Supplementary Figures and Tables

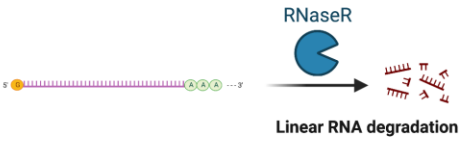
1. Total RNA extraction, DNAse treatment and quantification

•Phenol-Chlorophorm extraction •Column-based kit extraction

•DNAse treatment is mandatory for preventing unspecific amplification down the line

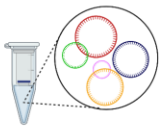
2. RNAse R treatment

- **Minimum starting RNA concentration:**
  1 µg of total RNA
- **Enzyme input:**
  10 units of RNAse R/reaction + Provided buffer
- **Incubation:**
  1 hr at 37°C



! Always include a mock treated control (no added enzyme) for each investigated sample, in order to asses the efficecny of the RNAse R treatment

3. RNA cleanup



• Column kit based purification of circRNA enriched fraction is required for the removal of degraded RNA fragments and other contaminants from the RNAse R treatment

Reccomended kit : RNAeasy cleanup kit (Qiagen)

4. RNA re-quantification and Bioanalyzer quality control (optional)

• Fluorimetric quantification is reccomended due to higher accuracy (Qubit)

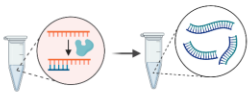
• Depending on input, a significant decrease in RNA concentration following the multiple processing steps is expected

5. cDNA synthesis

- **Starting concentrations**: 30 < ng

! Random hexamers should be used, and not OligoDTs

- **Reccomended kits:**
  AzuraFlex™ cDNA Kit
  Applied Biosystems™High-Capacity cDNA Kit



6. Primer validation

• Previously desgined primers should be validated via PCR and agarose gel electrophoresis, and the DNA purified from bands corresponding to the expected size of the amplicon can be sequenced to validate the expected amplified region

• A single peak from melting temperature resulted from the qPCR melting curve analysis can also confirm the specificty of the primers

! In some cases, a temperature gradient is required in order to confirm the correct annealing temperature of your

7. qPCR and circRNA enrichment analysis

• A standard curve of 5 cDNA dillutions is reccomended to determine the required amount for an efficient amplification

• The expression level of the parental gene (or a linear mRNA) should be investigated to determine both RNAse R treatment efficiency and calculate circRNA enrichment

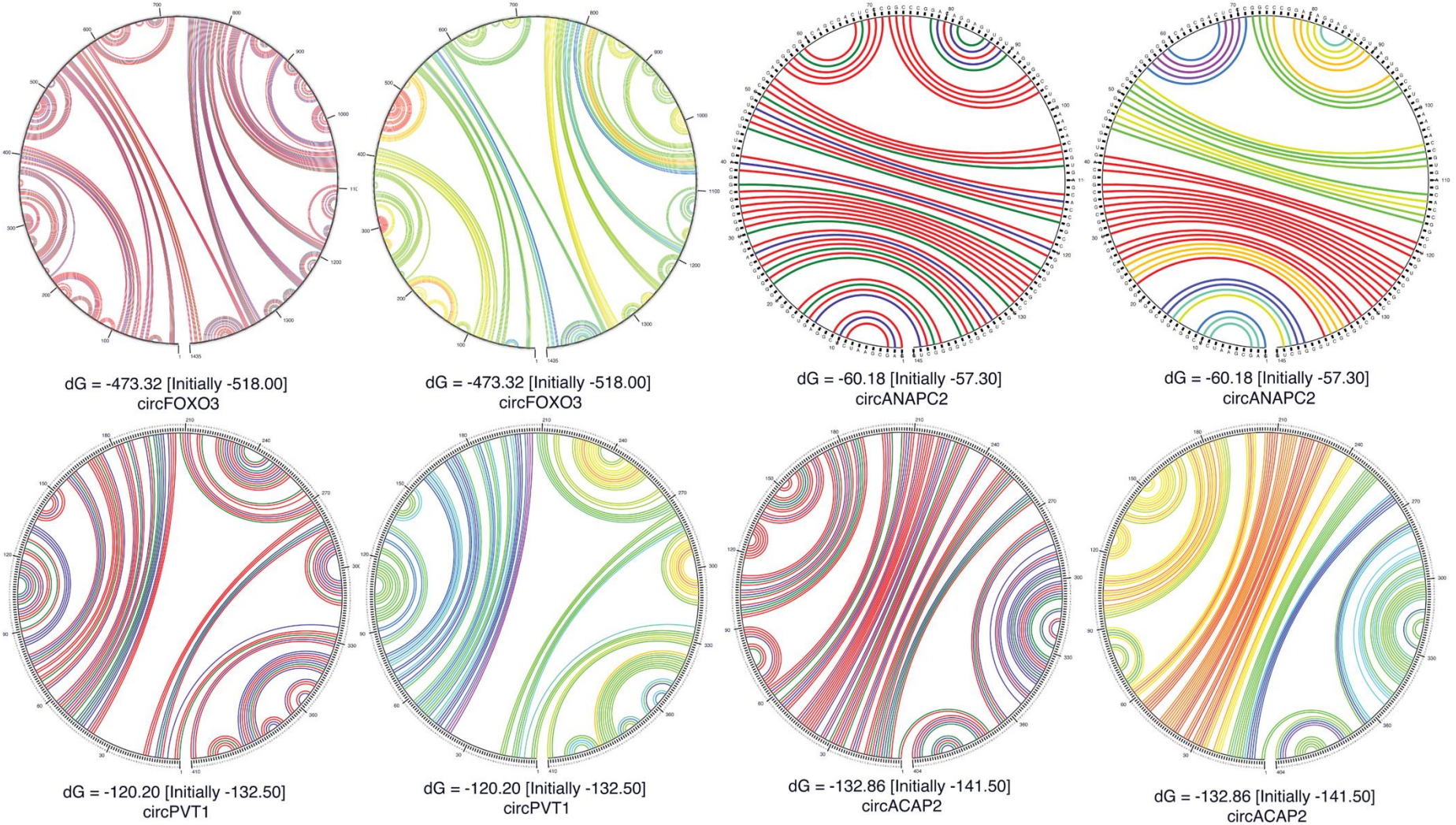
• Circular RNA enrichment following RNAse R treatment can be quanitifed as percentages, as by the formula indicated by Panda & Gorospe:

	Average CT Values		% of RNA in Control	% of RNA in RNase R treated sample
Target RNA	**Control**	**RNase R**	$2^{\Delta CT\ (Control-Control)} * 100$	$2^{\Delta CT\ (Control-RNase\ R)} * 100$
X mRNA	25,98	29,06	100	11.81
X circRNA	29,47	28,93	100	145.44

! Percentages can vary based on the transcript's specific susceptibility to RNAse R and circRNA enrcihment levels

! Linear RNAs should have a more significant decrease in CT values in comparison to the investigated circRNAs

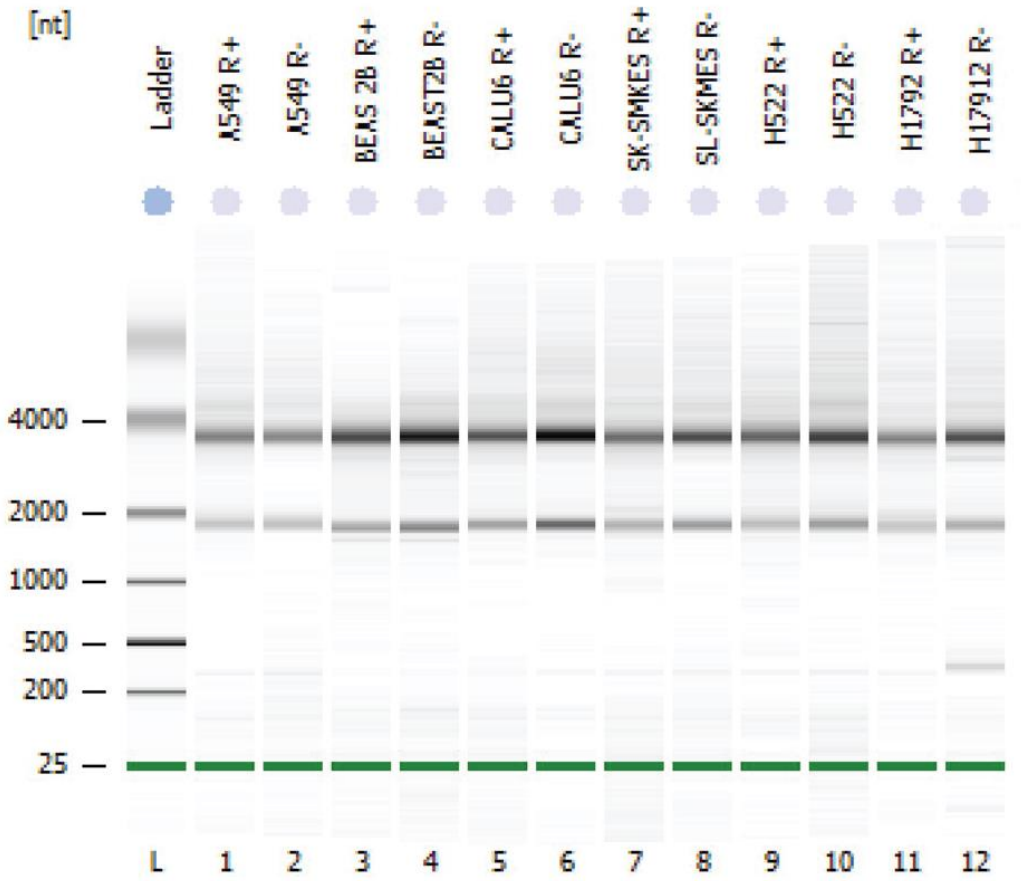
Supplemental Figure 1. Detailed step-by-step protocol for the techniques and steps required for the analysis of circRNA using qPCR



Supplemental Figure 2. Secondary structure formation prediction based on nucleotide complementarity (left, for each circRNA), the thermodynamic probability of their interaction (right, legend for interaction probability on far right) and calculated standard free energies (dG)

Supplemental Table 1. Nucleotide and GC% composition of the circRNAs.

Transcript	Total Bases	A	C	G	U	GC%
circFOXO3	1435	333	441	369	292	56,4
circPVT1	410	90	92	116	112	50,7
circANAPC2	145	20	37	65	23	70,3
circACAP2	410	82	104	125	93	56,7



Supplemental Figure 3. Bioanalyzer electrophoresis gel summary of RNAse R treated and untreated samples after cleanup

Supplemental Table 2. Primers utilized in the study

Circular RNA	Accession	Amplicon (bp)	Regions	Forward	Reverse
circFOXO3	NM_001455	183	Exon 2	GTGGGGAACTTCACTGGTGCTAAG	GGGTTGATGATCCACCAAGAGCTCTT
circPVT1	NR_003367	118	Exon 2	CTCTTCCTGGTGAAGCATCTG	CTGACAGGCACAGCCATCT
circANAPC2	NM_013366	162	Exon 3-1	AGAGGATGGAGGACCGTTG	TCCAGGCCACTAACAACTCC
circACAP2	NM_012287	229	Exon 3-1	TAATGATGCTGTCGTTGAGC	CCTTCAGACACTCCTCGAAA
FOXO3	NM_001455			GCAAGAGCTCTTGGTGGA	TGGGGCTGCCAGGCCACTTGGAGAG
PVT1	NR_003367			TGGAATGTAAGACCCCGACTCT	GATGGCTGTATGTGCCAAGGT
ANAPC2	NM_013366			TATGTTGCGCGGAGTCTTGT	CCAGCACTGTTGCTTGTCAC
ACAP2	NM_012287			TCCAGGAGCCGCACTTCTA	CTCCGGGATGTGGATCTTCA

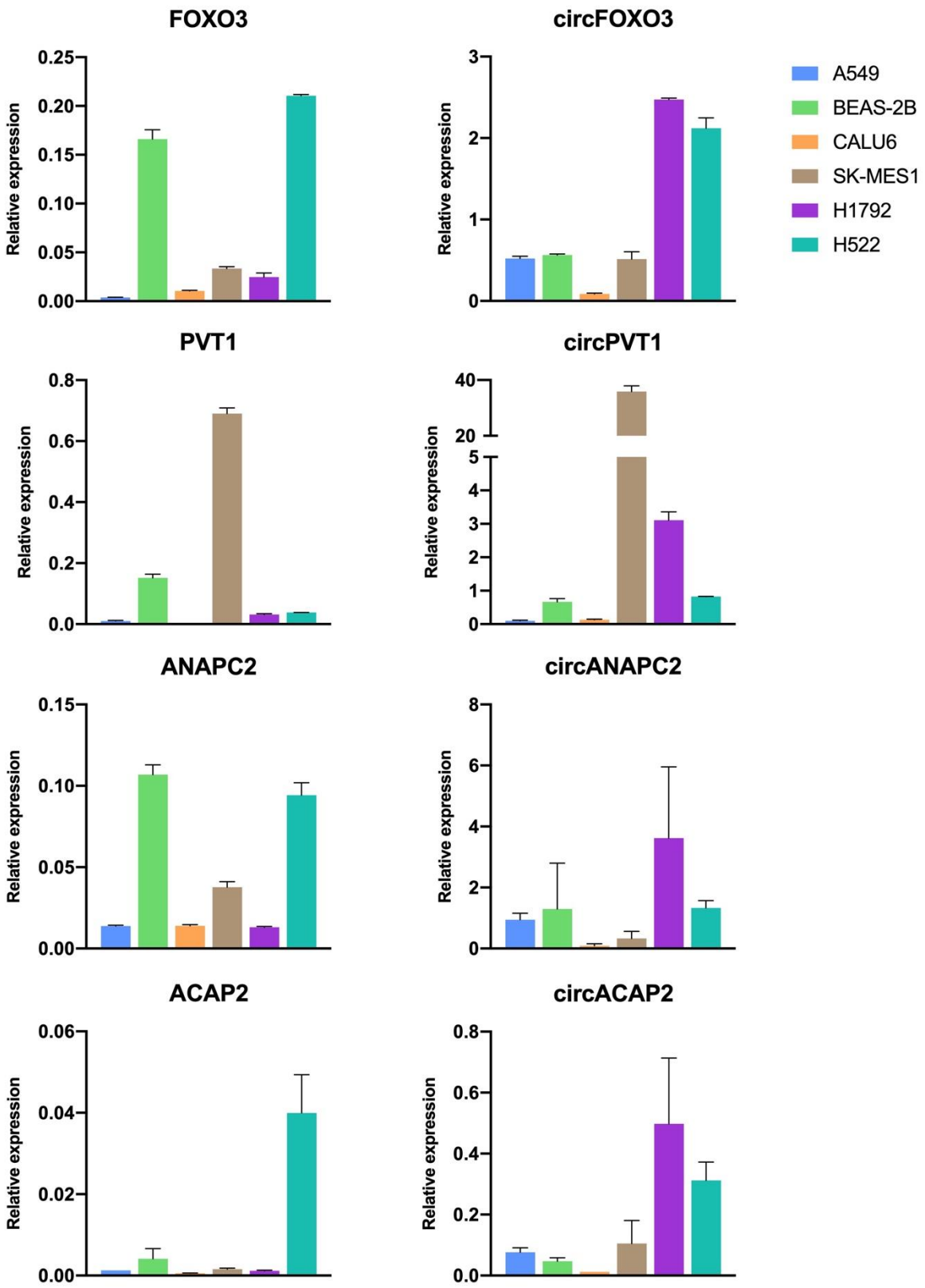
GAPDH	NM_002046	GTCTCCTCTGACTTCAACAGCG	ACCACCCTGTTGCTGTAGCCAA

Supplemental Table 3. Sample RNA concentrations following processing steps

Sample Characteristics			Concentration after RNAse Treatment and Cleanup				
Nr.	**Name**	**Starting Concentration (ng/uL)**	**RNAse R**	**Nanodrop (ng/uL)**	**Qbit (ng/uL)**	**RIN**	**cDNA Input**
1	A549	332	+	30.2	9,2	9.70	100
2	A549		-	10,2	3,4	9.4	
3	BEAS2B	220	+	30,4	11,26	9.2	
4	BEAS2B		-	13.2	4,4	9.9	
5	CALU6	566	+	30.6	3,76	9.9	
6	CALU6		-	10.3	2,94	10	
7	SK-MES1	272	+	29.1	3,4	9.80	
8	SK-MES1		-	9,6	4,94	10	
9	H522	174	+	27	7,96	8.8	
10	H522		-	11,2	2,64	10	
11	H1792	240	+	33.3	10,2	9.40	
12	H1792		-	10,3	1,4	8.40	

Supplemental Table 4. Exemplified method of calculating post-RNAse R enrichment of circRNAs and linear transcripts based on averaged CT values derived from the qPCR analysis of A549 RNAse R treated and untreated control RNA samples

		Average CT values		% of RNA in Control	% of RNA in RNase R
Sample	**Target RNA**	**Control (RNAse R-)**	**RNase R+**	**2^ΔCT (Control-Control) * 100**	**2^ΔCT (Control-RNase R) * 100**
A549	FOXO3	28,367	33,186	100%	3,54%
	circFOXO3	29,133	29,050		105,88%
	PVT1	26,984	33,125		1,42%
	circPVT1	33,033	31,268		340,06%
	ANAPC2	26,562	31,968		2,36%
	circANAPC2	28,735	28,490		118,53%
	ACAP2	29,995	30,820		56,42%
	circACAP2	31,562	31,831		83,00%



Supplemental Figure 4. Comparative relative expression levels of parental genes (left) and circRNAs (right) in the investigated cell lines.

Supplemental Table 5 Name and characteristics of the utilized circRNA databases utilized in the study

	Name	circRNA Annotation	Primer Design Tool	miRNA Binding	RBP Binding	ORF Information	Reference	Comments
1	circBank	×	-	×	-	×	[33]	Offers information about circRNA conservation and m6a modifications
2	circBase	×	-	-	-	-	[32]	Extensive; Includes main curated circRNA datasets available; Provides scripts to identify circRNA in sequencing data
3	circInteractome	circBase annotation	×	×	×	-	[44]	Extensive tool-wise; Includes both divergent primer and siRNA design tools
4	EncoRI (starbase)	circBase annotations	-	×	×	-	[41]	Database focused on miRNA and lncRNA interactions; circRNA-miRNA interactions based on AGO CLIP-seq Data
5	Circ2GO	Genomic coordinates	-	×	-	-	[43]	Contains RNAseq data from 60 human lung cell lines and 50 lung adenocarcinoma (LUAD) cell lines. ; Transcript map and expression level heatmaps generating tools
6	CSCD	Genomic coordinates	-	×	×	×	[42]	Detailed information about circRNA structure, cellular localization, and alternative splicing influence on circRNA composition.

"×" - yes; "-"- no;