VRIJE UNIVERSITEIT BRUSSEL

Universitair Ziekenhuis Brussel

PRENATAL DIAGNOSIS AND NEONATAL SCREENING

Alexander Gheldof

PRENATAL DIAGNOSIS – GENE TESTING

PRENATAL TESTING FOR MONOGENIC DISEASE – WHEN?

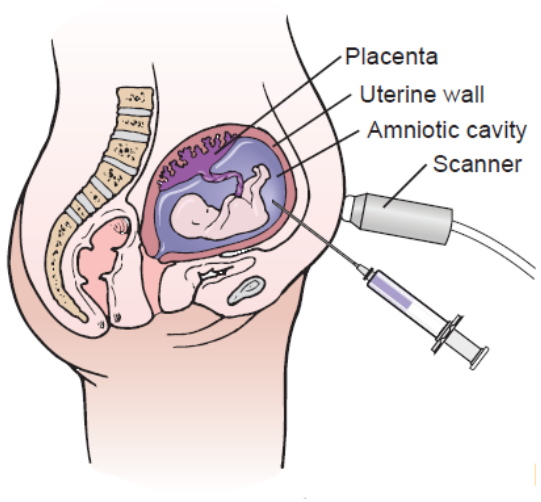
In case of familial history:

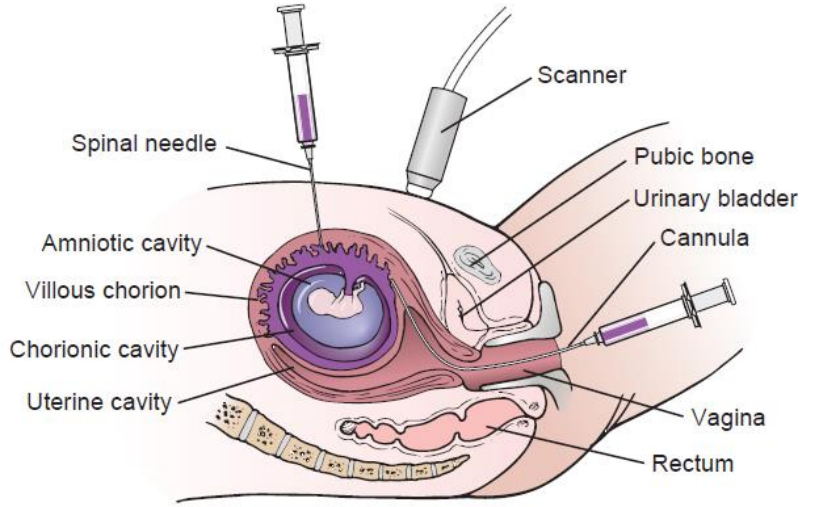
- Other child(ren) of couple is/are affected
- Family member(s) is/are affected
  - Important note: in case one partner of pregnant couple is a carrier => carriership test of other partner instead of PND
  - Only PND if partner was found to be carrier as well
- One of the partners of the pregnant couple is affected

In case of echographic abnormalities suggestive for monogenic disease

- For example:
- L1CAM (X-linked):
  - Hydrocephalus
  - Agenesis of corpus callosum
  - Macrocephaly
  - Corticospinal tract hypoplasia
- Achondroplasia (FGFR3, dominant) – *de novo* due to NM_000142.5(FGFR3):c.1138G>A, p.(Gly380Arg)
  - Shortened long bones
  - Paternal age effect

AMNIOCENTESIS (AC) – CVS - CHORDOCENTESIS





AC: 14-16 weeks of pregnancy

Shed fetal cells – originate from different sources:

Amniotic membranes, fetal respiratory system, skin, gastrointestinal and urinary tracts

Totally differentiated, lineage commited, pluripotent, highly multipotent stem cells
(Seyed et al. 2020)

CVS: 11-12 weeks pregnancy

Chorion villi cells: blood vessels and connective tissue from trophoblast and mesoderm (Larsen et al 2001)

Caveat: placental mosaïcism (see later)

AMNIOCENTESIS – DNA ANALYSIS

Direct analysis:

- After sampling: Centrifugation of one part of sample => DNA extraction => Testing
- Important to note: limited DNA quantity!
- In the past: complete Sanger sequencing of large genes was often compromised (eg L1CAM: 29 exons)
- Present with NGS: 1µg of high quality DNA is necessary and is most often available in the direct analysis

Analysis on cultured cells:

- After sampling: Centrifugation of other part => Cells grown in culture => DNA extracted after sufficient proliferation
- DNA most often obtained after 2-3 weeks in culture
- Why is this done?
  - As a backup in case direct analysis fails
  - Pool of available cells in case of meternal cell contamination (see later)

CVS – DNA ANALYSIS

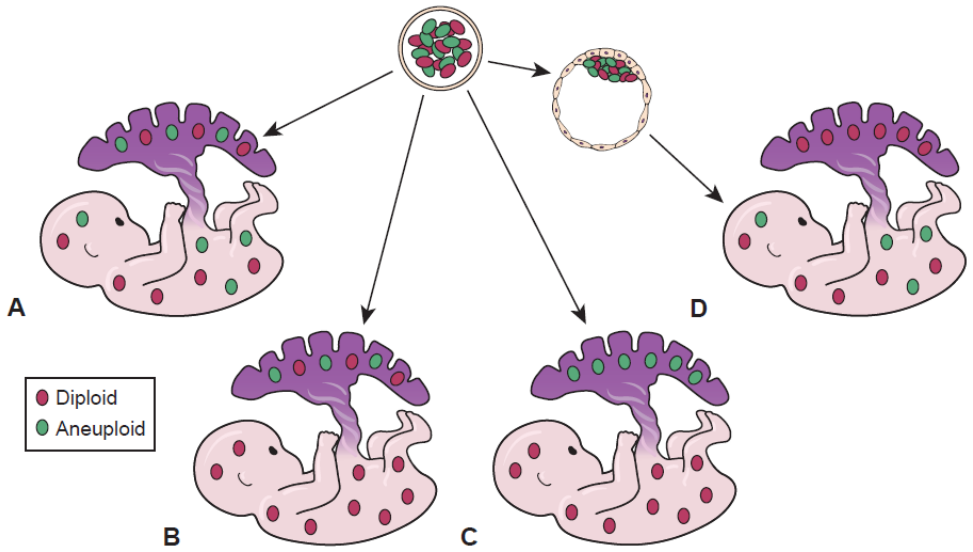
Direct analysis:

- After sampling: Manual mechanical dissection of CVS tissue => DNA extraction (longer protocol than direct AC) => testing
- Important to note: DNA quantity is larger than direct AC

Analysis on cultured cells:

- After sampling: Manual processing of CVS tissue (mechanical/enzymatic overnight dissection of sample => Cells grown in culture => DNA extracted after sufficient proliferation
- DNA most often obtained after 2-3 weeks in culture
- Why is this done?
  - As a backup in case direct analysis fails
  - Pool of available cells in case of meternal cell contamination (see later)

CVS TESTING – PLACENTAL MOSAÏCISM



Different outcomes of placental mosaicism

CVS result may not always reflect the true fetal status

For example: in 1-2% of pregnancies, discrepancies between karyotypes in trophoblast and fetus have been found

In AC samples: this discrepancy is highly unlikely

In case of non conforming results in CVS hinting at placental mosaïcism:

- Analysis can be redone on second part of the CVS sample
- Not always possible
- AC can be done after CVS
- Chordocentesis can be done after AC

MATERNAL CELL CONTAMINATION

We need to be sure we are examining fetal and not maternal DNA.

Distinction can be made by performing a maternal cell contamination test:

- A blood sample of the mother is compared to the AC or CVS sample
- Test is based on an STR marker analysis.
- STR: Short Tandem Repeat

- Intermezzo:
  - Repeat sequences make up 25-50% of mamalian genomes
  - Tandem repeats: short $(CTGA)_n$, long $(CTGAG…….CAAGGG)_n$ called minisatellites
  - Interspersed repeats: CTAGGGAAAAGGGGG-large genomic distance-CTAGGGAAAAGGGGG
    - Special case: retrotransposons => insertions of inactive viruses => can shift position

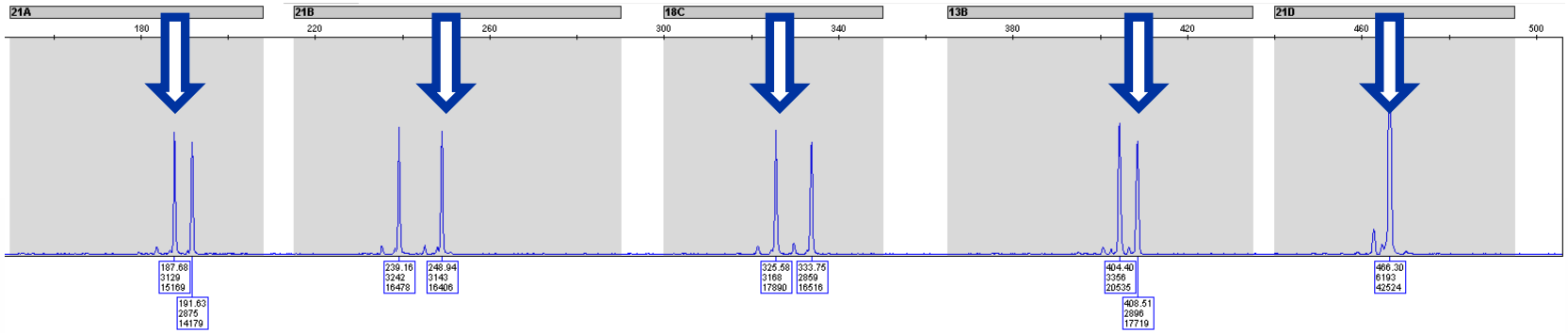
- STR: tri-pentanucleotides repeats with highly variable length across individuals
- Maternal cell contamination test: multiple STRs are tested, number can vary depending on kit (27 @ UZBrussel)

MATERNAL CELL CONTAMINATION

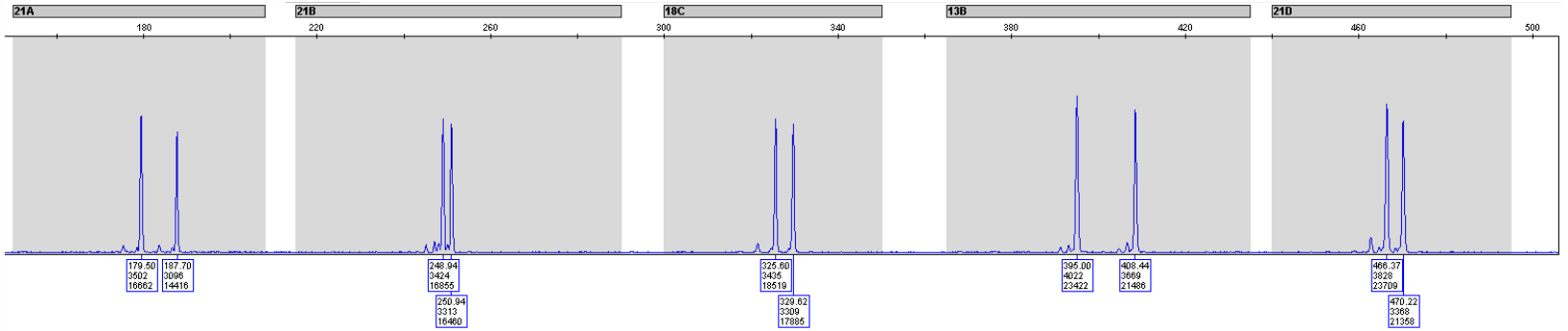
Example of STR test:

Arrows: maternal allele

Fetal sample:



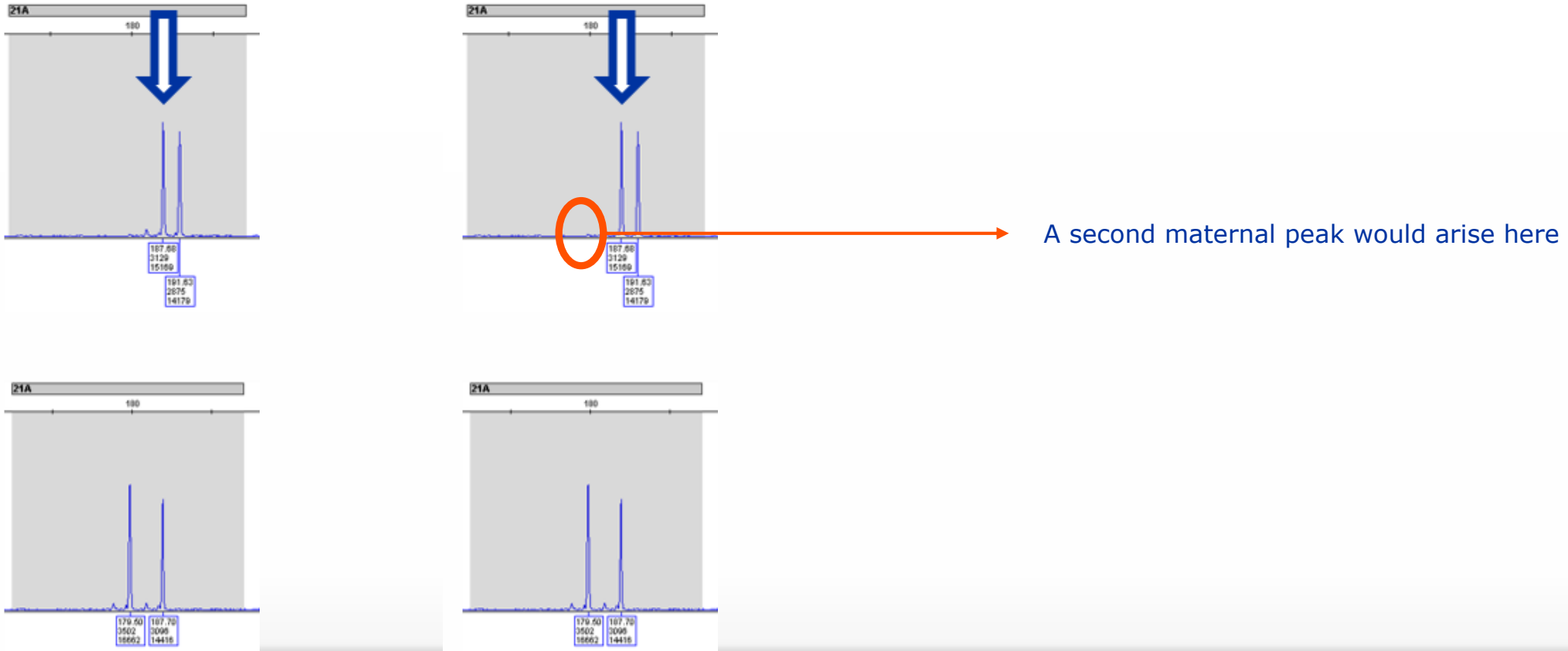
Maternal blood sample:



MATERNAL CELL CONTAMINATION

Informative STR markers: Markers where one can make a distinction between contamination or no contamination

Informative, because…. in case of contamination second maternal marker would pop up in fetus



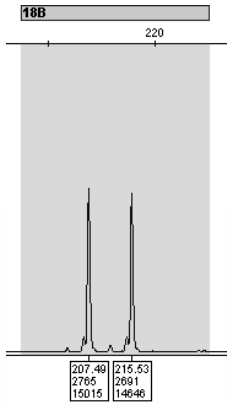
MATERNAL CELL CONTAMINATION

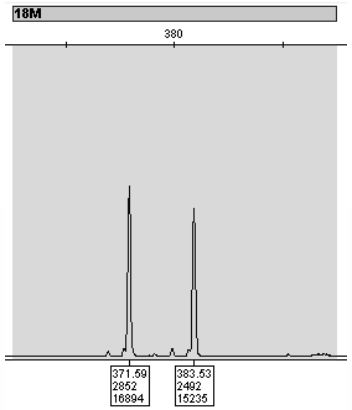
Non-Informative STR markers: Markers where one cannot make a distinction between contamination or no contamination

Example 1

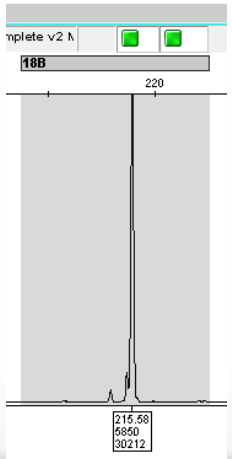
Example 2

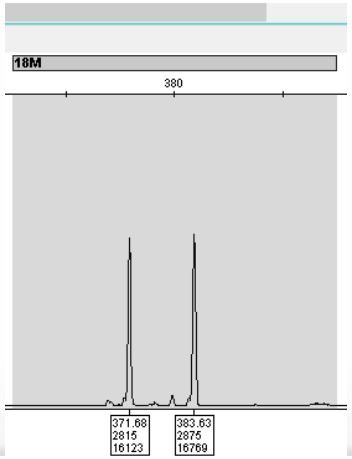
Sample of fetus





Maternal sample





Non informative in case of:

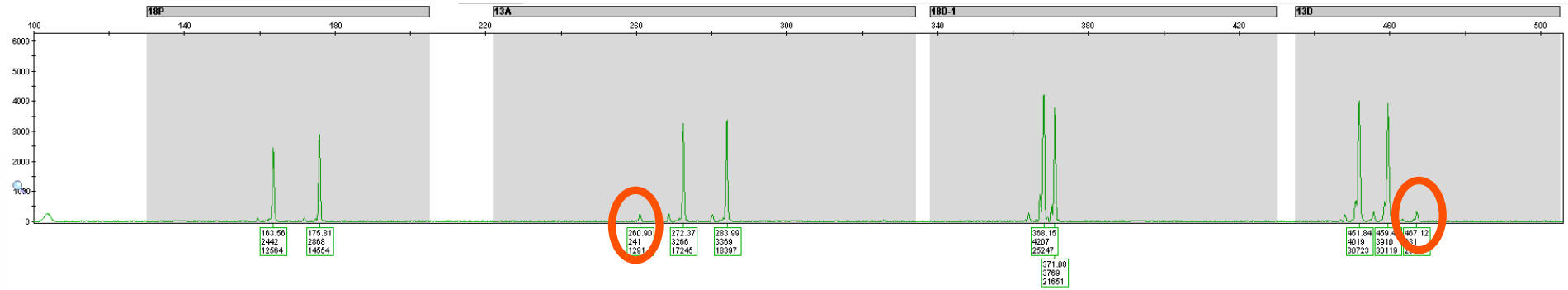
Homozygous allele in mother
Or
Mother has the same two alleles as fetus

MATERNAL CELL CONTAMINATION

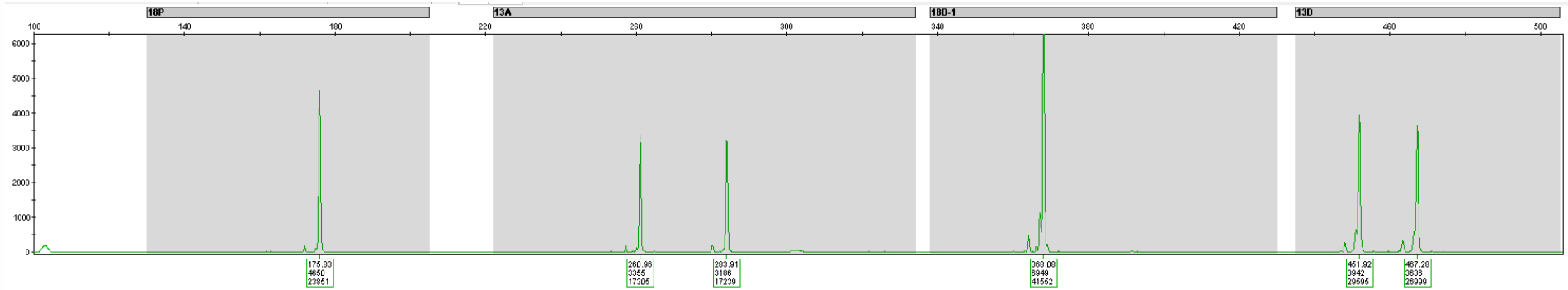
Example of sample with +/- 7% contamination

Non-informative | Informative | Non-informative | Informative

Sample of fetus

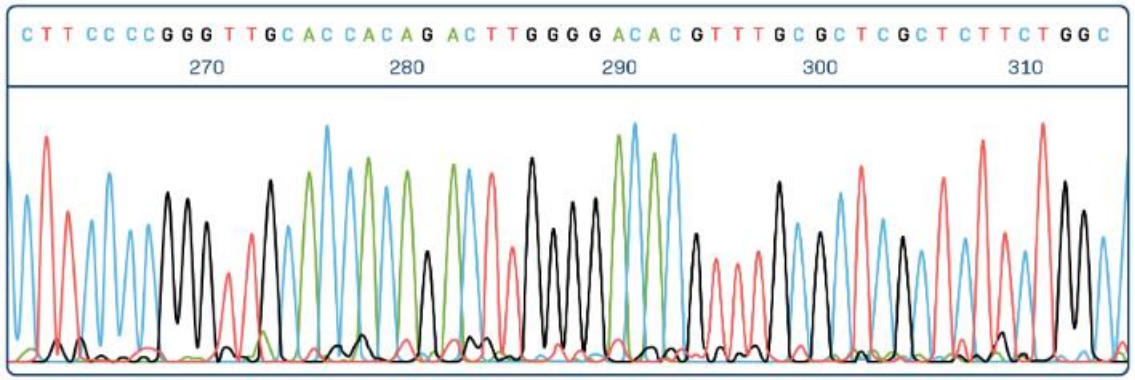


Maternal sample



MATERNAL CELL CONTAMINATION

- **Low** amounts of cell contamination are tolerated relatively well
- Sanger sequencing is robust enough to allow interpretation when contamination is below 10-15%
- Why?



  - Sanger sequencing often presents with a backround in the range of 10-15% in relation to the peak signals
  - Maternal cell contamination of 10-15% will thus be in the background of the test

- Nevertheless**: Maternal cell contamination should be avoided as it is most often > 15%!**

- Notable exceptions are FragileX (not detected with Sanger) and IKBKG (Incontinentia Pigmenti) testing
- These tests are highly sensitive to contamination

MATERNAL CELL CONTAMINATION

Not all tests are forgiving for small maternal contamination

- Notable exception with Sanger sequencing is IKBKG (Incontinentia Pigmenti)
  - IKBKG => Dominant inheritance (X-linked dominant – lethal for XY embryo's)
  - In > 80% of cases caused by a known 10 kb deletion
  - In other 20%: SNV's
  - However, *IKBKG* has a pseudogene *IKBKGP1*

  - As a consequence, 4 identical copies are present in women
  - Presence of a heterozygous (pathogenic) variant in *IKBKG* will emerge in a peak with a max height of 25% in in comparison to peak of other alleles

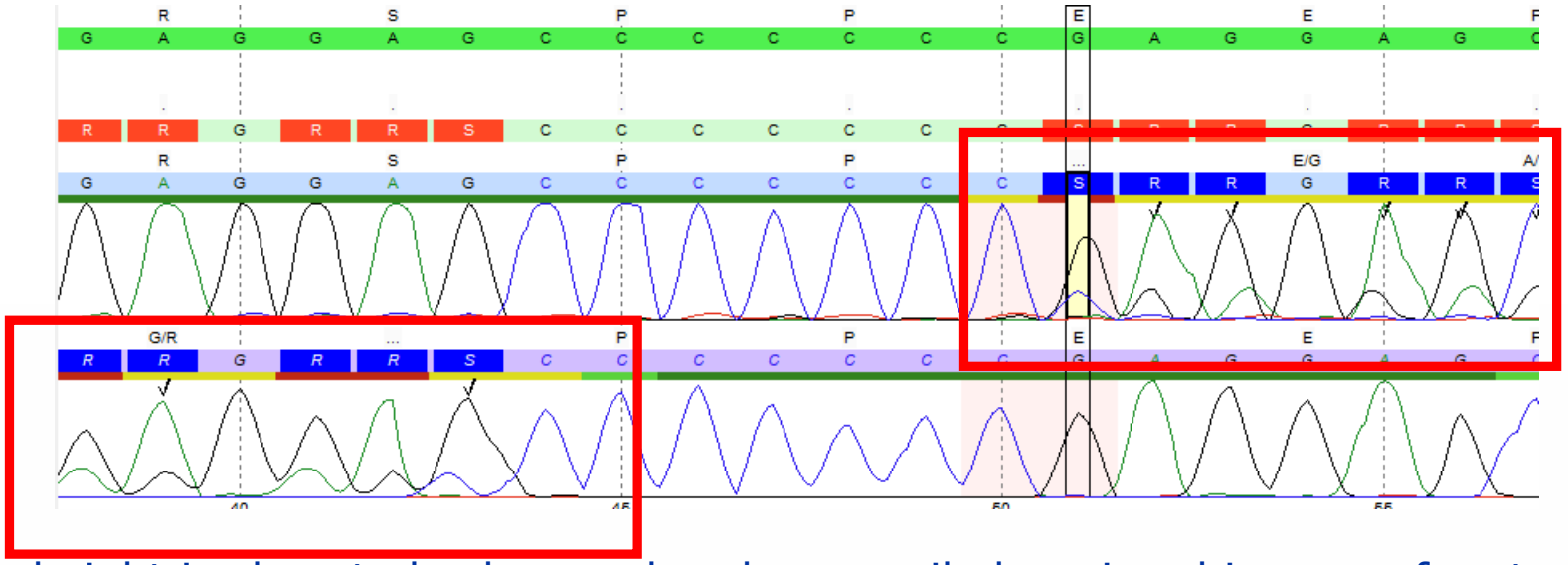
Maubach et al 2017

MATERNAL CELL CONTAMINATION

NM_003639.4(IKBKG):c.1167dupC, p.Glu390Argfs*5

Forward seq

Reverse seq



- 25% of peak height is close to background and can easily be missed in case of maternal contamination
- How to solve?
  - Long range PCR? Yes
  - NGS?
    - Short reads? Possible because less background. However, pipeline has to be adapted!
    - Long reads? Best solution because it is possible to discern whether the variant is located in coding or pseudogene

INTERMEZZO – GENE CONVERSION

Variant present in father in IKBKGP pseudogene

Daughter of couple is affected

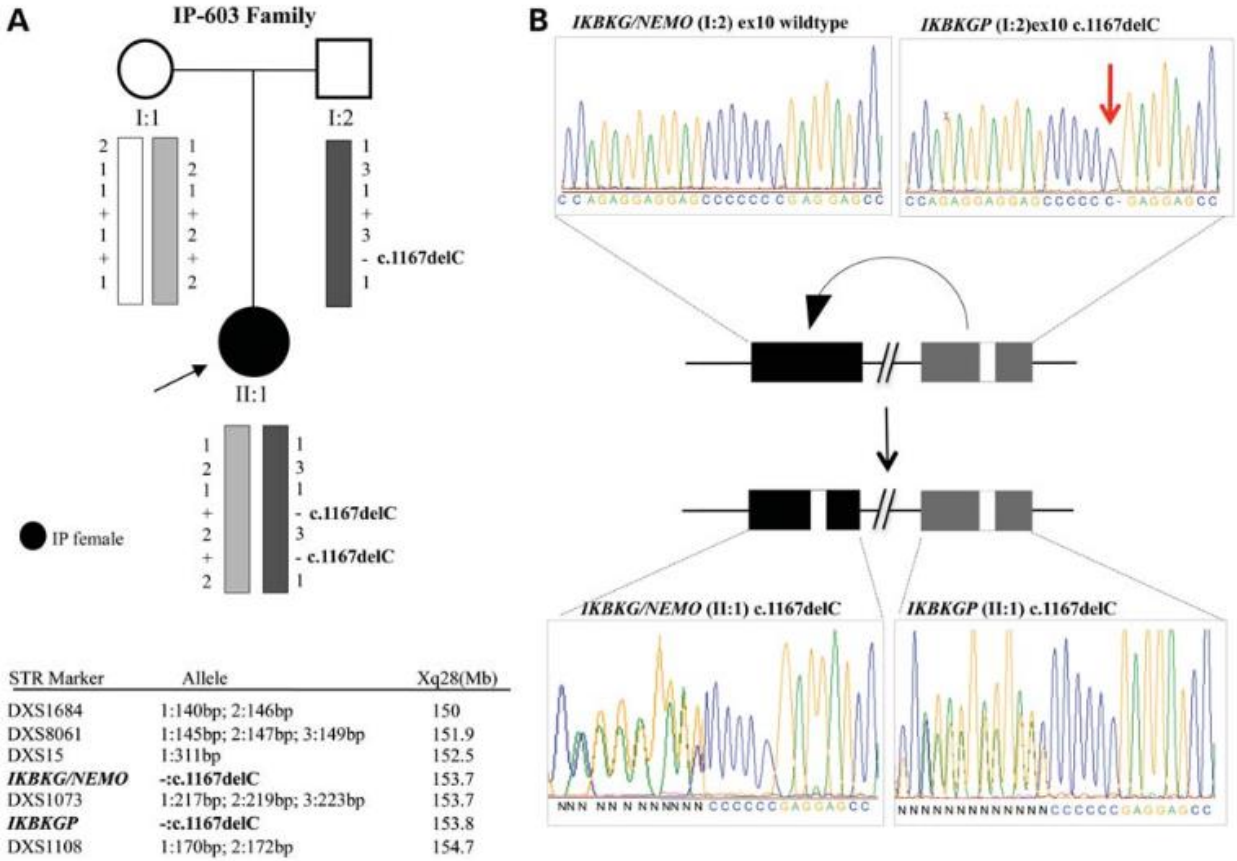
Reason?

Due to homologous recombination in the gametes of father, the variant has been "transferred" to the coding gene *IKBKG*

Daughter thus has the pathogenic variant in both the coding and the pseudogene

Gene conversion is an event which can take place in case of pseudogene presence

For example *GBA* (Gaucher disease), *IDS (*Hunter disease).



STR Marker	Allele	Xq28(Mb)
DXS1684	1:140bp; 2:146bp	150
DXS8061	1:145bp; 2:147bp; 3:149bp	151.9
DXS15	1:311bp	152.5
IKBKG/NEMO	**-:c.1167delC**	153.7
DXS1073	1:217bp; 2:219bp; 3:223bp	153.7
IKBKGP	**-:c.1167delC**	153.8
DXS1108	1:170bp; 2:172bp	154.7

Fusco et al., 2012

MATERNAL CELL CONTAMINATION

Fragile X:

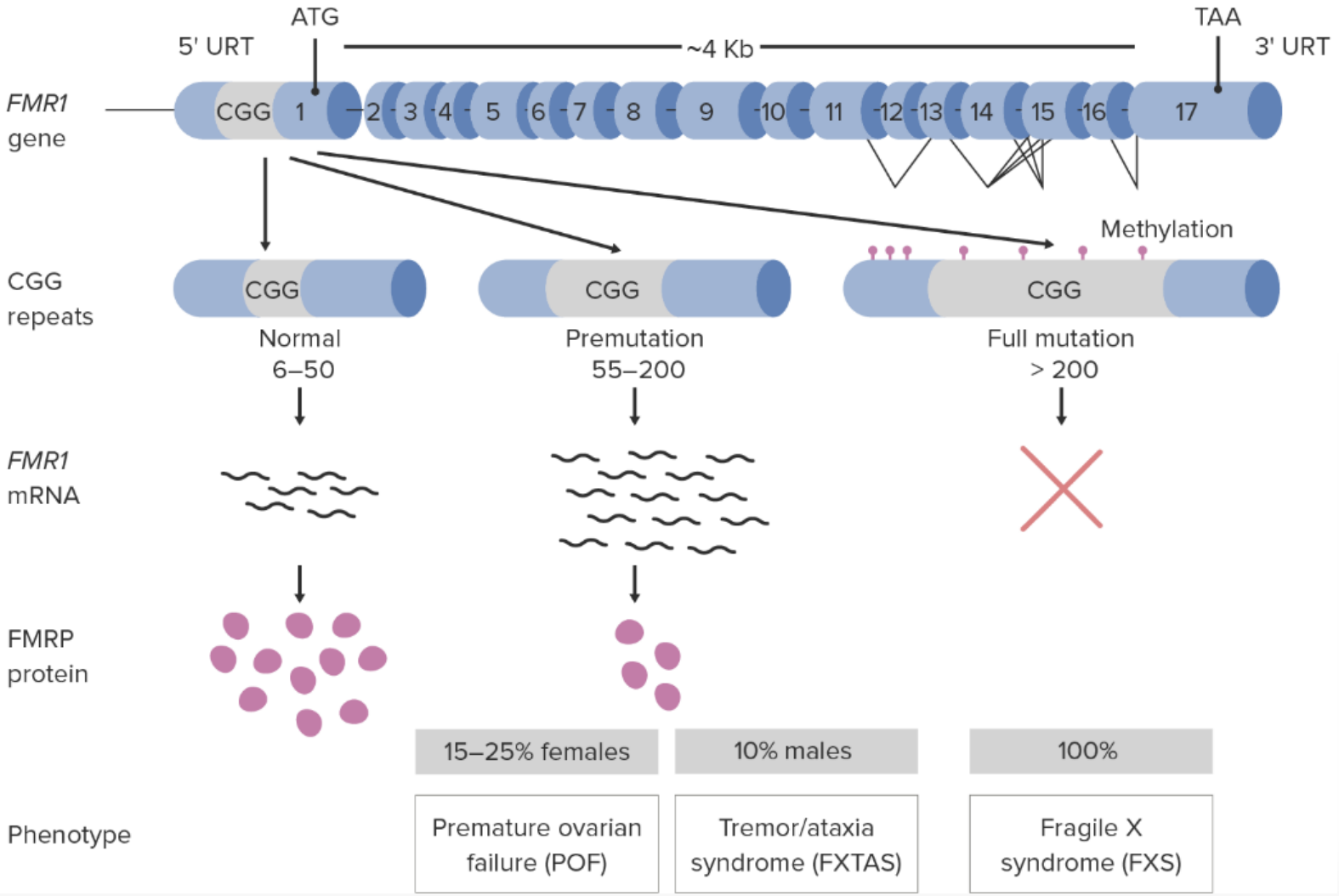
Triplet repeat CGG **expansion** in the 5’ UTR of the *FMR1* gene

X linked

Anticipation effect

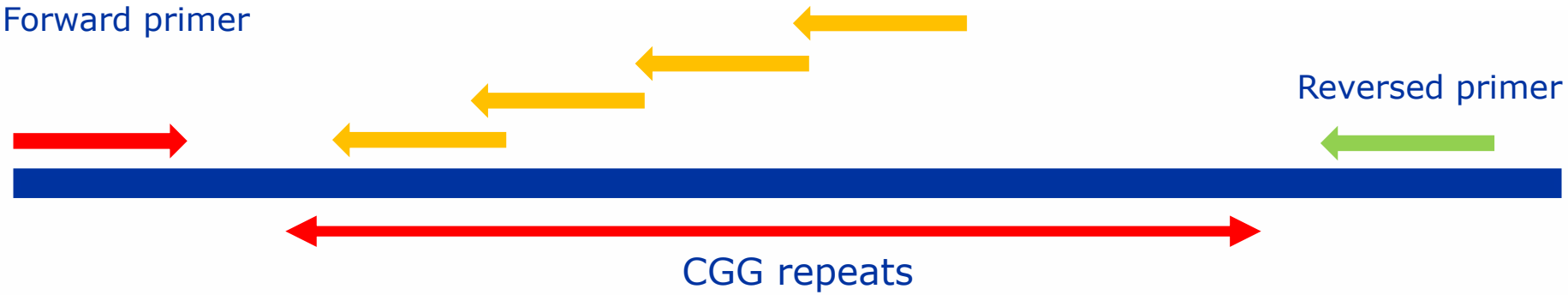
In case of full mutation:
Males:

- Elongated face
- Intellectual disability
- Autism
- Epilepsy (20% of cases)



MATERNAL CELL CONTAMINATION

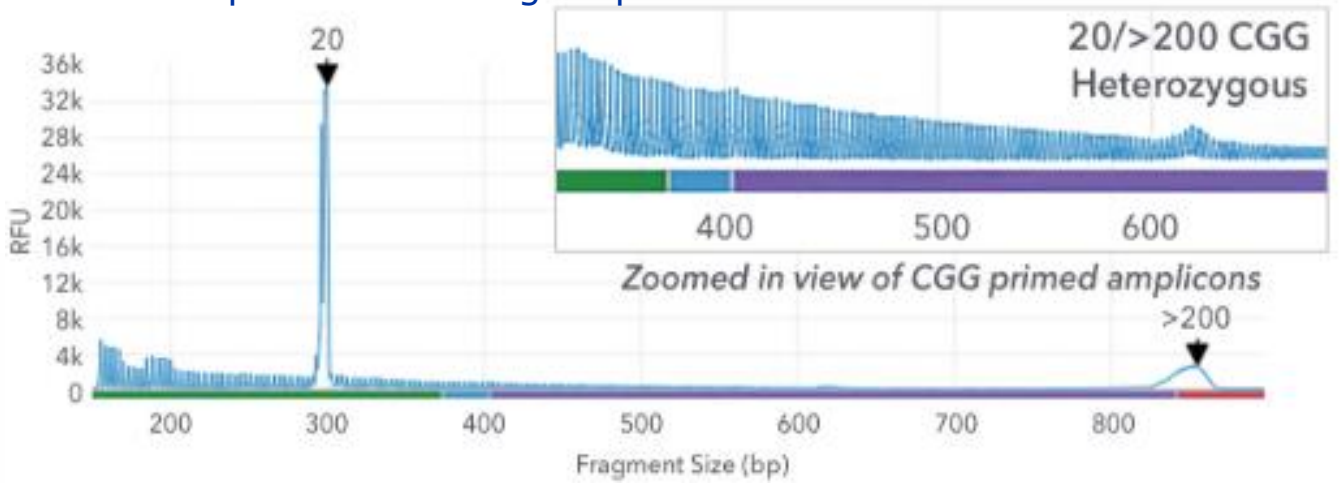
- CG rich sequences are notoriously difficult to amplify
- PCR with flanking primers is unable to amplify large repeat expansions
- How is this solved?
  - TP-PCR => Triplet Repeat PCR



- Ladder amplification
- Despite TP-PCR: large difference in sequencing amplification efficacy between short and long repeat lengths

MATERNAL CELL CONTAMINATION

- Fragile X: Large difference in amplification efficiency between normal and disease allele
- Due to TP-PCR: sensitive amplification of large repeats



- Implications: In case of an affected child: premutation of mother can be preferentially amplified instead of full mutation of fetus

X-INACTIVATION IN PRENATAL SETTING

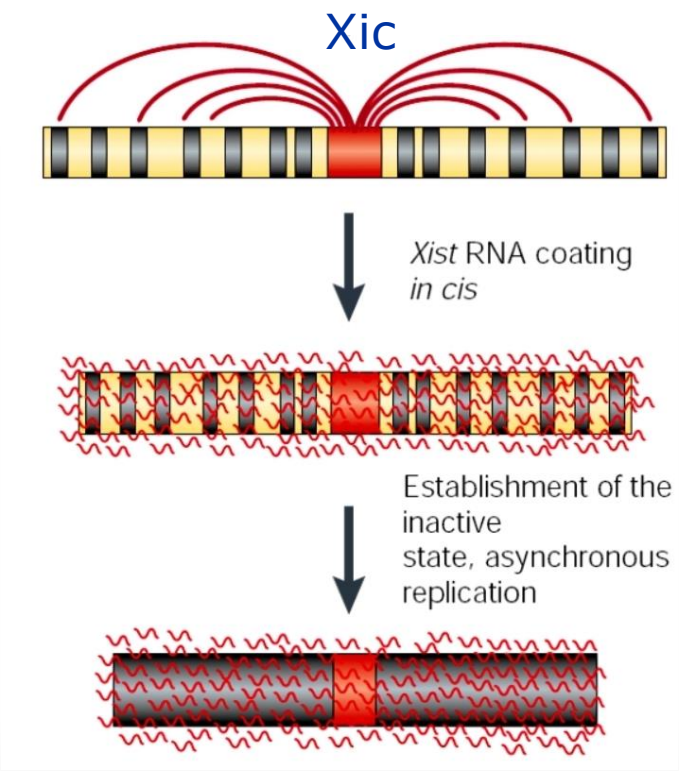
- The X-chromosome harbours +/- 1100 genes
- In both women (XX) and men (XY), gene dosage of X-linked transcripts is (should be) equal
- Solved by stochastic transcriptional inactivation of a single X-chromosome in each female cell (a process called by Lyonisation, X-inactivation or Xin)

Mechanism:

- Xist plays a central role: X-inactive specific transcript
- Xist: non coding RNA
- However, it is still unknown how randomnes is achieved

X-INACTIVATION IN PRENATAL SETTING

- Xist plays a central role: X-inactive specific transcript
- *Cis* regulatory mRNA, resulting in chromatin compaction (through DNA methylation and histone deacetylation)



Xic: X inactivation centre

X-chromosome

Xist transcripts

Inactive X-chromosome

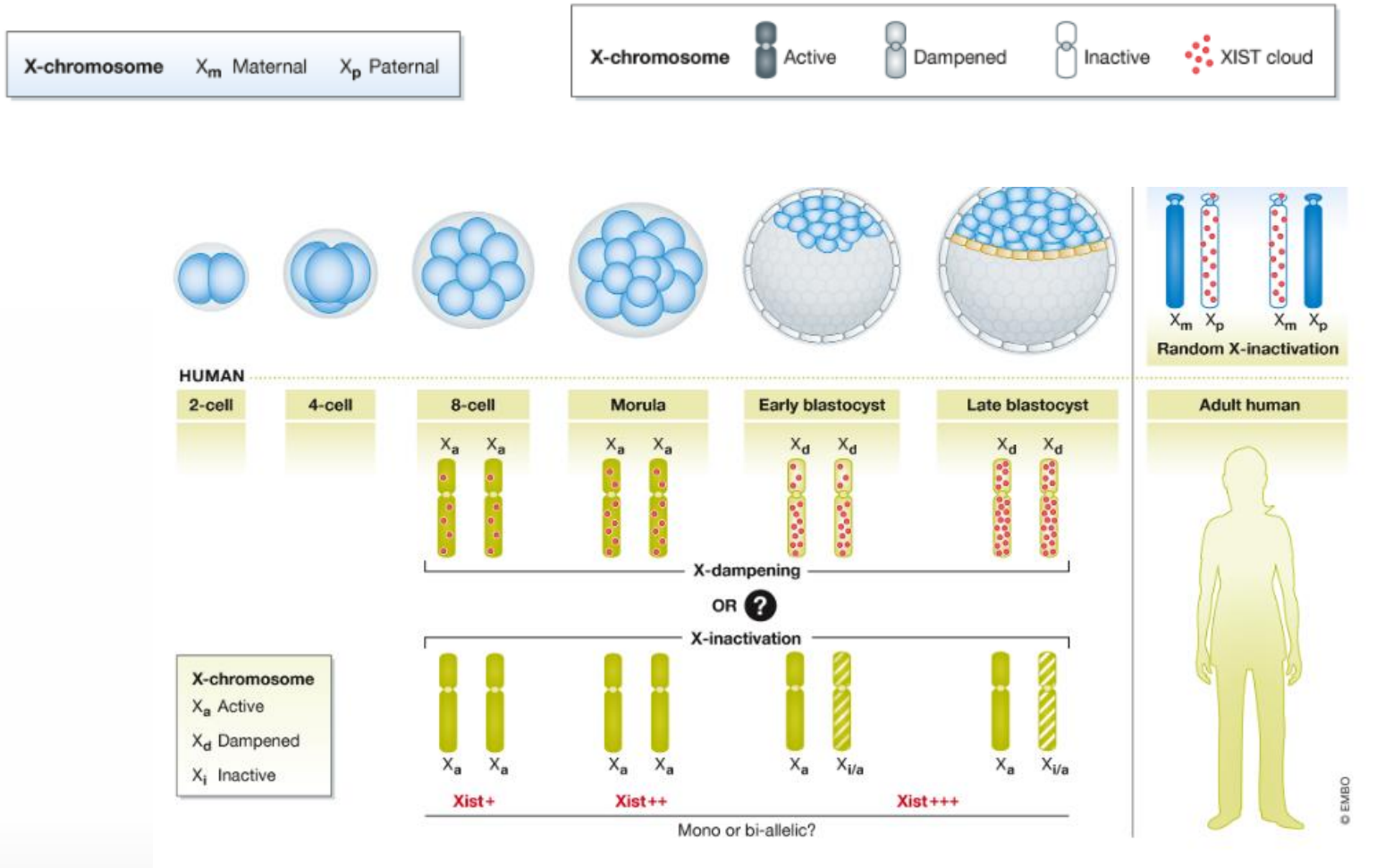
X-INACTIVATION IN PRENATAL SETTING

Xin is random

However, in humans, randomnes is only achieved after the blastocyst stage

All descendants of Xin cells maintain The same inactivation of paternal or maternal X-chromosome)

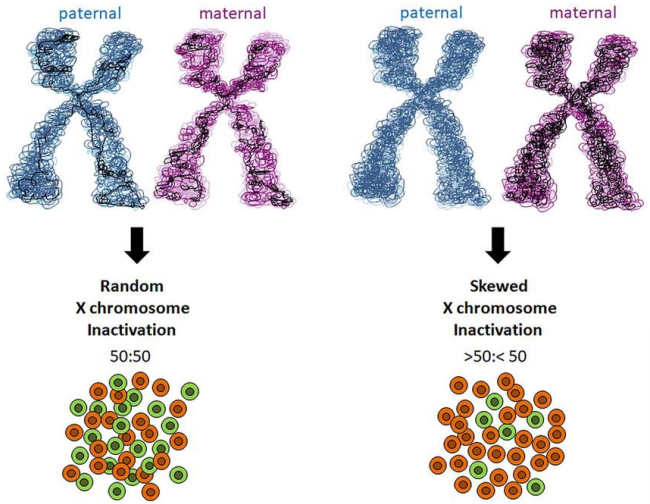
Different mechanism of transcript levelling in early embryonic stages (from EGA – late blastocyst stage)
Dampening is proposed



X-INACTIVATION IN PRENATAL SETTING

Xin and disease:

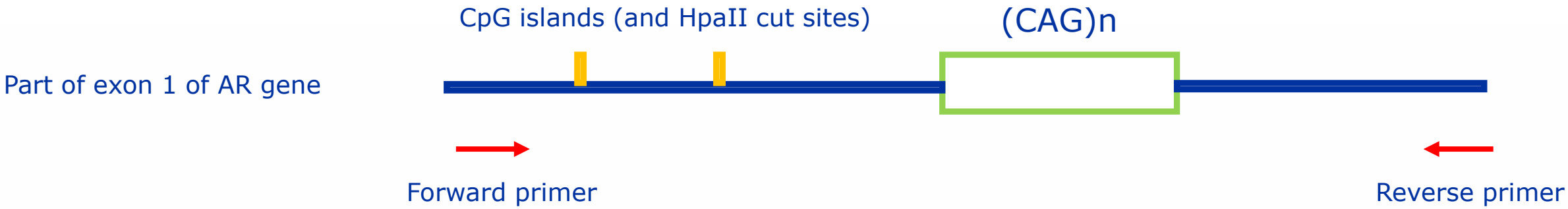
- Sometimes Xin does not occur stochastically
- The 1:1 ratio between paternal/maternal Xin is not maintained
- One particular X chromosome is more active than the other
- This is called X-skewing
- Spectrum: full skewing – light skewing



- For X-linked recessive diseases:
  - Heterozygous women (eg in Fabry disease – GLA gene) can be non affected
  - In case of random skewing
  - Or moderatley-severely affected in case of complete skewing where
  - only the chromosome with the pathogenic *GLA* allele is active

X-INACTIVATION IN PRENATAL SETTING

- Detection of Xin:
  - CpG island in exon 1 sensitive to methylation in the *AR* gene (androgen receptor – situated on the X-chromosome)
  - In combination with a downstream CAG repeat polymorphism (CAG)n to differentiate between the two alleles
  - **HpaII restriction enzyme: cuts unmethylated CpG islands, does not cut methylated CpG islands**

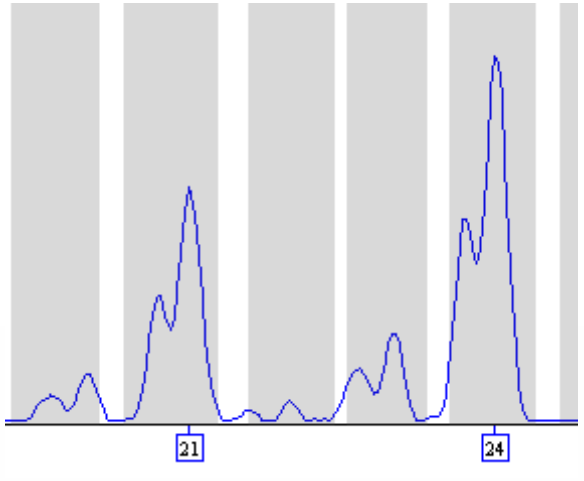


- Step 1: DNA incubated with and without HpaII
- Step 2: PCR is performed
- Step 3: fragment analysis

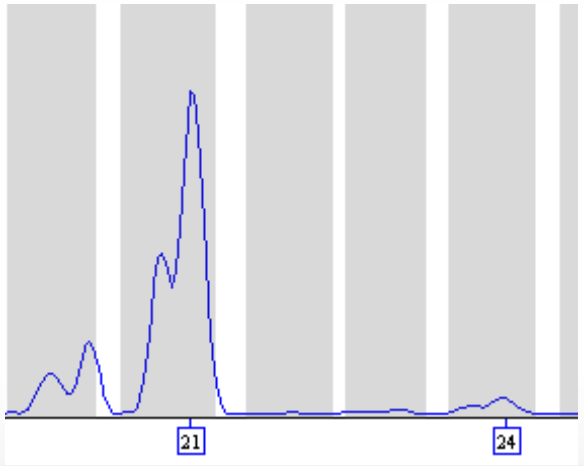
X-INACTIVATION IN PRENATAL SETTING

- Detection of Xin: Example

1) Undigested DNA
2) PCR



1) HpaII digested DNA
2) PCR



21 CAG repeat peak is not cut
=> Methylated => Inactive

24 CAG repeat peak is cut
=> NOT Methylated => Active

X-INACTIVATION IN PRENATAL SETTING

- How can Xin be used in a prenatal setting?
  - Example:
    - A VUS (variant of unknown significance) has been found in a prenatal case where genotype and gene
    - in which the VUS was found correspond
    - VUS is maternally inherited
    - Mother does not display a phenotye
    - **How to get a better interpretation of this VUS?**
    - Xin testing in mother:
      - If not skewed:
        - Both X-alleles are expressed equally
        - Mother has no phenotype and the VUS is still expressed
        - VUS **likely not** the cause of the prenatal phenotype
      - In case skewed:
        - Only one X-allele is active
        - Better argumentation that VUS could indeed be involved in prenatal phenotype
        - Mother has no phenotype, so the allele with the VUS could be inactivated

NEONATAL GENETIC SCREENING IN BELGIUM

- Currently, two conditions are genetically screened for in Belgium:
  - Mucoviscidosis (CFTR gene):
    - 1st tier: IRT (immunoreactive trypsinogen)
      - Neonates with CF have elevated levels of serum IRT
      - Tested for all neonates on dried blood spots
      - IRT testing has a high false positive rate
    - 2nd tier:
      - All IRT positives are genetically tested
      - 12 most prevalent pathogenic *CFTR* variants
      - Short Turn Around Time
      - Complete cycle of 1st and 2nd tier < 1 week

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NEONATAL GENETIC SCREENING IN BELGIUM

- Currently, two conditions are genetically screened for in Belgium:
  - SMA screening: spinal muscular atrophy

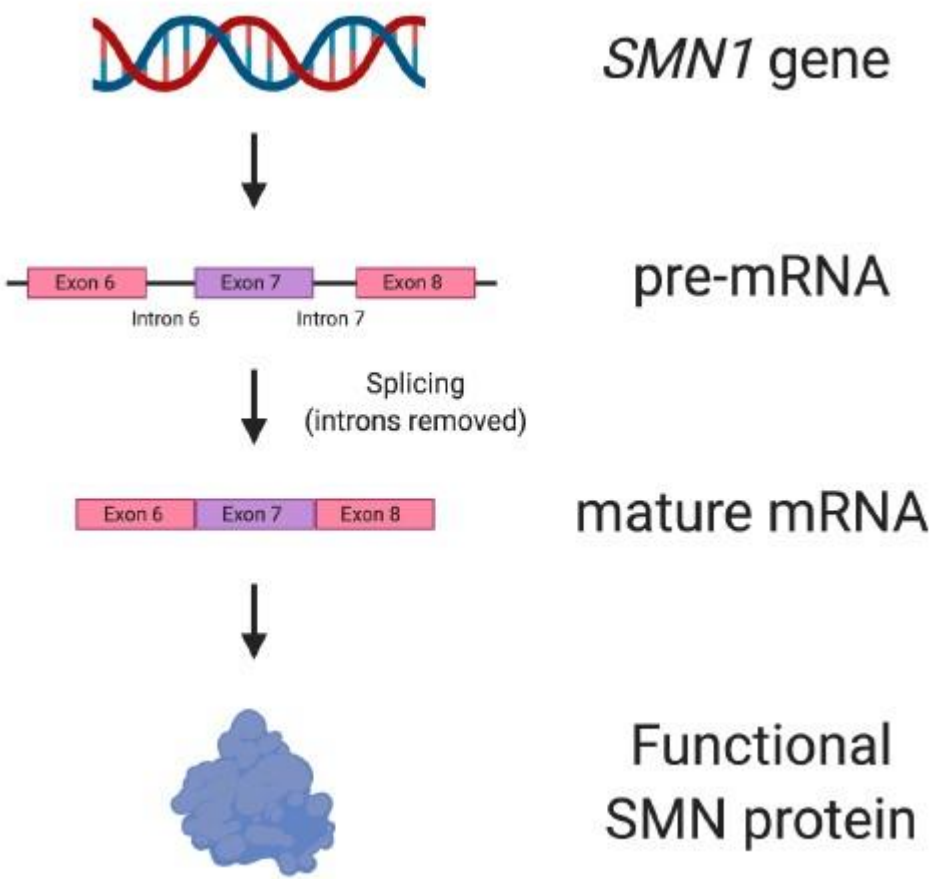
    - autosomal recessive muscular disorder (motor neurons)
    - highly heterogeneous phenotype: 1 – 4 types SMN1 gene
    - on chromosome 5q: 9 exons (1,2a, 2b, 3, 4, 5, 6, 7, 8)
    - > 94 - 96% patients homozygous ΔE7-8 (ΔE7 pathogenic)
    - residual compound heterozygous ΔE7-8 / variant of 2 variants
    - carrier status strongly depends on ethnicity (1/30 – 1/125)
    - incidence 1/6000 – 10000
    - Walloon carrier status: 1/42

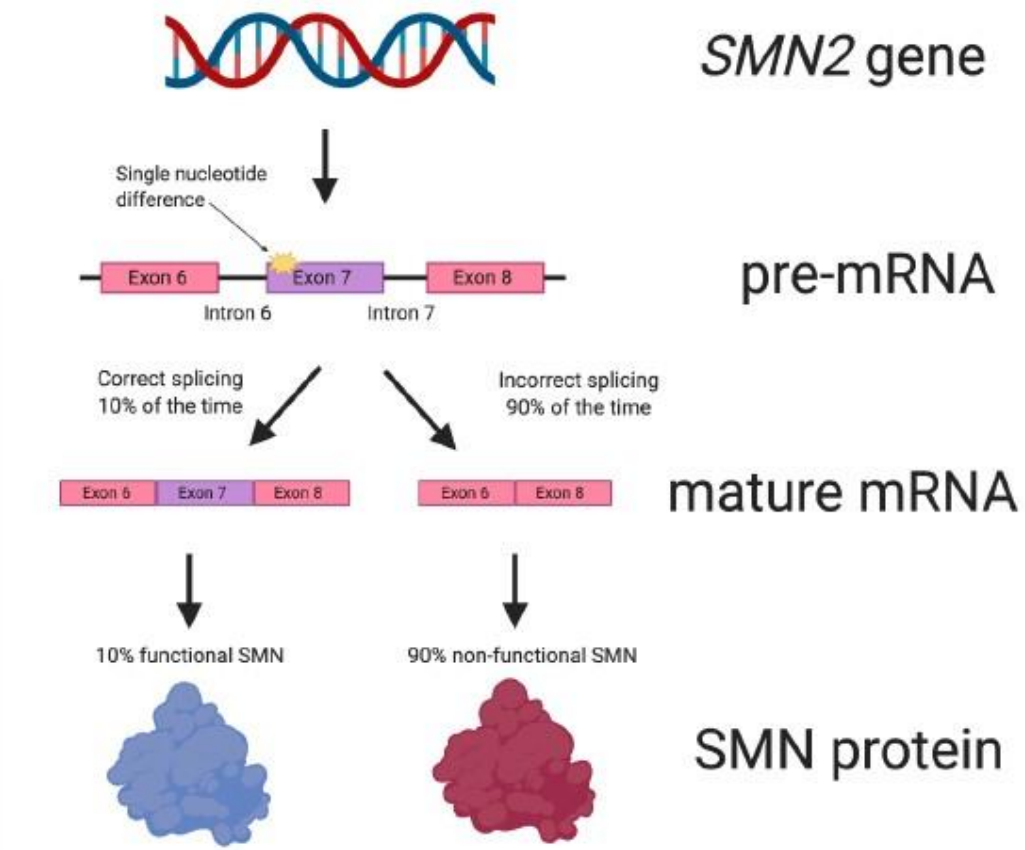
NEONATAL GENETIC SCREENING IN BELGIUM

- Currently, two conditions are genetically screened for in Belgium:
  - SMA screening: spinal muscular atrophy

    - progressive muscle weakness and atrophy
    - degeneration of motor neurons often fatal during the first 2 years of life
    - phenotype dependent on #SMN2 genes
    - SMN1 => active gene SMN2 (pseudogeneous, very similar)
    - only a few nt differences from SMN1: 1 causes splicing of exon 7
    - most transcripts not functional

NEONATAL GENETIC SCREENING IN BELGIUM





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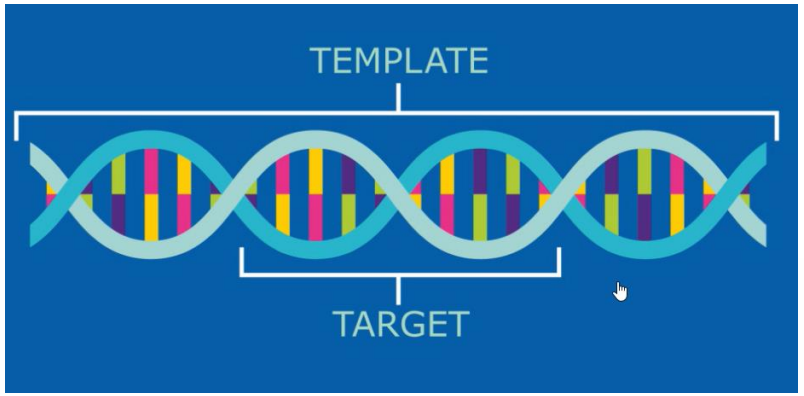
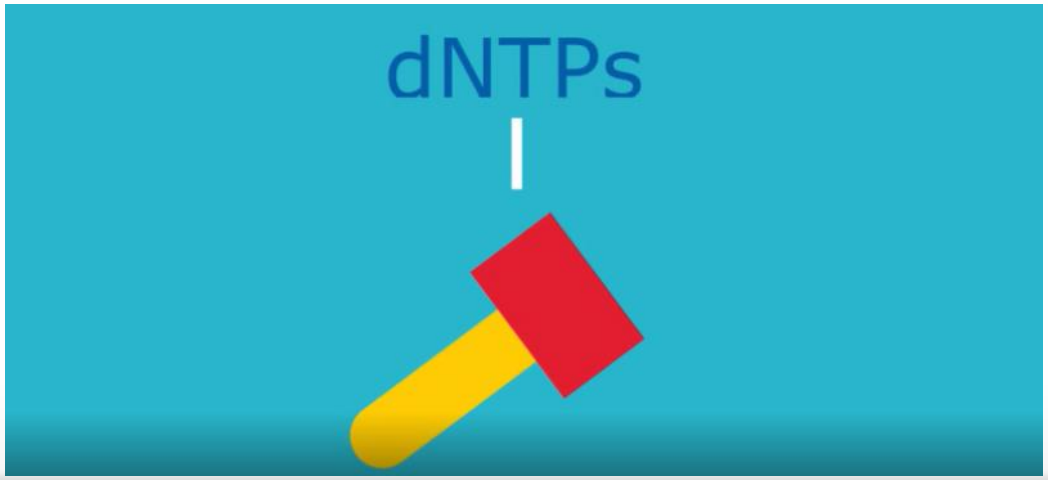
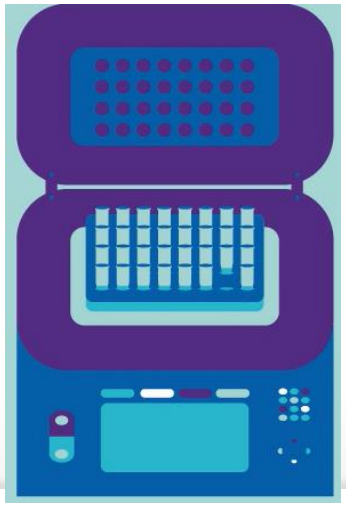
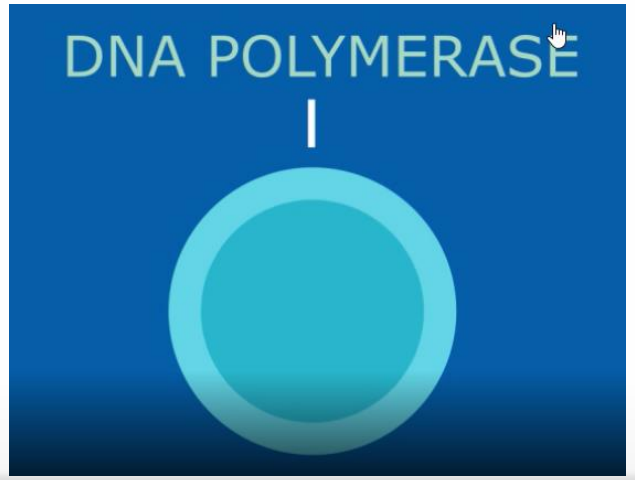
NEONATAL GENETIC SCREENING IN BELGIUM

qPCR with Taqman probes









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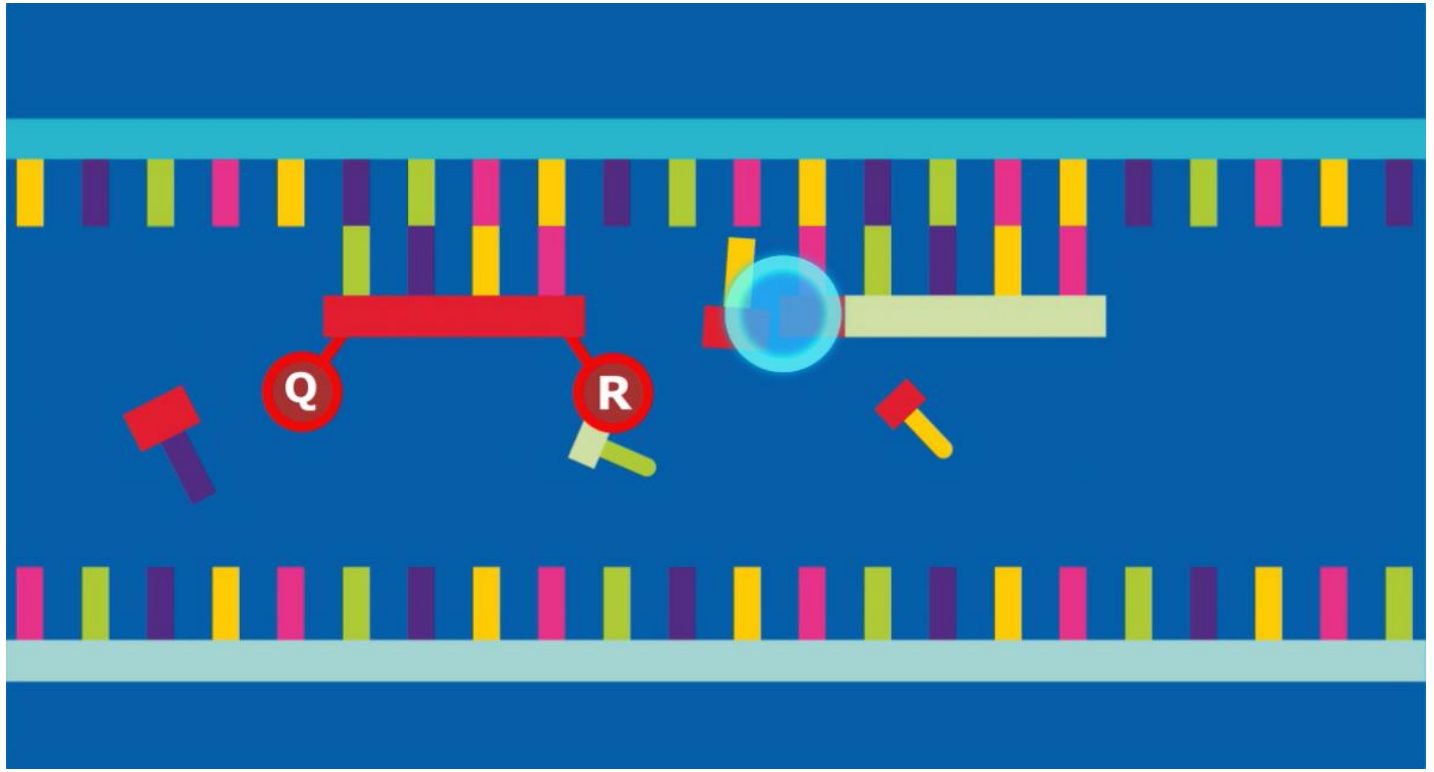
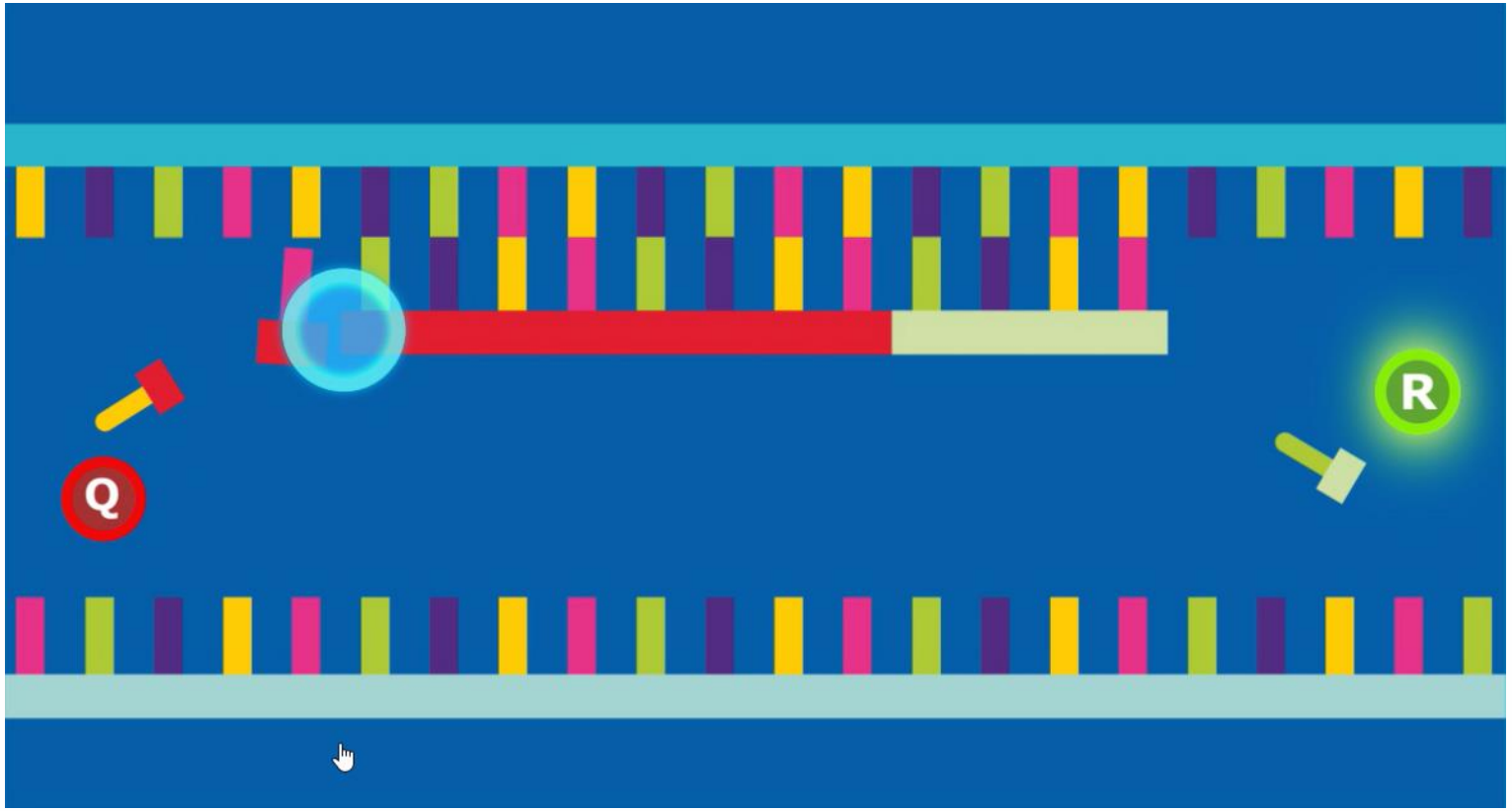
NEONATAL GENETIC SCREENING IN BELGIUM

PCR amplification at the E7/E8 region:



NEONATAL GENETIC SCREENING IN BELGIUM

PCR amplification at the E7/E8 region:



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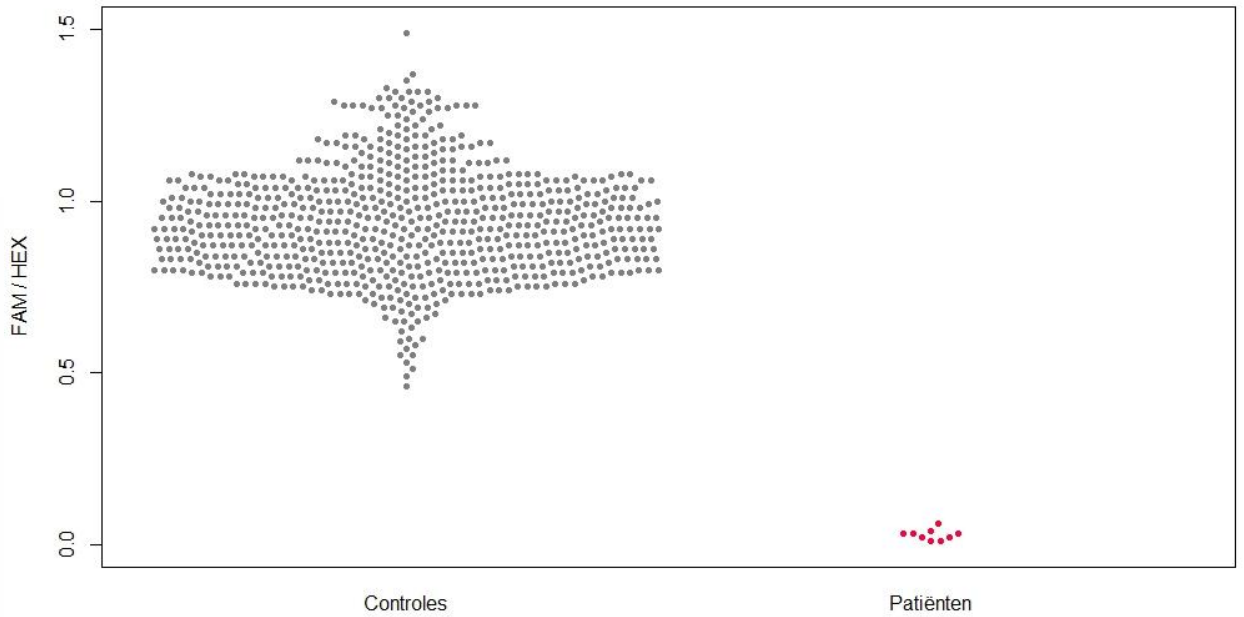
Final result:



THANK YOU

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