Evaluation of Capillary-Mediated Vitrification (CMV) as an Alternative to Traditional Cold Temperature Storage for Kinase Assay Reagents

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ABSTRACT

AssayQuant is a growing biotech company specializing in the development of sensor peptides that provide a quantitative picture of protein kinase activity over time via continuous measurement of chelation enhanced fluorescence. As a leader in kinome profiling and inhibitor potency and characterization, AssayQuant's -80°C freezers hold thousands of samples of over 400 different protein kinases in addition to various other reagents utilized for assay execution and optimization. The prevalence of freezer-dependent storage exposes any organization to space, time, and cost concerns ranging from freezer procurement to placement and routine maintenance. Inadequate freezer storage conditions can lead to temperature fluctuations, potentially affecting sample integrity, or even complete equipment failure, risking total sample loss. This is especially true for smaller evolving companies where growth often outpaces infrastructure, and the high costs of reagent procurement and freezer storage can, at times, be prohibitive. Although alternative methods to cold temperature storage exist, they rely largely on long sample preparation times, extensive product-specific optimization, and often employ high temperature or shear stress environments that are generally incompatible with the preservation of protein kinase activity. Capillary-mediated vitrification (CMV) is an emerging technique that offers a promising solution to eliminate dependence on cold-temperature reagent storage and shipment, and overcome its associated risks, while at the same time avoiding the concerns prevalent in other methods of sample stabilization. AssayQuant Technologies has partnered with Upkara, a global leader in biomolecule stabilization, to assess the potential of capillary-mediated vitrification as a viable alternative to its current cold-storage solutions. Assay reagents preserved via CMV were evaluated alongside conventionally stored samples in the context of a continuous enzymatic assay using AssayQuant's Phosphosens® technology. A viable alternative sample stabilization approach and workflow is presented along with prospective applications geared towards both in-lab storage and product distribution.

OVERVIEW

Cold Chain Considerations:

Space:

- Location (ease of access, room heat/humidity control)
- Supporting Infrastructure (power outlets, emergency backup)

Time:

- Routine Defrosting
- Experiment Delays (if frequent access leads to rising temps)

Cost:

- Freezer Procurement, Routine Service, and Repairs
- Temperature Fluctuations → Potential Sample Degradation
- Equipment Failure → Sample Loss

Shipping:

Dry Ice Shipments involve:

- Heavier Packages → Higher Cost
- Increased Logistical Complexity
- Greater Concern over Delays

Can we achieve freedom from the Cold Chain?

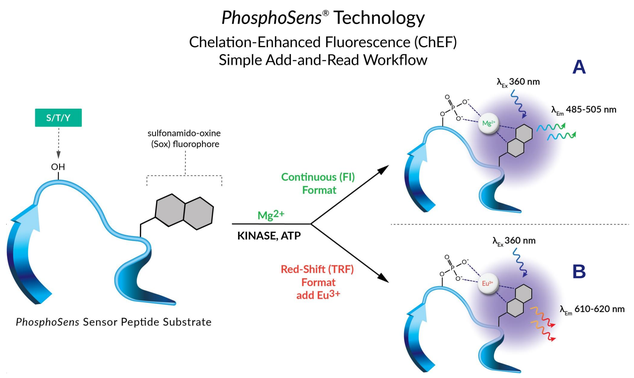
Let's consider capillary mediated vitrification (CMV)!

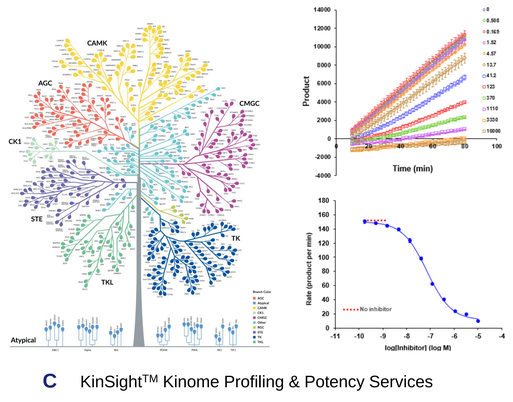
Proof-of-Concept studies were performed to evaluate the potential of CMV technology to overcome challenges of on-site reagent storage and shipment of traditionally cold chain restricted kinase assay reagents.

Future implementation of this technology could accelerate the pace of experimental research, enable higher throughput of samples, and extend the reach of our products by minimizing the hassles of intercontinental dry ice shipments.

INTRODUCTION

The *PhosphoSens*® Assay – Continuous & Endpoint/Red Formats





C KinSight™ Kinome Profiling & Potency Services

PhosphoSens® sensor peptide sequences are synthesized using solid-phase methods with the Sox fluorophore coupled through the sulfhydryl group of a cysteine residue proximal to a protein kinase phosphorylation site, such as a tyrosine, serine, or threonine. Addition of kinase results in phosphorylation of the sensor peptide. In the presence of magnesium ion, a chelation complex is formed with the phosphate group, resulting in fluorescence enhancement of the Sox fluorophore that can be monitored continuously as fluorescence intensity (**A**). Kinase inhibitors prevent phosphorylation and thus fluorescence. At any point, Europium ion can be added to displace the magnesium ion, resulting in a longer wavelength, time-resolved fluorescence (TRF) endpoint/Red format (**B**) that is useful for high-throughput or structure activity relationship (SAR) applications. This technology lends itself well to a variety of applications from determination of compound selectivity across the kinome to compound potency determination and further characterization (**C**).

Capillary Mediated Vitrification (CMV)

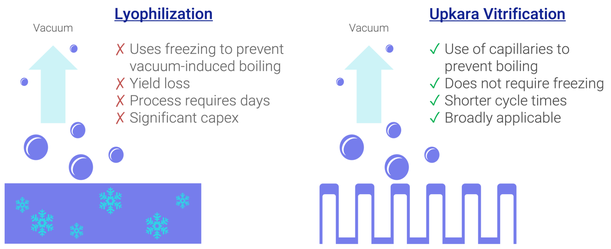
Capillary-mediated vitrification (CMV) is a process that leverages the mechanism of capillary evaporation to rapidly remove moisture from an aqueous matrix without freezing or boiling, transitioning biological reagents into a stable, glassy state. The pores within the scaffold act as capillaries, increasing the surface area and surface tension. The increase in surface tension prevents boiling, allowing the material to be dried under vacuum without freezing.

CMV Reagent Manufacturing

Step 1 Solution of buffer and reagent is mixed.

Step 2 Mixture is dispensed.

Step 3 Stabilized using a chamber.



Reagents are mixed with the Upkara BioFix™ Stabilization Buffer and applied to a solid, porous support. The supports are then processed in a vitrification chamber with a cycle time of less than one hour. This contrasts with lyophilization cycle times, which can require 12-72 hours to complete. The Upkara porous supports can be designed to work seamlessly with standard laboratory consumables or customized to meet the manufacturers and end-users' requirements. Additionally, the process is amendable to a variety of biomolecules, reducing R&D timelines and enabling the coformulation of complex mixtures.

MATERIALS & METHODS

Kinase Assay Reagents

Current evaluation kit consists of 6 components:

1. 10 X Reaction Buffer (RB)
2. 100 mM ATP
3. 1 M DTT
4. 550 mM EGTA
5. 1 mM sensor peptide
6. 5 X Kinase Dilution Buffer (KDB)

4 MasterMix components combined onto 1 scaffold

Vitrified Sample How to Use:

1. Open package. Uncap conical tube.
2. Pipette specified volume of elution buffer into centrifuge column.
3. Wait ~30s to saturate scaffold with elution buffer.
4. Centrifuge tube 1 min @ 1200 rpm.
5. Remove centrifuge column & discard.
6. Mix eluent to resuspend sample. Reagent is ready to use!

Components in Solution Require Cold Storage & Dry Ice Shipment

Vitrified Components Allow for Ambient Storage & Shipment

Protein Kinases

Two protein kinases were selected for proof-of-concept:

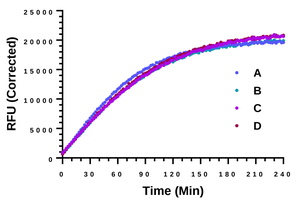
1. SRC proto-oncogene, non-receptor tyrosine kinase (**SRC)**
   Amino Acids [1-536(end)], N-terminal GST, Carna, 08-173
2. Bruton tyrosine kinase (**BTK)**
   Amino Acids [2-659(end)], N-terminal GST, Carna 08-180

Reagents typically stored on-site at -80°C were shipped to Upkara on dry ice for vitrification. Samples were sent in duplicate: one set was vitrified while the other set was stored at -80°C and then shipped back on dry ice to normalize for any differences between a sample stored on-site and one shipped back and forth on dry ice.

RESULTS

Master Mix Assay Reagents

15 µM AQT0104 with 0.32 nM SRC Comparison of Various MasterMix Conditions Corrected (Total - Background)



Condition	Condition Details	Reaction Rate ± Standard Deviation (RFU/pmol/min)		Reaction Rate ± Standard Deviation (RFU/min)		Reaction Rate Fold Difference
A	On-Site Cold Storage (-80°C)	23,880	220	191.0	1.7	1.0
B	Shipping Control (Dry Ice Transport)	21,380	130	171.0	1.1	0.9
C	Individually Vitrified (Ambient)	20,940	100	167.5	0.8	0.9
D	Jointly Vitrified (Ambient)	21,600	210	172.8	1.7	0.9

Vitrified MasterMix components stored at ambient temperature (both individually vitrified on several scaffolds and jointly vitrified on a single scaffold) performed equally well in kinetic assays with reaction rates comparable to those determined with traditionally stored reagents.

Kinase Inhibitor Potency Determinations

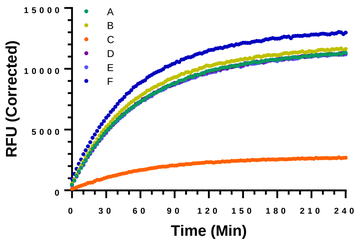
SRC Kinase Comparison

Condition	SRC Kinase Condition	IC50 (nM) 1mM ATP							
		Staurosporine @ 10µM				Staurosporine @ 1µM			
		Run 1	Run 2	Run 3	Average	Run 1	Run 2	Run 3	Average
A	On-Site (-80°C Storage)	66	77	99	81	75	89	103	89
B	Shipping Control (Dry Ice Transport)	62	72	105	79	76	96	102	92
C	Jointly Vitrified MasterMix Components with Vitrified Kinase in Biofix™ Buffer (Ambient Storage)	67	38	53	53	83	58	63	68
D	Individually Vitrified MasterMix Components with Vitrified Kinase in Biofix™ Buffer	70	51	67	63	95	83	76	84

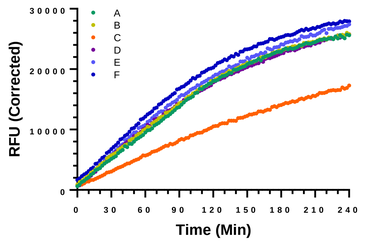
Staurosporine was used as a reference compound for IC_{50} determinations with SRC kinase. Reagents vitrified in BioFix™ buffer (C & D) showed similar IC_{50} values compared to frozen reagents (A & B). IC_{50} evaluation was not performed on SRC vitrified with Low-Binding BioFix™ Buffer.

Protein Kinase Reagents

15 µM AQT0101 Sensor Peptide with 1.0 nM BTK Kinase Condition Comparison

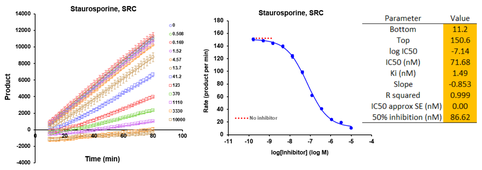


15 µM AQT0794 Sensor Peptide with 0.32 nM SRC Kinase Condition Comparison



Condition	Condition Details	BTK					SRC				
		Reaction Rate ± Standard Deviation (RFU/pmol/min)		Reaction Rate ± Standard Deviation (RFU/min)		Reaction Rate Fold Difference	Reaction Rate ± Standard Deviation (RFU/pmol/min)		Reaction Rate ± Standard Deviation (RFU/min)		Reaction Rate Fold Difference
A	On-Site (-80°C Storage)	6416	110	160.4	2.6	1.0	18750	210	150.0	1.7	1.0
B	Shipping Control (Dry Ice Transport)	6748	120	168.7	3.0	1.1	19880	210	159.0	1.7	1.1
C	Vitrified in *Biofix*™ Buffer (Ambient Storage)	1266	40	31.7	1.0	0.2	10300	180	82.4	1.4	0.5
D	Vitrified Jointly with KDB in *Biofix*™ Buffer (Ambient Storage)	6300	100	157.5	2.4	1.0	20990	260	167.9	2.1	1.1
E	Vitrified in *Low Binding Biofix*™ Buffer 1 (Ambient Storage)	6268	100	156.7	2.6	1.0	21860	250	174.9	2.0	1.2
F	Vitrified in *Low Binding Biofix*™ Buffer 2 (Ambient Storage)	7320	120	183.0	3.1	1.1	22010	300	176.1	2.4	1.2

Initial evaluation of vitrified protein kinases in BioFix™ Buffer showed decreased reaction rates for both SRC and BTK protein kinases (**C**). Modifying the vitrification buffer composition to a Low Binding BioFix™ formulation (**E** & **F**) or, alternatively, joint vitrification with Kinase Dilution Buffer (KDB) components (**D**) resulted in reproducibly higher reaction rates with kinetic progress curves comparable to those obtained with traditionally stored protein kinase at -80°C (**A**) or kinase shipped on dry ice (**B**).



Parameter	Value
Bottom	11.2
Top	150.6
log IC50	-7.14
IC50 (nM)	71.68
Ki (nM)	1.49
Slope	-0.853
R squared	0.999
IC50 approx SE (nM)	0.00
50% inhibition (nM)	86.62

BTK Kinase Comparison

Condition	BTK Kinase Condition	Elution Buffer	IC50 (nM) 1mM ATP	
			Ibrutinib	Fenetinib
A	On Site (-80°C Storage)	On Site Kinase Dilution Buffer	1.1	26
B	Shipping Control (Dry Ice Transport)	On Site Kinase Dilution Buffer	1.1	32
C	Vitrified (Round 1) in Biofix™ Buffer (Ambient Storage)	Vitrified Kinase Dilution Buffer	Weak enzyme activity; unable to obtain an IC50 value.	Weak enzyme activity; unable to obtain an IC50 value.
D	Vitrified (Round 2) in Biofix™ Buffer (Ambient Storage)	Vitrified Kinase Dilution Buffer	Weak enzyme activity; unable to obtain an IC50 value.	Weak enzyme activity; unable to obtain an IC50 value.
E	Vitrified Jointly with Kinase Dilution Buffer (Round 2) in Biofix™ Buffer (Ambient Storage)	Water	0.58	52
F	Vitrified (Round 2) in Low Binding Biofix™ Buffer 1 (Ambient Storage)	Vitrified Kinase Dilution Buffer	0.62	42
G	Vitrified (Round 2) in Low Binding Biofix™ Buffer 2 (Ambient Storage)	Vitrified Kinase Dilution Buffer	1.1	27

Ibrutinib & Fenetinib were used as reference compounds for IC_{50} determinations with BTK kinase. BTK kinase stored on-site at -80°C (A) and kinase shipped to and from Upkara on dry ice (B) yield nearly identical IC_{50} values. Kinase vitrified using BioFix™ buffer resulted in only weak assay activity from which an IC_{50} could not be determined. Subsequent rounds of kinase vitrification identified that Low-Binding BioFix™ Buffers (E and F), as well as custom vitrification of kinase with kinase dilution buffer components (D), result in comparable kinase activity and similar IC_{50} values for control compounds.

SUMMARY

- AssayQuant's PhosphoSens® assay components vitrified, stored at ambient temperature, and eluted in aqueous media can be used to generate kinetic assay data. Upkara's CMV technology can further simplify assay execution by consolidating multiple temperature sensitive components onto a single scaffold that can be stored or shipped without cold chain concerns.
- Protein kinases can be stabilized via CMV while preserving enzymatic activity. Assays performed using kinase stabilized with Upkara's Low Binding BioFix™ vitrification buffer, and subsequently stored at ambient temperatures, exhibit kinetic progress curves and reaction rates that are comparable to those generated with kinase stored at -80°C.
- Potency determinations performed with vitrified reagents yield IC_{50} values that are within 2-fold of traditionally cold stored reagents suggesting that potency and selectivity workflows are amenable to CMV stabilized reagents.