Appendix to application note:

Analysis of DNA integrity and stability using digital PCR

Does accurate and precise integrity calculation require the analysis of more than two targets located at the 5' and 3' end of the DNA of interest?

As shown in Figure 4 of the application note, the integrity analysis of partially intact samples benefits from a higher multiplexing grade analysis. This implies that an analysis solely targeting the ends of the AAV genome may not yield the same integrity values as when DNA integrity is assessed considering multiple targets present on the template, in this example, a 2-plex vs. a 5-plex reaction.

In Figure 4, a difference of 10 percentage points was observed. Where does this difference originate, and how can this result be mathematically explained?

In the past, only the targets at the 5' and 3' ends were usually used in a 2-plex reaction to calculate the integrity/linkage of a given template molecule. For the AAV template, outlined in Appendix Figure 1 (and more detailed in Figure 4), the respective 2-plex with target detection in the orange/red channels resulted in an average percentage intact value (integrity/linkage) of 45.88% (Appendix Table 1).

Note: The description of each column of the QIAcuity Software Suite version 2.5 output file for integrity (MultiOccupancy.csv) is stated in the QIAcuity User Manual (page 212).

Figure 1. Simplified representation of an AAV template. All five targets were addressed by assays labeled with different dyes. Detection took place in the five channels of the QIAcuity® dPCR System.

Table 1. Multiple occupancy output file (only relevant columns shown), including % intact values when using only the assays at the 5' and 3' ends of the AAV genome in triplicates (wells A1 to A3). The CMV enhancer region (ORANGE) was targeted at the 5' end and hGHpA (RED) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A1	ORANGE-RED	++	5719	2470	222.58	1520.42	46.06
A1	ORANGE-RED	+–	5719	626	–	853.71	–
A1	ORANGE-RED	–+	5719	688	–	926.57	–
A1	ORANGE-RED	––	5719	1935	–	–	–
A2	ORANGE-RED	++	7381	3101	289.43	1496.42	45.10
A2	ORANGE-RED	+–	7381	759	–	813.37	–
A2	ORANGE-RED	–+	7381	972	–	1008.11	–
A2	ORANGE-RED	––	7381	2549	–	–	–
A3	ORANGE-RED	++	7893	3315	286.59	1504.30	46.49
A3	ORANGE-RED	+–	7893	839	–	817.94	–
A3	ORANGE-RED	–+	7893	952	–	913.33	–
A3	ORANGE-RED	––	7893	2787	–	–	–

When adding the target labeled with green to the analysis, the mean integrity value for the triplicates is 42.54% (Appendix Table 2). A lower integrity value than in the 2-plex analysis is expected since the –++ group will be counted as intact/linked in the 2-plex case but not in the 3-plex case. Additionally, the green-only template molecules (+––) are not visible in the 2-plex analysis and are counted in the group without any fluorescence signal (––) (refer to Appendix Table 2 and the same groups highlighted in light grey in Appendix Table 5).

Table 2. Multiple occupancy output file (only relevant columns), including % intact when using the assays at the 5' and 3' ends and the gene of interest in the center in triplicates. The CMV enhancer region (ORANGE) was targeted at the 5' end, the gene of interest GFP (GREEN) at the center and hGHpA (RED) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A1	GREEN-ORANGE-RED	+++	5368	2293	181.73	1522.09	43.93
A1	GREEN-ORANGE-RED	++–	5368	343	–	459.41	–
A1	GREEN-ORANGE-RED	+–+	5368	407	–	531.70	–
A1	GREEN-ORANGE-RED	+––	5368	91	–	156.94	–
A1	GREEN-ORANGE-RED	–++	5368	57	–	36.91	–
A1	GREEN-ORANGE-RED	–+–	5368	214	–	356.97	–
A1	GREEN-ORANGE-RED	––+	5368	242	–	400.73	–
A1	GREEN-ORANGE-RED	–––	5368	1721	–	–	–
A2	GREEN-ORANGE-RED	+++	7381	3021	267.57	1456.96	41.91
A2	GREEN-ORANGE-RED	++–	7381	514	–	512.78	–
A2	GREEN-ORANGE-RED	+–+	7381	572	–	531.29	–
A2	GREEN-ORANGE-RED	+––	7381	126	–	158.20	–
A2	GREEN-ORANGE-RED	–++	7381	80	–	39.46	–
A2	GREEN-ORANGE-RED	–+–	7381	245	–	300.59	–
A2	GREEN-ORANGE-RED	––+	7381	400	–	476.81	–
A2	GREEN-ORANGE-RED	–––	7381	2423	–	–	–
A3	GREEN-ORANGE-RED	+++	7893	3212	281.41	1442.43	41.76
A3	GREEN-ORANGE-RED	++–	7893	559	–	499.82	–
A3	GREEN-ORANGE-RED	+–+	7893	605	–	523.67	–
A3	GREEN-ORANGE-RED	+––	7893	189	–	218.26	–
A3	GREEN-ORANGE-RED	–++	7893	103	–	61.88	–
A3	GREEN-ORANGE-RED	–+–	7893	280	–	318.13	–
A3	GREEN-ORANGE-RED	––+	7893	347	–	389.65	–
A3	GREEN-ORANGE-RED	–––	7893	2598	–	–	–

When adding the target labeled with yellow to the analysis, the mean integrity value for the triplicates is 40.30% (Appendix Table 3). A lower integrity value than in the 3–plex analysis is expected since the +–++ group will be counted as intact/linked in the 3–plex case but not in the 4–plex case. Additionally, the yellow–only template molecules (–+––) are not visible in the 3–plex analysis and are counted in the group without any fluorescence signal (–––).

Table 3. Multiple occupancy output file (only relevant columns), including % intact when using four assays in triplicates. The CMV enhancer region (ORANGE) was targeted at the 5' end, GFP (GREEN) and WPRE (YELLOW) at the center and hGHpA (RED) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A1	GREEN-YELLOW-ORANGE-RED	++++	5305	2229	182.29	1484.68	41.65
A1	GREEN-YELLOW-ORANGE-RED	+++–	5305	273	–	359.51	–
A1	GREEN-YELLOW-ORANGE-RED	++–+	5305	382	–	505.82	–
A1	GREEN-YELLOW-ORANGE-RED	++––	5305	58	–	98.68	–
A1	GREEN-YELLOW-ORANGE-RED	+–++	5305	38	–	37.28	–
A1	GREEN-YELLOW-ORANGE-RED	+–+–	5305	68	–	103.13	–
A1	GREEN-YELLOW-ORANGE-RED	+––+	5305	21	–	28.05	–
A1	GREEN-YELLOW-ORANGE-RED	+–––	5305	31	–	56.83	–
A1	GREEN-YELLOW-ORANGE-RED	–+++	5305	28	–	28.24	–
A1	GREEN-YELLOW-ORANGE-RED	–++–	5305	22	–	25.42	–
A1	GREEN-YELLOW-ORANGE-RED	–+–+	5305	44	–	59.95	–
A1	GREEN-YELLOW-ORANGE-RED	–+––	5305	53	–	96.53	–
A1	GREEN-YELLOW-ORANGE-RED	––++	5305	28	–	8.31	–
A1	GREEN-YELLOW-ORANGE-RED	––+–	5305	190	–	332.73	–
A1	GREEN-YELLOW-ORANGE-RED	–––+	5305	194	–	339.36	–
A1	GREEN-YELLOW-ORANGE-RED	––––	5305	1646	–	–	–
A2	GREEN-YELLOW-ORANGE-RED	++++	7145	2908	253.70	1449.24	40.45
A2	GREEN-YELLOW-ORANGE-RED	+++–	7145	394	–	398.58	–
A2	GREEN-YELLOW-ORANGE-RED	++–+	7145	537	–	520.93	–
A2	GREEN-YELLOW-ORANGE-RED	++––	7145	74	–	93.72	–
A2	GREEN-YELLOW-ORANGE-RED	+–++	7145	52	–	36.89	–
A2	GREEN-YELLOW-ORANGE-RED	+–+–	7145	102	–	118.01	–
A2	GREEN-YELLOW-ORANGE-RED	+––+	7145	19	–	14.87	–
A2	GREEN-YELLOW-ORANGE-RED	+–––	7145	45	–	61.34	–
A2	GREEN-YELLOW-ORANGE-RED	–+++	7145	36	–	30.90	–
A2	GREEN-YELLOW-ORANGE-RED	–++–	7145	7	–	0.44	–
A2	GREEN-YELLOW-ORANGE-RED	–+–+	7145	66	–	65.06	–
A2	GREEN-YELLOW-ORANGE-RED	–+––	7145	69	–	93.57	–
A2	GREEN-YELLOW-ORANGE-RED	––++	7145	29	–	0.00	–
A2	GREEN-YELLOW-ORANGE-RED	––+–	7145	218	–	286.52	–
A2	GREEN-YELLOW-ORANGE-RED	–––+	7145	322	–	414.47	–
A2	GREEN-YELLOW-ORANGE-RED	––––	7145	2267	–	–	–
A3	GREEN-YELLOW-ORANGE-RED	++++	7893	3128	296.29	1381.10	38.80
A3	GREEN-YELLOW-ORANGE-RED	+++–	7893	453	–	393.69	–
A3	GREEN-YELLOW-ORANGE-RED	++–+	7893	573	–	497.86	–

Table continued on next page. ▷

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Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A3	GREEN-YELLOW-ORANGE-RED	++––	7893	118	–	131.60	–
A3	GREEN-YELLOW-ORANGE-RED	+–++	7893	84	–	61.32	–
A3	GREEN-YELLOW-ORANGE-RED	+–+–	7893	106	–	106.12	–
A3	GREEN-YELLOW-ORANGE-RED	+––+	7893	32	–	25.81	–
A3	GREEN-YELLOW-ORANGE-RED	+–––	7893	71	–	86.66	–
A3	GREEN-YELLOW-ORANGE-RED	–+++	7893	44	–	31.58	–
A3	GREEN-YELLOW-ORANGE-RED	–++–	7893	25	–	17.51	–
A3	GREEN-YELLOW-ORANGE-RED	–+–+	7893	63	–	56.62	–
A3	GREEN-YELLOW-ORANGE-RED	–+––	7893	87	–	105.86	–
A3	GREEN-YELLOW-ORANGE-RED	––++	7893	59	–	30.30	–
A3	GREEN-YELLOW-ORANGE-RED	––+–	7893	255	–	300.62	–
A3	GREEN-YELLOW-ORANGE-RED	–––+	7893	284	–	333.04	–
A3	GREEN-YELLOW-ORANGE-RED	––––	7893	2511	–	–	–

When adding the target labeled with crimson, the mean integrity value for the triplicates is 35.80% (Appendix Table 4). A lower integrity value than in the 4–plex analysis is expected since the ++++– group will be counted as intact/linked in the 4–plex case but not in the 5–plex case. Additionally, the crimson–only template molecules (––––+) are not visible in the 4–plex analysis and are counted in the group without any fluorescence signal (––––).

Table 4. Multiple occupancy output file (only relevant columns), including % intact when using all assays in triplicates. The CMV enhancer region (ORANGE) was targeted at the 5' end, the CMV promoter (CRIMSON), GFP (GREEN), WPRE (YELLOW) at the center, and hGHpA (RED) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++++	5305	2107	218.08	1340.63	36.99
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	++++–	5305	122	–	144.04	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++–+	5305	261	–	340.01	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++––	5305	12	–	19.50	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–++	5305	82	–	87.89	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–+–	5305	300	–	417.93	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	++––+	5305	14	–	21.79	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–––	5305	44	–	76.89	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+++	5305	34	–	31.93	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–++–	5305	4	–	5.35	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+–+	5305	64	–	96.31	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+––	5305	4	–	6.82	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––++	5305	4	–	4.49	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––+–	5305	17	–	23.57	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–––+	5305	7	–	11.87	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––––	5305	24	–	44.96	–

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Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++++	5305	27	–	27.43	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+++–	5305	1	–	0.81	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++–+	5305	22	–	26.53	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++––	5305	0	–	0.00	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–++	5305	3	–	3.26	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–+–	5305	41	–	56.69	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+––+	5305	1	–	0.00	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–––	5305	52	–	96.58	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+++	5305	24	–	5.32	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	––++–	5305	4	–	2.98	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+–+	5305	171	–	297.08	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+––	5305	19	–	35.65	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––++	5305	6	–	3.76	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––+–	5305	188	–	335.59	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	––––+	5305	32	–	59.80	–
A1	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––––	5305	1614	–	–	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++++	7145	2723	297.16	1294.39	35.66
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	++++–	7145	185	–	154.85	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++–+	7145	370	–	369.04	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++––	7145	24	–	29.53	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–++	7145	94	–	74.54	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–+–	7145	443	–	446.39	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	++––+	7145	14	–	16.27	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–––	7145	60	–	77.45	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+++	7145	48	–	32.86	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–++–	7145	4	–	4.03	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+–+	7145	99	–	114.67	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+––	7145	3	–	3.34	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––++	7145	0	–	0.00	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––+–	7145	19	–	15.85	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–––+	7145	4	–	4.56	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––––	7145	41	–	56.78	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++++	7145	30	–	24.66	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+++–	7145	6	–	6.24	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++–+	7145	7	–	1.58	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++––	7145	0	–	0.00	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–++	7145	6	–	5.14	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–+–	7145	60	–	59.91	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+––+	7145	4	–	4.02	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–––	7145	65	–	89.54	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+++	7145	26	–	0.00	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	––++–	7145	3	–	0.00	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+–+	7145	189	–	246.26	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+––	7145	29	–	40.27	–

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Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−++	7145	7	–	2.66	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−+−	7145	315	–	411.81	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−−+	7145	34	–	47.16	–
A2	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−−−	7145	2233	–	–	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++++	7893	2961	341.51	1253.44	34.75
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	++++−	7893	167	–	127.67	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++−+	7893	430	–	369.74	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++−−	7893	23	–	23.95	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	++−++	7893	124	–	92.40	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	++−+−	7893	449	–	405.46	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	++−−+	7893	16	–	15.15	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	++−−−	7893	102	–	116.45	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−+++	7893	79	–	57.15	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−++−	7893	5	–	4.17	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−+−+	7893	99	–	98.46	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−+−−	7893	7	–	7.66	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−−++	7893	5	–	3.29	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−−+−	7893	27	–	22.53	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−−−+	7893	13	–	14.61	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	+−−−−	7893	58	–	72.05	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−++++	7893	40	–	28.22	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−+++−	7893	4	–	3.37	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−++−+	7893	24	–	17.52	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−++−−	7893	1	–	0.00	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−+−++	7893	1	–	0.00	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−+−+−	7893	62	–	56.62	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−+−−+	7893	1	–	0.00	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−+−−−	7893	86	–	106.25	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−+++	7893	54	–	28.35	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−++−	7893	5	–	1.94	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−+−+	7893	226	–	264.38	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−+−−	7893	29	–	36.24	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−++	7893	6	–	1.92	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−+−	7893	278	–	331.11	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−−+	7893	38	–	47.40	–
A3	GREEN-YELLOW-ORANGE-RED-CRIMSON	−−−−−	7893	2473	–	–	–

Appendix Table 5 shows an example well A3 where template molecules incorrectly allocated in the 2-plex scenario compared to the 3-plex, 4-plex and 5-plex scenarios are highlighted in light and dark grey.

Table 5. (%) intact values for well A3 when analyzed in 2-plex, 3-plex, 4-plex and 5-plex scenarios. Template molecules incorrectly allocated in the 2-plex scenario are highlighted in light and dark grey. Groups highlighted in light grey are incorrectly allocated in the 2-plex scenario and in the next higher grade multiplex scenario (e.g., the group –+–– is incorrectly allocated in the 2-plex scenario and the 3-plex scenario). Groups highlighted in dark grey are incorrectly allocated in the 2-plex scenario but correctly allocated in the next higher grade multiplex scenario (e.g., group +–––, which is not correctly allocated in the 2-plex scenario but already correctly allocated in the 3-plex scenario.

Scenario	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
2-plex	ORANGE-RED	++	7893	3315	286.59	1504.30	46.49
	ORANGE-RED	+–	7893	839	–	817.94	–
	ORANGE-RED	–+	7893	952	–	913.33	–
	ORANGE-RED	––	7893	2787	–	–	–
3-plex	GREEN-ORANGE-RED	+++	7893	3212	281.41	1442.43	41.76
	GREEN-ORANGE-RED	++–	7893	559	–	499.82	–
	GREEN-ORANGE-RED	+–+	7893	605	–	523.67	–
	GREEN-ORANGE-RED	+––	7893	189	–	218.26	–
	GREEN-ORANGE-RED	–++	7893	103	–	61.88	–
	GREEN-ORANGE-RED	–+–	7893	280	–	318.13	–
	GREEN-ORANGE-RED	––+	7893	347	–	389.65	–
	GREEN-ORANGE-RED	–––	7893	2598	–	–	–
4-plex	GREEN-YELLOW-ORANGE-RED	++++	7893	3128	296.29	1381.10	38.80
	GREEN-YELLOW-ORANGE-RED	+++–	7893	453	–	393.69	–
	GREEN-YELLOW-ORANGE-RED	++–+	7893	573	–	497.86	–
	GREEN-YELLOW-ORANGE-RED	++––	7893	118	–	131.60	–
	GREEN-YELLOW-ORANGE-RED	+–++	7893	84	–	61.32	–
	GREEN-YELLOW-ORANGE-RED	+–+–	7893	106	–	106.12	–
	GREEN-YELLOW-ORANGE-RED	+––+	7893	32	–	25.81	–
	GREEN-YELLOW-ORANGE-RED	+–––	7893	71	–	86.66	–
	GREEN-YELLOW-ORANGE-RED	–+++	7893	44	–	31.58	–
	GREEN-YELLOW-ORANGE-RED	–++–	7893	25	–	17.51	–
	GREEN-YELLOW-ORANGE-RED	–+–+	7893	63	–	56.62	–
	GREEN-YELLOW-ORANGE-RED	–+––	7893	87	–	105.86	–
	GREEN-YELLOW-ORANGE-RED	––++	7893	59	–	30.30	–
	GREEN-YELLOW-ORANGE-RED	––+–	7893	255	–	300.62	–
	GREEN-YELLOW-ORANGE-RED	–––+	7893	284	–	333.04	–
	GREEN-YELLOW-ORANGE-RED	––––	7893	2511	–	–	–
5-plex	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++++	7893	2961	341.51	1253.44	34.75
	GREEN-YELLOW-ORANGE-RED-CRIMSON	++++–	7893	167	–	127.67	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++–+	7893	430	–	369.74	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+++––	7893	23	–	23.95	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–++	7893	124	–	92.40	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–+–	7893	449	–	405.46	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	++––+	7893	16	–	15.15	–

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Scenario	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
5-plex	GREEN-YELLOW-ORANGE-RED-CRIMSON	++–––	7893	102	–	116.45	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+++	7893	79	–	57.15	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–++–	7893	5	–	4.17	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+–+	7893	99	–	98.46	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–+––	7893	7	–	7.66	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––++	7893	5	–	3.29	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––+–	7893	27	–	22.53	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+–––+	7893	13	–	14.61	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	+––––	7893	58	–	72.05	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++++	7893	40	–	28.22	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+++–	7893	4	–	3.37	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++–+	7893	24	–	17.52	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–++––	7893	1	–	0.00	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–++	7893	1	–	0.00	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–+–	7893	62	–	56.62	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+––+	7893	1	–	0.00	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–+–––	7893	86	–	106.25	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+++	7893	54	–	28.35	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	––++–	7893	5	–	1.94	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+–+	7893	226	–	264.38	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	––+––	7893	29	–	36.24	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––++	7893	6	–	1.92	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––+–	7893	278	–	331.11	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	––––+	7893	38	–	47.40	–
	GREEN-YELLOW-ORANGE-RED-CRIMSON	–––––	7893	2473	–	–	–

The above results show that using more than two targets for the integrity analyses decreases the risk of overestimating the integrity value because of template types incorrectly allocated within the 2-plex scenario. Therefore, it is recommended to increase coverage of the fully intact/linked template by targets in a reasonable manner, considering the direct proportionality between the target count and interference risk while prioritizing the amplification performance (e.g., signal-to-noise ratio and amount of rain).

There is an exception with inherently remarkably intact templates, such as plasmids (>90% intact; Appendix Figure 2). A linearized AAV transfer plasmid containing four targets was analyzed using dPCR. The QIAcuity integrity feature calculated a linkage score for the 2-plex, 3-plex and 4-plex analysis of 97–98%.

Figure 2. Plasmid integrity values comparable among different multiplexing grade levels. A An AAV transfer plasmid containing an upstream and downstream ITR, CMV enhancer (HEX), CMV promoter (ROX), GFP (Cy5) and WPRE (FAM) as targets was linearized using *EcoR*I and analyzed by dPCR. The targets were amplified using the QIAcuity CGT dPCR Assays. **B** dPCR quantification of the CMVe, CMVp, GFP and WPRE targets in a 4-plex reaction is shown. dPCR was performed on 8.5K Nanoplates using the QIAcuity Probe PCR Kit. 48 replicates were quantified, and genome integrity was analyzed using the QIAcuity Software Suite version 2.5. Coefficients of variation (CV) within the replicates (intra-assay) of one target as well as between the targets (inter-assay) are shown. **C** The plasmid integrity score was calculated for the 4-plex reaction, including all assays used and additional target combinations throughout the genome. Integrity scores (%) are indicated for all analyzed combinations. **D** Representative 1D scatterplots for all analyzed targets are shown.

Here, the differences between the multiplexing levels are minimal to negligible due to a low number of incorrectly allocated template types (as shown in Tables 6, 7 and 8).

Table 6. Multiple occupancy output file (only relevant columns), including % intact when using the assays at the 5' and 3' ends. The CMV enhancer region (YELLOW) was targeted at the 5' end and WPRE (GREEN) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
H1	GREEN-YELLOW	++	8221	1474	0.03	603.98	97.86
H1	GREEN-YELLOW	+–	8221	17	–	7.73	–
H1	GREEN-YELLOW	–+	8221	12	–	5.46	–
H1	GREEN-YELLOW	––	8221	6718	–	–	–
H2	GREEN-YELLOW	++	8222	1465	0.04	605.70	97.64
H2	GREEN-YELLOW	+–	8222	13	–	5.96	–
H2	GREEN-YELLOW	–+	8222	19	–	8.71	–
H2	GREEN-YELLOW	––	8222	6725	–	–	–
H3	GREEN-YELLOW	++	8170	1473	0.02	624.53	98.22
H3	GREEN-YELLOW	+–	8170	11	–	5.17	–
H3	GREEN-YELLOW	–+	8170	13	–	6.11	–
H3	GREEN-YELLOW	––	8170	6673	–	–	–

Table 7. Multiple occupancy output file (only relevant columns), including % intact when using the assays at the 5' and 3' ends and the gene of interest in the center. The CMV enhancer region (YELLOW) was targeted at the 5' end, the gene of interest GFP (RED) at the center and WPRE (GREEN) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Count random	Conc. per group [cp/µL]	% intact
H1	GREEN-YELLOW-RED	+++	8221	1474.00	0.03	603.98	97.51
H1	GREEN-YELLOW-RED	++–	8221	0.00	–	0.00	–
H1	GREEN-YELLOW-RED	+–+	8221	5.00	–	2.27	–
H1	GREEN-YELLOW-RED	+––	8221	12.00	–	5.46	–
H1	GREEN-YELLOW-RED	–++	8221	10.00	–	4.54	–
H1	GREEN-YELLOW-RED	–+–	8221	2.00	–	0.91	–
H1	GREEN-YELLOW-RED	––+	8221	5.00	–	2.28	–
H1	GREEN-YELLOW-RED	–––	8221	6713.00	–	–	–
H2	GREEN-YELLOW-RED	+++	8222	1465.00	0.02	605.70	97.28
H2	GREEN-YELLOW-RED	++–	8222	0.00	–	0.00	–
H2	GREEN-YELLOW-RED	+–+	8222	2.00	–	0.91	–
H2	GREEN-YELLOW-RED	+––	8222	11.00	–	5.05	–
H2	GREEN-YELLOW-RED	–++	8222	11.00	–	5.04	–
H2	GREEN-YELLOW-RED	–+–	8222	8.00	–	3.67	–
H2	GREEN-YELLOW-RED	––+	8222	5.00	–	2.30	–
H2	GREEN-YELLOW-RED	–––	8222	6720.00	–	–	–
H3	GREEN-YELLOW-RED	+++	8170	1473.00	0.02	624.53	98.08
H3	GREEN-YELLOW-RED	++–	8170	0.00	–	0.00	–
H3	GREEN-YELLOW-RED	+–+	8170	3.00	–	1.41	–
H3	GREEN-YELLOW-RED	+––	8170	8.00	–	3.76	–
H3	GREEN-YELLOW-RED	–++	8170	10.00	–	4.70	–
H3	GREEN-YELLOW-RED	–+–	8170	3.00	–	1.41	–
H3	GREEN-YELLOW-RED	––+	8170	2.00	–	0.94	–
H3	GREEN-YELLOW-RED	–––	8170	6671.00	–	–	–

Table 8. Multiple occupancy output file (only relevant columns), including % intact when using the assays at the 5' and 3' ends, as well as the promoter and gene of interest in the center. The CMV enhancer region (YELLOW) was targeted at the 5' end, the CMV promoter (CRIMSON) and GFP (RED) at the center, and WPRE (GREEN) at the 3' end.

Well	Categories	Group	Valid partitions	Count categories	Conc. per group [cp/µL]	% intact
H1	GREEN-YELLOW-RED-CRIMSON	++++	8221	1467	600.81	96.99
H1	GREEN-YELLOW-RED-CRIMSON	+++–	8221	7	3.17	–
H1	GREEN-YELLOW-RED-CRIMSON	++–+	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	++––	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	+–++	8221	5	2.27	–
H1	GREEN-YELLOW-RED-CRIMSON	+–+–	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	+––+	8221	6	2.73	–
H1	GREEN-YELLOW-RED-CRIMSON	+–––	8221	6	2.73	–
H1	GREEN-YELLOW-RED-CRIMSON	–+++	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	–++–	8221	10	4.54	–
H1	GREEN-YELLOW-RED-CRIMSON	–+–+	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	–+––	8221	2	0.91	–
H1	GREEN-YELLOW-RED-CRIMSON	––++	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	––+–	8221	5	2.28	–
H1	GREEN-YELLOW-RED-CRIMSON	–––+	8221	0	0.00	–
H1	GREEN-YELLOW-RED-CRIMSON	––––	8221	6713	–	–
H2	GREEN-YELLOW-RED-CRIMSON	++++	8222	1462	604.34	97.06
H2	GREEN-YELLOW-RED-CRIMSON	+++–	8222	3	1.37	–
H2	GREEN-YELLOW-RED-CRIMSON	++–+	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	++––	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	+–++	8222	2	0.91	–
H2	GREEN-YELLOW-RED-CRIMSON	+–+–	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	+––+	8222	4	1.83	–
H2	GREEN-YELLOW-RED-CRIMSON	+–––	8222	7	3.21	–
H2	GREEN-YELLOW-RED-CRIMSON	–+++	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	–++–	8222	11	5.04	–
H2	GREEN-YELLOW-RED-CRIMSON	–+–+	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	–+––	8222	8	3.67	–
H2	GREEN-YELLOW-RED-CRIMSON	––++	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	––+–	8222	5	2.30	–
H2	GREEN-YELLOW-RED-CRIMSON	–––+	8222	0	0.00	–
H2	GREEN-YELLOW-RED-CRIMSON	––––	8222	6720	–	–
H3	GREEN-YELLOW-RED-CRIMSON	++++	8170	1473	624.53	98.08
H3	GREEN-YELLOW-RED-CRIMSON	+++–	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	++–+	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	++––	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	+–++	8170	3	1.41	–
H3	GREEN-YELLOW-RED-CRIMSON	+–+–	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	+––+	8170	4	1.88	–
H3	GREEN-YELLOW-RED-CRIMSON	+–––	8170	4	1.88	–
H3	GREEN-YELLOW-RED-CRIMSON	–+++	8170	0	0.00	–

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Well	Categories	Group	Valid partitions	Count categories	Conc. per group [cp/µL]	% intact
H3	GREEN-YELLOW-RED-CRIMSON	–++–	8170	10	4.70	–
H3	GREEN-YELLOW-RED-CRIMSON	–+–+	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	–+––	8170	3	1.41	–
H3	GREEN-YELLOW-RED-CRIMSON	––++	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	––+–	8170	2	0.94	–
H3	GREEN-YELLOW-RED-CRIMSON	–––+	8170	0	0.00	–
H3	GREEN-YELLOW-RED-CRIMSON	––––	8170	6671	–	–

Robust integrity determination over a broad input range

Up to 5 targets can be accurately and precisely analyzed in one reaction without extensive sample dilutions between a total λ of 0.02 and 5. The analysis over a wide dynamic range is particularly important, especially for samples with unknown concentrations. Additionally, errors are introduced with each pipetting and dilution step, and the stability or interaction of nucleic acids with certain plastic materials can also make a difference in highly diluted states.

A synthetic gBlock containing two targets, one at the 5' and one at the 3' end, was serially diluted in 10x steps (from 8000 copies/µL down to 8 copies/µL).

Quantification and integrity analysis were performed in a 2-plex reaction on a QIAcuity dPCR System. The integrity values were comparable across the four measured orders of magnitude, demonstrating that the analysis can be performed over a wide concentration range (Appendix Figure 3). This is mainly due to a low number of random co-localization events occurring during the calculation of template integrity, which occurs more frequently at higher λ values.

gBlock input into reaction (copies/µL)	Integrity 5'–3' (%)
8	72.3
80	72.0
800	72.4
8000	71.2

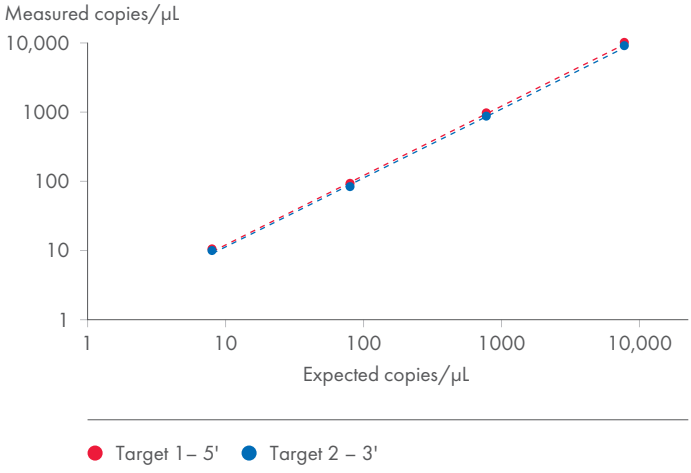


Figure 3. Samples of unknown concentrations can be analyzed within a broad dynamic range. Quantification of a serial dilution of a gBlock containing two targets of interest was measured in a 2-plex reaction on QIAcuity 8.5K Nanoplates using the QIAcuity OneStep Advanced Probe Kit. Concentrations of the dilution series were expected to range from 8 copies/µL up to 8000 copies/µL (10x dilutions). Integrity values (%) were analyzed using the QIAcuity Software Suite version 2.5 and are indicated for all dilution steps.

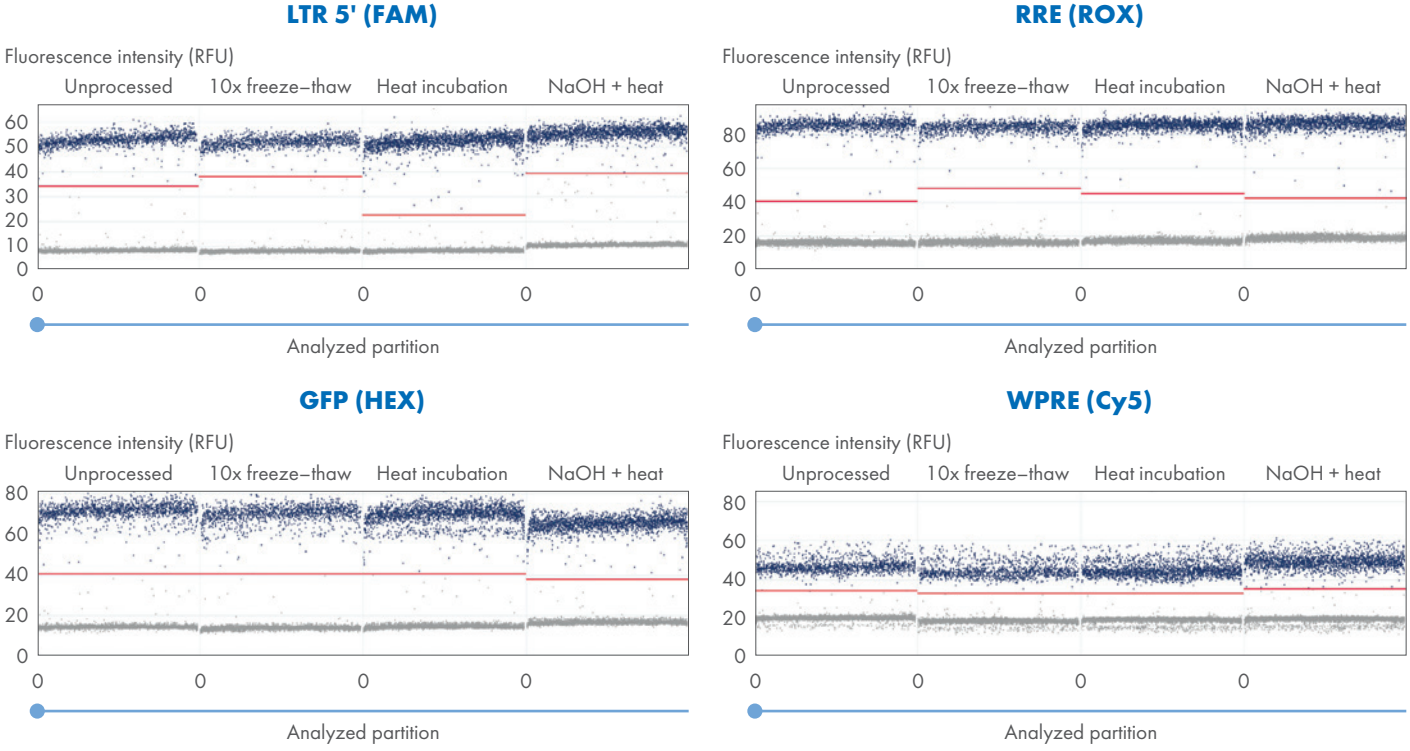
The QIAcuity software integrity feature provides insights into DNA stability

Storage of nucleic acids or various treatments (e.g., chemical, enzymatic, physical) can affect intactness. Digital PCR enables the analysis of the integrity of DNA templates.

The WHO standard for lentiviral vector integration was assessed for integrity using digital PCR. Genomic DNA (gDNA) was reconstituted and either directly analyzed or processed first before examination. The four lentiviral targets (LTR, RRE, GFP, and WPRE) were amplified in a 4-plex reaction. Both quantification and integrity analysis were repeated in 8 technical replicates. The analysis was performed using the QIAcuity Software Suite version 2.5.

The integrity value of the unprocessed gDNA was 72%. This value decreased after DNA processing (10x freeze-thaw, heat incubation, NaOH + heat incubation) (Appendix Figure 4).

Thus, the QIAcuity integrity analysis is an optimal tool for easily monitoring the quality of DNA synthesis, the effects of specific treatments on the sample or generally assessing the suitability of DNA starting materials and quality, just to name a few examples. It is important to note that the quantification may remain unchanged, whereas the integrity can already be affected as a result of, e.g., storage or processing.



WHO standard processing	Integrity LTR-RRE-GFP-WPRE (%)
unprocessed	72
10 freeze-thaw cycles	58
Heat incubation	57
NaOH + heat incubation	54

Figure 4. Storage and processing can impact DNA integrity.
The lyophilized WHO 1st Reference Reagent for Lentiviral Vector Integration Site Analysis (WHO, (NIBSC Code 18/144)) was reconstituted as recommended by the manufacturer and digested with PvuI before it was both directly used for quantification via dPCR (unprocessed) or further processed before measurement. Further processed Reference Reagent gDNA samples were either exposed to 10 consecutive freeze-thaw cycles or heat treated for 10 min at 95°C with and without 100 mM NaOH. The measurement was performed in a 4-plex setup with the following assay targets: 5' LTR (FAM), RRE (ROX), GFP (HEX) and WPRE (Cy5). The dPCR reactions were set up in 8.5K Nanoplates using the QIAcuity OneStep Advanced Probe Kit. 1D scatterplots for all analyzed targets are shown. Genome integrity was analyzed using the QIAcuity Software Suite version 2.5.

Supplementary protocols

Application Note Figure 3:

An AAV2 transfer plasmid containing a CMV promotor, a GFP and a WPRE sequence was quantified both via dPCR and via ddPCR with and without stepwise addition of a second AAV2 transfer plasmid (spike-in) containing solely the CMV promotor region as target. The dPCR measurement was performed on the QIAcuity dPCR System in a 3-plex reaction in technical triplicates using the QIAcuity Probe PCR Kit (QIAGEN, Cat. No. 250101) and the following QIAcuity CGT dPCR Assays: dPCR CGT Assay CMV promoter (FAM) (QIAGEN, Cat. No. 250247), dPCR CGT Assay GFP (Cy5) (QIAGEN, Cat. No. 250238) and dPCR CGT Assay WPRE (HEX) (QIAGEN, Cat. No. 250240). dPCR reactions were set up as described in the dPCR CGT Assays Quick-Start Protocol with one or two plasmid(s) as template DNA and with the addition of 0.25 U/µL *Eco*RI-HF restriction enzyme (New England Biolabs, R3101) directly to the dPCR reaction mixes. The concentration of the transfer plasmid was kept constant in all reactions while the copy number of the spike-in plasmid was step by step increased by 250 copies/µL. The dPCR reaction mixes were incubated for 10 min at RT before they were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011) for subsequent analysis. Cycling conditions on the QIAcuity dPCR System comprised of a 95°C initial hot start for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds. Imaging was performed using QIAcuity standard settings with 500 ms exposure and gain six on all channels. Integrity results were analyzed using the QIAcuity Software Suite version 2.5.

Application Note Figure 4:

AAV2 standard material (eGFP) (supplier P) was processed using the CGT Viral Vector Lysis Kit (QIAGEN, Cat. No. 250272) following the CGT Viral Vector Lysis Kit Quick-Start Protocol without Proteinase K and subsequently quantified using the QIAcuity CGT dPCR Assays (QIAGEN, Cat. Nos. 250230–250256) on a QIAcuity dPCR System. The processed AAV2 DNA was analyzed in a 5-plex setup in technical triplicates using the QIAcuity Probe PCR Kit (QIAGEN, Cat. No. 250101) and the following QIAcuity CGT dPCR Assays: dPCR CGT Assay GFP (FAM) (QIAGEN, Cat. No. 250236), dPCR CGT Assay CMV enhancer (Atto550) (QIAGEN, Cat. No. 250252 for FAM), dPCR CGT Assay CMV promoter (Cy5) (QIAGEN, Cat. No. 250249), dPCR CGT Assay WPRE (HEX) (QIAGEN, Cat. No. 250240) and dPCR CGT Assay hGH polyA (TexasRed) (QIAGEN, Cat. No. 250250-250251). dPCR reactions were set up as described in the dPCR CGT Assays Quick-Start Protocol, and Anza 93 HpaII restriction enzyme (Thermo Fisher Scientific, Cat. No. IVGN0936) was included in the dPCR reaction mixes at a concentration of 0.125 U/µL. dPCR reaction mixes were incubated for 10 min at RT before they were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011). Cycling conditions on the QIAcuity dPCR System consisted of a 95°C initial hot start for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds. Imaging was performed with QIAcuity standard settings at a 500 ms exposure and gained six on all channels. Vector genome quantification and integrity results were analyzed using the QIAcuity Software Suite version 2.5.

Application Note Figure 5:

A sample of AAV2 standard material (eGFP) (supplier P) was processed using the CGT Viral Vector Lysis Kit (QIAGEN, Cat. No. 250272) following the CGT Viral Vector Lysis Kit Quick-Start Protocol without Proteinase K to be then quantified using the QIAcuity CGT dPCR Assays (QIAGEN, Cat. Nos. 250230–250256) on a QIAcuity dPCR System as well as on a QX200 System (Bio-Rad). For the measurements on the QIAcuity dPCR System the viral vector lysate was measured in

4-plex reactions in technical triplicates using the QIAcuity Probe PCR Kit (QIAGEN, Cat. No. 250101) and the following QIAcuity CGT dPCR Assays: dPCR CGT Assay GFP (FAM) (QIAGEN, Cat. No. 250236), dPCR CGT Assay CMV enhancer (Cy5) (QIAGEN, Cat. No. 250254), dPCR CGT Assay WPRE (HEX) (QIAGEN, Cat. No. 250240) and dPCR CGT Assay hGH polyA (TexasRed) (QIAGEN, Cat. No. 250251 for HEX). The QIAcuity dPCR reactions were set as described in the dPCR CGT Assays Quick-Start Protocol with the addition of Anza 93 HpaII restriction enzyme (Thermo Fisher Scientific, IVGN0936) to the dPCR reaction mixes at a concentration of 0.125 U/µL. dPCR reaction mixes were incubated for 10 min at RT before they were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011). Cycling conditions on the QIAcuity dPCR System consisted of a 95°C initial hot start for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds. Imaging was performed with QIAcuity standard settings at a 500 ms exposure and gained six on all channels. Vector genome quantification and integrity results were analyzed using the QIAcuity Software Suite version 2.5. The quantitation of the extracted AAV DNA sample via ddPCR on the QX200 System (Bio-Rad) was performed in a duplex reaction in technical triplicates using the ddPCR Supermix for Probes (Bio-Rad, Cat. No. 1863023) together with the CGT dPCR Assay for CMV enhancer (HEX) (QIAGEN, Cat. No. 250253) and the dPCR CGT Assay hGH polyA (FAM) (QIAGEN, Cat. No. 250250) following manufacturers' settings and recommendations. Genome integrity for the ddPCR run was analyzed using the Bio-Rad QX Manager Software version 2.1.

Application Note Figure 6:

An unpurified in-process sample of AAV2 was processed directly after harvest with the CGT Viral Vector Lysis Kit (QIAGEN, Cat. No. 250272) following the CGT Viral Vector Lysis Kit Quick-Start Protocol (without Proteinase K) - QIAGEN before it was analyzed via dPCR on the QIAcuity dPCR System. The extracted AAV2 DNA was serially diluted (5-fold dilutions) and measured in a 3-plex setup in 4 technical replicates using the QIAcuity Probe PCR Kit (QIAGEN, Cat. No. 250101) and the following QIAcuity CGT dPCR Assays: dPCR CGT Assay GFP (Cy5) (QIAGEN, Cat. No. 250238), dPCR CGT Assay CMV enhancer (FAM) (QIAGEN, Cat. No. 250252) and dPCR CGT Assay CMV promoter (HEX) (QIAGEN, Cat. No. 250248). The dPCR reactions were set up as described in the dPCR CGT Assays Quick-Start Protocol with the addition of Anza 93 HpaII restriction enzyme (Thermo Fisher Scientific, Cat. No. IVGN0936) to the dPCR reaction mixes at a concentration of 0.125 U/µL. After incubation for 10 min at RT dPCR reactions were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011) and analyzed using the following cycling conditions on the QIAcuity dPCR System: Initial hot start at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds each. Imaging was performed with QIAcuity standard settings at a 500 ms exposure and gained 6 for all channels used. QIAcuity Software Suite version 2.5 was used to analyze the concentration and integrity values of measured samples.

Appendix Figure 2:

An AAV2 transfer plasmid containing a CMV enhancer, a CMV promoter, a GFP and a WPRE sequence flanked by ITRs was analyzed via dPCR. dPCR measurement was performed on the QIAcuity dPCR System in a 4-plex setup using the QIAcuity Probe PCR Kit (QIAGEN, Cat. No. 250101) and the following QIAcuity CGT dPCR Assays: dPCR CGT Assay CMV enhancer (HEX) (QIAGEN, Cat. No. 250253), dPCR CGT Assay CMV promoter (ROX) (QIAGEN, Cat. No. 250247 for FAM), dPCR CGT Assay GFP (Cy5) (QIAGEN, Cat. No. 250238) and dPCR CGT Assay WPRE (FAM) (QIAGEN, Cat. No. 250239). dPCR reactions were set up in 48 technical replicates following the dPCR CGT Assays Quick-Start Protocol with 0.25 U/µL *EcoR*I-HF restriction enzyme (New England Biolabs, R3101) in the reaction mixes for linearization of the transfer plasmid prior target amplification. After 10 min of incubation ▷

at RT, dPCR mixes were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011) and measured on the QIAcuity dPCR System using the following cycling conditions: a 95°C initial hot start for 2 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds each. Imaging was performed with the QIAcuity standard settings of 500 ms exposure and a gain of 6 on all four channels used. Quantification and integrity results were analyzed using the QIAcuity Software Suite version 2.5.

Appendix Figure 3:

A gBlock comprising two target sequences was quantified in a serial dilution in a 2-plex setup on the QIAcuity dPCR System with the 5' target detected in the FAM channel and the 3' target in the HEX channel. Reactions were set up in duplicates using the 4x OneStep Advanced Probe Master Mix (QIAGEN, Cat. No. 250131) as recommended by the manufacturer with 0.4 µM primer and 0.2 µM probe. They were transferred to QIAcuity 8.5K Nanoplates (QIAGEN, Cat. No. 250011) for measurement. Cycling conditions were as follows: 2 min 95°C for the initial hot start and subsequently 40 cycles of denaturation at 95°C for 1 5 seconds and a combined annealing and extension step at 60°C for 30 seconds each. For imaging, QIAcuity standard conditions were used (500 ms exposure, gain 6), and dPCR results were analyzed using the QIAcuity Software Suite version 2.5.

Appendix Figure 4:

The lyophilized WHO 1st Reference Reagent for Lentiviral Vector Integration Site Analysis (WHO, (NIBSC Code 18/144)) was reconstituted as recommended by the manufacturer before it was both directly used for quantification via dPCR (unprocessed) or further processed before measurement. Further processed Reference Reagent gDNA samples were either exposed to 10 consecutive freeze-thaw cycles or heat treated for 10 min at 95°C with and without the presence of 100 mM NaOH.

The measurement was performed in a 4-plex setup with the following assay targets: 5' LTR (FAM), RRE (ROX), GFP (HEX) and WPRE (Cy5). The dPCR reactions were set up with eight technical replicates using the 4x OneStep Advanced Probe Master Mix (QIAGEN, Cat. No. 250131) with 0.8 µM primer and 0.4 µM probe and with the addition of 0.025U/µL of the restriction enzyme Anza 27 PvuI (Thermo Fisher Scientific, IVGN0274) directly to the dPCR reactions. After setup, dPCR reaction mixes were incubated for 10 min at RT before they were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011) and analyzed via dPCR on the QIAcuity dPCR System. Cycling conditions consisted of a 95°C hot start for 5 minutes followed by 40 cycles at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds. Afterwards, imaging was performed with QIAcuity standard conditions of 500 ms exposure and gained six on all four channels analyzed. The genome integrity of unprocessed and processed gDNA was analyzed using the QIAcuity Software Suite version 2.5.

Application Note Table 1:

Both a sample of AAV2 standard material (eGFP) (supplier P) and an unpurified in-process sample of AAV2 were processed with the CGT Viral Vector Lysis Kit (QIAGEN, Cat. No. 250272) following the CGT Viral Vector Lysis Kit Quick-Start Protocol without Proteinase K before they were analyzed via dPCR on the QIAcuity dPCR System. The viral vector lysates were once analyzed directly after the lysis procedure (fresh) and after a storage period of 1 week at either –20°C or at 4°C. dPCR measurements of biological duplicates were performed in 3-plex reactions with three technical replicates using the QIAcuity Probe PCR Master Mix (QIAGEN, Cat. No. 250101) and QIAcuity CGT dPCR Assays (Cat. Nos. 250230–250256). dPCR reactions were set up following the dPCR CGT Assays Quick-Start Protocol, including Anza 93 HpaII restriction enzyme (Thermo Fisher Scientific, Cat. No. IVGN0936) at a concentration of 0.125 U/µL. After an incubation of 10 min at RT dPCR

reactions were transferred to an 8.5K Nanoplate (QIAGEN, Cat. No. 250011) and analyzed on the QIAcuity dPCR System using PCR cycling conditions as follows: Initial hot start at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 seconds and a combined annealing and extension step at 60°C for 30 seconds each. Imaging was performed using QIAcuity standard settings at a 500 ms exposure and gain 6 for all channels used. Titer quantification and integrity determination of measured samples were carried out using QIAcuity Software Suite version 2.5.

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