Supplementary Material

S1A. GenBank accession numbers of the *Austropotamobius torrentium* 16S rDNA sequences used to evaluate species-specificity of the *Ator* assay. Each sequence was trimmed to match only the region targeted by the assay. The sequences were downloaded on 2020-05-15. In bold are reported the local sequences.

AM181348, AM181347, AM181346, AF237599, AF235984, NC_033504, KX268734, JF293377, JF293379, JF293376, JF293374, JF293372, JF293371, JN683358, JN683357, JF293394, JF293389, JF293388, JF293378, JF293375, JF293373, JF293370, JF293368, JF293369, JF293393, JF293380, JF293400, JF293399, JF293397, JF293396, JF293395, JF293401, JF293398, JF293386, JF293384, JF293385, JF293392, JF293391, JF293390, JF293387, JF293402, JF293404, JF293403, JF293383, JF293382, JF293381, AJ242699, KP866115, **OM422805**, **OM422806**.

S1B. GenBank accession numbers of the *Austropotamobius pallipes* 16S rDNA sequences used to evaluate species-specificity of the *Apal* assay. Each sequence was trimmed to match only the region targeted by the assay. The sequences awere downloaded on 2020-05-15. In addition to sequences mined from the GenBank our alignment also included sequences from an in-house sequence database belonging to an ongoing study of phylogeography of *A. pallipes* in FVG. The in-house database includes sequences of 17 haplotypes detected within FVG [42], reported in bold.

JF430585, JF430583, JF430584, EF489427, NC_026560, KP205430, KP712875, KP712874, AY611201, Y611203, AY611202, AY611200, AY611199, JX446628JX446627, AF237610, AF237609, AF237595, AF237594, AF237597, AF237596, AF237602, AF237592, AF237591, AF237601, AF237590, AF237604, AF237607, AF237603, AF237600, AY611204, JF293367, AF237605, AY521287, AY521286, AY521285, AY521294, AY521293, AY521296, AY521290, AY521289, AJ242703, AJ242702, AY521295, AY521292, AY521288, AJ242701, AJ242700, AJ242706, AJ242705, AJ242707, AJ242708, EU308122, AJ242711, AJ242710, AJ242709, AJ242704, KX370586, KX370585, KX370584, KX370583, KX370582, KX370581, KX370580, KX370579, KX370578, KX370577, KX370576, KX370575, KX370574, KX370573, KX370572, KX370571, KX370570, KX370564, KX370563, KX370562, KX370561, KX370529, KX370528, KX370527, KX370526, KX370525, KX370524, KX370523, KX370522, KX370521, KX370520, KX370519, KX370518, KX370517, KX370516, KX370515, KX370514, KX370513, KX370512, KX370463, KX370462, KX370461, KX370460, KX370231, KX370230, KX370229, KX370228, KX370227, KX370226, KX370225, KX370224, KX370223, KX370222, KX370221, KX370220, KX370219, KX370218, KX370190, KX370189, KX370188, KX370187, KX370186, KX370176, KX370170, KX370133, KX370132, KX370131, KX370130, KX370129, KX370128, KX370105, KX370104, KX370103, KX370102, KX370101, KX370100, KX370099, KX370098, KX370097, AY521291, KX370560, KX370559, KX370558, KX370557, KX370556, KX370555, KX370554, KX370553, KX370552, KX370551, KX370550, KX370549, KX370548, KX370547, KX370546, KX370545, KX370544, KX370543, KX370542, KX370541, KX370540, KX370539, KX370538, KX370537, KX370536, KX370535, KX370534, KX370533, KX370532, KX370531, KX370530, KX370262, KX370261, KX370260, KX370127, KX370126, KX370125, KX370124, KX370123, KX370096, KX370095, KX370094, KX370093, KX370217, KX370213, KX370212, KX370211, KX370210, KX370209, KX370208, KX370207, KX370206, KX370205, KX370204, KX370203, KX370202, KX370201, KX370200, KX370199, KX370198, KX370197, KX370196, KX370194, KX370193, KX370192, KX370191, KX370184, KX370182, KX370181, KX370180, KX370179, KX370178, KX370175, KX370174, KX370161, KX370158, KX370157, KX370156, KX370155, KX370154, KX370153, KX370152, KX370151, KX370150, KX370149, KX370148, KX370147, KX370146, KX370145, KX370144, KX370143, KX370142, KX370140, KX370139, KX370138, KX370137, KX370136, KX370135, KX370134, KX370121, KX370120, KX370119, KX370118, KX370117, KX370116, KX370115, KX370114, KX370113,

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S1C. List of GenBank from 16S rDNA *Procambarus clarkii* and *Faxonius limosus* used to check species-specificity of both Ator and Apal GenBank accession numbers of *Procambarus clarkii* and *Faxonius limosus* 16S rDNA sequences used to evaluate species-specificity of the the *Ator* and *Apal* assays.

Procambarus clarkii	***Faxonius limosus***
KJ645835, KJ645834, KJ645833, KJ645832, KJ645831, KJ645830, KJ645829, KJ645828, KJ645827, KJ645826, KJ645825, KJ645824, KJ645823, KJ645822, KJ645821, KJ645820,	KT959482, JF293366, EU442690, GQ168834, NC_026561, KP205431

KJ645819, KJ645818, KJ645817, KJ645816, KC405653, JX120111, JX120110, JX120109, DQ666844, MK000282, MH300651, JX127829, AF436040, JF737227, AF235990, FJ619803, EF012352, EF012351, EF012350, GQ168838	

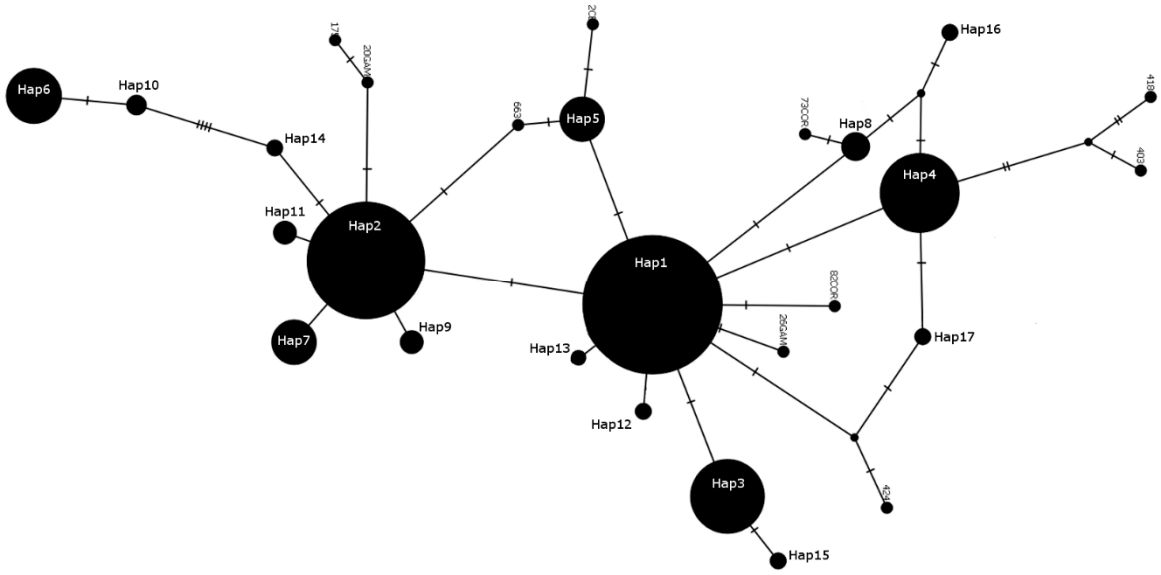

Figure S2. Haplotype network of *A. pallipes* 16S rDNA of sequences collected locally in the Friuli Venezia Giulia (FVG); sequences obtained from PhD thesis by Bertucci Maresca, [42]). Edge lengths are equal to pairwise distances between sequences and each dash on a branch represents a single mutation.

S3. List of 16S rDNA sequences used in the Ator Network.

Node label	GenBank of the matching sequences
AM181348	Ator, AM181348, AM181347, AM181346, AF237599, AF235984, NC033504, KX268734, JF293377, JF293379, JF293376, JF293374, JF293372, JF293371, JN683358, JN683357, JF293394, JF293389, JF293388, JF293378, JF293375, JF293370, JF293368, JF293393, JF293400, JF293399, JF293397, JF293396, JF293395, JF293401, JF293398, JF293386, JF293384, JF293385, JF293392, JF293391, JF293390, JF293387, AJ242699, KP866115, OM422805, OM422806
JF293369	JF293369, JF293380, JF293402, JF293404, JF293403
JF293383	JF293383, JF293382, JF293381
JF293373	JF293373

S4. List of 16S rDNA sequences used in the Apal Network.

Node label	GenBank of the matching sequences
JF293367	Apal, JF293367, AF237605, AY521293, KX370586, KX370585, KX370584, KX370583, KX370582, KX370581, KX370580, KX370579, KX370578, KX370577, KX370576, KX370575, KX370574, KX370573, KX370572, KX370571, KX370570, KX370564, KX370563, KX370562, KX370561, KX370529, KX370528, KX370527, KX370526, KX370525, KX370524, KX370523, KX370522, KX370521, KX370520, KX370519, KX370518, KX370517, KX370516, KX370515, KX370514, KX370513, KX370512, KX370463, KX370462, KX370461, KX370460, KX370231, KX370230, KX370229, KX370228, KX370227, KX370226, KX370225, KX370224, KX370223, KX370222, KX370221, KX370220, KX370219, KX370218, KX370190, KX370189, KX370188, KX370187, KX370186, KX370176, KX370170, KX370133, KX370132, KX370131, KX370130, KX370129, KX370128, KX370105, KX370104, KX370103, KX370102, KX370101, KX370100, KX370099, KX370098, KX370097, KX370217, KX370213, KX370212, KX370211, KX370210, KX370209, KX370208, KX370207, KX370206, KX370205, KX370204, KX370203, KX370202, KX370201, KX370200, KX370199, KX370198, KX370197, KX370196, KX370194, KX370193, KX370192, KX370191, KX370184, KX370182, KX370181, KX370180, KX370179, KX370178, KX370175, KX370174, KX370161, KX370158, KX370157, KX370156, KX370155, KX370154, KX370153, KX370152, KX370151, KX370150, KX370149, KX370148, KX370147, KX370146, , KX370145, KX370144, , KX370143, KX370142, KX370140, KX370139, KX370138, KX370137, KX370136, KX370135, KX370134, KX370121, KX370120, KX370119, KX370118, KX370117, KX370116, KX370115, KX370114, KX370113, KX370112, KX370111, KX370110, KX370109, KX370108, KX370590, KX370459, KX370458, KX370457, , KX370173, KX370172, KX370171, KX370106, KX370589, KX370588, KX370587, KX370511, KX370510, KX370509, KX370508, KX370507, KX370506, KX370505, KX370293, KX370292, KX370291, KX370290, KX370289, KX370288, KX370287, KX370286, KX370285, KX370284, KX370283, KX370282, KX370281, KX370280, KX370279, KX370278, KX370277, KX370276, KX370275, KX370274, KX370273, KX370272, KX370271, KX370270, KX370269, KX370268, KX370267, KX370266, KX370265, KX370264, KX370263, KX370259, KX370258, KX370257, KX370256, KX370255, KX370254, KX370253, KX370252, KX370251, KX370216, KX370215, KX370169, KX370168, KX370167, KX370163, KX370162, KX370195, KX370122, KX370185, KX370183, KX370177, KX370141, KX370107, OM422807, OM422808, OM422809, OM422810, OM422811, OM422813, OM422814, OM422815, OM422816, OM422817, OM422818, OM422819, OM422820, OM422821, OM422822, OM422823.
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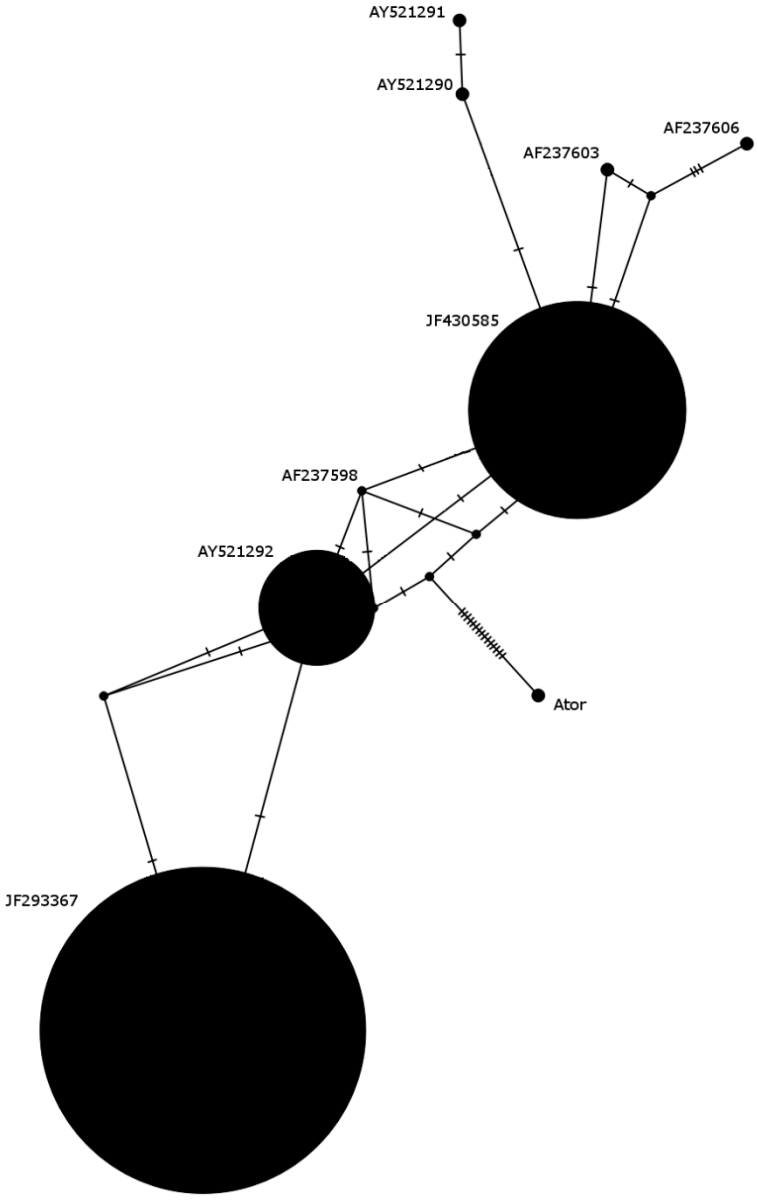

Figure S5. Haplotype network of the *A. pallipes* 16S sequences together with the Ator assay. Edge lengths are equal to pairwise distances between sequences and each dash on a branch represents a single mutation. Apal assay is identical to the JF293367 ID. Full list of the GenBank IDs for each trimmed haplotype is available in S1B.

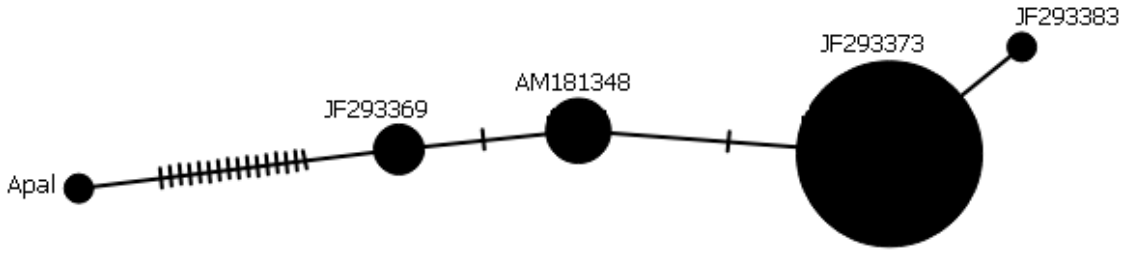

Figure S6. Haplotype network of the *A. torrentium* sequences together with the Apal assay. Edge lengths are equal to pairwise distances between sequences and each dash on a branch represents a single mutation. Ator assay is identical to the AM181348 ID. Full list of the GenBank IDs alongside the selected sequences are available at S1A.

S7.1. LOD calculation and data analysis

Preliminary limits of detection (LODs) for both the *Ator* and *Apal* assays were calculated to evaluate the efficiency of the primers for amplification of the target DNA and to define, with a reliable level, if the signals obtained by qPCR were within their limit of eDNA detection. Ten serial dilutions of *A. torrentium* gDNA, ranging from 1.215E+02 to 1.215E-07 ng, and of *A. pallipes* gDNA, ranging from 3.450E+01 to 3.450E-09 ng were run in qPCR reactions using the same PCR mix as reported in the manuscript for eDNA samples in section 2.6; the dilution series were run in two technical replicates.

The LOD was determined using a discrete threshold, whereby the lowest standard concentration of template DNA produced at least 95% positive replicates [52].

The LOD was performed on two positive controls using a dilution series up to 10^{-9}-fold. Cycle thresholds were recorded for each dilution. The detection limit of the qPCR reactions was comparable between the two positive controls and resulted in a dilution of $1/10^6$ and $1/10^5$ for *A. torrentium* and *A. pallipes*, respectively (S7.2 below).

Higher variability among replicates was obtained at the higher dilutions with some replicates not amplified in both samples (dilutions $1/10^7$ and $1/10^6$ for *A. torrentium* and *A. pallipes*, respectively).

Using the R script published by Klymus and colleagues [52], the LOD with 95% of consistency was set evaluated to be 1.215E-04 ng/µl for *Ator* and 3.450E-05 ng/µn for *Apal*.

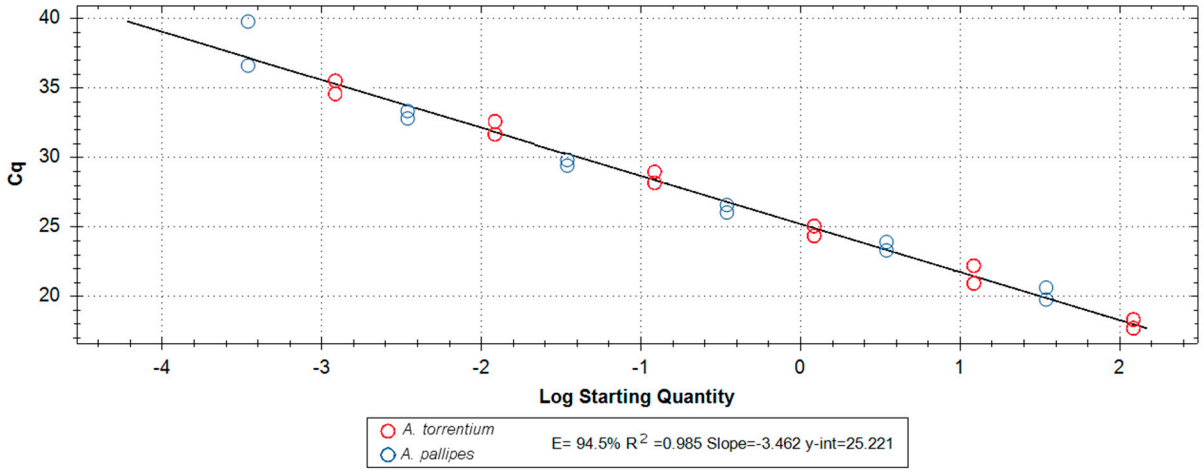

Figure S7. 2. Standard curve plot of the qPCR positive control samples. Y-axis gives the quantitative cycles (Cq), and x-axis the Log of the starting quantity of each dilution. gDNA from *A. torrentium* is reported in red circles, and gDNA from *A. pallipes* in blue ones. Figure edited with Maestro software (Bio Rad).

S8.1. Inhibition test

To evaluate possible effects of inhibitors on the amplification of eDNA samples, an "internal amplification control" (IAC) was used, namely IAC 1 in [53]. First, double-strand IAC (dsIAC) was produced in a 25 µl PCR mix containing 1X AccuStart II PCR ToughMix (Quantabio), 0.4 µM each oligonucleotide, and 50 ng of single-strand IAC (ssIAC), by using the following thermal profile: initial denaturation 95°C for 2 ', 35 cycles: 95°C for 30", 61°C for 30", 72°C for 1' and a final extension of 72°C for 10'. The annealed DNA was checked on a 3% TAE MetaPhor agarose gel and purified with 1.7X Mag-Bind® TotalPure NGS (Omegabiotek). The purified product was quantified in triplicate with Qubit® dsDNA BR (Broad-Range) Assay Kit (Thermofisher). Double-strand IAC was run in duplicate alongside a standard dilution series from $3x10^{-5}$ to $3x10^{-8}$ DNA copy number to perform a standard curve. Each reaction was performed in a final volume of 15 µl containing 1X KAPA Probe Force master mix (Kapa biosystems), 0.3 µM of the reverse primer and 0.15 µM of the forward primer, 1X EvaGreen® Dye (Biotium) and 1 µl selected IAC dilution, by using the following thermal profile: initial denaturation 95°C for 2 ', 35 cycles: 95°C for 30", 68°C for 30", followed by a melting curve from 65 °C to 95° with a temperature increment of 0.5 °C every 5". Triplicates PCR reactions containing $3x10^{-6}$ IAC copy numbers and 1µl of eDNA samples randomly taken from the sampled stations were run, at the same conditions, to evaluate possible inhibitors in the eDNA samples. From the inhibition test a Ct delay of the IAC mixed with the eDNA samples was observed at approximately 2 Ct's (25.80±0.10 SD) in respect to the same dilution amplified without eDNA (23.77±0.3 SD). Standard curve is provided in S8.2.

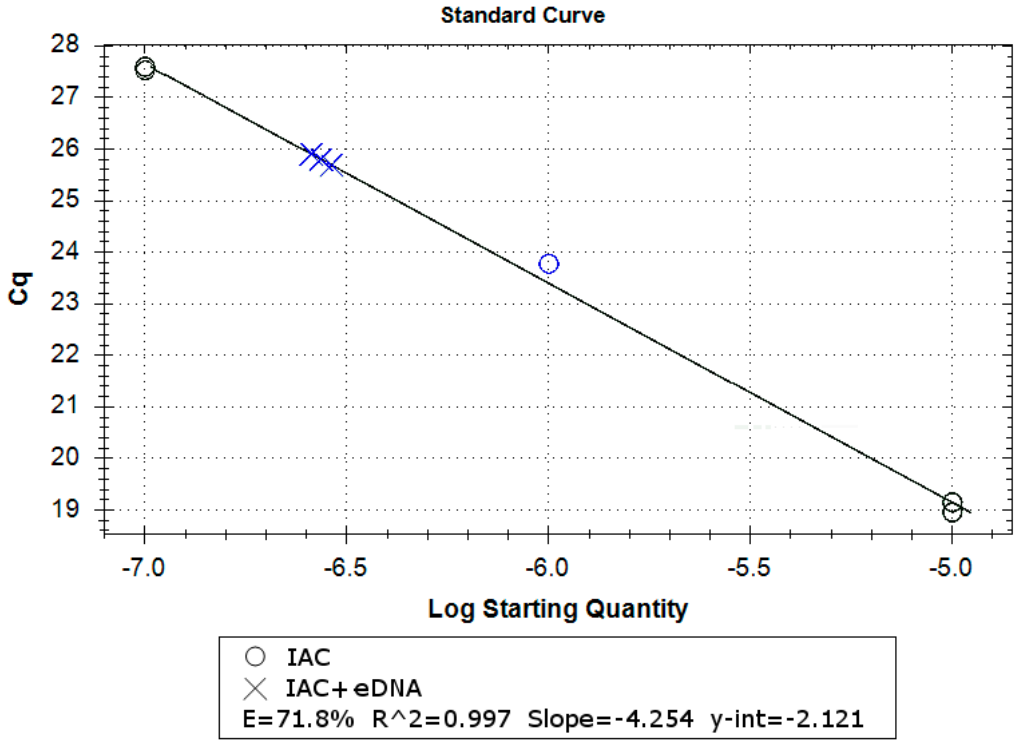

Figure S8.2. Detail of the Standard curve for Internal Amplification Control (IAC). Blue crosses indicate IAC within eDNA samples and blue circles IAC, both at IAC concentration of 3x104 DNA copy number. Cq stands for quantitation cycle.

S9. Detailed results obtained for each sampled site reported in Figures 4 and 5, along with the sampling date and initial Cycle threshold (Ct) from qPCR, '–' represents negative detection. The "corrected" Ct values calculated with the inhibition test. Asterisk (*) indicates positive sites published by Machino et al. (2015). Ct values are average lowered for two cycles when accounting for inhibition (Supplementary material S7).

ID site	Sampling date	System	Cycle thresholds PCR amplifications				
			Rep 1	Rep 2	Rep 3	Rep 4	Rep 5
1*	07.07.2017	Ator	33.1 (31.1)	32.8 (30.8)	31.0 (29.0)	30.4 (28.4)	
		Apal	-	-	-	-	-
2 *	07.07.2017	Ator	36.1 (34.1)	36.4 (34.4)	34.0 (32.0)	34.4 (32.4)	
		Apal	-	-	-	-	
3 *	07.07.2017	Ator	37.5 (35.5)	38.6 (36.6)	-	-	
		Apal	-	-	-	-	
	05.07.2018	Ator	34.9 (32.9)	34.1 (32.1)	34.2 (32.2)	34.3 (32.3)	33.6 (31.6)
		Apal	-	-	-	-	-

4 *	07.07.2017	Ator	-	-	-	-	
		Apal	-	-	-	-	
	05.07.2018	Ator	-	-	-	-	-
		Apal	-	-	-	-	-
5	11.07.2017	Ator	-	-	38.2 (36.2)	37.7 (35.7)	37.2 (35.2)
		Apal	-	-	-	-	
	05.07.2018	Ator	-	-	-	-	-
		Apal	-	-	-	-	-
6	11.07.2017	Ator	-	-	-	-	
		Apal	-	-	-	-	-
7	11.07.2017	Ator	-	-	-	-	
		Apal	-	-	-	-	
8 *	11.07.2017	Ator	-	-	-	-	
		Apal	-	-	-	-	-
9	11.07.2017	Ator	-	-	-	42.2 (40.2)	
		Apal	-	-	-	-	
	31.10.2017	Ator	-	-	-	-	-
		Apal	-	-	-	-	-
	05.07.2018	Ator	-	-	-	-	37.2 (35.2)
		Apal	-	-	-	-	-
10	17.08.2017	Ator	33.5 (31.5)	-	35.0 (33.0)	34.5 (32.5)	
		Apal	-	-	-	-	-
	31.10.2017	Ator	-	-	-	-	-
		Apal	-	-	-	-	-
11	17.08.2017	Ator	-	-	-	-	-
		Apal	-	-	-	-	-
	31.10.2017	Ator	38.2 (36.2)	-	-	39.0 (37.0)	39.3 (37.0)
		Apal	-	-	-	-	-
12	17.08.2017	Ator	35.0 (33.0)	35.6 (33.6)	-	-	
		Apal	-	-	-	-	
13	17.08.2017	Ator	-	-	-	-	

		Apal	-	-	-	-	
14	17.08.2017	Ator	-	-	-	-	
		Apal	-	-	-	-	
15	31.10.2017	Ator	-	-	-	-	
		Apal	37.5 (35.5)	-	37.2 (35.2)	34.7 (32.7)	
	05.07.2018	Ator	-	-	-	-	-
		Apal	33.6 (31.6)	34.0 (32.0)	32.5 (30.5)	34.4 (32.4)	33.1 (31.1)