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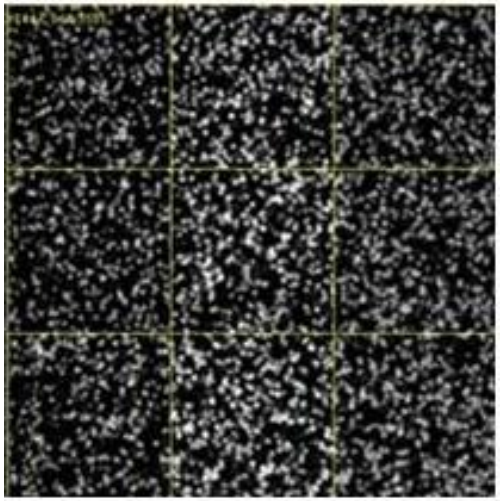
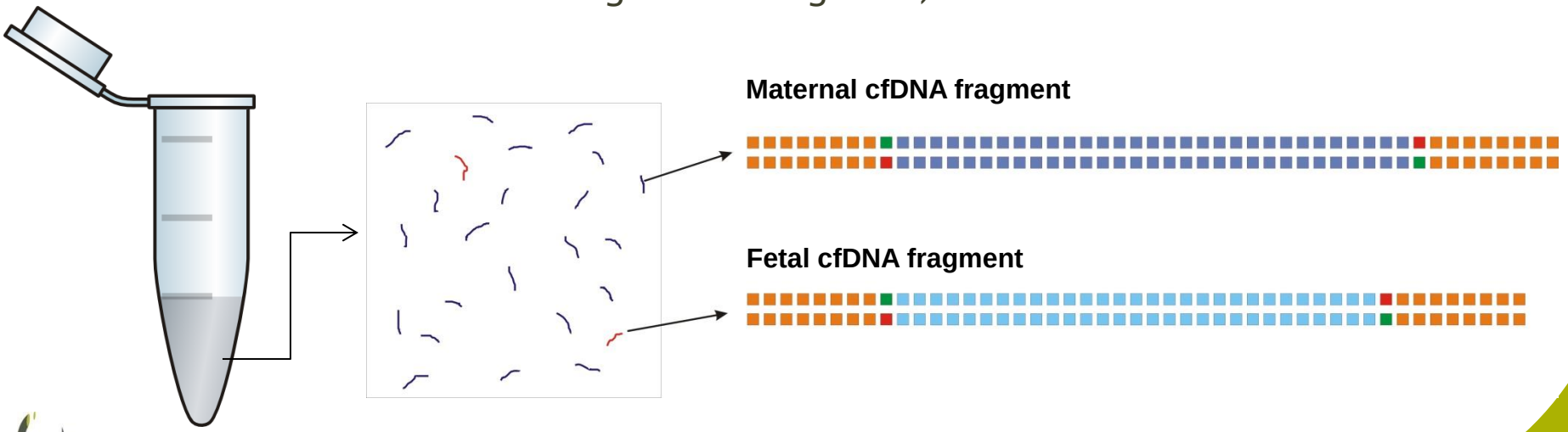


- The pooled library sample has to be attached to the glass slide (flowcell), so every unique fragment binds to a random but distinct zone on this slide
- Per zone, there will be 1 unique fragment that will be amplified, so multiple copies of the same fragment exist on that zone (=cluster generation)
  - → Amplification needed to overcome current detection limitations (not possible to detect/genotype a single DNA fragment)



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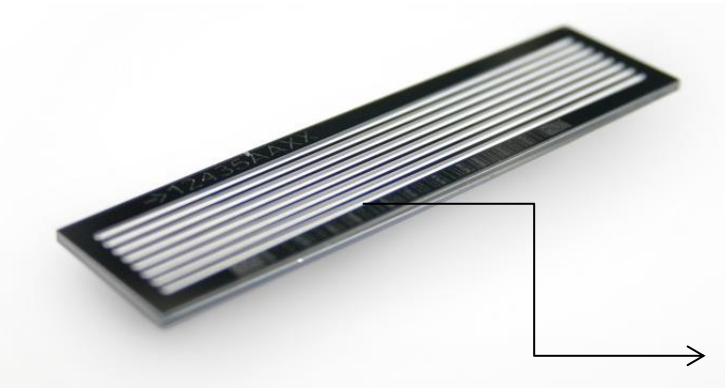
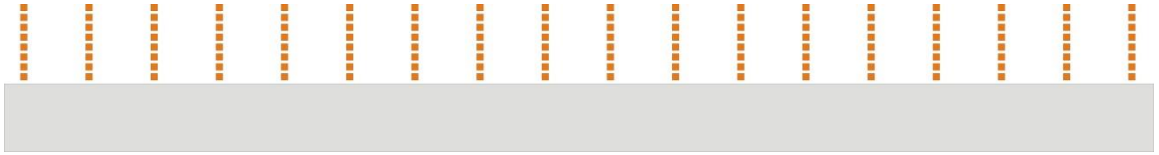


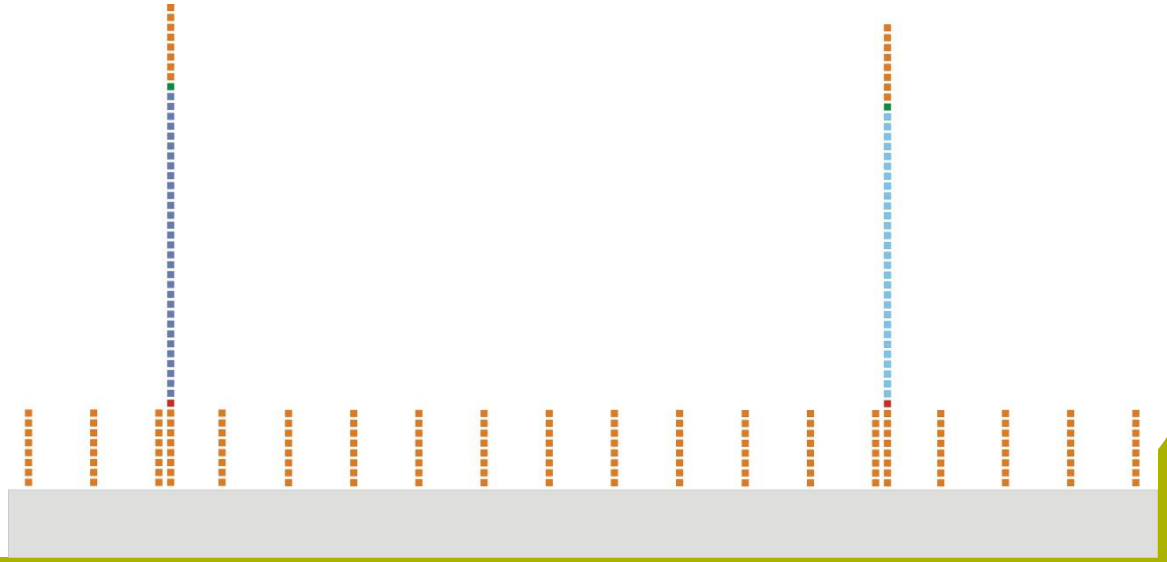
Glass slide coated with DNA fragments that recognize the adapters



Random binding of cfDNA fragments to probes on flowcell

Maternal cfDNA fragment

Fetal cfDNA fragment



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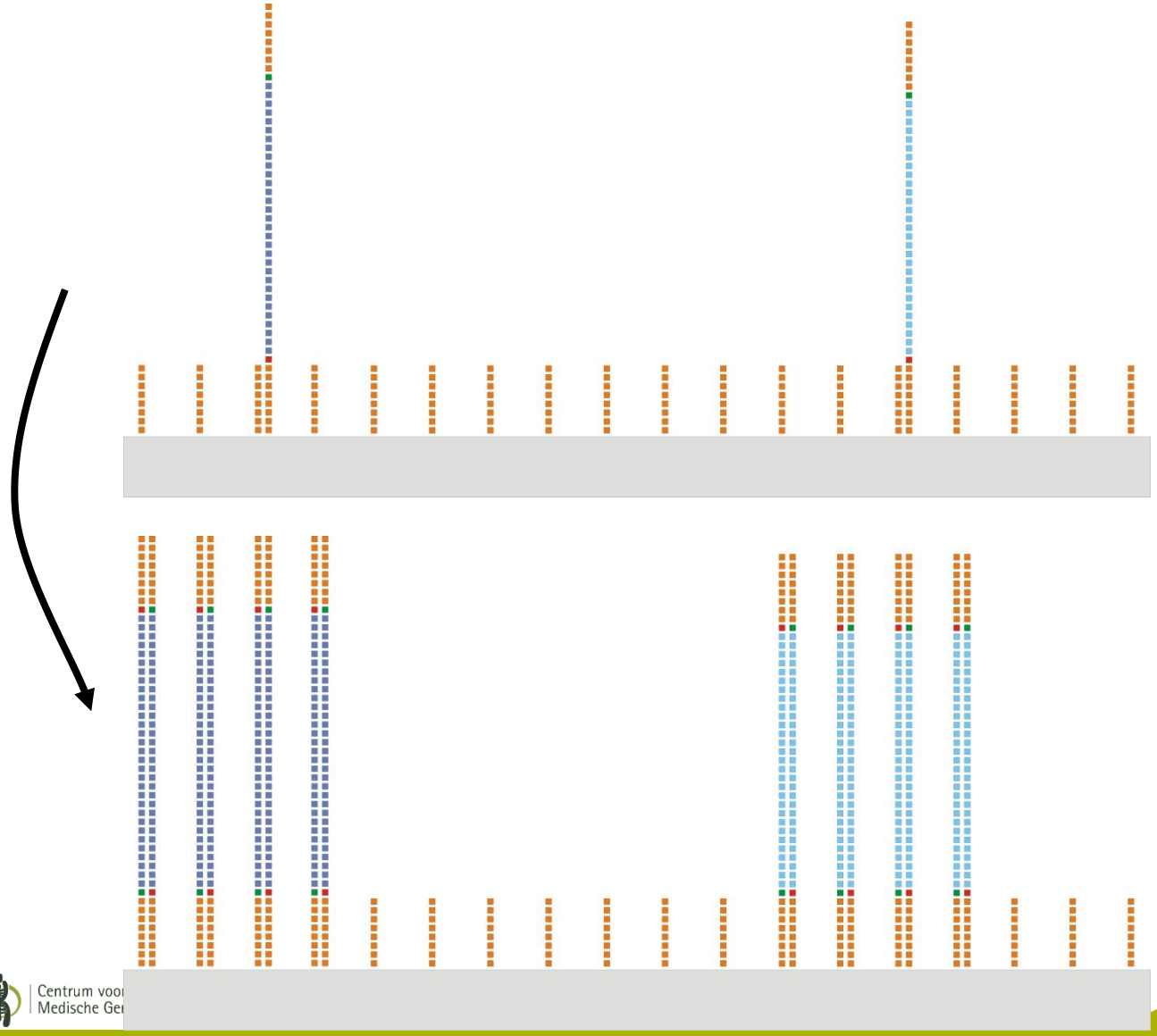
NIPT technique – Cluster gen. (3)

Isothermal amplification of each unique fragment per cluster (clonal amplification)



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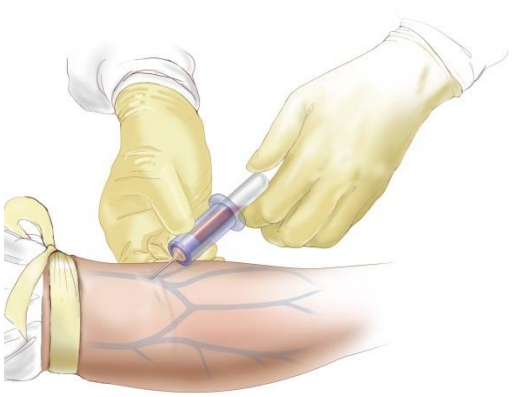
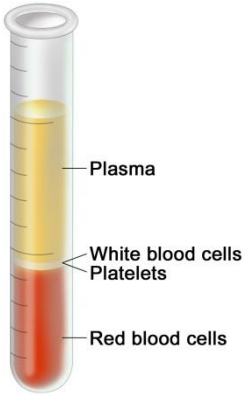
Overview NIPT technique

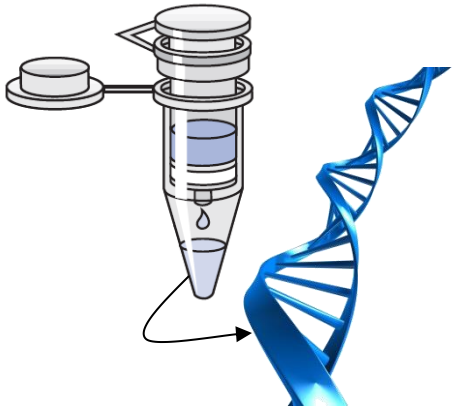
1. Phlebotomy



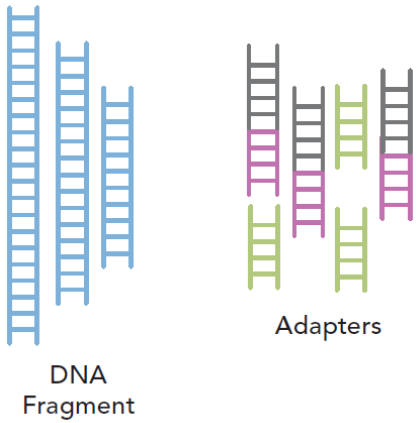
2. Plasma isolation



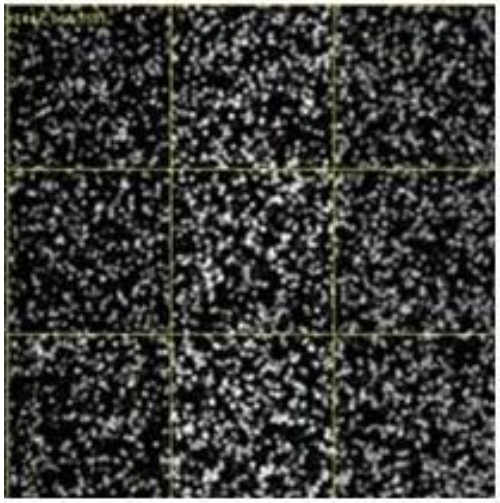
3. cfDNA extraction



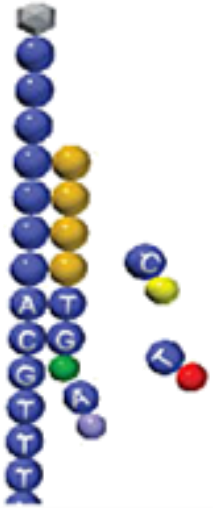
4. Library preparation



5. Cluster generation



6. Sequencing



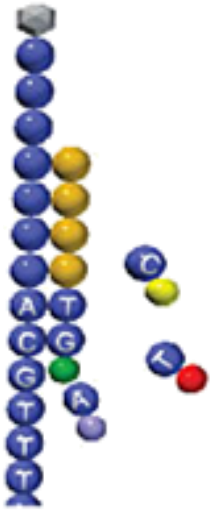
7. Data-analysis

"Data don't make any sense,
we will have to resort to statistics."

8. Reporting

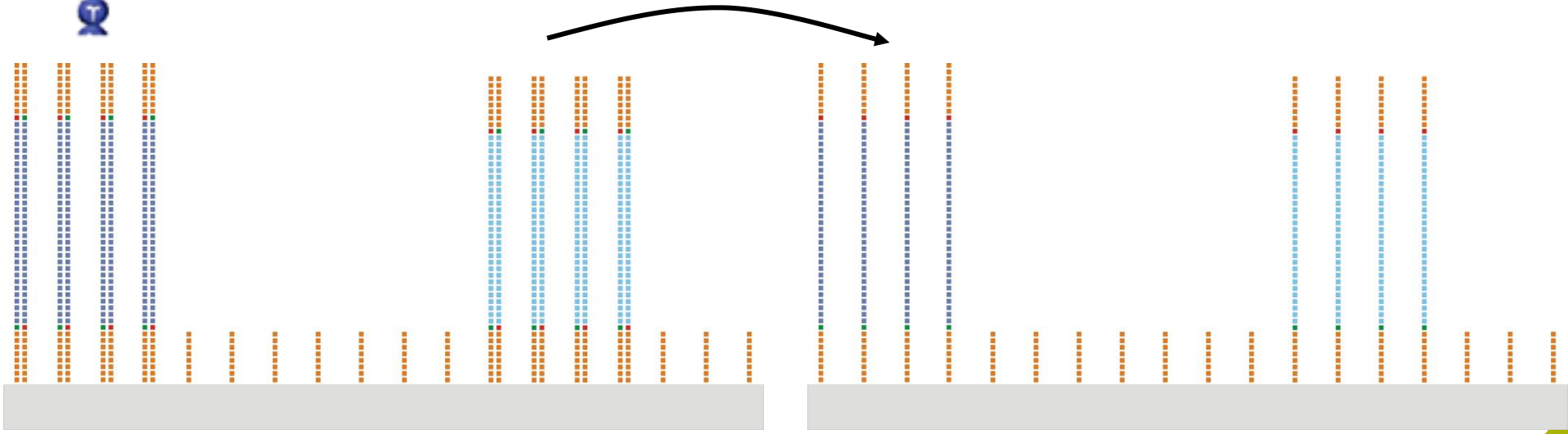
NIPT technique – Sequencing (1)

6. Sequencing



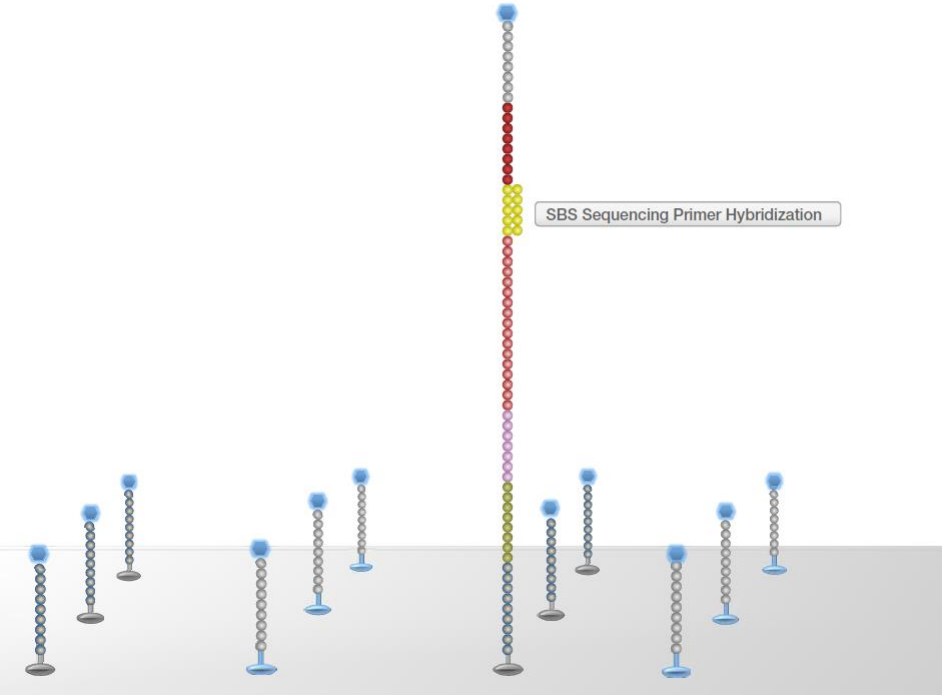
Each cluster is interrogated for its sequence

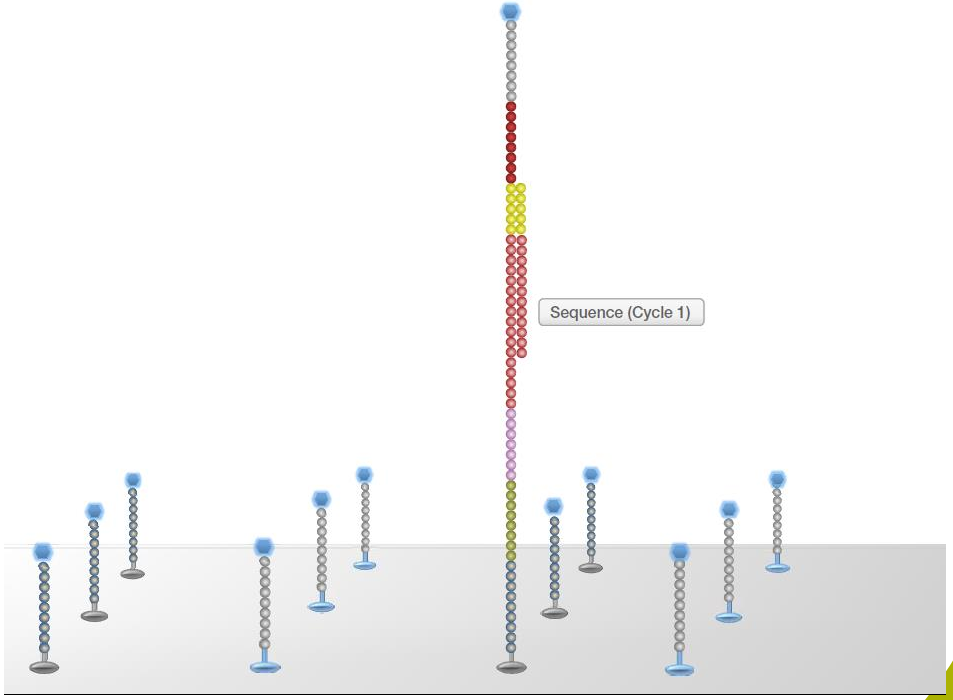
Create correct single stranded template for sequencing reaction



NIPT technique – Sequencing (2)

- Anneal sequencing primer
- Sequencing By Synthesis (SBS)
  - → Iteration of DNA polymerisation (1 base per cycle), laser scanning and decapping





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Overview NIPT technique

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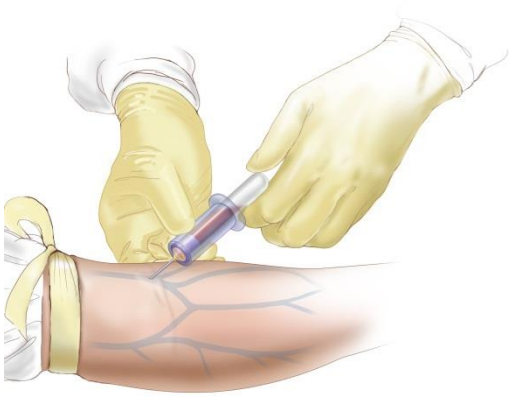

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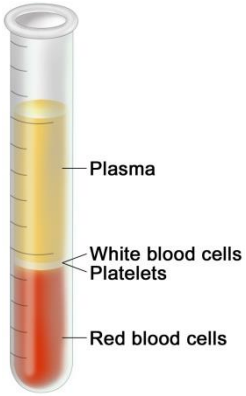

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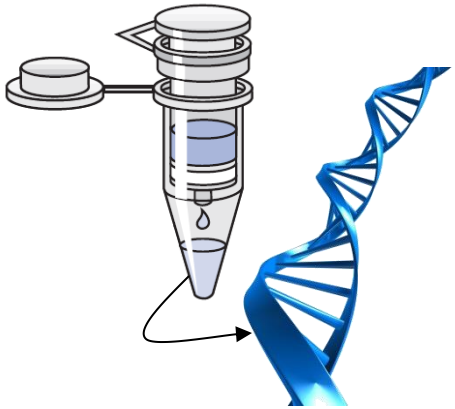

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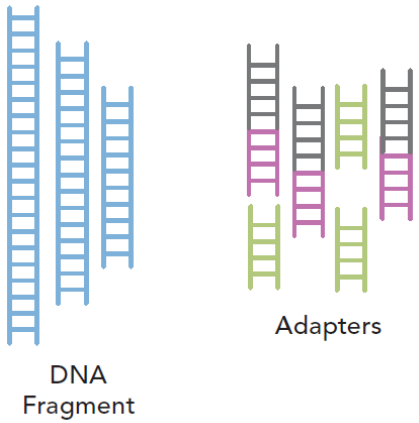

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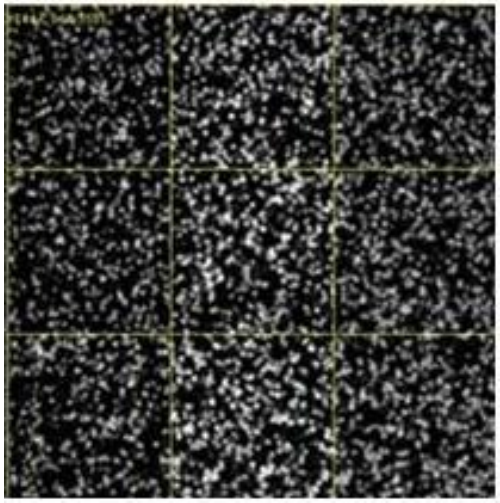
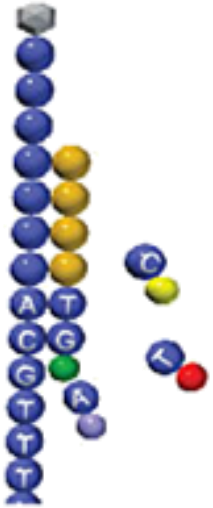


6. Sequencing



7. Data-analysis

8. Reporting

NIPT technique – Data analysis (1)

- Resolution of detection is determined by coverage (number of reads).
- Higher resolution has high impact on total cost.
- Range of currently implemented number of reads for detecting aneuploidies :
  - → Min : 2 x 10^6 reads
  - → Max : 25 x 10^6 reads

Yu et al, 2013, Noninvasive Prenatal Molecular Karyotyping from Maternal Plasma

NIPT technique – Data analysis (1)

Raw Data

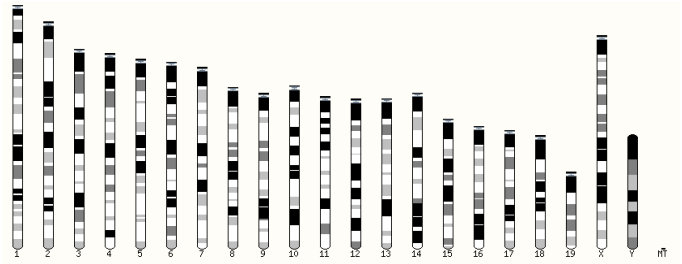
Maternal cfDNA fragment

Fetal cfDNA fragment

Raw sequencing data is aligned to the genome

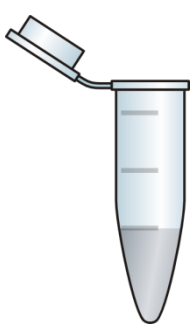
GC correction

Counting Statistics



NIPT technique – Data analysis (2)

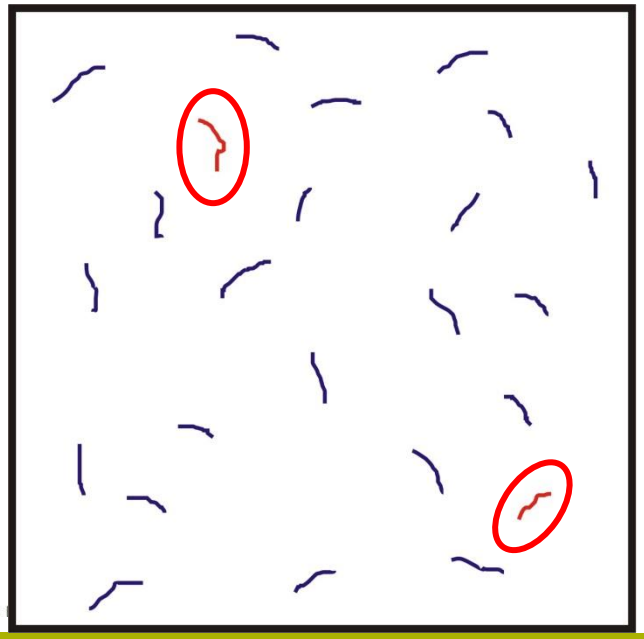
- Z-score calculation
  - → Step 1 : normalize for amount of data
    - Divide number of fragments for chr N by total number of fragments



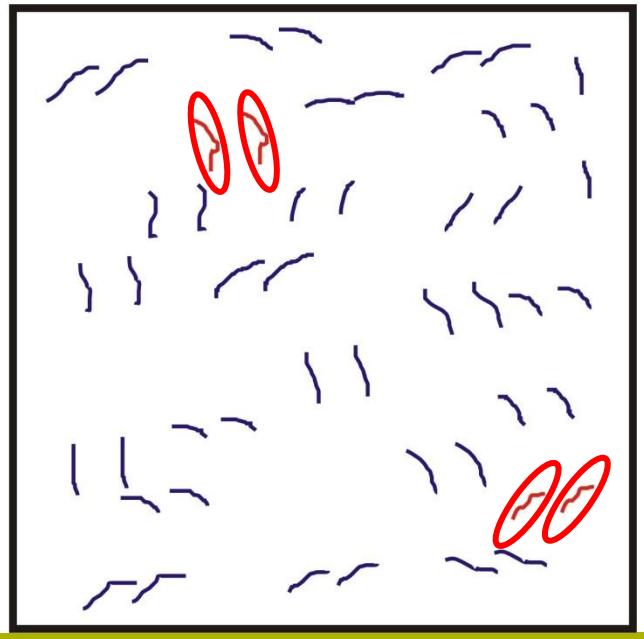
Red : 2
Total : 24

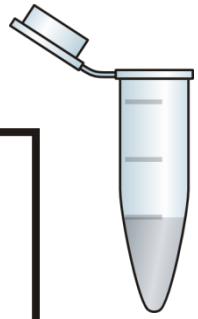
% red = 8,3%

Sample 1 : 1x representation



Sample 2 : 2x representation





Red : 4
Total : 48

% red = 8,3%

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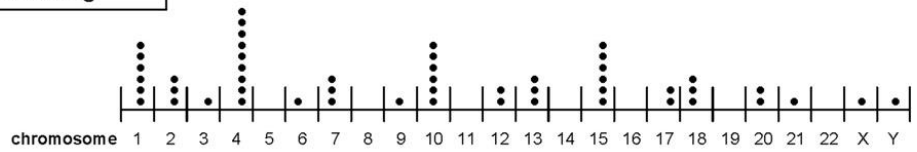
NIPT technique – Data analysis (3)

- Z-score calculation
  - → Step 2 : determine the number of standard deviations from mean (control SDs should be established for comparison purpose)

Sequence counting

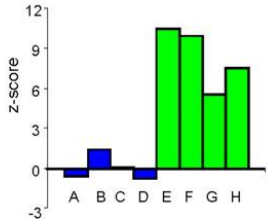


% representation of unique sequences mapped to a chromosome

$$\%\ \text{chrN} = \frac{\text{Unique count for chrN}}{\text{Total unique count}}$$

Disease status determination

$$\text{chrN z-score for test sample} = \frac{\%\ \text{chrN}_{\text{sample}} - \text{mean}\ \%\ \text{chrN}_{\text{reference}}}{\text{S.D.}\ \%\ \text{chrN}_{\text{reference}}}$$



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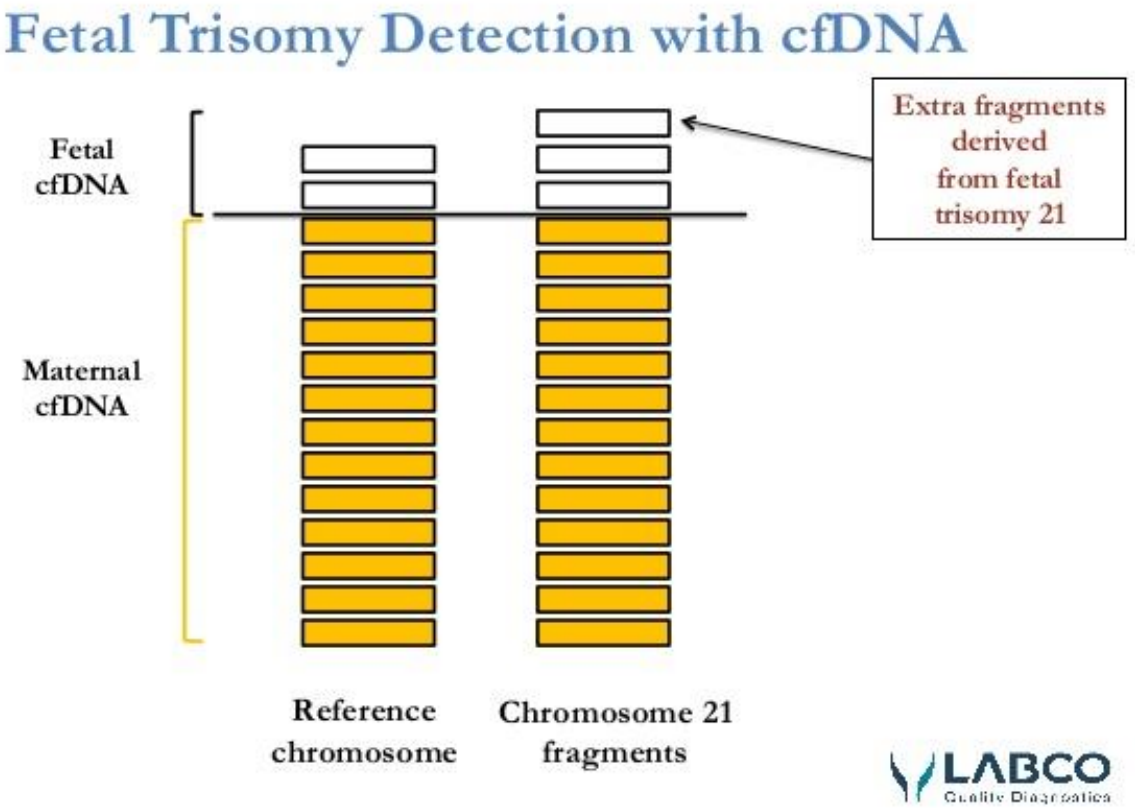
NIPT technique – Data analysis (4)

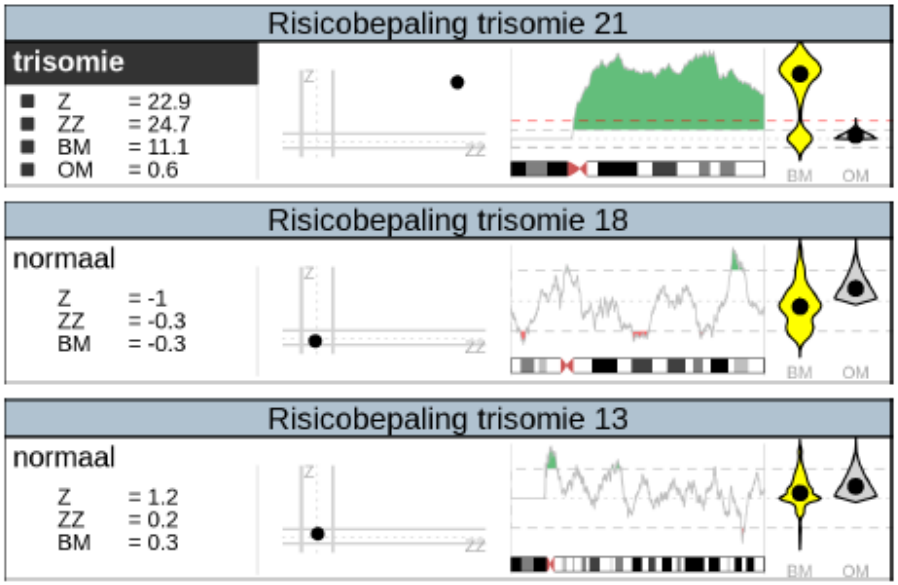
- Calculations in a nutshell :



NIPT technique – examples (1)

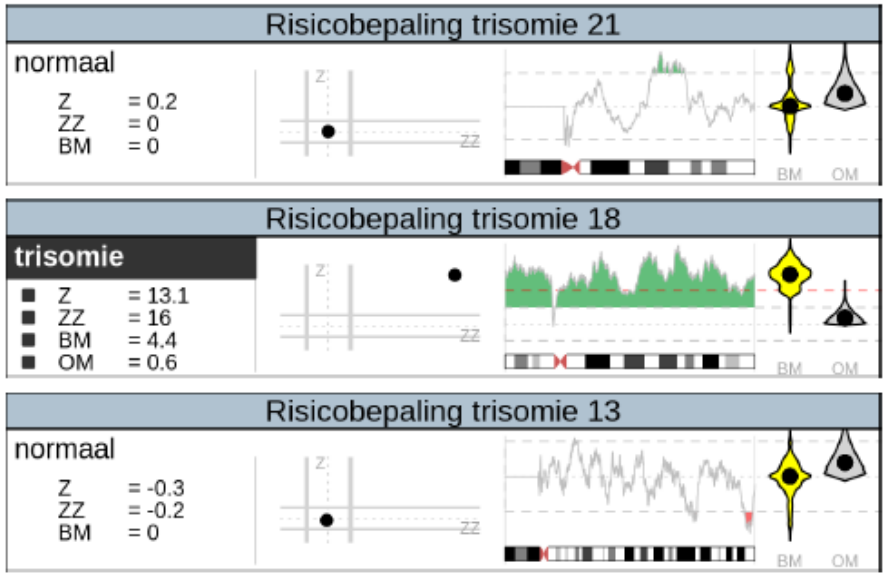
- Trisomy 21 + MX

Resultaat	Std dev	#Reads	#ReadsChrY	FF(ChrX)	FF(ChrY)	Geslacht
geslaagd	0.87	23558860	404(0)	13.77%	.48%	meisje



- Trisomy 18

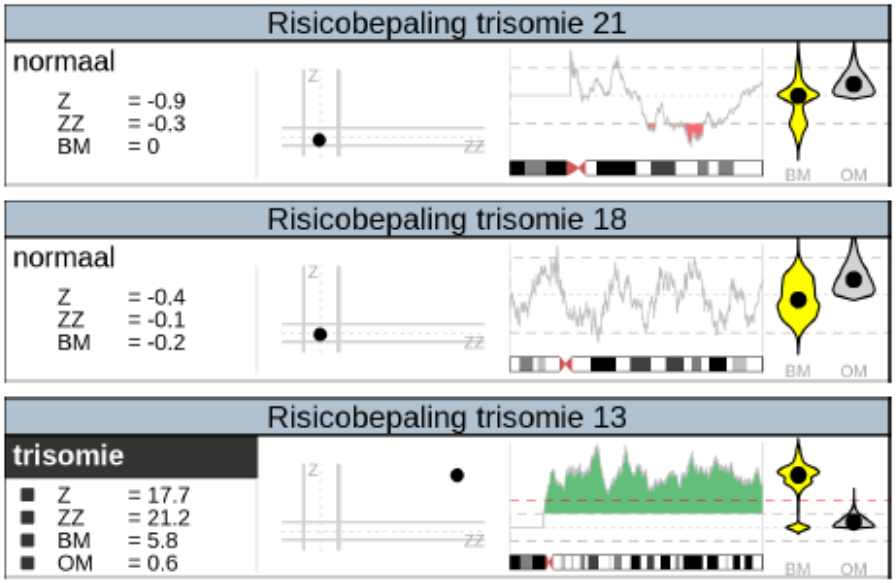
Resultaat	Std dev	#Reads	#ReadsChrY	FF(ChrX)	FF(ChrY)	Geslacht
geslaagd	0.76	11368540	2776(62)	10.94%	7.57%	jongen



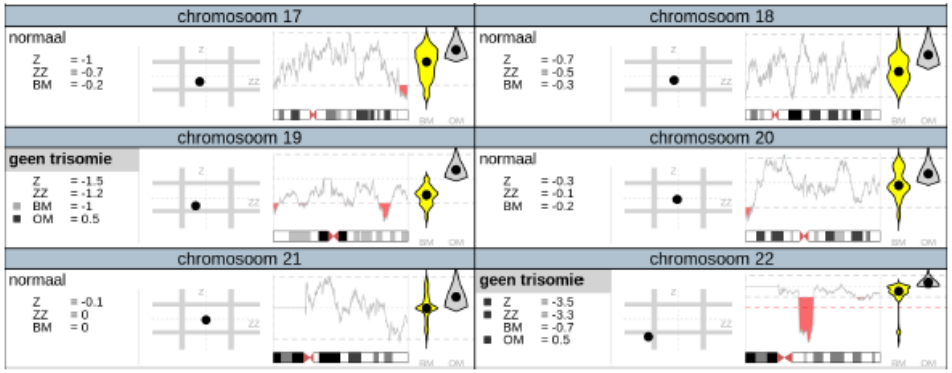
NIPT technique – examples (2)

- Trisomy 13

Resultaat geslaagd	Std dev 0.77	#Reads 10631117	#ReadsChrY 3022(66)	FF(ChrX) 12.41%	FF(ChrY) 9.07%	Geslacht man



- Microdeletion (22q11 – DiGeorge)



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Overview

- Detection of cell-free DNA
- NIPT technique
- Claimed accuracy
- Indications and limitations

Claimed accuracy (1)

The sensitivity and specificity of the test vary **per chromosome**

→ Near 100% sensitivity and specificity

Table 1 Results from four published clinical trials that measured NIPT's sensitivity and specificity in detecting common aneuploidies

	Trisomy 21		Trisomy 18		Trisomy 13		Monosomy X	
	Sensitivity (95 % CI)	Specificity (95 % CI)	Sensitivity (95 % CI)	Specificity (95 % CI)	Sensitivity (95 % CI)	Specificity (95 % CI)	Sensitivity (95 % CI)	Specificity (95 % CI)
Palomaki et al. 2011	98.6 % (95.9 - 99.7)	99.8 % (99.4 - 99.9)	———	———	———	———	———	———
Palomaki et al. 2012	———	———	100 % (93.9 -100)	99.7 % (99.3 - 99.9)	91.7 % (61-99)	99.1 % (98.5 - 99.5)	———	———
Bianchi et al. 2012	100 % (95.9 – 100)	100 % (99.1 – 100)	97.2 % (85.5 – 99.9)	100 % (99.2 – 100)	78.6 % (49.2 – 99.9)	100 % (99.2 – 100)	93.8 % (69.8-99.8)	99.8 % (98.7->99.9)
Norton et al. 2012	100 % (95.5-100)	99.97 % (99.8 -99.99)	97.4 % (86.5-99.9)	99.93 % (99.75 - 99.98)	———	———	———	———
Ashoor et al. 2012	———	———	———	———	80 % (49-94.3)	99.95 % (99.71-99.99)	———	———

1. Devers PL, Cronister A, Ormond KE, Facio F, Brasington CK, Flodman P. Noninvasive prenatal testing/noninvasive prenatal diagnosis: the position of the National Society of Genetic Counselors. J Genet Couns. 2013 Jun;22(3):291-5

Claimed accuracy (2) – meta analysis

Table 2 Accuracy estimates from sensitivity and subgroup analyses of the included studies by different study characteristics†

	Down (trisomy 21)			Edwards (trisomy 18)			Patau (trisomy 13)		
Variables	N	SN (95% CI)	SP (95% CI)	n	SN (95% CI)	SP (95% CI)	n	SN (95% CI)	SP (95% CI)
All studies	40	0.993 (0.989 to 0.996)	0.999 (0.999 to 1.000)	33	0.974 (0.958 to 0.984)	0.999 (0.999 to 1.000)	24	0.974 (0.861 to 0.996)	1.000 (0.999 to 1.000)
Sensitivity analyses									
Excluding outliers‡	37	0.993 (0.989 to 0.996)	1.000 (0.999 to 1.000)	32	0.977 (0.961 to 0.986)	0.999 (0.999 to 1.000)	22	0.977 (0.818 to 0.998)	1.000 (0.999 to 1.000)
Test failures									
Assuming all+ve	40	0.997 (0.990 to 0.999)	0.981 (0.972 to 0.988)	33	0.973 (0.956 to 0.983)	0.983 (0.974 to 0.990)	24	0.979 (0.873 to 0.997)	0.981 (0.966 to 0.989)
Assuming all−ve	40	0.962 (0.948 to 0.973)	1.000 (0.999 to 1.000)	33	0.942 (0.913 to 0.962)	0.999 (0.999 to 1.000)	24	0.885 (0.796 to 0.939)	1.000 (0.999 to 1.000)
Intention to diagnosis	40	0.976 (0.959 to 0.986)	0.981 (0.972 to 0.989)	33	0.958 (0.927 to 0.976)	0.983 (0.973 to 0.990)	24	0.903 (0.811 to 0.953)	0.981 (0.966 to 0.989)
Assuming all+ve	40	0.994 (0.989 to 0.997)	0.999 (0.999 to 1.000)	33	0.974 (0.958 to 0.985)	0.999 (0.999 to 1.000)	24	0.974 (0.863 to 0.996)	1.000 (0.999 to 1.000)
Assuming all−ve	40	0.993 (0.987 to 0.996)	0.999 (0.999 to 1.000)	33	0.970 (0.945 to 0.984)	0.999 (0.999 to 1.000)	24	0.976 (0.855 to 0.996)	1.000 (0.999 to 1.000)
Intention to diagnosis	40	0.993 (0.988 to 0.996)	0.999 (0.999 to 1.000)	33	0.972 (0.950 to 0.985)	0.999 (0.999 to 1.000)	24	0.976 (0.855 to 0.996)	1.000 (0.999 to 1.000)
Subgroup analyses									
Study design									
Cohort	5	0.932 (0.853 to 0.971)	0.999 (0.996 to 1.000)	4	0.868 (0.591 to 0.968)	0.998 (0.994 to 0.999)	3	0.851 (0.498 to 0.971)	0.999 (0.995 to 1.000)
Others	35	0.976 (0.963 to 0.985)	0.998 (0.997 to 0.999)	29	0.941 (0.914 to 0.960)	0.998 (0.997 to 0.999)	21	0.970 (0.852 to 0.994)	1.000 (0.999 to 1.000)
Population risk									
General	6	0.959 (0.874 to 0.987)	0.999 (0.998 to 1.000)	4	0.865 (0.627 to 0.961)	0.998 (0.997 to 0.999)	4	0.775 (0.135 to 0.987)§	1.000 (0.999 to 1.000)
High	22	0.973 (0.951 to 0.985)	0.997 (0.994 to 0.998)	19	0.930 (0.892 to 0.955)	0.997 (0.995 to 0.999)	11	0.953 (0.864 to 0.985)	0.999 (0.996 to 1.000)
Others	12	0.974 (0.940 to 0.989)	0.999 (0.998 to 0.999)	10	0.958 (0.907 to 0.982)	0.999 (0.999 to 1.000)	9	0.988 (0.547 to 1.000)	1.000 (0.999 to 1.000)
Population									
Others	36	0.977 (0.965 to 0.985)	0.998 (0.997 to 0.999)	31	0.943 (0.917 to 0.960)	0.998 (0.997 to 0.999)	23	0.974 (0.861 to 0.996)	1.000 (0.999 to 1.000)
Twins	4	0.894 (0.750 to 0.960)	0.996 (0.996 to 0.996)	2	0.737 (0.202 to 0.969)§	0.998 (0.986 to 1.000)	1*		
First trimester									
100%	7	0.960 (0.887 to 0.987)	0.999 (0.998 to 1.000)	5	0.925 (0.814 to 0.972)	0.998 (0.997 to 0.999)	5	0.850 (0.770 to 0.906)§	0.999 (0.998 to 0.999)
Others	33	0.973 (0.958 to 0.983)	0.998 (0.997 to 0.999)	28	0.939 (0.910 to 0.960)	0.998 (0.997 to 0.999)	19	0.966 (0.872 to 0.992)	1.000 (0.999 to 1.000)
Test types									
DANSR	9	0.958 (0.898 to 0.983)	0.999 (0.997 to 1.000)	6	0.948 (0.879 to 0.979)	0.998 (0.996 to 0.999)	3	0.606 (0.216 to 0.895)	1.000 (0.998 to 1.000)
MPSS	25	0.978 (0.963 to 0.987)	0.998 (0.997 to 0.999)	23	0.936 (0.899 to 0.960)	0.998 (0.997 to 0.999)	16	0.959 (0.989 to 0.991)	1.000 (0.999 to 1.000)
SNP technology	4	0.984 (0.937 to 0.996)	0.998 (0.993 to 1.000)	4	0.918 (0.751 to 0.976)	0.998 (0.994 to 1.000)	5	0.870 (0.647 to 0.960)	0.998 (0.992 to 0.999)
Publication year									
2007–2013	18	0.977 (0.958 to 0.988)	0.998 (0.995 to 0.999)	15	0.954 (0.919 to 0.975)	0.998 (0.995 to 0.999)	9	0.933 (0.799 to 0.980)	0.999 (0.993 to 1.000)
2014–2015	22	0.966 (0.939 to 0.981)	0.999 (0.998 to 0.999)	18	0.915 (0.853 to 0.952)	0.996 (0.998 to 0.999)	15	0.984 (0.770 to 0.999)	1.000 (0.999 to 1.000)

*Bivariate model inestimable for only one study in the subgroup.[23]
†Excluded studies with inestimable sensitivity (T21—Hall 2014; T18—Comas 2014, Hall 2014, Zhang (twins) 2015; T13—Sehnert 2011, Beamon 2014, Comas 2014, Bevilacqua 2015, Wax 2015, Zhang (twins) 2015).
‡Excluded outliers (T21—Dhallan 2007, Chiu 2011, Sparks 2012; T18—Chen 2011; T13—Chen 2011, Palomaki 2012).
‡p Value for subgroup differences <0.05 (statistically significant).
SN, sensitivity; SNP, single nucleotide polymorphism; SP, specificity.

Taylor-Phillips S, Freeman K, Geppert J, Agbebiyi A, Uthman OA, Madan J, Clarke A, Quenby S, Clarke A. Accuracy of non-invasive prenatal testing using cell-free DNA for detection of Down, Edwards and Patau syndromes: a systematic review and meta-analysis. BMJ Open. 2016 Jan 18;6(1):e010002. doi: 10.1136/bmjopen-2015-010002. PubMed PMID: 26781507; PubMed Central PMCID: PMC4735304.

Claimed accuracy (3)

The sensitivity and specificity of the test vary **with bioinformatics corrections**

	Standard z-score approach (repeat-masked genome)		Standard z-score approach (non-repeat-masked genome)		Z-score approach with GC correction (non-repeat-masked genome)	
	T13	T18	T13	T18	T13	T18
Sensitivity:	36.0%	73.0%	44.0%	83.8%	100.0%	91.9%
Specificity:	92.4%	97.2%	93.6%	98.0%	98.9%	98.0%
PPV:	31.0%	79.4%	39.3%	86.1%	89.3%	87.2%
NPV:	93.8%	96.1%	94.6%	97.6%	100.0%	98.8%

PPV: positive predictive value, NPV: negative predictive value, T13: trisomy 13, T18, trisomy 18. chr13 or 18 z-score >3 was used as the diagnostic cut-off.
doi:10.1371/journal.pone.0021791.t003

1. Chen EZ, Chiu RW, Sun H, Akolekar R, Chan KC, Leung TY, Jiang P, Zheng YW, Lun FM, Chan LY, Jin Y, Go AT, Lau ET, To WW, Leung WC, Tang RY, Au-Yeung SK, Lam H, Kung YY, Zhang X, van Vugt JM, Minekawa R, Tang MH, Wang J, Oudejans CB, Lau TK, Nicolaides KH, Lo YM. Noninvasive prenatal diagnosis of fetal trisomy 18 and trisomy 13 by maternal plasma DNA sequencing. PLoS One. 2011;6(7):e21791.

Claimed accuracy: PPV

- **Positive predictive value**, or **precision rate**, is the proportion of positive test results that are true positives (such as correct diagnoses).

- Reflects the probability that a positive test reflects the underlying condition being tested for. Its value strongly depends on the prevalence of the outcome of interest.

$$\mathrm{PPV} = \frac{\text{sensitivity} \times \text{prevalence}}{\text{sensitivity} \times \text{prevalence} + (1 - \text{specificity}) \times (1 - \text{prevalence})}$$

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Claimed accuracy: PPV

Table 3. Test Performance.*

Trisomy	No. of Cases	cfDNA Testing	Standard Screening
		% (95% CI)	
Trisomy 21	5		
Sensitivity		100 (47.8–100)	100 (29.2–100)
Specificity		99.7 (99.3–99.9)	96.4 (95.4–97.2)
Positive predictive value		45.5 (16.7–76.6)	4.2 (0.9–11.7)
Negative predictive value		100 (99.8–100)	100 (99.8–100)
Trisomy 18	2		
Sensitivity		100 (15.8–100)	100 (2.5–100)
Specificity		99.8 (99.6–100)	99.4 (99.0–99.7)
Positive predictive value		40.0 (5.3–85.3)	8.3 (0.2–38.5)
Negative predictive value		100 (99.8–100)	100 (99.8–100)

* Included in the test performance analysis for standard screening were 1912 patients who were tested for trisomy 21 (1909 unaffected patients plus 3 with true positivity) and 1906 patients who were tested for trisomy 18 (1905 unaffected patients plus 1 with true positivity). For the cfDNA test performance, results from standard screening were not required. Test analysis for cfDNA included 1952 patients who were tested for trisomy 21 (1947 unaffected patients plus 5 with true positivity) and 1952 patients who were tested for trisomy 18 (1950 unaffected patients plus 2 with true positivity).

N Engl J Med. 2014 Feb 27;370(9):799-808. doi: 10.1056/NEJMoa1311037. DNA sequencing versus standard prenatal aneuploidy screening. Bianchi DW(1), Parker RL, Wentworth J, Madankumar R, Saffer C, Das AF, Craig JA, Chudova DI, Devers PL, Jones KW, Oliver K, Rava RP, Sehnert AJ; CARE Study Group

Overview

- Detection of cell-free DNA
- NIPT technique
- Claimed accuracy
- Indications and limitations

Indications and limitations (1)

- NIPT is reimbursed in Belgium for all pregnancies (July 2017)

- Requirements
  - → Informed consent
  - → First trimester ultra sound result
  - → Blood sample (not taken before 12 weeks gestation, counted from last menstrual period)

- Not eligible for NIPT in case of:
  - → More than 2 fetuses
  - → Maternal blood transfusion, stem cell therapy, immunotherapy or transplantation (within last 3 months)

- NIPT is more difficult in case of:
  - → Heparin therapy (anti-blood clotting)
  - → High BMI (high maternal weight)
  - → Anomalies in parental genetic material
  - → Vanishing twins

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Indications and limitations (2)

- NIPT will fail on:
  - → balanced translocations
  - → polyploidy (e.g. triploidy)

- NIPT might fail on:
  - → unbalanced translocations
  - → sub chromosomal aberrations
  - → (placental) mosaicism
  - → maternal chromosomal abnormalities, …

- NIPT might not detect:
  - → micro-deletions or –duplications
  - → mosaic chromosomal aberrations

- NIPT is not able to detect monogenic abnormalities