Program #1946

Bacteriophage-Expressed Soluble Substrates for Protein Kinases

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1 Abstract

The human kinome comprises nearly 520 protein kinases that serve as key mediators of processes such as growth, metabolism, inflammation, cell division and apoptosis. Because kinases play crucial regulatory roles in so many processes, they are often linked to disease and represent attractive pharmacological targets. Despite extensive efforts, there remain numerous "orphan" kinases for which no substrate has yet been identified. Our goal is to develop a screening assay for the identification of new substrates for protein kinases.

We turned towards available M13 and T7 phage display systems since these bacteriophages exhibit fast and simple replication, and can be used as expression systems that physically link a protein target with its DNA coding sequence. We therefore constructed various M13 and T7 phages harbouring consensus phosphorylation sequences for the tyrosine kinase c-Src and the serine/threonine kinase Akt1. Normalized phage populations were used as substrates in kinase assays performed in well plates. The normalization of phage numbers was carried out using AlphaLISA® beads coupled to antibodies directed against bacteriophage structural proteins. This method is an effective substitute for the tedious, yet conventional phage titration.

In a similar bead assay setting, anti-phosphorylated consensus antibodies were used to determine the phosphorylation of phage-expressed substrates following kinase assays. We found the substrate constructs expressed at the surface of M13 and T7 bacteriophages to be phosphorylated by the kinases, whereas non-phosphorylatable mutants were not. These data suggest that phosphorylation occurs specifically on the expressed constructs and not on bacteriophage structural proteins.

Such tailored phages represent a simple, fast, self-replicative and inexpensive way of determining the kinase activity. Ultimately, this assay platform should enable us, not only to measure phosphotransferase activity, but also to identify new targets for virtually any protein kinase.

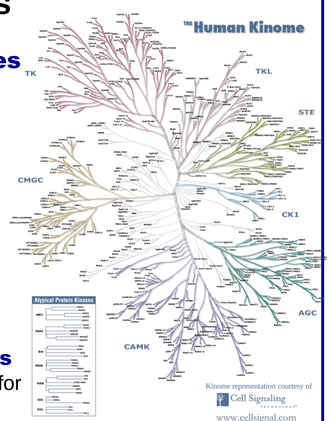
2 Project Goals

518 Protein kinases

~300 orphan kinases without any known substrate

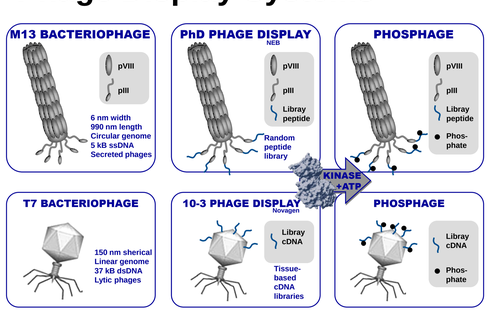
~ 90% Ser/Thr kinases

ORPHAN KINASE ? UNKNOWN SUBSTRATE



Because they physically link protein content to DNA sequence, **bacteriophages** could be used as substrates for protein kinases in a **de-orphanization strategy**

3 Phage Display Systems



4 Materials and Methods

Phage Titering

E. Coli bacteria were grown overnight and diluted 10-fold according to Manufacturer's Instructions. T7 phages (*10-3 T7 Phage display*, Novagen) were serial-diluted 10-fold, mixed with molten top agarose, poured on LB-Agar petri dishes and incubated overnight at 23C. M13 (*Ph.D. M13 Phage Display*, New England Biolabs) were processed similarly but X-gal and IPTG (Sigma-Aldrich) was added to the top agarose and incubation was done at 37 C. Blue M13 colonies or T7 lysis plaques were counted manually.

AlphaLISA titering

In a 384 well plate (PerkinElmer), phage preparations were incubated at 23°C with 3 nM biotinylated antibody (Anti-filamentous phage, Sigma-Aldrich; Novagen) and 20 µg/mL Acceptor beads (*Anti-M13* GE Healthcare, PerkinElmer; *Anti-T7* Novagen, PerkinElmer), before addition of streptavidin-coated Donor beads (PerkinElmer) for 30 min and subsequent reading on EnVision® multiplate reader(PerkinElmer).

Kinase assays

Normalized phage preparations were incubated at 23°C for 2h with 1 nM enzyme (*c-Src* CarnaBio USA; *Akt1* Millipore) and 10 µM ATP in a tris-based buffer containing 10 mM $MgCl_2$. 20 µg/mL Acceptor beads and 3 nM antibody (*Anti-phospho Akt consensus* Cell Signaling) were added 1 h prior to Donor beads. After a final 30 min incubation, plates were read. In some cases, an additional reading was performed overnight.

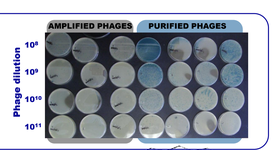
5 Phage-Expressed Proof-of-Concept Constructs

Kinase/ Consensus	c-Src E/D-E/D-X-X-Y-G/W	Akt1 R-X-R-X-X-S/T
wt substrate construct	Src wt KIEEPLYWMFG	Akt wt RKRNRNKSVEG
Phosphorylation site mutant	Src YF KIEEPLFWMFG	Akt SA RKRNRNKAVEG

6 M13 Phage Titering

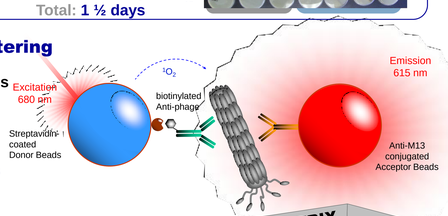
Conventional titering

Grow bacteria overnight
Infect bacteria
Plate on soft agarose
Grow overnight
Count colonies, manually
Total: 1 ½ days



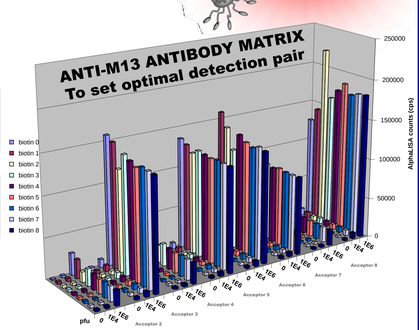
AlphaLISA titering

Dispense in wells
Add beads
Incubate 1h
Read plate
Total: 3 hours

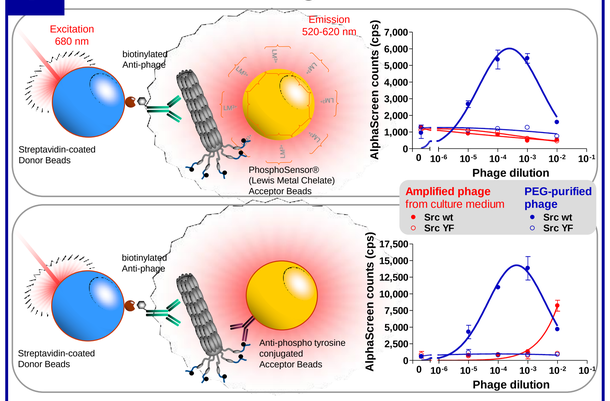


M13 can easily be titrated by AlphaLISA

Many antibody combinations work efficiently



7 c-Src M13 Phage Substrates



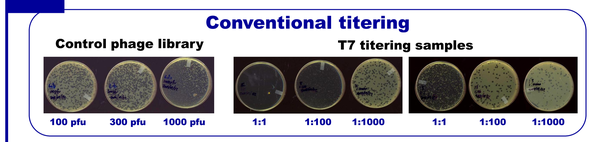
Constructs expressed at the surface of M13 can be specifically phosphorylated

c-Src phage phosphorylation can be monitored with anti-phospho tyrosine beads, even in amplified cultures

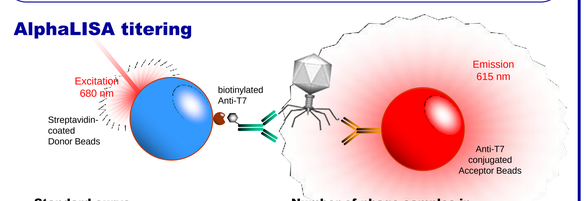
Purified M13 phosphorylation can also be detected by PhosphoSensor beads

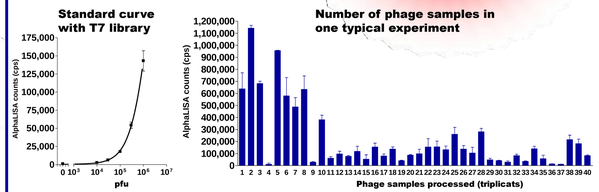
8 T7 Phage Titering

Conventional titering



AlphaLISA titering

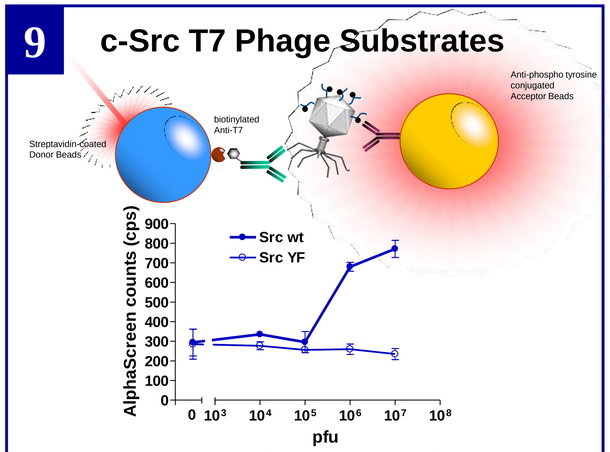




AlphaLISA is as accurate, and more precise, reliable and less time-consuming than conventional phage titering. Typical offset between the two methods is less than half a Log unit.

- AlphaLISA provides an accurate determination of total phage particles, whereas convential titration is a biofunctional assay measuring viable phage.
- We demonstrate a close correspondance between these two values under the phage purification conditions used here (Sequential PEG precipitation, 0.22µm filtration and Size-Exclusion Chromatography).

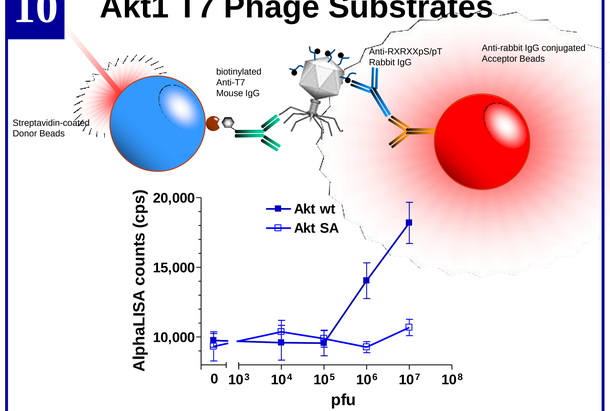
9 c-Src T7 Phage Substrates



T7-expressed constructs can be specifically phosphorylated by c–Src

This phosphorylation can be detected by anti-phosphotyrosine antibody-coupled beads

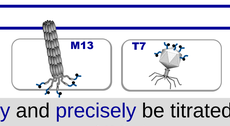
10 Akt1 T7 Phage Substrates



T7-expressed constructs can be specifically phosphorylated by Akt1

This phosphorylation can be detected by anti-phospho-consensus antibodies

11 Summary



Bacteriophages can easily and precisely be titrated by AlphaLISA in significantly less time (4X) than conventional methods

Phage colonies on solid support can be phosphorylated by kinases, and these experiments represent the first example for detection of bacteriophage phosphorylation in free solution

- Kinase assay and detection in the same well
- Constructs are specifically phosphorylated

Further purification of lytic phage T7 is required for PhosphoSensor detection