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Short Research Communication

External RNA Controls Consortium Beta Version Update

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Abstract

Spike-in RNAs are valuable controls for a variety of gene expression measurements. The External RNA Controls Consortium developed test sets that were used in a number of published reports. Here we provide an authoritative table that summarizes, updates, and corrects errors in the test version that ultimately resulted in the certified Standard Reference Material 2374. We have noted existence of anti-sense RNA controls in the material, corrected sub-pool memberships, and commented on control RNAs that displayed inconsistent behavior.

Key words: ERCC, spike-in controls, external RNA controls, NIST standard reference materials.

Advances in gene expression profiling technologies not only make it possible for individual groups to ask genome-wide questions, but properly controlled experiments with well-described metadata can be used over and over to make discoveries not envisaged by the data producers. Making these data robust and durable is greatly augmented by standard reference materials. The National Institute of Standards and Technology (NIST) as a part of the External RNA Controls Consortium (ERCC) developed 176 DNA plasmids that can be used as templates for RNA controls [1-3]. NIST Standard Reference Material (SRM) 2374 is a library composed of a subset of 96 plasmids. These same materials were used for commercially available ERCC RNA spike-in mixtures (Ambion/Thermo Fisher Scientific, Waltham, MA), which are formulations of 92 RNA molecules derived from the plasmids. The Commercial collection does not include ERCC-00007, -00018, -00023, and, -00128. One of the test versions that led to the SRM contained 96 RNA sequences transcribed from the plasmids, quantified, and mixed to form defined pools to be added to unknowns in transcription profiling experiments by array, sequencing, PCRs, or other assays. These test pools were widely distributed and were used by the human and model organisms Encyclopedia of DNA Elements projects [4, 5].

When "spiked" into an individual RNA sample, the readout from a single pool of ERCC controls can be used as a ruler. Each pool is designed to have dynamic range of 2^{20}. It is noteworthy that the actual linear range of their measurement depends on experimental platforms. Distribution of spike-in measurement fits to straight linear line in RNA-Seq and a monotonic sigmoidal pattern against actual abundance in microarrays or bead-arrays [6], consistent with data compression in hybridization-based techniques [7].

Addition of a single pool of ERCC controls

generates useful information, but their use can be enhanced when different pools of spike-in controls from different samples are directly compared. The "pools" of ERCC controls were mixed from multiple "subpools", such that comparisons between "subpools" that belong to different "pools" generate abundance ratios that can be used as differential expression standards. There were two distinct sets of pools in the test version. Pools 12-15 follow a modified Latin-square design, using 5 different subpools (A-E). The numbers of RNA molecules in subpool A are equal in pools 12-15, and thus subpool A molecules generate a constant 1-to-1 proportion between the pools. Subpools B-E have differing molar concentrations that produce a trend in relative abundance across the pools of 1, 1.5, 2.5, 4-fold. For example, if pool 12 and pool 13 were used for two different samples, the log2-transformed ratios between different subpools will be 0, -0.585, -0.687, -0.737, and 2 [6]. The second set of pools, 78A and 78B, provide a pair of samples with reciprocal changes in relative abundance, i.e. 1.5-fold up and down, producing log2 transformed ratios of 0, 0.585, and -0.585.

While production of the spike-in control RNAs was tightly controlled, it was a test set, and there are multiple cases where measurements of spike-in molecules do not match the original description and/or expectations. In this short note, we summarize data outlining problematic ERCC spike-ins. This information should be used in re-evaluating datasets using the test version, as well as any future work that may use remaining aliquots in circulation (Table 1, and Supplementary Material for more details).

The plasmid DNAs were sequenced and deposited in GenBank, however, the *in vitro* transcribed RNAs were not sequenced except during testing in RNA-Seq experiments. These experiments made it clear that seven ERCC controls had the complementary sequence indicating that the transcripts were from the other strand (ERCC-00009, -00014, -00057, -00059, -00099, -00108, and -00116). As a result, these spike-in controls would not be measurable in hybridization-based assays [6]. Similarly, they would not be aligned in a strand-specific RNA-Seq analysis unless strand specificity was "turned off" in read quantification steps, or complementary sequences were provided for alignment. Additionally, plasmids are replicated in bacteria, where errors can be introduced. Differences in the sequences of the actual RNAs and the plasmids used for transcript templates are known [4], suggesting that such mutations occurred during plasmid propagation in the test set. The certified values of SRM 2374 are the sequences of the plasmids as distributed in the final set, and were determined by exhaustive sequencing [8].

There were instances of pooling errors in the test set. From multiple experiments that used 78A and 78B, we recognized that ERCC-00085 behaves like Subpool "C", rather than the intended Subpool "B". Therefore, when pools 78A and 78B were compared, ERCC-00085 displayed 33.3% increased fold changes than the original description. We have not detected ERCC-00084 in our experiments and it is possible that this RNA was prepared from ERCC-00085 plasmid DNA, effectively increasing the measurement of ERCC-00085. Similarly, we have corrected pool membership of ERCC-00113 from Subpool C to Subpool D from pools 12-15. ERCC-00073 and ERCC-00144 did not provide accurate measurements [4, 6]. One reason for poor measurement may be due to the molecular properties of individual spike-in RNA species (e.g. size and secondary structure). Additionally, a previous study pointed out discrepancy in ERCC-00116 measurements between poly-dT based mRNA enrichment and rRNA depletion protocols [3, 9]. The polyA tails on the ERCC spike-ins are not optimal for PolyA+ selection, and using them prior to library production is not recommended [4]. While there could well be additional instances of unexpected behavior of ERCC spike-in measurements, the information we provide here explains the unexpected ERCC behaviors that we have encountered to date.

Table 1. Summarized information on NIST distributed ERCC spike-in control test version.

ERCC Control	GenBank[a]	DNA[b]	Length (nt)[c]	% GC[c]	MW	Subpool in pool 12 to 15	Subpool in pool 78
ERCC-00002[a]	DQ459430	Syn	1061	51	341,162	B	B
ERCC-00003[a]	DQ516784	Mjan	1023	33	327,530	A	A
ERCC-00004[a]	DQ516752	Mjan	523	34	167,216	C	C
ERCC-00007[1]	EF011068	Bsub	1135	46	362,636	D	A
ERCC-00009[d]	DQ668364	Bsub	984	47	316,584	E	C
ERCC-00012	DQ883670	Syn	994	51	320,263	A	A
ERCC-00013[a]	EF011062	Bsub	808	43	261,415	B	B
ERCC-00014[a,d]	DQ875385	Mjan	1957	44	631,409	C	B
ERCC-00016	DQ883664	Syn	844	48	271,684	D	A

ERCC-00017[a]	DQ459420	Syn	1136	51	367,042	E	C
ERCC-00018[a,l]	EF011065	Bsub	1026	43	330,493	C	C
ERCC-00019	DQ883651	Syn	644	49	207,543	B	B
ERCC-00022	DQ855004	Syn	751	47	241,178	C	C
ERCC-00023[l]	DQ516744	Mjan	273	31	88,186	D	A
ERCC-00024	DQ854993	Syn	536	46	173,128	E	C
ERCC-00025[a]	DQ883689	Syn	1994	50	640,941	A	A
ERCC-00028[a]	DQ459419	Syn	1130	51	364,285	B	B
ERCC-00031[a]	DQ459431	Syn	1138	48	365,732	E	C
ERCC-00033	DQ516796	Mjan	2022	33	651,534	D	B
ERCC-00034[a]	DQ855001	Syn	1019	49	328,139	E	A
ERCC-00035[a]	DQ459413	Syn	1130	51	364,378	A	A
ERCC-00039	DQ883656	Syn	740	49	238,322	B	B
ERCC-00040[a]	DQ883661	Syn	744	53	239,738	C	B
ERCC-00041	EF011069	Bsub	1123	45	363,007	D	C
ERCC-00042[a]	DQ516783	Mjan	1023	39	325,750	E	B
ERCC-00043[a]	DQ516787	Mjan	1023	33	330,122	A	C
ERCC-00044[a]	DQ459424	Syn	1156	50	372,347	B	B
ERCC-00046[a]	DQ516748	Mjan	522	35	168,087	C	C
ERCC-00048	DQ883671	Syn	992	48	320,110	D	B
ERCC-00051	DQ516740	Mjan	274	34	88,356	C	A
ERCC-00053[a]	DQ516785	Mjan	1023	31	327,971	A	C
ERCC-00054	DQ516731	Mjan	274	37	88,966	B	B
ERCC-00057[d]	DQ668366	Bsub	1021	50	328,287	C	A
ERCC-00058[a]	DQ459418	Syn	1136	50	366,548	D	C
ERCC-00059[d]	DQ668356	Bsub	525	48	168,750	E	A
ERCC-00060[a]	DQ516763	Mjan	523	31	168,195	A	C
ERCC-00061[a]	DQ459426	Syn	1136	50	366,454	B	B
ERCC-00062[a]	DQ516786	Mjan	1023	31	328,505	C	A
ERCC-00067	DQ883653	Syn	644	47	207,451	D	A
ERCC-00069[a]	DQ459421	Syn	1137	50	366,664	E	A
ERCC-00071	DQ883654	Syn	642	48	206,115	A	C
ERCC-00073[g]	DQ668358	Bsub	603	47	193,958	B	B
ERCC-00074[a]	DQ516754	Mjan	522	35	167,539	C	A
ERCC-00075[a]	DQ516778	Mjan	1023	36	325,442	D	B
ERCC-00076[a]	DQ883650	Syn	642	50	206,436	E	B
ERCC-00077	DQ516742	Mjan	273	33	87,694	A	A
ERCC-00078	DQ883673	Syn	993	50	320,094	B	B
ERCC-00079	DQ883652	Syn	644	49	207,757	A	C
ERCC-00081[a]	DQ854991	Syn	534	49	172,323	D	A
ERCC-00083[a]	DQ516780	Mjan	1023	35	325,668	E	A
ERCC-00084[e]	DQ883682	Syn	994	50	320,445	A	C
ERCC-00085[e]	DQ883669	Syn	844	49	271,323	B	B
ERCC-00086[a]	DQ516791	Mjan	1020	32	328,632	C	B
ERCC-00092[a]	DQ459425	Syn	1124	50	361,716	D	B
ERCC-00095[a]	DQ516759	Mjan	521	37	166,307	E	B
ERCC-00096[a,i]	DQ459429	Syn	1107	51	356,565	A	C
ERCC-00097[a]	DQ516758	Mjan	523	36	167,189	B	B
ERCC-00098[a]	DQ459415	Syn	1143	51	368,970	C	C
ERCC-00099[a,d]	DQ875387	Bsub	1350	41	434,408	D	A
ERCC-00104[a,k]	DQ516815	Mjan	2022	33	647,370	E	C
ERCC-00108[d]	DQ668365	Bsub	1022	49	328,424	A	A
ERCC-00109[a]	DQ854998	Syn	536	46	172,925	B	B
ERCC-00111	DQ883685	Syn	994	47	319,359	C	A
ERCC-00112[a]	DQ459422	Syn	1136	47	364,932	D	C
ERCC-00113[a,f]	DQ883663	Syn	840	50	270,697	D	A
ERCC-00116[d,j]	DQ668367	Bsub	1991	50	639,986	B	B
ERCC-00117[a]	DQ459412	Syn	1136	51	365,757	C	A
ERCC-00120[a]	DQ854992	Syn	536	48	172,605	D	A
ERCC-00123[a]	DQ516782	Mjan	1022	36	324,911	E	C
ERCC-00126[a]	DQ459427	Syn	1119	51	359,790	A	C
ERCC-00128[a,l]	DQ459428	Syn	1133	48	364,405	B	B
ERCC-00130	EF011072	Bsub	1059	46	342,268	C	C
ERCC-00131[a]	DQ855003	Syn	771	47	248,276	D	A
ERCC-00134[a]	DQ516739	Mjan	274	31	88,594	E	C
ERCC-00136[a]	EF011063	Bsub	1033	42	333,363	A	C
ERCC-00137[a]	DQ855000	Syn	537	50	173,218	B	B
ERCC-00138[a]	DQ516777	Mjan	1022	33	327,949	C	C
ERCC-00142[a]	DQ883646	Syn	493	50	159,090	D	C
ERCC-00143	DQ668362	Bsub	784	49	251,705	E	A
ERCC-00144[h]	DQ854995	Syn	538	46	173,404	A	C
ERCC-00145	DQ875386	Bsub	1042	44	336,179	B	B
ERCC-00147[a]	DQ516790	Mjan	1023	36	331,125	C	A

ERCC-00148	DQ883642	Syn	494	49	159,911	D	B
ERCC-00150	DQ883659	Syn	743	47	239,128	E	A
ERCC-00154[a]	DQ854997	Syn	537	50	173,317	A	C
ERCC-00156	DQ883643	Syn	494	49	159,199	B	B
ERCC-00157[a]	DQ839618	Syn	1019	50	328,635	C	C
ERCC-00158[a]	DQ516795	Mjan	1021	34	328,797	D	A
ERCC-00160[a]	DQ883658	Syn	743	46	239,437	E	C
ERCC-00162[a]	DQ516750	Mjan	523	36	166,409	A	A
ERCC-00163[a]	DQ668359	Bsub	543	47	174,949	B	B
ERCC-00164[a]	DQ516779	Mjan	1022	37	324,758	C	A
ERCC-00165	DQ668363	Bsub	872	50	279,788	D	C
ERCC-00168[a]	DQ516776	Mjan	1024	34	326,399	E	A
ERCC-00170[a]	DQ516773	Mjan	1024	34	330,808	A	B
ERCC-00171	DQ854994	Syn	505	48	163,022	B	B

(a) Sequence mismatches between the GenBank entries and the resequenced RNAs (see [4]).
(b) Syn: De novo synthetic design, Mjan: Methanocaldococcus jannaschii, Bsub: Bacillus subtilis.
(c) Length and GC content include poly(A) sequence.
(d) Reversed (anti-sense) in Pools 12 -15.
(e) ERCC-00084 is not detected. E.g. ERCC-00084 and ERCC-00085, may have both been prepared from ERCC-00085 plasmid. ERCC-00085 behaves as C in some batches of Pool 78A and 78B.
(f) Corrected Pool membership to D and corrected Pool concentrations accordingly.
(g) Poor performing.
(h) Consistently under-reports abundance.
(i) Consistently over-reports abundance in Pools 78A and 78B.
(j) Particularly unsuitable for polyA+ isolation.
(k) ERCC-00104 has a length of either 2202 nt or 2203 nt.
(l) Not present in current commercial collections.

Supplementary Material

Supplemental file 1.
http://www.jgenomics.com/v04p0019s1.csv

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Abbreviations

ERCC - External RNA Controls Consortium, NIST - National Institute of Standards and Technology, SRM - Standard Reference Material, ENCODE - Encyclopedia of DNA Elements.

Competing Interests

The authors declare no competing interests.

References

1. Baker SC, Bauer SR, Beyer RP, Brenton JD, Bromley B, Burrill J, Causton H, Conley MP, Elespuru R, Fero M *et al*: The External RNA Controls Consortium: a progress report. *Nature methods* 2005, 2(10):731-734.
2. ERCC: Proposed methods for testing and selecting the ERCC external RNA controls. *BMC genomics* 2005, 6:150.
3. Munro SA, Lund SP, Pine PS, Binder H, Clevert DA, Conesa A, Dopazo J, Fasold M, Hochreiter S, Hong H *et al*: Assessing technical performance in differential gene expression experiments with external spike-in RNA control ratio mixtures. *Nature communications* 2014, 5:5125.
4. Jiang L, Schlesinger F, Davis CA, Zhang Y, Li R, Salit M, Gingeras TR, Oliver B: Synthetic spike-in standards for RNA-seq experiments. *Genome research* 2011, 21(9):1543-1551.
5. [Internet] https://genome.ucsc.edu/ENCODE/protocols/dataStandards/
6. Pine PS, Munro SA, Parsons JR, McDaniel J, Lucas AB, Lozach J, Myers TG, Su Q, Jacobs-Helber SM, Salit M: Evaluation of the External RNA Controls Consortium (ERCC) reference material using a modified Latin square design. *BMC Biotechnol* 2016, 16(1):54.
7. Malone JH, Oliver B: Microarrays, deep sequencing and the true measure of the transcriptome. *BMC biology* 2011, 9:34.
8. [Internet] Standard reference material 2374; DNA sequence library for external RNA controls. https://www-s.nist.gov/srmors/certificates/view_certGIF.cfm?certificate=2374
9. Qing T, Yu Y, Du T, Shi L: mRNA enrichment protocols determine the quantification characteristics of external RNA spike-in controls in RNA-Seq studies. *Science China Life sciences* 2013, 56(2):134-142.