Optimisation of the let-7a iLock invader junction position

PBCV-1 DNA ligase failed to differentiate between closely related miRNAs (figure 6B). To establish Lock assay in this context, we designed iLocks for four *let-7* family isoforms (supplementary table 2). Using iLocks with symmetrical miRNA binding arms, *let-7f* and *let-7d* were specifically differentiated while *let-7a* miRNA was detected with 83 % specificity (supplementary figure S1), also generating 35 % background on *let-7e* miRNA. To see if the *let-7a* fidelity can be increased at higher temperatures, we incubated let-7 iLocks on *let-7e* miRNA in the 48 – 56 °C temperature range. According to our observations, there was no improvement in the specificity at higher temperatures; in fact, less signal was generated at 56 °C compared to lower temperatures (supplementary figure S2). We speculate that relatively low specificity of the let-7a probe on *let-7e* is probably due to two consecutive Gs', downstream of the -3 nucleotide mismatch. As a result, A/G mismatch (3 nucleotides upstream from a *let-7* central base) does not effectively hinder the formation of the invader structure that permits probe activation and misligation. To study how repositioning of the invader junction can influence the *let-7a/e* recognition, we designed new let-7a iLocks with the invader junction located at -3, -2, and -1 nucleotides upstream of the central base (called -3/-2/-1 respectively, supplementary figure S3). Considering the highest background for *let-7e* miRNA, we assessed fidelity of new let-7a iLock probes electrophoretically on the *let-7e* miRNA (supplementary figure S3). Misligation products were observed for the standard let-7a and let-7a -1 iLock, possibly due to the fact that *let-7e* mismatch did not hamper the invader structure formation. In let-7a -3 iLock, the invader junction was shifted 3 nucleotides upstream and positioned precisely over a -3 nucleotide mismatch in the *let-7e* miRNA. No background signal was seen on *let-7e* miRNA.

Supplementary table 1. Oligonucleotide sequences used in PBCV-1 DNA ligase genotyping fidelity

Name	*5' modification*	*Sequence (5'- 3')*
benchm_templ_C*		UCUCGCUGUCAUcCCUAUAUCCUCG
benchm_templ_A*		UCUCGCUGUCAUaCCUAUAUCCUCG
benchm_templ_G*		UCUCGCUGUCAUgCCUAUAUCCUCG
benchm_templ_U*		UCUCGCUGUCAUuCCUAUAUCCUCG
3'T_PLP	Phos	ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGGT
3'G_PLP	Phos	ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGGG
3'A_PLP	Phos	ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGGA
3'C_PLP	Phos	ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGGC
5'T_PLP	Phos	TATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'G_PLP	Phos	GATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'A_PLP	Phos	AATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'C_PLP	Phos	CATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
3'T_*i*Lock		TATATCCCTATAT**T**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG**T**
3'G_*i*Lock		TATATCCCTATAT**G**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG**G**
3'A_*i*Lock		TATATCCCTATAT**A**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG**A**
3'C_*i*Lock		TATATCCCTATAT**C**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG**C**
5'T_*i*Lock		TATATCCCTATATG**T**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'G_*i*Lock		TATATCCCTATATG**G**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'A_*i*Lock		TATATCCCTATATG**A**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG
5'C_*i*Lock		TATATCCCTATATG**C**ATGACAGCGAGA(10A)*AGTAGCCGTGACTATCGACT*(10A)CGAGGATATAGG

Decorator probe	Cy3	*AGTAGCCGTGACTATCGACT*[a]

**: RNA oligonucleotides; lowercase: centrally located polymorphic site; (10A): linker; bolded, underlined: nucleotides participating in Invader structure formation; [a]: last four bases of the decorator probe were 2' O-methylRNA to prevent oligo hydrolysis by Phi29 polymerase.*

Supplementary table 2. Oligonucleotide sequences used in miRNA detection

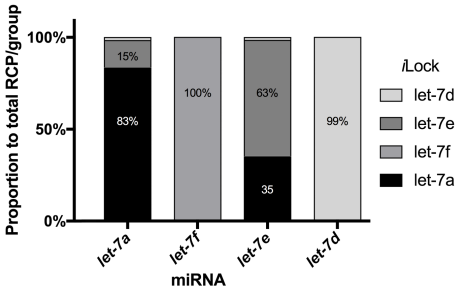
Name	*5' modification*	*Sequence (5'- 3')*
hsa_let-7a*		UGAGGUAGUAGGUUGUAUAGUU
hsa_let-7f*		UGAGGUAGUAGaUUGUAUAGUU
hsa_let-7e*		UGAGGUAGgAGGUUGUAUAGUU
hsa_let-7d*		aGAGGUAGUAGGUUGcAUAGUU
let-7a_PLP	Phos	CTACTACCTCA(7A)*CCTCAATGCACATGTTTGGCTCC(7A)*AACTATACAAC
let-7f_PLP	Phos	CTACTACCTCA(7A)*CCTCAATGCACATGTTTGGCTCC(7A)*AACTATACAAt
let-7e_PLP	Phos	CTcCTACCTCA (7A)*CCTCAATGCACATGTTTGGCTCC(7A)*AACTATACAAC
let-7d_PLP	Phos	CTACTACCTCt (7A)*CCTCAATGCACATGTTTGGCTCC(7A)*AACTATgCAAC
let-7a_iLock		CGCGTGTCGTTGC**C**CTACTACCTCA(10A)*CCTCAATGCACATGTTTGGCTCC*(10A)AACTATACAA**C**
let-7a-3_-iLock		CGCGATGCGATAC**A**CTACCTCA(10A)*CCTCAATGCACATGTTTGGCTCC*(10A)AACTATACAACCT**A**
let-7f_iLock		CGCGTGTCGTTGC**T**CTACTACCTCA(10A)*CCTCAATGCACATGTTTGGCTCC*(10A)AACTATACAA**T**
let-7e_iLock		CGCGTGTCGTTGC**C** CTCCTACCTCA(10A)*CCTCAATGCACATGTTTGGCTCC*(10A)AACTATACAA**C**
let-7d_iLock		CGCGTGTCGTTGC**C** CTACTACCTCT(10A)*CCTCAATGCACATGTTTGGCTCC*(10A)AACTATGCAA**C**
Decorator	Cy3	*CCTCAATGCACATGTTTGGCTCC*[a]

**: RNA oligonucleotides; lowercase: base substitutions in reference to the let-7a sequence; bolded, underlined: nucleotides participating in invader structure formation; [a]: last four bases of the decorator probe were 2' O-methylRNA to prevent oligo hydrolysis by Phi29 polymerase.*

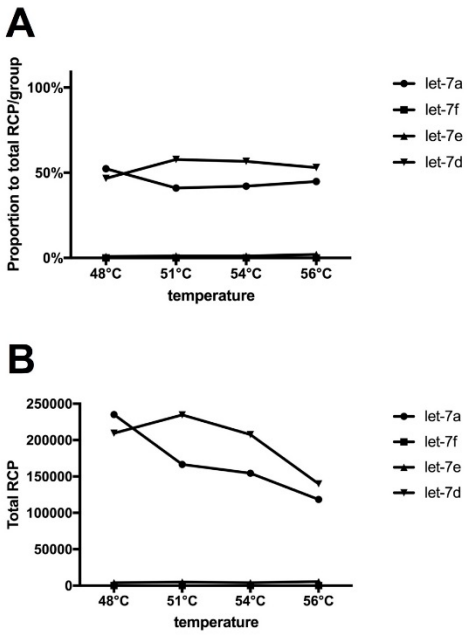
Supplementary table 3. Oligonucleotide sequences used in *KRAS* genotyping

Name	*5' modification*	*Sequence (5'- 3')*
hsa_KRASwt*		AACUUGUGGUAGUUGGAGCUgGUGGCGUAGGCAAGAGUGCC
KRASwt_PLP	Phos	AGCTCCAACTACCAC(10A)*AGTAGCCGTGACTATCGACT(10A)*CTTGCCTACGCCACC
KRAS_aGT_PLP	Phos	AGCTCCAACTACCAC(10A)*AGTAGCCGTGACTATCGACT(10A)*CTTGCCTACGCCACT
KRAS_tGT_PLP	Phos	AGCTCCAACTACCAC(10A)*AGTAGCCGTGACTATCGACT(10A)*CTTGCCTACGCCACA
KRAS_cGT_PLP	Phos	AGCTCCAACTACCAC(10A)*AGTAGCCGTGACTATCGACT(10A)*CTTGCCTACGCCACG
KRASwt_iLock		TATATCCCTATAT**C**AGCTCCAACTACCAC(10A)AGTAGCCGTGACTATCGACT(10A)CTTGCCTACGCCAC**C**
KRAS_aGT_iLock		TATATCCCTATAT**T**AGCTCCAACTACCAC(10A)AGTAGCCGTGACTATCGACT(10A)CTTGCCTACGCCAC**T**
KRAS_tGT_iLock		TATATCCCTATAT**A**AGCTCCAACTACCAC(10A)AGTAGCCGTGACTATCGACT(10A)CTTGCCTACGCCAC**A**
KRAS_cGT_iLock		TATATCCCTATAT**G**AGCTCCAACTACCAC(10A)AGTAGCCGTGACTATCGACT(10A)CTTGCCTACGCCAC**G**
Decorator probe	Cy3	*CCTCAATGCACATGTTTGGCTCC*[a]

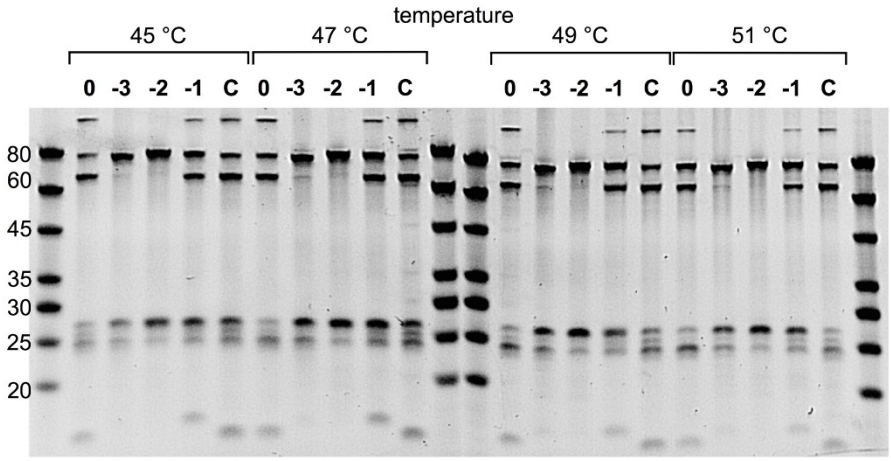
**: RNA oligonucleotides; highlighted: codon 12; lowercase: position 34, genotyped in KRAS mRNA; bolded, underlined: nucleotides participating i invader structure formation; [a]: last four bases of the decorator probe were 2' O-methylRNA to prevent oligo hydrolysis by Phi29 polymerase.*



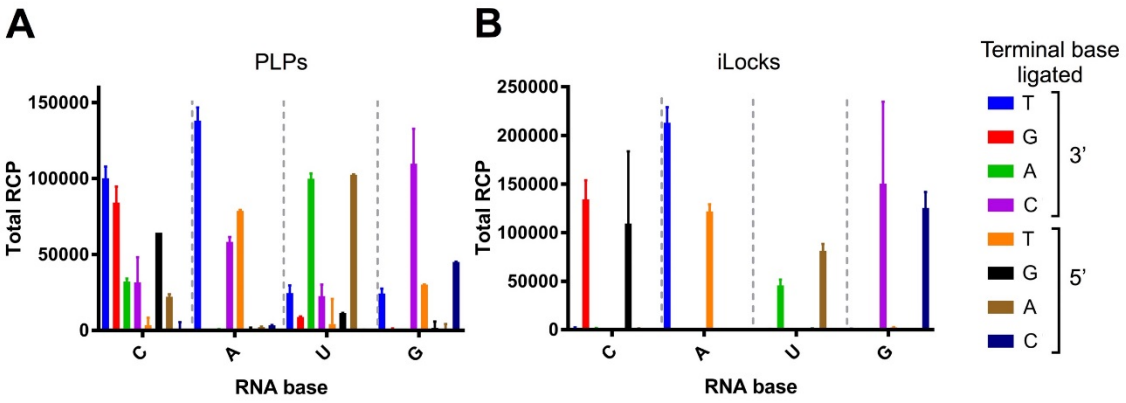
Supplementary figure S1. Detection of *let-7* miRNA family members using iLocks with symmetrical arms. For each iLock group, average number of RCPs was added and each iLock fidelity is presented as a percentage. Average number of RCPs in negative controls was subtracted from the total RCP counts.



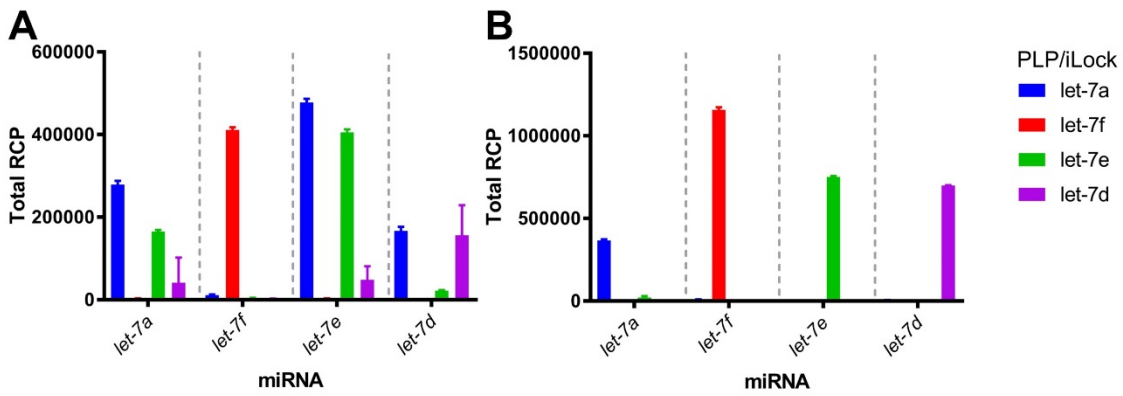
Supplementary figure S2. iLock miRNA detection assay optimisation. *let-7a* miRNA detection using all four iLocks in a 48 – 56 °C range; specificity (**A**) and assay efficiency (**B**). The average RCP number from negative samples (no template control) was subtracted from probe values. Error bars ± s.d.; n=2.



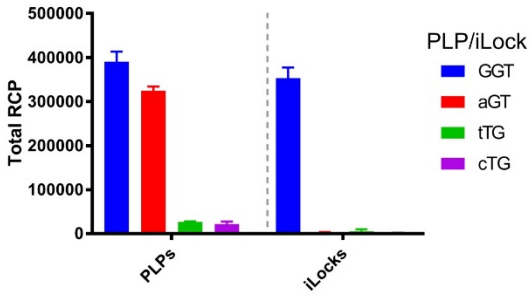
Supplementary figure S3. Analysis of the various let-7a iLocks and let-7e iLock on *let-7e* miRNA by PAGE. iLocks were activated in a 48 – 56 °C range and ligated using PBCV-1 DNA ligase. 0: traditional let-7a iLock with symmetrical miRNA binding arms; - n: positioning of the invader junction was shifted upstream by "n" nucleotides; C: control let-7e iLock.



Supplementary figure S4. **PBCV-1 DNA ligase end joining fidelity with 3' and 5' mismatches**. PBCV-1 DNA ligase specificity profiles using PLPs (**A,** figure 2) and iLocks (**A**, figure 5B) were created by adding the average number of RCPs for each PLP/iLock on one of four RNA templates and presenting the respective probe as a percentage within a group. The y-axis shows the number of RCPs and the x-axis, the polymorphic base in RNA target. The average RCP number from negative samples (no template control) was subtracted from probe values. Error bars ± s.d.; n=2.



Supplementary figure S5. ***let-7a* detection using PLP/iLock assays**. *let-7* miRNA detection using PLPs (**A,** figure 6B) and iLock assay (**B**). The y-axis shows the number of RCPs and the x-axis, the miRNA template. The average RCP number from negative samples (no miRNA control) was subtracted from probe average RCP count values. Error bars ± s.d.; n=2.

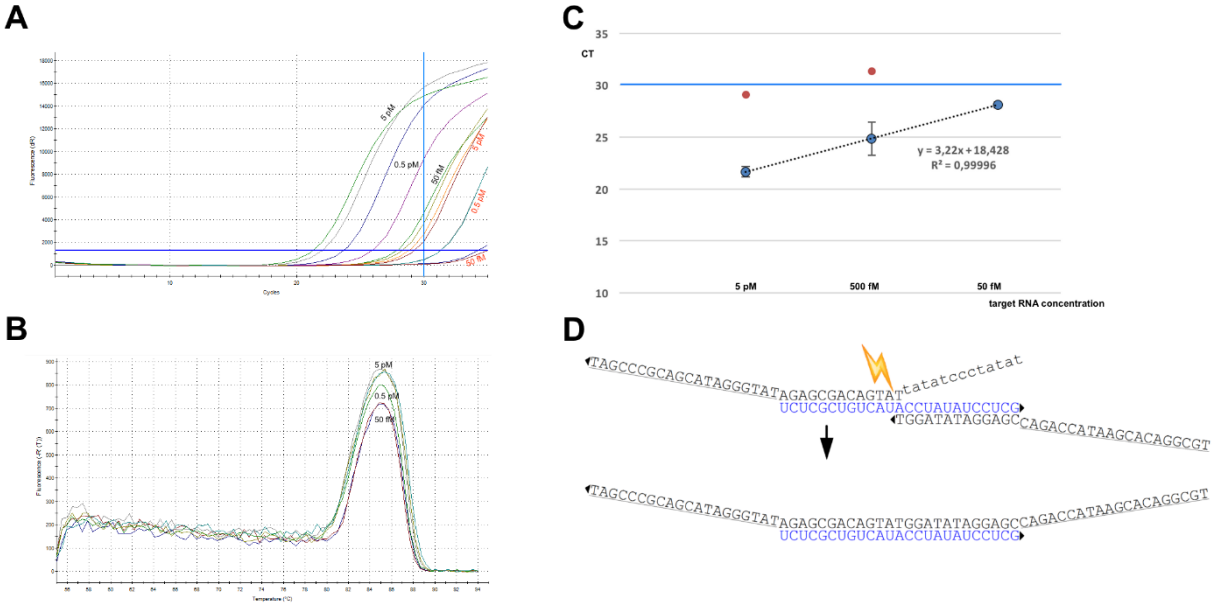


Supplementary figure S6. ***KRAS* codon 12 genotyping using PLP and iLock assays**. The y-axis shows the number of RCPs. Four SNPs (depicted as lowercase nucleotides in the key) were detected in *KRAS* wild type GGT codon 12 with PLPs (left part) and iLocks (right). The average RCP number from negative samples (no template control) was subtracted from probe values. Error bars ± s.d.; n=2.

Supplementary table 4. Oligonucleotide sequences used in Invader-PCR experiment

Name	*Sequence (5'- 3')*
benchm_templ_A*	UCUCGCUGUCAU**A**CCUAUAUCCUCG
INV_PCR_3'	TGCGGACACGAATACCAGACCGAGGATATAGG**T**
INV_PCR_5'	tatatccctatat**T**ATGACAGCGAGATATGGGATACGACGCCCGAT
PCR_R	CGGGCGTCGTATCCCATA
PCR_L	GCGGACACGAATACCAGAC

**: RNA oligonucleotides; lowercase: flap; bolded, underlined: nucleotides participating in invader structure formation; underlined: PCR primer binding sites*



Supplementary figure S7. Combination of Invader cleavage, RNA templated DNA ligation and exponential PCR. A: Amplification curves of the dilution series, including negative controls where Taq DNA polymerase was omitted during the probe-activation step (plots labelled in red). Ct of 30 is indicated with a blue, vertical line. Each dilution was assayed in duplicates. B: Melting curve of the dilution series. C: A standard curve was generated using a 10-fold dilution of a template Ct is plotted against the starting quantity of template for each dilution. Blue points: dilution series; red point: *Taq*- negative control. The equation for the regression line and the R-value are shown in the graph. Ct of 30 is indicated with a blue, horizontal line. D: Schematics of RNA templated probe activation, followed by ligation and PCR. Blue: RNA template; Underlined: primer hybridisation sides. Cleavage position is indicated with a spark. Template negative and ligase negative controls could not record any Ct value. The calculated amplification efficiency for dilution series was 104.4%.