Evaluation of Amplification Refractory Mutation System(ARMS) PCR when combined with the limiting dilution assay for K-ras mutation detection

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Introduction

Pancreatic cancer is the 5th leading cause of cancer death, largely because of its late detection, it has a very low (< 23%) 5 years survival rate even after surgical resection followed by chemotherapy [1]. Early diagnosis demands selection of particular cancer marker and an assay of high sensitivity and specificity. ARMS PCR can give high technical sensitivity and approaching 100% technical specificity for detection of point mutations, based on allelic discrimination by mutation specific primers. The incidence of K-ras mutation in pancreatic cancer is greater than75%, with >90% having a point mutation in codon 12 of the *K-RAS2* gene making it a convenient marker for cancer detection[2]. Unfortunately, K-ras mutation was also found in cases of chronic pancreatitis and even control patients[2]. Nevertheless, it is an important marker in combination with other tests.

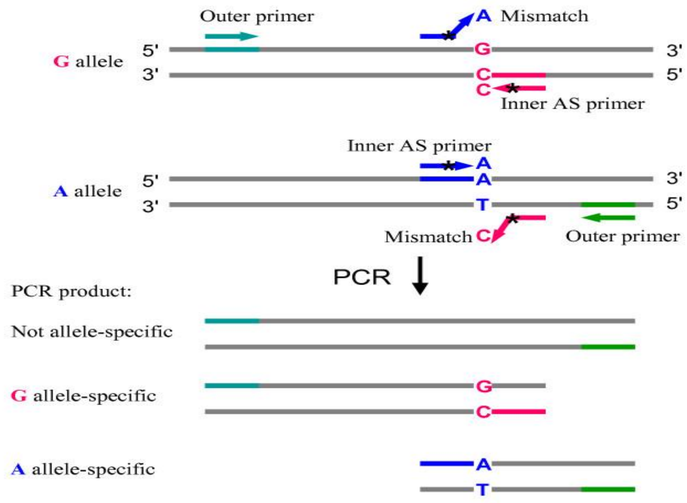

Figure 1. Schematic presentation of the tetra-primer ARMS-PCR method. A non-allele-specific amplicon is generated using control (any sequence) primers (indicated by indigo and green arrows). Allele specific primers (blue or red) are combined with either the indigo or green primes to produce allele specific product. A graph of quantity of template estimated using the allele specific primers against quantity of template estimated with the non-specific primers can be produced using varying concentrations of wild type or mutant template. This allows 98% confidence levels for specificity to be obtained. Results from a test sample are plotted on the same graph and points lying outside the 98% confidence for pure wild type are counted as mutant.

Aims and Objectives

To improve the sensitivity and specificity of ARMS for *K-ras2* mutation detection by combining it with a limiting dilution assay. To achieve this I had the following objectives:

1) The absolute quantification of *K-ras* and *K-RAS2* sequences in PANC-1 cell line and Genomic DNA respectively.

2) Absolute quantification of Mix 1:10 (mutant DNA: wild DNA), anticipating the reduction in mutant concentration in limiting dilution. Aiming to get a jackpot reaction (e.g. if a sample having 1 mutant template per 100 wild type templates if diluted to one molecule per reaction will by chance have just a mutant sequence once every 100 reactions).

Methodology

Quantitative real time PCR was performed with mutation specific (aspartate) primers and control primers for the isolated DNA from PANC-1 cell line and for genomic DNA and Mix1:10.

Data analysis was done by using standard curve, absolute second derivative method, fit point method and simple linear regression model.

Results

Ct values and detection limit was calculated for PANC-1 with aspartate primers (Fig. 1a) and with control primers (Fig. 1b).

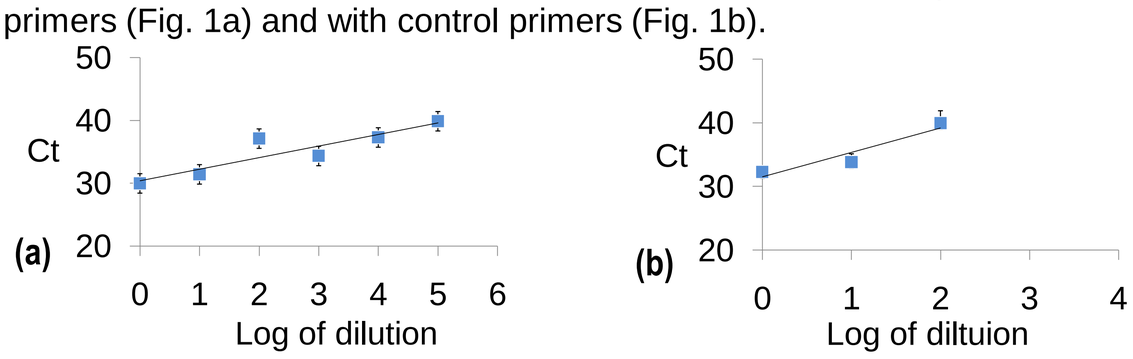

Figure 2. Standard curve was plotted (Ct against log of dilution.) (a) Maximum detection limit for PANC-1 was 0.00001 conc. (Ct =39.86). Error bars are standard error of mean for duplicates (b) Maximum detection limit was 0.01 conc., (Ct =47.13).

Ct values and detection limit were calculated for Mix 1:10 with aspartate primers (Fig. 3).

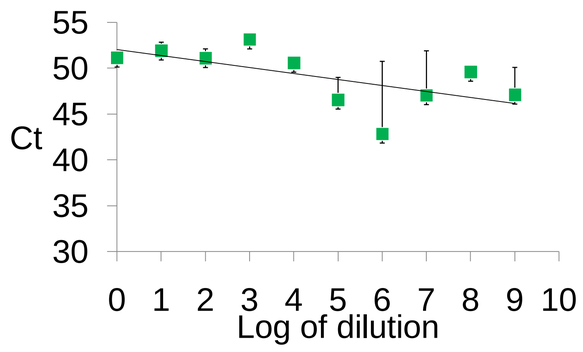

Figure 3. Standard curve was plotted, Ct against log conc. The maximum limit of detection was down to the dilution 10^{-9} .

Compared to C_p method (Fig. 4a), C_t method (Fig. 4b) proved to more effective in terms of data analysis for Mix 1:10

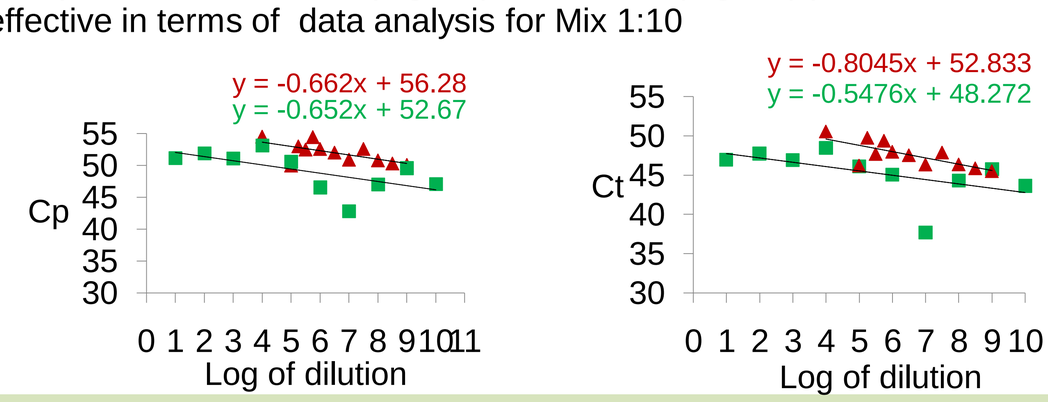

Figure 4. Standard curve (a) Cp method shows a difference of 0.010 in slope and (b) Ct method show a slope difference of 0. 265

Linear Regression model analysis for average of two experiments shows the negative slope (Table 1) for Mix 1:10 which states that higher dilution are having lower Cp compare to lower dilution followed by decreased PCR efficiency (Fig. 5).

Samples	Mutation primers		
	Slope	Intercept	r^2
PANC-1	2.143	32.26	0.885
Mix 1:10	- 0.648	54.75	0.410

Table 1. The positive slope, 2.143 for PANC-1 indicated a trend of increasing Ct with dilutions whereas , for Mix 1:10 the slope of -0.648 indicates a trend of decreasing Ct values with increasing dilution.

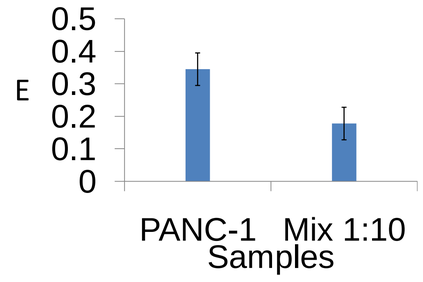

Figure 5. Columns represent the efficiency with standard error of mean. PCR efficiency for PANC-1 and Mix 1:10 was 0.345 and 0.178 units respectively.

Conclusions

✓Absolute quantification of mutant template in mixed samples depends on both mutant and wild type template concentration. Probably because wild template concentration affects PCR efficiency by inhibition.

✓Data analysis method has been formulated for mixed sample analysis, which is affected by the inhibition.

Future work

Investigation of how the specificity and sensitivity of ARMS assay can be improved by using LigAmp probe for the single nucleotide mutation. In LigAmp assay, one specific oligonucleotide binds to mutant base containing the probe and other will only hybridized with the previous one if the mutant base is present and can be quantified by RT PCR.

References

1. Neoptolemos JP, Stocken DD, Friess H, *et al.* N Engl J Med, 2004; **350**:1200-10.
2. Yan L, Greenhalf W, *et al. Gastroentrol., 2005;* ***128:*** *2124-2130.*