HTRF® CHECK KITS FOR GST AND 6HIS TAG ACCESSIBILITY

APPLICATION NOTE

ABSTRACT Tags are key elements for the purification of biomolecules. They also offer researchers flexibility in assay design.

This application note describes GST and 6HIS check kits for assessing tagged proteins during production processes and drug discovery.

INTRODUCTION

Tagged proteins are extensively used for the study of biomolecular interactions. The insertion of various peptidic motifs has enabled more straightforward purification and detection of recombinant proteins.

However, tag insertion sometimes alters protein activity and several constructs are generally necessary to obtain a viable protein. Independently, tag recognition may be hampered by protein conformation or may be affected by the position of the tag in the protein sequence. 6HIS tag, for instance, may not be detected with the same efficiency depending on its position at the N or C-terminal end of proteins. The accessibility of the motif by the corresponding detecting tool should therefore be assessed at the beginning of the assay development.

We have developed two HTRF® kits which enable

- Protein quantification: A fast and simple HTRF protocol makes it possible to measure GST or 6-HIS tagged proteins at each step of protein production and purification. These kits can be calibrated using the appropriate protein, making the assay very accurate and compatible with the SOPs used in industrial processes
- Drug discovery: 6HIS and GST check kits are useful after HTS to eliminate false positives. Compounds that interrupt the binding of tag-detection reagents (e.g. HTRF® MAb Anti-6HIS-Cryptate) will show strong signal inhibition in the check kit. These compounds can be eliminated from further characterization, saving time and resources.
- Tag Accessibility: The 6HIS check kit is a useful tool to determine whether the tag is accessible to antibodies and to predict the possibility of purifying the protein using a Nickel column. If a 6HIS fusion protein is not detected using our check kit, then the tag is inaccessible and not available for purification or other binding purposes.

The GST and 6HIS check kits are competitive immunoassays that use Cryptate-labeled anti-tag antibodies and XL665-labeled tags. The check kit assays can be run in a single microplate requiring only a two-hour incubation. Because of the ratiometric measurement of HTRF®, tagged proteins can be accurately detected in a variety of buffers and media.

As shown below, GST labeled with XL665 is detected by anti-GST Cryptate (GST-K) conjugate (Figure.1) and 6His labeled with XL665 is detected by anti-6His Cryptate (GST-K) conjugate (Figure.2).

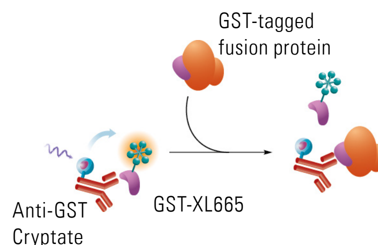

Figure 1: GST tag check kit assay principle

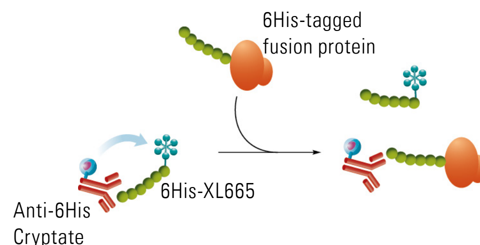

Figure.2: 6HIS tag check kit assay principle

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MATERIALS AND METHODS

The assays for GST and 6HIS were designed according to the same model, as described above.

Anti-tag antibodies were labeled with europium cryptate using standard SPDP-SMCC immunochemistry. Slightly larger amounts of cryptate were used in order to produce high specific activity conjugates (5 to 7 cryptates per antibody), and to simplify the procedure. The tag was directly labeled with XL665.

GST was obtained by desorption from a glutathione column following fusion protein cleavage by enterokinase. The dialyzed material was biotinylated with biotin-LC-NHS and dialyzed again to remove unreacted biotin. Part of the purified GST was left as is, to be used as a kit calibrator and control. 6HIS (GGGYGGGHHHHHH) motif was synthesized and N-terminally biotinylated. Three different purified GST fusion proteins were assessed to validate the GST assay. The respective constructs encompassed variable protein moieties (overall MW of 38.9, 40 and 42.8 kDa).

Controls for 6HIS inhibition consisted of polyhistidine peptide (Sigma, St Quentin-Fallavier, France), and two differently 6HIS-tagged RNA polymerase subunits from yeast.

The experimental procedure was similar for the two systems. Dilution for all components was made in 50 mM PO4 pH 7.0, 0.1 % BSA, 0.4 M KF. The table below gives volumes. Samples of tagged-moiety to be tested were introduced under 10 µl (replaced by 10 µl of assay buffer for the maximum binding control, making the final assay volume 20µl).

	SAMPLE: TAGGED PEPTIDE OR TAGGED PROTEIN (µL)	XL665 CONJUGATE (µL)	ANTI-TAG CRYPTATE CONJUGATE (µL)
GST check kit	10	5	5
6His check kit	10	5	5

Kinetics and temperature influence were optimized for the two assays. The influence of zinc, lithium and manganese concentrations was also studied for the 6HIS check kit.

All GST and 6HIS reagents were lyophilized for final check kit packaging. Reagent stability was therefore checked for liquid, lyophilized and reconstituted products.

RESULTS

GST CHECK KIT

GST preparation obtained by desorption from glutathione gel was found to be at 11.4 nmoles/ml. The native protein was used as a calibrator and sequentially diluted from 500 to 0.01 nM (final concentration). Optimization of reagent concentration led to the conditions defined earlier (see table). Reaction equilibrium was reached after 2 hours at R.T. and the standard curve was plotted as shown in Figure.3 beside. Under these assay conditions, repeatability was measured for all calibrators (10 times each). The detection limit was worked out by calculating $Mean_{cal1} - 2\sigma_{cal1}$. A detection limit of 0.26 nM was deduced by linear extrapolation between calibrators 1 and 3.

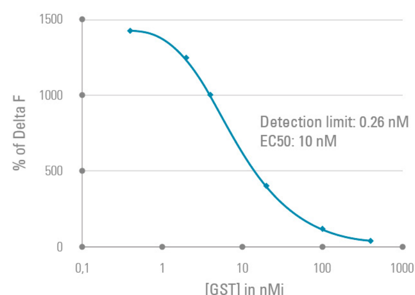

Figure. 3 : evaluation of GST check kit detection

GST CHECK KIT: MEASUREMENT CONSISTENCY

Several GST-tagged proteins were tested to verify the measurement consistency. As shown in Figure.4 beside, the 6 proteins behaved very similarly, which was consistent with the fact that they all bore the same GST moiety. Concurrently, the different protein moieties (more or less truncated) fused with GST had no influence on the anti-tag conjugate's recognition of GST.

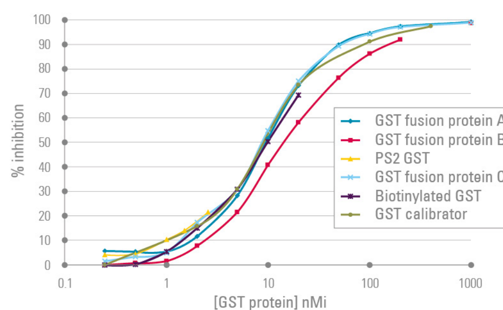

Figure. 4 : Inhibition of GST-XL665 by different GST proteins

GST CHECK KIT: TEST OF STABILITY

The last experiments carried out on final kit reagents (lyophilized) showed the excellent robustness of the kit (fig.5). Whatever the storage conditions, including the drastic AAP (accelerated ageing process: 2 days at 37°C + 3 days at –20°C + 2 days at R.T.), both lyophilized and reconstituted reagents remained within the ± 20 % variability range when compared to the reference material stored at –80°C.

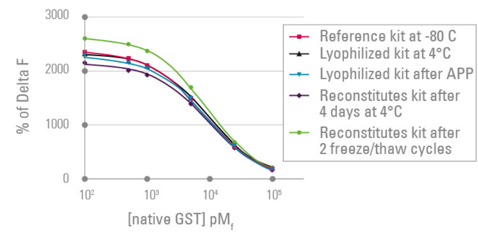

Figure. 5 : GST check kit stability

6HIS CHECK KIT: INFLUENCE OF THE INCUBATION TEMPERATURE

The 6HIS check kit performed similarly to the GST kit. However, experiments carried out at room temperature led to poor inter-assay reproducibility, essentially in terms of maximum delta F. Figure 6 beside shows the influence of incubation temperature in absence of competing tagged-protein. Best signals were obtained at 4°C. This also enabled signal normalization from one test to another. Slight modifications in temperature induced significant variations in delta F.

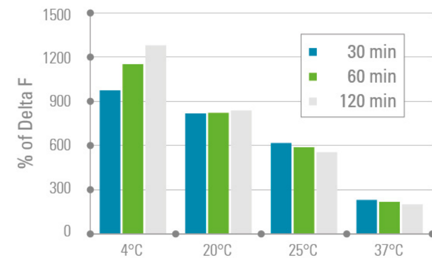

Figure 6 : influence of temperature on anti-6HIS antibody binding

6HIS CHECK KIT: INFLUENCE OF METAL ION

The system was also influenced by Zn2+ ions. As little as 0.1 µM ZnCl2 induced a 40 % inhibition of maximum signal, and binding was completely eliminated at 10 µM (Figure.7). Lithium and manganese had less significant effects, although a basic inhibition (10-15 %) remained for both and was slightly raised to 20 % for higher concentrations of Mn2+. This underlines the fact that the 6HIS motif is extremely sensitive to buffer composition.

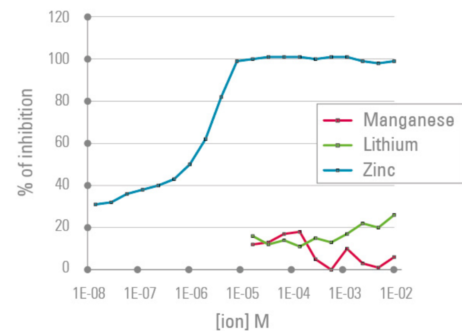

Figure 7: influence of metal ion concentration on anti-6His antibody binding

6 HIS CHECK KIT: TAG ACCESSIBILITY

This was also observed for the inhibitions carried out by various proteins. As shown in Figure. 8 beside, protein I (6HIS-tagged RNA polymerase) was tested either just after its purification (buffer A = imidazole) or once dialyzed (buffer B = phosphate). Results showed that the inhibition was much more pronounced with the phosphate solubilized protein. Due to the nature of the motif itself, it is very likely that the tag adopts a conformation that is recognized differently by the antibody, depending on medium conditions. This may lead to false negative results, since antibody binding may be prevented.

The same experiment confirmed that the number of motifs obviously accounts for the inhibition (polyHIS versus 6HIS peptide), as well as the construction itself, as shown by the slightly different inhibition curve obtained with the 6HIS-tagged RNA polymerase (protein II).

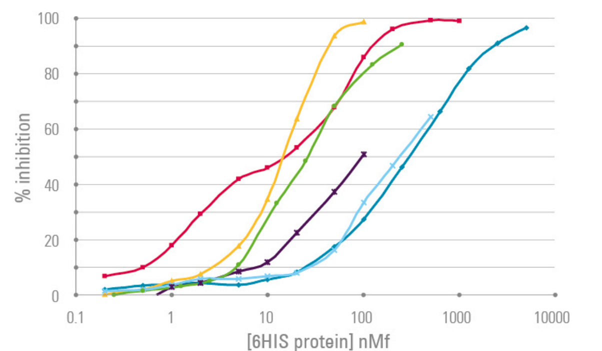

Figure 8: tag accessibility on different 6HIS-tagged proteins

CONCLUSION

Due to its larger size, GST tag accessibility is apparently less sensitive to protein conformation and the similar behavior of the proteins tested in this study makes GST check kit a potential quantitative assay for GST-fused proteins.

Concurrently, the position of the tag, the conformation of the protein and the assay buffer are important parameters for 6HIS detection and may affect assay recovery. Absolute quantification may be inaccurate (unless the same protein is used as a reference). However, the 6HIS check kit is a powerful tool for the verification of 6HIS tag accessibility.

Given the sensitivity of this motif to various external factors (e.g. metal ions), this kit can also be used to make sure that compounds tested or assay buffers do not interfere with the anti-6HIS antibody binding itself, thereby preventing possible false positive results.

The production of fusion proteins requires the confirmation of their structural integrity and the accessibility of the associated tag following isolation. The GST and 6HIS check kits are simple tools for assessing tagged proteins during production processes and drug discovery.

ORDERING INFORMATION

DESIGNATION	SIZE	CAT#
6HIS-tag check kit	1,000 tests	62HISPEB
GST-tag check kit	1,000 tests	62GSTPEB

RELATED INFORMATION

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