

# Information material on DiviTum

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## DiviTum overview

### Thymidine kinase

DiviTum is a novel assay in kit format for the measurement of thymidine kinase activity (TK) present in serum. TK is an enzyme expressed during G1-S stage (Fig 1) salvaging thymidine by its phosphorylation thus introducing it into the DNA metabolism (step 1 on Fig 2).

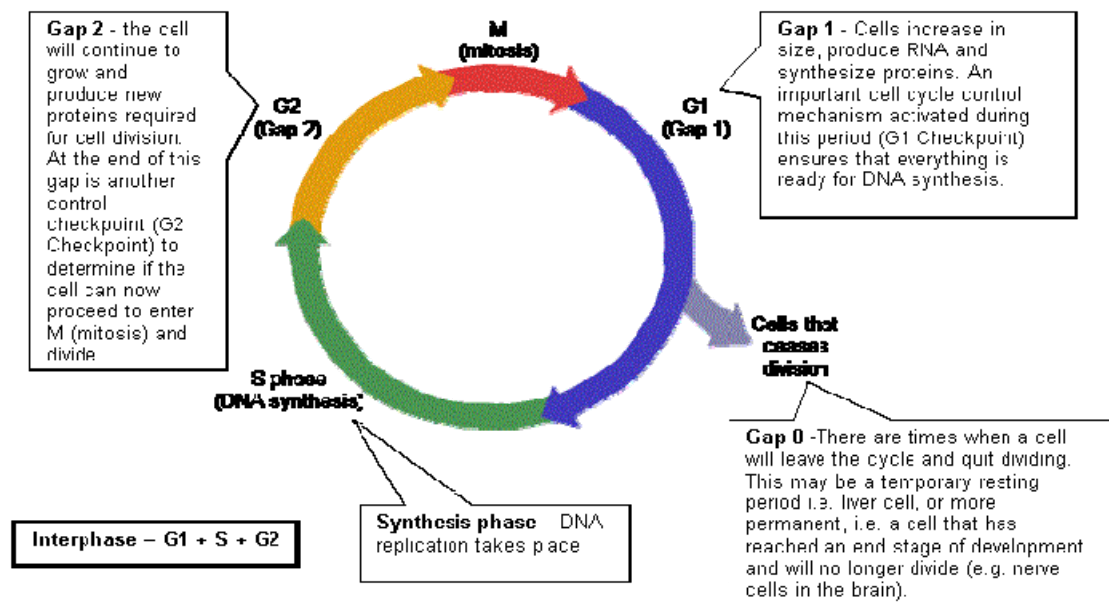


Fig. 1

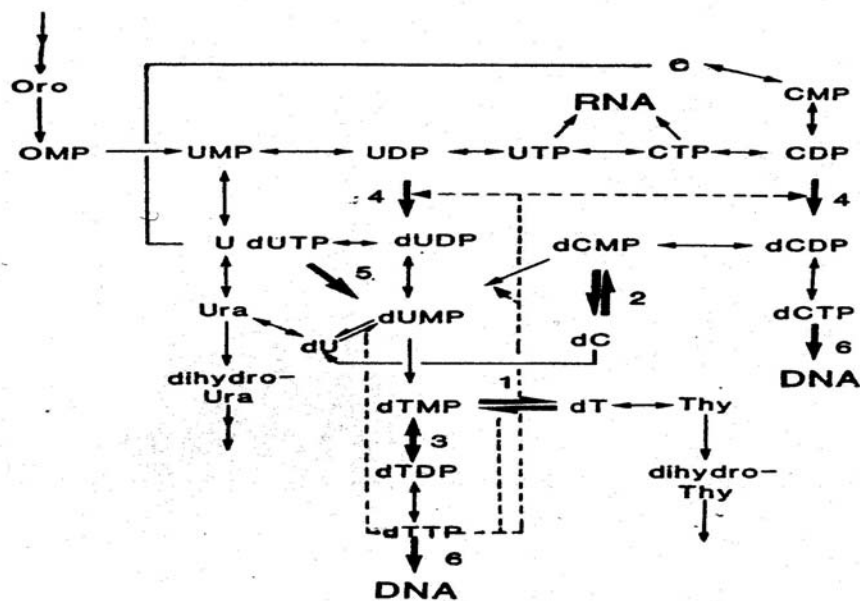


Fig. 2

The enzyme has a KEN box and is regulated by the ubiquitine system and disappears from the cell in mitoses (Fig 2). The differentiated cell in G<sub>0</sub> holds only 1/100 of the TK activity than the dividing cell. In Fig. 3 we have illustrated this by showing the amount of cells per well necessary to induce a signal for TK-negative CEM cells compared with wild type CEM cells. Our interpretation of this is that the presence of a mitochondrial isozyme (non-cell cycle specific) accounts for 1% of the signal in DiviTum.

### TK activity for wild type and TK- CEM cells

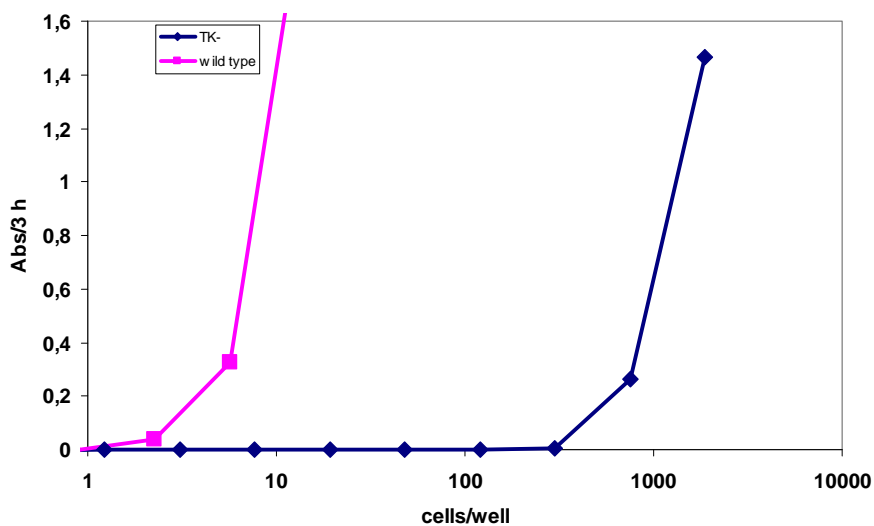


Fig 3.

## Metabolic impact

The TK works in consort with the de novo synthesis of TMP where thymidylate synthetase using folate converts dUMP to TMP, thus the TK expression and activity may be affected by B<sub>12</sub>/folate deficiency and drugs acting in this pathway such as 5-fluorouracil, methotrexate, pemedtrexed (Alimta) (Fig 2).

## Construction of assay

Original TK assays used <sup>3</sup>H-thymidine which after phosphorylation bound to DEAE paper, which in turn after wash and drying was put in scintillation cocktail was counted. Due to the long half-life of <sup>3</sup>H, and low counting efficiency, TK reference levels in serum of healthy could not be obtained. Changing the substrate to <sup>125</sup>I-iododeoxyuridine (Prolifigen assay) solved this problem and established the first standardised assay for TK activity determinations in clinical specimens.

To change from radioactive to non-radioactive assay the DiviTum was developed. DiviTum takes advantage of the natural metabolism (Fig 2) in order to immobilise the TK substrate which is 5-bromodeoxyuridine (BrdU). I.e. after phosphorylation the BrdUMP is processed to BrdUTP by yeast enzymes present (Fig 4), and the BrdUTP is immobilised by incorporation into a DNA strand immobilised in the bottom of the microtiter plate well. The last step is catalysed by a recombinant reverse transcriptase present in the DiviTum reaction solution. The novel DiviTum procedure avoids feed back inhibition of thymidine nucleotides present and gives a very sensitive assay requiring e.g. 0,2 ul serum per well detecting as little as 10<sup>-13</sup> gram of enzyme (Fig 5).

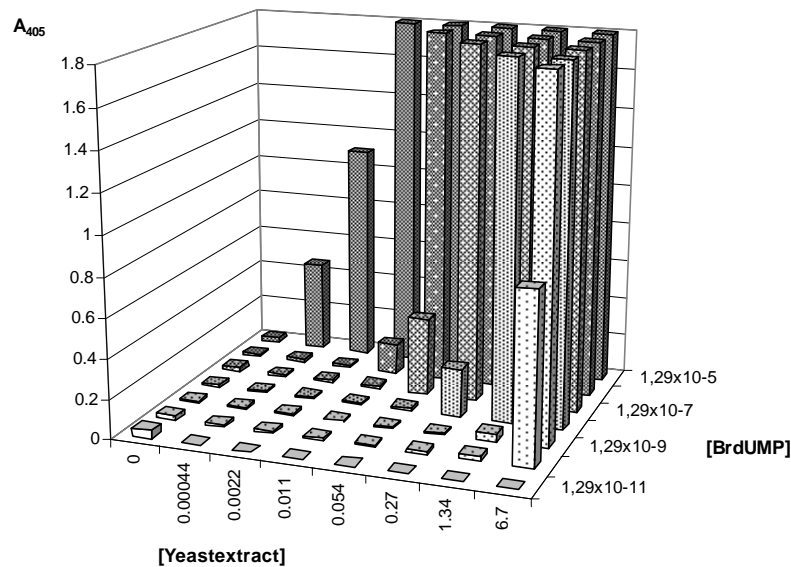


Fig 4.

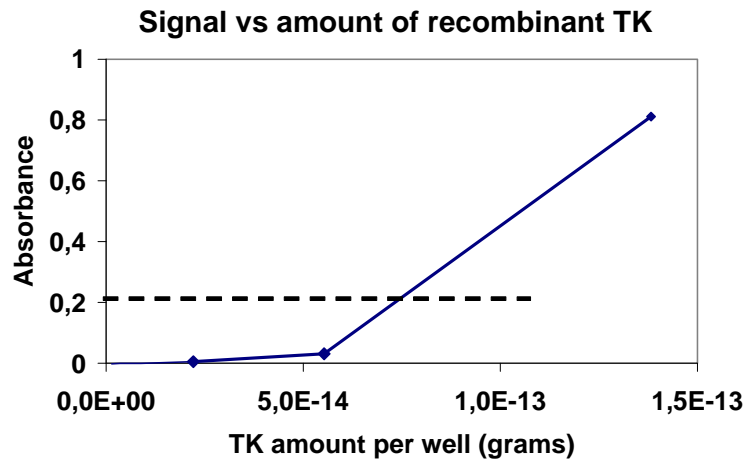


Fig. 5

### Correlation with TK-REA

The below graph illustrates the results of a correlation test between Prolifigen TK-REA and DiviTum. This test was performed on 20 year old samples that had been stored in -20 degrees centigrade. Although the TK-REA values were not rerun, the correlation is very high as one can see: 0.95. We expect the correlation to be even higher if a test is rerun on fresh sera that has not dried.

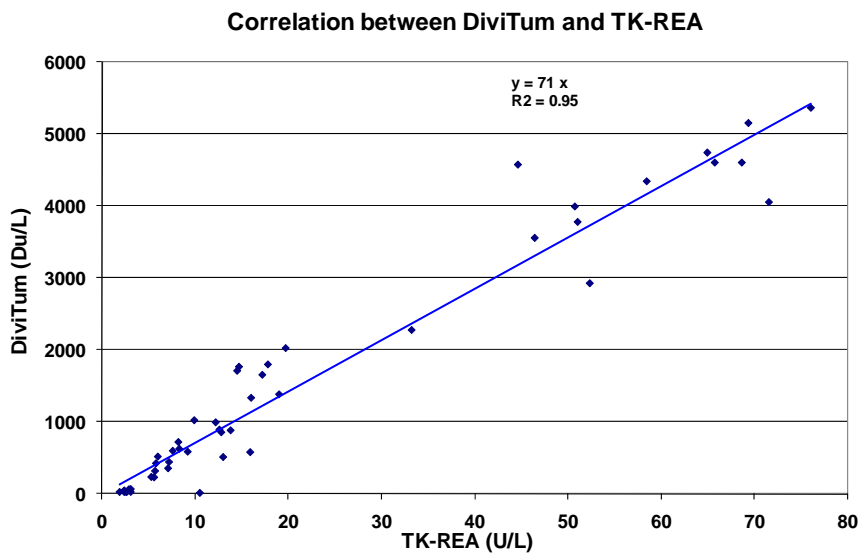


Fig. 6

From the slope of the curve you can see that the conversion factor between DiviTum units (Du/liter) is 71. Please note that all figures in this document that are scanned graphs from older publications use the old measurement unit. These are figures No: 8, 9, 10 and 11 which need to be multiplied with 70 to get corresponding DiviTum Du/L values.

However, since the TK activity measured is the sum of the normal level and the disease induced level, and with DiviTum we pick up much lower normal levels, this conversion factor is only valid from 8-10 units and upwards, i.e. about 600 Du/L.

Translated to DiviTum units (please see the note on units below) we see that the old test, TK-REA, could not resolve levels below 350-500. As seen on our study of blood donor (where a requirement to enroll is that the person has not been ill during the last 2-3 weeks) the spread of normal values ranges (90%) from 10 up to 80-90 Du/L. This is the basis for our statement that DiviTum is 10 times more sensitive, i.e. the signal induced by a tumor can be separated from the background level even if it is 10 times smaller.

In the study of blood donors (n=240) 10 individuals of each sex in each age interval (18 to 80 years of age) were asked if they would give a sample for research. There was no significant difference in s TK level between the sexes and no obvious age effect. (it was a tendency for younger and elderly people to have the highest values (larger than 90 Du/L) than the middle age group, and the hypothesis is that in the higher age cohort this is explained by slow growing not already detected tumors, and for the younger people this could be explained by a higher incidence of asymptomatic viral infections. As this was a closed study there was no way for us to follow up on the persons with elevated levels).

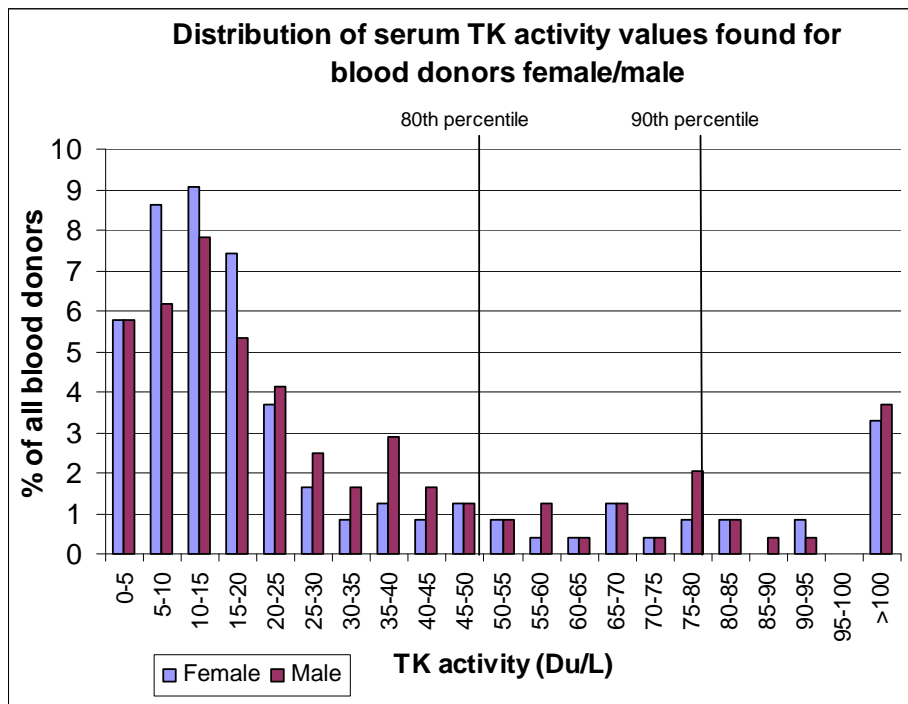


Fig. 7

**A note on the choice of units for DiviTum:**

DiviTum measures the TK activity and not protein amount. As such, the established unit of measurement should contain the name Units. We have chosen the name of our measurement unit to DiviTum units per liter (Du/L). This value corresponds to the

enzymatic activity obtained from a certain batch of recombinant TK-enzyme with the same protein concentration measured in ng/L.

### ***Manual or semi-automated version of DiviTum***

The first CE-labeled product version of DiviTum had the references freeze dried in the plate in the last two columns. The reason for us to do this was to eliminate a possible handling error.

However, our contacts with reference laboratories in central Europe led to the insight that there is a demand for a semi-automated procedure as this will shorten the time necessary to prepare each plate. Therefore we have changed the construction of the assay so that the references will be supplied separately in bottles. This will allow the robot (we are validating the assay on the Biotek Precision XS Microplate Sample Processor – catalogue number: PRC384/1M) to handle the references under the exact same conditions as the samples for determination (time, temperature etc). This second product version can also be used in manual preparation of the plates.



We recommend labs to switch to the robot when they run more than 100 determinations per week. The labs will not have to pay the cost of the robot upfront. Instead, there will not be the standard 15% discount for bulk orders the first three years (of course depending on the volume of the orders). After this time the robot is paid off and the ownership is transferred to the lab and the standard discount will be effectuated.

With the robot one technician can prepare more than 10 plates per day.

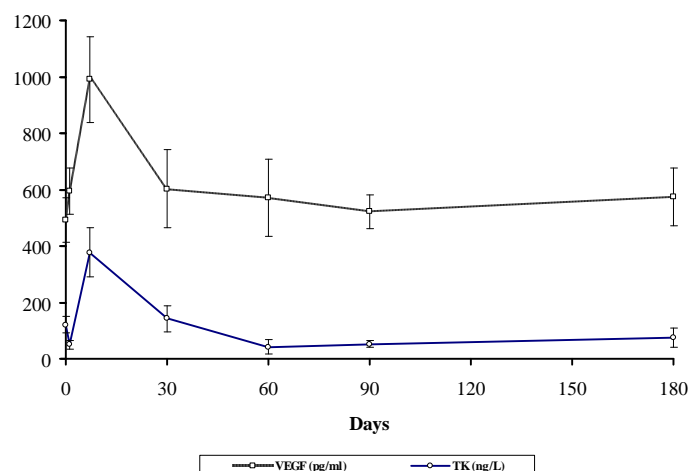
## **Pathological levels**

Pathological Serum TK (s-TK) levels occur in conditions involving increased cell division due to replenishment of destroyed cells, in connection with cell death in G<sub>1</sub>-S stage and in connection with tumour diseases.

## **Transient increases**

Transient increase occurs after trauma as illustrated by kidney removal in Fig 8 with normalisation after the wound healing which normally takes a few weeks.

## Trauma induced s-TK post kidney removal

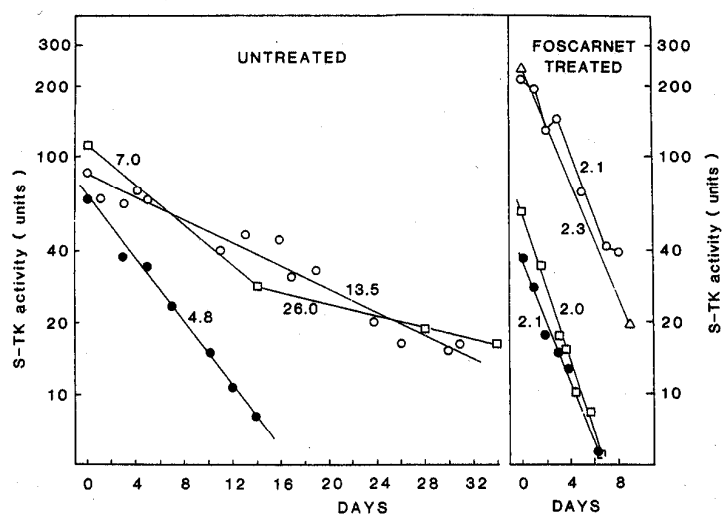


**Fig 8.**

Also certain infections, when generalised give increased s-TK, especially herpes viruses as illustrated in Fig 9 (left panel), and the normalisation occurs more rapid when antiviral drug is given Fig 9 (right panel).

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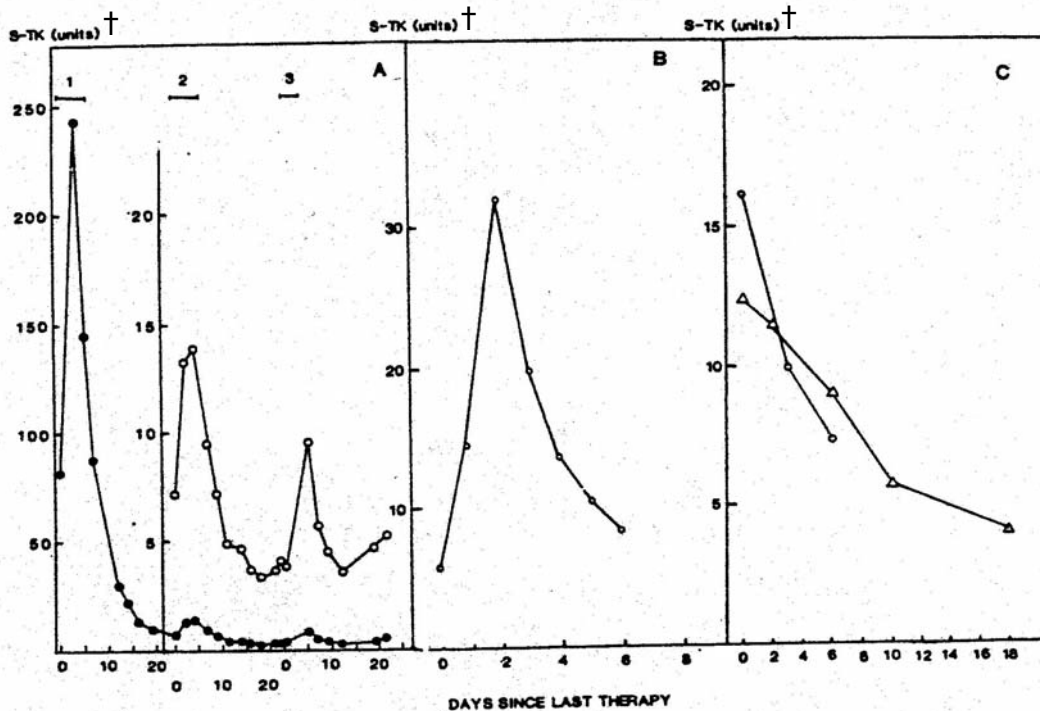


**Fig. 2.** The half-life of serum thymidine kinase (S-TK) activity in self-healing and foscarnet treated cytomegalovirus infected transplant patients. (Left lane) Untreated patients. Day 0 indicates only the first day the S-TK started to decrease. ●—●—● and ○—○—○ are renal recipients, and —□—□— is a bone-marrow transplant patient. (Right lane) Foscarnet treated patient. Treatment started at day 0. —○—○ and —□—□ renal recipients. —●—● and —Δ—Δ bone-marrow recipients. Numbers in the figure indicate  $t_{1/2}$  for S-TK calculated from the lines.

**Fig. 9**

Short time s-TK also occurs in connection with intermittent chemotherapy if substances killing tumour cells in division stage are included, as illustrated in the two left panels of Fig 10, i.e. intermittent treatment of leukaemia with POCAL and middle s-TK alteration

over one treatment cure of a colon cancer. The right panel of Fig 10 illustrates that no s-TK release is found upon treatment of prostate cancer with Estracyt or after starting treatment of hairy cell leukaemia with interferon. Long time transient increase of s-TK occurs after initiating therapies including chemical interfering with thymidylate synthetase and folic acid and in this case normalisation does not occur until treatment is ended.



†) Old units from the old test, must be multiplied by at least 70 to be comparable to DiviTum

Fig. 10

### Chronic increase in s-TK

Chronic increase occurs in severe B<sub>12</sub> deficiency giving megaloblastic anaemia and normalisation occurs after giving one injection of vitamin B<sub>12</sub> as illustrated in Fig 11 also showing the half life of TK in serum when removing cause of pathological level. Also, easily separated from tumour disease, auto immune diseases, like severe rheumatoid arthritis, increase the s-TK level.

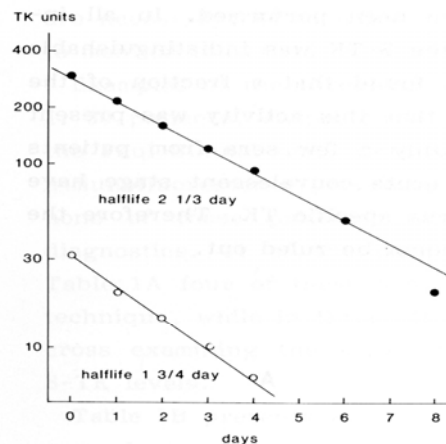


Fig. 11

Fig 3. Half life of S-TK. (●) Patient with pernicious anemia after B<sub>12</sub> injection; (○) patient with small cell carcinoma of the lung, decrease of therapy induced S-TK.

In tumour disease the pathological levels occur when the amount of dividing tumour cell is enough to cause a significant increase of s-TK above the normal level. Thus for slow growing tumours where only a fraction of cells are dividing a rather large mass of tumour is present e.g. 63% of prostate carcinoma found due to PSA increase are normal in s-TK as measured by DiviTum, while for other tumours like leukaemia pathological s-TK is almost always present upon the detection of the disease. In terminal tumour disease these differences in growth fraction is illustrated that one normally dies of prostatic carcinoma with s-TK less than 10000 DiviTum units while one can have at least a 10-fold more in acute leukaemia. TK can also be measured in other clinical specimens, spinal fluid, pleural effusion and ascites fluid to determine possible presence of tumour.

Using correctly sampled serum the DiviTum analyses gives complementing information to other medical investigation improving the base for different decisions, ranging from use of imaging technique or not, determination neoadjuvant therapy, of strength of prophylactic therapy or change of therapy or even if therapy may be postponed.

## **Recommended sampling procedures**

### **General protocol for serum sampling**

Serum for thymidine kinase activity determination using DiviTum™ should be collected in the following manner:

- All samples should be standard serum and the samples should be frozen as soon as possible after the coagulate is removed.
- Samples should not unnecessarily be exposed to higher temperatures than +30°C for more than an hour in order to avoid inactivation of the enzyme.
- The amount of sample required for analysis in automatic procedures is 200 µl (microliters).
- When running research material at Rönnerbol International, the dilutions are made manually and thus 70 µl per sample is appreciated.

### **Sampling healthy individuals (use in health control)**

- Sampling should be done from individual that have been without infection or other symptoms for the last three weeks.
- If pathological levels are found a new sample should be taken within three to four weeks to determine if the pathological level was transient or due to chronic disease.

### **Sampling in connection with evaluation of active disease (medical investigation)**

- If pathological levels are found, a second sample should be taken in convalescent state, normally after three to four weeks.

### **Sampling in patients with known disease, especially tumors**

- Samples should be taken in connection with the detection of the disease
- before commencing treatment, surgery or chemotherapy.
- Follow-up samples after surgery can normally be taken after 4 weeks for monitoring.
- Follow-up samples in intermittent chemotherapy should always be taken before starting the intermittent treatment. However, for heavy chemotherapy known to have immunosuppressive effect activating latent virus it is more reasonable to sample the first control for therapy efficiency after 60-90 days.
- For intermittent chemotherapies including drugs interfering with thymidylate synthetase and folate metabolism (see above) it is recommend to sample sera before starting therapy and 3 to 4 weeks after ending intermittent therapy
- After ending intermittent chemotherapy for the monitor of possible relapse of disease it is recommended to sample more frequent the two first years (every 4<sup>th</sup> month), thereafter every 6<sup>th</sup> month and after five years on annual basis. The interval can of cause be varied depending on the type of cancer, aggressiveness and probable time for relapse.
- For monitoring during constantly administered therapy, serum sampling can be performed at any time as long as the patient has been free of infections or other diseases during the last three weeks.