## SUPPLEMENTARY MATERIAL

# A blinded international study on the reliability of genetic testing for GGGGCC-repeat expansions in *C9orf72* reveals marked differences in results among 14 laboratories

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## Supplementary table 1. The results of 78 samples.

### **RP-PCR** results

All laboratories same results	50 samples (64.1%)	
1 laboratory different result	20 samples (25.6%)	13 samples (16.7%): 1 laboratory Q result 5 samples (6.41%):1 laboratory FP result 2 samples (2.56%): 1 laboratory FN result
2 laboratories different results	5 samples (6.41%)	4 samples (5.12%): 2 laboratories Q results 1 sample (1.28%): 1 laboratory FN and 1 laboratory Q results
6 laboratories different results	2 samples (2.56%)	1 sample (1.28%): 1 laboratory FN and 5 laboratories Q results 1 sample (1.28%): 6 laboratories Q results
7 laboratories different results	1 sample (1.28%)	1 sample (1.28%): 1 laboratory FN and 6 laboratories Q results

### Amplicon-length analysis results

All laboratories same results	72 samples (92.3%)	
1 laboratory	2 samples	1 sample (1.28%): 1 laboratory different peak number
different result	(2.56%)	1 sample (1.28%): 1 laboratory Q result
2 laboratories	1 sample	1 sample (1.28%): 1 laboratory different peak number and
different results	(1.28%)	1 laboratory Q result
3 laboratories	1 sample	1 sample (1.28%): 1 laboratory different peak number and
different results	(1.28%)	2 laboratories Q results
5 laboratories	2 samples	2 samples (2.56%): 4 laboratories different peak number and
different results	(2.56%)	1 laboratory Q result

## **RP-PCR**+ amplicon-length analysis results

All laboratories same results	53 samples (67.9%)	
1 laboratory different result	19 samples (24.4%)	16 samples (20.5%): 1 laboratory Q result 2 samples (2.56%): 1 laboratory FP result 1 sample (1.28%): 1 laboratory FN result
2 laboratories different results	3 samples (3.85%)	2 samples (2.56%): 2 laboratories Q results 1 sample (1.28%): 1 laboratory FP and 1 laboratory Q results
5 laboratories Different results	2 samples (2.56%)	1 sample (1.28%): 1 laboratory FN and 4 laboratories Q results 1 sample (1.28%): 5 laboratories Q results
6 laboratories Different results	1 sample (1.28%)	1 sample (1.28%): 1 laboratory FN and 5 laboratories Q results

FP: False positive, FN: False negative, Q: Undecided or unclassified sample.

	Sample	Allele 1	Allele 2	Sample	Allele 1	Allele 2
	1.1	7	Expanded	16.1	2	Expanded
	1.2	6	Expanded	16.3	2	Expanded
	2.1	2	Expanded	17.1	2	6
_	2.2	2	Expanded	17.2	2	5
	3.1	5	12	18.1	5	Expanded
	3.2	2	Expanded	18.2	2	Expanded
	4.1	4	Expanded	19.1	2	2
	4.2	4	Expanded	19.2	2	5
	4.3	8	Expanded	20.1	5	13
	4.4	4	Expanded	20.2	5	6
	5.1	2	2	21.1	5	Expanded
	5.2	2	2	21.2	5	Expanded
	6.1	5	5	22.1	11	Expanded
	6.2	2	Expanded	22.2	2	Expanded
	6.3	2	Expanded	23.1	8	Expanded
	6.4	2	Expanded	23.2	8	Expanded
	6.5	23-24	Expanded	24.1	2	Expanded
	6.6	8	Expanded	24.2	2	Expanded
	6.7	8	Expanded	25.1	2	2
	6.8	2	2	25.2	2	8
	7.1	2	6	26.1	2	Expanded
	7.2	2	5	26.2	2	Expanded
	8.1	2	6*	26.3	23-25	Expanded
	8.2	2	6*	27.1	2	Expanded
	9.1	2	10	27.2	8	Expanded
	9.2	2	8	27.3	5	Expanded
	10.1	5	Expanded	27.4	5	Expanded
	10.2	2	Expanded	28.1	2	7
	11.1	2	Expanded	28.2	5	Expanded
	11.2	2	Expanded	29.1	2	5
	12.1	2	5	29.2	2	6
	12.2	2	8	30.1	2	6
	12.3	2	5	30.2	2	5
	13.1	2	Expanded	31.1	7	Expanded
	13.2	2	Expanded	31.2	2	2
	14.1	2	2	31.3	2	Expanded
	14.2	2	8	32.1	6*	Expanded
	15.1	2	6	32.2	2	Expanded
	15.2	6	10	32.3	28-32	Expanded

## Supplementary table 2. The GGGGCC-repeat numbers on 78 samples from 32 families.

\*: mutant allele.

Laboratory	Α	В	С	D	Ε	F	G	Н	Ι	J	K	L	М	Ν
RP-PCR method														
Primers*	P1, 2, 3	P7, 8, 9	P1, 2, 3	P4, 13	P4, 5, 6	P10, 11, 12	P4, 5, 6	P1, 2, 3	P4, 5, 6	P4, 5, 6	No	P1, 2, 3	P4, 5, 6	P1, 2, 3
PCR reaction* Primer 1 dose (µM) Primer 2 dose (µM) Primer 3 dose (µM) Deaza-GTP (mM) DNA dose (ng/µl)	PR1 1.4 0.7 1.4 0.18 7.1	PR2 0.2 0.2 0.2 0.2 0.2 5.0	PR1 1.4 0.7 1.4 0.18 2.14	PR3 0.2 0.2 - 0.2 2.0	PR4 0.4 0.2 0.4 0.0375 20	PR5 0.33 0.33 0.33 0 10.0	PR6 1.0 1.0 0.25 12.5	PR1 1.4 0.7 1.4 0.18 7.1	PR7 1.4 0.7 1.4 0.18 12.0	PR8 2.5 2.5 0.25 0 3.3	No No No No No	PR1 1.4 0.7 1.4 0.18 7.1	PR6 1.0 1.0 0.25 5.0	PR9 1.4 0.7 1.4 0.16 3.3
PCR protocol* Extension time (min) Total cycle	PP1 3 40	PP2 8-18 45	PP3 3 40	PP4 3 32	PP5 40 sec 65	PP6 2-10 35	PP7 8-16 35	PP8 3 43	PP7 8-16 35	PP9 8-16 34	No No No	PP1 3 40	PP7 8-16 35	PP1 3 40
Analysis machine* PCR product conc. Analysis software*	M1 10% A1	M2 10% A2	M2 25% A1	M1 20% A1	M1 0.47% A1	M1 16.2% A5	M1 3.3% A2	M1 0.94% A2	M3 8.8% A2	M1 10% A2	No No No	M1 21% A1	M1 4.8% A1	M2 13.3% A3
Sensitivity Specificity Unclassified	100% 100% 0%	100% 100% 0%	100% 100% 0%	100% 100% 0%	91.3% 96.8% 5.1%	100% 87.5% 2.6%	97.8% 100% 0%	93.5% 100% 3.8%	93.5% 100% 3.8%	93.5% 93.8% 3.8%	71.7% 96.9% 14.1%	95.7% 100% 2.6%	89.1% 100% 5.1%	93.5% 87.5% 9.0%
Amplicon length analysis	s method													
Primers*	P14, 16	P14, 16	P14, 16	P15, 16	P14, 16	P17, 18	P14, 16	P14, 16	P15, 16	P14, 16	No	N.D.	N.D.	N.D.
PCR reaction* DNA dose (ng/µl)	PR10 2.2	PR11 5.0	PR12 2.4	PR13 4.2	PR10 2.0	PR14 3.3	PR15 3.35	PR16 7.1	PR13 2.0	PR17 3.3	No No	N.D.	N.D.	N.D.
PCR protocol* Extension time (min) Total cycle	PP10 1.5 34	PP11 1 38	PP12 2 30	PP13 2 35	PP14 1.5 35	PP15 1 33	PP16 1 36	PP17 3 40	PP18 2 35	PP19 8-16 34	No No No	N.D.	N.D.	N.D.
Analysis machine* PCR product conc. Analysis software*	M1 10% A1	M2 0.5% A2	M2 25% A1	4% gel	M1 0.47% A1	M1 16.2% A5	M1 0.17% A2	M1 0.47% A2	3% gel	M1 10% A2	No No No	N.D.	N.D.	N.D.

Supplementary table 3A. Total methods of 14 laboratories.

\*: Listed in supplementary table 3B and 3C, N.D.: not done. No: no method disclosed.

Supplementary table 3B. List of products and equipment.

Primers	s for RP-PCR
P1	FAM-AGTCGCTAGAGGCGAAAGC
P2	tacgcatcccagtttgagacgGGGGCCGGGGCCGGGGGCCGGGG
P3	tacgcatcccagtttgagacg
P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
P5	caggaaacagctatgaccGGGCCCGGCCCGACCACGCCCCGGCCCCGGCCCCGG
P6	caggaaacagctatgacc
P7	FAM-CAAGGAGGGAAACAACCGCAGCC
P8	ggataacaatttcacacaggGGGCCCGCCCGACCACGCCCCGGCCCCGGCCCCGG
Р9	ggataacaatttcacacagg
P10	FAM-AGTACTCGCTGAGGGTGAAC
P11	cgtacgcatcccagtttgagaGCCCCGGCCCCGGCCCCGG
P12	cgtacgcatcccagtttgaga
P13	cacgacgttgtaaaacgaCCCCGGCCCCGGCCCCGG

Primers	s for amplicon-length analysis
P14	FAM-CAAGGAGGGAAACAACCGCAGCC
P15	CAAGGAGGGAAACAACCGCAGCC
P16	GCAGGCACCGCAACCGCAG
P17	FAM-CAGGTGTGGGTTTAGGAGGT
P18	CCAGCTTCGGTCAGAGAAAT
Size sta	ndard
<b>S</b> 1	GeneScan 500XL ROX and 500ROX Size Standard (Applied Biosystems, USA)
S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
<b>S</b> 3	GeneScan 400HD Rox Size Standard (Applied Biosystems, USA)
<b>S</b> 4	GeneScan 600LIZ Size Standard (Applied Biosystems, USA)
Analysi	s machine
M1	3730 and 3730xl DNA Analyzer (Applied Biosystems, USA)
M2	3130 and 3130xl Genetic Analyzer (Applied Biosystems, USA)
M3	3500 Genetic Analyzer (Applied Biosystems, USA)
Analysi	s software

A1	Peak Scanner Software v1.0 or v2.0 (Applied Biosystems, USA)
A2	GeneMapper Software v4.0 or v4.1 (Applied Biosystems, USA)
A3	Geotyper v4 (Applied Biosystems, USA)
A4	Agarose gel
A5	In-house developed Tracl genotyping software

PCR reaction number	PR1	PR2	PR3	PR4	PR5	PR6	PR7	PR8	PR9
Laboratory	ACHL	В	D	Е	F	GM	Ι	J	N
Qiagen buffer *1	-	-	-	1x	-	1x	-	-	-
FastStart PCR Master *2	1x	-	-	-	-	-	-	-	-
Taq DNA polymerase (units)	-	-	1.8	0.75	-	2.5	1.0	-	-
Other buffer and polymerase	-	*4	*5	-	*6	-	*7	*8	*9
Primer 1 (µM)	1.4	0.2	0.2	0.4	0.33	1.0	1.4	2.5	1.4
Primer 2 (GGGGCC repeat) ( $\mu$ M)	0.7	0.2	0.2	0.2	0.33	1.0	0.7	2.5	0.7
Primer 3 (µM)	1.4	0.2	-	0.4	0.03	1.0	1.4	0.25	1.4
dCTP, dATP, dTTP (each) (mM)	-	0.2	0.2	0.05	-	0.25	0.18	-	-
7-deaza-2-deoxy GTP (mM)	0.18	0.2	0.2	0.0375	-	0.25	0.18	-	0.16
dGTP (mM)	-	-	-	0.0125	-	-	-	-	-
dNTPs (each) (mM)	-	-	-	-	0.5	-	-	-	-
DMSO	7%	5%	5%	-	-	5%	-	-	7%
Betaine (M)	-	-	1.0	-	2.0	1.0	-	1.75	0.83
Q solution *3	1x	-	-	1x	-	-	-	-	-
MgCl2 (mM)	0.89	2.0	-	-	-	-	-	-	0.83

Supplementary table 3C-1. Concentration of ingredients in RP-PCR.

\*1: *Taq* DNA polymerase (QIAGEN, Germany)

\*2: FastStart PCR Master mix (Roche, Switzerland)

\*3: Q solution (QIAGEN, Germany)

\*4: Taq Robust (Kapa Biosystems, USA), Kapa Enhancer 1x.

\*5: RG25 buffer (160mM NH4SO4, 670mM Tris-HCl (pH 8.8), 1% Tween, 25mM MgCl2)

\*6: Expand Long Template PCR system (Roche, Switzerland), Buffer 2 1x, Titanium Taq polymerase 2x.

\*7: AccuPrime GC-rich DNA polymerase (Invitrogen, USA), Buffer A 1x.

\*8: Extensor Mastermix (Thermo Scientific, USA)

\*9: True Allele PCR Premix (Applied Biosystems, USA), PCR master mix 1x.

PCR reaction number	PR10	PR11	PR12	PR13	PR14	PR15	PR16	PR17
Used laboratory	AE	В	С	DI	F	G	Н	J
Primer 4 (µM)	0.4	0.2	0.4	0.2	0.3	1.0	0.5	0.1
Primer 5 (µM)	0.4	0.2	0.4	0.2	0.3	1.0	0.5	0.1
dCTP, dATP, dTTP (each) (mM)	-	0.2	-	-	-	0.25	-	-
7-deaza-2-deoxy GTP (mM)	-	0.2	0.2	-	-	0.25	0.18	-
dNTP (each) (mM)	-	-	0.25	-	0.3	-	-	-
DMSO (%)	-	5.0	4.0	-	-	5.0	7.1	1.0
Betaine (M)	-	-	1.0	-	1.0	1.0	-	-
Q solution *1	-	-	-	-	-	-	1x	-
MgCl2 (mM)	-	2.0	-	-	-	-	0.89	-
Kit buffer	1x							
Kit polymerase (U)	-	0.2	1.25	0.4	0.3	2.5	-	-
Kit and polymerase	*2	*3	*4	*5	*6	*7	*8	*9

Supplementary table 3C-2. Concentration of ingredients in amplicon-length analysis PCR.

\*1: Q solution (QIAGEN, Germany)

\*2: One Taq 2X Master Mix with GC Buffer (New England BioLabs, UK)

\*3: KAPA Enhncer and Taq Robust (Kapa Biosystems, USA)

\*4: AmpliTaq and GeneAmp PCR buffer (Roche, Switzerland)

\*5: AccuPrime GC-Rich DNA Polymerase (Invitrogen, USA)

\*6: Kapa HiFi Hotstart PCR kit, GC buffer (Kapa Biosystems, USA)

\*7: *Taq* DNA Polymerase (QIAGEN, Germany)

\*8: FastStart PCR Master mix (Roche, Switzerland)

\*9: Megamix Blue (Microzone, UK)

PP1, Laboratory A, L, N			PP2, La	poratory B		PP3, L	aboratory (	2
Temp	Time	Cycle	Temp	Time	Cycle	Temp	Time	Cycle
95°C	15 min	1	98°C	10 min	1	95°C	8 min	1
94°C 70°C 72°C	1 min 1 min 3 min	2	98°C 65-58°C 72°C	35 sec 2 min 8 min	13 Every cycl -1°C	95°C 1e 70-56° 72°C	1 min C 1 min 3 min	8 Every cycle -2°C
94°C 68°C 72°C	1 min 1 min 3 min	3	97°C 58°C	35 sec 2 min	32 Every cycl	95°C 56°C le 72°C	1 min 1 min 3 min	32
94°C 66°C	1 min 1 min	4	72°C	8-18 min	+20 sec	72°C	10 mii	n 1
72°C	3 min		-12 C	hold	1	4°C	hold	1
94°C 64°C 72°C	1 min 1 min 3 min	5	40	lioid	1			
94°C 62°C 72°C	1 min 1 min 3 min	6	PP4, La	boratory D		PP5, Labor	ratory E	
94°C	1 min		Temp	Time	Cycle	Temp	Time	Cycle
60°С 72°С	1 min 3 min	7	95°C	5 min	1	95°C	5 min	1
94°C 58°C 72°C	1 min 1 min 3 min	8	95°C 68°C	30 sec 3 min 7 min	32	95°C 70-53.2°C 68°C	30 sec 30 sec 40 sec	49 Every cycle -0.35°C
94°C 56°C 72°C	1 min 1 min 3 min	5	12°C	hold	1	95°C 58°C 68°C	30 sec 30 sec 40 sec	16
72°C	10 min	1				68°C	10 min	1
4°C	hold	1				12°C	hold	1

## Supplementary table 3C-3. PCR protocols for RP-PCR (PP1-9).

PP6, Laboratory F

PP7, Laboratory G, I, M PP8, Laboratory H

	2		· · · ·	5	, ,		,	
Temp	Time	Cycle	Temp	Time	Cycle	Temp	Time	Cycle
98°C	10 min	1	98°C	10 min	1	95°C	5 min	1
97°C	35 sec		97°C	35 sec		95°C	30 sec	8
53°C	2 min	10	64°C	2 min	10	70 <b>-</b> 56°C	30 sec	Every cycle
68°C	2 min		68°C	8 min		72°C	3 min	-2°C
97°C	35 sec	25	97°C	35 sec	25	95°C	30 sec	
53°C	2 min	Every cycle	64°C	2 min	Every cycle	56°C	30 sec	35
68°C	2-10 min	+20 sec	68°C	8-16 min	+20 sec	72°C	3 min	
68°C	10 min	1	16°C	hold	1	72°C	20 min	1
4°C	hold	1				4°C	hold	1

## PCR protocols for RP-PCR (continued).

PP9, Laboratory J

Temp	Time	Cycle
95°C	5 min	1
95°C	30 sec	8
70-56°C	30 sec	Every cycle
72°C	8 min	-2°C
95°C	30 sec	26
56°C	30 sec	Every cycle
72°C	8-16 min	+20 sec
72°C	7 min	1
4°C	hold	1

## Supplementary table 3C-4. PCR protocols for amplicon-length analysis (PP10-19).

PP11, Laboratory B

PP12, Laboratory C

	-				•	,	-	
Temp	Time	Cycle	Temp	Time	Cycle	Temp	Time	Cycle
98°C	5 min	1	97°C	5 min	1	95°C	5 min	1
97°C	30 sec	10	97°C	30 sec		95°C	1 min	
65-56°C	30 sec	Every cycle	60°C	30 sec	10	56°C	1 min	30
68°C	1.5 min	-1°C	72°C	1 min		72°C	2 min	
97°C	30 sec		97°C	10 sec		72°C	10 min	1
55°C	30 sec	24	60°C	15 sec	28		10 11111	-
68°C	1.5 min		72°C	30 sec		4°C	hold	1
68°C	10 min	1	72°C	10 min	1			
12°C	hold	1	4°C	hold	1			

PP13,	Laborator	ry D	I
Tem	Time	Cycle	1
р			9
95°C	3 min	1	-
95°C	30 sec		e
68°C	30 sec	35	6
72°C	2 min		

PCR protocols for Amplicon length analysis (continued).

PP14, Laboratory E

Temp	Time	Cycle
98°C	5 min	1
97°C	30 sec	11
65-55°C	30 sec	Every
68°C	1.5 min	cycle -1°C
97°C	30 sec	
55°C	30 sec	24
68°C	1.5 min	
68°C	10 min	1
12°C	hold	1

## PP15, Laboratory F

Temp	Time	Cycle
95°C	5 min	1
98°C 59°C 72°C	20 sec 15 sec 1 min	33
72°C	5 min	1
4°C	hold	1

## PP16, Laboratory G

 $10 \min$ 

hold

1

1

72°C

12°C

Temp	Time	Cycle
94°C	4 min	1
94°C 62°C 72°C	30 sec 30 sec 1 min	36
72°C	10 min	1

PP17, Laboratory H	I
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Temp	Time	Cycle
95°C	4 min	1
95°C	30 sec	14
70-57°C	30 sec	Every cycle
72°C	3 min	-1°C
95°C	30 sec	
56°C	30 sec	26
72°C	3 min	
72°C	10 min	1
4°C	hold	1

## PP18, Laboratory I

Temp	Time	Cycle
95°C	3 min	1
95°C 63°C 72°C	30 sec 30 sec 2 min	35
72°C	10 min	1
16°C	hold	1

## PP19, Laboratory J

Temp	Time	Cycle
95°C	5 min	1
95°C	30 sec	8
70-56°C	30 sec	Every cycle
72°C	8 min	-2°C
95°C	30 sec	26
56°C	30 sec	Every cycle
72°C	8-16 min	+20 sec
72°C	7 min	1
4°C	hold	1

## Laboratory A

## **RP-PCR**

#### List

	LISt	
Primer 1	P1	FAM-AGTCGCTAGAGGCGAAAGC
Primer 2 (with GGGGCC repeat)	P2	tacgcatcccagtttgagacgGGGGCCGGGGCCGGGGCCGGGG
Primer 3	P3	tacgcatcccagtttgagacg
Size standard	<b>S</b> 2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730x/DNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

#### PCR reaction (PR1)

#### PCR protocol (PP1)

## Capillary electrophoresis

Products	Final conc.
FastStart PCR Master Primer 1 Primer 2 Primer 3 7-deaza-2-deoxy GTP DMSO Q solution MgCl2	1x 1.4 μM 0.7 μM 1.4 μM 0.18 mM 7 % 1x 0.89 mM
Total	7.1 ng/μ1 14.0 μl

Temp Time Cycle 95°C  $15\,\mathrm{min}$ 1 94°C 1 min 70-58°C  $1 \min$ 2-8\* 72°C  $3 \min$ 94°C 1 min 56°C 5 1 min 72°C 3 min 72°C  $10 \min$ 1 4℃ hold 1

PCR product	1.0 μl
Size standard	0.5 μl
Formamide	8.5 μl
Total	10.0 µl

95°C 5min and on ice. Analyse with DNA analyzer.

FastStart PCR Master (Roche, Switzerland) Q solusion (QIAGEN, Germany).

\* Every 2°C decrease add 1 cycle.

## Amplicon length analysis

	List	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730xl DNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

### PCR reaction (PR10)

Products	Final conc.
Primer 4 Primer 5 Kit buffer DNA	0.4 μM 0.4 μM 1x 2.2 ng/μl
Total	15 µl

Kit buffer: One *Taq* 2x Master Mix with GC Buffer (New England BioLabs, UK).

### PCR protocol (PP10)

Temp	Time	Cycle
98°C	5 min	1
97°C	30 sec	10
65-56°C	30 sec	Every
68°C	1.5 min	cycle -1°C
97°C	30 sec	
55°C	30 sec	24
68°C	1.5 min	
68°C	10 min	1
12°C	hold	1

#### **Capillary electrophoresis**

PCR product	1.0 μl
Size standard	0.5 μl
Formamide	8.5 μl
Total	10 µl

95°C 5min and on ice. Analyse with DNA analyzer.

## Laboratory B

RP-PCR		List					
Primer 1		P7	FAM-CAAGGAGGGAAACAACCGCAGCC				
Primer 2 (with GGGGCC repeat)		P8	ggataacaatttcacacaggGGGCCCGCCCCGACCACGCCCCGGC CCCGGCCCCGG				
Primer 3		P9	ggataacaat	ttcacacagg			
Size standard		S2	GeneScan	500 LIZ Si	ze Standard (Ap	plied Biosystems	s, USA)
Analysis machine		M2	3130xl Genetic Analyzer (Applied Biosystems, USA)				
Analysis software		A2	GeneMapper Software v4.0 (Applied Biosystems, USA)				
PCR reaction (PR2)			PCR pr	otocol (PP2	2)	Capillary elec	trophoresis
Products	Final	conc.	Temp	Time	Cycle	Dilute PCR pro	oduct
KAPA Enhancer	1x		98°C	10 min	1	PCR product:H	20=1:1
Primer 1 Primer 2 Primer 3 dCTP, dATP, dTTP (each)	0.2 μ 0.2 μ 0.2 μ	M M M	98℃ 65-58℃ 72℃	35 sec 2 min 8 min	13 Every cycle -1°C	PCR product Size standard Formamide	2.0 μl 0.1 μl 7.9 μl
7-deaza-2-deoxy GTP	0.2 m	ıΜ	97°C	35 sec	32	Total	10.0 µl
DMSO	5%		58°C	2 min	Every cycle	95°C 3min and	analyse

Total20 μl4°Chold1KAPA Enhancer and Taq Robust (KapaRamping up and down: 0.5°C/sec

72°C

72°C

 $2.0\,\mathrm{mM}$ 

 $0.2\,\mathrm{U}$ 

5 ng/µl

Biosystems, USA).

MgCl2

DNA

Taq Robust

### Amplicon length analysis

	List	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M2	3130xl Genetic Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper Software v4.0 (Applied Biosystems, USA)

## PCR reaction (PR11)

PCR protocol (PP11)

8-18 min

 $10\,\mathrm{min}$ 

+20 sec

1

## Capillary electrophoresis

with genetic analyzer.

Products	Final conc.	Temp	Time	Cycle	Dilute PCR product	-
KAPA Enhancer	1x	97°C	5 min	1	PCR product:H20=1:39	9
Primer 4 Primer 5 dCTP, dATP, dTTP (each)	0.2 μM 0.2 μM 0.2 mM	97°C 60°C 72°C	30 sec 30 sec 1 min	10	Diluted PCR product Size standard Formamide	2.0 μl 0.1 μl 7.9 μl
DMSO	5%	97°C	10 sec		Total	10.0 µl
MgCl2 Taq Robust DNA	2.0 mM 0.2 U 5 ng/μl	60°C 72°C	15 sec 30 sec	28	95°C 3min and analyse genetic analyzer.	with
Total	20 µ1	72°C	10 min	1		

KAPA Enhancer and *Taq* Robust (Kapa Biosystems, USA)

## Laboratory C

RP-PCR	List	
Primer 1	P1	FAM-AGTCGCTAGAGGCGAAAGC
Primer 2 (with GGGGCC repeat)	P2	tacgcatcccagtttgagacgGGGGCCGGGGGCCGGGGCCGGGG
Primer 3	P3	tacgcatcccagtttgagacg
Size standard	S2	GeneScan 500 LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M2	3130xl genetic analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

### PCR reaction (PR1)

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### PCR protocol (PP3)

## Capillary electrophoresis

Products	Final conc.	Temp	Time	Cycle
FastStart PCR Master	1x	95°C	8 min	1
Primer 1 Primer 2 Primer 3 7-deaza-2-deoxy GTP	1.4 μM 0.7 μM 1.4 μM 0.18 mM	95°C 70-56°C 72°C	1 min 1 min 3 min	8 Every cycle -2°C
DMSO Q solution MgCl2	7 % 1x 0.89 mM	95°C 56°C 72°C	1 min 1 min 3 min	32
DNA T-t-1	2.14 ng/µ1	72°C	10 min	1
I Olal	28 μi	4°C	hold	1

PCR product	2 <b>5</b> µ1
Size standard	2.5 μ1 0.25 μ1
Formamide	7.25 μl
Total	10.0 u1

95°C 2min and 4°C. Analyse with genetic analyzer.

FastStart PCR Master (Roche, Switzerland)

Q solusion (QIAGEN, Germany)

## **Amplicon length analysis**

<b>DOD</b> (1 (D)		
Analysis software	Al	Peak Scanner Software v1.0 (Applied Biosystems, USA)
Analysis machine	M2	3130xl genetic analyzer (Applied Biosystems, USA)
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Primer 5	P16	GCAGGCACCGCAACCGCAG
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
	List	

## PCR reaction (PR12)

PCR protocol (PP12)

### Capillary electrophoresis

Products	Final conc.	Temp	Time	Cycle	PCR product	2.5 µl
Primer 4	0.4 μM	95°C	5 min	1	Size standard Formamide	0.25 μl 7.25 μl
7-deaza-2-deoxy GTP	0.4 μM 0.2 mM	95°C 56°C	1 min 1 min	30	Total	10.0 µl
dNTPs (each) DMSO	0.25 mM 4 %	72°C	$2 \min$		95°C 2min and 4°C.	
Betaine	1.0 M	72°C	$10\mathrm{min}$	1	Analyse with ge analyzer.	enetic
AmpliTaq DNA polymerase DNA	1x 1.25 U 2.4 ng/μl	4°C	hold	1		
Total	25.0 μl					

AmpliTaq and GeneAmp PCR buffer

(Roche, Switzerland)

## Laboratory D

RP-PCR	List	
Primer 1	P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
Primer 2 (with GGGGCC repeat)	P13	cacgacgttgtaaaacgaCCCCGGCCCCGGCCCCGG
Primer 3	-	none
Size standard	S2	GeneScan 500LIZ Size Standard (Life Technologies, USA)
Analysis machine	M1	3730xlDNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v2.0 (Applied Biosystems, USA)

### PCR reaction (PR3)

### PCR protocol (PP4)

### **Capillary electrophoresis**

Products	Final conc.
RG25 buffer	1x
Primer 1 Primer 2	0.2 μM 0.2 μM
dCTP, dATP, dTTP (each)	0.2 mM
7-deaza-2-deoxy GTP	0.2 mM
DMSO	5%
Tag DNA polymerase	1.8 U
DNA	$2.0 \text{ ng}/\mu l$
Total	20.0 µl

Time	Cycle
$5 \min$	1
30 sec	32
3 min	
7 min	1
hold	1
	Time 5 min 30 sec 3 min 7 min hold

PCR product	2.0 μl
Size standard	0.5 μl
Formamide	7.5 μl
Total	10.0 µl

95°C 5min and analyse with DNA analyzer.

RG25 buffer (160mM NH4SO4, 670mM Tris-HCl (pH 8.8), 1% Tween, 25mM MgCl2)

### Amplicon length analysis

	List	
Primer 4	P15	CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Analysis system	A4	4 % agarose gel

### PCR reaction (PR13)

Primer 4 $0.2 \mu M$ Primer 5 $0.2 \mu M$ AccuPrime buffer A $1x$ AccuPrime Taq $0.4  U$ DNA $4.2  ng/\mu l$ Total $30.0 \mu l$	Products	Final conc.
Total 30.0 μ1	Primer 4 Primer 5 AccuPrime buffer A AccuPrime Taq DNA	0.2 μM 0.2 μM 1x 0.4 U 4.2 ng/μl
	Total	30.0 µ1

AccuPrime GC-Rich DNA Polymerase (Invitrogen, USA)

### PCR protocol (PP13)

Temp	Time	Cycle
95°C	3 min	1
95°C 68°C 72°C	30 sec 30 sec 2 min	35
72°C	10 min	1
12°C	hold	1

## Laboratory E

RP-PCR	List	
Primer 1	P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
Primer 2 (with GGGGCC repeat)	Р5	caggaaacagctatgaccGGGCCCGCCCGACCACGCCCCGGCCCCG GCCCCGG
Primer 3	P6	caggaaacagctatgacc
Size standard	S1	GeneScan 500ROX Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

### PCR reaction (PR4)

PCR protocol (PP5)

### **Capillary electrophoresis**

Products	Final conc.	Temp	Time	Cycle	Dilute PCR product	1.0.0
Qiagen buffer	1x	95°C	5 min	1	PCR product:H20=1.5:	100
Primer 1 Primer 2 Primer 3	0.4 μM 0.2 μM 0.4 μM	95°C 70-53.2°C	30 sec 30 sec	49 Every cycle	Diluted PCR product Size standard H2O	5.0 μl 0.07 μl 10.0 μl
ATP, CTP, TTP (each)	0.05 mM 0.0125 mM	08.0	40 sec	-0.33*C	Total	15.7 µl
7-deaza-2-deoxy GTP Q solution	0.0375 mM 1x	95°C 58°C 68°C	30 sec 30 sec 40 sec	16	95°C 5min and analyse DNA analyzer.	with
DNA DNA polymerase	0.75 0 20 ng/μl	68°C	10 min	1		
Total	25.0 µl	12°C	hold	1		

Taq DNA polymerase (QIAGEN, Germany)

## Amplicon length analysis

	List	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S1	GeneScan 500ROX Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

### PCR reaction (PR10)

Products	Final conc.
Primer 4 Primer 5 Kit buffer DNA	0.4 μM 0.4 μM 1x 2.0 ng/μl
Total	25.0 µl

Kit buffer: One Taq 2x Master Mix with GC Buffer (New England BioLabs, UK)

## PCR protocol (PP14)

-	,	-
Temp	Time	Cycle
98°C	5 min	1
97°C	30 sec	11
65-55°C	30 sec	Every cycle
68°C	1.5 min	-1°C
97°C	30 sec	
55°C	30 sec	24
68°C	1.5 min	
68°C	10 min	1
12°C	hold	1

#### **Capillary electrophoresis**

Dilute PCR product PCR product:H20=1.5:100

Diluted PCR product	5.0 μl
Size standard	0.07 μl
H2O	10.0 μl
Total	15.7 μl

95°C 5min and analyse with DNA analyzer.

## Laboratory F

### **RP-PCR**

	List	
Primer 1	P10	FAM-AGTACTCGCTGAGGGTGAAC
Primer 2 (with GGGGCC repeat)	P11	cgtacgcatcccagtttgagaGCCCCGGCCCCGGCCCCGG
Primer 3	P12	cgtacgcatcccagtttgaga
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A5	In-house developed Tracl genotyping software
Primer 1 Primer 2 (with GGGGCC repeat) Primer 3 Size standard Analysis machine Analysis software	P10       P11       P12       S2       M1       A5	FAM-AGTACTCGCTGAGGGTGAAC         cgtacgcatcccagtttgagaGCCCCGGCCCCGGCCCCGG         cgtacgcatcccagtttgaga         GeneScan 500LIZ Size Standard (Applied Biosystems, USA)         3730 DNA Analyzer (Applied Biosystems, USA)         In-house developed Tracl genotyping software

#### PCR reaction (PR5)

### PCR protocol (PP6)

#### Capillary electrophoresis

Products	Final conc.	Temp	Time	Cycle
Buffer 2 (kit)	1x	98°C	10 min	1
Primer 1 Primer 2 Primer 3 dNTP (each)	0.33 μM 0.33 μM 0.03 μM 0.5 mM	97°C 53°C 68°C	35 sec 2 min 2 min	10
Betaine Polymeras (TiTaq 50x) DNA	2.0 M 2x 10 ng/µl	97°C 53°C 68°C	35 sec 2 min 2-10 min	25 Every cycle +20 sec
Total	20.0 µl	68°C	10 min	1
Expand Long Template PCR system		4°C	hold	1

PCR product	2.0 μl
Size standard	0.35 μl
Formamide	10.0 μl
Total	12.35µl

95°C 1.5min and analyse with DNA analyzer.

(Roche, Switzerland)

### Amplicon length analysis

	List	
Primer 4	P17	FAM-CAGGTGTGGGTTTAGGAGGT
Primer 5	P18	CCAGCTTCGGTCAGAGAAAT
Size standard	S4	GeneScan 600LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A5	In-house developed Tracl genotyping software

### PCR reaction (PR14)

Product	Final conc.
Primer 4 Primer 5 dNTPs (each) Betaine Kapa HiFi GC buffer Polymerase (kit)	0.3 μM 0.3 μM 0.3 mM 1.0 M 1x 0.3U
DNA	3.3 ng/µ1
Total	15.0 µ1
10141	10.0 µ1

#### PCR protocol (PP15)

Temp	Time	Cycle
95℃	5 min	1
98°C 59°C 72°C	20 sec 15 sec 1 min	33
72°C	5 min	1
4°C	hold	1

### Capillary electrophoresis

PCR product Size standard Formamide	2.0 μl 0.35 μl 10.0 μl
Total	12.35µl
05001 5	. 1 1

95°C 1.5min and analyse with DNA analyzer.

Kapa HiFi Hotstart PCR kit (Kapa Biosystems, USA)

Ramping rate: 50%

**Capillary electrophoresis** 

## Laboratory G

1-1 CK	List	
Primer 1	P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
Primer 2 (with GGGGCC repeat)	Р5	caggaaacagctatgaccGGGCCCGCCCGACCACGCCCCGGCCCCG GCCCCGG
Primer 3	P6	caggaaacagctatgacc
Size standard	<b>S</b> 3	GeneScan 400HD Rox Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper v4.0 (Applied Biosystems, USA)
Primer 1 Primer 2 (with GGGGCC repeat) Primer 3 Size standard Analysis machine Analysis software	P4 P5 P6 S3 M1 A2	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC caggaaacagctatgaccGGGCCCGGCCCGACCACGCCCGGCCC GCCCCGG caggaaacagctatgacc GeneScan 400HD Rox Size Standard (Applied Biosystems, USA) 3730 DNA Analyzer (Applied Biosystems, USA) GeneMapper v4.0 (Applied Biosystems, USA)

#### PCR reaction (PR6)

#### PCR protocol (PP7)

#### Products Final conc. Temp Time Cycle Dilute PCR product PCR product:H20=1:5 Qiagen buffer 1x 98°C 10 min 1 $1.0\,\mu M$ Primer 1 Diluted PCR product 2.0 µl 97°C 35 sec Primer 2 1.0 µM Size standard 0.12 µl 10 64°C $2 \min$ 1.0 µM Primer 3 Formamide 7.88 µl 68°C 8 min dCTP, dATP, dTTP (each) 0.25 mM Total 10.0 µl 7-deaza-2-deoxy GTP 0.25 mM 97°C 35 sec 25 DMSO 5% 95°C 2min and analyse with 64°C 2 min Every cycle Betaine $1.0\,\mathrm{M}$ DNA analyzer. 68°C +20 sec 8-16 min Taq DNA polymerase 2.5 U DNA \*1 12.5 ng/µl Reduce ramping rate up and down to 0.5°C/sec. 20.0 µl Total

Taq DNA polymerase, buffer (QIAGEN, Germany)

\*1: Initial DNA concentration ~250 ng/µl.

### Amplicon length analysis

	List	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S3	GeneScan 400HD Rox Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper v4.0 (Applied Biosystems, USA)

### PCR reaction (PR15)

dCTP, dATP, dTTP (each)

7-deaza-2-deoxy GTP

Taq DNA polymerase

Products

Primer 4

Primer 5

DMSO

Betaine

DNA \*2

Total

Qiagen buffer

PCRp	PP16)	
Temp	Time	Cyc

4 min

30 sec

30 sec

1 min

10 min

Temp

94°C

94°C

62°C

72°C

72°C

#### Capillary electrophoresis

Dilute PCR product	
PCR product:H20=1:180	

Diluted PCR product	3.0 µl
Size standard	0.10 µl
HiDi Formamide	6.90 µl
Total	10.0 µl

95°C 2min and analyse with DNA analyzer.

Taq DNA polymerase, buffer (QIAGEN, Germany)

Final conc.

1.0 µM

1.0 µM

 $0.25\,\mathrm{mM}$ 

 $0.25\,\mathrm{mM}$ 

5%

1x

 $1.0\,\mathrm{M}$ 

2.5 U

20.0 µl

3.35 ng/µl

\*2: Initial DNA concentration ~67 ng/µl.

Cycle

1

36

1

2.0 µl

8.6 µl 10.6 µl

#### Supple table 3D. Methods of each laboratory

## Laboratory H

RP-PCR		List					
Primer 1		P1	FAM-AGTCGCTAGAGGCGAAAGC				
Primer 2 (with GGGGC	C repeat)	P2	tacgcatcccagtttgagacgGGGGCCGGGGCCGGGGCCGGGG				
Primer 3		P3	tacgcatcccagtttgagacg				
Size standard		S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)				
Analysis machine		M1	3730 DNA Analyzer (Applied Biosystems, USA)				
Analysis software		A2	GeneMapper v4.0 (Applied Biosystems, USA)				
PCR reaction (PR1)		РС	R protocol (PP8)		Capillary electrophoresis		
Products	Final conc.	Ten	emp Time Cvcle		<ul> <li>Dilute PCR product</li> </ul>		

Tioducts	i mai conc.	romp	THIC	Cycle	Diluter	_
FastStart PCR Master	1x	95°C	5 min	1	PCR product:H20=1:1	9
Primer 1 Primer 2	1.4 μM 0.7 μM	95°C 70-56°C	30 sec	8 Every cycle	Diluted PCR product Formamide mix	2.0 µ 8.6 µ
Primer 3 7-deaza-2-deoxy GTP	1.4 μM 0.18 mM	72°C	3 min	-2°C	Total	10.6
DMSO	7 %	95°C	30 sec		Formamide mix=8µl S	ize
MgCl2	1x 0.89	56°C 72°C	30 sec 3 min	35	standard + 850µlForm	amide
DNA	7.1 ng/µl	72°C	20 min	1	No denaturation.	alvzer
Total	28.1 µl	4°C	hold	1		ury 201.

FastStart PCR Master (Roche, Switzerland)

Q solusion (QIAGEN, Germany)

#### Amplicon length analysis Tiot

	LISU	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730 DNA Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper v4.0 (Applied Biosystems, USA)

### PCR reaction (PR16)

7-deaza-2-deoxy GTP

FastStart PCR master

Final conc.

 $0.5\,\mu M$ 

0.5 µM

7.1%

1x

1x

 $0.18\,\mathrm{mM}$ 

0.89 mM

7.1 ng/µl

28.0 µl

Products

Primer 4

Primer 5

DMSO

MgCl2

DNA

Total

Q solution

PCR protocol (PP17) Time

 $4 \min$ 

30 sec

30 sec

 $3 \min$ 

30 sec

30 sec

3 min

 $10 \min$ 

hold

Cycle

1

14

Every cycle

-1°C

26

1

1

Temp

95°C

95°C

72°C

95°C

56°C

72°C

72°C

4°C

70-57°C

## **Capillary electrophoresis**

Dilute PCR product PCR product:H20=1:39

Diluted PCR product	2.0 μl
Formamide mix	8.6 μl
Total	10.6 µl

Formamide mix=8µl Size standard + 850µ1Formamide

No denaturation. Analyze with DNA analyzer.

FastStart PCR Master (Roche, Switzerland)

Q solusion (QIAGEN, Germany)

## Laboratory I

RP-PCR	List	
Primer 1	P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
Primer 2 (with GGGGCC repeat)	Р5	caggaaacagctatgaccGGGCCCGCCCGACCACGCCCCGGCCCCG GCCCCGG
Primer 3	P6	caggaaacagctatgacc
Size standard	S1	GeneScan 500ROX Size Standard (Applied Biosystems, USA)
Analysis machine	M3	3500 Genetic Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper v4.0 (Applied Biosystems, USA)

#### PCR reaction (PR7)

PCR protocol (PP7)

**Capillary electrophoresis** 

1.5 µl 0.5 µ1

15.0 µl 17.0 µl

Products	Final conc.	Temp	Time	Cycle	PCR product	1.5 µl	
AccuPrime buffer A	1x	98°C	10 min	1	Size standard Formamide	0.5 μl 15.0 μ	
Primer 2	0.7 μM 97%	97°С 64°С	97°C 35 sec	10	Total	17.0 µ	
Primer 3 dCTP, dATP, dTTP (each)	1.4 μM 0.18 mM	68°C	8 min	10	95°C 5min and analyse		
7-deaza-2-deoxy GTP Taq DNA polymerase DNA	0.18 mM 1.0 U 12 ng/μl	97°C 64°C 68°C	35 sec 2 min 8-16 min	25 Every cycle +20 sec	with genetic an	analyzer.	
Total	25.0 µl	16°C	hold	1			

AccuPrime GC-rich DNA polymerase, buffer A (Invitrogen, USA)

Ramp rate adjusted to 64% for the denaturation and extension steps. Ramp rate adjusted to 25% for the annealing step.

## Amplicon length analysis

	List	
Primer 4	P15	CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Analysis system	A4	3% agarose gel

## PCR reaction (PR13)

Products	Final conc.
Primer 4	0.2 µM
Primer 5	0.2 µM
Kit buffer	1x
Kit polymerase	0.4 U
DNA	$2.0 \text{ ng}/\mu l$
Total	25.0 µl

PCR protocol (PP18)					
Temp	Time	Cycle			
95℃	3 min	1			
95°C 63°C 72°C	30 sec 30 sec 2 min	35			
72°C	10 min	1			
16°C	hold	1			

Kit: AccuPrime GC-rich DNA polymerase (Invitrogen, USA)

## Laboratory J

List	
P4 FAM-tgtaaaacgacggccagtCAAGGAGG	AACAACCGCAGCC
P5 caggaaacagctatgaccGGGCCCGCCCCG CCCCGG	CACGCCCCGGCCCCGG
P6 caggaaacagctatgacc	
S2 GeneScan 500LIZ Size Standard (Appli	Biosystems, USA)
M1 3730x/DNA Analyzer (Applied Biosyst	s, USA)
A2 GeneMapper v4.1 (Applied Biosystems	SA)
P4       FAM-tgtaaaacgacggccagtCAAGGAGGG         P5       caggaaacagctatgaccGGGCCCGGCCCGG         P6       caggaaacagctatgacc         S2       GeneScan 500LIZ Size Standard (Appli         M1       3730x/DNA Analyzer (Applied Biosystems)         A2       GeneMapper v4.1 (Applied Biosystems)	AACAACCGCAGCC CACGCCCCGGCCCCG Biosystems, USA) s, USA) SA)

### PCR reaction (PR8)

### PCR protocol (PP9)

Products	Final conc.		
Extensor Mastermix	1x		
Primer 1	2.5 μΜ		
Primer 2	2.5 μM		
Primer 3	0.25 µM		
Betaine	1.75 M		
DNA	3.3 ng/µl		
Total	15 µl		
Extensor Mastermix (Thermo			

Scientific, USA)

Temp	Time	Cycle
95°C	5 min	1
95°C	30 sec	8
70-56°C	30 sec	Every cycle
72°C	8 min	-2°C
95°C	30 sec	26
56°C	30 sec	Every cycle
72°C	8-16 min	+20 sec
72°C	7 min	1

## **Capillary electrophoresis**

PCR product Size standard Formamide	1.0 μl 0.5 μl 8.5 μl			
Total 10.0 μl				
95°C 2min and on ice. Analyse with DNA analyzer				

.

## Amplicon length analysis

	List	
Primer 4	P14	FAM-CAAGGAGGGAAACAACCGCAGCC
Primer 5	P16	GCAGGCACCGCAACCGCAG
Size standard	S2	GeneScan 500LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730xl DNA Analyzer (Applied Biosystems, USA)
Analysis software	A2	GeneMapper v4.1 (Applied Biosystems, USA)

### PCR reaction (PR17)

1 on protocon (1 1 12)
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Products	Final conc.
Primer 4	$0.1\mu\mathrm{M}$
Primer 5	$0.1\mu\mathrm{M}$
DMSO	1%
Megamix Blue	1x
DNA	3.3 ng/µl
Total	15 µl

Megamix Blue (Microzone, UK)

Temp	Time	Cycle
95°C	5 min	1
95℃	30 sec	8
70 <b>-</b> 56℃	30 sec	Every cycle
72°C	8 min	-2°C
95°C	30 sec	26
56°C	30 sec	Every cycle
72°C	8-16 min	+20 sec
72°C	7 min	1
4°C	hold	1

#### **Capillary electrophoresis**

1 1	-
PCR product Size standard Formamide	1.0 μl 0.5 μl 8.5 μl
Total	10.0 µl

95°C 2min and on ice. Analyse with DNA analyzer.

## Laboratory L

List	
21	FAM-AGTCGCTAGAGGCGAAAGC
2	tacgcatcccagtttgagacgGGGGCCGGGGCCGGGGGCCGGGG
<b>9</b> 3	tacgcatcccagtttgagacg
51	GeneScan 500XL ROX Size Standard (Applied Biosystems, USA)
И1	3730xlDNA Analyzer (Applied Biosystems, USA)
A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)
	List 1 2 3 1 1 1 1

### PCR reaction (PR1)

## PCR protocol (PP1)

Cycle

1

2-8\*

5

1

### **Capillary electrophoresis**

Products	Final conc.	Temp	Time
FastStart PCR Master	1x	95°C	15 min
Primer 1	1.4 μM	0.490	1 min
Primer 2	0.7 μM	94 0	
Primer 3	1.4 µM	/0-58	C I min
7-deaza-2-deoxy GTP	0.18 mM	72°C	3 min
DMSO	7 %	94°C	1 min
Q solution	1x	56°C	1 min
MgCl2	0.89 mM	72°C	3 min
DNA	100 ng		
Total	28.01	72°C	10 min
TOTAL	28.0 μI		220 1

1 2	1
PCR product	2.0 µl
Size standard	0.5 µl
HiDi Formamide	7.0 µl

9.5 µl

95°C 3min and analyse with DNA analyzer.

Total

FastStart PCR Master (Roche, Switzerland) Q solusion (QIAGEN, Germany)

\* Every 2°C decrease add 1

cycle.

Amplicon length analysis...not performed.

## Laboratory M

RP-PCR	List	
Primer 1	P4	FAM-tgtaaaacgacggccagtCAAGGAGGGAAACAACCGCAGCC
Primer 2 (with GGGGCC repeat)	P5	caggaaacagctatgaccGGGCCCGCCCGACCACGCCCCGGCCCCG GCCCCGG
Primer 3	P6	caggaaacagctatgacc
Size standard	<b>S</b> 2	GeneScan 500 LIZ Size Standard (Applied Biosystems, USA)
Analysis machine	M1	3730x/DNA Analyzer (Applied Biosystems, USA)
Analysis software	A1	Peak Scanner Software v1.0 (Applied Biosystems, USA)

PCR reaction (PR6)		PCR protocol (PP7)			Capillary electrophoresis	
Products	Final conc.	Temp	Time	Cycle	PCR product	1.0 µl
Qiagen buffer	1x	98°C	10 min	1	Size standard Formamide	0.1 μl 19.9 μl
Primer 1 Primer 2	1.0 μM 1.0 μM 1.0 μM 0.25 mM 0.25 mM 5% 1.0 M	97°С 64°С	35 sec 2 min	10	Total	21.0 µl
Primer 3 dCTP, dATP, dTTP (each)		68°C	8 min	10	95°C 5min and analyse with DNA analyzer.	
7-deaza-2-deoxy GTP DMSO Betaine		97°C 64°C 68°C	35 sec 2 min 8-16 min	25 Every cycle +20 sec		
Taq DNA polymerase DNA	2.5 U 5.0 ng/μl	Ramping up and down: 0.5°C/sec		•		
Total	20.0 µl					

*Taq* DNA polymerase, buffer (QIAGEN, Germany)

Amplicon length analysis...not performed.

## Laboratory N

RP-PCR	List	
Primer 1	P1	FAM-AGTCGCTAGAGGCGAAAGC
Primer 2 (with GGGGCC repeat)	P2	tacgcatcccagtttgagacgGGGGCCGGGGCCGGGGCCGGGG
Primer 3	Р3	tacgcatcccagtttgagacg
Size standard	S1	GeneScan 500ROX Size Standard (Applied Biosystems, USA)
Analysis machine	M2	3130 Genetic Analyzer (Applied Biosystems, USA)
Analysis software	A3	Genotyper v4 (Applied Biosystems, USA)

#### PCR reaction (PR9)

### PCR protocol (PP1)

### **Capillary electrophoresis**

Products	Final conc.
True Allele PCR premix	1x
Primer 1	$1.4  \mu M$
Primer 2	$0.7  \mu M$
Primer 3	1.4 µM
7-deaza-2-deoxy GTP	$0.16\mathrm{mM}$
DMSO	7%
Betaine	0.83 M
MgCl2	0.83 mM
DNA	3.3ng/µl
Total	30.0 µl

Temp	Time	Cycle		
95°C	15 min	1		
94°C	1 min			
70-58°C	1 min	2-8*		
72°C	3 min			
94°C	1 min			
56°C	1 min	5		
72°C	3 min			
72°C	10 min	1		
4°C	hold	1		

PCR product	2.0 µ1
Size standard	0.5 µl
Formamide	12.5 µl
Total	15.0 µl

95°C 2min and analyse with DNA analyzer.

True Allele PCR premix (Applied Biosystems, USA)

\* Every 2°C decrease add 1 cycle. Ramp speed: 1°C/sec

Amplicon length analysis...not performed.

## Supplementary table 4. The definitions of an expansion of the GGGGCC-repeat in

## C9orf72 as used in 13 laboratories.

## **GGGGCC-repeat number (4 laboratories)**

- > 24 repeats
- $\geq$  30 repeats
- > 30 repeats (2 laboratories)

## **RP-PCR shape (2 laboratories)**

Clear saw-tooth sloping pattern

## **GGGGCC-repeat number + RP-PCR shape (1 laboratory)**

>30 repeats + Stutter profile in RP-PCR

## **GGGGCC-repeat number + RP-PCR shape + Amplicon-length analysis result (1 laboratory)**

Saw-tooth pattern over 460 bp (30 repeats) in RP-PCR + One amplicon in amplicon-length analysis

## **RP-PCR** shape + Amplicon-length analysis result (1 laboratory)

Saw-tooth pattern in RP-PCR + One amplicon in amplicon-length analysis

## **RP-PCR** shape + Amplicon-length analysis result + Southern blot result (4 laboratories)

Saw-tooth pattern with concave shape in RP-PCR + One amplicon in amplicon-length analysis + Expanded band in Southern blot

One amplicon in amplicon-length analysis + Saw-tooth profile in RP-PCR + Expanded band in Southern blot

One amplicon in amplicon-length analysis + Long stutter in RP-PCR + Expanded band in Southern blot (2 laboratories)

	Laboratory I	Laboratory II	Laboratory III
Labelling	Radioisotope	Radioisotope	DIG system
Dose of gDNA	10 µg	10 µg	6-10 µg
Probe primer-F	TGACACACCAAGCGTCA	TATTAAGGTTCGCACAC	AGAACAGGACAAGTTGCC
	TCT	GCTA	
Probe primer-R	CACTGTGGGAGCAGTGT	GACGGCTGACACACCAA	AACACACACCTCCTAAAC
	CAT	G	С
Probe length	1000bp	210 bp	241 bp
Restriction enzyme	Xba I and Hind III	Xba I and Hind III	Xba I

## Supplementary table 5. Summary of the Southern blot methods of three laboratories.

## Supplementary Figure 1a,b,c,d



Four peaks of mutant allele.

129bp: Peak of wild type allele with 2 repeats.131bp: Peak of mutant allele with 6 repeats.

## В



## Supplementary figure 1a.b.c.d (cont.)



## Supplementary figure 1. Detailed analyses of the results of the three samples with mutations.

(A) The Rp-PCR and amplicon length analysis of sample 8.1. In RP-PCR there are four peaks before the wild-type allele peak at 290 bp. In amplicon-length analysis there are two peaks at 129bp (wild-type allele) and 131bp (the mutant allele), respectively and the two peaks are close together. As a consequence, four laboratories interpreted this sample as having one amplicon.

- (B) The sequence of sample 8.1. The wild-type allele has two GGGGCC-repeats and the mutant allele has six GGGGCC-repeats with a complex 15 deletion/17bp insertion mutation. Sample 8.1 and Sample 8.2 both carries this mutation.
- (C) The RP-PCR and amplicon length analysis results of sample 32.1. In RP-PCR there are four peaks before the 290bp peak (mutant allele) and a saw-tooth pattern peaks (expanded allele). In amplicon-length analysis there is one peak at 131bp (mutant allele) with 6 repeats.
- (D) The sequence of 32.1 illustrating the mutant allele which has six repeats with a complex 15bp deletion/17bp insertion mutation.

## Supplementary Figure 2 Laboratory I



+ + + +

## Supplementary Figure 2 (cont.)

## Laboratory II





Supplementary Figure 2. The Southern blot results in three laboratories.

Laboratory III ran out of DNA from one individual (sample 31.1). This laboratory therefore only

performed SB on 77 samples.

PC: Positive control, NC: negative control, +: sample with large repeat expansion, - :sample without large repeat expansion.